Ninth Quarterly Progress Report

NIH-N01-DC-3-1005

Protective Effects of Patterned Electrical Stimulation on the Deafened Auditory System

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October 1 – December 31, 2005

Contents:

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 of spiral ganglion neurons (SGNs) and the central auditory system (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 auditory nerve (AN) survival in both guinea pig and other mammalian models of sensorineural hearing loss (SNHL). This work will also provide insight into the role of neurotrophins in improving synaptic efficiency in the deafened auditory pathway.

b) The neurophysiological and neuroanatomical response to prolonged electrical stimulation of the auditory nerve following a neonatal SNHL. This work is designed to provide insight into the protective effects of electrical stimulation on SGNs and the plastic response of the CAS to temporally challenging stimuli presented chronically to one or two sectors of the AN. This work will also examine the ultrastructural changes evident at the AN/cochlear nucleus synapse in response to a neonatal SNHL and to chronic electrical stimulation of the AN.

c) The application of cell based therapies for rescue and replacement of SGNs following SNHL. These studies are designed to provide insight into the potential clinical application of cell-based therapies in the severe and profoundly deaf prior to cochlear implantation.

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 ninth quarter the following activities were completed:

2.1. Publications and conferences

The following paper was accepted for publication.

Coleman, B., Hardman, J., Coco, A., Epp, S., de Silva, M., Crook, J. and Shepherd, R.K. Fate of embryonic stem cells transplanted into the deafened mammalian cochlea. J. Cell Transplantation (in press).

A copy of this manuscript is attached (Appendix A).

2.2. Chronic electrical stimulation and neurotrophin delivery in the guinea pig

This work aims at developing techniques for SGN rescue based on the exogenous delivery of neurotrophins in combination with chronic depolarization via a cochlear implant.

Counts of SGN density were completed this quarter. The results will be prepared for publication in future quarters.

2.3. Chronic electrical stimulation in the cat

This work addresses the question of whether chronic depolarization alone, via a cochlear implant, can prevent SGN degeneration. Additionally, the question of whether patterned chronic electrical stimulation of the auditory nerve can produce plastic reorganization within the central auditory pathway is being addressed.

During this quarter, four neonatally deafened, implanted animals received daily chronic electrical stimulation. Each animal had electrically evoked auditory brainstem responses (EABRs) recorded monthly, with subsequent behavioral testing of maximum comfortable stimulus levels and minor adjustments of chronic stimulation levels as required. Daily impedance monitoring of the chronically stimulated animals is also ongoing, with development of automated analysis software for these measurements nearing completion this quarter (see section 2.3.1). All four chronically stimulated animals underwent acute electrophysiological experiments this quarter, which completes the acute electrophysiological experiments for two of our experimental cohorts and a thorough analysis of this data can now commence. We currently have four deafened un-implanted control animals that will undergo acute electrophysiological experiments over the next two quarters.

Following the completion of each acute electrophysiological experiment, the cochlea and CNS from each animal were harvested and prepared for subsequent analysis. Additionally, the cochlear nuclei from each animal were processed for both light microscopy and transmission electron microscopy and sent to Prof. David Ryugo for ultrastructural analysis of the end bulb of Held.

2.3.1 Software development

We have previously measured electrode impedance manually by reading electrode voltage and current directly from an electrically isolated cathode ray oscilloscope, or more recently using a computer based impedance monitoring system. Development of software for automated analysis of our chronic electrode impedance data continued this quarter. The impedance analysis software is implemented in Igor Pro 5.03. Our algorithm searches for the stimulating biphasic current pulse, which is used as a time reference to look for the peak voltage developed across the electrode ($V_{z)}$. When found, it is divided by the average amplitude of the current pulse to give the impedance (according to Ohm's Law, $Z=V_z/I$). This process is repeated for each valid impedance measurement (typically ~20/day) to give mean daily impedances.

The software scans the data files on startup to produces a list of cats, dates/times and electrodes. These can be selected to allow display of a raw waveform and single impedance for a particular date, or to compute and plot impedance for all measurements. The software can also extract and plot impedance data generated by the monitoring software.

