Eleventh Quarterly Progress Report

NIH-N01-DC-0-2109

Protective Effects of Patterned Electrical Stimulation On the Deafened Auditory System

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April 1st – June 30, 2003

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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) and 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 of the AN and CAS following chronic 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 deafened 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 eleventh quarter of this contract the following activities were completed:

- Responded to technical issues with regards to contract application for RFP 260-03-01.
- Prepared abstracts for the 2003 Conference on Implantable Auditory Prostheses. Abstracts from this meeting are included in Appendix A.
- Continued histological analysis of cochleae and auditory brainstem structures in both cats and guinea pigs following completion of their chronic electrical stimulation programs.

- Prepared and submitted a manuscript to Neuroscience Methods (see section 5, below).
- Commenced long-term neurotrophin/electrical stimulation study in profoundly deafened guinea pigs. This research is designed to test the hypothesis that electrical stimulation alone can maintain a long-term trophic advantage for spiral ganglion neurons following initial rescue using exogenous neurotrophins with electrical stimulation.
- Completed a long-term *in vivo* study "Rehabilitation of the deafened auditory nerve with Schwann cell transplantation". The present report presents a summary of this work (see Section 4, below).

3. Effects of chronic electrical stimulation and neurotrophin administration in deafened guinea pigs

We have continued our analysis of the refractory properties of electrically-evoked auditory brainstem responses (EABRs) measured at completion of the neurotrophin/electrical stimulation administration in deafened guinea pigs (see *Eighth Quarterly Progress Report*).

3.1 Results

Representative responses to paired pulse stimulation at 6, 12 and 18 dB about threshold are shown in Figure 1. All responses illustrate an initial refractory period of approximately 100-300 μs, during which the second of the stimuli (i.e. the probe stimulus) produce little to no response. As the interpulse interval is increased the response amplitude evoked by the probe stimulus increases, up to the normalized response at an inter-pulse interval (IPI) of 4000 μs. The exception to this trend is the response of the artificial perilymph/electrical stimulation (AP/ES) animals stimulated at 6 dB above threshold. These responses increased to a peak response, at an IPI of ≈ 1000 μs, which was greater than the responses recorded at an IPI of 4000 μs. Although the average response at this interpulse interval was greater than the response at 4000 μs, the increase was not significant (t-test, $p > 0.05$).

Direct intracochlear delivery of the neurotrophin brain-derived neurotrophic factor (BDNF) results in a significant reduction in the response to the second of a pair of stimuli delivered 6 dB above threshold with an interpulse interval of 1000 μs (2-way ANOVA with Bonferroni post-hoc, $p < 0.05$), as illustrated in Figure 2. There was no significant effect of chronic electrical stimulation, or an interaction of chronic stimulation with BDNF ($p > 0.05$), however the overall trend of the data is similar to the anatomical data presented in the *Ninth Quarterly Progress Report*.

