Evans AJ, Thompson BC, Wallace GG, Millard R, O'Leary SJ, Clark GM, Shepherd RK & Richardson RT (2008). Promoting neurite outgrowth from spiral ganglion neuron explants using polypyrrole/BDNF-coated electrodes. Journal of Biomedical Materials Research Part A 91 (1):241-50.

http://onlinelibrary.wiley.com/doi/10.1002/jbm.a.32228/abstract;jsessionid=302E7DF521E9412DBAAF6F50513D1B56.f02t04?deniedAccessCustomisedMessage=&userIsAuthenticated=false

Promoting Neurite Outgrowth from Spiral Ganglion Neuron Explants using Polypyrrole/BDNF-Coated Electrodes

ALISON J. EVANS[1,3], BRIANNA C. THOMPSON[2], GORDON G. WALLACE[2], RODNEY MILLARD[1,3], STEPHEN J. O'LEARY[1,3] GRAEME M. CLARK[1,2,3], ROBERT K. SHEPHERD[1,3] AND RACHAEL T. RICHARDSON[1,3]

1. The University of Melbourne, Department of Otolaryngology
2. Intelligent Polymer Research Institute, ARC Centre of Excellence for Electromaterials Science, University of Wollongong
3. The Bionic Ear Institute

Corresponding author: Rachael Richardson

The Bionic Ear Institute

384 Albert Street

East Melbourne,

Victoria, 3002

Australia

rrichardson@bionicear.org Tel: +613 9667 7500 Fax: +613 9663 1958

ABSTRACT

Release of neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF) from hair cells in the cochlea is essential for the survival of spiral ganglion neurons (SGNs). Loss of hair cells associated with a sensorineural hearing loss therefore results in degeneration of SGNs, potentially reducing the performance of a cochlear implant. Exogenous replacement of either or both neurotrophins protects SGNs from degeneration after deafness. We previously incorporated NT3 into the conducting polymer polypyrrole (Ppy) synthesized with *para*-toluene sulfonate (pTS) to investigate whether Ppy/pTS/NT3-coated cochlear implant electrodes could provide both neurotrophic support and electrical stimulation for SGNs. Enhanced and controlled release of NT3 was achieved when Ppy/pTS/NT3-coated electrodes were subjected to electrical stimulation. Here we describe the release dynamics and biological properties of Ppy/pTS with incorporated BDNF. Release studies demonstrated slow passive diffusion of BDNF from Ppy/pTS/BDNF, with electrical stimulation significantly enhancing BDNF release over seven days. A three-day SGN explant assay found neurite outgrowth from explants was 12.3-fold greater when polymers contained BDNF ($p<0.001$), although electrical stimulation did not increase neurite outgrowth further. The versatility of Ppy to store and release neurotrophins, conduct electrical charge and act as a substrate for nerve-electrode interactions is discussed for specialized applications such as cochlear implants.

KEYWORDS

Brain derived neurotrophic factor, polypyrrole, cochlear implant, electrical stimulation, sensorineural hearing loss, spiral ganglion neuron

INTRODUCTION

A recent worldwide survey estimated that 278 million people have a disabling hearing impairment (World Health Organisation, 2005). Deafness that results from loss of or damage to sensory hair cells and/or spiral ganglion neurons (SGNs) is defined as a sensorineural hearing loss. When hearing loss is severe or profound, conventional hearing aids provide no benefit and a cochlear implant is required to restore speech perception. Cochlear implant electrodes are implanted into the cochlea in a fluid-filled cavity known as the scala tympani positioning the electrodes close to the SGNs. Electrical stimulation via these electrodes initiates depolarization in proximal SGNs thereby evoking auditory perception. It is therefore important for SGNs to be well preserved for cochlear implant function. However, SGNs gradually degenerate after a sensorineural hearing loss due to the loss of neurotrophic support normally provided by hair cells. Application of exogenous neurotrophins such as neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF) preserves SGNs after hearing loss in experimental animals[1-3]. In addition to neural preservation, the peripheral dendrites of SGNs exhibit significant resprouting in response to neurotrophin treatment[4], leading to the question of whether these *de novo* dendrites can be brought closer to, or be in direct contact with, the implanted electrodes. This paper assesses whether an electroactive polymer (EAP), polypyrrole (Ppy) with incorporated BDNF, is a suitable electrode coating to provide a combination of electrical stimulation, neurotrophic support and a growth substrate for SGN dendrites.

Conducting EAPs such as polyaniline, polythiophene, polyacetylenes and Ppy are made up of a series of repeating monomers which have a conjugated backbone structure along

the polymer chains. Biologically relevant molecules can be integrated into the structure of some EAPs and these can be slowly released via diffusion or using electrical stimulation for enhanced and controlled release[5-7]. If the incorporated molecule is required to remain permanently within the polymer, it can be tethered via covalent bonds as demonstrated previously for nerve growth factor (NGF)[8]. Ppy is of particular interest for cochlear implants as a slow-release EAP due to the ability to enhance and control the release of incorporated neurotrophins using biologically relevant electrical stimulation[9].

The physical properties of Ppy are reliant on its redox state and are heavily influenced by the negatively-charged dopant used for synthesis[10]. For the work presented here, Ppy was synthesized with *para*-toluene sulfonate (pTS) as the dopant. Current research suggests that Ppy doped with pTS is a good candidate for bioapplications involving the electrical stimulation of Ppy because pTS produces strong and robust polymers, and the structural and mechanical integrity of the polymers are maintained throughout stimulation[6,9]. Previous work has shown that a range of molecules as diverse as antibodies, enzymes, DNA, growth factors and whole living cells can be incorporated into Ppy[11]. When reduced, the incorporated molecules can exit the polymer, a useful process for many applications[12-16]. However, each incorporated molecule needs to be tested individually for its uptake and release dynamics.

Several EAPs have been extensively studied for biocompatibility, cell adhesion and properties for use as biomedical or controlled drug-release devices. Ppy has traditionally been preferred for bioapplications as it is considered to be a stable and biocompatible

EAP. Ppy-coated electrodes or materials that were implanted either subcutaneously or in the brain or injected into the bloodstream did not evoke any more cellular reactions or inflammatory responses than control materials in rats, mice, rabbits and guinea pigs[17-20]. Many studies have analysed the responses of neurons to Ppy. *In vitro*, Schmidt and colleagues showed that an electrically stimulated Ppy substrate could aid in bridging the gap between two ends of a severed peripheral nerve explant by promoting significant increases in neurite lengths from the explant[21]. Similarly, stimulated Ppy was found to enhance neurite outgrowth in the PC-12 neuronal cell line[22]. A study in which Ppy was implanted into the sciatic nerve *in vivo* also reported nerve extension[23]. Furthermore, Ppy/pTS with incorporated NT3 has been shown to slowly release NT3 over a period of at least seven days, with enhanced release from Ppy/pTS/NT3 with electrical stimulation[6]. The released NT3 was shown to be bioactive as SGN explants growing on Ppy/pTS/NT3 displayed greater neurite outgrowth compared to explants grown on polymers without NT3 or unstimulated polymers[9].

