Published in final edited form as:

Exp Neurol. 2010 June ; 223(2): 464–472. doi:10.1016/j.expneurol.2010.01.011.

Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae

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Abstract

Sensory organs typically use receptor cells and afferent neurons to transduce environmental signals and transmit them to the CNS. When sensory cells are lost, nerves often regress from the sensory area. Therapeutic and regenerative approaches would benefit from the presence of nerve fibers in the tissue. In the hearing system, retraction of afferent innervation may accompany the degeneration of auditory hair cells that is associated with permanent hearing loss. The only therapy currently available for cases with severe or complete loss of hair cells is the cochlear implant auditory prosthesis. To enhance the therapeutic benefits of a cochlear implant, it is necessary to attract nerve fibers back into the cochlear epithelium. Here we show that forced expression of the neurotrophin gene BDNF in epithelial or mesothelial cells that remain in the deaf ear, induces robust regrowth of nerve fibers towards the cells that secrete the neurotrophin, and results in re-innervation of the sensory area. The process of neurotrophin-induced neuronal regeneration is accompanied by significant preservation of the spiral ganglion cells. The ability to regrow nerve fibers into the basilar membrane area and protect the auditory nerve will enhance performance of cochlear implants and augment future cell replacement therapies such as stem cell implantation or induced transdifferentiation. This model also provides a general experimental stage for drawing nerve fibers into a tissue devoid of neurons, and studying the interaction between the nerve fibers and the tissue.

Keywords

neurotrophin; transgenic BDNF; viral vector; nerve regeneration; guinea pig; deafness; adenovirus; adeno-associated virus; cochlea; spiral ganglion

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Note: Data were presented in part at the 2009 ARO Meeting, Baltimore, MD.

Introduction

Hair cells in the cochlea transduce acoustic auditory stimulation to activate the auditory nerve. In normal ears, the afferent auditory neurons, spiral ganglion neurons (SGNs), maintain peripheral nerve fibers that synapse with the hair cells (Rusznak and Szucs, 2009, Spoendlin, 1985, Spoendlin and Schrott, 1988), but the loss of hair cells may lead to degeneration of nerve fibers from the sensory epithelium, and eventually to degeneration of the SGNs (Bichler, et al., 1983, Jyung, et al., 1989, Koitchev, et al., 1982, Webster and Webster, 1981). Because lost mammalian auditory hair cells and neurons do not spontaneously regenerate, the hearing loss associated with their degeneration is permanent (Hawkins, 1973, Spoendlin, 1975).

Loss of inner hair cells is implicated in degeneration of SGN degeneration, but the causal relationship between the two is complex. The extent of SGN degeneration differs from lab animals, where it is common and severe (Dodson and Mohuiddin, 2000, Jyung, et al., 1989, Leake and Hradek, 1988, Sugawara, et al., 2005), and human ears, where it is much less common (Linthicum and Fayad, 2009, Nadol and Eddington, 2006, Spoendlin and Schrott, 1990). Moreover, the correlation between the outcome of cochlear implant prosthesis therapy and the survival of the SGNs was not found to be a strong in most cases (Blamey, 1997, Linthicum and Fayad, 2009). However, the loss of peripheral nerve fibers, the dendrites of the SGNs, is strongly correlated with the loss of inner hair cells and probably also the supporting cells in both animal and human ears (Linthicum and Fayad, 2009, Sugawara, et al., 2005). It is likely that outcome of the cochlear implant therapy is related to the condition of the remaining SGNs and the survival of peripheral dendrites.

In the absence of hair cells, cochlear implant electrodes can stimulate SGN bodies and possibly their central axons, providing partial restoration of hearing to patients with severe or profound hearing loss (Wilson and Dorman, 2008). Inspired by the prevailing concept that better survival of SGNs would enhance outcomes of cochlear implant therapy, several approaches have been used to preserve neurons in deaf ears. Of these, increasing the levels of neurotrophins in the cochlear fluids has been the most successful (Bowers, et al., 2002, Fritsch, et al., 2004, Ylikoski, et al., 1998). Neurotrophins were selected because studies had demonstrated that these growth factors have a role in development of afferent neurons in the organ of Corti (Altschuler, et al., 1999, Fritsch, et al., 1999) and in protecting SGN in ears where hair cells are lost (Aarnisalo, et al., 2000, Duan, et al., 2000, Nakaizumi, et al., 2004, Staecker, et al., 1996, Van De Water, et al., 1996).

Several methods have been used to elevate neurotrophin levels for protecting neurons in deafened ears. Infusion of trophic factors such as neurotrophins via mini-osmotic pumps has been shown to significantly enhance the numbers of surviving neurons (Glueckert, et al., 2008, Miller, et al., 1997, Wise, et al., 2005). Similar results were accomplished using forced expression of neurotrophins by introducing the genes for these growth factors into cells that line the perilymph (Bowers, et al., 2002, Miller, et al., 1997, Nakaizumi, et al., 2004, Staecker, et al., 1998). Short term secretion of a neurotrophin was also accomplished by implanting electrodes coated with a gel pre-soaked with the neurotrophin (Chikar, et al., 2008, Hendricks, et al., 2008).

