Fifth 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 fifth quarter of this contract the following activities were completed:

- Implanted and commenced chronic electrical stimulation on an additional three deafened kittens.
- Continued our terminal acute electrophysiological studies on the first group of cats that have been chronically stimulated.
- Deafened, implanted and chronically stimulated two adult guinea pigs. These animals received electrical stimulation with the simultaneous delivery of brain derived neurotrophic factor into the cochlea.
- Optimized protocols for the immunohistochemical detection of the three tyrosine protein kinase receptors (Trk) A, B, and C, and apoptosis in

tissues of the auditory pathway. This work will form the major part of the present report.

- Dr. Shepherd organized a meeting with Drs. Patricia Leake and Edwin Rubel to discuss auditory nerve degeneration following a SNHL, and potential protective effects of electrical stimulation. This meeting was held at UCSF Medical Center on 23/10/01 and provided useful discussion for future research in this area.

3. Chronic electrical stimulation studies in neonatally deafened cats

The three animals deafened during the previous quarter (NDC_14, NDC_15, NDC_16) were bilaterally implanted at eight weeks of age. Surgical details are outlined in our *Third Quarterly Progress Report*. All kittens made an uneventful recovery from surgery and commenced their chronic stimulation program approximately two weeks later. More details of our chronic stimulation study in neonatally deafened kittens will be provided in a future report.

A major undertaking during the quarter was the continuation of the terminal electrophysiological experiments we commenced last quarter. Full details of this work will be provided in future reports. Briefly, we performed six experiments during the quarter. All animals had been chronically stimulated for periods of up to eight months. In two partial-hearing animals we recorded single neuron activity from the primary auditory cortex in response to both acoustic and electrical stimulation. This work was performed in collaboration with Prof. D. Irvine and Dr. M. Brown of Monash University. Approximately 18 penetrations were made in each cortex in order to establish a topographic map of this structure. In a second series of experiments we recorded from the inferior colliculus of five of these animals to study spatial and rate plasticity in the central nucleus of the inferior colliculus. The procedures follow our previous work in this area (Shepherd et al., 1999). A total of 335 single and multi-units have been recorded to date and more experiments are planned for the following quarter. A major activity undertaken by Dr. Fellon (see Personnel; below) during the quarter was to upgrade the software used in the analysis of these data. A description of the upgrade will be given in the following quarter while a detailed analysis of the data awaits completion of the upcoming experiments.

At completion of these experiments each animal was killed with an overdose of anesthetic and its cochleae and brain were prepared for histological and immunohistochemical examination following the procedures described in (Hardie and Shepherd, 1999). Histopathological examination of the cochleae and brainstem will be performed over the next few quarters.

4. Electrical stimulation and neurotrophin administration in deafened guinea pigs

Two 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. Five days after deafening, the animals were

implanted with an electrode array/mini-osmotic pump assembly (Fig. 1; *First Quarterly Progress Report*). Surgical details are outlined in our *Fourth Quarterly Progress Report.* Both animals were chronically stimulated and received brain derived neurotrophic factor over a 28-day implant period. Upon completion of this period, functional studies were performed using electrically-evoked auditory brainstem responses; the animals were then killed with an overdose of anesthetic (*Fourth Quarterly Progress Report*). A further four animals are planned for inclusion in this study next quarter. Both the electrophysiological and histological data will be presented in a future report.

5. Neuroanatomical and neurochemical studies of the deafened auditory system

5.1 Trk and apoptosis immunoreactivity

As part of our studies of the neurobiological effects of electrical stimulation and neurotrophin delivery on the deafened auditory system, we have initially optimized protocols for the immunohistochemical detection of the three tyrosine protein kinase receptors (Trk) A, B, and C, and apoptosis (programmed cell death) in tissues of the auditory pathway.