This paper extends this research to another neurotrophin shown to preserve SGNs in the cochlea after deafness: BDNF. In the cochlea, both NT3 and BDNF are necessary for appropriate neuronal growth during development and for maintaining neuronal survival in adults. However, there are subtle differences in the spatio-temporal expression patterns of the neurotrophins and their receptors (TrkB receptor for BDNF and TrkC receptor for NT3)[24]. During development, BDNF is more critical for SGN survival in apical turns of the cochlea, while NT3 is more critical for basal turn SGN survival[25-27]. Interestingly, this phenotype was found to be reversed in adult animals, with basal turn SGNs being most

susceptible to loss of BDNF[28]. Since cochlear implants are inserted into the basal turn, an EAP electrode coating that releases BDNF would primarily influence the basal SGNs which have been shown to express TrkB receptors and to be responsive to BDNF.

In this report, Ppy was synthesized with pTS as a dopant, with and without BDNF, on gold electrodes (Ppy/pTS and Ppy/pTS/BDNF). Release of BDNF from Ppy/pTS/BDNF was examined in the presence or absence of applied electrical stimulation in the form of clinically-relevant biphasic current pulses. SGN explants were grown on Ppy/pTS or Ppy/pTS/BDNF in the presence or absence of electrical stimulation to examine SGN survival and neurite outgrowth. Compared to previous studies, much longer periods of electrical stimulation were used to emulate cochlear implant usage. The results are discussed in relation to cochlear implant applications.

MATERIALS AND METHODS

Polymer synthesis and plate preparation

Disposable 8-well Electric Cell-Substrate Impedance Sensing (ECIS) culture slides were used for polymer growth and explant culture (Applied BioPhysics). Each well contained a central circular 0.05 mm^2 sputter-coated gold working electrode and a 21.8 mm^2 common gold return electrode. The area of the working electrode was increased by removing the insulating layer with a short incubation in 20% NaOH followed by multiple rinses in Milli-Q water. The new working electrode size became 18 mm^2 in 6 of the wells and 21.3 mm^2 in 2 of the wells, the difference arising from the shape of the lead wire (Fig 1a).

In sterile conditions, Ppy polymerisation was carried out using an aqueous solution of 0.2 M distilled pyrrole (Merck), 0.05 M analytical grade pTS (Aldrich) and where specified, 2 μg/ml BDNF (Peprotech). A two-layered polymerisation approach was used in which Ppy/pTS was polymerised galvanostatically at a current density of 2 mA/cm^2 for 90 s initially, using the central gold electrode as the working electrode and a stainless steel mesh electrode placed in each well as a counter electrode. For polymers containing BDNF, 2 μg/ml BDNF was then added to the polymerisation mix before a further 60 min growth for all polymers. After polymerisation, wells were washed five times with 500 μl sterile Milli-Q water and stored at room temperature in sterile Milli-Q water until use. Polymers were used approximately 4 days after synthesis in most cases.

Neural explant culture

Polymer-coated ECIS slides or equivalent 8-well plastic culture slides (Lab-Tek, Nunc) were coated with cell adhesion molecules (CAMs) by incubating in 0.5 mg/ml poly-ornithine (Sigma; diluted in 0.15 M borate buffer pH 8.56) at room temperature overnight. On the day of culture, the poly-ornithine was removed and the culture slides washed three times with Hepes-buffered Eagle's media (HEM) containing penicillin and streptomycin. Culture slides were then incubated in 0.01 mg/ml laminin (Gibco; diluted in HEM) at 37^{o}C for at least 2 hr. Prior to plating of explants, the laminin was removed, the culture slides were rinsed once in HEM and then filled with 300 μl Dulbecco's modified Eagle's media supplemented with 25 mM Hepes buffer, N2 supplement (Invitrogen), 6 mg/ml glucose and antibiotics (Sigma) (DMEM/N2).

Time-mated Albino-Wistar rats were purchased from the University of Adelaide. Rat pups were used at post-natal days 4-6. National Institutes of Health (NIH) guidelines for the care and use of laboratory animals were observed (NIH Publication #85-23 Rev. 1985). The Animal Research Ethics Committee of the Royal Victorian Eye and Ear Hospital approved the care and use of the animals in this study (ethics #06/131A).

SGN explants were isolated from the upper basal turn of the rat pup cochleae as described previously[9] and placed in the prepared eight-well culture slides. Where indicated, BDNF (Peprotech; prepared as a stock solution in phosphate buffered saline (PBS) containing 1% BSA and then diluted to a working concentration in DMEM/N2) was added to the cultures at 0-100 ng/ml (in duplicate or quadruplicate) to make a final culture volume of 350 μl. The 0 ng/ml BDNF control consisted of PBS/1% BSA diluted exactly as per BDNF in DMEM/N2. Cultures were placed in a 37^{o}C humidified incubator containing 5% CO_2 for 3 days prior to fixing and staining.

Electrical stimulation

Explants were grown on the Ppy and CAM-coated gold electrodes in ECIS culture slides for 16 hr to allow settling and adhesion prior to stimulation. Culture slides were placed in an array holder (Applied BioPhysics) and connected to a pulse generator and oscilloscope[29]. Electrical connections were tested before the arrays were connected to stimulators designed to deliver biphasic current pulses to cochlear implants in experimental animals (backpack stimulators)[30]. Charged-balanced biphasic current pulses at 250 Hz were applied for three periods of 8 hr, with a 16 hr rest period between stimuli.

The waveform had ±0.2 mA current amplitude, 100 μs pulse width, 25 μs open-circuit interphase gap and 3.78 ms short-circuit phase between pulses (Fig 1b), resulting in a charge density of 0.1 $\mu C/cm^2$ per electrode. The shorting between current pulses reduces the build-up of direct current; a strategy employed clinically by cochlear implant stimulators[31].

Immunocytochemistry and neural analysis

Following the stimulation regime or standard culture, the DMEM/N2 was removed and explants were fixed in 100% ice-cold methanol for 10 min. One rinse in PBS was applied and blocking buffer (PBS/3% foetal bovine serum) was added for at least 1 hr at room temperature. Blocking buffer was then replaced with anti-neurofilament 200 kD primary antibody (Chemicon) diluted 1:2000 in blocking buffer. Incubation was for 1 hr at room temperature. Explants were washed 3 times with PBS and incubated at room temperature for 1 hr in Alexafluor 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Molecular Probes), diluted 1:400 in blocking buffer. Explants were rinsed 3 times in PBS. An anti-fade mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was applied and the explants viewed and photographed via fluorescent microscopy on a Zeiss Axioplan II fluorescent microscope.

The area of the central mass of tissue of all explants was measured using AxioVision software version 4.5 and compared across all groups. The number of NF200-labelled neurites sprouting from the explants was counted at the point at which they exited the

explant. Explants without any visible neurites were excluded from the study to ensure that only tissue able to grow neurites was dissected.

Scanning electron microscopy (SEM)

Explants grown on Ppy/pTS or Ppy/pTS/BDNF were fixed in 4% (w/v) paraformaldehyde for 15 min and then exposed to increasing concentrations of ethanol for 30 min each (30%, 50%, 70%, 90% and 100%). Samples were placed in a Balzers CPD 030 critical point dryer before being sputter-coated in gold (Edwards S150B gold sputter coater). Images were taken on a Philips XL30 FEG Field Emission Scanning Electron Microscope at the School of Botany, University of Melbourne, Australia.

