Fourth Quarterly Progress Report

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Protective Effects of Patterned Electrical Stimulation on the Deafened Auditory System

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1. Introduction

The goal of this contract is to develop methods of protecting the remaining portions of the auditory system from degeneration after loss of hair cells and to improve its effectiveness in extracting information provided by auditory prostheses. We have taken a broad neurobiological approach to this goal in order to study both the short and long-term response of the auditory system to loss of hair cells and the subsequent introduction of afferent input via an auditory prosthesis. Our studies are divided into three major areas of investigation:

(a) The neurophysiological and neuroanatomical response to prolonged electrical stimulation of the auditory nerve following a neonatal sensorineural hearing loss (SNHL). This work is designed to provide insight into the protective effects of electrical stimulation on the auditory nerve (AN) in addition to investigating the plastic response of the central auditory system (CAS) to temporally challenging stimuli presented chronically to one or two sectors of the AN.

(b) The neurophysiological and neuroanatomical response to the AN and CAS of deafened animals following prolonged intracochlear electrical stimulation in combination with neurotrophic support of the auditory nerve. This work is designed to investigate whether electrical stimulation and chronic administration of neurotrophins act in synergy to promote AN survival. This work will also provide insight into the role of neurotrophins in improving synaptic efficiency in the deafened auditory pathway.

(c) The neurophysiological and neuroanatomical response to acute electrical stimulation of the auditory nerve following a neonatal SNHL. These studies are designed to provide insight into the acute response of the AN and CAS to intracochlear electrical stimulation in deafened animals with little prior auditory experience.

While these studies are designed to provide insight into the plastic response of the deaf auditory pathway to re-activation via an auditory prosthesis, a major objective of this work is to apply our findings to the clinical environment.

2. Summary of activities for the quarter

During the fourth quarter of this contract the following activities were completed:

- Continued our deafening program as part of the feline chronic stimulation study.
- Implanted and commenced chronic electrical stimulation of an additional four deafened kittens.
- Commenced our terminal acute electrophysiological studies on the first group of cats that have been chronically stimulated.

- Deafened, implanted and chronically stimulated five adult guinea pigs. These animals are a control group, receiving electrical stimulation with the simultaneous delivery of Ringer's solution into the cochlea.
- Manufactured a second group of five electrode arrays for chronic stimulation studies in guinea pigs. These arrays can deliver pharmacological agents to the scala tympani via an osmotic pump/micro-tube assembly. The electrode assemblies will be implanted into deafened guinea pigs in the following quarter. They will form the first of our neurotrophin treated animals.
- Completed the development of a technique to allow cryo-embedding and sectioning of cochleas for future immunohistochemistry studies.
- RKS was an invited examiner, Oral PhD exam, University of Auckland, New Zealand, July 20.
- Invited speaker Audiological Society of Australia's continuing Education Program "Otoacoustic Emissions and Related Applications, St Vincent's Hospital, Melbourne, July 27. R. K. Shepherd, "A physiological overview of otoacoustic emissions".
- Presented an invited paper at the 2001 Conference on Implantable Auditory Prostheses, Asilomar, CA August 19-24. R.K. Shepherd, M.P. Colreavy, W.P.L. Hellier & S.J. O'Leary, "Morphological and functional studies of the mammalian cochlea: Implications for cochlear implants". A copy of the abstract is attached as an appendix.

3. Chronic electrical stimulation studies in neonatally deafened cats

3.1 Deafening

In addition to the four kittens deafened in the previous quarter (NDC_10-NDC_13, Table 1) we deafened a further three animals this quarter (NDC_14-NDC_16). The kittens were deafened at 12 days after birth using a single co-administration of kanamycin (KA) and ethacrynic acid (EA; Shepherd & Martin, 1995). All animals made an uneventful recovery from the procedure. Auditory brainstem responses (ABRs) were recorded two weeks following the deafening procedure in order to assess their hearing status using recording procedures outlined previously (Hardie and Shepherd, 1999).

The three animals deafened this quarter exhibited a profound hearing loss when evaluated using ABRs (Table 1). In order to take advantage of the increased susceptibility of the mature cochlea to ototoxic drugs (e.g. Shepherd and Martin, 1995) these animals were deafened at a slightly older age than the majority of kittens used in our study. It should be noted that our research plan calls for the use of *both* profound and severely deafened animals in order to model the range of hearing losses observed in an implant clinic. Animals with some residual hair cells in the apical turn will allow us to examine whether stimulus induced trophic support of SGCs is influenced by the presence of residual elements of the organ of Corti as suggested by earlier studies (e.g. Lousteau, 1987). This animal model will also allow us to study the plastic response of the auditory pathway to low frequency acoustic stimulation in combination with simultaneous electrical stimulation of a basal sector of auditory nerve. The organization of electrophysiological studies designed to examine this plastic response is in progress.

