

Critical Signaling Events during the Aminoglycoside-Induced Death of Sensory Hair Cells *In Vitro*

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ABSTRACT: Sensory hair cells undergo apoptosis following exposure to aminoglycoside antibiotics. In neurons, apoptosis is associated with a transient increase in intracellular Ca^{2+} , phosphorylation of the transcription factor c-Jun, and the release of cytochrome *c* from mitochondria into the cytosol, which along with other cofactors results in the activation of caspases. To examine the possible role of these events in the survival and death of the sensory receptors of the inner ear, we examined the effects of neomycin treatment on cytoplasmic calcium, activation of c-Jun-N-Terminal kinases (JNKs), cytochrome *c* release, and caspase-3 activation

in cultured vestibular hair cells. Increased numbers of phospho-c-Jun-labeled hair cells (a downstream indicator of JNK activation) were observed at 3–12 h after neomycin treatment, whereas increased numbers of cells with cytoplasmic cytochrome *c* were observed at 12–18 h following the onset of neomycin treatment. This was followed by an increase in the number of cells that contained activated caspase-3 and displayed pyknotic nuclei. Treatment with the general caspase inhibitor BAF did not affect the release of cytochrome *c* and the number of p-c-Jun-labeled cells, but reduced the number of cells with activated caspase-3 and pyknotic nuclei.

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In contrast, treatment with CEP-11004, an indirect inhibitor of the JNK signaling pathway, promoted hair cell survival following neomycin treatment and reduced the number of cells with phosphorylated JNK and c-Jun, cytoplasmic cytochrome *c*, and activated caspase 3. These results suggest that JNK activation occurs upstream of the release of cytochrome *c* and that cytochrome *c* release precedes caspase activation. Cytochrome *c* release and JNK activation were also preceded

by large changes in cytoplasmic calcium. Cytoplasmic calcium increases may be causally related to the release of cytochrome *c*, and may also be a potential pathway for activation of JNK in hair cells. © 2004 Wiley Periodicals, Inc. *J Neurobiol* 00: 000–000, 2004

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INTRODUCTION

Hair cells are mechanoreceptors within the inner ear that detect sound and head movement. In mammals, loss of these receptors through exposure to noise or treatment with ototoxic drugs can lead to permanent deficits in hearing and balance. Morphological evidence from many vertebrate species suggests that the loss of hair cells in response to treatment with aminoglycoside antibiotics occurs via apoptosis (Jørgensen, 1981, 1991; Forge, 1985; Li et al., 1995; Torchinsky et al., 1999; Forge and Li, 2000; Matsui et al., 2002).

Neuronal apoptosis is an evolutionarily conserved form of cell death that occurs through an orderly series of cellular events (e.g., Raff, 1998). The c-Jun–N-Terminal kinases (JNKs) are stress-activated protein kinases that have been implicated in neuronal cell death. Phosphorylation of JNKs leads to the activation of the transcription factor c-Jun (Virdee et al., 1997; Eilers et al., 1998), which can play a role in neuronal apoptosis (Estus et al., 1994; Ham et al., 1995). The JNKs are the only kinases known to activate c-Jun, and their activity is regulated through a sequential signaling cascade of upstream kinases including the mixed lineage kinases (MLKs). Several groups have demonstrated that synthetic indolocarbazole molecules (CEP-1347 and CEP-11004), inhibit MLKs and promote the survival of neurons in response to proapoptotic stimuli (Borasio et al., 1998; Maroney et al., 1998; Maroney et al., 1999; Murakata et al., 2002).

In some cases following JNK activation, the apoptotic signaling cascade converges at the mitochondria. Mitochondria are best known for their role in cellular metabolism, but also can release cytochrome *c* into the cytoplasm (Liu et al., 1996). Cytoplasmic cytochrome *c* with other cofactors then forms a multimeric complex called the apoptosome (Li et al., 1997; Zou et al., 1999), which activates caspases and initiates a proteolytic cascade that leads to the degradation of the cell's nuclear proteins. Recently, elevation of the cytoplasmic calcium concentration

($[Ca^{2+}]_{cyt}$) has been implicated in the release of cytochrome *c* (Schild et al., 2001).

Several studies have begun to elucidate the signaling pathways regulating aminoglycoside-induced hair cell death. Two studies have identified a role for the JNK signaling pathway in hair cell death (Pirvola et al., 2000; Ylikoski et al., 2002; Bodmer et al., 2002; Wang et al., 2003) and another study has identified a role for cytochrome *c* in the death pathway (Nakagawa and Yamane, 1999). General caspase inhibitors (e.g., BAF and zVAD) also promote hair cell survival following aminoglycoside treatment *in vitro* (Forge and Li, 2000; Cunningham et al., 2002; Matsui et al., 2002; Cheng et al., 2003) and *in vivo* (Matsui et al., 2003). Little is known, however, about the relative order of these events in relationship to other cell death events.

The primary objective of the present study was to characterize the temporal relationship of the phosphorylation of c-Jun, the translocation of cytochrome *c*, and the activation of caspase-3 in chick vestibular hair cells following treatment with neomycin. In addition, we monitored cells to detect the existence and time course of any neomycin-induced changes in $[Ca^{2+}]_{cyt}$. Preliminary reports of portions of these data were presented previously (Matsui and Warchol, 2001a, 2001b, 2003).

MATERIALS AND METHODS

Animals

White Leghorn chickens (*Gallus domesticus*) were obtained from Charles River SPAFAS (Charles River, CT) or Ideal Poultry Breeding Farms (Cameron, TX) and were housed in the Central Institute for the Deaf's animal care facility. The Central Institute for the Deaf Institutional Animal Care and Use Committee approved all experimental protocols and conform to the National Institutes of Health animal use guidelines.

Culture Techniques

Chicken Organ Cultures. Chickens (7–21 days posthatch) were euthanized with CO₂ 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 carried out 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 Corporation, Carlsbad, 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 μ L 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 Corporation). Explants were incubated at 37°C in a humidified 5% CO₂/95% air environment for 1 day *in vitro* (1 DIV), to allow the utricles to adjust to the culture environment. Cultures were then incubated under various conditions (see below) for another 1–24 h. In some experiments, utricles were thoroughly washed after 24 h of drug treatment and cultured in control medium for another 24 h.

Pharmacological Treatment of Organ Cultures. At the beginning of the second day *in vitro*, neomycin sulfate (Sigma Chemical Corporation, St. Louis, MO) was added to the culture medium, for a final dilution of 1 mM. Cultures containing aminoglycoside-free media but supplemented with 0.01% DMSO were maintained in parallel and served as controls. In experiments that examined caspase inhibition, Boc-Asp(Ome)-Fluoromethyl Ketone (BAF; Enzyme Systems Products, Livermore, CA) was added to the medium for a final concentration of 50 μ M. Caspase inhibitors were added to the cultures simultaneously with neomycin. Direct activation of the JNK-signaling pathway was achieved by culturing utricles in the presence of anisomycin (50 ng/mL; Calbiochem, San Diego, CA) for 3 h. The utricles were then thoroughly washed with control medium and cultured in control medium for another 21 h (total time 24 h *in vitro*). In experiments that examined JNK signaling inhibition, CEP-11004 (Cephalon, West Chester, PA) was added to the medium for a final concentration of 200, 400, 1600, or 4800 nM. CEP-11004 was added to the cultures 4 h before the addition of neomycin. The maximum final concentration of DMSO used 0.01%.

Cultures of Isolated Sensory Epithelia. To assess the direct effects of pharmacological agents on hair cells, sensory epithelia were isolated from chick utricles as described previously (Warchol, 1995, 2002). Utricles were removed from chicks, placed in sterile Medium-199, and the otoconia were removed. The utricles were then incubated in thermolysin (Sigma, 500 μ g/mL in Medium-199 with Earle's salts) for 1 h at 37°C in a humidified 5% CO₂/95% 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 membranes and associated connective tissue. Isolated sheets of sensory epithelia were transferred into fibronectin-coated culture wells (MatTek, Ashland, MA), containing 50 μ L of Medium-199/

10% FBS, cut into 8–10 pieces, and incubated for 3 days. The explants were then treated with 1 mM neomycin or control medium for another 24 h, fixed, and processed for immunohistochemistry. For experiments involving calcium imaging, utricles were removed from embryonic day 21 (E21) chicks and cultured for up to 7 days *in vitro*.

Tissue Processing

Cultured chick utricles and sensory epithelia were fixed with 4% paraformaldehyde for 15–20 min and then rinsed with PBS. Immunohistochemical steps were carried out at room temperature with thorough PBS washes between them, unless otherwise noted.