^{125}I BDNF-based release experiments

Ppy/pTS/^{125}I BDNF polymers were synthesised on strips of gold-coated mylar sheets and coated with CAMs as described earlier. The polymers were rinsed several times in MilliQ water and placed in a gamma counter for initial readings. The polymers were then placed in wells and incubated in 0.9% NaCl for 7 days at room temperature. Electrical stimulation (see Fig 1b) was applied continually to half of the electrodes for the entire period using a stainless steel mesh counter electrode. The NaCl was collected from each well on each day and replaced with new saline. At the end of 7 days, all NaCl and polymer samples were read in a gamma counter to assay the amount of ^{125}I BDNF present in the sample.

ELISA-based release experiments

Ppy/pTS/±BDNF was synthesised in ECIS culture slides, coated with CAMs as described and stored in MilliQ water for 4 days before starting the release assay. Wells were filled with 200 µL 0.9% NaCl and the slides placed at 37^{o}C for 16 hr. Half of the polymers were stimulated with charge-balanced biphasic pulses at 250 Hz using the waveform described (see Fig 1b) for 3 periods of 8 hr followed by 16 hr rest periods, as per the stimulation regime used in explant cultures. Saline samples were collected daily for 3 days, and stored at -20°C until all were collected.

BDNF was quantified using the BDNF E_{max} Immunoassay System (Promega) as per the manufacturer's instructions. 125 µl of the thawed samples were assayed alongside duplicate series of BDNF standards. BDNF concentration was determined by measurement of absorbance at 450nm with a Bio-Rad 550 spectrophotometric microplate reader (Hercules).

Outcome measures and statistical analyses

All data was tested for normal distribution using the Kolmogorov-Smirnov method and constant variance using a Spearman-Rank correlation between the absolute values of the residuals and the observed value of the dependent variable respectively. Confidence for all data analyses was set at 95% ($p<0.05$). All parametric neurite outgrowth data was analysed using one-way analysis of variance (ANOVA) with post-hoc analysis using the Holm Sidak method, while non-parametric data was analysed using Kruskal-Wallis ANOVA on Ranks with post-hoc analysis using Dunn's method. Data is presented as

means ± standard error of the mean or medians with 25%-75% range. In all cases, there were no significant differences in the area of the explant masses between groups.

RESULTS

BDNF release from Ppy/pTS/BDNF is enhanced by electrical stimulation

To gain an initial understanding of the dynamics of BDNF release from Ppy/pTS/BDNF, a ^{125}I-based BDNF release assay was conducted over 7 days, beginning immediately after synthesis and with stimulation applied throughout the period to the samples in the stimulated group. On average, 122.1 ± 3.28 ng/cm^2 ^{125}I BDNF was incorporated into the polymers (n=8). In the absence of electrical stimulation, there was a burst of ^{125}I BDNF release during the first day, after which the amount of released BDNF slowed down to 0.53 ng/cm^2 or 0.43% of total incorporated ^{125}I BDNF per day (n=2 per point) (Fig 2a). A burst of BDNF release was also evident when electrical stimulation was applied to the electrodes, however, in this case the ongoing electrical stimulation enhanced the release of ^{125}I BDNF from the polymer with an average of 2.15 ng/cm^2 or 1.8% of total incorporated ^{125}I BDNF released per day. Cumulative data show that stimulated polymers released 1.75 fold more ^{125}I BDNF over 7 days (23.1 ± 1.4 ng/cm^2 or 18.9% of total incorporated) compared with non-stimulated polymers (13.2 ± 1.4 ng/cm^2 or 10.8% of total incorporated) ($p<0.05$ One-way ANOVA) (Fig 2a).

In all subsequent assays, polymers were used 4 days after synthesis to avoid the burst of BDNF release identified from the ^{125}I release assay. Native BDNF was used in place of ^{125}I-labelled BDNF, stimulation was applied for 8 hr per day and experiment duration

was 3 days. An ELISA-based BDNF release assay was conducted to determine BDNF release under these experimental conditions. Stimulated Ppy/pTS/BDNF released BDNF at an average rate of 6.2 ng BDNF/cm^2/day while the unstimulated polymers released BDNF at a slightly reduced rate of 4.9 ng BDNF/cm^2/day over the 3 day period. At the end of this period, electrically stimulated polymers released 1.25 fold more BDNF than unstimulated polymers, but this difference was not statistically significant (n=3) (Fig 2b).

BDNF in solution promotes neurite outgrowth from SGN explants

To first understand how SGN explants respond to endogenous sources of BDNF, SGN explants were grown on plastic culture slides coated with CAMs in media containing 0, 1, 10 or 100 ng/ml BDNF. After 3 days, the explants were fixed and stained for SGN cell bodies and neurites using an anti-NF200 antibody. Between 14 and 20 explants were analysed in each treatment group across 4 independent experiments. In the absence of BDNF, explants showed some SGN survival and resprouting of neurites (6.4 ± 1.4 neurites per explant). The addition of 1 ng/ml or 10 ng/ml BDNF to the media was insufficient to significantly enhance neurite outgrowth (8.9 ± 2.4 and 13.9 ± 4.0 neurites per explant respectively). However, 100 ng/ml BDNF increased neurite outgrowth 3.9-fold compared to the 0 ng/ml BDNF control (24.8 ± 4.1 neurites per explant, $p<0.001$) (Figure 3).

SGN explant interactions with Ppy/pTS and Ppy/pTS/BDNF

Explants were grown on Ppy/pTS or Ppy/pTS/BDNF for 3 days and interactions of explants, neurites and other cells with the polymer were observed by SEM (n=2

experiments). The surfaces of Ppy/pTS and Ppy/pTS/BDNF were raised and globular, an observation that was especially obvious via a direct comparison with tissue culture plastic (Figure 4a). Explants were visible as a mass of tissue with radiating neurites and migratory cells such as fibroblasts (Figure 4b,c).

Polymers containing BDNF enhanced neurite outgrowth from explants

Explants were grown on Ppy/pTS and Ppy/pTS/BDNF in media containing no other neurotrophic support. Explants on Ppy/pTS/BDNF had a median (25%-75% range) of 37.0 (23.8-54.5) neurites per explant, approximately twelve times more neurites than those cultured on Ppy/pTS (3.0 (2.0-10.3)) ($p<0.01$ Dunn's method) (n=6; Figure 5).

Explants that were placed within 2.7 mm of Ppy/pTS/BDNF but not directly contacting the polymer were used to test whether the natural diffusion of BDNF was equally effective in promoting neurite outgrowth as direct contact. Explants growing near Ppy/pTS/BDNF showed median (25%-75% range) neurite outgrowth of 11.0 (6.3-26.3) neurites per explant compared to those grown near Ppy/pTS with 7.0 (3.0-15.0) neurites per explant ($p<0.05$). Neurite outgrowth from explants grown directly on Ppy/pTS/BDNF was not statistically different to neurite outgrowth from explants grown near Ppy/pTS/BDNF. However, the data in the 'near Ppy/pTS/BDNF' group was quite variable. There was no significant difference in median neurite outgrowth between explants grown on Ppy/pTS and near Ppy/pTS (Dunn's method, $p>0.05$).

