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Over-expression of BDNF by adenovirus with concurrent electrical stimulation improves cochlear implant thresholds and survival of auditory neurons

Jennifer A. Chikar^{a,b}, Deborah J. Colesa^a, Donald L. Swiderski^a, Adriana Di Polo^c, Yehoash Raphael^{a,b}, and Bryan E. Pfingst^{a,b}

^aKresge Hearing Research Institute, Department of Otolaryngology, University of Michigan Medical School, 1150 W. Medical Center Drive, Room 4605 Med Sci II, Ann Arbor, MI, USA 48109-5616

^bNeuroscience Program, 4137 Undergraduate Research Building, Box 2215, 204 Washtenaw Ave., Ann Arbor, MI, USA 48109-2215

^cDepartment of Pathology and Cell Biology, Université de Montréal, P. 6128 succursale Centre-ville, Montreal, Quebec, Canada H3C 3J7

Abstract

The survival of the auditory nerve in cases of sensorineural hearing loss is believed to be a major factor in effective cochlear implant function. The current study assesses two measures of cochlear implant thresholds following a post-deafening treatment intended to halt auditory nerve degeneration. We used an adenoviral construct containing a gene insert for brain-derived neurotrophic factor (BDNF), a construct that has previously been shown to promote neuronal survival in a number of biological systems. We implanted ototoxically deafened guinea pigs with a multichannel cochlear implant and delivered a single inoculation of an adenovirus suspension coding for BDNF (Ad.BDNF) into the scala tympani at the time of implantation. Thresholds to electrical stimulation were assessed both psychophysically and electrophysiologically over a period of 80 days. Spiral ganglion cell survival was analyzed at the 80-day time point. Compared to the control group, the Ad.BDNF treated group had lower psychophysical and electrophysiological thresholds as well as higher survival of spiral ganglion cells. Electrophysiological, but not psychophysical, thresholds correlated well with the density of spiral ganglion cells. These results indicate that the changes in the anatomy of the auditory nerve induced by the combination of Ad.BDNF inoculation and the electrical stimulation used for testing improved functional measures of CI performance.

Keywords

animal psychophysics; EABR; spiral ganglion cell; growth factors; electrical stimulation; gene therapy; adenovirus; Ad.BDNF

Corresponding Author: Yehoash Raphael, Ph.D., Kresge Hearing Research Institute, 1150 W Medical Center Drive, 9301 MSRB - 3, Ann Arbor, MI 48109-5648, Phone: (734) 936-9386, Fax: (734) 615-8111.

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1. Introduction

The most accepted treatment for sensorineural hearing loss (SNHL) at the level of the inner ear is the cochlear implant (CI), a neural prosthesis that electrically stimulates the auditory nerve and replaces the actions of lost hair cells and cochlear mechanisms. Current CIs provide a lower quality of hearing than acoustic hearing, especially for complex sounds such as music and speech in noisy background. One variable known to affect the perception of sound with a CI is the condition of the surviving auditory nerve (Colombo and Parkins, 1987; Pfingst et al., 1981; Pfingst and Sutton, 1983; Shepherd and Javel, 1997). In animal models of SNHL, degeneration of the spiral ganglion cells (SGCs), the primary neurons of the auditory pathway, often follows hair cell loss (Leake and Hradek, 1988; Spoendlin, 1975; Webster and Webster, 1981).

Trophic support of SGCs may be provided by electrical stimulation from the cochlear implant. Many studies that show an effect of electrical stimulation use chronic or continuous levels of electrical stimulation (several hours/day, every day of the week for several weeks or months) (Miller, 2001). There is, however, evidence that lower levels of electrical stimulation, such as that used in psychophysical or electrophysiological testing, can promote SGC survival, especially when that stimulation is initiated within a week of deafening (Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al., 1997). The combination of electrical stimulation with growth factor treatment has also been shown to be more effective than either treatment alone in promoting SGC survival (Hegarty et al., 1997; Kanzaki et al., 2002; Shepherd et al., 2005). Importantly, the post-deafening treatment of growth factors and electrical stimulation can improve the physiological response to cochlear implant stimulation (Shepherd et al., 2005; Shinohara et al., 2002).

Both *in vivo* and *in vitro* work indicate a number of growth factors can promote SGC survival, including brain-derived neurotrophic factor (BDNF) (Gillespie et al., 2003; Hartnick et al., 1996; Miller et al., 1997; Nakaizumi et al., 2004; Shinohara et al., 2002; Staecker et al., 1996), neurotrophin-3 (NT-3) (Bowers et al., 2002; Farinas et al., 2001; Staecker et al., 1995; Staecker et al., 1996), glial cell line-derived neurotrophic factor (Altschuler et al., 1999; Kanzaki et al., 2002; Yagi et al., 1999; Ylikoski et al., 1998), and ciliary-derived neurotrophic factor (Hartnick et al., 1996; Staecker et al., 1995). BDNF and NT-3 have also been shown to induce regrowth of auditory nerve peripheral processes (Malgrange et al., 1996; Staecker et al., 1996; Wise et al., 2005). Both BDNF and NT-3 are necessary for normal inner ear development, and there appears to be a longitudinal gradient of these proteins in the developing cochlea such that NT-3 is more robust in the basal end of the cochlea and BDNF more robust in the apex of the cochlea (Fritzsche et al., 1999). Knockout mice lacking the NT-3 protein show an incomplete development of the basal region of the cochlea, and BDNF-deficient mice show an incomplete development of the apical portion of the cochlea (Farinas et al., 2001; Fritzsche et al., 1997a; Fritzsche et al., 1997b). These data suggest auditory nerve survival in the basal region of the cochlea may be most influenced by NT-3. However, there is some evidence that the longitudinal gradients and actions of NT-3, BDNF, and their high-affinity receptors in the cochlea may reverse over time (Adamson et al., 2002; Schimmang et al., 2003). The ability of either growth factor to promote neuronal survival may therefore vary considerably from developing to adult tissue, and both NT-3 and BDNF are currently considered effective options for mature auditory nerve survival treatments.