Elevated levels of neurotrophins not only increased survival of SGNs, but also induced sprouting of peripheral nerve fibers (Glueckert, et al., 2008, Wise, et al., 2005). Growth or regeneration of nerve fibers, by itself, can further enhance SGN survival. To the extent that this new growth can be directed into the area of the auditory epithelium and the connective tissue beneath it (defined here as the basilar membrane area, BMA), and remain there for the long term, it could improve the function of the cochlear implant device by placing neurons closer to the electrodes and enhancing spatial selectivity of stimulation. Functional outcomes

of novel restorative methods that might be developed in the future (such as cell replacement by stem cell therapy) would also benefit from the presence of nerve fibers in the tissue.

The severely traumatized guinea pig cochlea serves as a model for profound hearing loss in humans. Here, we tested the response of the auditory nerve in severely deafened ears to elevated levels of neurotrophins in the BMA and its vicinity. Specifically, neurotrophin over-expression was produced by viral vector transduction of cells in the BMA of deafened ears. To eliminate hair cells in the guinea pig cochlear epithelium and generate profound deafness, we used a perilymphatic injection of neomycin. This ototoxic regimen quickly eradicates hair cells and leaves behind a severely traumatized cochlear epithelium that also lacks the typical morphology of non-sensory cells (Duckert, 1983, Kim and Raphael, 2007, Sapieha, et al., 2006). We refer to this severely lesioned tissue as the flat epithelium (FE). Presence of the FE has been observed after a long period of deafness following kanamycin treatment (Sugawara, et al., 2005), after genetic hearing loss (Pawlowski, et al., 2006) and other etiologies for animal deafness, and in human temporal bone studies of patients with congenital diseases, including some who also received a cochlear implant (Nadol, 1997, Nadol and Eddington, 2006, Teufert, et al., 2006). Patients with profound deafness and FE are among those who receive cochlear implants, and may be the first candidate population for future stem cell therapies. In both therapies, presence of nerves in the BMA would be beneficial.

To deliver a neurotrophin gene to cells of the BMA, we used one of two viral vectors with the *BDNF* gene insert. In one group of animals, we used an adenovirus (Ad.*BDNF*) because of the high efficiency of gene transfer of this vector. In another group, we used an adeno-associated virus with the combined *BDNF* and *GFP* inserts (AAV.*BDNF-GFP*) as the AAV vector has long lasting transgene expression and no known side effects (Sapieha, et al., 2006). We report that over-expression of *BDNF* in the BMA of deafened cochleae led to abundant regrowth of nerve fibers toward the cells that secreted the neurotrophin. The robust neurotrophin-induced neuronal regeneration into the BMA was accompanied by significant preservation of the SGNs.

Materials & Methods

Animals and groups

Animal care and handling and all procedures described in this work were approved by the University of Michigan Institutional Committee on the Use of Care of Animals and performed using accepted veterinary standards. Male pigmented guinea pigs weighing 300–350g were purchased from Elm Hill, Chelmsford, MA USA. Each animal was tested for normal Preyer's reflex before being included in the studies. Animal groups were Ad.*BDNF* (25), Ad.Empty (18), AAV.*GFP-BDNF* (13), and deafening only (7). The inner ears were assessed as whole-mounts by epifluorescence and/or confocal microscopy, or plastic cross-sections at the light or TEM levels.

Deafening and Inoculation surgery

Animals were deafened unilaterally (left ear) by infusing 10 µl of 10% (w/v) neomycin sulphate solution (Neo-Rx, Pharma-Tek, in saline) into the scala tympani perilymph via the round window membrane. The surgical method of deafening is described in previous reports (Izumikawa, et al., 2008, Kim and Raphael, 2007). This protocol resulted in a nearly complete degeneration of the organ of Corti in the basal and 2nd turn. Specimens with any residual differentiated supporting cells in the basal turn were excluded from the study. One week later, animals were inoculated with viral vectors into the scala media (Ishimoto, et al., 2002) or scala tympani (Yagi, et al., 1999).

Adenovirus and adeno-associated virus

Adenoviral vectors with mouse *BDNF* insert driven by a cytomegalovirus promoter have been described previously (Di Polo, et al., 1998). This vector was given to the animal group labeled *Ad.BDNF*. We injected 5 μ l of *Ad.BDNF* at a titer of 4×10^{12} adenoviral particle per ml. Control animals labeled as *Ad.Empty* received an adenoviral vector with no insert (a gift from GenVec, Inc., Gaithersburg, MD, USA). Adeno-associated virus vector with *BDNF* and *GFP* insert driven by a cytomegalovirus promoter, described by (Sapieha, et al., 2006), was given to the group designated *AAV.GFP-BDNF*. Viral suspension was preserved at -80°C and thawed on ice before use. We injected 5 μ l of viral solution containing 1.68×10^{12} AAV particles per ml.