Effect of electrical stimulation of Ppy/pTS/BDNF on neurite outgrowth from explants

Explants were cultured on Ppy/pTS/±BDNF as described. After 16 hr settling time, the electrical stimulus was applied for 8 hr followed by 16 hr of no stimulation. This pattern was repeated for 3 days. Experimental groups were stimulated Ppy/pTS, unstimulated Ppy/pTS, stimulated Ppy/pTS/BDNF and unstimulated Ppy/pTS/BDNF. There were between 28 and 52 explants per group from 6 independent experiments. There was no significant difference in neurite outgrowth between stimulated and unstimulated Ppy/pTS, but explant numbers in these groups were low making the power of the test also too low to make conclusions (4.0 (3.0-5.8) and 3.0 (2.0-10.3) neurites per explant respectively) (Dunn's method). Application of electrical stimulation to Ppy/pTS/BDNF enhanced neurite outgrowth 18.3 fold when compared to unstimulated Ppy/pTS (55.0 (29.0-68.0) neurites per explant, Dunn's method, $p<0.01$), but not significantly more than unstimulated Ppy/pTS/BDNF (Figure 6).

DISCUSSION

This paper assesses the applicability of a conducting polymer coating for cochlear implant electrodes with potential applications for other neural prostheses. In overview, it was found that the neurotrophin BDNF can be incorporated into Ppy and released slowly via diffusion or in greater quantities via an applied electrical stimulus. Polymers that contained BDNF promoted greater neurite outgrowth from explants growing on or near the polymer compared to polymers without incorporated BDNF. If applied to cochlear implant electrodes, released BDNF may prevent SGN degeneration after hearing loss and protect SGNs both during and after cochlear implantation to help preserve and regenerate

SGN peripheral dendrites, whilst still permitting electrical stimulation of the auditory nerve.

Ppy adheres very well to metals such as gold and platinum[32,33] and hence is an ideal candidate for coating cochlear implant electrodes which are made from platinum. It is noteworthy that the impedance of Ppy-coated gold electrodes is of the same order as uncoated gold electrodes, with or without neurotrophins and before or after electrical stimulation[9]. This is important for cochlear implantation applications as any increase in impedance and hence power requirements for the device are undesirable.

On average, 122.1 ± 3.28 ng BDNF was incorporated per square centimeter of Ppy/pTS/BDNF. This is comparable to Ppy/pTS/NT3 polymers in which 93.4 ± 6.6 ng/cm^2 NT3 was incorporated[6]. Also in line with Ppy/pTS/NT3, significantly greater release of ^{125}I BDNF was detected from 7-day stimulated Ppy/pTS/^{125}I BDNF compared to unstimulated Ppy/pTS/^{125}I BDNF. However, it is important to note that the Ppy/pTS/NT3 study used pulsed current (±0.5 mA/cm^2) while in this study the biologically safe biphasic current pulses employed by the cochlear implant were used to promote release of BDNF. This gives a better understanding of the release kinetics that would be expected in the cochlea if a Ppy-coated electrode array were implanted. From release studies using conditions more specific to SGN explant culture (unlabelled BDNF, 8 hr stimulation per day beginning 4 days after synthesis over 3 days, 37^{o}C incubation), no significant difference in the quantity of released BDNF between stimulated and unstimulated polymers was detected by ELISA, presumably due to the shorter incubation

period. Since similar quantities of released BDNF were detected by ^{125}I-based assay and ELISA, it can be presumed that the ^{125}I label does not affect the incorporation or release of the protein. The ^{125}I BDNF-based method has the benefit of being able to measure the amount of BDNF incorporated into the polymer and remaining in the polymer after release and appears to be a very sensitive method for quantification of released neurotrophin.

Previous studies have shown that other biological substances such as NGF and epinephrine exhibited enhanced release from Ppy upon electrical stimulation. A large voltage applied over 150 seconds increased streptavidin-bound NGF release 48-fold[34] while epinephrine release was increased over 5-fold when compared to non-stimulated Ppy[35]. Unlike the biphasic current pulses used here, none of these studies used biologically relevant stimulation parameters which avoid the generation of direct current that can damage tissue through the build-up of adverse electrochemical by-products[31,36]. The mechanism of BDNF release from Ppy/pTS/BDNF via electrical stimulation is still unclear. One theory is that the biphasic current pulses cause expansion and contraction (actuation) as the polymer cycles through oxidation and reduction, opening gaps within the overall structure through which neurotrophins near the surface of the polymer can escape[37,38]. In addition, ionic and hydrophobic properties of Ppy change during stimulation and may facilitate the ability of neurotrophins to escape from the polymer[39].

Neurite outgrowth was enhanced in SGN explants grown on Ppy/pTS/BDNF compared to Ppy/pTS indicating that biologically active BDNF is being released. While ELISAs

demonstrated that non-stimulated Ppy/pTS/BDNF released 19.76 ng/cm^2 BDNF after 3 days, equivalent to 4.66 ± 0.46 ng/ml BDNF, neurite outgrowth from explants grown on non-stimulated Ppy/pTS/BDNF (median 37 (23.8-54.5) neurites per explant) was greater than neurite outgrowth from explants grown in media containing 10 ng/ml or even 100 ng/ml BDNF (mean 13.9 ± 4.0 and 24.8 ± 4.1 neurites per explant respectively). This may indicate that slow release of BDNF from a polymer is more beneficial to neurons in culture than a single addition of BDNF at the start of the assay. Indeed continuous slow release of neurotrophins *in vivo* is more beneficial to SGNs than a single dose of neurotrophin[3], although the additional element of neurotrophin clearance from cochlear fluids must be taken into account here. Another explanation could be a proximity effect in which neurons directly contacting a material releasing neurotrophins receive a greater proportion of neurotrophin than neurons bathed in a solution of neurotrophins. In support of this, explants cultured near Ppy/pTS/BDNF (11.0 (6.3-26.3) did not have as many neurites as explants cultured directly on Ppy/pTS/BDNF (37 (23.8-54.5)). Similarly, greater neurite outgrowth was observed from SGN explants cultured directly on Ppy/pTS/NT3 that released a predicted 1.15 ng/ml NT3 compared to explants cultured in a solution of 40 ng/ml NT3[9].

Explants located near Ppy/pTS/BDNF are the most representative explants for modelling the current situation in an implanted cochlea as the implanted electrodes generally lie 1-2 mm away from the SGNs. Future cochlear implant designs might enable neurons to grow directly on the electrodes. In such a case, the electrode should be electroactive, promote neuron survival and encourage neurite extension as is the case for Ppy/pTS/BDNF.

Stimulation of SGNs directly contacting electrodes would require lower charge density and less power and create greater specificity due to reduced current spread compared to electrodes that are situated 1-2 mm away from neurons. This in turn could lead to electrode arrays designed with a greater number of smaller, more finely spaced electrodes to achieve more selective neuron firing patterns leading to potentially improved speech and music perception among cochlear implantees.