In animal models of SNHL, the most common method of introducing growth factors into the deafened cochleae is through osmotic pumps, which allow a short-term but continuous release of the chosen growth factor. While the continuous supply of drugs has advantages over a bolus injection, these pumps are susceptible to a number of problems, including rapid degradation of exogenous proteins at body temperature, potential infection from changing the pump, and

cannula clogging. It is possible that this exogenous growth factor treatment may need to be constantly maintained in order to preserve any benefit to SGC survival (Gillespie et al., 2003). Gene therapy may help overcome these issues by instigating a continuous supply of fresh and locally made growth factors. The morphology of the cochlea makes it a good candidate for gene therapy and previous work using viral vectors to upregulate growth factors in the cochlea has shown that transgene expression can be seen in the mesothelial cells that line the scala tympani (Yagi et al., 1999). This effect has been seen throughout the length of the cochlea (Nakaizumi et al., 2004; Raphael et al., 1996; Stover et al., 1999), indicating the ability of a single injection to transfect from the base to the apex.

An adenoviral construct containing a BDNF gene insert (Ad.*BDNF*) has been previously characterized both *in vivo* and *in vitro* (Di Polo et al., 1998; Nakaizumi et al., 2004; Rejali et al., 2007). Di Polo et al. (1998) showed the effect of this viral construct on the survival of retinal ganglion cells following axotomy, where transgene expression of Ad.*BDNF* was seen in Müller glial cells along with enhanced survival of retinal ganglion cells. This study was the first to provide *in vivo* evidence that Ad.*BDNF* transfected cells secrete bioactive BDNF (Di Polo et al., 1998). Rejali et al. (2007) more recently showed that Ad.*BDNF* transfected guinea pig fibroblast cells could release bioactive BDNF *in vitro*. The introduction of Ad.*BDNF* into the cochlea following aminoglycoside deafening has been shown to enhance SGC survival at both 28 days (Nakaizumi et al., 2004) and 48 days (Rejali et al., 2007) post-inoculation. However, no research to date has addressed the impact of Ad.*BDNF* treatment on functional measures. In the current study, we introduced Ad.*BDNF* to the aminoglycoside-damaged cochlea of guinea pigs, and assessed psychophysical and electrophysiological detection thresholds to cochlear implant stimulation. Data collected over 80 days showed that the combination of adenoviral-mediated growth factor upregulation and non-continuous electrical stimulation used for threshold testing resulted in improved auditory nerve survival and that this survival was associated with lower (better) functional thresholds.

2. Methods

2.1. Overview

Following operant conditioning with acoustic stimuli, animals were unilaterally ototoxically deafened, inoculated with one of two adenoviral constructs (Ad.*BDNF* or Ad.Empty), and implanted with a multichannel cochlear implant. Following implantation, psychophysical and electrophysiological thresholds were measured for 80 days. Animals were then euthanized and both cochleae were prepared for histological analysis. A separate group of animals was used to assess BDNF levels in cochlear fluids at one and two weeks following inoculation.

2.2 Subjects

Eight pigmented male guinea pigs (Elm Hill, Chelmsford, Mass, USA) were used in the first part of this study, four animals in an experimental group and four animals in a control group. Animals began psychophysical training with a free-feeding schedule until weights reached 400 g, at which time a restricted diet was instituted. This restricted diet was designed to keep animals at 80 % of free-feeding weight, encourage performance in a food-reward based psychophysical task, and maintain good health. Animal weights at time of inoculation and implantation ranged from 780–1200 g. Pure tone auditory brainstem response thresholds verified normal hearing in all tested animals prior to being placed in one of the treatment groups.

2.3. Adenoviral Vectors

Animals received one of two adenoviral constructs. Animals in the experimental group received an adenoviral vector with a mouse *BDNF* gene insert, driven by the cytomegalovirus promoter, labeled as Ad.*BDNF*, as previously described (Di Polo et al., 1998). Control animals

received an adenoviral construct containing no gene insert, labeled as Ad.Empty (a gift from GenVec, Inc., Gaithersburg, MD, USA).

2.4. Deafening

All animals were unilaterally deafened in the left ear via an aminoglycoside antibiotic (neomycin) introduced directly into the cochlea. For general anesthesia animals were given a ketamine (40 mg/kg) and xylazine (10 mg/kg) mix (IM), as well as atropine (0.05 mg/kg, SQ), and kept warm using a heating blanket. Lidocaine was used as a local anesthetic, and an incision was made in an arc caudal to the pinna of the left ear. The underlying muscle and tissue were gently pushed back to reveal the bulla and a small hole was made in the bulla with the tip of a scalpel blade. The round window was punctured and some perilymph was absorbed. Neomycin (10 % neomycin sulfate in sterile water, 60 μ L) was slowly infused into the scala tympani via the round window using a 100 μ L glass syringe and a 30 G needle. Following neomycin infusion, the hole in the bulla was sealed with carboxylate cement and the incision in the skin was stitched in layers. Although other methods of aminoglycoside deafening are effective (West et al., 1973; Xu et al., 1993), direct infusion of neomycin into the perilymph was chosen as the ototoxic method in this study to ensure a within-subject control, as this technique does not deafen the contralateral ear. Ototoxic deafening of only one ear also allows the option to implant the contralateral ear and continue behavioral experiments should the implant fail. Neomycin in volumes as low as 10 μ L has been shown to not only eliminate hair cells, but also create a flat, uniform epithelium from the organ of Corti within 4 days of injection into the perilymph (Kim and Raphael, 2007).

