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ARTICLE

Viral-mediated Ntf3 overexpression disrupts innervation and hearing in nondeafened guinea pig cochleae

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Synaptopathy in the cochlea occurs when the connection between inner hair cells and the auditory nerve is disrupted, leading to impaired hearing and nerve degeneration. Experiments using transgenic mice have shown that overexpression of NT3 by supporting cells repairs synaptopathy caused by overstimulation. To accomplish such therapy in the clinical setting, it would be necessary to activate the neurotrophin receptor on auditory neurons by other means. Here we test the outcome of NT3 overexpression using viral-mediated gene transfer into the perilymph versus the endolymph of the normal guinea pig cochlea. We inoculated two different *Ntf3* viral vectors, adenovirus (Adv) or adeno-associated virus (AAV) into the perilymph, to facilitate transgene expression in the mesothelial cells and cochlear duct epithelium, respectively. We assessed outcomes by comparing Auditory brainstem response (ABR) thresholds prior to that at baseline to thresholds at 1 and 3 weeks after inoculation, and then performed histologic evaluation of hair cells, nerve endings, and synaptic ribbons. We observed hearing threshold shifts as well as disorganization of peripheral nerve endings and disruption of synaptic connections between inner hair cells and peripheral nerve endings with both vectors. The data suggest that elevation of NT3 levels in the cochlear fluids can disrupt innervation and degrade hearing.

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INTRODUCTION

Molecular therapy has the potential to treat hearing loss and other diseases of the inner ear, but the risk of side effects that may compromise residual hearing or exacerbate the disease should be considered and studied in detail before adopting this therapy for clinical use.[1,2] One inner ear disease recently identified and defined is synaptopathy, where the connection between the sound transducing cell of the cochlea (inner hair cell) and the auditory nerve ending is disrupted.[3] Synaptopathy can be caused by overstimulation, and possibly by other factors such as aging and ototoxic aminoglycoside drugs, and results in short term disruption of suprathreshold parameters of hearing and long term degeneration of the auditory nerve.[4–6] Experiments using transgenic mice have shown that local overexpression of the neurotrophin NT3 by supporting cells that surround the inner hair cells can partially restore synaptic connectivity in mice exposed to loud noise.[7] To accomplish such therapy in the clinical setting, it will be necessary to activate the neurotrophin receptor on auditory neurons by other means. This can be done by infusing neurotrophins into the perilymph,[8] injecting therapeutic agonist antibodies that target the neurotrophic receptors[9] or inducing overexpression of a neurotrophin transgene using gene transfer methods such as injection of viral vectors into perilymph or endolymph.[9–12]

When neurotrophins are infused into the deaf cochlea with osmotic pumps, spiral ganglion neuron survival in deaf ears is enhanced[13,14]

and peripheral sprouting of neurons is observed. The sprouting of neurites is unregulated in direction and length, leading to a wide spread of fibers growing in different directions in the cochlea. When gene transfer techniques are used for overexpression of neurotrophins, the sprouting is better controlled because the target of the elongating neurites appears to be the cells that were transduced by the viral vector which then secrete neurotrophin.[9,11] When applied to ears with no hair cells, the goal of such gene transfer methods is to enhance the outcome of the cochlear implant by optimizing survival and health of the neurons and by reducing the distance between the electrode and the nerve ending; in this context, replicating the orderly arrangement in healthy ears may be less important. However, when considering treatment of synaptopathy in otherwise healthy ears, it is important to consider whether the occurrence of poorly regulated growth is likely and what effect it might have on cochlear function. The impact of viral-mediated overexpression of NT3 in ears with a full complement of hair cells and auditory neurons has not been reported in detail. In this study, we assessed the effect of using virus vectors to overexpress NT3 on the fates of hair cells, nerve endings, synapses and hearing in the normal guinea pig cochleae. NT3 was selected because of its positive effects on synaptopathy.[7] We determined that NT3 overexpression induced by viral vectors caused disruption of peripheral nerve endings and synapses, resulting in elevation of hearing thresholds without killing the hair cells.

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RESULTS

Adv.Ntf3 and AAV.Ntf3 activity in culture media

To determine activity of the adenovirus (Adv).Ntf3 and adeno-associated virus (AAV).Ntf3, we performed a set of short term in vitro experiments in which guinea pig fibroblasts were incubated with these vectors for 4 hours and then rinsed with fresh media. After 72 hours, we collected the culture media and analyzed it by enzyme-linked immunosorbent assay (ELISA) for NT3 concentration. The negative controls were media collected from cell cultures that were not treated with the viral vector. The average concentration of NT3 in media from cultures treated with Adv.Ntf3 was 1.33 ng/ml, which was ~1.3 times the ELISA standard and ~80 times the negative control. The average concentration of NT3 in media from cultures treated with AAV.Ntf3 was 0.272 ng/ml, ~0.27 times the ELISA standard and ~17 times the negative control. These data indicated that both viral vectors are active and produce NT3, with Adv.Ntf3 being more efficient than AAV.Ntf3 in guinea pig fibroblasts at this time point.

ABR thresholds after delivery of viral vectors into the cochlea

Multi-group multivariate analysis of variance (MANOVA) simultaneously analyzing baseline thresholds for all three frequencies confirmed no significant differences among groups assigned to the four treatment groups. After establishing baseline thresholds, solutions were injected into scala tympani via cochleostomy, for all groups. Thresholds for each group were compared with that group's baseline. Hearing did not change significantly after injection of normal saline (NS) ($F = 1.096$, $df = 6$, $P = 0.439$) (Figure 1a) or Adv.Empty ($F = 0.467$, $df = 6$, $P = 0.801$) (Figure 1b). In contrast, there was significant hearing loss after injection of Adv.Ntf3 ($F = 5.550$, $df = 6$, $P < 0.001$).