Our interest in the Trk's is driven in part by their suitability as markers of neurotrophin activity. They are therefore highly relevant to our electrical stimulation and neurotrophin administration study in the deafened guinea pig. The Trk's are high affinity receptors for nerve growth factor (NGF; Trk A), brain-derived neurotrophin factor (BDNF) and neurotrophin-4/5 (Trk B) and neurotrophin-3 (NT-3; Trk C). In addition, based on evidence for the activities of the various neurotrophins as well as their target receptors being intimately related, together with the specific function(s) of individual Trk receptors and their cognate ligands, we believe it will be useful to study all three receptor types.

Initial immunocytochemical studies have provided insight into the localization and relative levels of each receptor type in tissues of the mammalian cochlea and cochlear nucleus (Figs. 1, 3). Combined with the wealth of information concerning the histological, pharmacological and physiological properties of these auditory structures, the present findings and research methods will be applied to studies of the role of electrostimulation and chronic administration of neurotrophins in mammalian auditory system function, using rat, guinea pig and cat models.

In addition to Trk receptor studies, we have also established a protocol to detect and quantitate apoptotic cell death at single cell level in the auditory brainstem. Similar to Trk receptor studies, studies of apoptosis using TdT-mediated dUTP nick end labeling (TUNEL) will be applied to investigate the effects of electrical stimulation and chronic neurotrophin delivery on the deafened mammalian auditory system, using rat, guinea pig and cat models.

5.2 Trk Immunoreactivity in the Cochlear

Initial studies of Trk A, B and C receptors have been performed on 40 μm thick cryopreserved tissue sections of adult rat cochleae. The method developed within our laboratory for the processing of frozen cochlear sections (see *Fourth Quarterly Progress Report*) is compatible with immunocytochemistry (Fig. 1).

Figure 1 depicts Trk B and C immunolabeling, with high labeling of both receptor types associated with bipolar spiral ganglion cells and their peripheral processes, but negligible labeling of sensory hair cells within the organ of Corti. Importantly, these findings are consistent with our understanding of the relationship between the organ of Corti as a target organ and dependent primary sensory neurons of the spiral ganglia (Marzella and Clark, 1999). As a site of neurotrophin production and release, the organ of Corti would not be expected to express Trk receptors. Conversely, the trophic effects of the organ of Corti on spiral ganglion cells requires their concomitant expression of receptor protein.

In contrast to Trk B and C receptor findings, studies of the immunolocalisation of Trk A receptors indicate an absence of Trk A receptor protein throughout the cochleae. This finding is consistent with previous reports of a transient expression of Trk A mRNA in the developing inner ear neuroepithelium, including sensory neurons of the spiral ganglion and cells of the organ of Corti (Ylikoski et al., 1993; Schecterson and Bothwell, 1994; Qun et al., 1999).

5.3 Trk Immunoreactivity in the Dorsal Cochlear Nucleus

In addition to cochlear tissue studies, we have begun studies of Trk A, B and C immunolabeling in the cochlear nucleus. Initial studies have been performed using 40 μm thick cryopreserved tissue sections of Guinea Pig brain stem containing dorsal cochlear nucleus (DCN). Trk-reactive cells were examined in accordance with adjacent Nissl stained sections and neuronal areas and cellular types previously reported (Osen, 1969; Hackney et al., 1990).

From Nissl stained sections of DCN (Fig. 2), three major laminae could be identified including the outer most molecular layer (consisting of stellate cells and deeper cartwheel cells), a second and very prominent granule layer (comprised of many granule cells but also containing larger pyramidal cells), and a third polymorphic cell layer (including multipolar and giant cells).

From immunohistochemistry, a differential cell staining was observed between the different subtypes of Trk receptors. Trk A receptor immunstaining was largely restricted to the granule layer of DCN, with moderate staining of pyramidal cell somata (Fig. 3). Lower level diffuse staining was observed throughout surrounding neuropil.

