Regeneration and replacement in the vertebrate inner ear

ARTICLE in DRUG DISCOVERY TODAY · NOVEMBER 2005
Impact Factor: 6.69 · DOI: 10.1016/S1359-6446(05)83577-4 · Source: PubMed

4 AUTHORS, INCLUDING:

Mark Parker
Tufts University
20 PUBLICATIONS 280 CITATIONS

Brenda M Ryals
James Madison University
49 PUBLICATIONS 1,505 CITATIONS
<table>
<thead>
<tr>
<th></th>
<th>Title</th>
<th>Pages</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hearing loss and tinnitus: 250 million people and a US$10 billion</td>
<td>Pages 1263-1265</td>
<td>Munna M. Vio and Ralph H. Holme</td>
</tr>
<tr>
<td></td>
<td>potential market</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>RNA interference: new drugs on the horizon</td>
<td>Pages 1266-1267</td>
<td>Jane Bradbury</td>
</tr>
<tr>
<td>3</td>
<td>Reversing the evolution of antibiotic resistance</td>
<td>Page 1267</td>
<td>Nicole Johnston</td>
</tr>
<tr>
<td>4</td>
<td>KDI tripeptide from laminin: a novel approach to treat neurodegenerative disorders</td>
<td>Page 1268</td>
<td>Steve Carney</td>
</tr>
<tr>
<td>5</td>
<td>Keynote review: The auditory system, hearing loss and potential targets for drug development</td>
<td>Pages 1269-1282</td>
<td>Matthew C. Holley</td>
</tr>
<tr>
<td>6</td>
<td>Tinnitus: neurobiological substrates</td>
<td>Pages 1283-1290</td>
<td>Jos J. Eggermont</td>
</tr>
<tr>
<td>7</td>
<td>Compounds for the prevention and treatment of noise-induced hearing loss</td>
<td>Pages 1291-1298</td>
<td>Eric D. Lynch and Jonathan Kil</td>
</tr>
<tr>
<td>8</td>
<td>Local inner-ear drug delivery and pharmacokinetics</td>
<td>Pages 1299-1306</td>
<td>Alec N. Salt and Stefan K.R. Plontke</td>
</tr>
<tr>
<td>9</td>
<td>Regeneration and replacement in the vertebrate inner ear</td>
<td>Pages 1307-1312</td>
<td>Jonathan I. Matsui, Mark A. Parker, Brenda M. Ryals and Douglas A. Cotanche</td>
</tr>
</tbody>
</table>
| 10. | **Ototoxicity: therapeutic opportunities**  
   *Pages 1313-1321*  
   Leonard P. Rybak and Craig A. Whitworth |
|-----|---------------------------------------------|
| 11. | **Therapeutics of hearing loss: expectations vs reality**  
   *Pages 1323-1330*  
   Orna Atar and Karen B. Avraham |
| 12. | **Monitor – chemistry**  
   *Pages 1331-1332*  
   Paul Edwards |
“According to the World Health Organization 250 million people worldwide have a moderate-to-severe or greater hearing loss.”

Hearing loss and tinnitus: 250 million people and a US$10 billion potential market

The social and economic demand for therapeutic treatments for hearing loss and tinnitus is enormous. In spite of this, there are currently no FDA-approved drugs or cell-based therapies for these conditions. This special issue of Drug Discovery Today, produced in association with the Royal National Institute for Deaf People (RNID; see Box 1), highlights the remarkable progress that has been made towards understanding the molecular and cellular basis of hearing loss and tinnitus. The key components needed for the development of drug and cell-based therapies now exist, and the rewards that await pharmaceutical and biotechnology companies able to move this research to the clinic, where it can benefit millions of people worldwide, are huge.

Epidemiology

Millions of people all over the world have hearing loss or associated conditions, such as tinnitus, otitis media and Ménières disease. According to the World Health Organization 250 million people worldwide have a moderate-to-severe or greater hearing loss (www.who.int/pbd/deafness/facts/en/index.html). This figure more than doubles if people with mild hearing loss are included. The National Institute on Deafness and Other Communication Disorders estimates that 33 children are born every day in the USA with a significant hearing loss, which equates to 12,045 children annually*. In Europe, one in 51 people carry a specific version of the gene for the most common form of recessive deafness, connexin 26 [1].

Hearing loss becomes increasingly prevalent with age. RNID estimates that there are over 300 million people in the world with age-related hearing loss and this is expected to increase to 900 million by 2050†.

A common cause of hearing loss is repeated exposure to loud noise. 10 million people in the USA and 25–30 million people in Europe work daily in conditions that pose a potential risk to hearing [2]. People working in heavy industry and in the armed forces are at particular risk. Also, in the past 20 years, exposure to ‘recreational’ noise has increased with the advent of personal stereos and an increased numbers of bars and clubs playing loud music.

Social and economic burden

Hearing loss is the third most common chronic condition in the older population (after arthritis and...
Examples of biomedical research being supported by RNID:

- RNID-funded research is on the edge of identifying the very first genes that increase an individual’s susceptibility to age-related hearing loss. This discovery could lead to diagnostics to identify those at risk and to drug targets for the prevention of age-related hearing loss.
- RNID is supporting unique research exploring the therapeutic potential of human embryonic stem cells to treat hearing loss. This cutting-edge research could produce physiologically relevant cells for high throughput drug screening and cell-based therapies for restoration of hearing.
- RNID-funded research is improving our understanding of the neurobiology of the central auditory system. This vital research will lead to therapeutic strategies for alleviating tinnitus and other central auditory processing disorders.

BOX 1

About RNID

RNID (The Royal National Institute for Deaf People) is the largest not-for-profit organization representing the 9 million deaf and hard of hearing people in the UK. As a membership organization, we aim to achieve a radically better quality of life for deaf and hard of hearing people. We do this by campaigning and lobbying vigorously, by raising awareness of deafness and hearing loss, by providing services and through social, medical and technical research.

Examples of biomedical research being supported by RNID:

- RNID-funded research is on the edge of identifying the very first genes that increase an individual’s susceptibility to age-related hearing loss. This discovery could lead to diagnostics to identify those at risk and to drug targets for the prevention of age-related hearing loss.
- RNID is supporting unique research exploring the therapeutic potential of human embryonic stem cells to treat hearing loss. This cutting-edge research could produce physiologically relevant cells for high throughput drug screening and cell-based therapies for restoration of hearing.
- RNID-funded research is improving our understanding of the neurobiology of the central auditory system. This vital research will lead to therapeutic strategies for alleviating tinnitus and other central auditory processing disorders.

Visit www.rnid.org.uk for further information about RNID

high blood pressure; www.pamf.org/health/toyourhealth/hearingloss.html) [3] and has serious social and economic implications. It can cause considerable difficulties in communication with relatives, friends, colleagues and the outside world in general, and can lead to reduced physical and psychological well-being, social withdrawal, isolation, loneliness and depression [4]. Children who are born profoundly deaf face severe difficulty acquiring spoken language and are often taught in special schools. Deaf children educated in mainstream schools frequently face problems socially interacting with hearing children. Research suggests that such experiences might be detrimental to their mental well-being [5]. Communication disorders are thought to cost the US economy between US$154–186 billion per year [6] and noise-induced hearing loss causes significant economic losses of between 0.2–2.0% of the gross domestic product [7].

Current therapeutic interventions

Hearing loss frequently involves the loss of sensory hair cells and auditory neurons in the cochlea, and today’s treatments are limited to the use of prosthetics (e.g. hearing aids and cochlear implants).

Hearing aids

Hearing aids amplify the sounds people struggle to hear, improving individuals’ ability to communicate. The global hearing aid market is currently worth approximately US$8 billion. In recent years, the availability of programmable digital hearing aids, which allow tailored amplification to match users’ hearing loss, has increased their possible benefits. Improved directionality, background noise reduction and acoustic feedback suppression have also brought significant quality-of-life improvements to hearing aid users [8].

However, market penetration of hearing aids remains low, at around 20% of possible end users, even in the developed world. In many countries, a shortage of suitable audiological facilities with qualified audiologists has meant that potential users have not been able to gain access to hearing aids. Reimbursement issues and lack of awareness of the benefits of well-fitted hearing aids conspire to suppress market growth. Additionally, hearing aids can only offer benefit to people with residual hearing.

Cochlear implants

A cochlear implant bypasses the hair cells and directly stimulates the auditory nerve. They are only routinely provided to prelingual profoundly deaf children or postlingual deafened children and adults who do not benefit from hearing aids. Consequently, they are only suitable for a minority of people with hearing loss. Although the cochlear implant market is estimated to be worth approximately US$410 million, it is relatively small compared with the hearing aid market. The cost of assessment, implantation, intensive support and maintenance for the first three years alone is typically around US$100,000+. Whether or not the patient will receive a cochlear implant is dependent on the cause of the hearing loss, the reimbursement policy of the local healthcare provider or his/her ability to pay.

Although these prosthetics bring real benefit to those who use them, a considerable gap exists that could be addressed by pharmaceuticals focusing on the underlying biological causes of hearing loss.

Tinnitus

Tinnitus (commonly referred to as ringing in the ears or head) is often one of the first signs of potential damage to hearing, especially after exposure to loud noise. Fortunately, for many, tinnitus is a temporary phenomenon lasting for only a short period but 1 in 10 adults have clinically significant tinnitus (regular prolonged spontaneous tinnitus lasting 5 minutes or more), and for 1 in 100 adults tinnitus severely affects their ability to lead a normal life. RNID estimates that 13 million people in western Europe and the USA currently seek medical advice for their tinnitus. Over 4 million prescriptions are written each year for tinnitus relief but these are all for off-label drugs from a wide variety of therapeutic classes and most are associated with considerable side effects.

*Nottingham Cochlear Implant Programme Price list for 2003/2004, Queen’s Medical Centre Nottingham University Hospital NHS Trust.
Despite the significant unmet clinical need for a safe and effective drug targeting tinnitus relief, there is currently not a single FDA-approved drug on the market. RNID estimates that a novel tinnitus drug could have a product value of US$689 million in its first year of launch.

The time is right
So why, despite the existence of the vast potential markets described above and the significant unmet clinical need that could be addressed through novel drug intervention or cell-based therapies, have the pharmaceutical and biotechnology industries been slow to get involved? In the past, a lack of fundamental knowledge of the biological basis of hearing and hearing loss, and the absence of assays for drug screening were probably the main reasons. But, as Matthew Holley’s keynote review and the related reviews in this issue of Drug Discovery Today demonstrate, this no longer holds true: there has never been a better time for investment in translational hearing research.

There are already cases of compounds moving from preclinical to clinical development (see Lynch and Kil, this issue). A good example is ebselen, a compound that is thought to reduce damage caused by reactive oxygen species, which has been shown in animal models to reduce permanent hearing loss following noise exposure. Sound Pharmaceuticals have now secured FDA approval to conduct a Phase II clinical trial to test the ability of this compound to protect hearing in army recruits.

There are also opportunities for companies with expertise in developing gene- and cell-based therapies. Groundbreaking research has shown that Math1-expressing viruses produced some structural and functional recovery of the sensory structures in the cochlea of deafened guinea pigs. Other researchers have shown that mouse embryonic stem cells can give rise to sensory hair cells when transplanted into the developing chick ear. The possibility that such technology could be used to restore hearing is very exciting (see Matsui et al. in this issue).

In the field of tinnitus research, there are exciting opportunities for companies with an interest in neurosciences, particularly for those working on chronic pain. The neurobiology underlying these two conditions is remarkably similar and combining expertise in these areas could be very productive.

Government (e.g. the US National Institutes of Health, the UK Medical Research Council and the European Commission) and non-government agencies, including RNID, recognize the importance of hearing research and have been investing in this area for many years. Our own research grants programme (www.rnid.org.uk/research-grants) invests around US$1 million annually in research worldwide and we expect to see this level of support grow over the coming years. There is now a real opportunity to move this promising research from the laboratory to the clinic: the pharmaceutical and biotechnology industries are vital to this process. RNID is already working closely with industry to highlight the vast markets available for novel treatments, the current unmet clinical needs and the potential avenues of research that are open to them. We believe our considerable expertise in this field, our novel market reports (www.rnid.org.uk/marketreports) and links with both academia and industry have enabled a variety of key collaborations to date.

This special issue brings together an impressive body of evidence to demonstrate that the science now exists to deliver radical improvements in therapeutic interventions for hearing loss and tinnitus. By getting involved, industry will not only address the significant unmet clinical needs of millions of people but also be rewarded with at least US$10 billion worth of added revenue.

References

Munna M. Vio* Ralph H. Holme**
RNID,
19-23 Featherstone Street,
London EC1Y 8SL,
UK
*e-mail: munna.vio@rnid.org.uk
**e-mail: ralph.holme@rnid.org.uk

Researchers at Sirna Therapeutics (San Francisco and Boulder, USA) and Protiva Biotherapeutics (Burnaby, Canada) have taken an important step towards developing therapeutically active short interfering RNAs (siRNAs) [1]. By wrapping chemically modified siRNA in a specialized liposome, Sirna’s Senior Director of Biology, David Morrissey, and colleagues have greatly increased the in vivo potency and duration of action of a siRNA targeted against hepatitis B virus (HBV). ‘Our main clinical target is HCV’, explains Morrissey, ‘but this work is an important proof-of-principle for demonstrating the potency of our siRNAs.’

The therapeutic promise of RNA interference
RNA interference (RNAi) is a highly conserved, endogenous gene-silencing mechanism. Discovered in 1998, RNAi involves double-stranded RNA-mediated sequence-specific degradation of mRNA. Because the development of many diseases requires the production of specific proteins (for example, the replicative enzymes involved in viral infections or the oncoproteins that help to drive tumorigenesis), scientists quickly recognized the therapeutic potential of RNAi [2]. The fact that with siRNA-based drugs we would be tapping into an endogenous protein-driven mechanism,’ explains Morrissey, ‘makes the approach potentially very powerful.’

‘Wrapping the modified siRNA in lipid did a remarkably good job at reducing the amounts of siRNA needed to see an effect in vivo...’

But before siRNAs can be used therapeutically several challenges need to be addressed. For example, siRNAs are naturally degraded so their effect is only transient; consequently, ways must be found to prolong their gene-silencing activity. Furthermore, the delivery method must get the siRNAs to their target tissues efficiently and most importantly, once there, the cellular uptake of the siRNAs must be optimized and their cellular activity maximized.

Solving the delivery problems
Sirna scientists are working on these and other challenges and in June they reported that a daily intravenous dose of 30 mg/kg of a chemically optimized siRNA targeted to HBV RNA produced a 90% reduction of serum HBV DNA in a mouse model of HBV replication [3]. Recognizing that this is not a clinically viable dose, the Sirna scientists have now used lipid encapsulation to improve the pharmacology of their siRNA. In their current formulation, chemically modified siRNAs are covered with a lipid bilayer, designed to facilitate their cellular uptake and endosomal release, and then with a polyethylene glycol-lipid conjugate surface coating, to prevent rapid systemic clearance. This formulation produces a 95% reduction in HBV serum titers at doses 1–5 mg/kg per day (delivered in three intravenous doses). This reduction in HBV serum titers persists for seven days after the last dose and can be maintained by weekly doses of siRNA [1].

‘Wrapping the modified siRNA in lipid did a remarkably good job at reducing the amounts of siRNA needed to see an effect in vivo’, comments James Patton, Professor of Biological Sciences at Vanderbilt University, USA.

‘Although getting siRNAs to the liver is relatively straightforward’, comments Morrissey, ‘achieving significant uptake by hepatocytes together with long duration of effect is an important breakthrough. In addition, we can change the parameters of our formulation to target it to other tissues or decorate our liposomes with targeting ligands to get specific delivery.’ Sirna is now testing its current formulation in primate models of HCV and hopes to start Phase I clinical trials of an anti-HCV siRNA during 2006.

John Rossi, Chair of the Division of Molecular Biology at the Beckman Research Institute of the City of Hope and co-founder of Calando
News

Reversing the evolution of antibiotic resistance

Nicole Johnston, njohnston@rockefeller.edu

Bacteria develop resistance to antibiotics through gene mutations. Conventional wisdom has long held that these mutations are inevitable, caused by imperfect polymerases replicating a large genome under pressure. However, increasingly evidence suggests that bacteria are active players in the process, inducing proteins responsible for generating various types of mutations. Now, researchers at the Scripps Research Institute in La Jolla, CA, USA, led by Floyd Romesberg, are showing that interfering with these mutation-inducing pathways can reverse antibiotic resistance and render bacteria completely susceptible to the once ineffective antibiotic. ‘Inhibiting these mutation pathways from the outset’, says Romesberg, ‘could represent a novel approach to combating the evolution of antibiotic resistance’ [1].

‘Inhibiting these mutation pathways… could represent a novel approach to combating the evolution of antibiotic resistance’

Shutting down the SOS response

Resistance-conferring mutations arise from three recombination pathways involved in repairing DNA damage. The Scripps group examined the role of various genes encoding proteins involved in the bacterial SOS response to two antibiotics, ciprofloxacin and rifampicin (members of the important quinolone and rifamycin classes, respectively).

To test whether the SOS response is necessary for ciprofloxacin and rifampicin resistance to occur, they infected mice with pathogenic *Escherichia coli* containing either wild-type or mutant LexA (a protein induced during SOS repair of DNA damage). Autoproteolysis of LexA causes derepression of three polymerases (Pol II, Pol IV and Pol V) involved in generating mutations. They found that interfering with LexA prevented its autoproteolysis and the derepression of the LexA-controlled polymerases. As a result, *E. coli* was unable to turn on its SOS genes and could not evolve resistance to either ciprofloxacin or rifampicin. By contrast, *E. coli* with wild-type LexA resulted in the generation of *E. coli* subsequently becoming resistant to these antibiotics. They concluded that the LexA-controlled polymerases are necessary for the evolution of ciprofloxacin and rifampicin resistance.

Resistance to ciprofloxacin and rifampicin is, therefore, a consequence of proteins being induced to increase mutation rates significantly and not simply a chance occurrence of errors during genome replication. Interfering with the genes involved in these recombination pathways prevented the bacteria from becoming resistant.

Small-molecule inhibitors

‘The real motivation was for drug design and looking for novel targets’, says Romesberg. ‘Mutation plays a role and might be susceptible to this type of intervention. Inhibiting these pathways would have a significant impact on the evolution of resistance.’

He says they are currently trying to identify small molecules that could be administered in conjunction with antibiotics, preventing bacteria from acquiring resistance-conferring mutations in the first place.

‘This is immensely important’, says George Drusano, physician scientist at Albany Medical College and Ordway Research Institute in Albany, NY, USA. ‘We can roll back the past in certain circumstances and prevent the erosion of the susceptibility profile of clinically important organisms to this class of agents.’

Furthermore, the phenomenon might not be unique to bacteria. ‘The evolution of resistance in chemotherapy is a real problem in breast cancer’, says Romesberg. ‘In human cells, people just don’t know what the pathways are.’ He comments, ‘one approach would be to do genome-wide screens in yeast to identify genes that, when deleted, would render the yeast susceptible to mutation’. For now, he and his colleagues are trying to determine whether this is a universal phenomenon in bacteria and other antibiotic classes, at the same time as devising a chemical approach to reversing resistance.

Reference

KDI tripeptide from laminin: a novel approach to treat neurodegenerative disorders

Steve Carney, s.carney@elsevier.com

In 1979 George Martin and colleagues [1] reported on a novel specialised glycoprotein that they named laminin, which was extracted from EHS sarcoma, which produces abundant basement membrane. For many years, it was thought to be responsible solely for anchoring cells to components of the extracellular matrix. In the following years, however, it became clear that laminin had many other diverse biological functions including promoting cell survival, signalling through laminin receptors and tubulogenesis [2].

Laminin in the CNS
More recently, researchers, notably Päivi Liesi of the Department of Biological and Environmental Sciences and head of the Brain Laboratory at the University of Helsinki, have been working on the biological roles of laminin in the CNS. Interestingly, Liesi’s group was able to demonstrate that a peptide domain of laminin (KDI; lysine-aspartate-isoleucine) was capable of promoting functional regeneration of rat spinal cord injuries [3]. Liesi and colleagues, in a recent paper in the Journal of Neuroscience Research [4], have gone on to show, using patch-clamp electrophysiology in human cultured neocortical neurons, that the KDI tripeptide domain is a universal, potent and (in the case of the AMPA receptor) non-competitive inhibitor of ionotropic glutamate receptors (iGluRs). The receptors examined included the AMPA, kainate and NMDA subclasses of iGluR.

Glutamate excitotoxicity
The excessive release of glutamate following brain injury is responsible for the phenomenon of glutamate excitotoxicity, which is mediated through glutamate receptors and results in apoptotic cell death. The combination of KDI’s ability to ameliorate excitotoxicity and regenerate damaged nerve cells is particularly exciting, in that such a molecule could be expected to have benefit in a range of neurodegenerative disorders that could include Alzheimer’s disease, ALS, Parkinson’s disease and potentially other debilitating neurodegenerative diseases.

‘One of the wonderful things about this is that Dr Liesi’s discoveries are ready now for prime-time testing in patients.’

As Liesi commented: ‘The wider significance of his research is that KDI treatment may become the first natural and targeted therapy for people with central nervous system injuries resulting in paralysis and a range of diseases such as Alzheimer’s and ALS, for which there are currently no cures.’

Other researchers are equally excited at the prospect of proof-of-concept trials with the tripeptide. George Martin, one of the discoverers of laminin, and a former collaborator of Liesi’s, mentioned: ‘One of the wonderful things about this is that Dr Liesi’s discoveries are ready now for prime-time testing in patients. We do not have to go through a long drug development procedure that might take 10 years.’

Proof-of-concept trials
Hopefully, the proof-of-concept trials will establish the utility of the KDI tripeptide approach and that the tripeptide itself may be advanced as a potential drug candidate. However, even if the trials are positive, the KDI tripeptide may not be able to be developed as a drug in. There are many issues that can prevent the development of a potential active agent. However, it would appear that the target of KDI’s action on iGluRs could be of a size that would allow the production of small organic drugs capable of acting upon it. These findings may open up new avenues of research into the development of pan active iGluR modulators.

References
1 Timpl, R. et al. (1979) Laminin – a glycoprotein from basement membranes. J. Biol. Chem. 254, 9933–9937
There is a huge potential market for the treatment of hearing loss. Drugs are already available to ameliorate predictable, damaging effects of excessive noise and ototoxic drugs. The biggest challenge now is to develop drug-based treatments for regeneration of sensory cells following noise-induced and age-related hearing loss. This requires careful consideration of the physiological mechanisms of hearing loss and identification of key cellular and molecular targets. There are many molecular cues for the discovery of suitable drug targets and a full range of experimental resources are available for initial screening through to functional analysis in vivo. There is now an unparalleled opportunity for translational research.

There is a massive social and economic demand to develop therapeutic treatments for hearing loss. Deafness is one of the most widespread, costly and poorly understood disabilities in the world. It is also one of the most neglected. Its invisibility hides the suffering of many millions of people, who progressively lose their most important means of communication and who become socially isolated, especially in their later years.

In 2002, the World Health Organization (WHO) estimated that 250 million people have disabling hearing loss (www.who.int/pbd/deafness/en) and that two-thirds of them live in the developing world. The costs of communication disorders to the US economy have been estimated at US$ 154–186 billion per year [1]. In 1997 the cost of noise-induced hearing loss alone was estimated to be between 0.2% and 2% of the gross domestic product. In the UK in 2002 this fraction was equivalent to US$ 2.7–27 billion (Energy Information Administration, http://eia doe gov/emeu/international/other.html). The WHO described the scale of the problem, the primary causes and potential solutions in a series of conferences from 1994–1998 (www.who.int/pbd/publications/en/). It concluded that approximately 50% of hearing
loss is avoidable through careful management of noise exposure and of the administration of prescribed, ototoxic drugs. One of the key recommendations was investment in research, including transitional research to bring some of the remarkable, recent developments in basic research closer to the clinic. Investment in hearing research has been extremely modest in comparison with the measured social and economic costs but it has yielded results to be envied by many other disciplines. Widespread research activity has been underpinned by organizations, such as the National Institute for Deafness and other Communication Disorders (NIDCD) in the USA (www.nidcd.nih.gov), and large European consortia, such a GENDEAF (www.miteuro.org/gendeaf.htm) and the very recently launched EUROHEAR (www.eurohear.org). The global demand for therapeutic treatments is increasing dramatically with industrialization and lifespan. In developed countries the appetite for leisure noise among the young is expected to
The low-frequency region of a guinea pig cochlea, viewed by differential interference contrast microscopy. (b) Section through the cochlea of a guinea pig. (c) Diagram of a section through one turn of the cochlea. (d) The organ of Corti. The supporting cells form a single, continuous layer, in which every cell spans the epithelium from the basement membrane to the epithelial surface. IHCs are supported by phalangeal cells and along their lateral side, opposite the central axis of the cochlea, by a row of IPCs. IPCs form a continuous row and with a similar row of OPCs they form the tunnel of Corti, a long triangular shaped canal that runs along the length of the organ. Pillar cells are composed of tightly packed bundles of actin filaments and microtubules, embedded at each end in a dense actin mesh. They form a rigid frame, which is important for coupling the mechanics of the basilar membrane with the hair cell surfaces. Each row of OHCs is supported by rows of another type of supporting cell, called Deiters’ cells. These cells sit beneath the OHCs and connect them to the basilar membrane but they have narrow microtubular processes that project up to the epithelial surface. Lying over the tops of the hair cells is the tectorial membrane, which is a finely cross-linked structure of collagen fibers and tectorins. (e) Isolated outer hair cell from the low-frequency region of a guinea pig cochlea, viewed by differential interference microscopy. Scale bar = 10 μm. (f) Diagram of a hair cell flanked by two supporting cells. The hair cell bundle is composed of stereocilia, which regulate the flow of potassium ions (K⁺) from the endolymph through transducer channels attached to apical tip-links (arrowheads). Notch ligands (only jagged 2 shown) is in the hair cell activate notch in the adjacent supporting cell, which upregulates Her genes and suppresses hair cell differentiation. Release of this inhibition is likely to be necessary for regeneration. Notch signaling is reinforced by several other ligands that communicate between supporting cells and between supporting cells and hair cells. (See [67].) Abbreviations: BM, basilar membrane; C, cochlea; CN, cochlear nerve; DC, Deiters’ cell; E, inner ear; IPC, inner pillar cell; LSC, lateral semicircular canal; ME, middle ear; N, VIIIth nerve; NF, nerve fibres; OE, outer ear; OPC, outer pillar cell; PSC, posterior semicircular canal; RM, Reissner’s membrane; rw, round window; S, stapes; SG, spiral ganglion in Rosenthal’s canal; SM, scala media; SSC, superior semicircular canal; ST, scala tympani; SV, scala vestibule; SVA, stria vascularis; TM, tectorial membrane.

have a substantial, deleterious impact on hearing loss in older generations in the future. The aims of this review are to introduce the auditory system with a summary of the nature and scale of hearing loss and then to review recent research with a focus on the most likely cellular and molecular targets for drug development.