Table 1: Summary of hearing thresholds (in dB SPL). Click thresholds were determined for both ears while frequency specific ABRs were determined unilaterally

Animal	1 kHz	2 kHz	4 kHz	8 kHz	Click (l r)
NDC_1	-	-	-	-	>98 >98
NDC_2	-	-	-	-	>98 >98
NDC_3	63	71	91	>93	83 63
NDC_4	38	41	66	93	58 48
NDC_5	-	-	-	-	>98 >98
NDC_6	58	81.5	86	>93.5	72 -
NDC_7	43	56.5	76	77.5	- 57
NDC_8	-	-	-	-	>98 >98
NDC_9_c		66.5	>91	>93	83 83
NDC_10	N/A	N/A	N/A	N/A	88 88
NDC_11	N/A	N/A	N/A	N/A	73 78
NDC_12	N/A	N/A	N/A	N/A	88 88
NDC_13	N/A	N/A	N/A	N/A	93 >98
NDC_14	-	-	-	-	>98 >98
NDC_15	-	-	-	-	>98 >98
NDC_16	-	-	-	-	>98 >98

3.2 Implant surgery and the chronic stimulation program

During the quarter, four kittens (NDC_10-NDC_13; Table 1) were bilaterally implanted at seven to eight weeks of age. The left cochlea of each animal was implanted with a stimulating electrode array while the opposite ear was implanted with a control array. Details of the electrode array and surgical procedure can be found in our *Third Quarterly Progress Report*. All four kittens made an uneventful recovery from surgery, and their chronic stimulation program commenced approximately 2 weeks later.

The stimulator provides a temporally challenging stimulus waveform by delivering charge balanced biphasic current pulses non-simultaneously to one or two electrode pairs at 1200 pulses per second (pps) per channel. Each current pulse is 100 μs/phase with a 10 μs interphase gap. This waveform is amplitude-modulated (AM) to a depth of 50% at 30 Hz (see Fig. 2, *Second Quarterly Progress Report*). The minimum current amplitude of each channel is adjusted to the electrically evoked auditory brainstem response (EABR) threshold for that electrode pair (i.e. the maximum stimulus intensity is 6 dB above EABR threshold). These stimulus levels are confirmed as acceptable in the awake animal using basic behavioral criteria.

Each animal is stimulated approximately 6 h per day, five days per week for implant periods, to date, of up to 200 days. Both stimulus current and electrode voltage waveforms are monitored twice daily - just after the stimulator is turned on and just before it is turned off. Electrically isolated monitoring equipment is used for this purpose. Monitoring is used to confirm that the appropriate stimulation levels are set for each animal, and to determine electrode impedance (see the *Third Quarterly Progress Report*). An

example of the longitudinal monitoring of the electrode impedance is illustrated in Fig. 1.

