Neurotrophic treatment of the degenerating auditory nerve;

cochlear implants in deafened guinea pigs

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Neurotrophic treatment of the degenerating auditory nerve;
cochlear implants in deafened guinea pigs

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Neurotrophic treatment of the degenerating auditory nerve;

cochlear implants in deafened guinea pigs

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cochleaire implantaten bij doofgemaakte cavia's

(met een samenvatting in het Nederlands)

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Martijn Johannes Hermanus Agterberg

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Voor mijn ouders

en ter nagedachtenis aan mijn grootouders

“Water is wijzer dan mensen, het trekt zich van grenzen niets aan.

(Milan Kundera, De ondraaglijke lichtheid van het bestaan)”

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List of abbreviations

aABR, acoustically evoked auditory brainstem response

ABR, auditory brainstem response

aCAR , acoustically evoked correct avoidance response

BDNF, brain-derived neurotrophic factor

BSA, bovine serum albumin

CAP, compound action potential

CAR, correct avoidance response

CES, chronic electrical stimulation

CI, cochlear implant

CM, cochlear microphonics

CNTF, ciliary-derived neurotrophic factor

dB, decibel

eABR, electrically evoked auditory brainstem response

eCAR, electrically evoked correct avoidance response

ECoG, electrocochleography

GDNF, glial cell line-derived neurotrophic factor

ID, inner diameter

IHC, inner hair cell

IM, intramuscular

IV, intravenous

NGF, nerve growth factor

nSL, normal sound level

NT-3, neurotrophin 3

NT-4/5, neurotrophin 4/5

OHC, outer hair cell

PBS, phosphate-buffered saline

PeSPL, peak equivalent sound pressure level

SEM, standard error of the mean

SGC, spiral ganglion cell

SP, summating potential

SPL, sound pressure level

TDT, Tucker-Davis technologies

2wdu, two-weeks deaf untreated

6wdu, six-weeks deaf untreated

6wdBDNF, six-weeks deaf BDNF treated

6wdCES, six-weeks deaf chronic electrical stimulation

6wdnoCES, six-weeks deaf no chronic electrical stimulation

General introduction and overview

1

1.1 Cochlear Implants

A cochlear implant is an electronic device (schematically depicted in Fig. 1) that provides severely and profoundly hearing-impaired people with the possibility to perceive sound. Since the 1980s, implanted people are able to hear by means of direct electrical stimulation of the auditory nerve. In this way, the damaged sensory cells of the auditory periphery are bypassed. The auditory performance of implantees can be remarkable. Some of them reach a level of speech understanding that enables them to use the telephone. However, others are not able to understand speech at all (Fu and Galvin III, 2008).

In this introductory section, we will shortly describe the auditory pathway as far as it is relevant for the research described in this thesis. Subsequently, we will describe pathology of hearing, and which people can benefit from a cochlear implant. Then we will discuss the main subjects of this thesis: the spiral ganglion cells which constitute the larger part of the auditory nerve and which are the target cells for cochlear implants. Emphasis will be put on loss and rescue of these cells. After that we will present the objective and outline of this thesis. Finally, we will describe the main outcome parameters (morphological, electrophysiological and behavioral) that were investigated.

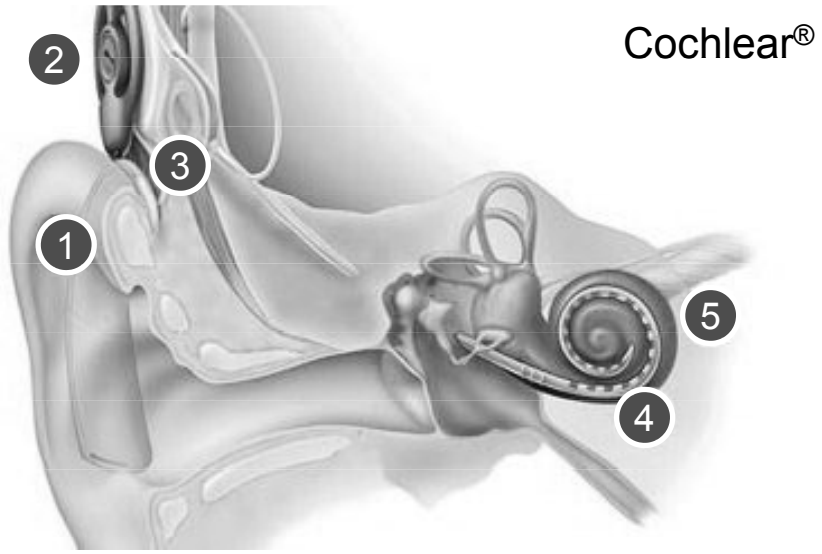

Fig. 1 Components of a cochlear implant. (1) Microphone behind the ear with speech processor. In the speech processor incoming sounds are analyzed and coded into digital signals. (2) External coil sending digital signals through the skin. (3) Internal coil, receiving signals and energy from the external coil. (4) Electrode array in the cochlea. (5) Auditory nerve.

1.2 The auditory periphery

The cochlea (Greek for snail or snail's house) is a crucial element in the auditory periphery. An important attribute of the cochlea, in fact of the entire auditory pathway, is that it has a tonotopic organization. This tonotopic organization refers to the neural spatial representation of frequency: in the cochlea nerve fibers sensitive to high frequencies innervate the base of the cochlea, and fibers sensitive to low frequencies the apex (Fig. 2A). Sounds reach the outer ear and are transported through the middle ear and oval window to the cochlea. In the cochlea, the mechanical vibration which constitutes the sound is converted into an electrochemical signal, a process that is called transduction. Crucial for this transduction are the sensory cells in the cochlea: the hair cells. In the mammalian cochlea one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) are present in the organ of Corti. This complex structure lies on a membrane, the basilar membrane, which traverses the entire cochlea. Figure 2B depicts these IHCs and the OHCs in a cross-section of the organ of Corti in a guinea pig. The electrochemical signal released by IHCs, evokes action potentials in the neurons which constitute the auditory nerve. These neurons are called spiral ganglion cells (SGC). SGCs transport the electrical activity to the central auditory system. The cell bodies of SGCs are located in Rosenthal's canal (Fig. 2C). This canal, recognizable by its bony boundaries (Rosenthal, 1823), starts at the basal part of the cochlea (location B1 in Fig. 2A) and spirals around the modiolus (the modiolus is the central core of the cochlea and consists of the beginning of the auditory nerve; N. VIII). There are two types of SGCs: (1) Type I SGCs (~90% of the total number of SGCs) innervating IHCs via peripheral processes (Fig. 3A) and conducting action potentials to their central target, the cochlear nucleus, which is the first station of the central auditory system, located in the brainstem. (2) Type II SGCs (~10% of the SGCs) with afferent fibers innervating the OHCs. The signals from the SGCs are processed in the various stations of the auditory brainstem and cortex eventually leading to an auditory percept.

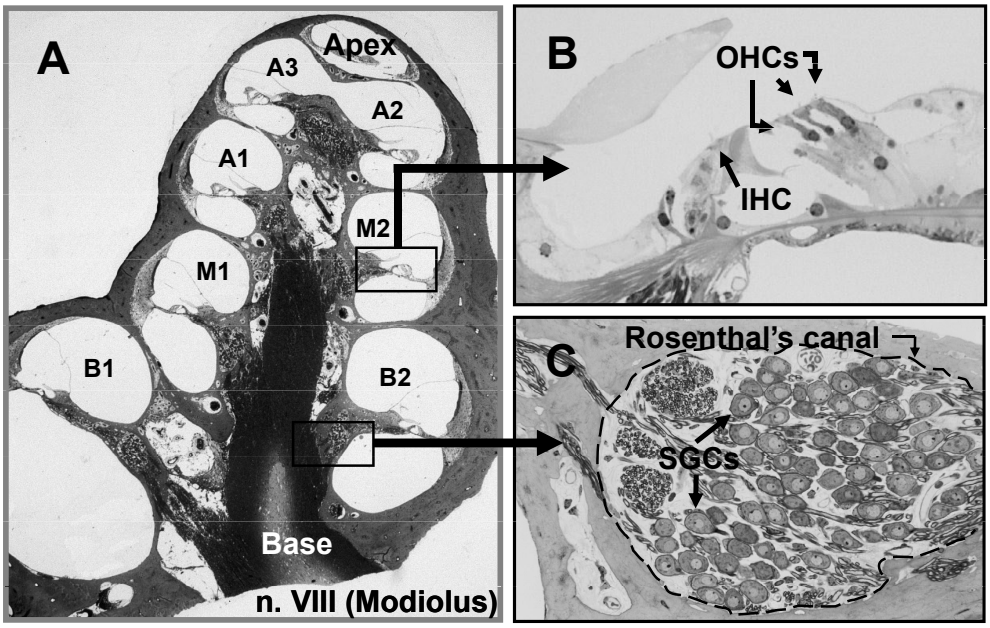

Fig. 2 Light micrographs of (A) a mid-modiolar section (1 µm) of an entire normal guinea pig cochlea showing the different cochlear locations as defined in this thesis (B1-A3), (B) the organ of Corti with inner and outer hair cells (IHC and OHCs) and (C) Rosenthal's canal packed with spiral ganglion cells (SGCs). N. VIII: auditory nerve.

1.3 Pathological hearing

For the normally hearing, it is difficult to imagine a life without hearing. Perceiving sound is very important in our highly communicative society, and sensorineural hearing loss, caused by damage or loss of cochlear hair cells, is the most common disability in humans. People with severe or profound hearing loss, which are typical cochlear implant candidates, almost always have a problem with the hair cells in the cochlea, not with their outer or middle ear or with so-called retrocochlear or more centrally located abnormalities. Common sources of sensorineural hearing loss with its origin in the cochlea are infections (such as meningitis), exposure to ototoxic substances or noise, specific diseases (such as Meniere's disease), autoimmune disorders, genetic hearing loss disorders and presbycusis (hearing loss due to aging). In the mammalian cochlea, hair cell loss is permanent because hair cells in the cochlea are unable to regenerate once they are lost.

1.4. Improvement of hearing with a cochlear implant

We can distinguish two major groups of people which are appropriate candidates for cochlear implantation: the prelingually deaf children, and a group of people (children and adults) that develops a severe hearing problem after they reached the ability to listen and speak. To date, more than 110,000 cochlear implants have been implanted in these two groups world wide (Fallon et al., 2007). However, there is a large variability in the auditory performance of cochlear implant recipients. At this time, in many countries, including the Netherlands, infants are screened for deafness and hearing impairments and prelingually deaf children sometimes receive a cochlear implant before they are one year old to provide input to the “auditory brain” as soon as possible (Cervera-Paz and Manrique, 2005; Deggouj et al., 2007; Wilson and Dorman, 2008). Many of these early implanted children are doing fine in the public education system, but others lag far behind in understanding speech. The majority of postlingually deaf children and adults receiving a cochlear implant, are able to perceive spoken language without visual cues (Leake et al., 2008). There are different factors explaining the variability in perceptual performance of cochlear implant users, and one of these possible factors, the vitality of the auditory nerve (Fig. 3B), is discussed in the next two paragraphs.

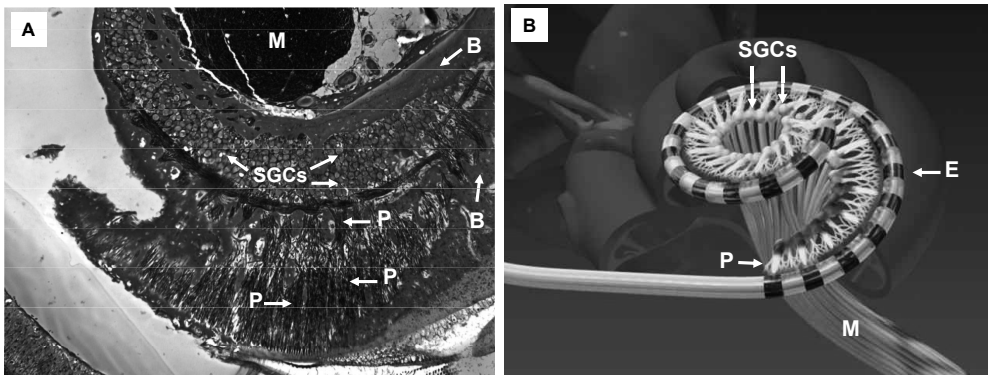

Fig. 3 (A) Light micrograph of a cross section of a guinea pig cochlea. SGCs: spiral ganglion cells in Rosenthal’s canal. B: Bony boundaries of Rosenthal’s canal. M: modiolus. P: peripheral processes. Photograph adopted with permission of G.T. Hradek and P.A. Leake, Epstein Hearing Research Laboratory, Department of Otolaryngology, University of California San Francisco. (B) Schematic representation of the electrode-nerve interface. SGCs: Cell bodies of two spiral ganglion cells. M: modiolus. P: peripheral processes. E: electrode. Reproduced with permission from Cochlear Ltd.

1.5 Spiral ganglion cell degeneration after loss of cochlear hair cells

In human post-mortem studies (Spoendlin, 1975; Nadol et al., 2001), and in animal models for sensorineural hearing loss, it was demonstrated that the auditory nerve degenerates progressively once the cochlear inner hair cells are lost (Webster and Webster, 1981; Leake and Hradek, 1988; Shepherd and Hardie, 2001). This degenerative process, which is related to the duration of hearing impairment (Nadol 1997; Incesulu et al., 1998), is much slower in humans than in animal models (Nadol et al., 2001; Leake and Rebscher, 2004; Fayad and Linthicum, 2006). The degeneration is evidently a consequence of the elimination of the spontaneous and evoked activity in the SGCs (Liberman and Kiang, 1978; Hartmann et al., 1984; Schepherd and Javel, 1997) and the loss of neurotrophic support from surrounding structures as the hair cells (Lefebvre et al., 1992; Ylikoski et al., 1993; Fritzch et al., 1999). Similar to the loss of cochlear hair cells the loss of SGCs is permanent because they can not regenerate.

1.6 Vitality of the auditory nerve and the main research question

It is obvious that a cochlear implant will not be effective when the auditory nerve is absent or dysfunctional, because action potentials (neural information) will not be carried to the brain. Therefore, it is thought that the performance of cochlear implant users is negatively affected by degeneration of SGCs. Indeed, two studies performed in monkeys, demonstrated that the performance with a cochlear implant was better in animals with more SGCs (Pfingst et al., 1981; Pfingst and Sutton, 1983). However, it should be noted that there is no explicit clinical evidence that enhanced SGC survival results in a better performance with a cochlear implant (as measured with speech perception scores; Nadol et al., 2001; Khan et al., 2005; Nadol and Eddington, 2006; Fayad and Linthicum, 2006). Still, we and others think that the current data sets are too limited to conclude that the extent of SGC survival does not influence cochlear implant performance (Leake et al., 2008).

The central research question that is addressed in this thesis is whether it is possible to maintain the vitality of the auditory nerve in an animal model in which degeneration of SGCs has already started. With this animal model we intend to mimic the clinical situation of postlingually hearing-impaired people, by deafening the guinea pigs after maturation of the auditory system. The next paragraph describes what our state of knowledge on the possibilities to improve the vitality of the auditory nerve is at the moment.

1.7 Electrical stimulation and application of neurotrophins can prevent degeneration of SGCs

To improve the hearing of people who lag behind in their performance several strategies have been proposed to prevent degeneration of the auditory nerve and subsequently tested in animal models (Roehm and Hansen, 2005; Pettingill et al., 2007; Hendricks et al., 2008). These neurotrophic therapies focus on chronic electrical stimulation (Lousteau, 1987; Hartshorn et al., 1991; Leake et al., 1991), delivery of neurotrophic factors to the cochlea (Steacker et al., 1996; Miller et al., 1997; Gillespie et al., 2004), or a combination of these two treatments (Kanzaki et al., 2002; Shepherd et al., 2005; Song et al., 2008; Scheper et al., 2008). In addition to basically improve hearing in people who lag behind in their performance these therapies may also improve more advanced auditory capabilities like speech perception in a noisy environment and perception of music for cochlear implant users in general.

In animal models, chronic electrical stimulation (CES) with a cochlear implant can help to reduce the degeneration of SGCs after deafening with ototoxic compounds (Lousteau et al., 1987; Hartshorn et al., 1991; Leake et al., 1991; Coco et al., 2007; Song et al., 2008). However, enhanced survival of SGCs in chronically electrically stimulated animals is not a consistent finding across laboratories (Li et al., 1999; Shepherd et al., 2005), and the most beneficial conditions with respect to various parameter settings (pulse rate, current, electrode configuration, duration of stimulation) for successful CES remain to be investigated.

Neurotrophic factors can also help to prevent SGCs from degeneration (Miller et al., 2002; Gillespie and Shepherd, 2005; Pettingill et al., 2007). Neurotrophic factors are neuropeptides that affect the development and survival of neurons in the peripheral and central nervous system. In the peripheral auditory system the neurotrophins, signaling through specific high-affinity binding Trk receptors (Gao, 1999), are the best characterized family of growth factors (Miller et al., 1997; Marzella and Clark, 1999; Gillespie, 2003; Gillespie and Shepherd, 2005; Pettingill et al., 2007). In contrast to other growth factors, neurotrophins exert their biological actions primarily on cells of the nervous system (Lewin and Barde, 1996). Loss of the continuous supply of neurotrophins by hair cells is considered to be the major factor leading to degeneration of the auditory nerve (Lefebvre et al., 1992), and in animal studies application of all four members of the neurotrophin family has been proven successful in preventing SGCs from degenerating after induced deafness: brain derived neurotrophic factor (BDNF; Staecker et al., 1996; Miller et al., 1997; Gillespie et al., 2003; Shepherd et al., 2005),

nerve growth factor (NGF; Shah et al., 1995), neurotrophin-3 (NT-3; Ernfors et al., 1996; Miller et al., 1997) and neurotrophin-4/5 (NT-4/5; Gillespie et al., 2004). Other neurotrophic factors, like glial cell-line derived neurotrophic factor (GDNF), have also been tested with success (Ylikoski et al., 1998). In all these studies infusion of neurotrophic factors was accomplished by means of an osmotic pump system (Alzet®) connected to a cannula (Brown et al., 1993) or a cochlear implant with integrated drug delivery microcannula (Shepherd and Xu, 2002). The neurotrophins were infused directly into the perilymph of the scala tympani to bypass the protective blood-brain barrier and to reach necessary concentrations.

It has been shown in vitro (Hegarty et al., 1997) and in vivo (Kanzaki et al., 2002; Shepherd et al., 2005; Song et al., 2008; Scheper et al., 2008) that the trophic support of electrical stimulation was additive to the trophic support of delivery of neurotrophins, indicating that different neurobiological mechanisms are involved in the prevention of SGC degeneration following a sensorineural hearing loss (Miller, 2001; Pettingill et al., 2007; Leake et al., 2008).

1.8 Objective and outline of the thesis

The objective of this thesis is the characterization of effects of neurotrophic treatments that may have the potential to prevent or decrease the degeneration of SGCs. Most experiments deal with the presence (morphology) and excitability (electrophysiology) of SGCs after deafening. These experiments were performed in normal-hearing and deafened guinea pigs.

The experiment described in **chapter 2** was performed to investigate the loss of hair cells and the time course of the secondary degeneration of SGCs after ototoxic deafening, during a period of 8 weeks. The aim of this study was to develop a guinea pig model suited to investigate the effect of CES and intracochlear infusion of neurotrophins, on SGC survival after sensorineural hearing loss.

In **chapter 3** the primary goal was to examine whether amplitude modulated CES was effective in preventing degeneration of SGCs in guinea pigs deafened with the ototoxic procedure described in chapter 2. The SGC packing density, size and circularity were determined and eABRs were recorded to examine whether the remaining SGCs were functional.

An established method to preserve SGCs is intracochlear infusion of neurotrophins with a cannula connected to an osmotic pump (Ernfors et al., 1996; Staecker et al., 1996;

Miller et al., 1997). It was not well known which morphological changes occurred in SGCs after deafening and subsequent delayed neurotrophic treatment. The study in **chapter 4** was performed to analyze the protective effect of delayed intracochlear BDNF-infusion with an osmotic pump, which has previously been reported to prevent degeneration of SGCs (Gillespie et al., 2004), and to examine the morphology of SGCs in detail using electron microscopy (de Groot et al., 1987). In contrast to the studies describing the trophic effect of CES (chapter 3) and the effect after cessation of BDNF-treatment (chapter 5), eABRs were not recorded.
The experiment described in **Chapter 5** was performed to examine the survival and functionality of SGCs after cessation of BDNF treatment. This study was important given the contradictory results in the literature. Gillespie et al. (2003) reported rapid degeneration of SGCs after cessation of neurotrophic treatment while Maruyama et al. (2008) demonstrated that SGC survival was maintained well after cessation of neurotrophic treatment.

