Published in final edited form as:
Neuroscience. 2008 March 27; 152(3): 821–828. doi:10.1016/j.neuroscience.2007.11.057.

Schwann cells genetically modified to express neurotrophins promote spiral ganglion neuron survival *in vitro*

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Abstract

The intracochlear infusion of neurotrophic factors via a mini-osmotic pump has been shown to prevent deafness-induced spiral ganglion neuron (SGN) degeneration; however, the use of pumps may increase the incidence of infection within the cochlea, making this technique unsuitable for neurotrophin administration in a clinical setting. Cell- and gene-based therapies are potential therapeutic options. This study investigated whether Schwann cells which were genetically modified to over-express the neurotrophins brain-derived neurotrophic factor (BDNF) or neurotrophin 3 (Ntf3, formerly NT-3) could support SGN survival in an *in vitro* model of deafness. Co-culture of either BDNF over-expressing Schwann cells or Ntf3 over-expressing Schwann cells with SGNs from early postnatal rats significantly enhanced neuronal survival in comparison to both control Schwann cells and conventional recombinant neurotrophin proteins. Transplantation of neurotrophin over-expressing Schwann cells into the cochlea may provide an alternative means of delivering neurotrophic factors to the deaf cochlea for therapeutic purposes.

Keywords

auditory; *ex vivo* gene transfer; BDNF; Ntf3

Sensorineural hearing loss (SNHL) is a common cause of deafness, and normally results from damage to or loss of the sensory auditory hair cells, which are a key step in the transmission of sound signals to the brain. Cochlear implants are currently the only therapeutic intervention for patients with a severe-profound SNHL. These devices bypass the degenerated hair cells to directly electrically stimulate residual spiral ganglion neurons (SGNs) to provide the auditory cues required for speech perception. However, SGNs undergo progressive degeneration following a SNHL, and evidence from animal studies indicates that ongoing SGN degeneration has the potential to compromise the efficacy of the electrode-neural interface, including elevated thresholds (Hardie and Shepherd, 1999), prolonged refractory behaviour (Shepherd et al., 2004), bursting activity (Shepherd and Javel, 1997), and slower recovery of the neural membrane following action potential generation (Sly et al., 2007). It is therefore anticipated that maintenance of a robust SGN population may improve the efficacy of the electrode-neural interface and enhance cochlear implant performance.

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Section Editor: Sensory Systems – Dr Richard Weinberg

Previous studies have shown that neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (Ntf3), which are important for the normal development and maintenance of the auditory system (Pirvola et al., 1992; Ylikoski et al., 1993; Farinas et al., 1994; Pirvola et al., 1994; Wheeler et al., 1994; Ernfors et al., 1995; Farinas et al., 2001; Fritzsch et al., 2004; Fritzsch et al., 2005), can also support SGN survival and prevent degeneration in models of deafness (Ernfors et al., 1996; Miller et al., 1997; Gillespie et al., 2003; Gillespie et al., 2004; Richardson et al., 2005; Wise et al., 2005). These anatomical changes have also been shown to correspond with functional improvements of the deaf auditory system (Shinohara et al., 2002; Yamagata et al., 2004; Shepherd et al., 2005), meaning the surviving neurons retain the electrophysiological properties required to transfer sound signals to the brain. Unfortunately, however, the survival effects are lost if delivery of the exogenous neurotrophin stops (Gillespie et al., 2003; Shepherd et al., submitted). Since the loss of neurotrophic support from the hair cells is considered a major contributing factor to the degeneration of SGNs in the deaf ear, these results are not surprising, and together these concepts suggest that ongoing neurotrophic factor support may be required for long-term or permanent maintenance of SGNs in deafness.

Furthermore, clinically safe and effective drug delivery methods are required. The finite delivery period of osmotic pumps mean that long-term neurotrophic factor treatment would require repeated replacement of the pumps, thereby increasing the risk of infection associated with such surgical procedures. While refillable pump-based systems have been shown to function adequately for up to eight months in rats (Praetorius et al., 2001), the long-term bioactivity of neurotrophic factors at body temperature has not been confirmed, and the regular refilling of these devices still poses a risk for the introduction of infection directly into the cochlea. While cerebrospinal fluid shunts and drug delivery pumps are routinely implanted in the central nervous system on a chronic basis, infection is a major cause of morbidity and mortality of these devices, with infection rates in the order of 8–10% for both (Albright et al., 2004; Ferguson et al., 2007; Kan and Kestle, 2007; Motta et al., 2007; Sciubba et al., 2007). Such outcomes would be totally unacceptable if translated to an intracochlear drug delivery system. Prevention of infection within the inner ear is extremely important, firstly, to minimise further damage to and loss of SGNs, and secondly, to avoid infection spreading via the cochlear aqueduct to the central nervous system.