The software can provide useful results in its current state of development; however there is additional information from the recorded voltage and current waveforms that is yet to be extracted. This includes: the access resistance, indicated by the resistive component of the electrode voltage waveform V_a; data from the second (inverted) portion of the voltage waveform; and curve fitting parameters.

The chronic impedance data is currently being analyzed to examine the following relationships and will be presented in detail in a future QPR:

- Overall change in impedance
- Total mean, variation and range in impedance
- Changes in impedance in relation to:-
    - MAP changes
    - Electrode Disconnections
    - Battery changes

2.4. Chronic electrical stimulation in the rat

This work aims to address the issue of whether early experience with simple forms of electrical stimulation enhances the ability to perceive differences between more complex patterns of electrical stimulation later in life. The experiments to examine this issue use a rat behavioral model in which rats with implanted stimulators are trained to discriminate different patterns of stimulation in a specially designed T-maze apparatus (described in previous reports).

The two chronically implanted rats for which data were presented in QPRs 7 and 8 were sacrificed after an implantation period of four months. The cochleae from these animals are currently being processed for histological assessment. At the completion of the stimulation program, threshold currents for eliciting a magnetically induced EABR remained close to the levels obtained two weeks and three months after implantation. Examination of the two implantable stimulators indicated that they were both in good condition. This work has demonstrated that our fully implantable small animal stimulator is suitable for chronic applications, including safety and behavioral studies.

Preliminary testing of hearing rats in the behavioral training maze was completed, and the maze has subsequently been modified to allow the presentation of cochlear electrical stimulation to chronically implanted rats. Presentation of cochlear electrical stimulation has also required the stimulus presentation software (originally written for the control of visual and acoustic stimuli) to be modified. These modifications have now almost been completed. Four rats were deafened, and one rat was implanted in preparation to commence preliminary training and testing of a group of deafened and implanted rats in the next quarter.

Work continued on optimization and construction of the fully implantable stimulators used in this study. A new batch of chronically implantable rat stimulators were assembled with professionally manufactured solder-masked, tinned and thru-hole plated circuit boards. These features allow the boards to be assembled more efficiently. Development is also underway on a more advanced design that will enable current levels to be adjusted (or turned off altogether) post-implantation. We are currently examining the feasibility of an infra-red transcutaneous interface, micro-controller and digital potentiometer in implementing this aim. A manuscript describing the stimulator is in preparation.

2.5. Cellular over-expression of neurotrophins

The aim of this study is to use cell transplantation techniques to deliver long-term/ongoing neurotrophic support to SGNs in animal models of deafness.

As reported previously, Schwann cells have been genetically modified to over-express either brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3). During this quarter, the first phase of *in vitro* experiments to determine the survival effects of neurotrophin over-expressing Schwann cells on SGNs were finalized. A detailed summary of the results from this study is included in Section 3.

The aspect of this work pertaining to the NT-3-Schwann cells was conducted by Miss Ricki Minter as the Honours component of her Bachelor of Science degree. Miss Minter completed her studies during this quarter, and achieved a H1 for her thesis.

We are still working on the genetic modification of male Schwann cells, which we anticipate will provide us with better identification methods for future *in vivo* studies. This work will continue over the coming quarters.

2.6. Analysis of gene-specific markers altered by deafening in the cochlea

The aim of this study is to investigate how the expression of genes related to neuronal survival and function in the mammalian auditory system are modified by sensorineural hearing loss and by re-activation via a cochlear implant.

In this quarter, we continued our efforts to choose a gel documentation system that can perform semi-quantitative documentation of gene expression. The current system that we are evaluating, Omega 12ic from Ultralum, is able to capture chemi-illuminescent pictures from Western blots but its sensitivity, as tested to date, is not convincingly better than the conventional method of X-ray film development. However, we have recently been successful in procuring additional top-up funding from the Trustees of Medical Research and Technology in Victoria, which we hope will enable us to find an appropriately sensitive system. Funding for this system will come from a grant from the Marion & E. H. Flack trust recently awarded to Drs. Tan and Shepherd.

