Novel Methods to Promote Survival and Regeneration of the Auditory Nerve and Improve Cochlear Implant Function

by

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Dedication

To my daughter Brielle, you've forever changed my perspective on life. Although you may not know it, you were the motivation I needed to finish this dissertation and to become a more productive and energetic person. I know you will continue to inspire me as you grow up, and I hope to be a source of inspiration for you.
I love you, little bee!

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Table of Contents

List of Figures

Figure

List of Appendices

Appendix

List of Abbreviations

BDNF	Brain – Derived Neurotrophic Factor
CI	Cochlear Implant
ES	Electrical Stimulation
NT – 3	Neurotrophic Factor – 3
PEDOT	Poly(3, 4 – ethylenedioxythiophene)
SGC	Spiral Ganglion Cell
SNHL	Sensorineural Hearing Loss
ST	Scala Tympani

Abstract

A principal cause of sensorineural hearing loss is injury to or loss of cochlear hair cells, which are critical components of sound transduction. These auditory hair cells do not naturally regenerate in mammals. Loss of hair cells often leads to a degeneration of the processes that innervate them along with the ganglion cell bodies of the auditory nerve. The only currently accepted treatment for hearing loss of this type is the cochlear implant, an auditory prosthesis that has been in clinical use for over 30 years. While the cochlear implant has been successful in providing or restoring hearing to over 100,000 patients, there are limitations to the hearing provided by the implant, particularly for complex sounds such as speech in noise and music. The peripheral processes and ganglion cell bodies receive and process the electrical stimulation from the implant, and thus the survival of these components of the auditory nerve is critical to the perception of sound from the cochlear implant. This dissertation presents two novel methods of promoting auditory nerve survival and regrowth following hair cell loss. The first method used an adenoviral construct containing a gene insert for brain – derived neurotrophic factor, designed to increase endogenous production of this growth factor. The introduction of this adenovirus into the cochlea led to a decrease in electrophysiological and psychophysical thresholds to cochlear implant stimulation and promoted long – term ganglion cell survival. This study was

unique in addressing the psychophysical effects of the anatomical changes induced by growth factor treatment. In the second method, a specialized implant coating was designed to attract growth of peripheral processes to make contact with a cochlear implant *in vivo*. In this study, a new histological technique was developed, which allowed visualization of peripheral processes and evaluation of their spatial relationship with an implant. This coating attracted significant neuronal growth in close proximity to the implant and decreased the impedance between implant electrodes. These studies together demonstrate the significant plasticity of the auditory nerve to survive following deafness, and indicate the potential for nerve regeneration efforts to improve cochlear implant performance.

Chapter 1

Introduction

Hearing loss is a major medical concern that typically presents in one of two forms. Conductive hearing loss is generally a problem with amplification, and the source of the hearing loss is in the middle ear or the outer ear. This type of hearing loss can usually be remedied by surgery or with a hearing aid, which is worn externally and enhances sound waves so they will reach the inner ear for normal neuronal processing. Sensorineural hearing loss (SNHL) occurs at the level of the inner ear, the auditory nerve, or higher levels of auditory system and is not easily remedied. When sound waves enter the inner ear, they vibrate the basilar membrane, to which cochlear hair cells are attached. These hair cells transduce the mechanical energy of sound waves into neural impulses and send temporal and frequency information via peripheral nerve fibers, to the primary ganglion cells of the auditory nerve, spiral ganglion cells (SGCs), located in the modiolus. The SGCs then send out central processes that form the auditory portion of the VIII cranial nerve, the vestibulocochlear nerve. Fig. 1.1 demonstrates the anatomy of the inner ear. In many cases of SNHL, the cochlear hair cells are missing or damaged and acoustic hearing is non – functional.

Auditory hair cells do not naturally regenerate in mammals, thus this form of hearing loss is permanent and irreversible.

The cochlear implant (CI) offers a viable and effective treatment option for those with severe to profound SNHL where hair cells are lost but some functional auditory nerve still exists. This prosthesis is an electrode array that is placed in one of the fluid – filled spaces of the cochlea and electrically stimulates the remaining auditory nerve. This implant bypasses the function of the cochlear hair cells by providing direct electrical energy to the SGCs. This stimulation can provide hearing to those who have never heard before, and restore hearing to those who have lost it. The CI functions well in quiet environments, but most users have trouble understanding complex sounds such as speech with background noise and music (Wilson et al., 2003; McDermott, 2004). Both clinical and basic research endeavors are underway with the goal of providing CI users with a more complete auditory experience. There are several CI models available, and the design of both the implant and the sound processor software has been greatly enhanced in the past several decades since the first implant (Rubinstein and Miller, 1999). In addition to implant engineering, several lines of research are aimed at improving the cochlear environment so that it is more receptive to electrical stimulation from the implant.

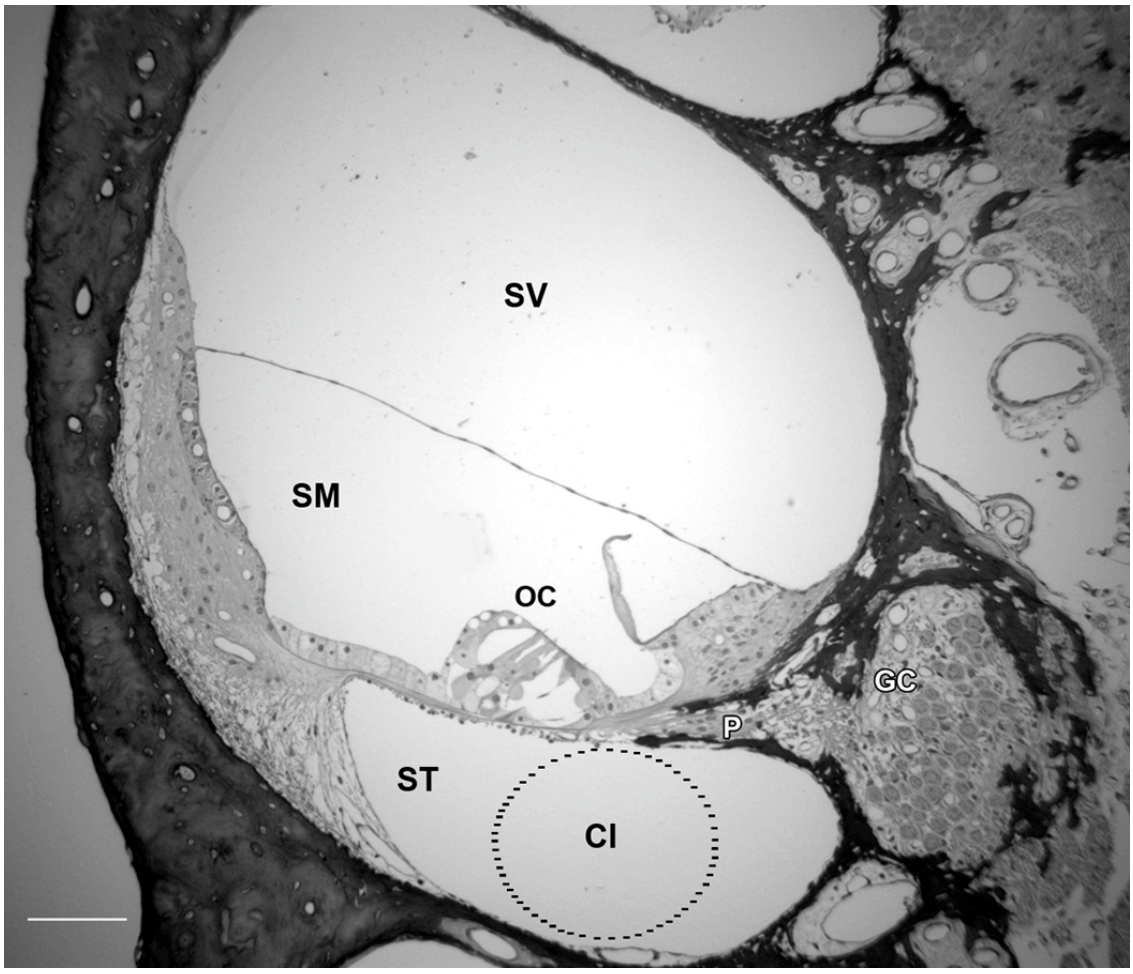


Fig. 1.1: Mid – modiolar section of guinea pig cochlea

A cross – sectional image of the guinea pig cochlea is shown to demonstrate the inner ear anatomy. The organ of Corti (OC), which contains cochlear hair cells, is located in the scala media (SM). This is one of the fluid – filled spaces of the cochlea, along with the scala vestibuli (SV) and the scala tympani (ST). Hair cells send out auditory nerve peripheral fibers (P) to ganglion cell bodies (GC). The cochlear implant (CI), indicated by the dashed circle, is placed in the ST, although exact lateral placement *in vivo* is variable. Scale bar = 100μm.

In humans, cochlear hair cell loss can be caused by a variety of genetic and environmental factors. Animal models of SNHL are similar to human pathologies and hair cell loss and nerve degeneration is typically induced by over – exposure to noise or ototoxic drugs. Aminoglycosides such as neomycin and kanamycin can destroy hair cells and the organ of Corti if applied either directly within the cochlea or given systemically (Prosen et al., 1978; Robertson and Johnstone, 1979; Kim and Raphael, 2007). The combination of an aminoglycoside and a loop diuretic, such as ethacrynic acid, given systemically, has also been proven effective at destroying hair cells and initiating neural degeneration bilaterally (West et al., 1973; Xu et al., 1993).

When inner hair cells are lost or damaged, a secondary and progressive degeneration of the peripheral processes, SGCs, and eventually the central auditory nerve often occurs (Spoendlin, 1975; Webster and Webster, 1981; Jyung et al., 1989). This degeneration could be due to a lack of electrical and chemical stimulation from the hair cells, as activity from both the peripheral and central sources is needed to maintain the integrity of SGCs. The level of degeneration is influential in the success of cochlear implantation, as the nerve needs to be viable and receptive to electrical stimulation. Fortunately, although hair cells do not naturally regenerate, both the peripheral processes and SGCs can be regenerated. A number of studies have shown that growth factor intervention following hair cells loss can slow or attenuate the loss of the auditory nerve (Staecker et al., 1996; Miller et al., 1997; Ylikoski et al., 1998; Marzella and Gillespie, 2002; Miller et al., 2002; Roehm and Hansen, 2005).

Perhaps the most effective and most studied growth factors that influence cochlear morphology are brain – derived neurotrophic factor (BDNF) and neurotrophic factor – 3 (NT – 3). Both BDNF and NT – 3 are members of the neurotrophin family of neurotrophic factors, and both are necessary for the normal development and innervation of the cochlea (Staecker et al., 1996; Pirvola et al., 1997; Fritzsch et al., 1999). These growth factors and their high affinity receptors, TrkC and TrkB, are present in longitudinal gradients in the developing cochlea. There is a high concentration of BDNF and TrkB in the apical end of the cochlea, with decreasing expression in the basal end. NT – 3 and TrkC has an opposite expression pattern in the young cochlea, with the highest concentration in the base (Fritzsch et al., 1997; Fritzsch et al., 1999). Despite this developmental difference, both BDNF and NT – 3 are effective at promoting SGC survival in both young and mature tissue (Staecker et al., 1995; Hartnick et al., 1996; Staecker et al., 1996; Miller et al., 1997). This effect can be seen if there is a delay in introducing growth factors to the deafferented nerve (Gillespie et al., 2004; Yamagata et al., 2004), but it is possible that the pro – survival effects of growth factor treatments cease when treatment ceases (Gillespie et al., 2003; Shepherd et al., 2008). Both NT – 3 and BDNF have been shown to promote peripheral processes growth after hair cell loss, *in vivo* and *in vitro* (Malgrange et al., 1996; Staecker et al., 1996; Cho et al., 1998; Wise et al., 2005).

While the effects of growth factors on cochlear morphology have been studied in some depth, the typical manner of introducing growth factors into the

cochlea in animal models is not clinically feasible. Most *in vivo* studies use single injections or an osmotic pump to deliver drugs to the inner ear. A single injection of growth factors at the time of cochlear implant surgery is feasible, but does not provide lasting benefits. While the osmotic pump can provide sustained release of drugs and is more effective at introducing drugs to the cochlea than a bolus injection (Brown et al., 1993; Prieskorn and Miller, 2000), it cannot be maintained long – term. The pump has an implanted finite reservoir of drugs, which requires surgery to replace. This creates an unacceptable level of infection risk and inconvenience for human use. A more permanent solution is needed to make growth factor intervention a relevant method to aid CI users.

The direct clinical relevance of CI research naturally dictates a systems level analysis, as any changes in cochlear morphology are only beneficial if the function of the CI is affected. The perception of sound from the CI should therefore be assessed physiologically or psychophysically. In this dissertation, the effects of two novel methods of introducing growth factors to the cochlea were determined using electrically – evoked auditory brainstem responses (EABRs) and psychophysical detection thresholds. The psychophysical paradigm has been developed over several decades and is well – established (Miller et al., 1995; Su et al., 2008). This paradigm is a simple go/no – go, positive reinforcement task that determines an animal's awake processing of electrical stimulation from the CI, and is further explained in Chapter 2.

The EABR is a staple in auditory system and CI performance assessment. Acoustically – evoked ABRs are commonplace in evaluating human hearing

levels, which makes this test particularly relevant in animal models of CI performance. The EABR is an evoked potential recording in response to CI stimulation, and is used as a general indicator of the status and functionality of the auditory pathway (Hall, 1990). Animals are typically deafened prior to EABR recordings to eliminate hair cells and any effect they may have on hearing levels (Brownell et al., 1985; Miller et al., 2006) and more accurately mimic the clinical stimulation of CI users. An example of a typical EABR waveform is presented in Fig. 1.2.

There are four peaks in the EABR waveform, which represent ascending regions of the auditory systems. Wave I is the response from the auditory nerve, Wave II is the response from the cochlear nucleus, Wave III is thought to arise from the superior olivary complex, and Wave IV is thought to arise from the inferior colliculus. Although Wave I may be the best indicator of the function of the cochlear nerve, this peak is often obscured by stimulus artifact. Wave II is therefore the first and most often analyzed peak, and changes in the amplitude of this peak are directly related to changes in stimulus intensity. Wave II amplitude decreases as stimulus intensity decreases and EABR thresholds are defined as the lowest current level at which Wave II is noticeably different from background noise, and typically have an predetermined amplitude (van den Honert and Stypulkowski, 1986; Miller et al., 1995). EABR thresholds to CI implant stimulation provide a routine, quantifiable, although coarse, test of the status of the auditory pathway.

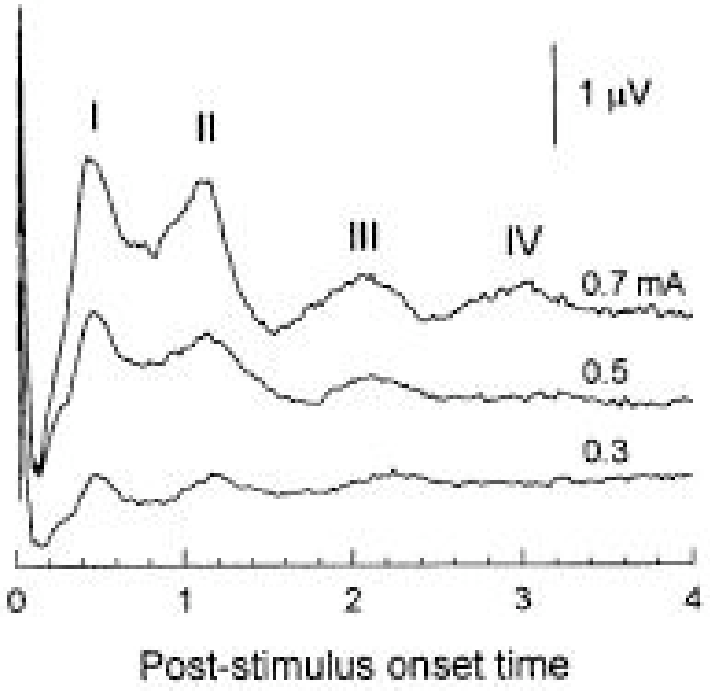


Fig. 1.2: Typical EABR response waveform.

The source of stimulation was from the intrascalar cochlear implant and responses can be recorded from either subdermal needle electrodes or screws placed within the skull. Each peak, labeled I – IV, represents a response from an ascending region of the auditory pathway, and Wave II is typically followed to determine EABR thresholds and changes in EABRs over time. Peak amplitude decreases with decreasing current level. From (Miller et al., 1995).

This dissertation presents two novel methods that were designed to gradually introduce growth factors to the cochlea, sustain growth factor expression over time, and improve physiological and/or psychophysical response to CI stimulation. The first method used an adenoviral construct to up – regulate the internal production of BDNF. A number of viral vectors, including adenoviruses, have been adapted for use in biological systems and modified so that they do not induce substantial rejection, immune response, or over – proliferation (Raphael et al., 1996; Weiss et al., 1997; Stover et al., 1999; Ishimoto et al., 2002). This viral technique is therefore a potential way of changing gene expression and providing a source of growth factors to the degenerating cochlea. These growth factors are internally produced and therefore less susceptible to degradation and could provide a longer duration of protection than exogenous proteins. The virus used here, labeled Ad.*BDNF*, was a replication – deficient recombinant virus with a gene insert for BDNF that was first used to promote survival of retinal ganglion cells following axotomy (Di Polo et al., 1998). It has also been shown to promote SGC survival following hair cell loss (Nakaizumi et al., 2004; Rejali et al., 2007). The current study was designed to test the effects of Ad.*BDNF* on long – term survival of SGCs in conjunction with a cochlear implant. This method and the results of this study are presented in Chapter 2, “Over – expression of BDNF by adenovirus with concurrent electrical stimulation improves cochlear implant thresholds and survival of auditory neurons”.

The second method used a conducting polymer and hydrogel coating on the cochlear implant to release growth factors into the cochlea. Conducting polymers have been developed in a number of engineering fields and have recently gained usage in the biomedical field (Green et al., 2008; Hendricks et al., 2008). When deposited around electrodes, the polymer creates a "fuzzy" coating which dramatically increases the surface area of the electrode (Yang et al., 2007). This improves charge transport and helps preserve the fidelity of the signal in stimulating electrodes and improves signal – to – noise ratio in recording electrodes (Cui et al., 2003; Ludwig et al., 2006). Conducting polymers can provide both controlled and sustained drug release, as drugs can be incorporated into the polymer and released through electrical stimulation. Hydrogels are also common in the biomedical field and are clinically used to aid in tissue formation and wound healing (Zimmermann et al., 2000). Hydrogels form an imitation extracellular matrix that supports tissue, skin, bone, and nerve growth and regeneration. Hydrogels are mostly water – based structures, with lightly linked polymer chains, which allow some drugs to be absorbed and diffused (Coviello et al., 2006; Mano et al., 2007). The combination of hydrogel and conducting polymer has not only the potential to aid in the long – term distribution of growth factors to the cochlea, but also increase the biocompatibility of the implant and facilitate electrical stimulus transmission. This method is investigated in Chapter 4, "Cochlear implant hydrogel coating promotes auditory nerve fiber growth within the scala tympani in direct vicinity of implant" and Chapter 5, "The effect of

PEDOT on cochlear implants electrophysiological thresholds and impedances *in vivo*".

Chapter 3, "Visualization of spiral ganglion neurites within the scala tympani with a cochlear implant *in situ*", presents a histological method that was developed over the course of this dissertation in response to a need to visualize the spatial relationship between the cochlear implant and nerve regrowth. This method was designed to aid the studies in Chapter 4 and 5, but could be applicable to several lines of auditory nerve regeneration research.

The studies described in this dissertation include multi – level analyses of a research topic that has significant clinical applicability. Novel techniques were developed and expanded which led to intriguing results that encourage further study. These projects provide an indication of the comprehensive effects of auditory nerve regeneration and survival on cochlear implant function.

Chapter 2

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

2.1. 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 (Pfingst et al., 1981; Pfingst and Sutton, 1983; Colombo and Parkins, 1987; 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 (Spoendlin, 1975; Webster and Webster, 1981; Leake and Hradek, 1988).

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 (Shinohara et al., 2002; Shepherd et al., 2005).

Both *in vivo* and *in vitro* work indicate a number of growth factors can promote SGC survival, including brain – derived neurotrophic factor (BDNF) (Hartnick et al., 1996; Staecker et al., 1996; Miller et al., 1997; Shinohara et al., 2002; Gillespie et al., 2003; Nakaizumi et al., 2004), neurotrophin – 3 (NT – 3) (Staecker et al., 1995; Staecker et al., 1996; Farinas et al., 2001; Bowers et al., 2002), glial cell line – derived neurotrophic factor (Ylikoski et al., 1998; Altschuler et al., 1999; Yagi et al., 2000; Kanzaki et al., 2002) and ciliary – derived neurotrophic factor (Staecker et al., 1995; Hartnick et al., 1996). 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 (Fritzsch 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 (Fritzsch et al., 1997; Fritzsch et al., 1997; Farinas et al., 2001). 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 (weeks) 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 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 up – regulate growth factors in the cochlea has shown transgene expression in the mesothelial cells that line the scala tympani (Yagi et al., 2000). This effect has been seen throughout the length of the cochlea (Raphael et al., 1996; Stover et al., 1999; Nakaizumi et al., 2004), indicating that even when the efficacy of gene expression decreases with distance from the base, the secreted growth factor may reach and influence more apical regions.

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. 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 up – regulation and the 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.

2.2. Methods

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 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.

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 their 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 the time of inoculation and implantation ranged from 780 to 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.

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).

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 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 µL) was slowly infused into the scala tympani via the round window using a 100 µL 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. There is a small possibility that contralateral hair cells may degenerate with this paradigm due to fluid leakage through the cochlear aqueduct. Contralateral ears were examined post – mortem to confirm that this did not occur. 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 µL 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).

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 a cochlear 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 reopened and the carboxylate cement removed. For treated animals, a single injection of 5 µL Ad.*BDNF* (approximately $4x10^{12}$ 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 µL glass syringe. In control animals, 5 µL of Ad.Empty was administered in a similar manner. Animals were left undisturbed for 20 min 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 basal turn of the cochlea. The letter G was used to identify an extracochlear ground electrode, which was placed in the post – auricular muscle. See Fig. 2.1 for details on the implant dimensions and labeling system.

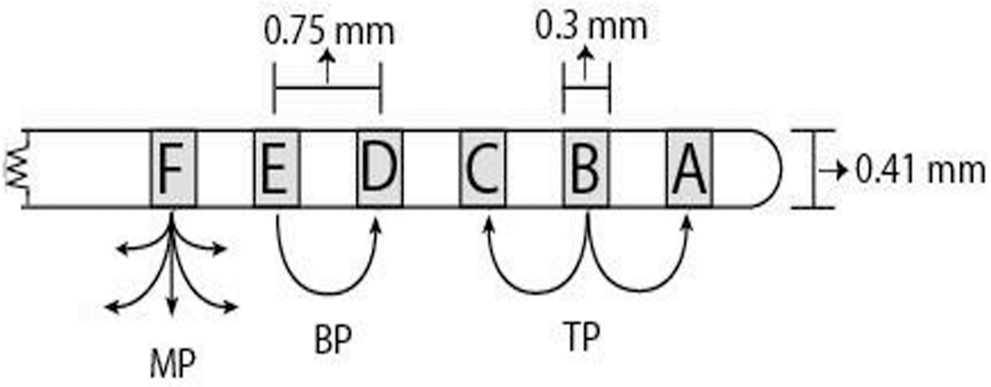


Fig. 2.1: Diagram of multichannel implant and electrode configurations.

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.

Psychophysical assessment

Animals were trained in a go/no – go positive reinforcement auditory stimulus detection task, similar to that described (Prosen et al., 1978; Miller et al., 1995; 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 min 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 apical monopolar electrode configuration (labeled B–G, see Fig. 2.1), 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 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 100 Hz sinusoid bursts (200 ms on, 100 ms 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 μA rms, 1 kHz sinusoidal stimulus.

EABR

Electrically – evoked auditory brainstem responses (EABRs) were recorded under general anesthesia 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 Fig. 2.1). Stimuli

were 50 μs phase duration monophasic alternating polarity square pulses. EABR threshold was defined as the lowest level at which there was a repeatable Wave II, as agreed upon by two unbiased observers.

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 2 % glutaraldehyde and placed in 3 % EDTA solution to decalcify until sufficiently soft for sectioning (approximately 1 month). Once decalcified, the implant was removed from the left ear, 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 μm 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 MetaMorph 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. Adobe Photoshop™ software was used to adjust contrast levels in all images.