Gene therapy is an alternative method for increasing neurotrophin expression within the cochlea, and in fact *in situ* transduction of the spiral ganglion with genes encoding BDNF, Ntf3 or glial-derived neurotrophic factor has been shown to support SGN survival (Staecker et al., 1998; Yagi et al., 2000; Bowers et al., 2002; Lalwani et al., 2002; Nakaizumi et al., 2004). However, long-term transgene expression would require the use of viral vectors, which also raises concerns regarding clinical applicability.

Ex vivo gene transfer, which involves the transduction of a host population of cells *in vitro* followed by transplantation of these cells *in vivo*, is an alternative for neurotrophin therapy within the inner ear. Importantly, previous studies have reported that cells genetically modified to over-produce neurotrophins can continue to express the transgene for at least 12 months (Winn et al., 1996; Tuszynski et al., 1998), providing further support for this technique as a potential therapy for prevention of SGN degeneration in deafness.

This study therefore aimed to investigate the combination of cell- and gene-based techniques on SGN survival. Specifically, Schwann cells were genetically modified to over-express either BDNF or Ntf3 and the survival effects of these cells on SGNs was determined *in vitro*.

EXPERIMENTAL PROCEDURES

In order to test the survival-promoting effect of neurotrophin over-expressing Schwann cells on SGNs, Schwann cells transfected with either BDNF or Ntf3 were co-cultured with primary SGNs. All experiments were performed under the approval of the Animal Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital, and in accordance with the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Expression vectors

Expression plasmids encoding for C-terminal enhanced green fluorescent protein (EGFP)-tagged rat prepro BDNF or human prepro Ntf3 were kindly provided by Dr Volkmar Lessmann, from the Johannes Gutenberg Universität, Mainz, Germany. The neurotrophin expression vectors had been constructed as previously described (Haubensak et al., 1998; Hartmann et al., 2001; Brigadski et al., 2005). Briefly, the complete sequence of either rat prepro BDNF cDNA or human prepro Ntf3 cDNA was introduced into the cytomegalovirus-promoter driven pEGFP-N1 expression vector (Clontech, Cambridge, UK), which also contains genes conferring resistance to kanamycin and neomycin. The original pEGFP-N1 vector was also provided as a control.

Transformation and plasmid collection

All constructs were introduced into TOP10 competent *Escherichia coli* bacterial cells (Invitrogen, Melbourne, VIC, Australia) at a concentration of 200ng plasmid per 50μl *E. coli* cells via heat shock, and cells were grown on kanamycin-containing Luria Broth agar plates. Plasmid DNA was purified from liquid cultures using the QIAprep Spin Miniprep Kit column-based method (QIAGEN, Melbourne, VIC, Australia) and stored at 20°C in Tris-EDTA (ethylene diaminetetra-acetic acid) buffer until required for transfection.

Schwann cell cultures

Schwann cell cultures were kindly provided by Dr Simon Murray and Professor Trevor Kilpatrick from the Howard Florey Institute, Melbourne, Australia. Briefly, Schwann cells were prepared from postnatal day (P) three rat sciatic nerve, and purified to >99.5% purity using the fibroblast inhibitor cytosine arabinoside followed by treatment with antiserum to the selective fibroblast antigen Thy-1, as previously described (Brockes et al., 1979). Schwann cells were grown on poly-lysine (25μg/ml; Sigma, Castle Hill, NSW, Australia) coated 75cm^2 flasks (Greiner Bio-One [Interpath Services, West Heidelberg, VIC, Australia]) in 10ml Schwann cell media (SCM; Dulbecco's modified Eagle's medium [DMEM; Thermo Electron Corporation, Noble Park, VIC, Australia] containing 2mM L-glutamine [Thermo], 50U/ml penicillin/streptomycin [Thermo], 10% fetal calf serum [FCS; Thermo], 0.08% bovine pituitary extract [Sigma] and 2μM Forskolin [Sigma]) at 37°C, 10% CO_2. The Schwann cells were maintained by sub-culturing when the cells were confluent (every 3–4 days) using 0.025% trypsin (Thermo) in 0.1M phosphate buffer with 0.018% EDTA (Merck Pty, Kilsyth, VIC, Australia).

Schwann cell transfection

On the day prior to transfection, Schwann cells were sub-cultured into two poly-lysine coated 6-well plates (Techno Plastic Products [Interpath Services, West Heidelberg, VIC, Australia]) at a concentration of 2×10^5 cells/well, ensuring cells would be in the log phase of differentiation on the day of transfection.

Four wells of Schwann cells were transfected with each of the three expression plasmids using the lipid-based transfection reagent Lipofectamine 2000 (LF2000; Invitrogen), according to

manufacturer's guidelines. Specifically, for each plasmid, 16μg DNA was gently diluted in 1mL Opti-MEM reduced serum media (Gibco [Invitrogen, Melbourne, VIC, Australia]), and 40μl of LF2000 was diluted with 960μl Opti-MEM and incubated at room temperature for 5 minutes. The diluted DNA and the diluted LF2000 were then combined in a 1:1 ratio and incubated at room temperature for 20–25 minutes.