Primary Antibodies. Activated (cleaved) caspase-3 was detected using a rabbit polyclonal anticlaved caspase-3 antibody obtained from Cell Signaling Technology (Beverly, MA). Cytoplasmic cytochrome *c* was labeled with a mouse polyclonal anticytochrome *c* from Pharmingen (Putcha et al., 1999). Hair cells were labeled with either a rabbit polyclonal anticalretinin obtained from Chemicon (Temecula, CA) (Rogers, 1989) or HCS-1 antibody (Gale et al., 2000, from Dr. Jeffrey Corwin, University of Virginia). Cytochrome oxidase was labeled with an antibody obtained from Molecular Probes (Eugene, OR). A polyclonal anti-rabbit phospho-c-Jun (Ser63) was obtained from Cell Signaling Technology. Phosphorylated (activated) JNK was detected using a mouse monoclonal anti-p-JNK (G-7) obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Activated Caspase-3, Cytochrome *c*, and Cytochrome Oxidase. To determine whether cells within the sensory epithelia contained activated caspase-3 or had released cytochrome *c* or cytochrome oxidase, utricles were incubated in a blocking solution consisting of PBS, 2% normal goat serum (NGS; Sigma), 1% BSA, and 0.2% Triton X-100 for 2 h. The tissue was then immediately placed into a primary solution of either: (1) anticlaved caspase-3 (1:50) or (2) anticytochrome *c* (1:2000), or (3) anticytochrome oxidase (1:500) antibodies and incubated overnight at 4°C. The next day, specimens were incubated in Cy-3-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at room temperature.

Phospho-c-Jun and HCS-1 Staining. To determine the time-course of c-Jun phosphorylation, fixed utricles were placed in a blocking solution consisting of PBS, 2% normal goat serum (NGS; Sigma), 2% normal horse serum (NHS), 1% BSA, and 0.2% Triton X-100 for 2 h, and then immediately incubated in a primary antibody solution containing anti-phospho-c-Jun (1:400) and HCS-1 (1:500) overnight at 4°C. The tissue was incubated in biotinylated goat antirabbit IgG antibody (1:150, in PBS/0.1% NHS/0.1% Triton X-100; Vector Laboratories Inc., Burlingame, CA) for 2 h, followed by goat-antimouse Cy-3-conjugated secondary antibody (1:500, Amersham Pharmacia Biotech, Piscataway,

NJ) and streptavidin-conjugated Alexa 488 (1:500, Molecular Probes) for 2 h.

Phospho-JNK and Activated Caspase-3. To determine the time-course of phosphorylated-JNK and caspase-3 activation, utricles were incubated in a blocking solution (described above) for 2 h and then immediately placed into a primary antibody solution of p-JNK (1:100) and cleaved caspase-3 (1:50) in PBS/2% NHS/2% NGS and incubated overnight at 4°C. Utricles were then incubated in biotinylated goat antirabbit IgG antibody (see above) for 2 h, followed by a goat-antimouse Cy-3 secondary antibody and Alexa 488-conjugated streptavidin for 2 h.

Fluorescent Nucleic Acid Staining. Utricles prepared for fluorescent immunohistochemistry were also immersed in bisbenzimidazole (Hoechst 33258; 20 µg/mL; Sigma) in the dark for 15 min and mounted on glass slides in glycerol-PBS (9:1).

Calretinin Labeling. To quantitatively assess the extent of hair cell survival, hair cells were identified using an antibody for calretinin (Rogers, 1989). Fixed utricles were incubated in 90% methanol with 0.03% H₂O₂ for 20 min, followed by incubation in a blocking solution (described above) for 2 h. The tissue was then immediately placed into a rabbit anticalretinin primary antibody (1:2000, in PBS/2% NHS) and incubated overnight at 4°C. Utricles were then incubated in biotinylated goat antirabbit IgG for 2 h (see above), followed by avidin-biotin-horseradish peroxidase complex (Vector Laboratories) for 90 min. Specimens were reacted with DAB (Vector Laboratories) for 5 min. Alternatively, if the specimens were viewed under a fluorescent microscope, the utricles were incubated in biotinylated goat antirabbit IgG (as described above) for 2 h, followed by incubation in Alexa 488-conjugated streptavidin (1:500) for 2 h.

Data Analysis

All counts of labeled-cells were conducted blinded with respect to pharmacological treatment.

Counts of Activated Caspase 3, Cytochrome c, Phosphorylated c-Jun, and p-JNK-Labeled Cells. Each labeled cell in eight randomly selected extrastriolar regions/organ was counted within a specified area (26,896 µm² area) using a reticule and a 60× objective. The regions were averaged and normalized to 25,000 µm² to obtain an estimate of the number of labeled cells/25,000 µm² for the extrastriolar region of each explant.

Counts of fluorescent nucleic acid staining bisbenzimidazole-labeled cells were counted in whole-mount preparations of the utricular maculae using fluorescent optics with a DAPI filter (excitation 346 nm, emission 460 nm). Each pyknotic nucleus within the regions described above was counted, averaged, and then normalized to 25,000 µm².

Pyknotic nuclei were easily detected by their condensed chromatin (Matsui et al., 2002).

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 using a Cohu CCD camera. Cell counts were made directly from the video monitor 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 borders 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 µm² for the striolar or extrastriolar region of each specimen.

Intracellular Calcium Imaging. After 5–7 days in culture sensory epithelia were rinsed with Medium-199 with Earle's salts and then incubated in 3 µM Fluo3-AM (Molecular Probes) for 45 min at 37°C in a humidified 5% CO₂/95% air environment incubator. Epithelia were washed with Medium-199 with Hank's salts and placed in 37°C chamber in the dark for 15 min before experimentation. Using HCS-1 labeling we estimated the hair cell density in 5–7-day-old epithelial cultures to be ~6 per 10,000 µm². The field of view for these experiments had an area of 36,000 µm², giving an average of 22 hair cells per field. Neomycin was added to the extracellular bath solution, to give a final concentration of 1 mM. Excitation light (450 nm) from a monochromator (Kinetic Imaging, UK) was coupled to the excitation port of an Axiovert TV100 microscope using a light guide and a Uniblitz shutter (Vincent Associates, Rochester, NY). Fluorescence emission at 520 nm was measured using a cooled CCD camera (Sensicam, PCO, Germany). Images were captured at 2-min intervals over a 6- to 7-h period. Regions of interest (ROI) were drawn around cells that responded to neomycin addition and mean fluorescence intensities in the regions of interest measured at all time points. To normalize data for analysis, fluorescence signals were computed as $(F - F_o)/F_o$, where F is the mean fluorescence intensity in the ROI and F_o is the fluorescence value in the ROI prior to addition of neomycin. The computed value $(F - F_o)/F_o$ is assumed to be proportional to the cytoplasmic free calcium concentration.

Statistical Analysis

All means shown in the figures are expressed as means ± S.E.; data given in text are means ± S.D. Data from fluorescent antibodies, hair cell counts, and bisbenzimidazole labeling experiments were subjected to either an unpaired two-sample *t*-test assuming unequal variances using Microsoft Excel 98 (Microsoft Corporation, Redmond, WA),

or a one-way analysis of variance using SigmaStat (Jandel Scientific Software Corp., Chicago, IL). Post hoc comparisons, when appropriate, used the Tukey-Kramer, Dunnett's, or Sheffe's test.

RESULTS

Caspase-3 Is Activated during Hair Cell Death

Caspase activation results in the activation of nucleases and the cleavage of nuclear structural proteins, leading to cell death. Aminoglycoside antibiotics, such as neomycin, selectively kill hair cells in cultures of the avian inner ear (Stone et al., 1996; Hirose et al., 1997; Kil et al., 1997; Warchol, 1999; Matsui et al., 2000; Matsui et al., 2002). Caspase inhibitors (e.g., Boc-Asp(Ome)-Fluoromethyl Ketone, BAF) can prevent neomycin-induced death in hair cells (Matsui et al., 2002). Because previous data have indicated that caspases were involved in aminoglycoside-mediated hair cell death (Forge and Li, 2000; Cunningham et al., 2002; Matsui et al., 2002; Cheng et al., 2003), we investigated whether caspase-3 was activated in dying vestibular hair cells. Utricles were cultured for various times in either: (1) 1 mM neomycin, (2) 1 mM neomycin/50 μ M BAF (general caspase inhibitor), or (3) control medium. Few activated caspase-3-labeled cells [Fig. 1(A)] were observed in control cultures; however, in neomycin-treated cultures, there was a marked increase in activated caspase-3-labeling, beginning about 12 h after neomycin was added to the culture medium [Fig. 1(D)]. The number of activated caspase-3-positive cells increased by fourfold following 12 h of neomycin treatment and by 7.5-fold following 18–24 h of neomycin treatment ($p < 0.001$). To determine whether caspase-3 activation was a transient event, utricles were cultured for 24 h in 1 mM neomycin, thoroughly washed, and cultured for an additional 24 h in neomycin-free (control) medium. At 24 h after neomycin treatment, the number of cells that were positive for activated caspase-3 returned to near control levels ($p = 0.9$). In contrast, cultures that had received both neomycin and BAF contained significantly fewer cells with activated caspase-3 ($p < 0.01$) after 18 h of neomycin treatment and similar densities of activated caspase-3-labeled cells were obtained from all specimens following 24 h of neomycin treatment ($p = 0.5$). These data suggest that aminoglycoside-induced vestibular hair cell death involves caspase-3 activation.

