First Quarterly Progress Report

NIH-N01-DC-3-1005

Protective Effects of Patterned Electrical Stimulation on the Deafened Auditory System

R. K. Shepherd, S. B. Epp, L.C.-C. Chen, A. Coco, R. E. Millard
and D. R. F. Irvine

The Bionic Ear Institute
384 Albert Street
East Melbourne 3002,
Victoria, AUSTRALIA

October 1 – December 31, 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 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 regeneration 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 first quarter of this contract the following activities were completed:

- Hired two post-doctoral fellows as key members of staff. These new recruits will play central roles in the research to be undertaken under this contract.
- Held a day retreat with the research group and Melbourne based consultants to review the research plan and initiate our new research directions.
- Manufactured guinea pig electrode arrays with chronic delivery systems.
- Attended and presented at the 34th Neural Prosthesis Workshop in Bethesda, MD.
- Manuscript preparation: During the quarter, one manuscript was revised in light of reviewer's comments and re-submitted to *Arch. Otolaryngol.*

- Completed and gained approval for all experiments proposed in this contract with our institutional Animal Research and Ethics Committee.
- Designed and developed a prototype fully implantable small animal stimulator suitable for use in rats. This work will be described in detail in a future QPR.
- Designed and began construction of rat test chamber for behavioral evaluation of the effects of raising deafened rats with simple patterns of electrical stimulation on their subsequent ability to perceptually distinguish more complex stimulation patterns.
- Devised the surgical techniques and initiated studies of the trophic effects of chronic neurotrophin delivery on the deaf rat cochlea.
- Completed deafening and initiated a second series of chronic stimulation /neurotrophin studies in the adult guinea pig. This work is designed to test the hypothesis that trophic advantage can be maintained by electrical stimulation alone following an initial period of co-delivery of neurotrophin and electrical stimulation (see Final QPR; Contract NIH-NO1-DC-0-2109).
- Developed a revised deafening technique for use in kittens based on multiple neomycin administration delivered subcutaneously.
- Held discussions with Dr. David Ryugo, Department of Otolaryngology, Johns Hopkins University, to initiate design of our collaborative research program investigating the plastic response of the end bulb of Held following chemical deafening and chronic electrical stimulation in kittens.
- Trialed Bionic Technology Inc. and University of Michigan recording electrodes for quality of recording single and multi-unit clusters in the cat primary auditory cortex.
- Completed the final two acute electrophysiological experiments in deafened control cats recording single and multi-unit activity from the inferior colliculus in order to examine spatial tuning curves and temporal response properties. (see Final QPR; Contract NIH-NO1-DC-0-2109). This work will now be prepared for publication.
- Continued histological analysis of cochleae and auditory brainstem structures from our neonatally deafened chronically stimulated cat series (see Final QPR; Contract NIH-NO1-DC-0-2109). This work is designed to test the hypothesis that chronic depolarization provides trophic support to SGNs following the loss of hair cells.

3. Electrical stimulation and neurotrophin administration in deafened guinea pigs

A major study associated with this contract is the investigation of the extent of protective effects of patterned electrical stimulation in association with simultaneous neurotrophin delivery to the deafened cochlea. We have documented both the trophic and functional advantages of combined electrical stimulation (ES) and neurotrophic treatment in work performed as part of our previous contract (see Final QPR, NIH-N01-DC-2109). In this report we describe the effects of this treatment on tissue response within the cochlea. Previous research has demonstrated that exogenous delivery of the neurotrophins brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) rescues SGNs following a SNHL (Ernfors et al., 1996; Gillespie et al., 2003; Miller et al., 1997; Staecker et al., 1996) Final QPR, NIH-N01-DC-2109). However, no study to

date, has examined the effects of chronic delivery of neurotrophins on non-neural tissue within the cochlea, despite the potentially important clinical implications of an untoward tissue response. In the present report we briefly present results examining the extent of fibrous tissue reaction associated with neurotrophin delivery. In future reports we will present further analysis of the effects of this treatment on non-neural tissue.

In the cochleae examined in the present study we were investigating whether the exogenous delivery of BDNF in concert with electrical stimulation (ES) provides a greater protective effect on SGNs than delivery of BDNF alone *in vivo*. The left cochlea of profoundly deafened guinea pigs was implanted with an electrode array and drug delivery system. BDNF or artificial perilymph (AP) was delivered continuously for 28 days. ES induced neural activity in two cohorts (BDNF/ES and AP/ES) while control animals received BDNF or AP without ES (BDNF/- and AP/-; Table 1). Further experimental details together with the results of SGN survival have been given in our Final QPR, NIH-N01-DC-2109.

As part of the present study, we reviewed the histological sections from the left cochleae of each treatment group for evidence of fibrous tissue growth within the scala tympani. Both normal control (NC) and deafened control (DC) cochleae were also examined for evidence of fibrous tissue growth. The extent of the tissue reaction was graded as illustrated in Table 2.

Table 1: Summary of treatment groups

Treatment group	Implant duration (days)	Chronic electrical stimulation	Contents of osmotic pump
BDNF/ES[1]	28	Yes	BDNF[2]
BDNF/-[3]	28	No	BDNF
AP/ES	28	Yes	Artificial perilymph[4]
AP/-	28	No	Artificial perilymph

Notes: [1] ES denotes chronic electrical stimulation; [2] 62.5 μg of BDNF/ml in 0.1% guinea pig albumin in 200 μl of Ringer's solution; [3]- denotes control (i.e. electrode assembly implanted but no chronic electrical stimulation); [4] 200 μl Ringer's solution.