Schwann cells were rinsed with phosphate buffered saline (PBS), and 1.5ml of fresh SCM was added to each well. The DNA/LF2000 solution (500μl) was added to each well of Schwann cells (four wells per plasmid), and mixed by gently rocking the plate. The plates were then incubated at 37°C, 10% CO_2 overnight.

The following day, the presence of the EGFP reporter gene under direct fluorescence microscopy was used to confirm successful transfection. Schwann cells were then sub-cultured at 1:10 into 6-well plates, maintaining four wells per plasmid type. After a further 24 hours, the selective reagent Geneticin (G418 sulphate; Gibco) was added to each well at a concentration of 400μg/ml. Previous experimentation in our laboratory determined this as the optimal concentration for selection of Schwann cell transformants over a 10–14 day period (data not shown). Following two weeks of selective pressure, cells transfected with each plasmid were pooled and purified by fluorescence-activated cell sorting, which isolated the top 3–5% of each cell type. The three resultant cell populations, BDNF-Schwann cells (BDNF-SCs), Ntf3-Schwann cells (Ntf3-SCs) and control EGFP-Schwann cells (EGFP-SCs), were grown in 75cm^2 flasks and selective conditions maintained with 200μg/ml Geneticin. Schwann cells were sub-cultured every 3–4 days at a 1:10 ratio, and conditioned media collected and stored at −80°C for analysis.

The concentration of neurotrophin secreted by the genetically modified cells was determined using enzyme-linked immunosorbent assays (ELISAs). Serial dilutions of conditioned media collected from the BDNF-SCs and EGFP-SCs was analysed for BDNF content (n=7), and media from Ntf3-SCs and EGFP-SCs was analysed for Ntf3 content (n=13), using an Emax Immunoassay System kit (Promega, Annandale, NSW, Australia) directed against the neurotrophin of interest, as per manufacturer's instructions. Standard curves were also performed for each experiment, in accordance with manufacturer's guidelines. The concentration of neurotrophin in the conditioned media was calculated from the respective standard curve.

Spiral ganglion neuron cultures

SGN cultures were prepared from P5–P6 albino Wistar rat pups, as previously described (Gillespie et al., 2001). Briefly, the rats were rendered unconscious on ice and rapidly decapitated. The bulla was dissected from each temporal bone, the bony otic capsule removed and the cochlea isolated, and the auditory nerve severed at the internal auditory meatus. The organ of Corti was then removed, and the central core of all cochleae, containing Rosenthal's canal, were pooled and digested in a solution of calcium-magnesium-free Hank's Buffered Salt Solution (Gibco) containing 0.025% trypsin (Merck) and 0.001% DNase (Roche, Castle Hill, NSW, Australia) for 30 minutes at 37°C. Trypsinisation was stopped with FCS and the solution was centrifuged at 2500 rpm for 5 minutes. The tissue was resuspended in HEPES buffered Eagle's medium (Gibco) containing 0.001% DNase, mechanically digested through a series of needles, and spun as above. The resultant pellet was resuspended in DMEM-SATO (DMEM with 100μg/ml human transferrin [Sigma], 16μg/ml putrescine [Sigma], 10μg/ml insulin [Sigma], 0.4μg/ml thyroxine [Sigma], 0.3μg/ml thyronine [Sigma], 0.06μg/ml progesterone [Sigma], 0.04μg/ml selenium [Sigma], 2mM glutamine [Gibco] and 4.5g/L glucose [Gibco]; a modification of the medium described by (Bottenstein and Sato, 1979)). The dissociated SGN suspension was pre-plated in a 6-well tissue culture plate and incubated for 30 minutes at 37° C.

Spiral ganglion neuron-Schwann cell co-cultures

After the pre-plate incubation, SGNs were collected and plated onto poly-ornithine (500μg/mL; Sigma) and laminin (10μg/mL; Gibco) coated 8-well chamber slides (Nalge Nunc International, Rochester, NY, USA) at a density of approximately 20,000 cells/well, based upon previous *in vitro* studies (Gillespie et al., 2001). Schwann cells were prepared for the co-cultures by trypsinising as previously described and resuspending in SCM. BDNF-, Ntf3- or EGFP-SCs were added to the SGNs at 20,000 Schwann cells/well. This concentration was calculated by scaling down the number of Schwann cells normally seeded into a $75cm^2$ flask. Control SGN cultures were grown in DMEM-SATO and treated with recombinant human (rh) BDNF or rhNtf3 (50ng/mL; PeproTech, Rocky Hill, NJ, USA); control Schwann cell cultures were grown in SCM without additional exogenous neurotrophins.

All cultures were fixed after three days *in vitro*. The Schwann cell control cultures were fixed with 4% paraformaldehyde (30 minutes), rinsed in PBS, the chambers were removed and the slides mounted in DAPI mounting medium (Vector Laboratories, Burlingame, CA, USA). The SGN controls and SGN-Schwann cell co-cultures were fixed with 100% ice-cold methanol (30 minutes) for immunostaining.