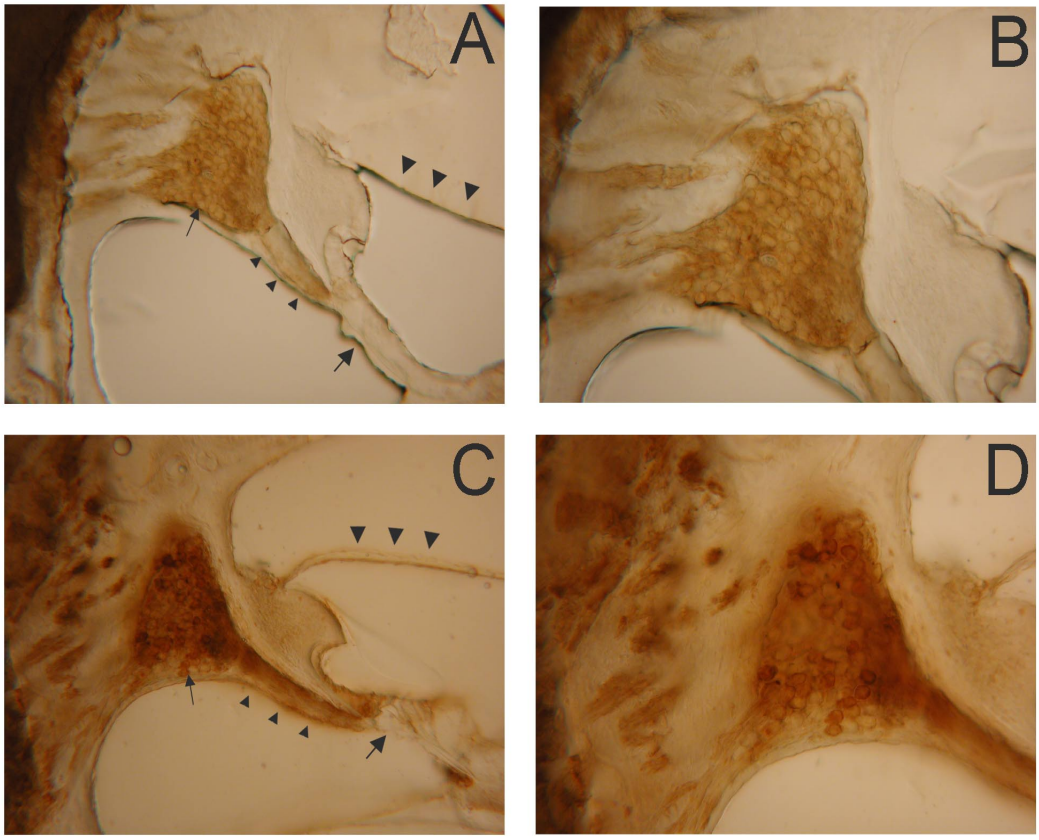

***Figure* 1.** Representative brightfield photomicrographs of Trk B (A: 10x; B: 20x) and C (C: 10x; D: 20x) receptor immunolabeling of 40 μm thick frozen sections in normal rat cochleae. Ressiner's membrane: large arrow heads; peripheral processes: small arrow heads; organ of Corti: large arrows; spiral ganglion cells: small arrows.

In contrast to Trk A, Trk B receptor immunoreactivity was prominent across all laminae of DCN, with strong labelling of both neuronal and non-neuronal somata. Particularly evident was the punctate immunostaining throughout granule layer neuropil, including intensely labelled and numerous pyramidal and granule cell bodies, and unidentified round medium-size cell soma (Fig. 3).

A unique pattern of intense Trk C immunoreactivity was demonstrated in the perisomatic region of round medium-size neural cell bodies and the proximal regions of their dendrites (Fig. 3). Thus the cytoplasm and nucleoplasm of these cells was not immunoreactive for Trk C. Interestingly, these cells are morphologically similar to a subgroup of Trk B labelled cells previously discussed. Finally, a concomitant immunolabeling of puncta was apparent throughout surrounding neuropil (Fig. 3).