Culture slides were electrically stimulated in eight hour periods with sixteen hours non-stimulation periods to simulate cochlear implant users who switch off their implants at night. While eight hours is a short period for stimulation when compared to most cochlear implant users, this duration was favourable as it is comparable with many *in vivo* animal studies that use the same stimulation duration or slightly less[30,40,41]. Electrical stimulation did not significantly enhance neurite outgrowth on polymers containing BDNF, and is perhaps not surprising given the results of the 3-day release assay in which there was no significant difference in BDNF release between stimulated and unstimulated polymers over this time period. Enhanced benefit to neurons may have been detected over longer periods of culture on stimulated Ppy/pTS/BDNF. Longer culture experiments were attempted, but these proved to be detrimental to neuron survival under these conditions. Stimulation alone (stimulated Ppy/pTS) was not shown to be beneficial or detrimental to neurite outgrowth from explants. We cannot be certain that neurons grown on Ppy/pTS/BDNF were depolarized in response to the electrical stimulus. However, given the charge densities employed here, together with the proximity of the explants to the electrodes, it is most likely that SGNs were depolarized. Greater release of BDNF and

hence enhanced response from SGNs may also be achieved by using different stimulation strategies, as was observed with stimulated NT3 release from Ppy/pTS/NT3 polymers[6]. Since our aim is to keep the stimulation biologically relevant, strategies would have to be limited to pulse duration, frequency and current densities used in cochlear implants for stimulating the auditory nerve.

Some *in vivo* studies have reported that chronic stimulation alone has no significant effect on neural preservation[30,42,43]. Conversely, other studies have reported greater SGN survival following chronic stimulation in aminoglycoside deafened cats[44] and GPs[1]. The different outcomes in these studies may be due to different implantation techniques or the method of deafening. More certain is that combining chronic electrical stimulation with neurotrophic factor treatment has been shown to be more beneficial than applying either of the treatments alone[30]. Furthermore, electrical stimulation in deafened guinea pigs can preserve SGNs following the removal of exogenous neurotrophins[45]. However, these *in vivo* studies only counted SGN soma and any effects on peripheral dendrites (the equivalent of the neurites quantified in this study) were not documented. A more comprehensive approach to measuring effects of neurotrophins and electrical stimulation on SGNs *in vivo*, such as SGN survival, myelination and effects on peripheral dendrites, is required to assess the impact on neurotrophins, electrical stimulation or a combination of both on SGNs after deafness.

It is clear that polymers such as Ppy/pTS/BDNF can only hold a finite quantity of neurotrophin before being depleted. One solution for longer-term biological effects is to

immobilize the neurotrophin in a way that renders the neurotrophin reusable. Indeed, enhanced neurite outgrowth was achieved with Ppy containing immobilized NGF and with electrical stimulation[8]. Immobilisation of the growth factor within the polymer overcomes the limited supply problem but can only be used with growth factors that do not need to be fully endocytosed to transduce signals. NGF has been shown to do this for some of NGF functions such as nerve survival, but not all of its functions[46]. Unlike NGF, other neurotrophins appear to require endocytosis and retrograde transport for function; hence neurotrophins will need to be released from polymers to benefit neurons. Other types of drug-eluting strategies may need to be employed for longer-term neurotrophin release, one example of these being cell-based therapy. Fibroblasts modified to express BDNF were placed in a protective agarose gel which in turn was applied to electrodes implanted in deafened guinea pig cochleae. SGN survival was observed in the vicinity of the implanted electrodes[47]. Viable cells have also been incorporated into polypyrrole and another conducting polymer; poly(3,4-ethylenedioxythiophene) (PEDOT)[48,49], and with further development it may be possible for neurotrophin-releasing cells to be incorporated into conducting polymers for extended release of neurotrophins. With this combination, the impedance of the electrodes is not compromised whilst providing a continuous supply of neurotrophins. Clinical uses for Ppy/pTS/BDNF need to be specific for the requirement of short-term slow release of BDNF. One example of this is protection of SGNs and hair cells from cochlear implant insertion trauma to preserving residual hearing and maximize hearing outcomes for cochlear implantees.

A combination of NT3 and BDNF promotes greater SGN survival in culture than either neurotrophin alone[50] and is very effective at promoting SGN survival and resprouting in the deafened cochlea[4]. We have now shown the benefits of Ppy/pTS polymers that release NT3 and BDNF individually. Extensions to these studies would include the synthesis of a polymer that incorporates and releases a combination of NT3 and BDNF and to examine the effects of all these polymers in the cochlea after sensorineural hearing loss.

CONCLUSIONS

This study demonstrated enhanced neurite outgrowth from auditory neuron explants grown on or near Ppy/pTS/BDNF. Release studies showed that this was due to diffusion of BDNF from the polymer. Although increased BDNF release was detected from electrically stimulated polymers over 7 days, electrical stimulation of Ppy/pTS/BDNF did not significantly increase neurite outgrowth from cultured explants in the shorter 3-day explant culture period. Electroactive/bioactive polymers such as Ppy/pTS/BDNF may find applications for cochlear implant electrodes as they do not impede electrical stimulation of the auditory nerve whilst providing BDNF to maintain the health of auditory neurons. By encouraging neurite outgrowth, there is the potential to increase the neural selectivity of the cochlear implant by bringing the peripheral dendrites of the neurons and the electrodes closer together. With this being the case, future cochlear implant development may include increasing the electrode number and implant channels with potential improvements to speech perception in noise and appreciation of music.

ACKNOWLEDGEMENTS

The authors would like to thank the following funding institutions associated with this research: The University of Melbourne, Department of Otolaryngology, Stavros S. Niarchos Foundation, John T Reid Charitable Trusts, Royal National Institute for Deaf People, Pierce Armstrong Foundation and Australian Research Council Centre of Excellence for Electromaterials Science.

FIGURE LEGENDS

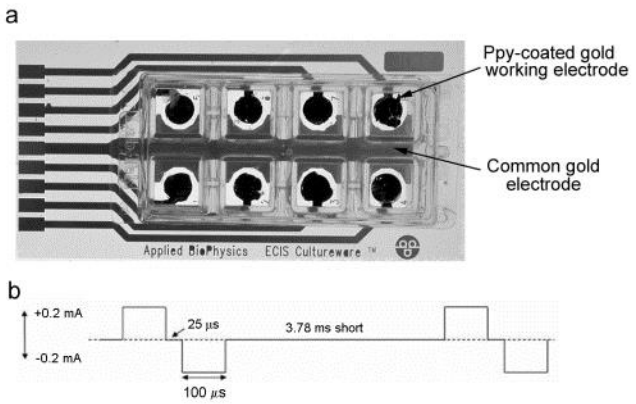

Figure 1. An ECIS culture slide with Ppy-coated gold electrodes and the electrical stimulus applied to the electrodes

(a) Top view of an eight-well ECIS culture slide consisting of 18-21 mm^2 gold active circular electrodes coated with Ppy and a gold common return electrode. **(b)** The biphasic waveform to stimulate the polypyrrole-coated electrodes had ± 0.2 mA amplitude, 100 μs pulse width, 25 μs open-circuit interphase gap and 3.78 ms short-circuit phase and was presented at 250 Hz.

