# New molecular therapies for the treatment of hearing loss

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## ARTICLE INFO

## ABSTRACT

An estimated 466 million people suffer from hearing loss worldwide. Sensorineural hearing loss is characterized by degeneration of key structures of the sensory pathway in the cochlea such as the sensory hair cells, the primary auditory neurons and their synaptic connection to the hair cells – the ribbon synapse. Various strategies to protect or regenerate these sensory cells and structures are the subject of intensive research. Yet despite recent advances in our understandings of the capacity of the cochlea for repair and regeneration there are currently no pharmacological or biological interventions for hearing loss. Current research focusses on localized cochlear drug, gene and cell-based therapies. One of the more promising drug-based therapies is based on neurotrophic factors for the repair of the ribbon synapse after noise exposure, as well as preventing loss of primary auditory neurons and regrowth of the auditory neuron fibers after severe hearing loss. Drug therapy delivery technologies are being employed to address the specific needs of neurotrophin and other therapies for hearing loss that include the need for high doses, long-term delivery, localised or cell-specific targeting and techniques for their safe and efficacious delivery to the cochlea. Novel biomaterials are enabling high payloads of drugs to be administered to the cochlea with subsequent slow-release properties that are proving to be beneficial for treating hearing loss. In parallel, new gene therapy technologies are addressing the need for cell specificity and high efficacy for the treatment of both genetic and acquired hearing loss with promising reports of hearing recovery. Some biomaterials and cell therapies are being used in conjunction with the cochlear implant ensuring therapeutic benefit to the primary neurons during electrical stimulation. This review will introduce the auditory system, hearing loss and the potential for repair and regeneration in the cochlea. Drug delivery to the cochlea will then be reviewed, with a focus on new biomaterials, gene therapy technologies, cell therapy and the use of the cochlear implant as a vehicle for drug delivery. With the current pre-clinical research effort into therapies for hearing loss, including clinical trials for gene therapy, the future for the treatment for hearing loss is looking bright.

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## Contents

## 1. Introduction

Over 5% of the world's population is living with a disabling hearing loss impacting on the individual's ability to communicate with others (WHO, 2018). Despite being a very important part of our lives, hearing is often taken for granted until it begins to deteriorate. The severity of the pathology ranges from loss of the synapse between the hair cells that convert sound input into electrical signals and the spiral ganglion neurons (SGNs) that transmit the electrical signals to the brain, to more severe cases with loss of hair cells and loss of SGNs. There are currently very few treatment options for people with hearing loss. Hearing aids may be used to amplify sounds for people with a mild or moderate hearing loss whereas cochlear implants are suitable for people with more significant hearing loss. While hearing aids and cochlear implants partially reverse the symptoms of hearing loss they do not restore the underlying pathology: cellular degeneration in the cochlea and loss of connection between the cochlea and the central auditory processing areas of the brain. At the pre-clinical level there is mounting evidence of therapeutic protection and/or regeneration of hair cells, SGNs and other affected cell types with numerous examples of hearing protection and hearing recovery. Therapeutic strategies focus on overcoming some of the obstacles of providing safe and efficacious localised drug delivery to the cochlea. This review will provide an overview of the auditory system, hearing loss, capacity for repair and regeneration and the current status of targeted therapies for drug delivery to the cochlea.

## 2. Normal cochlear anatomy and function

The peripheral auditory system consists of the external, middle and inner ear - or cochlea. The external auditory canal provides efficient transmission of the acoustic stimulus to the tympanic membrane or ear drum. The middle ear cavity, bound by the tympanic membrane laterally and the cochlea medially, efficiently couples sound energy from the air-filled external auditory canal to the fluid filled cochlea via the ossicular chain consisting of the malleus, incus and stapes (Peake & Rosowski, 1991). Fluid in the cochlea is displaced by movement of the stapes onto the oval window located in the base of the cochlea (Fig. 1A). The round window membrane, also located near the base of the cochlea, vibrates in opposite phase to the oval window as the fluid in the cochlea is displaced in response to acoustic stimulation. The round window provides a site for surgical access to the cochlea for both cochlear implants and drug delivery, as discussed later in the review, but the potential risks of additional hearing loss from targeting this membrane must be carefully considered when developing safe and effective drug delivery therapies (Fig. 1A).

The cochlea is a spiral shaped labyrinth embedded within the temporal bone. Three fluid-filled scalae form the membranous labyrinth (Fig. 1B). The basilar membrane separates the scala tympani from the scala media while Reissner's membrane separates the scala media from the scala vestibuli (Fig. 1C). Both the scala tympani and scala vestibuli contain perilymph, an extracellular fluid similar to cerebrospinal fluid (Salt & Hirose, 2018). In contrast, the scala media contains endolymph, a unique extracellular fluid which has a high
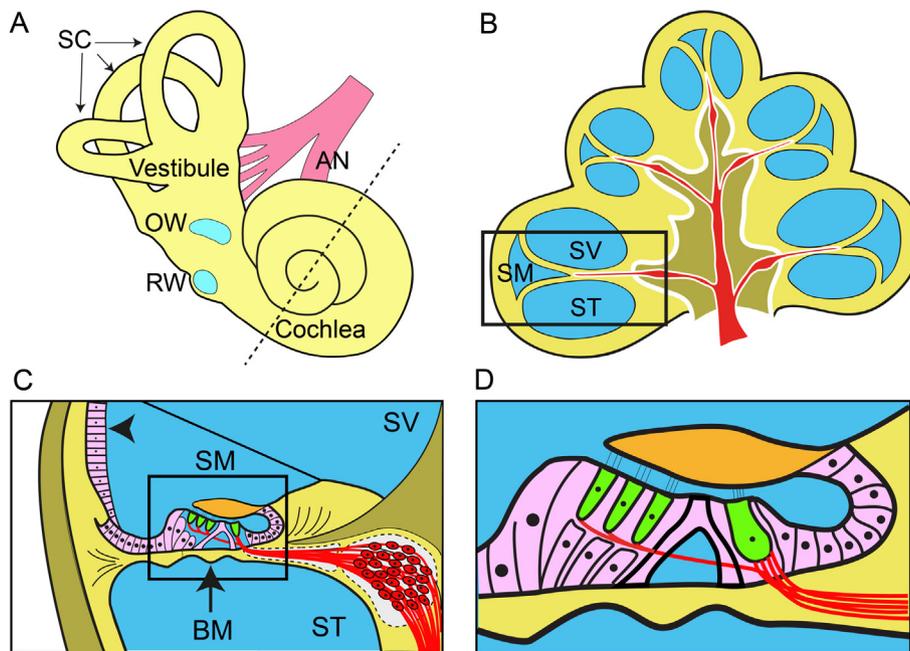


Fig. 1. (A) Schematic image of the inner ear depicting the three semi-circular canals (SC), the oval window (OW), the round window (RW) and the auditory nerve (AN). Drugs can be delivered to the inner ear via the semi-permeable round and oval windows, or directly into the semi-circular canals or cochlea via a small hole drilled through the bony wall or by piercing the round window. The dotted line bisects the cochlea in half which is depicted schematically in (B). (B) Transverse section of the human cochlea illustrating the three fluid-filled cochlear ducts shown in blue - scala vestibuli (SV), scala media (SM), and scala tympani (ST) spiralling around the modiolus containing the auditory nerve (red).The boxed region is shown in (C) in greater detail. (C) The organ of Corti (boxed) is located on the basilar membrane (BM, arrow) that separates the scala tympani from the scala media. The lateral wall of the scala media contains the stria vascularis (arrow head) which is responsible for the generation of the endocochlear potential. The SGN soma (red) are located in a small bony canal (Rosenthal's canal) that also spirals from the cochlear base to its apex. (D) The organ of Corti contains the two types of sensory hair cells - a single row of inner hair cells and three rows of outer hair cells (green) - together with supporting cells (pink). Peripheral fibres from spiral ganglion neurons (red) form synapses with the inner and outer hair cells.

concentration of potassium and a low concentration of sodium, a composition that is important for the normal process of transduction in the hair cells (Smith, Lowry, & Wu, 1954). The cells lining the perimeter of the scala media are joined by intercellular tight junctions that maintain the unique electrolyte composition of endolymph (Nadol, 1979). In the majority of cases, cochlear therapy techniques focus on delivery of a drug to the perilymph. In humans, the total perilymph volume is approximately 158 mm³ which includes the cochlea and semi-circular canals as there is free passage of fluids between these organs. The endolymph is sometimes targeted for therapies, with its volume being just 34 mm³ (Buckingham & Valvassori, 2001; Glueckert et al., 2018).

The sensory cells of the cochlea are located within the organ of Corti, a complex structure attached to the basilar membrane (Fig. 1D). In the human, the basilar membrane is approximately 32 mm in length from the cochlea base to the apex. The mechanical characteristics of the basilar membrane allow the cochlea to resolve different frequencies at different sites along its length; high frequencies in the base of the cochlea close to the middle ear, and lower frequencies at progressively more distal sites within more apical regions of the cochlea (Liberman, 1982).

The sensory hair cells consist of a single row of inner hair cells and three rows of outer hair cells (Fig. 1D). The human cochlea contains approximately 3200 inner hair cells and 10,000 outer hair cells (Schuknecht, 1974a). Both inner and outer hair cells contain up to 150 stereocilia protruding from their apical surface. On displacement of the basilar membrane, mechanically sensitive ion channels located in the stereocilia initiate depolarisation of the hair cell. This in turn results in the release of the excitatory neurotransmitter glutamate, depolarizing primary afferent neurons innervating the hair cell - the SGNs (Guinan Jr., Salt, & Cheatham, 2012; Patuzzi & Robertson, 1988). Both the hair cells and the SGNs are the most common target cell types for therapies to prevent or reverse hearing loss.

Inner and outer hair cells are innervated by specific SGN populations; the difference in their afferent innervation reflects the different roles these two hair cell types play in the transduction process. Type I SGNs, which represent ~90% of the total SGN population, exclusively innervate inner hair cells while Type II SGNs exclusively innervate outer hair cells (Lorente de Nó, 1933; Ryugo, Dodds, Benson, & Kiang, 1991; Spoendlin, 1978). The two hair cell populations play different and unique roles in auditory perception; inner hair cells convey auditory stimuli to the central auditory pathway via their innervation with the primary SGN population while outer hair cells provide biological amplification within the cochlea that increases hearing sensitivity to low intensity stimuli (Patuzzi & Robertson, 1988). The central fibres of the SGNs form the auditory nerve and projects to the cochlear nucleus - the first relay centre within the central auditory pathway (Lorente de Nó, 1933). There are approximately 30,000 SGNs in a normal human cochlea (Spoendlin, 1984).

The peripheral fibres of both Type I and II SGNs project from their soma in Rosenthal's canal, through a small opening in the bony wall called the habenula perforata, to the organ of Corti. Each Type I peripheral fibre makes a single synaptic connection with the closest inner hair cell. This synapse, known as a ribbon synapse, promotes the rapid release of neurotransmitter and can maintain high firing rates important for the temporal fidelity of sound processing (Griesinger, Richards, & Ashmore, 2005). In contrast, Type II peripheral fibres cross the floor of the organ of Corti and synapse with multiple outer hair cells (Spoendlin, 1984). The ribbon synapses in the inner hair cells have recently been identified as sensitive to even moderate noise exposure, preferentially affecting high-threshold neurons and is therefore also a specific target for drug therapies in addition to hair cells and SGNs more generally (Kujawa & Liberman, 2009; Lin, Furman, Kujawa, & Liberman, 2011; Liu et al., 2012; Shi et al., 2013), as outlined later in the review.

## 3. Deafness

### 3.1. Impact on people

Over 432 million adults and 34 million children have a disabling hearing loss, making it a major health and economic burden on society (WHO, 2018). Hearing impairment can result in significant communication disorders including poor development of spoken and written language in children, leading to significant educational, social and vocational ramifications that can adversely affect quality of life. In adults, hearing loss affects employment, income and social interaction and self-esteem (Theunissen et al., 2014; Wilson, Tucci, Merson, & O'Donoghue, 2017). Moreover, hearing loss is also a potential risk factor for accelerated cognitive decline and cognitive impairment in the elderly associated with increased social isolation (Curhan, Willett, Grodstein, & Curhan, 2019; Jayakody, Friedland, Martins, & Sohrabi, 2018; Lin et al., 2011; Lin et al., 2013; Tay et al., 2006). There are also links to the development of depression and its impact on the individual (Jayakody et al. 2018, Jayakody et al. 2018; Livingston et al., 2017; Thomson, Auduong, Miller, & Gurgel, 2017).