Immunohistochemistry analysis

Fourteen or 30 days after the inoculation, animals were decapitated under general anesthesia. Both temporal bones were extracted and the cochleae were perfused with 4% paraformaldehyde (PFA). After 2 hrs of fixation, cochleae were rinsed in phosphate buffered saline (PBS). Tissues were permeabilized with 0.3% Triton-X for 10 min, and then blocked against non-specific binding of secondary antibody by incubation in 5% normal goat serum for 30 min. Primary antibodies were monoclonal anti-neurofilament 200 KDa (Sigma) diluted 1:400 in PBS. Secondary antibodies were goat-anti mouse TRITC diluted to 1:200 in PBS. We counterstained the tissue for F-actin with Alexa Fluor 488 for 2 min, diluted to 1:300 in PBS. After the tissues were washed with PBS, whole-mounts of the BMA and surrounding tissue were obtained, mounted on glass slides and cover-slipped with Gel/Mount (Biomedex, Foster City, CA, USA). Whole-mounts were observed with a Leica DMRB epifluorescence microscope (Leica, Eaton, PA, USA) or a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany). Confocal images were acquired and processed with LSM image Browser (Carl Zeiss, Germany). Confocal images 2a–h, 5a, and 5d, are stacked set of z-plane images. Stacked planes ranged from 4 to 28 images (total depth ranging from 3.3 to 19.9 μm).

For ears observed in plastic sections, the tissues were first stained with anti-neurofilament as primary antibody (as described above) then by DAB secondary reagents following manufacturer's protocol (Vectastain ABC kit, Vector Labs), and then embedded in plastic and sectioned. The specimens were observed with a Leica DMRB light microscope (Leica, Eaton, PA, USA) and photographed with a CCD Cooled SPOT-RT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). Specimens for TEM were prepared as in a previous report (Beyer, et al., 2000) except the tissue was not stained en bloc with uranyl acetate. Grids with tissue sections were analyzed and images obtained using a Philips CM-100 TEM.

Plastic sections for SGN counts

The tissues were decalcified in 3% EDTA for 7–28 days, and embedded in JB-4 (Electron Microscopy Sciences, Hatfield, PA, USA) or Epon (Electron Microscopy Sciences, Hatfield, PA, USA). JB-4 blocks were sectioned with glass knives (3 μm) at a near-mid-modiolar plane, which provided six profiles of measurement. SGNs in Rosenthal's canal of the first and second turns were assessed.

Quantification and statistical analysis

We counted nerve fibers that extended more than 50 μm from the habenula perforata (HP) in each segment of the BMA using whole-mounts. Five images were selected randomly from the half turns of the 1st or 2nd turns of deaf ears receiving *Ad.BDNF* or *Ad.Empty*. Comparison of treatments was made at 14 and 30 day time points using Student's t test. Images of SGNs in the Rosenthal's canal were obtained using SPOT imaging software (Diagnostic Instruments). SGNs that contained a nucleus were counted by a blinded observer using ImageJ software. We

compared the difference of average SGNs in the 1st and 2nd turn in Ad.*BDNF* or Ad.Empty using one way ANOVA. P value of < 0.05 was considered significant.

Results

The “ deaf ” epithelium is devoid of nerve fibers

The normal cochlea contains sensory hair cells, supporting cells and other accessory cells (Fig. 1a–b) (see review for more detail (Forge and Wright, 2002, Raphael and Altschuler, 2003)). To determine the fate of nerve fibers after hair cell loss, we injected neomycin into perilymph of guinea pig ears. A week later we observed complete elimination of inner and outer hair cells throughout the cochlear duct, and FE in the base and 2nd turn (Fig. 1c–d). In a normal cochlea, nerve fibers exit the HP and extend to the sensory epithelium in a well organized pattern (Fig. 2a). The FE of neomycin-injected ears was generally devoid of nerve fibers (Fig. 2b–d), having only a few nerve fibers that entered near the HP, looped in this area, and exited (Fig. 2b). These conditions appear to be stable in the guinea pig through at least 30 days (Fig. 2c and d). Loops rarely extended beyond 50 μ m from the HP.

Regrowth of nerve fibers in the FE

We next investigated whether nerve fibers can grow into the BMA of the FE, in response to forced expression and secretion of a neurotrophin, which was accomplished by viral-mediated gene transfer. Vectors were delivered to endolymph or perilymph in separate experiments. To trace the growth pattern of regenerating nerve fibers in the FE, we double-stained whole-mounts of the auditory epithelium with markers for neurofilament and actin. Inoculation of Ad.*BDNF* into the endolymph 7 days after neomycin injection resulted in robust nerve fiber regrowth into the 1st and 2nd turns of the FE (Fig. 2e–h). Numerous nerve fibers grew past the HP and extended both laterally into the FE and medially toward the inner sulcus. The density of the nerve fibers generally decreased in areas lateral to the presumed location of Hensen cells, i.e., beyond the normal borders of the sensory epithelium (Fig. 2e and h). Nerve fibers extending into the FE were oriented in several directions, from radial to longitudinal. The nerve fibers that had a longitudinal orientation often appeared to have a smaller diameter than the radially-oriented nerve fibers (Fig. 2e). These data demonstrated successful nerve regrowth into a tissue completely devoid of hair cells and neuronal elements.

We found that nerve fibers passed between the FE cells, often circling them. The regenerated nerve fibers often exhibited an enlarged neurofilament-positive area adjacent to the HP (Fig. 2f). Additionally, nerve fiber growth could be observed extending into the spiral limbus and looping back to the HP area (Fig. 2g). Ad.*BDNF* inoculation into the perilymph (Fig. 2h) resulted in similar nerve fiber growth as that seen with inoculation into endolymph (Fig. 2e–g). Under both conditions, nerve fibers could be seen to cross from epithelial to mesothelial areas of the BMA whole-mounts (data not shown).