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 days test period were averaged to determine a threshold value for each animal for each electrode configuration (six values for each animal) (Figs. 2.3 and 2.5). 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 (Figs. 2.4 and 2.6). 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 deviation 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 deviation. 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 2.1). 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).

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 time point), 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 μL of cochlear fluids were extracted from each cochlea. Samples were diluted 100 fold using the sample diluent from a ChemiKine™ BDNF, Sandwich ELISA Kit (Millipore). This kit was used to determine BDNF concentration in the cochlear fluids. 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.

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, Int.).

2.3. Results

BDNF levels *in vivo*

The BDNF – ELISA revealed average concentration of BDNF in the right, non – inoculated cochleae of 0.78 ng/ml (± 0.86) (Fig. 2.2). The concentration of BDNF in the cochlear fluids one week following Ad.*BDNF* inoculation was 0.60 ± 0.66 ng/ml, similar to the non – inoculated ears. There was a marked increase in BDNF concentration 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 ($p < 0.05$).

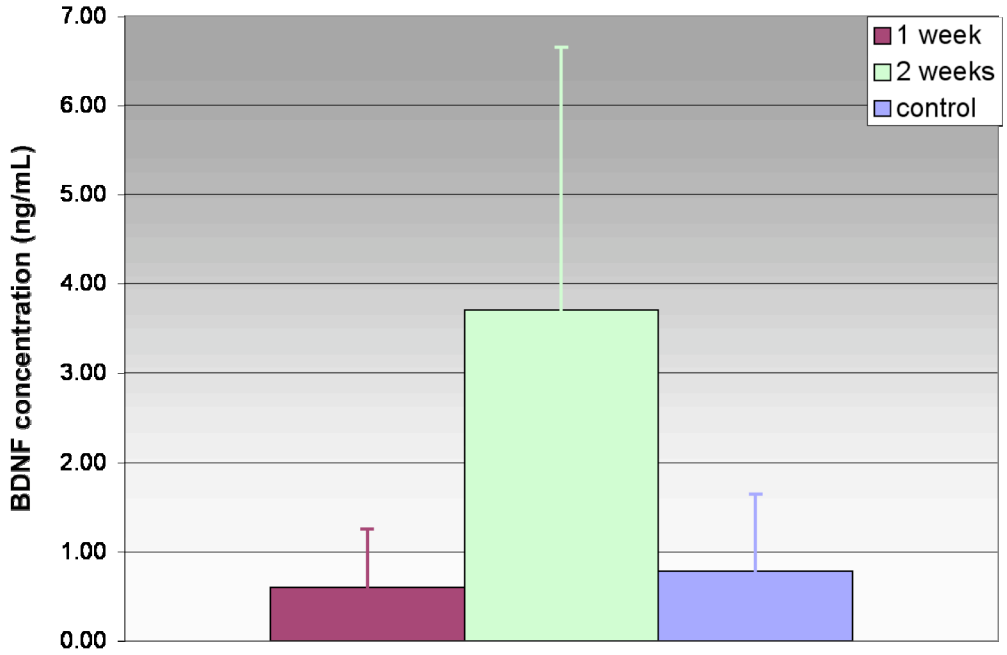


Fig. 2.2: BDNF concentration within cochlear fluids.

At two weeks post – inoculation, the concentration of BDNF within the cochlea was significantly higher than the concentration of BDNF after one week, and significantly higher than the right, non – inoculated ear.
One – way ANOVA, $p < 0.05$.

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. 2.3 shows both individual psychophysical detection thresholds, averaged over the entire testing period (closed symbols), as well as group averages ± 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.

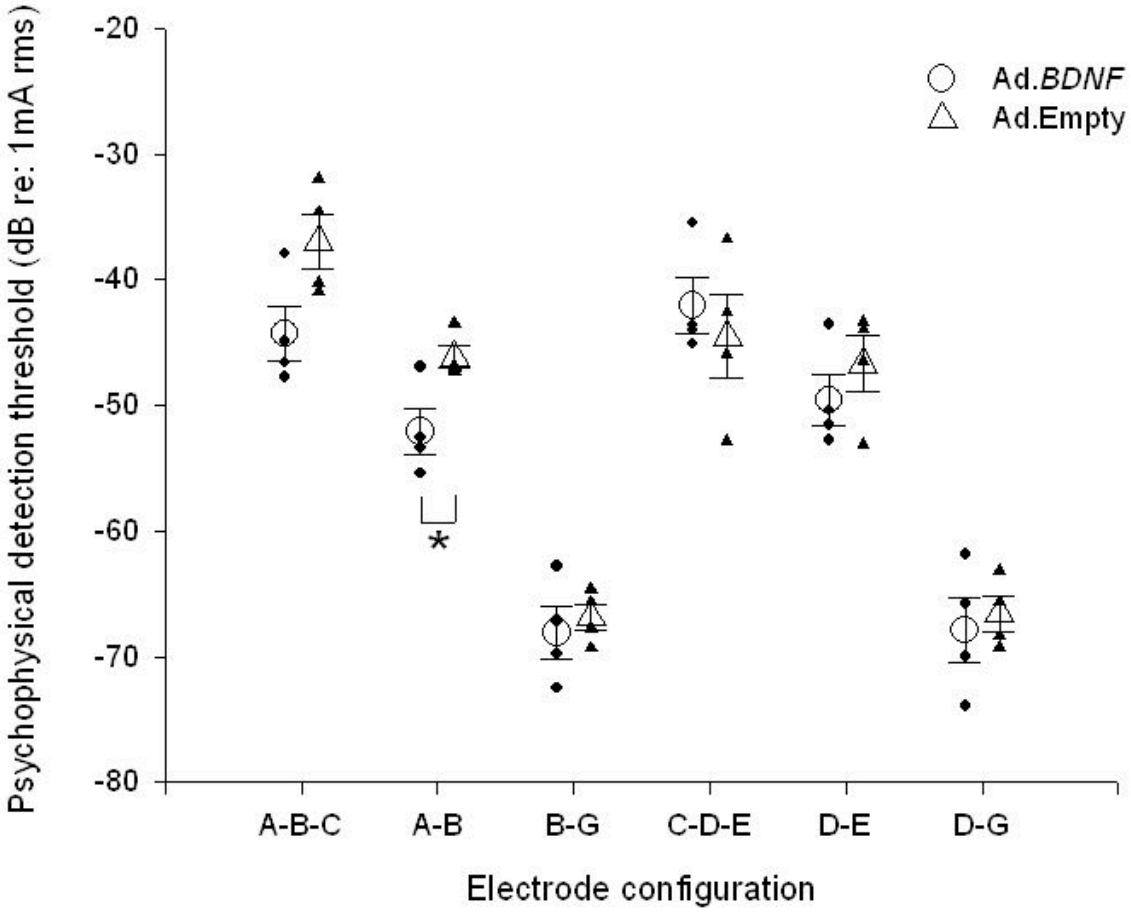


Fig. 2.3: Psychophysical detection thresholds averaged over time.

Each column represents a different electrode configuration. 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 ± 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: $p = 0.68$.

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. 2.4 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. 2.3, which were thresholds averaged over the entire experiment time, were lower throughout the length of the experiment and not dependent on time post – implantation.

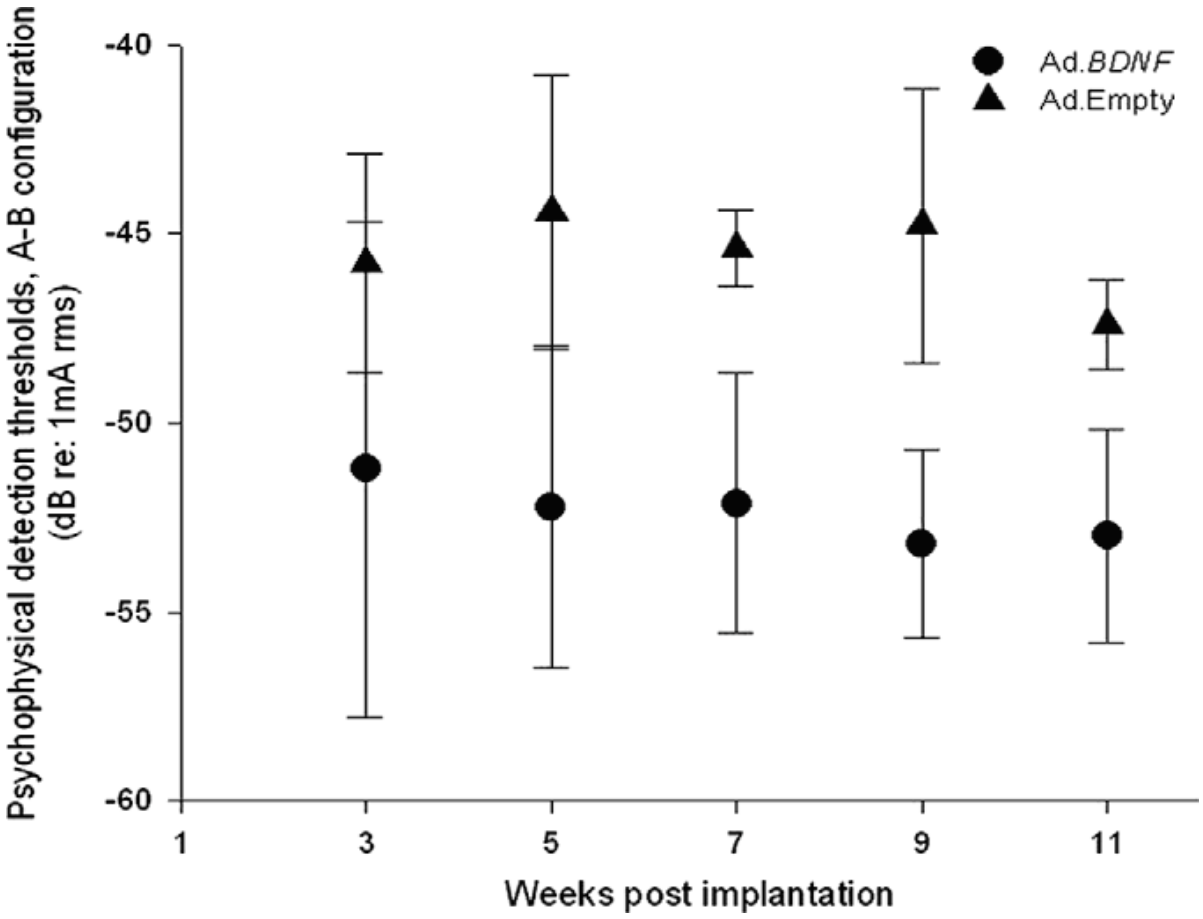


Fig. 2.4: Psychophysical detection thresholds over time, A–B configuration.

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 analyses therefore began 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 ± 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.

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 = 0.03 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. 2.5 shows both individual EABR thresholds, averaged over the testing period (closed symbols), and group averages ± one SEM (open symbols) for each electrode configuration tested. Labels on the x – axis in Fig. 2.5 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.

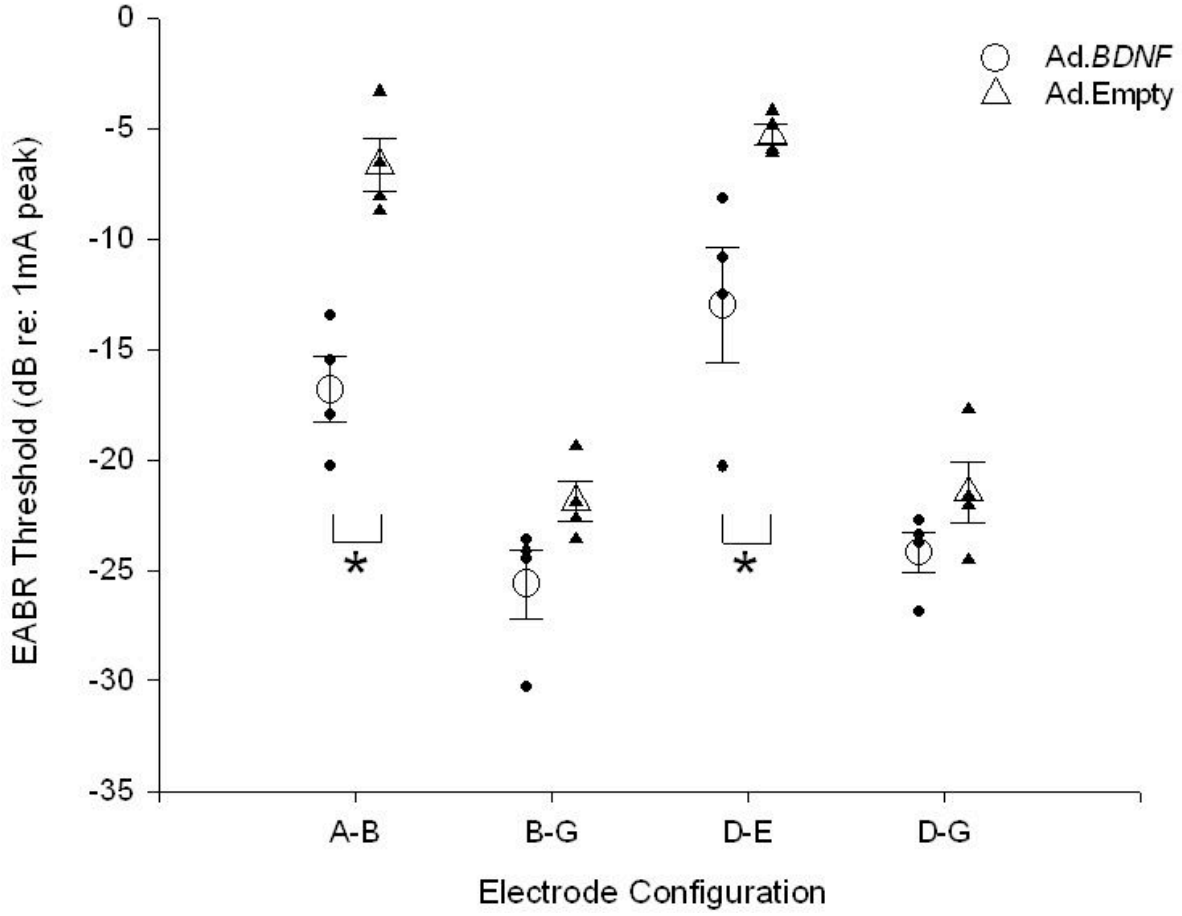


Fig. 2.5: EABR thresholds averaged over time.

Symbols and statistical tests as in Fig. 2.3. 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.

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. 2.6 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 weeks 3 through 11 post – implantation, the Ad.*BDNF* treated animals had significantly lower thresholds than the Ad.Empty control group ($p < 0.05$).

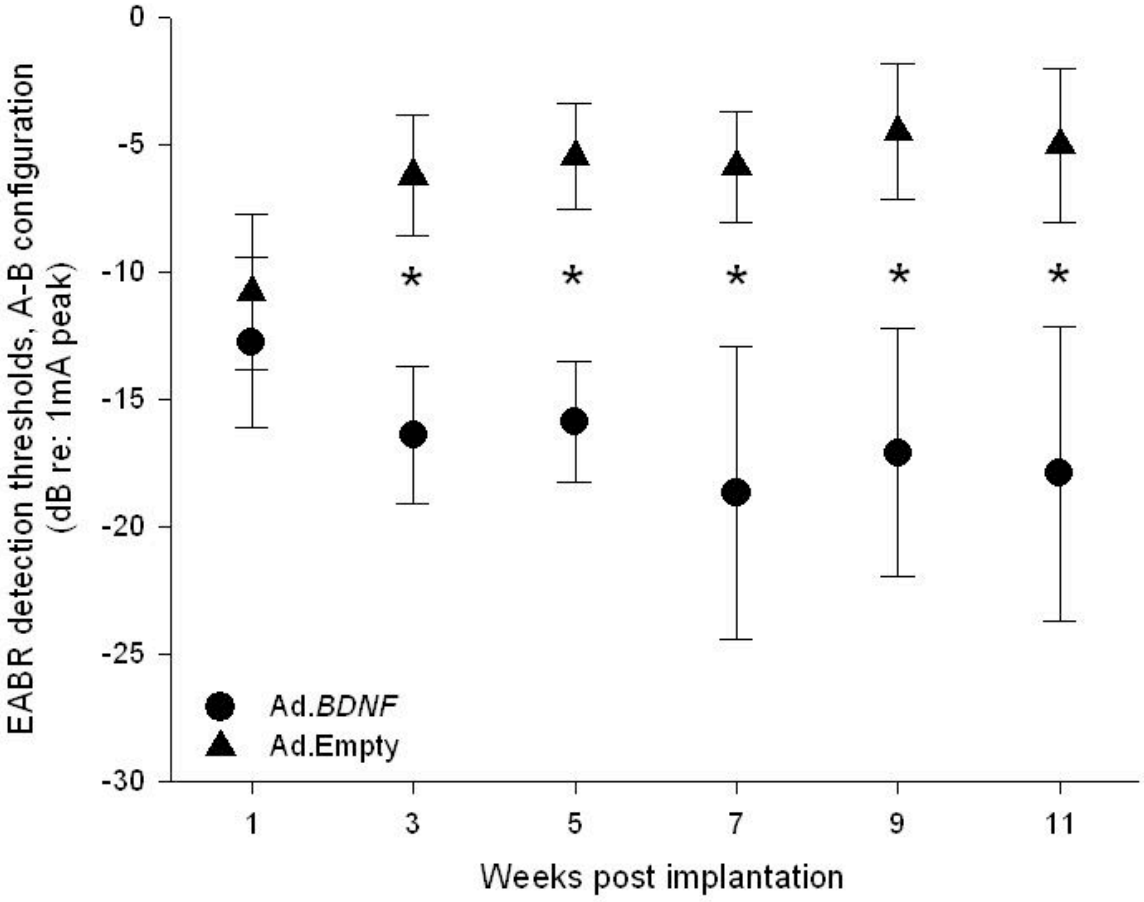


Fig. 2.6: EABR detection thresholds over time, A–B configuration.

Each data point represents a group average of thresholds obtained at each time point indicated. Symbols as in Fig. 2.4. 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.

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. 2.7). Image 2.7A shows the organ of Corti of an Ad.*BDNF* treated animal, 2.7B shows the organ of Corti of an Ad.Empty treated animal, and 2.7C 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.

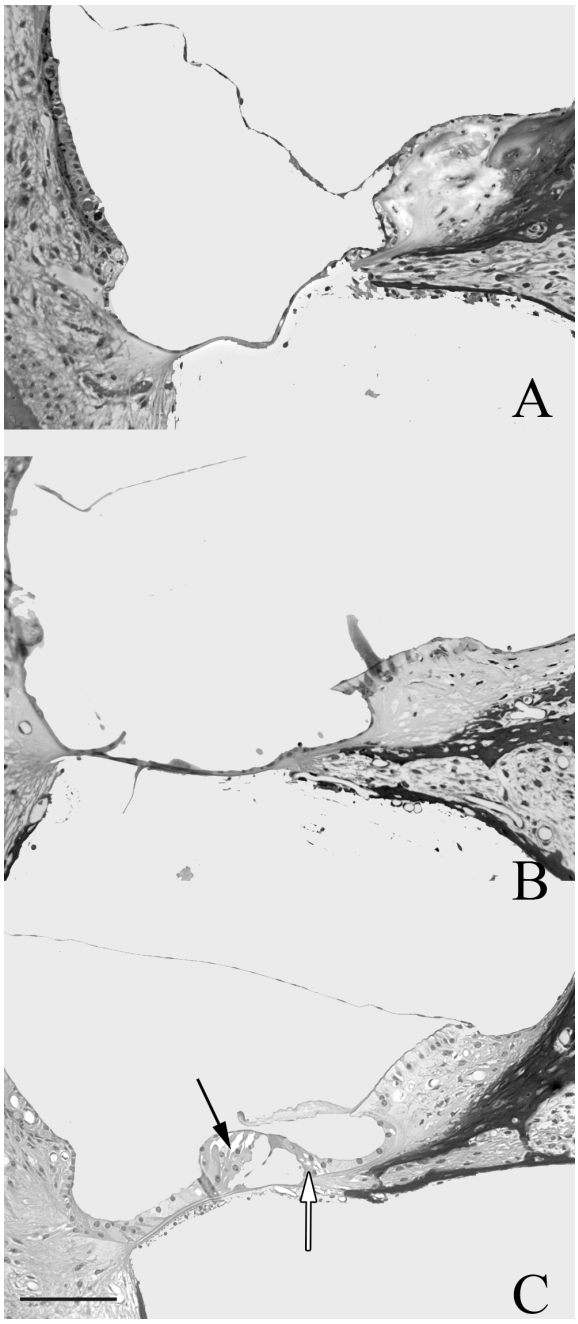


Fig. 2.7: Light microscopy images of the organ of Corti structure

The basal turn organ of Corti is shown. (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 – deafened ears (C). Scale bar = 50 μm.

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 (Figs. 2.8 and 2.9). 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 cochlear regions.

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. 2.9 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 SGCs and Rosenthal's canal.

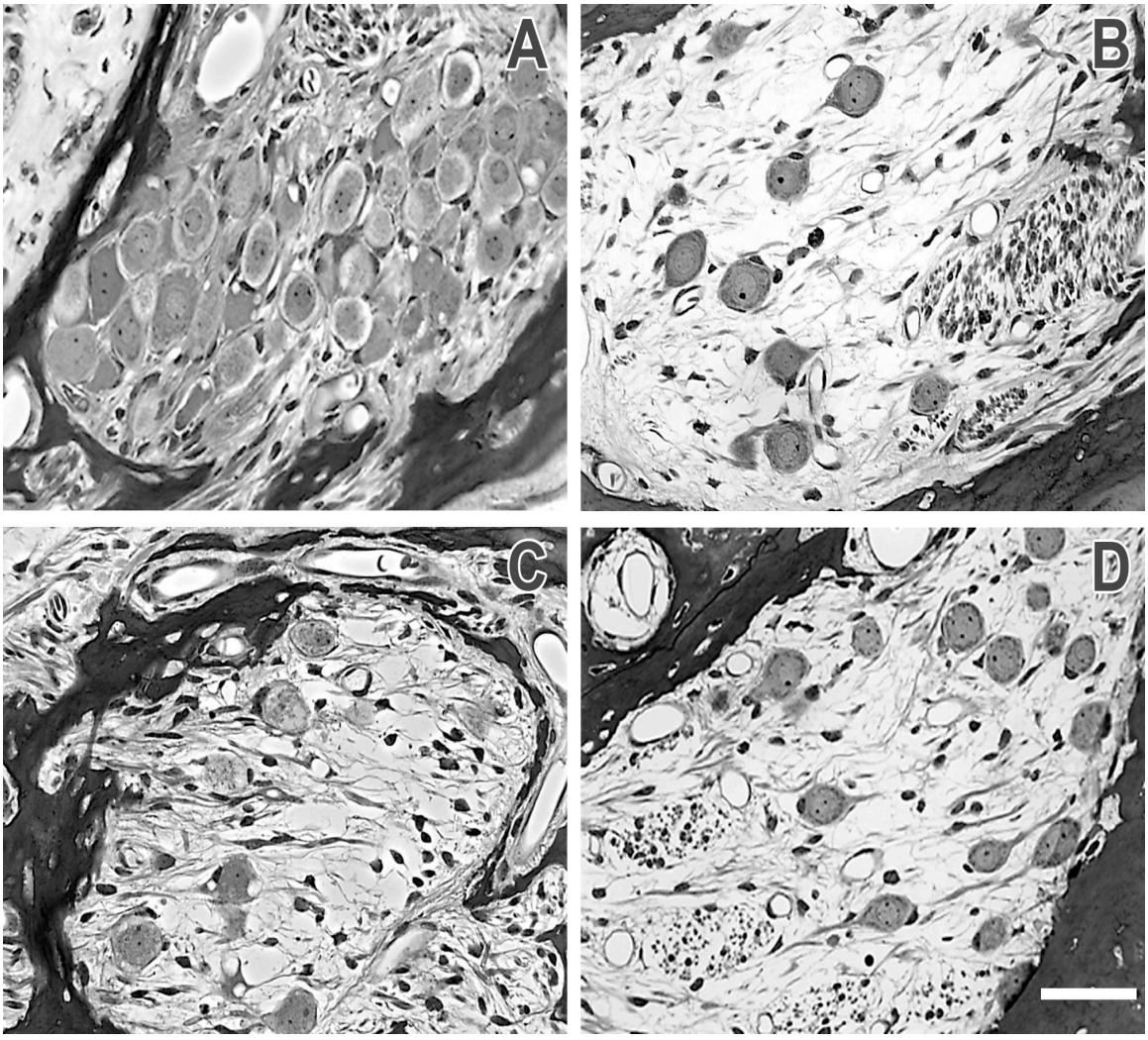


Fig. 2.8: Light microscopy images of the upper basal and middle turn of an Ad.*BDNF* (A, C) and an Ad.Empty (B, D) inoculated animal.