### 3.2. Rehabilitation

There are two forms of deafness; conductive and sensorineural. Conductive deafness is a mechanically based hearing loss associated with the external or middle ear. It can occur as a result of middle ear infections or ossification of middle ear bones that affect the capacity of the auditory structures to transmit sound information to the inner ear. It is typically a partial hearing loss and surgery and/or amplification via a hearing aid are the standard treatments. Sensorineural hearing loss (SNHL) is the most common form of deafness and typically occurs following damage to, or dysfunction of, the sensory hair cells within the inner ear (Schuknecht, 1974b), their synapse with primary auditory neurons (termed synaptopathy) (Kujawa & Liberman, 2015) or the stria vascularis (Wangemann, 2006). Extensive hair cell damage can result in a hearing loss that encompasses the important frequency range for human speech (~300 Hz-8 kHz). While a mild SNHL can be clinically managed using hearing aids, people with severe-to-profound SNHL may benefit from a cochlear implant which uses electrical stimulation to activate the auditory nerve. Cochlear implants enable recipients to understand speech in good listening conditions but results can vary considerably and performance in challenging or noisy environments is typically poor (Roche & Hansen, 2015; Wilson & Dorman, 2008).

Auditory rehabilitation, including the use of hearing aids and cochlear implants, results in positive improvements in terms of educational outcomes in children (Leigh, Dettman, Dowell, & Briggs, 2013; Nikolopoulos & Vlastarakos, 2010; Verhaert, Willems, Van Kerschaver, & Desloovere, 2008) as well as a decline in social isolation, depression and cognitive deterioration in the elderly (Castiglione et al., 2016). However, a perceived stigma of hearing loss and their limited effectiveness in challenging environments often prevents people from taking up these technologies (Wilson et al., 2017). The implementation of drug therapies for hearing loss has the potential to not only reach a greater portion of affected individuals but also to improve the outcomes when used in conjunction with devices.

### 3.3. Causes of SNHL

SNHL may have hereditary causes or may be acquired as a result of infection or injury, long-term exposure to loud noise or drugs that are toxic to hair cells. There is also a strong correlation between ageing and hearing loss suggesting that many factors increase the risk or susceptibility to SNHL later in life (Gates & Mills, 2005). As this review will discuss, the aetiology of hearing loss has a major impact on the potential for repair and regeneration and shapes the development of technologies for the treatment of hearing loss.

### 3.3.1. Genetic factors

Over 60% of cases of SNHL can be attributed to a genetic cause (Marazita et al., 1993), classified as non-syndromic (most common) or syndromic conditions affecting auditory function at different sites along the auditory transduction pathway (for review see (Angeli, Lin, & Liu, 2012)). For instance, mutations to cochlear gap junctions is a common cause of genetic hearing loss, in particular connexin 26 that enables the passage of potassium ions during normal cochlear function (Estivill et al., 1998). Indeed, connexin 26 mutations are responsible for ~50% of cases of non-syndromic hearing loss (Wingard & Zhao, 2015). Other causes of genetic hearing loss include mutations to genes important for hair cell function, for example the structural protein Myo7A (Weil et al., 1995), or to genes that encode for transmembrane channels essential for mechanotransduction in the hair cell stereocilia such as Transmembrane channel-like protein 1 (TMC1) (Pan et al., 2013).

### 3.3.2. Infections and injury

Hearing loss can result from infections contracted or injuries sustained at any stage of life. Viral infections such as cytomegalovirus are a major cause of congenital hearing loss (Goderis et al., 2014) and traumatic injuries to the temporal bone may cause significant hearing loss. Diseases such as auditory nerve neuromas may require surgical intervention that can have significant impact on hearing.

### 3.3.3. Ageing

Hearing loss associated with ageing, termed presbycusis, can have significant implications to heathy ageing. Disentangling the specific effects of ageing on cochlear health can be problematic with life-long environmental factors such as exposure to noise in combination with genetic predisposition to hearing loss making the task difficult (Kujawa & Liberman, 2006). Nevertheless, there is a clear link between progression of hearing loss and ageing with nearly two thirds of people over 70 having significant hearing impairment as assessed by standard audiometry testing (Lin, Thorpe, Gordon-Salant, & Ferrucci, 2011). Typically hearing loss is symmetrical with a progressive loss of hearing sensitivity over time. The characteristics of hearing loss are variable between people. Similar to the effects of excitotoxic injury from noise, it is likely that presbycusis manifests as a combination of loss of hearing sensitivity brought about by loss of ribbon synapses, hair cells and SGNs. Animal models of presbycusis can exhibit significant SGN loss without appreciable hair cell loss (Sergeyenko, Lall, Liberman, & Kujawa, 2013; Viana et al., 2015) suggesting that auditory neuropathy is a likely contributor to the loss of hearing function in humans (Makary, Shin, Kujawa, Liberman, & Merchant, 2011). Dysfunction to cochlear homeostasis, brought about by metabolic changes that affect the stria vascularis lining the outer wall of the scala media, are also likely to contribute to age-related hearing loss (Shi, 2016).

### 3.3.4. Noise exposure

Noise exposure can result in a temporary loss of hearing sensitivity that can resolve over days and weeks following the noise exposure. This temporary threshold shift was previously thought to reflect temporary changes to the structure of the outer hair cell stereocilia, the tectorial membrane and other changes in the organ of Corti (Nordmann, Bohne, & Harding, 2000). More recently, evidence has begun to emerge that more permanent changes occur in the cochlea despite a return to normal hearing thresholds (Ryan, Kujawa, Hammill, Le Prell, & Kil, 2016). As noted above, this phenomenon has been called synaptopathy or hidden hearing loss (Schaette & McAlpine, 2011) as the deficit in hearing cannot be detected in common audiometry testing where hearing thresholds are monitored at specific frequencies using a pure tone audiogram. The mechanism for hidden hearing loss is thought to be the loss of synaptic connections between the SGN peripheral fibres and the inner hair cells (Kujawa & Liberman, 2009, 2015; Lin, Furman, et al., 2011; Valero et al., 2017). In particular, SGNs with higher activation thresholds are more susceptible to damage while the low threshold

neurons are more resilient to this noise induced damage (Furman, Kujawa, & Liberman, 2013). The resulting outcome is that hearing sensitivity is maintained but the capacity to process auditory information in more challenging environments is impacted due to the loss of information provided by the higher threshold neurons. There is degradation in the capacity to process temporal information of suprathreshold input. This type of deficit can manifest itself as difficulty in understanding speech in challenging sound environments such as a noisy restaurant. In terms of the impact on the SGNs, significant loss of synaptic contact can result in ongoing and progressive degeneration of the nerve fibres and ultimately death of the SGN (Kujawa & Liberman, 2006), irrespective to the survival of the inner hair cells (Liberman, 2017).

Longer noise exposure or exposure to louder noise can lead to excitotoxic hair cell damage and loss due to excessive glutamate release at ribbon synapses (Pujol & Puel, 1999). There is a release of reactive oxygen species, increases in calcium levels in cochlear hair cells and a cascade of intracellular events within the hair cell leading to functional damage and death via apoptotic or necrotic mechanisms in the days to weeks following damaging noise exposure (Ping Yang, Henderson, Hua Hu, & Nicotera, 2004). Local cochlear inflammation and the upregulation of pro-inflammatory cytokines also occur following noise exposure (Tan, Thorne, & Vlajkovic, 2016). Excessive noise exposure can also result in mechanical damage to the organ of Corti such as rupture of the reticular lamina that can allow the endolymph and perilymph to mix leading to further damage of the hair cells, supporting cells and nerve fibres (Bohne, Kimlinger, & Harding, 2017). The outer hair cells are typically damaged and lost prior to the loss of the inner hair cells (Chen & Fechter, 2003).

### 3.3.5. Ototoxic drugs

Cochlear hair cells are sensitive to exposure to ototoxic drugs such as platinum-based chemotherapeutics (e.g. cisplatin) and aminoglycoside antibiotics (e.g. neomycin and kanamycin). Cisplatin is a chemotherapeutic agent widely used in the treatment of several forms of cancer. Ototoxicity is a common adverse side effect associated with the administration of this drug, particularly affecting the higher frequencies (Anniko & Sobin, 1986). Cisplatin enters the hair cell cytoplasm mainly via mammalian copper transport 1 and organic cationic transporter, where it Increases the oxidative stress of the cell promoting apoptotic and necrotic cell death (Sheth, Mukherjea, Rybak, & Ramkumar, 2017). It is anticipated that targeting these transporters and/or the application of antioxidants locally within the cochlea would reduce the incidence of hearing loss while maintaining the efficacy of cisplatin as a chemotherapy agent (Sheth et al., 2017).

The broad-spectrum antibacterial activity of aminoglycoside drugs are used to treat Gram-negative bacterial infections and are widely used throughout the world, often as a last resort option. The ototoxic compounds enter the cochlea via the stria vascularis and then enter into the apical surface of hair cells via the mechanotransduction channels located in the stereocilia. The compounds affect metabolic function of the hair cells by damaging mitochondrial ribosomes leading to the production of oxygen free radicals and initiating cell death mechanisms (Forge & Schacht, 2000). In animal models ototoxic drugs can result in profound deafness and elimination of all cochlear hair cells. The ototoxic drugs can directly affect the cochlear synapses, causing rapid loss of the ribbon synapse and subsequent retraction of the peripheral nerve fibres (Liu et al., 2013). The peripheral nerve fibres degenerate and retract within their myelin sheath followed by the breakdown and loss of myelin sheath (Wise, Pujol, Landry, Fallon, & Shepherd, 2017).

### 3.3.6. Strial/metabolic hearing loss

The stria vascularis, located in the scala media (Fig. 1C), maintains the high potassium concentration (150 nM $K^+$) of the endolymph via the functioning of ion pumps, producing the highly positive endocochlear potential ($+80$ to $+100$ mV). There are three main layers to the stria vascularis composed of marginal, intermediate and basal

cells. Any dysfunction of the endocochlear potential could impact on hair cell depolarisation in response to acoustic input with the overall effect of reduction in hearing sensitivity. For example, mutations in the potassium channel subunit KCNQ1 in marginal cells disrupt the endocochlear potential and cause severe congenital deafness; Jervell and Lange-Nielsen syndrome (Jervell & Lange-Nielsen, 1957). Strial dysfunction is also thought to contribute to age related hearing loss (Kujawa & Liberman, 2019) with the cochlear apex (low frequency) less susceptible to changes in the endocochlear potential compared to the basal (high frequency) region. Consequently the loss in hearing sensitivity due to strial dysfunction is likely to result in a gradual reduction in hearing sensitivity in the hearing frequency range (Dubno, Eckert, Lee, Matthews, & Schmiedt, 2013; Heeringa & Koppl, 2019).

### 3.3.7. Loss of residual hearing with cochlear implantation

The indication criteria for cochlear implantation have expanded over the last decade to include patients with useable low frequency hearing. Typically, patients that combine the residual low frequency acoustic hearing with the high frequency electric hearing from the cochlear implant have superior performance compared to profoundly deaf patients (Gantz, Turner, Gfeller, & Lowder, 2005). A significant concern and risk for these patients is the loss of residual hearing that can occur following cochlear implant surgery. The loss can occur immediately post-surgery (Adunka, Roush, Grose, Macpherson, & Buchman, 2006), presumably due to factors such as altered cochlear mechanics, acute trauma or immediate inflammatory effects of implantation (Fayad & Linthicum, 2006; Fayad, Makarem, & Linthicum Jr, 2009; Lee, Nadol Jr., & Eddington, 2010; Nadol Jr. et al., 2001; Nadol Jr. & Eddington, 2006). However, some patients can lose residual hearing months following implantation via mechanisms that are not well understood. Additional research is required to elucidate the causes of delayed loss of residual hearing. Other research has shown correlations between the extent of fibrous tissue in the cochlea (especially new bone growth), the population of remaining auditory neurons and cochlear implant performance (Kamakura & Nadol, 2016).

