The Journal of Neuroscience, February 15, 2002, 22(4):1218–1227

Inhibition of Caspases Prevents Ototoxic and Ongoing Hair Cell Death

Jonathan I. Matsui,[1,2] **Judith M. Ogilvie,**[1,3] **and Mark E. Warchol,**[1,2,4,5]

[1]*Central Institute for the Deaf, Fay and Carl Simons Center for Biology of Hearing and Deafness,* [2]*Division of Biology and Biomedical Sciences, Neuroscience Graduate Program, and Departments of* [3]*Ophthalmology and Visual Sciences,* [4]*Otolaryngology, and* [5]*Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110*

Sensory hair cells die after acoustic trauma or ototoxic insults, but the signal transduction pathways that mediate hair cell death are not known. Here we identify several important signaling events that regulate the death of vestibular hair cells. Chick utricles were cultured in media supplemented with the ototoxic antibiotic neomycin and selected pharmacological agents that influence signaling molecules in cell death pathways. Hair cells that were treated with neomycin exhibited classically defined apoptotic morphologies such as condensed nuclei and fragmented DNA. Inhibition of protein synthesis (via treatment with cycloheximide) increased hair cell survival after treatment with neomycin, suggesting that hair cell death requires *de novo* protein synthesis. Finally, the inhibition of caspases promoted hair cell survival after neomycin treatment.

Sensory hair cells in avian vestibular organs also undergo continual cell death and replacement throughout mature life. It is unclear whether the loss of hair cells stimulates the proliferation of supporting cells or whether the production of new cells triggers the death of hair cells. We examined the effects of caspase inhibition on spontaneous hair cell death in the chick utricle. Caspase inhibitors reduced the amount of ongoing hair cell death and ongoing supporting cell proliferation in a dose-dependent manner. In isolated sensory epithelia, however, caspase inhibitors did not affect supporting cell proliferation directly. Our data indicate that ongoing hair cell death stimulates supporting cell proliferation in the mature utricle.

Key words: auditory; hair cell; vestibular; tissue culture; apoptosis; proliferation; caspase inhibitors; regeneration

The sensory hair cells of the inner ear detect sound and head movements. In mammals, hair cells can be lost through disease, aging, infection, or exposure to noise or ototoxic drugs, leading to permanent balance and/or auditory deficits. Morphological evidence from many vertebrate species suggests that the loss of hair cells occurs via programmed cell death (PCD; Jørgensen, 1981, 1991; Forge, 1985; Li et al., 1995; Forge and Li, 2000). Consistent with these observations, *in situ* terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling of fragmented DNA (TUNEL labeling) identifies apoptotic hair cells in chicks (Kil et al., 1997; Nakagawa et al., 1997c; Torchinsky et al., 1999), rodents (Lang and Liu, 1997; Nakagawa et al., 1997a,b, 1998a,b,c; Usami et al., 1997; Zheng and Gao, 1997; Nishizaki et al., 1998; Zheng et al., 1998, 1999; Forge and Li, 2000; Pirvola et al., 2000), and humans (Jokay et al., 1998), suggesting that hair cell death occurs, at least in part, by PCD.

Programmed cell death via apoptosis occurs via an orderly series of cellular events (Raff, 1998), and some forms of PCD require new RNA and protein synthesis (Martin et al., 1988). Once PCD is initiated, a cascade of intracellular events culminates in the activation of caspases, triggering a proteolytic cascade that leads to the degradation of the nuclear proteins of the cell (Salvesen and Dixit, 1997). Caspase inhibitors prevent PCD in many types of neurons (Salvesen and Dixit, 1997) and in auditory and vestibular hair cells (Liu et al., 1998; Forge and Li, 2000).

In the present study, chick utricles were cultured in media supplemented with selected pharmacological agents that influence signaling in identified cell death pathways. We determined that both cycloheximide and caspase inhibitors promoted hair cell survival after aminoglycoside exposure. Also, the mature avian vestibular sensory epithelia exhibit a low, ongoing level of cell proliferation (Jørgensen and Mathiesen, 1988; Roberson et al., 1992; Warchol and Corwin, 1993; Kil et al., 1997; Wilkins et al., 1999), which is accompanied by a comparable rate of spontaneous hair cell death (Kil et al., 1997; Wilkins et al., 1999). It is not known, however, whether the loss of hair cells stimulates supporting cell proliferation or whether proliferation triggers the apoptotic cell death of hair cells. Results indicate that ongoing hair cell death is causally related to ongoing supporting cell proliferation.

A preliminary report of portions of these data was presented previously (Matsui et al., 2000b).

MATERIALS AND METHODS

Animals

White Leghorn chickens (*Gallus domesticus*) were obtained from Truslow Farms (Chestertown, MD) or Charles River SPAFAS (Charles River, CT) and were housed in the animal care facility of the Central Institute for the Deaf. All experimental protocols were approved by the Central Institute for the Deaf and the Washington University Institutional Ani-

Received Oct. 4, 2001; revised Nov. 20, 2001; accepted Nov. 28, 2001.

This work was supported by the Division of Biology and Biomedical Sciences and the National Organization for Hearing Research Foundation (J.I.M.), by the Foundation Fighting Blindness (J.M.O.), and by National Institutes of Health Grant DC03576 and National Aeronautics and Space Administration Grant NAG2-1364 (M.E.W.). We thank Jaclynn Lett for her excellent histological and photographic assistance and Dr. Miriam Burton for help with fluorescent microscopy. We also thank Drs. Anne Hennig, Kenneth Lee, and Eugene Johnson Jr for their helpful discussions. Finally, we thank Karla Marz, David Fashena, and Drs. Edwin Rubel and Eugene Johnson Jr for their critical review of this manuscript.

Correspondence should be addressed to Mark E. Warchol or Jonathan I. Matsui, Central Institute for the Deaf, 4560 Clayton Avenue, St. Louis, MO 63110-1549. E-mail: mwarchol@cid.wustl.edu or jmatsui@cid.wustl.edu.

mal Care and Use Committee and conform to the Society for Neuroscience animal use guidelines.

Culture techniques

Organ cultures. Chicks (7–21 d posthatch) were killed with CO_2 and decapitated. The lower jaw and the skin covering the head were removed, and the heads were immersed in 70% ethanol to kill surface pathogens. The remaining dissection was performed in a laminar flow tissue culture hood. The utricles were removed and transferred to chilled Medium 199 containing Hank's salts and HEPES buffer (Invitrogen, San Diego, CA). The otoconia were removed, and the utricles were cultured as organotypic explants in 48-well tissue culture plates (Costar, Cambridge, MA). Each well contained 200 μl of culture medium, which consisted of Medium 199 supplemented with Earle's salts, 2200 mg/l sodium bicarbonate, 0.69 mM L-glutamine, 25 mM HEPES, and 10% fetal bovine serum (FBS; Invitrogen). Explants were incubated at 37°C in a humidified 5% CO_2/95% air environment for 1 d *in vitro* (1 DIV) to allow the utricles to adjust to the culture environment.

Pharmacological treatment of cultures. At the beginning of the second day *in vitro*, neomycin sulfate (Sigma, St. Louis, MO) was added to the culture medium for a final dilution of 0.2, 0.5, or 1 mM. Cultures containing aminoglycoside-free medium were maintained in parallel and served as controls.

