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The effect of deafness duration on neurotrophin gene therapy for spiral ganglion neuron protection

Abbreviated Title: Gene therapy for the cochlea

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Abstract

A cochlear implant can restore hearing function by electrically exciting spiral ganglion neurons (SGNs) in the deaf cochlea. However, following deafness SGNs undergo progressive degeneration ultimately leading to their death. One significant cause of SGN degeneration is the loss of neurotrophic support that is normally provided by cells within the organ of Corti (OC). The administration of exogenous neurotrophins (NTs) can protect SGNs from degeneration but the effects are short-lived once the source of NTs has been exhausted. NT gene therapy, whereby cells within the cochlea are transfected with genes enabling them to produce NTs, is one strategy for providing a cellular source of NTs that may provide long-term support for SGNs. As the SGNs normally innervate sensory cells within the OC, targeting residual OC cells for gene therapy in the deaf cochlea may provide a source of NTs for SGN protection and targeted regrowth of their peripheral fibers. However, the continual degeneration of the OC over extended periods of deafness may deplete the cellular targets for NT gene therapy and hence limit the effectiveness of this method in preventing SGN loss. This study examined the effects of deafness duration on the efficacy of NT gene therapy in preventing SGN loss in guinea pigs that were systemically deafened with aminoglycosides. Adenoviral vectors containing green fluorescent protein (GFP) with or without genes for Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin-3 (NT3) were injected into the scala media (SM) compartment of cochleae that had been deafened for one, four or eight weeks prior to the viral injection. The results showed that viral transfection of cells

within the SM was still possible even after severe degeneration of the OC. Supporting cells (pillar and Deiters' cells), cells within the stria vascularis, the spiral ligament, endosteal cells lining the scala compartments and interdental cells in the spiral limbus were transfected. However, the level of transfection was remarkably lower following longer durations of deafness. There was a significant increase in SGN survival in the entire basal turn for cochleae that received NT gene therapy compared to the untreated contralateral control cochleae for the one week deaf group. In the four week deaf group significant SGN survival was observed in the lower basal turn only. There was no increase in SGN survival for the eight week deaf group in any cochlear region. These findings indicated that the efficacy of NT gene therapy diminished with increasing durations of deafness leading to reduced benefits in terms of SGN protection. Clinically, there remains a window of opportunity in which NT gene therapy can provide ongoing trophic support for SGNs.

1 Introduction

Sensorineural hearing loss (SNHL) can result from damage to the organ of Corti (OC) caused by a number of different factors including noise, aging, ototoxic drugs or can arise from genetic origins. For people with severe to profound SNHL a cochlear implant can be used to electrically excite the spiral ganglion neurons (SGNs) and restore some hearing function. However, SNHL leads to the progressive degeneration of the SGNs (Hardie et al., 1999; Webster et al., 1981) which could compromise the performance of the cochlear implant. Therefore, strategies to prevent, or reverse SGN degeneration may lead to improvements in the clinical benefits achieved by these devices.

One factor contributing to SGN degeneration is the removal of neurotrophic support that is normally provided by cells within the OC, in particular, the hair cells (Ernfors et al., 1992; Ylikoski et al., 1993) and supporting cells (Stankovic et al., 2004). Neurotrophins (NTs), for example Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin-3 (NT3), are known to play an important role in the development and maintenance of neural innervation within the cochlea (Altschuler et al., 1999; Fritzsch et al., 1997). Administering an exogenous supply of NTs to the cochlear fluids of deafened guinea pig (GP) cochleae can prevent SGN degeneration if given shortly after deafness (Agterberg et al., 2008; Ernfors et al., 1996; Glueckert et al., 2008; McGuinness et al., 2005; Miller et al., 1997; Staecker et al., 1996; Wise et al., 2005) or halt continued SGN degeneration if the administration is delayed following deafness (Gillespie et al., 2004; Wise et al., 2005). Experimentally,

mini-osmotic pumps have been used to provide the NTs. However, mini-osmotic pumps have a finite capacity and once the supply of NT has been exhausted the protective effects of NT are abolished (Gillespie et al., 2003) or may persist for a short period (two weeks) following NT exhaustion (Agterberg et al., 2009). The implication is that long-term NT delivery is required to achieve sustained SGN survival (Pettingill et al., 2007).

Gene therapy has the potential to provide a long-term source of NTs in the cochlea (Lalwani et al., 1998). Previous studies have shown that injection of viral vectors into the scala tympani compartment of the cochlea for expression of NT genes lead to the protection of SGNs from deafness-induced degeneration (Chikar et al., 2008; Lalwani et al., 2002; Nakaizumi et al., 2004; Staecker et al., 1998; Yagi et al., 2000). Furthermore, the introduction of NT genes into the scala media (SM) compartment also protected SGNs from deafness-induced SGN loss in addition to providing target-derived guidance cues that attracted resprouting SGN peripheral fibers (Wise et al., 2010; Shibata et al., 2010). However, the effectiveness of NT gene therapy is likely to depend on the viability of the cochlear cells targeted for viral transfection. In cases where the OC is selected as the target (Wise et al., 2010) its ongoing degeneration as a consequence of the pathology may limit the capacity of NT gene therapy to provide neurotrophic support necessary for the protection of SGNs. This is an important issue if gene therapy is to become clinically viable given that implant recipients may be deaf for long periods of time before they receive a cochlear implant and thus there is likely to be significant loss of cochlear cells that are viable for viral transfection.