Immunostaining

Immunostaining was performed using the avidin-biotin complex method of a standard Vectastain kit (Vector), as per manufacturer's instructions. Following fixation, cultures were rinsed with PBS and immunostained by incubation with, in series: 2% FCS in PBS (30 minutes); rabbit anti-neurofilament 200 kDa (Chemicon, Boronia, VIC, Australia; 1:400; 30 minutes); 2% FCS in PBS (3 × 5 minutes); biotinylated anti-rabbit secondary antibody (1:200; 30 minutes); PBS (3 × 5 minutes); avidin-biotin (1:100; 30 minutes); PBS (3 × 5 minutes); diaminobenzidine chromogen substrate (Vector; 5 minutes); and distilled water (3 × 5 minutes). Following immunostaining, the chambers were removed and the slides were dehydrated in 100% alcohol (3 × 1 minute), cleared in Histoclear (3 × 1 minute), and cover-slipped with DePeX mounting medium (Merck).

Analysis and Statistics

SGNs were co-cultured with EGFP-SCs (n=20), BDNF-SCs (n=9), rhBDNF (n=16), Ntf3-SCs (n=13) or rhNtf3 (n=13), across 10 independent experiments. For each experiment, all surviving SGNs – identified by immunopositivity for neurofilament – were counted in each well of the control EGFP-SC co-cultures, and the average SGN survival (per well) elicited by the EGFP-SCs was standardised to 100%. The number of surviving SGNs in each of the other treatment conditions was then counted (per well) and expressed as a percentage of the control. This was repeated for each individual experiment, and the average percentage SGN survival for each treatment condition was then calculated.

Statistical analyses were performed using the SigmaStat software package. Significant differences were identified using one way analysis of variance, and a Bonferroni test was used for all pairwise multiple comparisons. A difference was considered to be statistically significant at $p<0.05$.

RESULTS

Schwann cells can be genetically modified to over-express the neurotrophins BDNF and Ntf3

Successful transfection of the Schwann cells with each of the plasmids was initially confirmed 24 hours post-transfection by fluorescence microscopy. Transfected cells appeared green due to the presence of the EGFP reporter gene (Figure 1).

Transfection resulted in three Schwann cell lines: a) EGFP-SCs: Schwann cells that were genetically modified to express the fluorescent marker EGFP and serve as controls; b) BDNF-SCs: Schwann cells that were genetically modified to express EGFP-tagged BDNF; and c) Ntf3-SCs: Schwann cells that were genetically modified for EGFP-tagged Ntf3.

In addition, successful transfection was confirmed via ELISA analysis of conditioned media from each of the three cell types. Conditioned media was collected three days after sub-culturing when cells were confluent, at approximately 3×10^6 cells/flask. The control EGFP-SCs produced only a very small amount of BDNF (15.7±5 pg/day/10^6 cells [mean±SEM]), and no detectable amount of Ntf3. In contrast, the BDNF-SCs produced a significantly greater amount of BDNF (221±14 pg/day/10^6 cells) in comparison to the control Schwann cells ($p<0.05$). The Ntf3-SCs also produced significantly more Ntf3 than the control Schwann cells, producing 708±54 pg Ntf3/day/10^6 cells ($p<0.001$).

Neurotrophin over-expressing Schwann cells enhance SGN survival in vitro

SGNs were grown in co-culture with each of the three Schwann cell lines in order to assess the biological activity of the secreted neurotrophins and determine the survival-promoting effects of these cells in comparison to commercially available neurotrophins.

Co-culture of SGNs with the control EGFP-SCs resulted in the survival of 40±4 (mean±SEM) SGNs/well, which was standardised to 100% within each individual experiment.

Co-culture with the neurotrophin over-expressing Schwann cells significantly enhanced SGN survival over that elicited by the control EGFP-SCs. In comparison to the survival effects of the EGFP-SCs, the Ntf3-SCs increased SGN survival by 2-fold to 195±15% (mean±SEM; $p<0.01$). The BDNF-SCs further enhanced SGN survival, leading to a survival rate of 384±30% (mean±SEM; $p<0.001$), a 4-fold increase in survival over the control EGFP-SCs, and a 2-fold increase over the Ntf3-SCs. In addition, while there was no significant difference in the survival effects of the Ntf3-SCs versus either rhBDNF or rhNtf3, the BDNF-SCs significantly improved SGN survival as compared to both the rhBDNF (178±23%; mean±SEM) and rhNtf3 (223 ±20%; mean±SEM) treatments ($p<0.001$), (Figures 2 and 3).

DISCUSSION

Schwann cells were successfully genetically modified using a lipid-based technique to over-express the neurotrophins BDNF and Ntf3. Both BDNF-SCs (221±14 pg/day/10^6 cells) and Ntf3-SCs (708±54 pg/day/10^6 cells) produced significantly greater amounts of the respective neurotrophin as compared to the control Schwann cells, which were modified to express the reporter gene EGFP only.