2.5. Inoculation and Implantation

All animals were unilaterally implanted in the deafened left ear. Four days after neomycin deafening, animals were given general and local anesthesia as detailed above, and an incision was made down the midline of the head. Muscle and connective tissue were gently pushed apart to reveal the skull. Six screws were placed in the skull; three for electrically-evoked auditory brainstem response recordings and three screws formed a triangle around bregma and held the head of a restraining bolt, which held the implant in place. A thin coat of acrylic was applied to these screws to aid in securing the restraining bolt. The incision from the deafening procedure was then re-opened and the carboxylate cement removed. For treated animals, a single injection of 5 μ L Ad.*BDNF* (approximately 4×10^{12} adenoviral particles/mL) was placed through the round window into the perilymph of the basal turn of the scala tympani via a bent tip 30 G needle and a 10 μ L glass syringe. In control animals, 5 μ L of Ad.Empty was administered in a similar manner. Animals were left undisturbed for 20 minutes to allow permeation of the adenoviral solution. A small cochleostomy was then made apical to the round window to expose the scala tympani, and a multichannel cochlear implant was placed into the scala tympani. All animals received an 8-electrode implant, which resembled the apical end of the Nucleus CI-22™ human implant and was manufactured by Cochlear Corporation (Nucleus Ltd., Lane Cove, Australia). The implants had an approximate center-to-center distance between electrodes of 0.75 mm, which allowed 5–6 electrodes to be inserted into the cochlea. Post-mortem evaluations of implant position revealed an insertion depth range of 2.25 – 5.90 mm, an average of 4.27 ± 1.09 mm, and no differences between experimental and control groups (Student's t-test $p = 0.63$). Only six of the eight implant electrodes were stimulated, and were labeled A through F where A was the most apical. All electrodes were within the first turn of the cochlea. The letter G was used to identify a ground electrode, which was placed in the post-auricular muscle. See Fig. 1 for details on the implant dimensions and labeling system.

2.6. Psychophysical assessment

Animals were trained in a go-no go positive reinforcement auditory stimulus detection task, similar to that described (Miller et al., 1995; Prosen et al., 1978; Su et al., 2008). Briefly, animals were placed in a wire cage inside a sound-attenuating chamber and trained to respond to acoustic stimuli (including both pulse trains and sinusoids) by releasing a button. A food reward was given for correct responses. The method of constant stimuli was used to vary sound pressure levels, and threshold was defined as the level at which correct releases occurred on 50 % of the trials. An animal was considered trained when 10 consecutive thresholds were within a range of ± 5 dB SPL.

The day after implantation, animals began testing with the same paradigm as described above, but with electrical stimulation from the cochlear implant. Animals were tested for thresholds to cochlear implant stimulation in one 90-minute session per day, 5 days/week, for 11 weeks. In our psychophysical detection paradigm, it can take up to 40 days for animals to obtain stable thresholds to electrical stimulation. We continued our experiment for double this time (80 days) in order to obtain enough threshold values in each electrode configuration for valid statistical tests. Testing began with the most apical monopolar electrode configuration (labeled B–G, see Fig. 1), which was tested until a threshold was obtained. Thresholds for each remaining configuration were then obtained on a random schedule for the remainder of the testing period. Six electrode location and configuration combinations were tested on each of the eight animals used in the study. These electrode combinations consisted of apical and basal end of the implant tripolar configurations (electrodes A–B–C and C–D–E), apical and basal bipolar configurations (A–B and D–E), and apical and basal monopolar (B–G and D–G) configurations.

The electrical stimulus for psychophysical testing was a train of 100Hz sinusoid bursts (200 msec on, 100 msec off), generated by a Tucker-Davis Technologies (TDT) digital signal processor. The stimuli were sent to a TDT programmable attenuator, then to a controlled-current stimulator and finally to the animal's implant. To monitor any changes in implant status, electrode impedances were measured daily using a 1 μ A rms, 1 kHz sinusoidal stimulus.

2.7. EABR

Electrically-evoked auditory brainstem responses (EABRs) were recorded every two weeks, beginning one week post-implantation. Electrical stimuli were generated by a TDT digital processor, sent to a programmable attenuator, fed to a controlled-current stimulator, and delivered to the implant through a percutaneous connector mounted on the animal's skull. Neural activity was recorded using alligator clips attached to screws that were placed in the skull 2 cm anterior to bregma, 1 cm lateral of bregma on the implanted side, and 1 cm posterior to bregma. A bipolar and monopolar electrode configuration was tested at both apical and basal locations on the implant, giving four stimulating electrode pairs (A–B, B–G, D–E, and D–G, see Figure 1). Stimuli were 50 μ sec phase duration monophasic alternating polarity square pulses. EABR threshold was defined as the lowest level at which there was a repeatable P3 wave, as agreed upon by two unbiased observers.