The auditory system

Sound travels in air along the outer-ear canal to the eardrum and is then transmitted via the bones of the middle ear to the fluid environment of the inner ear, where the sensory organ resides (Figure 1a). The neural output is conducted along the auditory nerve to the hindbrain and ultimately to the auditory cortex via the central auditory pathways. Complexity increases from the outer ear to the cortex and is inversely proportional to our understanding of auditory processing and to our ability to treat hearing problems. There are numerous diseases of the ear [2] but most forms of hearing loss are sensorineural, involving loss of the sensory hair cells and primary sensory neurons in the inner ear [3]. In addition, approximately one in seven people suffer from tinnitus, a complex condition involving endogenous generation of noise from the inner ear and central auditory pathways [4,5]. This article focuses largely on the potential for drug development to treat sensorineural hearing loss (SNHL). Drug discovery is often an opportunistic process. However, knowledge of potential cellular and molecular targets greatly enhances the chances of success in terms of discovery and of the assessment of safety and specificity. Knowledge of the relevant anatomy and physiology is crucial to the development of drug delivery systems [6].

Conductive hearing loss

Conductive hearing loss involves the attenuation of sound conduction through the outer ear and middle ear (Figure 1a). The most common problems involve accumulation of wax and various forms of infection or skin disease [2]. The outer-ear canal leads to the eardrum and middle ear, which contains a series of three small bones named the malleus, incus and stapes. These bones couple the tympanic membrane to the oval window of the inner ear, focusing the sound energy so that it can be transmitted efficiently from air to fluid. Conductive hearing loss in the middle ear is caused by a wide variety of problems, such as infection and inflammation, otosclerosis, carcinoma, head injuries and sometimes genetic defects. Most of these conditions can be treated with drugs or surgery, including replacement of the ear ossicles, if necessary. Otitis media is one of the major causes of treatable hearing loss in children but, if it is ignored, it can have a serious impact on learning and social interactions [2]. With the exception of cochlear implants, which have now been fitted to more than 80,000 patients with severe or profound SNHL, otolaryngologists do not often venture beyond the oval window and into the inner ear, where the most important cellular and molecular targets for treatment of SNHL are located.

The inner ear

The inner ear contains six anatomically separate mechanosensory epithelia, which are adapted to interpret different forms of mechanical stimulus. Five of them are part of the vestibular system. The posterior, superior and lateral semicircular canals project from the dorsal region of the inner ear (Figure 1a). At one end of each canal there is a chamber that contains a small sensory epithelium, known as a crista ampullaris, and the three cristae detect angular acceleration of the head in three planes. Two separate macular epithelia located within the sacculo and the utricle detect vertical and horizontal linear acceleration, respectively. All of these sensory epithelia are positioned centrally within the inner ear. The auditory epithelium is located ventrally and is coiled into the characteristic structure of the cochlea.

The cochlea

The human cochlea is a coiled tube 30–35 mm long containing a collagenous basilar membrane, which is relatively narrow and thin at the basal end and which increases...
progressively in width and thickness towards the apex (Figure 1b). Sound energy is absorbed maximally at the part of the membrane that shares a similar resonant frequency and results in oscillatory motion of the basilar membrane. Thus, the mechanical properties of the basilar membrane determine the range of frequencies that we can hear, which is from ~18 kHz in the base to ~20 Hz in the apex. A cross-section of the cochlear tube shows the basilar membrane as an extension of the bony spiral lami na with the sensory epithelium, or organ of Corti, on top (Figure 1c). One of the most important specializations of the cochlea is its division into three parallel chambers, the scala vestibuli and scala tympani, which contain perilymph, and the scala media, which contains endolymph. The maintenance and circulation of these fluids are critical for cochlear function and the dynamics of drug delivery. Perilymph is similar to the cerebrospinal fluid (CSF), as it contains ~138 mM sodium and only ~7 mM potassium. In fact, it communicates with the CSF via the cochlear aqueduct, which is located in the scala tympani at the basal end of the cochlea. Endolymph contains ~1 mM sodium and ~154 mM potassium. Potassium ions are pumped into the scala media by cells of the stria vascul aris, which lies against the lateral wall of the cochlear duct. The ionic difference provides the driving force for mechano-electrical transduction, because the electrical potential in the scala media is ~80 mV compared with 0 mV in the scala vestibuli and scala tympani. This endocochlear potential occurs across the epithelial boundaries between the scalae. Endolymph is separated from perilymph in the scala vestibuli by the Reissner’s membrane and in the scala vestibuli by the organ of Corti and the adjacent non-sensory epithelium (Figure 1c). The endocochlear potential is essential for mechano-electrical transduction in hair cells and its demise is a critical factor in many forms of hearing loss.

The organ of Corti, as with all mechanosensory epithelia, is composed of supporting cells and hair cells (Figure 1d). There are major differences between mammalian and non-mammalian auditory epithelia, because mammalian supporting cells and hair cells are structurally adapted for a highly specialized mechanism of mechanical tuning [8]. This mechanism appears to allow mammals to hear much higher frequencies, but this might have come at a price in terms of the loss of regenerative capacity.

There are ~15,500 hair cells in each human cochlea. These include 3,500 inner hair cells (IHCs) and 12,000 outer hair cells (OHCs) (Figure 1d and 2a). The IHCs generally form one or two rows along the inner edge of the organ of Corti and are the primary sensory receptors, innervated by ~95% of the primary sensory afferent neurons in the spiral ganglion. Their hair bundles are composed of 50–100 stereocilia organized in two or more linear rows, which increase in height away from the cochlear axis. OHCs are cylindrical and usually organized into three rows along the outer edge of the organ of Corti. They receive only 5% of the afferent innervation but the majority of the efferent fibers, which originate from the superior olive in the brainstem [9].

Stereocilia resemble large microvilli ~300 nm in diameter and 2–5 μm long, the longer bundles being located at the apical low-frequency end of the cochlea. They are made of a semi-crystalline array of actin filaments cross-linked with fimbrin and a host of other actin-binding proteins. Neighboring stereocilia are connected by short extracellular links, which maintain the integrity of the bundle [10]. Transduction is thought to involve specialized tip-links that connect the tips of the shorter stereocilia to the shafts of longer neighbors (Figure 1f). Appropriate displacement of a hair bundle increases or decreases the stress on the tip-links and regulates the gating of a small number of mechanosensitive ion channels in the stereociliary membrane. The molecular anatomy of the hair bundle involves hundreds of different proteins [11]. Recent evidence suggests that the tip-link is composed of cadherin 23 [12] and that the mechanotransducer channel is TRPA1, a member of the transient receptor potential family of ion channels [13]. The transducer channel is a non-selective cation channel that regulates the flow of potassium ions into the cell. It also allows calcium entry, which is important for sensory adaptation and for active mechanical responses in the hair bundle [14]. Receptor potentials in IHCs regulate glutamate release at high-speed ribbon synapses in the basolateral membrane, thus modulating the activity of auditory nerve fibers [15]. Stimulus intensity is encoded by the number of channels activated, which influences the size of the receptor potential, and by firing rate in low- and high-threshold sensory nerve fibers. Each IHC receives ~20 afferent endings but each afferent fiber innervates a single IHC. OHCs respond quite differently to changes in membrane potential. Their membranes include a semi-crystalline array of a protein called prestin, which alters its conformation with the membrane potential and forces cell length changes at acoustic frequencies [16, 17]. The hair bundles also generated mechanical forces [14] and the two mechanisms are thought to amplify and tune the mechanical responses of the basilar membrane. The responses of the OHCs are modulated by the efferent innervation, primarily via activation of acetylcholine receptors, which permit calcium entry and subsequent activation of calcium-activated potassium channels [18]. Thus, there is a clear division of labor between the two types of hair cell. Without IHCs one would be totally deaf but the amplification provided by OHCs is extremely important and enhances our hearing sensitivity by 40–60 dB [16]. The innervation to the hair cells passes along the bony spiral lamina and into Rosenthal’s canal, where the spiral ganglion is located (Figure 1c). The spiral ganglion includes all the cell bodies of the primary sensory neurons, whose axons project via the VIIIth nerve to the cochlear nuclei in the brainstem.
The mechanical coupling between the basilar membrane, hair cells and hair bundles is highly specialized and places challenging constraints on regeneration. The tips of the OHC bundles are attached to a loosely woven, collagenous tectorial membrane. The two membranes are hinged separately at different levels so that vertical motion of the organ of Corti leads to shear displacements between the hair cell apices and the lower surface of the tectorial membrane. This causes oscillatory, planar displacements of the hair bundles and generates receptor potential modulation in the hair cells. IHC bundles are indirectly coupled to the tectorial membrane by fluid displacement in the restricted space between the tectorial membrane and epithelial surface.

**Pathology and treatment of hearing loss**

Treatments for sensorineural hearing loss can be divided into three categories, preventative, prosthetic and regenerative. Inexpensive drugs have already been tested to protect the auditory system from the deleterious effects of noise or prescribed, ototoxic drugs [19,20]. Protein kinase inhibitors that block apoptosis via c-Jun N-terminal kinases provide effective protection against acoustic trauma and ototoxicity [21,22]. Cochlear implants can partially replace the function of lost, auditory sensory cells and even the primary sensory innervation [23]. Regenerative treatments are not available. However, during the past few years our knowledge of the development and genetics of the auditory system has increased dramatically and research has revealed clear potential for gene and cell therapies [24]. These advances allow us to search more effectively for potential drug targets.

**Noise and ototoxic drugs**

Noise-induced hearing loss (NIHL) is the major cause of avoidable, permanent hearing loss, accounting in part for about a third of affected people in developed countries. Although protection from excessive noise is desirable, uncontrolled exposure will remain a serious problem for the foreseeable future. Despite the fact that the prevalence of hearing loss could be cut in half by responsible care within social and industrial environments, there remains a substantial need for curative as well as preventive treatments. Very recent warnings from the Royal National Institute for Deaf People (RNID) urge music fans to limit the maximum volume of their iPods, because the enthusiasm for MP3 players promises irreversible hearing damage for this generation of users (www.rnid.org.uk). In some circumstances, for example in the military, exposure to noise is hard to avoid. Thus, it is important to uncover the pathogenic mechanisms of NIHL and to develop effective preventive medications.

Prescribed, ototoxic drugs, principally the aminoglycoside antibiotics, account for ~3–4% of hearing loss in children and adults in developing countries and a significant number of adults in developed countries [25]. The use of ototoxic drugs is justified in the face of life-threatening conditions and, as with NIHL, greater knowledge of the molecular mechanisms will help in the discovery of effective preventive medications.

Noise and aminoglycoside antibiotics have been used to produce animal models of hearing loss for some time and now we have a reasonably clear picture of the immediate pathology. Excessive noise can cause structural damage to the hair bundles and can generate excitotoxic effects on the sensory nerve terminals [26]. Hair cells die by apoptosis and are removed as the apices of the surrounding supporting cells converge to seal the epithelium without compromising the composition of the endolymph [27,28] (Figure 2). Unlike supporting cells in non-mammalian epithelia, mammalian supporting cells in the organ of Corti do not proliferate to replace lost hair cells and they do not naturally change their phenotype. Loss of hair cells leads to loss of spiral ganglion neurons.
(SGNs) [29], which depend on hair cells for the production of survival factors such as the neurotrophin NT-3 and the brain-derived neurotrophic factor (BDNF). Otoxic drugs can cause death of SGNs directly, although for amino-glycosides the effects appear to be indirect and related to loss of hair cells [30,31]. The degeneration of SGNs following hearing loss in humans is variable and rarely complete [32,33].

Surprisingly, little is known about the molecular events associated with hair cell degeneration in the longer term and it is not clear whether the failure to replace hair cells is predominantly a function of inhibitory signals, as occurs in the spinal cord [34], or a lack of regenerative potential in the supporting cells. Nevertheless, accumulation of free radicals, excitotoxicity mediated by glutamate receptors and activation of apoptosis are predictable players in the loss of cells. Animal experiments show that growth factors and drugs directed against apoptosis, excitotoxicity and oxidative stress can provide valuable protection from hearing loss if applied during exposure [19]. For predictable exposure to noise or ototoxic drugs preventative treatments are already available and the market for them will remain substantial for the foreseeable future.

**Age-related hearing loss**

Age-related hearing loss (AHL or presbyacusis) is extremely complicated and includes the effects of both NIHL and ototoxic drugs [35]. Figures for the UK population reflect those for developing countries (www.mrid.org.uk). Moderate hearing loss, for which hearing aids are usually recommended, affects 1.6% of people from 16–60 years old, 16.5% of those between 61–80 years old and 57.9% of those over 80 years old. Combined with the effects of increasing lifespan and of leisure noise on younger generations, these numbers reveal the huge scale of the problem in the future.

AHL shares many of the features of classical neurodegenerative diseases, such as Parkinson’s disease, motor neuron disease or Alzheimer’s disease. Functional deficits are associated with irreversible losses of specific cell types. AHL is consistently associated with a loss of OHCs and a smaller decrease in the numbers of IHCs, which are lost progressively from the high-frequency end of the cochlea. These losses are usually associated with a decrease in the number of SGNs. As noted in the context of NIHL, these cells are susceptible to oxidative stress and they can be protected to some degree by antioxidants or possibly growth factors. However, the underlying causes of AHL are not known and the long timescales involved preclude the use of preventive drugs such as those used to treat NIHL.

The only way to treat AHL biologically is to replace lost hair cells and SGNs. This might be ineffective if the cause of cell death is indirect and has not been treated. Hair cells and SGNs often die first because they are the most vulnerable cells rather than because they malfunction [36]. AHL must certainly have a genetic component but is it simply a function of continuous accumulation of insults, such as noise, or part of a programmed decline? Intriguingly, most forms of inherited deafness are related to mutations in connexin genes [37,38]. Connexins are membrane proteins that provide low resistance electrical pathways between cells and permit exchange of small molecules, such as calcium ions, potassium ions and ATP. Hair cells do not express connexins but supporting cells do and they use them to form a functional syncytium, from which the hair cells are electrically isolated. One proposed function of supporting cells is to take up potassium ions that are pumped out across the hair cell basolateral membrane and recycle it back to the blood system or the scala media [39]. In mouse models of connexin mutations the hair cells die by apoptosis [40]. Replacing them or the SGNs would not solve the problem. Cochlear homeostasis is crucial and it is important to assess the contribution of other cells such as fibrocytes, which are distributed throughout inner-ear tissues [41,42]. Similarly, if the blood supply to the stria vascularis becomes less efficient with age, it can influence the endocochlear potential, which in turn will affect the hair cells. Ischemia is quite quickly followed by hair cell death and progressive degeneration of the stria could be an important factor in AHL [43]. However, despite suggestions that conditions such as atherosclerosis and hypertension cause hearing loss, the relationship might only be due to a shared association with ageing [44,45]. Bearing this issue in mind for future research programs, hair cell and SGN regeneration remains a potential option for AHL, which is a substantial, increasing cause of hearing loss worldwide and particularly in developed nations.

**Genetics of hearing loss**

Studies on the genetics of deafness have had a huge impact on our understanding of the development and physiology of the inner ear [39,46,47]. Hundreds of genes are involved in syndromic and non-syndromic hearing loss. Van Camp and Smith created an extremely useful database entitled the hereditary hearing-loss homepage (http://dnalab-www.uia.ac.be/dnalab/hhh/), which provides key information about deafness loci, genes, markers, published references and some gene-expression patterns. During the past 10 years nearly 60 autosomal recessive genes and a similar number of autosomal dominant genes have been discovered and many more remain to be identified. Some late-onset or progressive-deafness genes have been identified recently and much greater attention is now being given to risk factors that underlie susceptibility to noise, ototoxicity and age [48–50]. In therapeutic terms there is unlikely to be a single solution for the many forms of inherited deafness. Apart from mutations in connexin genes, the numbers of people suffering from mutations in any specific gene are small, often involving only a few families.
Cellular targets for regeneration

To stimulate regeneration it is important to identify the potential source of new cells, not only in the healthy ear but also in ears that have degenerated over a period of time. Furthermore, we must consider where any new cells must be located, if they are to be functionally useful. Non-mammalian vertebrates, especially birds and amphibians, regenerate lost hair cells naturally [51,52]. Supporting cells and hair cells share a common progenitor during development [53] and supporting cells are the natural source of new hair cells, either by straightforward conversion [54–56] or by a single asymmetric cell division [57–59]. There is evidence that mammalian vestibular hair cells can be replaced relatively slowly [57,60–62] but no evidence for replacement in the organ of Corti [62,63]. Interestingly, the very limited regenerative capacity of the vestibular epithelia, which are structurally similar to non-mammalian auditory epithelia, suggests that the specialized mechanism of mechanical tuning in the organ of Corti is not the sole reason for its resistance to regeneration. Developmental and genetic studies have revealed a long list of regulatory genes that control morphogenesis, cell proliferation and cell differentiation in the mammalian inner ear [64,65]. It is logical to assume that knowledge of development will inform therapeutic approaches to regeneration. This must be true to some extent but there are differences between the two processes. Tissue environment, including molecular interactions with adjacent cells and connective tissue, has a substantial impact on cell identity and cell fate. Many regulatory genes are expressed transiently during development and the tissue environment changes dramatically with time, so the adult organ of Corti has a very different molecular profile to that of earlier developmental stages. This is reflected in response to extrinsic signals, for example retinoic acid and thyroid hormone [66,67]. Nevertheless, useful information for regenerative purposes is likely to emerge from studies on that period of development, when progenitors are being selected as hair cells, supporting cells or neurons.

The nature of cochlear degeneration and the molecular profile and developmental competence of the cells that remain after longer term loss of hair cells are important factors (Figure 2). This has been studied in the short term following acute insult [68,69] and in animals that suffer from AHL [42], although we still know very little about molecular changes that might influence regenerative responses. Another factor is coupling new sensory cells with the sensory input. Hair cells must be located above the basilar membrane with some mechanical coupling between their hair bundles and the tectorial membrane. Considering all of these issues, in the organ of Corti the target cell population must be the supporting cells, which include inner phalangeal cells, pillar cells and Deiters’ cells.

Spiral ganglion neurons

Less attention has been given to neural regeneration because it tends to be viewed as secondary to the loss of hair cells. Spiral ganglion cell loss is variable and rarely complete in humans, even after long periods of deafness [33]. There is evidence that new hair cells can direct their own innervation from existing SGNs [70] but they are unlikely to be able to stimulate neuronal regeneration. Noise and prescribed drugs can damage SGNs directly and there is a therapeutic interest in regenerating auditory innervation alongside other treatments, such as cochlear implants [71,72]. It might be possible to stimulate proliferation and differentiation of replacement SGNs, either from existing SGNs or the glia but there is currently little experimental evidence to support this idea.

Stem cells

An excellent recent review describes the current state of research on inner-ear stem cells and their therapeutic potential [73]. There are three reasons for studying stem cells. The first is that they can potentially be transplanted into host tissue to replace lost cells. A surprisingly large number of exploratory cell transplantation experiments to the inner ear have already been carried out [74–82]. Cell transplantation is an unpredictable science, in which many experiments are conducted in a highly exploratory manner. The number of variables involved in a given experiment is so great that few studies can be compared directly. This has proved to be a major issue for analysis of cell therapies in Parkinson’s disease [83]. Cells for transplantation include embryonic (ESCs) [78], neural (NSCs) [84], mesenchymal (MSCs) or hematopoietic (HSCs) stem cells [73,85]. There are numerous different stem-cell lines that are prepared and treated in different ways before transplantation. The procedures for transplantation and the state of the host tissue provide further variation. However, there are some excellent animal models for the auditory system, and studies show that transplanted cells can reach the critical areas for repair, particularly the spiral ganglion and the cochlear duct [75,86].

The second reason for studying stem cells is of greater interest in the context of drug discovery. It involves the activation of endogenous, tissue-specific stem cells to effect repair. Growth-factor treatment does appear to awaken dormant stem cells in the hippocampus, following ischemic injury [87]. Stem cells require controlled environments to ensure that they remain undifferentiated and multipotent [88]. Only recently have such cells in a defined ‘niche’, for example in the eye, been discovered [89]. The challenge is thus to uncover a potential niche in the ear and then to find ways of activating the cells within it. The only evidence so far has come from an analysis of cells from the mouse utricular macula [90]. Nothing similar has been discovered in the cochlea. The third reason for working on stem cells is to find out how to differentiate them into the target cell type. Mouse ESCs can be cultured in vitro and transferred into chick otocysts, where they subsequently differentiate as hair cells [91]. The conditions used to prepare these cells before transplantation
are based upon knowledge of early development and are designed to induce gene-expression profiles similar to those of the early otic vesicle. It is not clear how important this conditioning process is or in what way the otocyst influences the ESCs but the preparations should allow these questions to be addressed. Most recently, progenitors for SGNs have been isolated from adult guinea and human auditory tissue and, if they can be cultured reliably and repeatedly, they should provide excellent material for transplantation as well as for studies on neuronal differentiation [92].

Molecular targets for regeneration
Impressive progress has been made in uncovering the genes that regulate proliferation and differentiation in auditory sensory cells. However, many of these regulatory molecules are not suitable as drug targets, which normally include membrane receptors, ion channels, proteases and other enzymes. Nevertheless, they do indicate the relevant molecular mechanisms and provide clues to the signaling pathways, within which suitable drug targets might be identified.

Proliferation of hair cells and supporting cells
In mammalian ears, the hair cells, supporting cells and sensory neurons differentiate during embryonic development (Figure 3). The most detailed studies come from the mouse [93], which has a gestation period of 19–21 days. At embryonic day E9.5, a patch of cells in the neural ectoderm has invaginated to form the otocyst (Figure 3),

FIGURE 3
Development of a mouse inner ear from embryonic day E8 to post-natal day P14 when the ear becomes fully functional. The top row of figures shows the formation of the darkly shaded otic placode (OPI), a patch of cells in the neural ectoderm that are destined to form the inner ear. These cells invaginate to form an otic pit (OPi) and then an enclosed otocyst (O). At E9–E10 the sensory neuroblasts (N) delaminate from the anteroventral region of the otocyst. At E13 the structures of the adult ear are apparent, including the endolymphatic duct (ED), semicircular canals (SCC), saccule (S) and cochlea (C). The lower row of figures shows the otocyst at E10 with delaminated neuroblasts. Hair cells, supporting cells and neurons cease proliferation at E12–14 and start to differentiate. Note that the SGNs migrate away from the tissue in which they are specified to form separate ganglia. The dashed lines in the lower part of the figure indicate the time and location from which some conditionally immortal cell lines have been derived. Lines derived from the locations indicated can be used as in vitro models for the differentiation of neuroblasts and sensory epithelial cells.
a ball of epithelial cells that quickly establishes dorsoventral, mediolateral and anteroposterior axes [65, 94]. Cochleovestibular neurons are selected from the anteroventral region from E9.5 and are among the first cells to differentiate. The cochlea forms from a tubular, ventral projection of the otocyst from E10.5–11.5. The first molecular marker for the organ of Corti in the ventral otic epithelium is the cyclin-dependent kinase inhibitor p27kip1 [95]. Precursors of hair cells and supporting cells at the tip of the cochlear projection exit the cell cycle at about E12.5 and become located in the apical low-frequency region of the cochlea. Proliferation continues at the base and the epithelium elongates until E14.5, when the last cell precursors exit the cell cycle at the basal end.

Cells that differentiate as hair cells selectively down-regulate p27kip1 but they subsequently express another cell cycle inhibitor, p19ink4d [96] and the retinoblastoma protein (pRb) [97,98]. In the null mouse for p27kip1, the organ of Corti develops with a few extra rows of hair cells and supporting cells, as if the proliferation of progenitors has been able to overrun for a short time before being inhibited [99]. Supporting cells normally maintain expression of p27kip1, so it was thought that if the protein could be inactivated in adult cells it might induce proliferation followed by differentiation of a daughter cell as a hair cell. p19ink4d is coexpressed with p27kip1 in the sensory precursors and persists in differentiating hair cells. Mice lacking p19ink4d do not form an abnormal epithelium but, within the first few weeks of birth, hair cells enter the cell cycle and die by apoptosis [96]. The pRb, encoded by the gene Rb1, also regulates the exit of hair cells from the cell cycle [97,98]. Related members of the same family include p107 and p130, which are encoded by Rbl1 and Rbl2, respectively. All three genes can cause cell-cycle arrest if they are overexpressed. Through the critical period of hair cell differentiation in the mouse utricle, from E12.5 to full functional maturity at post-natal day P12, Rb1 is expressed constantly, Rbl1 is upregulated and Rbl2 is downregulated. Hair cells express Rb1 but when this gene is deleted they can re-enter the cell cycle and produce new, functionally mature hair cells. It might be possible to manipulate these cell cycle regulators therapeutically, although it could be hard to produce a coherent effect by targeting them individually. We need to know more about cell-cycle regulation in supporting cells. The function of Rb1 could be therapeutically valuable but the main caveat is that the cellular targets are more likely to be supporting cells, because there is probably less inclination to stimulate regeneration before the hair cells have been lost.

Drugs that influence the cell cycle are focused predominantly on cancer, where the aim is to inhibit rather than to induce cell proliferation, largely with cyclin-dependent kinase inhibitors (CDKIs) applied as anti-tumor agents [100–102]. To stimulate regeneration of hair cells it is necessary to release the inhibitory effects of endogenous inhibitors transiently and to allow limited proliferation of supporting cells. Organotypic cultures of mammalian inner-ear epithelia provide excellent models for investigating these questions and have been used to screen the proliferative effects of growth factors [103–106]. For example, in the mammalian utricle, forskolin activates adenyl cyclase, increases cAMP and leads some cells to enter the cell cycle. This response is enhanced by human recombinant glial growth factor 2 (hrGGF2) and is blocked by inhibitors of membrane-receptor recycling [107]. Such preparations are suitable for larger scale screening to identify cell-specific targets that regulate the cell cycle. CDKIs are also involved in other cellular processes such as apoptosis, cell differentiation and transcription, so research in this area might have an impact beyond that of cell-cycle control [100,108].