One week after being given this vector, thresholds were elevated at each frequency (P-values for post-hoc univariate tests: 8 kHz < 0.001, 16 kHz < 0.001, and 32 kHz = 0.037). At 3 weeks, thresholds remained elevated at 8 kHz ($P = 0.002$) and 16 kHz ($P < 0.001$), but not at 32 kHz ($P = 0.212$) (Figure 1c). Hearing thresholds also changed in animals given AAV.Ntf3 ($F = 4.082$, $df = 6$, $P = 0.002$). As expected for the slower "activation" of AAV vectors, thresholds did not change at 1 week after inoculation (P-values for post-hoc univariate tests: 8 kHz = 1.000, 16 kHz = 0.599, and 32 kHz = 0.512) but were significantly elevated at 3 weeks (P- values < 0.001 for each tested frequency) (Figure 1d). These data suggest that surgical trauma and presence of Adv vector do not elevate ABR thresholds, rather, gene overexpression due to the neurotrophin insert led to deterioration of hearing in these animals.

Hair cell survival with Adv.Ntf3 and AAV.Ntf3

Analysis of the proportions of missing outer and inner hair cells found only minor (< 10%) hair cell loss, spread throughout the cochlea for animals treated with either Adv or AAV (Figure 2). The morphology of remaining hair cells did not reveal major qualitative changes throughout the cochlea and did not differ between Adv or AAV treatment groups (Figures 3 and 4). Thus, the significant functional deficit caused by these neurotrophin-expressing viral vectors was likely not due to changes in the number or morphology of the sensory hair cells, but was due to other causes.

Nerve endings of Adv.Ntf3 and AAV.Ntf3 treated animals

We assessed peripheral nerve fibers in the organ of Corti visualized by staining for neurofilament. In the basal turn of Adv.Ntf3 treated cochlea at 3 weeks after inoculation, we observed enlarged
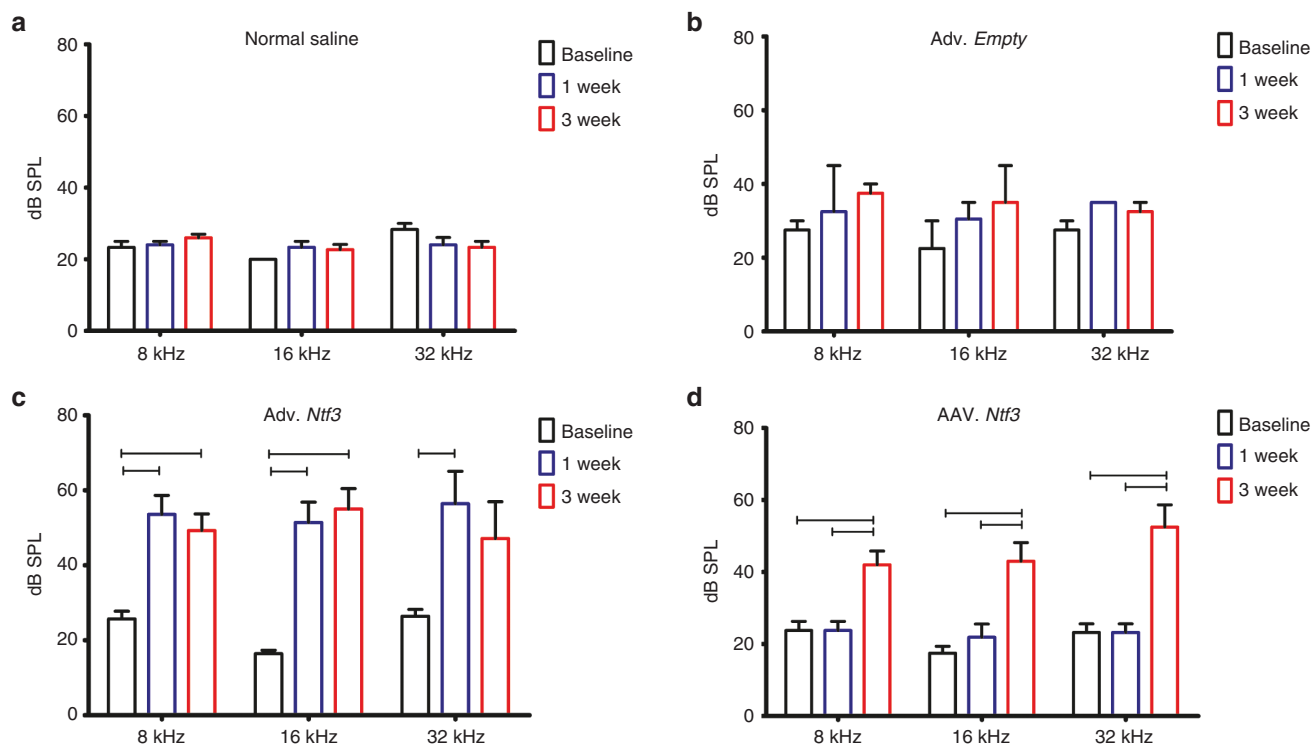

Figure 1 ABR thresholds after injection. Animals treated with normal saline (n = 3) showed similar ABR thresholds to baseline at 1 week and 3 weeks, at all frequencies (**a**). Animals treated with Adv.Empty (n = 2) also did not show ABR threshold changes at 1 and 3 weeks after surgery (**b**). Animals treated with Adv.Ntf3 (n = 7) showed significantly elevated ABR thresholds in all frequencies at 1 and 3 weeks (**c**). Animals treated with AAV.Ntf3 (n = 10) showed significantly elevated ABR thresholds only at 3 weeks (**d**). AAV, adeno-associated virus; Adv, adenovirus; ABR,

neurofilament-positive structures that appeared to be bulging nerve endings medial to the inner hair cells (Figure 3a). These features were not observed in the contralateral (noninjected) control ears (Figure 3b). Similar findings were observed in second turn and third turn (Figure 3c,d). In the fourth turn, peripheral nerve structures appeared normal and bulging nerve endings were not found (Figure 3e).

