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DEVELOPMENT OF A CELL-BASED TREATMENT FOR LONG-TERM NEUROTROPHIN EXPRESSION AND SPIRAL GANGLION NEURON SURVIVAL

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Abstract—Spiral ganglion neurons (SGNs), the target cells of the cochlear implant, undergo gradual degeneration following loss of the sensory epithelium in deafness. The preservation of a viable population of SGNs in deafness can be achieved in animal models with exogenous application of neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3. For translation into clinical application, a suitable delivery strategy that provides ongoing neurotrophic support and promotes long-term SGN survival is required. Cell-based neurotrophin treatment has the potential to meet the specific requirements for clinical application, and we have previously reported that Schwann cells genetically modified to express BDNF can support SGN survival in deafness for 4 weeks. This study aimed to investigate various parameters important for the development of a long-term cell-based neurotrophin treatment to support SGN survival. Specifically, we investigated different (i) cell types, (ii) gene transfer methods and (iii) neurotrophins, in order to determine which variables may provide long-term neurotrophin expression and which, therefore, may be the most effective for supporting long-term SGN survival *in vivo*. We found that fibroblasts that were nucleofected to express BDNF provided the most sustained neurotrophin expression, with ongoing BDNF expression for at least 30 weeks. In addition, the secreted neurotrophin was biologically active and elicited survival effects on SGNs *in vitro*. Nucleofected fibroblasts may therefore represent a method for safe, long-term delivery of neurotrophins to the deafened cochlea to support SGN survival in deafness.

Key words: neurotrophin, nucleofection, lipofection, lentivirus, spiral ganglion neuron, sensorineural hearing loss.

INTRODUCTION

Sensorineural hearing loss (SNHL), the most common form of deafness, is typically caused by the loss of cochlear hair cells. The only therapeutic treatment for patients with severe-profound SNHL is a cochlear implant – a neural prosthesis that electrically stimulates the residual spiral ganglion neuron (SGN) population to provide the rate and pitch cues necessary for speech perception. In the normal cochlea, the hair cells and supporting cells of the organ of Corti support the survival of SGNs through endogenous neurotrophin secretion (Fritzsch et al., 2004; Stankovic et al., 2004; Green et al., 2012; Zilberstein et al., 2012), and therefore damage to the organ of Corti and loss of this neurotrophin support, as occurs in SNHL, leads to the loss of SGNs. Since SGNs are the target cells for the cochlear implant, the loss of a significant population of SGNs may compromise the function of the device (Pfingst and Sutton, 1983; Shepherd and Javel, 1997; Hardie and Shepherd, 1999). Furthermore, future developments in device and software design may also benefit from an enhanced SGN population (Wise and Gillespie, 2012).

Exogenous delivery of neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) can support SGN survival in models of deafness (Ernfors et al., 1996; Miller et al., 1997; Gillespie et al., 2003, 2004; Yamagata et al., 2004; Richardson et al., 2005; Shepherd et al., 2005). However, the cessation of exogenous neurotrophin treatment can result in a loss of these survival effects (Gillespie et al., 2003; Shepherd et al., 2005). While others have reported continued auditory neuron survival for 2 weeks post-treatment (Agterberg et al., 2009), long-term outcomes remain unknown. Chronic electrical stimulation via a cochlear implant extends neurotrophin-based survival effects past the end of neurotrophin treatment; however, to maximize

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Abbreviations: BDNF, brain-derived neurotrophic factor; D10, DMEM plus FCS, L-glutamine, penicillin and streptomycin; D10M, D10 medium plus bovine pituitary extract and forskolin; DMEM, Dulbecco's Modified Eagle's Medium; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IQR, inter-quartile range; NT3, neurotrophin-3; PBS, phosphate-buffered saline; pEGFP, plasmid encoding EGFP; pBDNF-E, plasmid encoding EGFP-tagged BDNF; pNT3-E, plasmid encoding EGFP-tagged NT3; pLV-EGFP, lentiviral plasmid encoding EGFP; pLV-BDNF, lentiviral plasmid encoding BDNF; pLV-NT3, lentiviral plasmid encoding NT3; SGNs, spiral ganglion neurons; SNHL, sensorineural hearing loss; TBS-T, Tris-buffered saline with Tween 20.

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SGN rescue, long-term neurotrophin delivery is desirable (Shepherd et al., 2005, 2008).

Current methods of neurotrophin delivery into the cochlea, such as osmotic pumps, are not considered suitable for clinical application (Pettingill et al., 2007). Alternative pump-based delivery systems must be re-filled at regular intervals, necessitating multiple surgical procedures. This poses a small but significant risk of infection, which could result in labyrinthitis and meningitis (Wei et al., 2008). Furthermore, neurotrophins have a short half-life (Lindholm et al., 1988; Matsuoka et al., 1991; Poduslo and Curran, 1996; Kishino et al., 2001), meaning that the use of long-term pump delivery systems with high volume capacities may be complicated by the unknown bioactivity of neurotrophins maintained at body temperature for long periods.