In addition, the neurotrophin over-expressing Schwann cells significantly enhanced SGN survival *in vitro* when compared to the control EGFP-SCs, with Ntf3-SCs increasing the number of surviving SGNs by 2-fold, and BDNF-SCs supporting the survival of four times as many SGNs.

These results indicate that Schwann cells could successfully process and release the EGFP-tagged neurotrophins, and that these neurotrophins retained biological activities, as has been previously reported (Haubensak et al., 1998; Hartmann et al., 2001; Brigadski et al., 2005).

BDNF-SCs support SGN survival in vitro better than Ntf3-SCs

While both types of neurotrophin over-expressing Schwann cells significantly enhanced SGN survival in comparison to the control Schwann cells, the BDNF-SCs elicited the most potent survival effects. In fact, the BDNF-SCs supported the survival of twice as many SGNs as any

of the other neurotrophin treatments, including the Ntf3-SCs, despite the fact that Ntf3-SCs produced a significantly greater amount of Ntf3 than the BDNF-SCs did of BDNF. In addition, the BDNF-SCs enhanced SGN survival 2-fold over that elicited by the recombinant human neurotrophins, even though the concentration of these proteins (50ng/ml) was significantly greater than the amount of BDNF produced by the BDNF-SCs over the course of the experiments.

There are a number of arguments that could be used to explain these effects. Firstly, BDNF and Ntf3 are differentially expressed within the cochlea during development. Specifically, BDNF mRNA expression in SGNs is greatest in embryonic and early postnatal stages of development, while Ntf3 is only present in low levels during the early postnatal period (Ylikoski et al., 1993; Wheeler et al., 1994), suggesting that BDNF is more important for supporting the survival of immature SGNs. Also, the survival of early postnatal SGNs (P2–P10) has been shown to be greater in response to BDNF than Ntf3 (Mou et al., 1998). Therefore, since our SGN cultures were prepared from early postnatal (P6) rats, the SGNs may have been more responsive to the BDNF-SCs than the Ntf3-SCs, leading to the differences observed in terms of SGN survival. In comparison, the expression of Ntf3 seems essential for the maintenance of a robust SGN population in the mature adult cochlea (Ylikoski et al., 1993; Stankovic et al., 2004; Wissink et al., 2006). As such, it will be interesting to determine the response of SGNs to these neurotrophin over-expressing Schwann cells in adult animal models of deafness; in particular, whether mature SGNs are more responsive to Ntf3-SCs. Such experiments will also be imperative in ascertaining the most appropriate approach for clinically translational studies.

Secondly, Schwann cells have an intimate relationship with neurons and nerve fibres, with a number of important functions. For example, Schwann cells inherently produce small amounts of a variety of neurotrophic factors, including the neurotrophins BDNF, Ntf3, neurotrophin-4/5 and nerve growth factor, and the cytokines ciliary-derived neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF), to promote or ensure neuronal survival (Reynolds and Woolf, 1993; Frostick et al., 1998; Terenghi, 1999). It has also been reported that the survival effects of BDNF and Ntf3 on SGNs can be enhanced by combined application of CNTF or LIF. For example, CNTF can act in an additive fashion with each of BDNF and Ntf3 to improve SGN survival and neurite length (Staecker et al., 1995; Hartnick et al., 1996), while LIF can enhance the effects of both BDNF and Ntf3 in SGN cultures (Marzella et al., 1999; Gillespie et al., 2001), with BDNF and LIF acting synergistically to be the most potent stimulator of SGN survival *in vitro* (Marzella et al., 1999). This therefore suggests that the BDNF expressed by the BDNF-SCs may act in combination with other neurotrophic factors produced intrinsically by the Schwann cells, to synergistically boost the survival-promoting effects on SGNs. In contrast, the Ntf3 produced from the Ntf3-SCs may only act in an additive fashion with other inherently produced neurotrophic factors.

Furthermore, Schwann cells can influence the differentiation and growth state of neurons and control axon guidance by providing an environment that is favourable for growth (Reynolds and Woolf, 1993). Specifically, in addition to the production of neurotrophic factors, Schwann cells also produce and secrete extracellular matrix proteins such as laminin and fibronectin, and express cell adhesion molecules on their surface (Reynolds and Woolf, 1993; Frostick et al., 1998). Despite the fact that, during development, inner ear sensory neurons can differentiate in the absence of Schwann cells (Morris et al., 2006), nerve survival and growth in response to injury is facilitated and enhanced by the expression of these trophic and tropic molecules by Schwann cells. Furthermore, Schwann cells within the deafened cochlea can survive, at least for a limited period of time, despite SGN degeneration (Leake and Hradek, 1988; Hurley et al., 2007), and it has been suggested that Schwann cells can support their own survival by autocrine circuits involving neurotrophic signals (Mirsky and Jessen, 1999). The ability of

Schwann cells to respond to neurotrophic factors therefore suggests that Schwann cells have the capacity to stimulate and enhance reparative responses within the damaged cochlea.