The peripheral nerve fibers of the AAV.*Ntf3* treated cochlea were relatively normal (Figure 4), lacking the bulging structures seen in Adv.*Ntf3* inoculated ears. However, the number of peripheral nerve fibers in the area of the outer hair cells appeared qualitatively reduced in the basal turn (Figure 4a) compared with control (Figure 4b). Moreover, some nerve fibers were sprouting in different directions within the epithelium. These features were limited to the basal turn, with the second to fourth turns of the cochlea having normal morphology (Figure 4c–e).

These data show that 3 weeks after Adv.*Ntf3* treatment there were extensive changes in the organization of neurons in the organ of Corti in the first to third turns, whereas the AAV.*Ntf3* treated ears revealed less severe changes that were mostly restricted to nerve fibers in the area of the outer hair cells in the first turn.

Synaptic connections of Adv.*Ntf3* and AAV.*Ntf3* treated animals

To further assess the outcome of the changes induced by the neurotrophin vectors, we evaluated the synaptic connections between hair cells and peripheral nerve fibers in the basal turn using the presynaptic marker (RIBEYE; CtBP2).[15] In the contralateral (non-injected)

a Adv.*Ntf3*

b AAV.*Ntf3*

Figure 2 Cytocochleograms of hair cells in Adv.*Ntf3* and AAV.*Ntf3* treated animals. Cytocochleograms of the animals at 3 weeks after injection of viral vectors. There was no major loss of hair cells throughout the cochlea in either Adv.*Ntf3* (n = 5) (**a**) or AAV.*Ntf3* (n = 5) (**b**). AAV, adeno-associated virus; Adv, adenovirus.

control ears, there were abundant puncta that were stained for CtBP2 (Figure 5a). Similar results were observed in the NS treated animals (Figure 5b). In the animals treated with Adv.*Ntf3* (Figure 5c), the density of CtBP2-positive puncta appeared to be reduced compared with the contralateral and NS treated ears; but AAV.*Ntf3* treated ears did not appear to have reduced density of puncta (Figure 5d). Quantitative analysis confirmed that the number of CtBP2 puncta per inner hair cell was statistically different between groups (NS, Adv.*Ntf3* and AAV.*Ntf3*) ($F = 4.736$, $df = 6$, $P = 0.011$). Only the Adv.*Ntf3* group had fewer puncta than the NS group (Figure 5e); post-hoc univariate analysis found significant reduction in 16 and 32 kHz areas (*P*- values versus NS: 0.001 and 0.013; and *P*- values versus AAV.*Ntf3*: 0.001 and 0.006 respectively). Significant differences were not found in the 8 kHz area (*P*- values versus NS: 0.292, and AAV.*Ntf3*: 0.318), probably because this region was farther from the site of viral vector injection.

DISCUSSION

Summary of the results

We evaluated the structural and functional outcomes of viral-mediated overexpression of NT3 in the normal guinea pig cochlea. We used two vectors, Adv and AAV, both injected into the perilymph, a delivery target which is feasible for clinical use in human ears. The vectors were selected to target different cell populations for transgene expression when injected into perilymph, with Adv infecting mesothelial cells lining the perilymph space[9,16,17] and AAV infecting epithelial cells in the organ of Corti.[18–20] We observed hearing threshold shifts as well as morphological changes and disorganization of peripheral nerve endings and disruption of synaptic connections between inner hair cells and peripheral nerve endings with Adv, and lesser negative effect with AAV. This suggests that *Ntf3* gene delivery in normal cochlea has a potential to disrupt innervation patterns and cause hearing loss, especially when *Ntf3* is delivered by Adv.

Outcomes with Adv.*Ntf3* and with AAV.*Ntf3*

Delivery of NT3 by both of the viral vectors caused elevation of ABR thresholds; however, Adv.*Ntf3* induced ABR threshold shifts as early as 1 week after the inoculation. Although we did not measure NT3 levels at earlier time points, gene expression with Adv vectors is known to start and peak within 3–5 days[21–23] and the rapid onset of cochlear degradation is in line with the early timing of Adv gene expression.