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 μm.

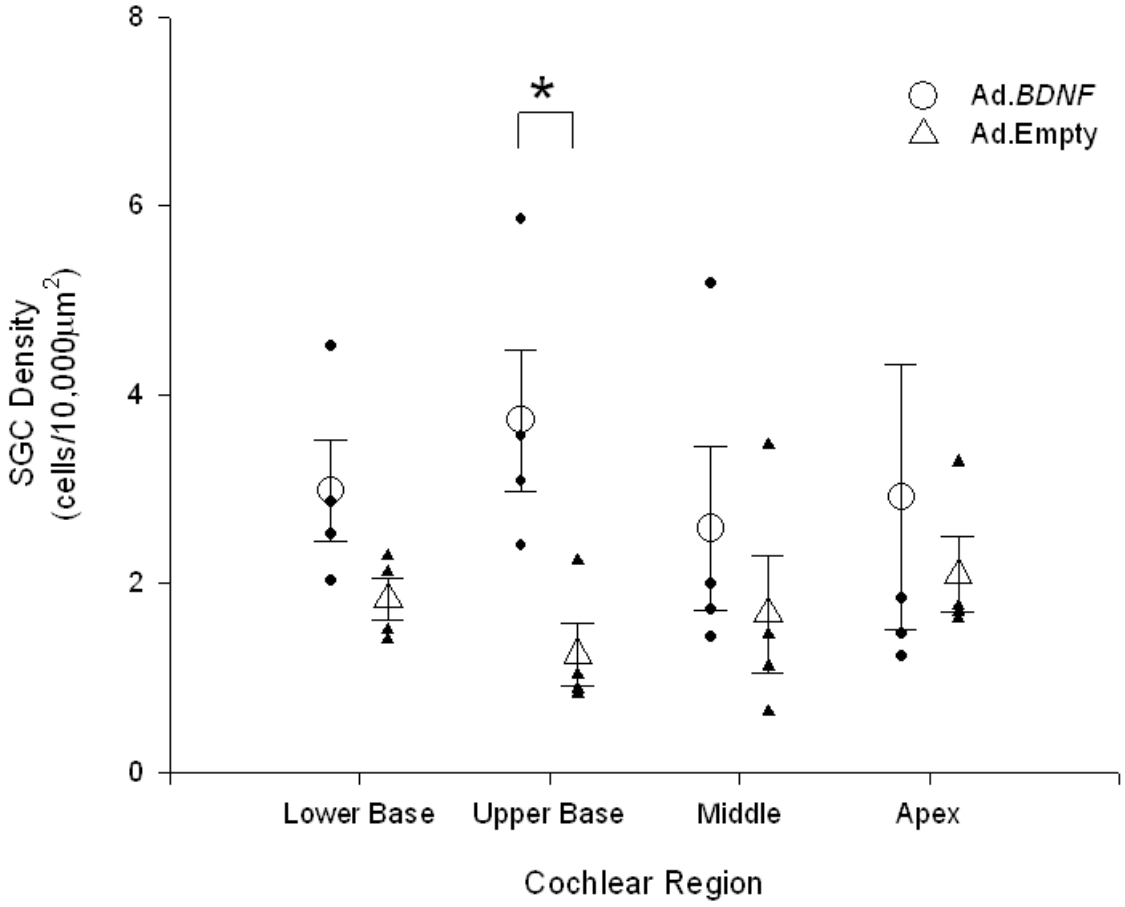


Fig. 2.9: SGC density per cochlear turn.

Symbols as in Fig. 2.3. (*) 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: $p = 0.60$.

Treatment Group	Cochlear region	Spiral ganglion cell cross – sectional area (μm^2)	Rosenthal's canal cross – sectional area (μm^2)
Ad.*BDNF*	Lower Base	302.0 +/ – 55.5	64671.2 +/– 4909.4
Ad.Empty	Lower Base	248.4 +/ – 45.3	74156.5 +/– 26004.3
Non–deafened control	Lower Base	216.3 +/ – 46.9	59350.6 +/– 16377.2
Ad.*BDNF*	Upper Base	286.5 +/ – 91.1	51404.9 +/– 5776.9
Ad.Empty	Upper Base	223.4 +/ – 12.2	58849.8 +/– 6383.7
Non–deafened control	Upper Base	170.8 +/ – 19.7	44544.9 +/– 6962.1
Ad.*BDNF*	Middle	289.1 +/ – 118.2	45012.7 +/– 4427.3
Ad.Empty	Middle	215.3 +/ – 44.8	45785.0 +/– 3562.7
Non–deafened control	Middle	178.7 +/ – 35.8	46140.5 +/– 7044.0
Ad.*BDNF*	Apex	266.5 +/ – 137.2	49895.4 +/– 29493.1
Ad.Empty	Apex	201.9 +/ – 36.2	52449.1 +/– 11231.7
Non–deafened control	Apex	202.5 +/ – 41.7	87128.1 +/– 32769.5

Table 2.1: Two – dimensional ganglion cell and Rosenthal's canal characteristics.

Listed are the group averages +/ – 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.

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 values 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. 2.10 shows the psychophysical – SGC comparison (2.10A) and the EABR – SGC comparison (2.10B). 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.

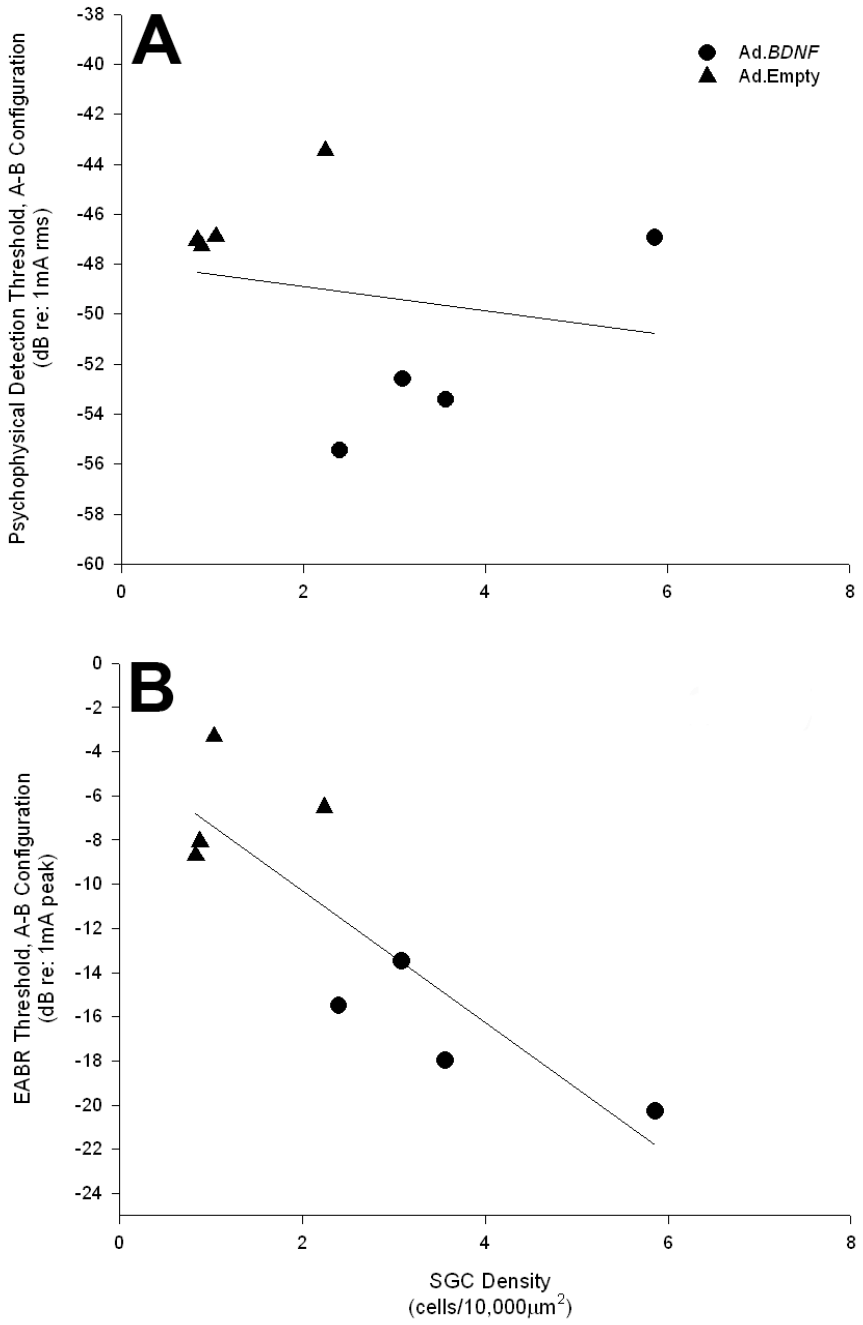


Fig. 2.10: Relationship between thresholds and number of surviving SGCs in (A) psychophysical testing and (B) electrophysiological testing.

Psychophysical detection thresholds and EABR thresholds from the A–B configuration and SGC density values 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).

2.4. 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 two weeks post – inoculation, providing a link between the introduction of the adenovirus into the cochlea and our observed morphological, physiological, and behavioral changes.

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 (Shinohara et al., 2002; Shepherd et al., 2005), 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 led to increased 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 density and function by preventing post – deafening degeneration and/or promoting neural regrowth, either of which would have a positive effect on cochlear implant function.

Psychophysical and electrophysiological thresholds

Our data show an effect of adenoviral – mediated up – regulation 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 implant 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 with 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 with 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.

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; Mitchell et al., 1997). 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., 2008).

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 (Raphael et al., 1996; Ishimoto et al., 2002). 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 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. The developmental patterns of BDNF and NT – 3 expression are well established (Fritzsch et al., 1999; Farinas et al., 2001). However, there is some evidence that pattern of expression changes location over time (Adamson et al., 2002; Schimmang et al., 2003). 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. Concentration gradients related to preferential transgene expression in the basal

cochlea may have also contributed to the difference seen here between effects in basal and more apical regions of the cochlea.

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 EABR 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.

Chapter 3

Visualization of spiral ganglion neurites within the scala tympani with a cochlear implant *in situ*

3.1. Introduction

In mammals, cochlear hair cell loss is a permanent and irreversible event, which often initiates auditory nerve deterioration. This nerve degeneration begins at the level of the afferent and efferent processes that innervate the hair cells, and may continue with the primary ganglion cell bodies (Spoendlin, 1975). In such cases of severe hearing loss, cochlear implants (CIs) offer an effective treatment option. CIs function by electrically stimulating the remaining auditory nerve and bypassing the function of lost hair cells. The status of the nerve, and its ability to receive and process electrical signals, is therefore a critical factor in the success of cochlear implantation. While mammalian hair cells cannot currently be regenerated to a functional level, there is well – established evidence that the introduction of growth factors can promote auditory nerve ganglion cell survival following hair cell loss (Marzella and Clark, 1999; Roehm and Hansen, 2005). In addition, it has been shown that an increase in ganglion cell survival is

correlated with improved electrophysiological thresholds to CI stimulation (Yamagata et al., 2004; Shepherd et al., 2005).

Once distal processes are regressed, the primary auditory neurons remain relatively far from the CI stimulation source. It may be beneficial for CI users if the peripheral processes of the auditory nerve could be preserved or directed to regrow towards and make contact with a CI. Decreasing the distance between the auditory nerve and the CI has been shown to lower thresholds and decrease the spatial spread of excitation in human CI patients (Cohen et al., 2005), animal subjects (Shepherd et al., 1993), and model systems (Briaire and Frijns, 2006). By stimulating nerve fibers instead of the more distant cell bodies, a lower level of current may be required to elicit a neural response, which may increase the battery life of the implant and reduce cellular damage. Battery power is a limiting factor in the parameters of electrical stimulation available from a CI; increasing the battery life could allow for more complex stimulation strategies. In addition, there is the potential for increased specificity of electrical stimulation and reduced channel interaction when neural impulses are initiated at nerve fibers instead of the cell body. Any of these improvements in electrical signal processing could improve the hearing experience for CI recipients.

Spontaneous regeneration of auditory nerve peripheral processes has been reported in mammals following both acoustic trauma (Bohne and Harding, 1992; Lawner et al., 1997) and aminoglycoside ototoxic deafening (Webster and Webster, 1982). Growth factor – induced regeneration of nerve fibers has also been seen both *in vivo* and *in vitro* (Malgrange et al., 1996; Staecker et al., 1996;

Cho et al., 1998; Wise et al., 2005; Glueckert et al., 2008). *In vivo* studies have shown fiber growth both within the habenula perforata, where processes are normally located (Wise et al., 2005; Glueckert et al., 2008), as well as within the scala tympani (ST), the fluid – filled space within the cochlea where CIs are placed (Leake and Hradek, 1988; Staecker et al., 1996). While several lines of research are aimed at promoting peripheral processes regrowth into the ST to contact a CI, current histological assays are not well suited to visualizing the results. If future work on auditory nerve regeneration proceeds to the level of directing growth to make contact with a CI, it will become necessary to visualize the implant and the regenerated fibers simultaneously while minimizing the perturbation of the regrown nerve fibers.

Typical cochlear preparations involve decalcification, embedding, and sectioning to produce mid – modiolar images of the interior of the cochlea. These mid – modiolar sections do not allow visualization of both the implant and the scala tympani because highly specialized sectioning tools are required to cut through the metal wire used in the construction of CIs, and the implant must therefore be removed prior to analysis. Surface preparations of various cochlear structures are also commonly used, but this technique typically exposes only one surface. Any microscopic analysis would require plating and coverslipping the specimen, eliminating the ability to examine the space within the ST. Inverted confocal microscopes are also unsuitable because the magnification levels are typically too high for proper visualization of the entire implant and its spatial relationship with the cochlea. We have therefore developed a technique that

utilizes a stereoscope to view the intact cochlear and preserves the placement of the CI, yet allows visualization of fluorescently – labeled neuronal fibers and preserves the spatial relationship between the two. The method detailed in this paper includes both a specialized cochlear dissection and the subsequent tissue processing. This method represents a novel visualization technique that provides a means for characterizing the presence and location of nerve fibers within the scala tympani with respect to a CI in a way that preserves the fidelity of the *in vivo* relationship.

3.2. Methods and materials

Subjects

Eleven male, pigmented guinea pigs (Elm Hill, Chelmsford, MA) were used in this study. Guinea pigs have cochleae that are mature at birth, similar to humans, which makes them a better animal model for cochlear implant research than other rodents. Animal weights ranged from 400 – 600 g at the time of implantation. Following systemic deafening, animals were implanted with two – electrode CIs through a cochleostomy near the round window. Animals retained their implants for four weeks, after which they were sacrificed for temporal bone removal and cochlear dissections. 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. The Unit for Laboratory Animal Medicine at the University of Michigan provided veterinary care and animal husbandry, in facilities certified by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, Intl).

Deafening procedure

Animals were systemically deafened using a combination of kanamycin (400 mg/kg, SQ) and ethacrynic acid (40 mg/kg, IV, two hours following kanamycin). This procedure is a standard means of mimicking human hearing loss, and effectively eliminates cochlear hair cells bilaterally (West et al., 1973; Xu et al., 1993). Hearing loss was confirmed with auditory brainstem responses (ABRs). ABRs were measured prior to deafening and four weeks post – deafening and hearing loss was defined as a shift of 40 dB SPL between the two measurements. Hair cell loss was confirmed with post – mortem histological analysis. Animals were deafened prior to implantation to initiate a regression of peripheral processes and increase the likelihood of fiber growth into the ST.

Implant construction

Implants consisted of two stimulating electrodes and were constructed in – house, as previously described (Miller et al., 1995). Briefly, a wire electrode was wrapped in a helix (300μm length and diameter) around an insulated wire leading to ball electrode (450μm diameter). All wires were platinum/iridium (A & M Systems, Sequim, WA). The lead wires from the electrodes were threaded through a piece of silicone tubing 35 mm long and connected to a base pedestal. The implant was inserted approximately 2 mm into the ST (Fig. 3.1 and Fig. 4.1).

Implantation

All animals were unilaterally implanted in the left ear. For general anesthesia, animals were given a mix of ketamine (40 mg/kg) and xylazine (10 mg/kg) (IM). Animals were also given atropine (0.05 mg/kg, SQ) to aid in respiration, 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 ear to be implanted. The underlying muscle and tissue were pushed back to reveal the bulla and a small hole was made in the bulla with the tip of a scalpel blade, exposing the middle ear space, the basal turn of the cochlea, and the round window. A bone drill and a 0.7 mm diamond burr was used to make a cochleostomy in the wall of the ST apical to the round window, and the two – electrode CI was placed into the ST. Implants were inserted approximately one –

third to one – half of the way into the basal turn of the cochlea. A secondary hole was made in the bulla, and a silk suture was threaded through the two bulla holes and used to tie the implant to the temporal bone and prohibit movement of the implant within the cochlea. Carboxylate cement covered both holes in the bulla and secured the position of the implant. The silicone tubing containing the lead wires of the implant was tunneled under the skin from the incision behind the ear to the midline of the skull where the implant base pedestal was secured to the skull of the animal using screws and dental acrylic. The skin incision was sutured in two layers and the animal was given warm subcutaneous saline and yohimbe (1 mg/kg) to reverse the effects of xylazine and aid in recovery.

Sacrifice and initial dissection

Four weeks post – implantation, animals were given an overdose of anesthesia (as above) and decapitated. Excess tissue and muscle from the neck and head was carefully removed until the silicone tubing of the implant leading into the left temporal bone was visible. The implant was cut with scissors outside the temporal bone and the remainder of the connective tissue and muscle attached to the temporal bone was removed so that the entire left (implanted) temporal bone could be dissected out. The right temporal bone was also removed. Each temporal bone was immediately submerged in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH = 7.3). While in 4 % PFA, the temporal bone was carefully trimmed to reveal the cochlea. The

region of the temporal bone that contained the carboxylate cement and silk suture, which held the implant in place, was left undisturbed. A small portion of the bone covering the apex of the cochlea was removed and one to two mL of PFA were locally perfused into the fluid spaces of the cochlea via this apical hole. The entire temporal bone, with the implant held in place by the suture tie in the bulla, was left in 4 % PFA for 24 hours to allow thorough fixation.

Basal cochlear bone removal

Following fixation, temporal bones were rinsed in PBS and placed in a 35 mm Petri dish in PBS. The temporal bone was trimmed such that the cochlea and the portion of the temporal bone that held the implant in place remained intact and the implant was immobile. The bone where the basal turn of the cochlea meets the temporal bone wall (black arrow, Fig. 3.1) was carefully thinned using a bone drill and a 3.0 mm carbide cutting burr (Osteon™). Once the bone was thin and translucent, a 1.0 mm diamond burr (Osteon™) was used to carefully create a cochleostomy in the basal cochlear wall, apical to the original implant cochleostomy. This hole was carefully expanded (dashed line, Fig. 3.1), using either the 1.0 mm diamond burr or fine – tipped forceps, to approximately 3 mm long, until the entire CI within the ST could be visualized.

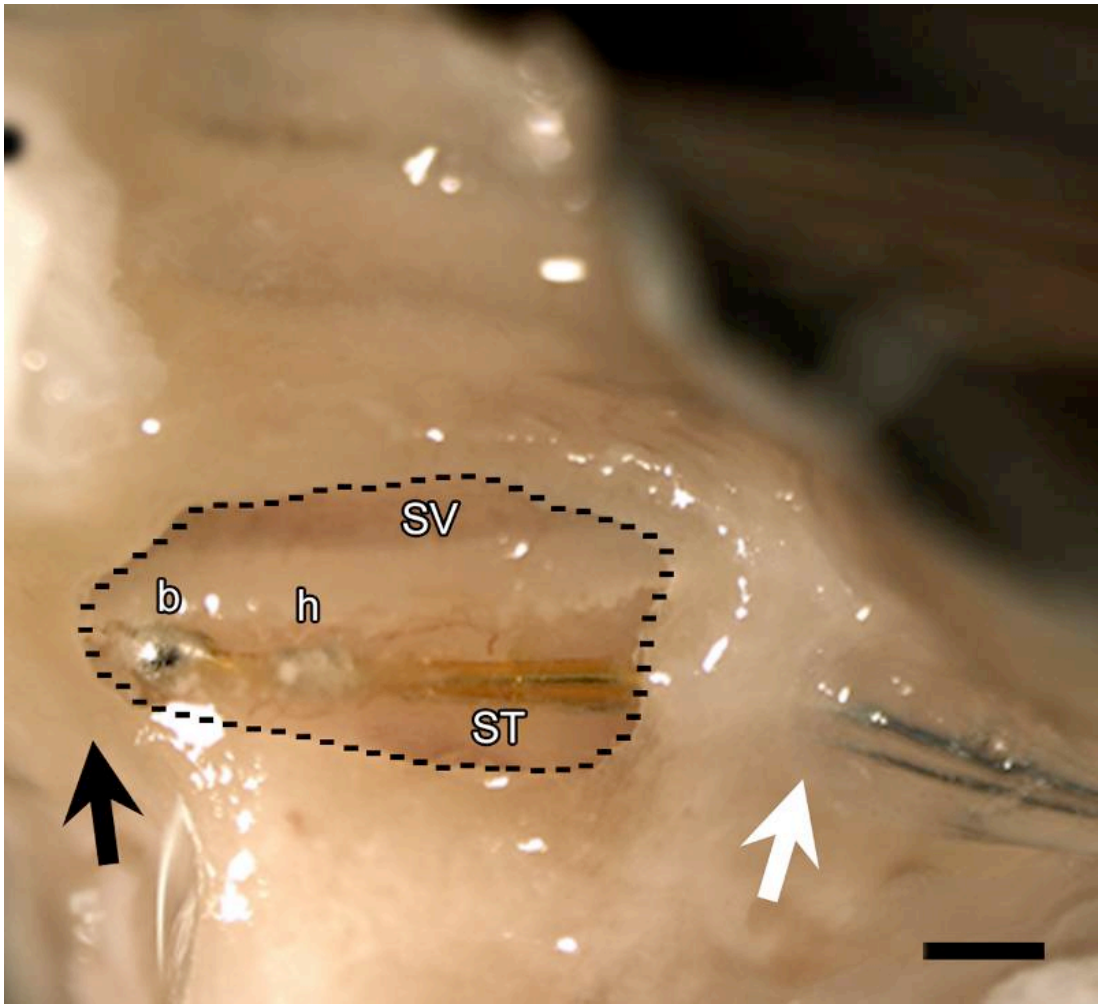


Fig. 3.1: Basal cochlear dissection with implant in place.

The left cochlea of a guinea pig is shown with the in – house constructed cochlear implant within the scala tympani four weeks post – implantation. The basal cochlear bone around the implant was carefully removed to reveal the location of the implant within the scala tympani without disturbing the implant or tissue within the cochlea. The dashed line indicates the perimeter of the area of bone removal. The black arrow shows the dissection starting point, where the temporal bone meets the cochlea and the bone is very thick relative to the rest of the cochlear bone. The white arrow indicates the original cochleostomy where the implant was inserted into the cochlea. Labels: b = ball electrode, h = helix electrode, ST = scala tympani, SV = scala vestibuli. Scale bar = 500μm.

Immunohistochemistry and fluorescent stereoscopy

Following removal of the basal turn outer wall, the entire temporal bone specimen was rinsed by submerging it twice in PBS for 5 minutes each. Specimens were permeabilized in 0.3 % Triton X – 100 (Sigma) for 10 minutes and rinsed with PBS for 5 minutes. Specimens were then exposed to a mouse monoclonal anti – neurofilament antibody (160kD, Millipore) diluted 1:100 in PBS / 0.1 % bovine serum albumin (BSA, Sigma) / 1 % goat serum, for one hour at room temperature. The 160kD neurofilament subunit was chosen for this study because it labels both type I and type II ganglion cells and their processes and labeling is not dependent on phosphorylation (Berglund and Ryugo, 1991). For this primary antibody step, which would have required a large amount of antibody to fully submerge the entire temporal bone, specimens were placed cochlear side down in a glass vial and enough solution was added to fully cover the cochlea but not all of the temporal bone (approximately 1 mL). During the soaking time, several local perfusions of the primary antibody solution were rinsed directly into the cochlea. Specimens were rinsed twice in PBS / 0.1 % BSA / 1 % goat serum, for 5 minutes each, and then placed into a solution containing the secondary antibody: goat anti – mouse FITC (Jackson), diluted 1:200 in PBS / 0.1 % BSA / 1 % goat serum. Specimens were left in the secondary antibody solution overnight at 4°C. As with the primary antibody solution, temporal bones were not completely submerged in the secondary antibody solution, but instead only the cochlear portion was covered. Following the overnight incubation, the tissue was

rinsed three times with PBS for 5 minutes each. Specimens were observed and photographed under a Leica MXFL III stereo fluorescence microscope (Leica, Eaton, PA) equipped with a 10x eyepiece, a 1.6x lens, and 0.8 – 10x focus knob. This allowed for a magnification range of 12.8x – 160x. Adobe Photoshop™ software was used to adjust contrast levels in all images.