Chapter 6 provides the results of a pilot study. In this study a clinically safer mode of delivery of BDNF to the cochlea was tested. Absorbable gelatin sponge soaked in BDNF solution was positioned on the round window of deafened guinea pigs. This method with less risk of infections does not include the opening of the cochlea which is necessary for BDNF-treatment with a drug delivery cannula.

The aim of the work presented in **chapter 7** was to investigate whether deafened guinea pigs were able to detect intracochlear electrical stimulation. This was examined in a modified shuttle-box paradigm (Phillippens et al., 1992). Guinea pigs, implanted with an electrode array in the cochlea, had to learn to avoid a strong stream of air on guidance of electrical stimulation provided with the implanted electrode array.

Chapter 8 provides a summary and a discussion of the presented results. This chapter also provides some recommendations for future studies.

1.9 Morphology, electrophysiology and behavior

The three main outcome parameters investigated in the studies described in this thesis are the SGC packing density (a morphological parameter), the amplitude of the electrically evoked auditory brainstem response (an electrophysiological parameter), and an avoidance response of acoustical and electrical stimulation of the cochlea (a behavioral parameter).

1.9.1 Morphology

The presence (or absence) of SGCs in the spiral ganglia of treated and untreated cochleae was determined along the length of the cochlea. Other morphological features, especially the SGC size, their shape (circularity), the electron density of their perikaryon, and the thickness of their myelin sheath, were also used in our investigations. Because it is difficult to analyze the ultrastructures of SGCs in light micrographs, these features were analyzed in one experiment (chapter 4) with transmission electron microscopy (de Groot et al., 1987).

It is difficult to draw conclusions on the functionality of remaining SGCs solely based on morphological parameters. For this reason, electrically evoked auditory brainstem responses (eABRs) and other evoked potentials were often recorded to further characterize the cells (see next paragraph).

1.9.2 Electrophysiology

To monitor the state of hearing at the moment the animals were included in the studies, compound action potentials (CAPs) and auditory brainstem responses (ABRs) were recorded. Sound stimuli were presented in an open-field configuration. The method to record the CAP is known as electrocochleography (Ruth et al., 1988). The response that is measured with electrocochleography occurs within the first 4 ms after a stimulus onset. The amplitude of the CAP is related to the number of auditory nerve fibers simultaneously generating an action potential. A disadvantage of this method is that it is necessary to position an electrode in or on the cochlea, with the risk of causing trauma to the inner ear, to obtain stable measurements of the CAP in awake animals. However, an advantage is that the amplitude of the CAP is a factor 100 larger than the amplitude of the ABR response, since the recording electrode is much closer to the source. Therefore, the CAP is more precise. We measured the CAP only in the experiment described in chapter 2 of this thesis (Versnel at al., 2007). In this experiment the electrode was positioned in the round window niche of the right cochlea (Klis et al., 2000). In the other experiments we used ABRs to monitor hearing thresholds.

ABRs are the stimulus-evoked potentials of the auditory nerve and various brainstem nuclei, recorded from superficial electrodes, with the advantage that hearing thresholds can be monitored without the invasive placement of the recording electrode in or on the cochlea. In humans the superficial electrodes are positioned on the skin of the skull. In animals it is possible to implant these electrodes in the skull to improve the electrode-brain interface. The

neural origin of the first peak (wave I) of the ABR is the auditory nerve. The sources of the other potentials, wave II-V, are not exactly known. It is thought that they are generated in the different nuclei in the auditory brainstem (Yost, 2000).

To monitor the functionality of the SGCs in normal-hearing animals or in animals after deafening we used electrically evoked ABR (eABRs). For the recordings of an eABR it is necessary to position a stimulus electrode in or on the cochlea. The eABRs presented in this thesis were evoked with an electrode array designed for guinea pigs (Cochlear®, Mechelen, Belgium). This electrode array was inserted through a cochleostomy in the base of the cochlea. Again, the neural origin of the first peak (wave I) of the eABR complex is the auditory nerve. This is the peak of interest. Yet, it was often obscured by the electrical artifact and for this reason the first negative peak (N_1) and positive peak (P_2) on each site of the fast rising part of the eABR complex were analysed. These peaks are indicated in a representative example of a series of eABR recordings in Figure 4. The amplitude of this part of the eABR complex correlates well with the number of SGCs and is thought to be a summation of the electrically evoked action potentials in the SGCs (Dobie and Kimm, 1980; Miller et al. 1983; Smith and Simmons 1983; Hall 1990).

It should be clarified, that when in this thesis ABRs were evoked electrically, we always named them eABRs, while ABRs and aABRs both refer to acoustically evoked ABRs.

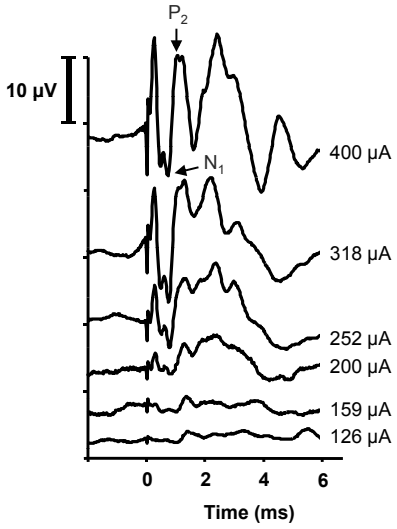

Fig. 4 Series of eABR recordings evoked with current pulses from well above threshold down to threshold. The first negative peak (N1) and the second positive peak (P2) which were widely used in this thesis as a measure for the eABR, are indicated with an arrow.

1.9.3 Behavior

To estimate whether deafened animals were able to detect electric pulse trains, delivered with the electrode array, we measured their avoidance responses in a shuttle box. The shuttle box test is a behavioral test in which animals can learn to gain a reward or avoid a punishment, by shuttling to the other compartment. Similar to Philippens et al. (1992), we used an unpleasant stream of air as punishment.

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Time course of cochlear electrophysiology and morphology after combined administration of kanamycin and furosemide

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Abstract

In animal models of deafness, administration of an aminoglycoside in combination with a loop diuretic is often applied to produce a rapid loss of cochlear hair cells. However, the extent to which surviving hair cells remain functional after such a deafening procedure varies. In a longitudinal electrocochleographical study, we investigated the variability of cochlear function between and within guinea pigs after combined administration of kanamycin and furosemide. Concurrently, histological data were obtained at 1, 2, 4 and 8 weeks after deafening treatment. The main measures in our study were compound action potential (CAP) thresholds, percentage of surviving hair cells and packing density of spiral ganglion cells (SGCs). One day after deafening treatment, we found threshold shifts widely varying among animals from 0 to 100 dB. The variability decreased after 2 days, and in 18 out of 20 animals threshold shifts greater than 55 dB were found 4 to 7 days after deafening. Remarkably, in the majority of animals, thresholds decreased by up to 25 dB after 7 days indicating functional recovery. As expected, final thresholds were negatively correlated to the percentage of surviving hair cells. Notably, the percentage of surviving hair cells might be predicted on the basis of thresholds observed one day after deafening. SGC packing density, which rapidly decreased with the period after deafening treatment and correlated to the percentage of surviving inner hair cells, was not a determining factor for the CAP thresholds.

key words deafness; ototoxicity; recovery; cochlear hair cells; spiral ganglion cells; electrocochleography

1. Introduction

Various animal models are used to study cochlear dysfunction or sensorineural deafness. For instance, congenitally deaf animals can be bred or the cochlea can be mechanically destroyed. More often, however, ototoxic drugs are used for this purpose. A common ototoxic procedure, first described by West et al. (1973), is a single administration of an aminoglycoside (e.g., kanamycin) in combination with a loop diuretic (ethacrynic acid or furosemide). This method has been applied in studies investigating neural degeneration (Webster and Webster, 1981; Xu et al., 1993), neurotrophic treatment to preserve the cochlear

nerve (Staecker et al., 1996; Miller et al., 1997; Gillespie et al., 2003), electrical stimulation with a cochlear implant (Lousteau, 1987; Mitchell et al., 1997), and hair cell regeneration (Izumikawa et al., 2005). However, the extent to which hair cells remain functional after the deafening procedure, i.e., the actual extent of deafness, can vary. A significant variability among animals was demonstrated by Nourski et al. (2004), who found that 12 hours after administration of kanamycin in combination with ethacrynic acid 4 out of 6 guinea pigs showed the intended loss of cochlear response (to click stimuli of 80 dB above normal threshold) and 2 out of 6 animals showed a substantial response. For this reason, auditory evoked potentials are often used to assess the result of the deafening procedure. For instance, using auditory brainstem responses in an experiment involving preservation of the nerve after hair cell loss, Shepherd et al. (2005) reported exclusion of animals because of insufficient increases of thresholds. A deafness check is especially relevant when long-term and/or expensive studies involving neurotrophic treatment are performed. It is then assumed that the condition of the cochlea does not change significantly after the animal passes the electrophysiological deafness check. However, variability within an animal might exist. For instance, Aran and Darouzzet (1975) demonstrated recovery from a 40-dB loss in the second week after 8-day treatment with kanamycin. Also, Klis et al. (2000, 2002) found substantial recovery over periods up to three weeks after long-term cisplatin treatment.

In the present study, we examined differences between animals and the within-animal changes during a period of up to 8 weeks after after administration of kanamycin in combination with furosemide. In guinea pigs with a permanent round window electrode, cochlear potentials in response to click and tone stimuli were recorded. These recordings were performed frequently during the first week after deafening and on a once-weekly basis thereafter. Clicks were included in the stimulus set as these are commonly used for electrophysiological checks (e.g., auditory brainstem responses). We measured the time course of the compound action potential (CAP) thresholds in order to address changes over time and differences between animals. The CAP thresholds were related to histological measures, i.e., the percentage of surviving hair cells and the spiral ganglion cell (SGC) packing density. A specific issue was the predictability of hair cell loss on the basis of thresholds during the first week after deafening.

2. Materials and methods

2.1. Animals and surgery

Twenty-five female albino guinea pigs (strain: Dunkin Hartley; supplier: Harlan Laboratories, Horst, The Netherlands) weighing 270-600 g were used. Animals had free access to food and water, and were kept under standard laboratory conditions. All experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC # 03.04.036).

The animals were injected with a non-ototoxic antibiotic (chloramphenicol sodium succinate; 60 mg/ kg, im) and anesthetized with 40 mg/kg ketamine hydrochloride (Ketanest®, im) and 10 mg/kg xylazine hydrochloride (Sedamun®, im). Local anesthetic (1% lidocaine) was used in areas to be incised. An electrode consisting of a stainless steel wire with a gold-ball tip was positioned in the round window niche of the right cochlea and sealed into the bulla (for details, see Klis et al., 2000, 2002). Stainless steel screws were inserted through the skull near the bregma to be used as reference and ground electrodes.

2.2. Deafening procedure

At least two weeks after electrode implantation, allowing for recovery from surgical trauma, 20 animals were concomitantly treated with kanamycin and furosemide (West et al., 1973). After electrocochleography, the animals were anaesthetized with ketamine (40 mg/kg, im) and xylazine (10 mg/kg, im). Kanamycin sulphate in isotonic saline (400 mg/kg, sc) was administered and, subsequently, the external jugular vein was exposed and cannulated and, 15-60 minutes after kanamycin injection, furosemide (100 mg/kg, Centrafarm®) was slowly infused. The dose of kanamycin is the same as in numerous studies that apply kanamycin in combination with ethacrynic acid (e.g., Staecker et al., 1996; Miller et al., 1997; Nourski et al., 2004) or furosemide (e.g., Gillespie et al., 2003; Shepherd et al., 2005; Wise et al., 2005) for deafening purposes. The dose of furosemide is the same as applied in the latter studies.

In five control animals a sham procedure was performed with isotonic saline without the ototoxic drugs.

2.3. Electrocochleography and acoustic stimuli

Recordings were performed as described in detail by Hamers et al. (2003). The animal, positioned in a sound-proof chamber, was awake; it was lightly restrained in a custom-made tight cloth jacket with the eyes covered and the right pinna and ear canal exposed. Sound generation and ECoG recordings were controlled by a personal computer and a DT3010/32 data acquisition board (Data translation). Acoustic stimuli were tone bursts of 2, 4, 8, 11.3 and 16 kHz with a duration of 8 ms (1 ms rise/fall times) and biphasic rectangular pulses (100 μs/phase). The sound stimuli were presented in an open-field configuration with a Fane tweeter (J-104) positioned at 10 cm from the pinna. Using a pair of attenuators (PA4, Tucker-Davis Technologies), sound levels were varied from about 100 dB SPL (frequency dependent) down to below threshold in 10-dB steps. The sound levels were calibrated by a sound level meter (Brüel & Kjaer, 2203) and a 1-inch condenser microphone (Brüel & Kjaer, 4132). Stimuli were presented with alternating polarity at 99 ms intervals. The responses were amplified (5,000x; EG&G Instruments model 5113), bandpass-filtered (1 Hz - 30 kHz), and sampled at 33 kHz.

The CAP and summating potential (SP) were obtained by adding the responses evoked by stimuli of opposite polarity, cochlear microphonics (CM) by subtracting these responses. The amplitude of the CAP was defined as the difference between the SP and the N1 peak. The amplitude of the CM was measured peak-to-peak. Response thresholds were assessed by deriving an iso-response level from the input/output curves (Fig. 1), specifically, the sound level at which the amplitude is 3 μV. In general, the CAP was used to assess the threshold.

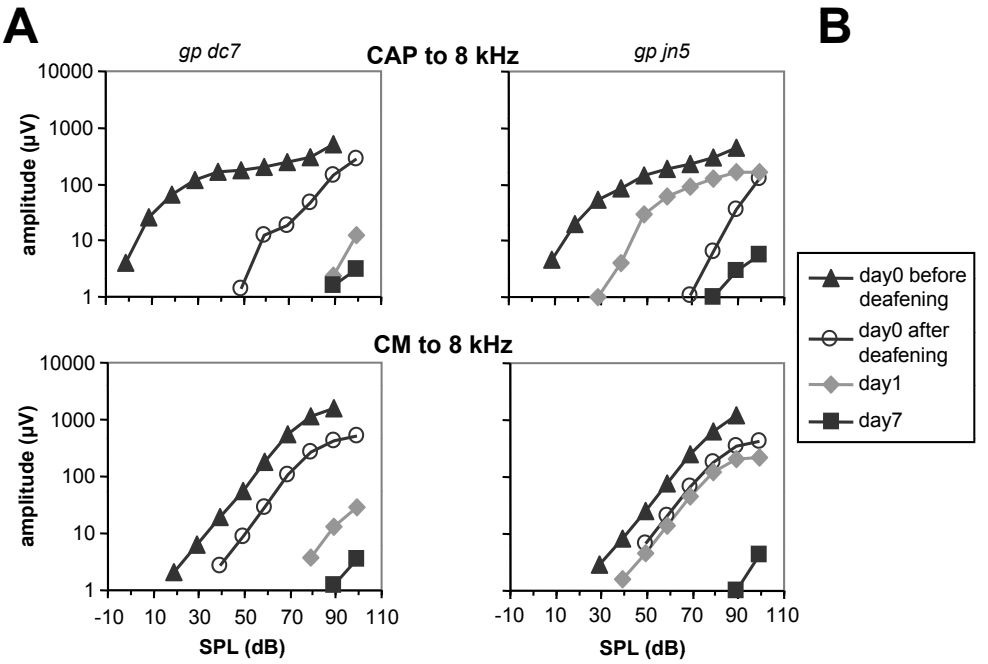


Figure 1 Input/output curves of CAP (*top*) and CM (*bottom*) to an 8-kHz tone pre- and three times post-deafening: immediately before, immediately after, 1 day after and 7 days after deafening.

(A) Input/output curves in guinea pig *dc7*. The curves monotonically shift with time after deafening towards higher stimulus levels.

(B) Input/output curves in guinea pig *jn5*. One day after deafening, the CAP curves show partial recovery, whereas the CM curves show a small decline.

2.4. Histology

Immediately after the final ECoG measurements, the left and right cochleas were fixed by intralabyrinthine perfusion with a tri-aldehyde fixative. Cochleas were processed as described in detail by De Groot et al. (1987). Cochleas were embedded in toto in Spurr's low-viscosity resin and divided into two halves along a standardized midmodiolar plane (Van Ruijven et al., 2004). Semithin (1 µm) sections were stained with methylene blue and azur II in sodium tetraborate and examined with a Zeiss Axiophot light microscope. The number of inner hair cells (IHCs) and outer hair cells (OHCs) was counted in each individual transection of the respective half-turn (b1, b2, m1, m2, a1, a2, a3; see Fig. 6A), as described in Van Ruijven et al. (2004). Digitized light-microscopical images of the spiral ganglia from each individual transection were analysed using the software program NIH Image, version 1.63 (http://www.

rsb.info.nih.gov/nih-image). The bony boundaries of Rosenthal's canal were outlined using a pressure-sensitive stylus on a digitizer tablet, and its cross-sectional area was calculated. The number of SGC perikarya was counted in each transection of Rosenthal's canal. SGC packing density was calculated by dividing the number of perikarya by the cross-sectional area (in mm^2).

2.5. Experimental groups

We used four experimental groups and one control group, with each group consisting of five guinea pigs. Experiments were performed in several series of 3 to 6 animals each; the assignment of an animal to an experimental group was random with the restriction that in each series of experiments the experimental groups were evenly represented. The four experimental groups were sacrificed for histological preparation after four different periods following the deafening procedure: 1, 2, 4 and 8 weeks. The control group was sacrificed 8 weeks after the sham treatment.

In all animals, ECoG was performed immediately before deafening and at the following times after deafening: immediately after (day 0), after 1 day, and after 2, 4 and 7 days. Subsequently, recordings were continued once-weekly (up to 2, 4 or 8 weeks) in so far the animals were not killed for histology.

3. Results

3.1. Time course in the first week after deafening

Twenty guinea pigs were treated with kanamycin and furosemide in order to cause severe or profound hearing loss. One day after the treatment, the differences of the effect of the deafening treatment among these 20 animals was marked, which is illustrated in Figs. 1 and 2.

Figure 1 shows input/output characteristics of CAPs and CMs for two different guinea pigs at one time before and at various times after the deafening treatment. In both animals, immediately after deafening, the CAP curves showed a large shift to higher levels (about 60 dB), whereas the CM curves showed only a small shift (about 15 dB); these immediate effects were found in all deafened animals. Large differences between these two examples were seen on day 1: in *dc7* (Fig. 1A) the CAP curve continued to worsen (by an additional 40 dB), but in *jn5* (Fig. 1B)

the CAP curve ameliorated (by 40 dB). Although this recovery was not seen in the CM curves, the development of the CM curves differed substantially between the two animals in that the shift in *dc7* (40 dB) was significantly greater than in *jn5* (3 dB). This difference between CM responses concurred with the difference between CAP responses. Across all animals, the CM threshold shifts at day 1 correlated strongly to the CAP threshold shifts at day 1 (linear regression, R^2>0.75, p<0.0001; for each tone frequency used). Seven days after deafening the responses had almost disappeared in both animals; only at the highest stimulus levels (about 100 dB SPL) small CAP and CM waveforms (<10 µV) were found. The great difference between the animals at day 1 was not apparent at day 7.

The time courses of thresholds that are shown in Fig. 2 for two individual guinea pigs, reflect the great difference in thresholds between animals at day 1 after deafening. In animal *dc7* (Fig. 2A) the thresholds were high after 1 day for all frequencies and continued to shift the second day. In animal *dc1* (Fig 2B) the thresholds were low at day 1, in particular for the lower frequencies (2-8 kHz), but substantially increased the second day (by 40-70 dB) and continued to increase until day 4. The frequency dependence of the thresholds at day 1 was observed in most animals.

Considering the large changes within two days as illustrated in Fig. 2B, it is conceivable that the thresholds at day 1 critically depend on the precise period between deafening and recording. Across all animals, the recordings at day 1 were performed between 20 and 28 hours after deafening. We found that over this time interval, the threshold shift was not correlated with the actual period (Fig. 2C). This indicates that probably recovery occurred before 20 hours and subsequent decline after 28 hours. The threshold shifts at day 1 were widely, and rather uniformly, distributed from 0 to 100 dB (Fig. 2C, D). This distribution moved towards large threshold shifts with increasing stimulus frequency (about 10 dB per octave) while the spread was similar across frequencies with distribution's standard deviations of 26-36 dB.