Representative photomicrographs from the basal turn of four cochleae are illustrated in Fig. 1. We typically observed a more extensive fibrous tissue reaction in the BDNF treated cochleae compared with the AP cohorts. This reaction was usually restricted to the basal turn (turn 1) of the scala tympani in the vicinity of the electrode array. By qualitatively grading the extent of the tissue reaction (Table 2), we were able to examine the statistical significance of the tissue reaction across cohorts using two-way analysis of variance (ANOVA) in which treatment and cochlear turn (upper turn 1, UT1; lower turn 2, LT2; upper turn 2, UT2; lower turn 3, LT3; upper turn 3, UT3) were tested.

Table 2: Fibrous tissue grading for each cochlear turn

Fibrous tissue grade	Description
0	No evidence of a fibrous tissue reaction
1	Tissue response restricted to ≤ 25% of the area of the scala tympani
2	Tissue response restricted to ≤ 50% of the area of the scala tympani
3	Tissue response restricted to ≤ 75% of the area of the scala tympani
4	Tissue response restricted to > 75% of the area of the scala tympani

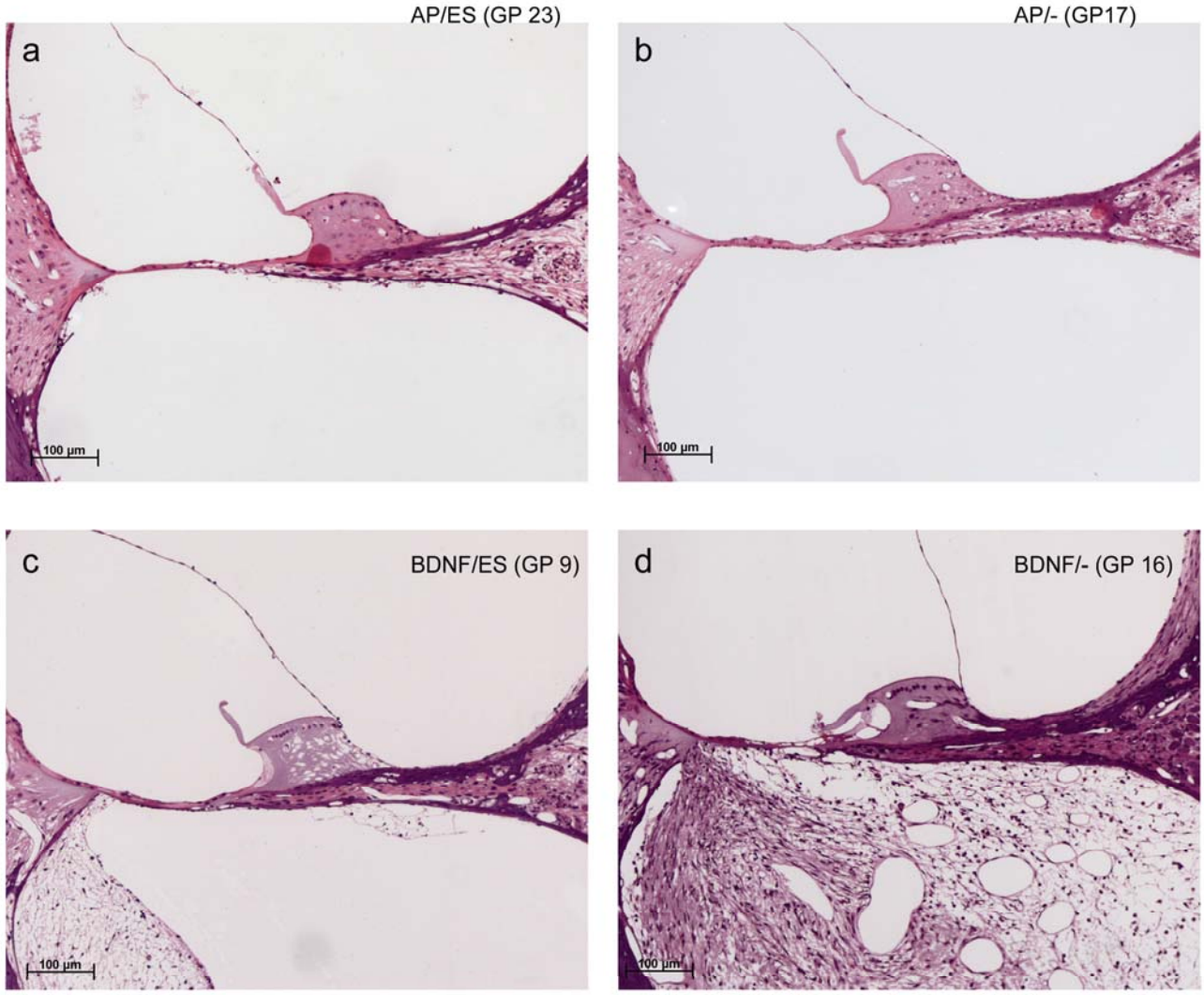


Figure 1. Representative photomicrographs illustrating the range of fibrous tissue response observed in the basal turn of deafened/implanted guinea pig cochleae. BDNF treated cochleae consistently showed a higher incidence of tissue response compared with the AP cohorts. The tissue grading in these examples were: a & b grade 0; c grade 1, d grade 4.