3.3. Results

Our neurofilament immunohistochemistry protocol was successful in labeling the auditory nerve and the spiral ganglion neurites in the scala tympani with a cochlear implant *in situ*. These processes could be visualized in the same field of view as the implanted electrode with a low – magnification stereoscope, following our cochlear dissection, which left the implant intact within the cochlea.

Immunohistochemistry of the central auditory nerve

The auditory nerve central processes in each temporal bone were examined under fluorescent light to verify successful labeling of neurons with neurofilament. Each temporal bone was turned cochlear side down (apex down) to reveal the auditory nerve as it exited the temporal bone at the internal auditory meatus. This was the region of the auditory pathway where the nerve would pass from the internal auditory meatus to the cochlear nucleus in an intact system.

However, because only the temporal bone itself was preserved for dissection and immunohistochemistry, the auditory nerve was severed from the rest of the auditory pathway and could be visualized. Images of the nerve were taken in both bright light and fluorescent light, and Fig. 3.2 shows an example of fluorescing auditory nerve fibers. This image contains two control specimens that verify the specificity of our immunohistochemistry protocol. The specimen in Fig. 3.2A & B received the full immunohistochemical protocol, the specimen in Fig. 3.2C & D did not receive the primary antibody step of the protocol, and the specimen in Fig. 3.2E & F did not receive any staining. There were no fluorescing fibers seen in the auditory nerve of any specimen that did not receive the full immunohistochemical protocol. The images under fluorescent light (right panel, Fig. 3.2B, D, F) show the auto – fluorescence of the temporal bone. There was a noticeable lack of illumination in the region where the auditory nerve was (black dashed lines) without the full immunohistochemical protocol (Fig. 3.2D, F) but a significant increase above background levels in the auditory nerve that received the full protocol (Fig. 3.2B). Fluorescing central auditory nerve fibers were verified in all specimens prior to further analyses.

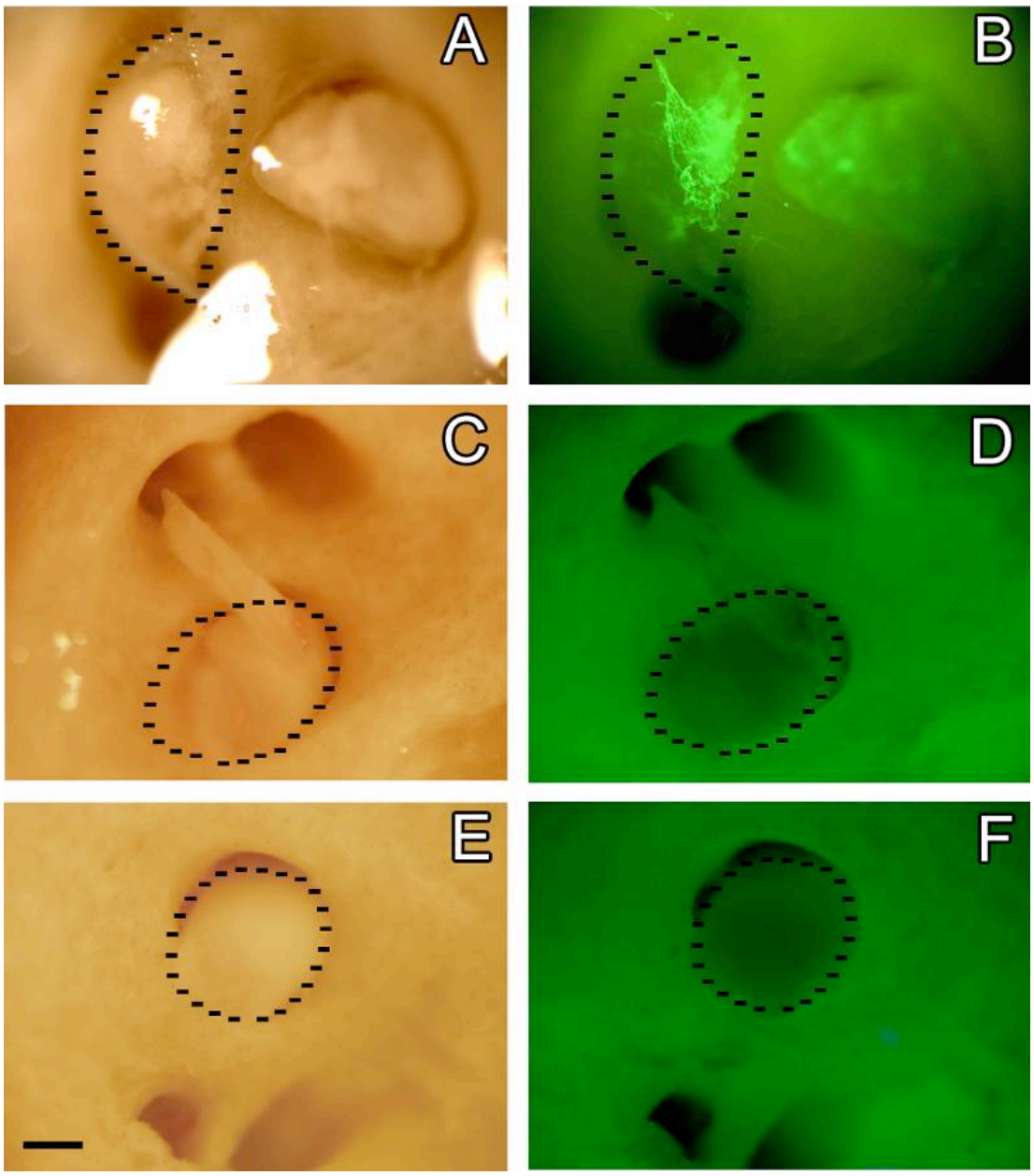


Fig. 3.2: Neurofilament staining of the central auditory nerve.

The auditory nerve as it exits the internal auditory meatus is shown under bright light (left panel) and fluorescent light (right panel). Dashed lines indicate the auditory nerve. Neurofilament – labeled fibers are visible within the auditory nerve under fluorescent light at mid – level magnification (51.2x) only in tissue samples that underwent the entire immunohistochemical protocol (A, B) and not in samples that did not receive the primary antibody (C, D) or did not receive any staining (E, F). Scale bar = 200μm

Immunohistochemical visualization of neurofilament – labeled processes within the scala tympani

Fig. 3.3 shows both bright light and fluorescent light images of the exposed ST from one animal at two magnification levels. At lower magnification levels (Fig. 3.3A & B), neurofilament – labeled processes are visible where the central auditory nerve exits the temporal bone (black arrow), as well as within the scala tympani (white arrow). As the magnification level was increased (Fig. 3.3C & D), neurofilament – labeled processes could be seen more clearly (white arrows). The scala tympani from three additional animals are shown in Fig. 3.4. Fig. 3.4A and 3.4B demonstrate that it was possible to view single fibers and the implant in the same view. At the highest level of magnification possible with the Leica stereoscope, 160x, neurofilament – labeled fibers) are visible in the direct vicinity of the implant (white arrows, Fig. 3.4B). Fig. 3.4C shows a normal non – deafened, non – implanted cochlea following our immunohistochemical protocol, where no neurofilament – labeled fibers were visible within the scala tympani.

Six out of the eleven implanted cochleae (55 %) that were examined exhibited neurofilament – labeled processes within the scala tympani. Our visualization technique allowed us to see neurofilament – labeled processes in various locations throughout the basal turn of the cochlea, including near the lateral cochlear wall (Fig. 3.4A), near the cochlear implant (Fig. 3.4B), near the original cochleostomy, and near the basilar membrane. This diversity was expected, as peripheral processes growth can be random and disorganized

(Bohne and Harding, 1992; Staecker et al., 1996), and there is currently no method to consistently attract nerve growth into the scala tympani. In addition, some cochleae exhibited fibrotic tissue growth within the scala tympani, which is common with long – term cochlear implantation (Ni et al., 1992; Shepherd et al., 1994). This tissue growth did not label with the neurofilament antibody, indicating that our labeling process was specific to neuronal processes only.

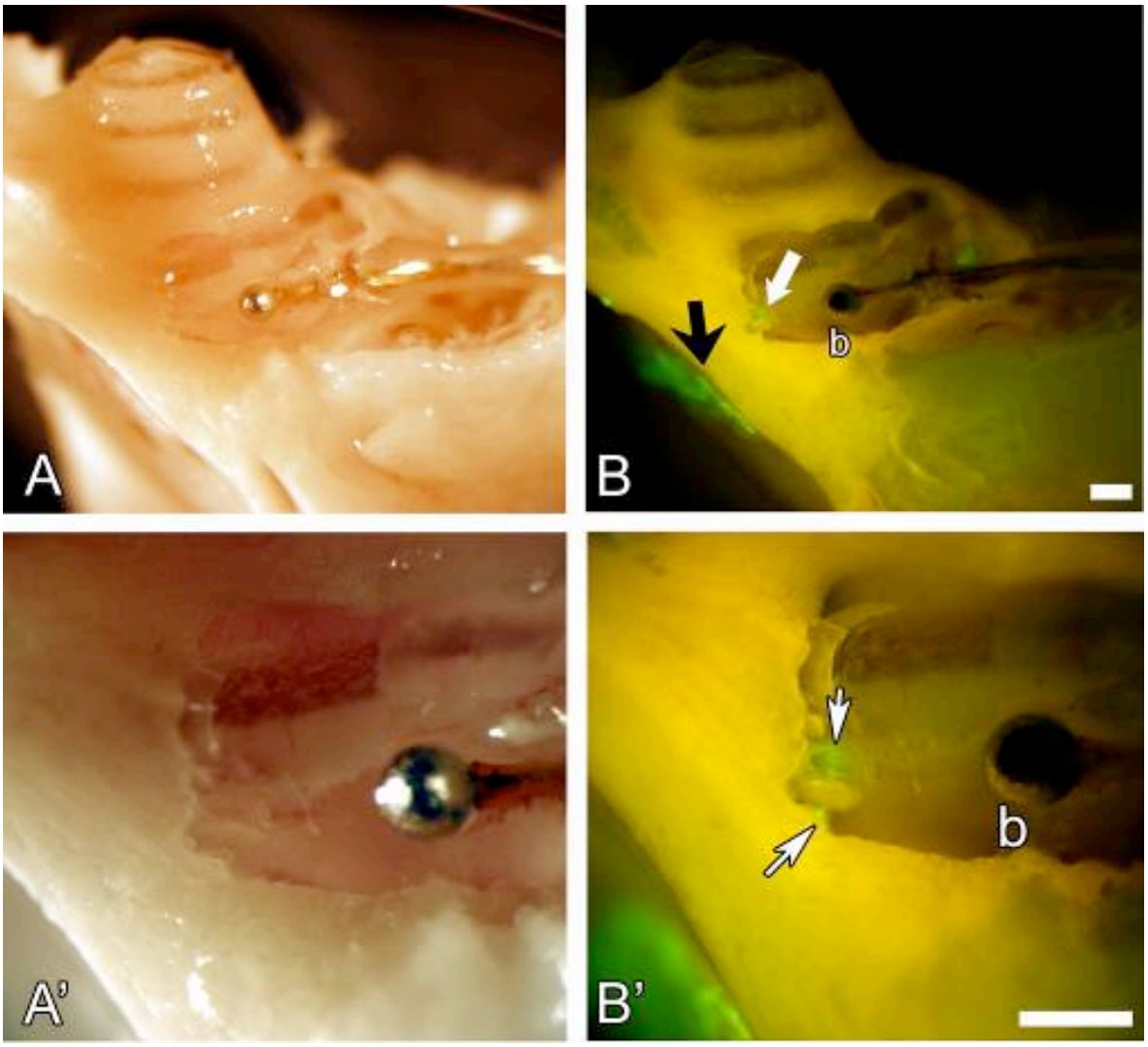


Fig. 3.3: Neurofilament staining within the cochlea.

The interior of the cochlea and the cochlear implant (b = ball electrode) following our dissection from the same specimen is shown at two magnification levels, under bright light and fluorescent light. (A) and (B) are low magnification images that show the entire cochlea, and the central auditory nerve exiting the temporal bone shows neurofilament – positive fibers (black arrow). (A') and (B') are middle magnification level images and neurofilament – labeled processes are visible within the scala tympani under fluorescent light (white arrows).
Scale bar = 500μm.

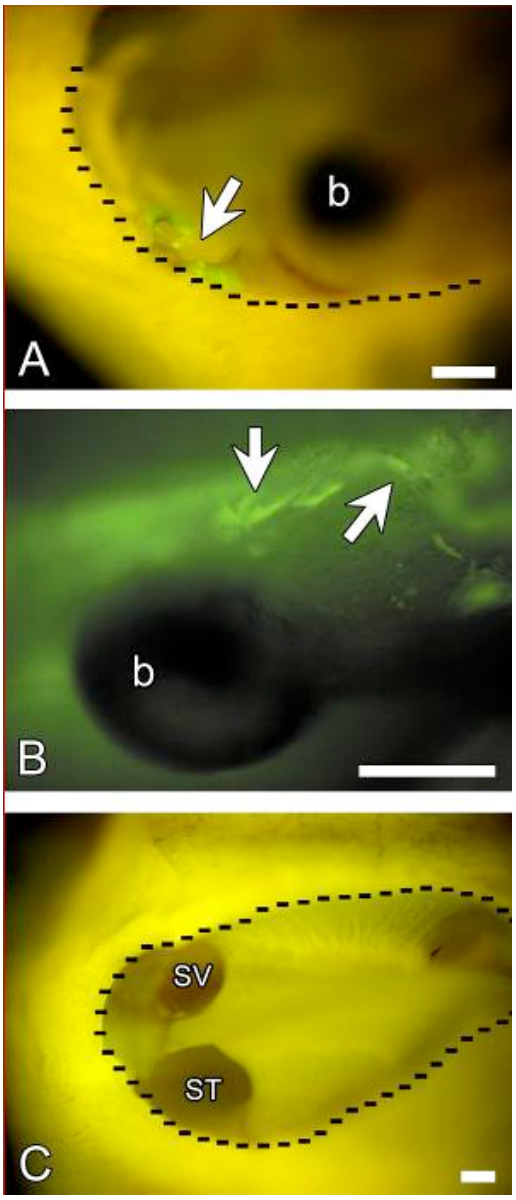


Fig. 3.4: Examples of neurofilament labeling within the cochlea.

Images from three different animals are shown. In (A), the ball electrode of the cochlear implant (b) and neurofilament – positive processes (white arrow) can be seen in the same view, although the implant was at a lower focal plane than the processes. In (B), at the highest magnification level possible with the stereoscope used here (160x), the implant electrode was clearly recognizable and individual processes can be seen (white arrows). In (C), a normal, non – deafened, non – implanted cochlea does not show any neurofilament – positive fibers within the scala tympani, ST = scala tympani, SV = scala vestibuli. Scale bar = 200μm in all images, dashed lines indicate the area where the lateral wall of the cochlea was removed to view the implant.

3.4. Discussion

In this study, we show that it was possible to visualize neuronal fibers at stereoscopic levels in relation to a CI *in situ*. Our technique leaves the CI in place within the cochlea, allows for very little movement of the implant during processing, allows visualization of the space within the ST, and reduces damage to cochlear tissue by performing minimal dissection. This represents a new technique to aid experiments that need to characterize the physical relationship between neurites and a CI *in vivo*.

The highest magnification level available with the stereoscope used in this study was 160x, a relatively low level compared to other histological methods. However, this level was sufficient to show individual nerve processes within the scala tympani (Fig. 3.4B). Lower magnification levels also showed fibers in both in the central auditory nerve (Fig. 3.2) and the scala tympani (Fig. 3.3). These figures demonstrate that the method described here can simultaneously provide images of the small nerve fibers and the larger implant, as well as the three – dimensional space around them.

The method described here may be best employed in conjunction with other cochlear histology methods. Due to the size of the implant, a stereoscope is necessary to visualize the entire implant within the cochlea, but the coarse nature of stereoscopic focus may make it difficult to determine the exact location of fluorescently – labeled fibers within the ST. Finer resolution could be found using mid – modiolar sectioning, whole mount preparations of the basal turn, or

confocal microscopy, any of which could be implemented following the method described in this paper. For example, a series of z – stacks using a confocal microscope could provide images that are more accurate and include more detail of the location of fluorescently – labeled fibers. However, the stereoscope must first be used to determine the general location of fibers because the high magnification of the confocal microscope does not permit visualization of the entire implant and the fibers in the same view. The technique described in this paper could therefore be an important preliminary histological step in auditory nerve regeneration studies.

This technique should be used as qualitative assessment of neurites within the cochlea and not a quantitative metric, for several reasons. First, the focal adjustments on the stereoscope are not fine enough to ensure accurate imaging of neurites. Second, there is limit to the time spent capturing images of fluorescing fibers, and anti – fade medium cannot be applied to these specimens because of air exposure. Individual variability in the strength of labeling is quite possible with images taken without anti – fade medium due to the length of light exposure prior to image capture. This can be seen in the difference in signal strength between the central nerve fibers seen in Fig. 3.2 and the peripheral fibers seen in Fig. 3.3. The images of the central nerve fibers were taken prior to the images of the peripheral nerve fibers, to verify successful immunohistochemical processing. The processes in the scala tympani images therefore received longer light exposure and did not fluoresce as brightly as the central nerve fibers. Finally, there is the possibility that fibers grew medial to the

cochlear implant and were blocked from view by the implant. These neurites could be seen in cross sections of the cochlea, and potentially counted in these images, but these sections are taken after implant removal, which will most likely disrupt or shear any fibers that have grown within the scala tympani. Any of these complications would likely make any neurite count an underestimate.

While we focused on neurofilament labeling, this protocol could be easily adapted for use with a host of neuronal and synaptic markers. Neurofilament labeling does not tell us whether the nerve growth seen within the scala tympani was afferent or efferent, regenerated fibers, or whether the nerve is functional. However, other markers, such as ChAT, synapsin, or GAP43, that could further define the fiber growth. Additional immunohistochemistry could be performed following this initial characterization, allowing gross visualization of nerve processes while not precluding the use of sectioning if microanalysis is also desired. Modifications to the immunohistochemical protocol, such as the choice of blocking agent and antibody hosts, should be considered if further labeling would occur.

The current paper does not attempt to regrow auditory nerve peripheral processes, but rather develop a method for visualizing nerve fibers and their spatial relationship to a cochlear implant. Animals were deafened prior to implantation and tissue processing to disconnect peripheral processes from hair cells and encourage growth into the scala tympani. Indeed, neurofilament – labeled processes were only seen within the deafened cochleae, not within the non – deafened cochleae (Fig. 3.4). Growth of these processes into the scala

tympani following hair cell loss has been seen previously (Leake and Hradek, 1988; Staecker et al., 1996). The fibers seen in these previous studies and in the images of this paper (Fig. 3.3 and Fig. 3.4) exhibited uncontrolled and indistinct growth patterns. The technique detailed in this study could provide a valuable tool for viewing nerve growth and help to refine future experiments directed at controlling and promoting the regrowth of auditory nerve fibers. This method provides an easily adaptable means to view fibers within the three – dimensional space of the scala tympani in conjunction with a cochlear implant.

Chapter 4

Cochlear implant hydrogel coating promotes auditory nerve fiber growth within the scala tympani in direct vicinity of implant

4.1. Introduction

Cochlear hair cells are the sensory transducing cells of the auditory system and innervate the primary cell bodies of the auditory nerve, spiral ganglion cells (SGCs). Peripheral processes extend from the hair cells in the basilar membrane through the habenula perforata, osseous spiral lamina, and connect to SGCs. Hair cell loss is a common cause of hearing loss, is caused by a number of environmental and genetic variables, and is irreversible in mammals. Once hair cells are lost or no longer functional, the peripheral processes and SGCs often degenerate as well (Spoendlin, 1975), due to the loss of both electrical and chemical stimulation. While the regeneration of mammalian cochlear hair cells is currently under investigation (Izumikawa et al., 2005), there is presently no clinical approach to restore hearing through these cells. In cases of severe to profound hearing loss, cochlear implants (CIs) offer a way to bypass the function of hair cells and directly stimulate the auditory nerve. CIs have been quite effective in providing hearing to patients for several decades, but users still have difficulty processing complex sounds such as music.

Compared to other implantable prostheses, the cochlear implant is located relatively far from the stimulated tissue. The implant is placed into the scala tympani, one of three fluid – filled space of the cochlea. Spiral ganglion cells are located within the center of the cochlea and separated from the implant by bone, tissue, and fluid. Thus, a large amount of current is needed to stimulate the auditory nerve, which can lead to diffuse stimulation from each electrode, overlapping current fields, and channel interaction. In addition, high current levels consume more battery power. However, if the distance between the nerve and the electrodes were reduced, more discreet stimulation and lower battery consumption are possible, which could provide better processing of complex sounds. Physiological studies of cochlear implant stimulation have shown that a shorter distance between the ganglion cells and the implant can lower thresholds to electrical stimulation (Shepherd et al., 1993; Cohen et al., 2005), and model studies suggest that stimulating the nerve fibers instead of cell bodies can improve the fidelity of the signal and lower thresholds (Stypulkowski and van den Honert, 1984; Colombo and Parkins, 1987)

Spontaneous resprouting of the auditory nerve peripheral processes has been seen in mammals following both acoustic trauma and aminoglycoside induced hair cells loss (see Chapter 3). These regenerated fibers are quite variable in quantity and location, but give evidence that there may be opportunity for interventions to promote nerve fiber survival and growth in addition to improving SGC survival. In animal models, the introduction of neurotrophic factors, particularly neurotrophic factor 3 (NT – 3) and brain – derived

neurotrophic factor (BDNF), when introduced following hair cell loss, can promote somatic survival (Hartnick et al., 1996; Staecker et al., 1996; Gillespie et al., 2003), and regrow peripheral processes (Malgrange et al., 1996; Wise et al., 2005). While these studies show that it is possible to regrow the deafferented auditory nerve, it has not yet been shown that this regrowth can be directed toward a cochlear implant *in vivo*.

Attempts to direct growth of nerve processes to make contact with an intracochlear implant are hindered by the location of the implant, within the scala tympani in perilymphatic fluid, which does not provide a substrate for neurites to grow on or give directional cues. We have therefore developed a cochlear implant coating that was designed to provide both an extracellular matrix on which neurites can attach and extend processes as well as a chemotropic target to direct growth. This coating was composed of an alginate hydrogel that has been modified to include the arginine – glycine – aspartate (RGD) motif (Rowley et al., 1999) and was applied to the implant prior to implantation and encircles the implant. This gel forms a lining between the implant and the cochlea, which increases biocompatibility of the implant and aids neurite growth by creating a scaffold within the scala tympani on which neurites can grow. In addition, the composition of the hydrogel makes it an excellent vehicle for drug delivery, as the gel can be hydrated in a solution containing a drug of interest, which was absorbed into the gel and slowly released from the gel once the implant was placed in the scala tympani. BDNF was the growth factor of choice in this study, and we hypothesized that the slow release of BDNF from the coating would

provide a chemoattractant source to direct auditory nerve process regrowth toward the cochlear implant.

4.2. Methods and Materials

Implant Construction and Coating

Cochlear implants were made in – house as previously described (Chapter 3) (Miller et al., 1995). Briefly, a platinum – iridium wire (A & M Systems, Sequim, WA) was used to construct a ball electrode (450 μm diameter), around which another wire was wrapped to create a helix shape (300 μm length and diameter). A piece of polyimide 2 mm long separated and insulated the two wires (Fig 4.1). The lead wires from the two electrodes were threaded through silicone tubing and connected to a pedestal that held the wires in place. Liquid silastic was used to seal the junction between the ball electrode and the polyimide and between the silicone tubing and the two lead wires.