The CAP and CM threshold shifts found after one day were not correlated to the immediate threshold shifts (linear regression across all animals, R^2<0.2, p>0.1) for either frequency used, apart from one condition (CM at 16 kHz: R^2=0.3, p<0.05). Finally, the weight of the animal, which partly reflects its age, was not a significant factor determining the threshold shifts after one day (R^2<0.2, p>0.05).

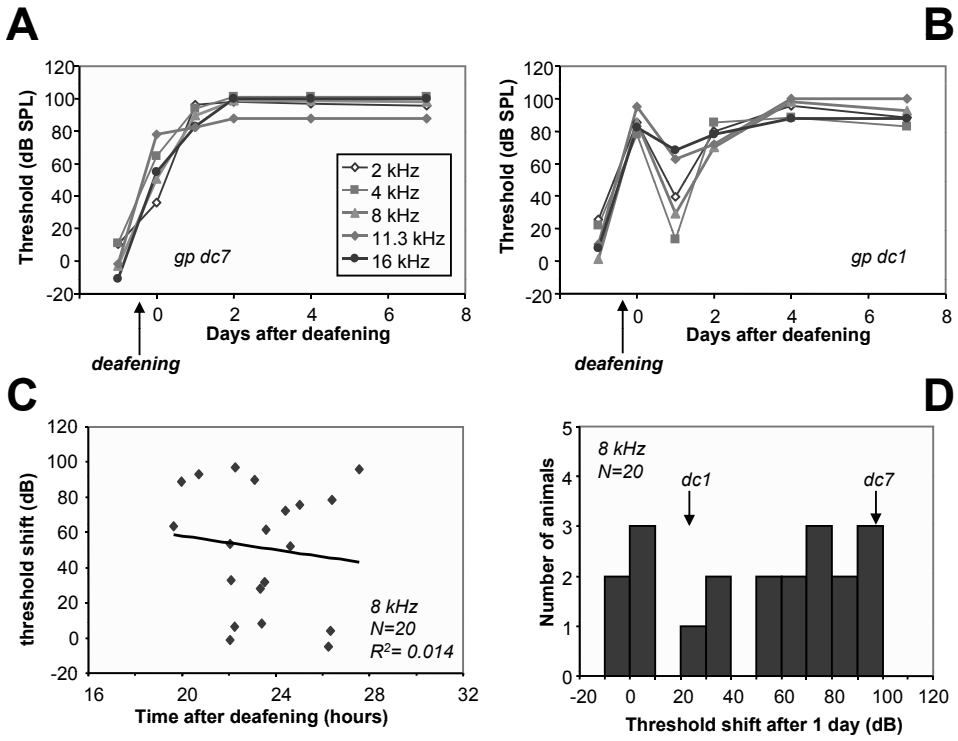


Figure 2 (A, B) Time courses of CAP thresholds up to 7 days for 5 different tone frequencies (2-16 kHz) in two guinea pigs. The CAP thresholds before deafening were recorded on day 0, just before the deafening surgery. In A) the thresholds at 4, 11.3 and 16 kHz reached ceiling values from day 2 to day 7.

(C) Threshold shift at "one day" after deafening versus the actual one-day period in hours.

(D) Distribution of threshold shifts for 8 kHz at one day after deafening.

3.2. Thresholds 7 days after deafening

The examples in Figs. 1 and 2 showed that thresholds at 7 days after deafening were sufficiently high according to the aim of a deafening treatment (shifts > 50-60 dB, Mitchell et al., 1997; Shepherd et al., 2005). We found that in 18 out of 20 animals threshold shifts at day 7 were larger than 55 dB for clicks (Fig. 3B). Threshold shifts in the other two animals were small (< 35 dB for clicks), which would be considered insufficient for a deafness model. These two animals have been excluded from various analyses in following sections of this report, since we are particularly interested in variability between and within animals with high thresholds.

Figure 3 shows that the threshold shifts at day 7 were significantly correlated to the threshold shift at day 1 (linear regression, $p<0.01$). This was found at all frequencies applied (in Fig. 3A: 2 kHz) and clicks (Fig. 3B).

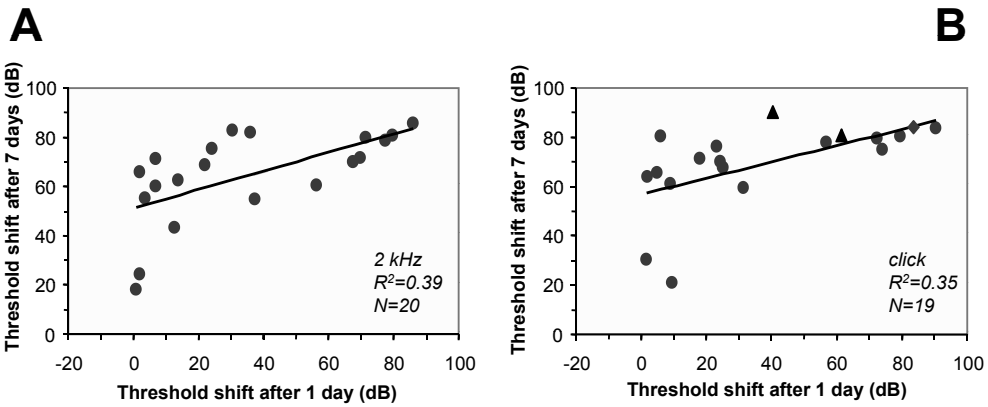


Figure 3 CAP threshold shifts 7 days after deafening versus threshold shifts 1 day after deafening. (A) 2-kHz tones, (B) click stimuli. In 3 cases (all for clicks, B) the thresholds reached ceiling values: triangles indicate data above ceiling values after 7 days; the diamond indicates ceiling values for both 1 day and 7 days; the circles indicate the remaining regular data (thresholds below ceiling). Statistics for 2-kHz: $R^2 = 0.39$, $F(1,18) = 11.5$, $p = 0.003$; for clicks: $R^2 = 0.35$, $F(1,17) = 9.3$, $p = 0.007$.

3.3. Long-term time courses of thresholds

Unexpectedly, we found significant threshold decreases after 7 days indicating a recovery of cochlear function. Figure 4 shows two examples of time courses where the threshold decreased during the second week after deafening. In one example (*jn4*, Fig. 4A), where the first-week time course was characterized by high thresholds at day 1, the thresholds decreased by about 10 dB at the higher frequencies (8-16 kHz) in the second week. In the second example (*jn5*, Fig. 4B), where the first-week time course was characterized by relatively low thresholds at day 1, the thresholds decreased during the second week at all frequencies used (2-16 kHz) by 5 to 25 dB. In fact, this decrease already started after day 4. After day 14, a slow increase of thresholds was observed in *jn4* (Fig. 4A), which was monitored for up to 56 days. This late decline starting after 2 to 4 weeks was found in 3 out of 5 animals that were monitored during 8 weeks. Linear regression analysis shows that averaged across the five animals the threshold increase from 4 to 8 weeks was small but significant (2 dB/week,

R^2>0.75, p<0.05; frequencies 2, 4, and 8 kHz, and clicks). Between 2 and 4 weeks significant changes were not found for any stimulus (< 5 dB; Wilcoxon test, p>0.05).

The threshold change during the second week is shown as a function of the threshold shift at day 1 in Fig. 5. Two points arise from this figure. Firstly, both for 2 kHz (Fig. 5A) and for clicks (Fig. 5B), the majority of threshold changes were negative, reflecting lower thresholds at day 14 than at day 7. Thirteen of 15 animals that were monitored during two weeks (note that 5 of the initial 20 animals were prepared for histology at day 7) had high thresholds at day 7 (see section 3.2). Wilcoxon statistics in these 13 animals shows that the thresholds significantly decreased for tones of 4 and 11.3 kHz (p<0.05) and for clicks (p<0.01). For clicks the threshold decreases were greater than 5 dB in 7 out of 12 animals. Secondly, close inspection of the data in Fig. 5 suggested that large second-week threshold decreases occurred in those animals which had relatively low thresholds one day after deafening. This correlation, however, was not significant (linear regression, p>0.1).

The thresholds of the control animals remained stable over the course of 8 weeks with the final thresholds not significantly different from the initial thresholds (average changes <4 dB; p>0.2).

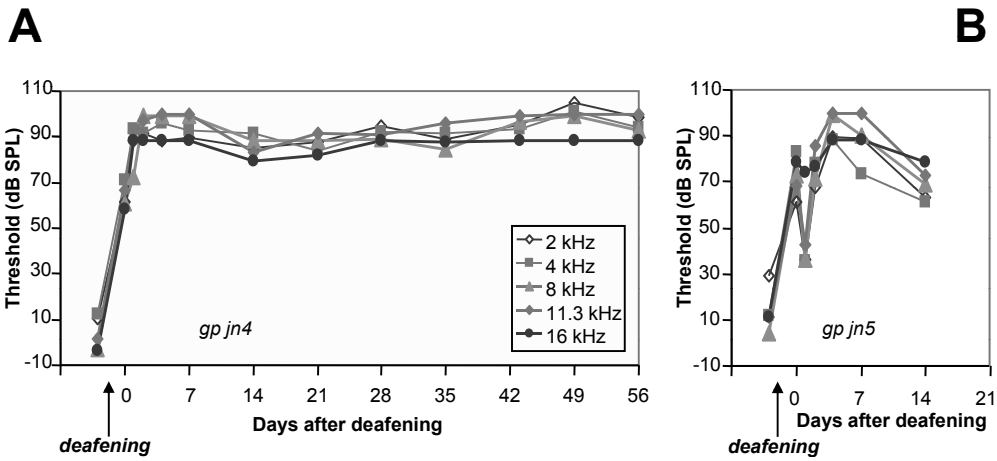


Figure 4 Time courses of CAP thresholds up to 56 days (A) or 14 days (B) for 5 different tone frequencies (2-16 kHz) for two individual guinea pigs. The CAP thresholds before deafening were recorded on day 0, just before the deafening surgery. In A) thresholds were above ceiling values at several measurements, for instance for 8, 11.3 and 16 kHz at day 4, day 7 and day 49. In B) ceiling values were reached for 11.3 and 16 kHz at day 4 and day 7.

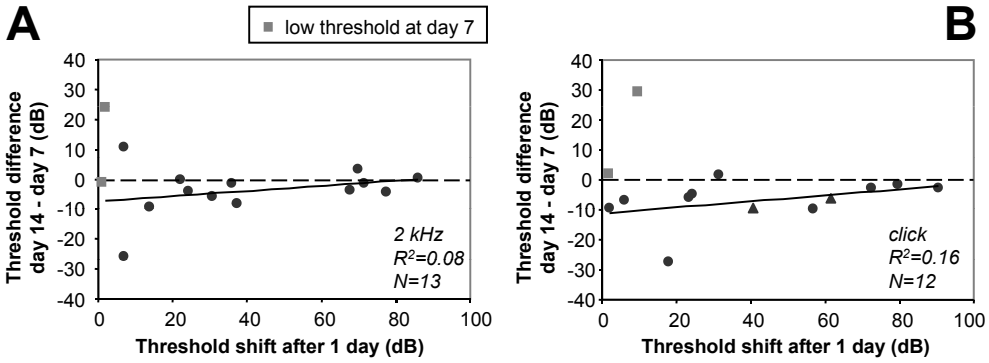


Figure 5 The difference between thresholds at 14 and at 7 days after deafening versus threshold shifts 1 day after deafening. Negative values of the threshold difference reflect a lower threshold after 14 days than after 7 days (indicating recovery), positive values reflect the opposite (indicating decline). The grey squares indicate the guinea pigs with relatively low thresholds, the triangles indicate data with thresholds above the ceiling value on day 7. Regression analysis is performed on all data apart from the low-threshold data (squares). (A) 2-kHz tones, (B) click stimuli.

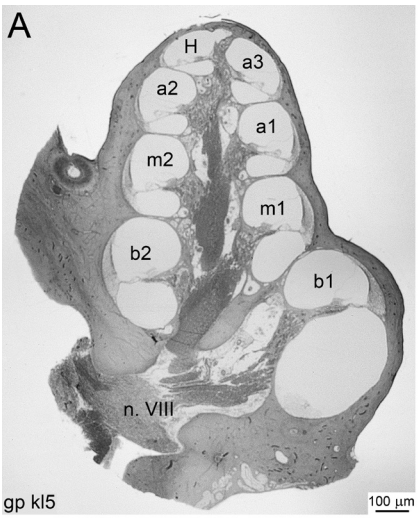
3.4. Hair cell survival

Midmodiolar sections of the cochleas of deafened and control animals were examined with light microscopy (Fig. 6). Figure 6B shows a normal organ of Corti and Fig. 6C shows a typical example of the effect of the combined treatment with kanamycin and furosemide on the morphology of the organ of Corti. The organ of Corti was collapsed with both the OHCs and IHCs missing. The amount of nerve fibers in the spiral osseous lamina was largely diminished.

The general effect of deafening on hair cell loss is reflected by the distribution of surviving hair cells along the basilar membrane (Fig. 7). On the average, OHC loss was complete in the basal turns and the percentage of surviving OHC increased along the basilar membrane towards the apical turns (Fig. 6A). In 12 animals no IHCs were found, in the 8 remaining animals IHC survival was found in all cochlear turns. The hair cell survival did not significantly vary with the period after deafening treatment (Kruskal-Wallis test, $p>0.5$; IHC, OHC).

The final CAP thresholds (recorded on the day of histological preparation of the cochlea) were significantly correlated to the surviving hair cells (linear regression, $p<0.01$) as shown in Fig. 7B, C. Multiple regression analysis showed that other factors such as duration

after deafening and SGC packing density, were not significant (p>0.5) in determining the CAP thresholds. Four animals showed a large HC survival (about 40 to 70 %). As expected, two out of these four were the animals that showed relatively low thresholds at day 7 (Fig. 3). One of the remaining two animals showed a relatively large recovery during the second week (animal jn5, see Fig. 4B).



Location	Distance from apex (mm)	CF (kHz)
b1	16.5	26.2
b2	12.5	10.4
m1	9.5	5.1
m2	7.5	2.7
a1	5.5	1.3
a2	3.5	0.7
a3	1.5	0.3

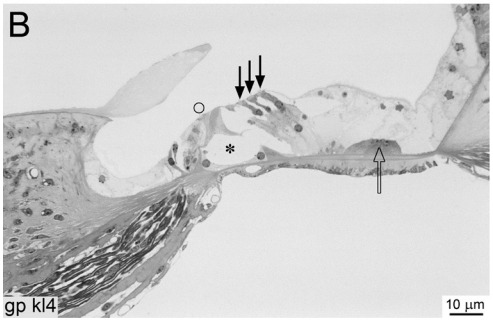


Figure 6(A) Midmodiolar section (1 µm) of cochlea of guinea pig *kl5* (1 week after deafening). Transections of the cochlear half-turns are labeled b1 and b2 for the basal turns, m1 and m2 for the middle turns, and a1, a2, and a3 for the apical turns. The table (Van Ruijven et al., 2005) indicates the position along the basilar membrane and characteristic frequency (CF) which correspond to the particular location of the transactions. H: helicotrema; n.VIII: cochlear nerve.

(B) Organ of Corti of normal cochlea of guinea pig *kl4*, in cochlear half-turn b1. o: inner hair cell; arrows: outer hair cells; asterisk: tunnel of Corti; open arrow: Boettcher's cells.

(C) Organ of Corti in b1 eight weeks after deafening (guinea pig *ap1*). The organ of Corti is collapsed with both the IHCs and OHCs missing. The amount of nerve fibers in the spiral osseous lamina (arrow) is clearly diminished. Asterisk: tunnel of Corti; open arrow: Boettcher's cells.

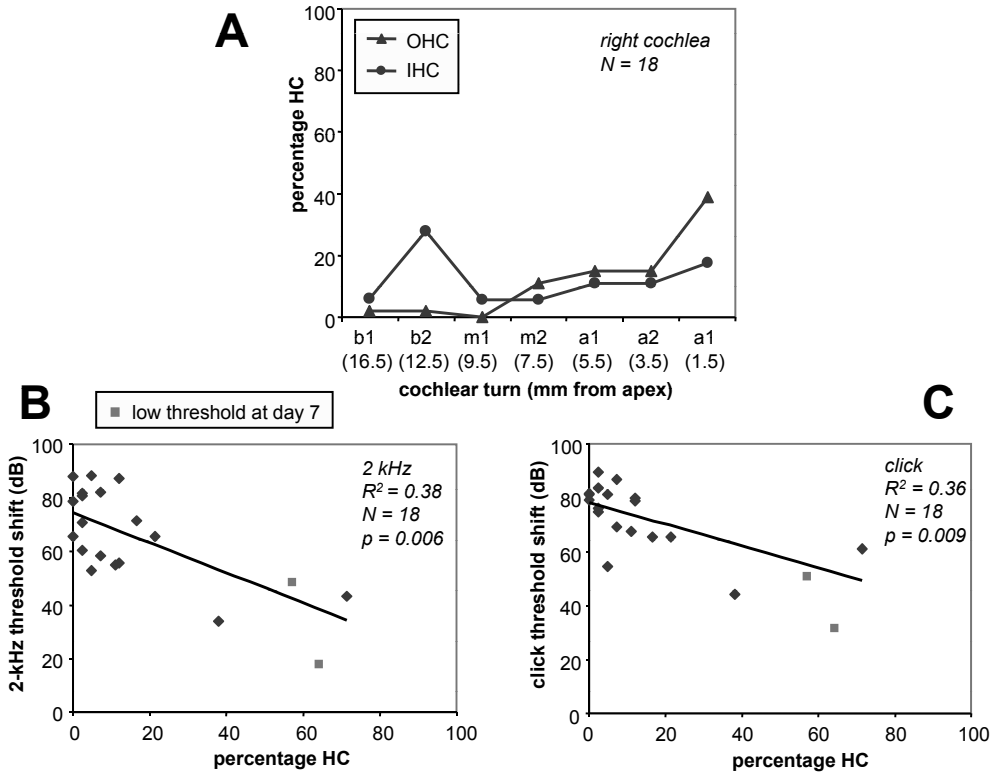


Figure 7 (A) Hair cell survival averaged across animals with high thresholds at 7 days after deafening (threshold shift for clicks > 55 dB; n=18). OHC and IHC percentages are plotted separately as a function of cochlear half-turn.

(B, C) CAP thresholds versus average HC survival. OHC and IHC numbers have been averaged across cochlear half-turns, and subsequently the obtained OHC and IHC percentages have been averaged into one data point. The grey squares indicate the guinea pigs with relatively low thresholds at 7 days after deafening (threshold shifts < 35 dB). (B) Thresholds to 2-kHz tones. For animals with high thresholds at 7 days (n = 18): $R^2 = 0.38$, $F(1,16) = 9.9$, $p = 0.006$; for all animals (n = 20): $R^2 = 0.56$, $F(1,18) = 22.7$, $p < 0.001$. (C) Thresholds to click stimuli. For animals with high thresholds at 7 days: $R^2 = 0.36$, $F(1,16) = 8.9$, $p = 0.009$; for all animals: $R^2 = 0.59$, $F(1,18) = 26.1$, $p < 0.001$.

3.5. Spiral ganglion cell survival

The light micrographs in Fig. 8 illustrate the progressive degeneration of SGCs with the period after the deafening treatment. Some loss of SGCs is visible two weeks after deafening; the loss is severe after four and eight weeks.

The SGC packing density was measured in the three most basal half-turns (b1, b2, m1). Figure 9 shows the time course of the SGC packing density measured in both cochleas of the normal-hearing animals (n=5) and the deafened animals that showed high thresholds at day 7 (n=18). The SGC packing density decreased with the period after deafening. This decrease can be described by an exponential decay function with a time constant of 7 weeks (1/e=37 % survival after 7 weeks).

Furthermore, the SGC packing density depended on the survival of IHCs. Figure 10 shows the data separately for each survival time, indicating a modest increase of SGC density with IHC survival (increase of about 200 SGCs/mm^2 from 0 to 100 % IHC). Multiple regression analysis with IHC percentage and duration after deafening as independent variables showed, apart from the strong correlation with the duration after deafening, that the SGC packing density was significantly correlated to the IHC survival (p<0.05).