2.7. The application of stem cells for SGN replacement

The aim of this study is to develop clinically feasible techniques for the application of stem cell therapy for SGN replacement in the profoundly deaf.

This quarter a manuscript describing delivery strategies for stem cell replacement of SGNs was accepted for publication (Appendix A). This work highlighted the fact that the injection of a cell suspension into the scala tympani is not effective due to widespread cell dispersal throughout the cochlea and into the central nervous system. We therefore commenced a pilot study transplanting stem cells in a hydrogel matrix into the deafened guinea pig cochlea. The matrix is designed to hold the cells within their target site (Rosenthal's canal), thereby minimizing the risk of cell dispersion. All animals were deafened, underwent transplantation surgery, and were sacrificed. The tissue is currently being prepared for histological analysis.

Two manuscripts are in preparation. The first relates to the effects of co-culture on the differentiation of mouse embryonic stem cells into neurons *in vitro*; the second manuscript details a novel cell counting methodology.

3. Gene- and cell-based therapies for spiral ganglion neuron rescue

SGNs are the target cells of the cochlear implant and, as such, the loss of SGNs that occurs in deafness may impinge upon the benefits cochlear implant patients can receive from their devices. A means of preventing SGN degeneration in deafness is therefore of great clinical significance. The neurotrophic factors brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are known to be important for the development and maintenance of the auditory system, and have also been demonstrated to have protective effects on SGNs in animal models of deafness [1-5]. Unfortunately, it has also been shown that these survival effects are not maintained beyond the treatment period, with cessation of treatment leading to a rapid loss of survival effects [4]. It is therefore important to develop clinically relevant methods for the long-term protection of SGNs from deafness-induced degeneration. Gene- and cell-based therapies are potential therapeutic options for the treatment of many neurodegenerative conditions, including hearing loss. Importantly, it has been demonstrated that genetically modified cells can secrete neurotrophins for over 12 months [6].

The aim of this project is to genetically modify Schwann cells (SCs) to over-express either BDNF or NT-3, and test the survival-promoting effects of these SCs on SGNs in animal models of deafness, over both the short- and long-term.

3.1. Preparation of neurotrophin over-expressing Schwann cells

The first aspect of this study involved the preparation of control and neurotrophin over-expressing SCs.

Schwann cells were obtained from our collaborators at the Howard Florey Institute in Melbourne, and were derived from postnatal day (P) 3 rat sciatic nerve.

Expression vectors were provided by Dr Volkmar Lessmann from the Johannes Gutenberg Universitat, Mainz, Germany (Lessmann *et al.*, 2003). These vectors encoded:

(i) Enhanced green fluorescent protein (EGFP)
(ii) Rat pre-pro BDNF
(iii) Rat pre-pro BDNF plus EGFP
(iv) Rat pre-pro NT-3
(v) Rat pre-pro NT-3 plus EGFP

Schwann cells were genetically modified with the expression vectors using the lipid-based transfection reagent Lipofectamine 2000 (Invitrogen), and stable transformants were selected using the neomycin sulphate analogue Geneticin (Gibco). Transfections resulted in five different Schwann cell lines:

(i) E-SCs: Schwann cells that express the fluorescent marker EGFP
(ii) B-SCs: SCs that express BDNF
(iii) BE-SCs: SCs that express BDNF plus EGFP
(iv) N-SCs: SCs that express NT-3
(v) NE-SCs: SCs that express NT-3 plus EGFP

Successful transfection was confirmed by the presence of the EGFP reporter gene (Figure 1; attached) and/or via enzyme-linked immunosorbent assay (ELISA) for BDNF or NT-3 production (Table 1).

Table 1. ELISA analysis of neurotrophin production from genetically modified Schwann cells.