### 3.4. Potential for repair or regeneration

Given the diversity of causes of hearing loss, the pathology in the cochlea will also be extremely varied in terms of cells affected and the severity of damage. Damaged cells and debris are eliminated by resident and migrating macrophages (Hirose, Rutherford, & Warchol, 2017). If damage is severe and widespread, a complete loss of the sensory epithelium can occur resulting in a so-called flattened sensory epithelium. Such profoundly damaged cochleae represent a potentially insurmountable obstacle for regenerative therapies; essentially trying to recapitulate the highly structured and complex sensory epithelium from scratch (Taylor, Jagger, & Forge, 2012). Consequently, research efforts have been focused towards the development of protective therapies and therapies designed to return hearing function in cochleae that have not sustained profound sensory cell loss. The main targets for these therapies are the hair cells, SGNs and the ribbon synapse.

### 3.4.1. Hair cells

It has long been known that hair cells in birds and non-mammalian vertebrates have the capacity to regenerate following damage (Balak, Corwin, & Jones, 1990; Corwin & Cotanche, 1988; Ryals & Rubel, 1988). Regeneration occurs via either direct transdifferentiation, whereby neighbouring supporting cells are converted to hair cells, or by mitotic regeneration in which supporting cells divide and are subsequently converted to a hair cell. Conversely, in the mature mammalian auditory system, spontaneous regeneration of auditory hair cells following damage to the sensory epithelium is extremely limited and short lived (Cox et al., 2014). Attempts to induce hair cell regeneration in the mature mammalian cochlea by cellular reprogramming have had

varied success. Some studies have reported conversion of supporting cells to hair cells by increasing the expression of the Atoh1 gene, a basic helix-loop-helix transcription factor, in supporting cells via gene therapy or molecular techniques with associated recovery of hearing thresholds (Izumikawa et al., 2005; Kraft, Hsu, Brough, & Staecker, 2013). Manipulation of the Notch signalling pathway is another method of increasing Atoh1 gene expression and this has also successfully regenerated hair cells in the cochlea with recovery of hearing after noise injury (Du et al., 2018; Mizutari et al., 2013; Yeh, Chiang, Rees, Edge, & Liu, 2018). Others have reported hair cell conversion that was either too limited, short-lived or incomplete to result in recovery of hearing (Atkinson, Wise, Flynn, Nayagam, & Richardson, 2014; Izumikawa, Batts, Miyazawa, Swiderski, & Raphael, 2008; Kawamoto, Ishimoto, Minoda, Brough, & Raphael, 2003; Kelly, Chang, Pan, Lin, & Chen, 2012; Kuo, Baldwin, Layman, Taketo, & Zuo, 2015; Liu et al., 2012; Liu, Fang, Dearman, Zhang, & Zuo, 2014; Yang et al., 2012; Yang, Cong, Han, Huang, & Chi, 2013). Factors that may affect the efficacy of hair cell regeneration and hearing recovery include the degree of pathology in the cochlea, maturity of the organ of Corti or age-related epigenetic modifications, timing of the therapy and the expression dynamics of the transfected gene (Zhang et al., 2018). The sub-type of supporting cell being converted may also impact on outcomes with pillar and Deiters cells being more resistant to conversion (Walters et al., 2017). Newly regenerated hair cells have been noted in some cases to attract neuronal fibres and express synaptic markers suggesting that the hair cells can be reconnected (Gubbels, Woessner, Mitchell, Ricci, & Brigande, 2008; Kawamoto et al., 2003; Kuo et al., 2015; Walters et al., 2017). It is also noteworthy that recovery of vestibular function has also been observed for Atoh1 gene therapy following vestibular hair cell ablation as a result of vestibular hair cell regeneration (Schlecker et al., 2011; Staecker, Praetorius, Baker, & Brough, 2007).

A clinical trial is underway to investigate the potential for Atoh1 gene therapy to restore hearing in patients with profound hearing loss (NCT02132130). An adenovirus carrying the human Atoh1 transcription factor gene is being assessed for safety, tolerability and efficacy in patients with severe-to-profound hearing loss. An experimental dose of 20–60 μL is delivered via the intra-labyrinthine route in order to access the cochlear supporting cells in the sensory epithelium.

A single-factor approach to reprogramming of supporting cells may too simplistic (Jahan, Pan, & Fritzsch, 2015) with some reports of increased conversion and improved survival and function of converted hair cells with a combinatorial approach. Co-factors to the Atoh1 gene have included beta-catenin; a component of the Wnt signalling pathway (Kuo et al., 2015), transcription factors Gfi1 and Pou4f3 (Costa et al., 2015; Walters et al., 2017), deletion of p27KIP1; a factor associated with quiescence of hair cells and supporting cells (Walters et al., 2017) and the GATA3 transcription factor (Walters et al., 2017).

Direct transdifferentiation of supporting cells to hair cells depletes the supporting cell population that normally plays a critical mechanical role in auditory transduction and homeostasis. To overcome this, the supporting cell population may first require proliferation prior to trans-differentiation to hair cells (mitotic regeneration). Recent research has shown that the inner ear contains a distinct population of progenitor cells that can form new hair cells and neurons in culture (Oshima et al., 2007). These resident cells serve as hair cell precursors during development (Chai et al., 2012; Shi, Kempfle, & Edge, 2012). By altering intracellular signalling pathways such as Wnt and Notch activation the progenitor cells can be 'switched on' and stimulated to divide and differentiate into cochlear hair cells (McLean et al., 2017). In explanted cochlear tissue depleted of hair cells by aminoglycosides, the addition of small molecules that activate Wnt and Notch signalling pathways resulted in an increase in the number of hair cells by stimulating the division and differentiation of Lgr5-positive cells without the loss of supporting cells that occurs with Atoh1 gene therapy (McLean et al., 2017).

### 3.4.2. SGNs

Following loss of hair cells, there is retraction of the SGN peripheral fibres. The SGN cell bodies within Rosenthal's canal shrink before the loss of the neuronal cell body via apoptotic or necrotic processes (Wise et al., 2017). Interestingly, once the neuronal cell body has degenerated the cochlear Schwann cell that provided myelin to the SGN appears to alter its phenotype towards a non-myelinating form and interacts with remaining neurons (Wise et al., 2017). This dynamic behaviour of the remaining Schwann cells is likely to facilitate the capability of remaining neurons to resprout their peripheral fibres, which often occurs spontaneously after a cochlear insult such as aminoglycoside damage, but can be enhanced by the delivery of neurotrophic growth factors. In terms of functional changes in the deafened cochlea as a consequence of neural and myelin degeneration, changes in auditory nerve functioning in response to electrical stimulation are more subtle. Auditory neurons maintain the ability to respond to electrical stimuli, however, reductions in refractory periods and increases in electrical thresholds have been reported in long-term deafened animals (Shepherd, Roberts, & Paolini, 2004) and myelin-deficient mice (Zhou, Abbas, & Assouline, 1995).

The relative slow decline of SGNs (compared to hair cell loss) makes them an amenable target for drug therapies in the cochlea. Given that the number of SGNs is an important factor influencing post-implant hearing performance clinically (Kamakura & Nadol, 2016; Seyyedi, Viana, & Nadol, 2014), it is important to develop effective strategies aimed at their rescue. There is a large body of work on the use of neurotrophins in the cochlea for the protection of SGNs, regrowth of the peripheral fibres and repair of the hair cell ribbon synapse (see Section 3.4.3. and review (Ramekers, Versnel, Grolman, & Klis, 2012)).

### 3.4.3. Neurotrophins and their role in the treatment of hearing loss

Neurotrophins are a family of proteins that play a major role in both the development and maintenance of neurons in the central and peripheral nervous systems (Kashyap, Roberts, Waseem, & Tyagi, 2018). During cochlear development, SGNs receive neurotrophic support, including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), from hair cells, supporting cells of the organ of Corti and second order cochlear nucleus neurons (Fritzsch, Pirvola, & Ylikoski, 1999; Schecterson & Bothwell, 1994; Stankovic et al., 2004; Ylikoski et al., 1993). The high-affinity receptors for these neurotrophins (TrkB and TrkC, respectively) are expressed on SGNs (Schecterson & Bothwell, 1994; Ylikoski et al., 1993). Importantly, the recent identification of BDNF and its high affinity receptor in developing human cochleae suggests a similar role in human SGNs (Johnson Chacko et al., 2017).

Both BDNF and NT-3 also play a role in the maintenance of SGNs in the adult inner ear (Qun, Pirvola, Saarma, & Ylikoski, 1999; Ylikoski et al., 1993). Removal of this support leads to the gradual degeneration of SGNs via apoptotic cell death (Alam, Robinson, Huang, & Green, 2007; Fritzsch et al., 1999). Significantly, the neuroprotective effects of exogenous neurotrophins on SGNs *in vivo* have been demonstrated (Gillespie,

Clark, & Marzella, 2004; Leake, Hradek, Hetherington, & Stakhovskaya, 2011; McGuinness & Shepherd, 2005; Miller et al., 1997; Shinohara et al., 2002; Staecker, Kopke, Malgrange, Lefebvre, & Van de Water, 1996) (Fig. 2). Associated with this rescue effect is regrowth of peripheral SGN peripheral fibres compared with deafened controls (Budenz et al., 2015; Leake et al., 2011; Richardson et al., 2007; Wise, Richardson, Hardman, Clark, & O'Leary, 2005), with implications in reducing excitation thresholds when electrically stimulated with a cochlear implant (Landry, Fallon, Wise, & Shepherd, 2013). Finally, exogenous neurotrophins have been shown to promote synaptic regeneration of the SGN peripheral fibres to the hair cell (i.e. the ribbon synapse) and rescue of hearing function in adult animals following acoustic trauma (Sly et al., 2016; Suzuki, Corfas, & Liberman, 2016; Wan, Gomez-Casati, Gigliello, Liberman, & Corfas, 2014). While protective effects of neurotrophin administration have been observed for at least 2 weeks post-therapy (Agterberg, Versnel, van Dijk, de Groot, & Klis, 2009; Sly et al., 2016), it appears that long-term exogenous neurotrophin delivery to the cochlea may be required for ongoing SGN protection (Gillespie, Clark, Bartlett, & Marzella, 2003). In contrast, promoting SGN peripheral fibres to re-synapse with sensory hair cells via exogenous neurotrophin delivery would probably not require long durations of therapy as the connection would presumably be maintained by the endogenous supply via the hair cell and supporting cells of the organ of Corti (Sly et al., 2016; Suzuki et al., 2016).

These pre-clinical studies have shown that there are a number of opportunities for drug therapies for hearing loss that each presents a set of unique requirements, such as specific cellular targeting or slow-release delivery, as well as universal requirements such as the need to protect residual cochlear function and for reliable dosing. The next sections will focus on current and new technologies being developed to meet the demand for a drug therapy that can be applied to the cochlea for preservation and regeneration of hair cells, SGNs, ribbon synapses or other affected cell types.

## 4. Delivery of drugs to the inner ear

Drug based therapies targeting inner ear disease have been used clinically for over 60 years, initially using systemic administration to deliver aminoglycosides for the treatment of severe bilateral Meniere's disease, and more recently the application of steroids for sudden SNHL. Although still in clinical practise, these therapies exhibit significant limitations including highly variable pharmacokinetics due to the blood-cochlear barrier and clinical variability (e.g. patient age; renal function; aetiology; previous inner ear pathology; genetic disposition), and potential undesirable side-effects associated with systemic drug administration (Shepherd, 2011).