Therefore, the aim of this study was to examine the effects of deafness duration on the efficacy of viral-mediated NT transfection in preventing SGN loss in the deafened cochlea. GPs were ototoxically deafened and then unilaterally injected with adenovirus (Ad) vectors into the SM of the cochlea after one, four or eight weeks of deafness. The Ad vectors contained the gene for green fluorescent protein (Ad-GFP) with or without genes for the NTs BDNF and NT3 (Ad-GFP-NT) and three weeks post-injection the cochleae were collected for histology. The results showed enhanced SGN survival with deafness periods of one and four weeks, but not eight weeks, indicating that there is a critical window for SM viral injections to provide therapeutic benefits in preventing SGN loss.

2 Materials and Methods

2.1 Ad production

Viral production and efficacy is described in Wise et al., (2010). Briefly, E1/E3/polymerase/terminal protein-deleted Ad type 5 vectors containing GFP under the control of a cytomegalovirus promoter with or without mouse BDNF or NT3 expressed via an IRES sequence (Ad-GFP, Ad-GFP-BDNF and Ad-GFP-NT3) were generated using the AdEasy system (Stratagene, La Jolla, CA). The virus was produced in C7-HEK 293 cells and then purified using commercially available ion exchange chromatography membranes (Vivascience, Littleton, MA), and stored in Ad storage buffer (25% glycerol, 10 mmol/l Tris pH 8.0, and 2 mmol/l MgCl2) at −80 °C. The virus was titered on HT1080 cells by spectrophotometry [>10^{11} optical particle units (OPU)/ml by OD_{260}](Mittereder et al., 1996). Viral stocks were verified by PCR to be

free of replication-competent Ad. Before the SM injection, the Ad vectors were diluted 1:5 in artificial endolymph (120 mM KCl, 2.5 mM NaCl, 0.5 mM $MgCl_2$, 0.28mM $CaCl_2$, 7.6 mM K_2HPO_4, 2.7 mM KH_2PO_4, pH 7.4) to final concentrations of $1.1x10^{11}$ OPU/ml (Ad-GFP), $3.0x10^{10}$ OPU/ml (Ad-GFP-NT3) and $4.33x10^{10}$ OPU/ml (Ad-GFP-BDNF).

Ad samples were tested to ensure production of NTs by the infected cells (Wise et al., 2010).

2.2 Animals

Female or male adult pigmented Dunkin–Hartley GPs were used (n=36 average weight 370 g). All procedures were approved by the Animal Research Ethics Committee of the Royal Victorian Eye and Ear Hospital in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85-23 Rev. 1985). GPs were randomly assigned to the experimental groups described in Table 1.

[*Table 1 near here*]

2.3 ABR recordings

Auditory Brainstem Responses (ABRs) were measured prior to deafening using procedures described previously (Shepherd et al., 1985; Wise et al., 2010). Only animals with normal hearing thresholds (ABR threshold <43 dB peak-equivalent sound pressure level) were used.

2.4 GP deafening

Three pre-injection deafness durations were used in this study (1, 4 and 8

weeks – Table 1). Normal hearing GPs were deafened under anesthesia via an intravenous 100 mg/kg frusemide (Troy Laboratories, Smithfield, Australia) and subcutaneous 400 mg/kg kanamycin sulfate (Sigma-Aldrich, Castle Hill, Australia) injections according to procedures described previously (Wise et al., 2005). Following the pre-injection deafness duration (1, 4 or 8 weeks) GPs received the SM injection of the viral vector.

2.5 Scala media injection of viral vectors

Viral vectors (Ad-GFP or Ad-GFP-NT) were unilaterally injected into the SM of the left cochlea of the deafened or normal hearing GP using aseptic techniques as described previously (Wise et al., 2010). Briefly, a retroauricular incision exposed the bulla and a small cochleostomy was made into the basal turn. The basilar membrane was visualized and a quartz glass recording micropipette (World Precision Instruments, Sarasota, FL) with a 5–10 µm tip diameter was inserted through the membrane and into the SM. An endocochlear potential (EP) was recorded (average EP 78.9 mV ±SEM 5.2; $n = 26$) to verify entry into the SM (Sellick et al., 2008). Two microliters of the viral preparation was injected into the SM over 5 minutes. The cochleostomy was sealed with a small piece of muscle, the bulla sealed with dental cement, and the wound closed with sutures.

2.6 Histological processing

Following the three week post-injection treatment period, GPs were euthanized with 1.5 ml pentobarbitone and intracardially perfused with 0.9% (wt/vol) saline containing 0.1% (vol/vol) heparin sodium and 0.025% (wt/vol) sodium nitrite, followed by 10% (vol/vol) neutral buffered formalin (NBF).

Cochleae were removed and postfixed in 10% (vol/vol) NBF overnight and then decalcified in 10% (wt/vol) EDTA in 0.1 mol/l phosphate buffer. Cochleae were embedded in OCT (Tissue-Tek, Torrance, CA) and sectioned on a cryostat at 12 μm through pre-modiolar and mid-modiolar planes and mounted onto SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany), leaving the final half of the cochleae intact. The remaining half-cochlea contained the viral injection site and was cut into half-turn surface preparations as described previously (Wise et al., 2005; Wise et al., 2010). Standard immunofluorescent protocols were followed using antibodies to neurofilament-200 (NF-200; Chemicon International, Boronia, Australia) for staining SGNs and peripheral fibres, anti-calretinin (Chemicon International) and phalloidin (Molecular Probes, Eugene, OR) for cells in the OC and AlexaFluor secondary antibodies (Molecular Probes). Sections were examined on a Zeiss Axioplan II fluorescence microscope (Carl Zeiss, Jena, Germany). Cochlear half-turn surface preparations were viewed on a Zeiss confocal microscope.