AAV vector gene expression is generally thought to peak 14 days after infecting cells.[24–26] This relatively slow onset and late peak of viral-mediated gene expression may account for the lower levels of NT3 we observed, compared with Adv at the time point of measurement. In the *in vitro* studies, culture media was collected 72 hours after inoculation of the viral vector, which is well before the peak of AAV.*Ntf3* gene expression. Thus, we cannot tell if the relatively minor impact of AAV.*Ntf3* on cochlear function and structure are due to the lower concentration of NT3 or to the gene expression pattern (location), being secreted by auditory epithelium cells rather than the more remote mesothelial cells. The practical point however is that even a vector that yields gene expression in the auditory epithelium such as AAV.*Ntf3*, leads to changes in organization of the cochlear neural network. Thus, to be able to produce positive results for healing of synaptopathy, vectors will have to specifically and exclusively infect cells in the area of the supporting cells near the inner hair cell, or the inner hair cell itself.
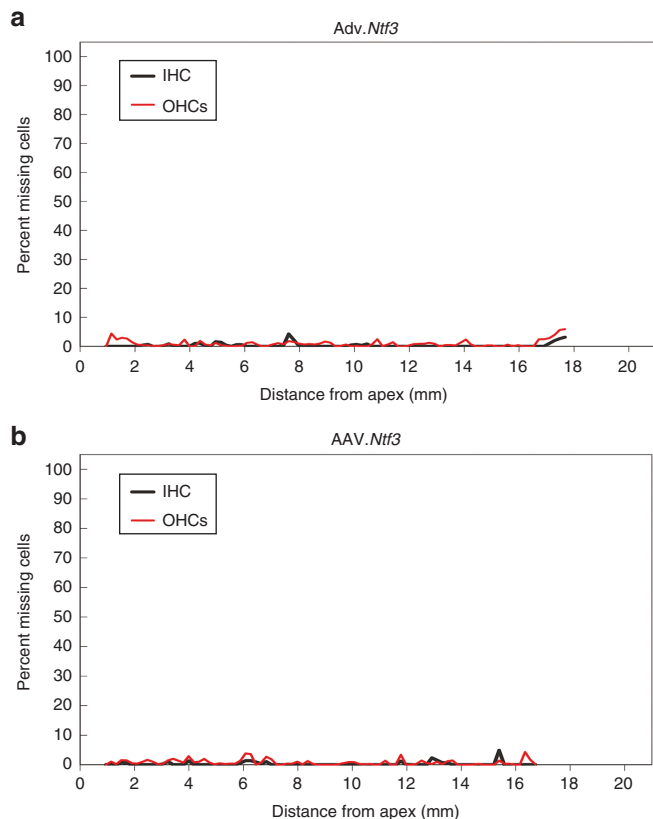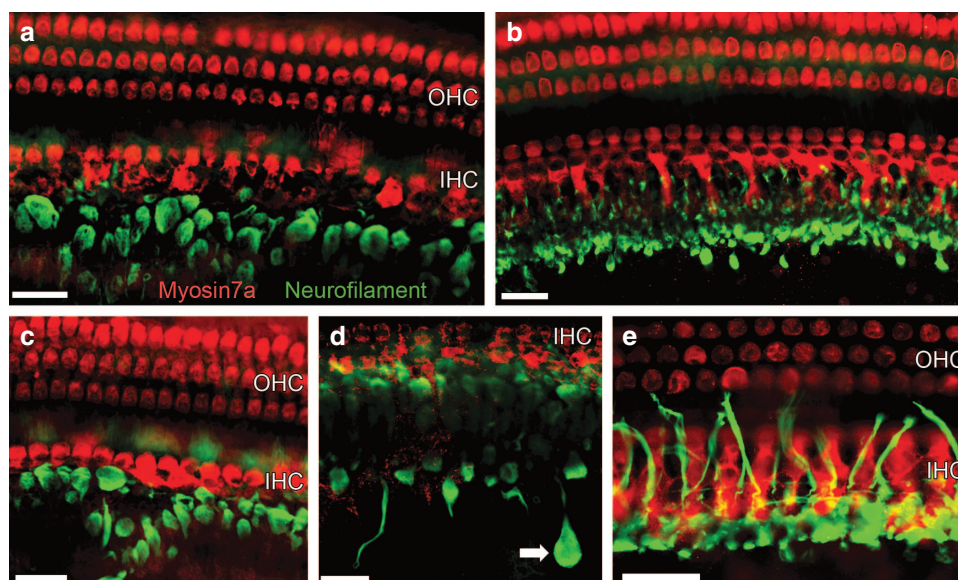
Figure 3 Epi-fluorescence of hair cells and nerve endings in the Adv.*Ntf3* treated cochlea. Expanded nerve endings appearing vesicular, seen by neurofilament staining (green) were found in the basal turn of the cochlea (**a**), but not in the contralateral (noninjected) control ears (**b**). Expanded nerve endings were also observed in the second (**c**) and third (**d**) turns, and enlarged neurofilament-stained structures were occasionally observed medial to the inner hair cells (arrow). Normal configuration of nerve endings was observed in fourth turn (**e**). Both inner hair cell (IHC) and outer hair cell (OHC) (red; Myosin VIIa) density and their morphology appeared normal at 3 weeks after the injection of Adv.Ntf3 **a**, **c–e**. Scale bars indicate 20 μm. Adv, adenovirus.
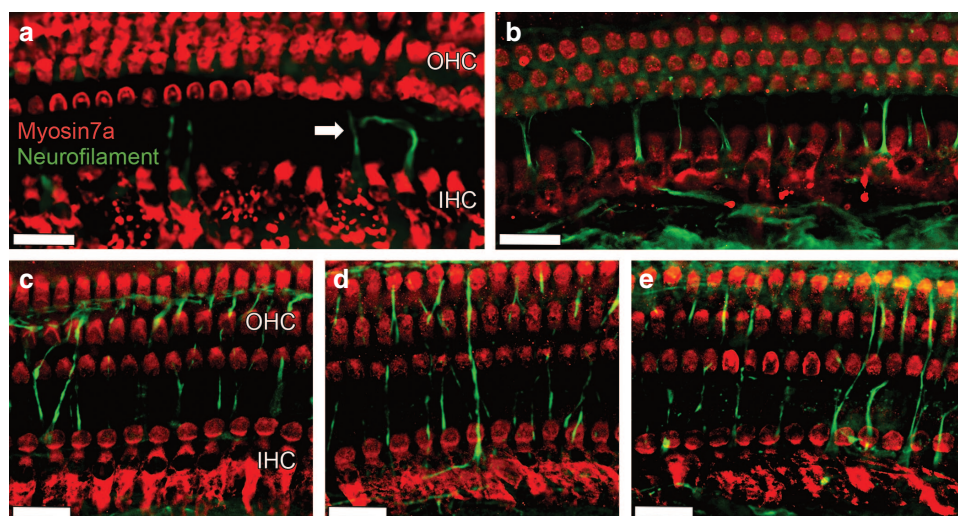

Figure 4 Epi-fluorescence of hair cells and peripheral nerve fibers in the AAV.*Ntf3* treated cochlea. Abnormal features of the peripheral nerve fibers (green; neurofilament) observed in the basal turn included reduced number of nerve fibers contacting the outer hair cells (**a**) compared with contralateral (noninjected) control ears (**b**), and nerve fibers sprouting in different directions within the epithelium (arrow). These features were limited to the basal turn and were not observed in second (**c**), third (**d**) and fourth turns (**e**) of the AAV.*Ntf3* treated cochlea. Both inner and outer hair cell (red; MyosinVIIa) density and their morphology appeared normal at 3 weeks after the injection of AAV.*Ntf3* (**a**, **c–e**). Scale bars indicate 20 μm. AAV, adeno-associated virus.

