Mechanisms of hair cell death and protection
Alan G. Cheng, Lisa L. Cunningham and Edwin W Rubel

Purpose of review
Sensory hair cells are mechanotransducers of the inner ear that are essential for hearing and balance. Hair cell death commonly occurs following acoustic trauma or exposure to ototoxins, such as the aminoglycoside antibiotics and the antineoplastic agent cisplatin. Loss of these inner ear sensory cells can lead to permanent sensorineural hearing loss, balance disturbance, or both. Currently, the only effective clinical intervention is prevention from exposure to known ototoxic insults. To help improve therapeutic strategies, a better understanding of the molecular mechanisms underlying hair cell degeneration is required. Current knowledge of these cell death mechanisms and potential therapeutic targets are discussed in this review.

Recent findings
Studies have shown that caspase-9 and caspase-3 are key mediators of hair cell death induced by noise, aminoglycosides, and cisplatin. The Bcl-2 family consists of a group of proapoptotic and antiapoptotic molecules that act upstream of and regulate caspase activation. Recent studies have shed light on the roles of molecules acting more upstream, including mitogen-activated protein kinases and p53.

Summary
The mechanisms of sensory hair cell degeneration in response to different ototoxic stimuli share a final common pathway: caspase activation. Inhibition of caspases prevents or delays hair cell death and may preserve hearing/balance function. Inhibition of mitogen-activated protein kinases protects against noise-induced and aminoglycoside-induced but not cisplatin-induced hair cell death, which suggests divergent upstream regulatory mechanisms.

Keywords
apoptosis, auditory, degeneration, hair cell, vestibular

Abbreviations
JNK c-jun NH2-terminal kinase
MAP mitogen-activated protein

Introduction
Programmed cell death is a physiologically normal event during development. The correct cell density is achieved and maintained by tightly controlled levels of generation and degeneration of cells. Studies of cell death have identified these cellular events to be regulated by genes that are highly conserved from nematodes to humans. A toxic insult to a cell can activate a cascade of cell death genes, thus leading to the cell’s demise. Molecules that are important mediators of cell death in other tissues are now known to play key roles in sensory hair cell degeneration.

Caspases
Caspases constitute a family of proteases that normally exist as inactive enzymes. These are cysteine-dependent, aspartate-specific proteases that function to mediate apoptotic destruction of the cell. A total of 14 caspase family members have been identified in mammals [1,2]. Independent studies have shown that general caspase inhibitors are able to promote hair cell survival after treatment with cisplatin [3,4] and aminoglycosides [5–9]. Only a subset of caspases mediates apoptosis, and those members can be divided into upstream and downstream members [2]. In general, upstream caspases are activated by a death-inducing signal, and downstream caspases are activated by upstream caspases. All caspases are activated by cleavage of an inactivating prodomain to produce the mature enzyme.

Caspase-8 is an upstream family member that is tightly linked to the membrane-associated death domain-containing receptors. When respective ligands (including Fas ligand and tumor necrosis factor-α) bind these receptors, pro-caspase-8 is recruited at the intracellular level. This recruitment allows for clustering and autoactivation of one caspase-8 molecule by another [10]. Activated caspase-8 then activates the downstream caspases, including caspase-3, caspase-6, and caspase-7. Although caspase-8 activity has been detected in hair cells after noise exposure [11], aminoglycoside treatment [6,7], and cisplatin treatment [12], three direct lines of evidence suggest that...
caspase-8 is not a key mediator of sensory hair cell death. First, in neomycin-treated utricles in vitro, inhibition of caspase-8 does not prevent hair cell death or caspase-3 activation [4,6]. Second, when utricles from lpr mutant mice – mice lacking a functional Fas receptor – are treated with neomycin in vitro, no protection from aminoglycoside-induced hair cell death is observed [13]. Finally, caspase-8 activation is not observed in cisplatin-treated cochleae, and a caspase-8 inhibitor failed to protect hair cells from cisplatin-induced death in vivo [4]. These results suggest that the death receptor pathway does not play a key role during aminoglycoside-induced or cisplatin-induced sensory hair cell death.

Another major upstream caspase is caspase-9, which is activated by a signal from the mitochondria. Its activation requires the release of cytochrome c from the mitochondria, which then binds the cytoplasmic apoptotic protease activating factor, dATP, and procaspase-9 [2,14]. Assembly of this multiprotein unit results in cleavage and activation of caspase-9. Activated caspase-9 can then cleave and activate the downstream caspases, resulting in apoptotic destruction of the cell. Activation of caspase-9 has been detected in aminoglycoside-treated utricles and cochleae in vitro, cisplatin-treated auditory cells and cochlear cell lines, and cochleae from noise-exposed animals [4,6,7,11,12,15]. When mouse utricles are co-treated with neomycin and a semi-selective inhibitor of caspase-9 in vitro, both hair cell death and caspase-3 activation are reduced [6]. Similar results are observed in cochlear hair cells treated with cisplatin and a caspase-9 inhibitor [4].