In an attempt to improve clinical outcomes, investigators developed drug delivery techniques specifically targeting the inner ear by delivering drugs directly to the round window membrane at the base of the cochlea. Intra-tympanic clinical delivery techniques include direct trans-
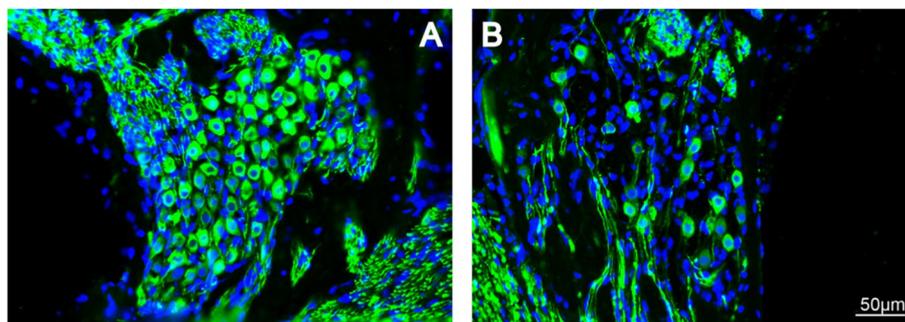


**Fig. 2.** Neurotrophin therapy results in SGN survival after hearing loss in guinea pigs. (A) An intra-cochlear BDNF therapy applied 1 week after ototoxic hearing loss maintains the survival of SGN cell bodies (green) in Rosenthal's canal as well as the peripheral fibres over a 4 week period. (B) The SGN population deteriorates over 5 weeks in deafened guinea pigs that receive a control therapy (Wise, Tan, Wang, Caruso, & Shepherd, 2016).

tympanic injection into the middle ear or the use of Gelfoam™ as a means to maintain drug contact with the round window membrane. Similarly, the Silverstein Microwick™ (Summit Medical Inc.) is a device that is placed onto the round window via a small incision in the tympanic membrane allowing drugs to be transported from the external auditory meatus to the round window. These techniques have been used to provide a more targeted approach in the treatment of Meniere's disease and sudden SNHL after systemic treatment has failed (McCall et al., 2010; Swan, Mescher, Sewell, Tao, & Borenstein, 2008). This route offers a short-term delivery strategy that is suitable for some clinical applications but also has a number of limitations including loss of drug through the eustachian tube, variability in pharmacokinetics due to variability and thickening of the round window membrane (Goycoolea, 2001), and the very slow diffusion of the drug through the cochlea (Salt & Plontke, 2009; Swan et al., 2008).

The most efficient route is delivery of the drug directly into the inner ear. The anatomy of the cochlea makes it relatively isolated and local therapy can be applied with few off-site effects. The scala tympani provides a small, relatively contained fluid-filled compartments into which drugs can be delivered at high doses to reach all target cells including hair cells, supporting cells, SGNs and the stria vascularis (Richardson et al., 2004; Richardson et al., 2019; Richardson, O'Leary, Wise, Hardman, & Clark, 2005); only the scala media is not accessible via this route due to the presence of tight junctions (Swan et al., 2008). In addition to the clear advantage of delivering predictable amounts of a drug into the inner ear, this technique offers the potential for safe delivery of drugs that have a history of adverse side effects when administered systemically. While efficient, direct administration of drugs to the cochlea brings with it increased risks of both short and long-term damage to inner ear structures and therefore requires careful preclinical investigation to develop safe surgical access and delivery techniques prior to clinical application. This is particularly important in mild forms of SNHL where further loss of sensory hair cells or their peripheral fibres could result in a profound hearing loss. In cases involving cochlear implantation, where surgical access to the inner ear is part of the procedure and there is little or no residual hearing, the risks of "doing harm" are significantly reduced. It is for this reason that a major clinical application of therapeutic drugs into the cochlea, at least in the short term, will be performed in association with cochlear implantation with a focus on preserving residual hearing and/or promoting SGN survival and peripheral fibre regeneration (Plontke, Gotze, Rahne, & Liebau, 2017; Wise et al., 2011).

## 5. Novel cochlear therapy technologies

The key features of a clinically relevant drug delivery strategy are that the system must be capable of delivering a therapeutic dose of the drug safely to the inner ear over durations that are required for a clinically meaningful outcome. The active agent is typically delivered to the round window membrane or to the intracochlear environment depending upon the clinical indication that is being targeted. While there are sophisticated microfluidic based drug delivery pumps under development with application to the cochlea (Peppi, Marie, Belline, & Borenstein, 2018) the present review concentrates on non-pump based delivery techniques. Some of the considerations for these systems are described in the following section.

### 5.1. Biomaterials

The observations in preclinical studies that neurotrophin delivery (often using mini-pump systems) can protect and promote re-sprouting of SGNs in deafened models have been the motivation for the development of new strategies to deliver neurotrophins that are suitable for clinical application. As neurotrophins have a short biological half-life, poor pharmacokinetics and poor permeability across biological barriers (Bartus, 2012; Khalin, Alyautdin, Kocherga, & Bakar, 2015) it is

difficult and expensive to systemically administer them for therapy in the cochlea. Thus localized biocompatible nanotechnology-based ?thyc=5?> neurotrophin delivery systems are being developed such as nanogels, hydrogel, micelles, microspheres, electrospun nanofibers, nanoparticles and supraparticles. Neurotrophins can be immobilized in nanotechnology-based systems via physical entrapment, adsorption or electrostatic interaction. The sustained release of neurotrophins from the drug carriers can then be achieved via the diffusion of neurotrophins and/or the degradation of the carriers. Controlled and sustained release systems can increase the residence time of neurotrophins in the middle ear/cochlea. In this section, we will focus on the most commonly used delivery strategies under investigation for neurotrophin therapy (or other therapeutic molecules) for hearing impairment: hydrogels, nanoparticles and supraparticles.

#### 5.1.1. Hydrogels

Hydrogels are comprised of a porous network of lightly hydrophilic cross-linked polymers, which enables them to easily swell or dehydrate in aqueous environments based on triggering mechanisms such as pressure, temperature, electrical potential or pH, thus achieving drug loading and release (Aurand, Lampe, & Bjugstad, 2012). Hydrogels can be synthesized by various polymerization approaches (Aurand et al., 2012; Khademhosseini & Langer, 2007) and have been widely used as drug delivery reservoirs in tissue engineering and neuroscience applications. For the treatment of hearing loss, hydrogels loaded with neurotrophins are typically applied onto the round window membrane (Havenith et al., 2011; Noushi, Richardson, Hardman, Clark, & O'Leary, 2005). Because of their high viscosity and viscoelastic properties, they prevent the neurotrophins from rapidly flowing through the eustachian tube in the middle ear, thus increasing the residence time to achieve more uniform delivery to the inner ear. Release of the drug is often rapid and occurs by diffusion (Burdick, Ward, Liang, Young, & Langer, 2006; Noushi et al., 2005). The injection of hydrogels onto the round window membrane is less invasive compared to the intracochlear administration route and is therefore a potential platform to translate this approach into clinical application.

Collagen, gelatin and agarose are natural biomaterials that can easily form a hydrogel or be simply modified into a synthetic injectable hydrogel (Madduri & Gander, 2012). Pre-clinical studies using hydrogels infiltrated with neurotrophins have evaluated the technique for the protection or rescue of SGNs post-hearing loss. For example, encapsulation of BDNF in a biodegradable hydrogel, and its implantation onto the round window membrane of deafened guinea pigs resulted in BDNF delivery to the cochlea over a 7 day period, protecting SGNs and significantly lowering electrically evoked auditory brain-stem response thresholds compared to controls (Endo et al., 2005). Similarly, Gelfoam™, a biodegradable gelatin matrix, loaded with BDNF and positioned onto round window membrane of a deafened guinea pig, promoted survival of SGNs in the basal region of the cochlea after 2 weeks treatment (Havenith et al., 2011) and after 4 weeks of treatment with NT-3 via alginate (Noushi et al., 2005).

For more sustained drug release, researchers have looked towards layering technology. Sustained release of lysozyme, a similar protein in size, molecular weight and isoelectric point to BDNF, for up to a month was reported from an agarose hydrogel where the protein was encapsulated in poly(ethylene glycol)/poly (acrylic acid) which was assembled layer-by-layer (Mehrotra et al., 2010).

The ease of application of hydrogels delivered transtympanically for treatment of hearing loss is offset by some potential challenges, such as the relatively quick release profile of most hydrogels which might not be applicable for long term drug delivery and the variations in the thickness of the round window membrane observed in the clinical setting.
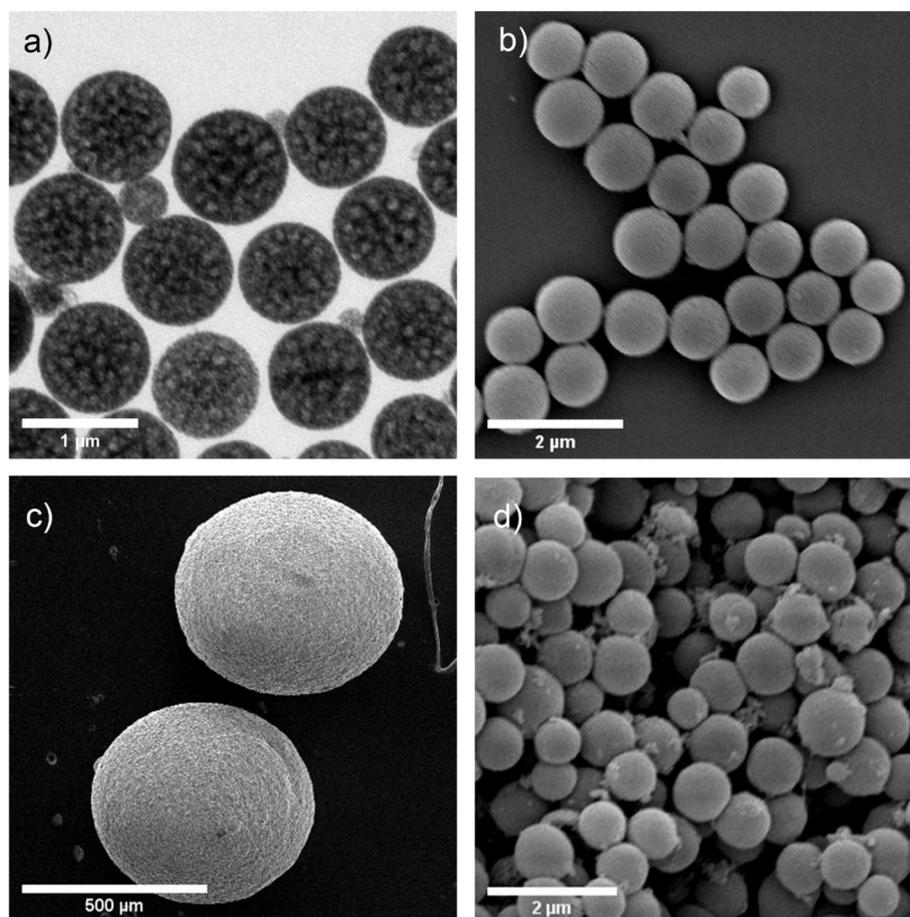
#### 5.1.2. Nanoparticles

Since neurotrophins are rapidly released from most hydrogels, it is essential to develop other drug delivery carriers that form strong

bonds with neurotrophins such as electrostatic interaction for longer term release. Nanoparticle/nanocarrier systems are promising drug delivery technologies that have the ability to encapsulate and transport neurotrophins to the inner ear (Fig. 3A, B). Encapsulation of neurotrophins results in stabilization of the protein and controlled and sustained drug release, avoiding neurotoxicity of highly concentrated neurotrophins (Scharfman, Goodman, Sollas, & Croll, 2002; Suzuki et al., 2016; Swan et al., 2008).

Specific targeting of nanoparticles to auditory hair cells and SGNs has been achieved via surface modifications. For instance, poly (d,l)-lactide-*co*-glycolide acid (PLGA) nanoparticles functionalized with hydrophilic molecules like poloxamer 407, showed greater cellular uptake in outer hair cells compared to unmodified PLGA nanoparticles and PLGA nanoparticles with chitosan and methoxy poly(ethylene glycol) modifications (Wen et al., 2016). Moreover, poly(ethylene glycol)-b-poly (ε-caprolactone) nanoparticles with surface modifications of nerve growth factor derived peptides demonstrated specificity for SGNs (Roy et al., 2010). Recently, a peptide (termed A666) with specific binding affinity for prestin, an abundant outer hair cell protein, was conjugated to poly(lactide) nanoparticles in order to specifically target outer hair cells. The peptide was coupled via the SH-terminal to maleimide groups of poly(ethylene glycol) on the surface of the nanoparticles. The aim was to use the nanoparticles to deliver a protective payload of dexamethasone to outer hair cells for protection of hearing during cisplatin-based cancer treatment, where ototoxicity is a common side-effect (Anniko & Sobin, 1986). As a result, dexamethasone loaded A666-nanoparticles enhanced outer hair cell viability and afforded some hearing protection following cisplatin therapy in guinea pigs, with a slight advantage over

dexamethasone or non-coated dexamethasone loaded nanoparticles. The nanoparticles were confirmed to cross the intact round window membrane and targeted to outer hair cells, with internalisation of the nanoparticles shown in an inner ear cell line HEI-OC1 (Wang et al., 2018).