The influence of NT3 overexpression on nerve endings

The ability of neurotrophin overexpression to induce sprouting and attract auditory nerve fibers to remote areas has been shown in experiments using osmotic pumps.[14] Similar sprouting has also been found in ears devoid of hair cells that received neurotrophins expressing viral vectors.[9–11] Nerve sprouting with gene transfer was more targeted to the cells that express the neurotrophin transgene and therefore less diffuse than that observed with miniosmotic pumps. The results of these previous studies in the deaf cochlea demonstrated the concept that nerve fibers are attracted to grow toward areas of higher neurotrophin concentrations in ears devoid of inner hair cells. However, in this study, examining the effect of NT3 overexpression in the normal guinea pig cochlea, nerve endings were connected to their target cells in the auditory epithelium at the time we injected the viral vector. The results therefore raise the possibility that high levels of neurotrophins at some distance away from the inner hair cell can attract neurons that have already formed synapses away from their natural target, disrupting normal innervation patterns.

In addition to disconnecting from the inner hair cell, the nerve ending structures also appear swollen. Similar results have been reported
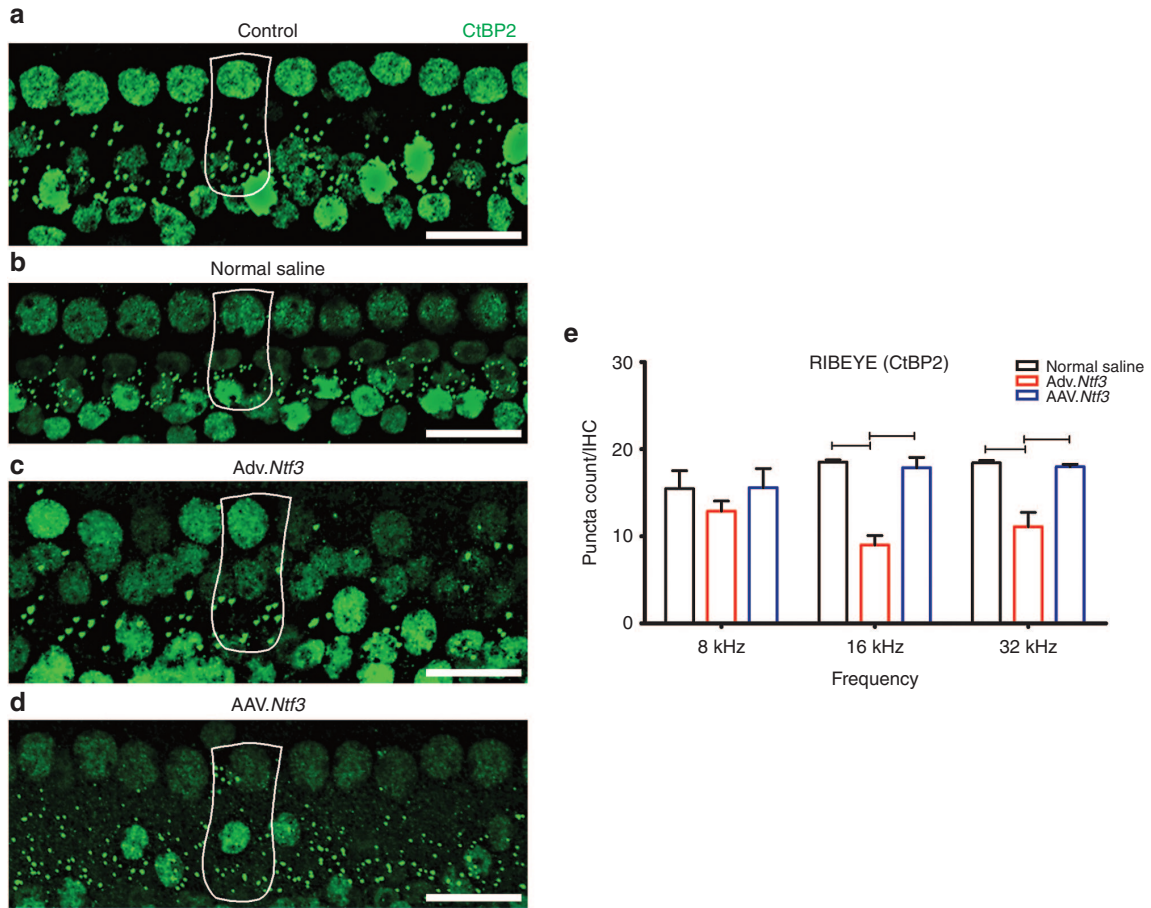
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Figure 5 Epi-fluorescence and quantitative analysis of synaptic puncta density. Whole-mounts of the auditory epithelium (representative images for the 16 kHz region, ~15 mm from the apical end), stained for presynaptic puncta (CtBP2) in control ears (**a**, **b**), ears that received Adv.*Ntf3* (**c**) and ears that received AAV.*Ntf3* (**d**). Solid lines are used to outline inner hair cells. The number of CtBP2 puncta per inner hair cell was not significantly different between contralateral ears (no injection) **a** and ears injected with normal saline **b**. In ears injected with Adv.*Ntf3*, the number of puncta was smaller than in control ears **c**. AAV.*Ntf3* injection resulted in no reduction in the number of puncta **d**. The number of CtBP2 puncta per inner hair cell was significantly smaller in Adv.*Ntf3* compared with the normal saline and AAV.*Ntf3* treated animals in the 16 and 32 kHz regions (**e**). Scale bars indicate 20 μm. AAV, adeno-associated virus.
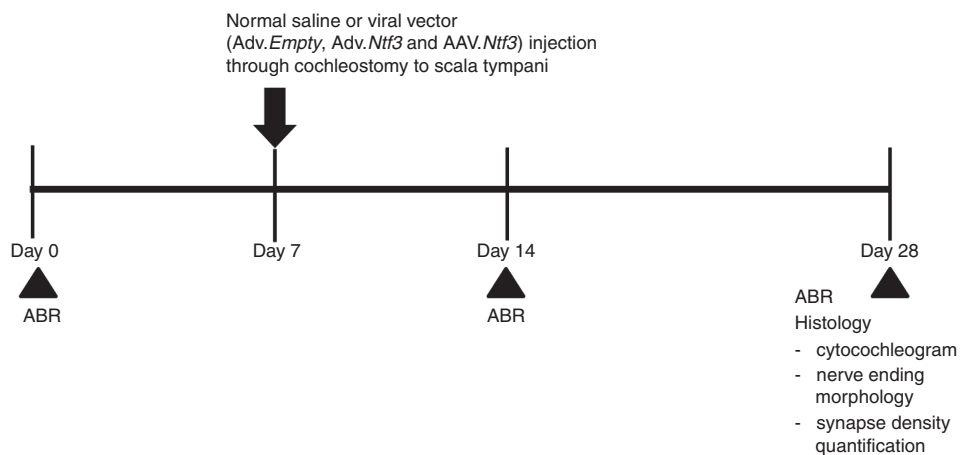