2.7 Data analysis

All results are presented as the mean ± the standard error of the mean (SEM).

2.7.1 GFP expression

The GFP reporter gene in Ad-GFP-NT and Ad-GFP treated cochleae was used to examine gene expression in mid-modiolar sections. The location of GFP expression from up to nine non-consecutive mid-modiolar sections (over 324 μm) was analyzed and used to form a composite image represented on a standard mid-modiolar GP cochlea section. In addition, GFP expression was

examined in cochlear half-turns from the one, four and eight week deaf groups and the normal hearing control groups.

2.7.2 SGN survival and organ of Corti degeneration

SGN density was analyzed from up to nine non-consecutive mid-modiolar sections from each cochlea in a blinded manner using the same protocol as previously described (Wise et al., 2010). SGN density was determined by counting NF-200-positive SGN cell bodies within Rosenthal's canal and by measuring the area formed by the perimeter around the outermost SGNs using a Zeiss Axioplan II microscope and AxioVision software. SGN densities were measured for six cochlear locations; lower basal (LB), upper basal (UB), lower middle (LM), upper middle (UM), lower apical (LA) and upper apical (UA) turns in the injected (left) and non-injected (right) cochleae. Statistical analysis of SGN density data was performed using a two-way repeated measures ANOVA using treated (left) versus untreated (right) cochlea and cochlear turn as factors. Post hoc comparisons were carried out using the Holm-Sidak method. The difference in SGN density between the injected and non-injected cochleae of each GP was calculated (termed SGN survival when positive or SGN loss when negative) and used for the graphical representation of the data thus allowing normalised comparisons between groups.

To determine whether NT gene therapy had an effect on the size of the surviving SGNs (which may therefore lead to biasing in the SGN density measurements), the soma area of SGNs was measured in the Ad-GFP-NT treated (left) and control (untreated right) cochleae from the one week group. Soma area was measured in the lower and upper basal region by randomly

selecting 10 NF200 labeled SGNs in three non-consecutive mid-modiolar sections. A grid and a random number generator were used for the selection process. The outer perimeter of the cell body was traced in Image J (NIH, USA) and the area calculated. Average area was (± SEM) determined for a total of 30 SGNs for the treated (left) and untreated (right) cochlea and compared using a repeated measures (RM) ANOVA. There was no difference in the soma area between the left (average 228.5 μm^2 ±1.2 μm^2) and the right cochlea (average 226.2 μm^2 ±3.3 μm^2) measured in the lower basal turn (one way RM ANOVA, P=0.65).

In order to characterize the extent of damage to the organ of Corti following aminoglycoside deafening, the cross-sectional area of any residual organ of Corti structures was measured in the lower basal turn of right control cochleae of the one, four and eight week deaf cochleae, in addition to a cohort of normal hearing animals. Area measurements were taken from three non-consecutive mid-modiolar sections and presented as averages ±SEM and statistical analysis was carried out using a one way ANOVA.

2.7.3 SGN peripheral fibers

Surface preparations were used to examine the projection of SGN peripheral fibers from confocal images that contained GFP+ cells using a protocol described previously (Wise et al., 2010). Only a subset of GFP-expressing cells were suitable for detailed analysis and the selection of these cells was restricted to GFP-expressing cells within the OC region as colocalization of GFP-expressing cells and resprouting fibers was not observed elsewhere. Furthermore, the GFP+ cells were required to be distal to the inner spiral

bundle in order to avoid counting peripheral fibers that would normally be located within this structure. As described previously (Wise et al., 2010) pixel density occupied by NF-200 labeling was measured within a boundary 10 µm from the edge of GFP+ cells. Four consecutive Z-planes with the highest pixel density were averaged. Measurements from Ad-GFP-injected GPs and Ad-GFP-NT-injected GPs were compared using a t-test.

3 Results

3.1 Viral vector expression profile

The GFP reporter gene in the Ad-GFP-NT and Ad-GFP was used to examine the expression patterns in mid-modiolar sections of GPs that were deafened one, four and eight weeks before injection with the viral vector. As reported previously, there was no observable difference in the pattern of expression between Ad-GFP and Ad-GFP-NT groups (Wise et al., 2010) and therefore the GFP expression data was combined across groups to form a composite image (Figure 1). The general expression profile was similar between experimental groups and comparable to that previously reported for the one week deafened group (Wise et al., 2010). The GFP expression was predominantly observed in the lower and upper basal turn. However, in one case from the four and eight week group expression was also observed in the middle and apical regions.

[*Figure 1 near here*]

3.2 Viral vector transfection in the normal cochlea

GFP expression was examined in the OC of cochleae from normal hearing GPs. The aim here was to evaluate viral transfection in cochleae that were undamaged by aminoglycoside deafening in order to identify specific cell types that were transfected. Expression of GFP was observed in all cell types of the OC (Figure 2). These cell types included both inner and outer hair cells, inner and outer pillar cells, and supporting cells including Hensen's cells and Deiters' cells. Furthermore, GFP labelling was consistently observed in the interdental cells of the spiral limbus and occasionally in the stria vascularis and Reissner's membrane. Expression was prominent in the OC and was detected throughout the lower basal (Figure 2 C) and upper basal turn (not shown).

