



The feasibility of an encapsulated cell approach in an animal deafness model

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ABSTRACT

For patients with profound hearing loss a cochlear implant (CI) is the only treatment today. The function of a CI depends in part of the function and survival of the remaining spiral ganglion neurons (SGN). It is well known from animal models that inner ear infusion of neurotrophic factors prevents SGN degeneration and maintains electrical responsiveness in deafened animals. The purpose with this study was to investigate the effects of a novel encapsulated cell (EC) device releasing neurotrophic factors in the deafened guinea pig.

The results showed that an EC device releasing glial cell line-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factor (BDNF) implanted for four weeks in deafened guinea pigs significantly preserved the SGNs and maintained their electrical responsiveness. There was a significant difference between BDNF and GDNF in favour of GDNF. This study, demonstrating positive structural and functional effects in the deafened inner ear, suggests that an implanted EC device releasing biologically protective substances offers a feasible approach for treating progressive hearing impairment.

1. Introduction

The World Health Organization (WHO) estimates that 360 million people worldwide, or > 5% of the world's population suffers from disabling hearing loss [1]. Loss of auditory function is caused primarily by damage or loss of sensory cells (hair cells) in the inner ear cochlea as a result of acoustic trauma, exposure to ototoxic drugs, cochlear infection, genetic abnormalities, or aging. Loss of sensory cells results in a subsequent degeneration of spiral ganglion neurons (SGNs) [2,3], which are primarily bipolar neurons that relay auditory information from the sensory cells of the organ of Corti to the central auditory system. There are two types of SGNs, Type I and Type II. In mammals, neither the sensory cells nor the SGNs have the ability to regenerate, and there are currently no effective interventions for their repair. Currently, the only available therapeutic intervention for patients with a profound hearing loss is a cochlear implant (CI). With a CI, the malfunctioning sensory cells are bypassed and the subsequent functional level of the auditory system is directly stimulated via an electrode inserted into the fluid-filled cochlea. Type I neurons constitute 90–95% of SGNs and are the targets of CI.

The effectiveness of CI is thought to be related to the number and functional state of SGNs [4,5]. Several animal studies have shown a correlation between electrical responsiveness, measured by obtaining electrically-evoked auditory brainstem responses (eABRs), and the number of remaining SGNs in deafened animals [6–8]. Consequently,

extensive experimental research has explored the possibilities of protecting and maintaining SGNs and their electrical responsiveness in order to optimize the beneficial effect of implanted CI. However, due to the natural barriers between fluid compartments accessible via systemic infusions and the compartments related to the inner ear (e.g., the blood-perilymph barrier and the intrastrial fluid-blood barrier), it has proven difficult to pharmacologically target the inner ear. Especially the intrastrial fluid-blood barrier restricts the transport of drugs into the inner ear [9]. Recent studies have therefore focused on developing methods for local inner ear drug delivery mainly using osmotic pumps connected to a cannula positioned in scala tympani [6,7,10,11]. However, approaches using adenoviral transfection [12,13] and encapsulated cells [14] have been explored. It has been shown that neurotrophic factors released by osmotic pumps preserve and maintain SGNs in traumatized inner ear. Moreover, it has been shown that neurotrophic factor treatment results in a significant decrease in eABR thresholds, suggesting that the functionality of SGNs is also preserved [6,7,10,11,15]. However, even though experimental intracochlear treatment using neurotrophic factors has been successful, the administration techniques need further development in order for this therapeutic concept to be tested in a clinical setting.

An interesting approach, which has already been tested clinically for the treatment of neurodegenerative disease [16,17], is using a delivery system based on implantation of an encapsulated cell (EC) device. Briefly, the EC device [18,19] consists of a semi-permeable membrane

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enclosing genetically modified human cells that release neurotrophic factors. The semi-permeable thermoplastic membrane protects the encapsulated cells by preventing the entry of damaging elements of the host immune system while allowing the cell-produced neurotrophic factors to diffuse out into the surrounding host tissue. This EC device offers not only sustained local neurotrophic factor delivery, but also the possibility to terminate the treatment by removing the retrievable device [20].

The EC device was designed to fit the guinea pig inner ear and implanted in a CI experimental model in order to monitor the functional effects of neurotrophic factors released from the EC. It is demonstrated that the EC device can preserve the functionality of SGNs in the deafened guinea pig.