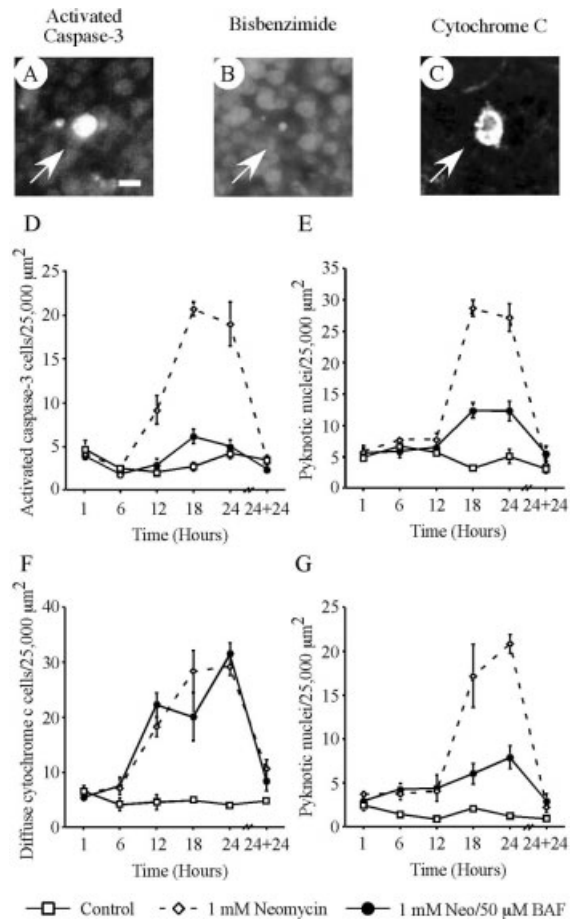


Figure 1 Aminoglycoside treatment results in activation of caspase-3, release of cytochrome *c*, and changes in nuclear morphology. Chick utricles were cultured for various times in either: (1) 1 mM neomycin, (2) 1 mM neomycin/50 μ M BAF (a general caspase inhibitor), or (3) control medium. Specimens were fixed and processed to visualize activated caspase-3, cytochrome *c*, and nuclear chromatin. Immunoreactivity for activated caspase-3 (A) and cytoplasmic cytochrome *c* (C) was observed in cells in the sensory region of the utricle. Dying cells had irregularly shaped and pyknotic nuclei, while healthy cells had nuclei that were diffuse and uniformly stained (B). Cells with cytoplasmic cytochrome *c*, activated caspase-3-positive cells, and pyknotic nuclei were normalized to 25,000- μ m² regions throughout the extrastrial region of the sensory epithelium (eight regions/organ). More cells with activated caspase-3 were observed after 12 h of neomycin treatment, while treatment with the general caspase inhibitor BAF significantly reduced the number of cells with activated caspase-3 (D) and pyknotic nuclei (E). The number of cells that had cytoplasmic cytochrome *c* staining increased over time in both neomycin-treated and neomycin/BAF-treated cultures (F); treatment with BAF did not prevent the release of cytochrome *c* into the cytoplasm. Significantly more pyknotic nuclei were observed in neomycin-treated cultures as opposed to BAF/neomycin and control cultures (E and G). Results are mean (\pm S.E.M.) for three experiments from 8–13 organs. Data points may obscure error bars. Scale bar = 10 μ m.

Changes in Nuclear Morphology Parallel Caspase-3 Activation

One of the hallmarks of apoptosis is a morphological change in the cell's nucleus, including nuclear condensation and fractionation (Wyllie et al., 1980). To examine morphological changes in hair cell nuclei following neomycin treatment, utricles that were examined in the previous series of experiments were also costained with the DNA-binding dye, bisbenzimidide [Fig. 1(B)]. The numbers of pyknotic nuclei were quantified from the same microscopic fields that were used to quantify caspase-3 activation. Nuclei of cells in control utricles appeared oval and homogeneously stained with moderate intensity [Fig. 1(B)]. Few pyknotic nuclei were observed in control cultures (data not shown). In contrast, following 18–24 h of neomycin treatment, many nuclei were intensely stained, branched, and irregularly shaped, indicating substantial structural changes in nuclear chromatin (Matsui et al., 2002). Quantitative data indicate that there was a five- to sevenfold increase in the number of pyknotic nuclei when compared to control cultures [$p < 0.001$; Fig. 1(E)]. Many cells that were labeled for activated caspase-3 [Fig. 1(A)] also had pyknotic nuclei [Fig. 1(B)], and the increase in the density of pyknotic nuclei corresponded with the increase in the number of activated caspase-3-labeled cells [Figs. 1(D,E)]. Simultaneous addition of BAF with neomycin significantly reduced the number of pyknotic nuclei ($p < 0.001$), but did not completely block cell death. There was a 2.5–3.5-fold increase in the number of pyknotic nuclei in BAF–neomycin-treated cultures, when compared to controls. This observation is consistent with previously reported data (Matsui et al., 2002).

Neomycin-Treated Hair Cells Release Cytochrome *c* into the Cytoplasm

Recent studies of NGF-deprived sympathetic neurons have shown that cytochrome *c* release from mitochondria results in the activation of caspases, particularly caspase-3 (Srinivasan et al., 1998; Putcha et al., 1999). A recent study reported that caspase-9, an upstream caspase that is often associated with the mitochondrial-mediated cell death pathway, was activated in mammalian vestibular hair cells following neomycin treatment (Cunningham et al., 2002). To determine if cytochrome *c* is released in vestibular hair cells after ototoxic insult, utricles were cultured in 1 mM neomycin for various times using the conditions described above. Specimens were immunoreacted to detect diffuse cytoplasmic cytochrome *c*

staining [Fig. 1(C)] as opposed to the normal punctate staining pattern, which would indicate that the cytochrome *c* is still within the mitochondria (data not shown). Following 6 h of neomycin treatment, few cells had cytoplasmic cytochrome *c*, but that number increased by fourfold following 12 h of neomycin treatment and by nearly sixfold following 18 h of neomycin treatment [Fig. 1(F)]. To determine whether the release of cytochrome *c* from the mitochondria was a transient event following neomycin treatment, utricles were cultured for 24 h in 1 mM neomycin, thoroughly washed, and maintained in control medium for an additional 24 h *in vitro*. The number of cells with cytoplasmic cytochrome *c* decreased by 65% relative to utricles fixed immediately after neomycin treatment [Fig. 1(F)]. Similar increases and decreases in the number of cells with cytoplasmic cytochrome *c* were observed in BAF/neomycin-treated cultures, indicating that caspase inhibition had no effect upon the release of cytochrome *c*. Few cells in control cultures had cytoplasmic cytochrome *c* at any time point ($p > 0.5$).

The utricles that were immunoreacted to detect cytochrome *c* were also stained with bisbenzimidide. After the cytoplasmic cytochrome *c* cells were quantified, the same field was used to quantify pyknotic nuclei. Significantly more pyknotic nuclei were found in neomycin-treated cultures, when compared to control cultures [$p < 0.001$; Fig. 1(G)]. Although BAF treatment had no effect upon cytochrome *c* release in neomycin-treated cultures, simultaneous application of BAF resulted in significantly fewer cells with pyknotic nuclei ($p < 0.001$). The increase in pyknotic nuclei occurred about 6 h after the release of cytochrome *c*, indicating that the release of cytochrome *c* precedes changes in nuclear morphology.

An additional set of experiments was conducted in order to confirm that the cells with cytoplasmic cytochrome *c* were hair cells. Isolated sensory epithelia, which contain a relatively pure population of hair cells and supporting cells, were cultured for 24 h with either 1 mM neomycin or control media (Warchol, 1995, 2002). Specimens were processed to detect calretinin (to identify hair cells) [Fig. 2(A,C)] and cytochrome *c* [Fig. 2(B,C)], and visualized using a confocal microscope. About twice as many cells in neomycin-treated cultures had cytoplasmic cytochrome *c*, when compared to control cultures [Fig. 2(D)]. Nearly all of the cells that had contained cytoplasmic cytochrome *c* were also calretinin positive, indicating that dying hair cells release cytochrome *c* from mitochondria.