[*Figure 2 near here*]

3.3 Viral vector transfection in the deafened cochlea

The progressive degeneration of the OC observed in the longer durations of deafness was associated with a decrease in the level of viral transfection of the residual OC cells. Antibodies to calretinin (stains inner and outer hair cells, and Deiters' cells) and the f-actin stain phalloidin (stains pillar cells, hair cell stereocilia and the cuticular plate) were used to assist in the identification of transfected cells in the degenerated OC of deafened cochleae. In mid-modiolar sections from the deafened cochleae, although no hair cells were evident, residual supporting cells in the OC were often observed and some were transfected by the viral vector. These cells were predominantly Deiters' cells and pillar cells (Figure 3 A, B) and interdental cells on the spiral limbus

(Figure 3 C). Additionally, endosteal cells lining the perilymphatic spaces and cells in Reissner's membrane were also transfected (data not shown).

Cochlear surface preparations (Figure 3 D-F) were used to examine the viral transfection profile and also to examine the responses of resprouting peripheral fibers. As previously reported for the one week group, the number of NF-200 positive pixels surrounding transduced cells from Ad-GFP-NT-injected GPs (171.3 ± 27.1, n = 13 cells) was significantly greater compared to transduced cells from Ad-GFP-injected GPs (71.8 ± 24.9, n = 7 cells) (P < 0.05, t-test) (Wise et al., 2010). However, in the four and eight week groups, there was less cellular expression of the GFP in comparison to the one week group and consequently too few GFP+ cells that met the selection criteria for peripheral fiber analysis (see Methods). The combination of smaller numbers of transfected cells within the residual OC and fewer resprouting fibers therefore meant that the sample size was too limited for statistical comparison with sufficient power between experimental groups. Qualitatively, there was no trend for a greater density of resprouting fibers around Ad-GFP-NT transfected cells compared to Ad-GFP transfected cells.

[*Figure 3 near here*]

3.4 Organ of Corti degeneration

In order to characterize the extent of aminoglycoside-induced damage to the sensory epithelium the cross-sectional area of the residual structures of the OC was measured in the right (untreated) cochleae from the one, four and eight week groups and also from cochleae with normal hearing. Example

micrographs represent the typical degeneration profile observed in each group (Figure 4). In all of the deafened cochleae there was loss of the inner and outer hair cells and a more gradual loss of supporting cells that was progressive over time. A flattened epithelium was commonly observed in the eight week group. Statistical analysis of the cross-sectional area of the OC between experimental cohorts indicated a significant difference in area between the normal hearing cochleae and cochleae in all of the three deafened groups (ANOVA, $P<0.001$). There was a statistical difference between the one and eight week group and between the four and eight week group (ANOVA, $P<0.05$).

[*Figure 4 near here*]

3.5 Spiral ganglion neuron density

The density of SGNs was measured and statistical comparisons made between the Ad-injected cochlea and the non-injected control cochlea for each cochlear region. Example micrographs of the SGNs within Rosenthal's canal and the SGN density data for the lower basal turn represented as an SGN density difference (Ad-injected minus non-injected cochlea) are presented for the one, four and eight week pre-injection deafness duration (Figure 5). The density of SGNs was also measured for normal hearing cochleae and was 1578.9 SGN/mm^2 (± 69.5 SEM).

3.5.1 One week deafened group

Following a pre-injection deafness period of one week and injection of Ad-GFP-NT into the SM there was a significantly greater density of SGNs in the basal region of the injected cochlea compared to the non-injected cochlea

(repeated measures 2 way ANOVA, $P<0.05$). Post hoc analysis showed significant differences in both the lower basal (see Figure 5) and upper basal (data not shown) cochlear turns (Holm-Sidak, $P<0.05$). Conversely, there was no effect on SGN survival in the lower basal turn following injection of Ad-GFP into the one week deafened cochlea (Figure 5). However, post hoc analysis indicated a significant decrease in SGN density in the Ad-GFP-injected cochlea compared to the non-injected cochlea in the upper basal turn only (Holm-Sidak, $P<0.05$, data not shown).

3.5.2 Four week deafened group

Injection of Ad-GFP-NT into the SM in the four week deafened group resulted in a significantly greater density of SGNs in the lower basal turn, but not the upper basal turn, when compared to the non-injected cochlea (repeated measures ANOVA, Holm-Sidak, $P<0.05$) (Figure 5). There was no significant difference in the density of SGNs in the lower basal region of the Ad-GFP-injected cochlea compared to the non-injected control cochlea.

3.5.3 Eight week deafened group

Following injection of either Ad-NT or Ad-GFP into the SM after eight weeks of deafness there was no statistical difference in SGN density for the lower (or upper) basal turns for injected cochleae compared to non-injected cochleae (Figure 5).

[*Figure 5 near here*]

3.5.4 Middle and apical cochlear regions

Statistical comparisons of SGN densities in the four week and eight week deafened GPs indicated that there were no differences in the Ad-injected cochleae compared to the non-injected cochleae for the middle or apical turns (data not shown). In the one week deaf group, differences in SGN densities between injected and non-injected cochleae for the middle and apical turn have been reported previously (Wise et al., 2010).

4 Discussion

The findings from this study indicated that adenovirus transfection of cells in the SM was observed following one, four and eight weeks of deafness in GPs. However, the level of gene expression and the SGN survival benefits of NT gene therapy diminished as a function of pre-injection deafness duration. The progressive degeneration of the OC observed proceeded from base to apex, and was particularly evident in the longer eight week duration of deafness (Figure 4), which was associated with a decrease in the level of viral transfection of the residual OC cells. With an increased duration of deafness before the injection of the viral vector, the SGN survival promoting effects of Ad-GFP-NT treatment decreased. Treatment after eight weeks of deafness, when there was a significant reduction in the size of the sensory epithelium compared to the other groups (Figure 4), was no longer effective in promoting SGN survival.