Selection and differentiation of hair cells and supporting cells
Proliferation and differentiation are not necessarily separate processes, although hair cells and supporting cells can clearly differentiate in the absence of p27kip1, p19ink4d and Rb1. The selection of neuroblasts from the early otic epithelium, the development of prosensory epithelial patches and the selection of hair cells and supporting cells from within a sensory patch are regulated by notch signaling [67,109–111]. It is possible that the key to regeneration lies in stimulating proliferation and relying on endogenous interactions between notch receptors and their ligands to select an appropriate pattern of hair cells and supporting cells. Notch signaling can instruct cell differentiation by influencing expression of the bHLH genes Math1, Hes1 and Hes5 [67,109,110,112] (Figure 1g). Numerous studies on mutant and null mice reveal that these genes regulate the numbers and pattern of hair cells and supporting cells within the sensory epithelium. Cell fate can potentially be modified by drugs targeted against the notch signaling pathway [113,114]. Mammalian hair cells express relatively low levels of notch1 receptor but the levels in supporting cells are much higher. If inhibition of notch signaling were enough, then loss of hair cells should be sufficient to trigger a fate change in the supporting cells. This appears to happen in birds [115] and it might be possible to trigger it therapeutically in mammals.

The discovery that the POU domain transcription factor Brn3c is necessary for differentiation of all hair cells in the inner ear presented some exciting possibilities [116]. Subsequent experiments revealed that Brn3c is actually a survival factor [117], which regulates expression of gfi (growth factor independent) [118] and BDNF [119]. Brn3c is not able to drive hair cell differentiation when transplanted into sensory epithelia. However, the bHLH transcription factor Math1 (Atoh1), the mouse homolog of Drosophila ‘atonal’, is also required for hair-cell differentiation [120]. There is some debate about whether Math1 is functional in sensory progenitors [121] as opposed to nascent hair cells [122], which is relevant in terms of its influence on hair-cell differentiation during development.
Most exciting, however, is that if it is transfected into cochlear or vestibular sensory epithelia in vitro, then it can induce hair-cell differentiation [123,124]. More dramatically, gene transfection to the guinea pig cochlea in vivo stimulates hair-cell differentiation [70,125] and in adults it leads to measurable functional recovery [125]. Gene transfection is not without its problems therapeutically [126] but these results are extremely promising. New hair cells attract dendrites from existing neurons and thus have the potential to be wired up to the cochlear nerve [70]. Cells transformed in the organ of Corti appear to be derived from Deiters’ cells and retain their contact with the basilar membrane [125]. Most studies to date have been carried out on animal models that have suffered acute loss through chemical or noise-induced damage and it will be important to try the same approach in animal models of longer term hearing loss [42].

Given the fact that Math1 transfection can induce hair cell differentiation in adult ears [125], it is worth looking for drugs that might activate expression of this gene. There are some extremely important tools available to do this, including a Math1 reporter construct that has been used to create transgenic mice [127]. The reporter can be incorporated into inner-ear cell lines [128,129] for HTS of drugs that might activate expression. Interestingly, Id proteins, which regulate various aspects of cell proliferation and differentiation, have been identified as potential drug targets for cancer therapy [130]. They are expressed in inner-ear epithelia and SGNs [131] and might interact with the function of Math1 [132].

Although some functional recovery is possible with Math1 transfection, existing supporting cells are converted into abnormal hair cells without proliferation and this could limit the potential therapeutic benefit. With this in mind, some argue that genes normally expressed earlier in development might have the potential to regenerate a complete sensory epithelium. Mice lacking the transcription factor Sox2 lack hair cells and, based on expression of p27Kip1, also supporting cells [133]. Sox2 is known for its expression in stem cells [133] and might be an important regulator of pluripotency in early sensory progenitors [134]. Whether its expression in adult epithelia, in a cellular environment quite different to that during embryonic development, can lead to regeneration of the whole epithelium remains to be seen. Interestingly, the Sox2-null phenotype in the cochlea is similar to that of a mutant for fibroblast growth factor receptor 1 (FGFR1), which is required for the production of the sensory precursor population [135], and FGFRs are upregulated in supporting cells during regeneration of the chick auditory epithelium [136].

Growth factors and other signaling molecules can clearly change cell fate decisions. The protein sprouty2 (Spry2) is a negative regulator of receptor tyrosine kinases and it appears to antagonize FGF signaling during development of the organ of Corti [137]. In the absence of Spry2 the first row of Deiters’ cells develops as a row of pillar cells. Constitutive activation of the canonical Wnt/β-catenin signaling pathway during embryonic development can convert sensory epithelium in the chick from an auditory to a vestibular phenotype [138]. There is a considerable body of evidence concerning the roles of different growth factors during inner-ear development [139]. Some growth factors have protective effects against predictable hearing loss [140] and there is evidence that they can stimulate a limited regenerative response in mammalian vestibular epithelia [141,142]. Their effects are generally mediated by receptor tyrosine kinases, whose ligand-binding sites and kinase domains present potential drug targets [143]. It is thus important to study these receptors and their downstream signaling pathways in targeted cell types within the inner ear [144,145]. The therapeutic application of growth factors is complicated by widespread side effects and the need to deliver them locally and for long periods. In this context, the inner ear has the advantage of being a relatively enclosed system, even though there is a direct link to the CSF [6].

**Spiral ganglion neurons**

Numerous transcription factors regulate differentiation and survival of SGNs. These include the bHLH factors neurogenin 1 [146], which is equivalent to Math1 in hair cells [147], and NeuroD [148,149], the t-box protein Tbx1 [150], the LIM/homeodomain protein islet-1 [151], the POU-domain factor Bm3a [152] and the zinc finger factor Gata3 [153–155]. Furthermore, during early stages of cochlear neuroblast development, FGF1, FGF2 and the insulin-like growth factor 1 (IGF-1) are important for proliferation, differentiation and survival [156–160]. There is also a substantial literature on BDNF and NT-3 [161–164], which are secreted by hair cells and some supporting cells and which not only influence neuronal survival but also some of the more subtle electrical properties that differ between the apical and basal ends of the cochlea [165,166]. Applied together, the two factors can reduce SGN degeneration and enhance dendritic growth several weeks after deafening in adult guinea pigs [72]. This kind of treatment has potential applications for cochlear implants not only to enrich the interface between the dendrites and the implant electrodes but also to minimize surgical trauma during implant insertion. Survival of postnatal SGNs also depends upon neuregulin, which mediates reciprocal interactions between them and adjacent supporting cells [167].

**Gene arrays and proteomics**

Gene arrays and proteomics provide the opportunity to look for relevant signaling pathways and functionally related groups of molecules [168]. Affymetrix oligonucleotide arrays were used to profile gene expression during development of the mouse utricular macula and this provided clues to the recent work on retinoblastoma proteins...
as regulators of hair cell division [98]. Similar arrays were also used to identify the gene for growth factor independent 1 (gfi1) as a downstream target of the POU domain factor Brn3c [118]. cDNA arrays have been used to study gene expression in different development compartments of the early mouse otocyst [169]. Custom-made human cDNA arrays have been used to analyze differences in gene expression in the regenerating and quiescent chick cochlea [170]. Plasticity of the central auditory pathways has also been studied with cDNA arrays [171,172].

In complex tissues, these studies are challenging because many regulatory genes have different functions in different cells at different stages of development. It thus becomes difficult to look at specific processes in individual cell types. This issue can be addressed by establishing cell lines from the inner ear [129]. There are now many lines available and the majority of them are conditionally immortalized [128,173–176]. Conditional immortalization allows cells to be isolated and transformed from specific times and locations during development (Figure 3) [177]. The cells can be cloned and expanded but then ‘differentiated’ under controlled conditions after inactivation of the immortalizing gene. This approach has been used with affymetrix oligonucleotide arrays to plot temporal profiles of gene expression with time [178]. Genes that share similar temporal expression profiles are likely to be functionally related, even if they are not linked directly by transcriptional regulation. The transcription factor Gata3 is essential for the development of the mammalian ear [153,155] and is especially important for the formation of the spiral ganglion [154,155]. It is also upregulated during regeneration of the chick cochlea [170]. It has been functionally linked to the control of proliferation in hematopoietic cells and in a cochlear epithelial cell line it shares a close temporal profile to the CDKIs p27kip1 (Figure 4) and p21. These cell lines can provide not only important information on gene networks but also the tools for studying in vitro interactions among proteins for undertaking large-scale drug screens [179]. Proteomics approaches are still in their infancy in the auditory system [180], although it is now a priority for funding at the National Institutes of Health [181].

Animal models and the move to human tissue

Hearing research is endowed with a wide range of animal models, which can be used to explore the nature of deafness and to assay the functional effects of experimental treatments, including gene transfection, drug delivery and cell transplantation. Rodents provide models for NIHL, drug-induced hearing loss, specific loss of SGNs, progressive and age-related hearing loss. Additional animal models such as the zebrafish [182] help us to understand genes involved in various forms of human deafness and this will be true for the worm, Caenorhabditis elegans, and Drosophila, which have provided clues to some of the most important developmental genes. The newt, a master of regeneration, offers preparations in which one can study the molecular mechanisms regulating cell proliferation and differentiation of new hair cells in adult vertebrates [55,183]. Nevertheless, there is a clear need to work with human tissue where possible. Human progenitors of SGNs have been cultured as neurospheres and can differentiate as neurons in vitro [92]. These cells will provide candidates for transplantation but will also be important for studies on proliferation and differentiation.

Conclusion

Deafness presents one of the largest global markets for drug development, and basic research has opened up many promising lines of research for preventive and regenerative treatment. This is backed by an impressive repertoire of experimental preparations from the most elementary in vitro models of cell proliferation and differentiation to functional analysis of the auditory system in vivo. Cell lines, stem cells, organotypic cultures and a wide range of animal models provide the key components for drug discovery and development. There is now an unparalleled opportunity to take the highly productive basic research of the past 10–20 years and focus very firmly on its translation to clinical application.


Naito, Y. et al. (2004) Transplantation of bone marrow stromal cells into the cochlea of chinchillas. *Neuroreport* 15, 1–4


Tatematsu, K. et al. (2000) Fate of neural stem cells grafted into injured inner ears of mice. *Neuroreport* 14, 1677–1681


Erkan, L. et al. (1996) Role of transcription factors Bm-3.1 and Bm-3.2 in auditory and visual system development. *Nature* 381, 603–606

Xiang, M. et al. (1998) Requirement for Bm-3c in maturation and survival, but not in fate determination of inner ear hair cells. *Development* 125, 3935–3946


Branca, M.A. (2005) Gene therapy: cursed or...
146 Ma, Q. et al. (1998) neuropegin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20, 469–482
149 Kim, W.Y. et al. (2001) NeuroD-null mice are deaf due to a severe loss of the inner ear sensory neurons during development. Development 128, 417–426
151 Li, H. et al. (2004) Ileit-1 expression in the developing chicken inner ear. J. Comp. Neurol. 477, 1–10
152 Huang, E.J. et al. (2001) Bm3a is a transcriptional regulator of soma size, target field innervation and axon pathfinding of inner ear sensory neurons. Development 128, 2421–2432
Tinnitus: neurobiological substrates

Jos J. Eggermont

Tinnitus is an auditory phantom sensation of ringing in the ears that is experienced when no external sound is present. It is a prevalent disorder that is frequently caused by insults to the peripheral auditory and somatosensory systems, especially in the elderly. This creates an imbalance between inhibitory and excitatory transmitter actions in the midbrain, auditory cortex and brainstem (where neural activity from somatosensory and auditory stimuli interact). This imbalance causes hyperexcitability often leading to the perception of phantom sounds. Although changes in transmitter–receptor systems have become better documented, there are currently no proven drug treatments for humans. Methods for preventing tinnitus have been demonstrated in animal studies.

Animal models

The neural substrate of tinnitus can only be adequately studied in animal models that show evidence of tinnitus under similar conditions to humans. Behavioral test models have been devised for rats [1–3], hamsters [4] and mice [5]. The findings have been taken as evidence that conditions that cause tinnitus in humans and these particular animal models will also cause tinnitus in other experimental animals, such as chinchillas, guinea pigs and cats. In cats, rats, mice and hamsters, changes in spontaneous neural activity in auditory nerve fibers (ANFs), the dorsal cochlear nucleus (DCN), the inferior colliculus (IC) and the auditory cortex have been recorded following the application of a tinnitus-inducing agent.

Tinnitus-inducing agents include excessively loud noise, salicylates, quinine, aminoglycoside antibiotics and cisplatin. In general, the spontaneous firing rates (SFRs) in ANFs decrease or stay the same after administration of these agents [6–9], although a near-toxic dose of salicylate has been shown to cause increased spontaneous firing rates in ANFs [10].

Contrasting with this reduced firing in the auditory periphery is the general finding of increased spontaneous activity in central auditory system structures after noise trauma or low doses of ototoxic drugs.
These structures include the DCN [11–15], the external nucleus of the inferior colliculus (ICx) [16,17] and the secondary auditory cortex (AII) for salicylate and quinine [18], and the primary auditory cortex (AI) for noise trauma [19,20]. However, in the central nucleus of the inferior colliculus (ICc) in mice, no changes in SFR were found months after chronic salicylate administration or noise trauma [21]. Figure 1a shows the various findings superimposed on a simplified wiring diagram of the auditory nervous system. These findings of increased SFR have been attributed to reduced levels of central inhibition [probably γ-aminobutyric acid-(GABA)-ergic] in central auditory structures [2,22,23] leading to neural hyperactivity in IC [24].

In contrast to the lack of change in SFR [21], strong c-Fos immunostaining has been found in the ICc of rats, with little in the DCN and none in the ventral cochlear nucleus (VCN) after five days of chronic application of salicylate [25]. However, after one large dose of salicylate very little c-Fos- or arg3.1-related activity was found in the IC, whereas elevated levels were evident in auditory cortex and amygdala [26]. The findings cited above potentially support the previously proposed contribution of the extralemniscal pathway (DCN, ICx and AII) in acute salicylate- and quinine-induced tinnitus [27]. This is somewhat different from noise-induced tinnitus, which shows a nearly immediate, [20] as well as long-term [19], increase in the spontaneous firing rate in primary auditory cortex (AI) but not in ICc [21]. Presumably, the changes in SFR might originate in the AI, propagate to the AII and then centrifugally affect the ICx and DCN. Clearly, more studies are needed to address this issue.

**Transitont and chronic tinnitus**

It is likely that there are different causes for immediate and long-term changes in SFR after the application of tinnitus-inducing agents. Most drug studies cited above have been acute and neural changes have been recorded within a few hours after drug application. These studies could have overlooked certain effects that only manifest after chronic sound or drug application and slow induction of tinnitus-like phenomena. Although large one-time doses of salicylate will cause transient tinnitus in humans, chronic use of low therapeutic doses of salicylate (e.g. in rheumatic arthritis) will cause tinnitus only in the long run, which is typically reversible and does not inevitably lead to hearing loss. Notable studies that explored the long-term effects of salicylate application in guinea pigs using the average frequency-spectrum of round-window electric noise (known to be generated by ANF spiking activity [28]) showed that the spectrum level went down in the first few days after the start of the application, in agreement with SFR results. However, in the course of the first few weeks of application, the spectrum level increases substantially without changes in the hearing threshold. This change in spectrum level, particularly manifested at frequencies around 1 kHz, has been credited to increased synchronization of nerve fibers spiking. An alternative explanation is an enhanced subthreshold resonance in the ANF dendrites that is caused by the activation of voltage-controlled Na⁺ channels [29]. A consequence of this resonance might be an increased probability of doublet-spike firing, as observed following noise trauma in cat ANF [9].

**Peripheral cause, central effect**

In the AI, SFR recordings have been made from the same neurons before and up to six hours after noise exposure. The immediate effects of a one-hour exposure to very loud pure tones were an increase in threshold for the characteristic frequency range above the tone frequency but without an immediate change in SFR. However, after approximately two hours after exposure, SFR had increased significantly whereas response-threshold values improved to ~25 dB above pre-exposure levels [20]. Several weeks after the exposure, hearing losses had typically recovered further but SFR remained increased [30], even in regions where no significant hearing loss could be measured [19].

A notable finding was the increased synchrony in the spike firing by neurons immediately after the trauma [20], which increased in the following hours. This neural synchrony decreased after several weeks to slightly, but still significantly, elevated levels compared to controls [19]. A similar increase in spike-firing synchrony was found 45 min to 2 h after quinine administration [31]. It is not clear at present if the increased spike-firing synchrony has a causal relationship with tinnitus but in the cases cited it was always a consistent firing even without concomitant increases in SFR.

It is intriguing that in the DCN the increase in SFR only became significant 2–3 days after exposure to 140 dB sound pressure level (SPL) noise [12]. This could indicate that these changes are truly plastic and result from a homeostatic adjustment to a reduced drive from the auditory periphery, whereas the more immediate effects in the cortex could be caused by a fast downregulation of

**TABLE 1**

<table>
<thead>
<tr>
<th>Epidemiology of tinnitus¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of subjects</strong></td>
</tr>
<tr>
<td><strong>Traumatic</strong></td>
</tr>
<tr>
<td>Noise (long duration or transient)</td>
</tr>
<tr>
<td>Head and Neck Injury including whiplash</td>
</tr>
<tr>
<td><strong>Medical</strong></td>
</tr>
<tr>
<td>Otolologic</td>
</tr>
<tr>
<td>Drugs, medication</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Other</td>
</tr>
</tbody>
</table>

¹Data from the Tinnitus Archive, second edition, Oregon Health and Science University (www.tinnitusarchive.org).
²Factors as reported in questionnaires by tinnitus sufferers interviewed between December 1981 and August 1994.
GABAergic activity. It is also possible that corticofugal activity has a role in the gradual changes observed in the DCN [32].

After long-term administration of cisplatin [12] it was found that, as long as the outer hair cells (OHCs) were intact, there was no increase in spontaneous activity in the DCN. In cases of severe damage of the OHCs, the spontaneous activity in the DCN increased, but less so if the inner hair cells (IHCs) were also damaged. This led to the hypothesis, as suggested previously [33], that tinnitus only arises after selective damage of the OHCs and putatively by a loss of activation of the granule cells in the DCN by type II ANFs that innervate the OHC. This in turn would lead to reduced activation of the cartwheel and
stellite cells and thereby in a disinhibition of the fusiform cells, which form the output of the DCN to the IC.

The findings of Cazals et al., [28] showing that the noise spectrum recorded from the round window during chronic salicylate treatment changes only after several days, also opens the possibility that the late changes in the DCN after noise trauma [12] are caused by slow increases in the spontaneous activity (or burst-firing) in ANFs. The increased SFR in the DCN must ultimately become independent of ANF input because subsequent sectioning of the auditory nerve had no effect on the SFR in the DCN [34].

Because most salicylate studies have been acute (i.e. recordings were made within a few hours after administration), the findings of an unchanged SFR in the ANFs [6,7] and increased SFR in the ICx [15–17] can not rule out a peripheral component for tinnitus caused by chronic salicylate treatment [3,28]. However, the effects of noise trauma on ANFs were all investigated after the establishment of the permanent threshold shift, so the decrease of spontaneous activity in these ANFs, combined with increases in the AI, requires a central source of the ensuing tinnitus. This source is probably not the ICx [21].

**Causes of tinnitus might be multisensory**

The second largest cause of tinnitus (after insults to the cochlea) is putative abnormal activity in the somatosensory system [35–37] resulting from head and neck injuries, whiplash and various mandibular and dental problems [38]. Nerve fibers from the trigeminal ganglion, dorsal column nuclei and trigeminal nuclei innervate the CN, superior olivary complex (SOC) and IC. The ophthalmic and mandibular divisions of the trigeminal ganglion innervate the magnocellular and granular regions of the VCN, respectively. In addition, the cuneate nucleus forms the source of the mossy fibers in the DCN. The mandibular division is partly in the middle-ear reflex circuit. The trigeminal circuit is also part of the olivocochlear feedback loop. In combination, the interaction of the somatosensory systems with the auditory system provides for powerful feedback loops that regulate peripheral sensitivity (Figure 1a).

The DCN is an important integration site for auditory and somatosensory information (e.g. from the pinnae [39]) but influences of trigeminal nerve activity are also evident in the VCN [40]. Imbalances between the auditory and somatosensory input can lead to imbalances between excitation and inhibition, either by reduced auditory input (as caused by noise trauma) [13,14] or, putatively, after increased somatosensory input following injury or inflammation.

**A role for calcium**

Intracellular Ca$^{2+}$ has a role in regulating the balance between inward and outward currents in neurons and hair cells. The function of the hair cells also depends on the Ca$^{2+}$ signaling pathways governing the fast neurotransmitter exocytosis of IHCs and the slow motility changes of the OHCs. There is increasing evidence of a role for Ca$^{2+}$ in the fast transduction process in hair cells [41]. The effects of noise, salicylate and quinine include a sustained increase in the Ca$^{2+}$ concentration in hair cells [42]. Salicylates also cause a dose-dependent decrease in the free perilymphatic Ca$^{2+}$ concentration [43]. Decreasing the extracellular Ca$^{2+}$ concentration [44] can result in burst-firing behavior in neurons. Increased burst-firing was observed after salicylate application in ICx [16] but not in ICx [21]. During noise exposure, there is a very large transient increase in the endolymph Ca$^{2+}$ concentration, similar to the sustained Ca$^{2+}$ increase observed in animals with experimentally induced endolymphatic hydrops (the animal model for Ménière’s disease) [45]. Tinnitus, sustained as well as transient, is one of the defining characteristics of Ménière’s disease.

**Glutamate neurotoxicity**

Excess glutamate, kainate and α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA) all cause ANF dendrite swelling followed by membrane disruption, whereas N-methyl-α-aspartate (NMDA) application does not. Continuous release of glutamate from intact IHCs induces growth of new dendritic processes after noise trauma damage [46]. This regrowth is probably the cause of a reduction in noise-induced hearing loss following recovery in an enriched acoustic environment compared with recovery in a quiet environment [20]. Guitton et al. [3,47] suggest that salicylate-induced tinnitus results from inhibition of cyclooxygenase activity resulting in altered arachidonic acid metabolism, which potentiates NMDA-receptor currents in the cochlea. The increased opening probability of NMDA receptors can result in burst or epileptiform firing activity in ANFs, potentially leading to tinnitus. Such bursting activity has been found in some ANFs after noise trauma [9].

**Glycine and GABA downregulation and glutamate strengthening**

Noise exposure lowers GABA-mediated inhibition in the IC [48]. Glutamic acid decarboxylase (GAD) levels in the IC increased immediately after noise exposure but returned to lower than control values 30 days after exposure [22]. Because GAD is the rate-limiting enzyme in the formation of GABA, an increase in GAD concentration suggests an initial upregulation of the reservoir pool of GABA after the trauma (probably as a compensatory mechanism) but a downregulation later. In the first week after exposure to unilateral noise trauma [49], electrically evoked glutamatergic transmission in the ipsilateral VCN slice increased, whereas its uptake was depressed. In the DCN, glutamate release was increased and uptake was unchanged. At 14 days after exposure, glutamatergic release and uptake were lowered, probably because of the degeneration of ANFs. At 90 days after exposure, glutamatergic release and
AMP-A receptor binding were sharply increased. This was understood to be caused by neuro-plastic mechanisms similar to those observed after unilateral cochlear ablation. The findings are consistent with a noise-induced strengthening of glutamatergic transmission in VCN and DCN leading to hyperexcitability in the auditory pathways [24]. Surprisingly, spontaneous glutamate release measured by hyperfusion in slice was not affected by noise exposure.

After salicylate application, an upregulation of GAD and a decrease in GABA receptor affinity was observed in the IC of rats showing behavioral evidence for tinnitus [2]. Interestingly, in aging animals, there was an upregulation in the number of GABA receptors, probably to compensate the significant loss of presynaptic GABA release [50]. The reduced GABA release might explain the increasing incidence of tinnitus in the elderly who have suffered moderate noise-induced hearing loss earlier in life.

A more drastic alteration of cochlear output, compared with the usually partial noise- or drug-induced hearing loss, is found after unilateral removal of one cochlea. In that case, there occurs a downregulation of bilateral glycine release in DCN and a reduction in the number of glycine receptors in VCN and lateral superior olive (LSO), as well as a strengthening of glycinergic activity in the medial superior olive (MSO) [51,52]. In the IC, GABA uptake is downregulated and D-aspartate uptake is bilaterally upregulated [53]. The commonality of the effects is shown in Figure 2b.

Tinnitus reflects the nasty side of neural plasticity
Animal research, as reviewed above, has shown the response properties of neurons following ototoxic drugs and hearing injuries, and pointed to changes occurring in the balance of excitation and inhibition at multiple levels of the auditory pathway [2]. It is reasonable to assume that the effect of this change in balance in the central nervous system and the auditory cortex contributes in some way to tinnitus. One change that has been well documented is alteration of tonotopic maps in the AI after noise-induced cochlear damage (Figure 2). In the intact auditory cortex, there is an orderly representation of spectral frequency across the auditory cortex in a caudal-rostral direction; the tonotopic map reflects place-coding of sound frequency by the cochlea. After noise trauma, and probably also after other traumatic hearing losses, the tonotopic organization in the cortex is changed such that cortical neurons with characteristic frequencies (CFs) in the frequency region of the hearing loss no longer respond according to their place in the tonotopic map, but reflect instead the frequency tuning of their less affected neighbors (Figure 2b [20,30]). Neurons with CFs in the affected region also show increased spontaneous activity and increased neural synchrony [19,20]. These results point to a potential link between reorganization of the cortical tonotopic map, changes in neuron SFRs and tinnitus [32].

These changes in response properties of neurons, and changes in cortical tonotopic map organization, which are induced by noise exposure and other tinnitus-inducing agents, do not occur in isolation of one another. Decreases in intracortical inhibition and increases in SFRs after the loss of peripheral input to central neurons can promote the development of synchronous spiking activity [19,20] by prolonging postsynaptic depolarization and increasing the likelihood of temporally coincident inputs converging on synapses. In the normal central auditory system surround inhibition (the inhibition surrounding the excitatory part of the receptive field of a neuron) produced by thalamocortical input would be expected to restrict synchronous activity to neurons tuned to properties of the acoustic stimulus, thereby leading to normal auditory perception. However, when the constraints of intracortical inhibition are weakened, distributed synchronous spike-firing activity can develop [20] and stabilize over wider cortical territories, leading to the perception of sounds that are physically absent (tinnitus).

Chronic tinnitus and chronic pain display considerable similarities, including plastic changes in the central nervous system leading to hypersensitivity to sensory stimuli and a change in the way those stimuli are perceived. Involvement of the sympathetic nervous system has been postulated in chronic pain and tinnitus [54]. Tinnitus has been classified among the positive symptoms that arise after lesions of the nervous system [55], sharing with neurogenic pain the phenomenon of low-threshold calcium spike-burst firing in the medial thalamus. Another example of similarities in tinnitus and pain is that the vanilloid receptor type 1 (VR1) is expressed in the spiral ganglion of rats [56]. VR1 is commonly expressed in dorsal root and trigeminal ganglion cells and allows us to appreciate the painful effect of hot peppers. In case of an inflammatory response, arachidonic acid can be metabolized by lipooxygenase, and its metabolites act as agonists at the VR1 binding site. This could provide another mechanism for hyperacusis and tinnitus.