Cell type	Neurotrophin expressed	Quantity of neurotrophin (mean±SEM) over 3 days
E-SCs	BDNF or NT-3	None detected
B-SCs	BDNF	112±9 pg/ml
BE-SCs	BDNF	188±15 pg/ml
N-SCs	NT-3	None detected
NE-SCs	NT-3	590±34 pg/ml

3.2. Survival-promoting effects of neurotrophin over-expressing Schwann cells in vitro

Co-culture of each of the Schwann cell types with SGNs was subsequently used to determine the survival effects of these genetically modified cells on SGNs.

SGN cultures were prepared from P5-P6 rat pups, as previously described [7]. SGNs were co-cultured with each of the SC types (E-SCs, B-SCs, BE-SCs, N-SCs or NE-SCs), with SCs added to the neuronal cultures at 20,000 cells per well. Control SGN cultures were treated with a commercially available recombinant human (rh) form of either BDNF or NT-3 (50ng/ml; PeproTech), to be used for comparative purposes since these neurotrophins are known to elicit survival effects. Control SC cultures were grown without additional exogenous neurotrophins. All cultures were fixed after three days *in vitro*. Control SC cultures were mounted in DAPI mounting media (Vector). The SGN control cultures and the SGN-SC co-cultures were immunostained with Neurofilament 200kD (Chemicon) and the chromogen substrate DAB, and the number of surviving SGNs per well were counted.

One series of experiments was conducted to test the BDNF over-expressing SCs (B-SCs & BE-SCs), while a separate series of experiments was performed for the NT-3 over-expressing SCs (N-SCs & NE-SCs). In each case, the average SGN survival elicited by the rh neurotrophin was arbitrarily defined as 100%, and the survival effects of each of the SC types was expressed as a percentage of the relative rh neurotrophin.

In the BDNF experiments, E-SCs resulted in 75±15% SGN survival (mean±SEM) as compared to rhBDNF; B-SCs led to SGN survival of 205±44% (mean±SEM); and BE-SCs supported the survival of 272±62% (mean±SEM) of SGNs in comparison to rhBDNF. The survival effects of the BE-SCs were significantly greater than those of the E-SCs and rhBDNF ($p<0.05$). These results are shown in Figure 2 (attached).

In the NT-s experiment, in comparison to rhNT-3, E-SCs resulted in 45±5% auditory neuron survival (mean±SEM); N-SCs led to auditory neuron survival of 80±11% (mean±SEM); and NE-SCs supported the survival of 89±11% (mean±SEM) of auditory neurons in comparison to rhNT-3. The survival effects elicited by the NE-SCs were similar to those of rhNT-3, and significantly greater than the survival effects of the control E-SCs ($p<0.05$). These results are represented in Figure 3 (attached).

The main purpose for testing two expression vectors for each neurotrophin (BDNF and BDNF-EGFP; NT-3 and NT-3-EGFP) was to ensure that the inclusion of the EGFP reporter gene, which allows for confirmation of genetic modification, and will also enable tracking of the neurotrophin *in vivo*, does not alter the biological activity of the neurotrophins. Indeed, we found that there was no statistically significant difference in SGN survival elicited by the B-SCs and the BE-SCS; likewise there was no statistically significant difference in survival effects of the N-SCs and NE-SCs. Therefore, importantly, the presence of the EGFP gene does not impair the production, secretion or biological activity of either of the neurotrophins. As such, due to the added benefit of the presence of the EGFP reporter gene, namely, visualization of neurotrophin secretion *in vivo*, future experiments will make use of the BE-SCs and NE-SCs.

An interesting finding was that the N-SCs did not produce any detectable amounts of NT-3, but that these cells still had a trophic influence significantly greater than that of the control E-SCs. For completeness, further analysis will be required to determine if perhaps the expression vector was for a neurotrophin other than NT-3.

3.3. Conclusion

These results indicate that Schwann cells that have been genetically modified to produce either BDNF (BE-SCs) or NT-3 (NE-SCs) can elicit survival effects on SGNs. Future studies will assess the longevity of the production of neurotrophins from these cells, and will test the capacity of neurotrophin over-expressing Schwann cells to support SGN survival following transplantation into the cochlea of deafened animals.