Importantly, Schwann cells have previously been suggested as a possible source of neurotrophic support for SGNs (Hansen et al., 2001; Andrew et al., 2007), and *in vitro* experiments have reported improved SGN survival in cultures containing non-neuronal tissue, with the enhanced effects attributed to the direct cell-cell contact between Schwann cells and SGNs, and the Schwann cells serving as a substrate for neuronal attachment and growth (Whitlon et al., 2006). Therefore, in the current study, the microenvironment produced by the Schwann cells is also likely to have played a role in the enhanced survival effects elicited by the neurotrophin over-expressing Schwann cells in comparison to the recombinant neurotrophin proteins.

Ex vivo gene transfer for clinically relevant SGN rescue

One of the current limitations to research attempting to prevent deafness-induced SGN degeneration is the method by which to deliver trophic support in a clinically relevant manner. *Ex vivo* gene transfer, which involves the transplantation of cells that have been genetically modified to express exogenous therapeutic gene products, may serve this purpose. Numerous studies have in fact attempted *ex vivo* gene transfer as a potential therapeutic option to deliver neurotrophic factors, in a variety of systems, and using a variety of cell types. For example, Schwann cells genetically modified to over-express CNTF enhance axonal regeneration from rat retinal ganglion cells following optic nerve transection (Hu et al., 2005). In addition, both Schwann cells and fibroblasts over-expressing either nerve growth factor or BDNF can elicit robust axonal outgrowth following transplantation into the chronically injured rat spinal cord (Tuszynski et al., 1996; Grill et al., 1997b; Menei et al., 1998; Weidner et al., 1999; Tobias et al., 2005). Furthermore, cellularly delivered Ntf3 induces significant growth of corticospinal axons and also improves sensorimotor functional deficits after spinal cord injury in the rat (Grill et al., 1997a; Blits et al., 2000). Importantly, a recent study has reported that a cochlear implant electrode array coated with BDNF-secreting fibroblasts could reduce auditory nerve degeneration in the deaf guinea pig (Rejali et al., 2007).

Several criteria are essential for *ex vivo* gene transfer to be considered clinically viable. Such a technique would require that the cell type used (i) is readily attainable, (ii) can survive grafting for extended periods of time, (iii) is a suitable target for genetic modification, and (iv) pose no ethical controversy (Hendriks et al., 2004).

Based on these specifications, Schwann cells can be considered a suitable candidate cell type for the *ex vivo* delivery of neurotrophic factors. Firstly, Schwann cells can be obtained by peripheral nerve biopsy, allowing for autologous transplantation and thereby eliminating concerns of immunological rejection and inflammatory reactions, as well as eliminating ethical concerns. In addition, Schwann cells can be cultured *in vitro* such that sufficient numbers of cells can be obtained for implantation. Secondly, Schwann cells can survive transplantation into the guinea pig cochlea, and have been demonstrated to enhance SGN survival in comparison to deaf, untreated controls (Andrew et al., 2007), presumably as a result of the secretion of neurotrophic factors. Schwann cells have also been demonstrated to chronically survive grafting in other systems (Tuszynski et al., 1998), suggesting that there is also the potential for long-term survival within the fluid spaces of the cochlea. Finally, Schwann cells can be successfully genetically modified to over-express neurotrophins, and in fact, can maintain neurotrophin transgene expression for at least 12 months (Tuszynski et al., 1998). Most importantly, as demonstrated in the present study, neurotrophin over-expressing Schwann cells can enhance SGN survival over that achieved with both normal Schwann cells and traditional recombinant neurotrophin proteins.

However, *ex vivo* gene transfer within the cochlea may require combined and/or co-ordinated therapies, such as electrical stimulation via a cochlear implant, for ongoing, permanent rescue of SGNs from deafness-induced degeneration. While it has previously been shown that neurotrophin-induced SGN survival is not maintained beyond the treatment period (Gillespie et al., 2003), a recent study has demonstrated that the initial trophic advantage afforded by osmotic pump-based BDNF infusion can be maintained by chronic electrical stimulation (Shepherd et al., submitted). Interestingly, BDNF secretion can be triggered by electrical stimulation (Lessmann et al., 2003), which may have promising implications for combined neurotrophin and cochlear implant therapies. Future studies will need to assess the effects of cell-based neurotrophin delivery with electrical stimulation, in particular in terms of whether transient neurotrophin expression is sufficient or if stable production is required. The use of inducible promoters, in which transgene expression can be regulated and switched on and off, may also prove useful.

CONCLUSION

This study demonstrates that Schwann cells genetically modified to over-express the neurotrophins BDNF or Ntf3 can enhance SGN survival *in vitro* compared to both control Schwann cells and recombinant neurotrophin proteins. Transplantation of these neurotrophin over-expressing Schwann cells into the cochlea may provide a clinically relevant means of providing neurotrophic support and preventing SGN degeneration following a sensorineural hearing loss.