3.6. CAP thresholds and hair cell survival

Figure 11A shows the thresholds at one day after deafening as a function of hair cell survival, comparable to Fig. 7B. The thresholds at one day after deafening appeared to show a relation to hair cell survival which could be best described by a power function (R^2=0.60, p<0.0001). It might be attractive to predict hair cell survival on the basis of an early electrophysiological assessment. Therefore, hair cell survival is plotted as a function of the day-1 threshold shifts (Fig. 11B). The data show that for threshold shifts less than 3 dB hair cell survival is greater than 10 %, and for threshold shifts greater than 30 dB hair cell survival is less than 10 %.

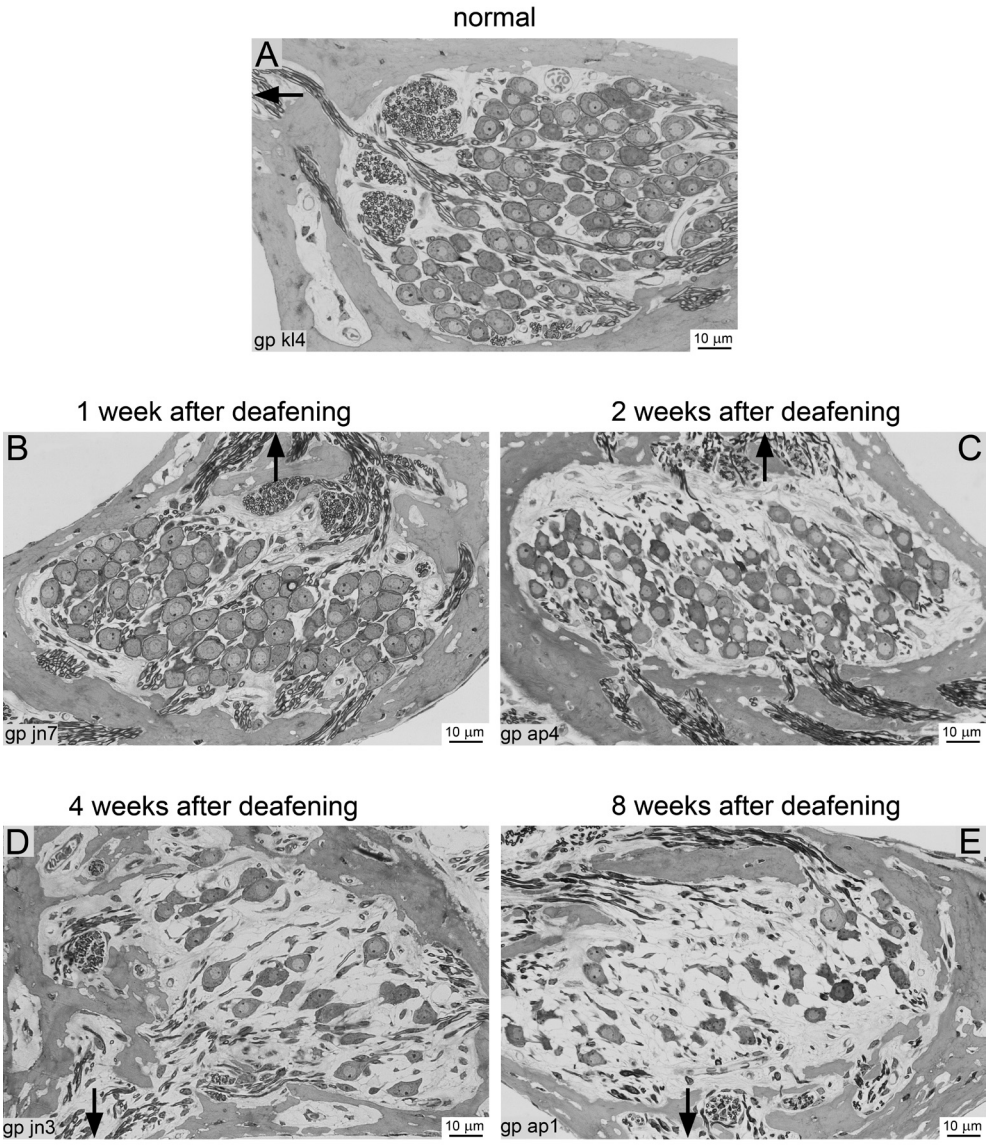


Figure 8 Spiral ganglion cells in normal cochlea and in cochleas following concomitant kanamycin and furosemide administration. The arrows in the micrographs indicate the direction of the organ of Corti. (A) Normal cochlea, lower basal turn b1 (guinea pig *kl4*) (B) 1 week after deafening (guinea pig *jn7*). (C) 2 weeks after deafening (guinea pig *ap4*). (D) 4 weeks after deafening (guinea pig *jn3*). (E) 8 weeks after deafening (guinea pig *ap1*). B-E: in lower middle turn m1.

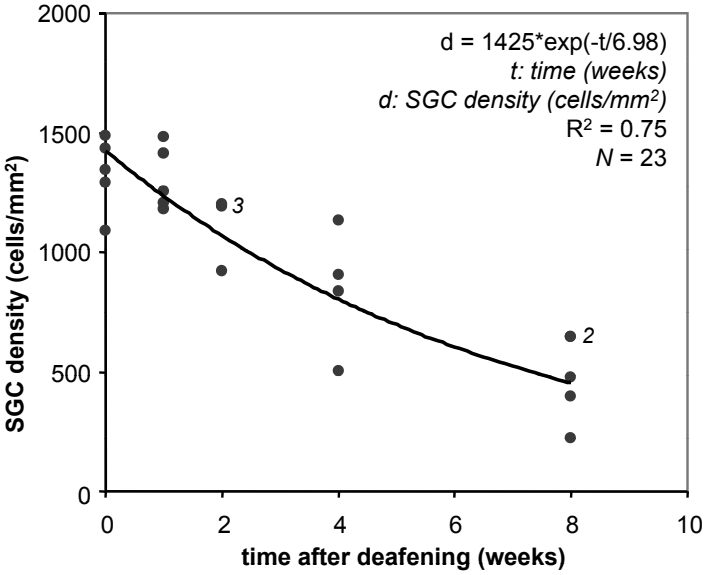


Figure 9 SGC packing density as a function of survival time after deafening. The data include 5 normal-hearing animals and 18 deafened animals. Like the deafened animals, the normal control animals had an electrode implanted on the right cochlea. The SGC packing density is obtained by averaging SGC densities across the three most basal half-turns (b1, b2, m1), and by averaging over both ears. The italic digit near a data point indicates the number of overlapping data points. An exponential function has been fitted to the data: the time constant is 7.0 weeks, indicating that after that period 37 % (1/e) of the SGCs are still present. Linear regression of logarithm of SGC density versus survival time: $R^2 = 0.75$, $F(1,22) = 64.6$, $p < 0.0001$.

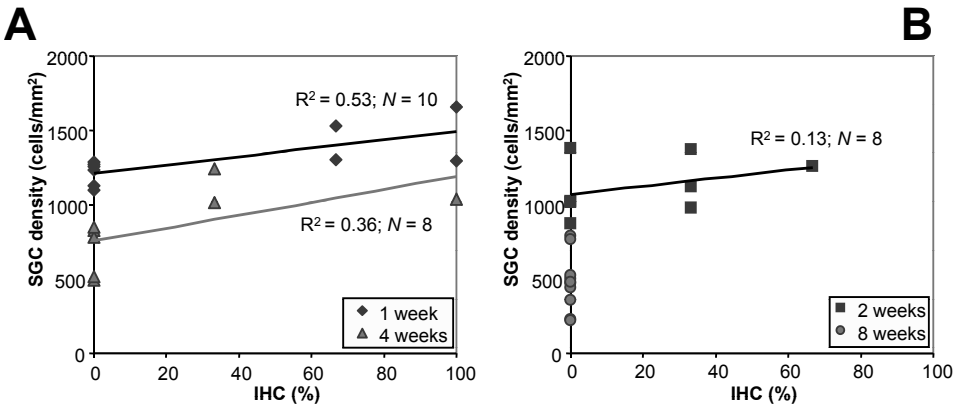


Figure 10 SGC packing density as a function of percentage surviving IHCs. SGC packing density and IHC numbers have been averaged across the three most basal half-turns (b1, b2, m1). For clarity, the data are shown in two plots: 1 week and 4 weeks after deafening (A), 2 and 8 weeks after deafening (B).

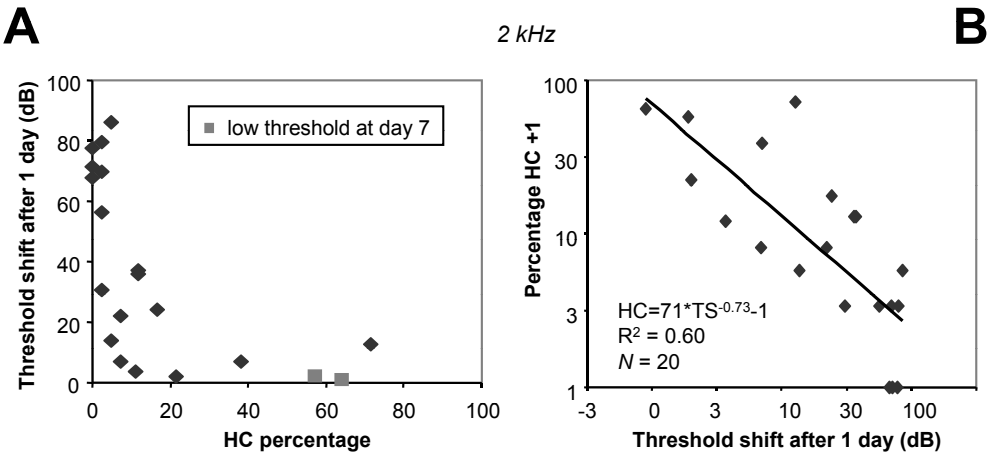


Figure 11 Threshold shift after 1 day for 2-kHz tones related to percentage surviving hair cells. OHC and IHC numbers have been averaged across all cochlear half-turns, and subsequently the obtained OHC and IHC percentages have been averaged. (A) Thresholds as a function of HC percentage, plotted as in Fig. 7B. (B) HC percentage as a function of thresholds, plotted on a double logarithmic scale. Linear regression: $R^2 = 0.60$, $F(1,18) = 27.0$, $p < 0.0001$.

4. Discussion

The time courses of the CAP thresholds registered after the deafening procedure with standard doses of kanamycin and furosemide contained two interesting features. First, the threshold shifts at one day after deafening were highly variable among animals (100 dB range). Second, there was a small but significant recovery during the second week following substantial threshold shifts measured 4 to 7 days after deafening (55-85 dB).

4.1. First-day thresholds

The immediate threshold shifts we found after the deafening treatment agree with other studies applying administration of an aminoglycoside in combination with a loop diuretic (West et al., 1973; McFadden et al., 2004; Nourski et al., 2004). This early decline of the cochlear response is attributed to the reversible effect of furosemide, including the smaller effect on the CM than on the CAP (*CAP*: Sewell, 1984; Hu et al., 2003; *CM*: West et al., 1973; Van Emst et al., 1997). A drop of the endocochlear potential is thought to cause the decline of hair cell current (CM) and cochlear nerve activity. Recording cochlear potentials, West et

al. (1973), Nourski et al. (2004) and McFadden et al. (2004) found a recovery of the response within one hour, which can be attributed to a recovery of the endocochlear potential (Sewell, 1984; Van Emst et al., 1997). This recovery typically lasted only briefly (less than 4 hours) and, subsequently, a second decline of the response occurred. However, in some animals (two of six in Nourski et al.) the recovery lasted for at least 12 hours. The current findings indicate that the recovery may last for even 24 hours, in which case the response either strongly declines during the second 24-hour period or it weakly declines and does not reach a high threshold (as was found in 2 out of 20 animals). Thus, the second phase of the threshold increase occurs within 5 hours, or between 24 and 48 hours. In the former case the threshold shift is always large (> 30 dB/day), and in the latter case in the shift is large or small. A schematic of the three possible time courses in the first two days after deafening, combining the data of Nourski et al. and ours, is illustrated in Fig. 12. This second phase can be attributed to severe kanamycin-induced damage to OHCs as observed within 6 hours by Russell et al. (1979). The high correlation we found between CM and CAP thresholds at day 1 also indicates that during the first day the main damage is inflicted upon the OHCs. In most animals, the thresholds reached a maximum after four days (Figs. 2A,B). This concurs with histological studies indicating that hair cell loss, which is initially limited to OHC loss, has stabilized in three to five days after deafening (Webster and Webster, 1981; Dodson, 1997).

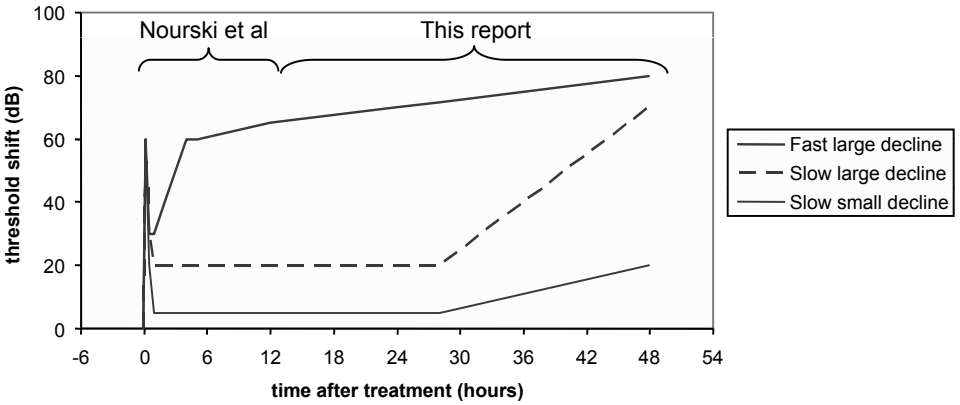


Figure 12 Schematic of possible early time courses after the deafening procedure. The course during the first 12 hours is based on data of Nourski et al. (2004) and the course between 24 and 48 hours is based on the data presented in this report. The immediate threshold shift after deafening is found in both reports.

The wide distribution of threshold shifts at day 1 (Fig. 2D) can be explained by a variability in either the degree (in dB) or the duration of the recovery. The former might contribute more than the latter, considering the lack of a correlation between threshold shifts and period after deafening (Fig. 2C).

The correlations between CAP thresholds after 7 days and those after 1 day (Fig. 3) and between surviving hair cells and CAP thresholds after 1 day (Fig. 11) imply that the faster the kanamycin reaches its cellular target the larger the final effect. The relatively high correlation between hair cell survival and thresholds after 1 day, in spite of the long period between histology and recording, might be surprising. However, one should consider the distribution of threshold shifts at day 1 which is uniform and wide compared to the later distributions (Figs. 2, 3). The measuring errors can be assumed to be constant over time, however, the measuring error relative to the spread of thresholds is smaller at day 1 than at later moments. This small relative error might counterbalance the effect of the early timing of the measurement. In other words, animals can be distinguished on the basis of the thresholds at day 1 no worse than on the basis of thresholds measured later. It allows for a fair assessment after only one day of the long-term effect of the deafening treatment.

4.2. Second-week recovery

Various studies using the deafening method according to West et al. (1973) apply an inclusion criterion based on absolute thresholds or threshold shifts (50 – 60 dB) after 4 to 7 days (Mitchell et al., 1997; Kanzaki et al., 2002; Wise et al., 2005; Shepherd et al., 2005) . According to those criteria 18 out of 20 guinea pigs in our study had sufficient threshold shifts (>55 dB for clicks). With such high thresholds, which indicate severe damage to the organ of Corti, a recovery might not be likely. However, our data strongly suggest some recovery between 7 and 14 days after deafening (Figs. 4, 5). Similarly, in a longitudinal CAP study in guinea pigs Aran and Darrouzet (1975) found recovery from kanamycin treatment after 8 to 10 days, also in the second week. They attributed the recovery to clearance of kanamycin from the cochlea. Alternatively, the ototoxic drug might hamper strial function, thereby reducing the endocochlear potential (EP). Recovery of the EP would improve the function of the surviving hair cells. Klis et al. (2002) presented evidence for this mechanism to explain recovery from a 60 dB hearing loss after cisplatin treatment.

This late functional recovery implies that caution should be taken in studies in which total hair cell loss is required. In that case, the threshold shift after 7 days or earlier is not an appropriate inclusion criterion and thresholds after at least two weeks or the post-hoc histological examination of the organ of Corti might be used instead. Treatment during the second week with for instance neural growth factors aimed at enhancing SGC survival, might be confounded by the spontaneous functional recovery. In particular, in studies with treatment to induce hair cell regeneration one should be aware of, and preferably avoid, the possibility of spontaneous recovery not related with the treatment.

The slow decline following the second-week recovery (Fig. 4A) is reminiscent of data reported by Aran and Darrouzet (1975). They found a deterioration phase two months after the start of the recovery phase (discussed above). Similarly, Stengs et al. (1997) reported some deterioration of cochlear potentials 2-4 months after recovery from cisplatin-induced hearing loss in guinea pigs.

4.3. SGC degeneration

The degeneration of SGC after cochlear trauma induced by aminoglycosides has been studied extensively (Ylikoski, 1974; Webster and Webster, 1981; Xu et al., 1993; Dodson, 1997; Leake and Rebscher, 2004). It is generally thought that the degeneration of SGCs is secondary to loss of IHCs and the afferent nerve terminal, since SGC trauma has never been observed in the presence of IHCs. Conditions in which OHCs are lost while IHCs are intact do not lead to SGC loss (Ylikoski, 1974). The correlations between SGC packing density and IHC percentage (Fig. 10) are congruent with this proposition. Various mechanisms might play a role in the degeneration such as absence of neurotrophic support, absence of neural activity, and glutamate excitotoxicity (discussed in Dodson, 1997).

In global terms, the time course we found (Fig. 9) concurs with findings of others. The time constant varies between studies depending on various factors such as species and type and/or dosage of insult (e.g., Dodson and Mohuiddin, 2000; Leake and Rebscher, 2004; McFadden et al., 2004). For instance, a time constant of about 3 weeks has been found in guinea pigs deafened by an intracochlear injection with gentamicin (Dodson and Mohuiddin, 2000) and a time constant of about 8 months has been found in cats neonatally deafened with neomycin (Leake and Rebscher, 2004); in humans the degeneration is generally even slower. The shape of the time course might vary as well. We chose to fit the data with an

exponential function with a single time constant. Data obtained over longer periods than in the current study (8 weeks) indicate that a single time constant does not suffice (Leake and Rebscher, 2004, in cat, study of 7 years; Dodson and Mohuiddin, 2000, in guinea pig, study of 7 months). As various mechanisms might play a role in the degeneration (see above), multiple time constants are plausible.

4.4. Implications

The recordings at day 1 are important to predict final thresholds and hair cell survival. If very limited or no survival is required, e.g. for regeneration research, then the first-day threshold should be high (>30 dB for 2 kHz tones). On the other hand, if some residual hearing is needed, e.g., in the context of the combination of electrical and acoustical stimulation, then thresholds should be low. In general, the thresholds at day 1 can serve as a useful independent variable.

Acknowledgements

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Chronic electrical stimulation of the degenerating auditory nerve; morphology and functionality of spiral ganglion cells

3

Abstract

Some studies have demonstrated that treatment with intracochlear chronic electrical stimulation (CES) protects spiral ganglion cells (SGCs) from degeneration in deafened animals. In contrast, other studies reported no effect of CES. The present study examined whether CES in a mode as presented in human cochlear implants (amplitude modulated, high pulse rate) affected SGC survival, morphology and functionality in deafened guinea pigs. Eleven guinea pigs were implanted in the right cochlea with an electrode array, and deafened four weeks later. To monitor the functionality of the auditory nerve, electrically evoked auditory brainstem responses (eABRs) were recorded. Five animals received monopolar CES through three electrodes in the basal cochlear turn. CES was started two days after deafening and lasted 4h/day, five days per week, for a period of six weeks. The packing density of the SGCs was not affected by CES. The only effect found was that SGCs of implanted cochleae, irrespective of whether they received CES, were larger and more circular than SGCs in unimplanted cochleae. Probably, brief electrical stimulation during eABR recordings and a limited behavioural experiment, but not CES itself, affected the morphology of surviving SGCs. A less likely alternative is that the implantation itself caused the alterations. The amplitudes of the suprathreshold eABR responses and the eABR thresholds in CES treated animals were not significantly different from those in untreated animals. In conclusion, brief electrical stimulation might have an effect on the morphology of SGCs, but substantial stimulation does not per se affect either the number or the morphology of these cells.