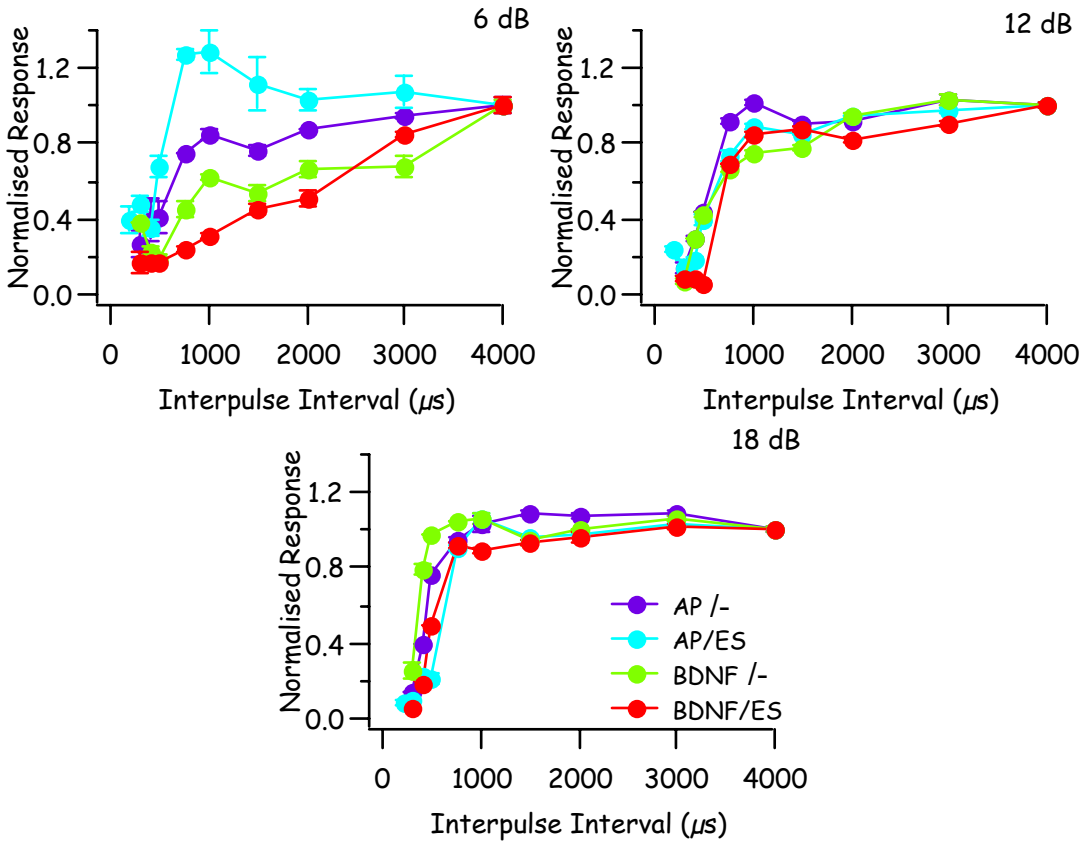

Figure 1. Representative EABR amplitudes evoked by a probe stimulus versus IPI for three masker/probe intensities (6, 12 and 18 dB above EABR threshold). The data are normalized to the probe-evoked EABR amplitude at an IPI of 4000 μs. These data are based on wave III of the EABR, and each graph is the average (± STD) of two responses (see our *Eighth Quarterly Progress Report* for further details). AP/- artificial perilymph/no electrical stimulation; AP/ES artificial perilymph/chronic electrical stimulation; BDNF/- BDNF infusion/no electrical stimulation; BDNF/ES BDNF infusion/ chronic electrical stimulation.