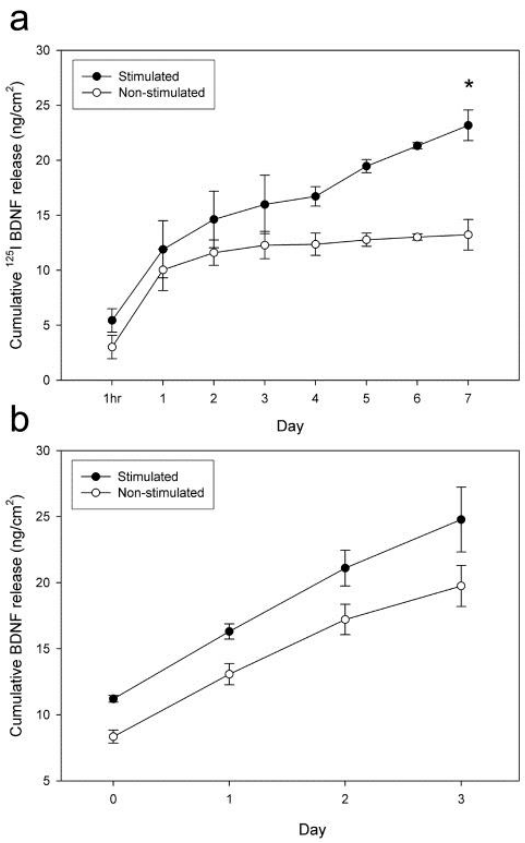

Figure 2. Electrical stimulation of Ppy/pTS/BDNF enhances the release of BDNF from the polymer compared to unstimulated Ppy/pTS/BDNF

(a) ^{125}I BDNF-containing polymers were synthesised on gold mylar and incubated for 7 days in normal saline at room temperature. Each day the entire saline sample was removed and replaced with fresh 0.9% saline. Samples were read in a gamma counter and converted to BDNF mass. Cumulative released BDNF masses are shown for stimulated and unstimulated polymers (n=2 experiments per point). *$p<0.05$ stimulated vs non-stimulated after 7 days cumulative data **(b)** Ppy/pTS/BDNF polymers were grown on gold electrodes on ECIS slides and used 4 days after synthesis. Incubations were at 37^{o}C. Three periods of 8 hr electrical stimulation were applied to half of the polymers with a 16 hr period of no stimulation and the saline was sampled daily over 3 days. Samples were assayed for BDNF using ELISA. Cumulative released BDNF concentrations are shown for stimulated and unstimulated polymers (n=3 experiments per point with 9 independent samples per point).

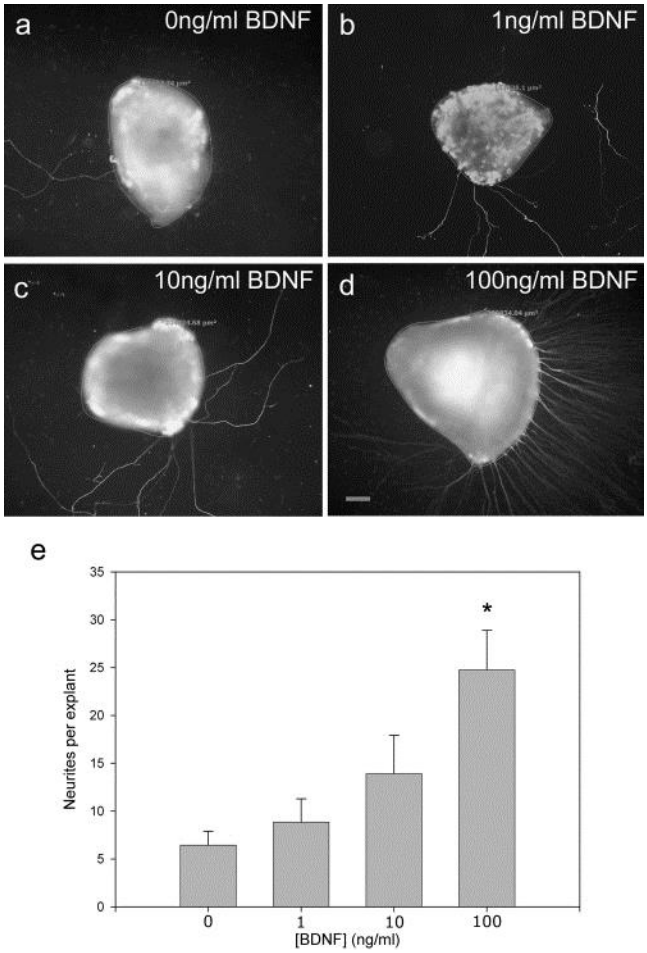

Figure 3. Exogenous BDNF enhances neurite outgrowth in SGN explants

(a-d) Representative images of SGN explants grown on tissue culture plastic with various concentrations of BDNF added to the culture media. In the absence of BDNF and with the addition of 1 ng/ml and 10 ng/ml BDNF, very few neurites were observed from explants. Explants grown in 100 ng/ml BDNF demonstrated greater numbers of resprouting neurites. Scale bar is 100 μm and applies to all images. **(e)** Graph of mean neurites per SGN explant after 3 days culture in 0, 1, 10 or 100 ng/ml BDNF. Error bars represent the standard error of the mean. *$p<0.05$ compared to the 0 ng/ml BDNF control.

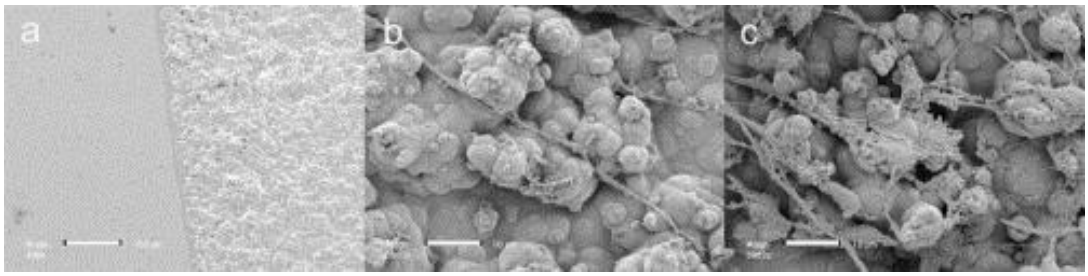

Figure 4. The surface topography and cellular interactions with Ppy/pTS/BDNF via SEM

(a) A comparison of the surface topography between plastic (left) and Ppy/pTS (right). The surface of Ppy/pTS consisted of raised, uneven globules. Scale bar is 100 μm. **(b, c)** Neurites and cells growing out from the explant tissue mass on the uneven surface of Ppy/pTS/BDNF. Scale bars are 10 μm.