Keywords: Chronic electrical stimulation, Inner hair cell, Guinea pigs, Spiral ganglion cells

1. Introduction

Loss of inner hair cells results in a progressive loss of spiral ganglion cells (SGCs) (Spoendlin, 1975; Webster and Webster, 1981; Leake and Hradek, 1988; Shepherd and Hardie, 2001). When cochlear hair cells are lost there is a rapid decline in endogenous neurotrophic support (Ylikoski et al., 1993; Fritzsch et al., 1999) and a decline of the spontaneous and evoked activity in the SGCs (Liberman and Kiang, 1978; Hartmann et al., 1984; Shepherd and Javel, 1997). The loss of metabolic activation of SGCs has been found to result in secondary loss

of neurotrophic factor secretion by SGCs themselves (Hyson and Rubel, 1989). The loss of the continuous supply of neurotrophins by hair cells and SGCs is considered to be the major factor leading to degeneration of the auditory nerve (Lefebvre et al., 1992; Roehm and Hansen, 2005). Together with the loss of neurotrophic support by presynaptic cells (hair cells), there are also morphological and functional changes in other parts of the auditory system. These occur initially in the cochlear nucleus, which is the central target of the auditory nerve (Leake et al., 2006; Stakhovskaya et al., 2008), and then extend into the deeper regions of the auditory brain, the inferior colliculus (Vollmer et al., 2007), and finally into the auditory cortex (Ryugo et al., 1997; Kral and Tillein, 2006). These changes may also contribute to the degeneration of SGCs since both presynaptic and postsynaptic efferent connections are necessary for SGC survival in vivo (Hegarty et al., 1997).

Intracochlear chronic electrical stimulation (CES) prevented degeneration of SGCs in several studies. These studies examined the effect of CES in guinea pigs (Lousteau, 1987; Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al., 1997; Kanzaki et al., 2002; Miller et al., 2003), rats (Song et al., 2008) and cats (Leake et al., 1991; Araki et al., 1998; Coco et al., 2007). However, CES was not always successful (Shepherd et al., 1994; Araki et al., 1998; Li et al., 1999; Shepherd et al., 2005). In the studies that were successful the effect of CES on SGC survival was not as prominent as the effect of exogenous neurotrophic treatment (Miller, 2001; Pettingill et al., 2007). Compared to the SGC packing density in cochleae of normal-hearing guinea pigs, SGC survival was at most 50% (Miller, 2001; Leake et al., 2008). In contrast, intracochlear infusion of neurotrophins, initiated within the first week after deafening, yielded greater than 90% SGC survival compared to the population of SGCs in cochleae of normal-hearing guinea pigs (Ernfors et al., 1996; Steacker et al., 1996; Gillespie and Shepherd, 2005; Richardson et al., 2006). Remarkably, experiments performed in vitro show the opposite. In SGC cell cultures depolarization was more effective than application of neurotrophins (Hegarty et al., 1997).

There are many methodological differences between the studies that addressed the neurotrophic effect of CES. The studies differ in the species that was used, the intensity of stimulation, the duration of stimulation, the duration of deafness before the start of electrical stimulation and the electrode configuration. To make a careful comparison between the conditions during CES in our study, and the conditions of CES in other studies, the experiments performed in guinea pigs will be further discussed in the next paragraph.

To our knowledge, there are currently 8 studies describing the effect of electrical stimulation in deafened guinea pigs (Lousteau, 1987; Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al., 1997; Li et al., 1999; Kanzaki et al., 2002; Miller et al., 2003; Shepherd et al., 2005). The parameter settings (pulse rate, current, electrode configuration, duration of stimulation) and results obtained in these studies are summarized in Table 1. An effect of CES on SGC density was reported in 5 of the studies shown in Table.1. In 7 of the studies CES was started within the first eight days after deafening. Shepherd et al. (2005) started CES ten days after deafening, and Miller and Altschuler (1995) started CES one moth after deafening in an additional group of animals. Miller and Altschuler (1995) reported a reduced protective efficacy in this additional group. The majority of studies applied a monopolar stimulus configuration by placing a single-ball electrode into the scala tympani via the round window membrane and the reference electrode on the bulla. Shepherd et al. (2005) delivered amplitude modulated stimuli to a bipolar intracochlear electrode pair. Mitchell et al. (1997) concluded that high-rate (2750 pulses/s) stimulation provides the same benefits to SGC survival as low-rate (250 pulses/s) stimulation. Intensities varying from 100 to 400 μA and durations varying from 1 h/day to 24 h/day were effective. They reported also that even brief electrical stimulation, applied during their eABR recordings (electrically evoked Auditory Brainstem Responses) that were performed once a week, reduced the degeneration of SGCs. This was consistent with findings of Miller and Altschuler (1995), who also reported a reduced degeneration of SGCs in cochleae receiving brief electrical stimulation during eABR recordings. These results indicate that a wide range of settings was effective and that it is uncertain which factors exactly determine the efficacy of electrical stimulation (Miller, 2001; Leake et al., 2008). For similar reasons it is not possible to conclude which parameter settings caused the ineffectiveness of CES in the studies of Li et al. (1999) and Shepherd et al. (2005).

The current study was undertaken to investigate the effect of amplitude modulated CES in deafened guinea pigs on survival, morphology and functionality of SGCs. We applied amplitude-modulated high rate CES in freely moving animals in order to approach the situation in real cochlear implant users. CES was started two days after deafening because Miller and Altschuler (1995) reported that delayed CES was less effective in preventing degeneration of SGCs. Three electrodes in the basal turn were activated. The variables we studied were SGC packing density, perikaryal area, cell circularity, eABR amplitude and eABR thresholds. In addition, the area of Rosenthal's canal was analysed because CES might cause bone growth in Rosenthal's canal affecting the SGC packing density (Li et al., 1999).

Table 1. Summary of the main variables of chronic electrical stimulation (CES) that can affect SGC packing density in deafened guinea pigs. 8 studies that investigated the effect of CES in deafened guinea pigs are listed. The studies 2-6 are performed in the same lab. D = day of deafening, CI = day of cochlear implantation, CES = day that chronic electrical stimulation was started, ICEEP = inferior colliculus electrically evoked potential. The arrow (↑) indicates that CES affected the SGC density. 0 indicates that CES did not affect the SGC density.

Reference	Age / Weight	D/CI/CES	Duration	Stimuli	Intensity	Electrode	Stimulation	SGC density
1) Lousteau et al., 1987	200-300 g	D 0 CI -2 CES 0	1 h/day 45 days	Constant current square wave 250 µs per phase 200 pps, monopolar	100 µA	3 mm Teflon wire (stainless steel .007 inch diameter), intracochlear	CES during sedation (chloral hydrate)	↑
2) Hartshorn et al., 1991	300-450 g	D 0 CI -? CES 5	2 h/day 5 days/ week 63 days	1 kHz sinus on a 50% duty cyclus, monopolar	400, 300, 200, 100 µA	250-µm bal 2 mm in scala tympani Teflon wire (stainless steel .007 inch diameter)	CES during fixation	↑
3) Miller and Altschuler, 1995	300-450 g	D 0 CI 5 CES 5 and 28	7 h/day	Charge-balanced 200 µs pulses, 50 or 200 Hz, monopolar	100, 200 µA (6-12 dB above eABR threshold)	250-µm bal 90% platinum 2 mm in scala tympani	CES with battery in harness	↑
4) Mitchell et al., 1997	225-425 g	D 0 CI 5 CES 8	24 h/day 42 days	1 kHz sinus on a 40% cyclus 40 µs on 60 µs of, monopolar	250, 100, 25 µA	250-µm bal 90% platinum 2 mm in scala tympani	CES with battery in harness	↑
5) Kanzaki et al., 2002	240-380 g	D 0 CI 5 CES 8	24 h/day 36 days	100 µs/phase, 250 Hz on a 40% cyclus, monopolar	100 µA	250-µm bal 90% platinum 3 mm in scala tympani	CES with battery in harness	↑
6) Miller et al., 2003	200-300 g	D 0 CI 4 CES 7	24 h/day 36 days	Charge-balanced 100 µs/ph, 250 Hz, monopolar	100 µA	250-µm bal (90% platinum) electrode/cannula 3 mm in scala tympani	CES with battery in harness	↑
7) Li et al., 1999	± 300 g	D 0 CI -7 CES 0	5 h/day 5 days/ week 56 days	Biphasic 200 µs/phase 100 pps, monopolar	ICEEP -2 dB ICEEP +2 dB ICEEP +6 dB	0.4 mm bal 90% Pt, cochleostomi, intracochlear and extracochlear	CES with battery in harness	0
8) Shepherd et al., 2005	332-816 g	D 0 CI 5 CES 10	6 h/day 5 days/ week 23 days	100 µsec/phase charge-balanced biphasic current 1,200 pps amplitude modulated 50% at 30 Hz, bipolar	0.39-1.6mA (max 6 dB above eABR threshold)	Three Pt band electrodes on a 0.6 mm silicone carrier, intracochlear	CES bipolar	0

2. Material and Methods

2.1. Animals and experimental design

Eleven female albino guinea pigs (strain: Dunkin Hartley; weighing 250-350 g) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the animal care facility of the Rudolf Magnus Institute of Neuroscience. All animals had free access to both food and water and were kept under standard laboratory conditions. Lights were on between 7:00 am and 7:00 pm. Temperature and humidity were kept constant at 21°C and 60%, respectively. In all animals the right cochleae were implanted with an eight-electrode array four weeks before deafening. The eABRs were recorded once or twice per week. In one animal eABRs could not be recorded with the same electrode anymore in later sessions due to electrode failure. With the exception of one guinea pig the animals were additionally tested in a pilot behavioural study (see Chapter 7 of this thesis). During the behavioural training the animals were trained to detect intracochlear electrical stimulation and received brief electrical stimulation for maximally 5 minutes per day during the last 5 days of the experiment. Five animals received CES, which started two days after deafening and lasted six weeks. The animals were killed for histology six weeks after deafening and both left and right cochleae were analyzed using light microscopy.

Four cohorts of cochleae were studied, namely the right implanted cochleae of animals that received brief electrical stimulation, but did not receive CES (6 weeks deaf no CES, *6wdnoCES*), the left untreated cochleae of these animals (6 weeks deaf untreated 1, *6wdu1*), the right implanted cochleae of animals receiving CES (6 weeks deaf CES, *6wdCES*), and a second group of left untreated cochleae of the animals receiving CES (6 weeks deaf untreated 2, *6wdu2*).

Data of SGC packing density and morphology were compared to data from normal cochleae presented in a previous paper (Agterberg et al., 2008). All experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC # 03.04.036).

2.2. Implantation and cochlear function

Animals were anesthetized with Domitor® (medetomidine hydrochloride; 10 mg/kg, im) and Ketanest-S® ((S)-ketamine; 40 mg/kg, im). The right cochleae were implanted with an eight-electrode array (platinum ring electrodes of 0.3 mm width, inter-electrode distance: 0.75 mm,

Cochlear®, Mechelen, Belgium). The right bulla was exposed retro-auricularly and a small hole was created with a scalpel, to visualize the cochlea. The array was inserted 3-4 mm through a cochleostomy in the basal turn near the round window. The array was fixed on the bulla with dental cement (Ketac-Cem Aplicap, ESPE Dental Supplies, Utrecht, The Netherlands) and connected to the skull with a screw (Brown et al. 1993) and fixed with dental cement. The incision was closed in two layers with vicryl.

Acoustically evoked auditory brainstem responses (aABRs) and eABRs were measured in freely moving animals. The aABRs were recorded to check hearing thresholds and the eABRs to assess the excitability of the SGCs. All electrophysiological recordings were performed in a sound-attenuated chamber. They were recorded with three stainless steel screws (8.0x1.2mm) inserted into the skull bone 1 cm posterior to bregma, 2 cm anterior to bregma and 1 cm lateral from bregma, respectively (Mitchell et al., 1997). Stimulus generation and signal acquisition were controlled with custom-written software and a personal computer. The stimuli were synthesized and attenuated using a Tucker-Davis Technologies TDT3 system (modules RP2, PA5 (2x) and SA1). The responses were amplified differentially using a Princeton Applied Research 113 pre-amplifier (amplification: 5,000; band pass filter: 0.1-10 kHz) with the posterior and anterior screws as active and reference electrodes, respectively, and the lateral screw as ground electrode. The amplified signal was digitized by the TDT3 system (module RP2) and made available for off-line analysis.

For aABR recordings broadband click stimuli consisting of monophasic rectangular pulses (20 μs, 10 pulses/s) were presented in free field from 75 dB above threshold in normal-hearing animals (~110 dB peSPL) down to threshold in 10-dB steps.

For recordings of eABRs, alternating monophasic current pulses (20 μs, 10 pulses/s) were presented to the most apical intracochlear electrode using the lateral screw in the skull as return electrode (monopolar eABR recordings). Stimuli were presented from 400 μA down to threshold in 2 dB steps. Fig. 1 shows an eABR evoked with 400 μA. The first positive peak (P1) in the eABR recordings was often obscured by the electrical artifact. The first negative peak (N1) and the second positive peak (P2) were always clearly visible and not obscured by the electrical artifact, nor by the digastric muscle response (see Hall, 1990), which appeared in approximately half of our measurements at high stimulus levels.

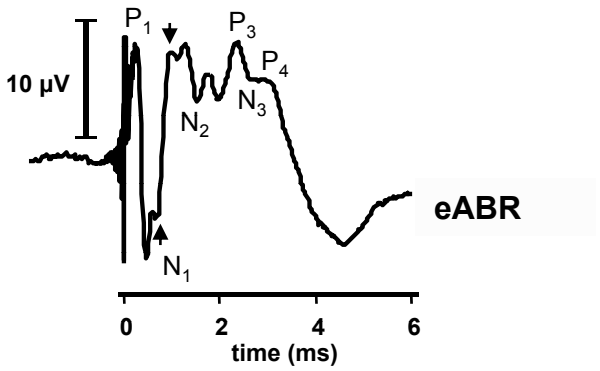

Fig. 1. eABR evoked with 400 µA pulses. Stimulus onset is at time 0. When N_1 and/or P_2 consisted of more than one sub peak, the first sub peak on each side of the fast rising part of the eABR complex (indicated with the arrows) was used in the analysis.

For these reasons wave N_1-P_2 was used as measure of the amplitude of the eABR. Hall (1990) reported that the amplitude evoked with suprathreshold current levels correlated well with SGC density, and therefore we considered wave N_1-P_2 an appropriate parameter to examine the excitability of the SGCs. When N_1 and/or P_2 consisted of more than one sub peak, the first sub peak on each site of the fast rising part of the eABR complex (indicated with the arrows) was used for the analysis.

The threshold was defined as the stimulus level that evoked a 2 µV reproducible waveform of N_1-P_2.

2.3. Deafening Procedure

Animals were anesthetized with Domitor® (10 mg/kg, im) and Ketanest-S® (40 mg/kg, im) and injected subcutaneously with kanamycin (400 mg/kg, sc) followed (15-60 minutes later) by slow intravenous infusion of furosemide (100 mg/kg, iv). This procedure, originally reported by West et al. (1973), has been shown to eliminate almost all the hair cells (Gillespie et al., 2003; Versnel et al., 2007). For the intravenous infusion of furosemide, the left or right jugular vein was exposed and a catheter was inserted. A successful insertion was confirmed with withdrawal of blood into the catheter. Animals were included in the study when they demonstrated a threshold shift of > 50 dB, 14 days after deafening.

2.4. CES

Chronic electrical stimulation (CES) was started the second day after deafening. Freely moving animals, connected with a cable to a commutator (SL6CM7/07, Plastic One Inc, Roanoke, USA) were stimulated 4 hours per day, 5 days per week, and CES was continued for six weeks. Stimuli were biphasic pulses with 20 μs pulse width/phase and an interphase gap of 6 μs. Stimuli were presented via a commercially available implant interface (implant-in-a-box; Cochlear®, Mechelen, Belgium).

The input to the interface was amplitude-modulated white noise (amplitude modulation: low-pass noise, cut-off: 30 Hz) generated in a pc and processes by a sound card (Creative® USC sound blaster 5.1). The noise was converted to pulses by a Freedom processor (Cochlear®) which were delivered to the implant-in-a-box. Threshold was set at 80 current units (74 μA) and the maximum level was 170 current units (377 μA). With these settings, each electrode delivered 1200 pps (~20% below threshold (no pulses), ~15% in a range of 100-200 μA, ~40% in a range of 200-300 μA, ~25% in a range of 300-400 μA). An example of a stimulus pattern during 30 ms is illustrated in Figure 2. Three electrodes were active per animal, using the caudal screw in the skull as return electrode (monopolar electrical stimulation).

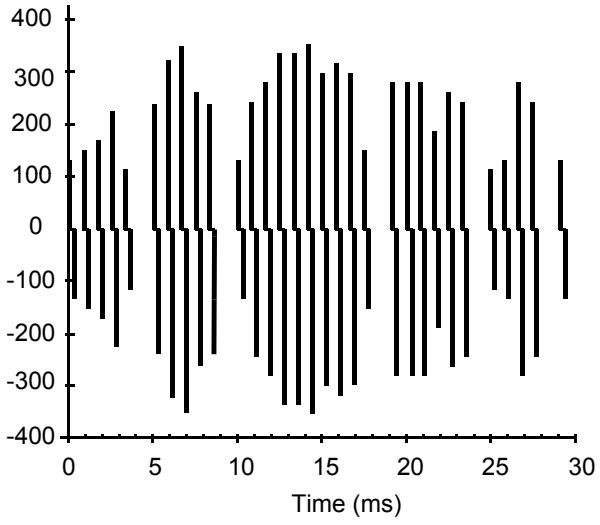

Fig. 2. Illustration (30 ms) of amplitude modulated high pulse rate (1200 pps) electrical stimulation. Pulses are biphasic with 20 μs pulse width/phase and an interphase gap of 6 μs, thus, the pulses in the illustration are wider than actually presented.

2.5. Hair cell counts and determination of SGC packing densities

Immediately after the final ABR measurements, the left and right cochleae were fixed by intralabyrinthine perfusion with a fixative consisting of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% DMSO in 0.08 M sodium cacodylate buffer (pH 7.4), followed by immersion in the same fixative for 3 h at room temperature and subsequent histological processing for light microscopy (for further details, see De Groot et al., 1987). Light microscopical assessment and quantitative analyses (hair cell counts and SGC packing densities) were performed using semithin (1 μm) midmodiolar sections stained with methylene blue and azur II in sodium tetraborate. The efficacy of the deafening procedure was assessed by measuring ABR threshold shifts, and by counting the inner hair cells and outer hair cells in all cochleae, at seven different locations along the basilar membrane (B1-A3; Fig. 4B).

SGC counts were performed in digitized light microscopical images of Rosenthal's canal were taken from midmodiolar sections (1 μm). The bony boundaries of Rosenthal's canal were outlined and its cross-sectional area calculated. The number of perikarya of the SGCs was counted in each individual transection of Rosenthal's canal. This procedure was performed for those half-turn transections that were present in all animals, which was up to and including the most basal part of the apical half-turn, at 5.5 mm from the apex (location A1 in Fig. 4B). SGC packing density was calculated by dividing the number of SGCs by the area of Rosenthal's canal.

2.6. Determination of perikaryal area and circularity

Of all cells at cochlear location B2 containing an obvious nucleus, the perikaryal area and circularity were measured. Perikaryal area as a measure of cell size was determined by outlining the myelin sheath of the SGCs. For outlining, a pressure-sensitive stylus on a digitizer tablet was used. Cell circularity is a feature of cell shape that can be measured directly in *NIH Image* (Version 1.63; US National Institutes of Health, Bethesda, Maryland) after delineating the perimeter of the cell. It is calculated as follows: $4\pi \cdot A/L^2$, where A is area and L is perimeter (circularity is 1 for a perfect circle and less than 1 for an imperfect circle, e.g. 0.78 for a square).

2.7. Statistical analyses

Statistical analyses were performed using SPSS® for Windows (version 15.0.1). The data of SGC densities, perikaryal area and cell circularity, area of Rosenthal's canal and eABR measures were analyzed using repeated-measures analysis of variance (RM ANOVA). Differences were considered statistically significant when $p<0.05$.