Following injections of Ad-GFP-NT into the one week deafened cochleae there was a significant increase in SGN density in both the lower (Figure 5)

and upper (data not shown) basal turns compared to the contralateral control cochleae. A significant increase in SGN survival was also observed following injections of Ad-GFP-NT into cochleae that were deafened four weeks prior to the injection. However, the overall level of survival was lower and restricted to the lower basal turn only (the region in the closest proximity to the viral injection site) when compared to the data from the one week group. This finding is likely to be a consequence of the reduced level of gene expression in this group. When Ad-GFP-NT was injected into eight week deafened cochleae no significant SGN survival was observed.

The GFP expression was primarily localised in the basal region (Figure 1) with data from only two animals exhibiting expression in middle and apical regions (in addition to basal region expression). The expression profile, coupled with the localised SGN survival effects in the one week and four week deafened groups indicated that enhanced SGN survival coincided with the location of NT expression. Although there was degeneration and collapse of the OC, gene expression was evident in remaining OC cells, particularly in the one week deafened group and, with reduced transfection in the four week deafened group. Transfected cells were residual supporting cells in the degenerating OC that were likely to be pillar cells and Deiters' cells (Figure 3 A-C). However, in the totally degenerated (flattened) OC, commonly observed in the eight week group, transfection was rarely observed on or near the basilar membrane but was apparent in other cells such as interdental cells (Figure 3 C and F) and cells in the Reissner's membrane (data not shown). Therefore, the expression profile was variable in terms of the cell types labeled in the

deafened cochlea (Figure 3) and even in the normal cochlea (Figure 2).

A consequence of the reduced Ad-GFP-NT transfection would be a reduction in the levels of NT produced, a factor likely contributing to the loss of SGN survival effects seen in the longer-term deafened animals. An additional factor contributing to the reduced SGN survival observed with longer pre-injection durations may relate to the cell types that were transfected with the genes for NTs. Due to the loss of the OC, particularly in the eight week deaf cochleae that commonly exhibited a flattened sensory epithelium without Ad transfection, cells that were transfected were predominantly interdental cells, cells within the stria vascularis and cells in the endosteal lining of the SM. These cells are located further away from the SGNs compared to the cells in the residual structures of the OC. Consequently, SGNs would have reduced access to the NT supply and would be less likely to have direct interactions with transfected cells via resprouting SGN peripheral fibers. However, a previous study has reported Ad transfection in the severely degenerated (flattened) OC (Shibata et al., 2010). In this study viral inoculations were given one week following deafness, with survival times of 14 or 30 days. An important distinction is that deafness was induced with direct ST injections of neomycin, a technique that leads to much faster degeneration than the systemic kanamycin and frusemide administration used here. Furthermore, Shibata et al., (2010) used a larger volume and higher viral titers (5 µl of $4x10^{12}$ viral particles) for their viral inoculations. It is likely that the pre-inoculation deafness duration, the degeneration timecourse and viral volume and titer are factors in the efficiency of viral transfection in the damaged

sensory epithelium.

The extent of gene expression was severely reduced in the deafened cochleae (Figure 3) compared to that detected following injections into the SM of normal hearing GPs (Figure 2). Nevertheless, the findings reported in this study indicated that gene therapy remains a possibility after longer periods of deafness. This indicates that the adenovirus transfection mechanisms (binding of adenovirus to the coxsackie adenovirus receptor (CAR) (Coyne et al., 2005) and internalization via integrins $\alpha v\beta 3$ / $\alpha v\beta 5$ (Waehler et al., 2007) are functional in OC cells at different stages of degeneration.

The responses of resprouting peripheral fibers to Ad-GFP and Ad-GFP-NT viral transfection were examined in this study and have previously been reported for the one week group (Wise et al., 2010). In this group there was significantly greater density of neurofilament-labeled peripheral fibers in close proximity to the Ad-GFP-NT transfected cells compared to the Ad-GFP transfected cells. The implication of this finding is that the NT gene therapy provided localised trophic support for the resprouting fibers. The current study aimed to extend these findings in order to examine the effects of NT gene therapy on peripheral fiber resprouting in more severely degenerated cochleae. Only transfected cells on or near the basilar membrane were selected for the peripheral fiber analysis as this is typically where resprouting fibers were observed in the deafened cochlea. However, due to the low viral expression in the residual cells of the OC in the four and eight week deafened group a robust statistical comparison of the Ad-GFP and Ad-GFP-NT was not possible. Qualitatively, although resprouting peripheral fibers were observed on the

basilar membrane and also projecting over the inner sulcus, there was no tendency for these fibers to appear in higher density around the transfected cells expressing the NT genes. It is possible that the resprouting peripheral fibers in cochleae deafened for longer periods of time are less responsive to the localised NT supply or that the lower levels of NT production as a consequence of reduced NT expression meant that there was a decrease in the extent of NT-mediated peripheral fiber regrowth. The longer-term fate of resprouting fibers in the deafened cochlea is not known, but it is likely that the ongoing loss of the SGN over time would result in diminished resprouting activity.