Nerve regeneration into the mesothelium

To better visualize the association of nerve fibers in the FE with the connective tissue beneath it, we sectioned Ad.*BDNF* or Ad.Empty inoculated cochleae (Fig. 3a). Ad.Empty inoculated cochleae contained no nerve fibers on the moving part of the BMA, consistent with results seen in whole-mounts (Fig. 2b–d). In contrast, Ad.*BDNF* inoculated animals exhibited nerve fiber growth into the FE and the connective tissue of the BMA. Similarly, regions medial to the BMA did not contain nerve fibers in control animals, whereas Ad.*BDNF* treated ears did. The FE, itself, did not appear to differ between controls and neurotrophin-treated ears, suggesting that the morphology of these cells was not influenced by the BDNF.

To define the structural interaction between regenerated nerve fibers and the FE, including its associated extracellular matrix (ECM), we examined plastic sections with transmission electron microscopy (TEM). Nerve fibers were identified based on their elongated shape and the presence of filaments matching in size to neurofilaments and microtubules, and on the absence of any such cells in control tissues. Nerve fibers that regenerated into the FE were unmyelinated and grew under and between the cuboidal cells, but not adjacent to the luminal surface. These nerve fibers also crossed into the ECM (Fig 3b and c, **compare to 3a Ad.BDNF**). Nerve fibers did not reach the luminal surface, which appeared to be maintained by junctional complexes comprised of apical tight junctions, adherens junctions and desmosomes (Fig. 3b and d). FE cells associated with regenerated nerve fibers exhibited irregular cell borders and membrane-bound projections beneath the apical junctions (Fig. 3d). Occasionally, mitochondria were seen in these nerve fibers (Fig. 3e).

Quantification of regenerated nerve fibers

To assess the long-term persistence of regenerated nerve fibers induced to grow into the FE by neurotrophin over-expression, we counted the nerve fibers extending at least 50 μm away from the HP at 14 and 30 days after inoculation. Control deafened ears, which received no vector or Ad.Empty vector, exhibited few or no nerve fibers at this distance from the HP at either 14 or 30 days. In contrast, Ad.BDNF-inoculated animals exhibited strikingly higher numbers of nerve fibers at both time points (Fig. 4). The mean number of nerve fibers was slightly lower at 30 days than at 14 days, but the difference was not significant. Decreasing nerve fiber number would be consistent with declining adenovirus-mediated BDNF production over time after vector inoculation. Previous reports show that surviving SGNs require sustained presence of BDNF to maintain their morphology (Gillespie, et al., 2003).

Localization of transduced cells with AAV-GFP-BDNF

To investigate the relationship between cells in the BMA that express BDNF and the regenerating nerve fibers, we used an adeno-associated virus (AAV) vector containing a GFP-tagged *BDNF* expression cassette. The efficiency of transduction with AAV vectors is lower than that of adenovirus vectors, presumably resulting in a smaller number of BDNF sources that collectively attract a smaller number of nerve fibers. These smaller numbers are more convenient for assessing the trajectory of the regenerating nerve fibers and their co-localization with the transduced cells. Epithelial cells transduced by the AAV vector were green (GFP-positive) (Fig. 5a–d). In some cases, nerve fibers appeared to deviate or change their projection route to reach the GFP-positive cells, and to terminate adjacent to, or attached to the neurotrophin-producing cell. Preferential orientation of nerve fibers towards GFP-positive cells was noted after inoculations of AAV.BDNF to both scala tympani (Fig. 5a–c) and scala media (Fig. 5d). These data indirectly suggest that GFP expressing cells attract the nerve fibers toward the source of BDNF, and raise the possibility that the nerve fibers move up the concentration gradient.

SGN protection via Ad.BDNF

To determine the influence of neurotrophin secretion mediated by viral vectors on the neurons' somata, both qualitatively and quantitatively, we assayed the density and the qualitative morphology of surviving SGNs using near-mid-modiolar cross sections of ears deafened with 10% neomycin and treated with Ad.BDNF or Ad.Empty. In the Ad.Empty group, severe degeneration of SGNs was apparent in the 1st and 2nd turns (Fig. 6a and c). Remaining neurons were smaller than normal, their cytoplasm stained darker, and their outer perimeter was ruffled and irregular. A bundle of efferent nerve fibers was often present (Fig. 6a **arrow**). In these Ad.Empty treated ears, the fluid spaces were intact and the supporting cells were degenerated into a FE. In the Ad.BDNF inoculated ears, we observed a marked preservation of SGNs in

the 1st two turns (Fig. 6b and d). Surviving cells appeared large and collectively filled the canal. Their cytoplasm had normal density and their nuclei were well demarcated, easy to distinguish, and normally shaped. The FE was similar to that seen in Ad.Empty animals, but the fluid spaces contained cells and possibly debris. The density of SGNs in BDNF treated ears was significantly higher than in Ad.Empty ears (Fig. 6e). This difference was noted in both 1st and 2nd turns of the cochlea.

Discussion

We investigated the outcome of forced expression of transgenic BDNF by cells lining the cochlear fluids in ears that were first severely deafened with neomycin and had a total loss of the organ of Corti with only a FE layer remaining. We determined that robust growth of nerve fibers into the deafened epithelium occurred after inoculating with either Ad.BDNF or AAV.BDNF, but not in control groups. Transduced cells appeared to serve as focal targets toward which nerve fibers grew. Furthermore, the survival of SGNs was significantly enhanced in ears receiving transgenic BDNF.