Implants received either a hydrogel + BDNF coating, a hydrogel – only coating, or no coating at all. Hydrogel coatings were applied by dipping the implant into two solutions. The first solution was 1 % RDG – alginate in PBS. The second solution was 2 % $CaCl_2$ in PBS, which cross – linked the polysaccharide chains of the alginate. Implants were dipped in this combination of solutions four times, to build up a coating on the implant that was approximately 50 μm thick

(Fig. 4.1). The gel was then dehydrated in open air and the implant was sterilized for surgery using ethylene oxide.

Immediately prior to implantation, implants from the hydrogel + BDNF group were placed in 10 µl of 400 ng/ml human recombinant BDNF (Millipore) for 45 minutes in a 1.5 mL microcentrifuge tube. This volume was sufficient to completely cover the hydrogel – coated end of the implant. Implants were dehydrated for 20 minutes prior to implantation to allow ease in insertion and to avoid dislodging the hydrogel coating from the implant during surgery.

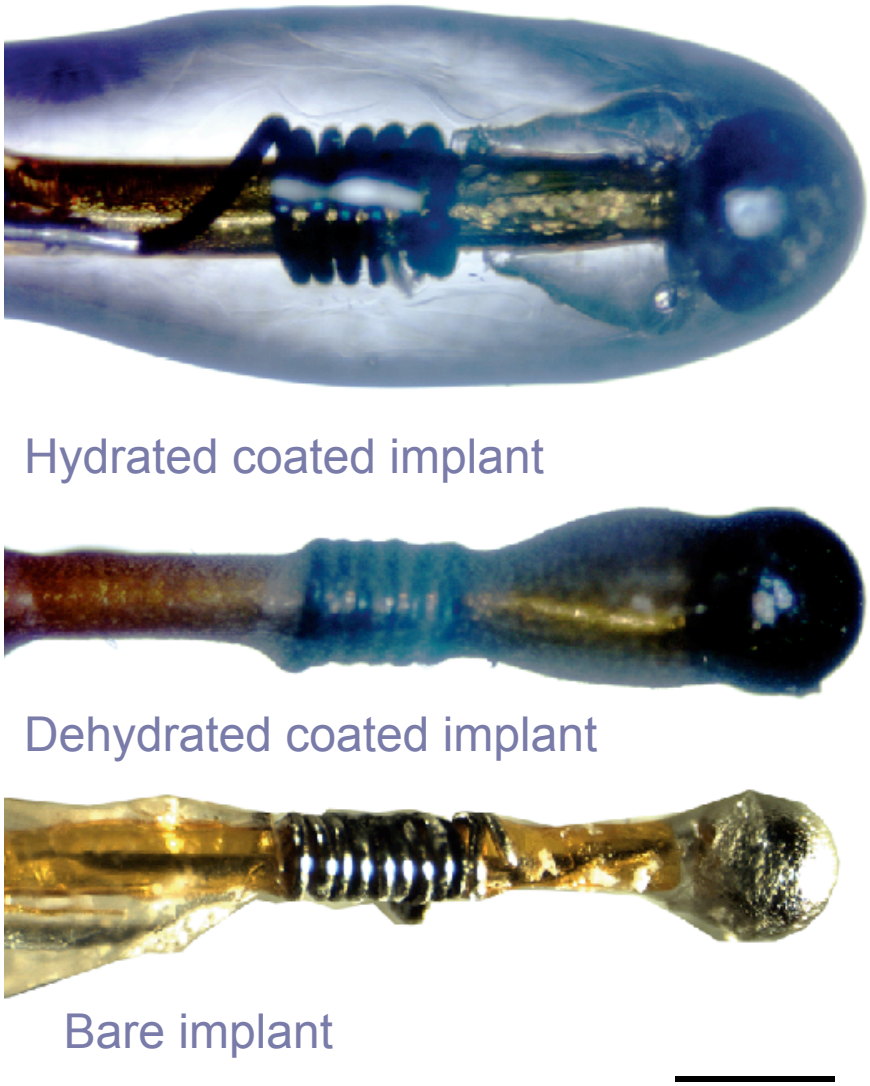


Fig. 4.1: Two – electrode cochlear implant used in the study.

Implants were constructed in – house using Pt/Ir wire. The ball electrode was insulated with polyimide tubing and the helix electrode was wrapped around the polyimide. Silastic insulated the space between the two electrodes. For treatment groups that received hydrogel coated implants, the implant was dipped in alginate hydrogel that formed a coating around both electrodes (top panel). This coated could be dehydrated (middle panel), to allow a cochlear implantation similar to an uncoated implant (bottom panel), and the coating swelled once placed into the fluid – filled scala tympani. Scale bar = 500µm.

Subjects

Eleven male pigmented guinea pigs (Elm Hill, Chelmsford, Mass, USA) were used in this study, and weights ranged from 400 – 600 g at time of implantation. Pure tone auditory brainstem responses were recorded pre – and post – deafening to ensure normal hearing levels and thoroughness of the deafening procedure. 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).

Deafening and implantation

Animals were systemically deafened using a combination of kanamycin (400 mg/kg, SQ), and ethacrynic acid (40 mg/kg, IV, two hours following kanamycin) one week prior to implantation. Animals were given a ketamine (40 mg/kg) and xylazine (10 mg/kg) mix (IM) for general anesthesia and lidocaine was used as a local anesthetic. For cochlear implantation, an incision was made down the midline of the head and muscle and connective tissue were gently pushed apart to reveal the skull. Three screws were placed in the skull to form a

triangle around bregma and these screws held the head of a restraining bolt. The base of the cochlear implant was connected to the restraining bolt to secure the implant to the animal's head. An incision was made in an arc caudal to the pinna of the ear to be implanted (left ear for all animals) and the underlying muscle and tissue were gently pushed back to reveal the bulla. A small hole was made in the bulla with the tip of a scalpel blade and a small cochleostomy was made just apical to the round window to expose the scala tympani. Implants were inserted approximately one – third to one – half of the way into the basal turn of the cochlea. Carboxylate cement was applied to the hole in the bulla to seal the middle ear space and secure the position of the implant. The skin incision was sutured in two layers and the animal was given warm subcutaneous fluids and allowed to recover.

Neurofilament assessment

Four weeks post – implantation, animals were deeply anesthetized and decapitated. Both temporal bones were removed and processed as previously described (Chapter 3). Briefly, the temporal bone was trimmed to reveal the cochlea and the implant remained immobile by the carboxylate cement attaching it to the temporal bone. With the implant still in place, each cochlea received a local perfusion of 4 % paraformaldehyde and allowed to soak in 4 % paraformaldehyde overnight. The wall of the basal turn of the cochlea was carefully trimmed using a bone drill and diamond burrs and/or forceps to reveal

the implant in place within the scala tympani. Immunohistochemistry was performed using neurofilament 160kD (1:100) as the primary antibody and FITC as the second antibody (1:200). Specimens were viewed under a Leica MXFL III stereo fluorescence microscope (Leica, Eaton, PA).

4.3. Results

Stereoscopic view

Stereoscopic images from one animal from each of the three treatment groups are shown in Fig. 4.2 (hydrogel + BDNF), Fig. 4.3 (hydrogel – only), and Fig. 4.4 (bare). The images on the left (A) are under bright light for orientation purposes, and the images on the right (B) are under fluorescent light and are magnified views of the basal scala tympani. Neurofilament – labeled processes (white arrows) were visible within the scala tympani in the hydrogel + BDNF and hydrogel – only groups. These fibers were located in various locations within the scala tympani, including near the basilar membrane (Fig. 4.2B) and near the outer scala tympani wall (Fig. 4.3B). There was no observed consistency of fiber location within groups. There were no neurofilament – positive processes seen in control cochleae.

In addition to the neurofilament – positive processes seen near the basilar membrane and the scala tympani wall, fibers were also seen in close vicinity to

the cochlear implant. A higher magnification image of the ball electrode from an animal in the hydrogel + BDNF group is shown in Fig. 4.5. Individual fluorescent fibers are clearly visible within 50 μm of the ball electrode, within the hydrogel complex. There were fluorescing fibers also seen in different focal planes, indicating more neurites throughout the hydrogel, surrounding the ball electrode.

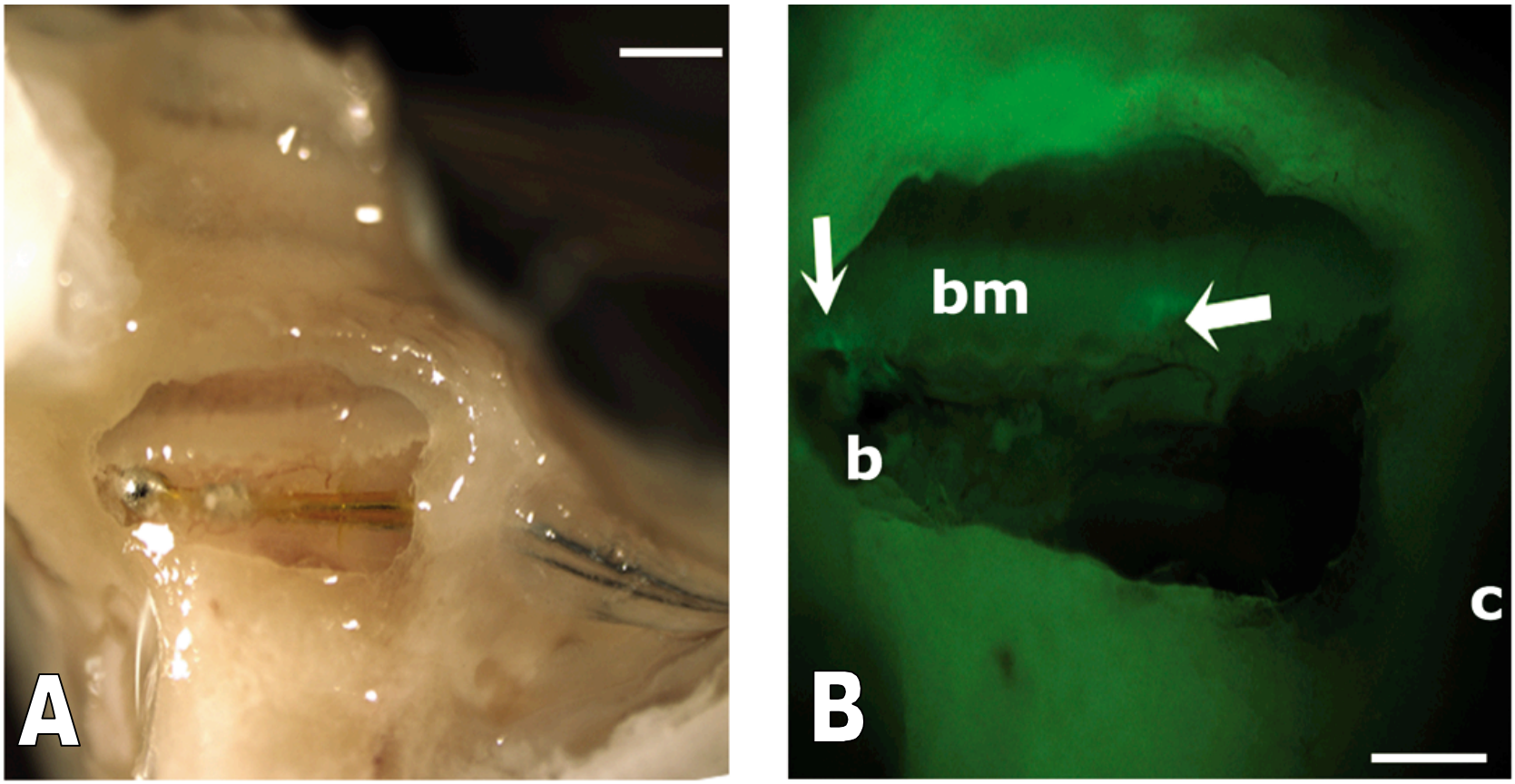


Fig. 4.2: Neurofilament positive fibers within the scala tympani of a cochlea implanted with a hydrogel and BDNF coated implant.

(A) shows the entire cochlea under bright light and (B) shows a magnified view of the basal turn of the cochlea under fluorescent light. The cochlear implant (b = ball electrode) and neurofilament – labeled fibers (white arrows) are both visible within the scala tympani. Scale bar in A = 1mm, scale bar in B = 500µm,
bm = basilar membrane, c = original cochleostomy.

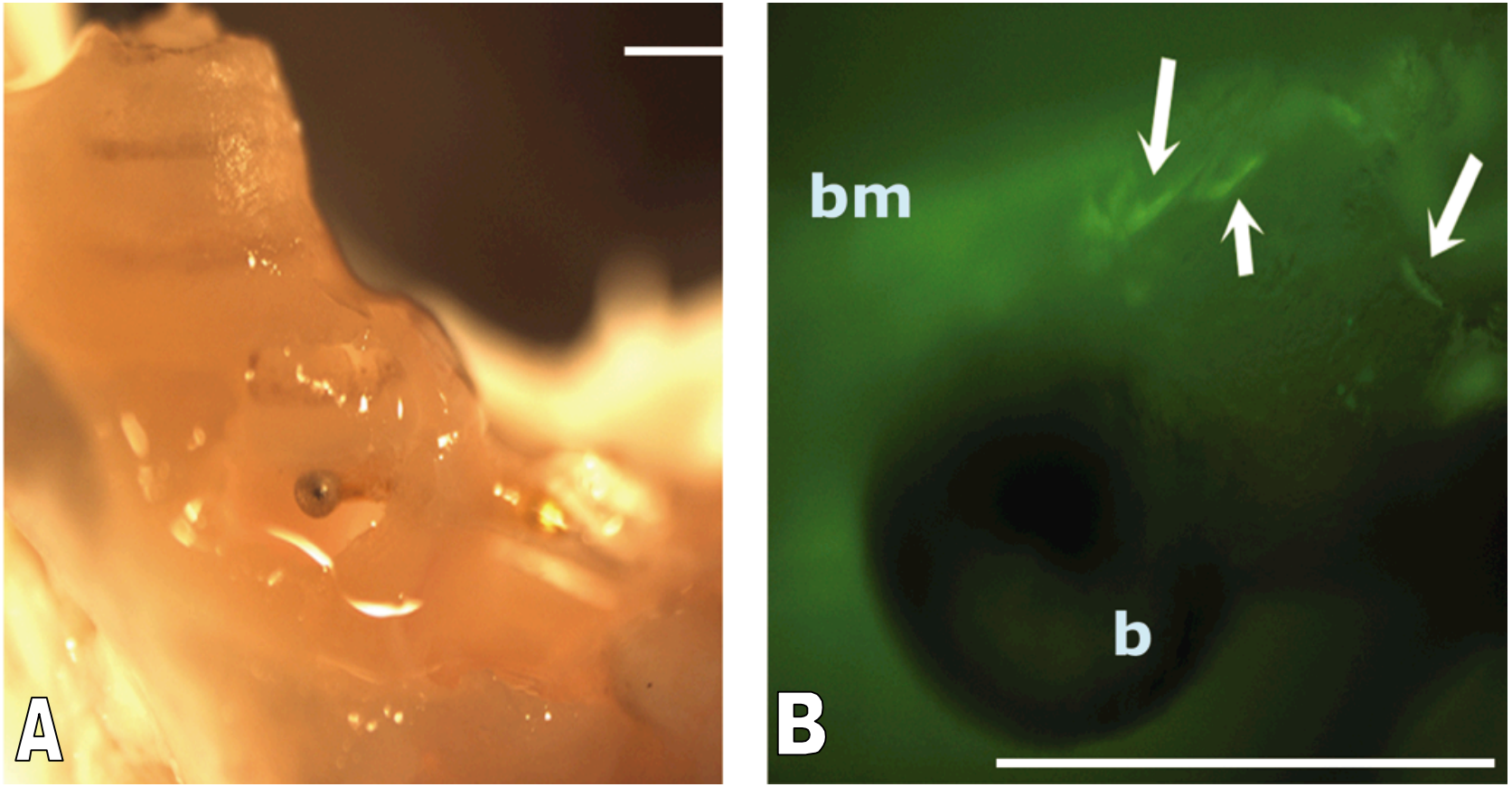


Fig. 4.3: Neurofilament positive fibers within the scala tympani of a cochlea implanted with a hydrogel – only coated implant.

(A) shows the entire cochlea under bright light and (B) shows a magnified view of the basal turn of the cochlea under fluorescent light. The cochlear implant (b = ball electrode) and neurofilament – labeled fibers (white arrows) are both visible within the scala tympani. Scale bar in A = 1mm, scale bar in B = 500µm, bm = basilar membrane.

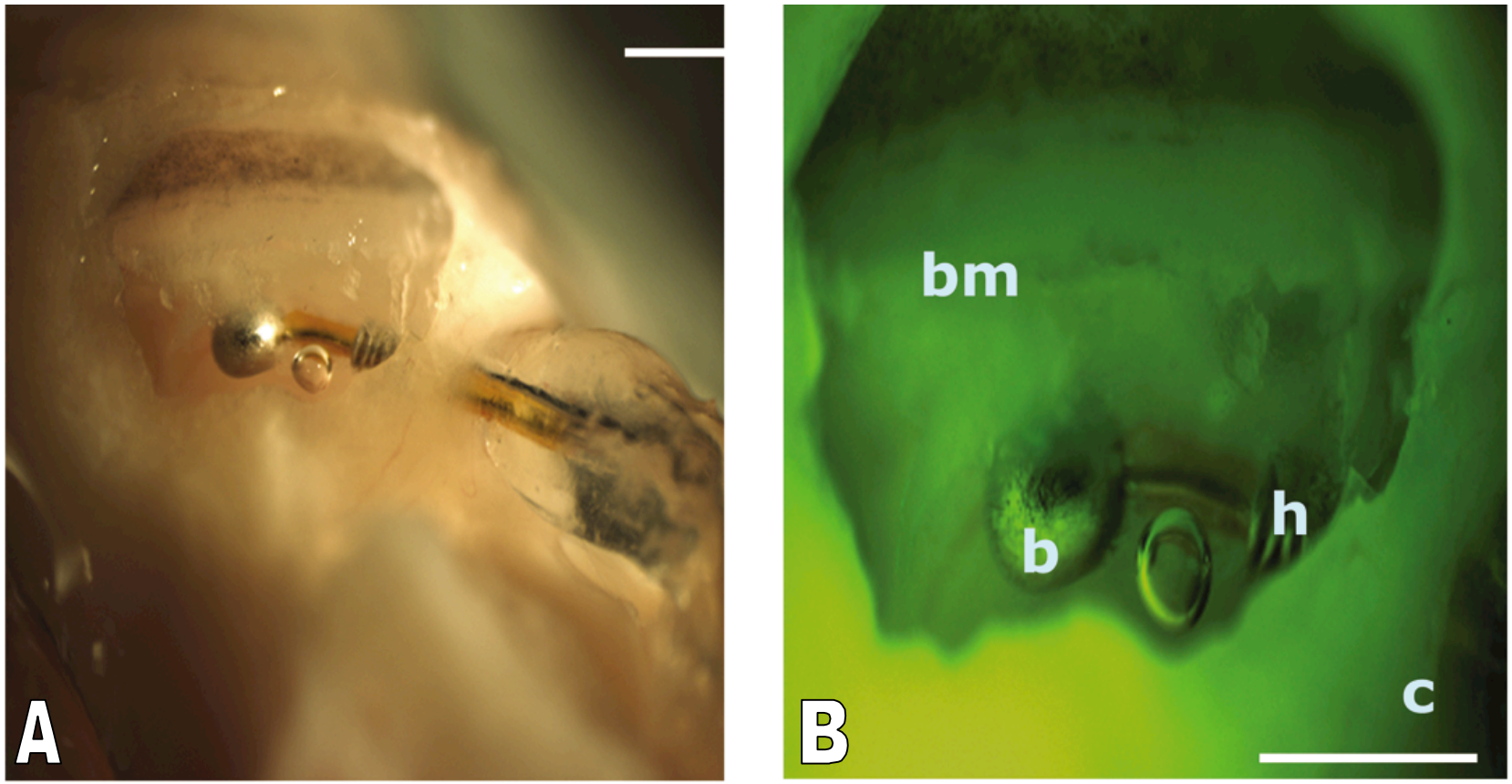


Fig. 4.4: Neurofilament positive fibers within the scala tympani of a cochlea implanted with an uncoated implant.

(A) shows the entire cochlea under bright light and (B) shows a magnified view of the basal turn of the cochlea under fluorescent light. The cochlear implant (b = ball electrode, h = helix electrode) is visible within the scala tympani, but no neurofilament – positive processes were evident. Scale bar in A = 1mm, scale bar in B = 500µm, bm = basilar membrane, c = original cochleostomy.

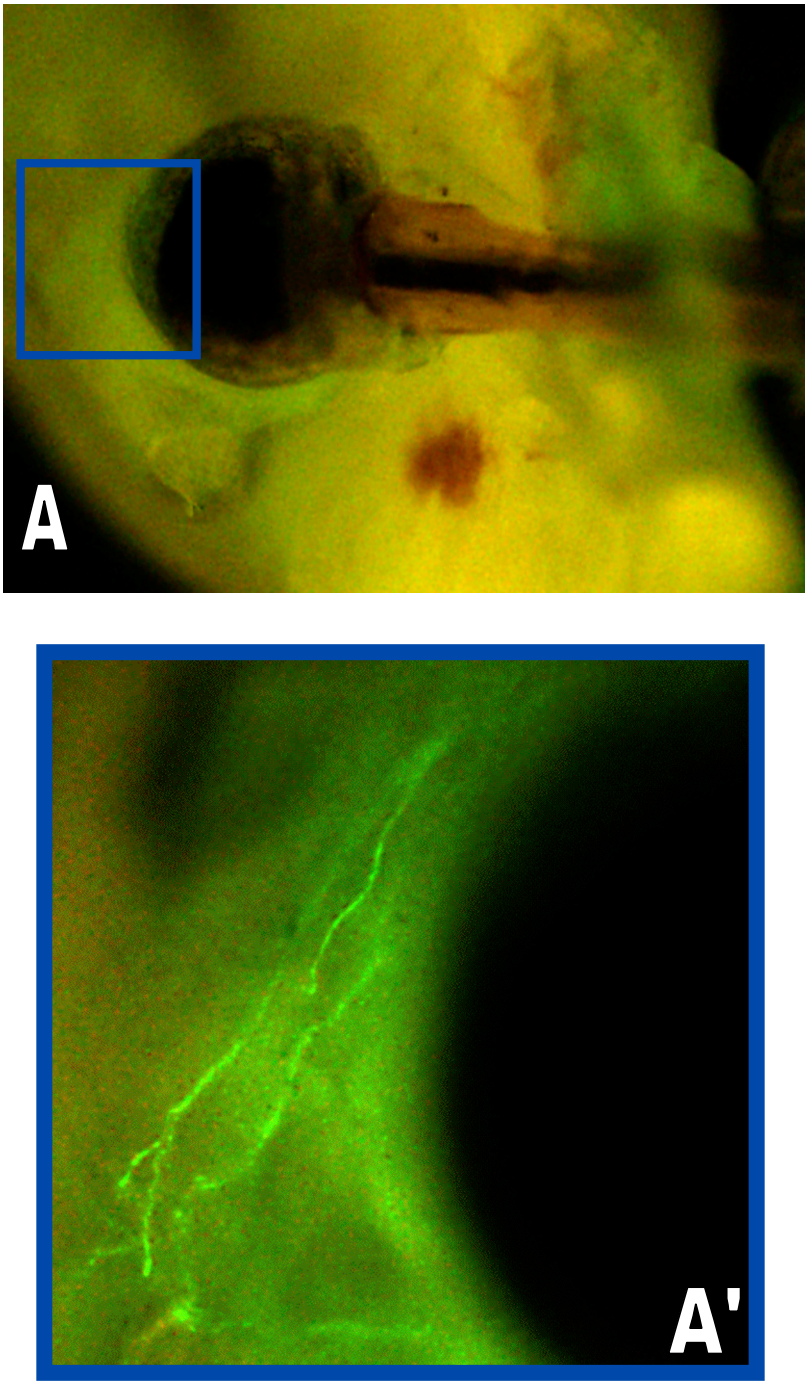


Fig. 4.5: Magnified view of the ball electrode within the scala tympani.

(A) shows the ball electrode from an animal that received a hydrogel and BDNF coated implant, and (A') shows a magnification view of the region highlighted in blue. Several neurofilament – labeled processes are visible in the immediate vicinity of the electrode. Other fibers in lower focal planes are visible as a glow, also close to the electrode.

The number of animals who showed neurofilament – labeled processes within the scala tympani is quantified in Fig. 4.6. Three out of four animals (75 %) in both the hydrogel + BDNF and hydrogel – only groups showed fluorescing neurites, and no animals in the bare implant group showed fluorescing neurites within the scala tympani. All animals showed fluorescing auditory nerve as it exited the temporal bone, which verified successful staining (see Chapter 3 for details on central auditory nerve fluorescence).