The stabilization and sustained drug release properties of nanoparticles have made them an attractive means for the delivery of neurotrophins to the cochlea as a hearing loss therapy. For example, Tan et al. demonstrated poly (L-glutamic acid) nanoparticles loaded with BDNF could achieve sustained delivery for 70 days although nearly 90% of the BDNF was released in the first 30 days. The biological activity of BDNF was not affected by interaction with the nanoparticles and when applied to the cochlea of deafened guinea pigs prevented the degeneration of SGNs. However, the long-term stability of the BDNF in the nanoparticles was not measured (Tan et al., 2012). Similarly, phytantriol lipid-based crystalline nanoparticles were shown to load a large amount of nerve growth factor and improve the availability of nerve growth factor in the cochlea following placement on the round window membrane of the cochlea as compared to application of nerve growth factor in the absence of nanoparticles (Bu et al., 2015). Recently, Schmidt et al. has used silica nanoparticles with an amino group surface modification to encapsulate BDNF and demonstrated sustained release for a period of 80 days. The BDNF released from the silica nanoparticle-based drug delivery system was shown to retain bioactivity for at least 39 days that was equivalent to or greater than the addition of 50 ng/mL BDNF to cultured SGNs (Schmidt et al., 2018). Technologies that maintain the activity of neurotrophic factors is an important consideration in the development of successful slow-release delivery systems.



**Fig. 3.** Characterization of silica nanoparticles and silica supraparticles. (a) Transmission electron microscope image of silica nanoparticles (b) Scanning electron microscope (SEM) image of the silica nanoparticles. (c) SEM image of silica supraparticles. (d) SEM image of the surface structure of a silica supraparticle where individual nanoparticles can be seen clustered together. Modified with permission (Ma et al., 2018).

Exosomes are cell-derived membrane lipid nanoparticles secreted by many cells to mediate cell to cell communication by delivering proteins, nucleic acids or metabolites. They are now being considered as a drug delivery tool due to their intrinsic biocompatibility, stability, targeting properties and ability to overcome natural barriers (Valadi et al., 2007). Exosomes can be used to package and deliver genetic material such as RNA, DNA, gene editing tools and viral gene therapy as well as small molecules and peptides. In the auditory system, exosomes have been shown to enable delivery of adeno-associated virus to otherwise hard-to-transfect outer hair cells of the cochlea, outperforming conventional viral vectors (Gyorgy et al., 2017).

Small nanoparticles have the potential to migrate from the cochlea to the brain via the cochlear aqueduct– a small canal connecting the basal turn of the scala tympani to the subarachnoid space of the cranium (Salt & Hirose, 2018), presenting a safety concern that is not unique to nanoparticles (Kho, Pettis, Mhatre, & Lalwani, 2000; Zhang et al., 2013). Moreover, some nanoparticle technologies exhibit a rapid release profile over the first few days followed by prolonged release of only very small amounts of neurotrophin (Tan et al., 2012; Wise & Gillespie, 2012). Many therapeutic applications in the auditory system have a need for a more regulated release of neurotrophins over a longer period of time.

### 5.1.3. Supraparticles

Supraparticles consist of thousands of colloidal nanoparticles with a total size range of 10 μm to 1000 μm. The overall larger size of supraparticles together with the large surface area provided by the individual porous nanoparticles and the pores between the nanoparticles provide an opportunity for a large drug loading capacity compared to individual nanoparticles (Sperling & Gradzielski, 2017) (Fig. 3C, D). The larger size of supraparticles also addresses the safety concerns of nanoparticle delivery systems where nanoparticles may unintentionally flow into cerebral spinal fluid through the cochlear aqueduct (Zhang et al., 2013). Furthermore, BDNF-loaded supraparticles synthesized by an evaporation-induced self-assembly method also showed a reduction in the initial burst of release commonly seen with other biomaterials and demonstrated sustained BDNF release over at least 70 days in vitro, presumably due to the highly convoluted path that the neurotrophin must take to elute from the particle (Wang et al., 2014). After 70 days there was still about 89% of the initial amount remaining in the particle and with the release rate still steady. When applied to deafened guinea pig cochleae, the BDNF-loaded particles maintained SGN numbers at near-normal levels after 28 days treatment (Wang et al., 2014; Wise et al., 2016). Co-delivery of two drugs important for hearing loss therapies, BDNF and dexamethasone, was demonstrated over 30 days using gelatin hybrid mesoporous silica supraparticles synthesized via a polydimethylsiloxane mould approach (Maina et al., 2014). In an important step towards clinical translation and commercialization, large porous silica supraparticles shown in Fig. 3 were prepared by an automatable electrospray technique prior to lysozyme or

BDNF encapsulation as an approach for long-term drug delivery to treat inner ear disease (Ma et al., 2018). The large porous silica supraparticles (~550 μm) are biocompatible, they can be loaded with large amounts of lysozyme (10 μg per supraparticle) or BDNF (7 μg per supraparticle), and showed sustained release profiles (lysozyme >110 days; BDNF >40 days; Fig. 4). The cumulative BDNF release of the supraparticles was 4000-fold larger than the reported silica nanoparticles after 40 days (Schmidt et al., 2018).

Overall, the studies on the silica supraparticles have played an important role in the development of controlled and sustained neurotrophins delivery. However, the pharmacokinetics of neurotrophins in the cochlea released from supraparticles has not been well studied. In addition, the therapeutic dose of neurotrophins and the distribution of the degradation components from supraparticles in different organs have not been investigated. These studies are required in order to translate this drug delivery system into clinical applications.

### 5.2. Gene therapy

Recent developments in the field of cochlear molecular therapies are showing exciting trends towards clinical therapies for hearing loss, particularly for the repair of genetic defects that result in loss of hair cells. Molecular therapies include gene replacement, gene augmentation, gene editing, post-translational modifications and alternative splicing. The transfer of genetic material into cells can be achieved via viral or non-viral vectors, including adenovirus, adeno-associated virus (AAV), herpes simplex virus I, vaccinia virus and non-viral technologies such as lipid gene transfer systems. The challenges for the field of gene therapy have been to prove that the technique applied to the cochlea is safe, highly efficacious and targeted. Recent advances in the field are stepping up to meet these goals through improvements in the mode of delivery to the cochlea and advances in viral vector technology.

### 5.2.1. Viral vectors

AAVs are generally considered to have the best profile of safety and efficacy for clinical translation (Colella, Ronzitti, & Mingozzi, 2018). AAVs are non-pathogenic, have low immunogenicity and provide long-term gene expression in non-dividing cells. The safety profile of AAVs has improved with all viral genes removed from the current generation of viruses. The multitude of serotypes gives diverse targeting capability which means that specific AAV serotypes can be matched to different indications. The main limitations of AAVs are a restriction on the size of the gene of interest and pre-existing immunity to AAV which may render the treatment ineffective.

The limited cargo capacity of AAVs was previously an obstacle for genes such as otoferlin, also known as DFNB9. Mutations in the large otoferlin gene cause profound hearing loss due to defects in vesicle exocytosis (Roux et al., 2006). Researchers have overcome this limitation through a dual AAV approach where large genes have been split
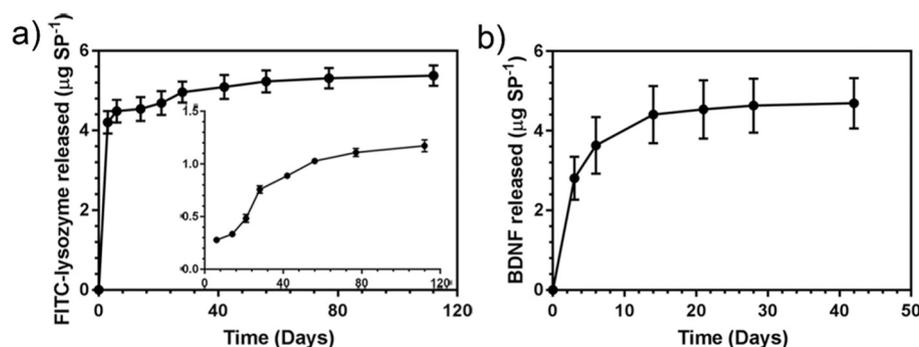


Fig. 4. In vitro cumulative release profiles of (a) FITC-lysozyme and (b) BDNF release from silica supraparticles. Insert in (a) shows the in vitro FITC-lysozyme release profile starting from day 6. Adapted with permission (Ma et al., 2018).

between two AAV vectors. The gene is then reassembled in the transfected cells. Partial restoration of hearing thresholds was observed in otoferlin knockout mice following delivery of dual AAV vectors into the cochlea (Al-Moyed et al., 2019). Full length otoferlin was observed in an average of 19 or 30% inner hair cells (based on two different dual AAV strategies) with restoration of fast vesicle exocytosis. Weaker expression was observed in outer hair cells. With further improvements to the dual AAV vectors to achieve higher full-length protein expression, this study demonstrates the potential for this technique for transferring large genes into cells within the auditory system.

Work is also underway to address the problem of pre-existing immunity to AAVs. The synthetic vector, AAV2/Anc80L65, first described by Zinn, is comprised of ancestral sequences of AAV capsids (Zinn et al., 2015). Cross reactivity to AAV2/Anc80L65 was partially reduced in AAV8 pre-immunized animals whereas AAV8 was completely neutralized (Zinn et al., 2015). The AAV2/Anc80L65 vector also heralded an important breakthrough in terms of cellular targeting in the cochlea. AAV2/Anc80L65 targeted both inner and outer hair cells of the cochlea with high efficiency, while other viral vectors had previously shown poor uptake in outer hair cells. Furthermore, AAV2/Anc80L65 transfected SGNs more efficiently than had been previously achieved following gene therapy into the fluids of the cochlea (Landegger et al., 2017). The vector also had faster transfection kinetics compared to other AAVs with expression visible as early as 1 day post-therapy (Carvalho et al., 2018).

AAVs are the subject of numerous clinical safety and efficacy studies for various diseases including inherited blindness, Parkinson's disease and cystic fibrosis to name a few. Many studies are showing promising results such as improvements in visual acuity in blind patients with choroideremia (MacLaren et al., 2014). However, an adenovirus is the subject of the only current clinical trial (NCT02132130) for the treatment of hearing loss, as discussed previously.

### 5.2.2. Application and distribution

Typically, gene therapy viral vectors are delivered directly to the cochlear fluid compartments: the perilymph or the endolymph. The perilymphatic compartment can be accessed via the round window or oval window membranes of the cochlea or by drilling a cochleostomy through the otic capsule of the cochlea to directly access the scala tympani (Fig. 1A). The round window approach is reported to be the least damaging to residual hearing (Chien, McDougald, Roy, Fitzgerald, & Cunningham, 2015), sometimes with no reported loss of hearing thresholds following surgical access and injection of viral vectors (Gyorgy et al., 2019). While early studies on gene therapy to the cochlea were often hampered by low transfection efficiency, largely a result of the method of delivery to the cochlea or the cell specificity of the viral serotype, recent developments in mode of delivery and viral vector technology in cochlear gene therapy research are eliminating this problem (Lustig & Akil, 2018).

Enormous inter-subject and inter-study variation in transfection levels following direct injection into the cochlear perilymph fluid highlight the difficulty of this approach experimentally. Reported transfection rates to inner hair cells following round window membrane or cochleostomy injection of viral vectors varied from 15% to 100% at the base of the cochlea, with declining transfection rates towards the apex (Akil et al., 2012; Askew et al., 2015; Chien et al., 2015; Chien et al., 2016). Up to 50% inter-subject variation was also reported (Askew et al., 2015). A major cause of variation is the rapid efflux of the perilymphatic fluid that occurs once the otic capsule or the round window membrane has been opened whilst gaining access to the cochlea for application of gene therapy. During efflux of perilymph from the cochlea, cerebrospinal fluid flows into the cochlea via the cochlear aqueduct to maintain fluid homeostasis (Salt & Hirose, 2018). Injection of a viral vector into the cochlea is therefore against the perilymph/CSF flow with inevitable loss of the injected material, unknown dosing and difficultly in transfecting the apical turns of the cochlea. Techniques

designed to carefully seal potential fluid leaks from the cochlea during the injection such as with gels or glues have shown increased efficacy (Plontke, Hartsock, Gill, & Salt, 2016). Another group addressed low transfection rates by creating a flow-system during injection in which the cochlea was inoculated with AAV2/9 via the round window membrane while perilymph was drawn out from a distal location in the semi-circular canals (Yoshimura, Shibata, Ranum, & Smith, 2018). The resulting near complete transfection in inner hair cells was consistent throughout the cochlea in both young (P15–16) and adult (P56–60) mice, while the effect on hearing was minimal. In direct comparison, injection through the round window membrane without distal venting resulted in 68% inner hair cell transfection in the base, only 21% in the middle turn and as little as 2% in the apex with high variability between animals (Yoshimura et al., 2018). Finally, other causes of variation in transfection may come from the use of different AAV serotypes, different doses, animal age/strain differences and variations in surgical experience.