Prevention and treatment of tinnitus
Drug treatment of tinnitus in humans has been largely unsuccessful, although Xanax® (Pfizer) has been shown to reduce the loudness of tinnitus slightly [57], the only consistent (but short-lived) relief being that provided by lidocaine infusion. In an animal model [17], the effect of lidocaine on IC neurons that showed increased SFR after salicylate application was short-lived (~5 min) and did not affect all neurons similarly. Successful prevention of tinnitus in animal models includes: administration of an L-type Ca2+ channel blocker (nimodipine) that prevented quinine-induced tinnitus [58]; dietary supplements of CaCl2 in drinking water three days before application of salicylate in guinea pigs [43]; application of NMDA antagonists in the cochlear perilymph of rats blocked the behavioral evidence of tinnitus after salicylate
application [3]; and post-trauma rearing of cats in an enriched acoustic environment that spectrally matches the inverse of the hearing loss region [59].

**Implants and tinnitus**

Cochlear implants can reduce tinnitus volume and awareness in 86–92% of patients and rarely (<10%) enhances it.
Cochlear implants did slightly better than hearing aids in reducing tinnitus: 54% in cochlear implant patients versus 48% in hearing-aid users [62]. As yet, the mechanism of action remains unknown, but it probably provides a more balanced cross-frequency input to the brain, perhaps similar to that provided by an enriched acoustic environment [59].

**Conclusions**

Transient and long-standing tinnitus probably have different underlying mechanisms. Findings on acute tinnitus point to a neuroexcitotoxic effect that increases glutamatropic pathway activity, whereas long-standing tinnitus requires changes that include plastic as well as homeostatic mechanisms that resemble those of chronic pain. These mechanisms also cause changes, which have been linked to tinnitus, in the organization of the cortical place-frequency map. In transient and long-standing tinnitus, SFRs are increased in the auditory central nervous system. The non-lemniscal auditory system might have a key role in tinnitus generation because it is more sensitive to drug-induced tinnitus and provides a substrate for interaction between the auditory and somatosensory systems. Prevention of tinnitus in animal models shows promise, but drug treatment of long-standing tinnitus in humans has so far been unproven.

**Acknowledgements**

Support was provided by the Campbell McLaurin Chair for Hearing Deficiencies.
51 Suneja, S.K. et al. (1998a) Plastic changes in glycine and GABA release and uptake in adult brain stem auditory nuclei after unilateral middle ear ossicle removal and cochlear ablation. Exp. Neurol. 151, 273–288
56 Balaban, C.D. et al. (2003) Type I vanilloid receptor expression by mammalian inner ear ganglion cells. Hear. Res. 175, 165–170
62 Mo, B. et al. (2002) Tinnitus in cochlear implant patients—a comparison with other hearing-impaired patients. Int. J. Audiol. 41, 527–534
Compounds for the prevention and treatment of noise-induced hearing loss

Eric D. Lynch and Jonathan Kil

Noise-induced hearing loss (NIHL) is the leading occupational disease and a major contributor to the development of age-related hearing loss. The pharmacological prevention and treatment of NIHL has been under preclinical investigation for the past 20 years. Promising treatments have now been identified and entered into clinical development. Within the next five years, safe and effective drugs could be approved as the first generation of otoprotectants. This review covers strategies that are under investigation for NIHL. Drugs that effectively prevent and treat NIHL will have a significant impact on medical costs, disability compensation and several issues affecting the quality of life.

Noise is the greatest causative factor among the defined etiologies of hearing loss. Traditionally, prevention of noise-induced hearing loss (NIHL) has been addressed by providing wearable hearing protection and reducing noise emissions. However, for many occupations this has been insufficient, especially when noise levels exceed 130–140 decibels (dB).

According to the National Institute for Deafness and Communication Disorders (NIDCD), the American Speech, Language and Hearing Association (ASHA), and the Occupational Safety and Health Administration (OSHA) >30–40 million Americans are exposed to hazardous sound or noise levels on a regular basis. NIHL affects ~10–15 million people, of all age groups, in the USA [1,2]. NIHL is the leading occupational disease, a significant cause of disability and a major cost to society.

Many noisy occupations such as the military [3,4], construction [5,6], manufacturing, mining [7], forestry [8], farming [9], aviation [10,11], rail [12] and trucking [7] report the urgent need to develop hearing-conservation programs. Whereas annual surveillance and compliance remains an ongoing issue, the efficacy of hearing-protection devices (e.g. earplugs) and hearing-protection measures (i.e. reduced noise exposure time) could be augmented by pharmacological agents that might reduce NIHL more effectively.

In Table 1, OSHA-regulated exposures to noise are detailed according to sound pressure level, which is measured in dB as a logarithmic scale of sound intensity. Every 3 dB increase is a doubling of sound intensity. For practical purposes, OSHA states that every 5 dB increase in sound exposure level requires a 50% reduction in exposure time or duration. OSHA recommends that no one should be exposed to >140 dB of sound, even for brief periods.

Personnel in certain military and industrial occupations are at extreme risk of developing NIHL as a result of noise exposure levels often exceeding 120 dB. For example, the M16 rifle (routinely used during basic training and annual weapons qualification exercises) discharges at 156 dB. A common result is the development of a temporary threshold shift (TTS). With multiple, cumulative exposure events, significant irreversible hearing loss can occur that produces a permanent threshold shift (PTS).
Recent acute-noise-exposure data from a US Army Special Forces study reported 11% of 72 subjects who underwent live-fire weapons-training exercises for three days sustained permanent hearing threshold shifts [13]. A survey of US Marines exposed to live-fire exercises for 3–5 days indicated that 11% of this group had noise-induced PTS [13]. These data are consistent with the 1994 findings of Attias et al. [14], where 11% of 150 basic training recruits from the Israel Defense Forces experienced significant threshold shifts following 56 days of live-fire exercises. Evaluating hearing within a few hours of exposure to measure TTS (a dangerous condition in high-risk, communication-intensive environments) could double or triple this number. Furthermore, analyzing hearing loss at even one important speech frequency (e.g. 2, 3 or 4 kHz) would generate a much higher incidence level.

Another inner-ear disorder that is highly correlated with acute and chronic NIHL is tinnitus [15,16]. In a retrospective study of 3466 claimants who sought compensation for occupational NIHL, the prevalence of those reporting tinnitus as a function of hearing loss at 4 kHz saturation for occupational NIHL, the prevalence of those reporting tinnitus as a function of hearing loss at 4 kHz ranged from 41.7 to 56.5%, regardless of the amount of exposure to measure TTS (a dangerous condition in high-risk, communication-intensive environments) could double or triple this number. Furthermore, analyzing hearing loss at even one important speech frequency (e.g. 2, 3 or 4 kHz) would generate a much higher incidence level.

### Occupational hearing loss and hearing conservation

Military hearing-conservation programs (HCPs) are limited in their ability to control exposure to high level noise, generated from weapons fire and explosives [18], partly as a result of inadequate sound attenuation by hearing-protection devices (HPDs). Personnel required to wear HPDs are often noncompliant, citing poor fit, restricted head movement, discomfort and reduced communication ability among their reasons. In combat situations, military personnel are less likely to use HPDs because of impaired communication and the need for enemy detection. Hearing loss increases with increasing noise exposure and number of years in service, especially for those in armor, artillery and infantry branches [19].

In a survey of 12,492 medical records to evaluate the Navy’s HCP, Wolgemuth et al. [3] found that the incidence of significant threshold shift (STS) was 29% (SD = 11.1%). In the absence of criteria establishing acceptable levels of STS in HCPs the authors concluded that the incidence of STS ‘may be too high’, especially in certain job categories.

<table>
<thead>
<tr>
<th>SPL (dB)</th>
<th>Duration</th>
<th>Sound source</th>
<th>Industry</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>&lt;1 min</td>
<td>Firearms, jet engine</td>
<td>Military, aviation</td>
</tr>
<tr>
<td>130</td>
<td>&gt;1 min</td>
<td>Drop forge, jackhammers</td>
<td>Manufacturing, mining, construction</td>
</tr>
<tr>
<td>120</td>
<td>&gt;5 min</td>
<td>Amplified speaker</td>
<td>Musicians, recreational</td>
</tr>
<tr>
<td>110</td>
<td>&gt;15 min</td>
<td>Engines</td>
<td>Rail, trucking</td>
</tr>
<tr>
<td>100</td>
<td>&gt;1 h</td>
<td>Woodshops, chainsaws</td>
<td>Forestry</td>
</tr>
<tr>
<td>90</td>
<td>&gt;4 h</td>
<td>Motorcycles, lawnmowers</td>
<td>Recreational</td>
</tr>
<tr>
<td>85</td>
<td>&gt;8 h</td>
<td>Interior plane cabins</td>
<td>Aviation</td>
</tr>
</tbody>
</table>

Whereas audiogram compliance was in the 80–92.9% range, follow-up compliance was 62%, suggesting that the customary means used to evaluate HCPs were not highly correlated with STS levels. Bohnker et al. [4] analyzed 68,632 monitoring audiograms of enlisted personnel from the US Navy and the US Marine Corps. (1995–1999). They found that ‘hearing deterioration continues to be a significant issue in force health protection’ and stated a requirement for ‘better programmatic processes to prevent hearing deterioration.’ Looking at the industrial sector, Dobie [20] reviewed occupational HCPs and found little evidence of efficacy, which he concluded was usually because of flaws in study methodologies (e.g. treatment and control groups were not matched for age, history of hearing loss, non-occupational noise exposure or use of HPDs, among others). Irrespective of reported limitations, the epidemiology provided by HCPs reveals an urgent need to enhance current levels of hearing protection without additional restriction, discomfort or communication interference.

A drug to prevent and treat NIHL will improve military force performance and readiness, especially in combat where hearing ability can impact on soldier ‘survivability’. OSHA states that on a daily basis, 40 million people in the USA are exposed to hazardous noise levels that might permanently damage hearing. Pharmacological protection will augment hearing conservation in routine training and occupational situations where most NIHL occurs, reducing the compensation costs associated with NIHL across all industries.

### Pathogenesis

In response to sound waves traveling through the cochlea, auditory hair cells in the organ of Corti depolarize following the opening of mechanotransduction channels caused by the physical deflection of the stereocilia on their apical surface. The organ of Corti contains two types of auditory hair cell: inner and outer hair cells (IHC and OHC, respectively). OHCs are organized into three rows and are usually the first hair cells affected. Healthy OHCs contract in response to acoustic stimulation, resulting in an increase in sensitivity (or gain) of ~40–50 dB (active cochlear amplification) [21,22]. Mitochondria are some of the first and most affected intracellular organelles in models of NIHL. IHCs are predominantly sensory in nature and are heavily innervated by the eighth cranial (auditory) nerve.
The constitutive activity of IHCs and OHCs is dramatic given our noisy environments. The amount and type of hair cell damage depends on the frequency, intensity and duration of the noise exposure. Above a specific intensity level, OHCs show signs of metabolic exhaustion with the accumulation of reactive oxygen and reactive nitrogen species (ROS and RNS, respectively). When OHCs are permanently damaged or lost, the threshold sensitivity of the IHC increases (loss of active cochlear amplification) and it is often recorded as a threshold shift or as hearing loss.

Over the past decade, much progress has been made in our understanding of the cellular and biochemical basis of NIHL. Acute exposure to loud noise affects several structural elements in auditory hair cells, including cell membrane and intracellular biochemical pathways [23]. These changes can evoke the formation of free radicals (in particular ROS and RNS) that overwhelm resident detoxification and antioxidant mechanisms [24–27]. Others have shown a greater susceptibility to NIHL in animals and humans with dietary magnesium (Mg) deficiency [28]. Mechanistically, low Mg might contribute to a loss of membrane potential, resulting in altered or decreased sensorineural function.

A major intracellular antioxidant pathway that can detoxify free radicals and attenuate ROS and/or RNS involves the tripeptide glutathione (GSH) [29,30]. Loud noise can reduce GSH and increase the level of oxidized glutathione in the inner ear [31] leaving it prone to ROS- and/or RNS-mediated cell damage. GSH interacts with glutathione peroxidase (GPx), which catalyzes the ability of GSH to act as an antioxidant. Intriguingly, GPx activity also decreases following noise exposure [27].

The additive effect of increased ROS and/or RNS and depleted antioxidant capacity can lead to cell injury or death. Some of the most damaging ROS and/or RNS are those that can oxidize lipids such as hydroxynonenal (4-HNE) and peroxynitrite (ONOO−). These free radicals degrade lipids and damage membrane-bound organelles such as mitochondria and nuclei. Excess ROS and/or RNS generated by elevated hair cell metabolic activity during intense noise exposure could overwhelm the antioxidant buffering capacity of the cell, leading to permanent loss or injury of hair cells [13,32,33].

Otoprotection in preclinical development
Recent studies with antioxidants, N-methyl-D-aspartate (NMDA) antagonists, caspase or cell death inhibitors, and growth factors have some significant design limitations that restrict their direct clinical application [34]. Among these strategies, the use of antioxidants to neutralize ROS and/or RNS is an appealing early intervention step in the prevention of cellular damage in the cochlea. At present, there are no FDA-approved drug products that can reduce or prevent NIHL. However, animal work demonstrates that NIHL can be attenuated by agents that reduce the level or activity of ROS and/or RNS or of free radicals.

One of the earliest compounds tested for prevention of NIHL was allopurinol, a hypoxanthine analogue that acts as an inhibitor of xanthine oxidase and a scavenger of free radicals. Allopurinol is a prescription drug that is FDA-approved for the treatment of gout and hyperuricemia induced by cancer chemotherapy. Systemic injections of high-dose allopurinol (50 mg/kg) into live animals before, during, and after 60 h of continuous noise exposure (90 dB), reduced the level of hearing loss immediately after noise exposure had finished [35]. However, Franzé et al. [36] found that the same dose of allopurinol could only reduce the TTS after intense noise exposure and not the PTS measured at 15 and 30 days post-noise.

Other compounds that have demonstrated some efficacy in preventing NIHL are GSH precursors such as N-acetylcysteine (NAC) and methionine (MET). NAC is a GSH prodruk that, upon de-acetylation to l-cysteine by the liver and local tissues, enhances GSH production [30]. High-dose NAC is FDA-approved as a mucolytic agent for respiratory diseases and can reverse acute hepatic toxicity following acetaminophen overdose. It is given orally or intravenously (i.v.) at 70 mg/kg for 24–48 h. In persons that have normal GSH levels, NAC is well tolerated and is taken orally at 1–3 g per day. NAC has been shown to be ototoprotective when injected intraperitoneally (i.p.) at 325 mg/kg [13]. MET has also been shown to act as an otoprotectant when injected at 200 mg/kg [13,37]. MET is an essential amino acid and can be converted to cysteine, the rate-limiting substrate for GSH production. Racemic MET (d and l isoforms) is FDA-approved to acidify urine. It is well tolerated at 500–1000 mg per day when administered orally.

Other groups have focused on reducing hair cell apoptosis by disrupting mitogen-activated protein kinase (MAPK) cell death signaling through peptide inhibition of c-Jun N-terminal Kinase (JNK). d isoforms of a 20 amino acid HIV–TAT peptide to facilitate cellular uptake have been shown to protect against NIHL in guinea pigs when delivered subcutaneously or locally (intracochlear) via catheter before and following noise exposure [38–40]. Most recently, D-JNKI-1 peptides have shown otoprotection when delivered locally to the round window membrane of the cochlea within 24 h of noise exposure, although this is less effective than intracochlear administration [40].

Although promising, these initial discoveries have several limitations that could restrict their ability to enter human clinical trials for otoprotection. A major limitation is the route of administration and bioavailability. All of the aforementioned studies involved systemic or local injections of the compound. For human utility in an outpatient setting oral dosing is preferred to improve patient compliance, especially for more chronic therapies. A chronic therapeutic strategy is consistent with a noisy occupational or recreational environment. Another notable limitation is the level of dosing. All of the preceding studies used very high-dose levels. Although scientifically important,
most of these compounds exhibited poor oral bioavailability, required high-dose levels or did not offer long-lasting protection. In addition, the enzymes or rate-limiting catalytic proteins involved in these antioxidant pathways had not been tested. There are several limitations to this proof-of-concept work that could inhibit their direct translation into clinical testing.

Recently, three publications involving a mimic of GPx showed excellent otoprotection using an oral route of administration [41–43]. Efficacy in guinea pig and rat models of NIHL under TTS and PTS conditions has been reported. These data are consistent with the observation that GPx activity is decreased in the cochlea after noise exposure [27] and that deletion of the GPx1 gene confers increased susceptibility to noise damage [44]. In addition, this significant otoprotection was achieved in the low mg/kg range.

In normal cells, GPx functions at near-maximal levels. Augmentation of GPx activity with the enzyme itself is not practical because of its large size and relative instability. However, small-molecule mimics of GPx have been synthesized and had high GPx activity in vitro and in vivo [45–47]. Among the GPx mimics that have been developed, ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is the most advanced and has been shown to have excellent oral availability and low toxicity making it a suitable candidate drug for the prevention of NIHL (Figures 1 and 2).

**FIGURE 1**

Representative auditory brainstem response (ABR) tracings from vehicle (control), 3-day, and 14-day ebselen (GPx mimic) treated F-344 rats at 15 weeks post-noise exposure. Rats were exposed for 4 h to 113 dB noise centered at 8 kHz with a 1 octave band width. Treatment was by oral gavage and began 1 day before noise exposure. The amount of ABR threshold shift or hearing loss is indicated by the upper tracing (*) for each animal, whereas the lower tracing confirms the absence of threshold or an evoked response at 5 dB below threshold at (a) 8 kHz and (b) 16 kHz. X-axis in milliseconds (ms); Y axis in decibels (dB); treatment (Rx) = 4 mg/kg ebselen orally twice daily for the duration indicated. Bar charts show mean ABR threshold shifts at (c) 8 kHz and (d) 16 kHz for control, 3-day and 14-day ebselen treated groups (n=8 ears/group, SEM shown).
Ebselen has strong activity against peroxynitrite (ONOO−), a super ROS and/or RNS formed by the combination of two free radicals: super oxide anion and nitric oxide [48–50]. It reduces cytochrome c release from mitochondria and nuclear damage [51] during lipid peroxidation. Because ebselen acts as a catalyst and is not consumed during detoxification reactions [52], low doses might prove to be effective at reducing NIHL. Preclinical studies in rats and guinea pigs indicate that noise-induced TTS and PTS can be reduced by ebselen when it is administered orally in the 4–10 mg/kg range [41,42]. This corresponds to a 280–700 mg dose in humans, a dose previously shown to be well-tolerated [53,54].

In addition to its function as a GPx mimic, ebselen has been described as having the properties of: thioredoxin reductase [55], dehydroascorbate reductase and thioltransferase [56], anti-inflammatory compounds [45,57] and anti-apoptotic compounds [51,58]. In general, ebselen is capable of reducing oxidative stress levels in various cell types through a variety of mechanisms. Intense noise exposure can lead to increased oxidative stress causing OHC loss via activation of apoptotic and necrotic pathways. Noise exposure causes the release of cytochrome c from mitochondria in apoptotic and necrotic cells. The release of cytochrome c in a subpopulation of OHCs takes place early in the cell death process, before any outward signs of necrosis or apoptosis [59]. Ebselen might exert its protective effect in the cochlea through the inhibition of cytochrome c release from mitochondria in OHCs, as has been demonstrated in other systems [51,60]. Membrane-lipid peroxidation in the cochlea of animals exposed to high levels of noise has been demonstrated to be a predisposing factor in the permanent loss of OHC [32]. The precise biochemical mechanism(s) of ebselen-mediated protection in the cochlea of animals is unknown but is probably associated with the attenuation of ROS- and/or RNS-mediated damage.

**Otoprotection in clinical development**

**Mg**

In a double-blind placebo-controlled study involving 300 young, healthy military recruits, those supplemented daily with 4 g of oral Mg granulate verum (6.7 mmol Mg aspartate) showed significantly less PTS than those in the placebo control group (11.2% versus 21.5%) one week post noise [14]. Analysis of Mg levels in serum, erythrocytes and mononuclear cells showed a strong negative correlation between mononuclear Mg levels and the development of PTS that was independent of treatment group. However, a weak correlation between serum Mg levels and PTS was reported in a study of 68 male soldiers that had been exposed to high-level weapons noise over an 8–18 year period [61]. Analysis of Mg supplementation in soldiers exposed to low-level noise also shows reduced TTS levels, although no significant changes in serum or mononuclear cell Mg levels were identified between treated and placebo groups [62].

**NAC**

In a double-blind placebo-controlled study involving 600 young, healthy US Marine recruits, 900 mg NAC (effervescent tablet) was dosed orally, three times daily for two continuous weeks to reduce PTS during weapons training. At present the data from this trial are being analyzed. In a recent study, normal-hearing adults were dosed orally with placebo or 900 mg NAC 30 min before entering a nightclub where they were exposed to two hours of loud music. Personal dosimeters recorded a mean noise level of 98.1 dB (A-weighted). An average of 14 dB TTS at 4kHz was reported in subjects immediately after exposure (within 15 minutes). No significant differences between groups were identified [63]. This observation might be related to the requirement of high dose NAC to effectively prevent NIHL in animal models [13,37] or the limited ability of NAC to prevent TTS.

**Ebselen**

In an upcoming double-blind placebo-controlled Phase II study of oral ebselen, 60 young, healthy US Army recruits will receive doses of ebselen twice daily for two continuous weeks during weapons training. They will be assessed within six hours to determine TTS and subsequently at two and four weeks post-noise to determine PTS.
Although limited, these initial clinical studies of NIHL indicate that the incidence of TTS, PTS and tinnitus can be determined quickly. This allows for short clinical trial periods when compared with the clinical trial periods of other debilitating neurosensory diseases. This observation is a clear advantage in developing novel drugs for NIHL and tinnitus.

**Summary**

Several pathways are worth considering for the development of otoprotective compounds (summarized in Table 2). In general, the population utilizing these drugs will be healthy individuals who are at risk of developing permanent hearing loss as a result of occupational exposure to noise. Any significant, adverse events will not be tolerated. It is also unlikely that healthy individuals would undergo surgical procedures, receive repeated systemic injections or ingest beverages to prevent NIHL.

Finally, the characteristics of the drug itself, with regards to manufacturing costs and stability, will need to be considered. If these criteria can be met, market acceptance and support will be driven largely by the clear medical need for a drug and the savings associated with reduced medical and disability costs and training of personnel.

### TABLE 2

Summary of compounds tested to prevent NIHL

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>Effective dose</th>
<th>ROA</th>
<th>Comments</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antioxidants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH prodrug</td>
<td>Allopurinol</td>
<td>50–100 mg/kg</td>
<td>i.p.</td>
<td>TTS reduction</td>
<td>[35,36,64]</td>
</tr>
<tr>
<td></td>
<td>ALCAR</td>
<td>100 mg/kg</td>
<td>i.p.</td>
<td>PTS reduction; limited study</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>Edavarone</td>
<td>17 mM</td>
<td>local</td>
<td>PTS reduction</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>lipoic acid</td>
<td>50–200 mg/kg</td>
<td>i.p., p.o.</td>
<td>TTS and PTS reduction</td>
<td>[67]</td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>430 μg/kg</td>
<td>p.o.</td>
<td>TTS and PTS reduction; limited study; extensive pretreatment</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>R-PIA</td>
<td>50 mM</td>
<td>local</td>
<td>PTS reduction; limited study</td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td>α-tocoferol</td>
<td>10–50 mg/kg</td>
<td>i.p.</td>
<td>PTS reduction</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>200 mg/kg</td>
<td>i.p.</td>
<td>PTS reduction</td>
<td>[13,71]</td>
</tr>
<tr>
<td></td>
<td>Monoethylester</td>
<td>50–150 mM</td>
<td>local</td>
<td>PTS and TTS reduction</td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td>NAC</td>
<td>325 mg/kg</td>
<td>i.p.</td>
<td>TTS and PTS reduction; Ph-III NIHL completed</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>OTC</td>
<td>735 mg/kg</td>
<td>i.p.</td>
<td>Limited PTS protection</td>
<td>[72]</td>
</tr>
<tr>
<td><strong>Antioxidant enzymes</strong></td>
<td>GPx</td>
<td>Ebselen/SPI-1005</td>
<td>4–30 mg/kg</td>
<td>p.o.</td>
<td>TTS and PTS reduction; acute stroke studies halted; NIHL Ph I/II upcoming</td>
</tr>
<tr>
<td></td>
<td>SOD</td>
<td>SOD–PEG</td>
<td>2000 μg</td>
<td>i.m.</td>
<td>Limited study; TTS reduction; potential for SOD paradox</td>
</tr>
<tr>
<td><strong>Calcineurin inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A</td>
<td>10 μg/ml</td>
<td>local</td>
<td>PTS and TTS reduction; limited study</td>
<td>[73]</td>
</tr>
<tr>
<td></td>
<td>FK506</td>
<td>1–10 μg/ml</td>
<td>local</td>
<td>PTS and TTS reduction; limited study</td>
<td>[73]</td>
</tr>
<tr>
<td><strong>Diuretics</strong></td>
<td>Mannitol</td>
<td>15 mg/kg</td>
<td>i.p.</td>
<td>Limited study; PTS reduction</td>
<td>[74]</td>
</tr>
<tr>
<td><strong>Glucocorticoids</strong></td>
<td>Dexamethasone</td>
<td>100 ng/ml</td>
<td>local</td>
<td>Limited study; PTS reduction is U-shape</td>
<td>[75]</td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
<td>aFGF</td>
<td>1000 ng/ml</td>
<td>local</td>
<td>PTS reduction; limited study</td>
<td>[76]</td>
</tr>
<tr>
<td></td>
<td>GDNF</td>
<td>100 ng/ml</td>
<td>local</td>
<td>PTS reduction; higher dose was ototoxic</td>
<td>[77,74]</td>
</tr>
<tr>
<td><strong>Iron chelators</strong></td>
<td>Deferoxamine</td>
<td>100 mg/kg</td>
<td>s.c.</td>
<td>Limited study; PTS reduction; clinically observed ototoxicity</td>
<td>[74]</td>
</tr>
<tr>
<td><strong>JNK Inhibitors</strong></td>
<td>CEP-1347</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td>PTS reduction, limited study; Parkinson’s Disease studies halted</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>D-JNKI-1</td>
<td>1–100 μM</td>
<td>local</td>
<td>TTS and PTS reduction</td>
<td>[40]</td>
</tr>
<tr>
<td><strong>Magnesium</strong></td>
<td>Mg</td>
<td>4 g in humans</td>
<td>p.o.</td>
<td>TTS and PTS reduction; efficacy correlates with Mg deficiency versus treatment</td>
<td>[14,61,62]</td>
</tr>
<tr>
<td><strong>NMDA antagonists</strong></td>
<td>Carbamathione</td>
<td>5.6 mg/kg</td>
<td>i.p.</td>
<td>PTS reduction; limited study</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>Caroverine</td>
<td>1.6–12.8 mg/ml</td>
<td>local</td>
<td>PTS reduction; transient block of sound transmission</td>
<td>[78]</td>
</tr>
<tr>
<td></td>
<td>MK-801</td>
<td>1 mg/kg</td>
<td>i.p.</td>
<td>PTS reduction; limited study</td>
<td>[32,79]</td>
</tr>
<tr>
<td></td>
<td>PD 174494</td>
<td>10 mg/kg</td>
<td>i.p.</td>
<td>Limited PTS protection</td>
<td>[32]</td>
</tr>
<tr>
<td><strong>NOS inhibitors</strong></td>
<td>L-NAME</td>
<td>1 mg/kg</td>
<td>i.p.</td>
<td>Limited study; some ototoxicity seen at higher frequencies</td>
<td>[32]</td>
</tr>
</tbody>
</table>

Abbreviations: ROA, route of administration; i.p., intraperitoneal; p.o., oral; i.m., intramuscular; s.c., subcutaneous.