Statistical examination of the data showed that there was a highly significant difference across both treatment groups ($P < 0.001$) and cochlear turn ($P < 0.001$). Moreover there was a statistically significant interaction between treatment and cochlear turn ($P < 0.001$; Table 3). The *post hoc* Holm-Sidak pair wise comparison procedure revealed that BDNF/ES and BDNF/- treatment groups showed significantly greater fibrous tissue reaction when compared to AP/ES, AP/-, NC and DC groups. Finally, the same *post hoc* test revealed that this tissue reaction was restricted to the most basal turn tested (UT1). There was no significant difference across treatment groups for any turn apical to this site.

Table 3: 2-way ANOVA fibrous tissue reaction in scala tympani

Source of variation	DF	SS	F	P
Cochlear treatment	5	15.290	16.196	<0.001
Cochlear turn	4	4.942	6.544	<0.001
Treatment x turn	20	10.600	2.807	<0.001
Residual	140	26.433		
Total	169	58.124		

This work has focused on the effects of exogenous delivery of a neurotrophin on non-neural tissue within the cochlea. The results suggest both an increased incidence and extent of fibrous tissue growth within the implanted/BDNF treated scala tympani compared with implanted/AP treated or un-implanted control cochleae. It is noteworthy that the extent of tissue response was restricted to turn 1 of the guinea pig cochlea, suggesting that the tissue response may be dependent on the concentration of BDNF. It should also be noted that a more extensive tissue reaction was associated with the BDNF/ES compared with the BDNF/- cochleae, implying that ES may enhance the promotion of fibrous tissue generated by the BDNF. The source of this interaction is presently unknown.

Although the tissue response was relatively mild and localized to the scala tympani in the vicinity of the electrode array, such a response is undesirable, as it will lead to an increase in electrode impedance (Xu et al., 1997) and result in increased power consumption for cochlear implants. A vigorous tissue reaction may also reduce the efficacy of the neurotrophin being delivered to the SGNs by restricting access to the neural tissue, and may also lead to difficulties associated with the eventual replacement of an electrode array (Shepherd et al., 1995). Finally, it is possible that the tissue response observed here is a result of an immune response in the guinea pig to the recombinant human BDNF, and that the extent of tissue reaction would be much less when delivered in patients.

4. Development of new deafening techniques

We hypothesize that the trophic effects of electrical stimulation on SGNs is only evident in neurons stimulated after relatively short periods of hair cell loss; longer periods of deafness do not lead to SGN rescue via depolarization because the majority of SGNs have passed some critical point down the apoptotic pathway. As a result of the lack of trophic effects of electrical stimulation observed in our previous feline studies (Final QPR, Contract NIH-N01-DC-0-2109), we have decided to deafen our kittens - in this contract - using daily-injections of neomycin (NE) rather than the co-administration of kanamycin (KA) and ethacrynic acid (EA) we have used previously. Multiple NE administration has been successfully used by Leake and colleagues previously (Leake et al., 1988; Leake et al., 1999; Leake et al., 1992). It is possible that at the age of cochlear implantation (~7 weeks old), greater SGN loss will be evident following the rapid loss of hair cells associated with KA/EA treatment in 10-14 day old kittens, compared with the relatively slow, delayed hair cell loss that occurs in response to multiple administration of NE (Shepherd et al., 1985).

Our Animal Research and Ethics Committee required us to develop a revised deafening procedure based on a subcutaneous (sc) rather than intramuscular delivery of NE. All other aspects of this procedure followed that of Leake et al., (1999). This technique was developed using two newborn kittens. Briefly, NE (Sigma) was administered daily from post-natal day 1 (P1; no drug is given on the day of birth). NE was dissolved in sterile saline at a concentration of 30 mg/ml and administered at a dose rate of 60 mg/kg body weight sc. Throughout this procedure the animals were monitored for evidence of renal function and their weight and general health was recorded daily (Fig. 2).

Hearing status was assessed by recording click-evoked Auditory Brainstem Responses (ABRs) at P16-18 (Figs 3-4). Kittens with no evidence of an ABR to a 98 dB peak equivalent SPL click would receive no further NE administration. Neomycin administration was continued in animals with a recordable ABR and their hearing was again tested at three-four day intervals until they were profoundly deaf.

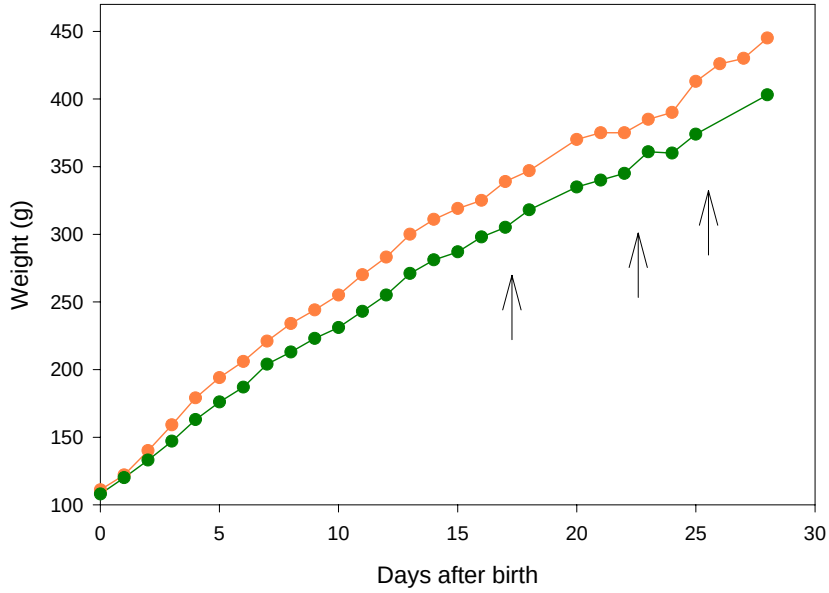


Figure 2. The weight of each kitten recorded daily over the first 28 days of life. NE administration commenced at P1 and continued daily until P25 (kitten 03_925; green) or P28 (kitten 03_924; orange). Both kittens continued to gain weight over the period of NE administration. Note that the arrows indicate dates when ABRs were performed.