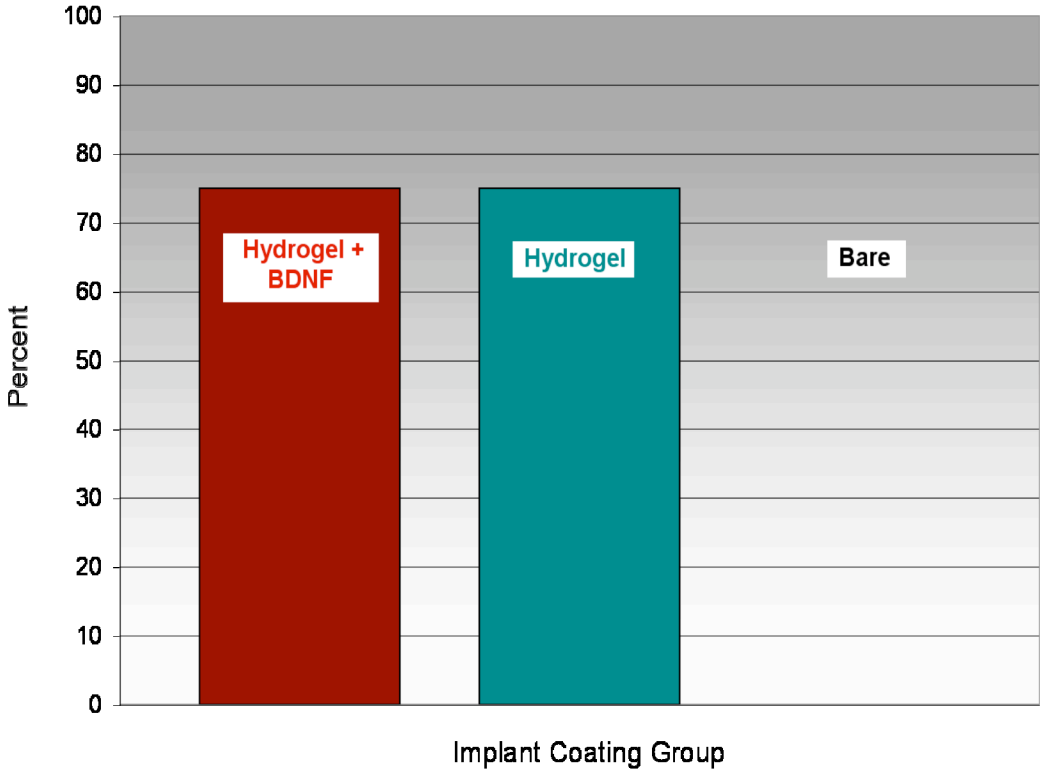


Fig. 4.6: Percent of cochleae within each treatment group with neurofilament – positive fibers within the scala tympani.

Three out four cochleae in both the hydrogel + BDNF and hydrogel – only groups showed neurofilament – labeled processes within the scala tympani. There were no processes seen in the cochleae from the bare group.

4.4. Discussion

We found that the application of an RGD – modified hydrogel to the cochlear implant could create an environment within the cochlea that supported and attracted neurite growth near the cochlear implant. The introduction of BDNF to gel coating did not improve neurite growth compared to hydrogel alone.

Previous studies have shown that the regeneration and growth of auditory nerve peripheral processes is possible. However, these studies have looked at the processes in their normal locations within the cochlea, the osseous spiral lamina and the habenula (Wise et al., 2005; Glueckert et al., 2008). Spontaneous neurite growth has shown that fibers can extend briefly into the scala tympani (Leake and Hradek, 1988; Staecker et al., 1996), but what we show here is significant neurite growth over several hundred micrometers within a fluid – filled space with the aid of a hydrogel scaffold. We saw this growth with hydrogel coatings that both did and did not contain BDNF, indicating that the addition of a growth factor was not necessary to attract neurites. The hydrogel used in this study was customized to include an RGD motif, which could have provided attractant cues that non – modified hydrogel would not have.

The type of neurite growth seen here could be quite beneficial for cochlear implant stimulation. Stimulating the nerve endings could lower the current required to reach threshold (Stypulkowski and van den Honert, 1984), which could lead to decreased channel interaction and improved speech – processing strategies. In addition, the hydrogel coating could improve the biocompatibility of

the cochlear implant by providing a physical buffer between the metal of the implant and the soft tissue of the cochlea and the delicate neurites.

The nature of the stereoscopic images in this study does not allow accurate quantification of the number of neurites within the scala tympani (see Chapter 3). The three – dimensional view leads to several different focal planes with fluorescent fibers in one image, as seen in Fig. 4.5. However, we demonstrate in this study that a hydrogel can attract and maintain (up to four weeks) neurite survival in fluid – filled space, over a distance of several hundred micrometers. Several hydrogels are already approved for clinical use in a number of biological systems, particularly for wound healing and tissue regeneration (Zimmermann et al., 2000). This study indicates the potential for hydrogel coatings to facilitate and improve CI performance. The growth of neurofilament – labeled processes within the scala tympani could improve the perception of sound from the CI if these fibers are functional and can be stimulated. The decrease in distance between the nerve and the electrode could decrease current levels, cause less cellular damage and consume less battery power. Future work should address the function of these nerves if these coating materials are to be of benefit to cochlear implant patients.

Chapter 5

The effect of PEDOT on cochlear implants electrophysiological thresholds and impedances *in vivo*

5.1. Introduction

Cochlear implants (CIs) stimulate the auditory nerve and provide hearing to people with severe to profound hearing loss. In the undamaged cochlea, sound is processed by sensory hair cells, which initiate action potentials within the auditory nerve. For many CI patients, the hair cells are missing or degenerated to a non – functional level and thus acoustic hearing is lost. CIs provide electrical hearing through bypassing the function of these lost hair cells and initiating action potentials at the level of the primary ganglion cells of the auditory nerve (spiral ganglion cells, SGCs). While current implant models function well and can provide hearing where there once was none, there is room for improvement. In particular, many CI users have difficulty understanding complex sounds, such as speech with background noise and music.

The location of the CI is unusual for neural prostheses and presents a challenge for biomedical technologies. Most implantable prostheses are placed in

direct contact with the tissue or neural structures that are stimulated. However, CIs are placed into the scala tympani, one of the fluid – filled spaces of the cochlea, and at a distance of up to 1 mm from the target of electrical stimulation, SGCs. The stimulation from the implant must pass through perilymph, bone, and soft tissue before reaching SGCs, all of which can affect impedance and current levels. In addition, the exact location of the implant within the scala tympani can vary between the lateral wall and the modiolus. The distance from the nerve and the implant affects the physiological thresholds to stimulation (Shepherd et al., 1993; Cohen et al., 2005; Briaire and Frijns, 2006). Reduced distance between the nerve and the electrodes can decrease thresholds and the spatial spread of excitation, potentially leading to more independent channels of stimulation and more complex speech processing strategies. Advancements in the processing of sound could improve the quality of hearing for CI users.

Conducting polymers, including polyaniline, polpyrrole and poly(3, 4 – ethylenedioxythiophene) (PEDOT) have been used in recent years in neural probes and prostheses as a means of improving the electrode – tissue interface (Green et al., 2008). These polymers create a fibrous coating around individual electrodes that substantially increases the surface area of the electrode. The application of conducting polymers on both stimulating and recording electrodes leads to improved signal – to – noise ratio, decreased impedances, reduced scar tissue formation, and improved charge transport (Cui et al., 2001; Ludwig et al., 2006). For cochlear implants, conducting polymers offer the ability to manipulate

the environment immediately surrounding the implant in ways that could improve electric hearing.

Hydrogels have also been used extensively in clinical and basic research to aid in tissue and organ regeneration and engineering (Coviello et al., 2006; Mano et al., 2007). Hydrogels are mostly water – based materials with cross – linked polymer chains that can both swell and dehydrate. These gels imitate an extracellular matrix by providing scaffolds to support neuronal and tissue growth and are porous enough to allow drug delivery. Hydrogels are highly biocompatible, as they can be made from natural materials, such as polysaccharides, and can degrade over time. Alginate is one polysaccharide used to form hydrogels that has been used in several biological systems. In the cochlea, alginate hydrogels have been used to effective deliver neurotrophic factors and transfected cells into the cochlea (Endo et al., 2005; Noushi et al., 2005; Rejali et al., 2007).

This study addresses the effects of a combination of a conducting polymer (PEDOT) and hydrogel coating on cochlear implant stimulation. When PEDOT is grown galvanostatically in the presence of a hydrogel, an outgrowth of the polymer through the gel matrix is achieved, effectively extending the reach of the electrode (Kim et al., 2004). This extension of the electrodes will decrease the initial distance between the nerve and the electrode. The hydrogel also provides a stabilizing force for the implant, as the gel encircles the implant and will extend to the walls of the scala tympani and center the implant within that space. This could reduce variability in distance from the implant and improve the fidelity of

the stimulation. The combination of conducting polymer and hydrogels to form a cochlear implant coating should enhance their individual effects on implant performance. The current study tested the *in vivo* effects of this combined cochlear implant coating on electrophysiological thresholds to cochlear implant stimulation and on impedances between implant electrodes.

5.2. Methods

Implant Construction and Coating

Cochlear implants were made in – house as previously described (Chapter 3). Implants received a hydrogel + PEDOT coating (labeled GelDOT), a PEDOT coating (labeled PEDOT), or no coating at all (labeled Bare). PEDOT was first electrochemically deposited on the two electrodes of the implant as previously described (Cui et al., 2001). The solution used for deposition was a 0.1 % EDOT / 0.2 % polystyrene sulfonate (PSS) in distilled water. Hydrogel coatings were applied by dipping the implant into two solutions a 1 % RDG – alginate in PBS (Rowley et al., 1999) and 2 % $CaCl_2$ in PBS (Kim et al., 2004). Implants were dipped in the gel solutions four times to build up a coating on the implant that was approximately 50 μm thick. After hydrogel coating, the implants were placed in a 0.1 % EDOT / 0.2 % PSS / 2 % $CaCl_2$ / PBS solution for additional deposition, which formed the PEDOT – PSS extensions through the

gel. GelDOT coated implants were also soaked in 400 ng/mL of brain – derived neurotrophic factor (BDNF) as part of a separate experiment. Implants were sterilized for surgery using ethylene oxide, and the hydrogel coated implants remained dehydrated until implantation into the cochlea.

Subjects

Twelve male pigmented guinea pigs (Elm Hill, Chelmsford, Mass, USA) were used in this study. Weights ranged from 400 – 600 g at the time of implantation. Animals were ototoxically deafened so that hair cell function would not interfere with the electrophysiological threshold recordings. Pure tone auditory brainstem responses were recorded pre – and post – deafening to ensure normal hearing levels and thoroughness of the deafening procedure. 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).

Surgery

Animals were systemically deafened using a combination of kanamycin (400 mg/kg, SQ), and ethacrynic acid (40 mg/kg, IV, 2 hours following kanamycin) one week prior to implantation. Animals were given a ketamine (40 mg/kg) and xylazine (10 mg/kg) mix (IM) for general anesthesia and lidocaine was used as a local anesthetic.

For cochlear implantation, an incision was made down the midline of the head and 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 that formed a triangle around bregma. These screws held the head of a restraining bolt, which connected to the base pedestal of the implant. The screws for electrically – evoked auditory brainstem response recordings were located 2 cm anterior to bregma, 1 cm lateral of bregma on the implanted side, and 1 cm posterior to bregma.

An incision was made in an arc caudal to the pinna of the ear to be implanted (left ear for all animals) and the underlying muscle and tissue were gently pushed back to reveal the bulla. A small hole was made in the bulla with the tip of a scalpel blade and a small cochleostomy was made below the lip of the round window to expose the scala tympani. Implants were inserted approximately one – third to one – half of the way into the basal turn of the cochlea (2 – 3 mm). Carboxylate cement was applied to the hole in the bulla to seal the middle ear space and secure the position of the implant. A ground

electrode (500 μm ball electrode, Pt – Ir) was placed in the post – auricular muscle. The skin incision was sutured in two layers and the animal was given warm subcutaneous fluids and allowed to recover.

Impedances

The impedance between the ball and helix electrodes, the ball and ground electrodes, and the helix and ground electrodes, was measured following implantation. Values were obtained several times a week and an average value per week was used for analyses. Impedances were measured using a 1 μA rms, 1 kHz sinusoid wave from an in – house constructed impedance meter.

Electrically – evoked auditory brainstem response

Electrically – evoked auditory brainstem responses (EABRs) were recorded as previously described (Chapter 2), under general anesthesia once a week for four weeks beginning one week post – implantation. Electrical stimuli were 50 μs phase duration monophasic alternating polarity square pulses. Neural activity was recorded using alligator clips attached to screws that were placed in the skull. One bipolar (ball – helix electrode) and two monopolar (ball – ground and helix – ground) electrode configurations were tested. EABR threshold was defined as the lowest level at which there was a repeatable Wave II.

5.3. Results

Impedance values

Impedances averaged over the entire testing period were significantly lower for both GelDOT and PEDOT than for the Bare group (Fig. 5.1). This difference was seen in both monopolar and bipolar configurations and was evident within the first week of implantation (one – way ANOVA for each configuration, $p < 0.05$). There were no differences between the GelDOT and PEDOT groups in any configuration.

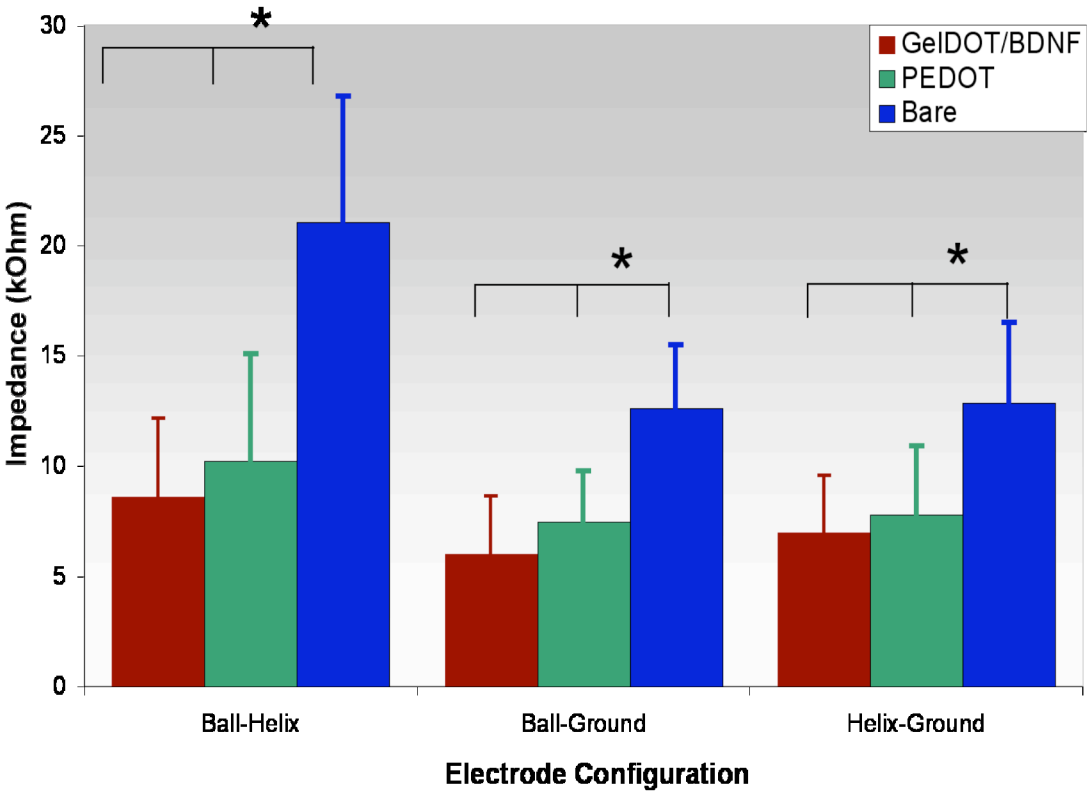


Fig. 5.1: Average impedance values at 1kHz

Weekly impedance values for three electrode configurations were averaged over the four week testing period and compared between groups. GelDOT/BDNF and PEDOT coated implants had significantly lower impedances than the uncoated group in all configurations, and there were no differences between the GelDOT and PEDOT groups. One – way ANOVA was performed on each electrode pair, (*) indicates $p < 0.05$.

Over time, impedances increased for all groups, as is typical for chronic implantation. However, the rate of increase varied between groups (Fig. 5.2). For the bipolar (ball – helix) configuration, repeated measures two – way ANOVA revealed a significant effect of both group and time ($p = 0.01$ and $p < 0.01$ respectively) but no interaction between group and time ($p = 0.07$). This indicates that there were significant changes within groups over time, but that the groups remained distinct from each other. There was a statistically significant difference between thresholds at 1 and 2 weeks post – implantation for the Bare group. A significant difference was not seen at these times points for the PEDOT and GelDOT groups. Instead, the difference in thresholds was significant between 1 and 3 weeks post – implantation in the PEDOT group and 1 and 4 weeks post – implantation in the GelDOT group. Similar patterns for all groups and time points were seen in both of the monopolar configurations.

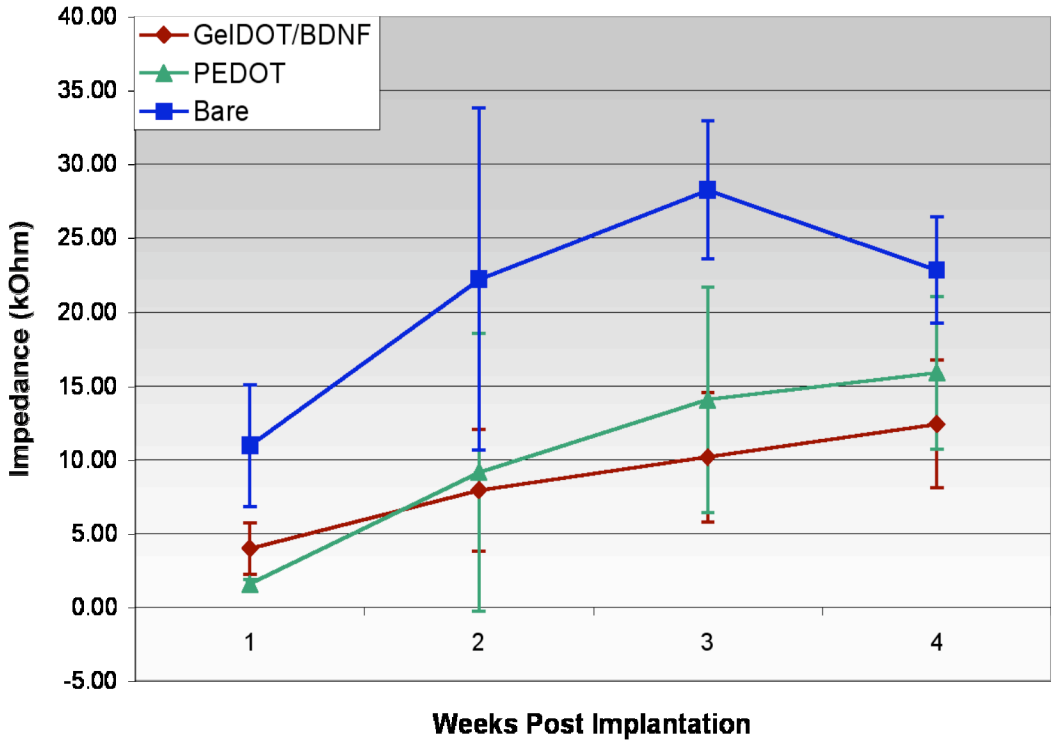


Fig. 5.2: Impedance values averaged over time for the bipolar (ball – helix) electrode configuration.

Data represent weekly averages per group +/ – one standard deviation. A repeated – measures two – way ANOVA revealed a significant effect of group and time but no interaction between groups. The impedance between GelDOT/BDNF and PEDOT coated electrodes was initially lower than the impedance between bare electrodes, and the increase in impedance was not as steep as the increase with bare electrodes.

EABR thresholds

There was a trend towards lower EABR thresholds in the bipolar configuration, averaged over four weeks, for the Bare group than either the GelDOT and PEDOT groups, but this was not a statistically significant difference (one – way ANOVA, $p = 0.07$) (Fig. 5.3). There were no observable or statistical differences in either of the monopolar configurations.

There were no differences seen between groups in thresholds over time. Fig. 5.4 represents EABR thresholds for the ball – ground monopolar electrode configuration, separated into weekly intervals. There was a significant effect of time ($p < 0.01$) but not group ($p = 0.51$) and no interaction between group and time. These data indicate that all groups showed similar changes in thresholds over time, which was a slight decrease from initial thresholds. A similar pattern was seen in the second monopolar configuration (helix – ground). The bipolar configuration did not show an effect of time or group, and all three groups had thresholds that were stable over time but not statistically significant from each other.

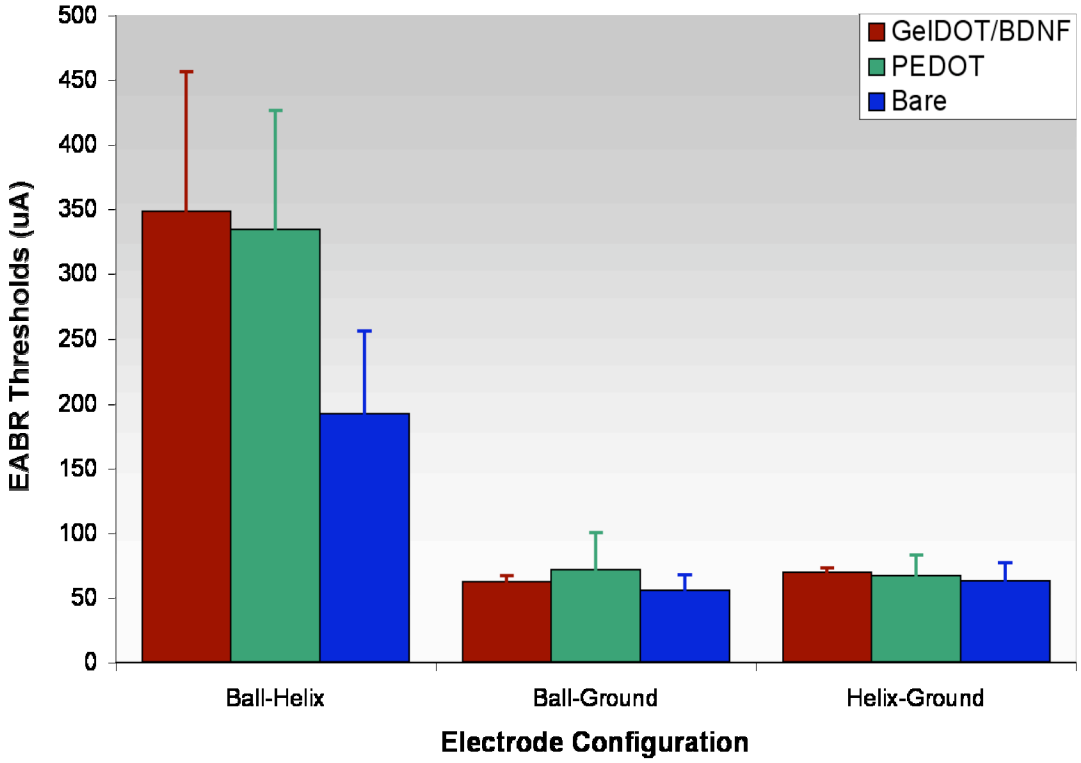


Fig. 5.3: Average EABR Thresholds for each electrode configuration.

Weekly EABR thresholds were averaged per group and one – way ANOVA was performed for each configuration. No differences were found in any of the three electrode configurations tested.