To determine whether protein synthesis was necessary for aminoglycoside-induced hair cell death, we cultured the utricles for 24 hr with 1 mM neomycin and 1 μg/ml cycloheximide (Sigma). In experiments that examined caspase inhibition, Boc-Asp(Ome)-fluoromethyl ketone (BAF; Enzyme Systems Products, Livermore, CA) or z-Val-Ala-Asp(Ome)-fluoromethyl ketone (zVAD; Enzyme Systems Products) was added to the medium for a final concentration of 10, 50, or 100 μM for BAF or 25 μM for zVAD. Cycloheximide and the caspase inhibitors were added to the cultures simultaneously with neomycin. Control cultures also received 0.1% dimethyl sulfoxide (DMSO). To examine the effects of cell cycle inhibition on ongoing cell death and supporting cell proliferation, we added 25 μM aphidicolin (Calbiochem, San Diego, CA) to the culture medium.

To determine whether certain pharmacological agents had deleterious effects on hair cells, we cultured the utricles for 48 hr with neomycin and putative cell death inhibitors (e.g., BAF), but without neomycin. Additional experiments examined the acute effects of caspase inhibitors on long-term survival of hair cells. After a 1 d treatment with neomycin and 50 μM BAF the utricles were rinsed three times and maintained in Medium 199 and 10% FBS for an additional 5 d (total time, 7 DIV). One-half of the culture medium was changed every other day.

Sensory epithelial cultures. To assess the direct effects of pharmacological agents on supporting cell proliferation, we isolated sensory epithelia from chick utricles as described previously (Warchol, 1995, 1999). Utricles were removed from chicks and placed in sterile Medium 199; the otoconia were removed. Then the utricles were incubated in thermolysin (500 μg/ml in Medium 199 with Earle's salts; Sigma) for 1 hr at 37°C in a humidified 5% CO_2/95% air environment incubator and then returned to chilled Medium 199 for further dissection. A 27-gauge needle was used to dissociate the sensory epithelia from the basement membrane and associated connective tissue. Isolated sheets of sensory epithelia were transferred into fibronectin-coated culture wells (MatTek, Ashland, MA) containing 50 μl of Medium 199 and 10% FBS and incubated for 3 d to allow the sensory epithelium to adhere to the fibronectin substrate. Then the explants were maintained in 50 μl of Medium 199 and 10% FBS in the presence of 0.1% DMSO, 50 μM BAF, or 25 μM zVAD for an additional 2 d (total, 5 DIV). One-half of the medium was changed every other day.

Tissue processing

Cultured utricles and sensory epithelia explants were fixed with 4% paraformaldehyde for 20 min and then were rinsed with PBS unless otherwise stated. Also, immunohistochemical steps were performed at room temperature with thorough PBS washes between them, unless otherwise stated.

Primary antibodies. Mouse monoclonal anti-BrdU antibody was obtained from Becton Dickinson (San Jose, CA). Dr. J. H. Rogers (University of Cambridge, UK) generously donated the rabbit monoclonal anti-calretinin. Additional rabbit polyclonal anti-calretinin was obtained from Chemicon (Temecula, CA).

Fluorescent nucleic acid staining. Fixed utricles were immersed in bisbenzimide (Hoechst 33258; 20 μg/ml; Sigma) in the dark for 1 hr. Specimens were washed thoroughly with PBS and mounted on glass slides in glycerol/PBS (9:1).

TUNEL labeling. The ApopTag kit (Intergen, Purchase, NY) was used to label dying hair cells, following the protocol described by Kil et al. (1997). Fixed utricles were incubated in 90% methanol with 0.5% hydrogen peroxide (H_2O_2) for 15 min and immersed in a blocking solution consisting of 5% nonfat dairy dry milk and 2% bovine serum albumin (BSA; Sigma) for 20 min; this was followed by 1× equilibrium buffer for 10 min. Then the tissue was incubated in TdT enzyme in a humidified oven for 30 min at 37°C. A stop/wash buffer was added to the wells, and the tissue was incubated in the humidified oven for 30 min. The specimens were exposed to digoxigenin for 30 min and then were reacted with 0.5 mg/ml diaminobenzidine (DAB) in 0.05 M Tris buffer and 0.03% H_2O_2/$NiCl_2$ for 5 min. Utricles were mounted onto slides as whole mounts in glycerol/PBS.

Calretinin labeling. To assess the extent of hair cell survival quantitatively, we identified hair cells by using an antibody for calretinin (see Fig. 1) (Rogers, 1989). Fixed utricles were incubated in 90% methanol with 0.03% H_2O_2 for 20 min, followed by incubation in a blocking solution consisting of PBS, 2% normal horse serum (NHS; Sigma), 1% BSA, and 0.2% Triton X-100 for 20 min. Then the tissue was placed immediately into a rabbit anti-calretinin primary antibody (1:2000; in PBS and 2% NHS) and incubated overnight at 4°C. Utricles were incubated in biotinylated goat anti-rabbit IgG antibody (1:150; in PBS and 0.1% NHS; Vector Laboratories, Burlingame, CA) for 2 hr, followed by avidin-biotin-horseradish peroxidase complex (Vector Laboratories) for 90 min. Specimens were reacted with DAB for 5 min and mounted on microscope slides in glycerol/PBS.

Bromodeoxyuridine immunohistochemistry. To assay supporting cell proliferation, we added the mitotic tracer bromodeoxyuridine (BrdU; 3 μg/ml; Sigma) to cultures for the last 4 hr *in vitro*. Specimens were processed for BrdU immunohistochemistry by following a standard protocol (Warchol and Corwin, 1996). Fixed utricles were incubated in 90% methanol with 0.03% H_2O_2 for 20 min, 2N HCl for 30 min, and blocking solution (PBS, 2% NHS, 1% BSA, 0.2% Triton X-100) for 20 min; then they were incubated overnight in mouse anti-BrdU monoclonal antibody (1:50; in PBS, 2% NHS, 0.1% Triton X-100) at 4°C. After being rinsed thoroughly in PBS, the tissue was incubated in biotinylated horse anti-mouse IgG antibody (1:150; in PBS, 0.1% NHS, 0.1% Triton X-100; Vector Laboratories) for 2 hr, followed by avidin-biotin-horseradish peroxidase complex (Vector Laboratories) for 90 min. Specimens were reacted with DAB for 6 min and mounted on microscope slides in glycerol/PBS.

Transmission electron microscopy. To assess morphological changes in hair cells qualitatively, we fixed the utricles in 2.5% glutaraldehyde and 2% paraformaldehyde overnight at 4°C. Tissue was post-fixed for 1 hr in osmium tetroxide, stained en bloc with 1% uranyl acetate for 1 hr, serially dehydrated through an acetone series, and embedded in Epon Araldite. Ultrathin sections were poststained with 1% uranyl acetate and lead citrate. The specimens were imaged with a Philips EM-300 transmission electron microscope.

Data analysis

All counts of labeled cells were conducted "blind" with respect to pharmacological treatment.

Counts of calretinin-labeled cells. Whole-mount preparations were visualized on a Zeiss Axiovert 135 microscope, and video images of microscopic fields were displayed on a Sony monitor via a Cohu CCD camera. Cell counts were made directly from the video monitor by using calibrated templates that outlined fields of 100 × 100 μm. Selected regions from either the striolar or extrastriolar regions of the utricle were displayed on the video monitor. Calretinin-labeled cells were counted from six regions in the central extrastriolar region and four regions distributed along the striolar region of each utricle. Care was taken to avoid the lateral limits of the sensory epithelium because these regions frequently contained areas of epithelial damage resulting from the surgical dissection. The regions were averaged to obtain an estimate of the number of surviving hair cells/10,000 μm^2 for the striolar or extrastriolar region of each specimen.