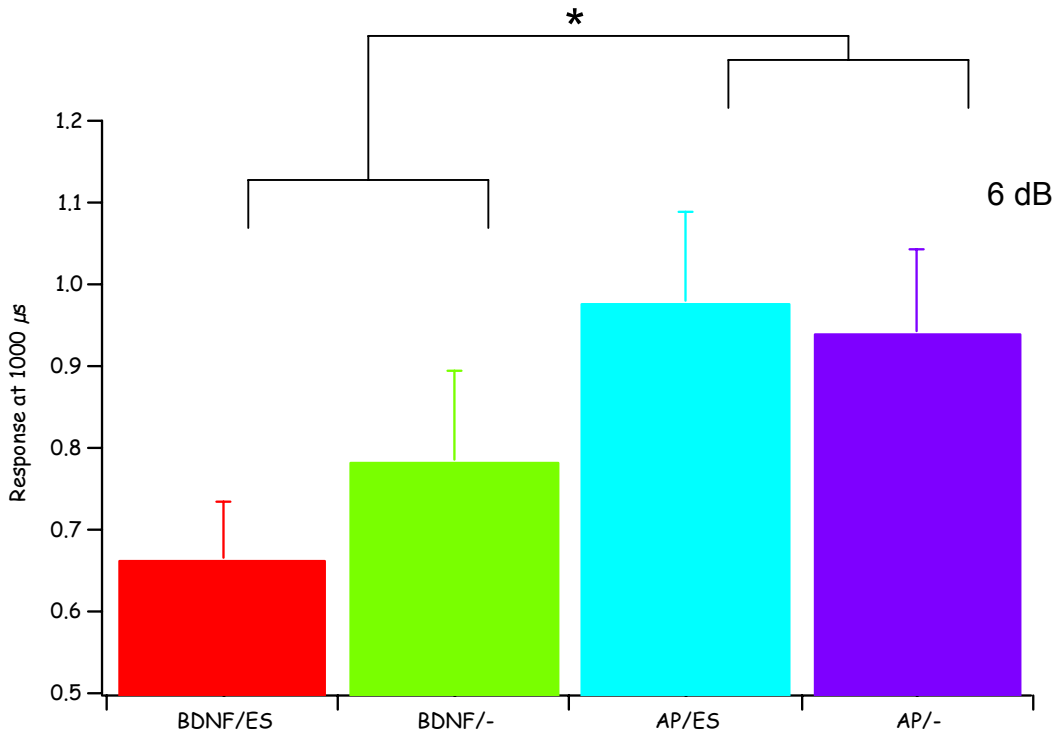

Figure 2. Mean (± SEM) response to the second of a pair of stimuli delivered at 6 dB above threshold with an interpulse interval of 1000 µs. * = $p<0.05$.

With the stimuli at 12 dB and 18 dB above threshold there was no significant effect of either BDNF or chronic stimulation (Figure 3).

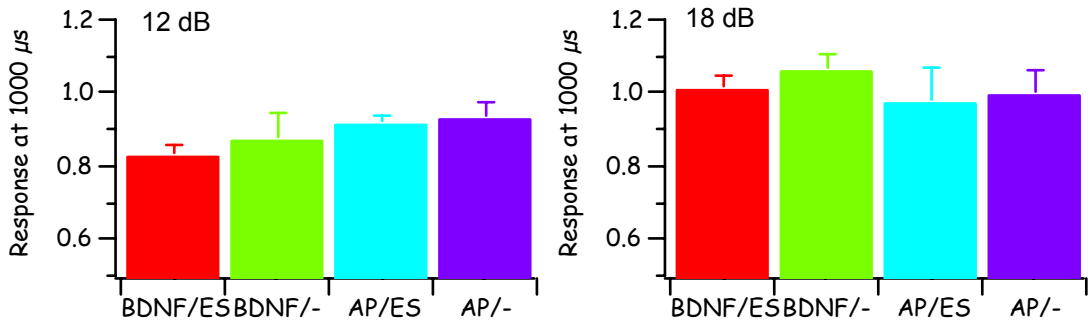

Figure 3. Mean (± SEM) response to the second of a pair of stimuli delivered at 12 and 18 dB (left panel, right panel respectively) above threshold with an interpulse interval of 1000 µs.

3.2 Discussion

The histological data presented in the *Ninth Quarterly Progress Report* suggests that not only does intracochlear delivery of BDNF significantly increase spiral ganglion neuron (SGN) survival, concurrent chronic electrical stimulation enhances this effect. The anatomical findings are not completely reflected in the functional data; while a significant effect of intracochlear delivery of BDNF was observed in terms of reduced EABR thresholds (*Eighth Quarterly Progress Report*), there were no clear significant differences among these treatment groups based on the present EABR refractory data.

A reduction in the response at an interpulse interval of 1000 μs is correlated with an increase in SGN survival, which at first may seem paradoxical. Two points that should be noted however, are that the refractory measurements where taken relative to EABR threshold, which was significantly lower in the BDNF treated animals (*Eighth Quarterly Progress Report*); and that as the stimulus level was increased (compare Figs. 2 and 3) the normalized response approached unity. In fact for the BDNF/ES group there is a statistically significant dependence on stimulation level (One-way ANOVA, $p < 0.001$). The larger responses in the non-BDNF treated animals at 6 dB above threshold, may therefore be a result of a narrower effective dynamic range.