3 Results

3.1. Hair cell loss and aABR threshold shifts

After deafening all eleven animals demonstrated threshold shifts >60 dB for click-evoked aABRs. Thus, hearing loss was well above our criterion of a 50 dB shift. This severe hearing loss was microscopically confirmed. All inner and outer hair cells were lost at all cochlear locations, except in two cochleae. In the right cochlea of one animal receiving brief electrical stimulation during eABR recordings and in the right cochlea of one animal receiving CES, a few IHCs remained.

3.2. SGC packing density and area of Rosenthal's canal

Figure 3 shows light micrographs of Rosenthal's canal at cochlear location B2, providing typical examples of SGCs of the four cohorts (*6wdu1, 6wdnoCES, 6wdu2* and *6wdCES*). In all examples a remarkable loss of SGCs was evident as compared to the SGC population in a normal-hearing animal (Fig. 3E). There was no protective effect of CES (compare D to C). However, in the cochleae that were briefly electrically stimulated (*6wdnoCES*) or received CES (*6wdCES*), the SGCs appeared to have a more ovoid shape compared to the non-stimulated contralateral ears (*6wdu1* and *6wdu2*). In the *6wdu1* and *6wdu2* groups the SGCs had lost their characteristic ovoid shape and acquired a more elongated or dendritic appearance.

Figure 4A shows the mean SGC packing densities in the groups at cochlear locations from B1 through A1. The data confirm the individual results shown in Fig. 3. Densities in all four experimental groups were considerably lower than the SGC packing densities in cochleae of normal-hearing animals. SGC densities were not affected by CES. RM ANOVA revealed no statistically significant differences in SGC packing density between the *6wdu2* and *6wdCES* groups. Furthermore, ANOVA revealed no statistically significant difference in SGC packing density between the *6wdu1* and *6wdnoCES* groups, indicating that brief electrical stimulation

did not affect the SGC packing density either. It is possible that brief electrical stimulation had induced local effects at the basal cochlear locations in which the electrode array was positioned. Therefore, we statistically compared the SGC density of the stimulated implanted ears (*6wdnoCES*) to non-stimulated ears (*6wdu1*) in the same animals at cochlear location B2. A paired t-test showed that the SGC packing density at location B2 was not statistically different (p>0.1).

Figure 5 shows a histogram of the mean area of Rosenthal's canal in all groups of cochleae. The area of Rosenthal's canal of the cochlear locations B1 through A1 was not affected by CES or brief electrical stimulation.

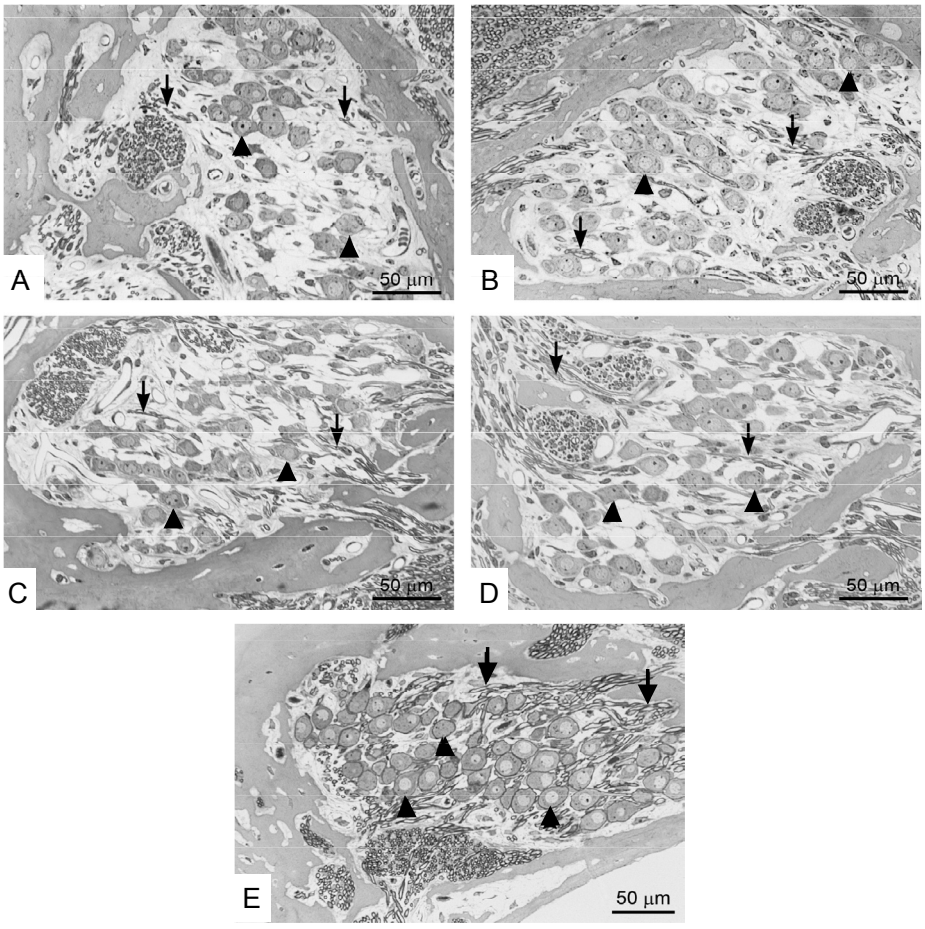

Fig. 3. Light micrographs of Rosenthal's canal at location B2. SGCs are indicated with arrowheads and nerve fibers with arrows. (A, B) SGCs and nerve fibers in the spiral ganglion in the *6wdu1* (A) and *6wdnoCES* (B) groups. (C, D) SGCs and nerve fibers in the spiral ganglion in *6wdu2* (C) and *6wdCES* (D) groups. (E) SGCs and nerve fibers in a normal-hearing control.

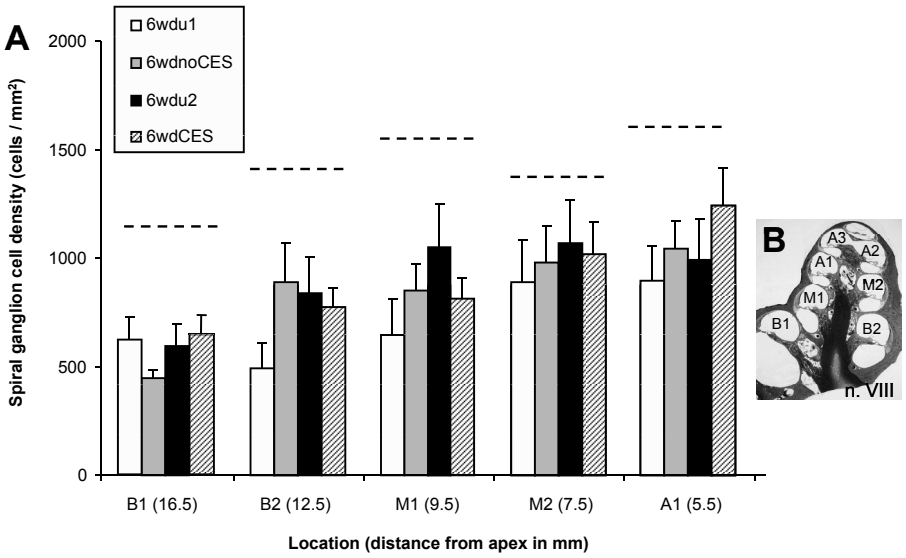

Fig. 4. (A) Mean SGC packing densities at locations B1, B2, M1, M2, and A1 in the *6wdu1* (n=6 cochleae), *6wdnoCES* (n=6 cochleae), *6wdu2* (n=5 cochleae) and *6wdCES* (n=5 cochleae) cohorts. The distance from the apex (in mm) of the corresponding locations B1 through A3 is indicated according to Greenwood (1990). Dashed lines: SGC densities in normal-hearing animals at locations B1-M2; data taken from a previous study (Agterberg et al., 2008). Error bars: SEM. (B) Midmodiolar section (1 µm) of a normal guinea-pig cochlea showing the different locations at which SGCs were examined. N. VIII: cochlear nerve.

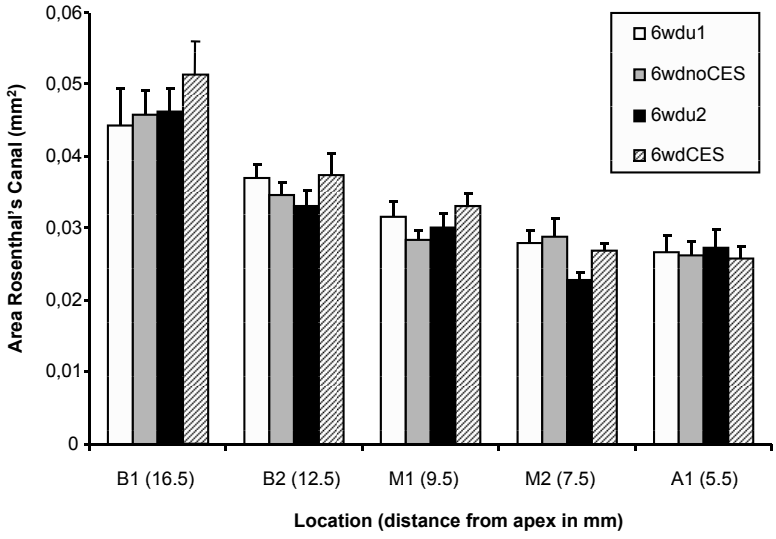

Fig. 5. Mean area of Rosenthal's canal at locations B1, B2, M1, M2, and A1 in the four cohorts of cochleae *6wdu1* (n=6), *6wdnoCES* (n=6), *6wdu2* (n=5) and *6wdCES* (n=5).

3.3. Perikaryal area and circularity

Figure 6 shows the mean values of the perikaryal area (A) and circularity (B) of SGCs at location B2. Perikaryal area and circularity of SGCs in the right implanted cochlea, receiving brief (*6wdnoCES*) or extensive (*6wdCES*) electrical stimulation, were larger than in the left unstimulated cochleae (ANOVA; F = 6.2, df = 1, 9, p<0.05 and F = 6.9, df = 1, 9, p<0.05 respectively).

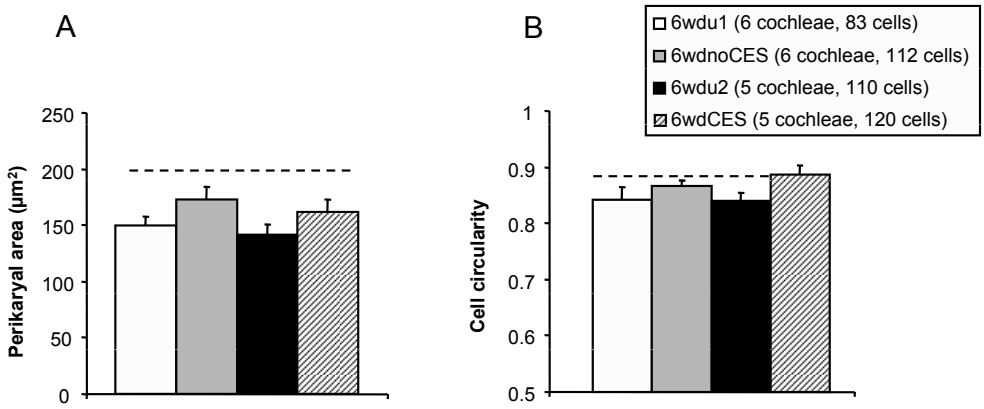

Fig. 6. Perikaryal area (A) and circularity (B) of SGCs in the *6wdu1, 6wdnoCES, 6wdu2* and *6wdCES* cohorts. Data shown were obtained by averaging all individual SGC measurements within one spiral ganglion, followed by averaging over cochleae. Dashed lines represent measurements in normal-hearing animals (Data from Agterberg et al., 2008). Error bars: SEM.

3.4 eABR recordings

Figure 7 shows the mean time course of eABR amplitudes elicited with 400 µA pulses for the two groups of animals. The eABR recordings performed immediately after implantation (- 4 weeks) and just before sacrificing (week 6) are not presented in the figures because these measurements were not performed in awake animals as were all other recordings. There were no significant differences in amplitude of wave N_1-P_2 between the *6wdnoCES* and *6wdCES* groups. Both treatment groups demonstrated a gradual increase of the amplitude (RM ANOVA: F = 7.1, df = 3, 24, p<0.01) during the first 4 weeks after implantation; this is a consistent finding in our laboratory and remains unexplained. After deafening a gradual decrease of the amplitude of wave N_1-P_2 was found (RM ANOVA: F = 7.0, df = 5, 40, p<0.001).

Figure 7B shows the amplitudes after normalization with respect to the data before deafening. Analysis of the normalized data confirmed the similarity in amplitudes between the *6wdnoCES* and *6wdCES* groups (F = 11.4, df = 1, 4, p<0.001).

Both the *6wdnoCES* group and the *6wdCES* group demonstrated a significant decrease in eABR threshold the first week after implantation (RM ANOVA: F = 7.0, df = 3, 24, p<0.01). After deafening no significant threshold shifts or differences between these two groups were observed.

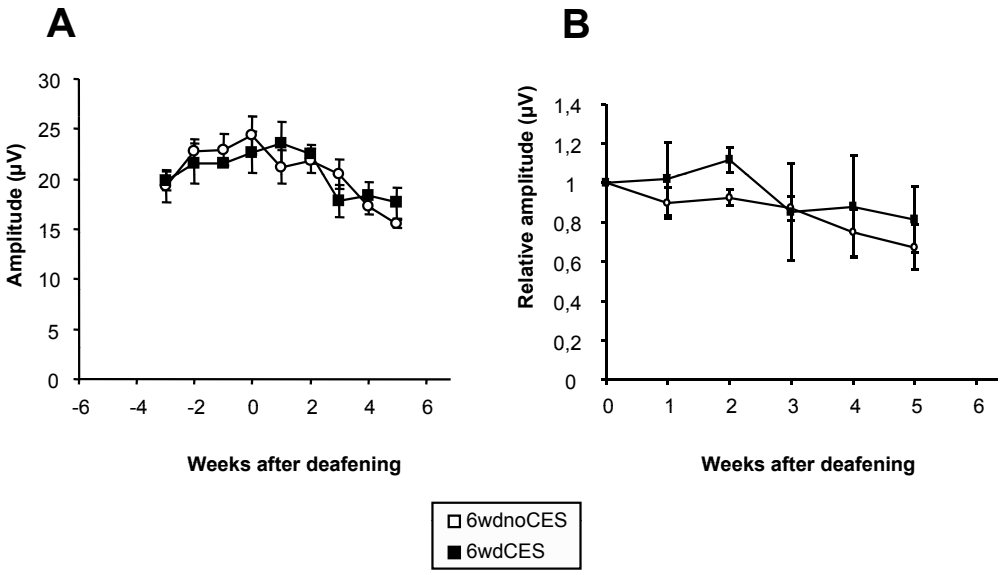

Fig. 7. (A) Mean amplitudes of eABRs recorded to 400-µA pulses in control (*6wdnoCES*, n=5) and experimental (*6wdCES*, n=5) animals. The animals are implanted at -4 weeks and deafened at 0 weeks. Error bars: SEM. (B) Mean amplitude shift relative to baseline eABR measurements on day 0 and day -7. Error bars: standard deviation.

4 Discussion

The most important finding in this study is that high pulse rate amplitude modulated CES did not prevent degeneration of SGCs (Figs. 3, 4). Furthermore, CES by itself did not affect the morphology and the excitability of the SGCs. However, the SGCs in all untreated left cochleae were smaller than normal. SGCs in the right implanted cochleae, receiving either brief electrical

stimulation or CES, were larger and more circular than SGCs in the left cochleae, approaching normality (Figs. 3, 6). The latter indicates an effect of brief stimulation on the morphology of SGCs, or an effect of the implantation itself. Because CES did not affect the SGC packing density, it is not remarkable that CES did not affect the excitability of the auditory nerve, as measured by eABR recordings (Fig. 7). Both eABR amplitudes at suprathreshold stimulation and eABR thresholds were similar whether or not an animal received CES.

4.1. Inconsistent effects of CES

The present results are in agreement with Li et al. (1999) and Shepherd et al. (2005), but in disagreement with several other studies (Lousteau, 1987; Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al., 1997; Kanzaki et al., 2002; Miller et al., 2003). Several factors that might have contributed to the variability across animal studies have been proposed. It has been suggested that electrical stimulation at high stimulus rates may lead to neural degeneration (Tykocinski et al., 1995). However, Mitchell et al. (1997) demonstrated that high-rate (2750 pulses/s) stimulation provides the same benefits to SGC survival as low-rate (250 pulses/s) stimulation. Furthermore, Leake et al. (1995, 2008) suggested that monopolar electrical stimulation might directly activate the auditory nerve axons within the modiolus, and that this mode of stimulation may not prevent degeneration of the SGC cell body. This seems unlikely since it was demonstrated in several studies that monopolar electrical stimulation can be effective in reducing the degeneration of SGCs (Lousteau, 1987; Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al., 1997; Kanzaki et al., 2002; Miller et al., 2003). We investigated the effect of amplitude modulated CES on SGC density to approach the situation in real cochlear implant users. Although a wide range of settings was effective in reduction of SGC degeneration (Miller, 2001; Leake et al., 2008) and it is reported that amplitude modulated CES was effective in bilaterally deafened kittens (Leake et al., 1999), we found no effect of CES on SGC densities. Thus, it remains uncertain which factors are critical for a successful intervention with CES.

4.2. Presence of hair cells in relation to the effect of CES

The first study that demonstrated a protective effect of CES on SGC survival in deafened guinea pigs was reported by Lousteau (1987). They reported increased survival of SGCs in deafened guinea pigs with recognizable structures in the organ of Corti, while they reported

no effect of CES in animals with a complete loss of identifiable structures in the organ of Corti (these structures were not specified in this study). They argued that electrical stimulation may prevent or slow down IHC degeneration and thus secondarily protect SGC degeneration. This suggestion is not supported in a study performed in cats (Coco et al., 2007). In this study, the authors also demonstrated enhanced survival of SGCs in animals with remaining hair cells, but they reported that there was no significant difference in hair cell survival between the stimulated and non-stimulated groups. It might be that remaining hair cells facilitate the protective effect of CES. Hair cells express neurotrophins (Ylikoski et al., 1993), and it was demonstrated in a number of studies that exogenous delivery of neurotrophins does enhance the effect of CES (Kanzaki et al., 2002; Shepherd et al., 2005; Song et al., 2008). In other words, the effect of CES was additive, or even synergetic, to the effect of infusion of neurotrophins. This indicates that at least partly different neurobiological mechanisms are involved in the protection of SGCs from degeneration. Both neurotrophic factors, expressed by remaining hair cells, and depolarization, may be required for the survival of SGCs (Hegarty et al., 1997). On the other hand, several studies demonstrated enhanced survival of SGCs in the stimulated ears of guinea pigs with essentially complete hair cell loss (Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al., 1997; Kanzaki et al., 2002).

In conclusion, it might be that the effects of CES are less prominent or absent in cases of complete loss of cochlear hair cells, and that CES was not effective in our study because of the almost complete loss of cochlear hair cells in all cochlear turns.

4.3. Morphology of SGCs

It has been previously reported that CES affected the SGC soma size (Araki et al., 1998; Coco et al., 2007), which is supported by our data with respect to brief electrical stimulation. Assuming that a closer to normal cell size and shape is an indication of greater cell vitality, which has been suggested by others who investigated the size of SGCs after intracochlear neurotrophic treatment in deafened guinea pigs (Shah et al., 1995; Steacker et al., 1996; Gillespie et al., 2004; Richardson et al., 2005; Miller et al., 2007; Agterberg et al., 2008), the present data (Fig. 6) indicate that brief electrical stimulation provided trophic support. However, in the present study the animals were implanted before deafening. Although previous animal studies reported that the presence of an electrode in the cochlea at the time of deafening, did not affect the SGC density (Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al.,

1997; Miller, 2001), it can not be ruled out that the presence of an electrode array during and after the deafening procedure had minor effects on the morphology of surviving SGCs.