2. Materials and methods

2.1. Experimental design

Normal hearing guinea pigs were experimentally deafened by locally infusing an ototoxic drug, neomycin, into the middle ear. Three days after the injection, the animals were tested for hearing loss using acoustically-evoked auditory brainstem response (ABR). After three weeks, when ABR recordings demonstrated significant hearing loss, the animals were implanted with an EC device and a stimulus electrode mimicking a CI. The animals were then divided into four groups based on the cellular contents of the implanted EC device: 1) glial cell-derived neurotrophic factor (GDNF)-releasing cells (GDNF/ARPE-19); 2) brain-derived neurotrophic factor (BDNF)-releasing cells (BDNF/ARPE-19); 3) non-modified cells of the parental cell line (ARPE-19); and 4) devices not containing cells (Cell-free). A custom-made stimulus electrode was implanted into the scala tympani of the inner ear in order to elicit evoked potentials (eABRs) that reflect SGN responsiveness. After four weeks, the animals were sacrificed and the EC devices retrieved to measure the final level of neurotrophic factor release.

2.2. EC devices

The EC device contained cells of a genetically modified human cell line enclosed within a semi-permeable fibre membrane having a unique isoreticulated pore structure that allows influx of oxygen and nutrients to nourish the cells, and an outflow of therapeutic factor(s) into the surrounding tissue (Fig. 1A and B). The semi-permeable membrane protects the encapsulated cells from an immune response and rejection, and therefore no immunosuppression is required [16,18]. The release of BDNF and GDNF was confirmed in vitro in a separate study (Dash-Wagh et al., in preparation), in which the encapsulated cell devices were co-cultured with tissue explants from the newborn rat spiral ganglion (postnatal days 3–5). After a three-day period, there was a significant effect on neuronal survival and neurite outgrowth.

The EC device used in the present study was designed to fit the guinea pig cochlea. The diameter was 0.4 mm and the length was 4 mm excluding the tether. The tether was used to steer the device and precisely position it without damaging the membrane. In the present study the devices contained ARPE-19 neurotrophic-factor producing cell line. The parental ARPE-19 cell line is a spontaneously immortalized human retinal pigment epithelial cell line. The native cells were transfected with plasmid DNA encoding GDNF or BDNF using the sleeping beauty technique [21]. Both BDNF and GDNF were cloned from human DNA sequence. Growth factor release levels were measured using enzyme-linked immunosorbent assay (ELISA). A total of 30 EC devices were implanted in four experimental groups: GDNF/ARPE-19 ($n = 9$), BDNF/ARPE-19 ($n = 8$), ARPE-19 (parental cell line, $n = 7$), and Cell-free (empty device, $n = 6$).

The EC devices were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; 1:1) and 10% human endothelium serum free medium (Invitrogen). Before implantation,

neurotrophic factor release from the majority of the EC devices was measured using ELISA. After the final eABR measurement, the EC device was retrieved from the subjects for a second release measurement.

2.3. Deafening

Normal hearing animals were deafened by transtympanic injections of the ototoxic drug neomycin [6]. Within 48 h, most sensory cells had degenerated. The sensory cell loss resulted in a secondary degeneration of SGNs due to loss of neurotrophic support. In order to mimic the conditions of hearing impaired patients, the animals were deafened three weeks prior to device implantation. In some subjects, neomycin was administered repeatedly (maximum three extra doses) in order to produce significant damage to the auditory sensory cells. For this study, only animals with a threshold shift of 45–65 dB sound pressure level (SPL) were used.

2.4. Implantation surgery

The guinea pigs were deeply anaesthetized using ketamine (40 mg/kg i.m.) and xylazine (10 mg/kg i.m.). An ophthalmic ointment was then applied to the eyes to prevent corneal ulcers due to ketamine-induced suppression of the blink reflex. Marcain, a local anesthetic, was injected subcutaneously on the head, neck, and behind the ear. The middle ear cavity was opened to expose the cochlea and a cochleostomy was made in the basal part of the cochlea in order to get access to the scala tympani. Using the tether connected to the EC device, the device was then inserted into the scala tympani. To prevent perilymph leakage from scala tympani, a small piece of fascia was put around the tether to seal the cochlea hole. The electrode mimicking a CI has previously been described [22]. Briefly, a stimulus electrode was inserted through the round window membrane into scala tympani and the ground electrode was placed against the wall in the middle ear cavity.

After the last eABR measurement, the temporal bone was again opened to retrieve the GDNF- and BDNF-releasing devices (e.g., experimental groups GDNF/ARPE-19 and BDNF/ARPE-19). A final measurement was made to verify the release of neurotrophic factors throughout the experiments (in a few animals, it was not possible to retrieve the EC device as the tether detached or the device was damaged during retrieval).