In sum, while we have reported significant rescue of SGNs associated with exogenous BDNF delivery into the cochlea, and the enhanced effects that chronic electrical stimulation has on this treatment, we do not observe any clear functional advantage in terms of reduced refractory effects using the EABR. We plan further studies to investigate these issues using single viiith nerve fiber recordings.

4. Rehabilitation of the deafened auditory nerve with Schwann cell transplantation

The target population of cochlear implants, the SGNs, undergo persistent, irreversible damage following SNHL. A wide range of rehabilitative techniques have been applied with the aim of arresting these degenerative effects. These include depolarization, neurotrophins, genetic viral vectors and, in this research, the use of cell-based therapies. In the auditory system the cellular therapy approach is in its most preliminary stages, with researchers predominantly aiming to regenerate hair cells with stem cell technology (Ito et al., 2001; Iguchi et al., 2003).

Schwann cell therapy is an established experimental tool for neural rehabilitation following spinal cord injury and is in Phase-I clinical trials to treat demyelinated lesions caused by multiple sclerosis. The current study sought to harness the natural trophic and remyelinating properties of Schwann cells to protect SGNs following deafness.

4.1 Why use Schwann cell therapy following SNHL?

The survival of normal peripheral nerves relies, in part, on trophic support from surrounding Schwann cells. Following acute trauma to peripheral nerves the natural secretions and physical support of activated Schwann cells are crucial for neural regeneration (Hall, 1999). Schwann cells revert to an immature phenotype and up-regulate the synthesis and secretion of various growth-permissive molecules such as nerve growth factor, ciliary derived neurotrophic factor, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, neurotrophin-3, extracellular matrix molecules and cellular adhesion molecules (Frostick et al., 1998). Following extensive damage this endogenous reparative system breaks down and clinical intervention is required.

Current clinical strategies involve transplantation of a whole segment of peripheral nerve, however, experimental results show that Schwann cells alone can deliver substantial trophic support (Guenard et al., 1992; Levi and Bunge, 1994; Levi et al., 1994; Ogden et al., 2000; Fornaro et al., 2001; Mosahebi et al., 2002). The resulting regeneration of axons is robust, precisely directed and concentration dependent, with more Schwann cells leading to greater regeneration (Hadlock et al., 1998). Furthermore, new axons are functionally remyelinated by the Schwann cells (Levi et al., 1994). Similar results have been reported in various models of central nervous system damage (spinal cord injury) and demyelination (multiple sclerosis).

4.2 Hypothesis

Given that following SNHL the peripheral portion of the SGN undergoes degeneration and demyelination (Shepherd and Hardie, 2001) and is known to respond to neurotrophin treatment (e.g. see *Ninth Quarterly Progress Report*), it is hypothesized that Schwann cell transplantation into the cochlea will provide trophic support to deafened SGNs.

Rehabilitation may be achieved by providing long-term neurotrophic support, remyelinating the demyelinated soma of the surviving neural population and thereby, increasing the chance of SGN survival. Furthermore, Schwann cells may assist in the regeneration and remyelination of peripheral processes lost following SNHL. Specifically, this pilot study aimed to develop methods to deliver Schwann cell transplants into the cochlea following a severe-profound SNHL, and to evaluate the extent of any protective effects this treatment may have on SGNs.

4.3 Materials & methods

Twelve adult GPs (400-600g) with an ototoxically induced (Kanamycin 520 mg/kg; Frusemide 130 mg/kg), bilateral SNHL received an infusion of Schwann cells two or four weeks after deafening and were sacrificed two weeks later (Table 1).