Counts of fluorescent nucleic acid staining. Bisbenzimide-labeled cells were counted in whole-mount preparations of the utricular maculae by using fluorescent optics with a DAPI filter (excitation, 346 nm; emission, 460 nm). Each pyknotic nucleus in eight randomly selected regions of each utricular macula was counted, using a reticule and a 40× objective, and then normalized to 25,000 μm^2. Pyknotic nuclei were detected easily

by their condensed chromatin. The number of pyknotic cells per 25,000 μm^2 of sensory epithelium was calculated for each explant.

Counts of TUNEL-labeled cells. Each TUNEL-labeled cell in six extrastriolar regions of each utricular macula was counted, using a reticule and a 60× objective. The number of TUNEL-labeled cells per 25,000 μm^2 of sensory epithelium was calculated for each explant. Again, care was taken to avoid the lateral limits of the sensory epithelium to avoid counting cells damaged by the surgical dissection. Such regions were distinguished by the obvious discontinuities in the apical surface of the sensory epithelium and large numbers of labeled cells in and immediately surrounding the regions of damage.

Counts of BrdU-labeled cells in whole mounts. BrdU-labeled epithelial cells in whole-mount preparations of the utricular maculae were counted by light microscopy. Each BrdU-labeled cell was counted from six randomly selected regions of the extrastriolar region of each utricular macula, using a reticule and a 60× objective. The number of BrdU-labeled sensory epithelial cells per 25,000 μm^2 of sensory epithelia was calculated for each explant.

Counts of BrdU-labeled cells in epithelial cultures. Quantification of cell proliferation in the epithelial cultures was performed directly from the video monitor by using calibrated templates that outlined fields of 100 × 100 μm. Previous studies have shown that the proliferation of supporting cells in epithelial cultures depends on local cell density (Warchol, 1998). To control for this effect, we performed all quantification from confluent regions of the cultures with a cell density of 20–40 cells/10,000 μm^2. A proliferation index (BrdU-labeled cells/total cells) was computed for each sampled region. Proliferation indices were obtained from four to seven regions within each individual culture.

Statistical analysis

Data from hair cell counts, bisbenzimide labeling, and BrdU labeling experiments were subjected to either an unpaired two sample *t* test assuming unequal variances with Microsoft Excel 98 (Microsoft, Redmond, WA) or a one-way ANOVA with SigmaStat (Jandel Scientific Software, Chicago, IL). *Post hoc* comparisons, when appropriate, used the Tukey–Kramer or Scheffe's test.

RESULTS

Morphology of the utricle

The utricle contains both sensory hair cells and nonsensory supporting cells. Hair cells can be identified by their structural features (cuticular plate, stereocilia, and kinocilium) and the location of their nuclei in the lumenal stratum of the sensory epithelium. Two types of hair cells, designated type I and type II, are present in the avian utricular macula and can be differentiated on the basis of morphology and innervation (Jørgensen, 1989). Type I hair cells are located exclusively in the striolar region of the utricular macula, whereas type II hair cells are found throughout the extrastriolar region and in a narrow region of the striola (Jørgensen, 1989). Antibodies recognizing calretinin selectively label hair cells, but not supporting cells or peripheral epithelial cells, in the avian and mammalian vestibular system (Rogers, 1989; Zheng and Gao, 1997). Labeling was particularly evident in the stereocilia bundles (Fig. 1). If the stereocilia bundle was missing, the apical portion of the remaining hair cell was labeled (data not shown). To determine the baseline density of hair cells in the chick utricle in our culture system, we removed utricles from undamaged animals and cultured them for 48 hr in control medium. Hair cells in both the striolar and extrastriolar regions were quantified and expressed as the mean number of calretinin-labeled cells/10,000 μm^2 ± SD. Calretinin-labeled cells were counted from six regions that were distributed throughout the central extrastriolar portion (cotillus) of each utricle and four regions distributed throughout the striolar region. Greater hair cell densities were observed in the extrastriolar region (105.1 ± 12.5; $n = 4$) than in the striolar region (45.6 ± 2.2; $n = 4$).

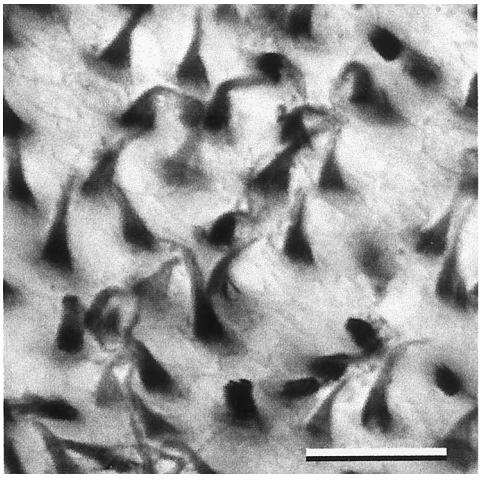

Figure 1. Hair cells labeled for immunoreactivity to calretinin. Utricles from control animals were fixed and processed for immunohistochemistry with the use of an antibody directed against calretinin. Stereocilia bundles and the cuticular plate are labeled. Scale bar, 20 μm.

Changes in cell survival and nuclear chromatin after neomycin treatment

Aminoglycoside antibiotics, such as neomycin, selectively kill hair cells in cultures of the avian ear organs (Navaratnam et al., 1996; Stone et al., 1996; Hirose et al., 1997; Kil et al., 1997; Warchol, 1999; Matsui et al., 2000a). To characterize the pattern of hair cell loss induced by neomycin, we incubated utricles in control (aminoglycoside-free) medium for 24 hr and then in 1 mM neomycin-supplemented medium for another 24 hr. Then the utricles were fixed and processed for calretinin immunohistochemistry. Approximately 40 calretinin-positive cells/10,000 μm^2 were present in the extrastriolar regions of the neomycin-treated specimens, whereas ~18 hair cells/10,000 μm^2 were found in the striolar regions. This pattern of hair cell loss was similar to that observed in utricles exposed to comparable concentrations of streptomycin sulfate both *in vivo* (Weisleder and Rubel, 1992, 1993) and *in vitro* (Matsui et al., 2000a).

To examine changes in the nuclear morphology in hair cells after neomycin treatment, we incubated utricles in 1 mM neomycin-supplemented medium for 24 hr. Fixed specimens were stained with the DNA-binding dye bisbenzimide (Hoechst staining) (Witte et al., 2001). Many cell nuclei in neomycin-treated utricles were intensely stained, branched, and irregularly shaped, indicating massive structural changes in nuclear chromatin (Fig. 2*A*). In contrast, nuclei of cells in control utricles appeared oval and homogeneously stained with moderate intensity (Fig. 2*B*). Bisbenzimide-labeled supporting cells could be distinguished from the underlying connective tissue by cell morphology and location within the utricle. Significantly more pyknotic nuclei were found in neomycin-treated cultures when compared with control cultures ($p < 0.001$; Fig. 2*C*).