The choice of BDNF and gene transfer for its over-expression

The present study shows that transgenic BDNF over-expression can induce ample regrowth of nerve fibers into the BMA of deafened ears with a FE in place of the organ of Corti. A neurotrophin was selected for the experiment because these molecules are well known for their capacity to promote neuronal growth both *in vitro* and *in vivo* (Davies, 2000, Edwards, et al., 1989, Gillespie, et al., 2001, Purves, et al., 1988). Previous studies investigated the influence of neurotrophins on SGN survival (Gao, 1998, Roehm and Hansen, 2005, Staecker, et al., 1996). Exogenous BDNF and other neurotrophins (e.g. FGF and NTF3), have also been shown to promote nerve fiber regrowth or sprouting in deafened guinea pig cochleae when delivered by an osmotic pump (Altschuler, et al., 1999, Glueckert, et al., 2008, Wise, et al., 2005). Under these conditions, the nerve fibers appeared to grow sporadically, with no orientation toward the auditory epithelium, probably because the highest concentration of the neurotrophin was in the fluid, near the tip of the cannula. Nevertheless, those studies established that the auditory nerve can respond to increased levels of BDNF and exhibit regrowth of nerve fibers. An important implication of our study is that delivery of BDNF by gene transfer may have several advantages over the mini-osmotic pump, including the directional growth of nerve fibers towards cells in the FE, more robust growth of the nerve fibers, and stability over time with one application of an AAV vector.

We demonstrate robust nerve fiber regeneration induced by BDNF in this study, but other neurotrophins or combinations of growth factors should be considered for optimal nerve regeneration and survival. During development, hair cells are thought to secrete BDNF and/or NTF3 and attract auditory nerve fibers (Pirvola and Ylikoski, 2003, Roehm and Hansen, 2005, Rubel and Fritzsche, 2002). Thus, NTF3 is another candidate for promoting nerve survival and inducing nerve fiber regeneration in deaf ears. A combination of the two neurotrophins may be even more efficient at attracting nerve fibers to the BMA, but given the dense nerve regeneration seen with BDNF alone, this may not be necessary. Another potential application for use of two different neurotrophins is to induce preferential growth of neurons into different frequency areas of the BMA. During development, NTF3 is preferentially expressed in the basal cochlea (high frequency area) and BDNF in the apical cochlea (low frequency) (Farinas, et al., 2001, Ylikoski, et al., 1993). If this differential affinity is preserved in the mature ear, the transgenes could potentially be delivered preferentially to the respective areas, such that the corresponding neurons could be induced to grow nerve fibers into the specific areas. This could improve function of cochlear implant therapy using existing technology and could inspire

a re-design of the electrode array to better deliver a larger number of specific frequencies to the appropriate target areas.

Areas of cell transduction and nerve fiber growth pathways

Cell transduction by the viral vectors introduced into the scala media is not restricted to the auditory epithelium in the area of the organ of Corti. Rather, epithelial cells that flank the sensory area are also transduced, including the interdental cells in the spiral limbus (Kawamoto, et al., 2003). After vector introduction into the scala tympani, most transduced cells are mesothelial cells lining the perilymphatic space. With both routes of vector inoculation, we determined that nerve fibers extended into the FE and the tissue under the epithelium in the BMA. Nerve fibers also extended into regions flanking the BMA, in the spiral limbus (medial to the BMA) and the outer sulcus (lateral to the BMA). Within the FE of the BMA, nerve fibers were able to weave their way between the cuboidal cells, and in some cases looped around them. This is consistent with previous reports showing a similar growth pattern in other areas of the tissue lining the scala media (Wise, et al., 2005) and with similar observations made in other tissues (Rajnicek and McCaig, 1997). Taken together, these results indirectly suggest that the nerve fibers are sensitive to gradients of neurotrophin concentration within the FE layer, and that the epithelium is permissive to intercalation of nerve fibers between the cuboidal cells. This is important for future attempts to populate the deaf auditory epithelium with stem cells, in part because it suggests the FE is amenable to insertion of cells under the right conditions, and because it shows that the inserted cells will be able to attract auditory nerve fibers after differentiation to the hair cell phenotype.

The pattern of nerve fiber growth in the region medial to the BMA was different from that in the FE proper. Nerve fibers were observed exiting the HP and reaching the spiral limbus. Some of these nerve fibers looped back towards the HP for unknown reasons. Among possible reasons for looping out of the interdental cell area are turnover in this tissue leading to loss of cells that secrete BDNF, or presence of factors that inhibit sprouting or even repel neurons. Some of the medially directed nerve fibers also exhibited areas with enlarged diameter, appearing nearly spherical. Enlarged nerve fibers were previously reported outside Rosenthal's canal following mini-osmotic pump infusion of a neurotrophin (Glueckert, et al., 2008, Staecker, et al., 1996). Our analysis of whole-mounts demonstrates the prevalence of these enlarged nerve fibers in the areas medial to the HP and underline the importance of studying these cells. Enlarged nerve fibers were not observed in the vibrating part of the BMA. For now, the nature of these enlarged nerve fibers and the reasons for their appearance remain to be elucidated.