2.8. Histology

Eighty days post-implantation, the animals were deeply anesthetized and perfused intracardially with 2 % glutaraldehyde and both cochleae were removed. Tissue processing was completed as previously described (Yagi et al., 2000), modified as described below. Each cochlea was locally perfused with 4 % paraformaldehyde and placed in EDTA solution to decalcify until sufficiently soft for sectioning (approximately 1 month). Once decalcified, the implant was removed from the left ears, and each cochlea was embedded in JB-4 resin and sectioned in the mid-modiolar plane, which provided 6 measurable profiles of Rosenthal's canal (Kanzaki et al., 2002). Sections were 3 μ m thick, and every third section was kept and

stained with 1 % Toluidine Blue in 1 % Sodium Borate. These sections were evenly divided into a caudal, middle and rostral group and one slide from each of these groups was chosen by a random number generator for evaluation. Each profile of Rosenthal's canal in each of these three slides was evaluated by an observer who was blinded to the treatment groups, using SPOT Imaging™ software for data acquisition and Meta-Morph Offline™ software for data analysis. The outer edge of each region of Rosenthal's canal was traced using MetaMorph Offline™ software and the two-dimensional area was calculated by the software. The two-dimensional somatic area of SGCs with clearly defined nuclei was assessed in the same way, and the number of these SGCs per Rosenthal's canal region was counted.

2.9. Data Analysis

Log transformations on all psychophysical and electrophysiological data points were completed to normalize psychophysical and electrophysiological thresholds. For individual animal data, there were no differences seen in either change over time (slope) or range (maximum – minimum values) in psychophysical and electrophysiological thresholds. Therefore, all data points throughout the 80-day test period were averaged to determine a threshold value for each animal for each electrode configuration (six values for each animal) (Figure 2 and Figure 4). Each of the electrode configurations tested represents an independent variable, and Student's t-test was used to determine differences between experimental and control groups for each configuration.

The data for one configuration (A–B) was separated by date post-implantation, and a two-way repeated measures ANOVA test was performed on both psychophysical and EABR data (Fig. 3 and Fig. 5). Student's multiple comparison post-hoc tests determined specific differences between groups and over time. EABRs were collected every two weeks and an average threshold and standard error were determined for each of these dates for control and treated groups. Psychophysical detection thresholds were collected on a daily basis, but not every configuration was run each day. In order to compare to electrophysiological data, psychophysical threshold levels for each animal within a two-week timeframe (i.e. data collected between 7 and 21 days post-implantation) were averaged, and these values were used to determine a psychophysical detection threshold average and standard error.

To determine differences in morphology, SGC density and cross-sectional somatic area were compared between groups. The data from the three slides of each individual were averaged to determine a mean cross-sectional somatic area for each turn of the cochlea (lower basal, upper basal, middle, and apical) (Table 1). Density was calculated for each animal by dividing the average number of SGCs within each region of Rosenthal's canal by the average cross-sectional area of that region. To eliminate the possibility of individual animal differences in the area of Rosenthal's canal, we compared the areas of this region for each turn of the cochlea (Table 1). One-way ANOVAs and Student's t-tests were used to evaluate differences between the Ad.*BDNF*, Ad.Empty, and non-deafened groups for each turn of the cochlea. Our non-deafened control group was composed of the right, non-deafened, non-implanted, and non-inoculated cochleae of both Ad.*BDNF* and Ad.Empty inoculated groups. Linear regressions were used to determine correlations between psychophysical detection thresholds and SGC density as well as between EABR detection thresholds and SGC density. All analyses were completed using SigmaStat™ (Jandel Scientific).

2.10. Ad.*BDNF* activity in vivo

In a separate experiment, six guinea pigs were inoculated with Ad.*BDNF* (same concentration as above) in the left cochlea through the round window, using the same surgical procedure as detailed above. One or two weeks post-inoculation (n = 3 for each timepoint), animals were sacrificed, decapitated, and both left and right temporal bones were removed. The basal turn

of the cochleae from each ear was carefully thinned using fine-tipped forceps until a hole was made in the basal turn outer wall. A micro-capillary tube was gently inserted into this hole, and 1–2 μ l of cochlear fluids were extracted from each cochlea. Samples were diluted 100 fold using the sample diluent from a ChemiKine™ BDNF Sandwich ELISA Kit. This kit was used to determine BDNF levels in the cochlear fluids, following the instructions in the kit. The non-inoculated right ears of both groups ($n = 6$) were used as controls. A one-way ANOVA was performed to determine differences between the one week inoculated ears, the two week inoculated ears, and the right, non-inoculated control ears.

2.11. Animal Care

This study was performed in accordance with National Institutes of Health Guidelines (Guide for the Care and Use of Laboratory Animals, 1996). The University Committee on the Use and Care of Animals at the University of Michigan approved the experimental protocols. Veterinary care and animal husbandry were provided by the Unit for Laboratory Animal Medicine, in facilities certified by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, Intl.)

3. Results

3.1 BDNF levels in vivo

The BDNF-ELISA kit revealed average levels of BDNF in the right, non-inoculated cochleae of 0.78 ng/ml (± 0.86). The levels of BDNF in the cochlear fluids one week post-inoculation were similar (0.60 ± 0.66 ng/ml). There was a marked increase in BDNF levels in the cochlear fluids tested two weeks post-inoculation (3.7 ± 2.95 ng/ml). A one-way ANOVA revealed a statistically significant difference between groups ($p = 0.05$), and a post-hoc multiple pairwise comparison revealed that the two week group was significantly different from the control group ($p = 0.05$)

3.2. Psychophysical detection thresholds

The Ad.*BDNF* treated group showed significantly lower psychophysical detection thresholds than the Ad.Empty group in the apical bipolar configuration (labeled A–B) (Student's t-test, $p = 0.03$). Mean thresholds for the Ad.*BDNF* group were also lower for the apical tripolar configuration (A–B–C), but this was not significant ($p = 0.06$). For the monopolar configuration at the apical end of the implant as well as all configurations at the basal end of the implant, thresholds for the two groups were similar and not significantly different. Fig. 2 shows both individual psychophysical detection thresholds, averaged over the entire testing period (closed symbols), as well as group averages $\pm$ one standard error of the mean (SEM) (open symbols with bars) for each electrode configuration tested. The labels are arranged left to right along the x-axis from the apical end of the implant for tripolar, bipolar, and monopolar configurations to the basal end of the implant for tripolar, bipolar, and monopolar configurations.