One main downstream caspase is caspase-3, which carries out the apoptotic program by cleaving proteins necessary for cell survival, including Bcl-2, inhibitors of deoxyribonucleases, and cytoskeletal proteins [16–19]. Caspase-3 activation has been detected in hair cells damaged by aminoglycosides [6,7,15*,20**], cisplatin [21], and acoustic trauma [11,22,23].

In the central nervous system, certain caspase-inhibited cells have been noted to undergo a delayed, caspase-independent cell death [24*]. In the inner ear, when vestibular hair cells were evaluated 5 drug-free days after an initial 24-hour co-treatment with neomycin and a general caspase inhibitor, hair cell survival was maintained [9]. When caspase-protected hair cells are examined for translocated cytochrome c and caspase-3 activation 24 hours after the initial insult (neomycin exposure), the extent of both of these two cell death-associated events in damaged organs is comparable with that in control, untreated organs [25]. These two lines of evidence suggest that caspase-protected hair cells do not undergo delayed degeneration. By contrast, further studies are needed to determine whether functional hair cells remain for longer periods.

To examine whether caspase-protected hair cells remain functional in the short term, Matsui et al. [8] examined utricles of chickens treated systemically with gentamicin. When a general caspase inhibitor was added locally by an osmotic pump, the utricular organs were found to be functionally normal by vestibular-ocular reflex testing [8]. These results are consistent with those of Wang et al. [4], who found that specific inhibitors of caspase-3 and caspase-9 protect against cisplatin-induced hair cell death and hearing loss. These studies suggest that caspase-protected hair cells are functionally intact, at least over the short term.

**Bcl-2 family and mitochondria**

The Bcl-2 family consists of a group of proteins that function to regulate cell death. Studies in a variety of other systems have shown that these molecules are key mediators of cell death acting upstream of caspase activation [1,26,27]. These molecules function as a checkpoint for cell death and survival signals at the level of the mitochondria. Bcl-2 family members can be characterized as either antiapoptotic or proapoptotic.

Anti-apoptotic Bcl-2 family members include Bcl-2 and Bcl-XL [26,28*]. Bcl-2 family members that function to promote cell death include Bax, Bak, Bcl-X, Bid, Bad, and Bim [26,28*]. Bcl-2 family members form heterodimers or homodimers within the cell. When a cell is challenged, the balance between the proapoptotic and antiapoptotic Bcl-2 family members serves as a primary control checkpoint regulating cell death and survival [29]. Antiapoptotic Bcl-2 family members are able to form heterodimers with proapoptotic members, resulting in a neutral signal [30]. When the balance tilts in favor of apoptosis, the proapoptotic Bcl-2 member Bax can translocate from the cytoplasm to the mitochondria and promote the formation of pores in the mitochondrial membrane [26,27]. These cellular events lead to loss of the mitochondrial transmembrane potential, the generation of reactive oxygen species, and leakage of cytochrome c into the cytoplasm.

In numerous systems in which Bcl-2 is overexpressed, cell death is inhibited [31,32]. By contrast, deletion of Bax has been found to promote cell survival and to decrease cytochrome c translocation and caspase-3 activation in the central nervous system [33]. Although the Bcl-2 family is known to contain key regulators of cell death, only a few studies have examined its role in cell death in the inner ear.

Immunohistochemical studies of aged gerbils showed diminished expression of Bcl-2 and increased expression of Bax in auditory hair cells [34]. Similar results were reported in auditory hair cells exposed to cisplatin in vivo [35]. More recently, when Devarajan et al. [12]
treated a mouse cochlear cell line with cisplatin, these cells demonstrated translocation of Bax from the cytoplasm to the mitochondria. A similar observation was made in auditory hair cells from rats treated with cisplatin [4]. To examine the effect of Bcl-2 overexpression on hair cell survival, Cunningham et al. [36*] used an in-vitro preparation of adult mouse utricles. When utricles were treated with neomycin, hair cells from mice overexpressing Bcl-2 were protected against cell death and caspase-9 activation. These data support a protective role for Bcl-2 in the inner ear and indicate that Bcl-2 acts upstream of the caspase cascade.