Outer hair cells of the cochlea are typically more difficult to transfect with viral vectors compared to inner hair cells. As mentioned previously, the AAV2/Anc80L65 synthetic vector drastically improved targeting to outer hair cells compared to other AAV serotypes (Landegger et al., 2017; Suzuki, Hashimoto, Xiao, Vandenberghe, & Liberman, 2017). Another group employed an exosome delivery technique to improve targeting to both inner and outer hair cells with AAV1 or AAV9. The in vitro data demonstrated that exosome-AAV1 vectors could improve inner hair cell transfection (65% transfected) and outer hair cells (50% transfected) compared to non-exosome based AAV vectors in cochlear explant cultures (as low as 20% for AAV1 for both inner and outer hair cells). Up to 95% outer hair cells were targeted with exosome-AAV9, demonstrating significantly enhanced efficiency of gene delivery with the exosome packaging (Gyorgy et al., 2017). Similar enhanced transfection of hair cells was demonstrated by injecting exosome-AAV1 vectors into the cochlear perilymph of neonatal mice with 88% inner hair cell transfection (compared to 65% for conventional AAV1) and 28% outer hair cell transfection (compared to 14% for conventional AAV1). The gene delivery technique was tested in Lhfpl5$^{-/-}$ mice, a strain of mice with profound hearing loss and vestibular dysfunction due to deficiency in a protein integral to mechanotransduction in inner and outer cells (Xiong et al., 2012). Presence of the correctly localised Lhfpl5 protein was demonstrated in inner and outer hair cells, with overall improved hair cell survival, functional mechanotransduction, partial improved hearing thresholds in 9 out of 12 animals (thresholds at ~70 dB SPL), a detectable startle response and rescue of balance in 5 animals (Gyorgy et al., 2017).

A few studies have investigated delivery to the endolymph within the scala media (Fig. 1B) which is much smaller and more difficult to access surgically. Access is achieved by injection through the basilar membrane but causes more hearing damage compared to perilymphatic injection (Shibata, Di Pasquale, Cortez, Chiorini, & Raphael, 2009; Wise et al., 2010); or via a cochleostomy through the stria vascularis, also with the potential to damage the strial cells responsible for cochlear fluid dynamics and homeostasis (Wangemann, 2006). In the guinea pig, injection of an adenovirus carrying neurotrophin genes directly into the endolymph resulted in greater transfection in the hair cells and supporting cells of the organ of Corti compared to perilymphatic injection, attributable to the more contained nature of the endolymphatic space compared to the perilymph (Shibata et al., 2009, Wise et al., 2010). Endolymphatic injection was also successfully applied to mice for the correction of a genetic defect resulting in hearing loss. KCNQ1 is a potassium channel subunit essential for the generation of the endocochlear potential and the high potassium content of endolymph which are crucial for the transduction of sound by hair cells (Casimiro et al., 2001; Lee et al., 2000). Mutations in KCNQ1 are responsible for Jervell and Lange-Nielsen syndrome resulting in severe congenital deafness (Jervell & Lange-Nielsen, 1957). Neonatal mice lacking the KCNQ1

gene were injected with an AAV carrying the KCNQ1 gene via the scala media with resulting expression in 61–75% of cochlear marginal cells of the stria vascularis in all cochlear turns. KCNQ1 gene therapy to the endolymph, but not the perilymph, corrected the severe morphological defects caused by the mutation, restored the endocochlear potential and improved hearing thresholds (Chang et al., 2015).

The risk of loss of residual cochlear function is obviously a significant issue when it comes to therapies that aim to protect or restore hearing function. One method employed to minimise the impact on hearing function was gene therapy via the posterior semi-circular canal, first trialled with adenovirus carrying a reporter gene with a significant advantage in hearing preservation (Kawamoto, Oh, Kanzaki, Brown, & Raphael, 2001). AAV2/Anc80L65 containing a reporter gene was injected into the posterior semi-circular canal of 6 week old mice, with resulting transfection of nearly 100% of cochlear inner hair cells and high transfection of outer hair cells throughout the cochlea without affecting hearing thresholds (Suzuki et al., 2017). Furthermore, up to 10% of SGNs in the cochlea were also transfected, with greater efficiency in the apical turn. Vestibular hair cells, supporting cells and ganglion cells were also transfected without any obvious vestibular dysfunction or changes in vestibular evoked thresholds. This technique therefore has merit for drug therapies targeting both cochlear and balance disorders (Suzuki et al., 2017). A similar finding was reported for neonatal mice injected with AAV8 via the posterior semi-circular canal that showed moderately high cochlear inner hair cell and vestibular hair cell transfection without balance or hearing dysfunction (Guo et al., 2017; Isgrig & Chien, 2018).

### 5.2.3. Gene replacement

In animal models of genetic loss-of-function hearing loss, gene therapy has demonstrated successful repair of genetic defects in hair cells and partial or near complete restoration of hearing, although often the therapy is required at early stages of hearing development with obvious translational challenges.

The Whirlin gene is required for actin polymerisation in hair cell stereocilia. As the name suggests Whirler mice exhibit circling and head tossing behaviour indicative of vestibular dysfunction as well as a profound hearing loss due to a frameshift mutation in the Whirlin gene (Lane, 1963). Delivery of a viral vector containing the Whirlin gene to the perilymph of the cochlea via the round window of neonatal Whirler mice restored the morphology of the stereocilia in transfected cells (Chien et al., 2016). However, there was no improvement in overall hearing sensitivity as a result of the low transfection efficiency in this study (15% hair cells at the base of the cochlea) (Chien et al., 2016).

TMC1 is another gene required for hair cell transduction channel function (Pan et al., 2018). Delivery of an AAV2/1 through the round window membrane of neonatal mice that were bred for recessive TMC1 deafness resulted in nearly 60% inner hair cell transfection and the return of some hearing function (Askew et al., 2015). To target outer hair cells more efficiently, the group then used the synthetic AAV2/Anc80L65 serotype in the TMC1 model of hearing loss and found dramatically improved transfection in both inner (91%) and outer hair cells (50%). Restoration of hearing thresholds of up to 50 dB was observed in TMC1 mutant mice (Nist-Lund et al., 2019). Since the cochlear injection approach also resulted in expression in vestibular hair cells, the researchers tested whether gene therapy could restore balance and vestibular function in TMC1/TMC2 mutant mice, as vestibular hair cells express both TMC1 and TMC2 (Kawashima et al., 2011). Following neonatal injection of AAV2/Anc80L65 containing the TMC1 or TMC2 gene into the cochlea via the round window the mice showed vestibular function similar to wild type mice (Nist-Lund et al., 2019).

Vesicular glutamate transporter type 3 (VGLUT3) is required for synaptic transmission in hair cells. Mice lacking the VGLUT3 gene are profoundly deaf due to lack of glutamate release from hair cells (Seal et al., 2008). Restoration of auditory brainstem responses to within 10 dB of wild-type mice as well as a partial restoration of behavioural responses to auditory stimuli were demonstrated following injection of an AAV1 containing VGLUT3 into VGLUT3 knockout neonatal mice via the round window membrane. In this case, nearly every inner hair cell was transfected, demonstrating the enormous potential of gene therapy when efficient and timely targeting of cells is achieved (Akil et al., 2012). However, variable loss of hearing was then observed approximately 7 weeks after gene therapy suggesting transgene inactivation or a slow progressive hearing loss resulting from the surgical delivery of the transgene (Akil et al., 2012).

Ushers syndrome is responsible for 3–6% of childhood deafness (Boughman, Vernon, & Shaver, 1983). Patients with the most severe form experience profound hearing loss, balance issues and progressive blindness. A disrupted harmonin protein is responsible for this phenotype, causing disorganised hair cell stereocilia bundles and loss of mechanotransduction. Using the synthetic AAV serotype AAV2/Anc80L65, harmonin-a1 or harmonin-b1 was introduced into the cochlear and vestibular hair cells in a mouse model of Usher syndrome. Following neonatal cochlear injection, when there are still surviving hair cells, inner hair cell transfection rates were 80–90% with correctly localised harmonin protein at the tips of the stereocilia. Partial recovery of inner and outer hair cell function, auditory thresholds and behaviourally relevant auditory function were observed in the majority of mice injected with harmonin-b1 6 weeks after injection, particularly in the low frequencies (Pan et al., 2017). Out of five mice examined 6 months after injection, three showed continued low frequency hearing attributable to the survival of outer hair cells (Pan et al., 2017).

To summarise, gene therapy to the cochlea via AAV viral vectors has resulted in partial or sometimes near-complete correction of genetic defects in the auditory pathway. Several challenges are still remaining such as the timing of treatment with respect to the degenerative changes of the disease. In many cases experimentally, the gene therapy is administered to neonatal mice in order to repair the gene in the hair cell prior to hair cell degeneration. Given that the development of human hearing occurs prior to birth (Hall 3rd, 2000), the translation of these approaches would potentially require gene therapy in utero. Characterisation of long-term expression and outcomes will also be required for each type of gene therapy as long-term outcomes are variable.

### 5.2.4. Modulating gene translation

Not all genetic mutations require gene replacement for repair of the hearing defect. Some point mutations result in aberrant gene splicing such that the translated protein is truncated or frame-shifted and inactive. In such cases, manipulating splicing could potentially correct the defect by blocking the offending splice site and directing splice site selection to normal. As mentioned above, harmonin is a protein encoded by the USH1C gene and is located in stereocilia and ribbon synapses of hair cells in the cochlea and vestibular system where it has a role in stereocilia structure and mechanotransduction (Verpy et al., 2000). About 6–8% of mutations in Usher syndrome are in the USH1C gene, attributed to a USH1C c.216G > A mutation which causes mis-splicing and ultimately a truncated non-functional harmonin protein (Lentz et al., 2005; Verpy et al., 2000). Antisense oligonucleotide therapy was developed to base-pair at the position of the mutation, blocking access of splicing factors to the aberrant splice site and redirecting the splicing to the correct location in the gene. Deviating from many of the other gene therapy strategies described above where the gene therapy is localised to the cochlea, antisense oligonucleotide therapy in a mouse model of this particular Usher mutation (Lentz Jennifer, Pan Fuming, San Ng San, Deininger Prescott & Keats Bronya, 2007) was administered via systemic administration in neonates (Donaldson et al., 2018; Lentz et al., 2013; Vijayakumar et al., 2017) or embryonically via the amniotic fluids (Depreux et al., 2016) as there is a critical time period during the development of hearing in which therapy must be administered. Antisense oligonucleotide therapy afforded long-term significant rescue of hearing and correction of vestibular function associated with restoration of hair cell stereocilia morphology (Donaldson et al., 2018, Lentz

et al., 2013, Vijayakumar et al., 2017). Earlier treatment had a greater impact on hearing restoration consistent with the developmental pattern of expression of harmonin that occurs between P4 and P16 (Lentz et al., 2013).

The ease of delivery, specificity and efficacy of antisense oligonucleotides make this form of therapy an exciting option for altering gene expression in diseases with a known genetic component. Splice-switching oligonucleotide therapy is currently in clinical trials for the treatment of Duchenne muscular dystrophy and spinal muscular atrophy (Havens & Hastings, 2016) showing relatively low toxicity and good tolerability.