The paths neurons use to sprout through, and their final destination, are important for their function and survival in the tissue. The observation of nerve fibers crossing back and forth between the epithelium and the mesothelial layer, traversing the basilar membrane as they do so, is compatible with the biochemical continuity between the intercellular fluids in the FE and the perilymph in scala tympani. This is important for experimental and clinical applications, because it suggests that perilymphatic introduction of the viral vector is sufficient for inducing nerve fiber growth to the BMA, and scala tympani inoculation of the viral vector can be performed in human ears and other experimental animals. In the human ears, injecting the viral vector at the same time as inserting the cochlear implant electrode should be technically feasible and should suffice for attracting auditory nerve fibers into the FE.

Advantages of gene transfer for providing a cellular source of neurotrophin secretion

Parameters that need to be considered for enhancing clinically-meaningful nerve fiber regeneration into the BMA include safety, long-term stability, specificity to the site, efficiency of nerve regeneration and the types of neurons responding to the neurotrophin. The long-term persistence of regenerated nerve fibers is an especially important concern for developing

applicable therapies. To insure that regenerated nerve fibers remain for a meaningful period of time, it is necessary to maintain continued long-term secretion of the neurotrophin by the transgene-expressing cells. This is especially true considering that cessation of exogenous BDNF treatment in deafened cochleae accelerated degeneration of SGNs (Gillespie, et al., 2003, Shepherd, et al., 2008). Our data, while showing significant enhancement of SGN survival with Ad.*BDNF*, also found a tendency for long-term decrease of average nerve fiber count in similarly treated ears. Because the transgene expression of adenovirus peaks at 7 days after inoculation and gradually declines (Lundstrom, 2003), the decrease in nerve fiber count may correlate to the decline of BDNF transgene expression. For better long-term transgene expression and nerve fiber survival, it would be necessary to use viral vectors such as AAV, which typically maintain long-term transgene expression (Lalwani, et al., 1998, Sapieha, et al., 2006). AAV vectors are also expected to have no side effects as this virus is not a human pathogen (Daya and Berns, 2008). Our data showing usefulness of AAV.*BDNF* for inducing nerve fibers growth into the FE suggest that the AAV-neurotrophin vectors could be strong candidates for future treatments, including procedures for inducing nerve regrowth along with cochlear implantation. It is feasible that the AAV could be injected into the perilymph at the same time and through the same opening used for inserting the electrode array.

In addition to duration of gene expression and nerve fiber survival in the BMA, the location of nerve fiber regrowth is also an important parameter. We determined that the areas nearest to the site of viral vector inoculation exhibit the most efficient nerve fiber regrowth, providing strong but indirect causal relationship between the secretion of the neurotrophin and the regrowth. Since the likely practical site of inoculation will be the same as that used for inserting the cochlear implant electrode, the outcome is favorable for providing proximity between nerve fibers and electrode.

Side effects of and non-specific cell transduction by viral vectors should also be considered. One important side effect is the potential for spread to the contralateral ear. It has been demonstrated that viral vectors introduced into one ear can later be detected in the contralateral ear (Lalwani, et al., 1996). This was shown to occur via the cerebrospinal fluid through the cochlear aqueduct (Stover, et al., 2000), especially when the inoculated volume was especially large. It is less likely that small quantities of viral vector would spread to adjacent tissues or to the contralateral ear, but the influence of such eventuality needs to be considered. Another potential negative outcome would be growth of nerve fibers into areas other than the BMA. Although the area of the stria vascularis and Reissner's membrane did not appear to contain nerve fibers, further studies are needed to address the specificity of regrowth to the BMA. If necessary, vectors that have specificity for cells in the BMA can be developed.

The type of neurons growing into the BMA

Nerves regrowing in response to neurotrophin secretion into the deaf cochleae were previously identified as afferent nerve fibers (Glueckert, et al., 2008, Wise, et al., 2005). It is also important to assess the contribution of type I versus of type II auditory nerve fibers. Several observations indirectly suggest that the majority of nerve fibers are type I fibers. First, the majority of nerve fibers appear to be of a larger diameter, and only a small number of nerve fibers are narrow. The presence of a few narrow nerve fibers indirectly suggests that at least some type II neurons have regenerated into the BMA. Second, the quality and quantity of the preserved type I SGNs in Rosenthal's canal seen in the cross sections also corroborates the likelihood for a majority of type I nerve fibers growing back into the BMA. If indeed the majority of nerve fibers are type I SGNs, the nerve regeneration should be useful for sending auditory signals to the brain stem, either when stimulated by the cochlear implant electrode or when stimulated by new hair cells placed in the cochlea. Further work is needed to determine if other neurons such as

auditory efferents and vestibular afferents may respond to the elevated neurotrophins and grow towards their source.

Enhanced survival of SGNs with Ad.BDNF application

BDNF-induced survival of the SGNs in deaf ears has been described in various reports (Chikar, et al., 2008, Nakaizumi, et al., 2004, Wise, et al., 2005). The data we present differ from those reports in the deafening model, which in this study entails the use of neomycin and leads to a much more severe lesion. Compared to the kanamycin & ethacrynic acid model, neomycin causes hair cells to degenerate faster and also causes the supporting cells to degrade, leaving behind a FE. Neomycin also causes the neurons to degenerate faster and to a greater extent. The ability of the BDNF treatment to maintain a healthy-looking (by light microscopy) population of SGNs in Rosenthal's canal after treatment with neomycin underlines the efficacy of the BDNF treatment as a protective measure.