3.3. Psychophysical threshold changes over time

The psychophysical detection threshold values did not significantly change over time for either of the Ad.*BDNF* and Ad.Empty groups, but there was a difference between group thresholds that was present throughout the experiment. Fig. 3 shows psychophysical detection threshold averages for each two-week period of the experiment, using the A–B configuration data. A two-way repeated measure ANOVA did not reveal a significant interaction between group and time ($p = 0.76$) or a difference in time ($p = 0.63$), but did reveal a difference between groups ($p = 0.02$). This suggests that the lower thresholds seen in the A–B configuration for the Ad.*BDNF* group in Fig. 2, which were thresholds averaged over the entire experiment time,

were lower throughout the length of the experiment and not dependent on time post-implantation.

3.4. EABR thresholds

In both of the bipolar configurations (A–B and D–E), the Ad.*BDNF* group had significantly lower electrophysiological thresholds than the Ad.Empty group (Student's t-test $p = 0.002$ for A–B and $p = 0.028$ for D–E). The difference between groups was not significant in either of the monopolar configurations ($p = 0.08$ for B–G and $p = 0.16$ for D–G). Fig. 4 shows both individual EABR thresholds, averaged over the testing period (closed symbols), and group averages $\pm$ one SEM (open symbols) for each electrode configuration tested. Labels on the x-axis in Figure 4 are arranged left to right from the apical end of the implant for bipolar and monopolar configurations to the basal end of the implant for bipolar and monopolar configurations.

3.5. EABR changes over time

The differences in EABR detection thresholds between treated and control groups as a function of time were slightly different from those seen in psychophysical detection thresholds. Fig. 5 shows the EABR detection thresholds at each test date for the A–B configuration. A two-way repeated measures ANOVA revealed a statistically significant interaction between group and time ($p = 0.01$). Although the detection thresholds for both the Ad.*BDNF* and Ad.Empty group were initially similar, there was a decrease in EABR thresholds for the Ad.*BDNF* group as well as an increase in EABR thresholds for the Ad.Empty group over time. For week 3 through 11 post-implantation, the Ad.*BDNF* treated animals had significantly lower thresholds than the Ad.Empty control group ($p \leq 0.05$).

3.6. Morphological changes

Upon dissection, each cochlea was visually inspected for markers of trauma or infection, including broken bone, bony growth, opaque cochlear fluids, and/or fluid in the middle ear space. There were no observed signs of a major infection in any of the animals used in the study. In all animals included in this study there was a loss of the organ of Corti structure following neomycin infusion (Fig. 6). Image 6A shows the organ of Corti for an Ad.*BDNF* treated animal, 6B shows the organ of Corti of an Ad.Empty treated animal, and 6C shows the organ of Corti of a right, non-deafened cochlea. These images are representative of the basal, middle, and apical turns of all groups. A flat epithelial layer with no hair cells or differentiated supporting cells, typical of ototoxic deafening, was observed in all animals in the Ad.*BDNF* and Ad.Empty groups.

SGC data were divided into lower basal, upper basal, middle, and apical turns of the cochlea for analysis. There was a higher density of surviving neurons in Ad.*BDNF* treated cochleae than in the Ad.Empty treated cochleae in the upper basal turn region, but there was not a significant difference between these groups in the lower basal, middle, or apical turns (Fig. 8). There are two animals in the Ad.Empty group whose data for the lower basal turn are near the level of that seen in the Ad.*BDNF* group. Because these animals received no growth factor treatment, the level of SGC survival in this region of the cochlea, which is physically closest to the implant, may reflect the influence of electrical stimulation alone on SGC survival. Although it may appear that there are outliers in the Ad.*BDNF* group (animals with high density values), the animal with the highest SGC number is not the same in all turns.

The density for both Ad.*BDNF* and Ad.Empty groups was significantly lower than the density for the non-deafened control group in all turns of the cochlea (one-way ANOVA $p < 0.01$ for all cochlear turns); therefore, Fig. 8 shows data from only Ad.*BDNF* and Ad.Empty groups. There were no differences seen between the non-deafened control group, the Ad.*BDNF* group,

and the Ad.Empty group in either cross-sectional SGC somatic area or cross-sectional area of Rosenthal's canal (Table 1). These data suggest that the differences in SGC density between the Ad.*BDNF* and Ad.Empty groups was due to an increase in the number of SGCs in the Ad.*BDNF* group, and not due to a difference in the relative sizes of the SGCs and Rosenthal's canal.

3.7. Correlation of results

The density of surviving SGCs was compared to both psychophysical detection thresholds and EABR thresholds. Thresholds for the A–B configuration were compared to SGC density for the upper basal turn of the cochlea. This electrode configuration and region of the cochlea showed the greatest difference between the Ad.*BDNF* and Ad.Empty groups. We expected to see lower thresholds for those cochleae that showed a higher density of surviving neurons if the survival status of the auditory nerve directly affected cochlear implant performance. Fig. 9 shows the psychophysical-SGC comparison (9A) and the EABR-SGC comparison (9B). Regression analysis found a significant relationship between the density of surviving SGCs and EABR thresholds ($F = 15.64$, $df = 1, 6$, $p = 0.008$) and a non-significant relationship between surviving SGCs and psychophysical detection thresholds ($F = 0.25$, $df = 1, 6$, $p = 0.64$). The relationship between EABR thresholds and the density of SGCs was also highly correlated ($r^2 = 0.72$). The slope of this linear regression was negative (-2.97), indicating lower thresholds with increasing SGC number.