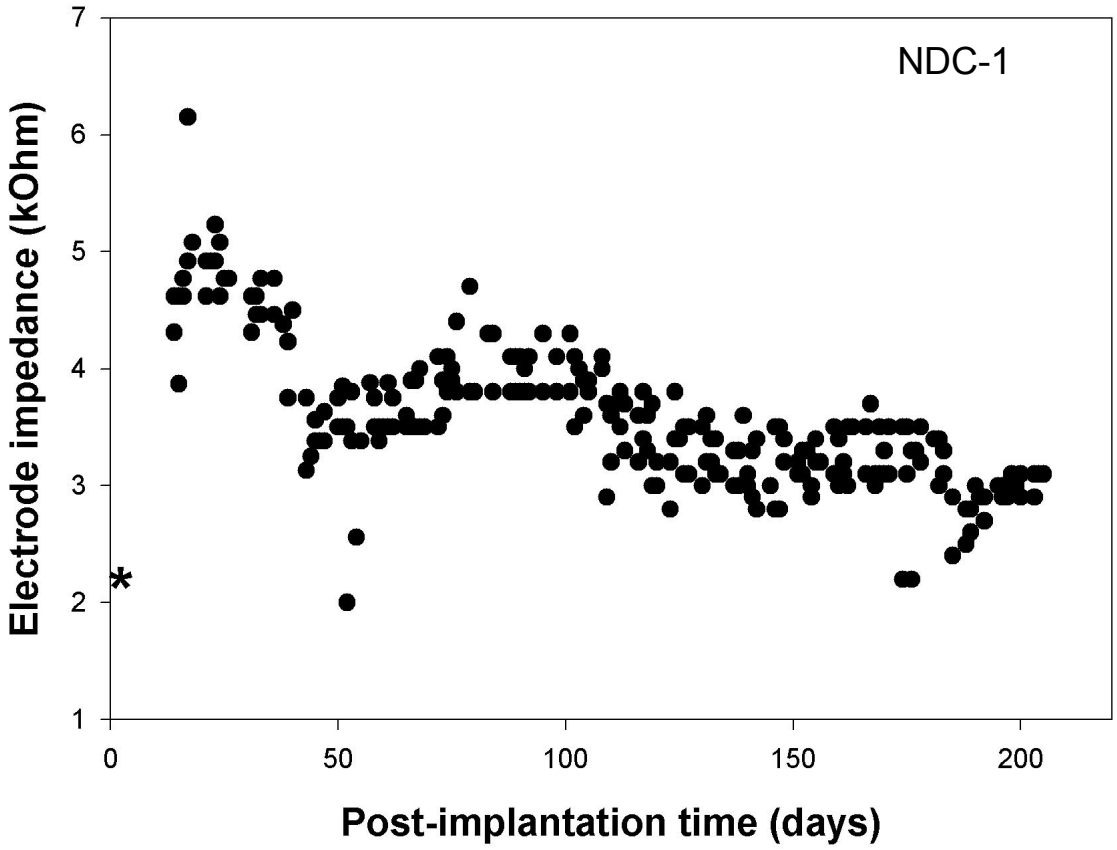


Figure 1. Longitudinal electrode impedance data from NDC_1, the longest implanted animal in the present study. Impedance measurements commenced approximately 2 weeks following implantation of the electrode array. The * illustrates the typical value of electrode impedance measured *in vitro*. Presumably the initial post-operative increase in impedance reflects tissue growth within the cochlea. These data are derived from both minimum and maximum current amplitudes in the AM waveform.

Finally, in the *Third Quarterly Progress Report*, we described a systematic 5-8% *decrease* in electrode impedance as a function of electrical stimulation. Longitudinal comparison of the electrode impedance measured just prior to, and on completion of the daily stimulation program, showed that this stimulus induced change was highly statistically significant. We continue to monitor this effect and will present a more comprehensive analysis in a future report.

4. Electrical stimulation and neurotrophin administration in deafened guinea pigs

4.1 Deafening

A total of five adult guinea pigs were profoundly deafened using a single intravenous injection of 100 mg/kg of Frusemide followed by a subcutaneous injection of 400 mg/kg of Kanamycin. All animals made an uneventful recovery from the procedure. ABRs recorded five days following deafening confirmed that all animals were profoundly deaf (click thresholds >92 dB SPL).

4.2 Implant surgery

Five days following deafening each guinea pig was unilaterally implanted with an electrode array/mini-osmotic pump assembly (Fig. 1; *First Quarterly Progress Report*). Surgery was performed under sterile conditions. Each animal was anesthetized using ketamine (40 mg/kg) and xylazine (4 mg/kg) Supplemental doses were administered during surgery at a level sufficient to maintain the animal in an areflexic state. Rimadyl (4 mg/kg) was administered to provide long-term analgesia and Baytril (10 mg/kg) was used as a broad-spectrum antibiotic. A dorsal approach was used to expose the bulla and the underlying cochlea. The round window membrane was incised using a fine needle and the intracochlear electrode array was gently inserted into the scala tympani for a distance of ~ 4.5 mm. The round window was then sealed with muscle, the connector assembly was placed into the bulla cavity the leadwire proximal to the electrode array was fixed using dental cement. The distal leadwire was fixed to the parietal bone with an additional Dacron mesh tie, then passed subcutaneously to exit the skin via a small incision in the neck. A 200 μl mini-osmotic pump (Alzet 2004), loaded with sterile Ringer's solution and incubated in sterile saline at 37^{o}C for 24 hours prior to surgery, was connected to the polyurethane delivery tube and implanted into a subcutaneous pocket between the scapulae. This pump has a flow rate of 0.25 μl/hour, providing a continuous infusion period of 28 days. The wounds were then sutured in two layers and the wound sites sprayed with Opsite®. Each animal was given 10 ml of Hartmann's solution subcutaneously. EABRs were recorded immediately following surgery using techniques and stimulus parameters described previously (*Third Quarterly Progress Report*).

During surgery the animal's temperature was maintained at 37^{o}C using a heating pad. All five guinea pigs made an uneventful recovery from surgery, and their chronic stimulation program commenced 5 days following surgery. All animals were stimulated in a bipolar mode using an identical stimulus protocol to that used in the feline studies (*Third Quarterly Progress Report)*.

4.3 Chronic stimulation program

The amplitude of the AM stimulus waveform was set so that the minimum current level equaled the EABR threshold (i.e. the maximum stimulus intensity was 6 dB above EABR threshold). These stimulus levels were subsequently confirmed to be acceptable in the awake animal using basic behavioral

indicators. To date, maximum stimulus current amplitudes used in this study have been in the range 0.35-1.2 mA at 100 μs/phase, developing charge densities in the range 7.5-24.4 $\mu C.cm^{-2}$ geom. per phase.

The stimulators are carried in a harness worn by the guinea pig to enable continuous stimulation without confining the animal's activities. Each animal is stimulated approximately 6 h per day, five days per week for implant periods of 28 days. Both stimulus current and electrode voltage waveforms are monitored twice daily - just after the stimulator is turned on and just before it is turned off. The electrode impedance Z_p, measured longitudinally in four animals stimulated over the 28 day implant period is illustrated in Fig. 2 (see *Third Quarterly Progress Report* for further details). These impedances are within the normal range observed for this type of electrode assembly (e.g. Xu et al., 1997).

During the chronic stimulation program one guinea pig died. Autopsy showed that the animal had congested lungs consistent with an upper respiratory infection. The remaining four guinea pigs maintained good health throughout the stimulation program.