Translocation of Bax molecules to the mitochondria promotes the formation of pores that render the mitochondrial membrane permeable, which in turn leads to release of cytochrome c into the cytoplasm and loss of mitochondrial transmembrane potential [26,27]. In the inner ear, loss of the mitochondrial membrane potential has been reported after gentamicin treatment of auditory hair cells [37]. In-vivo and in-vitro studies of aminoglycoside-treated auditory and vestibular hair cells demonstrated release of cytochrome c to the cytoplasm [15*,20**,25,38]. A similar observation was made in cisplatin-treated auditory hair cells [4] and cochlear cell lines [12]. Cytochrome c translocation has also been reported in hair cells from animals exposed to noise trauma [11,39]. To examine the relationship between cytochrome c translocation and caspase activation, Matsui et al. [25] administered a general caspase inhibitor, boc-aspartyl-(OMe)-fluoromethylketone (BAF), and neomycin to utricles in vitro. They found that general caspase inhibition did not prevent this cytochrome c translocation, which supports the notion that the caspase cascade acts downstream or independently of cytochrome c translocation [25].

**Reactive oxygen species**

It is well established that reactive oxygen species are generated in hair cells exposed to cisplatin [40,41], aminoglycosides [42–45], and noise [46,47]. Numerous studies have reported that enhancing antioxidant levels (through drug application or genetic manipulation) promotes hair cell survival while preserving function [48,49*,50]. Hair cell death is potentiated when ototoxic drugs are applied to knockout mice lacking the enzymes responsible for maintaining antioxidant homeostasis [51,52]. The relationship between reactive oxygen species and other cell death events is not fully understood. Recent studies in the central nervous system have shown that mitochondria-associated oxidants are involved in pathways regulating cytochrome c translocation and caspase activation [53*].

**p53**

Cisplatin-induced cytotoxicity occurs by way of its ability to induce formation of DNA adducts [54]. When the resulting DNA damage overwhelms the cell’s intrinsic DNA repair mechanisms, apoptosis is initiated. One important mediator of DNA damage-induced cell death is the p53 tumor suppressor gene. When the DNA repair mechanisms fail, p53 is phosphorylated [55,56]. Phosphorylation activates and stabilizes p53, which in turn upregulates the proapoptotic Bcl-2 family member, Bax, at the transcriptional level [57]. Activated nuclear p53 can also translocate directly to and damage the mitochondria [58]. Neurons from p53 knockout mice are protected against cell death induced by diverse forms of damage [59,60]. Cell death events, including Bax and cytochrome c translocation and caspase-3 activation, have been shown to be downregulated in these p53-deficient cells, which suggests that p53 activation functions in early in the regulation of cell death [30,61]. Independent studies have also shown p53 to be upregulated in cisplatin-treated hair cells [12,21]. Recent studies from our laboratory have shown that deletion of the p53 gene protects sensory hair cells from cisplatin-induced cell death, caspase-3 activation, and cytochrome c translocation [62]. These lines of evidence support the hypothesis that p53 is an upstream regulator of cisplatin-induced hair cell death. The role(s) of p53 in aminoglycoside-induced or noise-induced hair cell death has not been examined to our knowledge, but certainly warrants attention.

**c-jun NH2-terminal kinase pathway**

Another important group of cell death mediators is the mitogen-activated protein (MAP) kinases. The c-jun NH2-terminal kinases (JNKs) constitute a group of MAP kinases that are activated in response to a variety of cellular insults, including excitotoxicity, radiation, and inflammatory cytokines [63,64*]. There are three isoforms of JNK (JNK 1, 2, and 3), with splicing variants of each. JNKs become activated by way of phosphorylation by MAP kinase kinases [64*,65*]. When activated, JNK in turn phosphorylates and activates the transcription factor, c-jun. Phosphorylation of JNK and c-jun have been demonstrated in inner ear hair cells treated with neomycin and cisplatin [4,25,66**]. Small-molecule inhibitors of the family of mixed lineage kinases, which are upstream regulators of MAP kinases, protect hair cells from noise-induced and aminoglycoside-induced death [66***,67]. Toxic effects of these inhibitors were noted at high doses, however, which suggests that a narrow therapeutic dose exists.

An independent study examining a specific inhibitor of JNK also demonstrated protection of hair cells from aminoglycosides and acoustic trauma, and that hair cell function was preserved [68]. Interestingly, JNK inhibition does not protect against cisplatin-induced sensory hair death, nor does it prevent redistribution of Bax and cytochrome c [4], which suggests that divergent upstream mechanisms underlie cisplatin-induced and aminoglycoside-induced hair cell death.
Table 1. Summary of studies examining cell death events and various protective agents in sensory hair cell death induced by aminoglycosides, cisplatin and acoustic trauma.