2.5. Electrically-evoked Auditory Brainstem Response (eABR)

The animals were anaesthetized as previously described and placed in a sound proof box. The eABRs were recorded using a SigGen system 2 signal analyzer (Tucker-Davis Technologies, FL), as previously described [23]. Briefly, responses to monophasic current pulses (50- μ s long; presented at 50 pps) with alternative polarity were recorded between a permanent electrode placed at the vertex (active), a subdermal needle electrode placed subcutaneously above the bulla on the deafened ear (reference) and a ground electrode placed in the hind leg. The thresholds were monitored and defined as the lowest stimulus level in 10- μ A steps that elicited a reproducible waveform.

2.6. Histology

After the final eABR measurement, the animals were deeply anaesthetized with pentobarbital sodium (25 mg/kg i.p.) and transcardially perfused with saline (37 °C) followed by cold glutaraldehyde (2.5% in 0.1 M phosphate buffer). The temporal bone was removed and opened to expose the cochlea. Small fenestrations were made in the round window membrane and at the apex in order to gently flush glutaraldehyde through the cochlea. After fixation the cochleae were decalcified in 0.1 M ethylenediaminetetraacetic acid (EDTA) in 0.1 M phosphate buffer, and prepared for histology as previously described [6]. In short, the cochleae were rinsed, dehydrated and embedded in