Table 1 Summary of Experimental Animals

Animal Number	n	Deafened	Duration prior to implantation (weeks)	Post-implantation survival Times (weeks)
GP 1-6	6	✓	2	2
GP 7-12	6	✓	4	2

The round window was exposed via a dorsal approach to the cochlea through the middle ear. A sterile delivery tube was attached to a 5 µL glass Hamilton syringe and front-filled by lowering the tip into Schwann cell suspension and using a micro delivery pump. The tip of the delivery system was then inserted through an incision in the round window into the scala tympani and 200,000 cells were gradually (0.5 µL per minute) delivered in a 2 µL suspension.

Two weeks following surgery the animals were euthanased and the cochleae prepared for histological examination. The density of SGNs was quantified by calculating their survival in Rosenthal's canal in Turns 1-3 in mid-modiolar sections. The number of surviving SGNs was determined by counting each SGN with a visible nucleus. The total count divided by the area measured indicates SGN density (cells/mm^2). Neuronal densities for each cochlear turn in the Schwann cell treated groups were statistically compared with the corresponding region in the untreated contralateral control cochlea using the non-parametric Mann-Whitney U test.

4.4 Preliminary results

Figure 4 (2-week deafened) and Figure 5 (4-week deafened) illustrate SGN density in upper turn 1 of Rosenthal's canal for each experimental animal. Figure 6 graphically compares the mean SGN densities for turns 1, 2 and 3 between Schwann cell treated (right column) and untreated contralateral control cochleae (left column). All statistical data presented is mean + standard error of the mean. Statistical analysis of SGN densities demonstrates a significant difference between the treated (967.7 + 30.5) and control cochleae (835.2 + 28.5) in turn 1 of two week deafened animals (Mann-Whitney U test $p<0.05$). No significant difference was detected (Mann-Whitney U test $p>>0.05$) between the density of surviving SGNs in turn 2 or 3 for the 2-week deafened animals or any turns for the 4-week deafened animals.