4. Additional activities

- Having reached the half-way point in the current NIH contract, a Strategic Planning Retreat was held to discuss and develop our short, medium and long term goals and the resources that we can use to achieve them. This retreat was coordinated by PhD student Ms. Jacqueline Andrew.

  Specifically, team leaders from each of the distinctive research groups presented their plans for the future, covering goals for the Short term, End of contract and Beyond 2007.

  Time was also dedicated to discussing the resources we have to help us achieve our goals, including personnel, equipment and budget.

- Dr Patricia Hurley submitted her PhD thesis. A copy of her thesis abstract is attached (Appendix B).

- Miss Ricki Minter completed the Honours year of her Bachelor of Science degree. A copy of her thesis abstract is attached (Appendix C).

5. Plans for next quarter

Plans for the following quarter include:

a) Continued manuscript writing and submission, and preparation for attending conferences.

b) Analysis of data from the Chronic electrical stimulation and neurotrophin delivery in the guinea pig study.

c) Analysis of data from the deafened, chronically stimulated cats, including acute electrophysiological data.

d) Commence preliminary training and testing of a group of deafened and implanted rats in the T-maze.

e) Continued fabrication of electrode assemblies for use in our chronic stimulation studies.

f) Test methods of encapsulating Schwann cells *in vitro*, in preparation for *in vivo* transplantation studies.

g) Refinement of the protocols for the transfection and selection for stable transformants of the male Schwann cells.

h) Continues investigation of the short- and long-term effects of deafness on neuronal and trophic markers in the cochlea neurons.

i) Immunohistochemical analyses and cell quantification will be performed on tissues from the stem cell/hydrogel pilot study.

6. Personnel

Dr Steven Backhouse has joined the group as a TWJ (Thomas Wickham-Jones) Otology Fellow, which is a 12-month position in clinical otology and research. The TWJ Foundation is an educational charitable trust whose aim is to support the relief of deafness through awarding research and educational grants to otolaryngologists and other associated health professionals working in the (United Kingdom) National Health Service.

Dr Backhouse will be involved in our cell-based therapies studies, with the specific aim of developing appropriate surgical approaches for the delivery of cells into the mammalian cochlea. Three clinically viable approaches to access SGNs and the auditory nerve for cell-based therapies will be investigated. These are:

(i) Scala tympani via round window
(ii) Rosenthal's canal of cochlear basal turn
(iii) Auditory nerve via translabyrinthine approach

Outcomes from this work will hold high significance for current and future cell-based therapy experimentation in the cochlea of deafened animals.

7. Acknowledgements

We gratefully acknowledge the important contributions made by our Histologist, Maria Clarke; Veterinarian Dr Sue Peirce; Elisa Borg for management of our animal house; Helen Feng for electrode manufacture; Frank Nielsen for engineering support; Prof. Trevor Kilpatrick and Dr. Simon Murray from the Howard Florey Institute for their collaboration in obtaining Schwann cells, Dr Volkmar Lessmann from the Johannes Gutenberg Universitat, Mainz, Germany for generously providing the expression vectors and Dr. Tony Paolini from La Trobe University for advice in using the rat test chamber.

8. References

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9. Appendix A (attached)

Coleman, B., Hardman, J., Coco, A., Epp, S., de Silva, M., Crook, J. and Shepherd, R.K. Fate of embryonic stem cells transplanted into the deafened mammalian cochlea. J. Cell Transplantation (in press).

10. Appendix B (attached)

Hurley, P.A. Molecular changes to the auditory nerve following sensorineural hearing loss. Ph.D. abstract; The University of Melbourne.

11. Appendix C (attached)

Minter, R. Survival effects of Schwann cells modified to over-express neurotrophin-3 on spiral ganglion neurons *in vitro*. B. Sci (Honours) abstract; The University of Melbourne.