**References**

72 Yamasoba, T. et al. (1998) Role of glutathione in protection against noise-induced hearing loss. Brain Res. 784, 82-90
74 Yamasoba, T. et al. (1999) Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. Brain Res. 815, 317-325
76 Sugahara, K. et al. (2001) The role of acidic fibroblast growth factor in recovery of acoustic trauma. Neuroreport 12, 3299-3302
77 Shoji, F. et al. (2000) Glial cell line-derived neurotrophic factor has a dose dependent influence on noise-induced hearing loss in the guinea pig cochlea. Hear. Res. 142, 41-55
Local inner-ear drug delivery and pharmacokinetics

Alec N. Salt and Stefan K.R. Plontke

Several drugs that are applied directly to the inner ear are in widespread clinical use for the treatment of inner-ear disorders. Many new substances and drug delivery systems specific to the inner ear are under development and in some cases are being evaluated in animal experiments and in clinical studies. However, the pharmacokinetics of drugs in the inner ear is not well defined and the field is plagued by technical problems in obtaining pure samples of the inner-ear fluids for analysis. Nevertheless, a basic understanding of the mechanisms of drug dispersal in the inner ear has emerged, which facilitates the design and interpretation of future pharmacokinetic studies.

In recent years there has been increasing interest in the treatment of inner-ear disorders by local rather than systemic application of drugs. Substances are applied intratympanically, which means they are injected into the middle-ear cavity. This procedure is based on the premise that the drug will contact the round window membrane (RWM) of the cochlea, enter the scala tympani (ST) and spread throughout the ear. The target tissues of such treatments might include the sensory hair cells, the afferent nerve fibers and supporting cells of the cochlea (hearing) or vestibular (balance) portions of the inner ear. The idea of a topical application of drugs to the inner ear is not new. Local anesthetics and aminoglycosides were applied decades ago to treat inner-ear disorders [1–3]. The present, most widely used form of intratympanic therapy is the injection of gentamicin into the middle ear in patients with Menière’s disease [3–8]. Gentamicin is toxic to the sensory cells of the balance system and thereby suppresses the vertigo in these patients by partially ablating their vestibular system. There is also an increasing number of clinical reports related to the local application of glucocorticoids for acute hearing loss [9–16], glucocorticoids for Menière’s disease [17–20] or for tinnitus [21–25]. Other substances that have been tested in humans include local anesthetics, neurotransmitters and neurotransmitter antagonists [26,27], and the use of growth factors, antioxidants, apoptosis inhibitors and antisense oligonucleotides is also increasing. Animal experiments have shown promising results by using locally applied drugs to provide otoprotection from noise and drug toxicity [28–36]. An extension of such studies is local viral and nonviral gene transfer for the sustained treatment of inner-ear disorders [37–42]. It has recently been shown that Atoh1, a key regulator gene of hair cell development also known as Math1, induces regeneration of hair cells and substantially improves hearing thresholds in the mature deaf inner ear after delivery to nonsensory cells through adenoviral vectors [43]. Although the above examples show that local therapy has many advantages over systemic...
therapy, it should be noted that no drug to date has been approved anywhere in the world for local application in the treatment of inner-ear disorders.

Local application of drugs to the inner ear is based on the rationale that, despite the lower total amount of drug given, medications applied topically to the RWM can result in higher concentrations in the inner-ear fluids than with systemic application. Pharmacokinetic studies have confirmed this principle [9,34,44–47]. Potential side effects of systemic treatment and complications from a long-lasting higher-dose therapy can be avoided through topical application therapy. Substances applied locally at a low dose can be administered if there are major restrictions or even contraindications associated with systemic application.

Although in theory the local application of drugs to the inner ear has great potential, in practice there are numerous technical difficulties to overcome. Important issues that have so far received only limited consideration are:

(i) Which parts of the ear do drugs reach, in what concentration and with what time course?
(ii) How do different delivery methods or application protocols influence the drug levels at each time point at the different locations in the ear?
(iii) How variable are the drug levels achieved with different delivery protocols and what are the major sources of variation?

Currently, doses, protocols and application systems are empirically justified. This approach has led to varying results in the therapy of Menière’s disease by intratympanic gentamicin treatment, which serves as an example of the uncertainties associated with different application strategies. Although some studies reported few patients with deafness as an unwanted side effect of local gentamicin treatment [7], others found complete deafness of the treated ear in more than 20% of patients [48] and in one study, in which drugs were applied for a prolonged period, 80% of the patients were deafened [49]. It is therefore necessary to acquire an understanding about the quantitative drug distribution in the inner-ear fluids when medications are applied locally with different delivery protocols.

**General principles of drug distribution in the inner ear**

The inner ear represents a geometrically complex structure, with characteristic large fluid-filled extracellular spaces (scalae), each with multiple interfaces with other scalae and with outside compartments, such as the systemic blood circulation and the middle ear cavity (Figure 1). ST and scala vestibuli (SV) contain perilymph, a fluid similar in ionic composition to other extracellular fluids, whereas the endolymphatic space (ELS) contains fluid with a unique, high potassium composition. In contrast to most other body fluids, the inner-ear fluids do not move or flow appreciably and are not actively ‘stirred’. As a result, the spread of locally applied drugs through the ear occurs only slowly and predominantly by passive diffusion [50,51]. The diffusion coefficient, which governs the rate at which drugs spread, depends on the physical characteristics of the diffusing particles or molecules, with their molecular weight playing a major role [52].

Transfer of substances through the RWM to the ST of the inner ear also appears to be primarily a passive process. Active transport processes have been assumed particularly for larger molecules and particles, but have not been confirmed so far [53]. The rate at which medications cross the RWM to the inner ear depends on the size, geometry and tissue permeability characteristics of the RWM. Animal experiments have shown that, despite its three-layered nature, the RWM behaves as a semipermeable membrane. Many agents have been applied to the RWM and their transition into ST has been assessed either by histological methods, by direct measurement of concentrations or by indirect methods, such as through an influence on hearing thresholds [53]. The permeability of the RWM can also be influenced by simultaneous application.
of other substances [54,55] or by intracochlear pressure changes that alter the distension of the membrane [56].

The processes underlying drug distribution in the ear have been subdivided into ‘radial’ (with respect to the modiolus, the central core of the cochlea) and ‘longitudinal’ processes (Figure 1) [51,57,58]. Radial distribution processes include communications between the parallel scalae of the same turn (Figure 1, open arrows) and communications with the vascular system (Figure 1, solid arrows). Communication between ST and SV via the lateral wall (the tissues at the right of Figure 1a) appears to be particularly rapid [58–60]. Communication with the blood is through the endothelial cells of the capillary beds in the lateral wall that provides a tight ‘blood–labyrinth barrier’, comparable with the blood–brain barrier. Longitudinal processes include diffusion and longitudinal flow along the scalae. In the normal, unopened cochlea the flow rates of endolymph and perilymph are extremely low so the effect of flow can be regarded as negligible compared with diffusion [57,61]. Other longitudinal communications include those between ST and SV through the helicotrema at the cochlear apex and the open communication between the basal part of SV and the vestibulum.

A major process that influences drug distribution in the inner ear is that of clearance, which refers to the removal of substance from the cochlear fluids via the capillary beds in the lateral wall and modiolus. Broadly speaking, clearance includes all processes that lead to the reduction of substance levels in the cochlear fluids, such as losses to the fluid spaces of the modiolus, uptake into intercellular spaces and inactivation of the applied drug by metabolism or by binding to tissues. The interplay between diffusion and clearance is key to determining the distribution in the inner ear of substances applied to the RWM. Substances cleared from the fluids at high rates will rapidly reach a steady state in which drug diffusion is balanced by clearance, so the drug can never reach the apical regions of the cochlea in appreciable concentration. Substances with slower clearance rates will diffuse further along the cochlea. The magnitude of longitudinal drug gradients following local delivery is therefore highly dependent on the rate the drug is cleared from perilymph. The greatest technical problem associated with local drug administration is the large gradients of drug concentration, with highest levels near the site of application and decreasing levels at more distant sites. These gradients can be predicted by computer simulations [59,62] and have been demonstrated experimentally by ion-electrode measurements [50] and by histological methods [41,63].

**Preclinical studies of pharmacokinetics in the inner ear**

The development and approval of medications and drug-application systems requires the study of the pharmacokinetics, toxicity and efficacy of medications applied locally to the RWM. Preclinical animal studies lay the foundation for introducing specific drug-delivery protocols into clinical practice. Only a few studies have quantitatively analyzed drug concentrations in the inner-ear fluids [9,34,45,46,63–65]. However, data from two of these studies show very different perilymph concentrations of a drug after using similar application modes [9,46]. Large deviations in pharmacokinetic profiles in animal experiments considerably limit their applicability to the human situation. For this reason, it is important to understand some of the technical problems associated with preclinical pharmacokinetic studies of the inner ear.

The withdrawal and analysis of samples from the inner ear represent a considerable technical challenge because the fluid volumes in the inner ear are extremely small. For example, the entire perilymph volume in the cochlea of the guinea pig is less than 10 µl [66,67]. In previously published studies, it was necessary to take sample volumes close to or greater than the total cochlear perilymph volume in order to have sufficient volume for pharmaceutical analysis [9,34,45,46,64,65]. When fluid is aspirated from the basal turn of the ST, such as through the RWM, it is replaced by cerebrospinal fluid (CSF), which enters the ST through the cochlear aqueduct and contaminates the perilymph [56,62,68,69]. As greater volume is taken, the sample will contain an increasing amount of CSF so that the volume taken no longer represents pure perilymph. In one study, multiple samples, each larger than the cochlear perilymph volume, were taken over time [9]. The assessment of pharmacokinetic profiles based on such fluid samples can therefore be misleading. In a recent study that used a chemical marker, the marker concentration of fluid samples taken through the RWM was compared with the perilymph concentration of the marker measured before sampling with an ion-selective microelectrode sealed into the scala. It was calculated that even a 1 µl sample taken through the RWM was contaminated with 20% CSF [56]. Larger sample volumes, such as the 10 µl samples used in some prior studies, were estimated to contain >15% perilymph and <85% CSF. A consequence is that the drug levels reported in some pharmacokinetic studies do not accurately represent the real drug concentration in the perilymph. It is therefore important to scrutinize carefully the methodology used before accepting that published values described as ‘perilymph concentrations’ are reliable.

Interpretation of specific delivery and sampling protocols is aided by a finite-element computer model (http://oto.wustl.edu/cochlea/model.htm) that considers the anatomy of the inner ear, general pharmacokinetic principles and solute distribution processes. This model allows the calculation of solute movements associated with a variety of drug delivery protocols. It also simulates several measurement methods, including measurement at a specific point (comparable with an ion-selective microelectrode), and several fluid-sampling techniques, including microdialysis. Volume flows associated with the aspiration of samples are incorporated into the
Potential misinterpretation of fluid sample measurements.
Calculation of the methylprednisolone (MP) concentration in the basal turn of ST in the absence of sampling (b) that would account for the amount of MP detected in the four 10 µl samples taken through the RW membrane by Parnes et al. [9](a). Note that the perilymph MP level must be substantially higher and the MP must remain in the fluid space for longer time than the sample measurements alone suggest (data derived from [62]).

Simulations. This simulator is therefore useful for the interpretation of a variety of experimental configurations. A detailed analysis of the repeated sampling study mentioned above [9] has shown that perilymph concentration of methylprednisolone (MP) in ST must have been more than 10 times higher than the presented sample concentrations, as shown in Figure 2 [62]. This analysis reconciles the disparate concentrations of steroids in the ear reported by different studies [9, 46, 62]. It also confirms that the major hazard of sampling perilymph from ST is that the actual perilymph drug levels might be substantially underestimated by the concentrations detected in samples.

Other studies in which perilymph was sampled from the vestibulum [52, 70, 71] are more readily interpretable in terms of the basic mechanisms by which drugs distribute within the inner-ear fluids. The amount and time-course concentration change of gentamicin in the vestibulum is consistent with drug entering the basal region of ST and spreading quickly to SV and vestibulum by radial diffusion pathways across the membranous structures [59]. The amount of drug reaching the apical regions of the cochlea was predicted to be substantially lower than that at the base, which is consistent with the limited hearing loss seen when moderate gentamicin doses are applied locally. Other studies have adopted sampling methods in which the animal was first sacrificed, the temporal bone removed and then the fluids aspirated from the cochlea. In this method, the perilymph withdrawn is replaced by air, so that sample contamination with CSF cannot occur [46]. The choice of sampling location is constrained by the aim of the study, specifically those studies that are interested in the vestibular effects of drugs need to sample from the vestibulum, whereas for studies focused on the auditory effects of drugs the drug concentration in ST is of greater relevance.

An alternative to fluid sampling from the cochlea is the use of microdialysis, which involves sealing a permeable probe into the scala, through which fluid is continually perfused. Analysis of repeated samples of fluid efflux from the probe allows a drug concentration time-course in the cochlea to be monitored without volume disturbance. Time courses for several substances including gentamicin and dexamethasone following application to the RWM have been presented [47, 72, 73]. Although this is an excellent method to quantify RWM permeability, the drug time course measured with this technique also does not accurately represent the true perilymph kinetics. This is because the leakage of drug from perilymph into the dialysis probe (which is an essential part of the method) represents a non-physiological clearance of the drug from the cochlea fluid spaces. It has been demonstrated that dialysis of small compartments in vitro caused rapid clearance of solute from the compartment, which could be accurately modeled [72]. In a subsequent analysis of in vivo dialysis experiments using dexamethasone and fluorescein it was further demonstrated that the dialysis probe significantly contributed to solute clearance from perilymph [74], making it impossible to define the physiological rate of drug clearance. The high rates of drug clearance reported in prior dialysis studies are therefore unlikely to represent the perilymph kinetics that would exist in the absence of dialysis. It is unfortunate that the errors in dialysis and sampling studies underestimate the drug concentration as well as the time the drug remains in the perilymph.

There are only few pharmacokinetic studies that use fluids samples taken from the inner ear of humans. In patients undergoing labyrinthectomy, perilymph was sampled from the vestibulum at various times following local delivery of gentamicin to the middle ear [75]. Such data will be valuable in the extrapolation of pharmacokinetic studies in animals to the situation in humans.

Drug application systems
As more candidate substances for the treatment of inner-ear disorders are being discovered, it is necessary to develop appropriate strategies for their delivery in the least invasive manner possible. In controlled-release systems, such as biopolymers or pumps that might be implanted or external, the rate of release is determined largely by the design of the device itself and is not dependent on environmental conditions. By contrast, sustained-release systems provide prolonged release but the release rate is significantly affected by environmental conditions [76]. For the local drug delivery to the inner ear, a variety of strategies exists, ranging from intratympanic injections of fluids to the use of pumps, polymers and gels.
Single or repeated intratympanic injection with or without volume stabilization and with or without visualization of the RWM

The most frequently used method for local drug application is an intratympanic injection of drug solution [1,26,77,78]. The limitation of this method is the lack of control of the drug concentration reaching the RWM and of the duration of contact of the drug with the RWM, which are important factors in determining the drug level achieved in the cochlea. During and after injection the patient usually lies with the treated ear upwards. The time the drug remains in the middle ear is uncertain, however, as drug is lost by drainage of the solution via the eustachian tube (during swallowing) or by resorption through the middle ear mucosa. Several methods have been developed to increase the time drugs remain in the middle ear. In animal experiments, fibrin glue was employed to stabilize the applied volume as a gel [45], whereas in humans hyaluronic acid [14,79,80] or resorbable gelatin sponges [81] have been used. Despite this, the doses, dosing intervals and therapeutic durations required to achieve a specific therapeutic goal are hard to predict. Other factors that contribute to variation include anatomic obstacles, such as plugs of connective or adipose tissue, or so-called pseudomembranes of the round window (RW) niche. It has been estimated that approximately one-third of patients have obstructions of the RWM [82]. Inspection of the RWM before drug application is possible using a microotoscope (explorent®, Tuttlingen, Germany) [83]. Another approach to maintain drug in contact with the RWM is the use of the ‘microwick’ [84], in which a ‘wick’ is positioned in the RW niche via a tympanostomy tube. The external end of the wick is located in the external auditory canal, to which the patient can intermittently apply medications. Although it is possible that the device acts as a wick when it is initially placed and is dry, it remains uncertain how much volume flows along the wick when the fibers are already fluid-saturated. It is more likely that drug diffusion within the fluid spaces of the wick will dominate drug movement towards the inner ear, and this is likely to be a slow process.

Continuous or discontinuous drug application via partly or fully implantable pump systems

During brief drug applications, small permeability variations in the face of large drug gradients across the RWM, combined with variations in the brief application time, contribute significantly to variations of perilymph drug levels. In an attempt to control better the drug level at the RWM, continuous drug application has been employed, via partially or fully implantable catheter systems and pumps. In animals, the mini-osmotic Alzet pump® (Durect, Cupertino, USA) has been widely used for drug delivery to the middle ear [85] and for intracochlear delivery of drugs [29,36,86].

A device for discontinuous drug delivery that has been evaluated in animals but that is not yet approved for use in humans is the TI-DDS® (Totally Implantable Drug-Delivery-System) [87,88]. The manually operated pump releases a defined volume of 5 or 10 µl upon push-button activation. A subcutaneously implanted reservoir can be refilled transcutaneously.

In humans, although a variety of experimental catheter systems have been employed, the round window microcatheter from Durect (RWµCath™, Durect, Cupertino, USA) represents the best characterized system to date [10,13,27,48]. In a recent nonconcurrent cohort study it was found that local continuous glucocorticoid delivery via the RWµCath™ in patients with acute, severe or profound hearing loss and failure of standard systemic therapy showed a significant improvement in hearing compared with a historical control group without local treatment [16]. However, for unknown reasons, commercial production of the Durect RWµCath™ was halted in 2004.

There remains an intense interest in the development of safe, effective and minimally invasive drug-delivery systems for the inner ear, with several groups working on intracochlear catheter-based application systems. One approach has been to combine drug delivery with an existing device, such as by incorporating a drug delivery cannula into a cochlear implant electrode [89]. Other groups are working on specific implantable drug delivery devices usable in normal ears, as for example Fiering et al. [90]. This group is hoping to combine developments in biomedical engineering that will allow the incorporation of more elements from micro- and nanotechnology (sensors, processors, effectors and actuators) into a delivery device specifically for the inner ear.

Biodegradable biopolymers

In addition to the use of gels to stabilize the volume of drug-containing solutions in the middle ear, there is also enormous interest in the use of biodegradable biopolymers for the controlled delivery of substances to the inner ear. The two main roles of these polymers are diffusion control of active agents and disintegration control of the polymer, which results in release of the active agent.

Advantages of polymeric controlled-release devices include a specific release kinetic for prolonged delivery and the possibility of drug targeting. Of special interest are so-called ‘smart polymers’ that are able to respond to chemical or physical changes of the environment, such as pH, temperature and electric field, that might allow them to be incorporated into auto-regulated implantable drug-delivery systems. Disadvantages are the limited amounts of drug that can be incorporated in a given application form [76,91,92].

Two recent publications have reported on the use of biopolymers for local drug delivery to the inner ear [65,93]. Another application in this area is the coating of cochlea implant electrodes with biodegradable carrier substances to release drugs. In this application, glucocorticoids, antioxidants, apoptosis inhibitors or neurotrophins could be employed to counteract side effects of electrode
insertion, to aid survival of the spiral ganglia or to stimulate neurite growth towards the implant electrodes [94].

Influence of the application system on pharmacokinetics with RW application

Based on analyses and simulations of experimental inner-ear pharmacokinetic studies in animals [59,62], it has been established that the application protocol is a major factor in determining the absolute drug level reached in the inner ear. The time that the drug remains in the middle ear plays a primary role, with highest intracochlear drug levels found with continuous delivery and lower levels found with brief applications. In addition, the relative distribution of drugs in the ear varies with application protocol because of the interactions between the duration of application with clearance processes.

Conclusions for clinical applications

The applied concentration of drug and the delivery protocol required to achieve a specific goal in humans is likely to differ substantially from that demonstrated in experimental animals. As drug concentrations in the inner-ear fluids depend on dispersion by diffusion, they are influenced by the differing scala lengths and volumes of the inner ear in different species. For example, to achieve the same active dose in the vestibulum of humans, higher middle-ear and basal-turn drug levels will be required compared with experimental animals with smaller cochlear fluid volumes. This is particularly important in the therapy of Menière’s disease by intratympanic drug application, where one also has to consider the relative vestibulotoxicity and cochleotoxicity of specific protocols. A major factor affecting the drug level in the ear is the time the drug remains in the middle ear. It is therefore of major importance that application methods are developed in a way that the amount of drug in the middle ear and the application duration are closely controlled.

Basal to apical concentration gradients generated by local drug application are also of clinical relevance. Concentration gradients will be greater in the human cochlea than in experimental animals because of the longer cochlear spiral of the human. Whereas drug applied to the RWM of mice will readily reach the cochlear apex, the same is not true for humans, as the cochlea is substantially longer. Gradients in drug concentration between the basal (high-frequency) regions compared with apical (low-frequency) regions of the cochlea partially account for the limited hearing loss in humans with Menière’s disease treated with vestibulotoxic levels of gentamicin. Drug gradients can be exploited for the treatment of other inner-ear disorders, such as high-frequency tinnitus and high-frequency hearing loss. By contrast, it will be difficult to treat hearing disorders in the middle and lower frequency range by present-day intratympanic drug application methods.

Acknowledgement

This work was supported by NIH/NIDCD DC01368.

References

1 Ersner, M.S. et al. (1951) Transtympanic injection of anesthetics for the treatment of Meniere’s Syndrome. AMA Arch. Otolaryng. 54, 43-52
2 Völger, G. (1952) Beseitigung von Labyrinthausschwellungen bei dem Menièr’sen Symptomkomplex durch das Hyaluronidasepräparat Kinetin. HNO 3, 142-147
9 Parnes, L.S. et al. (1999) Corticosteroid pharmacokinetics in the inner ear fluids: an animal study followed by clinical application. Laryngoscope 109, 1-17

replacement and hearing improvement by inoculation. Between the perilymphatic scalae of the cochlea. *J. Neurosci.* 23, 141–151

Hydrogensuccinat. *runden Fenstermembran für Prednisolon-21-


87 Lehner, R. et al. (1997) A totally implantable drug delivery system for local therapy of the middle and inner ear. Ear Nose Throat J. 76, 567–570
Hair cells are the mechanoreceptors found within the inner ear that detect sound and head movements. Serious hearing and balance impairments can occur through the loss of hair cells by aging, environmental stresses, such as loud noises, or exposure to chemotherapeutic drugs, such as cisplatin or aminoglycoside antibiotics. At least 28 million Americans have a hearing impairment but only one out of five people who could benefit from a hearing aid actually wears one (www.nidcd.nih.gov). Because a large proportion of hearing loss involves the loss of hair cells, regeneration or replacement of these cells is a possible alternative to prosthetic devices.

Scientists once believed that warm-blooded animals had a full complement of hair cells at birth and, if lost, the damage was permanent. Over 15 years ago, several studies demonstrated that avians can regenerate their sensory hair cells [1–4]. Other studies demonstrated that the regenerated sensory hair cells were functional (reviewed in [5,6]). Nowadays, the regeneration phenomenon is better understood but the signaling mechanisms regulating hair cell regeneration remain unknown.

The sensory epithelium of the inner ear comprises two different general cell types: sensory hair cells and nonsensory supporting cells. There are several specialized types of hair cells in the mammalian inner ear: in the auditory system (inner and outer hair cells) and in the vestibular system (type 1 and 2 hair cells). These cells can be distinguished by their location in the organ, their morphologies and by the type of neurons that innervate the hair cell. There are also different types of supporting cells found within the mammalian auditory system (e.g. Deiters’ cells and pillar cells) that each express unique structural and molecular signatures. However, in the vestibular system, the supporting cells appear to be relatively homogeneous, and scientists have yet to find morphological, molecular or physiological differences between the supporting cells.

Many events occur when the hair cells in the inner ear are damaged or killed. For example, the sensory epithelium is capable of repairing itself when hair cells in the sensory epithelium are damaged with a sub-lethal stimulus [7–9]. However, if the damage is more severe, it normally leads to the death of some or all of the hair cells in the sensory epithelium. Dying hair cells undergo programmed cell death (apoptosis) [10,11] and are either ejected from the sensory epithelium [12] or engulfed by
neighboring cells. Following the death of the hair cells, the neurons from the VIIIth cranial nerve retract their synaptic terminals. In birds and lower vertebrates, a signal from the dying hair cell induces regeneration by triggering the neighboring supporting cells to either proliferate or transdifferentiate into an immature hair cell. Proliferating cells then respond to environmental, molecular or genetic cues to differentiate into hair cells or supporting cells. Finally, nerve fibers from the VIIIth cranial nerve reconnect the hair cell to the central nervous system so that the animal can process the sensory information.

**Fish and chicks: 'lower vertebrate' model systems to study hair cell regeneration**

In the past fifteen years, two different mechanisms have been proposed for sensory hair cell regeneration in avian and other non-mammalian species: mitotic proliferation and direct, nonmitotic, transdifferentiation. Many studies indicate that the supporting cells adjacent to a dying hair cell receive a signal to enter the cell cycle. The supporting cells that undergo mitotic proliferation migrate to the luminal surface of the sensory epithelium, duplicate their DNA and then divide into two daughter cells (reviewed in [6,13–15]). The daughter cells then proceed through symmetrical differentiation to produce two hair cells or two supporting cells [16–18], or through asymmetrical differentiation to produce one hair cell and one supporting cell [17–20]. Alternatively, hair cells can transdifferentiate from neighboring supporting cells by non-mitotic mechanisms [21–26]. Transdifferentiation is a switch in gene expression in the supporting cell so that it expresses markers that are characteristic of a developing hair cell. Although non-mitotic transdifferentiation of supporting cells is a simpler way of replacing lost hair cells, it results in the loss of the supporting cells; and often large numbers of hair cells and supporting cells are needed to repopulate the sensory epithelia. Mitotic cell division maintains the structural integrity of the organ by producing hair cells and supporting cells, whereas direct transdifferentiation results in a significant loss of supporting cells if they all transdifferentiate into hair cells.