Figure 6 Schematic time-line of *in vivo* experiment. The surgical delivery of the viral vector or control substance (normal saline; NS) was performed at day 7 (7 days after the baseline ABR tests). Postoperative ABR tests were done day 14 (postoperative day 7) and day 28 (postoperative day 21), and animals were sacrificed on day 28 for the histology.

after acute noise exposure or ischemia[27,28] and in deaf animals in which Adv.*BDNF* was injected into the cochlea.[11] This swelling of nerve fiber endings appears to be more frequent after neurotrophin gene transfer than after the noise exposure or ischemia. In contrast to Adv-mediated NT3 overexpression, no major changes of nerve endings were observed in AAV.*Ntf3* treated animals. The difference in target cells of the two different viral vectors could be responsible for this, with AAV infecting cells in the auditory epithelium.

Another factor to consider is the timing of gene expression onset. AAV peak gene expression is ~2 weeks later than Adv,[29,30] such that the 3-week time point used in these experiments may be too early for detecting nerve fiber changes with AAV. Still, we noted a reduced number of nerve fibers in the outer hair cell area after AAV.*Ntf3* injection, which might be responsible for the ABR threshold shift recorded in these cochleae.

Mechanism of lower synaptic densities in ears with NT3 overexpression

Inoculation of Adv.*Ntf3* resulted in a reduced density of pre-synaptic puncta on inner hair cells. The pattern and extent of synapse disruption are similar to those seen in synaptopathy induced by acute noise exposure.[28] We have no reason to assume that elevated levels of NT3 in the cochlea can cause disruption in glutamate release or recycling, and therefore we suspect that synaptic ribbons are down-regulated because nerve endings are relocating away from the inner hair cells, attracted by elevated levels of NT3 in adjacent areas.

Alternative methods of neurotrophin receptor activation for future clinical applications

Despite the anatomical location of the inner ear, deep in the temporal bone, delivering therapeutic agents into the cochlea is feasible via routes through the tympanic membrane or mastoid bone into the middle ear cavity. Neurotrophins have been considered for protection against several types of inner ear trauma,[31,32] and for improving the neural substrate in deaf ears to enhance the outcome of cochlear implant prosthesis therapy.[12,33–35] With the evidence that elevation of NT3 levels can be beneficial for treating synaptopathy due to acoustic trauma,[7] it is worthwhile to explore methods of adapting this concept in clinically-applicable ways. The candidate ears for treatment with NT3 would typically have a normal or nearly-normal population of hair cells and auditory neurons. Here we determined that viral-mediated overexpression of NT3 can lead to detrimental side effects including disruption of normal physiology and anatomy. For safe and efficacious clinical application, these drawbacks should be minimized either by restricting the location of NT3 expression to the inner hair cell region and/or by activation of TrkC receptors using means other than diffusible NT3. This can be done by enhancing viral vector specificity with cell-specific promotors or endogenous miRNAs.[36] Alternatively, TrkC antibodies, which have been shown to act as NT3 agonists, can target auditory nerve endings with high specificity and may enhance repair of synapses and nerve survival.[37] Such an approach may treat synaptopathy without the side effects reported in this study.

One limitation of therapies based on siRNA or monoclonal antibody injections is the limited activity time and the likely need for repeated injections. Sustained levels of NT3 expression can best be accomplished via viral gene therapy. But to be able to use this technology, it will be necessary to have vectors that can infect a specific target cell, most likely the supporting cells that surround the inner hair cell or the inner hair cell itself. Better characterization of gene expression and surface receptors specific to the inner and outer phalangeal cells as well as the inner hair cells should provide tools for the design of such vectors. Similarly, it is possible that the duration and concentration of NT3 also are critical. Viral vectors that can be regulated for duration and extent of gene expression could help accomplish and optimize the gene expression profile for NT3 treatment of synaptopathy.