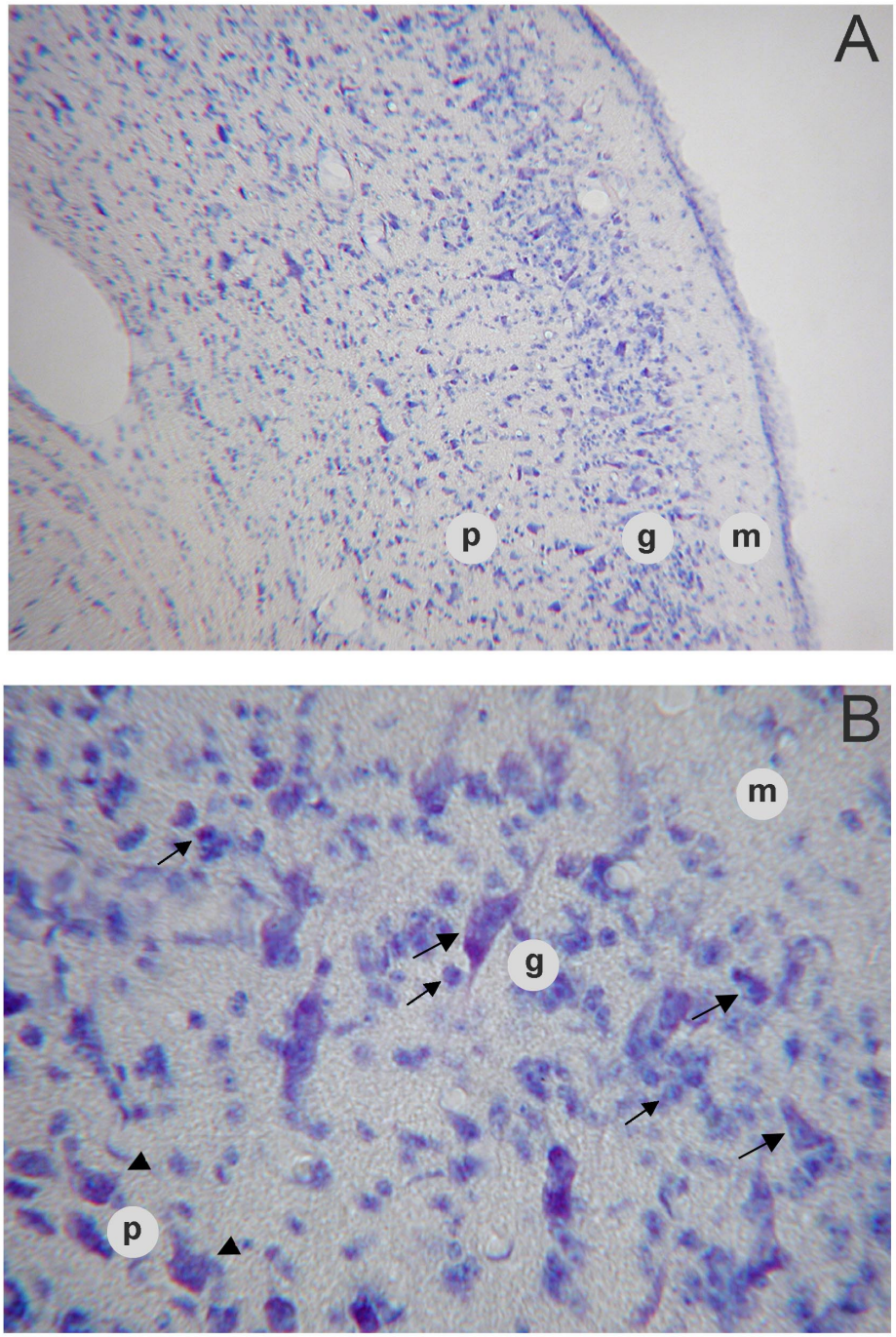

***Figure* 2.** Representative brightfield photomicrographs of 40 μm thick frozen Nissl stained sections of guinea pig DCN. m: molecular layer; g: granule layer; p: polymorphic layer. A: low-power (10x) photomicrograph illustrating staining across all laminae of DCN. B: high-power (40x) photomicrograph illustrating staining in the granule layer, as well as deep and superficial molecular and polymorphic layers respectively. granule cells: small arrows; pyramidal cells: large arrows; multipolar cells: large arrow heads.