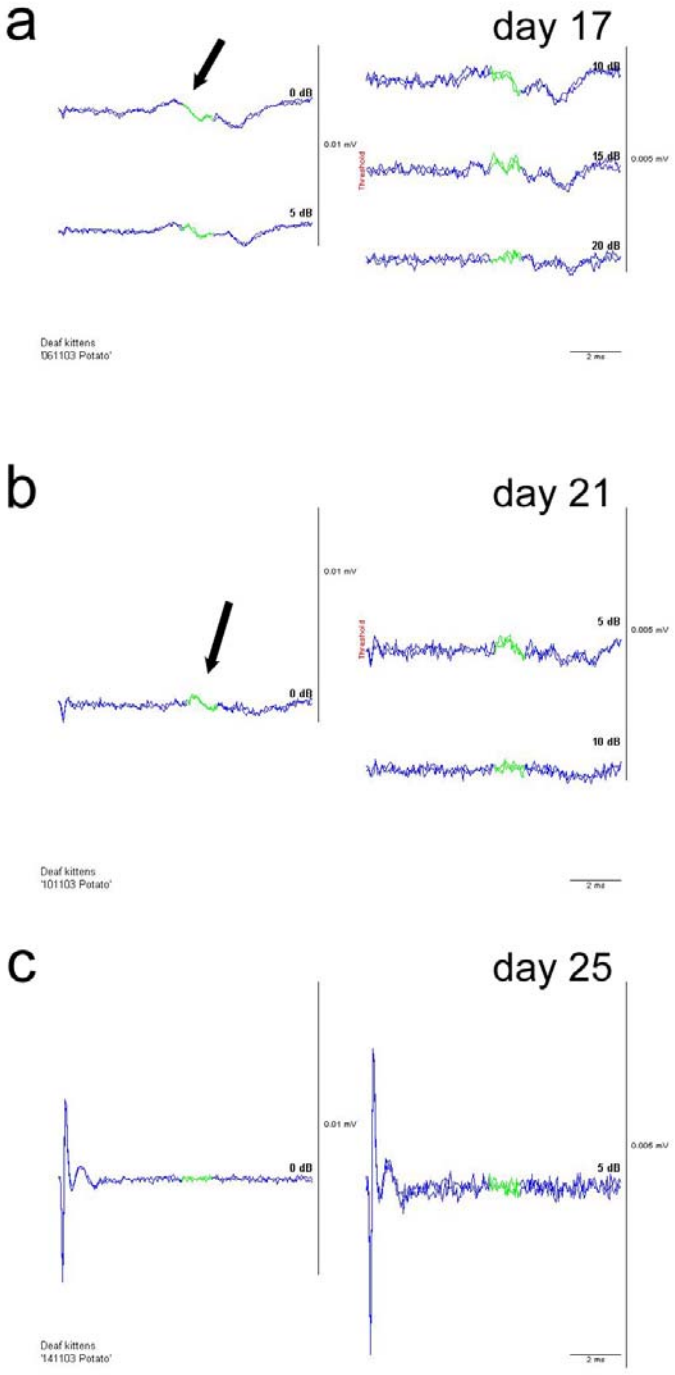


Figure 3. Click-evoked ABRs recorded from kitten 03_925. Wave IV is indicated by the arrow. Stimulus intensity is given in dB attenuation (dBA; re a 98 dB peak equivalent SPL click). At day 17 (a) clear ABR was evident to a 98 dB click (0 dBA; arrow). A response was also evident at intensities of up to 20 dBA below that. The daily administration of NE was maintained. By 21 days of age (b) the amplitude of the response at 98 dB (0 dBA) was significantly smaller, and threshold was 93 dB (5 dBA). Again, the daily administration of NE was continued. By P25 there was no evidence of an ABR at 98 dB (0 dBA; C), and the administration of NE ceased. The large response in the first ms of this figure is a stimulus artifact.