5 Clinical considerations

This research has shown that NT gene therapy, designed to promote SGN survival and provide cellular targets accessible to resprouting peripheral fibers, is most effective when applied soon after the onset of hearing loss. The continued degeneration of the OC and loss of residual cells seen here in the GP cochlea decreased the efficacy of gene therapy when applied directly to the SM. However, in humans the loss of the OC and the SGNs may take many years (Nadol et al., 1989), depending on the etiology of hearing loss. Therefore, the clinically relevant window for NT gene therapy is likely to be longer than that observed in this study. In addition, if stable transfection can be achieved, then NT gene therapy, when initiated early after deafness, may slow or prevent the subsequent loss of structure of the OC. This may be an important strategy to consider in slowly progressive human pathologies which, unlike the present experimental model, involve hearing loss that worsens over

months to years. Nevertheless, since NT gene therapy was still possible even in long-term deafened cochleae future advances in the efficacy of NT gene therapy may lead to increased benefits in terms of SGN protection in the long-term deafened cochlea.

NT gene therapy may also provide therapeutic benefits when combined with cochlear implant use. A previous study has shown that chronic electrical stimulation from a cochlear implant enhanced SGN survival following the exogenous supply of NTs with mini-osmotic pumps (Shepherd et al., 2005). Furthermore, significant SGN survival effects were maintained with continued chronic electrical stimulation following the removal of the NT supply (Shepherd et al., 2008). Therefore, combining electrical stimulation from a cochlear implant along with NT gene therapy may enhance SGN survival and fiber resprouting and prolong the neuroprotective effects of NT gene therapy over time.

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Figures

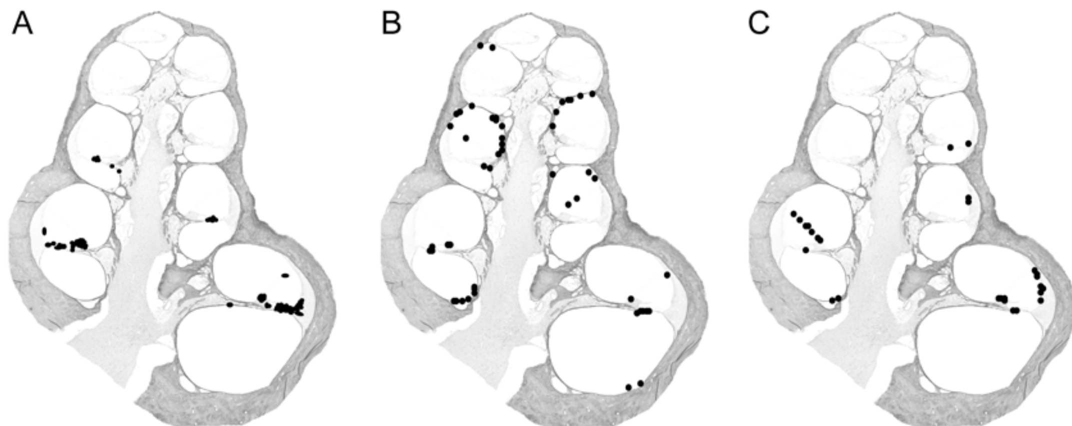

Figure 1. Composite images of GFP expression in GP cochleae. (A) Three weeks following injection of Ad-GFP or Ad-GFP-NT into the SM of GPs which had been deaf for one week, GFP expression was predominantly detected in the lower and upper basal turn. Limited expression was also observed in the lower and upper middle turns. **(B)** Three weeks after injection of Ad-GFP or Ad-GFP-NT in GPs which had been deaf for four weeks, GFP expression was predominantly detected in the lower and upper basal turn but was also observed in the middle and apical regions of one GP. GFP was located in the spiral limbus and OC in basal region and also in the endosteal cells lining the perilymphatic spaces in the middle and apical regions. **(C)** Following injections into cochleae that had been deaf for eight weeks, expression was detected in the OC in the basal region, spiral limbus, endosteal cells and along Reissner's membrane. Limited expression in middle and apical regions was observed in one case.

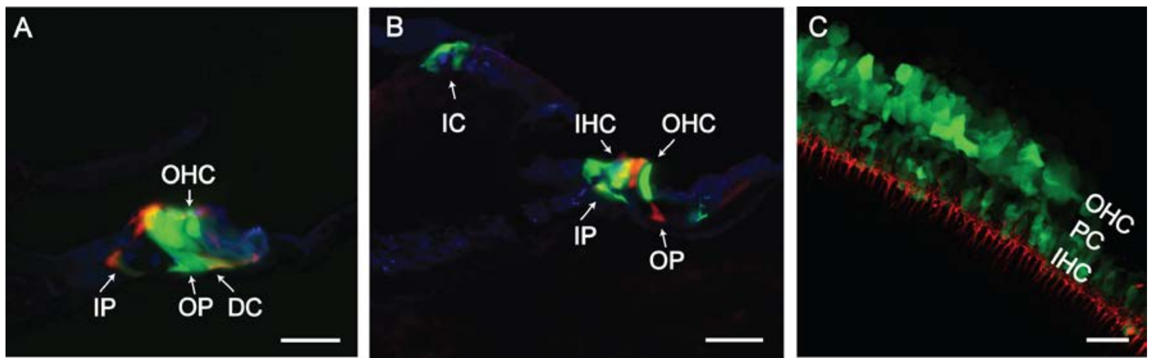

Figure 2. **GFP expression in the normal OC. A**) GFP (green) was observed in the outer hair cells (OHC), Deiters' cells (DC) and the outer pillar cell (OP) in this example. The inner pillar cell (IP – stained red with phalloidin) was not transfected. **B**) In this example GFP expression was observed in the inner hair cell (IHC), the outer hair cells, the inner pillar cells and the interdental cells (IC) on the spiral limbus. The outer pillar cell (red) was not transfected. **C**) A surface preparation of the lower basal turn of a normal OC exhibiting widespread GFP expression within the OC. The peripheral fibres of the SGNs are labelled with anti-neurofilament (red). Scale bar 50 μm.