4.4. Possible overestimation of the effect of CES on SGC packing densities

The studies that reported an effect of CES on SGC density (Lousteau, 1987; Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al., 1997; Kanzaki et al., 2002; Miller et al., 2003) relied on counts that might have included biased stereological methods and did not take into account the possible differences in cell size (Coggeshall and Lekan, 1996; Agterberg et al., 2008; Leake et al., 2008). For this reason it might be that those studies (Lousteau, 1987; Hartshorn et al., 1991; Miller and Altschuler, 1995; Mitchell et al., 1997; Kanzaki et al., 2002; Miller et al., 2003) overestimated the effect of CES on SGC packing densities. For an example of the correction of SGC packing densities for size, see Fig. 7 in Chapter 4 (Agterberg et al., 2008). In this study SGCs were larger because of intracochlear infusion of brain-derived neurotrophic factor (BDNF). This example demonstrates that after correction for size, SGC packing densities in cochleae treated with BDNF were still significantly larger than in untreated cochleae. Since the reported effects of CES on SGC size are smaller (~10%; Coco et al., 2007) than the reported effects of intracochlear infusion of BDNF on SGC size (~30%; Shepherd et al., 2005; Agterberg et al., 2008), we conclude that a possible overestimation can not fully explain the reported effect of CES on SGC density.

4.5. Clinical observation in relation to the effect of CES on SGC density

Finally, we would like to mention that the absence of an effect of CES on SGC density, as presented in this study and by others (Li et al., 1999; Shepherd et al., 2005), is in line with the observation that in human temporal bones of people who received a cochlear implant during life, SGC survival between implanted and non-implanted ears did not differ (Fayad and Linthicum, 2006). This indicates that the mode of electrical stimulation as used in cochlear implant recipients and in the present study, might not be the optimal mode to protect the auditory nerve from degeneration.

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Morphological changes in spiral ganglion cells after intracochlear application of Brain-Derived Neurotrophic Factor in deafened guinea pigs

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Abstract

When guinea pigs are deafened with ototoxic drugs spiral ganglion cells (SGCs) degenerate progressively. Application of neurotrophins can prevent this process. Morphological changes of rescued SGCs have not been quantitatively determined yet. It might be that SGCs treated with neurotrophins are more vulnerable than SGCs in cochleae of normal-hearing guinea pigs. Therefore, the mitochondria and myelinisation of type-I SGCs were studied and the perikaryal area, cell circularity and electron density were determined. Guinea pigs were deafened with a subcutaneous injection of kanamycin followed by intravenous infusion of furosemide. Brain-derived neurotrophic factor (BDNF) delivery was started two weeks after the deafening procedure and continued for four weeks. Four cohorts of cochleae were studied: (1) cochleae of normal-hearing guinea pigs; (2) of guinea pigs two weeks after deafening; (3) six weeks after deafening; (4) cochleae treated with BDNF after deafening. The deafening procedure resulted in a progressive loss of SGCs. Six weeks after deafening the size of mitochondria, perikaryal area and cell circularity of the remaining untreated SGCs were decreased and the number of layers of the myelin sheath was reduced. In the basal part of the cochlea BDNF-treatment rescued SGCs from degeneration. SGCs treated with BDNF were larger than SGCs in normal-hearing guinea pigs, whereas circularity had normal values and electron density was unchanged. The number of layers in the myelin sheath of BDNF-treated SGCs was reduced as compared to the number of layers in the myelin sheath of SGCs in normal-hearing guinea pigs. The morphological changes of SGCs might be related to the rapid loss of SGCs that has been reported to occur after cessation of BDNF treatment.

Keywords: Brain-Derived Neurotrophic Factor; Electron microscopy; Guinea pig; Perikaryal area; Spiral ganglion cells

1. Introduction

One of the factors explaining the variability in perceptual performance of cochlear implant users might be the amount of surviving spiral ganglion cells (SGCs) in relation to the extent and duration of the sensorineural hearing loss. Animal studies have shown that loss of inner hair cells (IHCs) results in progressive loss of SGCs (Ylikoski et al., 1974; Webster and Webster,

1981), possibly as a consequence of loss of neurotrophic support (Lefebvre et al., 1992). Most studies report a decrease in perikaryal area of SGCs after hair cell loss (Staecker et al., 1996; Leake et al., 1999; Shepherd et al., 2005; Richardson et al., 2005). Behavioral studies in monkeys have presented strong evidence that a greater survival of SGCs is associated with improved performance with a cochlear implant (Pfingst et al., 1981, 1983). Studies in rats and cats have shown that progressive loss of SGCs reduces the efficacy of electrical stimulation, as measured by electrically evoked auditory brainstem responses (Hall, 1990; Miller et al., 1994; Hardie and Shepherd, 1999; Shepherd et al., 2004). Although there is no explicit clinical evidence from post-mortem studies that enhanced SGC survival results in a better performance with a cochlear implant (Nadol et al., 2001; Khan et al., 2005), it might be clinically important to preserve the integrity of the peripheral neural system in the cochlea of cochlear implant candidates and recipients.

A potential way to preserve SGCs after deafening via ototoxic drugs, is the exogenous intracochlear application of neurotrophic factors (for overviews, see Miller et al., 2002; Gillespie et al., 2005; Pettingill et al., 2007). Intracochlear application of neurotrophic factors, such as neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF), prevents loss of auditory neurons in the guinea pig (Staecker et al., 1996; Miller et al., 1997). When neurotrophic treatment was started after degeneration had set in, 2-6 weeks after deafening, the remaining SGCs could still be rescued (Gillespie et al., 2004; Yamagata et al., 2004; Richardson et al., 2005; Wise et al., 2005; Miller et al., 2007). According to the neurotrophin hypothesis and based upon findings in other sensory systems (Montero and Hefti, 1988; Mansour-Robaey et al., 1994), it is expected that cessation of BDNF treatment results in degeneration of the SGCs. Gillespie et al. (2003) have confirmed this expectation. Remarkably, they reported an accelerated degeneration of SGCs after cessation of BDNF treatment as compared to degeneration without BDNF. This abnormally rapid degeneration suggests that the SGCs have changed during BDNF treatment and that rescued SGCs are more vulnerable than SGCs at the start of treatment. Some studies qualitatively describe BDNF-treated SGCs with terms as: “large”, “round” and “healthy” in order to assess the size, shape, and condition of the SGCs (Shah et al., 1995; Gillespie et al., 2004; Miller et al., 2007). Although two studies have quantitatively shown that BDNF-treated SGCs in deafened guinea pigs are larger than untreated SGCs (Shepherd et al., 2005; Richardson et al., 2005) and one study reported a reduction in the number of individual layers in the myelin sheath of SGCs treated with BDNF

and acidic fibroblast growth factor (Glueckert et al., 2008), the shape and condition of the SGCs rescued with BDNF have not been quantitatively determined.

The aim of this study was to characterize and quantify morphological changes in SGCs after deafening and subsequent BDNF treatment. Light microscopy was used to examine the amount of degeneration of SGCs after delayed BDNF treatment. Electron microscopy was used to describe the morphology of the SGCs in detail and quantify their size, circularity and electron density.

2. Materials and methods

2.1. Animals and experimental design

Albino female guinea pigs (strain: Dunkin Hartley; weighing 250-350 g) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the animal care facility of the Rudolf Magnus Institute of Neuroscience. All animals had free access to both food and water and were kept under standard laboratory conditions. Lights were on between 7:00 am and 7:00 pm. Temperature and humidity were kept constant at 21°C and 60%, respectively.

We used one control group and two experimental groups of animals. In these groups both left and right cochleae were analyzed using light- and electron microscopical procedures. The control group consisted of four normal-hearing guinea pigs (*normal*). The right cochleae of these animals were implanted with a cannula two weeks after a sham deafening procedure. Phosphate-buffered saline (PBS) was infused into the cochleae for four weeks, and the animals were killed for histology immediately after finishing the PBS infusion. The first experimental group contained three guinea pigs killed for histology two weeks after deafening (*two-weeks deaf untreated, 2wdu*), of which both the left and right cochlea were used. The second experimental group consisted of six animals, killed six weeks after deafening, of which the left cochleae were untreated (*six-weeks deaf untreated, 6wdu*). The right cochleae of these animals were implanted with a cannula two weeks after deafening and treated with BDNF for four weeks (*six-weeks deaf BDNF treated, 6wdBDNF*). These animals were killed for histology immediately after finishing the BDNF treatment. Summarizing, four cohorts of cochleae were studied (*normal, 2wdu, 6wdu, and 6wdBDNF*). All implanted animals included in this study had an open cannula at termination and were without any trace of otitis media. All experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC # 03.04.036).

2.2. Deafening procedure and ABRs

Animals were anesthetized with xylazine (Sedamun®, i.m. 10 mg/kg) and ketamine (Ketanest®, i.m. 40 mg/kg). Before the deafening procedure, three stainless steel screws (8.0x1.2mm) were inserted into the skull bone to record auditory brainstem responses (ABRs). The screws were inserted 1 cm posterior to bregma, 2 cm anterior to bregma and 1 cm lateral from bregma (Mitchell et al., 1997). Before, during and after deafening we recorded ABRs to monitor hearing thresholds. Measurements were performed in a sound-attenuated chamber. Broadband click stimuli consisting of biphasic rectangular pulses (100 μs/phase) were presented in free field using a tweeter (Fane J-104) positioned 10 cm above the unanesthetized guinea pig. Stimulus generation and signal acquisition were controlled with custom-written software and a personal computer (for details, see Versnel et al., 2007). Stimuli were presented from 86 dB above threshold in normal-hearing animals down to threshold in 10-dB steps. Threshold was defined as the sound level at which the ABR was just noticeable upon visual inspection of the response.

When initially normal hearing thresholds were confirmed, kanamycin (400 mg/kg) was injected subcutaneously followed by slow intravenous infusion of furosemide (100 mg/kg) as a loop diuretic. This procedure, first described by West et al. (1973) using ethacrynic acid (40 mg/kg) as the loop diuretic, has been shown to eliminate almost all cochlear hair cells. Gillespie et al. (2003) and Versnel et al. (2007) confirmed this procedure using furosemide instead of ethacrynic acid. For the intravenous infusion of furosemide, the left jugular vein was exposed and a catheter was inserted. A successful insertion was confirmed with withdrawal of blood into the catheter. The control animals received isotonic saline (subcutaneously and intravenously), instead of kanamycin and furosemide. After deafening, the ABRs were measured on days 1, 7 and 14 to assess the extent of hearing loss. All animals of the experimental groups included in this study demonstrated a threshold shift of ≥50 dB, measured 14 days after deafening.

2.3. Cannula implantation and BDNF treatment

Two weeks after the deafening procedure, six deafened animals and the four control animals were implanted with a cannula in the right cochlea. The cannula consisted of medical vinyl tubing (90 mm, ID: 0.8 mm) with a tip of polyimide tubing (11 mm, ID: 0.12 mm); the total volume was ~40 μl. Alzet mini-osmotic pumps (model 2004; flow rate 0.25 μl/h; reservoir 200 μl) were filled with BDNF (PeproTech®) solution (100 μg/ml), and then incubated in sterile saline

for 48 h at 37°C to guarantee a constant flow rate at implantation. The BDNF concentration of 100 µg/ml was chosen because it is in the range of dosages (50 to 100 µg/ml) that have been proven to be effective in several studies; Gillespie et al. (2003, 2004) infused 62.5 µg/ml, Shepherd et al. (2005) 62.5 µg/ml, Wise et al. (2005) 50 µg/ml, and Miller et al. (2007) used 100 µg/ml. Cochleae were treated with BDNF for four weeks. For the normal-hearing animals, the mini-osmotic pumps were loaded with PBS, pH 7.4. Bovine serum albumin (BSA, 1%) was added to the BDNF and PBS solutions.

The animals were anesthetized with xylazine and ketamine, and the right bulla was exposed retro-auricularly. A small hole was drilled to visualize the cochlea. The round window membrane was perforated and the tip of the cannula was inserted into the scala tympani through the round window (Prieskorn and Miller, 2000). The cannula, filled with BDNF solution (~40 µl; 100 µg/ml) or PBS, was fixed onto the bulla with dental cement (Ketac-Cem Aplicap; ESPE Dental Supplies, Utrecht, the Netherlands). The mini-osmotic pumps were attached to the cannula and inserted into a subcutaneous pocket. The cannula was connected to the skull with a screw (Brown et al., 1993) and fixed with dental cement. The wound was closed in two layers with Vicryl®.

2.4. Hair cell counts and determination of SGC packing densities with light microscopy

Immediately after the final ABR measurements, the left and right cochleae were fixed by intralabyrinthine perfusion with a fixative consisting of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% DMSO in 0.08 M sodium cacodylate buffer (pH 7.4), followed by immersion in the same fixative for 3 h at room temperature and subsequent histological processing for light and electron microscopy (for further details, see De Groot et al., 1987). Light microscopical assessment and quantitative analyses (hair cell counts and SGC packing densities) were performed using semithin (1 µm) midmodiolar sections stained with methylene blue and azur II in sodium tetraborate. The efficacy of the deafening procedure was assessed in addition to ABR thresholds by counting the inner hair cells (IHCs) and outer hair cells (OHCs) in all cochleae. The number of hair cells present in one midmodiolar plane was counted at seven different locations along the basilar membrane at a half-turn spacing (B1, B2, M1, M2, A1, A2, and A3; Fig. 1), as described by Van Ruijven et al. (2004). Hair cell counts were performed by two well-trained investigators, independently of one another, in a single-blind fashion.

Mean SGC packing densities were determined as described previously (Van Ruijven et al., 2004). Digitized light microscopical images of the spiral ganglia taken from five different locations in the cochlea (B1, B2, M1, M2, and A1; Fig. 1) were imported in *NIH Image* (Version 1.63; US National Institutes of Health, Bethesda, Maryland). SGC packing densities could not always be determined for the most apical locations (A2 and A3), due to tangential sectioning of Rosenthal's canal at this level. The bony boundaries of Rosenthal's canal were outlined and its cross-sectional area (in mm^2) was calculated. The number of perikarya of the SGCs was counted at each location. SGC packing density was calculated by dividing the number of SGCs by the cross-sectional area of Rosenthal's canal and expressed as the mean number of SGCs per mm^2.

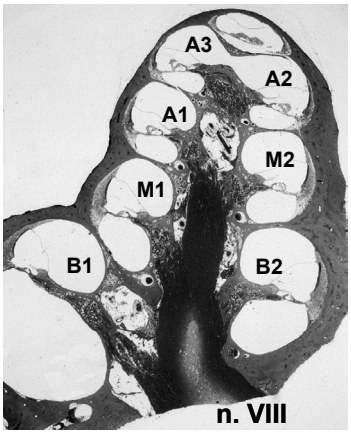
2.5. Electron microscopy

In order to study the ultrastructural morphology of the SGCs and to quantify morphological changes, ultrathin (60-90 nm) sections of re-embedded quarter turns were contrast-stained with methanolic uranyl acetate and Reynolds' lead citrate and examined in a JEOL 1200EX transmission electron microscope (De Groot et al., 1987). The morphology of the SGCs was studied in the basal turn of the cochlea, a location where the effect of BDNF on SGC packing density is most prominent. Because there is a considerable intracochlear regional variation in perikaryal area (Leake et al., 1999), and possibly also in other cellular features, SGCs at a specific location (12.5 mm from the apex; location B2 in Fig.1) were examined and photographed for quantitative ultrastructural analysis.

To obtain an overview of the spiral ganglion, the entire Rosenthal's canal was photographed in a series of electron micrographs that were printed at a final magnification of x3200, and these were assembled into a complete photomontage. Based upon close inspection of photomontages of spiral ganglia from deafened and BDNF-treated cochleae, several cellular features of the myelinated (type-I) SGCs were selected for further quantitative analysis: (1) perikaryal area; (2) cell circularity; and (3) electron density. All cells demonstrating morphological determinants typical of type-I SGCs (for details, see Romand and Romand, 1984) were counted and measured. The morphological determinants included a myelin sheath and cytoplasm containing large aggregates of cisternae of the rough endoplasmic reticulum, stacks of the Golgi apparatus and large numbers of mitochondria. However, only data obtained from type-I SGCs with an obvious nucleus were included in these analyses. Mean perikaryal

area, circularity and electron density were obtained by averaging all individual measurements within one spiral ganglion, and then by averaging all data of the individual cochleae.

From the photomontages several SGCs from all cohorts were selected for close inspection of the mitochondrial size and number of layers in the myelin sheath. Electron micrographs of the selected SGCs were printed at a final magnification of x32,000.



Location	CF (kHz)	Distance from apex (mm)
B1	26.2	16.5
B2	10.4	12.5
M1	5.1	9.5
M2	2.7	7.5
A1	1.3	5.5
A2	0.7	3.5
A3	0.3	1.5

Fig. 1. Midmodiolar section (1 µm) of a normal guinea-pig cochlea showing the different locations at which SGCs were examined. The table (adapted from: Van Ruijven et al., 2005) gives the characteristic frequencies (CF; Greenwood, 1990) and the distance from the apex (in mm) of the corresponding locations B1 through A3. N. VIII: cochlear nerve.

2.5.1. Determination of perikaryal area

Perikaryal area delineated by the innermost layer of the myelin sheath of the type-I SGCs with an obvious nucleus was outlined. In case of cell shrinkage the area delineated by the plasma membrane of the SGC perikaryon was outlined. For outlining a pressure-sensitive stylus on a digitizer tablet was used.

2.5.2. Determination of cell circularity

Cell circularity is a feature that can be measured directly in *NIH Image* after delineating the perimeter of the cell. It is calculated as follows: $4\pi \cdot A/L^2$, where *A* is area and *L* is perimeter (circularity is 1 for a perfect circle and less than 1 for an imperfect circle, e.g. 0.78 for a square).

2.5.3. Determination of electron density

Electron density was measured in the perikaryon of the SGC, including both the nucleus and the cytoplasm. A gray level value between 0 (white) and 255 (black) was assigned to each image pixel, and then gray levels in the images were calculated. The same program settings, such as image contrast and brightness, were used for all images analyzed. The data were normalized by comparing them with the densitometric data averaged from four rectangular sampling fields in the modiolar bone in the same photomontage.

2.6. Correction of packing densities for size

When the SGCs do not have the same size, an error is introduced in calculating the SGC packing densities (Coggeshall and Lekan, 1996; Leake et al., 1999). In all four cohorts of cochleae (*normal, 2wdu, 6wdu, and 6wdBDNF*) the mean perikaryal area at location B2 was determined and the SGC packing densities in the experimental cohorts were corrected for size by using the equation:

$$b_{cor} = b \cdot \sqrt{\frac{A_n}{A}}$$

Where b_{corr} is the corrected packing density, b is the actually counted packing density, A_n is the perikaryal area of SGCs in the *normal* group, and A is the perikaryal area of SGCs in the experimental groups. Since we performed electron microscopic analysis at one location (B2 in Fig. 1) we could not correct for size at the other locations.

2.7. Statistical analyses

Statistical analyses were performed using SPSS® for Windows (version 12.0.1). The data of SGC densities at the different cochlear locations (B1-A1) from the *normal, 6wdu* and *6wdBDNF* groups were analyzed using repeated-measures analysis of variance (RM ANOVA). To identify differences between these groups at specific cochlear locations t-tests were used. Statistical comparisons of the morphological parameters (perikaryal area, cell circularity and electron density) at location B2 between *normal, 2wdu* and *6wdBDNF* groups were made using unpaired samples *t*-tests, and paired samples *t*-tests were used to compare the morphological parameters between the *6wdu* and *6wdBDNF* groups.

3. Results

3.1. Hair cell loss and ABR threshold shifts

Seven out of the nine deafened animals demonstrated threshold shifts of ≥70 dB for click-evoked ABRs, two weeks after the deafening procedure. Two animals (one from *2wdu* and one from *6wdu/6wdBDNF*) had a threshold shift of ~50 dB, two weeks after the deafening procedure. The ABR thresholds in the *6wdu/6wdBDNF* group remained stable during BDNF treatment and were the same six weeks after deafening as two weeks after deafening. In the animals with a threshold shift of ≥70 dB, almost all IHCs and OHCs were lost (2-4% remaining). In the animals with a threshold shift of ~50 dB some IHCs and OHCs, mainly at the apical locations, were still present (~20%). All animals were included in the analysis because the inclusion criterion was a threshold shift of ≥50 dB. In normal-hearing guinea pigs, implantation of a mini-osmotic pump filled with PBS only, caused substantial damage to both IHCs (25% remaining) and OHCs (42% remaining) at the lower basal location (B1), while almost all hair cells were present at the other cochlear locations (~90%).