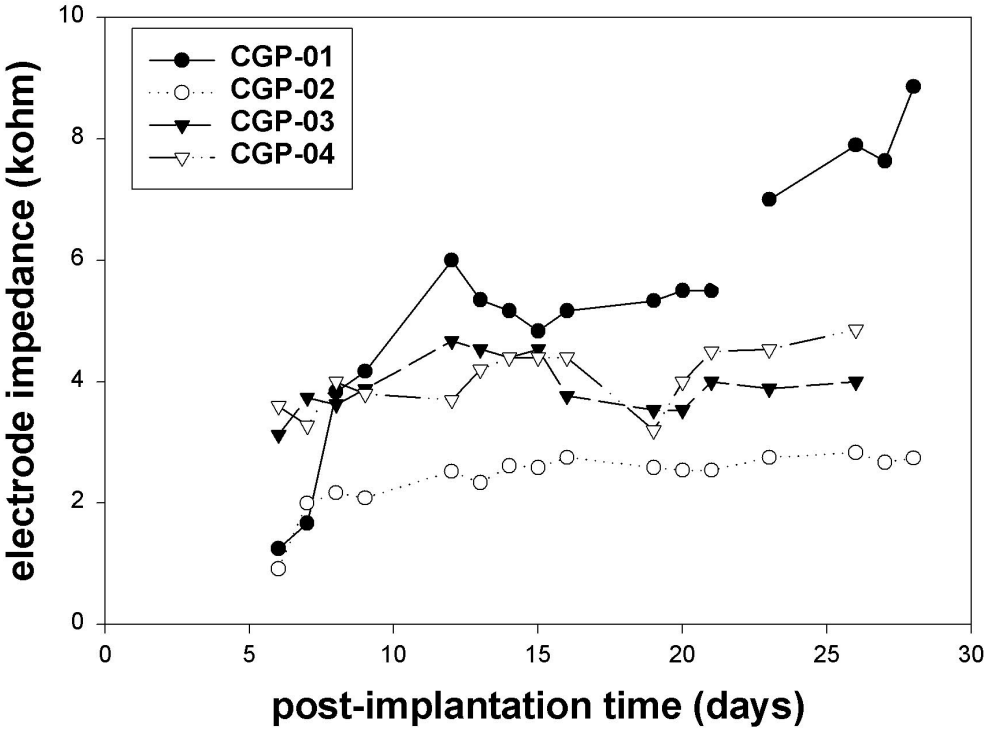


Figure 2. Longitudinal electrode impedance (Z_p) data illustrated for four guinea pigs stimulated over an implant period of 28 days. These data were derived from the electrode voltage measured in response to the peak current of the AM waveform. Three of the four animals exhibited relatively stable impedances over the implant period. The break in data for CGP-01 reflects a shift in stimulating electrodes from pair 1-2 to pair 2-3.

At completion of the 28 day implantation period EABRs were recorded from each animal in order to assess the functional status of the auditory pathway. Refractory properties of the EABR were measured following procedures that have been described previously (van den Honert and Stypulkowski, 1986; Abbas and Brown, 1991; Zhou et al., 1995). An example of these responses is illustrated in Figs. 3 and 4. We anticipate that these data will provide a useful technique to assess the functional properties of auditory neurons in varying stages of degeneration.