Ultrastructural changes after neomycin treatment

The morphological hallmarks of apoptotic PCD are structural changes such as cell body shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation (Kerr et al., 1972). The effects of neomycin on the morphology of hair cells were studied using transmission electron microscopy. We sampled approximately eight ultrathin sections per organ from four to five organs per culture condition (total, ~32–40 sections per condi-

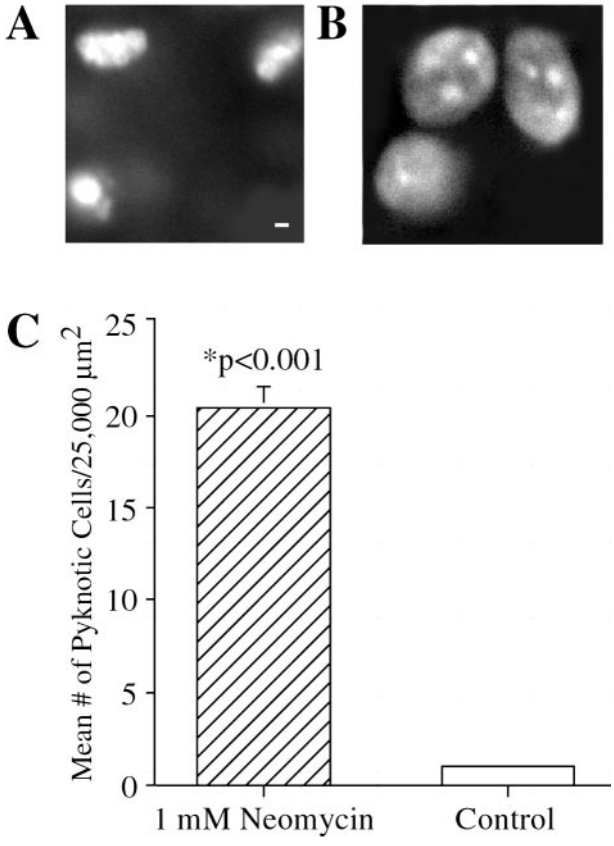

Figure 2. Neomycin induces nuclear chromatin changes in hair cells. Utricles were cultured for 24 hr with 1 mM neomycin (*A*) or control medium (*B*), and then they were fixed and stained with bisbenzimide. Dying cells in neomycin-treated cultures were shaped irregularly and had pyknotic nuclei, whereas cells in control cultures were stained uniformly. Pyknotic cells were quantified in 25,000 μm^2 regions throughout the organ (8 regions per organ; *C*). Results are the mean ± SEM for three experiments from 13 organs. Data point may obscure error bar. Scale bar, 1 μm.

tion). After exposure to neomycin for 24 hr, the hair cell nuclei shrank, nuclear chromatin condensed, and cytoplasm became more electron-dense (Fig. 3*A*). There was also a noticeable increase in vacuolization, lipid inclusions, and the formation of intracellular membrane whorls presumably caused by lysosomes (Fig. 3*A*). Despite these changes, mitochondria were well preserved within the degenerating cell. In control utricle cultures the hair cells appeared normal (Fig. 3*B*), although some increase in vacuolization was seen when compared with *in vivo* tissue. There was no evidence of PCD among supporting cells.

Inhibition of protein synthesis promotes hair cell survival

Apoptosis is an active process in which macromolecules are synthesized to bring about cell death. Previous data indicate that inhibition of protein synthesis prevents death in some types of neurons (Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990). To determine whether protein synthesis was necessary for aminoglycoside-induced hair cell death, we cultured utricles for 24 hr with neomycin and 1 μg/ml cycloheximide, a macromolecule synthesis inhibitor. This treatment nearly doubled the number of surviving calretinin-positive cells (Fig. 4). Hair cell densities in the extrastriolar region were 72.2 ± 17.4 in cycloheximide-/neomycin-treated tissue and 43.5 ± 8.0 in neomycin-treated tissue. In the striolar regions the hair cell densities were 37.2 ± 10.1 in cycloheximide-/neomycin-treated tissue and 19.2 ± 3.6 in neomycin-treated tissue. It appears that hair cell loss was reduced, but not stopped, by cycloheximide treatment.

An additional experiment examined whether cycloheximide was toxic to hair cells. Utricles were cultured without neomycin in the presence or absence of 1 μg/ml cycloheximide for 24 hr. Hair cell densities were 88.6 ± 14.2 in the extrastriolar region of cycloheximide-treated tissue versus 88.3 ± 14.4 hair cells in control tissue. In the striolar region the hair cell densities were 51.0 ± 9.3 hair cells in cycloheximide-treated tissue and 47.8 ± 9.4 hair cells in control tissue ($n = 8$, cycloheximide; $n = 9$, controls). There was no significant difference in hair cell density between cycloheximide-treated cultures and control cultures ($p > 0.5$), indicating that short-term exposure to cycloheximide is not toxic to hair cells.

Caspase inhibitors increase hair cell survival after treatment with neomycin

Caspase induction results in the activation of nucleases and the cleavage of nuclear structural proteins, which can cause cell death. Application of caspase inhibitors can prevent PCD in neurons (Salvesen and Dixit, 1997). In one set of experiments, we investigated whether BAF, a general caspase inhibitor, could reduce the number of pyknotic nuclei in the sensory regions of the utricle after neomycin treatment. Utricles were cultured for 24 hr with 1 mM neomycin and 50 μM BAF or 0.1% DMSO (vehicle) and then were stained with bisbenzimide. Significantly fewer pyknotic nuclei ($p < 0.001$) were observed in BAF/neomycin-treated utricles when compared with neomycin-treated cultures (Fig. 5*A*).

Caspase activation also can cause DNA strand breaks during PCD, and DNA fragmentation has been observed to occur before pyknosis. Dying cells can be detected by enzymatically labeling the free 3′-OH termini of fragmented DNA with modified nucleotides (TUNEL labeling). Utricles were cultured for 24 hr with 1 mM neomycin and 50 μM BAF or 0.1% DMSO and then were processed for TUNEL labeling (Kil et al., 1997). The number of TUNEL-labeled cells decreased significantly in BAF-treated cultures, relative to controls (Fig. 5*B*). To control for possible DMSO toxicity to hair cells, we cultured utricles in control medium or 0.1% DMSO-supplemented medium and found that there was no significant difference ($p = 0.3$) in the number of TUNEL-labeled cells/25,000 μm^2 with either medium condition (26.9 ± 4.7, DMSO; 23.2 ± 6.2, controls).

In addition to TUNEL labeling, we assayed hair cell survival by counting calretinin-labeled hair cells. Utricles were cultured for 24 hr with three different concentrations of neomycin (0.2, 0.5, and 1 mM) and either 50 μM BAF or 0.1% DMSO. Approximately 40% more hair cells were present in both the extrastriolar and the striolar regions of BAF-treated utricles than in controls (Figs. 6, 7*A*, Table 1).