Conclusions

We showed that overexpression of NT3 in normal guinea pig cochleae leads to impaired hearing, reduction in the number of synaptic ribbons on inner hair cells and deterioration of the pattern of innervation in the organ of Corti. These outcomes were especially prominent after Adv.*Ntf3* injection in perilymph, and demonstrated that auditory nerve endings are lured away from inner hair cells when elevated levels of NT3 arise elsewhere. The data suggest that development of NT3 therapy for synaptopathy should focus on methods of elevating NT3 secretion in specific areas or cells that are normal targets of innervation or on methods that use agonists targeting the receptors on auditory nerve endings. More generally, the results indicate that the pattern of innervation in the normal ear can be disturbed by manipulating levels of neurotrophins in the cochlea.

MATERIALS AND METHODS

Analysis of NT3 concentration in culture

We used an established cell line culture of guinea pig fibroblast. The cells were seeded into 6-well culture plates, 120,000 cells/well. Culture media was removed after 24 hours and 1 ml of new fresh media was added. Cultures were transfected with *Ntf3*-expressing viral vectors as described.[10,38] Cells were exposed to Adv.*Ntf3* or AAV.*Ntf3* for 4 hours, and then incubated with fresh media. The viral vectors were Adv.*Ntf3* and AAV.*Ntf3*. Adv.EFα-*NTF3* (rat *NTF3*, and the Rat NTF3 and the protein tag FLAG are driven by the elongation factor-1 α promoter, lot# AYR050407, titer 2×10^{12})[39] was kindly provided by H. David Shine (Baylor College of Medicine). AAV2 smCBA-gpNTF3-myc (ref# Ω3923, titer 3.14×10^{13}) was purchased from the Vector Core at the University of Florida. In this vector, the Myc tag was placed at the C-terminus of the NT3 cDNA, because this is normally the safest end on which to put a tag without interference with gene function (personal communication, Dr William Hauswirth, the University of Florida). Media (supernatant) collected from cells cultured without the viral vector was used as negative control. Media was collected for ELISA analysis, 72 hours after the vector-containing media was replaced and samples were stored at −20 °C until analysis.

Concentrations of NT3 in media were evaluated using the Human NT3 ELISA kit (Catalog# BE69051, IBL-America, Minneapolis, MN), according to the manufacturer's instructions. The manufacturer-provided NT3 solution was used as a positive control (highest concentration prepared) and to set the standard curve to infer concentrations of unknowns. For the analysis of unknowns, we obtained a standard curve with a high correlation ($r^2 > 0.9$) between optical density and NT3 concentration of serially diluted standard and inferred the concentrations of the treated culture media from the curve.

Animals and experimental procedures

A total of 26 pigmented, specific pathogen free guinea pigs from the KHRI specific pathogen free Colony were used for this study. The animals were between 1 and 2 months old (250–400 g) at the onset of the experiment. This study was approved and performed according to the guidelines of the University of Michigan Institutional Animal Care and Use Committee (IACUC). These animals were allocated to four treatment groups as described below. The administered solutions included NS (n = 3), Adv.*Empty* (control vector, E1 and partial E3 deleted, elongation factor-1 α promoter, bovine growth hormone polyA site, titer 5×10^{12}) (n = 3), Adv.*Ntf3* (n = 11) (see the description above), and AAV.*Ntf3* (n = 10) (see the description above). At the beginning of experiment, hearing was evaluated by ABR to establish baseline thresholds. All animals had thresholds within the normal range. Experimental solutions were injected into the cochlea 7 days after the ABR test. After the injection, ABR thresholds were evaluated at 1 and 3 weeks post operatively, and then animals were sacrificed for histology (Figure 6).

Surgery

The cannula used for injecting the reagents to the inner ear was prepared prior to surgery, as follows: a polyethylene tube connected to a fine polyimide tube at one end, and to a 5 µl Hamilton syringe with a needle filled with sterile water at the other end. The animals were anesthetized with ketamine HCl 40 mg/kg (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine 10 mg/kg (AnaSed, Shenandoah, IA) and the left postauricular area

was prepared for surgery. The animals were placed in the lateral position. 0.5 ml of 1% lidocaine HCl was injected subcutaneously in the postauricular area for local anesthesia. The basal turn of the cochlea was exposed through a postauricular incision and opening of the temporal bulla. A cochleostomy was made at the basal turn of the cochlea inferior to the round window niche. Then, 4 μl of solution were infused through the cochleostomy into the scala tympani at 1.0 μl/min, using a motorized pump to control the infusion speed. After the inoculation was complete, the tube was left in place for an additional 10 to 15 minutes. After the tube was removed, the cochleostomy was sealed with soft tissue plug and bulla wall was sealed with bone chip or muscle plug with tissue adhesive (Vetbond, 3M, St. Paul MN) and carboxylate cement (Durelon, 3M ESPE, St. Paul MN). The muscle incision was closed with a 5-0 Vicryl suture and the skin incision was closed with 5-0 Ethilon suture. The procedures took ~30 minutes to complete including the additional 10 to 15 minutes after the injection. Later, the animals were allowed to awaken from anesthesia, hydrated with subcutaneous injection of NS and their pain was controlled with carprofen (4 mg/kg, s.c., Rimadyl, New York, NY).