Acknowledgments

The authors would like to thank Dr Volkmar Lessmann from the Johannes Gutenberg Universität, Mainz, Germany, for generously providing the expression plasmids used for this study, and Dr Simon Murray and Professor Trevor Kilpatrick from the Howard Florey Institute, Melbourne, Australia, for providing the Schwann cells. We are also grateful to Dr Rachael Richardson and Dr Justin Tan for technical advice regarding the molecular biology aspects of the experiments. This work was supported by the NIDCD (N01-DC-3-1005), the Macquarie Bank Foundation, the State Government of Victoria, and the Bionic Ear Institute.

ABBREVIATIONS

BDNF
: brain-derived neurotrophic factor

BDNF-SCs
: Schwann cells over-expressing BDNF

CNTF
: ciliary-derived neurotrophic factor

DMEM
: Dulbecco’s modified Eagle’s medium

EGFP
: enhanced green fluorescent protein

EGFP-SCs
: Schwann cells expressing EGFP

ELISA
: enzyme-linked immunosorbent assay

FCS

fetal calf serum LF2000, Lipofectamine 2000

LIF
leukaemia inhibitory factor

Ntf3
neurotrophin 3

Ntf3-SCs
Schwann cells over-expressing Ntf3

P
postnatal day

PBS
phosphate buffered saline

rh
recombinant human

SCM
Schwann cell media

SGN
spiral ganglion neuron

SNHL
sensorineural hearing loss

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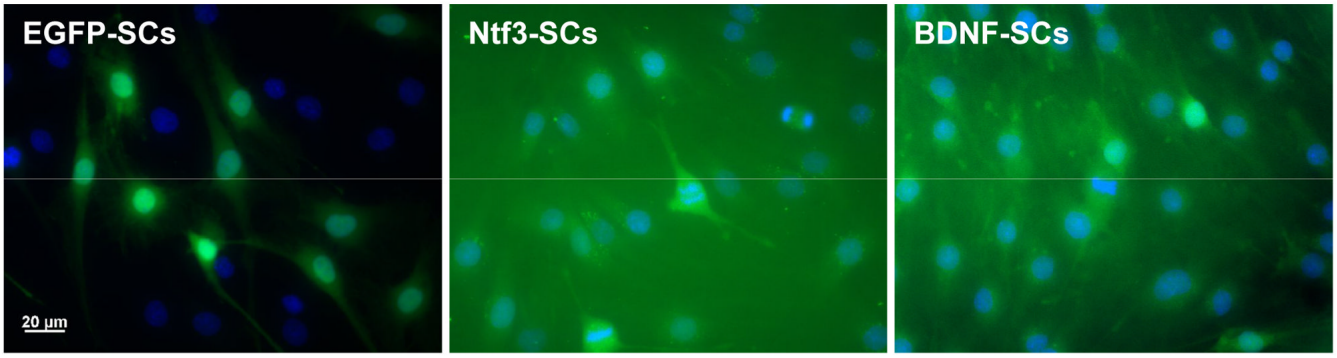

Figure 1.

Successful transformation of Schwann cells was confirmed by the presence of the EGFP reporter gene. ELISA analysis indicated that the BDNF-SCs produced 221±14 pg BDNF/day/10^6 cells, while the Ntf3-SCs produced 708±54 pg Ntf3/day/10^6 cells, significantly greater amounts of either neurotrophin than the control EGFP-SCs.