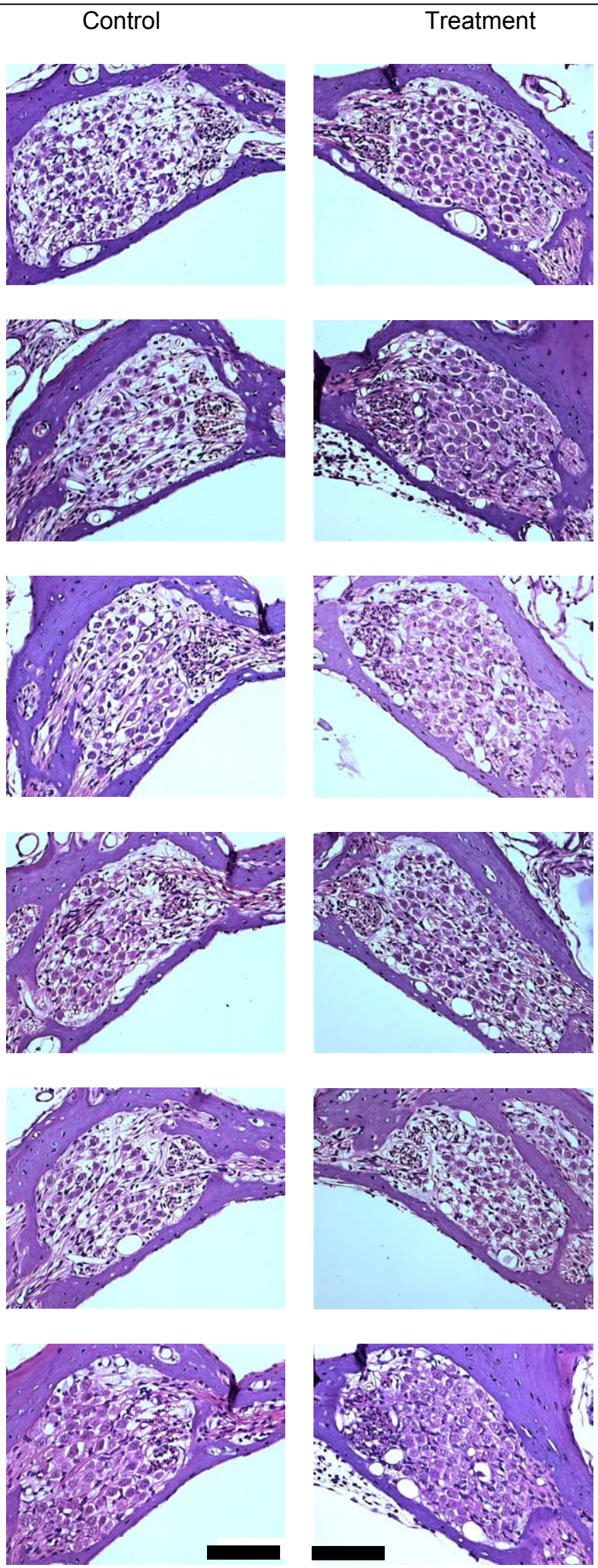

Figure 4. Rosenthal's canal of upper turn 1 for contralateral untreated control (left) and Schwann cell treated (right) cochleae from each animal in the 2-week deafened group. Scale bar = 100μm

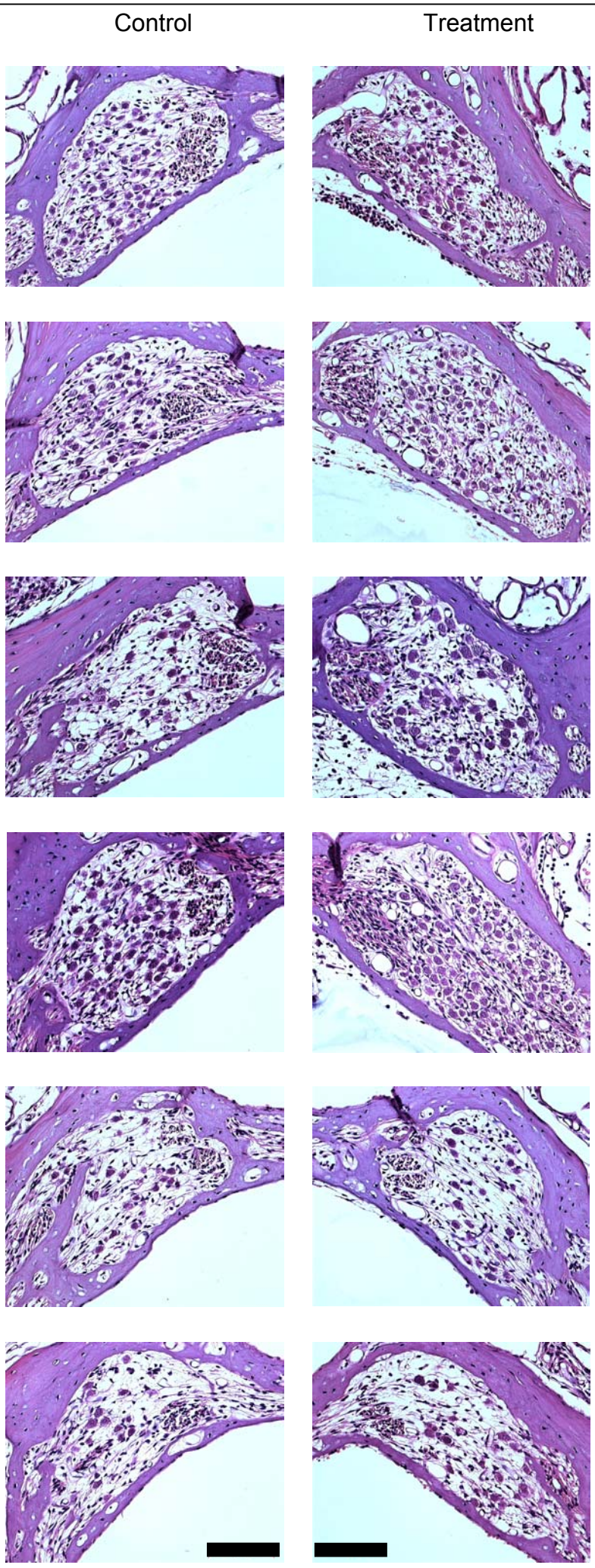

Figure 5. Rosenthal's canal of upper turn 1 for contralateral untreated control (left) and Schwann cell treated (right) cochleae from each animal in the 4-week deafened group.