RNA interference is a technique that can be used to silence specific genes linked to a disease, particularly for gain-of-function mutations. Gain-of-function mutations occur when a mutation results in an altered gene product which then interferes with the normal functioning of a biological pathway. An example in the auditory system is connexin-26, encoded by the Gap Junction Protein Beta 2 gene (GJB) which is a part of a larger complex of proteins that form a connexon, the pore-forming unit of gap junctions (Kumar, 1999). A point mutation in connexin-26 results in a protein that still complexes with the other five connexins but interferes with the function of gap junctions, preventing hearing transduction in the cochlea through deficient potassium recycling (Cohen-Salmon et al., 2002; Denoyelle et al., 1998; Richard et al., 1998; Rouan et al., 2001). To suppress the mutant protein, a short-interfering RNA was complexed to a liposome delivery system and applied to the intact round window membrane of a mouse model of the connexin point mutation. The RNA interference treatment significantly and specifically suppressed the mutant GJB mRNA, preventing hearing loss in the mouse model (Maeda, Fukushima, Nishizaki, & Smith, 2005). Similarly, in a mouse model of a gain-of-function TMC1 mutation known as Beethoven (Vreugde et al., 2002), a single intracochlear injection of a microRNA delivered by an AAV slowed the progression of hearing loss through suppression of the mutated gene. At the 35-week time point, mice in the RNA interference group had a 40 dB improvement in hearing thresholds over non-treated controls (Shibata et al., 2016). Other gain-of-function mutations in the auditory system that may benefit from RNA interference include missense mutations of the KCNQ4 gene that affect potassium channels (Coucke et al., 1999; Kubisch et al., 1999) and the DFNA5 gene which produces a protein of currently unknown function (Van Laer et al., 1998; Van Laer et al., 2004).

As discussed in Section 3.4.1, new hair cells can be generated in mammals by increasing expression of the Atoh1 transcription factor, decreasing Notch signalling or regulating other pathways following hearing loss (Samarajeewa, Jacques, & Dabdoub, 2019). Silencing of hairy and enhancer of split-1 (Hes1), a down-stream effector of Notch signalling and inhibitor of Atoh1 (Lanford et al., 1999; Zine, Van de Water, & de Ribaupierre, 2000), is also sufficient to promote transdifferentiation of supporting cells to hair cells in the mouse cochlear tissue (Du et al., 2013; Korrapati, Roux, Glowatzki, & Doetzlhofer, 2013; Slowik & Bermingham-McDonogh, 2013). To test these results in vivo, a Hes1 small interfering RNA was delivered to adult guinea pig cochleae via PLGA nanoparticles 72 h after noise-induced hearing loss by mini-osmotic pump infusion into the perilymph over 24 h. As a result of the treatment there were fewer missing inner and outer hair cells across the entire length of the cochlear spiral, some correctly positioned and some ectopic. There was also progressive recovery of hearing of >10 dB, particularly in the epicentre of the noise-induced damage and near the infusion site (Du et al., 2018).

In an exciting development for gene modulation therapies, the US Food and Drug Administration has approved the first gene-silencing technology based on RNA interference: the drug, patisiran, which has improved outcomes in patients with a rare heart and nerve function condition (Adams et al., 2018).

### 5.2.5. Cell targeting

The majority of experimental cochlear gene therapies to date aim to target cells in the sensory epithelium such as the hair cells or supporting cells. SGNs have proven to be relatively resistant to transfection. There is recent interest in transducing SGNs, particularly for optogenetics research which requires expression of a light-sensitive ion channel in SGNs. The aim is to develop an optical cochlear implant for improved spectral resolution during SGN stimulation in the cochlea (Jeschke & Moser, 2015; Richardson, Thompson, Wise, & Needham, 2017). At first embryonic gene therapy was performed in order to achieve efficient SGN transfection (Hernandez et al., 2014). When E11.5–12.5 embryos were transfected, there was 40% SGN transfection which was sufficient to evoke activity within the central auditory pathway to optical stimulation of the transfected SGN. Clinical translation of this technique requires a solution in which post-natal, preferably adult SGNs, are transfected. One solution was the use of a novel AAV serotype which improved SGN transfection in post-natal mice. In 5–7 day old early postnatal mice, strong SGN expression of the opsin was achieved across all turns (about 55% in base and 75% in apex) when using a novel serotype with improved neural transfection, AAV-PHP.B (Deverman et al., 2016) via a round window membrane injection (Keppeler et al., 2018) or 74% transfection when using the AAV2/Anc80L65 viral vector in P4 mice (Duarte et al., 2018). Interestingly, another solution came in the form of a novel mode of delivery to the cochlea. In adult gerbils, AAV2/6 was injected directly into the modiolus of the cochlea. This technique resulted in up to 30% transfection in SGNs in all turns of the cochlea in about half of the injected animals. The technique was damaging to SGNs though, with approximately 25% loss of SGNs (Wrobel et al., 2018). Subsequent optical stimulation of SGNs expressing the light-sensitive ion channels resulted in auditory percepts and behavioural responses in transfected gerbils (Wrobel et al., 2018).

### 5.2.6. Gene editing

Usher syndrome is an inherited disorder causing deafness and blindness with up to 13 associated genes. >50% of Usher syndrome cases harbour a mutation in the USH2A gene. The large size of the USH2A gene makes it difficult to develop a conventional gene therapy for these patients, but the CRISPR/Cas9 gene editing technology now offers an alternative strategy. The Cas9 nuclease is guided with precision by small RNAs to the target sequence for editing. Successful targeting and repair of the USH2A mutation was demonstrated in fibroblasts in vitro (Fuster-Garcia et al., 2017). More recently CRISPR/Cas9 technology has been used to restore hearing in animals with a genetic form of deafness that normally results in loss of hair cells. The faulty TMC1 gene present in cochlear hair cells was targeted by the gene-editing tool to repair a single base-pair mutation. In a novel take on the CRISPR/Cas9 technology, the authors used a special DNA-free, virus-free delivery technique in which the Cas9 complex was encapsulated in lipid packaging to directly deliver the Cas9 complex inside the hair cell rather than having the cell make the Cas9 from DNA (Zuris et al., 2015). This reduced the off-target editing thereby improving the precision of the editing. The outcome in these mice was significantly greater hair cell survival and prevention of loss of hearing (Gao et al., 2018).

Genome editing with nucleases such as Cas9 can be subject to extraneous insertions and deletions (indels) and is largely restricted to mitotic cells (Cox, Platt, & Zhang, 2015). To overcome these limitations, researchers have used a nuclease-free base editing technique which increased the ratio of editing compared to indels (Komor, Kim, Packer, Zuris, & Liu, 2016). Lipid-mediated delivery of the beta-catenin base editor to the mouse inner ear resulted in the replacement of Ser 33 to Tyr in the beta-catenin gene. This resulted in altered post-translational protein modification (prevention of phosphorylation) and therefore, upregulation of Wnt signalling. This in turn promoted mitosis in supporting cells of the cochlea and reprogramming of the cells towards the hair cell lineage (Yeh et al., 2018).

### 5.2.7. Gene augmentation

Viral gene therapy is not just limited to the correction of hereditary disorders. Gene therapy can be utilised to augment gene expression, for

example to restore neurotrophin levels in the cochlea following the loss of hair cells and supporting cells that occurs with SNHL (Atkinson et al., 2012; Budenz et al., 2015; Fukui et al., 2012; Wise et al., 2010; Wise et al., 2011). When neurotrophin gene therapy was administered to the scala media of guinea pigs that were deafened by aminoglycosides there was a preservation of the SGN population along with outgrowth of the peripheral fibres towards neurotrophin-expressing cells in the sensory epithelium (Wise et al., 2010, Wise, Fallon, et al., 2011) (Fig. 5). It is yet to be determined whether neurotrophin gene therapy results in improvements in cochlear implant function as has been shown with long-term recombinant BDNF delivery to the cochlea (Shepherd, Coco, Epp, & Crook, 2005). Guinea pig cochleae that were inoculated with AAV containing the NT-3 gene and then implanted with an electrode array showed measurable and long-lasting SGN survival, but variability in transfection made electrophysiological assessment difficult (Pfingst et al., 2017).

Finally, the potential to use the cochlear implant to drive transfection of DNA into resident cells lining the scala tympani via brief but intense pulses from the electrode array provides a unique transfection mechanism for gene therapy. Using this electroporation technique Housley and colleagues were able to transfect mesenchymal cells with a complementary DNA (cDNA) gene construct resulting in the expression of BDNF (Pinyon et al., 2014). Using a deafened guinea pig model, they were able to show reduced electrical thresholds and improved SGN survival with significant regrowth of the nerve fibres within the scala tympani. The resultant improvement in the electrode-neural interface may lead to clinical improvements for cochlear implant recipients following completion of an upcoming Phase 1 clinical trial.

### 5.3. Cochlear implant combination therapies

Cochlear implant electrode arrays are implanted into the scala tympani of severe-to-profoundly deaf patients. There is therefore the potential to use the device to deliver therapeutics to the cochlea. The validity of this approach was first investigated by manufacturing a cannula internally to the electrodes which was connected to a pump for the continuous delivery of neurotrophins to the guinea pig cochlea following hearing loss (Shepherd & Xu, 2002). SGN survival was enhanced compared to deaf controls and further improvement in the SGN density was observed in animals that were simultaneously given electrical stimulation (Shepherd et al., 2005; Shepherd, Coco, & Epp, 2008). However, the presence of a cannula or any cavity in the electrode array is problematic as it increases the infection risk and significantly alters the mechanical characteristics of the electrode array. For this reason some studies examined drug release from a cochlear implant coating that has been modified to be loaded with drugs for subsequent release into the cochlea. These have included a coating of cells that have been modified to express and release neurotrophins and degradable coatings.

In one study cochlear implant electrodes were coated with the electro-active polymer polypyrrole. The polypyrrole could be doped with NT-3 or BDNF that was only released during electrical stimulation (Richardson et al., 2007; Thompson et al., 2006; Thompson et al., 2010; Thompson, Moulton, Richardson, & Wallace, 2011). Arrays that were coated with polypyrrole containing NT-3 were implanted in guinea pigs that had been ototoxically deafened and electrical stimulation was provided for 4 weeks. It was found that there was greater nerve survival in guinea pigs implanted with coated compared to control arrays (Richardson et al., 2009) although the technique is limited to a relatively small and finite payload of neurotrophin.

Inflammation and fibrosis following cochlear implantation is believed to be the cause of some post implantation-related hearing loss that can occur in recipients with residual hearing (Chang et al., 2009; Eastwood et al., 2010). Pharmacological interventions to reduce the immune response are being investigated with a particular emphasis on the anti-inflammatory drug dexamethasone. Local delivery of dexamethasone to the cochlea can prevent some of the loss of residual hearing and reduces the tissue response around the implanted array (Lee et al., 2013). Some researchers have been looking to the cochlear implant itself as a means to elute drugs like dexamethasone over the time frame of inflammation and fibrosis which occurs in stages during the days and weeks following cochlear implantation. The dexamethasone was added during the polymerisation of silicone elastomer such that the drug is an integral part of the implant (Farahmand Ghavi, Mirzadeh, Imani, Jolly, & Farhadi, 2010). When tested in guinea pigs the dexamethasone-eluting cochlear implant attenuated some inflammatory cell infiltration in the cochlea between 3 and 13 days post-
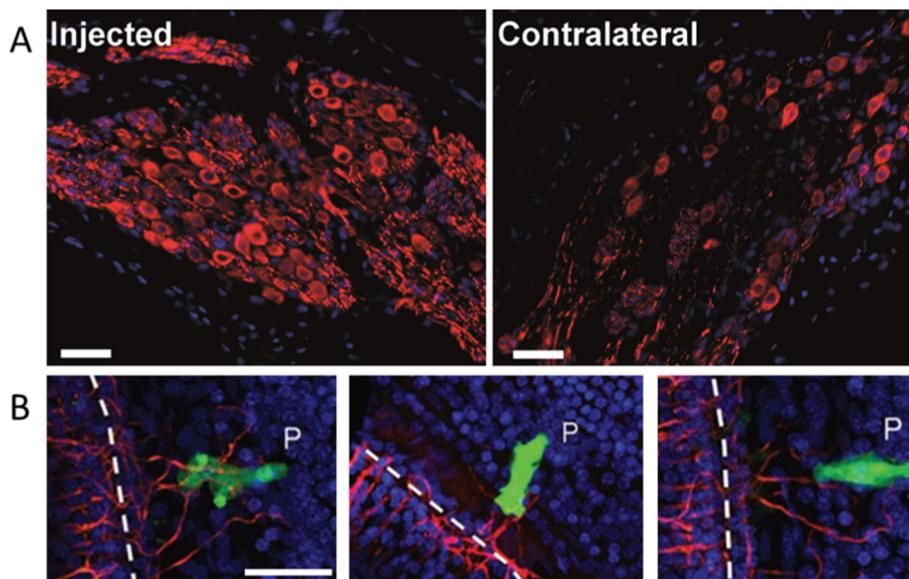


**Fig. 5.** Neurotrophin gene therapy provides cellular sources of neurotrophins for SGN survival after hearing loss (A) Comparison of spiral ganglion neuron cell bodies (red) in a cochlea treated with neurotrophin gene therapy and the contralateral non-injected cochlea. Guinea pigs were deafened with aminoglycosides 1 week prior to gene therapy with adenoviral vectors containing BDNF and NT3 genes into the scala tympani. After three weeks there was a dramatic difference in the number of surviving spiral ganglion neurons between the treated and non-treated cochleae. Scale bars are 50 μm. (B) Surface view of the organ of Corti with peripheral fibres of spiral ganglion neurons (red) attracted towards pillar cells (P) that have been transfected with neurotrophin genes (as labelled by a green-fluorescent protein reporter gene). A DAPI counterstain is in blue. Scale bars are 50 μm. The dotted line indicates the position of the habenula perforata. Adapted from (Wise, Fallon, et al., 2011).

implantation (Farhadi et al., 2013). This approach has also demonstrated a trend towards hearing protection, however the results to date have not been statistically significant and may depend on the level of damage during insertion (Astolfi et al., 2016; Stathopoulos et al., 2014).