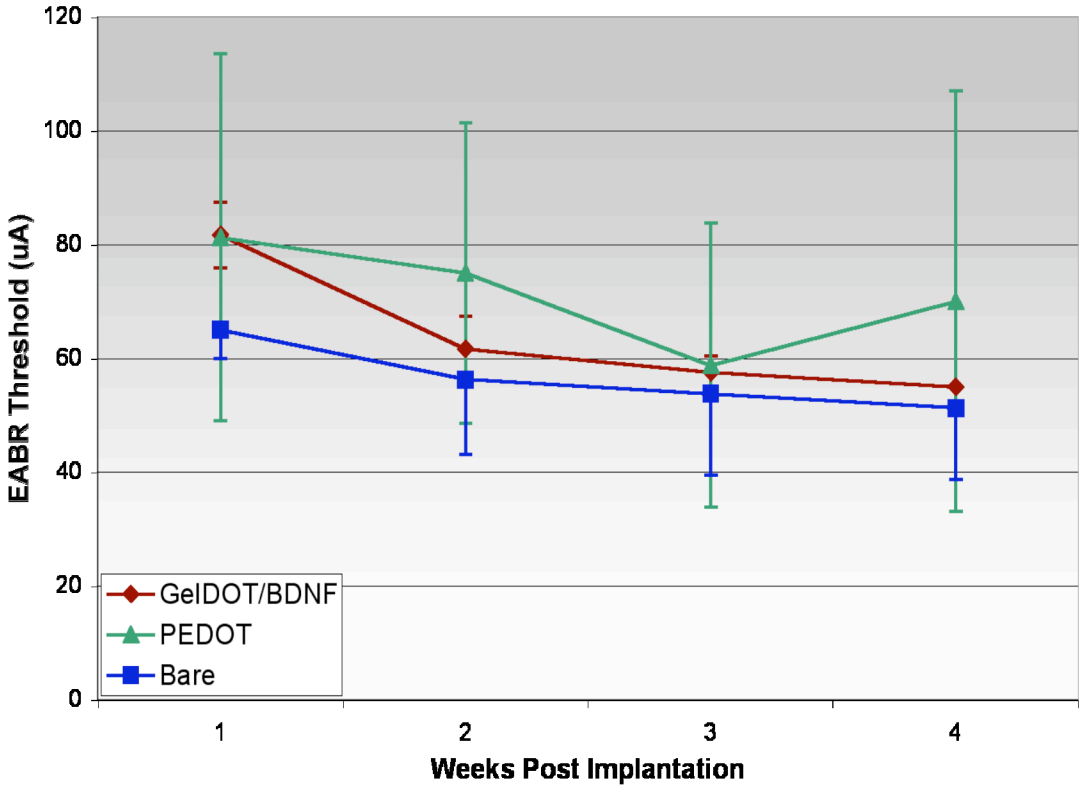


Fig. 5.4: EABR thresholds in a monopolar configuration over time.

Weekly thresholds were averaged per group and a two – way repeated measures ANOVA was performed to determine changes over time. There was no interaction between group and time, but there was an effect of time.

5.4. Discussion

The PEDOT and GelDOT coatings applied to the cochlear implant prior to implantation affected long – term *in vivo* impedance measures but did not affect electrophysiological thresholds. The greatest effect of the coatings on impedances was not seen in averages over time, but rather in the changes over time. This may be reflective of how each element of the coating affects either the electrical signal itself or the tissue response to cochlear implantation and electrical stimulation.

Impedance values are a function of both the characteristics of the electrodes and the status of the environment immediately surrounding the electrodes. The PEDOT coating on the electrodes created greater surface area; this most likely accounts for the overall decrease in impedance values with both the GelDOT group and the PEDOT group. This has been shown in cortical recordings and *in vitro* stimulation, but this is first evidence that the addition of a conducting polymer on the electrodes of a chronic cochlear implant can improve impedances *in vivo*. In the cochlea, increases in the impedance at frequencies such as the one used here (1 kHz), soon after implantation, can be attributed to changes in the immediate vicinity of the electrode (Duan et al., 2004). The differences between the treatment groups seen here in the rate of change are therefore most likely due to differences in the electrode – cochlea interface caused by the coating. The GelDOT group had a slower rate of impedance increases than the PEDOT group, which in turn had a slower rate of impedance

increases than the Bare group. The slower rate of increasing impedances over time for both the GelDOT and PEDOT groups may indicate that the conducting polymer slowed or reduced the intrascalar tissue growth that often accompanies cochlear implantation, and that the addition of hydrogel coating further enhanced this effect.

Although we saw a decrease in impedances with the PEDOT and GelDOT coatings, we did not see a corresponding decrease in EABR thresholds. Previous research indicates that lower physiological thresholds are correlated with improved auditory nerve survival (Shepherd et al., 2005). The lack of a difference in EABR thresholds seen here may therefore be an indication that the implant coatings did not affect the survival or the function of the auditory nerve or pathway, but instead merely changed the properties of the implant itself. The tissue – implant interface is a critical facet of research focused on improving cochlear implant function, and it is important to address both the tissue and implant aspects of the interface. While the PEDOT and GelDOT coatings may not have changed the cochlear environment in a significant manner, they did improve the stimulation from implant, and that may have significant effects on the perception processing of electrical stimulation.

The combination of a PEDOT and alginate hydrogel around the cochlear implant *in vivo* has great potential to improve electric hearing. The lower impedances and the slower rate of impedance increases over time in the GelDOT & PEDOT coatings could be important to future implant design and construction. Lower impedances could lead to lower voltage requirements,

increasing battery life and allowing the use of more complex speech processing software. A shallower rate of impedances increases over time could prohibit some of the issues that arise with high impedances, including signal compliance and compromised fidelity of the stimulation signal. Future physiological testing is required to fully characterize the effects of this coating on the processing of cochlear implant signals, as threshold levels alone may not be a sensitive enough measure. Although further refinements are necessary to fully characterize the clinical benefits of such a coating, this study demonstrates the feasibility, biocompatibility, and potential of the PEDOT and hydrogel cochlear implant coating.

Chapter 6

Conclusions

This dissertation presents several methods for improving auditory nerve survival following hair cell loss that are relevant to cochlear implant function. In an animal model of sensorineural hearing loss, these methods improved spiral ganglion cell survival, attracted peripheral processes growth into the scala tympani, lowered psychophysical detection threshold to cochlear implant stimulation, lowered electrophysiological thresholds to cochlear implant stimulation, and reduced impedances between implant electrodes. Any of these changes individually would be of interest to cochlear implant research, but the combination provides novel, exciting and clinically relevant data. The studies included in this dissertation employed molecular, cellular, physiological, and behavioral level metrics that provide a more complete understanding of the effects of the techniques used.

6.1. Adenoviral mediated up – regulation of BDNF

In the first study, an adenoviral construct containing a gene insert for the growth factor BDNF promoted spiral ganglion cell survival 80 days post –

inoculation. This inoculation also led to lower EABR thresholds and lower psychophysical detection thresholds with focused electrical stimulation. Spiral ganglion cell survival was highly correlated with EABR thresholds, but not psychophysical detection thresholds. This study showed an effect of a molecular level change on a systems level response and highlighted the need for several modes of cochlear implant functional assessment.

Ad.*BDNF* was inoculated into the cochlea at the time of implantation, four days after ototoxic lesioning. An increase in BDNF concentration in perilymph was seen between seven and fourteen days following Ad.*BDNF* inoculation (Figure 2.2). This time course allowed at least eleven and up to eighteen days of neural degeneration between initial trauma and potential effects of BDNF protection. Aminoglycoside – induced hair cell loss is seen within four days following introduction into the cochlea (Kim and Raphael, 2007), and significant ganglion cell degeneration can be seen within two weeks of ototoxic deafening (Webster and Webster, 1981). This suggests considerable cell body degeneration could have occurred before BDNF production was increased significantly. The timing of these events suggests that the Ad.*BDNF* treatment may have not only preserved some SGCs but also promoted growth of new neurons. The idea that growth factors can regenerate SGCs is supported by other studies that show SGC survival following a delay between deafening and growth factor treatment (Gillespie et al., 2004; Yamagata et al., 2004) as well as studies that show an increase in SGC density above normal levels after growth factor treatment (Shepherd et al., 2005; Glueckert et al., 2008).

Neuronal regeneration may also be indicated by the changes in EABR thresholds over time (Fig 2.6). The control group's thresholds increased over time, which may be attributable to a degeneration of the auditory nerve over time. Likewise, the decrease in EABR thresholds in the Ad.*BDNF* group over time may by attributable to a regeneration of the nerve. A similar trend was seen in a study by Shinohara et al. (2002), where EABR thresholds declined over time in a animal group that received growth factor treatment. In this study, growth factors were introduced to the cochlear continuously over the experimental timeframe using an osmotic pump; in the current study a single Ad.*BDNF* inoculation produced similar results. A separate longitudinal study assessing SGC density at different time points could further elucidate this result.

It is possible that the changes seen in EABR thresholds were not related simply to SGC density, but rather SGC function. Although not assessed in this study, growth of peripheral processes in addition to cell bodies following Ad.*BDNF* inoculation is feasible. These processes may have facilitated the transmission of electrical stimulation, particularly if the processes were myelinated. Future studies could include more detailed histological examination of the osseous spiral lamina and Rosenthal's canal, as well as the myelination of the central auditory nerve.

6.2. Neurite growth within the scala tympani

In the second study, a hydrogel coating promoted neurite growth within the scala tympani near a CI *in vivo*. Neurite growth was seen within the scala tympani of cochleae that received a hydrogel coated implant both with and without BDNF. This indicates that the presence of a scaffold within the scala tympani was sufficient to support growth, without a chemoattractant source. While previous research has shown that auditory nerve processes can regenerate following hair cell trauma, (Cho et al., 1998; Wise et al., 2005; Glueckert et al., 2008), anatomical assessments have been restricted to the osseous spiral lamina and habenula; that is, the normal, expected sites of these processes. This study was able to show fiber growth in the fluid – filled space of the scala tympani, a distance of several hundred micrometers from the normal location. Growth was seen within the hydrogel complex in various regions of the scala tympani, including the direct vicinity of the cochlear implant. The hydrogel coating promoted significant neurite growth, as nine out of twelve animals that received a hydrogel – coated implant showed some neurofilament – labeled processes within the scala tympani.

The hydrogel used in this study was not a simple alginate gel, but rather was modified prior to use to include an arginine – glycine – aspartic acid (RGD) motif. RGD is an integrin – binding site for a vast number of extracellular matrix ligands, which effectively promotes cell adhesion (Ruoslahti, 1996). This sequence has been coupled to the polysaccharide chains of the alginate gel, and

has been shown to promote muscle and bone tissue growth (Rowley et al., 1999; Alsberg et al., 2003). This modification may be vital to the neuronal growth that we have seen in this study, by providing a source of support for neurite ingrowth within the hydrogel. This modification may also explain the neurite growth seen without BDNF, because the RGD served as an attractant when BDNF was not available.

With the immunolabeling system used in this study, we cannot be sure that the fibers seen within the scala tympani are in fact auditory nerve fibers. Neurofilament is found in all nerves and it is possible, although unlikely due to the relative isolation of the cochlea, that the fibers seen here came from a source other than the auditory nerve. We also do not know if these fibers are afferent or efferent, and this will be a major topic to address if this type of neural regeneration is to be of use to cochlear implant function. These issues, however, do not negate the exciting results that we have developed a method to attract nerve fibers to grow through cochlear fluids over a distance of several hundreds micrometers toward a cochlear implant *in vivo*.

The concentration of BDNF within the cochlea over time should also be assessed in future studies. Our hydrogel coated implants were soaked in 10 μL of 400 ng/mL BDNF, which is a relatively high concentration of the growth factor. Neurotrophins, including BDNF and NT – 3, are effective at promoting spiral ganglion cell survival when introduced into the cochlea in lower concentrations [i.e. 50 ng/mL, (Miller et al., 1997; Wise et al., 2005)]. However, the supply of low concentration growth factors is continually replenished if introduced through

osmotic pumps, as in the above studies. The passive release of growth factors from the hydrogel – coated implants in the current study provided only one non – renewable source of growth factors; we increased the concentration to compensate for this. Interestingly, when neurite regrowth was seen in the scala tympani by Staecker et. al. (1996), a growth factor concentration of 1 mg/ml was used. In comparison to this study, the current results show significant neurite growth within the scala tympani using four orders of magnitude lower concentration of BDNF.

A novel histological method was developed in the course of this study to determine the spatial relationship between neurites and a cochlear implant *in situ*. This technique was sensitive enough to visualize individual neurites, yet broad enough to view the implant and the cochlea around it. The development of this technique was crucial to the results of this study. Our goal was to determine if we could induce neurite growth near the cochlear implant, which necessitated viewing both the implant and the neurites simultaneously. No other established technique allowed us this opportunity, and therefore a portion of this dissertation was dedicated to the development of this technique. This method is not limited to this study, but could be easily applied to future research involving intra – scalar morphological changes induced by or directly related to cochlear implants.

6.3. PEDOT coating reduces *in vivo* impedances

In the third study, a conducting polymer/hydrogel coating on the cochlear implant reduced both bipolar and monopolar impedances but did not affect EABR thresholds. The conducting polymer, PEDOT, has previously been used in recording electrodes (Ludwig et al., 2006), but this is the first published data showing the effect of PEDOT on stimulating electrodes. A reduction of *in vivo* impedances is important to cochlear implant performance for several reasons. With low impedances, a lower voltage is required to produce the same current level needed to activate neurons, and a lower voltage could lead to lower battery usage. Battery life and replacement is an issue with implantable prostheses in general. A reduction in the need to replace batteries would make life simpler for implant users and a decrease in the battery consumption for stimulation could set aside more battery power for speech processing software. Increased speech processing strategies are theoretically available, but not yet utilized because of their high battery demands, but decreasing impedances could indirectly aid this process.

A reduction in impedances is also important for long – term implant characteristics because high impedance values affect implant compliance and can degrade the fidelity of the electrical signal. This can disrupt or distort the perception of sound for cochlear implant users. Impedances typically increase with time following implantation, as demonstrated by the control group in Fig. 5.2. The PEDOT coating slowed the rate of impedance increases over time,

potentially allowing longer implantation times. Future longitudinal studies could further reveal the effects of this coating on *in vivo* impedance changes following implantation. This study demonstrated that the PEDOT and/or GelDOT coating made the implant more biocompatible (as evidenced by the decrease in impedances) yet did not attenuate the electric hearing process (as evidenced by the lack of difference in EABR thresholds).

6.4. Summary

Both the adenoviral inoculation and the hydrogel/conducting polymer coating offer long – term, highly effective means of delivering growth factors to the cochlea and promoting survival and regeneration of the auditory nerve. This is an important consideration for cochlear implant work because the stimulation from the implant is dependent on a functional auditory nerve, yet the nerve is susceptible to degeneration over time. Although the studies in this dissertation presented growth factor treatment at the time of implantation, both inoculation and hydrogel insertion to the cochlea prior to implantation are also feasible. This approach to growth factor treatment may support nerve survival until implantation can occur.

The ultimate goal of this and other research is to improve the processing of complex sounds so that cochlear implant users receive more complete auditory perception. The increase in SGC density, directed growth of nerve fibers to make contact with a cochlear implant, and reduced *in vivo* impedances

present several potential physiological and perceptual benefits to cochlear implant users. The results from these three studies could lead to a lower level of current required to stimulate the nerve. Lower current levels could decrease the spread of excitation from each electrode of the implant, allowing more independent channels of stimulation. Of course, lowering current levels is just one way to improve cochlear implant function, and will likely need to be combined with other advances in hardware and software engineering to provide more complete perception of sound. Future morphological, physiological, psychophysical testing is required to fully develop the clinical applicability of the treatments described in this dissertation. However, the data presented in these studies provide evidence that changing the morphology of the cochlea to make it more receptive to cochlear implant stimulation is necessary, feasible, and beneficial.

Appendices

Appendix A

Color Images from Chapter 3

Visualization of spiral ganglion neurites within the scala tympani with a cochlear implant *in situ*

Fig. 3.1: Basal cochlear dissection with implant in place.

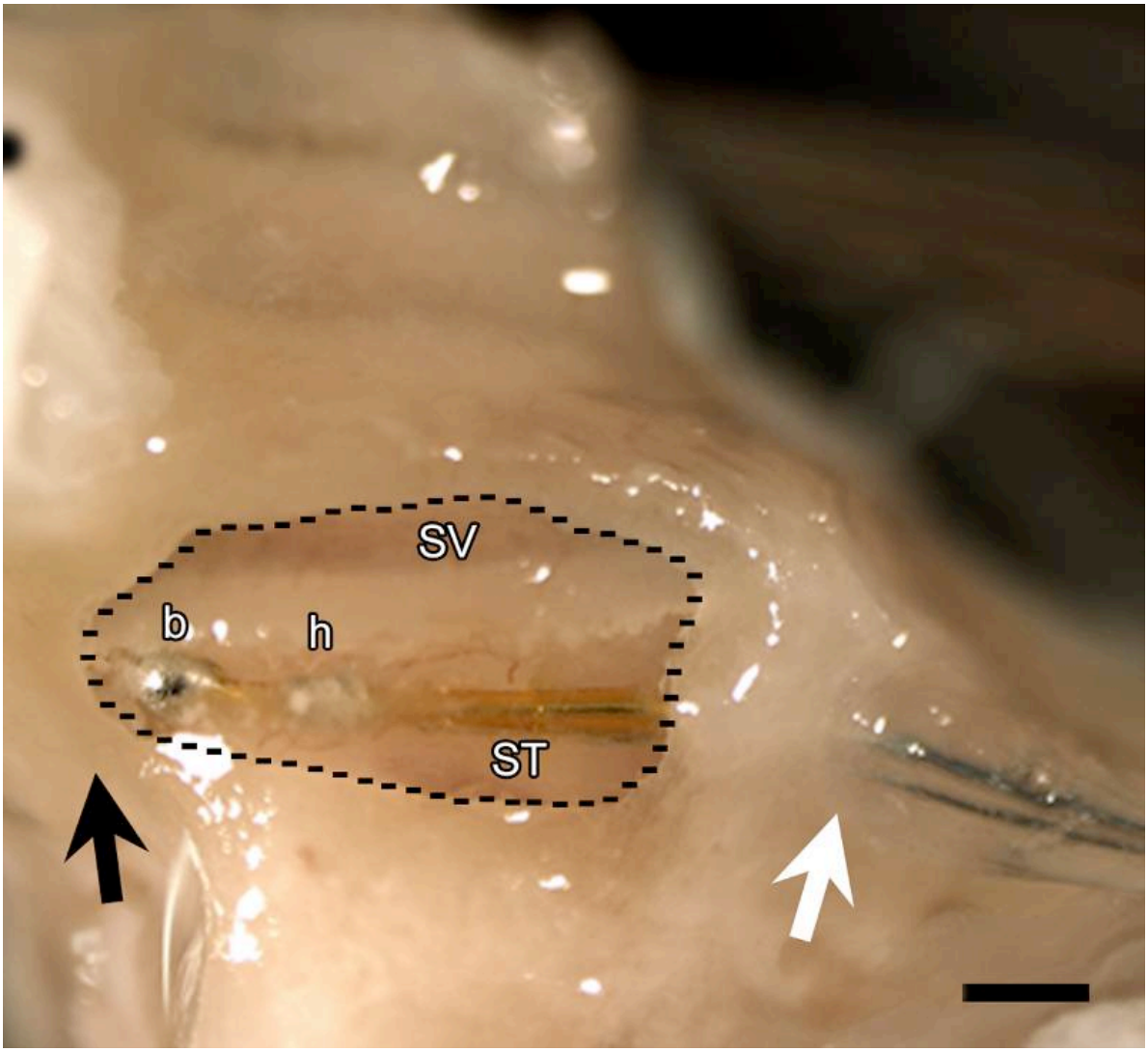


Fig. 3.2: Neurofilament staining of the central auditory nerve.

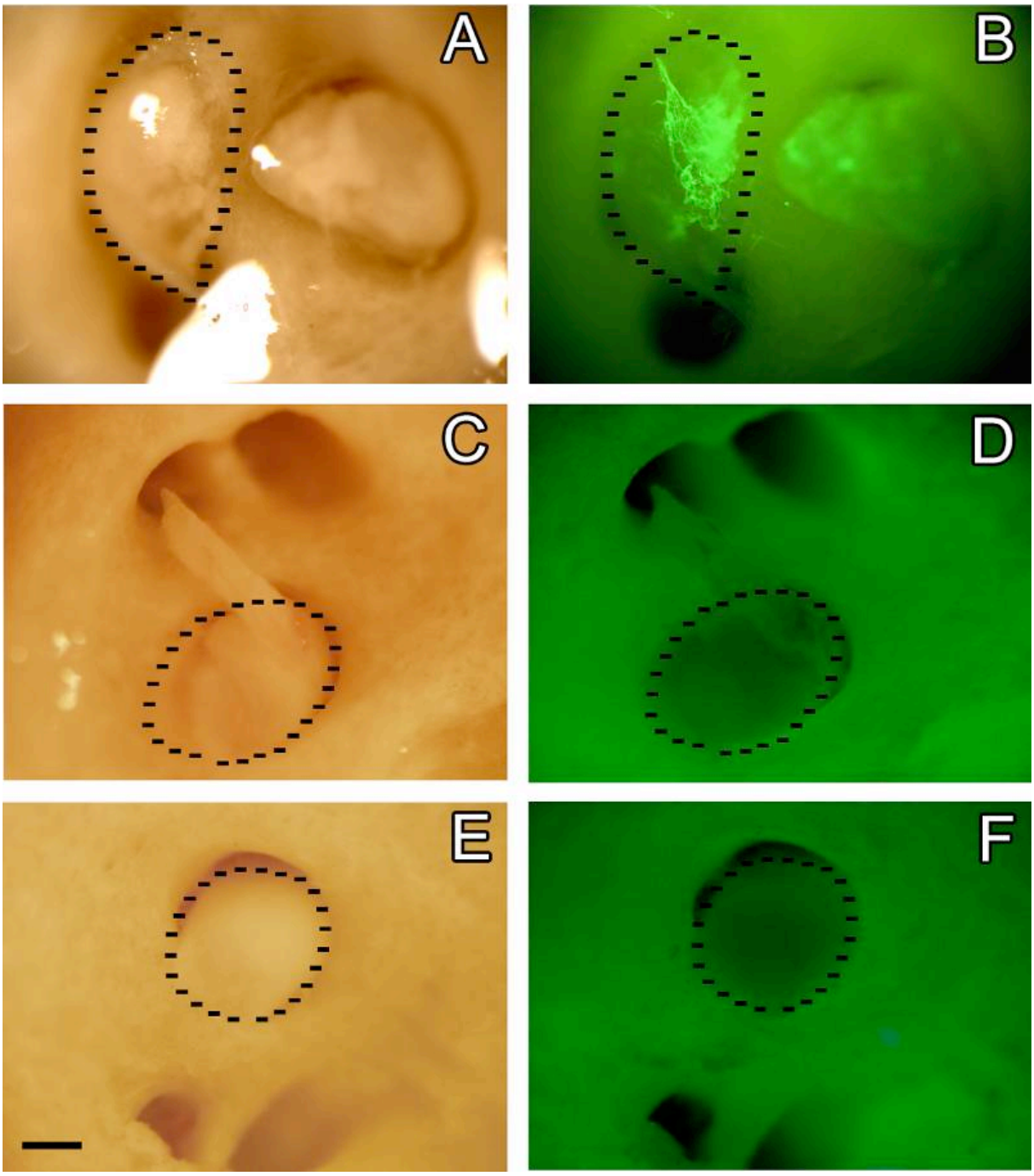


Fig. 3.3: Neurofilament staining within the cochlea.