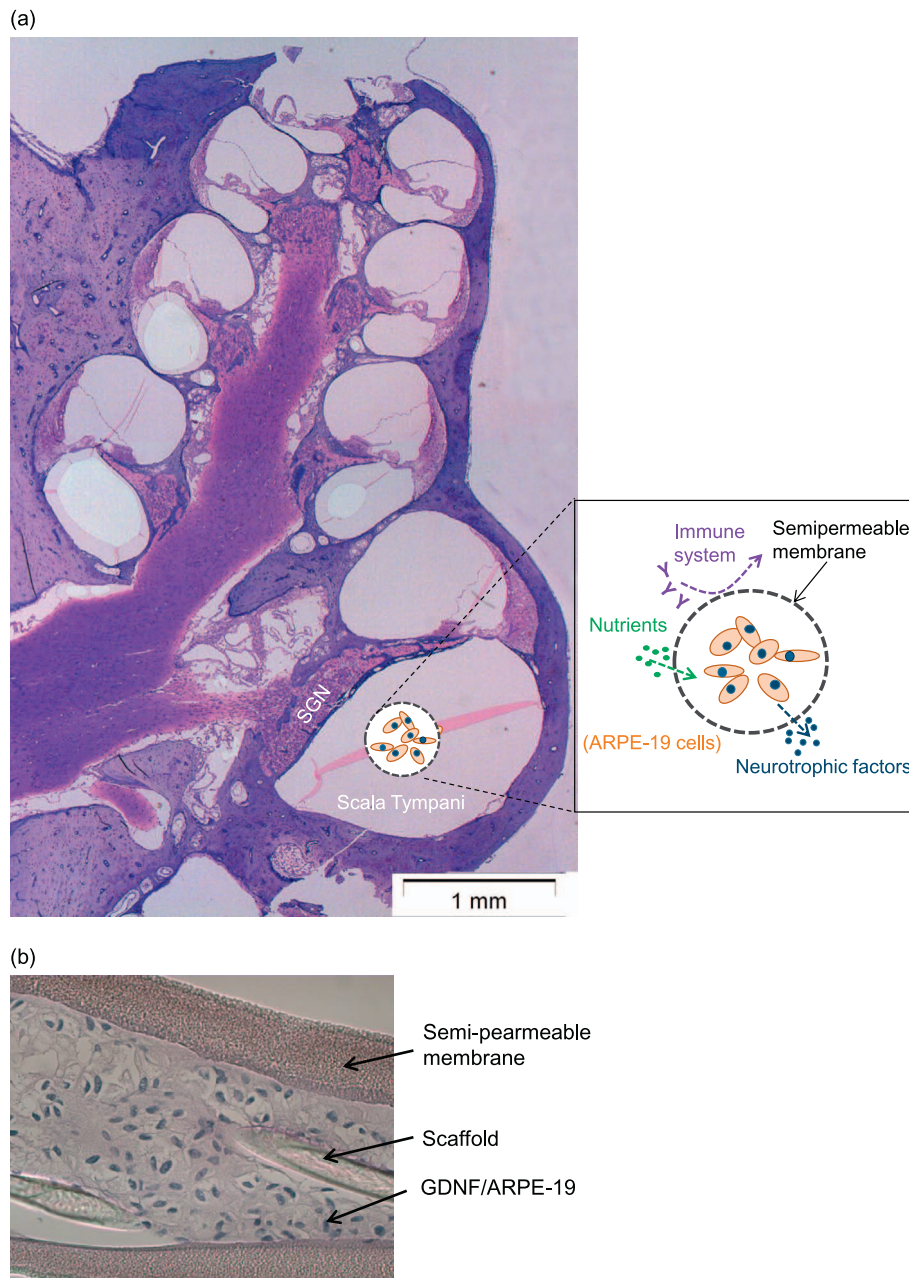


Fig. 1. A Micrograph showing a mid-modiolar cross-section of a normal cochlea. The location and size of an EC device implanted in scala tympani are illustrated schematically. B. Micrograph showing part of an unused EC device embedded in paraffin, sectioned, and stained (20 \times). The EC device in the micrograph contained GDNF/ARPE-19 cells stained with haematoxylin and eosin.

JB-4 plastic (Polyscience Ink., PA) and sectioned at 4- μ m thick sections. When reaching the mid-modiolar plane, every third section was mounted and stained with toluidine blue. By selecting every third section it was ensured that each neuron was counted only once. Six mid-modiolar sections from each animal were chosen. The outline of the Rosenthal's canal was traced (ZEN Imaging Software, Zeiss) to estimate the area, the number of remaining SGNs that were counted, and the measured SGN area. The SGN density was expressed as the number of SGNs per 10,000 μ m², and the average density of SGNs was calculated. A total of 36 Rosenthal's canal sections were examined from each animal. Additionally, sensory cell regions were examined in order to confirm cellular degeneration. All hair cells were missing or severely damaged except in a few animals where the remaining hair cells were found in the most apical part of the cochlea.

The results used to display conditions for normal animals (Figs. 4–6) originate from a previous study [6]. The data illustrating SGN density in normal animals (not deafened, no treatment) shown in Fig. 4 has been previously published [6]. However, the SGNs have been re-measured

and analysed using the ZEN Imaging software (ZEN Imaging Software, Zeiss) used in the present study. The results related to the normal situation (Figs. 5A and 6) have not been published previously.

2.7. Subjects

A total of 30 guinea pigs of both sexes (200–330 g; Harlan Laboratories Inc., Netherlands) were used. The animals were kept in an enriched environment and housed in small groups with lights on between 7 am and 7 pm, and with temperature maintained at 21 °C and a humidity of 60%. All animal procedures were performed in accordance with the ethical guidelines of Karolinska Institutet and consistent with national regulations for care and use of animals. The local ethics committee approved the experimental procedures (Stockholms Norra Djurförsöksetiska Nämnd; approval N165/13).

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical analysis of the eABR study and evaluation of SGN density and area. Data are presented as mean \pm SEM. Statistically significant difference was considered at $p < 0.05$.

3. Results and discussion

A few models of encapsulated cells for inner ear treatment such as alginate capsules [24] or devices based on nanoparticles [25–27] have been presented in the last decade. In the present study, the device contained encapsulated cells (EC) that were genetically modified for long-term overexpression of BDNF or GDNF. A similar EC device has previously been tested in the clinic on patients with Alzheimer's disease [16,17]. In these studies, an EC device delivering nerve growth factors was surgically implanted into the lateral basal forebrain. It remained in place for twelve months before being retrieved. The studies showed that the surgery was safe and well tolerated by the patients. Konerding et al., [28] recently studied the combined effect of repeated long-term electrical stimulation (suggested to reduce SGN degeneration) and an implanted GDNF-releasing EC device on the inner ear. The effect on electrical responsiveness of the auditory system was relatively weak, and the preservation of SGNs was only evident in animals that were also subjected to electrical stimulation.

3.1. Cochlear implant model

In humans, the time period between the first signs of hearing impairment caused by malfunctioning sensory cells and finally being selected as a candidate for cochlear implantation can be quite long. Fortunately, secondary degeneration of human SGNs occurs over several years following sensory cell damage, thus usually leaving a sufficiently large neural population that can be electrically stimulated using a CI. We have established an experimental animal model that mimics the clinical situation but with the degenerative process being much faster [29]. Using an implanted stimulus electrode, eABRs could be measured for several weeks. The present study used three-week deafened guinea pigs that already had a 40% SGN loss [6,30]. The functional impairment was confirmed using ABR recordings prior to implantation of the CI in order to avoid any significant differences between the experimental groups. The threshold shifts were 47.7 ± 1.5 (GDNF/ARPE-19), 49.4 ± 1 (BDNF/ARPE-19), 52.9 ± 3.4 (ARPE-19), and 49.2 ± 2.7 (Cell-free).