In addition to the significantly increased numbers of survival SGNs, the morphology of the neurons suggests that they remain in healthy functional condition, as also shown in other systems in which BDNF was used for protection (Luther and Birren, 2009). The healthy morphology could be a direct result of the elevated BDNF levels, and may also be influenced by the presence of regrown peripheral nerve fibers. It is likely that both factors influence the survival of neurons and the preservation of their morphology, but at present, it is difficult to dissect out the relative contribution of each of these two factors.

The deaf cochlea as a model for nerve sprouting and regeneration

This model for inducing nerve regeneration into a tissue devoid of innervation may also be useful for studies of regenerative neurogenesis in other systems. Our data show that nerve fibers can successfully regrow into a permissive area, suggesting that attraction by BDNF is sufficient to induce directional growth into the target. The ability of nerve fibers to cross the basal lamina and freely weave between cells in the BMA demonstrates the exploratory nature of growth cones and their ability to carve their own path, guided by the attractive cues in the tissue. This model may allow testing of additional neurotrophins and their combinations with other growth factors, and of molecules that may participate in path finding by providing attractive or repelling cues.

Clinical potential of nerve regeneration in the cochlea

Currently, cochlear implant auditory prostheses provide as many as 22 distinct channels of frequency-specific electrical stimulation to the implanted cochlea. However, patients with these implants receive benefit from only 4 to 8 channels (Friesen, et al., 2001). This is probably because the site of neural activation is at the cell body or central process of the SGNs, since the peripheral processes have degenerated. Because the electrodes in the scala tympani must activate neurons that are some distance away and separated by porous bone, the population of neurons stimulated by any given electrode is large and overlaps considerably with the population stimulated by neighboring electrodes. If peripheral processes of the SGNs can be regenerated to attain close proximity to the individual electrodes, many more functional channels of stimulation should be achievable, which would result in considerable improvement in speech recognition in quiet and noise, and improved tone perception (Friesen, et al., 2001, Xu and Pfingst, 2008).

While on the proximity of nerve fibers to the stimulating electrodes is theorized to enhance cochlear implant function, the process of nerve regeneration we now describe involves the risk that some nerve fibers will grow to areas remote from the BMA, resulting in increased distance and impedance and less specific stimulation. Further development of the technology of gene transfer would allow restricting the delivery of transgenes to the areas of the BMA.

Presence of nerve fibers in the BMA would also benefit future stem cell therapy, where transplanted stem cells would replace lost hair cells. Presence of neurons in such ears will be essential for the new hair cells to send auditory signals to the brain, and may also provide reciprocal positive influence on survival and function of the transplanted cells. The combination of stem cells and robust re-innervation is presently the most likely biological treatment for deaf cochleae with a FE, as transdifferentiation therapies have so far failed in this tissue (Izumikawa, et al., 2008).

Conclusions

Our data show that nerve fibers can be induced to grow into the FE, with a concomitant increase in the density of SGNs in severely deafened ears. This outcome will likely enhance the function of cochlear implant auditory prostheses. The ability to preserve SGNs and induce regrowth of their nerve fibers into the FE also has important implications for the restoration of hearing by replacement therapies, because regenerated nerve fibers and preservation of the auditory neurons would enhance innervation of the new sensory cells. The method for eliminating neuronal fibers from the auditory epithelium and inducing their regeneration back into the tissue may also serve as a general model for research on re-innervation of tissues devoid of neurons.

Acknowledgments

We are grateful to Stephan Kang, Donald Swiderski, Mark Crumling and Cathy Krull for assistance and helpful commentary. We thank GenVec for Ad.Empty adenovirus vectors. This work was supported by the A. Alfred Taubman Medical Research Institute, the Berte and Alan Hirschfeld Foundation, the R. Jamison and Betty Williams Professorship, and NIH/NIDCD Grants R01-DC01634, R01 DC007634, T32DC005356 and P30-DC05188.

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Figure 1.

The auditory epithelium from normal cochleae (a–b) or cochleae analyzed one week after an insult with neomycin (c–d) and viewed by epi-fluorescence (a, c) or light microscopy of plastic sections (b, d). The left side of the plastic sections corresponds to the lower aspect of the whole-mounts. (a) Phalloidin labeled whole-mount shows the epithelial mosaic containing hair cells and supporting cells. The approximate site of the HP is depicted by a dotted line. (I, inner hair cell; P, pillar cells; 1, 2, 3, 1st 2nd and 3rd rows of outer hair cells). (b) Sensory hair cells and non-sensory supporting cells and other cells and non-cellular components of the organ of Corti. The site of the HP is depicted with an arrow. (c) In the deaf cochlea, hair cells are absent and instead, a sheet of polymorphic cells with abundant actin in the intercellular junctions line the scala media and form the FE (FE). The dotted lines show the area of the HP. (d) The FE lines the scala media. The presumed site of the HP is depicted with an arrow. Scale bars are 20 μ m and also apply to both images above.

Figure 2.