To determine whether BAF treatment resulted in the long-term survival of hair cells or simply delayed their death, we cultured utricles for 1 d in 1 mM neomycin and 50 μM BAF or 0.1% DMSO. Then the cultures were washed thoroughly and maintained *in vitro* for an additional 5 d in control medium (total 7 DIV) and processed for calretinin immunohistochemistry. After 7 DIV, many cultured utricles had curled up, and the striolar region was difficult to quantify, so only the extrastriolar region was analyzed. After 7 DIV, there were similar hair cell densities

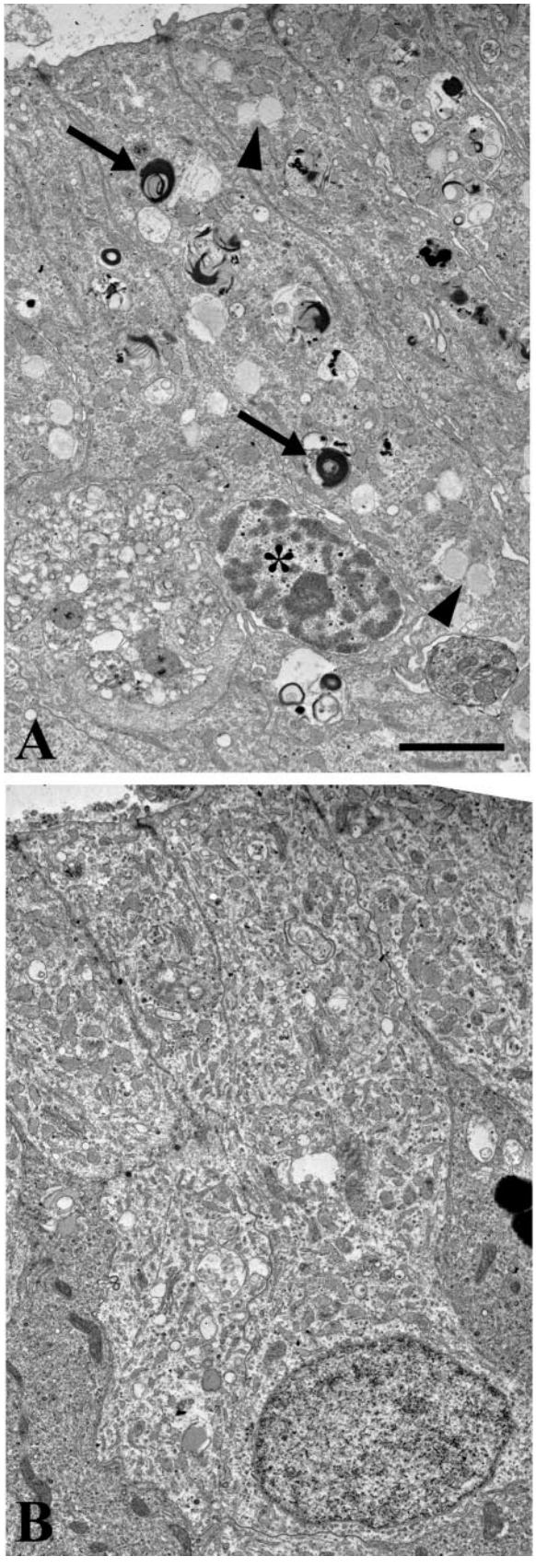

Figure 3. Ultrastructural changes in hair cells after 24 hr of neomycin treatment. Shown are transmission electron microscopy micrographs of hair cells from utricles cultured in 1 mM neomycin (*A*) or control medium (*B*). Hair cells in neomycin-treated cultures had condensed and fragmented nuclear chromatin (*). Vacuoles, intracellular membrane whorls (*arrows*), and lipid inclusions (*arrowheads*) were observed also. In control cultures, the hair cells appeared normal, with no significant morphological changes. Mitochondria were intact in both control and degenerating cells. Scale bar, 2 μm.

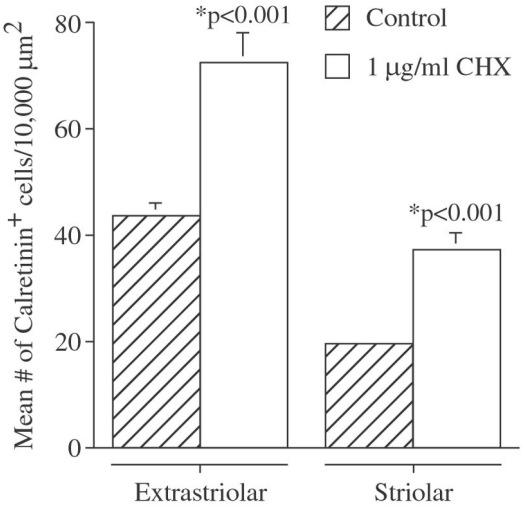

Figure 4. Inhibition of macromolecule synthesis promotes hair cell survival in the presence of neomycin. Utricles were cultured for 24 hr with 1 mM neomycin and 1 μg/ml cycloheximide or control medium. Calretinin-labeled cells were quantified in 10,000 μm^2 regions of both the extrastriolar (6 regions per organ) and striolar (4 regions per organ) areas. Results are the mean ± SEM for three experiments from 10–12 organs. Data point may obscure the error bar.

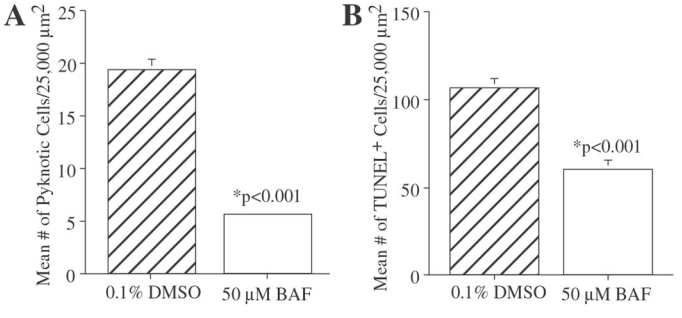

Figure 5. Caspase inhibitors prevent hair cell death after neomycin treatment. Utricles were cultured for 24 hr with 1 mM neomycin and 0.1% DMSO (carrier) or 50 μM BAF. Cultures were fixed, and the nuclei were stained with Hoechst (*A*) or were processed for TUNEL labeling (*B*). Pyknotic cells were quantified in 25,000 μm^2 regions throughout the organ (8 regions per organ). $TUNEL^+$ cells were quantified in 25,000 μm^2 regions throughout the organ (6 regions per organ). Results are the mean ± SEM for three experiments from 10–13 organs. Data point may obscure the error bar.

in utricles that were treated with BAF and neomycin and control cultures. Hair cell density in BAF-/neomycin-treated cultures was 85.9 ± 3.6 versus 46.1 ± 5.6 in neomycin-treated cultures or 88.7 ± 2.7 in control cultures that were treated with 0.1% DMSO (Fig. 7*B*). This indicates that hair cells saved by BAF can survive for at least 5 d.

Finally, we tested a different caspase inhibitor to determine whether it promotes hair cell survival. Utricles were cultured with 1 mM neomycin and 25 μM zVAD or 0.1% DMSO, fixed, and immunoreacted to detect calretinin. The addition of zVAD to the

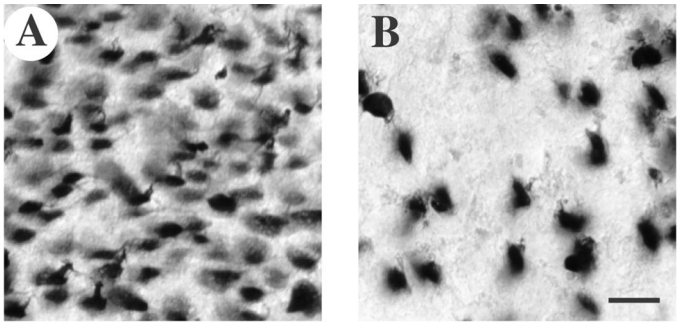

Figure 6. Photomicrograph of hair cells that were treated with neomycin and with or without BAF. Utricles were cultured for 24 hr in 1 mM neomycin and 0.1% DMSO (*A, B*) and 50 μM BAF (*A*). Enhanced numbers of hair cells were present in BAF-treated cultures compared with cultures that contained neomycin alone. Scale bar, 10 μm.

cultures had a protective effect comparable with that of BAF (Fig. 7*C*).