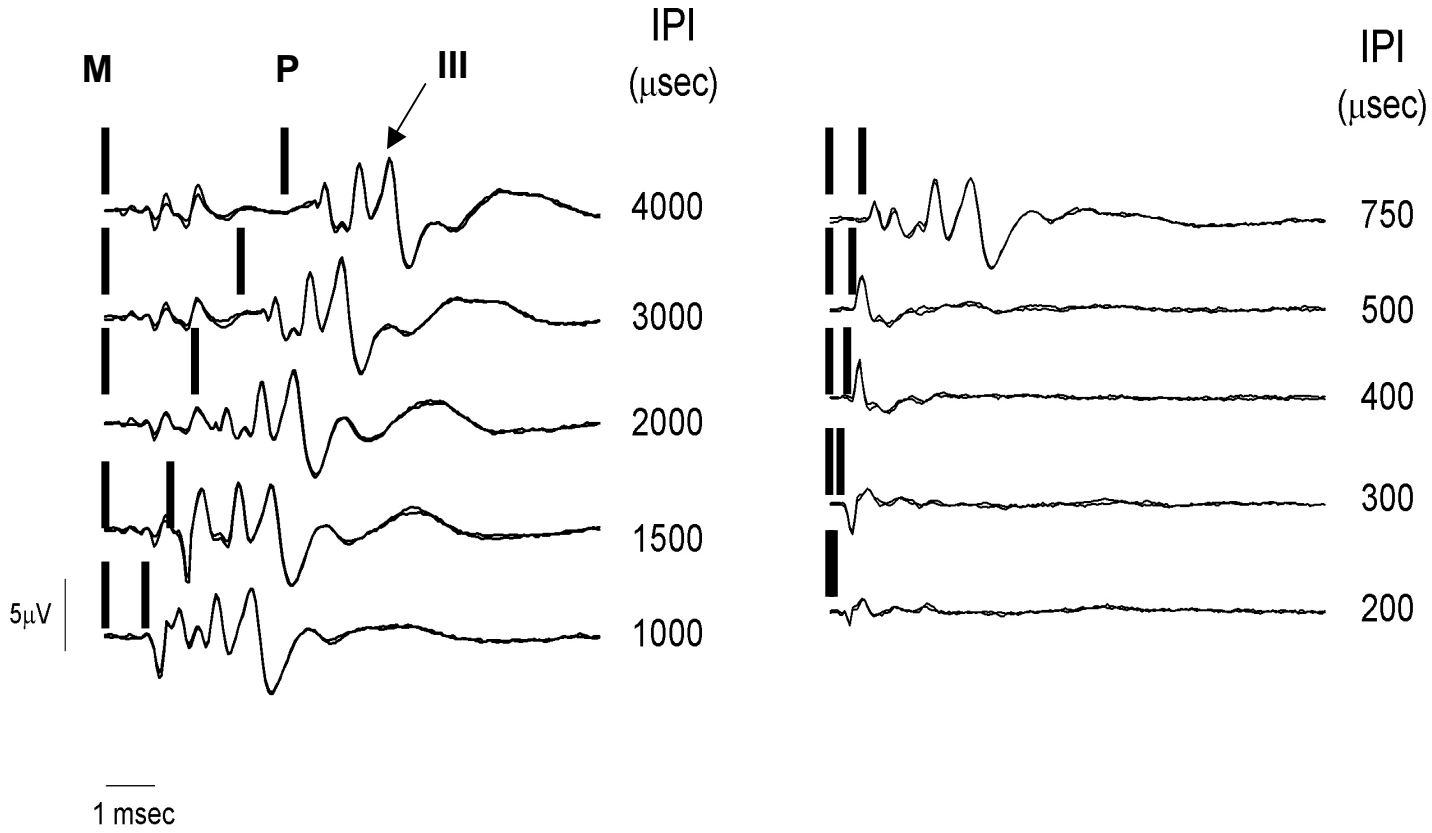


Figure. 3. Refractory properties of the EABR in the deafened guinea pig. This figure illustrates a typical set of derived EABR responses obtained by subtracting the masker (M) alone response from a masker + probe (P) response. This derived response effectively illustrates the response to the probe stimulus only. In these experiments the inter-pulse period (IPI) between the masker and probe is reduced from 4000-200 μs. When the IPI is less than 1000 μs, the refractory state of neurons excited by the masker result in a dramatic reduction in the amplitude of the probe-evoked derived response. Both the probe and masker were held at the same amplitude above threshold (15.5 dB in this example). Wave III of the guinea pig EABR is measured from the peak (III) to the following trough (see Fig. 4).

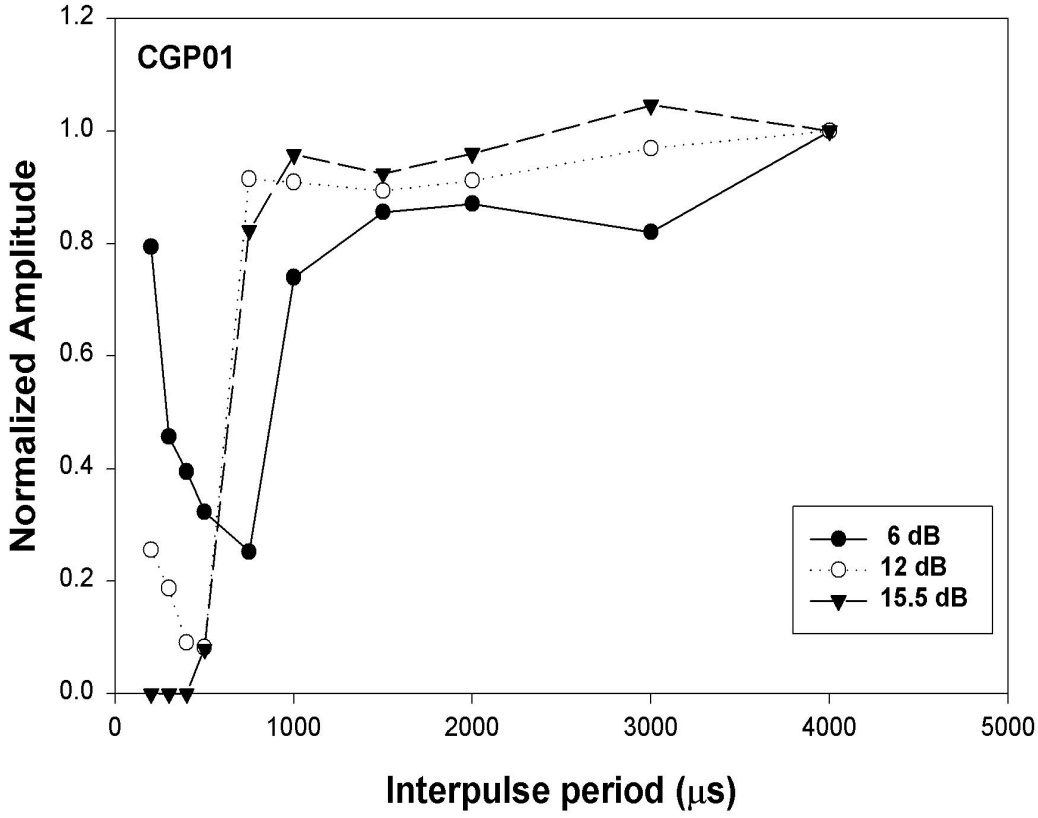


Figure 4. Normalized wave III amplitude (see Fig. 3) of the derived probe–evoked EABR plotted as a function of interpulse period for three masker/probe stimulus intensities (6, 12 and 15.5 dB above EABR threshold). These data were normalized to the wave III amplitude at an IPI of 4000 μs. Note the partial recovery in the response amplitude at narrow IPIs for 6 and 12 dB above threshold stimuli. There is no evidence of this partial recovery at 15.5 dB.