To confirm the integrity of the mitochondria following the release of the cytochrome *c* into the cyto-

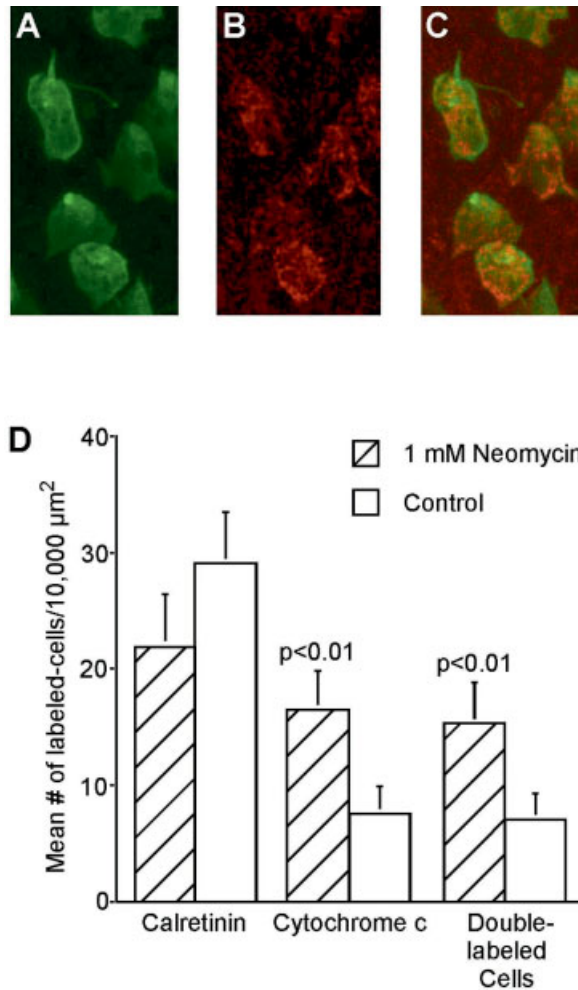


Figure 2 Cytochrome *c* release is mainly restricted to hair cells. Isolated sensory epithelia were cultured for 24 h with either 1 mM neomycin or control medium. Specimens were then processed to detect calretinin (A,C) and cytochrome *c* (B,C). Confocal images of regions of high hair cell density were obtained. All single- and double-labeled cells were counted within a specified area ($16,641 \mu\text{m}^2$) and then normalized (D). Significantly more cells in neomycin-treated cultures had cytoplasmic cytochrome *c*, when compared to controls. Nearly all of the cells that contained cytoplasmic cytochrome *c* were also calretinin positive, indicating that those cells were hair cells. Results are mean (\pm S.E.M.). Images were obtained from two to three regions per explant. A total of eight explants per culture condition were used.

plasm, we examined the distribution of cytochrome oxidase following neomycin treatment. Cytochrome oxidase is a membrane bound enzyme found in the inner membrane of the mitochondria, which catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen. Unlike cytochrome *c*, cytochrome oxidase is not released from mitochondria during the

early stages of apoptosis (Putcha et al., 2000). Utricles were cultured for either 12 or 24 h in: (1) 1 mM neomycin, (2) 1 mM neomycin and $50 \mu\text{M}$ BAF, or (3) control medium, and were then immunolabeled for both cytochrome *c* and cytochrome oxidase. Following 12 h of neomycin treatment, there was a significant rise ($p < 0.01$) in the number of cells with cytoplasmic cytochrome *c*/ $25,000 \mu\text{m}^2$ in both neomycin-treated cultures (16.0 ± 6.7) and neomycin/BAF-treated cultures (15.0 ± 5.7), when compared to control cultures (5.2 ± 1.1). After 24 h of treatment there were even more cells with cytoplasmic cytochrome *c* in neomycin (19.5 ± 6.0) and neomycin/BAF-treated cultures (19.7 ± 6.5), when compared to control cultures (4.8 ± 2.2). In contrast, very few cells at either time point had diffuse cytoplasmic cytochrome oxidase staining, but rather maintained their normal punctate staining (range 0.09 – 0.35). This indicated that, although cytochrome *c* was released from the mitochondria, there was almost no exposure of the inner mitochondrial membrane to the intracellular environment.

Time Course of Phosphorylation of c-Jun following Aminoglycoside Treatment

c-Jun is a component of the AP-1 transcription factor. Extracellular signals including growth factors and other stimuli can cause the phosphorylation of serine residues 63/73 of c-Jun and activate c-Jun dependent transcription. Previous studies have implicated the c-Jun N-Terminal kinase (JNK) pathway in aminoglycoside-induced hair cell death (Pirvola et al., 2000; Ylikoski et al., 2002). To more precisely determine when c-Jun is phosphorylated relative to caspase activation, utricles were cultured for various time points in either: (1) 1 mM neomycin, (2) 1 mM neomycin and $50 \mu\text{M}$ BAF (a general caspase inhibitor), or (3) control medium. The utricles were then fixed, and processed for immunohistochemistry using antibodies that specifically recognize c-Jun that is phosphorylated at Serine residue 63 [Fig. 3(A)] and hair cells [HCS-1, Fig. 3(B)]. Nuclei were also costained with bisbenzimidazole [Fig. 3(C)]. Little phospho-c-Jun labeling was observed in control cultures; however, in neomycin- or neomycin/BAF-treated cultures there was a marked increase in nuclear phospho-c-Jun staining, beginning at approximately 3 h after neomycin was added to the culture medium [Fig. 3(A)] and colocalized to hair cells [Fig. 3(B)]. By 12 h, there was a fivefold increase in the number of phospho-c-Jun-positive cells in neomycin-treated cultures when compared to controls [Fig. 3(D)]. Treatment with BAF did not reduce the number of phospho-c-Jun

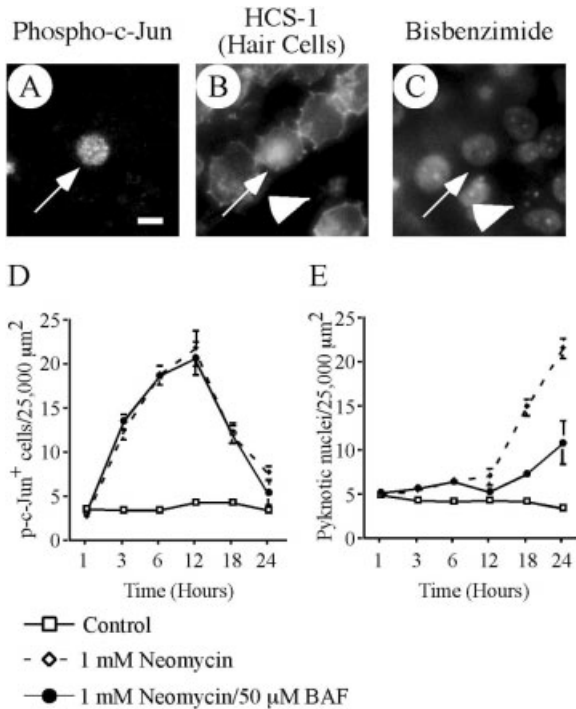


Figure 3 Aminoglycoside treatment results in the phosphorylation of c-Jun and changes in nuclear morphology. Chick utricles were cultured for various times in either: (1) 1 mM neomycin, (2) 1 mM neomycin/50 μM BAF (a general caspase inhibitor), or (3) control medium. Specimens were fixed and immunohistochemically processed to detect (A) phosphorylated c-Jun, (B) hair cells (HCS-1 antibody), and (C) nuclear chromatin (bisbenzamide). Phosphorylated c-Jun-labeled cells and pyknotic nuclei were quantified from eight regions/organ throughout the extrastriolar region of the sensory epithelium. Results are given as mean \pm S.E.M. for three experiments (10–13 organs). Data point may obscure error bar. (A,B) c-Jun phosphorylation was colocalized to hair cells in the sensory epithelia (arrow). (C) Dying cells in neomycin-treated cultures had irregularly shaped and pyknotic nuclei (arrowhead), while nuclei in healthy cells were diffuse and uniformly stained. (D,E) More cells labeled with phosphorylated c-Jun were observed in neomycin treated cultures, beginning 3 h after application of neomycin. The number of phospho-c-Jun-labeled cells peaked at 12 h and returned to near control levels by 24 h. This time course correlated with a significant increase in the number of pyknotic nuclei at 18 h in neomycin-treated utricles (E). Treatment with the general caspase inhibitor BAF significantly reduced the number of pyknotic nuclei in neomycin-treated cultures ($p < 0.001$).

positive cells. In both experimental conditions, the number of cells that were phospho-c-Jun-positive peaked at 12 h following the addition of neomycin and returned to near control levels by 24 h [Fig. 3(D)].