**Functional studies using avians**

When it was discovered that birds could regenerate their sensory hair cells, the next logical question was: are the regenerated cells functional? Not only do the supporting cells need to differentiate into hair cells but the newly regenerated hair cells also need to be re-innervated by the VIIIth cranial nerve fibers. Moreover, the animal must use the new sensory information to produce behaviorally meaningful responses. Two recent reviews on functional recovery after sound and drug-induced damage have comprehensively covered these issues [5,6].

Although many studies have examined the physiology of recovered sensory hair cells, few have examined the complex properties of perceptual processing and behavioral plasticity. The recognition and production of vocal signals depend on hearing and are necessary for communication. Budgerigars, Melopsittacus undulatus, have been used to examine the renewal of vocal production and complex auditory perception after hair cell regeneration [27,28]. These birds mimic sounds and readily learn new vocalizations throughout life, which has been likened to language acquisition in humans. The birds were trained to match precisely their vocalizations to specific acoustic templates. Aminoglycoside treatment disrupted auditory perception and vocal production. Behavioral tests of auditory sensitivity showed that audiometric thresholds returned to near-normal levels (within 20 dB) within four weeks of deafening. More-complex perceptual tasks, such as vocal call discrimination and/or recognition, took up to five months to return to normal levels. Precision in vocal production initially declined but was restored to pre-treatment levels before the recovery of auditory function. Therefore, relatively little acoustic feedback from a few regenerated hair cells was necessary to guide full recovery of vocal precision.

Another series of studies examined complex communication behavior in male Bengalese finches, Lonchura striata, which learn a single sequence of ‘syllables’ early in life and reliably produce the same song throughout their lifespan [29–32]. After recording each bird’s song and verifying its stability, they were treated with a combination of aminoglycosides and sound exposure to induce hearing loss and their songs rapidly deteriorated [33]. Once hearing was restored by hair cell regeneration [30], the song returned to its pre-exposure structure [31]. Restoration of hearing allowed each bird to access a stored ‘template’ of its own learned vocalization, and gradually match this new vocalizations to the stored memory.

Compensatory behaviors, such as gaze, oculomotor and postural responses, that occur during movement largely depend on a functioning vestibular system. The vestibular ocular reflex and the vestibular colic reflex disappear after the vestibular hair cells of birds are destroyed with aminoglycoside antibiotics [34–38]. However, these reflexes reappear as the hair cells regenerate [35–37,39]. Dickman and Lim [40] trained adult pigeons to run along a chamber and peck an illuminated key. Multiple behavioral measures assessing performance, posture, and head stability were quantified. Once normative values were obtained, the animals received aminoglycosides, which killed the vestibular hair cells and resulted in severe postural and head instability. As the regeneration process progressed, the tremor and head shakes diminished and spatial orientation and navigation ability improved to pretreatment levels.

**Zebrafish: a new model system to study apoptosis and hair cell regeneration**

Recently, there has been much interest in using zebrafish, Danio rerio, as an animal model for studying the inner ear. Zebrafish have sensory hair cells in the vestibular organs
that include two maculae (the utricle and saccule) and the three semicircular canals. Although the zebrafish does not have an auditory organ, such as a cochlea, per se, adult zebrafish can detect sound frequencies from ~200 to 4000 Hz [41,42]. Zebrafish also have a lateral line system which comprises neuromasts that reside along the head and body in a stereotyped manner [43,44]. Each neuromast contains a central cluster of hair cells (surrounded by nonsensory supporting cells) that function to detect water currents relative to the animals’ body [45,46].

Zebrafish embryos can be easily manipulated experimentally because they develop rapidly ex utero (the first hair cells can be detected 24 h after fertilization [47]). For example, ‘small molecules’ can be added to the aqeous environment to determine which molecules affect inner ear development, or whether these chemicals could have a protective effect against toxins, such as aminoglycoside antibiotics. This makes zebrafish a very amenable model for small-molecule chemical screens.

Zebrafish provide a genetically tractable vertebrate because progeny can be chemically mutated and the subsequent inner-ear mutants can be isolated with forward-genetic screens using behavioral assays and observation [48,49]. Many essential genomics resources, including genome maps, large-insert genomic libraries, radiation hybrid panels and expressed sequence tag databanks, have been developed for the zebrafish and continue to improve (www.zfin.org).

Several studies have examined the death and subsequent regeneration of hair cells in the lateral line [50–52]. In two studies, zebrafish larvae were treated with neomycin [51,52]. In one study, supporting cell proliferation increased 12 h after treatment and regenerated hair cells were observed 1–2 days later [51]. Future studies will use this preparation to identify genes that influence vertebrate hair cell death, survival and regeneration following ototoxic insults.

The mammalian cochlea: to grow where none has grown before

Although all non-mammalian vertebrates regenerate hair cells, two crucial questions remain about the mammalian cochlea: why have only mammalian vestibular hair cells shown any capacity for regeneration and why are hair cells (and supporting cells) in the auditory portion of the inner ear apparently unable to regenerate after damage? The supporting cells in the mammalian auditory system undergo terminal mitosis during embryogenesis, whereas the supporting cells in the mammalian vestibular system retain some limited capacity to regenerate in adulthood [53,54]. However, there is a window of time during embryonic development when additional hair cells can be induced to develop in the immature cochlea. Undifferentiated progenitor cells that normally give rise to the mouse organ of Corti form supernumerary hair cells if they are treated with retinoic acid between embryonic days 13 and 16 [55,56]. Furthermore, if existing hair cells are killed by laser ablation during this developmental period, adjacent uncommitted progenitor cells will change their fates and differentiate into hair cells to replace those that were lost. Surprisingly, labeling experiments with markers for DNA synthesis indicates that the additional and/or replacement hair cells do not arise from proliferation of the existing hair cells. Rather, it appears that existing progenitor cells within the developing organ of Corti are able to change their developmental fates in response to changes in their local environment. Thus, at least for a short time during cochlear development, the progenitor cells are multipotent and have the ability to develop into many different cell types.

It has been proposed that the mammalian cochlea shuts down its proliferative and regenerative capacity after embryogenesis to establish a more stable and complex auditory processor. Although this has functional benefits, it leaves the system vulnerable to genetic mutation and the more-recently evolved societal assaults of noise-induced hearing loss and ototoxic drug damage. Given that it appears that the mammalian cochlea is unable to regenerate on its own, scientists have developed three general strategies intended to artificially force the inner ear to regenerate or replace lost sensory cells, including: (1) the experimental manipulation (knockouts, transgenics) of genes that inhibit proliferation in the organ of Corti; (2) the intentional transfection (gene therapy) of cochlear cells with viruses carrying genes for inducing hair cell differentiation; and (3) the replacement of lost cochlear cells with either intrinsic or extrinsic (transplanted) stem cells.

Genetic manipulation in the mammalian inner ear

Several genes negatively regulate cellular proliferation in mature, highly differentiated tissues to stop uncontrolled growth (i.e. to stop them becoming cancerous). The p27/Kip1 and pRb genes are activated in the mammalian cochlea at the time of terminal mitosis in the sensory epithilum [57–60]. Targeted deletions of these two genes lead to a prolonged period of mitotic activity in the sensory epithilum and an overproduction of hair cells and supporting cells [57–59]. Following an initial extended period of proliferation of progenitor cells in the p27 knockouts, proliferation terminates by postnatal day six and is soon followed by extensive hair cell death [58] and the loss of auditory function [57,58]. The pRb knockouts exhibit a robust and continued proliferation of progenitor cells and an extensive overproduction of hair cells [59,60]. Moreover, the hair cells show clear mitotic activity [59,60], although this often results in aberrant or multinucleated hair cells [60]. Unfortunately, these pRb mutants die at birth, so the effect of the overproduction of hair cells on cochlear function and the maturation of the organ of Corti are not known. Similarly, targeted deletion of p19/ink4d, another inhibitor of cell cycle progression, causes the initiation of DNA synthesis in hair cells but then leads to the precocious apoptosis of these cells and subsequent hearing loss [61].
Thus, these cell cycle inhibitors are used by the cochlear tissues to downregulate mitosis in the sensory epithelium and establish a mature, non-proliferating sensory epithelium. The elimination of these blockers initially leads to continued proliferation and overproduction of hair cells. However, these cell cycle inhibitors must also have another critical developmental function because eliminating them results in premature death of the hair cells or even the entire animal. Although these studies are a proof-of-principle that regeneration in the mammalian cochlea can be activated by elimination of specific mitotic blockers, there are additional consequences to the loss of these inhibitors that need to be understood before they can be used as a therapeutic approach to hearing loss.

Gene therapy in the mammalian cochlea

Recently, several studies have demonstrated that the mammalian homolog of the Drosophila transcription factor atonal1 – Math1 – is sufficient for hair cell genesis in inner-ear tissues. Math1 is expressed in developing hair cells [62,63], and transgenic mice that are homozygous for the targeted deletion of the Math1 gene lack vestibular and cochlear hair cells [63]. Moreover, transfection of Math1 (or its human analog, HATH1) into mouse organ of Corti or utricular cultures [64,65] is sufficient to induce new hair cell development. Recent in vivo experiments have demonstrated that Math1 transfected into the cochleas of guinea pigs deafened by kanamycin led to an extensive structural and functional recovery of the organ of Corti [66,67]. In these cases, the new hair cells appear to arise from direct transdifferentiation of existing supporting cells, rather than through the mitotic production of new cells. Although these studies are in their beginning stages, transfecting Math1 into the deafened cochlea could prove to be a promising option for gene therapy. Target specificity is problematic in many gene therapy paradigms [68]. For example, when Math1-encoding viral particles were successfully introduced into guinea pig cochlea, there was an ectopic production of hair cells in places other than the organ of Corti [66]. Therefore, to re-establish the proper connections after damage, a model must be established where Math1 can be more effectively delivered directly to the site of hair cell loss.

Stem cells in the inner ear

Stem cells have received considerable attention both in the scientific community and in the popular press because of their potential to repair or regenerate damaged cells and tissues in the human body. These cells have the capacity to give rise to any cell type in the body, and the fate of their progeny is determined by the microenvironment in which the stem cells reside. Thus, it has been proposed that if intrinsic stem cells in the inner ear could be activated or if extrinsic stem cells could be transplanted into the ear, they could produce new hair cells and/or neurons to replace those that have been lost. Recently, a population of cells from the utricles of mice have been isolated that have been described as end-organ stem cells [69]. These isolated cells exhibit the capacity to self-renew and to produce cell types with the morphologies and biological markers of hair cells (myosin VIIa and Bm3.1) and supporting cells (pancytokeratin and p27Kip1). Additionally, these mouse cells were able to incorporate into muscle and liver when transplanted into the developing embryo of a chicken, which suggests a multipotency characteristic of stem cells. However, it is not completely clear that these stem cells were derived from cochlear epithelial cells, because there is a possibility that they could have arisen from mesenchymal or vascular stem cells in the tissue. It is also worth noting that, although these stem cells apparently reside in the vestibular sensory epithelia, there is no evidence that they contribute to recovery in the cochlea.

In other studies, researchers have manipulated stem cell populations to get them to express characteristics of neural or hair cell precursors. Mouse embryonic stem cells have been induced down a pathway that resembles neural progenitor cells [70]. When these cells were transplanted into an embryonic chick otocyst, they gave rise to new hair cells. Other investigators [71] were able to induce mouse embryonic stem cells to form neuron-like cells by overexpression of bHLH. Similarly, treating mouse bone-marrow stem cells with sonic hedgehog and retinoic acid enabled the stem cells to differentiate into neuron-like cells [72]. Strategies like these will allow readily available stem cells to be primed to have a more cochlear fate, and could be used for transplantation into damaged cochleas.

A few groups have attempted to transplant neural stem cells into the inner ears of mammals. Rat hippocampal stem cells have been injected into the cochleas of newborn rats, which exhibited some integration of the stem cells into the cochlear epithelium [73]. Adult neurospheres were transplanted into the cochleas of normal or neomycin-treated guinea pigs [74]. There was a higher survival rate of stem cells in the neomycin-treated cochleas, and it was reported that some of the surviving cells were beginning to express neuronal markers. Experiments in our laboratory [75,76] have transplanted an immortalized line of neural stem cells into the noise-damaged cochleas of mice and guinea pigs. These stem cells survived for up to six weeks, integrated into the spiral ganglion and organ of Corti in the damaged regions, and differentiated into neurons, glia, hair cells and supporting cells. These various stem cell studies are all still preliminary but they do indicate that there might be a resident mammalian stem cell population, at least in the vestibular end organs. Moreover, the introduction of pluripotent stem cells into a damaged ear could result in these cells integrating into the remains of the sensory epithelium, thereby deriving the necessary signals for their differentiation into hair cells, supporting cells and neurons that...
could repopulate and repair the damaged sensory epithelium. However, the same difficulties exist for transplantation as for gene therapy experiments. Integrating the transplanted cells into damaged epithelium and generating the correct numbers of cells in the correct parts of the organ of Corti will be a challenge. Given that much of cochlear function depends on the mechanical properties of the organ of Corti, excess or inappropriately placed cells are likely to cause problems.

**Summary**

Regeneration in the vertebrate inner ear offers an exciting opportunity to explore the cellular, molecular and functional mechanisms that are involved in rebuilding the exquisitely sensitive sensory receptor complexes of the vestibular and cochlear end organs and their connections to the central nervous system. Regeneration occurs naturally or in response to damage or trauma in the bird cochlea and in the vestibular epithelia of mammals, birds and lower vertebrates. Thus, the regulatory pathways that control regeneration are readily available for defining and manipulation. Regeneration in non-mammalian systems offers a unique pharmacological opportunity to explore and modulate the pathways that lead to hair cell regeneration and could give important insights into the potential for inducing mammalian hair cell regeneration. The mammalian cochlea has not yet shown an innate capacity for regeneration but current techniques involving genetic manipulation, gene therapy and stem cell transplantation could enable us to unlock the cochlea’s ability for structural repair and recovery of function. These research opportunities offer an exciting potential for developing cell- or drug-based therapies for treating hearing loss and balance disorders in humans.

**References**

14. Cotanche, D.A. (1999) Structural recovery from...
65 Li, H. et al. (2003) Pluripotent stem cells from the adult mouse inner ear. Nat. Med. 9, 1293–1299
Ototoxicity: therapeutic opportunities

Leonard P. Rybak and Craig A. Whitworth

Two major classes of drugs currently in clinical use can cause permanent hearing loss. Aminoglycoside antibiotics have a major role in the treatment of life-threatening infections and platinum-based chemotherapeutic agents are highly effective in the treatment of malignant disease. Both damage the hair cells of the inner ear, resulting in functional deficits. The mechanisms underlying these troublesome side effects are thought to involve the production of reactive oxygen species in the cochlea, which can trigger cell-death pathways. One strategy to protect the inner ear from ototoxicity is the administration of antioxidant drugs to provide upstream protection and block the activation of cell-death sequences. Downstream prevention involves the interruption of the cell-death cascade that has already been activated, to prevent apoptosis. Challenges and opportunities exist for appropriate drug delivery to the inner ear and for avoiding interference with the therapeutic efficacy of both categories of ototoxic drugs.

Aminoglycosides

Aminoglycoside antibiotics were developed in 1944 to treat Gram-negative bacteria that were not responsive to conventional antibiotics, such as penicillin. These compounds can be characterized by amino sugars that have glycosidic linkages. Subsequently, a number of similar compounds have been developed and are still commonly used. However, their clinical use is limited by toxic side effects that include cochlear toxicity, vestibular toxicity and nephrotoxicity. The aminoglycoside antibiotics include streptomycin, kanamycin, tobramycin, neomycin, gentamicin, amikacin and netilmicin. All display ototoxicity but vary in their preferential damage to the cochlea or vestibule.

Reactive oxygen species

The generation of reactive oxygen species (ROS) is believed to be the initiating step of aminoglycoside ototoxicity in a cascade of events that ultimately results in cell death. The formation of ROS by aminoglycosides appears to involve iron. Aminoglycoside compounds can form complexes with iron [1] that then react with unsaturated fatty acids to form superoxide (O$_2^-$) radicals and lipid peroxides [2]. Typically, O$_2^-$ is converted to hydrogen peroxide (H$_2$O$_2$) by superoxide dismutase (SOD) and detoxified into water and oxygen by catalase. However, in highly oxidative conditions, endogenous antioxidant pathways can become overwhelmed, and free oxygen radicals can become abundant. Aminoglycosides, such as gentamicin, can activate inducible nitric oxide synthase (iNOS) in inner ear tissues, triggering an increase in nitric oxide [3]. Under these conditions, O$_2^-$ can react with available nitric oxide to form the destructive peroxynitrite radical or it can directly initiate cell death. Additionally, recent evidence suggests that genes for some antioxidant enzymes...
might be downregulated by aminoglycosides. Organ of Corti (OC) cultures exposed to gentamicin displayed a 2.16-fold downregulation of a catalase gene [4]. If \( \text{H}_2\text{O}_2 \) is not degraded by catalase, iron can catalyze the conversion of \( \text{H}_2\text{O}_2 \) to highly reactive hydroxyl radicals (\( \cdot\text{OH} \)) by the Fenton reaction [5]. Ternary complexes of \( \text{Fe}^{2+/3+} \), gentamicin-phosphatidylinositol 4,5-bisphosphate \( (\text{PIP}_2) \) and arachidonic acid (AA), which can be oxidatively damaged to release arachidonic acid, can form. Arachidonic acid can also form a ternary complex with iron and gentamicin, which, reacts with lipid peroxides and molecular oxygen, leading to the propagation of arachidonic acid peroxidation and further cellular damage [6]. Free radicals can rapidly react with cell constituents, including cell membranes and DNA. The resulting oxidative stress can trigger apoptotic cell death (Figure 1).

Aminoglycosides preferentially damage the outer hair cells (OHCs) of the OC and/or the type I vestibular hair cell. Intriguingly, supporting cells and inner hair cells (IHCs) of the OC are mostly unaffected. One possible reason for this discrepancy is the activation and translocation of the nuclear factor (NF) \( \kappa_B \), which is believed to have a role in ROS-induced cell signaling. Systemic administration of kanamycin to rats resulted in increased lipid peroxidation in all cell types of the OC, as indicated by immunostaining for 4-hydroxynonenal (4-HNE). This was accompanied by increased NF\( \kappa_B \) levels in nuclei of IHCs and supporting cells. However, NF\( \kappa_B \) was absent from the nuclei of OHCs, indicating that this nuclear factor is translocated to the nuclei of cells resistant to kanamycin, but not in cells that are sensitive to kanamycin. However, co-administration of salicylate or 2,3-dihydroxybenzoate facilitated translocation of NF\( \kappa_B \) into the OHC nuclei and protected them from kanamycin-induced cell damage [7].

In addition to differences in NF\( \kappa_B \) gradients within OC cell types, OHCs have lower antioxidant capacity compared with other OC cell types [8]. The level of glutathione, an endogenous intracellular antioxidant, in OHCs is lower than that of the level in other cell types in the OC, and there is a gradient of OC glutathione levels from the base to the apex of the cochlea. Apical OHCs have much higher levels of glutathione than basal OHCs [9]. Aminoglycoside-induced OHC damage originates in the basal region of the cochlea and can progress to middle and apical turns.

**Apoptotic cell death**

An excess of ROS is believed to trigger cell death by apoptosis (programmed cell death). Two forms of apoptosis are currently recognized: an extrinsic death-receptor-mediated apoptosis; and an intrinsic mitochondrion-mediated cascade. Current literature, which is based predominantly on acute *in vitro* studies using cell lines or explants from neonatal rodents, supports the intrinsic apoptosis pathway as the major pathway induced by aminoglycosides in the cochlea (but it remains to be seen whether this view will prevail with additional chronic *in vivo* studies). The intrinsic apoptosis pathway is characterized by activation of G proteins, such as Ras, and GTPases, such as Rac. These events can result in activation of a family of stress-activated protein kinases, such as mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK). The increased activity of these enzymes is accompanied by increased intracellular Ca\(^{2+}\) concentrations and release of cytochrome \( c \) from mitochondria. Cytochrome \( c \) release appears to be mediated by Bax (a protein that enhances apoptotic cell death), which causes mitochondrial membrane damage and pore formation. However, recent evidence from hepatic tumor cells suggest that \( \text{O}_2^- \) can directly cause a profound release of cytochrome \( c \), without damage to mitochondrial membranes, through a voltage-dependent anion transport channel. This phenomenon appears to modulate pore formation, but is not dependent on it [10]. Similarly, OC explants from adult guinea pigs displayed gentamicin-induced OHC death that was preceded by changes in mitochondrial membrane potentials. Co-administration

![FIGURE 1](https://www.drugdiscoverytoday.com)

**FIGURE 1**

**Proposed mechanisms for aminoglycoside-induced cell death in the cochlea.** Aminoglycosides have been shown to form ternary complexes with lipid components of the cell membrane, including phosphatidylinositol (4,5)-bisphosphate (PIP\(_2\)) and arachidonic acid (AA) and iron (\( \text{Fe}^{2+} \)). The complex of AG, PIP\(_2\), and \( \text{Fe}^{2+} \) can be oxidized by molecular oxygen to produce superoxide anion (\( \text{O}_2^- \)). Superoxide can then react with other cellular components or can dismutate to form hydrogen peroxide (\( \text{H}_2\text{O}_2 \)). The latter can react with complexed \( \text{Fe}^{3+} \) to produce hydroxyl radicals (\( \cdot\text{OH} \)). AA can be released from oxidative damage to PIP\(_2\), and can also form ternary complexes with \( \text{Fe}^{2+} \) and AG. OH\(^-\) can react with AA in the AG–\( \text{Fe}^{2+} \)-form carbon radicals, which can directly initiate peroxidation or can combine with molecular \( \text{O}_2 \) to form a peroxy radical with AA [6]. These various ROS can then activate cell-death pathways in the OHCs in the cochlea.
of the iron chelators, 2,2′-dipyridyl, salicylate, or cyclosporine A, a blocker of mitochondrial permeability transition, resulted in partial OHC protection. However, none of these compounds prevented gentamicin-induced condensation of chromatin in the nuclei of these cells [11]. These studies suggest that release of cytochrome c from mitochondria and subsequent activation of caspases-8, 9 and 3 are key steps in the cell death pathway, although other separate events also occur in the nucleus.

Aminoglycosides have been directly linked to many steps of the intrinsic apoptotic pathway in in vivo and in vitro studies. Gentamicin administration results in in vitro blocked using GTPase or Rac inhibitors [13]. The next stage of aminoglycoside-induced apoptosis appears to be the stress-activated protein-kinase cascade. This includes various protein kinases, such as JNK. These kinases are stored in the cytoplasm by a scaffold protein, c-Jun-interacting protein-1 (JIP1), that regulates their activity. This protein is believed to organize components of the JNK signal cascade and facilitate their phosphorylation. Activated JNK can then mediate activation of c-Jun, c-Fos, ELK-1 and activating transcription factor 2 (ATF2) in nuclei and Bcl-2 in mitochondria. Increases in JNK, c-Jun, c-Fos and Bcl-2 compounds have all been observed after aminoglycoside administration, as have cytoplasmic levels of cytochrome c and morphological indicators of apoptosis [14].

**Approaches to protection**

Approaches to otoprotection have included ‘upstream’ protection using antioxidants, free-radical scavengers and metal chelators. In addition, ‘downstream’ methods of protection have been investigated using compounds that inhibit various stages of apoptosis.

**Upstream protection**

Several promising agents have been investigated that prevent the initial stages of lipid peroxidation and cell damage by blocking the formation of ROS or scavenging ROS once they are formed. These include vitamin E [15], β-methionine [16] and α-lipoic acid [16]. Ebselen, an effective antioxidant and scavenger of peroxynitrite, has been shown to reduce gentamicin ototoxicity [18].

Because of the interaction of aminoglycosides with iron to form ROS, metal chelators have been investigated as protective agents. Deferoxamine, 2,2′-dipyridyl, salicylate and 2,3-dihydroxybenzoate are effective iron chelators that also function as antioxidants, and have been demonstrated to protect against aminoglycoside ototoxicity in animal studies [11,19]. Salicylate and 2,3-dihydroxybenzoate have been shown to facilitate the translocation of NFκB into the nuclei of OHCs, thus triggering anti-apoptotic pathways in these cells [7].

Flavonoids have been identified in a number of Chinese herbal extracts and have been shown to be protective against oxidative stress. These compounds possess numerous properties, including antioxidant effects, enhancement of antioxidant enzymes, free-radical scavenging and calcium stabilization. Herbal extracts that have been investigated as otoprotectants against aminoglycosides in animal studies include Ginkgo biloba [20], Gu Gui Bu (GSB) [21] and Tanishinone, a phenolic acid derivative of Danshen [22].

Other compounds that display antioxidant properties and have recently been investigated as protective agents against aminoglycoside ototoxicity include corticosteroids and neurotrophic growth factor. Dexamethasone, a corticosteroid, protected isolated OHCs from aminoglycoside ototoxicity, presumably by the inhibition of nitric oxide synthesis and free radical formation [23,24]. Neurotrophic growth factors have been shown to increase antioxidant enzyme activity, reduce NO formation and increase anti-apoptotic Bcl-2 proteins while inhibiting pro-apoptotic proteins. This family of compounds has shown considerable promise as protective agents. Gene therapy with transforming growth factor (TGF)-β1 and glial-cell-derived neurotrophic factor (GDNF) was shown to protect auditory function in guinea pigs [25].

Augmentation of endogenous antioxidant enzymes has been helpful in defining mechanisms of aminoglycoside ototoxicity as well as providing novel potential approaches to otoprotection. The SOD mimetic, M40403, has recently been shown to protect organotypic OC cultures from gentamicin ototoxicity [26]. Surprisingly, M40403 was not effective in protecting against the toxic effects of cisplatin in this system. Perhaps cisplatin has greater intrinsic toxicity than gentamicin, causing a greater depletion of glutathione than gentamicin, or it might cause an imbalance in complementary enzyme systems in this tissue, or it might act by pathways other than through ROS [26].

Guinea pigs whose cochlea were inoculated with adenoviral vectors for catalase, SOD1 or SOD2 were partially protected from aminoglycoside ototoxicity. Catalase and SOD2 overexpression were more effective than SOD1 [27].

Alternative otoprotective strategies have been used for aminoglycosides, including ‘cell toughening’ and modifying aminoglycoside kinetics with loop diuretics. The phenomenon of cell toughening in the OC, where preconditioning to low levels of stress can prepare cells to tolerate higher levels, has been studied as a means of protection from acoustic trauma. Toughening can increase levels of endogenous antioxidants, thus bolstering cellular protective mechanisms. Preconditioning with low doses of amakacin could protect against ototoxic dosages of amikacin in a guinea pig study [28].