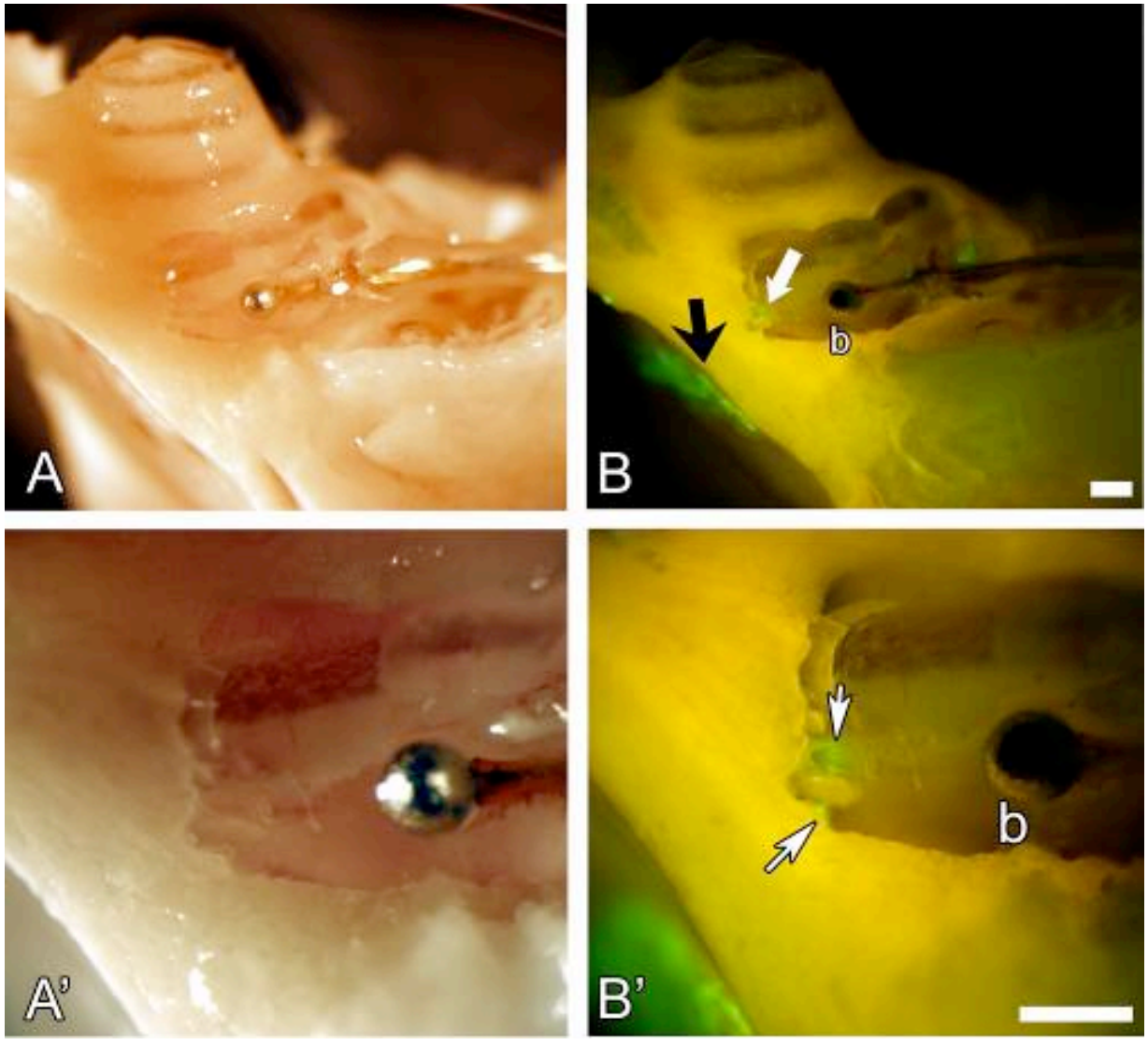
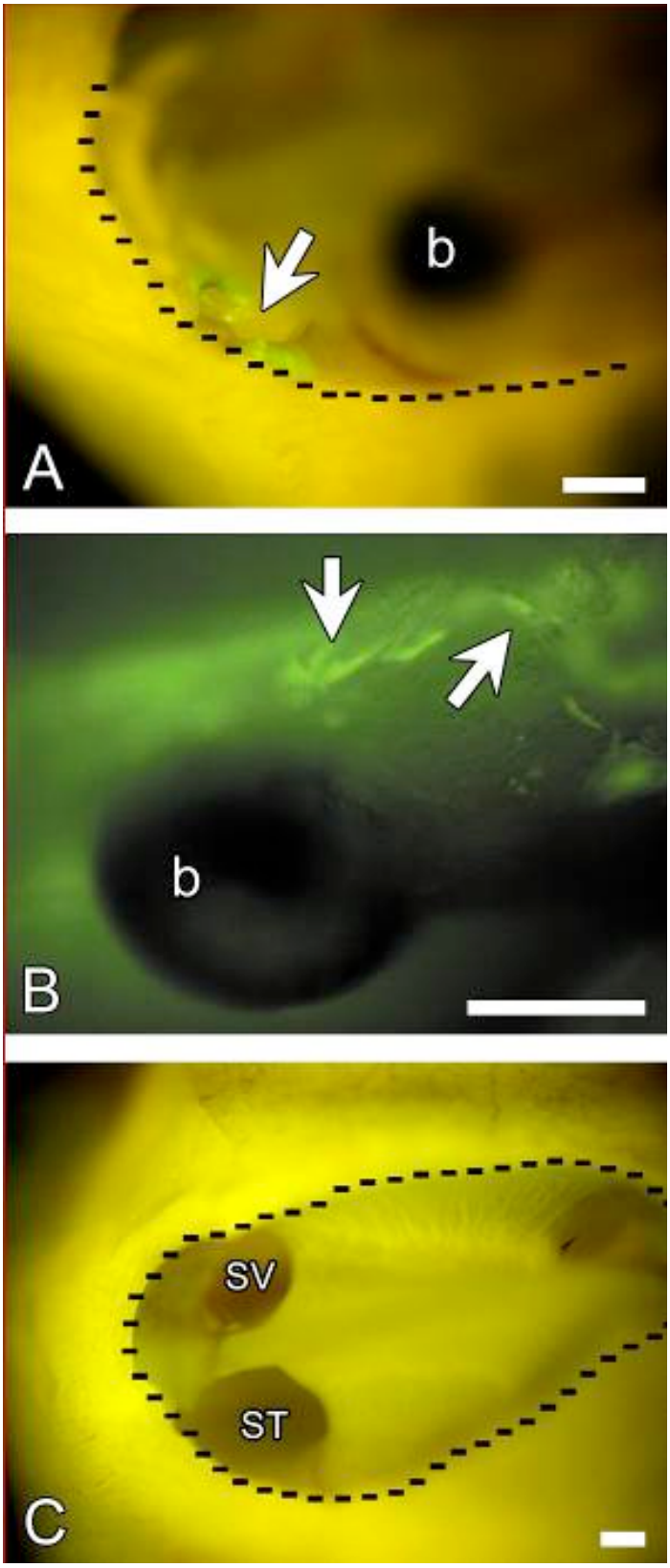


Fig. 3.4: Examples of neurofilament – labeling within the cochlea.



Appendix B

Color Images from Chapter 4

Cochlear implant hydrogel coating promotes auditory nerve fiber growth within the scala tympani in direct vicinity of implant

Fig. 4.1: Two – electrode cochlear implant used in the study.

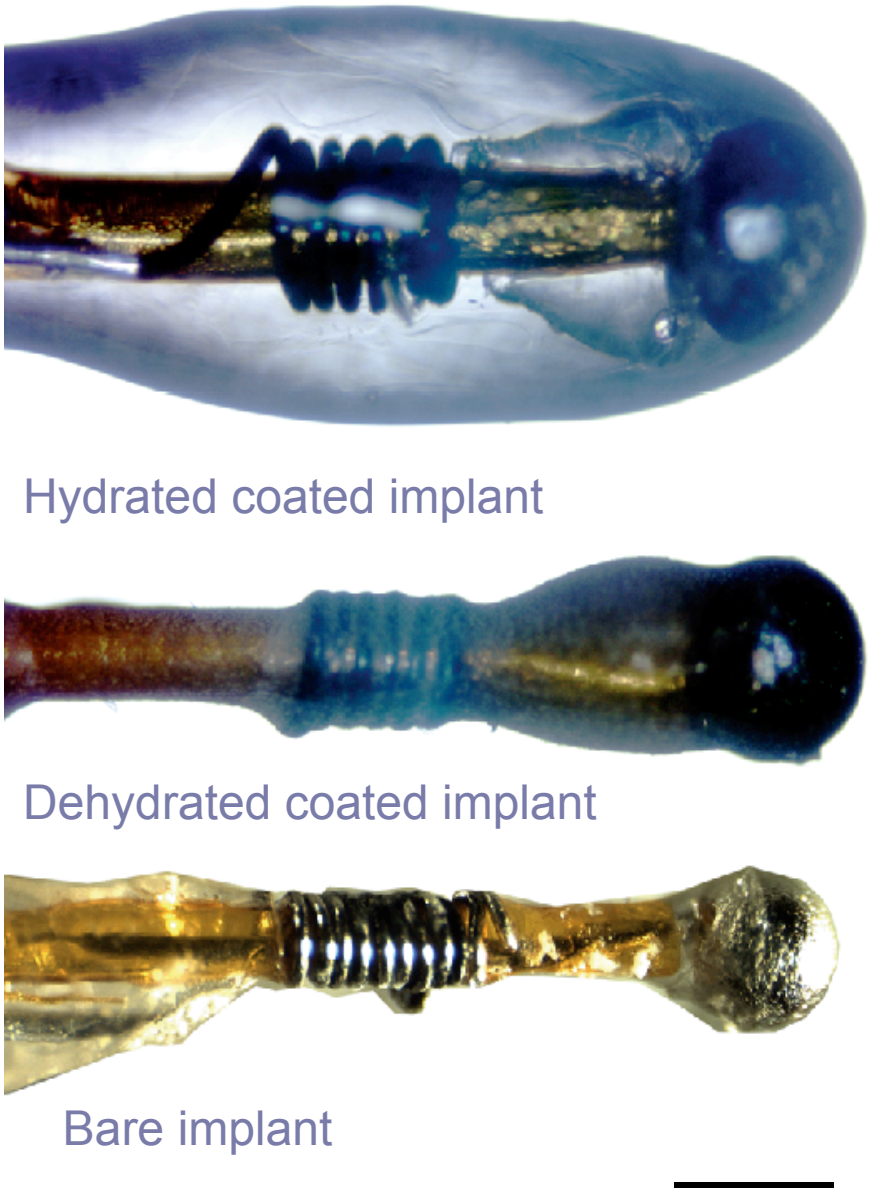


Fig. 4.2: Neurofilament positive fibers within the scala tympani of a cochlea implanted with a hydrogel and BDNF coated implant.

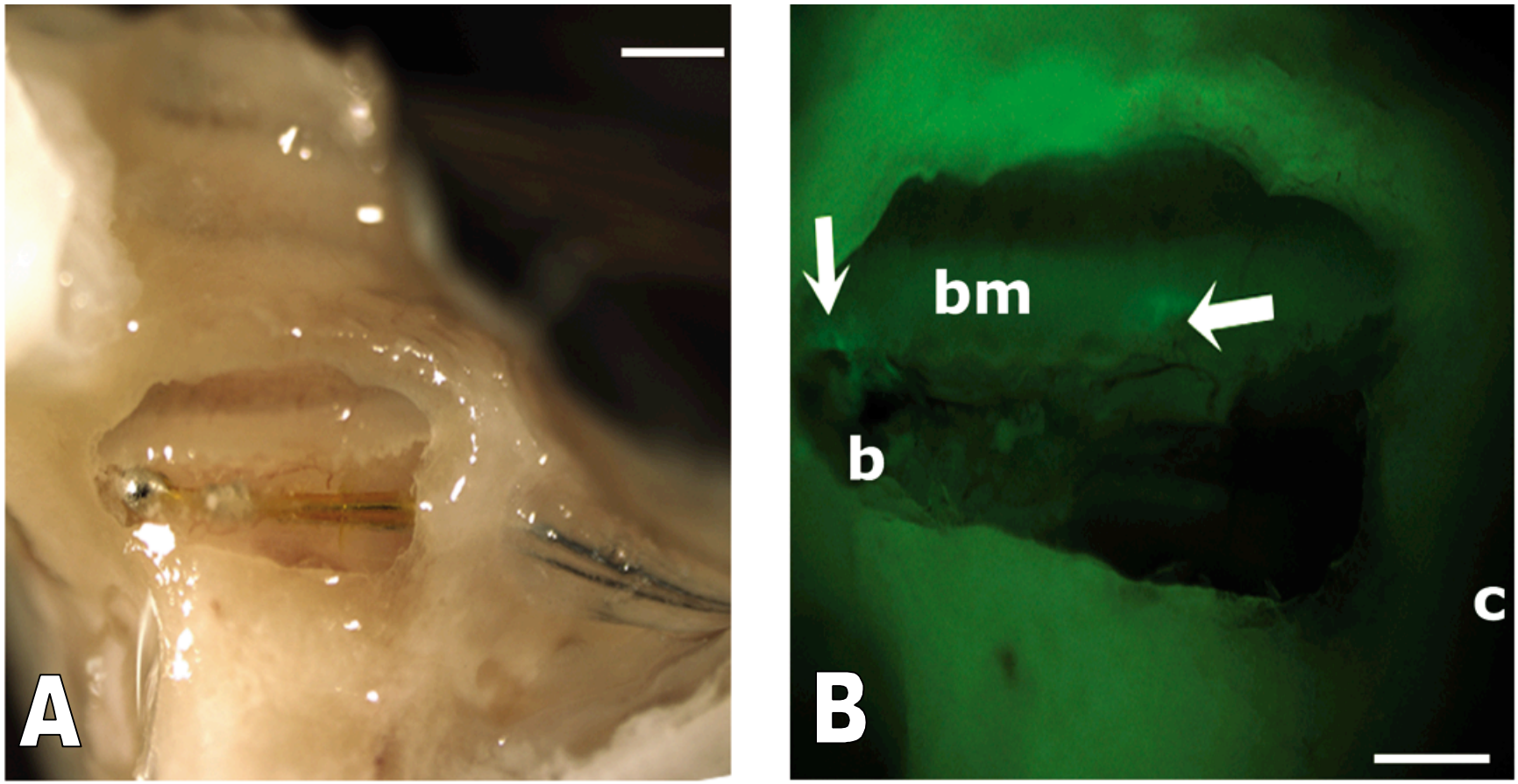


Fig. 4.3: Neurofilament positive fibers within the scala tympani of a cochlea implanted with a hydrogel – only coated implant.

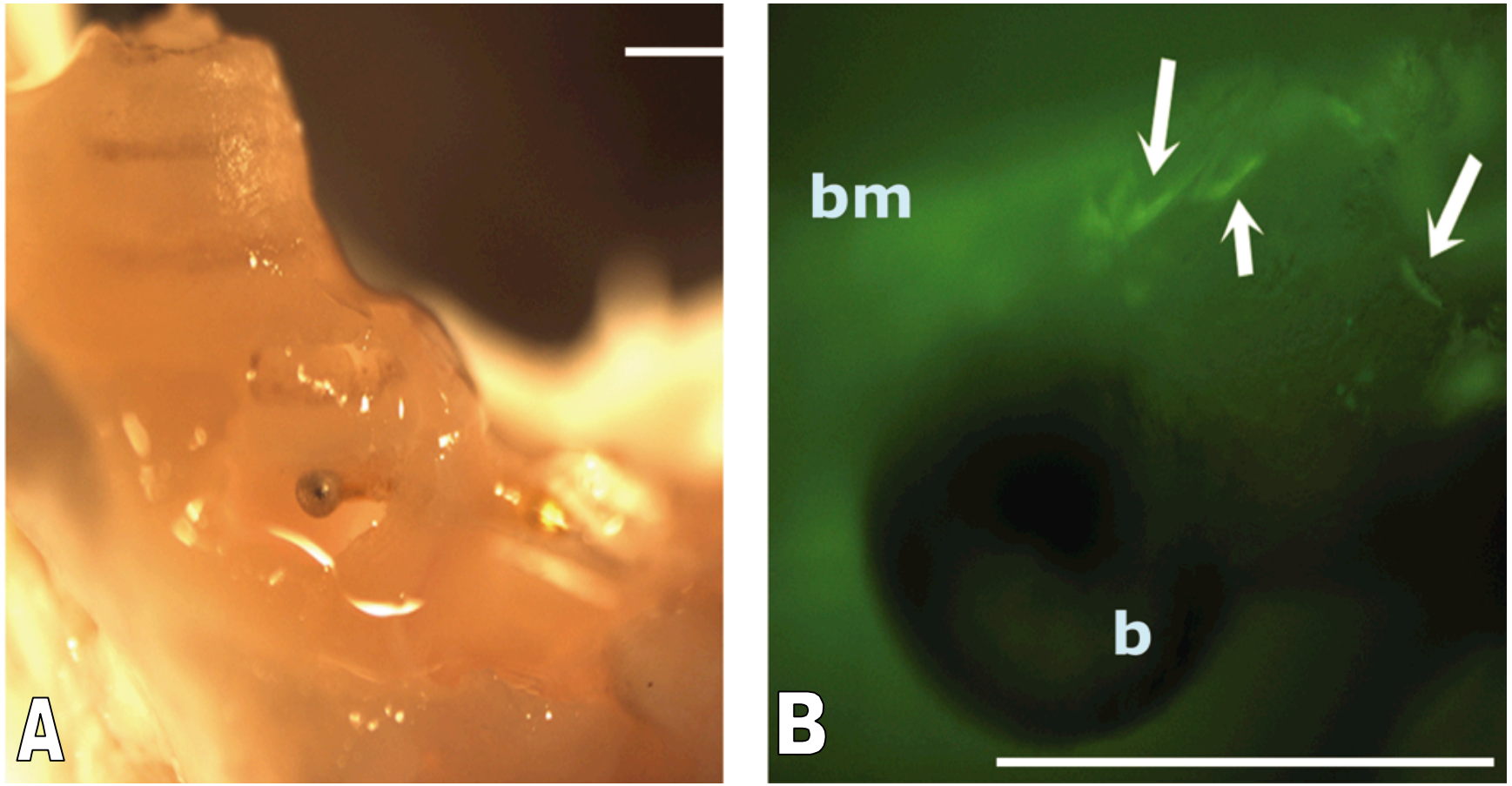


Fig. 4.4: Neurofilament positive fibers within the scala tympani of a cochlea implanted with an uncoated implant.

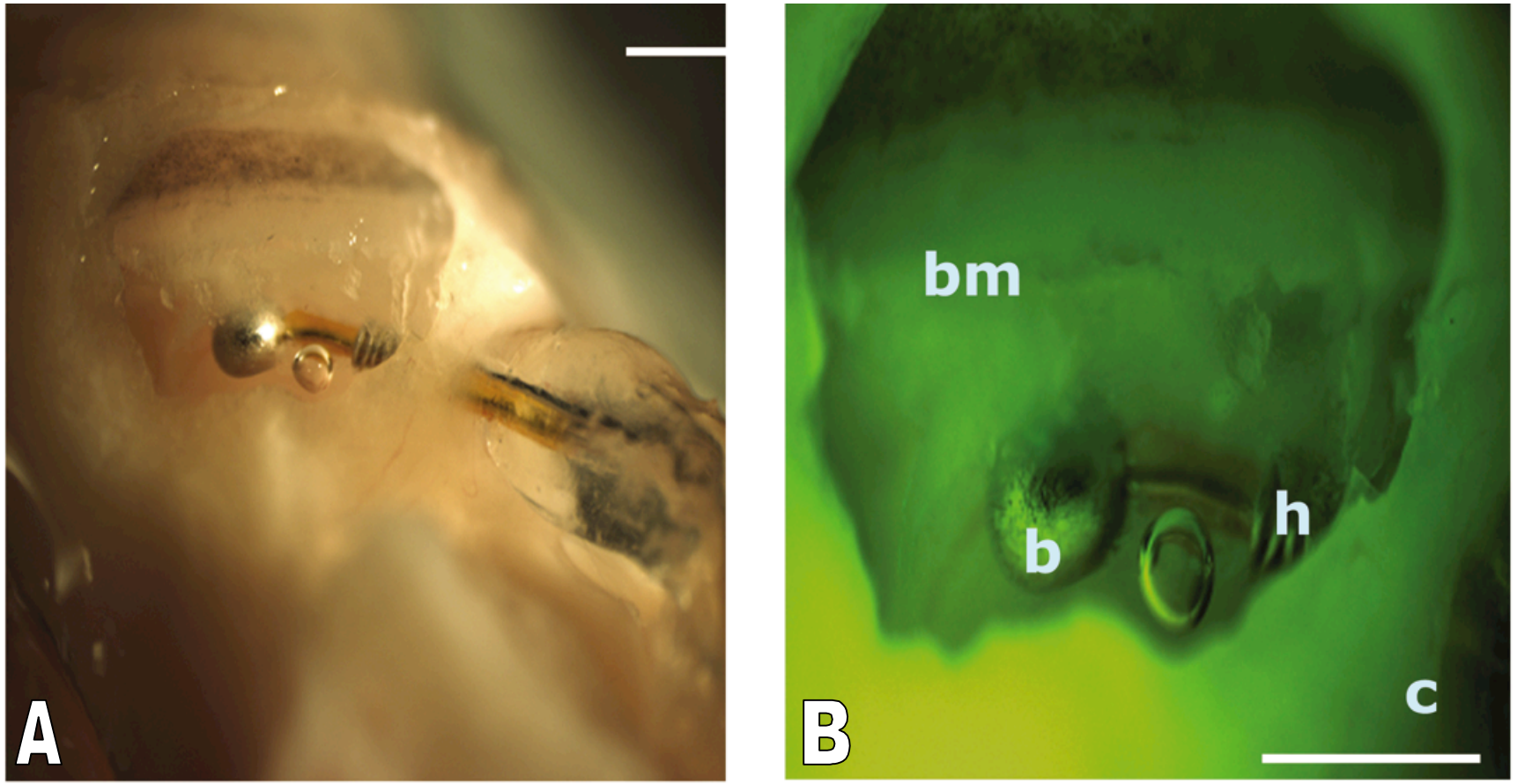
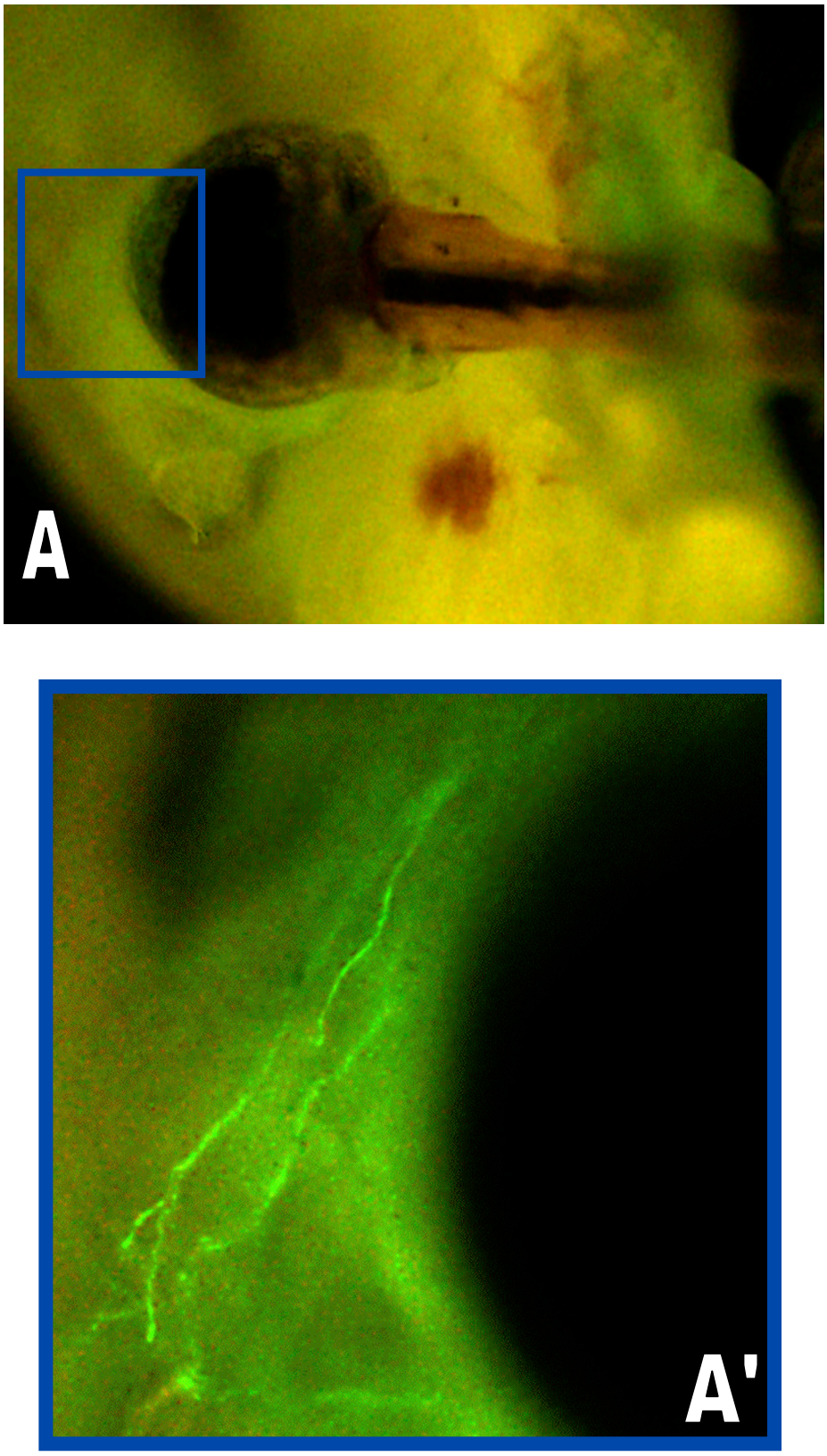


Fig. 4.5: Magnified view of the ball electrode within the scala tympani.



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