4. Discussion

In this study, a single inoculation of Ad.*BDNF* in combination with non-continuous electrical stimulation promoted SGC survival 84 days post-deafening and lowered psychophysical and electrophysiological thresholds for cochlear implant stimulation for specific electrode configurations and locations. Previous work on the effects of growth factors and electrical stimulation in the cochlea have shown similar results with EABR and SGC measurements, but this study was, to our knowledge, the first to address the effects of these post-deafening treatments on psychophysical detection thresholds. This functional measurement may be more clinically relevant and reflective of an animal's ability to hear with a cochlear implant than the electrophysiological assessment typically collected from animal models of cochlear implant function. We have also shown that Ad.*BDNF* inoculation into the cochlear fluids via the round window leads to an increase in BDNF production within 2 weeks post-inoculation, providing a link between the introduction of the adenovirus into the cochlea and our observed morphological and physiological changes.

4.1. Long-term effects of adenoviral treatment and electrical stimulation

The current study assessed the morphological status of the auditory nerve almost 12 weeks post-inoculation of Ad.*BDNF*, a relatively long time for experiments on neurotrophic factors and auditory nerve survival. Previous *in vivo* work addressing the trophic effects of BDNF on the damaged auditory nerve have examined SGC status at 2 weeks (Miller et al., 1997), 4 weeks (Shepherd et al., 2005; Shinohara et al., 2002), and 8 weeks (Staecker et al., 1996) following treatment initiation. Importantly, all of these studies used osmotic pumps to supply a constant source of exogenous growth factors to the cochlea throughout the testing period. The current study used one inoculation of Ad.*BDNF* and non-continuous electrical stimulation and showed a high level of surviving SGCs in the basal turn of the cochlea after 12 weeks. Although it appears that there was an effect of Ad.*BDNF* treatment and electrical stimulation in both the lower and upper basal regions, the difference between treatment groups was not significant in the lower basal turn. This is the region of the cochlea where the implant was located, and the similarities between groups in this region may reflect the influence of direct electrical stimulation. Similar levels of electrical stimulation have been shown to improve SGC survival,

particularly in the vicinity of the implant (Hartshorn et al., 1991). The greatest effect of our post-deafening treatment was seen in the upper basal turn, and this may reflect either the spread of the adenoviral transfection or the region of the cochlea in which BDNF has its greatest influence.

In addition to long-term morphological effects, our treatment of a single inoculation of Ad.*BDNF* in combination with electrical stimulation also had long-term functional effects. Electrophysiological threshold levels assessed at regular time intervals within the entire experiment showed an effect of time in the A–B configuration. The thresholds for the Ad.Empty group worsened (increased) over time, whereas the thresholds for the Ad.*BDNF* group improved (decreased) over this same period. Presumably, the auditory nerve was progressively degenerating in the Ad.Empty group over this time period (Leake and Hradek, 1988), and this is what lead to the increasing EABR thresholds. The decrease in EABR thresholds for the Ad.*BDNF* group indicates that the Ad.*BDNF* inoculation in combination with the electrical stimulation from the cochlear implant was maintaining auditory nerve status and function by preventing post-deafening degeneration and/or promoting neural regrowth, either of which would have a positive effect on cochlear implant function.

4.2. Psychophysical and electrophysiological thresholds

Our data show an effect of adenoviral-mediated upregulation of BDNF and electrical stimulation on two measures of the cochlear implant function, psychophysical detection thresholds and EABR thresholds. However, the results for these two measures are not identical. The greatest effect on psychophysical detection of cochlear implant stimulation was seen at the apical end of the implant. Electrode configuration A–B was the only tested configuration where the introduction of Ad.*BDNF* led to a statistically significant improvement in psychophysical thresholds. This configuration used the two most apical electrodes on the implant as stimulating and return channels. The insertion method used in this study (through a cochleostomy) typically places the electrodes at the apical end of the implant close to the modiolus, as this end of the implant fills the scala tympani. Depending on the individual implantation, the basal end of the implant could either hug the modiolus or arc outward. The location of the electrodes at the basal end of the implant therefore probably varied across animals between close to modiolus and close to the outer scala tympani wall. This means that the A and B electrodes were more likely than other electrodes to be physically closest to Rosenthal's canal and surviving SGCs, and would probably stimulate a more restricted portion of the nerve. The apical tripolar configuration, labeled A–B–C, also used the most apical electrodes in the implant, and the data for this configuration showed a similar trend to that seen in the A–B configuration, where psychophysical detection thresholds were lower in the Ad.*BDNF* treated group than in the Ad.Empty group. However, the monopolar configuration at the apical end of the implant (B–G) did not show a difference between groups. Because of the distance between stimulating and return electrodes, bipolar and tripolar configurations typically stimulate a more discrete group of neurons than monopolar configurations. These data suggest that for psychophysical detection thresholds to cochlear implant stimulation, focused electrical stimulation nearest to the auditory nerve is most likely to show differences in SGC survival.

Electrode configuration, but not necessarily the distance between the implant and the nerve, played a role in determining effects of our post-deafening treatments on EABR thresholds. In our EABR data, the greatest difference between the Ad.*BDNF* and Ad.Empty groups was seen in the A–B and D–E configurations. These were bipolar configurations that used electrodes at both the apical (A–B) and basal (D–E) end of the implant. We did not see an effect of treatment on either monopolar configuration tested. These data suggest that for electrophysiological thresholds to cochlear implant stimulation, a smaller spread of excitation is most likely to show

differences in SGC survival, even if the source of that excitation is farther from the nerve itself, as in the basal end of the implant bipolar configuration (D–E).