The non-monotonic behavior of the refractory properties of the EABR at intensities of 6 and 12 dB above EABR threshold (Fig. 4) has been observed previously (Stypulkowski and van den Honert, 1984). These authors argued that the partial recovery in the amplitude of the probe-evoked EABR at low IPIs is most likely due to the temporal integration of the masker and probe stimuli. This behavior disappears at supramaximal stimuli for the neural population, i.e. the masker is above threshold for all neurons, placing them all in an absolute refractory state and therefore unresponsive to the probe (for short IPIs). We concur with this interpretation.

During the next 12 months we will obtain further EABR refractory data in order to determine the effectiveness of this technique in assessing subtle functional differences in cochleae with varying degrees of SGC pathology. These data will include animals treated with chronic stimulation and neurotrophin delivery.

4.4 Cochlear Histopathology

Immediately following the recording of EABRs, each animal was killed with an overdose of anesthetic (sodium pentobarbital) and systemically perfused with heparinized saline followed by phosphate buffered 4% paraformaldehyde. Both cochleae and the brain were removed for histology. The brain was sectioned on a cryostat for subsequent anatomical and immunocytochemical studies. This work will be presented in a future progress report. The cochleae were decalcified, dehydrated, embedded in resin and serially sectioned at 2 μm. Sections every 126 μm were stained with either thionine or haematoxylin and eosin. In subsequent reports we will present quantitative studies of SGC density measurements in both stimulated and control cochleae. Here we illustrate the upper basal turn of two of our implanted/stimulated animals (Fig. 5), showing the absence of the sensory epithelium, reduced ganglion cell survival, and a minimal tissue response to the implanted electrode array following chronic electrical stimulation and delivery of sterile Ringer's solution. The minimal tissue response observed in these cochleae is consistent with that observed using the same electrode assembly delivering neomycin (*First Quarterly Progress Report*), and supports our earlier observation that this electrode assembly is biocompatible. Minimizing the extent of tissue response around the electrode array is an important issue if we are to successfully deliver neurotrophins throughout all cochlear turns.

5. Neuroanatomical and neurochemical studies of the deafened auditory system

As part of our studies of the effects of deafness on the auditory system, we will use immunocytochemical techniques to study the pathological response of SGCs at the cellular level following loss of the sensory epithelium. To this end we have developed over the last quarter, a method for producing 30-60 μm thick frozen sections of the cochlea that is compatible with immunocytochemical techniques. Typical cryostat sections of the normal rat cochlea are illustrated in Fig. 6. These sections show relatively little tissue distortion. Being frozen sections this tissue has not been exposed to the elevated temperatures or the harsh chemical environments associated with other histological techniques. These sections should therefore offer optimal conditions in which to perform our immunocytochemistry studies. More details of this research will be presented in future progress reports.