Ongoing cell death occurs in cultured utricles

Hair cells in the mature avian vestibular system die spontaneously and are replaced continuously (Jørgensen and Mathiesen, 1988; Roberson et al., 1992; Kil et al., 1997; Wilkins et al., 1999). To characterize the amount of ongoing cell apoptosis in vestibular organs, we cultured utricles in control medium for 24 hr and then processed them for TUNEL labeling. Specimens contained 15.3 ± 1.4 TUNEL-labeled cells/25,000 μm^2. TUNEL-labeled cells were randomly distributed throughout the sensory epithelia. Both the number and the density of the TUNEL-labeled cells were in agreement with the data of Kil et al. (1997).

In light of this observation, we wished to determine whether ongoing hair cell death in utricles resulted from the activation of caspases. Specimens were incubated for 24 hr in medium supplemented with 10, 50, or 100 μM BAF or 0.1% DMSO. Significantly fewer TUNEL-labeled cells were observed in 50 μM BAF-treated organs when compared with DMSO-treated organs ($p < 0.001$; Fig. 8*A*). Ongoing apoptosis was reduced by ~50% in 50 μM BAF-treated cultures and by ~80% in 100 μM BAF-treated cultures.

Preventing ongoing cell death reduces supporting cell proliferation

It has been hypothesized that the death of hair cells triggers the proliferation of nearby supporting cells (Corwin and Cotanche, 1988; Girod et al., 1989; Raphael and Altschuler, 1992; Roberson et al., 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994; Warchol and Corwin, 1996). To investigate the relationship between ongoing cell death and the level of supporting cell proliferation in the chick vestibular organs, we inhibited hair cell death by treatment with BAF. Proliferating cells were labeled by the addition of BrdU to the medium for the final 4 hr *in vitro*. Significantly fewer BrdU-labeled cells were observed in cultures that were treated with 50 μM BAF compared with control cultures ($p < 0.001$; Fig. 8*B*). In fact, supporting cell proliferation decreased by >50% in 50 μM BAF-treated cultures and by nearly 90% in 100 μM BAF-treated cultures. This result suggests that reducing hair cell death causes a reduction in supporting cell proliferation. It was important, however, to demonstrate that BAF and zVAD did not inhibit cell proliferation directly. To address this issue, we studied the effects of caspase inhibitors on supporting cell proliferation in cultures of isolated pieces of utricular sensory epithelium maintained on fibronectin substrates. Isolated sheets of utricular sensory epithelia were cultured for 48 hr in medium supplemented with 50 μM BAF, 25 μM zVAD, or 0.1% DMSO (controls). In addition, BrdU was added to the medium for the final 4 hr *in vitro*. After fixation and processing for BrdU immunohistochemistry, the numbers of BrdU-labeled cells were quantified from confluent regions with cell densities of 20–40 cells/10,000 μm^2, using methods described by Warchol (1999). A proliferation index, defined as the number of BrdU-labeled cells/total number of cells per 10,000 μm^2, was calculated for each sampled region. Similar levels of supporting cell proliferation were observed in control, BAF-treated, and zVAD-treated cultures. The proliferation index was 0.20 ± 0.03 in control cultures, 0.20 ± 0.01 in BAF-treated cultures (Fig. 8*C*), and 0.23 ± 0.01 ($p = 0.9$) in zVAD-treated cultures. These results indicate that treatment with caspase inhibitors does not directly inhibit the proliferation of vestibular supporting cells.

In a separate series of experiments, we treated utricles with 25 μM aphidicolin, which prevents DNA synthesis after entry into S phase for 24 hr, and then we processed the tissue for TUNEL labeling. Treatment with aphidicolin had no effect on ongoing apoptosis. Dying cell densities were 4.9 ± 1.3 in aphidicolin-treated cultures versus 3.4 ± 0.7 in control cultures ($p = 0.8$; n = 8–9). Parallel experiments with BrdU showed that aphidicolin inhibited supporting cell proliferation to 7% of control values. Taken together, these results indicate that blocking supporting cell proliferation is not sufficient to induce hair cell death.

DISCUSSION

Results presented here demonstrate that hair cells exposed to neomycin exhibit classically defined apoptotic morphologies, as assessed by light microscopy and transmission electron microscopy. Additionally, the death of neomycin-treated hair cells requires *de novo* protein synthesis. Caspase activation is required for both ongoing- and neomycin-induced hair cell death. Finally, our results suggest a causative relationship between ongoing (spontaneous) hair cell death and supporting cell proliferation.

Aminoglycosides kill vestibular hair cells *in vitro*

Previous work has shown that neomycin kills hair cells in organ cultures of inner ear sensory organs (Richardson and Russell, 1991; Warchol et al., 1993; Saffer et al., 1996; Kil et al., 1997; Quint et al., 1998). In the present study, treatment with neomycin resulted in increased numbers of pyknotic and condensed nuclei and enhanced TUNEL labeling. In addition, observations with transmission electron microscopy revealed morphological changes characteristic of apoptosis, including condensed, marginated, and fragmented nuclei and intact mitochondria (Wyllie et al., 1980). These observations are consistent with previous studies of the effects of aminoglycosides on hair cells in mammals (Forge, 1985; Kotecha and Richardson, 1994; Li et al., 1995; Forge and Li, 2000).

Treatment with three different pharmacological agents (cycloheximide, BAF, and zVAD) increased hair cell survival by 40–50% after exposure to 1 mM neomycin. Significantly, none of these agents completely prevented hair cell death. Rather, a substantial number of hair cells died regardless of the death-blocking pharmacological agent that was used. One plausible explanation for this observation is that exposure to 1 mM neomycin for 24 hr causes some chick vestibular hair cells to die via necrosis. Although no necrotic hair cells were observed in a recent study of the effects of aminoglycosides on cultured mam-

Table 1. Protective effects of BAF on hair cell death induced by neomycin *in vitro*

	0.2 mM Neomycin		0.5 mM Neomycin		1 mM Neomycin	
	0.1% DMSO	50 μM BAF	0.1% DMSO	50 μM BAF	0.1% DMSO	50 μM BAF
Extrastriolar	68.5 ± 2.8	101.2 ± 4.6	60.2 ± 4.1	94.7 ± 5.1	50.9 ± 3.4	89.2 ± 4.0
Striolar	40.0 ± 1.4	59.5 ± 2.4	23.5 ± 2.4	38.4 ± 2.8	18.5 ± 1.3	31.3 ± 1.8

Utricles were cultured for 24 hr with 0.2, 0.5, or 1 mM neomycin and 0.1% DMSO or 50 μM BAF. Calretinin$^+$ cells were quantified in 10,000 μm^2 regions of both the extrastriolar (six regions/organ) and striolar (four regions/organ) areas. Results are the mean ± SEM for three experiments from 10–13 organs.

malian vestibular organs (Forge and Li, 2000), another study reported some necrotic hair cells in control cultures after prolonged incubation (Li and Forge, 1995). Alternatively, a caspase-independent pathway could mediate some hair cell death. In cerebellar granule cells, general caspase inhibitors have only a marginal survival-promoting effect, suggesting the presence of a caspase-independent death pathway (Miller et al., 1997). Multiple cell death pathways also have been demonstrated in sympathetic neurons (Deshmukh and Johnson, 2000). In that study, treatment with staurosporine resulted in two distinct forms of neuronal death, depending on the concentration of staurosporine (Deshmukh and Johnson, 2000). Low concentrations of staurosporine induced degeneration that resembled the apoptotic death induced by nerve growth factor deprivation. In contrast, treatment with high concentrations resulted in a caspase-independent form of death, with chromatin changes that were neither TUNEL-positive nor necrotic. These results illustrate the need to assess cell death by multiple criteria; further studies are needed to identify more components of the aminoglycoside-induced death pathway in hair cells.