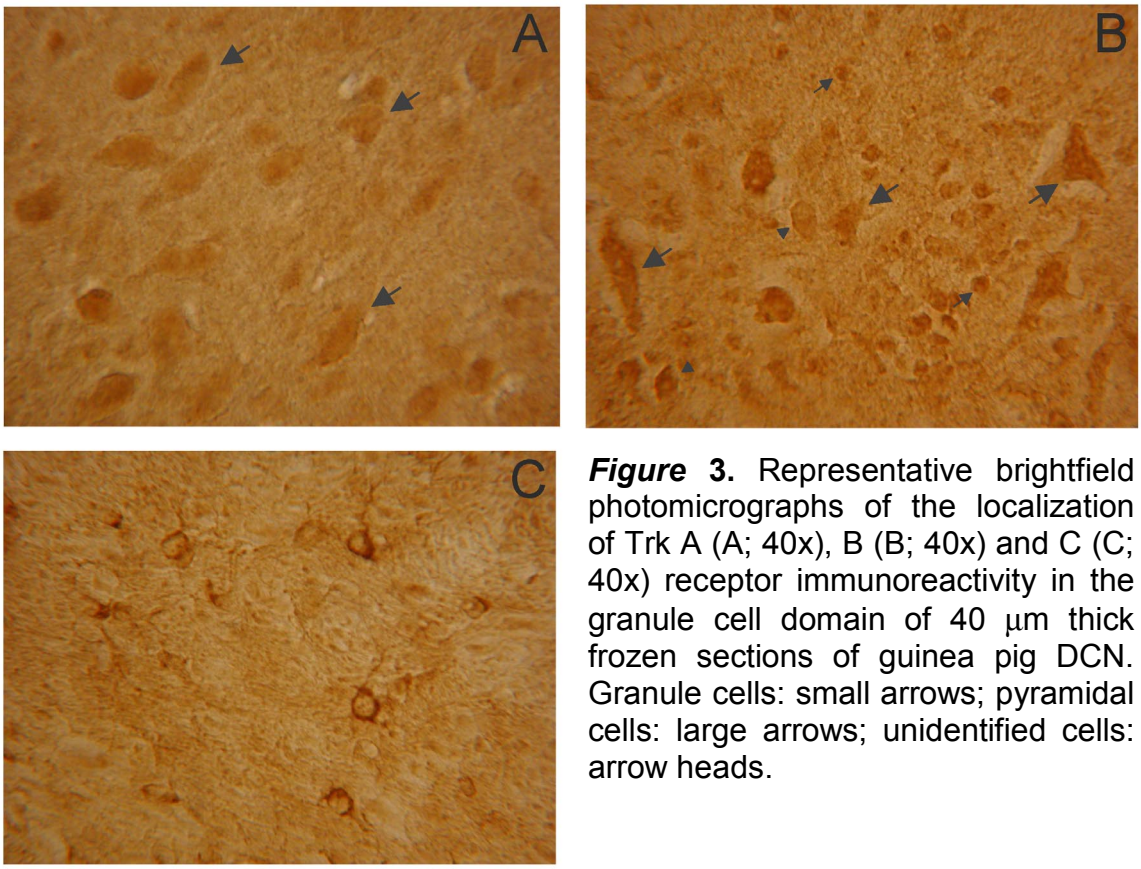

***Figure* 3.** Representative brightfield photomicrographs of the localization of Trk A (A; 40x), B (B; 40x) and C (C; 40x) receptor immunoreactivity in the granule cell domain of 40 μm thick frozen sections of guinea pig DCN. Granule cells: small arrows; pyramidal cells: large arrows; unidentified cells: arrow heads.

5.4 Apoptosis in the Dorsal Cochlear Nucleus

We have successfully used TUNEL to identify apoptotic cells in 40 μm thick cryopreserved tissue sections of normal Guinea Pig brain stem containing DCN. Apoptotic neurons with clearly fragmented nuclei could be observed in DCN (Fig. 4). Chromatin condensation was apparent in cell somata (Fig. 4). As expected, low numbers of labeled cells were found in each section examined.