Few pyknotic nuclei were observed between 1–12 h after the addition of neomycin, but there was a

significant rise in the number of pyknotic nuclei at 18 and 24 h in neomycin-treated cultures [$p < 0.001$; Fig. 3(E)]. At 24 h after the addition of neomycin to the cultures, there was a fivefold increase in the number of pyknotic nuclei when compared to control cultures.

Anisomycin Activates the JNK Pathway in Hair Cells

The bacterial compound anisomycin activates the MEKK/MKK/JNK pathway at the level of, or above, MEKK (Kawasaki et al., 1996; Meier et al., 1996). To examine the direct effects of JNK activation on hair cell survival, utricles were treated with 50 ng/mL anisomycin for 3 h, and then either immediately fixed or thoroughly washed with control medium, and cultured for another 21 h in control medium (total 24 h *in vitro*). The utricles that were fixed at 3 h were processed for phospho-c-Jun immunohistochemistry and costained with bisbenzamide. Specimens treated for 3 h with anisomycin contained nearly three times as many c-Jun-positive cells/25,000 μm² (11.7 ± 3.7), as did controls (4.3 ± 0.5). Most cells that were positive for p-c-Jun were also colabeled with HCS-1, indicating that these cells were hair cells.

In an additional experiment, utricles that were cultured for an additional 21 h after anisomycin treatment were fixed and processed for calretinin immunohistochemistry. 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. There was about a 40% reduction in the number of hair cells in both sensory regions of the utricle in anisomycin-treated cultures, when compared to controls ($p < 0.001$). This reduction in hair cell density was similar to that observed in utricles after treatment with the aminoglycoside antibiotic neomycin (1 mM) for 24 h (Matsui et al., 2002) suggesting that JNK activation is sufficient for hair cell death.

To determine whether there was a synergistic effect between neomycin and anisomycin, utricles were cultured for 3 h in either: (1) control medium, (2) 1 mM neomycin, (3) 50 ng/mL anisomycin, or (4) 1 mM neomycin and 50 ng/mL anisomycin. After 3 h of pharmacological treatment, the utricles were thoroughly rinsed with control medium and then cultured in control medium or medium containing 1 mM neomycin (if the medium originally contained neomycin) for an additional 21 h (total 24 h neomycin treatment). The utricles were then fixed and processed for calretinin immunohistochemistry. There were significantly fewer hair cells in

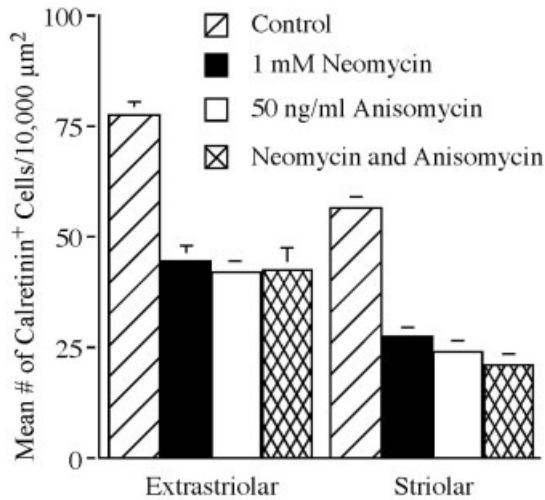


Figure 4 Treatment with anisomycin activates the JNK signaling pathway and kills hair cells. Utricles were cultured for 3 h in either: (1) control medium, (2) 1 mM neomycin, (3) 50 ng/mL anisomycin, or (4) 1 mM neomycin and 50 ng/mL anisomycin. After 3 h of pharmacological treatment, the utricles were thoroughly rinsed with control medium and then cultured in control medium or in medium containing 1 mM neomycin (if the medium originally contained neomycin) for an additional 21 h (for a total of 24 h treatment). The utricles were fixed and processed for calretinin immunohistochemistry, and calretinin-positive cells (hair cells) were quantified from 10,000 μm^2 regions of both the extrastriar (six regions/organ) and striar (four regions/organ) areas. Significantly fewer hair cells were present in both regions of neomycin-, anisomycin-, and neomycin/anisomycin-treated cultures, compared to control cultures ($p < 0.001$). There was no significant difference in hair cell densities between the three experimental conditions ($p > 0.5$). Results are the mean (\pm S.E.M.) for three experiments from seven to eight organs.

both the extrastriar and striar sensory regions in all three pharmacological treatments when compared to controls ($p < 0.01$; Fig. 4). Moreover, there was no significant difference ($p > 0.5$) in the percentage of hair cells lost in any of the three pharmacological treatments, indicating that there was no synergistic effect between neomycin and anisomycin. This suggests that both pharmacological agents use the same signaling pathway to trigger hair cell death.

CEP-11004 Promotes Hair Cell Survival from Treatment with Neomycin

Recent studies have shown that the indolocarbazole molecule CEP-11004, a mixed-lineage kinase (MLK) inhibitor and indirect inhibitor of JNK activation, can promote neuronal survival (Murakata et al., 2002). To

determine whether CEP-11004 could promote hair cell survival, chick utricles were cultured for 24 h in either: (1) control medium, (2) 1 mM neomycin, (3) 1 mM neomycin and 1600 nM CEP-11004, or (4) 1600 nM CEP-11004. The utricles were fixed and processed for calretinin immunohistochemistry [Figs. 5(A–C)]. Baseline hair cell densities were obtained from utricles cultured in control media [Fig. 5(A,D)]. Hair cell density was greater in the extrastriar region ($75.0 \pm 24.4/10,000 \mu\text{m}^2$; $n = 8$) than in the striar region ($46.6 \pm 13.5/10,000 \mu\text{m}^2$; $n = 8$). These densities were in agreement with a previously published report (Matsui et al., 2002). Treatment with 1 mM neomycin resulted in about a 48% decrease of hair cells in the extrastriar region and 21.4% decrease in the striar region when compared to untreated controls [$p < 0.001$; Fig. 5(B,D)]. Simultaneous addition of 1600 nM CEP-11004 promoted hair cell survival in both sensory regions [Fig. 5(D)]. Treatment with CEP-11004 resulted in about 95% hair cell survival in the extrastriar region and 79% hair cell survival in the striar region, when compared to controls. Almost twice as many hair cells were present in the extrastriar and striar regions of CEP-11004/neomycin-treated cultures than in utricles cultured with neomycin alone ($p < 0.001$).

To establish a dose–response relationship, utricles were cultured for 24 h with 1 mM neomycin and varying concentrations of CEP-11004 (200–4800 nM) or 0.01% DMSO (vehicle). Specimens were then fixed and processed for calretinin-immunohistochemistry and hair cell densities were obtained from the extrastriar [Fig. 5(E)] and striar regions [Fig. 5(F)]. Hair cell densities in both sensory regions of control cultures and neomycin-alone-treated cultures were similar to those described above (data not shown). Low concentrations of CEP-11004 (200 nM) provided no protection in either the extrastriar or striar regions. Increasing the concentration of CEP-11004 to 400 nM increased hair cell survival by 139% in the extrastriar region and by 266% in the striar region when compared to neomycin-treated cultures ($p < 0.001$). Maximal protection was observed using 1600 nM CEP-11004, which yielded similar densities as those shown in Figure 5(D). High concentrations of CEP-11004 (4800 nM) had no protective effects on hair cell survival in either sensory region. Instead, hair cell densities in cultures treated with 4800 nM CEP-11004 were similar to those in neomycin-treated cultures ($p > 0.5$).