Whole-mounts of the auditory epithelium stained for neurofilaments (red) and actin (green) and viewed with confocal microscopy, showing normal (a), neomycin-deafened (b–d), or Ad.*BDNF* treated ears 14 days after neomycin deafening (e–h). Dotted line depicts presumed area of HP in a–e, and h. Ad.*BDNF* was inoculated via scala media (e–g) or scala tympani (h) route. (a) The neurofilament positive nerve fibers exit the HP and reach inner (I) and outer hair cells (1–3), forming the inner (open arrowhead) and outer spiral bundle (arrow-head). (b) 7 days after neomycin deafening, nerve fibers are absent in the FE except for the area near the HP and a few loops (arrows) near the HP. (c–d) Fourteen (c) or 30 days (d) after Ad.Empty vector inoculation, no nerve fibers are seen in the epithelium beyond the HP area. (e) The diameter and orientation of nerve fiber extension into the FE varied. Narrower nerve fibers appeared further away from the HP (top of image) in a longitudinal (spiral) orientation. A nerve fiber that terminated not far from the HP (open arrow-head) exhibited an enlarged portion at the termination site. Most if not all nerve fibers weaved between the junctions of the FE cells (arrow-heads). (f) Enlarged neurofilament-positive areas appeared as spherical regions (dotted line) above and slightly medial to the HP, in a region corresponding to the inner sulcus or spiral limbus of a normal ear. (g) Nerve fibers in the interdental cell area projected while changing orientation (arrow). The surface of the nerve fibers in the interdental cell area appeared coarser than nerve fibers in the BMA (compare to e and h). (h) Following inoculation into the scala tympani, nerve fibers extended to the auditory epithelium weaving between FE cells. Scale bar=50 μ m.

Figure 3.

Cross-sections of the BMA observed with light (a) or TEM of ears treated with Ad.BDNF via the scala media and obtained for histology 14 days later (b–e). (a) Radial section of the FE after inoculation of Ad.Empty (top) or Ad.BDNF (bottom) stained for neurofilament with DAB (brown) and lightly counterstained with toluidine blue to reveal tissue structure. In Ad.Empty inoculated ears the nerve fibers rise near the surface of the HP (arrow) but do not extend further into the BMA. In Ad.BDNF inoculated ears, nerve fibers extend beyond the HP (arrow) and run in and under the FE, partially entering the ECM (arrow-heads). Scale barh 20 μ m. (b) An un-myelinated nerve fiber (N) is seen beneath the cuboidal cells (CE) in the BMA and above the surface of the ECM. Tight junctions (arrow) and desmosomes (arrow-head) are observed

between the cuboidal cells. An adherens junctional complex is seen between them. (c) A nerve fiber (N) is seen in contact with the ECM in an area devoid of basal lamina (area between black arrow-heads). The basal lamina can be observed adjacent to this area (open arrow-heads). (d) The area of contact between two flat epithelial cells above a nerve fiber (N) includes an irregular membrane structure (arrow-heads point to desmosomes). (e) Mitochondria (black arrow) are seen in the nerve fibers along with filaments and vesicles. Scale bars=500 nm.

Figure 4.

Quantitative analysis of the nerve fibers projecting at least 50 μm into the BMA peripheral (lateral) to the HP. Comparison of Ad.Empty and Ad.BDNF shows a significant difference of average nerve fiber count in the 1st and 2nd turn at 14 (gray bars) and 30 (black bars) days after deafening. (*) indicates $p < 0.01$. Deafened animals with no inoculation had similar counts to Ad.Empty animals. Bars are standard deviation and statistical comparison was done by Student t-test.

Figure 5.

Whole-mounts of ears treated with neomycin and then inoculated with AAV.*GFP-BDNF* via scala tympani (a–c) or scala media (d), observed with CFM (a and d) and epi-fluorescence (b and c). (a) Nerve fibers extend into the BMA and projections deviate toward the GFP-positive cells, and in some cases terminate near the cell (arrow-head). (b and c) Nerve fibers with large diameters are also seen to be attracted to GFP-positive cells in the residual Hensen cell area (H). (d) Nerve fibers deviate from outer spiral bundle area (dotted line) and move toward the GFP-positive cells. Scale bars=50 μ m.

Figure 6.

Light microscope micrographs of plastic sections through Rosenthal's canal in the 1st (a and b) and 2nd (c and d) cochlear turns 14 days after inoculation of Ad.Empty (a and c), or Ad.BDNF (b and d), and quantification of SGNs (e). (a and c) SGNs are scarce. A presumed efferent nerve fiber bundle is present (arrow). (b and d) Rosenthal's canal in Ad.BDNF inoculated ears exhibits a confluent population of neurons and no gliotic areas. In some of the Ad.BDNF inoculated ears, connective tissue could be observed in the scala tympani (arrow-head in b). Scale bars=100 μm. (e) Mean SGN density among the cochlear regions of basal and 2nd turn of Ad.Empty and Ad.BDNF inoculated animals. Comparison of SGN density in Ad.Empty (gray bars) and Ad.BDNF-inoculated groups (black bars) in all regions showed

significantly larger numbers of surviving SGN in the Ad.*BDNF* groups. Error bars are standard deviations. Statistical comparison was by one-way ANOVA. (*) indicates $p < 0.01$. (L=Lower and U=Upper).