3.2. Effects on electrical responsiveness

To test whether the release of neurotrophic factors from the implanted EC devices affected auditory function, eABRs were measured weekly throughout the 4-week treatment period. The results showed a significant difference in eABR thresholds between the two groups treated with neurotrophic factors (i.e., GDNF and BDNF released from the EC device) and the two control groups (Fig. 2). After experimental week 4 (one week of treatment) there was already a significant difference ($p < 0.001$) between the GDNF/ARPE-19 group and the group implanted with empty devices (Cell-free). From experimental week 5 onwards there was also a difference between the BDNF/ARPE-19 and the Cell-free group. Interestingly, there was initially also a significant difference between the two control groups ($p < 0.05$) in favour of the ARPE-19 group. However, after this first measurement (experimental week 4), no more differences were found between the control groups (ARPE-19 and Cell-free). The results suggest that the non-modified ARPE-19 cells may have also released protective factors, but that the positive effects were insignificant as neural degeneration progressed. The GDNF/ARPE-19 group showed significantly lower eABR thresholds compared to the BDNF/ARPE-19 group. There was a significant

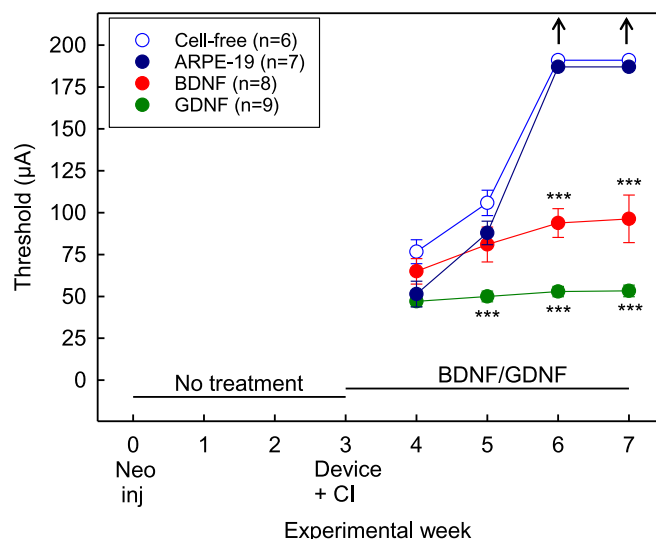


Fig. 2. Animals exposed to BDNF or GDNF released from the EC devices (BDNF/ARPE-19 and GDNF/ARPE-19, respectively) displayed significantly lower eABR thresholds compared to the two control groups (ARPE-19 or Cell-free). From experimental week 5, there was a statistically significant difference ($p < 0.01$) between the BDNF/ARPE-19 and GDNF/ARPE-19 groups, and between the two control, and GDNF/ARPE-19 groups ($p < 0.001$). The difference increased throughout the experiment with GDNF/ARPE-19 expressing the lowest eABR threshold. At experimental week 6 and 7 no electrical responses could be elicited in the control groups using the present equipment. *** $p < 0.001$.

difference at experimental week 5 ($p < 0.01$), which became more pronounced over time (experimental week 6 and 7; $p < 0.001$).

As illustrated in Fig. 3, there was considerable variation in the individual eABR thresholds within the BDNF/ARPE-19 group, a variation that was not observed in the GDNF/ARPE-19 group. After the EC device retrieval, neurotrophic factor release from the BDNF/ARPE-19 devices was considerably lower than the GDNF/ARPE-19 EC-devices. The pre-implantation release levels were approximately 32.5 ng/device/24 h and 7.2 ng/device/24 h for GDNF and BDNF, respectively. It is noteworthy that the release of GDNF increased (99.9 ng/device/24 h) over the 4-week implantation period, whereas BDNF release decreased (4.0 ng/device/24 h). However, for both GDNF and BDNF, there was a considerable spread within the groups. Except for a few devices where the tether came off while still implanted in scala tympani, all EC devices were retrieved and the concentration of neurotrophic factor release measured using ELISA. The measurements showed that two of the devices, one from the BDNF/ARPE-19 group and the other from the GDNF/ARPE-19 group, did not release any therapeutic protein. It is not possible to determine when the release ceased for these two devices. However, the final eABR thresholds obtained from these animals were not significantly different from the average in the respective group.