Finally, to determine whether CEP-11004 was toxic to hair cells, some utricles were cultured with either 1600 or 4800 nM CEP-11004 (without neomycin) for 24 h. There was no significant difference in

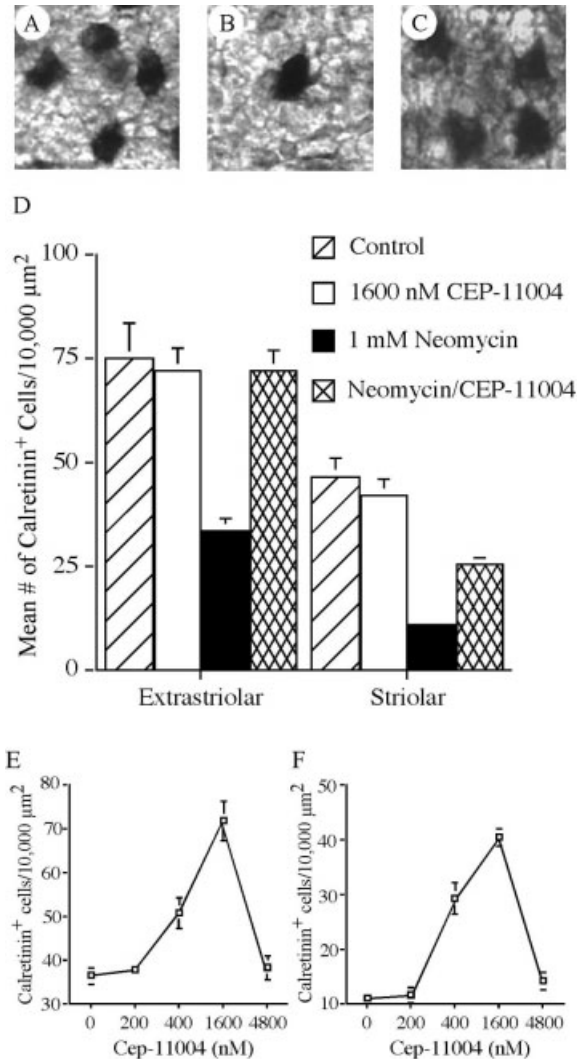


Figure 5 CEP-11004 treatment promotes hair cell survival in the presence of neomycin. Utricles were cultured for 24 h with (A) 0.01% DMSO, (B) 1 mM neomycin and 0.01% DMSO, (C) 1600 nM CEP-11004 and 1 mM neomycin, or 1600 nM CEP-11004. Cultured utricles were fixed and processed for immunohistochemistry using an antibody to calretinin, to label surviving hair cells [dark stain in (A–C)]. Calretinin-positive cells were quantified from 10,000 μm^2 regions of both the extrastriolar (six regions/organ) and striolar (four regions/organ) areas (D). Enhanced numbers of hair cells were present in both sensory regions of CEP-treated cultures, compared to cultures that received neomycin alone. To establish a dose-response curve for CEP-11004-mediated protection, utricles were cultured for 24 h with 1 mM neomycin and either 0, 200, 400, 1600, or 4800 nM CEP-11004, and then processed for calretinin immunohistochemistry. Increasing concentrations of CEP-11004 promoted hair cell survival, with maximum protection at 1600 nM in both the extrastriolar (E) and striolar regions (F). A higher concentration of CEP-11004 (4800 nM) had no protective effect in either sensory region. Results are the mean (\pm S.E.M.) for three experiments from 10–13 organs. Scale bar = 10 μm .

hair cell density between 1600 nM CEP-11004-treated cultures and control cultures, indicating that short-term exposure to 1600 nM CEP-11004 is not ototoxic [$p > 0.5$; Fig. 5(D)]. In contrast, treatment with 4800 nM CEP-11004 resulted in a significant decrease in the number of hair cells in both the extrastriolar region ($46.6 \pm 8.6/10,000 \mu\text{m}^2$) and striolar region ($32.1 \pm 11.3/10,000 \mu\text{m}^2$), when compared to control cultures ($88.1 \pm 11.8/10,000 \mu\text{m}^2$ and $64.0 \pm 7.9/10,000 \mu\text{m}^2$, respectively).

CEP-11004 Reduces JNK-Phosphorylation, c-Jun Phosphorylation, and Downstream Cell Death Signaling Pathways in Neomycin-Treated Cultures

CEP-11004 has been shown to be an indirect inhibitor of JNK activation (Murakata et al., 2002). Therefore, to determine whether CEP-11004 affected JNK activation, utricles were cultured for either 12 or 24 h in: (1) 1 mM neomycin, (2) 1 mM neomycin and 1600 nM CEP-11004, (3) 1600 nM CEP-11004, or (4) control medium. Utricles were processed for immunohistochemistry using an antibody that recognizes the activated (phosphorylated) form of JNK (data not shown). Following neomycin treatment, significantly more cells were immunopositive for activated JNK at both 12 and 24 h, compared to control cultures or cultures treated with neomycin and CEP-11004 [$p < 0.001$; Fig. 6(A)].

Once activated, JNK phosphorylates several transcription factors including c-Jun. To determine whether CEP-11004 inhibited phosphorylation of c-Jun, other utricles were fixed and processed for immunohistochemistry using an antibody that recognizes c-Jun that is phosphorylated at Serine residue 63. After both 12 and 24 h of treatment with neomycin, there was a significant increase in the number of cells that were immunoreactive for phosphorylated c-Jun in the neomycin-treated cultures, compared to either the control or CEP/neomycin-treated cultures [$p < 0.001$; Fig. 6(B)]. Similar densities of phosphorylated c-Jun-positive cells were identified in control cultures and CEP-11004/neomycin-treated cultures at both time points ($p = 0.12$ and 0.2 , respectively), indicating that CEP-11004 reduced the presence of phosphorylated c-Jun.

To determine whether the phosphorylation of c-Jun occurred upstream of the release of cytochrome *c*, the utricles used to quantify phospho-c-Jun were also immunolabeled to detect cytochrome *c*. After the phospho-c-Jun-labeled cells were quantified, the numbers of cells with cytoplasmic cytochrome *c* were quantified from the same microscopic fields. After

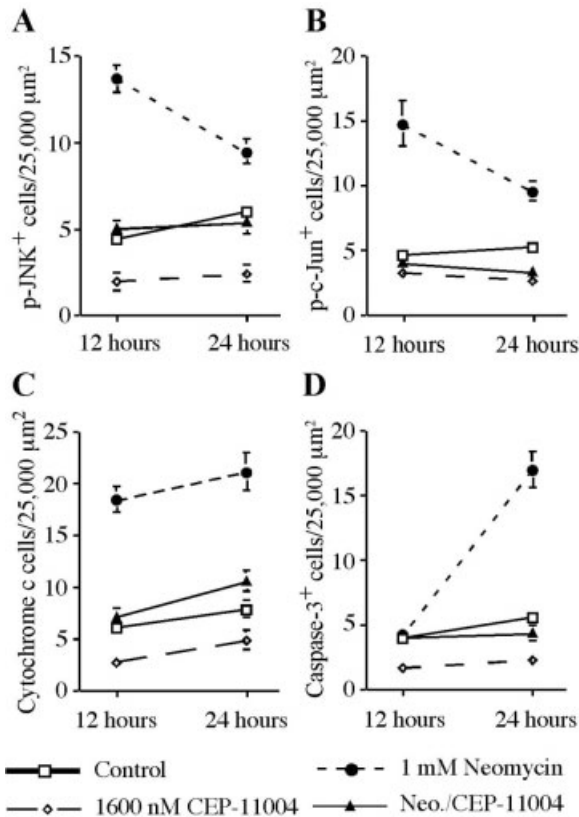


Figure 6 Effects of CEP-11004 on levels of p-JNK, p-c-Jun, cytochrome *c*, and activated caspase-3, following neomycin treatment. Utricles were cultured for either 12 or 24 h with 0.01% DMSO, 1600 nM CEP-11004, 1 mM neomycin, or 1600 nM CEP-11004 and 1 mM neomycin. Plots show the densities of cells that were immunopositive for (A) phosphorylated-JNK, (B) phosphorylated c-Jun, (C) cytoplasmic cytochrome *c*, and (D) activated caspase-3. Treatment with CEP-11004 significantly reduced the numbers of cells that were immunoreactive for all of these markers after exposure to neomycin ($p < 0.001$). Labeled cells were quantified from eight regions distributed throughout the extrastricular portion of each specimen. Results are mean \pm S.E.M. for three experiments from 7–12 organs. Data points may obscure error bars.

12 h of treatment, there was a 2.5-fold increase in the number of cells with cytoplasmic cytochrome *c* in the neomycin-treated cultures, compared to either the control or CEP/neomycin-treated cultures [$p < 0.001$; Fig. 6(C)]. Strikingly, similar densities of cells with cytoplasmic cytochrome *c* were identified in both control cultures and CEP-11004/neomycin-treated cultures at both time points ($p > 0.5$). Therefore, the phosphorylation of c-Jun occurs upstream of the release of cytochrome *c*. Activation of caspases results in the activation of nucleases and the cleavage of nuclear structural proteins that lead to cell death.

Although treatment with BAF did not prevent the activation of c-Jun in neomycin-treated cultures [Fig. 3(D)], BAF treatment reduced the number of pyknotic nuclei in the neomycin/BAF-treated cultures [Fig. 3(E)], indicating that caspases play a role in mediating hair cell death. Other studies have also indicated that caspase-3, a downstream caspase, mediates hair cell death (Matsui and Warchol, 2001b; Cunningham et al., 2002; Cheng et al., 2003). To determine whether caspase-3 activation occurred upstream or downstream of JNK activation, utricles that were processed for activated-JNK immunohistochemistry were also colabeled with an antibody for the activated (cleaved) form of caspase-3. After the cells that were p-JNK-positive were quantified, the number of cells with activated caspase-3 in the same field was also quantified [Fig. 6(D)]. Treatment with 1600 nM CEP-11004, in the presence of neomycin, reduced the number of activated-caspase-3-positive cells to control levels at 24 h ($p = 0.4$). Therefore, these data indicate that JNK activation occurs upstream of caspase-3 activation.