ABR measurement

ABR recordings were collected at 8, 16, and 32 kHz in the left ear of each animal. Animals were anesthetized intramuscularly with ketamine (58.8 mg/kg), xylazine (2.4 mg/kg), and acepromazine (1.2 mg/kg) and placed on a thermo-regulating heating pad to maintain body temperature. ABRs were recorded in an electrically and acoustically shielded chamber (Acoustic Systems, Austin, TX). Tucker Davis Technologies (TDT) System III hardware and SigGen/BioSig software (TDT, Alachua, FL) were used to present the stimulus and record responses. Neural activity in response to brief tone bursts were measured using needle electrodes inserted subcutaneously ventral to each pinna and at the vertex of the skull. The sound was delivered to an area just inside the tragus. Each tone burst was 15 milliseconds in duration, with 1 millisecond rise/fall times, presented 10 bursts per second through an EC1 driver (TDT, aluminum enclosure made in-house). Then, 1,024 responses were averaged for each stimulus level and each frequency. Responses were collected for stimulus levels in 10 dB steps at higher stimulus levels, and at 5 dB steps near threshold. Thresholds were interpolated between the lowest stimulus level where a response was observed, and 5 dB lower, where no response was observed. For statistical analysis of ABR data, MANOVA was used to test for differences in baseline thresholds between sets of animals assigned to the four treatments, analyzing all tested frequencies simultaneously, then to test for changes in threshold after inoculation in each treatment group. Subsequently, univariate ANOVA was performed to evaluate threshold differences between consecutive time points for each frequency, with sequential Bonferroni criterion used to correct for the number of post-hoc tests.

Tissue-preparation and immunocytochemistry

Animals were sacrificed after the last ABR test and their temporal bones were harvested. Samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for at least 2 hours, rinsed with PBS, and the area of the auditory epithelium and spiral limbus were dissected for whole-mount preparations. Tissues were stained for Myosin VIIa and neurofilament, to assess hair cell survival and the morphology of nerve endings and nerve fibers, respectively. Staining for CtBP2 was used to evaluate the density of synaptic ribbon puncta in inner hair cells. The dissected tissues were permeabilized in 0.3% Triton X-100 in PBS for 10 minutes, then incubated with blocking buffer (5% normal goat serum in PBS) for 30 minutes to block non-specific binding of secondary antibodies. After that, samples were incubated with primary antibodies. The following primary antibodies, dilutions and incubation conditions were used: rabbit anti-Myosin VIIa (Proteus 25–6,791, 1:200 dilution in blocking buffer, 1 hour, room conditions); mouse anti-neurofilament (Millipore/Chemicon AB9,563, Darmstadt, Germany 1:200 dilution in blocking buffer, 1 hour, room conditions); mouse anti-CtBP2 (BD Transduction Labs #612,044, San Diego, CA 1:500 dilution in 1% normal goat serum in PBS, overnight, room conditions).

After rinsing the primary antibody with PBS, tissues were incubated with a fluorescence-labeled secondary antibody for 30 minutes and rinsed with PBS before mounting on microscope slides. The secondary antibodies used for Myosin VIIa and neurofilament were Alexa Fluor 488 or 594 (Invitrogen/Molecular Probes, Carlsbad, CA) diluted 1:200 in PBS, incubated for 30 minutes and the secondary antibodies for CtBP2 was goat antimouse (IgG1)-488 (Life Technologies #A21121) diluted 1:1,000 in PBS, incubated for 30 minutes (two times). After PBS rinsing, stained tissues were mounted on glass slides with Fluoro-Gel mounting media (Electron Microscopy Sciences, Hatfield, PA).

For epi-fluorescence analysis, we used a Leica DMRB epi-fluorescence microscope (Leica, Eaton, PA) equipped with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI) and SPOT-RT software Ver.5.0. For confocal microscopy analysis, we used Olympus FV 500 Confocal microscope (Olympus, Center Valley, PA). Photographs were cropped and labeled with Adobe Photoshop version 14, 2.1x32 and Illustrator software (Adobe System, San Jose, CA).

Assessment of hair cell survival

Ears that received Adv.Ntf3 and AAV.Ntf3 were evaluated for hair cell survival 21 days after inoculation using whole-mounts of the auditory epithelium stained with Myosin VIIa-specific antibody. Tissues were viewed in a Leica DMRB epi-fluorescence microscope with 40× (1.25 × digital zoom) objective lens. Hair cell counts were analyzed using the Kresge Hearing Research Institute (KHRI) Cytocochleogram Program, Version 3.0. In each field of view, 0.20 mm scale grid which was placed in the 10× eyepiece was adjusted along the centers of the pillar cells. The percentage of hair cell loss for each 0.20 mm segment was calculated for each row. To compare between specimens differing in total cochlear length, each specimen's data were used to calculate the percent of hair cell loss in 75 intervals (each representing 1.33% of that specimen's cochlea). For graphical comparison of hair cell loss between treatment groups, the average outer hair cell and inner hair cell loss was calculated for each interval and plotted against distance (mm) from the apex.

Quantification of synaptic puncta

The quantification procedures were based on an established method[7] with modifications to accommodate guinea pig tissues. The cochlear distance from the apex was measured using ImageJ plugin (http://www.masseyeandear.org/research/otolaryngology/investigators/laboratories/eaton-peabody-laboratories/epl-histology-resources/imagej-plugin-for-cochlear-frequency-mapping-in-whole-mounts) and this distance was converted into frequency based on the calculation by Greenwood.[40] Confocal z-stacks of the 8, 16, and 32 kHz regions from each cochlea were taken with a 60× (1× digital zoom) oil immersion lens. For synaptic punctum counts, the z-stacks (0.25 μm step size) were set to include the entire length of inner hair cells so that all the synaptic puncta could be imaged. In these stacked images, we counted all puncta labeled for presynaptic marker (RIBEYE and CtBP2), using tpsDig software (Version 2.12; F. James Rohlf, Ecology & Evolution, SUNY at Stony Brook, NY). Counts in each z-stack were divided by the number of inner hair cell nuclei, which could be visualized by staining of CtBP2 antibody. Each image usually contained 20–27 inner hair cells. For Statistical analysis of punctum density, MANOVA was used to test for differences in punctum counts among the three groups (NS, Adv.Ntf3 and AAV.Ntf3), analyzing all tested frequency areas simultaneously. Subsequently, to test which groups were different, we performed pairwise comparisons of groups using MANOVA, then for those pairs with statistical significance, univariate ANOVA was performed to determine which frequency areas differed.

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