3.3. Effects on the SGN population

It has been hypothesized that the electrical responsiveness of the inner ear is closely related to the number of remaining SGNs in Rosenthal's canal. It is well established that neurotrophic factor treatment of the deafened inner ear does not only increase SGN survival, but also results in preservation of neuronal electrical responsiveness [6,31–35]. All experimental groups were analysed according to the density of SGNs. For comparison, a group of normal animals (no treatment) was included. The number of remaining SGNs was significantly higher in the GDNF/ARPE-19 implanted animals than not only the two control groups (ARPE-19, $p < 0.001$; Cell-free, $p < 0.01$), but also the BDNF/ARPE-19 group ($p < 0.05$). There were also significant differences in the amount of remaining SGNs along the length of the cochlea in both the BDNF and GDNF groups, with both

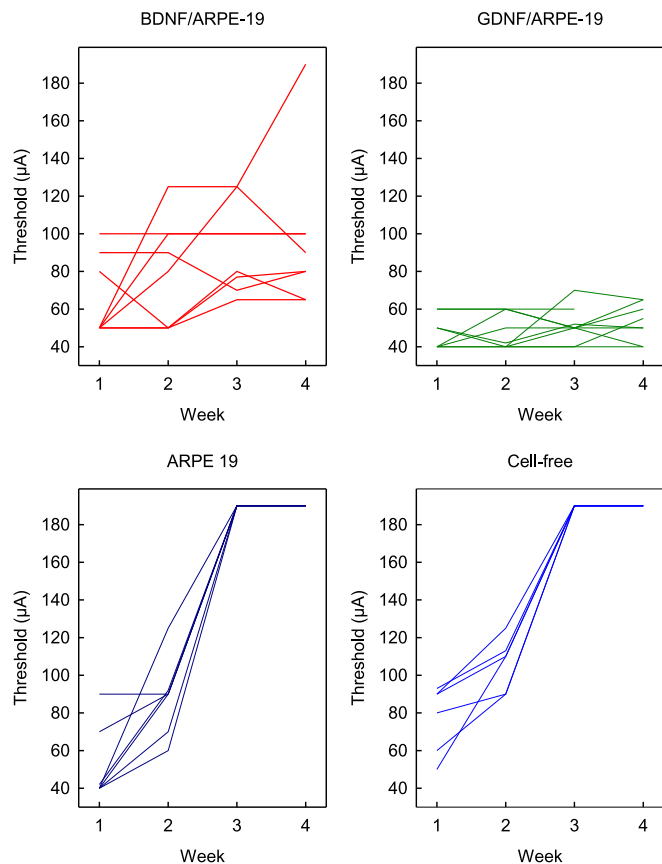


Fig. 3. Individual eABR thresholds obtained in each experimental group. Compared to the other groups, the BDNF/ARPE-19 group showed considerably more variation in the measured thresholds. The animals in the GDNF/ARPE-19 group showed a small or no increase in eABR thresholds throughout the experiment. The results from the two control groups showed a significant difference ($p < 0.05$) at experimental week 4 (one week of treatment) in favour of the group implanted with the EC devices with ARPE-19 cells. At experimental week 6 and 7 (treatment week 3 and 4), it was not possible to elicit any responses from either control group.

displaying a gradual decrease in cell density from base to apex (base vs. apex, BDNF $p < 0.001$; GDNF $p < 0.01$). No differences were found within the cochlea of the control (Cell-free) group. Similarly, no cell density differences along the cochlea were observed in the group of normal animals. Interestingly, a significant difference (less neurons remaining at apical locations) was seen in the ARPE-19 group, suggesting that the parental cell line also exerted a biological effect.

SGNs receive neurotrophic support from hair cells in the organ of Corti and target neurons within the cochlear nucleus during development [36–38]. BDNF is expressed in hair cells in the organ of Corti during embryonic development, but the levels decline in the prenatal period such that the mRNA levels are very low on postnatal day 1. In adult animals, the SGN expression of BDNF is higher in the basal part of the cochlea compared to more apically located regions [39]. In contrast, GDNF is only found postnatally, and is equally distributed along the cochlea [40]. The different pattern of BDNF and GDNF expression in the adult inner ear most likely contributes to the present observation that GDNF released within the cochlea resulted in more pronounced effects on eABR thresholds and SGN survival.

The structural effects of EC-devices releasing GDNF/ARPE-19 and BDNF/ARPE-19 compared to the control groups are shown in Fig. 5. A distinct difference in SGN densities in the four experimental groups is clearly seen and correlates well with the results shown in Fig. 4.