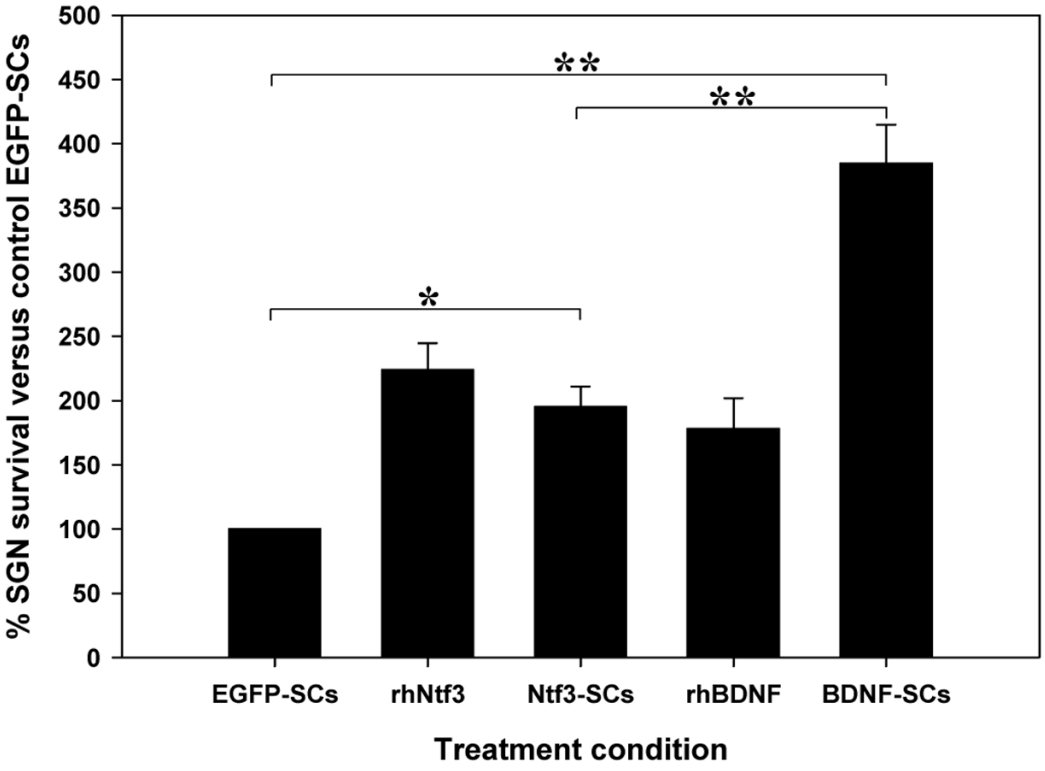

Figure 2.

Co-culture with either the BDNF-Schwann cells or the Ntf3-Schwann cells led to a significant increase in auditory neuron survival in comparison to that elicited by both the respective neurotrophin controls (rhBDNF and rhNtf3), and the control Schwann cells. * $p<0.01$; ** $p<0.001$.

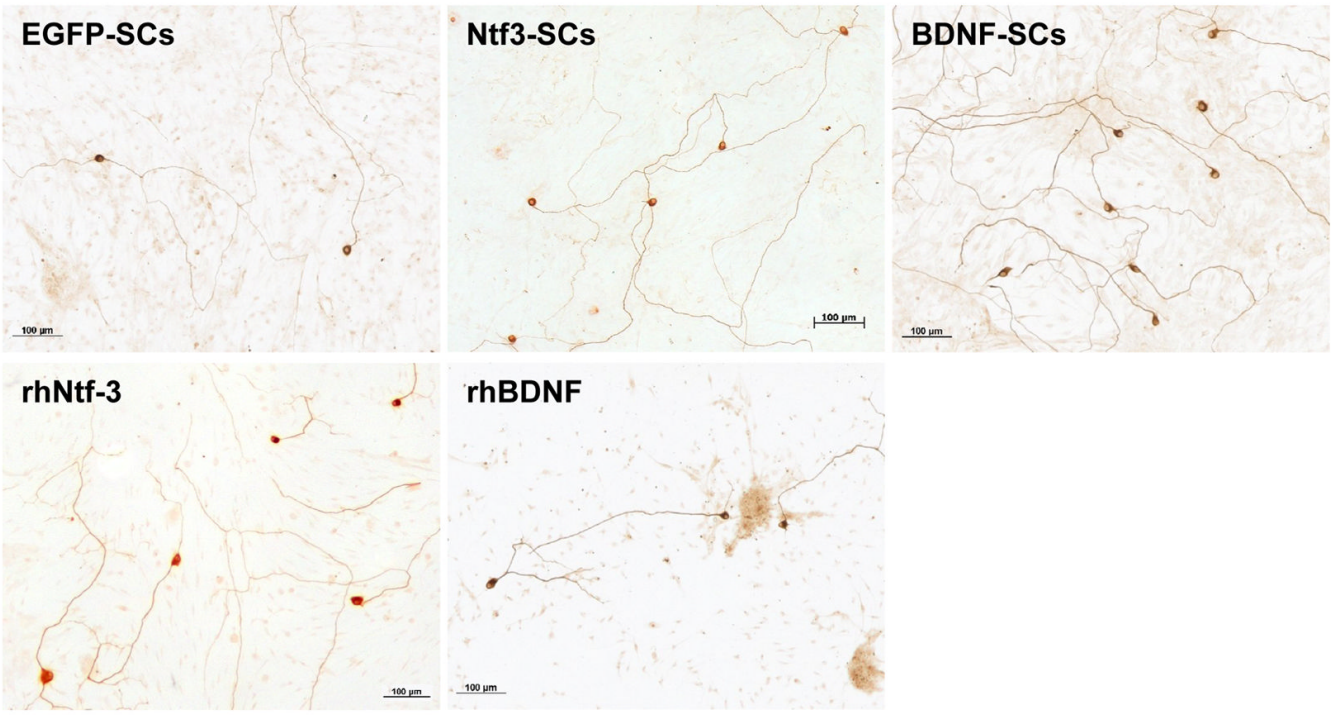

Figure 3.
Survival of postnatal day six rat SGNs when grown in co-culture with the various Schwann cell lines, or in the presence of recombinant human neurotrophin proteins. SGN survival was greatest in the BDNF-Schwann cell co-cultures ($p<0.001$).