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<td>+[11,39]</td>
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<td>p53 ND</td>
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<td>+[12,21]</td>
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N/A, not applicable; ND, no data; +, supporting evidence; —, evidence against; cyto c, cytochrome c; JNK, c-jun NH2-terminal kinase.

Matsui et al. [25] also examined several cell death events while using an indirect inhibitor of JNK in aminoglycoside-treated vestibular organs; however, this study indicated that the protected hair cells showed diminished cytochrome c translocation and caspase-3 activation [25] (Table 1). This study suggests that JNK activation functions upstream of cytochrome c redistribution and caspase activation.

**Conclusion**

Much progress has been made to define the mechanisms underlying hair cell degeneration in recent years. Intracellular damage caused by noise, aminoglycosides, and cisplatin seems to share a final common pathway: cytochrome c translocation and caspase activation. Inhibition of caspases protects hair cells against both aminoglycosides and cisplatin while preserving function. These data suggest that caspase activity may represent an appropriate therapeutic target.

Intracellular events occurring upstream of caspase activation include cytochrome c and Bax translocation. Although JNK and c-jun become activated in response to noise, aminoglycosides, and cisplatin, there is evidence indicating that JNK inhibition effectively protects hair cells from aminoglycoside-induced and noise-induced, but not cisplatin-induced, hair cell death. Thus, it seems that upstream cell death events diverge between these three ototoxic insults and that individualized treatment regimens may be necessary.

Although antioxidants are well established as otoprotectants, their connection with other cell death events remains to be determined. An effective otoprotective strategy might require intervention at several levels along the pathways regulating cell death and survival, and it is therefore essential to further our understanding of sensory hair cell death pathways.

A final cautionary note seems relevant here. As noted above, whether a cell lives or dies depends in part on the delicate balance between the proapoptotic and anti-apoptotic factors. It seems that once the live-or-die decision has been reached, positive feedback systems ensure rapid death or, possibly, equally rapid recovery. This means that the effects of dosage for both the ototoxic agent and the modulator (pharmacologic agent or gene product) and their interaction are likely to be nonlinear [69]. This is easy to understand if the ototoxic agent is tested only at the dose that causes one half of the hair cells to die. This will be the steepest part of the dose-response curve, where there is usually the most variability: the place where a modestly effective protective treatment will be likely to show a dramatic outcome. As we thoroughly evaluate the entire two-dimensional matrix of ototoxic agents and proposed protective treatments, the nonlinearities become apparent. One example is our own work (in progress) using an indirect inhibitor of JNK. In two model systems, mature utricles in vitro [70] and zebrafish lateral line neuromasts [71], we find that this inhibitor is protective over a very narrow range of neomycin doses. Thus, for a full understanding of biology as well as for any consideration of future clinical utilization, more complete dose-response relationships must be examined. That is, if data on any protective treatment against ototoxic insult are to be eventually translated into clinical use, it will be critical that the treatment is effective over a range of doses of the ototoxic agent and that the modulator itself be effective over a reasonable range.

**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


16 This study reinforces previous findings of caspase activation and cytochrome c redistribution in aminoglycoside-treated vestibular hair cells.


22 The time course of cell death events, including caspase activation, cytochrome c translocation, T cell-restricted intracellular antigen-related protein translocation, and nuclear condensation, in the cochleae of chickens treated with gentamicin was examined in detail.


27 This article is an up-to-date review of the roles of caspases in neuronal cell death.


32 This review article summarizes our current knowledge of the Bcl-2 family in neuronal cell death and survival.


41 Vestibular organs from adult wild-type and Bcl-2 overexpressing mice were treated with neomycin in vitro. Bcl-2 overexpression both confers hair cell protection and prevents caspase-9 activation, which indicates that Bcl-2 acts more upstream in relation to caspase activation.


55 Adenoviral vectors were used to deliver superoxide dismutase genes into the inner ear. This pretreatment was able to prevent hair cell death and hearing loss.
64 Zine A, van de Water TR. The MAPK/JNK signalling pathway offers potential therapeutic targets for the prevention of acquired deafness. Curr Drug Targets CNS Neurol Disord 2004; 3:325–332. This is a current review of the roles of MAP kinase in cell death with special focus on the inner ear.
66 Pirvola U, Xing-Qun L, Virkkala J, et al. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. J Neurosci 2000; 20:43–50. This is a landmark article examining the protective role of JNK in the inner ear. In-vitro and in-vivo experiments found that inhibition of JNK protected against hair cell death induced by neomycin and noise.