To estimate possible differences in soma size of the remaining cells, the cross-sectional area of SGNs was measured in all groups (Fig. 6). A significant difference was found in the GDNF/ARPE-19 group compared

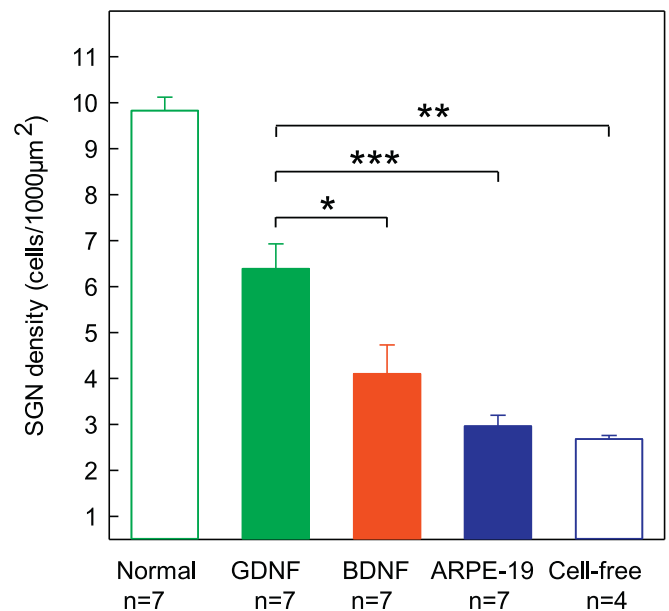


Fig. 4. SGN density in the experimental groups and normal animals. The GDNF/ARPE-19 implanted animals showed a significantly larger population of remaining SGNs compared to the BDNF/ARPE-19, ARPE-19, and Cell-free device groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

to the groups implanted with devices containing either ARPE-19 or Cell-free devices ($p < 0.01$ and $p < 0.05$, respectively). The smallest soma size was found in the group with normal animals. Significant differences were seen in the BDNF/ARPE-19 vs. Normal ($p < 0.05$), ARPE-19 vs. Normal ($p < 0.001$), and Cell-free vs. Normal ($p < 0.05$) groups. However, there was no significant area difference between the GDNF treated and cells in the normal group. This may suggest a positive effect of GDNF at the cellular level as indicated by the observation of maintained electrical responsiveness shown in Fig. 4.

It is well known that the SGN soma size changes after deafening, but the dynamics of these size changes are not clear. The results from measurements of SGN soma area vary between different laboratories [6,10,30,32–34]. This depends in part on using different techniques for measurements, deafening, and treatment, and not least for how long the animals have been deaf. It is also possible that different neurotrophic factors affect SGNs differently.

In animals that still had the EC device implanted at the end of the experiment (i.e., the ARPE-19 and Cell-free groups and in the cases where the tether came off) it was observed that some of the EC devices were surrounded by layers of fibrous tissue. Similarly, Konerding et al., [28] reported extensive fibrotic encapsulation of a GDNF releasing device after a six-month period. In the present study, when examining the eABR results obtained from animals with fibrous tissue around the EC device, there was no indication that fibrous tissue had affected the functionality of the EC devices. However, as the present study monitored auditory function only for a four-week period, it is necessary to design experiments running over longer periods of time to evaluate the functionality of a permanently implanted device.

4. Conclusion

The results of the current study showed that implanting a device containing encapsulated cells (EC device) releasing neurotrophic factors (BDNF or GDNF) into the inner ear significantly protected auditory SGNs from neomycin-induced cell death, and maintained their electrical responsiveness. The study thus demonstrated that an implanted EC device releasing biologically protective substances offers a feasible approach for treating progressive hearing impairment.