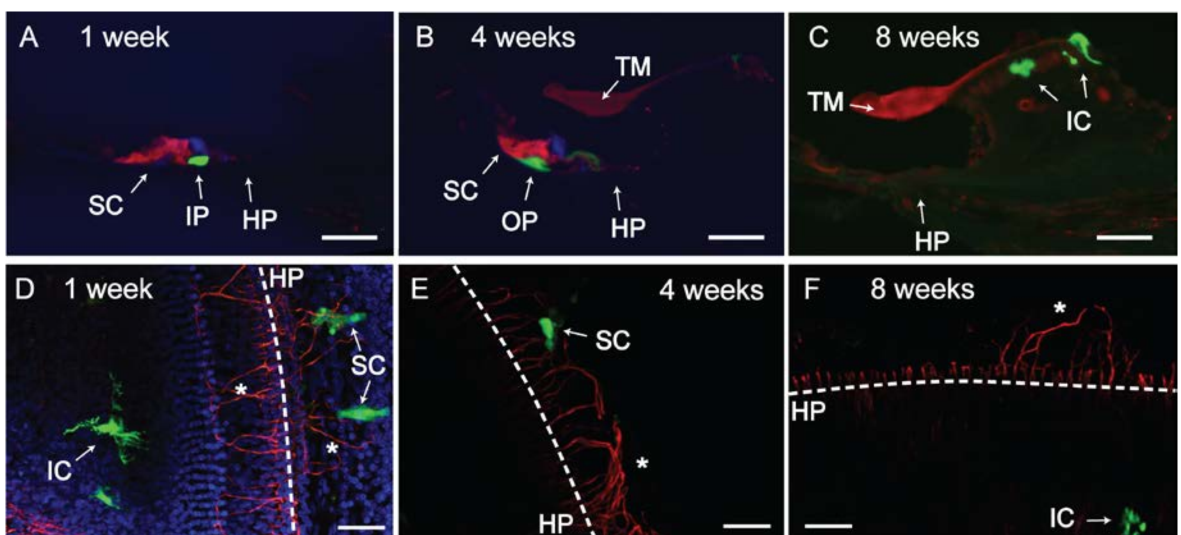

Figure 3. GFP expression and peripheral fiber responses in deaf GP

cochlea. (A-C) Cochlear mid-modiolar sections from one, four and eight week deafened groups were stained with anti-calretinin (red) and phalloidin (blue) to identify transfected cells (green) in the OC. In each case, the location of the habenula perforata (HP) is shown. **(A)** An example of the degenerating OC from the lower basal turn of one week deafened GP showing GFP expression in an inner pillar cell (IP) around a collapsed tunnel of Corti. Degenerating supporting cells (SC, most likely Deiters' cells) are stained red. **(B)** A section from lower basal turn of a four week deafened GP showing GFP expression within an outer pillar cell (OP) in the degenerated OC. In this example degenerated SCs (red) were not transfected and the tectorial membrane (TM) can be observed. **(C)** A section from lower basal turn of an eight week deafened GP that exhibited total loss of the OC and no GFP expression in this region. However, interdental cells (IC) of the spiral limbus were transfected with GFP. **(D)** Surface preparation of the lower basal turn from a one week deafened GP showing Ad-GFP-NT transfection in SCs in the degenerating OC and in ICs of the spiral limbus. Neurofilament-labelled resprouting peripheral fibers (* red) can be seen to project towards the Ad-GFP-NT expressing cells (green) and also on the inner sulcus towards the spiral limbus. DAPI staining shows cell nuclei (blue) **(E)** A surface preparation of the lower basal turn from a four week deafened GP showing Ad-GFP-NT transfection in SCs in the degenerating OC. Although neurofilament-labelled resprouting peripheral fibers were evident (*) there was no tendency for them to project towards the Ad-GFP-NT transfected cells. **(F)** In this example of a surface preparation from the lower basal turn from the eight week deafened GP there were no GFP transfected cells in the region of the basilar membrane. GFP expression was

observed in the ICs on the spiral limbus. Neurofilament-labelled resprouting peripheral fibers (*) were also observed. Scale bar 50 µm.