Both of the functional measures used in this study suggest that focal stimulation of the surviving auditory nerve may best reveal an effect of growth factor and electrical stimulation treatment. Both psychophysical and electrophysiological thresholds are important functional indicators of the effect of neurotrophic treatments on auditory nerve survival. The EABR is a gross field potential measurement, and requires the synchronized activation of a large number of neurons. Psychophysical responses may or may not require a similar degree of synchronicity as in the EABR. Our EABR thresholds correlated better with the density of surviving SGCs than did the psychophysical detection thresholds. By requiring a high level of synchrony between neurons in order to obtain a response, EABR thresholds may be a better indicator of the pathological disruptions in the timing of the surviving nerve activation. However, this coarse metric of auditory nerve status may not be completely indicative of an animal's ability to perceive and process sound from the cochlear implant.

4.3. Ad.BDNF transgene expression and electrical stimulation

The levels of electrical stimulation provided to the cochlea to obtain the data for our functional measures are similar to levels that have previously been shown to improve SGC survival (Hartshorn et al., 1991). This stimulation may have helped maintain auditory nerve survival throughout our experiment, as viral constructs are effective in the cochlea for a few weeks at most (Raphael et al., 1996; Weiss et al., 1997). Using the same vector as in this study, Di Polo et al. (1998) saw a peak of Ad.BDNF expression at 7 days after inoculation into the retina and no expression beyond 14 days post-inoculation. As our study continued until almost 12 weeks, it is unlikely that transfected cells were still secreting BDNF at the end of the experiment. Rather, BDNF levels were probably high in the first few weeks after viral vector inoculation, as indicated by our ELISA data, and returned to baseline levels later. At that point, electrical stimulation may have contributed to the survival and functionality of the neurons. Our results are consistent with the idea that continued electrical stimulation could provide significant protective effects once growth factor secretion or application is diminished (Shepherd et al., 2007).

Substantial immune response is not typically seen in the cochleae of guinea pigs after one inoculation of adenovirus vectors, especially when the animals are specific-pathogen-free and have not experienced an adenovirus infection prior to the experiment (Ishimoto et al., 2003; Raphael et al., 1996). The empty vector control used here was an advanced generation vector and less likely to cause an immune reaction than the first generation vector Ad.BDNF, although we did not see any major indications of an immune response in either the Ad.BDNF or the Ad.Empty groups. Still, a mild and not easily detectable response to the virus may occur, which we expect is more likely with first generation vectors than with later generation vectors. Our outcome may therefore be an under-estimate of the protective ability of BDNF, because the adenovirus itself may have elicited some negative side effect. Indeed, the variations in the construction of viral vectors (deleted regions, promoters, etc.) can make comparison of viral vector efficacy somewhat difficult. However, it is clear that gene therapy such as that used in this study can be effective in treating inner ear damage and in influencing auditory nerve function.

There is an ongoing discussion regarding the cochlear gradients of BDNF and NT-3 and their respective high-affinity receptors in the adult cochlea (Adamson et al., 2002; Schimmang et al., 2003), although the developmental patterns of BDNF and NT-3 are well-established (Farinas et al., 2001; Fritsch et al., 1999). It may be possible that the increased SGC survival we have seen exclusively in the basal turn is related to the choice of neurotrophic factor in our viral construct. The use of Ad.NT-3 or a combination of Ad.BDNF and Ad.NT-3 may provide

more survival in the middle and apical turns if there is a greater effect of NT-3 than BDNF in these regions of the adult cochlea.

4.4. Conclusions

We found that the introduction of Ad.*BDNF* in conjunction with the electrical stimulation required for psychophysical and EABR testing led to decreased psychophysical and electrophysiological thresholds as well as increased SGC survival in deafened guinea pigs. The current results add to previous research supporting the use of neurotrophins in combination with cochlear implants to aid in rehabilitation for severe sensorineural hearing loss. We also extend the clinical relevance of this approach by including multiple functional assessments and by using relatively long-term treatments in the deafened cochlea. We found a demonstrable behavioral effect of adenoviral-mediated changes in BDNF gene expression and electrical stimulation of the deafened cochlea over a period of 80 days. In addition, the results from this study highlight the need for several measurements of auditory nerve function, as EABR and psychophysical thresholds showed different patterns following treatment. Attention should be paid to both electrode configuration and the location of the electrode with respect to the nerve when assessing the effects of growth factor treatment on cochlear implant function.

Abbreviations Used

BDNF, Brain-derived neurotrophic factor; CI, Cochlear implant; EABR, Electrically-evoked auditory brainstem response; NT-3, Neurotrophic factor-3; SEM, Standard error of the mean; SGC, Spiral ganglion cell; SNHL, Sensorineural hearing loss.

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Figure 1. Diagram of multichannel implant and electrode configurations

The banded electrodes are labeled A through F, where A is at the most apical (first inserted) end of the implant. MP = monopolar stimulation where the return electrode was a ground wire placed in the post-auricular muscle. BP = bipolar stimulation where the return electrode was immediately adjacent to the stimulating electrode. TP = tripolar stimulation where the two electrodes surrounding the stimulating electrode were return electrodes. All stimulation configurations are placed on arbitrary electrodes for illustration purposes.

Figure 2. Psychophysical detection thresholds averaged over time

Each column represents a different electrode configuration (A-B-C, A-B, B-G, C-D-E, D-E, D-G). Closed symbols represent averages over time of individual animal data points for each configuration. Open symbols represent the group average ($n = 4$) and error bars are $\pm$ standard error of the mean (SEM). Circles represent animals that received Ad.BDNF inoculation and triangles indicate animals that received Ad.Empty inoculation. Differences between groups were assessed via Student's t-test; (*) indicates $p \leq 0.05$. P-values for each configuration are as follows: A-B-C: $p = 0.06$, A-B: $p = 0.03$, B-G: $p = 0.61$, C-D-E: $p = 0.56$, D-E: $p = 0.38$, D-G: $p = 0.68$.