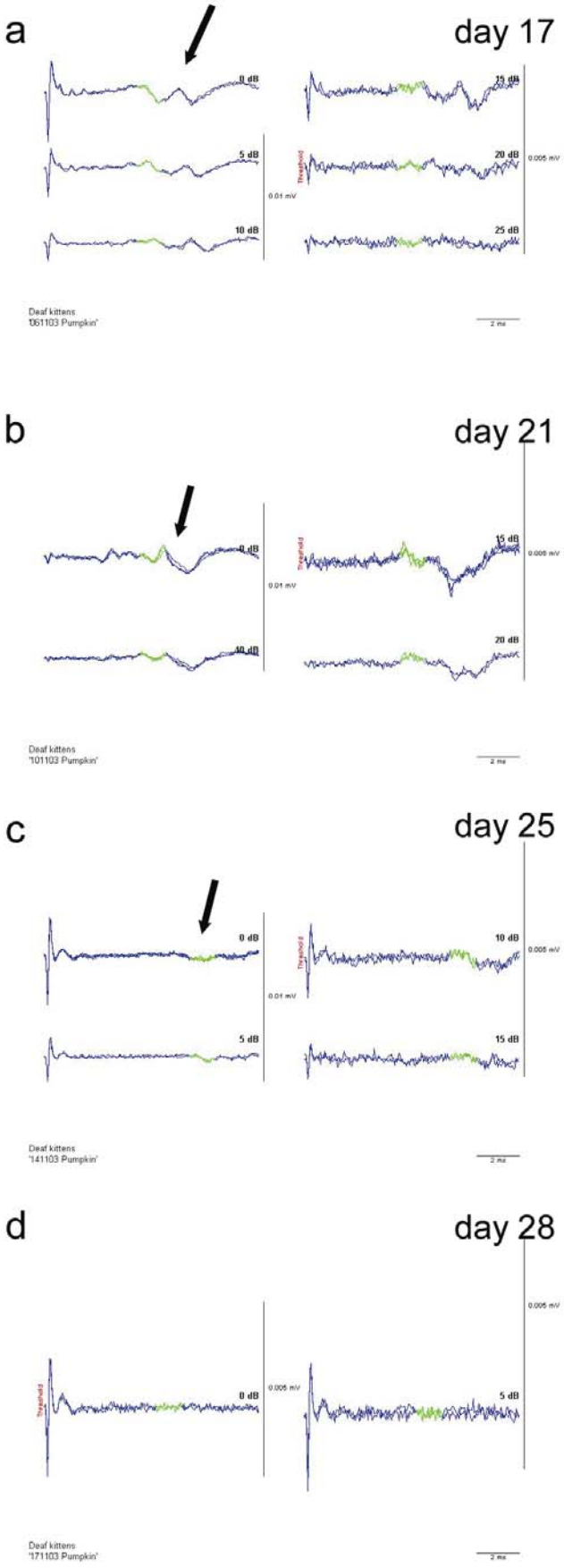


Figure 4. Click-evoked ABRs recorded from kitten 03_924. Wave IV is indicated by the arrow. Stimulus intensity is given in dB attenuation (dBA; re a 98 dB peak equivalent SPL click). At P17 a clear ABR was evident to a 98 dB click (arrow). A response was also evident at intensities of up to 20 dBA below that. The daily administration of NE was maintained. At 21 days of age (b) the amplitude of the response at 98 dB (0 dBA) was smaller, but the threshold was similar (15-20 dBA). Again, the daily administration of NE continued. At P25 the response amplitude had decreased significantly (c) but was still apparent at 88 dB (10 dBA). The daily NE administration was continued. By P28 there was no evidence of an ABR at 98 dB (0 dBA; d), and the administration of NE ceased. The large response in the first ms of this figure is a stimulus artifact.

This work has demonstrated that we can successfully deafen kittens using the technique of Leake et al., (1999) while delivering the aminoglycoside subcutaneously rather than intramuscularly. The animals were healthy, exhibited continued weight gain, and showed no clinical evidence of renal impairment. This technique will be adopted as our standard feline deafening protocol in this contract.

5. Study of the plastic response of the central auditory pathway following chronic electrical stimulation in neonatally deafened cats

This research, which will form a major effort within the present contract, is designed to examine plastic changes within the primary auditory cortex (AI) of neonatally deafened kittens. Specifically, the objective of these experiments is to examine the spatiotemporal reorganization of the AI in response to afferent input via localized electrical stimulation of the AN and to determine the extent to which AI can undergo further plastic change in response to a shift from two-channel simultaneous to two-channel non-simultaneous AN stimulation.

Most previous studies investigating the effects of chronic stimulation on central auditory pathway reorganization have focused on the inferior colliculus. This is largely for the practical reason that the spatial organization of this nucleus can be determined in individual penetrations through the nucleus and only one or two penetrations have to be made in an individual animal to obtain the required data. In contrast, conventional recording of the spatial organization of the AI has required penetrations into the middle cortical layers at multiple recording sites (Fig. 5), and is consequently very labor intensive: approximately 40-50 penetrations are needed to cover in reasonable grain the full extent of the cat AI (which lies on the middle ectosylvian gyrus [MEG]) between the anterior and posterior ectosylvian sulci [AES and PES], and on the rostral bank of PES.

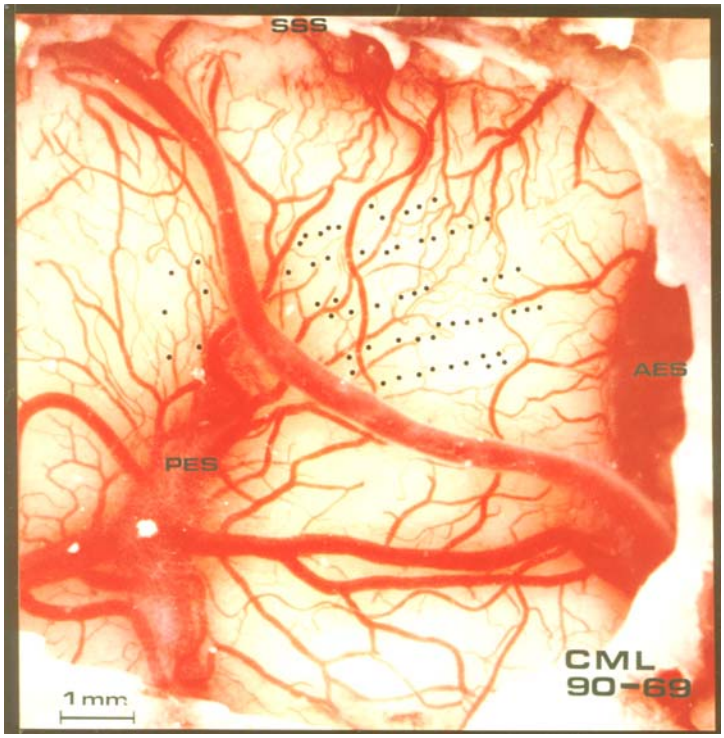


Figure 5. Photograph of cortical surface, showing positions of microelectrode penetrations (black dots) into the right AI of the cat. AES, anterior ectosylvian sulcus; PES, posterior ectosylvian sulcus; SSS, suprasylvian sulcus. (from D. Irvine).