Cell-based therapies, in which cells secreting a therapeutic substance are implanted into a patient, are an alternative mechanism for continuous delivery of neurotrophins into the cochlea (for review see Zanin et al., 2012). Cell-based therapies may utilize cells which naturally secrete therapeutic agents (Wise et al., 2011), or may be combined with gene transfer techniques to genetically modify cells to secrete the desired therapeutic agent(s) (Pettingill et al., 2008, 2011). Cell-based therapies provide an avenue for delivering neurotrophins at physiological levels and in a consistent manner, and also overcome issues of infection (Shepherd, 2011) and longevity of survival effects (Gillespie et al., 2003; Shepherd et al., 2005) associated with other experimental delivery methods. In addition, cell-based therapies have the potential for long-term neurotrophin expression (Winn et al., 1996). For these reasons, cell-based therapies are considered clinically viable, and have already been implemented for therapeutic drug delivery in clinical trials for various neurodegenerative conditions (for review see Zanin et al., 2012).

Previously, we successfully genetically modified Schwann cells using lipofection to express BDNF or NT3 and demonstrated that these cells could support SGN survival *in vitro* (Pettingill et al., 2008). Furthermore, we reported that the implantation of encapsulated, BDNF-secreting Schwann cells into the deaf guinea pig cochlea successfully supported SGN survival over 2- and 4-week implantation periods (Pettingill et al., 2011). While promising, longer term studies, using cells with a greater duration of neurotrophin secretion, are required in order to best assess the potential of this therapy for ongoing SGN survival (Pettingill et al., 2011).

There are numerous experimental parameters that may play an important role in achieving longer term neurotrophin expression from a cell-based treatment. In the current study, we investigated different (i) cell types, (ii) gene transfer techniques and (iii) neurotrophins, with the aim of developing a population of cells that reliably secreted neurotrophin for periods of time significantly greater than 4 weeks.

EXPERIMENTAL PROCEDURES

We utilized an array of test conditions in order to develop cells with long-term neurotrophin expression. Specifically, Schwann cells and fibroblasts were genetically modified using lipofection or nucleofection to express BDNF or NT3. Schwann cells were also genetically modified using lentiviral vectors to express these neurotrophins. The resultant neurotrophin-expressing cells were compared in terms of transfection efficiency, and duration and quantity of neurotrophin expression. The bioactivity of the secreted neurotrophins was assessed by quantifying survival effects on SGNs *in vitro*.

All experiments involving animals were carried out with approval from the Animal Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital, Australia (Project # 09/179AB), in accordance with The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997), and the National Institute of Health Guide for the Care and Use of Laboratory Animals (1996). All efforts were made to minimize pain and the number of animals used.

Preparation of primary Schwann cell and fibroblast cultures

Schwann cells and fibroblasts were isolated from rat sciatic nerve explants using established protocols (Morrissey et al., 1991; Plant et al., 2002; Godinho et al., 2013). Briefly, 8–10-week-old rats were killed using sodium pentobarbitone (150 mg/kg intraperitoneally) and the sciatic nerves removed. The perineurium was dissected from the nerve and the nerve was cut into segments of approximately 1-mm length and placed into 60-mm dishes containing 1 mL D10 medium [Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) with 10% fetal calf serum (FCS; Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin (Invitrogen) and 100 mg/mL streptomycin (Invitrogen)], and incubated at 37 °C in 5% CO_2. Fibroblasts began to migrate from the nerve explants first (Plant et al., 2002; Godinho et al., 2013), and could be collected by repeatedly re-plating the explants into new tissue culture plates to avoid Schwann cell contamination. Specifically, 1 week after the initial plating, the explants were transferred to a new dish containing D10, and the remaining adherent cells were washed with phosphate-buffered saline (PBS; pH 7.2), the medium was replenished, and the fibroblasts were cultured at 37 °C in 5% CO_2. This procedure was repeated on a weekly basis for 3–4 weeks, until Schwann cells were identified growing out from the explants, as based upon their spindle-like morphology. All tissue culture dishes containing only fibroblasts were then trypsinised and the cells pooled and grown in D10 in 75-mm tissue culture flasks.

The Schwann cell-containing explants were then collected and dissociated using a solution of dispase (1.25 U/mL) and collagenase (0.05%), spun, re-suspended in D10 containing the mitogens bovine pituitary extract (20 μg/mL) and forskolin (2 μM/mL) [D10M], and grown in tissue culture flasks coated with poly-L-lysine (100 μg/mL; Sigma).

Immunostaining of Schwann cell and fibroblast cultures. Confluent cells grown on 2-well chamber slides were fixed with ice-cold methanol (30 min), rinsed with PBS and immunostained with antibodies to S100 and