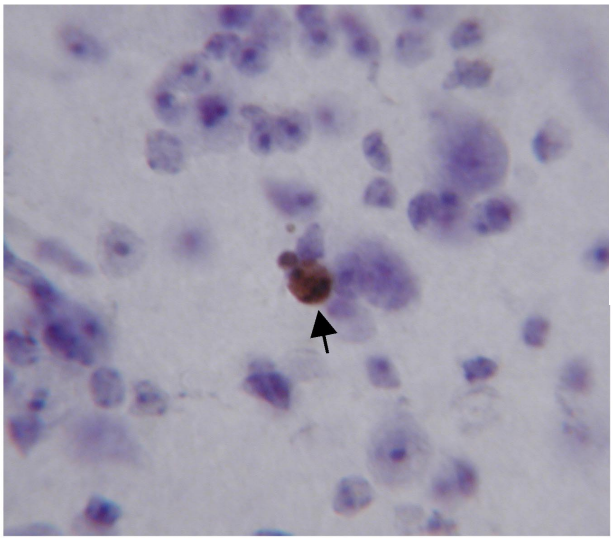

***Figure* 4.** Representative bright-field photomicrograph of an apoptotic cell in DCN (40x). Samples were counterstained with haematoxylin. Chromatin condensation was apparent (arrow).

5.5 Summary

In summary, we have now completed the optimization of protocols for immunohistochemical studies of the three Trk receptor subtypes and apoptosis in tissues of the auditory pathway. Our initial experiments will pave the way for future investigation of the cellular and neurochemical mechanisms that underlie re-activation of the deafened auditory pathway via a cochlear implant. Together with other avenues of investigation, including studies of cell proliferation, we believe that research findings will assist understanding of the potential benefits and pitfalls to the treatment of human deafness.

6. Personnel

Two new members joined the team during the quarter.

Dr. James Fellon has joined our group, having recently completed a PhD at Monash University where he designed and constructed a multi-channel recording system and used this system to investigate stochastic resonance in biological receptors. Dr. Fellon also holds a B.Sc. and B. Eng. (Hons) from Monash University. Dr. Fellon will participate in the acute electrophysiological studies performed at completion of our chronic stimulation studies in cats. He will also be responsible for software development including upgrading of our single unit data analysis and the development of single unit identification routines from multi-unit data. Dr. Fellon will be working in our group until July 2002.

Mr. Pascal Peloquin joined the group as a Research Assistant for this quarter as part of a Student Work Abroad program. Mr Peloquin has a B.Sc with previous experience as a research assistant in electrophysiology with the Faculty of Medicine & Dentistry at the University of Western Ontario, London, Ontario, Canada. Mr. Peloquin made an important contribution to our research, particularly in relation to our chronic stimulation studies. A full-time research assistant will join the team next quarter.

7. Publications

During the quarter two chapters were completed and submitted for inclusion in a new book on neural prostheses: “Neuroprosthetics: Theory and Practice”. K. Horch & G. Dhillon (Eds), World Scientific Publishing.
Shepherd, R.K. “The Auditory System”. and Seligman, P.M. & Shepherd, R.K. “Cochlear Implants”.

8. 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.
- Perform immunohistochemical studies of Trk receptors and apoptosis of cochlear and brainstem structures in cats and guinea pigs following completion of the chronic stimulation program.
- Continue developing immunochemistry protocols for other markers of auditory system function using normal hearing animals.
- Begin studies of the deafened rat auditory system using immunohistochemistry and radioligand binding.

9. Acknowledgements

We gratefully acknowledge the important contributions made by our Veterinarian Dr Sue Pierce, Elisa Borg for management of our animal house, Dr. James Fellon for software development and data collection, Pascal Peloquin and Damon Shepherd for research assistance, Helen Feng for electrode manufacture, Maria Clarke for histology support, Dr. Phillip Marzella and Lisa Gillespie for advice on neurotrophin delivery systems and Rodney Millard and Frank Nielsen for engineering support.

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