The practical advantage of IC recording is offset by some disadvantages. Not only are some forms of plasticity less apparent in IC than in AI (Irvine et al., 2003; Rajan et al., 1993), but activity in AI is much more closely related to perceptual experience than is activity in IC and thus provides a more secure basis from which to draw conclusions about the relationship between neural and behavioral/perceptual changes.

It is for these reasons that we propose to examine the plastic effects of chronic electrical stimulation of the AN at the level of the AI. The practical difficulty of recording at multiple sites will be overcome by using a microelectrode array assembly manufactured by Bionic Technologies LLC (Fig. 6) together with a 16 Channel single shank recording electrode manufactured by the Center for Neural Communication Technology, University of Michigan (http://www.engin.umich.edu/facility/cnct/index.html). It is envisaged that the Bionic Technology array will cover much of AI on the surface of MEG, while the Michigan probe will be used to record from low-frequency AI sites in the rostral bank of PES.

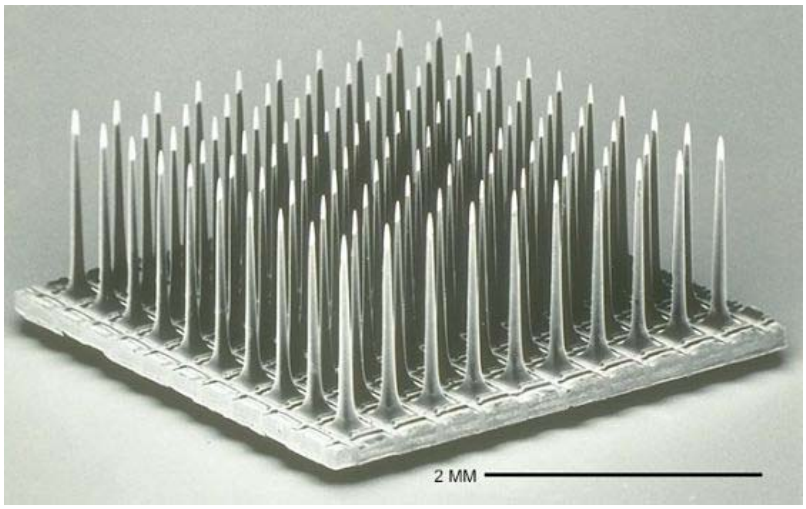


Figure 6. An example of the Bionic Technologies acute electrode array we propose to use to record from the AI.
http://www.cyberkineticsinc.com/

During the present quarter we assessed prototype Bionic Technology and University of Michigan recording electrodes in cat AI. Briefly, two profoundly deafened cats were used in two-day acute experiments in which the inferior colliculus (IC) was mapped as part of another study (see 6th QPR Contract NIH-N01-DC-0-2109). Prior to exposing the IC the AI was exposed to enable electrically-evoked single- and multi-unit activity to be recorded. Anesthetic and surgical procedures have been described previously (Shepherd et al., 1999). The recording electrodes were mounted on a micromanipulator (Narishige) and advanced into the AI in 10 μm steps at an angle of approximately 90^{o} to the cortical surface. The electrical stimulus consisted of 100 μs per phase charge balanced biphasic current pulses with variable current amplitude to 4 mA, delivered to a bipolar electrode array located in the contralateral scala tympani. The unit activity was amplified and band pass-filtered (typically 150 Hz-3 kHz) before being displayed on an oscilloscope. The stimulus artifact was eliminated using a sample-and-hold circuit placed prior to the filter in order to minimize ringing. The output of the filter was fed to a MacLab and spike waveforms were

digitized using Chart at a sample rate of 40 kHz. Examples are illustrated in Figs. 7 & 8.

The three single Bionic Technology sample electrodes that were used in these experiments to record from the gyral surface had impedances of 0.24 to 0.59 MΩ. The Michigan probe used to record from the bank of PES in these experiments had impedances at different recoding sites in the range 1.2 to 2.5 MΩ.