Otopotoxic synergism between aminoglycosides and loop diuretics has been well documented. However, delayed administration of ethacrynic acid has been suggested as a means of lowering perilymphatic concentrations of
aminoglycosides, thus limiting the extent of cochlear damage. Cochlear damage was reduced when ethacrynic acid was administered 12 to 18 h after the last dose of gentamicin in guinea pigs [29].

**Downstream protection**

In addition to protecting the cochlea from oxidative stress and free radicals that lead to cell death, it could also be possible to slow or reverse the process of cell death. As discussed earlier, aminoglycosides have been shown to place cochlear cells into a pro-apoptotic state. As a result, trials with compounds that can block aminoglycoside-induced apoptosis have been the focus of recent research. For example, inhibition of early promoters of apoptosis, GTPases, Rho and Rac, by *Clostridium difficile* toxin B provided dose-dependent protection against aminoglycoside ototoxicity in vitro. This toxin also reduced the levels of c-Jun phosphorylation [13].

Phosphorylation of c-Jun via the JNK cascade appears to be a key turning point for OHC apoptosis. An inhibitor of the JNK cascade, CEP1347, has been shown to reduce aminoglycoside ototoxicity [30]. A synthetic inhibitor of JNK phosphorylation (D-JNKI-1) has shown some promise in protecting against neomycin ototoxicity. Co-administration of D-JNKI-1 resulted in nearly complete protection against neomycin-induced OHC mortality in OC explants. D-JNKI-1 also reduced neomycin-induced expression of c-Fos, a nuclear transcription factor involved in apoptosis, to near control levels. These results were also observed in vivo [14].

Blocking later events in the apoptotic pathway could also provide otoprotection. Minocycline, a tetracycline antibiotic, is a known inhibitor of caspases and cytochrome-c-release into the cytoplasm. Minocycline has recently been shown to protect against gentamicin ototoxicity in vitro [31]. The preservation of cell viability by minocycline is

**TABLE 1**

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
<th>Protective agent</th>
<th>In vivo</th>
<th>In vitro</th>
<th>Species</th>
<th>Efficacy</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>2,2-DPD or cyclosporine A</td>
<td>X</td>
<td></td>
<td>Guinea pig</td>
<td>++</td>
<td>[11]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>G protein inhibitor (GDP-βs)</td>
<td>X</td>
<td></td>
<td>Rat</td>
<td>+++</td>
<td>[12]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Ras inhibitor (FTI277)</td>
<td>X</td>
<td></td>
<td>Rat</td>
<td>+++</td>
<td>[12]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Ras inhibitor (B581)</td>
<td>X</td>
<td></td>
<td>Rat</td>
<td>++</td>
<td>[12]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>C. difficile toxin</td>
<td>X</td>
<td></td>
<td>Rat</td>
<td>++</td>
<td>[13]</td>
</tr>
<tr>
<td>Neomycin</td>
<td>D-JNKI-1</td>
<td></td>
<td></td>
<td>Mouse</td>
<td>+++</td>
<td>[14]</td>
</tr>
<tr>
<td>Neomycin</td>
<td>D-JNKI-1</td>
<td></td>
<td></td>
<td>Intracochlear</td>
<td>Guinea pig</td>
<td>+++</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>α-Tocopherol</td>
<td></td>
<td></td>
<td>Oral gavage</td>
<td>Guinea pig</td>
<td>+++</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>d-methionine</td>
<td>i.p.</td>
<td></td>
<td>Guinea pig</td>
<td>++</td>
<td>[16]</td>
</tr>
<tr>
<td>Amikacin</td>
<td>Lipoic acid</td>
<td>i.m.</td>
<td></td>
<td>Guinea pig</td>
<td>+++</td>
<td>[17]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Ebselen</td>
<td>i.p.</td>
<td></td>
<td>Guinea pig</td>
<td>+++</td>
<td>[18]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Salicylate</td>
<td>s.c.</td>
<td></td>
<td>Guinea pig</td>
<td>+++</td>
<td>[19]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td><em>Ginkgo biloba</em> extract</td>
<td>Round window</td>
<td>Guinea pig</td>
<td>+++</td>
<td>[20]</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Gu Siu Bu</td>
<td>i.m.</td>
<td></td>
<td>Guinea pig</td>
<td>++</td>
<td>[21]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Danshen</td>
<td>s.c.</td>
<td></td>
<td>Mouse</td>
<td>++</td>
<td>[22]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Danshen</td>
<td>X</td>
<td></td>
<td>Mouse</td>
<td>+++</td>
<td>[22]</td>
</tr>
<tr>
<td>Kanamycin + Ethacrynic acid</td>
<td>Dexamethasone</td>
<td></td>
<td></td>
<td>Guinea pig</td>
<td>+</td>
<td>[23]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Dexamethasone + liver extract</td>
<td>X</td>
<td></td>
<td>Chinchilla</td>
<td>+++</td>
<td>[24]</td>
</tr>
<tr>
<td>Kanamycin + Ethacrynic acid</td>
<td>GDNF + TGF-β1</td>
<td></td>
<td></td>
<td>Intracochlear adenovirus</td>
<td>Guinea pig</td>
<td>++</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>SOD analog (M40403)</td>
<td>X</td>
<td></td>
<td>Mouse</td>
<td>++</td>
<td>[26]</td>
</tr>
<tr>
<td>Kanamycin + Ethacrynic acid</td>
<td>SOD1 or SOD2</td>
<td></td>
<td></td>
<td>Intracochlear adenovirus</td>
<td>Guinea pig</td>
<td>++</td>
</tr>
<tr>
<td>Amikacin</td>
<td>Amakacin preconditioning</td>
<td>i.m.</td>
<td></td>
<td>Guinea pig</td>
<td>++</td>
<td>[28]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Ethacrynic acid</td>
<td>i.v.</td>
<td></td>
<td>Guinea pig</td>
<td>+</td>
<td>[29]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>CEP1347</td>
<td>s.c.</td>
<td></td>
<td>Guinea pig</td>
<td>+</td>
<td>[30]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Minocycline</td>
<td>X</td>
<td>Rat</td>
<td>++</td>
<td>[31]</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Minocycline or p38 MAPK inhibitor (SB203580) + caspase 3 inhibitor (DEVD or ZVAD)</td>
<td>X</td>
<td>Rat</td>
<td>+++</td>
<td>[32]</td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations: i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous.

*Key: X denotes that in vitro studies have been carried out.

*Key: +, low efficacy; ++, moderate efficacy; +++ high efficacy.
accompanied by blocking gentamicin-induced caspase-3 activation and release of cytochrome c into the cytoplasm of hair cells. However, the use of a caspase-3 inhibitor alone only partially protects against gentamicin ototoxicity. Therefore, the effectiveness of minocycline might be attributed to its inhibition of upstream enzymes, such as p38 MAPK, in combination with caspase-3 inhibition. In fact, it has recently been demonstrated that caspase inhibitors combined with a p38 MAPK inhibitor, provided synergistic protection against gentamicin ototoxicity in vitro [32]. The most recent experimental studies of protective agents against aminoglycoside ototoxicity in vitro and in vivo are summarized in Table 1.

**Platinum compounds**

Cisplatin was first synthesized by Peyrone in 1845, and thus it is sometimes referred to as ‘Peyrone’s chloride’. In 1965, Rosenberg and Cavaleri discovered that an electrical current delivered between two platinum electrodes inhibited the proliferation of Escherichia coli [33]. They found that platinum complexes were formed in the presence of ammonium and chloride ions. Cisplatin was found to be the most active platinum compound in experimental tumor systems, and was introduced into clinical chemotherapy in the early 1970s. It is a highly effective agent for the treatment of a wide variety of soft-tissue neoplasms, including testicular, ovarian, cervical, bladder and lung cancer and squamous cell cancer of the head and neck. Unfortunately, nephrotoxicity, neurotoxicity and ototoxicity can occur. The clinical presentation of cisplatin includes tinnitus and high frequency sensorineural hearing loss, which can be permanent and progressive, involving the lower frequencies. With escalation of the dose of cisplatin in treatment protocols, nearly every patient can develop at least some hearing loss [34]. Cisplatin ototoxicity can involve the production of ROS [35]. Administration of ototoxic doses of cisplatin to experimental animals results in depletion of glutathione and antioxidant enzymes (SOD, catalase, glutathione peroxidase and glutathione reductase) in cochlear tissues, with a corresponding increase in malondialdehyde levels [36]. A potential source for the production of toxic, free radicals in the cochlea following cisplatin exposure is NADPH oxidase, the enzyme that catalyzes the formation of superoxide radicals. A particular isoform of NADPH oxidase, NOX3, is highly expressed in the inner ear as demonstrated by real-time PCR. In situ hybridization studies demonstrated that NOX3 is localized to vestibular and cochlear sensory epithelia and spiral ganglion. NOX3-transfected human embryonic kidney 293 cells pre-incubated with cisplatin showed markedly enhanced superoxide production. These exciting new findings suggest that NOX3 is an important source of ROS generation in the cochlea, which might contribute to hearing loss [37]. Because this enzyme is only expressed in the inner ear, a specific inhibitor of this enzyme could possibly be developed and then administered either systemically or locally to the round window membrane to protect against hearing loss and cochlear damage caused by cisplatin.

The superoxide radicals generated by cisplatin exposure can be transformed into hydrogen peroxide, which can be catalyzed by iron to form the highly reactive hydroxyl radical. This can react with polyunsaturated fatty acids to generate the toxic aldehyde 4-hydroxynonenal (4-HNE). After local application of cisplatin to mouse cochlea, 4-HNE and nitrotyrosine (NT) were detected immunohistochemically in auditory epithelia and in neurons damaged by cisplatin. However, in auditory hair cells, only 4-HNE but not NT-immunoreactivity was detected. These findings suggest that the hydroxyl radical might play a crucial role in cisplatin-induced hearing loss and hair cell degeneration [38].

Cisplatin-induced ototoxicity in animal models is characterized by high-frequency hearing loss in guinea pigs, as demonstrated by changes in compound action potential thresholds after 5 daily intraperitoneal (i.p.) injections of 2mg/kg of cisplatin. This was accompanied by selective loss of outer hair cells in the basal and middle turns of the cochlea. The outer hair cells in the basal region of the cochlea demonstrated TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling)-positive staining and pathologic staining with Hoechst 33342 dye, indicating apoptotic cell death [39].

A new model for the study of cisplatin ototoxicity has been developed. This consists of a two-cycle treatment in rats, rather than an acute single high dose of cisplatin as used in other studies. Each cycle consists of four days of cisplatin injections (1 mg/kg i.p., twice daily) separated by 10 days of rest. Hearing threshold elevations and hair cell loss occurred only after the second cycle, and no mortality was reported. This model serves to eliminate potentially confounding factors that can determine the survival of a special cohort of animals [40].

Important signaling events that regulated the cell death of cisplatin-damaged cochlear cells included: the activation and redistribution of cytosolic Bax and the release of cytochrome c from injured mitochondria; activation of caspases-9 and 3 but not caspase-8; and the cleavage of fodrin by activated caspase-3 within the cuticular plate in damaged hair cells [41]. Because perfusing the cochlea with a caspase-8 inhibitor was not effective in preventing either cisplatin-induced hair cell death or hearing loss, it appears that the apoptosis of cochlear hair cells caused by cisplatin in guinea pigs is caspase-8 independent. Moreover, because caspase-9 and caspase-3 are activated in cisplatin-damaged hair cells, and because the intracochlear perfusion of the inhibitors of these caspases prevented apoptosis and hearing loss, it is likely that cisplatin ototoxicity is mediated by mitochondrial damage in the affected hair cells, with sequential activation of initiator and effector caspases, resulting in apoptosis, hair cell destruction and hearing loss (Figure 2).
Cisplatin-treated animals were found to have significant increases in JNK, and perilymphatic perfusion of D-JNKI-1 prevented neither the activation and cellular redistribution of Bax nor the release of cytochrome c from mitochondria [41].

**Upstream protection**

A number of potentially protective agents containing thiol groups have been tested for efficacy against cisplatin ototoxicity in animal studies. These include: sodium thiosulfate, D- or L-methionine, diethyldithiocarbamate, methylthiobenzoic acid, lipoic acid, N-acetylcysteine, tiopronin, glutathione ester and amifostine. Perfusion of sodium thiosulfate into the cochlea of guinea pigs completely prevented cisplatin-induced hearing loss, and cochlear hair cells were well preserved in protected animals [42]. However, chronic round window application of sodium thiosulfate with an osmotic minipump provided no protection against cisplatin ototoxicity [43]. D-methionine provided excellent protection against outer hair cell loss, antioxidant enzyme depletion and auditory threshold elevations in rats pretreated with this agent prior to cisplatin administration [44]. Glutathione ester, but not glutathione, protected rats against cisplatin ototoxicity [45].

Amifostine afforded dose-dependent protection against cisplatin ototoxicity in hamsters. However, the protective agent that produced moderate to complete protection against ototoxicity also caused neurotoxicity at higher doses, manifested by prolongations in the auditory brainstem response (ABR) interpeak latency [46]. α-Tocopherol has been reported to reduce cisplatin-induced outer hair cell damage and auditory brainstem threshold elevations in rats following systemic pretreatment [47]. It blocked lipid peroxidation in the cochlea, and prevented apoptosis and outer hair cell loss accompanied by ABR threshold elevations in guinea pigs [48].

However, in another study of cisplatin ototoxicity in guinea pigs, only partial protection was seen with α-tocopherol pretreatment. When tiopronin (N-(2-mercaptopropionyl)-glycine) was combined with α-tocopherol, more effective protection was obtained. The greater effect seen with this combination was explained by the prevention of free radical formation by two different mechanisms: the thiol compound, tiopronin, by improving the antioxidant defenses of the cochlea to scavenge ROS, and α-tocopherol by inhibiting lipid peroxidation and preventing chain reactions [49]. In rats, tiopronin administered with cisplatin resulted in a significant protective effect on hair cells in the basal half and in the lower half of the middle turn of the cochlea. This cytoprotective effect was associated with a significant increase in the distortion product otoacoustic emissions (DPOAE) elicited in protected rats compared with rats treated with cisplatin alone [50]. In contrast with sodium thiosulfate, which interferes with the tumoricidal effects of cisplatin, tiopronin showed no reduction of tumor cell cytotoxicity in vitro. In tumor-bearing mice, tiopronin substantially reduced tumor growth in animals treated with a nontoxic dose of cisplatin, suggesting that tiopronin might enhance the antitumor properties of cisplatin [51]. Trolox® (Oxis), a water-soluble form of vitamin E, was found to be effective in reducing the ototoxicity of cisplatin when applied topically to the round window membrane of guinea pigs. Reduction in ABR threshold elevation and hair cell loss was observed in animals protected with Trolox® compared with those treated with cisplatin alone [52].

**FIGURE 2**

**Proposed mechanisms for cell death in OHCs exposed to cisplatin.** Cisplatin can generate O$_2^-$, perhaps through NADPH oxidase or other enzymes in the cochlea. Through the Fenton reaction, OH$^-$ can be produced. The latter can interact with polyunsaturated fatty acids (PUFA) in the cell membrane to generate toxic aldehydes, such as 4-hydroxynonenal (4-HNE), leading to cell death. O$_2^-$ can activate inducible nitric oxide synthase (iNOS) to generate nitric oxide (NO) which can interact with O$_2^-$ to form peroxynitrite (OONO$^-$). The latter can react with proteins to form nitrotyrosine (NT). These toxic intermediates can then trigger cell death by causing the release of cytochrome c from the mitochondria, resulting in activation of downstream caspases (caspase-9 and -3), leading to apoptosis.
Administration of cisplatin (1mg/kg twice daily) for two cycles of four days each, separated by 10 days of rest produced significant hearing loss of 40 to 50 dB by ABR testing in rats receiving cisplatin alone. When sodium salicylate was added (100mg/kg subcutaneous, twice daily) cisplatin-induced threshold shifts were reduced and the antioxidant levels in the cochlea were restored or preserved. Salicylate might provide an antioxidant effect that antagonizes cisplatin ototoxicity [40]. Previous studies in tumor-bearing animals showed no antitumor interference in animals cotreated with cisplatin and salicylate at dosage levels where cisplatin ototoxicity was prevented by salicylate pretreatment [53].

Aminoguanidine is an inhibitor of iNOS. It is also an antioxidant that can scavenge hydroxyl radicals. Pretreatment of rats with aminoguanidine reduced the ototoxicity of cisplatin, resulting in significantly less malondialdehyde production in the cochlea and less elevation of ABR thresholds, but did not reduce the amount of nitric oxide produced. Thus, it might act as a free-radical scavenger, rather than as an iNOS inhibitor, in protecting the cochlea against cisplatin injury [54].

A significant reduction in acute ototoxicity of cisplatin in rats was observed in rats pretreated with a combined oral formulation of allopurinol and ebselen (a glutathione peroxidase mimic). Outer hair cells and auditory thresholds were preserved in rats administered the protective agents [55].

The antioxidant defense mechanisms in the cochlea might be mediated, at least in part, by adenosine receptors (ARs). The A1AR, and possibly, the A3AR might provide cytoprotection in the cochlea [56]. Local instillation of R-phenylisopropyladenosine (R-PIA) results in significant increases in cochlear glutathione peroxidase and superoxide dismutase within 90 min [57]. The application of cisplatin to the round window membrane of chinchillas results in an increase in A1AR in the cochlea at 24 and 72 h [57]. Pretreatment with the A1AR agonists, R-PIA or 2-chloro-N-cyclopentyladenosine (CCPA) provided significant protection against the loss of cochlear hair cells and ABR threshold elevations observed after cisplatin application to the round window membrane of chinchillas. These protective effects were blocked by prior application of the specific A1AR antagonist, 8-cyclopentyl-1,3-dipropylxanithane (DPCPX). Furthermore, application of the A2AR agonist, 2-[4-(2-carboxy-ethyl)phenylamino]-5′-N-ethylcarboxamidoadenosine (CGS) to the round window membrane prior to cisplatin actually increased cochlear damage and threshold shift compared with cisplatin alone. These findings are consistent with the concept that the A1AR contributes significantly to cytoprotection in the cochlea, therefore protecting against hearing loss [58]. Although A1ARs in cochlear tissues are upregulated following cisplatin exposure, antagonists to this receptor do not afford protection against cisplatin ototoxicity. Rather, the agonists for the A1AR provide good protection against hearing loss and cochlear damage from cisplatin. This could occur because the upregulation of adenosine receptors represents an abortive attempt of the cochlea to protect itself from cisplatin toxicity, rather than a noxious effect of the cisplatin exposure. The interaction between adenosine A1 agonists and their receptor increase the antioxidant defenses in the cochlea and thereby afford protection of the structure and function.

**Downstream protection**

Intracochlear perfusion with caspase-3 inhibitor (z-DEVD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) dramatically reduced the incidence of apoptosis, hair cell loss and hearing threshold change that would otherwise occur after cisplatin administration in the guinea pig [41]. On the other hand, the intracochlear perfusion of D-JNKI-1, a cell-permeable peptide that blocks JNK-mediated activation of c-Jun, failed to prevent the mitochondrial release of cytochrome c. Paradoxically, the presence of D-JNKI-1 increased the sensitivity of cochlear hair cells to damage by cisplatin [41].

Addition of the p53 inhibitor, pifithrin-α to cultures of organotypic OC cells exposed to cisplatin protected the hair cells from ototoxic damage. These findings suggest that ototoxicity of cisplatin involves activation of p53 in triggering apoptotic cell death [59]. The results of the various experimental studies using in vitro and in vivo methods are summarized in Table 2.

**Clinical implications and conclusions**

There are certain similarities, yet other unexplained differences, in the ototoxic effects of aminoglycoside antibiotics and cisplatin. Both cause high frequency sensorineural hearing loss, which is usually permanent, and associated with loss of outer hair cells in the basal turn of the cochlea. Animal experiments suggest that both groups of drugs produce ROS in the inner ear, and these intermediates can activate cell-death pathways. Current evidence suggests that both groups of ototoxins act predominantly through the intrinsic cell death pathway, although future experiments should exclude the possible role of other pathways in addition to the intrinsic pathway. Aminoglycoside and cisplatin ototoxicity can both be reduced by the use of protective agents that block the production of or scavenge ROS. These upstream protective agents, such as antioxidants, can be administered systemically to protect against aminoglycoside or cisplatin ototoxicity, provided that interference with desired therapeutic effects have been convincingly excluded. Protective agents acting on downstream pathways can also be effective, but they might require local administration to minimize systemic side effects. It is possible to administer chemoprotective agents, anti-apoptotic drugs and other pharmaceuticals to the round window in humans to effect downstream protection against ototoxicity [60], thereby avoiding systemic toxicity from the protective agent and obviating antagonism of the therapeutic effects.
of aminoglycosides or cisplatin. Future studies should further elucidate the similarities and differences between the ototoxic mechanisms underlying the ototoxicity of aminoglycosides and cisplatin in order to develop more selective and specific protective strategies to minimize their ototoxicity.

References

9 Sha, S.H. et al. (2001) Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. Hear. Res. 155, 1–8
12 Battaglia, A. et al. (2003) Involvement of ras activation in toxic hair cell damage of the mammalian cochlea. Neuroscience 122, 1025–1035
18 Takumida, M. et al. (1999) Free radicals in the guinea pig inner ear following gentamicin exposure. ORL J. Otorhinolaryngol. Relat. Spec. 61, 63–70
sha, s.h. and schacht, j. (1999) salicylate attenuates gentamicin-induced ototoxicity. lab. invest. 79, 807–813
20 jung, h.w. et al. (1998) effects of ginkgo biloba extract on the cochlear damage induced by local gentamicin installation in guinea pigs. j. korean med. sci. 13, 525–528
21 long, m. et al. (2004) flavanoid of drynaria fortunei protects against gentamicin ototoxicity. phytother. res. 18, 609–614
22 wang, a.m. et al. (2003) tanshinone (salviae miltiorrhize extract) preparations attenuate aminoglycoside-induced free radical formation in vitro and ototoxicity in vivo. antiinfect. agents chemother. 47, 1836–1841
23 hимеро, c. et al. (2002) intra-cochlear administration of dexamethasone attenuates aminoglycoside ototoxicity in the guinea pig. hear. res. 167, 61–70
24 park, s.k. et al. (2004) protective effect of corticosteroid against the cytotoxicity of aminoglycoside otic drops on isolated cochlear outer hair cells. laryngoscope 114, 768–771
25 kawamoto, k. et al. (2003) hearing and hair cells are protected by adenosinal gene therapy with tgf-beta1 and gdfn. mol. ther. 7, 484–492
26 mccadden, s.l. et al. (2003) m40403, a superoxide dismutase mimetic, protects cochlear hair cells from gentamicin, but not cisplatin toxicity. toxicol. appl. pharmaco. 186, 46–54
27 kawamoto, k. et al. (2004) antioxidant gene therapy can protect hearing and hair cells from ototoxicity. mol. ther. 9, 173–181
29 ding, d. et al. (2003) late dosing with ethacrynic acid can reduce gentamicin concentration in perilymph and protect cochlear hair cells. hear. res. 185, 90–96
31 corbacella, e. et al. (2004) minocycline attenuates gentamicin induced hair cell loss in neonatal cochlear cultures. hear. res. 197, 11–18
32 wei, x. et al. (2005) minocycline prevents gentamicin-induced ototoxicity by inhibiting p38 map kinase phosphorylation and caspase 3 activation. neuroscience 131, 513–521
33 rosenberg, b.h. and cavalieri, l.f. (1965) template deoxyribonucleic acid and the control of replication. nature 206, 999–1001
34 benedetti panici, p. et al. (1993) efficacy and toxicity of very high-dose cisplatin in advanced ovarian carcinoma: 4-year survival analysis and neurological follow-up. int. j. gynecol. cancer 3, 44–53
35 clerici, w.j. et al. (1996) direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. hear. res. 98, 116–124
36 rybak, l.p. et al. (2000) effect of protective agents against cisplatin ototoxicity. am. j. otol. 21, 513–520
37 banfi, b. et al. (2004) nox3, a superoxide-generating nadph oxidase of the inner ear. j. biol. chem. 279, 46065–46072
38 lee, j.e. et al. (2004) role of reactive radicals in degeneration of the auditory system of mice following cisplatin treatment. acta otolaryngol. 124, 1131–1135
39 alam, s.a. et al. (2000) cisplatin-induced apoptotic cell death in mongolian gerbil cochlea. hear. res. 141, 28–38
40 minami, s.b. et al. (2004) antioxidant protection in a new animal model of cisplatin-induced ototoxicity. hear. res. 198, 137–143
41 wang, j. et al. (2004) caspase inhibitors, but not c-jun nh2-terminal kinase inhibitor treatment, prevents cisplatin-induced hearing loss. cancer res. 64, 9217–9224
42 wang, j. et al. (2003) local application of sodium thiosulfate prevents cisplatin-induced hearing loss in the guinea pig. neuropharmacology 45, 390–393
43 wimmer, c. et al. (2004) round window application of d-methionine, sodium thiosulfate, brain-derived neurotrophic factor and fibroblast growth factor-2 in cisplatin-induced ototoxicity. otol. neurotol. 25, 33–40
44 campbell, k.c.m. et al. (2003) the effect of d-methionine on cochlear oxidative state with and without cisplatin administration: mechanisms of otoprotection. j. am. acad. audiol. 14, 144–156
45 campbell, k.c.m. et al. (2003) glutathione ester, but not glutathione protects against cisplatin-induced ototoxicity in a rat model. j. am. acad. audiol. 14, 124–133
46 church, m.w. et al. (2004) wr-2721 (amifostine) ameliorates cisplatin-induced hearing loss but causes neurotoxicity in hamsters: dose-dependent effects. j. assoc. res. otolaryngol. 5, 227–237
47 kalkanis, j.g. et al. (2004) vitamin e reduces cisplatin ototoxicity. laryngoscope 114, 538–542
48 teranishi, m-a. et al. (2001) effects of alpha-tocopherol on cisplatin-induced ototoxicity in guinea pigs. hear. res. 151, 61–70
50 fetoni, a.r. et al. (2004) the protective role of tiopronin in cisplatin ototoxicity in wistar rats. int. j. audiol. 43, 465–470
51 viale, m. et al. (1999) cisplatin combined with tiopronin or sodium thiosulfate: cytotoxicity in vitro and antitumor activity in vivo. anticancer drugs 10, 419–428
52 teranishi, m. and nakashima, t. (2003) effects of trolax, locally applied, on round windows, on cisplatin-induced ototoxicity in guinea pigs. int. j. pediatr. otolaryngolog. 67, 133–139
53 li, g. et al. (2002) salicylate protects hearing and kidney function without compromising its oncolytic actions. lab. invest. 82, 585–596
54 kelly, t.c. et al. (2003) aminoguanidine reduces cisplatin ototoxicity. hear. res. 186, 10–16
55 lynch, e.d. et al. (2005) reduction of acute cisplatin ototoxicity and nephrotoxicity in rats by oral administration of allopurinol and ebselen. hear. res. 201, 81–89
56 ford, m.s. et al. (1997) expression and function of adenosine receptors in the chinchilla cochlea. hear. res. 105, 130–140
57 ford, m.s. et al. (1997) up-regulation of adenosine receptors in the chinchilla cochlea by cisplatin. hear. res. 111, 143–152
59 zheng, m. et al. (2003) pilithrin-alpha suppresses p53 and protects cochlear and vestibular hair cells from cisplatin-induced apoptosis. neuroscience 120, 191–205
60 seidman, m.d. and van de water, t.r. (2003) pharmacologic manipulation of the labyrinth with novel and traditional agents delivered to the inner ear. ear nose throat j. 82, 276–300
Therapeutics of hearing loss: expectations vs reality

Orna Atar and Karen B. Avraham

With the completion of the sequencing of the human genome, the field of medicine is undergoing a dramatic and fundamental change. The identification of our genes and the proteins they encode and the mechanisms of mutations that are pathogenic will allow us to devise revolutionary new ways to diagnose, treat and prevent the thousands of disorders that affect us. Certainly, disorders of the auditory system are no exception. Revealing the molecular mechanisms of hearing and understanding the role of each player in the intricate auditory network could enable us to employ gene- or cell-based therapy to cure or prevent hearing loss. To this end, much emphasis has been placed on the identification and characterization of genes involved in human deafness, as well as research on mouse models for deafness. Ultimately, the effect of genomics on medicine will be dramatic, providing us with the ability to cure sensory defects, a tangible goal that is now within our reach.
Hereditary Hearing Loss

The genes known today to be involved in human hereditary NSHL encode a large variety of proteins, including molecular motors, gap junctions, ion channels, transcription factors and proteins that form the extracellular matrix of the inner ear (for updated list of genes see the Hereditary Hearing Loss Homepage, http://webhost.ua.ac.be/hhh/). However, many crucial genes in auditory transduction were found in cellular and model systems, with no apparent involvement in human disease. This could be due to the fact that some gene mutations will be lethal in humans or alternatively, a particular mutation has not been found in the human population. Therefore the use of deaf mouse models, whether spontaneous, ENU-induced or created by gene-targeted mutagenesis, have been priceless for the discovery of essential auditory genes. Much of this knowledge has been gained as a direct consequence of the unraveling of the human genome sequence, heralded as one of the most significant discoveries of modern times.