Hair cell densities in all cultured utricles were lower than the values obtained from utricles *in vivo* (Warchol, 2001), indicating that some death occurs even in the control cultures. Spontaneous death of mature hair cells in cultures of inner ear sensory organs has been reported in previous studies (Oesterle et al., 1993; Stone et al., 1996; Quint et al., 1998; Matsui et al., 2000a). In addition, avian vestibular hair cells have a relatively short lifespan (Kil et al., 1997; Goodyear et al., 1999; Stone et al., 1999; Wilkins et al., 1999), and this could be a causative agent for the density differences. Alternatively, the sensory epithelia could expand after placement in culture. Such expansion also would result in decreased hair cell density.

General caspase inhibitors promote hair cell survival

Apoptosis is mediated by proteases of the caspase family (Salvesen and Dixit, 1997). In the present study, the general caspase inhibitor BAF promoted hair cell survival when used in conjunction with three different neomycin concentrations. At best, no more than 50% of the original hair cell population was rescued by either BAF or zVAD. This is comparable with the protective effect of BAF in other sensory organs when maintained in culture (Ogilvie, 2001). In contrast, a similar study of mature mammalian utricles found that BAF treatment saved nearly all hair cells after 1 mM gentamycin treatment (Forge and Li, 2000). Different culture conditions, different aminoglycosides, and species differences could account for the discrepancy in hair cell survival. For example, chick vestibular hair cells have been estimated to live from ~1 to 15 weeks, whereas mammalian vestibular hair cells are capable of surviving for the entire lifetime of the animal (Roberson et al., 1992; Rubel et al., 1995; Kil et al., 1997; Goodyear et al., 1999; Stone et al., 1999; Wilkins et al., 1999). These observations suggest that the cell death pathway(s) in the avian vestibular organs may show some important differences from their mammalian counterparts.

Relationship between spontaneous hair cell death and ongoing cell addition

Several previous studies have demonstrated ongoing cell proliferation in mature vestibular organs (Jørgensen and Mathiesen, 1988; Roberson et al., 1992; Warchol and Corwin, 1993; Weisleder and Rubel, 1993; Rubel et al., 1995; Kil et al., 1997; Wilkins et al., 1999) and have demonstrated that the baseline level of proliferation appears to be counterbalanced by ongoing hair cell death (Kil et al., 1997; Goodyear et al., 1999; Stone et al., 1999; Wilkins et al., 1999). This dynamic pattern of ongoing cell death and proliferation differs from that observed in the chicken cochlea, in which the supporting cells are mitotically quiescent until the hair cells are lost because of trauma (Corwin and Cotanche, 1988; Oesterle and Rubel, 1993) or proliferate at a very low rate (Oesterle and Rubel, 1993). The results of these previous studies, however, leave the relationship between ongoing cell death and cell proliferation unclear. The loss of vestibular hair cells may stimulate supporting cell proliferation, but spontaneous supporting cell proliferation might also trigger the death of hair cells. In the present study, we examined the effects of caspase inhibition on spontaneous hair cell death in the chick utricle and showed that caspase inhibitors reduced the amount of ongoing hair cell death by ~50–85%. Significantly, caspase inhibition also reduced ongoing supporting cell proliferation to ~50–90% of control levels. Data obtained from isolated sensory epithelia indicate that caspase inhibitors do not directly affect supporting cell proliferation, which is consistent with previous data from a variety of mammalian cell types (Jacobsen et al., 1996). Taken together, these results suggest that ongoing hair cell death stimulates supporting cell proliferation in the mature utricle.

Receptor turnover in other sensory systems

The stimulus that causes hair cells to die spontaneously is not known. Ongoing turnover of sensory receptors has been demonstrated in other sensory systems. In the olfactory sensory epithelium new neurons continually replace olfactory receptor neurons that have been lost by apoptosis (Holcomb et al., 1995). A recent study has indicated that cells die in the olfactory sensory epithelium, via a ligand-mediated cell death pathway (e.g., fas and fas-ligand; Farbman et al., 1999). Another study has implicated the Bcl-2 protein in neuronal turnover under basal conditions (Hayward and Morgan, 1995). Mature olfactory receptor neurons normally live at least 90 d, but when mice are reared in a laminar flow hood to prevent rhinitis, olfactory receptor neurons can survive as long as 12 months (Hinds et al., 1984). Therefore, the turnover of olfactory receptor neurons may be regulated by environmental factors. Actively dying olfactory receptor neurons could provide a positive signal that stimulates neuronal precursor proliferation. Alternatively, differentiated neurons may produce