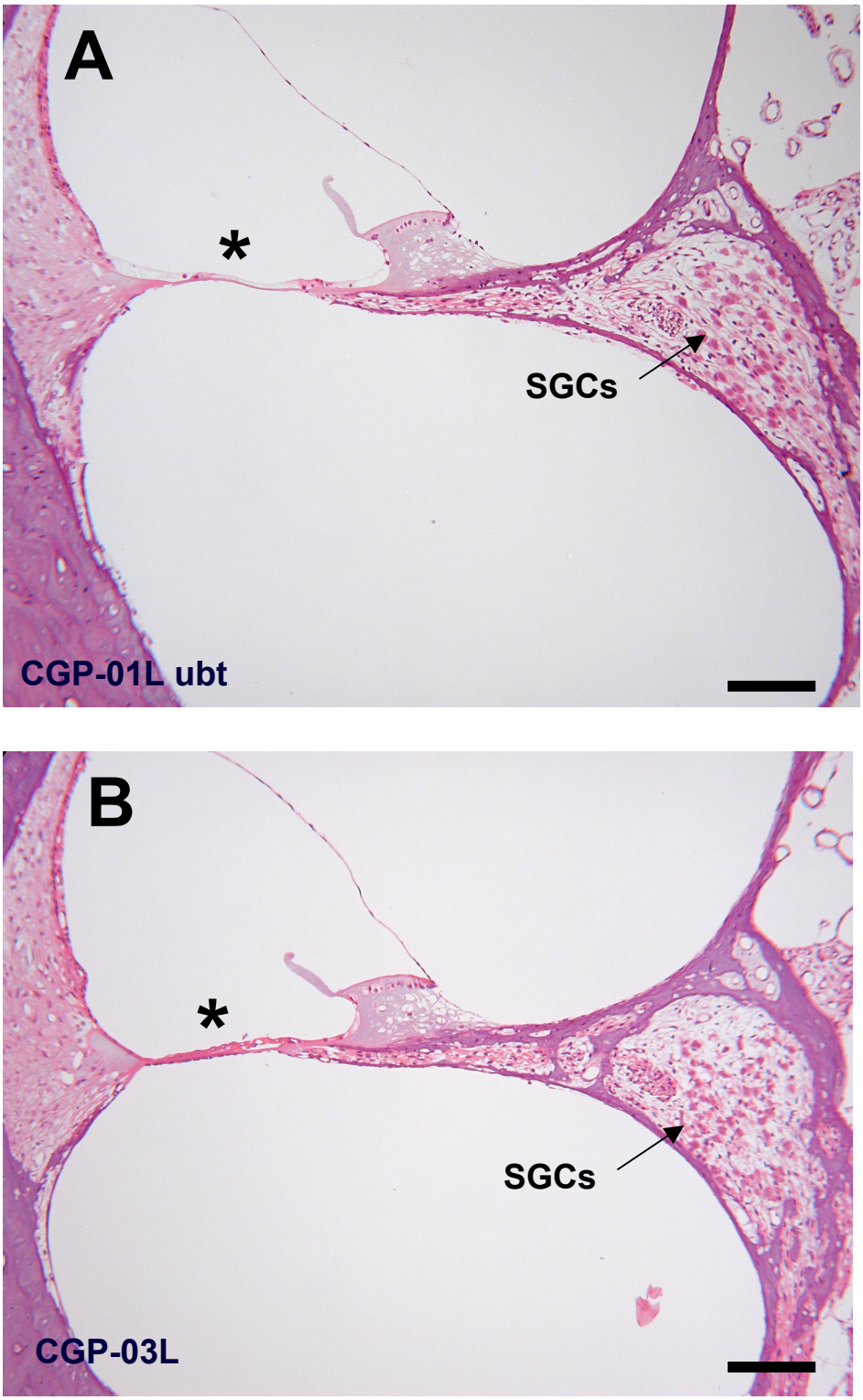
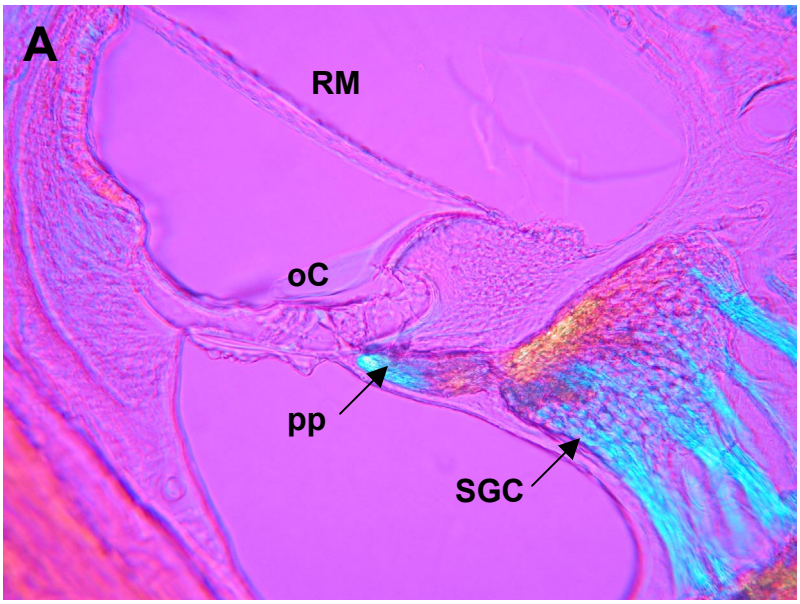


Figure 5. Photomicrographs illustrating the upper basal turn of two of the implanted guinea pigs used in the present study. Both animals had been deafened for 33 days, their left cochleae implanted with an electrode array and drug delivery assembly for 28 days. During this period the cochlea was electrically stimulated and 200 µl of sterile Ringer's solution was delivered to the scala tympani. Note the loss of the organ of Corti (*), the partial loss of SGCs, and the minimal tissue response to the electrode assembly within the scala tympani. Scale bar = 100 µm.