Despite the high prevalence of HL in our society, treatment today is limited to hearing aids and cochlear implants. These therapeutic tools do not completely restore our ability to hear, but for now, are the best options available. Studies are being conducted to provide alternative means of biological therapy to provide a more comprehensive treatment. There are several general approaches being considered for therapeutic intervention in HL: (1) prevention of cell death; (2) manipulation of expressed genes by gene therapy methods; (3) inhibition of negative regulators; and (4) stem cell therapy (Figure 2).

These therapeutics strategies for hearing impairment are attractive and promising for restoring HL of genetic origin. However, clinical application, particularly in the ear, is still limited. Most of the difficulties are technical ones concerning delivery of genes into the afflicted portion of the cochlea. This review will cover the current therapeutic approaches for rescuing HL by preventing hair cell death, gene manipulation and stem cell therapy.

Prevention of cell death

Most of the literature has dealt with prevention of hair cell death following acoustic trauma or aminoglycoside ototoxic damage. Protecting hair cells from irreversible degradation has been a primary objective because of the finite number of hair cells in the inner ear. Hair cells stop differentiating during development and are post-mitotic, so that the number of cells we are born with (~16,000) is our lifetime supply. Little is known about the mechanisms of cell death in heredity disease. It has been shown that in cases where hair cell damage can be predicted (although not in most genetic cases), there are several ways to moderate the damage by using neurotrophic factors [3–5], antioxidants [6,7] or anti-apoptotic agents [8,9]. It remains to be determined if any of these agents or factors can also rescue hair cells from death due to genetic disease. In this review, we will focus on anti-apoptotic agents as a promising avenue for restoring HL.

Caspase 3 inhibitors (e.g. zVAD, BAF) can promote hair cell survival in vivo [10] and in vitro [11] after treatment with aminoglycosides. It remains to be determined if anti-apoptotic agents can also rescue hair cells from death due to genetic disease. In a Mongolian gerbil model for age-related HL, it has been shown that the HL is associated with suppression of the bcl-2 protein and activation of caspase 3 [12]. In several mouse models for HL, several apoptotic factors have been shown to be involved. Caspase 3-deficient mice suffer from severe HL, hyperplasia of supporting cells and degeneration of sensory hair cells [13,14]. Apparently, different steps in development and preservation of the auditory system are mediated by caspase 3. The transcription factor POU4F3 has a critical role in the development of sensory hair cells in mouse [15–17] and in humans [18]. Pou4f3 knockout mice have no cochlear or vestibular hair cells, resulting in complete deafness. The hair cells in these mice progressively degenerate via apoptosis during late embryonic development [19]. Cellular and molecular mechanisms seem to
be similar in HL resulting from aging, drug ototoxicity and genetic mutations. A final common pathway could be apoptosis. It is likely that anti-apoptotic factors will increasingly be considered as important candidates for intervention strategy in sensorineural HL. A more comprehensive understanding of the molecular mechanism of hair cell death might lead us to employ new therapeutic anti-apoptotic agents to alleviate hereditary HL.

**Gene manipulation**

In non-mammalian vertebrates, lost hair cells can be replaced by newly generated hair cells. Many efforts have been made to reveal the progenitor cell for newly produced hair cells. Genes that regulate the proliferation and the differentiation of hair cells and supporting cells are rapidly being discovered. The main approach for generation of hair cells is to introduce genes into the cochlea using gene delivery methods to induce non-sensory cells or damaged hair cells to transdifferentiate into functional hair cells.

**Genes regulating hair cell differentiation**

During embryonic development, cell fate is determined by a sequence of events governed by intercellular signaling
and expression of specific genes. In the inner ear, there are several known genes that participate in hair cell differentiation. By altering genes in the existing sensory epithelia, stimulation of cell division and differentiation may occur. Among these genes is the mammalian atonal homolog 1 (Atoh1, previously known as Math1), a basic helix loop helix transcription factor, which is necessary for hair cell differentiation [20]. Mice deficient in Atoh1 fail to develop hair cells [20]. The delivery of the gene encoding Atoh1 into the inner ear resulted in new hair cells [21–24]. The major breakthrough of the past year was the study performed by the Raphael group [23]. They used drug-induced deaf guinea pigs and introduced the Atoh1 gene using adenoviruses into the cochlea of these guinea pigs. Surprisingly, they brought back lost cochlear hair cells. Not only did new hair cells appear, but they also had functional properties. Eight weeks after injection, hearing improved substantially in the infected ears. This outcome is the first functional restoration of a damaged mammalian hearing organ at the cellular level. The reappearance of hair cells was extensive and occurred apparently at the correct site and in the correct orientation. The new hair cells were able to attract innervation. The authors speculate that the new hair cells were derived from supporting cells or from other nonsensory cells close to the organ of Corti, indicating a transdifferentiation pattern. The overall cell number in the organ of Corti increased after Atoh1 expression, suggesting that either secondary cell proliferation occurred or that cell migration into the organ of Corti took place. Cells with a mixed morphology were observed, demonstrating that the transdifferentiation was incomplete. Further investigation is required to better switch off the supporting cell repertoire and switch on the hair cell repertoire of genes to gain full transdifferentiation.

Several genes other than Atoh1 can cause developmental defects when absent from inner ear sensory epithelia, and some of these genes could be applicable for gene therapy use. The genes Hes1 and Hes5 are negative regulators of Atoh1, and mice lacking either gene have increased numbers of hair cells when compared with wild-type animals [25,26]. Hes1 knockout animals have more inner hair cells (IHC), whereas Hes5 knockout animals have more outer hair cells (OHC) [25]. These experiments have a significant therapeutic value that will set a platform for the future development of inner ear gene therapy approaches based on the expression of key developmental genes.

**Cell-cycle regulation genes**

In the cell-cycle program, the level of cyclin-dependent kinase (CDK) activity controls signals that drive cells into S-phase. The products of at least three different gene families, Ink4, Cip/Kip (for CDK interacting protein/kinase inhibitory protein) and the retinoblastoma (Rb) pocket protein family, suppress S-phase entry [27]. These gene products are named CDK inhibitors (CKI). Once the important molecules that signal mitosis in the sensory epithelium are identified, the next major step toward accomplishing hair cell regeneration will be introducing and regulating the expression of these genes in the cochlea. The Cip/Kip family includes p21Cip1, p27Kip1 and p57Kip2. When the p27Kip1 gene is knocked out in mice, the result is ongoing cell proliferation in the mature organ of Corti, well after the developmental period when mitosis in the sensory epithelia ceases [28]. Targeted disruption of Ink4d in the mouse changes the maintenance state of sensory hair cells in post-natal mice. In Ink4d−/− animals, hair cells re-enter the cell cycle and consequently undergo apoptosis, resulting in progressive HL [29]. The gene encoding Rb is part of a tumor suppressor gene family. The major function of Rb is in the regulation of cell cycle progression. Its ability to regulate the cell cycle correlates with the state of phosphorylation of Rb.

It has recently been shown that hair cells lacking the gene for Rb continue to proliferate, with extra rows of inner hair and outer hair cells appearing, while expressing differentiation markers [30,31]. In summary, it appears that Ink4d, Kip1 and Rb are involved in maintaining cellular order, suggesting a potential use of CKIs in regeneration of hair cells in the inner ear. The risk of using cell cycle genes is that malignancy might accompany any change in cell cycle regulation, so that using these genes in gene therapy will require special attention to this potential problem.

**Delivery methods**

In some ways, the cochlea is particularly well-suited for gene therapy. It is isolated from the remainder of the body by the blood–labyrinth barrier, and the perilymph and endolymph fluids permit liquids to reach the entire cochlea quickly. The advantage of the small and enclosed structure of the inner ear, however, also poses a limitation because practice is still required on how to deliver vectors to the inner ear without causing damage to existing or residual hearing. Despite significant surgical challenges, the mouse is a useful model for studying gene therapy and multiple mutants with HL are available for study [32]. Developing strategies for local delivery into the inner ear is crucial for clinical therapies based on experimental findings.

A variety of viral and nonviral gene transfer vectors have been developed for implementation of gene therapy and the delivery of therapeutic genes. Non-viral vectors include liposomes, the traditional nonviral vector used in inner ear research. The components of cationic liposomes are positively charged, whereas nucleic acids are negatively charged, so that the two materials are able to form a stable particle. The liposomes can be mixed with DNA of virtually any size. They are easily prepared in large amounts and one of their most promising features is that they are non-immunogenic (for review see [33]).
approach used has been to transfect a liposome mixture with LacZ or GFP reporter genes into mice and guinea pig cochleae in vivo [34–36], but this technique appears to be inefficient for successful gene delivery. There are other non-viral methods, such as the gene gun [37] and electroporation [22], but their rate of transfection is low. These methods have yielded significant results so far only in vitro.

There is extensive interest in the development of viral vector delivery systems of genes to the cochlea to cure hearing impairment. Recent studies have focused on vectors based on the herpes simplex virus [38–40], lentivirus [41], adenovirus [42–44], and adeno-associated virus (AAV) [45,46]. The expression patterns of vector-encoded transgenes have been found to differ significantly between vectors.

Adenoviral vectors (Ad) are being used most widely today for cochlear gene transfer. Some of their advantages are infection of dividing and non-dividing cells, ease of production, availability of high titers and high transduction efficiency. On the other hand, they do not integrate into the genome, leading to transient expression, and they can cause a strong immune response that will be toxic to the recipient cell [47]. Hair cell transduction in vivo has been made with the replication defective (E1-, E3-, pol-) or (E1-, E3-, E4-) Ad vector [24,48]. One must note the differences in the specific expression between studies. This variation might result from different Ad vector generations, Ad concentrations, methods of injection and differences in detection assays. The major drawback is in the immune response [49,50]. Recent studies have demonstrated limited hair cell damage [51] or protection from side effects by using immunosuppression treatment [52]. For these reasons, adenoviral vectors are still considered to be an optimal delivery tool in gene therapy of the cochlea.

AAV vectors for gene therapy are associated with several attractive features [53]. AAV vectors induce a much less potent immune response than Ad vectors [54]. AAV is able to infect and integrate into non-dividing cells with a high rate of occurrence and lead to stable integration. A major drawback of AAV is the packaging limit of 4.5 kb of foreign DNA in AAV particles, even when almost all the genome is replaced by the gene of interest (for review see [47]). Despite this limitation, the reduced immune response makes AAV attractive for further exploration. Recently, several studies found that different AAV serotypes can act differently on cochlear explants or in vivo and yield different transduction efficiencies (Figure 3a) [55–57]. The differences between in vivo and in vitro transduction must be taken into account when planning to use a particular serotype.

In the future, it is likely that we will see the fusion of vectors that will combine the infectivity and stability of the viral vectors and the safety of non-viral vectors. These ‘super’ vectors might lead to promising application of gene therapy for hearing disorders.

**Targeting expression**

The success of gene therapy depends on the ability of gene delivery systems to selectively deliver therapeutic genes to a sufficient number of target cells, yielding expression levels that influence the diseased state. There are several methods to target cells, specifically through the use of specific promoters or receptors.

The use of tissue-specific promoters makes it feasible to selectively express the therapeutic gene at the target cell [58]. Modification of promoters yields different expression patterns as well as differences in degrees of expression. The Atoh1 enhancer had been isolated and used to analyze the expression pattern of this gene [59]. The myosin VIIa hair cell-specific promoter was identified using transgenesis (Figure 3B) [60]. Myosin VIIa driven GFP expression was restricted to the hair cells of the inner ears derived from these transgenic mice. These promoters and/or enhancers can be used to deliver therapeutic gene products to the sensory hair cells.

In the case of surface receptors, which are unique to the target cells, a ligand that serves as a gene delivery vehicle would efficiently bind to the target cell surface receptor. To date, no specific hair cell receptor has been described as a ligand for gene targeting. Finding targeted ligands and more hair cells specific promoters could improve our delivery systems for the sensory epithelia.

**Stem cell therapy**

There is a great deal of hope and promise in stem cell research. The ability to differentiate stem cells into multiple cell types has been successfully applied in the generation of dopaminergic neurons for Parkinson disease [61,62] and insulin-secreting cells for diabetes [63,64]. In 2003, there was a major breakthrough in the use of stem cells for the replacement of hair cells with the discovery of embryonic stem (ES) cells, adult inner ear stem cells and neural stem cells that can generate hair cells in vivo [65–67]. Recent studies have shown that
auditory [68] and vestibular [66] sensory epithelia can be a source for stem or progenitor cells.

A well-established technique to generate stem cells is by using adult organs (for review see [69]). It has been shown that either neural stem cells [65,70] or inner ear stem cells [66] have the ability to differentiate to different inner ear cell types in vitro [66] and in vivo [65,70]. The generated cell types and the specific markers expressed were different between these two adult stem cells. The potential for using these stem cells in therapeutic treatment lies in the ability to induce the cells to proliferate and differentiate into sensory hair cells to rescue or restore HL. ES cells are pluripotent cells derived from the inner cell mass of the blastocyst. The Heller group succeeded in deriving inner ear progenitors from ES cells in vitro [67]. The progenitor cells expressed hair cell-specific markers such as the transcription factors Atoh1 and Pou4f3 and hair cell structural proteins such as myosin VIIa, epsin and parvalbumin 3. The ES-inner ear progenitors were implanted into the inner ear of chick embryos. Only cells in the inner ear sensory epithelia expressed hair cell-specific markers, demonstrating that the surroundings influenced the ES-derived cells. This is the first time that ES cells have successfully generated hair cells in vivo. The next step is to see whether the same observations will repeat themselves in a mouse inner ear, which is not identical to the avian ear.

However, it has not yet been proven that stem cells can become functional hair cells and integrate in the auditory epithelium. For hearing to be restored, it is necessary to form the appropriate neural pathways. Stem cells as cell therapy must, first of all, answer several questions: is the location of the newly generated hair cells important? Do we need to reconstruct the exact architecture of the inner ear to gain functional hearing? Will randomly oriented hair cells transfer correct information to the brain? Based upon the success of using cochlear implants, we can hope that any new hair cell that is connected to the nervous system will improve hearing abilities. Among the complex issues we will face is the need to prevent the stem cells from growing into tumors. Further experiments must be performed in vivo with an appropriate animal model for HL, which will need to face the challenges of surgical complications. Although there are ideal mouse models for hereditary [71,72] and drug-induced [73] HL, the mouse inner ear dimensions limit potential applications. Clearly, the next step in this field is to use the knowledge gathered from mouse (and other animals) and adapt it to human stem cells as a potentially therapeutic tool.

The future of therapeutics for hearing loss

To date, much of the work done thus far in the field has focused on gene therapy, that is, adding a ‘missing’ component by genetic methods. Unfortunately, gene therapy has not fulfilled expectations and many efforts to cure disease by this method have failed. Rather, modulating key components by inhibition could be a more favorable approach. For example, improving techniques for antisense or RNA interference (RNAi) are essential for the inner ear research field, as both these techniques have been heralded as having great potential for disease therapy. A new study performed by the Smith group succeeded in silencing the expression of the R75W allele variant of the GJB2 gene (connexin 26), which causes autosomal dominant NSHL. By silencing the mutant gene using the RNAi technique in vitro and in vivo, they restored hearing loss in a genetic mouse model [74]. The use of RNAi in clinical therapy still faces many obstacles. These include finding a suitable delivery method, establishing continuous and stable silencing, and coping with the interferon response (for review see [75]). Nevertheless, there is hope that RNAi can be successfully used as a therapeutic tool for HL. Furthermore, as delivery methods improve, modifying negative regulatory genes and cell cycle genes, discussed above, could be the method of choice to induce the growth of new hair cells.

Concluding remarks

The expanding knowledge of the molecular basis cell death, cell cycle and differentiation, along with advances in gene transfer technology, should aid in the development of methods for hair cell regeneration. A combination of genomic techniques will continue to reveal the regulatory networks of auditory transduction. For example, the microarray approach for elucidating regulatory pathways has proven to be useful [76] in identifying potential survival molecules, whereas the yeast two-hybrid approach has proven invaluable in elucidating the structural components of the stereocilia [77].

It is most reasonable to assume that gene therapy for HL will not be limited to one approach, but rather will integrate stem cells and gene modifications, along with drug treatment. For example, such methods might involve stem cells that will secrete a drug or express a gene that will restore functional hair cells.

The limited availability of hair cells has impeded their study and has made it logistically difficult to carry out many molecular biological and biochemical assays. For further research, improvements of some technical tools, such as the generation of more reliable hair cell lines, will be necessary to achieve significant progress in hair cell gene therapy. For clinical application, safe, effective and direct methods of gene delivery to the inner ear need to be developed to rescue HL.

Acknowledgements

We would like to thank Amiel Dror for creating the figures, Yehoash Raphael, Haim Sohmer and Andre Rosenthal for critical reading of the review and B. and A. Hirschfield for their support.
65 Tateya, I. et al. (2003) Fate of neural stem cells grafted into injured inner ears of mice. *Neuroreport* 14, 1677–1681
77 Boeda, B. et al. (2002) Myosin VIIa, harmonin and cadherin 23, three Usher I gene products that cooperate to shape the sensory hair cell bundle. *EMBO J.* 21, 6689–6699
The 5-HT_7 receptor (5-HT_7R) is the most recently identified subtype of the serotonin G-protein-coupled receptor superfamily. It possesses about 36–53% homology with the other human 5-HT receptors. The 5-HT_7R has been found to be positively coupled to adenylate cyclase, that is, agonists at the receptor cause a dose-dependent increase in intracellular cyclic AMP (cAMP). Using in situ hybridization techniques, it has been shown that 5-HT_7R is found both centrally and peripherally. The prominent expression of 5-HT_7 receptor in the thalamus, limbic and cortical regions of the brain, in addition to its having a high affinity for several antipsychotic and antidepressant agents, suggest that it could be involved in mental disorders such as schizophrenia [1] and depression [2].

There are many different compound classes capable of binding to the 5-HT_7R. A relatively large group of these ligands contain several fragments that additionally bind to other GPCRs (e.g. 5-HT_1A, 5-HT_2A, D_2). For example, an amine moiety (mostly 4-N-arylpiperazine,tetrahydroisoquinoline or 4-substituted tetrahydropyridine), which is connected by a different length alkyl chain (2–5 carbon atoms) to a terminal aromatic fragment [3]. Recently, workers have applied solid phase parallel chemistry for the generation of a focused arylpiperazine library [4], targeted at 5-HT_7 receptors on a solid support (SynPhase Lanterns: www.mimotopes.com, mimotopes, Pty). These authors designed a structurally related 64-member library of sulphonamide and carboxamide L- and D-proline derivatives. The library synthesis was carried out on BAL linker functionalized polyamide SynPhase Lanterns (Mimotopes, Pty) using a split-and-pool approach. The lanterns were equipped with coloured cogs and spindles (corresponding to building blocks) to produce a convenient visual tagging system [5]. The library members were evaluated for their in vitro affinity at central serotonin 5-HT_7 receptors; additionally, the affinity of 12 compounds for D_2 receptors was assessed, and 17 selected compounds were tested for their ability to bind to 5-HT_1A receptors.

The compounds selected were screened in radioligand binding assays. One of the most potent compounds against 5-HT_7 discovered was (i), which possessed a K_i of 183 nM at the 5-HT_7 receptor. This library also uncovered a number of compounds with good potency at the 5-HT_1A receptor: (ii), for example, has a K_i of 29 nM for 5-HT_1A. Thus, this work has developed an efficient solid supported method for the synthesis of novel sulphonamide and carboxamide proline derivatives. A 64-member library was obtained on SynPhase Lanterns and screened for the compounds biological evaluation for 5-HT_7 and 5-HT_1A serotonin, and D_2 dopamine receptor affinities. Further work is warranted as this study provides initial data for further investigations concerning 5-HT_7 receptor agents.

### MOLECULES

**Carboxamide proline derivatives as CNS agents**

The 5-HT_7 receptor (5-HT_7R) is the most recently identified subtype of the serotonin G-protein-coupled receptor superfamily. It possesses about 36–53% homology with the other human 5-HT receptors. The 5-HT_7R has been found to be positively coupled to adenylate cyclase, that is, agonists at the receptor cause a dose-dependent increase in intracellular cyclic AMP (cAMP). Using in situ hybridization techniques, it has been shown that 5-HT_7R is found both centrally and peripherally. The prominent expression of 5-HT_7 receptor in the thalamus, limbic and cortical regions of the brain, in addition to its having a high affinity for several antipsychotic and antidepressant agents, suggest that it could be involved in mental disorders such as schizophrenia [1] and depression [2].

There are many different compound classes capable of binding to the 5-HT_7R. A relatively large group of these ligands contain several fragments that additionally bind to other GPCRs (e.g. 5-HT_1A, 5-HT_2A, D_2). For example, an amine moiety (mostly 4-N-arylpiperazine,tetrahydroisoquinoline or 4-substituted tetrahydropyridine), which is connected by a different length alkyl chain (2–5 carbon atoms) to a terminal aromatic fragment [3]. Recently, workers have applied solid phase parallel chemistry for the generation of a focused arylpiperazine library [4], targeted at 5-HT_7 receptors on a solid support (SynPhase Lanterns: www.mimotopes.com, mimotopes, Pty). These authors designed a structurally related 64-member library of sulphonamide and carboxamide L- and D-proline derivatives. The library synthesis was carried out on BAL linker functionalized polyamide SynPhase Lanterns (Mimotopes, Pty) using a split-and-pool approach. The lanterns were equipped with coloured cogs and spindles (corresponding to building blocks) to produce a convenient visual tagging system [5]. The library members were evaluated for their in vitro affinity at central serotonin 5-HT_7 receptors; additionally, the affinity of 12 compounds for D_2 receptors was assessed, and 17 selected compounds were tested for their ability to bind to 5-HT_1A receptors.

The compounds selected were screened in radioligand binding assays. One of the most potent compounds against 5-HT_7 discovered was (i), which possessed a K_i of 183 nM at the 5-HT_7 receptor. This library also uncovered a number of compounds with good potency at the 5-HT_1A receptor: (ii), for example, has a K_i of 29 nM for 5-HT_1A. Thus, this work has developed an efficient solid supported method for the synthesis of novel sulphonamide and carboxamide proline derivatives. A 64-member library was obtained on SynPhase Lanterns and screened for the compounds biological evaluation for 5-HT_7 and 5-HT_1A serotonin, and D_2 dopamine receptor affinities. Further work is warranted as this study provides initial data for further investigations concerning 5-HT_7 receptor agents.

### Diketopiperazines as potential inhibitors of calpain

Calpains are a class of intracellular cytoplasmic non-lysosomal cysteine proteases expressed ubiquitously in mammalian cells. Of the 16 different kinds of calpain identified to date, two of the most studied are µ-calpain (calpain I) and m-calpain (calpain II). They differ in their sensitivity with respect to calcium ion activation: Calpain I requires micromolar concentrations of calcium, in contrast with calpain II, which responds only to millimolar concentrations [6]. Both isoforms are heterodimers with identical 30 kDa subunits, but differing 80 kDa subunits. Calpain overactivation is implicated in many conditions, particularly stroke and myocardial infarction [7]. Thus, selective calpain inhibitors might represent useful pharmacological probes and possibly therapeutic agents. Recent work [8] has centred on the identification of selective non-peptide inhibitors of calpain. These authors developed a one-pot cyclization procedure for the synthesis of diketopiperazine derivatives as calpain inhibitors. A small library of diketopiperazines was synthesized in solution and evaluated for inhibition of calpain I in a continuous fluorescence assay, using recombinant calpain I produced by the
baculovirus expression system, and Suc-Leu-Tyr-AMC23 as the fluorogenic substrate. When the library compounds were tested, only negligible activity against calpain I was observed: one of the most potent compounds isolated (ii) displayed an IC$_{50}$ of 0.1 mM. Compounds screened possessed IC$_{50}$s in the range 0.1–1.0 mM. This work has identified a small library of 2,5-diketopiperazines with potentially promising activity as calpain inhibitors. However, no potent calpain analogues were discovered from this methodology. Further work, as indicated by the authors, can now proceed in this important search for calpain inhibition using structure-based methods and an HTS approach.


Paul Edwards
paul.edwards@santhera.com