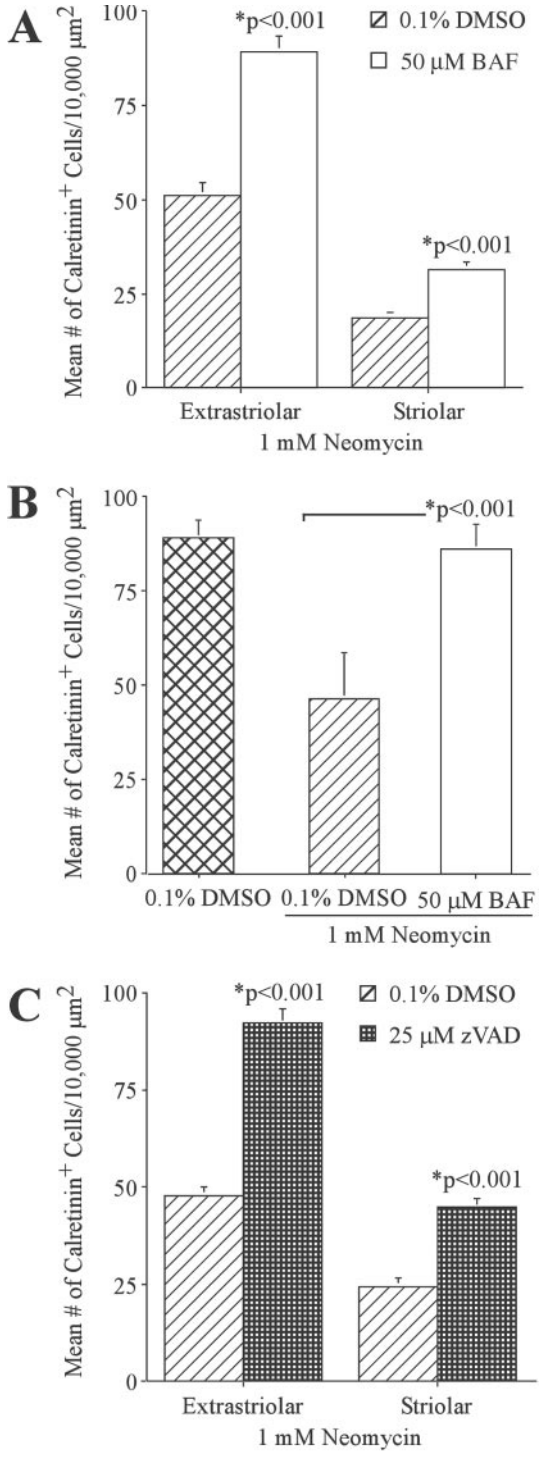

Figure 7. Caspase inhibitors promote hair cell survival after neomycin treatment. In short-term experiments the utricles were cultured for 24 hr with 1 mM neomycin and 0.1% DMSO (*A, C*), 50 μM BAF (*A*), or 25 μM zVAD (*C*). In long-term experiments (*B*) the utricles were cultured for 24 hr with 0.1% DMSO, 1 mM neomycin, or 1 mM neomycin and 50 μM BAF; they were washed and then cultured for 5 d in control medium. Calretinin$^+$ cells were quantified in 10,000 μm^2 regions of both the extrastriolar (6 regions per organ) and striolar (4 regions per organ) areas. In the long-term cultures the calretinin$^+$ cells were quantified in the extrastriolar region only. Similar levels of hair cell survival were observed in BAF/neomycin-treated cultures and control cultures after 5 DIV. Results are the mean ± SEM for three experiments from 10–13 organs.

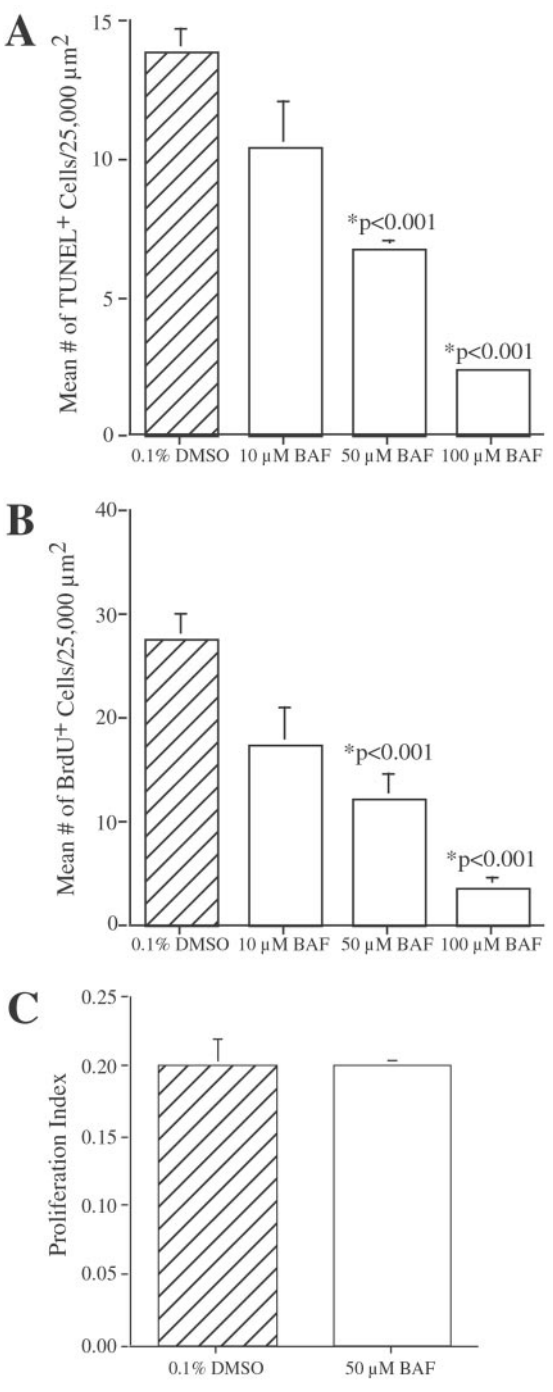

Figure 8. BAF reduces ongoing cell death in the avian inner ear. Utricles (*A, B*) or isolated sensory epithelia (*C*) were cultured for 24 hr with 0.1% DMSO (controls) or 10, 50, or 100 μM BAF. Fixed specimens then were processed for TUNEL labeling (*A*) or BrdU immunohistochemistry (*B, C*). TUNEL$^+$ or BrdU$^+$ cells were quantified in the extrastriolar regions in each organ and then were normalized to 25,000 μm^2. Results are the mean ± SEM for three experiments from seven to nine organs. Significantly fewer TUNEL$^+$ cells were present in BAF-treated specimens (*A*). In addition, correspondingly fewer BrdU$^+$ cells were present in BAF-treated cultures compared with controls (*B*). *C*, Plot of proliferation index (BrdU$^+$ cells/total cells) in a 10,000 μm^2 region, demonstrating that BAF does not affect supporting cell proliferation directly. Combined results from these experiments suggest a causative relationship between ongoing hair cell death and ongoing supporting cell proliferation.

an inhibitory signal that prevents progenitor cells from dividing and generating new neurons as long as the normal compliment of neurons is intact. Several studies support the former hypothesis by demonstrating an increase in progenitor cell proliferation after injury (for review, see Murray and Calof, 1999). No studies, however, have been able to rule out the latter mechanism completely.

Gustatory receptors also have a limited lifespan and must be replaced to maintain the structure of the sensory epithelium. In rodents, the sensory cells in taste buds are replaced every 9–14 d (Beidler and Smallman, 1965; Farbman, 1980; Delay et al., 1986; Zeng and Oakley, 1999). Because each taste bud contains ~50–75 cells, this turnover rate implies that approximately three to five cells are replaced each day. This estimate corresponds to the number of apoptotic cells that are observed under normal conditions in lingual taste buds (Takeda et al., 1996). A recent study suggested that gustatory cells die via a p53/Bax-dependent cell death pathway (Zeng and Oakley, 1999), but the precise signals that trigger the programmed death of cells in the adult gustatory sensory epithelium are not known.

Turnover of sensory receptors also has been demonstrated in certain hair cell epithelia. For instance, hair cell death has been reported in lateral line organs of various fish (Jørgensen, 1991), and a recent report has indicated that hair cells in the postembryonic zebrafish lateral line also turn over naturally (Williams and Holder, 2000). Significantly, those authors also noted that zebrafish neuromasts that were treated with zVAD displayed decreased hair cell death and decreased ongoing supporting cell division. No mechanism has been proposed as the hair cell death stimulus, but environmental stressors may cause the turnover of lateral line hair cells because they are situated on the animal's external surface and are exposed to water constantly.

Summary

The results of the present study, in agreement with previous data, demonstrate that cultured hair cells undergo apoptosis after exposure to aminoglycosides (Li et al., 1995; Forge and Li, 2000) and that aminoglycoside-induced hair cell death can be reduced by the inhibition of caspases and protein synthesis. It is not clear, however, whether caspase inhibitors also promote hair cell survival *in vivo* and whether the surviving hair cells are functional. Additional studies should also elucidate other aspects of the aminoglycoside-induced cell death pathway. Because caspases are used to mediate cell death, identifying which caspases are necessary for hair cell death may determine whether there is a single cell death pathway or whether there are multiple parallel cell death pathways.

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