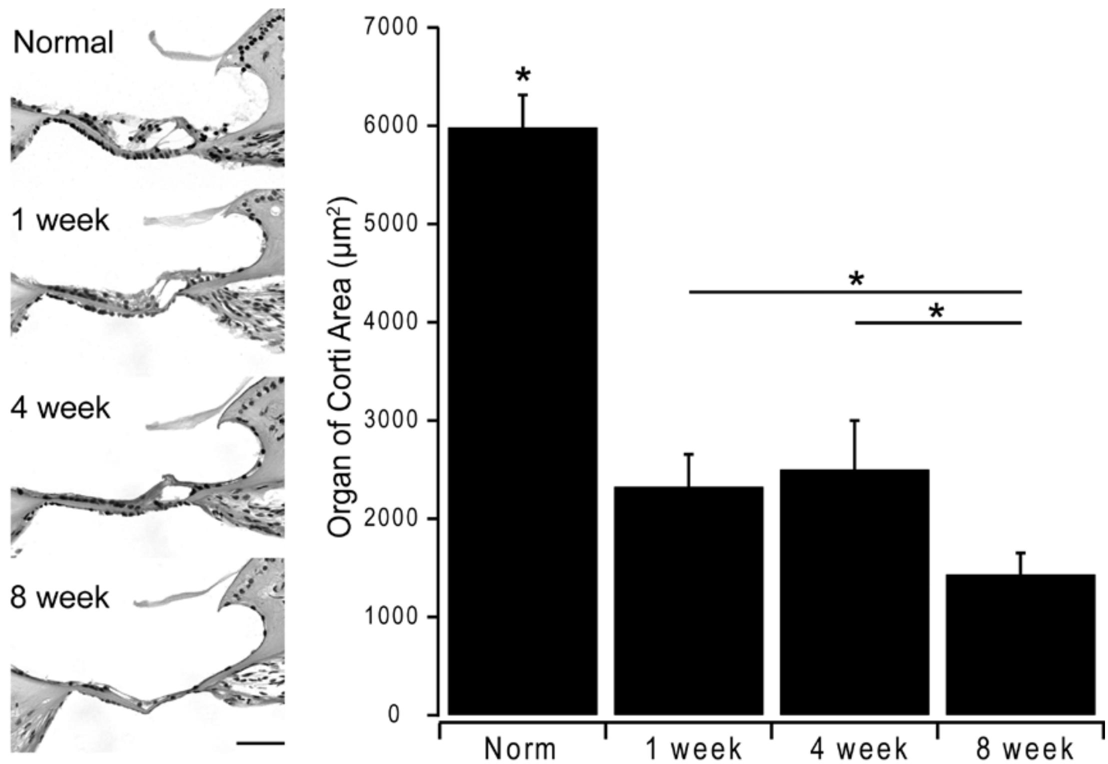

Figure 4. Degeneration of the OC following aminoglycoside deafening. Example micrographs of the OC in the normal cochlea and residual Corti structures in the one, four and eight week group from the right control cochlea. There was a progressive collapse of the OC with longer durations of deafness (scale bar 50µm). The area of the OC was measured and the average area (±SEM) is plotted for each experimental group. OC area was significantly greater in the normal hearing cochleae compared to the other groups. In the deafened cochleae there was no difference in area between the one and four week group, however in the eight week group the area was significantly smaller compared to the shorter deafness durations.

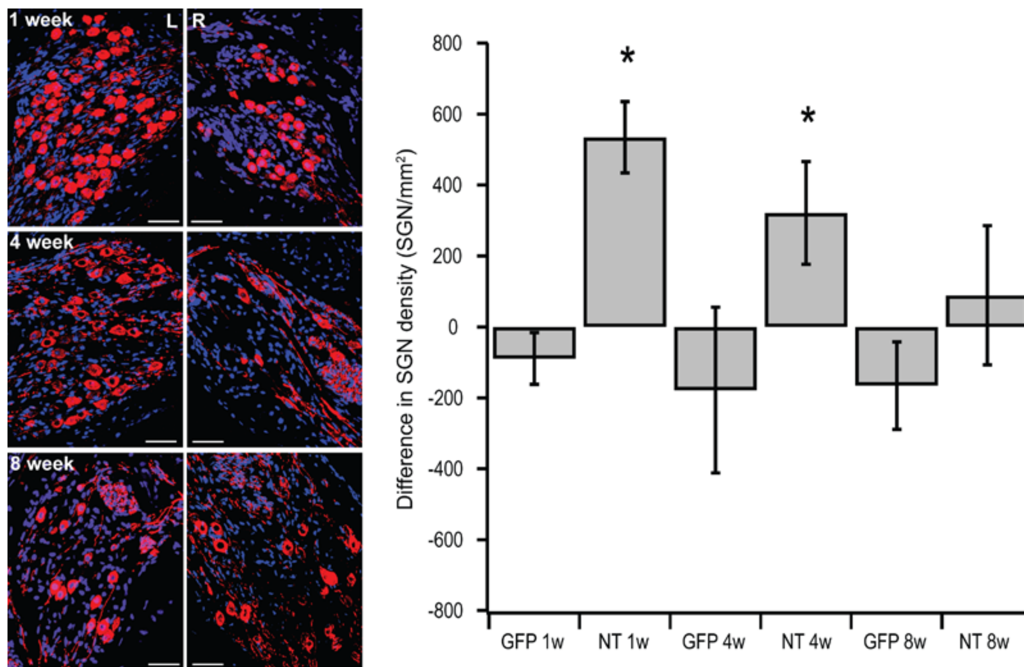

Figure 5. SGN survival or loss in the lower basal turn following viral injections. GPs were deafened for one week, four weeks or eight weeks prior to the injection with Ad-GFP (GFP) or Ad-GFP-NT (NT). Three weeks following the viral injections the density of SGNs was determined. Example confocal images of SGNs in the lower basal turn are presented for the one week, four week and eight week groups that received Ad-GFP-NT in the treated (left) cochlea. The right cochlea served as the untreated control. Cochleae were stained for NF200 (red) and DAPI (blue); scale bar 50µm. Data is presented as the average (±SEM) of the difference in the SGN density between the injected and the non-injected cochlea. A positive average difference indicates greater SGN survival in the injected cochlea. Injection of Ad-NT following one week and four weeks of deafness, but not eight weeks of deafness, resulted in significant SGN survival in the lower basal turn compared to the contralateral non-injected cochlea.