To determine whether blocking the MLK pathway affected known downstream cell death processes, the morphology of hair cell nuclei was examined using the DNA labeling dye bisbenzimidazole. Utricles that were processed for p-c-Jun immunohistochemistry were also costained with bisbenzimidazole. After the phospho c-Jun-positive cells were quantified, the number of pyknotic nuclei in the same field was also counted. More pyknotic nuclei/25,000 μm^2 were found in neomycin-treated cultures at both 12 and 24 h (6.5 ± 1.0 , 22.5 ± 2.5), when compared to CEP-11004 (2.9 ± 1.2 , 3.3 ± 1.2) and control cultures (4.4 ± 1.8 , 4.8 ± 0.8) at both time points. Similar densities of pyknotic nuclei were identified in control cultures and 1600 nM CEP-11004/neomycin-treated cultures (control: 5.3 ± 1.2 , CEP-11004: 7.7 ± 2.1).

CEP-11004 Inhibits Spontaneous Hair Cell Death

Hair cells in the mature avian vestibular system die spontaneously and are continuously replaced (Jørgensen and Mathiesen, 1988; Roberson et al., 1992; Kil et al., 1997; Wilkins et al., 1999; Matsui et al., 2002). Recent evidence indicates that ongoing hair cell death leads to supporting cell proliferation (Matsui et al., 2002). To investigate the mechanisms regulating ongoing cell death, utricles were cultured for 12 or 24 h in the presence or absence of 1600 nM CEP-11004. After 24 h, significantly fewer cells were immunopositive for phosphorylated c-Jun and p-JNK

in the CEP-11004-treated cultures, compared to control cultures [$p < 0.01$; Fig. 6(A,B)]. Also, there were fewer pyknotic nuclei in CEP-11004-treated cultures ($2.9 \pm 1.2/25,000 \mu\text{m}^2$, $3.3 \pm 1.2/25,000 \mu\text{m}^2$) when compared to control cultures ($4.4 \pm 1.8/25,000 \mu\text{m}^2$, $4.8 \pm 0.8/25,000 \mu\text{m}^2$) at both time points ($p < 0.05$). In addition, treatment with 1600 nM CEP-11004 reduced the number of cells with cytoplasmic cytochrome *c* [Fig. 6(C)] and activated caspase-3 [Fig. 6(D)] by 50% ($p < 0.001$). These results indicate that ongoing cell death is regulated, in part, by the phosphorylation of c-Jun and activation of caspase-3.

Intracellular Calcium Changes Precede Activation of Caspase-3 after Neomycin Treatment *In Vitro*

Intracellular calcium overload is known to act as an apoptotic trigger (Berridge et al., 2000). To determine whether increased intracellular calcium might be an upstream signal for the activation of caspase-3, we examined changes in $[\text{Ca}^{2+}]_i$ following neomycin treatment. Intracellular calcium was measured using Fluo3, a fluorescent indicator that emits with greater intensity when the concentration of free calcium increases. Neomycin (1 mM) was added to cultured sensory epithelia and $[\text{Ca}^{2+}]_i$ measured over the next 6–7 h. Results from five different experiments showed that a total of 28 cells responded to incubation in 1 mM neomycin with substantial increases in $[\text{Ca}^{2+}]_i$ (Fig. 7). This corresponds to approximately 30% of the total number of hair cells in the field of view (see Materials and Methods). The average time to the peak of the $[\text{Ca}^{2+}]_i$ response was 242 ± 87 min. The duration of the calcium “rise” was variable, lasting between 30 and 120 min. In addition to the large rise in $[\text{Ca}^{2+}]_i$, a slower and less substantial increase in $[\text{Ca}^{2+}]_i$ was noticeable over the first 180 min of neomycin exposure, in agreement with an earlier study (Hirose et al., 1999). We also monitored $[\text{Ca}^{2+}]_i$ in three control (no neomycin) cultures for 5 h. During this time, only one cell exhibited a calcium increase and this was much shorter in duration than the neomycin-induced response.

DISCUSSION

Results presented here demonstrate that neomycin treatment leads to activation of the JNK signaling pathway and release of cytochrome *c* from mitochondria into the hair cell cytoplasm. These events are followed by the activation of caspase-3 and the formation of pyknotic nuclei. The general caspase inhib-

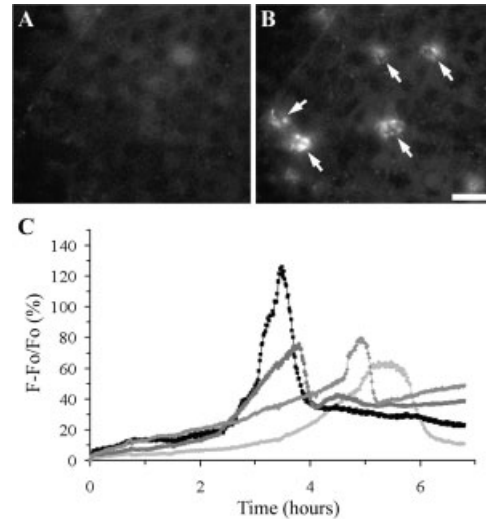


Figure 7 Changes in intracellular calcium levels in hair cells following treatment with neomycin. Cultured sensory epithelia from chick utricles were loaded with Fluo3-AM and then exposed to 1 mM neomycin. (A) Still images from an experiment showing Fluo3 emission at the beginning (A) and 300 min after (B) the addition of 1 mM neomycin to the medium. Some cells (B, arrows) show significant increases in intensity, which indicate changes in $[\text{Ca}^{2+}]_i$. (C) Quantitative data from another experiment showing the changes in intracellular calcium in four different cells. Intensity was measured using a ROI drawn around individual cells, and intensity measurements were calculated as percentage changes from the intensity at time zero. The time at which the $[\text{Ca}^{2+}]_i$ reached a maximum was measured for each cell that showed an increase of $>10 \times$ S.D. (standard deviation of the signal observed during the initial 20 min of recording). Increased in $[\text{Ca}^{2+}]_i$ typically occurred during the first 180 min after application of neomycin. Bar = 20 μm .

itor BAF significantly reduced the numbers of cells with activated caspase-3 and pyknotic nuclei, but did not prevent the activation of the JNK signaling pathway or the translocation of cytochrome *c*. Other data demonstrated that treatment with CEP-11004 promoted hair cell survival in the presence of neomycin and reduced the number of hair cells with translocated cytochrome *c* and had phosphorylated c-Jun, p-JNK, and activated caspase-3. This indicates that the JNK signaling pathway occurred upstream of the translocation of cytochrome *c* and caspase activation. Finally, an increase in intracellular calcium was observed prior to the release of cytochrome *c*.

Mitochondria and Cytochrome *c*

Our data indicate that mitochondria in chick vestibular hair cells release cytochrome *c* into the cytoplasm

following treatment with aminoglycosides. Similar findings have been reported in studies of mammalian vestibular hair cells following treatment with aminoglycosides (Nakagawa and Yamane, 1999). Concurrent treatment with the general caspase inhibitor BAF did not prevent the translocation of cytochrome *c*. This observation is in agreement with results of studies of sensory neurons responding to a variety of apoptotic stimuli (Kluck et al., 1997; Bobba et al., 1999). Furthermore, the increase in pyknotic nuclei occurred about 6 h after the release of cytochrome *c*, indicating that the release of cytochrome *c* preceded the activation of caspases and changes in nuclear morphology.

Several observations indicate that mitochondria may play a role in mediating aminoglycoside ototoxicity. First, patients with mutations of the mitochondrial ribosomal RNA are more susceptible to aminoglycoside-induced hearing loss (Prezant et al., 1993; Casano et al., 1999). Second, recent studies have shown that inhibition of caspase-9-like activity prevents caspase-3 activation in hair cells (Cunningham et al., 2002). Caspase-9 activation normally requires cytoplasmic cytochrome *c* in interaction with dATP (a cofactor) and the caspase regulatory protein, apoptosis protease activating factor-1 (Apaf-1; Li et al., 1997). Third, our data indicate that cytochrome *c* release is not a consequence of nonspecific degradation of the mitochondria, because the mitochondrial protein cytochrome oxidase was not released into the cytoplasm following aminoglycoside treatment. Very few cells in any of our specimens displayed cytoplasmic immunoreactivity for cytochrome oxidase. This result is consistent with cellular fractionation experiments showing that no cytochrome oxidase was found in the cytosol of sympathetic neurons deprived of nerve growth factor (NGF), whereas cytochrome *c* was found in abundance (Putcha et al., 2000).