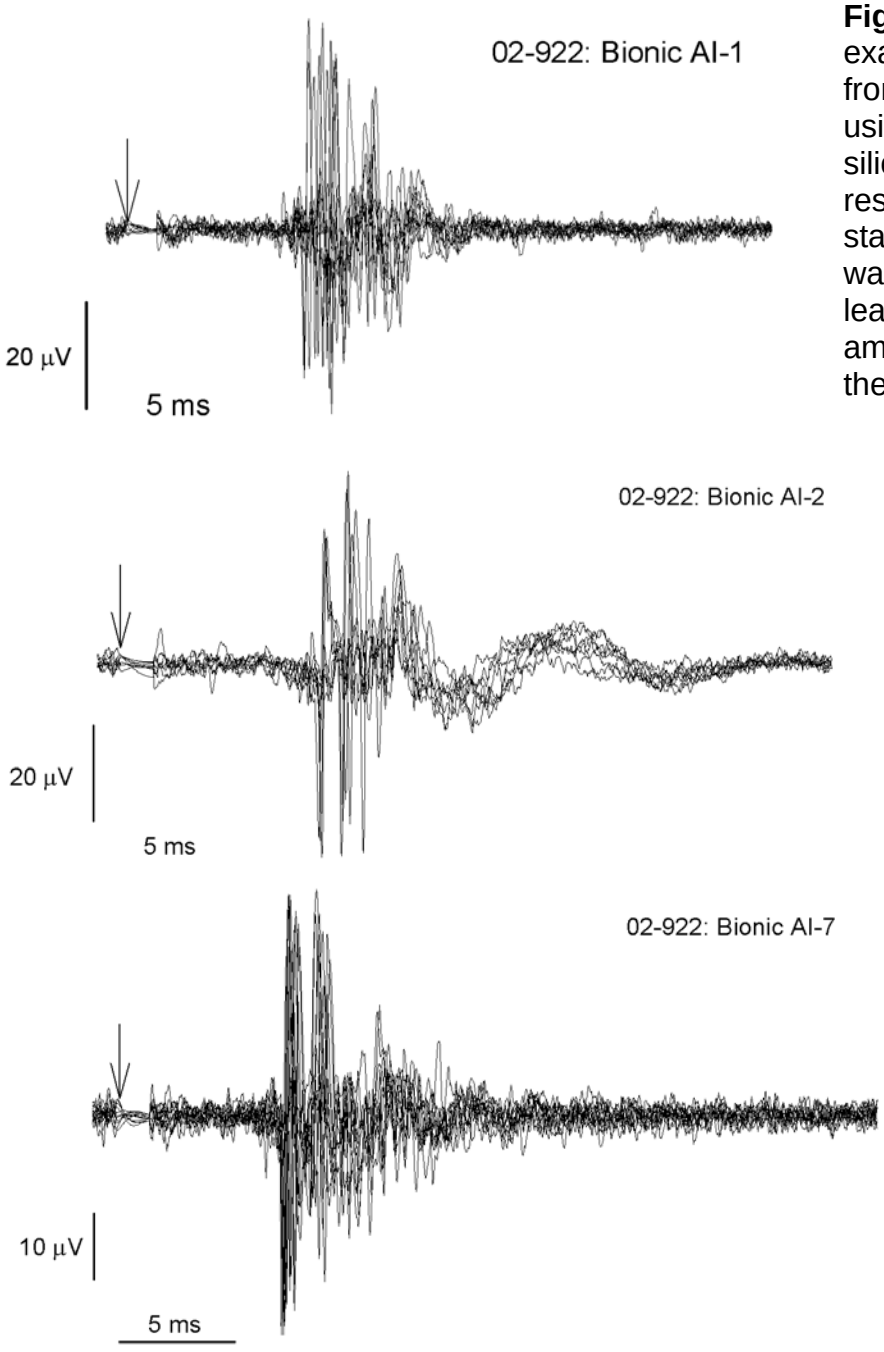
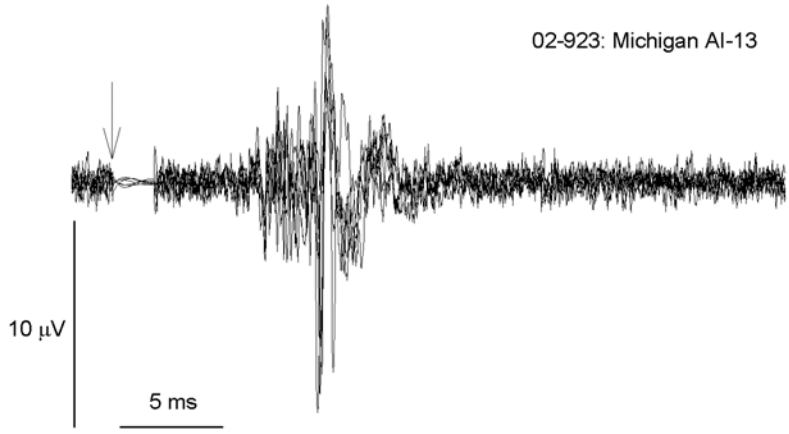


Figure 7. Representative examples of AI recordings from the anaesthetized cat using a Bionic Technology silicone electrode. The responses were very stable (single unit activity was held for periods of at least 45 minutes), spike amplitude was well above the noise floor and readily discriminable.



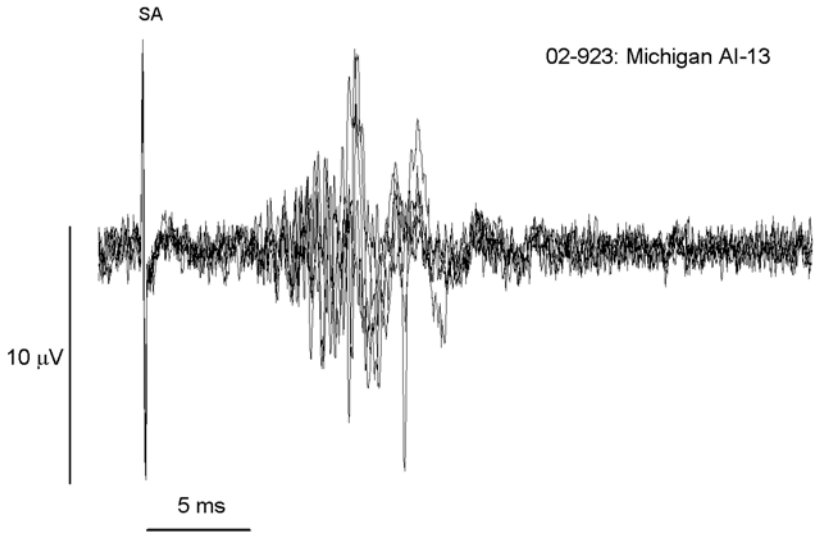


Figure 8. Representative examples of AI recordings from the anaesthetized cat using a Michigan probe. The responses were slightly smaller that the Bionic Technology electrodes but again were stable and the spike amplitude was well above the noise floor.