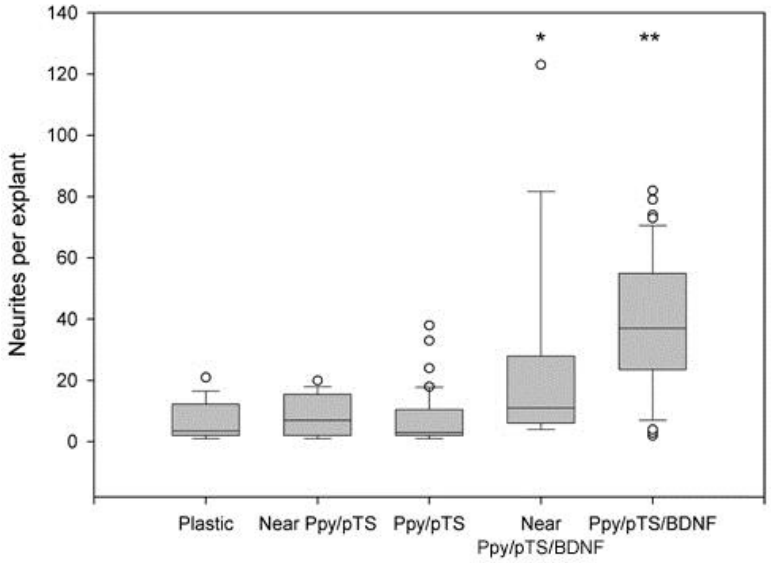

Figure 5. Ppy/pTS/BDNF enhances neurite outgrowth from SGN explants

Explants grown on or near Ppy/pTS/BDNF extended more neurites than those grown on or near plain Ppy/pTS. This implies that release of BDNF via natural diffusion from the polymer was benefiting SGNs within the explants, especially when the polymer was directly contacting the explant. (*$p<0.05$, **$p<0.01$ compared to Ppy/pTS control).

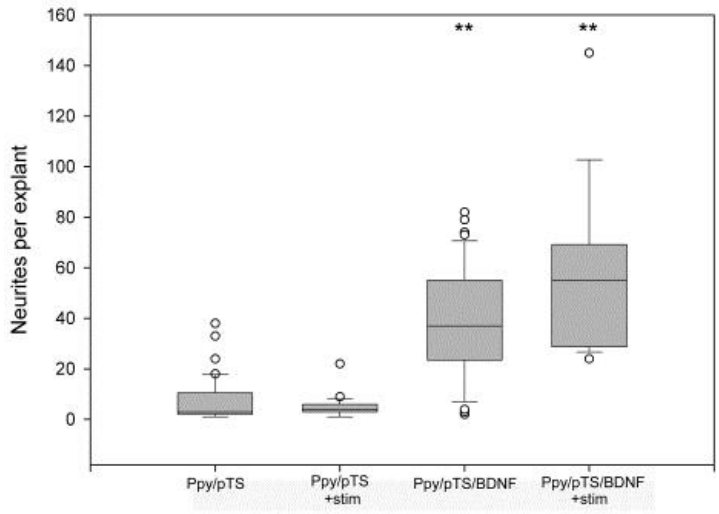

Figure 6. Electrical stimulation of Ppy/pTS/BDNF does not significantly enhance neurite outgrowth in SGN explants

Electrical stimulation (stim) of Ppy/pTS/BDNF further enhanced neurite outgrowth from SGN explants compared to Ppy/pTS ($p<0.01$), but neurite outgrowth was not significantly greater than the non-stimulated Ppy/pTS/BDNF group. (**$p<0.01$ compared to Ppy/pTS control).

REFERENCES

1. Miller JM, Chi DH, O'Keefe LJ, Kruszka P, Raphael Y, Altschuler RA. Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss. Int.J.Dev.Neurosci. 1997;15:631-643.
2. Gillespie LN, Clark GM, Marzella PL. Delayed neurotrophin treatment supports auditory neuron survival in deaf guinea pigs. Neuroreport 2004;15(7):1121-5.
3. Richardson RT, O'Leary S, Wise A, Hardman J, Clark G. A single dose of neurotrophin-3 to the cochlea surrounds spiral ganglion neurons and provides trophic support. Hear Res 2005;204(1-2):37-47.
4. Wise AK, Richardson R, Hardman J, Clark G, O'Leary S. Resprouting and survival of guinea pig cochlear neurons in response to the administration of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3. J Comp Neurol 2005;487(2):147-65.
5. Wadhwa R, Lagenaur CF, Cui XT. Electrochemically controlled release of dexamethasone from conducting polymer polypyrrole coated electrode. J Control Release 2006;110(3):531-41.
6. Thompson BC, Moulton SE, Ding J, Richardson R, Cameron A, O'Leary S, Wallace GG, Clark GM. Optimising the incorporation and release of a neurotrophic factor using conducting polypyrrole. J Control Release 2006;116(3):285-94.
7. Li Y, Neoh KG, Kang ET. Controlled release of heparin from polypyrrole-poly(vinyl alcohol) assembly by electrical stimulation. J Biomed Mater Res A 2005;73(2):171-81.
8. Gomez N, Schmidt CE. Nerve growth factor-immobilized polypyrrole: bioactive electrically conducting polymer for enhanced neurite extension. Journal of Biomedical Materials Research A 2007;81(1):135-49.
9. Richardson RT, Thompson B, Moulton S, Newbold C, Lum MG, Cameron A, Wallace G, Kapsa R, Clark G, O'Leary S. The effect of polypyrrole with incorporated neurotrophin-3 on the promotion of neurite outgrowth from auditory neurons. Biomaterials 2007;28(3):513-23.
10. Wallace GG, Innis PC. Inherently conducting polymer nanostructures. J Nanosci Nanotechnol 2002;2(5):441-51.
11. Wallace GG, Kane-Maguire LAP. Manipulating and monitoring biomolecular interactions with conducting electroactive polymers. Advanced Materials 2002;14:953-960.
12. Pyo M, Reynolds JR, Warren LF, Marcy HO. Long Term Redox Switching Stability of Polypyrrole. Synth Met 1994;68:71-77.
13. Zhao H, Price WE, Too CO, Wallace G, Zhou D. Parameters Influencing transport across conducting electroactive polymer membranes. Memb Sci 1996;119:199-212.
14. Murray P, Spinks G, Wallace G, Burford R. In-situ Mechanical Properties of Tosylate Doped (pTS) Polypyrrole. Synth Met 1997;84:847-848.
15. Jerome C, Martinot L, Strivay D, Weber G, Jerome R. Controlled exchange of metallic cations by polypyrrole-based resins. Synth Met 2001;118(1-3):45-55.