Scale bar = 100μm

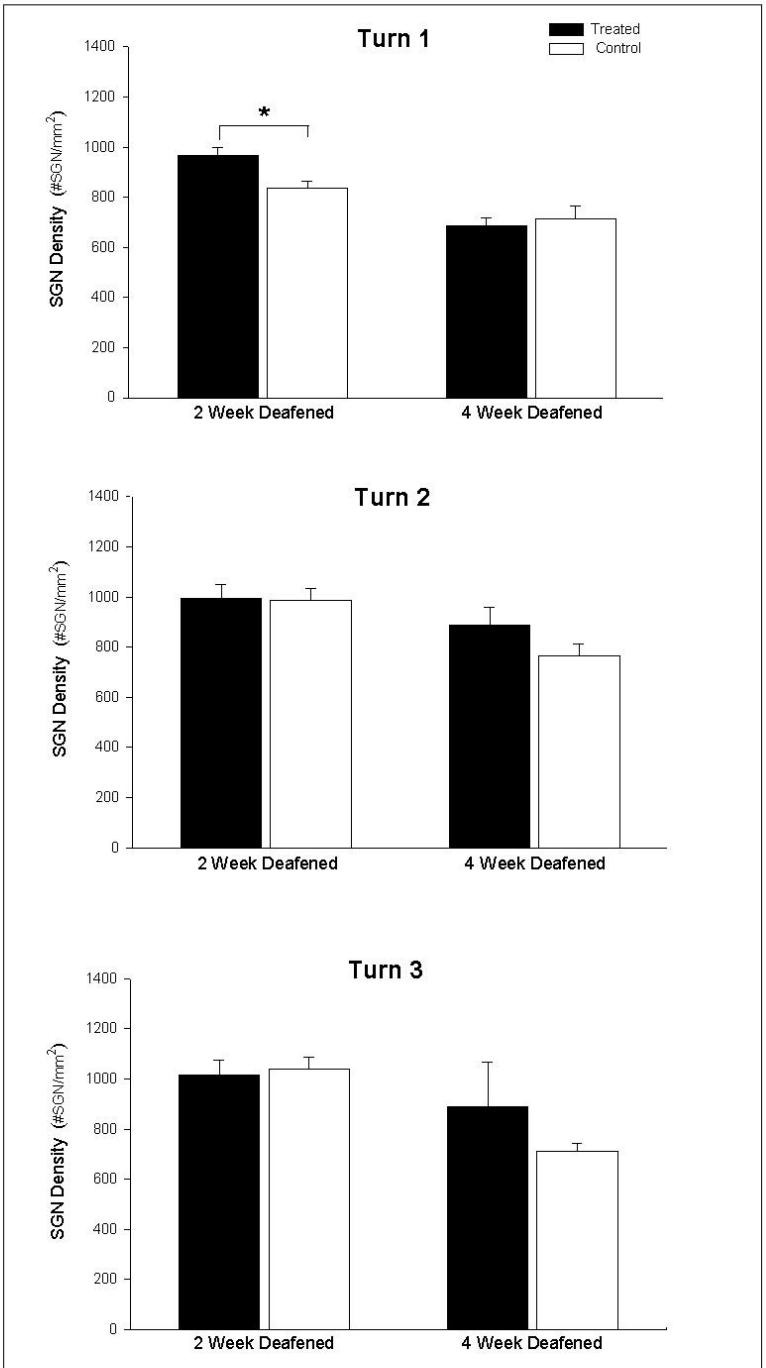

Figure 6. Comparison of mean SGN densities for Schwann cell treated and contralateral untreated control cochleae for two and four week deafened groups in 3 cochlear turns. Error bars denote the standard error of the mean; * statistically significant difference on Mann-Whitney U test, $P<0.05$.