Future studies will need to resolve what factors trigger cytochrome *c* translocation in hair cells. Recent data suggest that members of the BCL-2 oncogene family may participate in aminoglycoside-induced hair cell death (Cunningham et al., 2004). The BCL-2 family is comprised of proteins that form homodimers and heterodimers that either promote (e.g., BAX and BID) or inhibit (e.g., BCL-2 and BCL-X_L) cell death. The ratio of proapoptotic to antiapoptotic BCL-2 family members within a particular cell may determine whether the cell will die in response to a death signal (Adams and Cory, 1998). How BCL-2 family members lead to cytochrome *c* release is not known, and many possible mechanisms of action for proapoptotic BCL-2 family members have been proposed (reviewed in Chang et al., 2002).

Notably, an action of BCL-2 on intracellular calcium stores has been proposed (Pinton et al., 2002) and the time course of increases in cytoplasmic calcium data presented herein suggests a role in the release mechanism.

Cellular Stress, JNKs, Calcium, and Hair Cell Death

In this study, we demonstrate that there is a rapid increase in the phosphorylation of JNK and c-Jun in hair cells following treatment with neomycin. Our data also suggest that there is a strong correlation between the phosphorylation of the transcription factor c-Jun and the translocation of cytochrome *c*. Direct stimulation of the JNK signaling pathway (via treatment with anisomycin) resulted in about a 40% reduction in the number of hair cells in both sensory regions relative to controls. This decrease in hair cell density was similar to that observed with utricles cultured with 1 mM neomycin, and suggests that JNK activation is associated with hair cell death. When utricles were treated with both anisomycin and neomycin, there was no difference in hair cell density when compared to specimens in which each of these drugs were applied individually. This suggests that there was no synergistic effect between neomycin and anisomycin, and that both pharmacological agents use the same signaling pathway to regulate hair cell death. Other reports indicate that anisomycin can inhibit protein synthesis, which is also known to kill cells (Kardalinos et al., 1994; Zinck et al., 1995). A previous study, however, demonstrated that global macromolecule inhibition via treatment with cycloheximide for 24 h is not toxic to hair cells (Matsui et al., 2002). Therefore, protein synthesis inhibition was probably not the primary reason why anisomycin killed the hair cells.

Treatment with the MLK inhibitor, CEP-11004 (1600 nM), resulted in near complete protection of hair cells in the presence of neomycin. Recent studies used a different MLK inhibitor (CEP-1347) or a peptide inhibitor of JNK to promote both auditory and vestibular hair cell survival in mammals (Pirvola et al., 2000; Ylikoski et al., 2002; Wang et al., 2003). A similar dose-response curve was obtained using CEP-1347 and 100 μ M neomycin in neonatal mouse cochlear cultures (Ylikoski et al., 2002). That study also showed that high concentrations of CEP-1347 (5000 nM) had no protective effect on auditory hair cell survival, but did not report whether high concentrations of CEP-1347 were toxic to hair cells. In the present study, treatment with a high concentration of CEP-11004 (4800 nM) resulted in about a 50% re-

duction in the number of hair cells, when compared to control cultures. Given that our control experiments indicate that the DMSO vehicle alone is not ototoxic, this result suggests that high concentrations of CEP-11004 are toxic to hair cells.

Treatment with the general caspase inhibitor BAF reduced the number of pyknotic nuclei, but did not prevent the phosphorylation of c-Jun. In contrast, treatment with CEP-11004 reduced the number of cells with phosphorylated c-Jun, activated JNK, activated caspase-3, and pyknotic nuclei. These observations indicate that JNK signaling lies upstream of the activation of caspases. Interestingly, CEP-11004 had a greater protective effect on hair cells than did treatment with general caspase inhibitors (e.g., Matsui et al., 2002). Perhaps blocking early in the cell death pathway may be a more effective means of promoting hair cell survival. Activation of JNK, however, is unlikely to be the earliest event in the hair cell death pathway. Numerous studies suggest that an increase in the concentration of intracellular reactive oxygen species (ROS) is an early event in programmed cell death, and precedes the activation of JNK. For instance, depriving sympathetic neurons of NGF in cell culture caused an increase in ROS production within hours of growth factor withdrawal (Greenlund et al., 1995; Kirkland and Franklin, 2001; Kirkland et al., 2002), and treatment with free-radical scavengers (e.g., superoxide dismutase or glutathione) reduced ROS levels and promoted neuronal survival (Greenlund et al., 1995; Kirkland and Franklin, 2001). ROS are also produced in hair cells in response to aminoglycoside exposure (Clerici et al., 1996; Hirose et al., 1997, 1999; Sha et al., 2001), and free radical scavengers can protect hair cells against aminoglycoside-induced death (Garetz et al., 1994; Song and Schacht, 1996; Sinawat et al., 2000; Sha et al., 2001). The pathway(s) leading to JNK activation are complex, and involve multiple MAP kinases and other regulator proteins (Kyriakis and Avruch, 2001) as well as calcium mobilization (Hashimoto et al., 1998), but many studies have shown that JNK activation is correlated with cell death or apoptosis by agents that act, at least in part, via generation of ROS (Chen and Tan, 2000). These data suggest that ROS are important mediators of aminoglycoside-induced hair cell death, which may be an early death signaling mechanism that activates JNKs.

Calcium is a critical second messenger in many intracellular signaling pathways. Changes in intracellular free calcium have been extensively studied in the process of cell death, but the actual role of Ca^{2+} signaling in this process is unclear (Berridge et al., 2000). Prolonged periods of elevated intracellular cal-

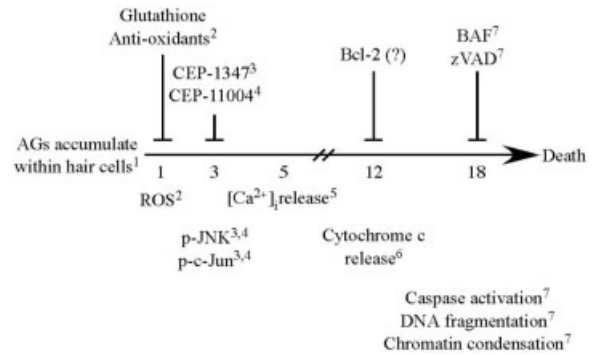


Figure 8 Summary diagram of critical events following the addition of neomycin to sensory hair cells, based upon currently available data. Numbers represent the approximate time (hours) the event occurs after the application of neomycin to the culture medium. ¹Desrochers and Schacht, 1982; Hayashida et al., 1985; Hiel et al., 1993; Aran et al., 1995; Hashino and Shero, 1995; Komeda and Raphael, 1996; Richardson et al., 1997. ²Reviewed in Forge and Schacht, 2000. ³Pirvola et al., 2000; Ylikoski et al., 2002. ⁴Bodmer et al., 2002, present study. ⁵Present study. ⁶Nakagawa and Yamane, 1999, present study. ⁷Forge and Li, 2000; Cunningham et al., 2002; Matsui et al., 2002; Cheng et al., 2003; Matsui et al., 2003, present study.

cium levels may initiate apoptotic pathways by activating endonucleases, phosphatases, and other calcium-dependent proteases. A previous study showed that slow and apparently irreversible changes in intracellular calcium occur in sensory hair cells at early time points following aminoglycoside treatment *in vitro* (Hirose et al., 1999). In the present study we monitored cytoplasmic calcium over much longer time periods. A large and prolonged increase in cytoplasmic calcium was observed, on average, 4 h after the application of 1 mM neomycin. In addition, we also observed a much smaller and slower increase in cytoplasmic calcium, which would correlate with that observed in explants of the chick cochlea (Hirose et al., 1999). Changes in intracellular calcium have been linked to activation of the JNK pathway. The onset of changes in intracellular calcium in hair cells in epithelial culture preparations are quite closely linked to the onset of p-c-Jun labeling in hair cells in cultured utricles (around 3–4 h). Changes in intracellular calcium have been implicated in activation of the JNK pathway in T cells, acting via the Ca^{2+} /calmodulin-dependent protein phosphatase, calcineurin (Werlen et al 1998). It is tempting to suggest a link between the observed calcium increase, p-c-Jun and cytochrome *c* release in neomycin-treated hair cells, but further studies are required to address this issue.

In summary, we have shown that neomycin-induced hair cell death in the chick utricle is mediated

by the phosphorylation of c-Jun, the translocation of cytochrome *c*, and the activation of caspase-3, with a potential early signaling event being changes in cytoplasmic calcium (Fig. 8). These findings further our understanding of the biology of hair cell death, and may suggest potential targets for therapeutic intervention.

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