We conclude that each of the electrodes gave high-quality multi-unit recordings with signal:noise ratios in the order of 10 or better, and with single unit spikes that could readily be separated using spike-sorting software. Importantly, the Bionic Technology electrodes produced quality recordings over a wide range in depth of the cat AI (400-2100 μm) – this is an important feature as these electrodes only have one recording site per silicone probe. Finally both recording electrodes provided stable recordings for periods in excess of 45 minutes. Given the present results we intend to use both recording electrodes to study the plasticity of the AI in neonatally deafened, chronically stimulated kittens.

6. Plans for Next Quarter

- Continue manuscript preparation and submission.
- Host the Inaugural Australasian Auditory Neuroscience Workshop.
- Complete the *in vivo* phase of our chronic ES and neurotrophin delivery study in guinea pigs where animals receive ES and BDNF for 28 days and then ES alone for periods of up to 10 weeks. The cochlear histology of these animals should be completed next quarter.
- Commence chronic delivery of BDNF to the deafened rat cochlea in order to test the hypothesis that this neurotrophin can rescue SGNs in rat when delivered exogenously.
- Initiate research involving the delivery of partially differentiated stem cells into the deaf guinea pig cochlea as the first phase of our SGN regeneration studies.
- Continue to deafen newborn kittens in preparation for our first chronic ES study in deafened kittens for this contract.
- Trial a prototype implantable stimulator and develop an electrode array and surgical protocol for chronic cochlear implantation in the rat.
- Complete and trial the rat behavioral test box using normal hearing subjects in response to acoustic stimulation.
- Purchase acute recording electrodes and inserter from Bionic Technology Inc.
- Obtain experience with AI recordings in rats using the Bionic Technologies and University of Michigan electrodes. We have considerable previous experience in recording from AI in other species (cats, guinea pigs, marmosets), but have not previously recorded from cortex in the rat.
- Complete the search process for our third post-doctoral fellow (molecular biology).

7. Personnel

In addition to the staff that worked full or part-time on our previous contract, we have recruited a number of new personnel to the present contract.

Prof. Dexter Irvine

Dr Irvine holds a Doctor of Philosophy degree, and currently has a half-time appointment as Professor in the Department of Psychology at Monash University. He has over thirty years experience in auditory neuroscience, and has published a monograph on auditory brainstem processing mechanisms in addition to 90 peer-reviewed journal articles and book chapters. Almost a third of his publications deal with the response characteristics and functional organization of auditory cortex, and his work over the last decade has been focused on auditory cortical plasticity as a consequence of partial hearing loss and its functional consequences. Dr. Irvine will spend 33% of his time working on this contract. His research will be directed towards cortical plasticity studies in deafened electrically stimulated kittens, and behavioral/electrophysiological studies in deafened electrically stimulated rats.

Dr. James Fallon

Dr. Fallon obtained his PhD from Monash University, from where he also holds degrees in Electrical Engineering and Physiology. He has 3 years experience working in neurophysiological research ranging from animal studies to microneurography, including 6 months with Dr. Shepherd as part of NIH contract NIH-N01-DC-0-2109 (2000-2003). He is currently based at the Prince of Wales Medical Research Institute where he his studying single motor units and cutaneous and muscle afferents in humans using microneurographic techniques. He has published 5 peer reviewed papers and book chapters, and 3 NIH Quarterly Progress Reports. Dr. Fallon will take up a full-time appointment in August as a Research Fellow and play a leadership role in electrophysiological studies performed in this contract.

Ms. Lisa Gillespie

Ms. Gillespie has a Bachelor of Science degree in Anatomy. She completed her Honours studying spinal motor neurons *in vitro* at Monash University. Lisa recently submitted her PhD, in which she investigated various aspects of neurotrophin survival effects on the deaf cochlea. Specific features of her studies have included: identification of neurotrophic factors capable of stimulating axonal growth from auditory neurons *in vitro*; the longevity of the survival effects of BDNF *in vivo*; effects of delayed neurotrophin treatment following deafening *in vivo*; and identification of axonal guidance molecules present within the cochlea. Prior to commencing her post-graduate studies, Ms. Gillespie was employed as a Research Assistant with the Bionic Ear Institute. She now has 6 years of experience studying the actions of neurotrophic factors within the ear, and is proficient in cell culture techniques, as well as deafening and surgical implantation procedures. Ms. Gillespie has published four papers and has presented results at national and international conferences since 1999. Ms. Gillespie will take up a full-time appointment as a Post-doctoral Research Fellow in March, and will play a key role in our *in vivo* neurotrophin studies.

Ms. Bryony Coleman

Bryony Coleman has a Bachelor of Science degree with a major in biochemistry and molecular biology. She completed her Honours in Neurobiology at the Clinical School, University of Tasmania. She is experienced in neuronal cell culture, immunohistochemical techniques, confocal microscopy and basic molecular biology. Bryony is currently undertaking her PhD in our laboratory investigating SGN regenerative techniques in the deafened cochlea via the application of stem cells.

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; and Frank Nielsen for engineering support.

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