Figure 3. Psychophysical detection thresholds over time, A-B configuration

Each data point represents the average of psychophysical detection thresholds collected in the preceding two-week period for the four animals in each experimental group. Most animals did not have valid thresholds in the first week following implantation; data therefore begin with the third week averages. Circles indicate Ad.BDNF treated animal averages and triangles indicate Ad.Empty treated animal averages at each time point. Error bars indicate $\pm$ one standard deviation. A two-way repeated measures ANOVA revealed an effect of group ($p = 0.02$) but not a significant effect of time ($p = 0.63$) or interaction between time and group ($p = 0.76$). Post-hoc multiple pairwise comparisons gave the following p -values: 3 weeks = 0.21, 5 weeks = 0.07, 7 weeks = 0.09, 9 weeks = 0.06, 11 weeks = 0.06.

Figure 4. EABR thresholds averaged over time

Symbols and statistical tests as in Figure 2. P-values for each configuration: A-B: $p = 0.002$, B-G: $p = 0.08$, D-E: $p = 0.03$, D-G: $p = 0.16$.

Figure 5. EABR detection thresholds over time, A-B configuration

Each data point represents a group average of thresholds obtained at each time point indicated. Symbols as in Figure 3. A two-way repeated measures ANOVA revealed a significant interaction between group and time ($p = 0.01$), and (*) indicates a significant difference between groups was found at that time point. Post-hoc multiple pairwise comparisons gave the following p-values: 1 week = 0.47, 3 weeks = 0.03, 5 weeks = 0.02, 7 weeks = 0.03, 9 weeks = 0.03, 11 weeks = 0.04.

Figure 6. Light microscopy images of the organ of Corti structure of the basal turn (A) represents the Ad.*BDNF* group, (B) represents the Ad.Empty group, and (C) represents the non-deafened, non-inoculated control group. There are no hair cells or supporting cells visible in (A) or (B), while inner hair cells (white arrow) and outer hair cells (black arrows) are observed in non-neomycin deafened ears (C). Scale bar = 50 μ m, Adobe Photoshop TM software was used to adjust contrast levels in all images.

Figure 7. Light microscopy images of the upper basal and middle turn of an Ad.BDNF (A, C) and an Ad.Empty (B, D) inoculated animal

The basal turn Rosenthal's canal (A, B) showed a greater number of surviving SGCs in the Ad.BDNF treated ear (A) than the Ad.Empty cochlea (B). The middle turn Rosenthal's canal (C, D) showed little difference between treatment groups in the number of surviving SGCs. Scale bar = 50 μ m, Adobe Photoshop TM software was used to adjust contrast levels in all images.

Figure 8. SGC density per cochlear turn for the Ad.BDNF and Ad.Empty groups

Symbols as in Figure 2. (*) indicates $p \leq 0.05$, Student's t-test p-values were: Lower Base: $p = 0.10$, Upper Base: $p = 0.02$, Middle: $p = 0.42$, Apex: $p = 0.60$.

Figure 9. Relationship between implant functional thresholds and number of surviving SGCs in (A) psychophysical testing and (B) electrophysiological testing

Psychophysical detection thresholds and EABR thresholds from the A–B configuration and SGC density from the upper basal turn are compared. Individual animal data points are plotted; circles represent animals that received Ad.*BDNF* inoculation and triangles indicate animals that received Ad.Empty inoculation. Lines represent linear regressions through all data points ($r^2 = 0.04$ in A and $r^2 = 0.72$ in B).

Table 1

Two-dimensional ganglion cell and Rosenthal's canal characteristics.

Treatment Group	Cochlear region	Spiral ganglion cell cross-sectional area (μm)	Rosenthal's canal cross-sectional area (μm)
Ad. <i>BDNF</i>	Lower Base	302.0 $\pm$ 55.5	64671.2 $\pm$ 4909.4
Ad.Empty	Lower Base	248.4 $\pm$ 45.3	74156.5 $\pm$ 26004.3
Non-deafened control	Lower Base	216.3 $\pm$ 46.9	59350.6 $\pm$ 16377.2
Ad. <i>BDNF</i>	Upper Base	286.5 $\pm$ 91.1	51404.9 $\pm$ 5776.9
Ad.Empty	Upper Base	223.4 $\pm$ 12.2	58849.8 $\pm$ 6383.7
Non-deafened control	Upper Base	170.8 $\pm$ 19.7	44544.9 $\pm$ 6962.1
Ad. <i>BDNF</i>	Middle	289.1 $\pm$ 118.2	45012.7 $\pm$ 4427.3
Ad.Empty	Middle	215.3 $\pm$ 44.8	45785.0 $\pm$ 3562.7
Non-deafened control	Middle	178.7 $\pm$ 35.8	46140.5 $\pm$ 7044.0
Ad. <i>BDNF</i>	Apex	266.5 $\pm$ 137.2	49895.4 $\pm$ 29493.1
Ad.Empty	Apex	201.9 $\pm$ 36.2	52449.1 $\pm$ 11231.7
Non-deafened control	Apex	202.5 $\pm$ 41.7	87128.1 $\pm$ 32769.5

Listed are the group averages $\pm$ one standard deviation for the SGC cross sectional somatic area and cross sectional area of Rosenthal's canal. Data are separated into a lower basal, upper basal, middle and apical turns of the cochlea. A one-way ANOVA analysis of each turn of the cochlea revealed no statistically significant differences between groups.