4.5 Schwann cells can increase SGN survival

This pilot study has shown that a single delivery of Schwann cells can provide trophic support to SGNs following aminoglycoside induced deafness. A single delivery of Schwann cells significantly improved SGN survival in the basal turn of GPs deafened for two but not four weeks. This finding provides the first *in vivo* evidence of a trophic influence of transplanted Schwann cells on SGNs.

The mechanisms by which transplanted Schwann cells effect these changes on SGN survival are unknown. It is likely to be a result of diffusible trophic factors secreted from the Schwann cell. The characteristics of such secretions are controlled by the interaction of the Schwann cell population with the specific biochemical environment, and may include various neurotrophins, extracellular matrix molecules and neural cell adhesion molecules.

4.6 Future Directions

Although further work is required to refine our knowledge of the mechanisms in play and the exact conditions required for SGN rehabilitation, these important results are worthy of continued experimental attention. The study will be expanded to explore the spatial and temporal restrictions of the trophic effects, the effects of vehicle alone (sham control) and the survival and distribution of the Schwann cells within the cochlea. This work will eventually tie in with other studies performed under the current contract, allowing the functional effects of Schwann cell transplantation to be analyzed with EABRs and the possibility of additive trophic effects - combining neurotrophins, electrical stimulation and cell-based therapies - to be assessed.

5. Publications

During the quarter, the following manuscript was submitted and accepted for publication.

Hurley P.A., Clarke, M., Crook, J.M., Wise A., and Shepherd R.K. Cochlear immunochemistry – A new technique based on gelatin embedding. *J. Neurosci. Methods* (in press).

6. Personnel

6.1 Ms. Jacqueline K. Andrew

Ms. Andrew holds both Bachelor of Science and Bachelor of Arts degrees and has recently completed an Honours thesis entitled ‘Rehabilitation of the Auditory Nerve by Schwann Cell Transplantation’ in The Department of Otolaryngology under the supervision of Dr Robert Shepherd and Dr. Jeremy Crook. As part of her upcoming PhD, Ms Andrew will play a major role in our *in vitro* and *in vivo* investigation of the merits of cell-based therapies in the cochlea. During her

undergraduate study she completed various auditory neurophysiology and neuroanatomy related research projects. She has also worked within this department over the past three years, as a Research Assistant and Research technician.

7. Plans for Next Quarter

- Complete histological preparation and analysis of cochleae and auditory brainstem structures in cats and guinea pigs following completion of the chronic stimulation program.
- Continue preparation for manuscript submission and conference presentations.
- Complete terminal acute electrophysiological experiments on neonatally deafened un-stimulated control cats.
- Complete long-term BDNF/electrical stimulation studies on deafened guinea pigs.
- Complete design and prototype testing of a fully implantable cochlear stimulator.
- Complete the sham controls (vehicle alone) for the Schwann cell transplantation study investigating the trophic support of these cells on SGNs.

8. Acknowledgements

We gratefully acknowledge the important contributions made by our Histologist, Maria Clarke; Veterinarian, Dr Sue Pierce; Elisa Borg for management of our animal house; Helen Feng for electrode manufacture; Rodney Millard and Frank Nielsen for engineering support, Dr. Jeremy Crook for immunohistochemical advice, and Dr. Trevor Kilpatrick and Ms. Tania Cipriani from the Walter and Eliza Hall Institute for supply of Schwann cells.

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10. Appendix A

The following abstracts, funded fully or partially under the present contract, were submitted and accepted for poster presentation at the 2003 Conference on Implantable Auditory Prostheses, Asilomar Conference Center, Pacific Grove, CA.