### 5.4. Cell-based therapies

Another potential solution to the damage or loss of hair cells or SGNs is cell-based therapies for the replacement or restoration of these cells. Stem cells are one of the most commonly used in the field of cell-based therapies due to their potential to differentiate into different cell types including hair cells and SGNs. To date, there are quite a few studies examining the differentiation of murine embryonic stem cells (Coleman, Fallon, Pettingill, de Silva, & Shepherd, 2007; Lin et al., 2009; Reyes et al., 2008) or human embryonic stem cells (Buckiova et al., 2012; Chen et al., 2009; Shi, Corrales, Liberman, & Edge, 2007) into functional hair cells or auditory neurons.

When transplanted into the cochlear environment, differentiated stem cells have been shown to survive and integrate into the tissue (Coleman et al., 2006; Tateya et al., 2003). Strikingly, transplanted neural progenitor cells appeared to respond to natural differentiation and guidance cues in the tissue environment with engrafted embryonic stem cells in the cochlear nerve trunk projecting fibres towards the sensory epithelium (Corrales et al., 2006). Human otic progenitor cells that were differentiated towards cells with morphological and electrophysiological features of hair cells and SGNs *in vitro* promoted recovery of auditory brainstem response thresholds when transplanted into SGN-depleted gerbils (Chen et al., 2012). Induced pluripotent stem cells (IPSCs), differentiated cells that have been reprogrammed to an embryonic state, also received much attention as they can be transplanted without immune response since they are generated from the patients' own cells (Takahashi & Yamanaka, 2006). In one study, mouse IPSC-derived neural progenitors could be differentiated into a neural lineage. After transplantation into the mouse cochlea *in vivo*, a small percentage of the transplanted IPSC-derived neural progenitors expressed VGLUT1, a marker for glutamatergic neurons (Nishimura et al., 2009).

In addition to the transplantation of differentiated neural stem cells to replace degenerated sensory cells or neurons in deafened cochlea, a second cell-based therapy approach involves the delivery of cells designed to rescue sensory cells or neurons from ongoing degeneration. For example, *in vitro* experiments indicated that genetically modified Schwann cells could maintain the survival of SGNs (Pettingill, Minter, & Shepherd, 2008). To avoid recognition by the immune system and cellular migration from the implantation site, BDNF-over-expressing Schwann cells were encapsulated by a semi-permeable alginate membrane where oxygen and nutrients could diffuse into the cells and the BDNF and waste could diffuse away from the cells (Pettingill, Wise, Geaney, & Shepherd, 2011). The results showed that alginate encapsulated BDNF-over-expressed Schwann cells could promote the survival of SGNs in deafened guinea pigs. Further, porcine choroid plexus cells, which naturally secrete a cocktail of neurotrophins (Skinner et al., 2009), encapsulated in an alginate membrane promoted survival of SGNs and their peripheral fibers for several months in deafened guinea pigs with a combination of chronic electrical stimulation via cochlear implantation with minimal tissue response to the grafted cells (Wise, Tu, et al., 2011).

There are still a number of challenges for clinical application of cell based therapies. It is essential that the transplanted cells survive for long periods of time and not interfere with normal cochlear function or affect residual hearing (Coleman, de Silva, & Shepherd, 2007; Hildebrand et al., 2008). Potential immunological responses to embryonic stem cells must be considered (Beisel, Hansen, Soukup, & Fritzsch, 2008) alongside the risk of tumorigenesis if they are not fully differentiated (Erdo et al., 2003; Gunewardene, Dottori, & Nayagam, 2012). An additional uncertainty is whether the generated cells have

the same functional response as normal SGNs or hair cells (Mittal et al., 2017; Takeda et al., 2018). It is also challenging to precisely inject the cells into the target without damage to existing structures and for the cell to integrate into the existing tissue and to form functional connections (Altschuler, O'Shea, & Miller, 2008; Backhouse, Coleman, & Shepherd, 2008; Coleman, de Silva, & Shepherd, 2007; Takeda et al., 2018).

### 5.5. How close are we and which delivery technique is best?

This review has covered a number of novel drug therapy technologies for the potential treatment of hearing loss. The therapies are varied by necessity to mirror the diverse aetiologies of hearing loss. A number of the therapies discussed have demonstrated partial reversal or slowed progression of hearing loss in relevant animal models. These have included hair cell regeneration following acquired damage to hair cells via the manipulation of transcription factor pathways by gene or drug therapies (Du et al., 2018; Izumikawa et al., 2005; Kraft et al., 2013; Mizutari et al., 2013; Yeh et al., 2018). The most promising hair cell regeneration therapies are those that use a combination of factors (Costa et al., 2015; Kuo et al., 2015; Walters et al., 2017) and those that promote mitotic regeneration such that the supporting cell population is maintained during hair cell regeneration (McLean et al., 2017). For acquired synaptopathy or hidden hearing loss, delivery of neurotrophic factors such as NT-3 to the cochlea has been shown to rescue hearing function via the regeneration of the ribbon synapse following noise trauma (Sly et al., 2016; Suzuki et al., 2016). The simplicity of the trans-tympanic delivery technique will make this therapy readily translatable, despite the short window of opportunity for effective treatment. Neurotrophins are also protective to spiral ganglion neurons that can otherwise degenerate following hearing loss and hence are a promising drug therapy that could be applied to a range of hearing loss conditions. In many cases, extended exposure to neurotrophins is beneficial to the therapy, hence research into drug delivery technologies such as biomaterials (incorporating hydrogels, nanoparticles, supraparticles) or cell-based therapies that can not only stabilise the drug over time but also provide sustained release of the drug. With so many tools available, gene therapy has the potential to treat a diverse range of SNHL such as gene editing of point mutations, replacement of defective genes, silencing of gain-of-function mutations, suppression of aberrant splice variants, alteration of post-translational modifications, manipulation of transcription factor pathways for regeneration and augmentation with therapeutic genes such as neurotrophins. Using mouse models of known human mutations or acquired damage to hair cells, there have been many reports of long-term correction of genetic defects ranging from partial to near-complete reversal/prevention of hearing loss with early intervention (Akil et al., 2012; Al-Moyed et al., 2019; Askew et al., 2015; Chang et al., 2015; Donaldson et al., 2018; Gao et al., 2018; Gyorgy et al., 2019; Lentz et al., 2013; Maeda et al., 2005; Nist-Lund et al., 2019; Pan et al., 2017; Shibata et al., 2016; Vijayakumar et al., 2017). Furthermore, with a direct read-out of transfection efficiency in the form of a fluorescent reporter gene, gene therapy research has led to significant improvements in cochlear delivery techniques that will benefit all forms of therapy to the cochlea. In contrast, there have been relatively few examples of hearing restoration by cell based therapies (Chen et al., 2012) compared to gene therapy techniques highlighting the challenges of differentiation stem cells into functional auditory sensory cells, integrating the cells into the auditory pathway and maintaining long-term survival in the cochlea.

While there are relatively large numbers of clinical trials incorporating trans-tympanic drug delivery into the middle ear (Peppi et al., 2018; Staecker et al., 2017) there are few trials incorporating drug delivery directly into the cochlea due to concerns with potential damage to residual hair cells. As noted above, the only clinical trial registered with ClinicalTrials.gov incorporating direct intra-labyrinthine drug delivery is designed to evaluate the safety, tolerability, and the potential ability

of the drug CGF166, an adenovirus containing the human Atoh1 gene, to improve hearing in patients with severe-profound SNHL (NCT02132130).

The various delivery systems have specific advantages and disadvantages that must be taken into consideration in developing a clinical based delivery system. For example, delivery using biomaterials – particularly nano- and supraparticles – can deliver large payloads including a cocktail of drugs delivered over different time-constants. These systems can deliver both native protein and appropriate synthetic drugs. Biomaterial-based delivery techniques typically have short or moderate release profiles and must be carefully tuned to ensure they do not produce high concentrations of the drug(s) at the target site. In contrast, cell and gene based delivery techniques offer potentially longer-term solutions, whether for gene augmentation, genetic correction or cell regeneration, with a high degree of cell specificity. However, many of the promising therapies for hereditary hearing loss would require early intervention before complete loss of the hair cells or irreversible morphological changes.

In addition to these technical considerations, it is also important when developing these systems to consider the manufacturing and clinical implications of the technology (Shepherd, Villalobos, Burns, & Nayagam, 2018). For example, in a clinical setting the technology will need to conform to Good Manufacturing Practice guidelines and recognized standards of sterilization for healthcare products and packaging systems, and show a reasonable shelf-life at room temperature. If drug, gene or cell loading is to be performed during surgery it must be undertaken using sterile techniques under appropriate guidelines for handling biological material.

Finally, it is likely that the initial delivery techniques will target hearing preservation (Lyu et al., 2018), SGN peripheral fibre regeneration (Landry et al., 2013; Pinyon et al., 2014) and a reduction in inflammation (Chambers et al., 2019) in cochlear implant patients. This work, together with novel techniques designed to safely target either the round or oval windows to treat disorders including Meniere's disease (King, Shepherd, Brown, & Fallon, 2017) and hidden hearing loss (Sly et al., 2016; Wan et al., 2014) are likely to dominate inner ear drug delivery in the near-term. With the clinical experience gained from these approaches, more trials evaluating direct drug delivery to the cochlea in patients are expected. Disorders including tinnitus, sudden hearing loss and hearing protection following exposure to loud noise or ototoxic drugs would be potential candidates for this route.

## 6. Conclusions

Hearing loss is a significant problem world-wide. The sensory hair cells and SGNs of the cochlea are often affected and do not spontaneously regenerate in humans. A number of factors have been identified that can preserve, repair or regenerate these cell types with a growing number of studies showing recovery of hearing in animal models of acquired and genetic forms of hearing loss. The anatomy of the cochlea presents some unique challenges for the development of a therapy for hearing loss. The cochlear tissues and membranes are extremely sensitive, difficult to access and consistent dosing is often hard to achieve. To overcome these limitations, researchers are using novel drug delivery techniques and technologies. The cochlear implant itself is also the subject of a range of drug delivery strategies, from electroactive biomaterial coatings to using electrical stimulation to transfect cochlear cells with DNA encoding neurotrophic factors. Hydrogel biomaterials are being considered for the simplicity of application to the round window of the cochlea for non-invasive and safe drug delivery to the cochlea. Nano- or supraparticle biomaterial technologies offer slow-release and high pay-load drug delivery properties that are attractive for neurotrophin delivery to the cochlea to protect SGNs from degeneration. There are now numerous examples of hearing restoration in genetic models of hearing loss following gene therapies due to new viral vector and gene editing technologies and improvements in delivery

techniques. The potential to activate progenitor cells in the cochlea have also been explored for hair cell regeneration alongside cell-based therapies for repopulating the SGN population or delivering neurotrophins for SGN protection after hearing loss. As the potential for a drug treatment to restore hearing becomes evident in pre-clinical studies, advancements in drug therapy technologies such as those described here will help ensure that translation of these discoveries can be achieved.

## Conflict of interest statement

The authors A.K.W, Y.M and R.K.S are named inventors on a patent describing drug delivery technique that is owned by the Bionics Institute. There is no financial conflict of interest by these or any author.

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