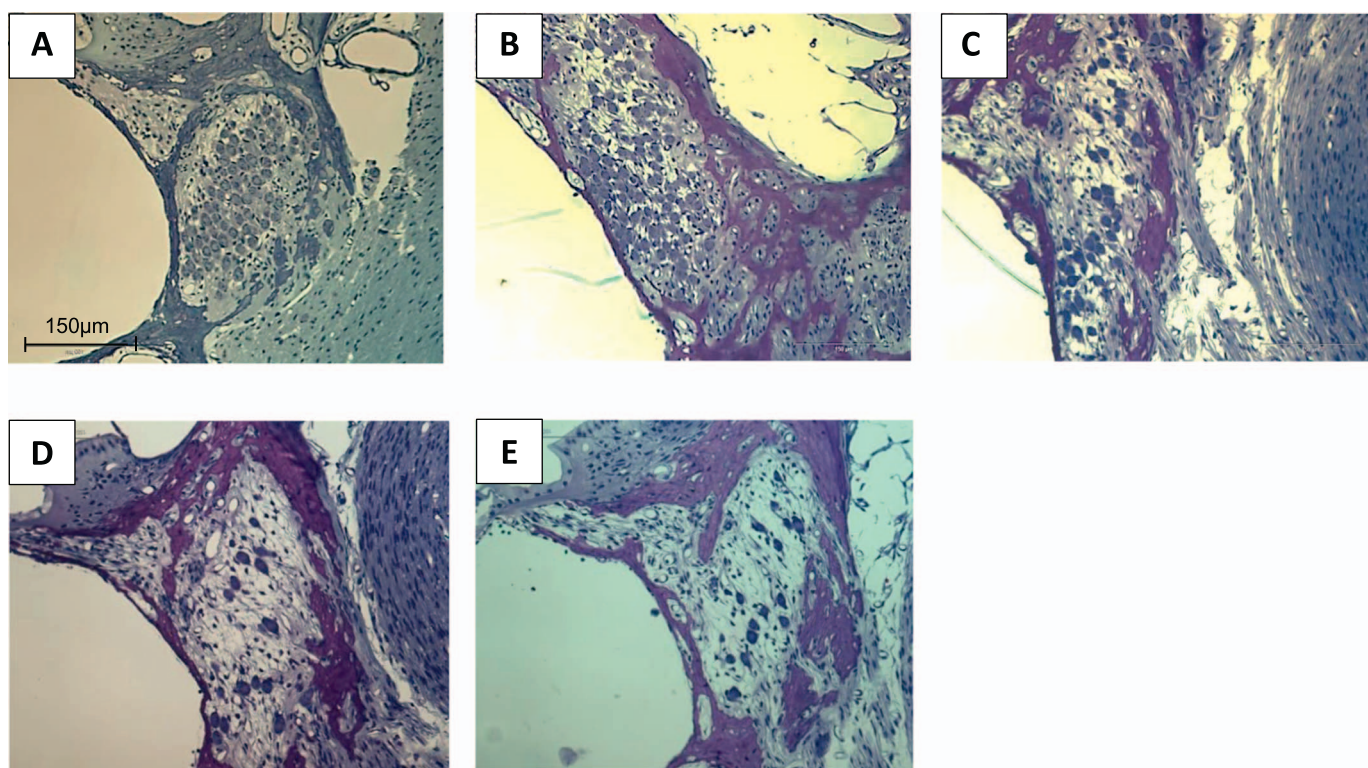


Fig. 5. Photomicrographs showing the SGN region in Rosenthal's canal at the middle turn of the guinea pig cochlea (10 ×). A) Normal (no treatment); B) Deafened and implanted with a GDNF/ARPE-19 device; C) Deafened and implanted with a BDNF/ARPE-19 device; D) Deafened and implanted with an ARPE-19 device; E) Deafened and implanted with a Cell-free device. Scale bar = 150 μm.

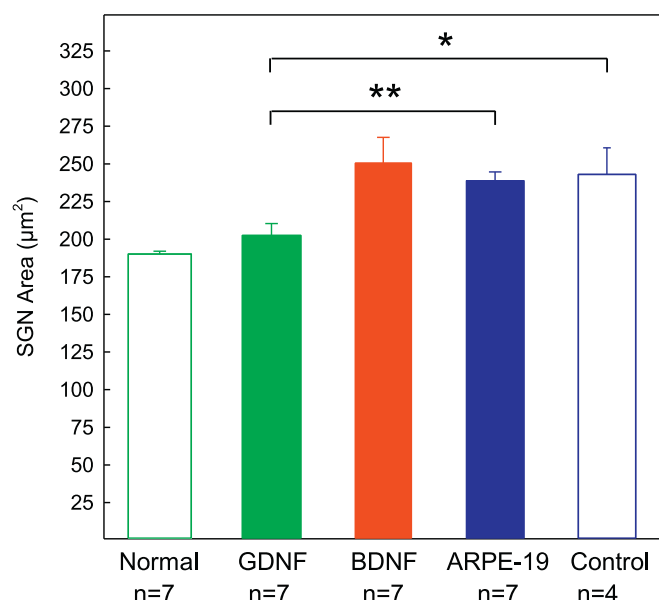


Fig. 6. SGN cell size expressed as cross sectional area. A significant difference was found in the GDNF/ARPE-19 group compared to the groups implanted with devices containing either ARPE-19 or Cell-free devices ($p < 0.01$ and $p < 0.05$, respectively). The size of the GDNF treated cells did not differ significantly from normal and untreated SGNs. * $p < 0.05$; ** $p < 0.01$.

Conflict of interest

JT and LUW were employed by NsGene.

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