16. Ansari Khalkhali RA, Price WE, Wallace G. Quartz crystal microbalance studies of the effect of solution temperature on the ion-exchange properties of polypyrrole conducting electroactive polymers. Reactive and Functional Polymers 2003;56(3):141-146.
17. Jiang X, Marois Y, Traore A, Tessier D, Dao LH, Guidoin R, Zhang Z. Tissue reaction to polypyrrole-coated polyester fabrics: an in vivo study in rats. Tissue Eng 2002;8(4):635-47.
18. Wang X, Gu X, Yuan C, Chen S, Zhang P, Zhang T, Yao J, Chen F, Chen G. Evaluation of biocompatibility of polypyrrole in vitro and in vivo. J Biomed Mater Res A 2004;68(3):411-22.
19. George PM, Lyckman AW, Lavan DA, Hegde A, Leung Y, Avasare R, Testa C, Alexander PM, Langer R, Sur M. Fabrication and biocompatibility of polypyrrole implants suitable for neural prosthetics. Biomaterials 2005;26(17):3511-9.
20. Cui X, Wiler J, Dzaman M, Altschuler RA, Martin DC. In vivo studies of polypyrrole/peptide coated neural probes. Biomaterials 2003;24(5):777-87.
21. Schmidt CE, Shastri VR, Vacanti JP, Langer R. Stimulation of neurite outgrowth using an electrically conducting polymer. Proc Natl Acad Sci U S A 1997;94(17):8948-53.
22. Kotwal A, Schmidt CE. Electrical stimulation alters protein adsorption and nerve cell interactions with electrically conducting biomaterials. Biomaterials 2001;22(10):1055-64.
23. Zhang Z, Rouabhia M, Wang Z, Roberge C, Shi G, Roche P, Li J, Dao LH. Electrically conductive biodegradable polymer composite for nerve regeneration: electricity-stimulated neurite outgrowth and axon regeneration. Artif Organs 2007;31(1):13-22.
24. Farinas I, Jones KR, Tessarollo L, Vigers AJ, Huang E, Kirstein M, de Caprona DC, Coppola V, Backus C, Reichardt LF and others. Spatial shaping of cochlear innervation by temporally regulated neurotrophin expression. Journal of Neuroscience 2001;21(16):6170-80.
25. Bianchi LM, Conover JC, Fritzsch B, DeChiara T, Lindsay RM, Yancopoulos GD. Degeneration of vestibular neurons in late embryogenesis of both heterozygous and homozygous BDNF null mutant mice. Development 1996;122(6):1965-73.
26. Fritzsch B, Farinas I, Reichardt LF. Lack of neurotrophin 3 causes losses of both classes of spiral ganglion neurons in the cochlea in a region-specific fashion. Journal of Neuroscience 1997;17(16):6213-25.
27. Fritzsch B, Barbacid M, Silos-Santiago I. The combined effects of trkB and trkC mutations on the innervation of the inner ear. International Journal of Developmental Neuroscience 1998;16(6):493-505.
28. Schimmang T, Tan J, Muller M, Zimmermann U, Rohbock K, Kopschall I, Limberger A, Minichiello L, Knipper M. Lack of Bdnf and TrkB signalling in the postnatal cochlea leads to a spatial reshaping of innervation along the tonotopic axis and hearing loss. Development 2003;130(19):4741-50.
29. Newbold C, Richardson R, Huang CQ, Milojevic D, Cowan R, Shepherd R. An in vitro model for investigating impedance changes with cell growth and electrical stimulation: implications for cochlear implants. J Neural Eng 2004;1(4):218-27.

30. Shepherd RK, Coco A, Epp SB, Crook JM. Chronic depolarization enhances the trophic effects of brain-derived neurotrophic factor in rescuing auditory neurons following a sensorineural hearing loss. J Comp Neurol 2005;486(2):145-58.
31. Huang CQ, Shepherd RK, Carter PM, Seligman PM, Tabor B. Electrical stimulation of the auditory nerve: direct current measurement in vivo. IEEE Trans Biomed Eng 1999;46(4):461-70.
32. Diaz AF, Kanazawa KK, Gardini JP. Electrochemical polymerization of pyrrole. Journal of the Chemical Society Chemical Communications 1979;14:635-636.
33. Schirmeisen M, Beck F. Electrocoating of iron and other metals with polypyrrole. Applied Electrochemistry 1989;19(3):401-409.
34. George PM, La Van DA, Burdick JA, Chen C-Y, Liang E, Langer R. Electrochemically controlled drug delivery from biotin-doped conductive polypyrrole. Advanced Materials 2006;18(5):577-581.
35. Son JH, Chang J-H, Kim DB, Lee S-K, Pak JJ. Application of polypyrrole(PPy) as the media material for the release and the detection of a neurotransmitter. In: Bar-Cohen Y, editor. Smart structures and materials 2005: Electroactive Polymer Actuators and Devices (EAPAD); 2005. p 423-430.
36. Brummer SB, Turner MJ. Electrochemical considerations for safe electrical stimulation of the nervous system with platinum electrodes. IEEE Trans Biomed Eng 1977;24(1):59-63.
37. Lewis TW, Moulton S, Spinks G, Wallace G. Optimisation of a polypyrrole based actuator. Synth Met 1997;85(1-3):1419-1420.
38. Hutchison AS, Lewis TW, Moulton S, Spinks G, Wallace G. Development of polypyrrole-based electromechanical actuators. Synth Met 2000;113:121-127.
39. Wallace G, Spinks G, Kane-Maguire LAP, Teasdale PR. Conductive Electroactive Polymers: Intelligent Materials Systems: CRC Press; 2003. 1-237 p.
40. Ryugo DK, Kretzmer EA, Niparko JK. Restoration of auditory nerve synapses in cats by cochlear implants. Science 2005;310(5753):1490-2.
41. Coco A, Epp SB, Fallon JB, Xu J, Millard RE, Shepherd RK. Does cochlear implantation and electrical stimulation affect residual hair cells and spiral ganglion neurons? Hearing Research 2007;225(1-2):60-70.
42. Shepherd RK, Matsushima J, Martin RL, Clark GM. Cochlear pathology following chronic electrical stimulation of the auditory nerve: II. Deafened kittens. Hear Res 1994;81(1-2):150-66.
43. Araki S, Kawano A, Seldon L, Shepherd RK, Funasaka S, Clark GM. Effects of chronic electrical stimulation on spiral ganglion neuron survival and size in deafened kittens. Laryngoscope 1998;108(5):687-95.
44. Leake P, Hradek, GT, Rebscher, SJ, Snyder, RL. Chronic intracochlear electrical stimulation induces selective survival of spiral ganglion neurons in neonatally deafened cats. Hearing Research 1991;54(2):251-271.
45. Shepherd R, Coco A, Epp S. Neurotrophins and electrical stimulation for protection and repair of spiral ganglion neurons following sensorineural hearing loss. Hear Res 2007;(in press).
46. MacInnis BL, Campenot RB. Retrograde support of neuronal survival without retrograde transport of nerve growth factor. Science 2002;295(5559):1536-9.

47. Rejali D, Lee VA, Abrashkin KA, Humayun N, Swiderski DL, Raphael Y. Cochlear implants and ex vivo BDNF gene therapy protect spiral ganglion neurons. Hear Res 2007;228(1-2):180-7.
48. Campbell TE, Hodgson AJ, Wallace GG. Incorporation of erythrocytes into polypyrrole to form the basis of a biosensor to screen for rhesus (D) blood groups and rhesus (D) antibodies. Electroanalysis 1999;11(4):215-222.
49. Richardson-Burns SM, Hendricks JL, Foster B, Povlich LK, Kim DH, Martin DC. Polymerization of the conducting polymer poly(3,4-ethylenedioxythiophene) (PEDOT) around living neural cells. Biomaterials 2007;28(8):1539-52.
50. Mou K, Hunsberger C, CLeary J, Davis R. Synergistic effects of BDNF and NT-3 on postnatal spiral ganglion neurons. The Journal of Comparative Neurology 1997;386:529-539.