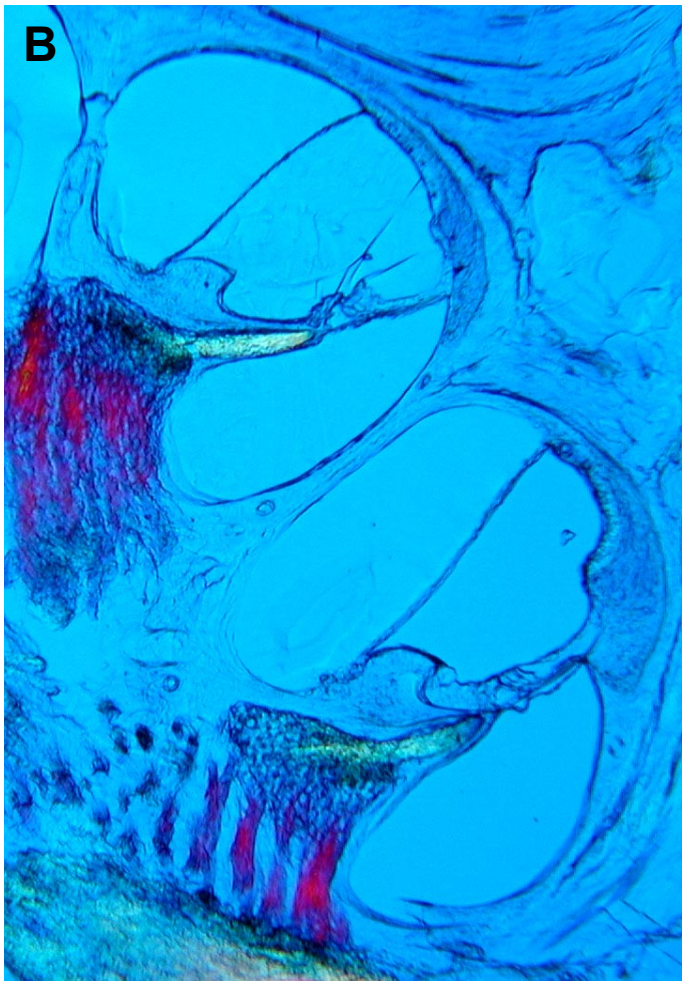


Figure 6. Photomicrographs of cryostat sections from normal rat cochleae illustrating lower middle turn (A), and the upper basal/upper middle turns (B). Both examples are unstained 60 μm thick sections imaged using differential interference contrast microscopy. RM, Ressiner's membrane; pp, peripheral processes; oC, organ of Corti; SGC, spiral ganglion cells.

6. Plans for Next Quarter

- Continue our chronic stimulation studies in deafened kittens and guinea pigs.
- Continue the manufacture of guinea pig and feline electrode assemblies.
- Continue terminal acute electrophysiology experiments on chronically stimulated cats and guinea pigs.
- Continue histological preparation and analysis of cochleae and auditory brainstem structures in cats and guinea pigs following completion of the chronic stimulation program.
- Continue developing our immunochemistry protocols using normal hearing animals.
- Prepare and present our progress report at the 32nd Neural Prosthesis Workshop, Bethesda MD.

7. Acknowledgments

We gratefully acknowledge the important contributions made by our Veterinarian Dr Sue Pierce, Elisa Borg for management of our animal house, Jacqueline Andrew for research assistance, Helen Feng for electrode manufacture, Dr. Phillip Marzella and Lisa Gillespie for advice on neurotrophin delivery systems and Frank Nielsen for engineering support.

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9. Appendix

Abstract form the 2001 Conference on Implantable Auditory Prostheses, Asilomar, CA, August 19-24.

MORPHOLOGICAL AND FUNCTIONAL STUDIES OF THE MAMMALIAN COCHLEA: IMPLICATIONS FOR COCHLEAR IMPLANTS

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The development of peri-modiolar electrode arrays and the use of combined electric-acoustic stimulation may lead to improved clinical outcomes for cochlear implant subjects. In this paper we describe the morphology of the modiolus with respect to these new generation electrode arrays, and describe the functional and morphological status of the stria vascularis (SV) following a long-term sensorineural hearing loss (SNHL) in light of electric-acoustic stimulation in implant subjects.

We examined the ultrastructure of the modiolar wall within the scala tympani using scanning electron microscopy. Part of the osseous spiral lamina (OSL), lies between the electrode array and the spiral ganglion cells. The OSL was examined in both human and cat cochleae. This bony structure consists of a series of tiny pores, the canaliculae perforantes (Schuknecht *et al.,* 1959). Our work confirmed the presence of canaliculae perforantes in the cat cochlea and demonstrated an extensive network within the human cochlea. The diameter of these pores typically varied from 1-10 μm. This work also highlighted the delicate nature of the OSL, with its thickness varying from a mean of 25 μm in the basal turn to 8 μm in the apical turn of the human cochlea. This extensive network of pores implies that the OSL is essentially electrically "transparent", however this structure is very delicate and therefore highly susceptible to electrode insertion trauma.

Endolymph within the scala media of the cochlea is maintained at a DC potential of ~80 mV. This so-called endocochlear potential (EP) is integral to the physiological mechanisms underlying normal hearing. We studied the EP over the course of a long-term SNHL as no study to date has investigated this relationship. Guinea pigs were profoundly deafened with kanamycin, and the status of the EP was studied at 2 days and 2, 8 and 16 weeks post-deafening. Compared with control animals there was a highly significant reduction in the mean EP in animals deafened for 2 days ($p<0.0001$, t-test). In animals deafened for a period of 2 weeks the EP had recovered to near normal levels, while animals deafened for 8 – 16 weeks exhibited complete recovery. Histological examination confirmed the absence of hair cells in these cochleae. Analysis of the SV, the specialised cells of the lateral cochlear wall responsible for the generation of the EP, revealed a significant reduction in SV area with duration of deafness. Despite these atrophic changes to the SV, the EP was maintained at normal levels. These results imply that residual hair cells remain capable of transduction via electrophonic or acoustic stimulation following a long-term SNHL.

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