3.2. SGC morphology and SGC packing densities

Figure 2 shows light micrographs of Rosenthal's canal in the upper basal turn (B2), providing typical examples of the four different cohorts of cochleae (*normal, 2wdu, 6wdu, and 6wdBDNF*). In the *normal* group, Rosenthal's canal contained the full complement of SGCs and nerve fibers embedded in a matrix consisting of vascularized connective tissue (Fig. 2A). Two weeks after deafening (*2wdu*) the cellular distribution within the spiral ganglia appeared unchanged (Fig. 2B). Six weeks after deafening (*6wdu*), a dramatic loss of SGCs was evident (Fig. 2C). The surviving SGCs had lost their characteristic ovoid shape and, instead, had acquired a more elongated or dendritic appearance. In cochleae treated with BDNF (*6wdBDNF*), the amount of SGCs appeared unchanged as compared to the amount of SGCs in the *normal* group, and the SGCs retained their ovoid shape (Fig. 2D). In general, the SGCs in the *6wdBDNF* group seemed to be larger and more tightly packed than the SGCs in the *normal, 2wdu* and *6wdu* groups.

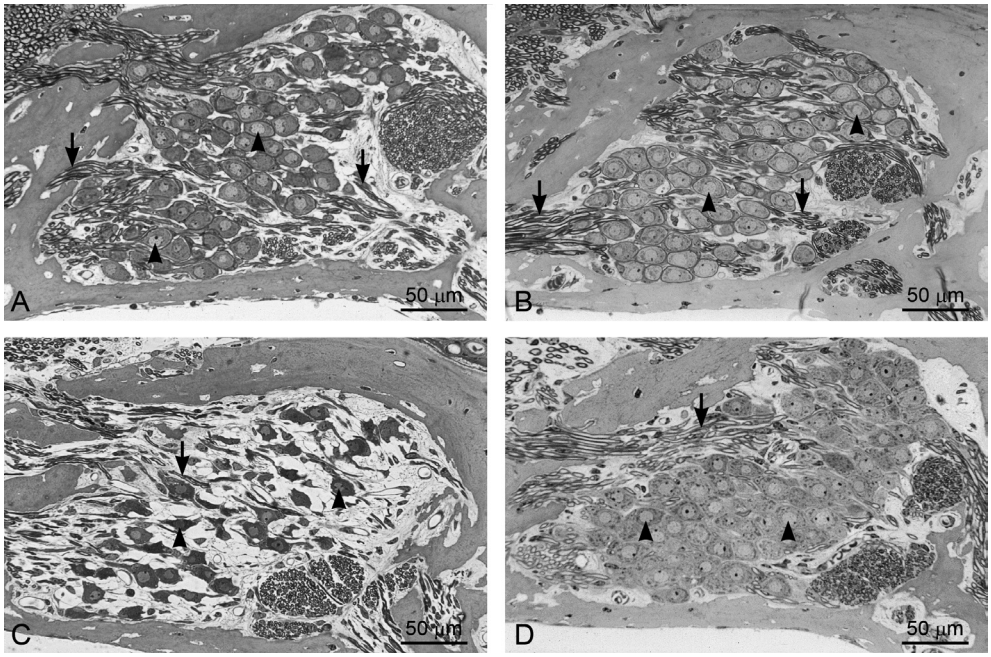


Fig. 2. Light micrographs of Rosenthal's canal at location B2 in the four different groups. (A) SGCs (arrowheads) and nerve fibers (arrows) in the spiral ganglion from a normal cochlea. (B) Two weeks after deafening (*2wdu*). (C) Six weeks after deafening (*6wdu*). (D) In BDNF-treated cochleae (*6wdBDNF*). C and D are from the same animal.

Figure 3 shows a histogram of the mean SGC packing densities in the normal *(n=8 cochleae), 6wdu (n=6 cochleae)* and *6wdBDNF (n=6 cochleae)* groups. The SGC packing densities of the cochlear locations from B1 through A1 are plotted. Packing densities were not corrected for size (see section 2.6). The data of the *2wdu* group are omitted, because these data were published in an earlier paper (Versnel et al., 2007). The mean SGC packing density in the *2wdu* group (averaged across locations B1, B2 and M1) was reduced by about 15% as compared to the mean SGC density in cochleae of normal-hearing guinea pigs. The SGC packing densities in the BDNF-treated cochleae (*6wdBDNF*) were significantly larger than those in untreated cochleae (*6wdu*) (RM ANOVA; main effect BDNF treatment: $p<0.001$). The difference was a factor 2 at locations B1, B2 and M1. After establishing this significant effect of BDNF treatment, we performed *t*-tests for each location separately. At the more apical locations, M2 (paired *t*-test, $p=0.08$) and A1 ($p>0.2$), this effect of BDNF was not statistically significant. Comparing the SGC packing densities of *6wdBDNF* with *normal*, RM ANOVA revealed a significant

interaction with cochlear location (p<0.01). At locations B1 and B2 there were no differences in SGC packing densities between *normal* and *6wdBDNF* (unpaired *t*-test, p>0.4). At the more apical locations (M1, M2 and A1), SGC packing densities were significantly lower in *6wdBDNF* than in the normal group (unpaired *t*-test, p<0.05). At all locations (B1-A1), the SGC packing densities in *6wdu* were significantly lower than those in *normal* (unpaired *t*-test, p<0.05). In summary, BDNF treatment resulted in preservation of SGC packing densities in the basal part of the cochlea, but it was much less effective in the apical part.

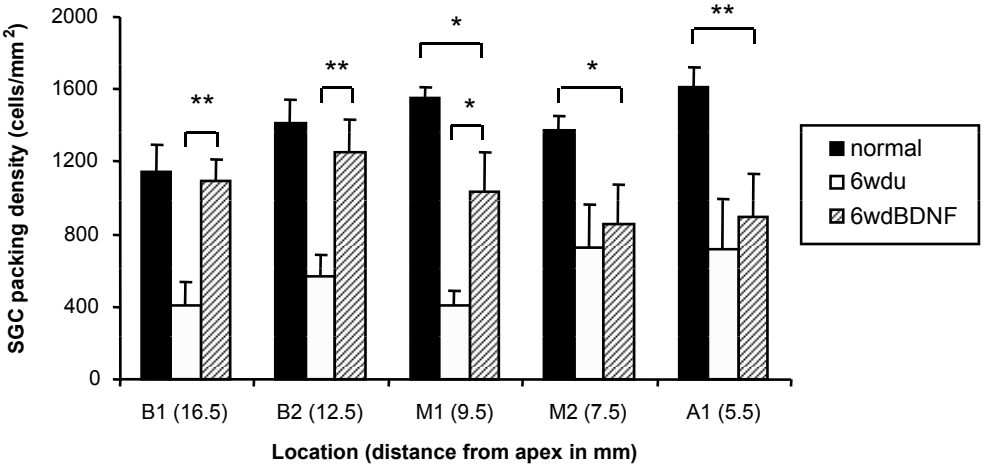


Fig. 3. Mean SGC packing densities at locations B1, B2, M1, M2, and A1 in the *normal (n=8 cochleae), 6wdu (n=6 cochleae)* and *6wdBDNF (n=6 cochleae)* groups. *:p<0.05; **:p<0.01. Statistical analyses between the *normal* and *6wdu* are not shown but are described in the text. Error bars: SEM.

3.3. SGC ultrastructural morphology

Figure 4 shows electron micrographs of SGCs in Rosenthal's canal at location B2 from the four different cohorts. The normal SGCs demonstrated a prominent myelin sheath formed by the enveloping Schwann cell and had an ovoid shape (Fig. 4A). The perikaryon contained a large, round and relatively electron-lucent nucleus with one or more prominent nucleoli. In the cytoplasm large aggregates of cisternae of the rough endoplasmic reticulum and stacks of the Golgi apparatus were present as well as large numbers of mitochondria. Two weeks after deafening (*2wdu*), most SGCs demonstrated a cell shape and ultrastructural appearance that was not different from their counterparts in the normal cochlea (Fig. 4B). Remnants of degenerated SGCs were present in all cochleae in the *2wdu* group. Six weeks after deafening

(*6wdu*) the amount of (type-I) SGCs had dramatically diminished, resulting in a high degree of free space between the individual cells (Fig. 4C). The SGCs had a shape that was different from normal type-I SGCs; the cells were generally more elongated or dendritic in shape, although some cells retained an ovoid shape. Most of the SGCs demonstrated a more compact distribution of organelles and intracellular content, resulting in an electron-dense appearance. The myelin sheath appeared to be thinner. The SGCs in this group appeared smaller and remnants of degenerated SGCs were present. After BDNF treatment (Fig. 4D), the SGCs were generally larger than those in the *normal* group, but they demonstrated a cell shape and an ultrastructural morphology similar to normal SGCs. The electron density of the cells was comparable to that of normal SGCs, but the myelin sheath appeared to be thinner. Remarkably, the SGCs in the *6wdBDNF* group appeared to be more tightly packed than in the *normal* group.

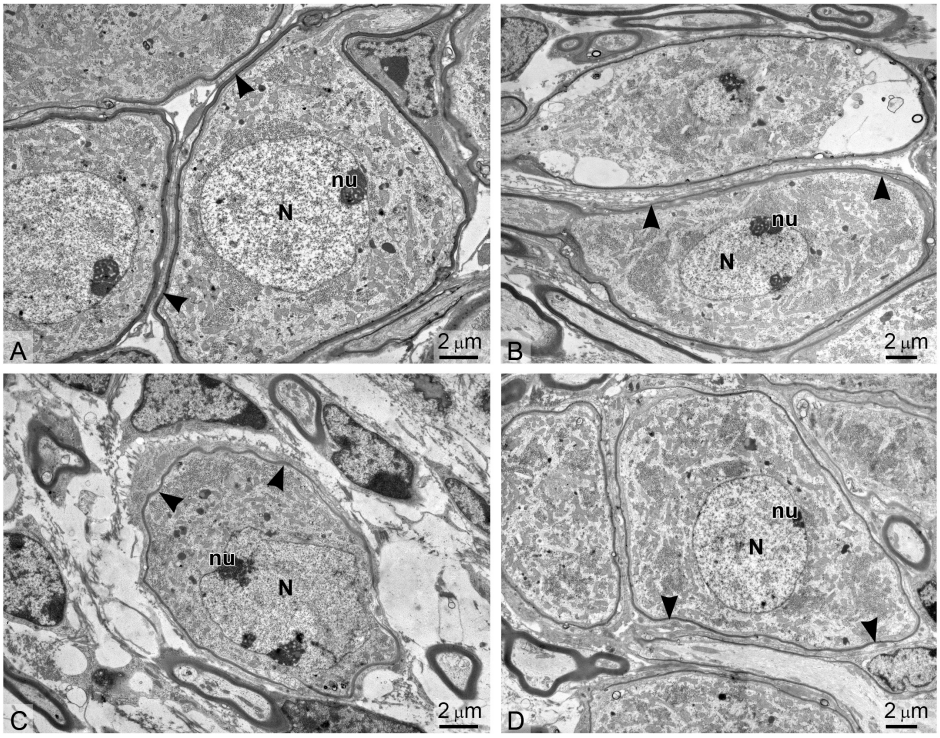


Fig. 4. Electron micrographs of type-I SGCs in Rosenthal's canal at location B2 from the four different groups. (A) SGC from a normal cochlea. (B) Two obliquely sectioned SGCs from the *2wdu* group. (C) Type-I SGC from the *6wdu* group. (D) SGCs from the *6wdBDNF* group. The myelin sheaths of the SGCs are indicated with arrow-heads. N = nucleus; nu = nucleolus.

Figure 5 shows electron micrographs (x32,000) of morphological structures at the subcellular level of representative SGCs from the four different groups. Figure 5A depicts the myelin sheath and mitochondria with a normal appearance in SGCs from a normal cochlea. Two weeks after deafening (2wdu) the myelin sheath and mitochondria appeared normal (Fig. 5B). Six weeks after deafening (6wdu) the number of layers of the myelin sheath was reduced and the mitochondria were smaller (Fig. 5C). After BDNF treatment (Fig. 5D) the number of layers in the myelin sheath was reduced, but the mitochondria appeared to have the same size as the mitochondria in the normal group.

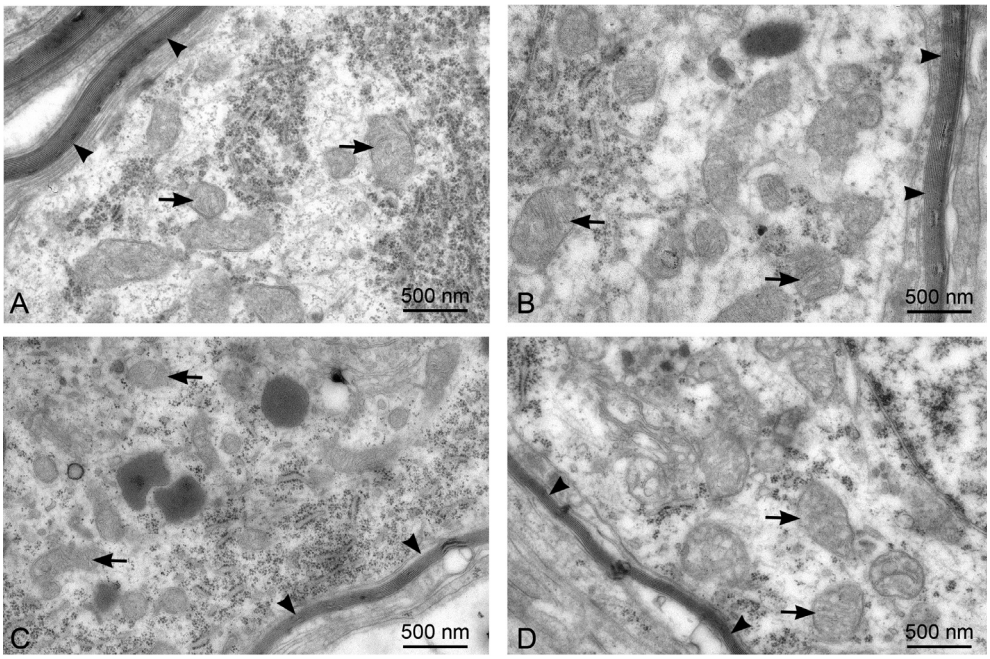


Fig. 5. Electron micrographs of morphological structures at the subcellular level of representative SGCs from the four different groups. (A) Myelin sheath (arrowhead) and mitochondria (arrows) in SGCs from a normal cochlea. (B) Normal appearance of the myelin sheath (arrowhead) and mitochondria (arrows) in SGCs from the *2wdu* group. (C) The myelin sheath (arrowhead) is thinner and mitochondria (arrows) are smaller in SGC from the *6wdu* group. (D) Thin myelin sheath (arrowhead) and mitochondria with a normal appearance (arrows) in SGCs from the *6wdBDNF* group. Note the similarity in size of mitochondria in the SGCs from the *normal* group and *6wdBDNF* group.

3.4. SGC size versus SGC packing density

Figure 6 compares the data of the mean SGC packing density and mean SGC perikaryal area at location B2 in all individual cochleae. The SGC packing density was corrected for size (see section 2.6). The scatter plot shows a distinct clustering of data points according to the four cohorts, except for the data point of one of the six BDNF-treated cochleae (*6wdBDNF*) that was found in the cluster of the *6wdu* group (low packing density, small perikaryal area). In the *normal* group there was no difference in perikaryal area between the left (non-implanted) and right (implanted) cochleae. This means that infusion with PBS did not affect SGC size and packing density. Compared to the *normal* group, the SGC packing density was decreased in the *6wdu* group, but not in the *2wdu* group. In both the *2wdu* and *6wdu* group the SGCs were smaller than in the *normal* group. Thus, after deafening there was an initial decrease in perikaryal area (i.e., the SGC shrank) followed by a decrease in the packing densities. In general, SGCs in BDNF-treated cochleae had a larger perikaryal area (i.e., the cells were larger) than normal SGCs, whereas the SGC packing density was in the same range. Thus, after deafening the perikaryal area decreased and after BDNF treatment it increased again, becoming even larger than normal.

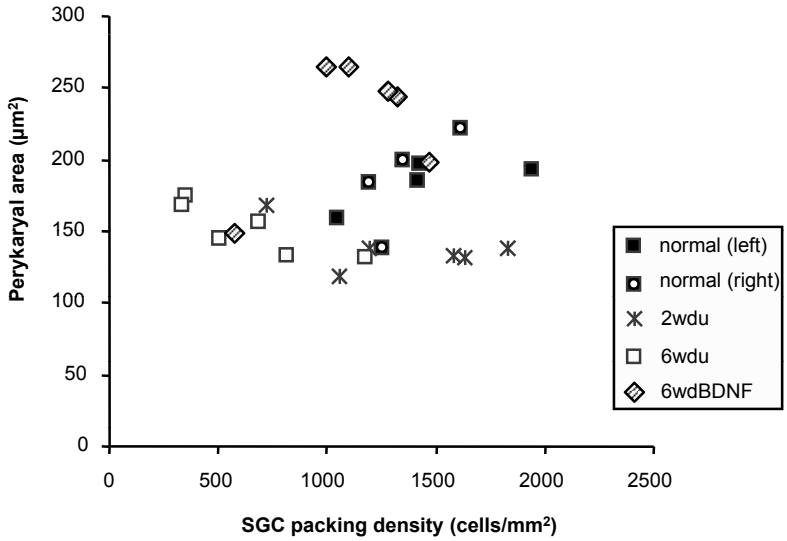


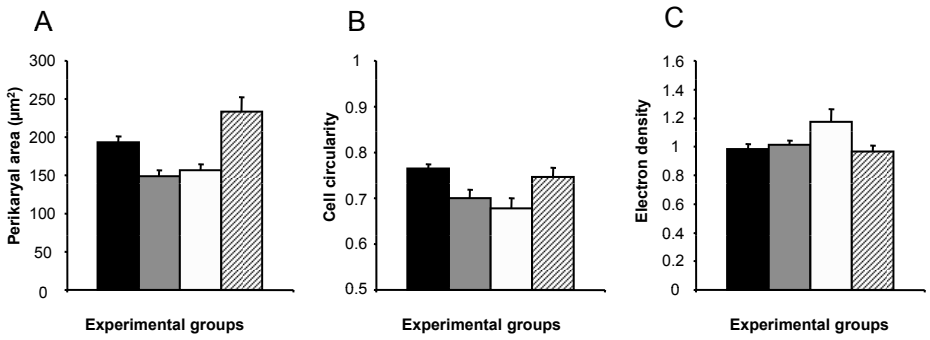
Fig. 6. Scatter plot of perikaryal area versus SGC packing density in the four groups. Data from implanted cochleae in the *normal* group are marked with a white dot. The SGC packing density is corrected for size. SGC packing density and perikaryal area have been determined in the upper basal turn (location B2).

3.5. Size, circularity and electron density of SGCs

Figure 7 shows the mean values of the perikaryal area, circularity and electron density of SGCs at location B2. Mean perikaryal area, circularity and electron density were obtained by averaging all individual measurements within one spiral ganglion, and then by averaging all data of the individual cochleae. Statistical analyses of the data confirm the observations made above: Perikaryal area of SGCs in deafened animals was significantly decreased in the *2wdu* and *6wdu* groups ($p<0.01$) as compared to the normal group. Perikaryal area of SGCs in BDNF-treated cochleae was significantly larger than in the *normal, 2wdu* and *6wdu* groups ($p<0.05$). The differences in size were considerable: 235 µm2 in the *6wdBDNF* group versus 150 µm2 in the *2wdu* and *6wdu* groups. Circularity in the *2wdu* and *6wdu* groups was significantly smaller (~10%) than in the *normal* group ($p<0.01$). There was no difference in circularity between the SGCs in the BDNF-treated cochleae and those in the *normal* group ($p>0.4$). Electron density in the perikaryon of the SGCs was not increased in the *2wdu* and *6wdBDNF* groups ($p>0.5$), but in the *6wdu* group there was a significant increase in electron density ($p<0.05$).

3.6. Correction of packing densities for size

We have demonstrated that BDNF-treated SGCs at location B2 are larger than normal SGCs. This introduces an error in the determination of the SGC packing densities (Coggeshall and Lekan, 1996; Leake et al., 1999). SGC packing densities at location B2 in the experimental cohorts (*2wdu, 6wdu,* and *6wdBDNF*) have therefore been corrected for size and are presented in Fig. 8. After this correction, the SGC packing densities in the BDNF-treated cochleae were still significantly larger than in the untreated (*6wdu*) cochleae ($p<0.05$).



p values

	Groups	Perikaryal area	Cell circularity	Electron density
Unpaired	*Normal–2wdu*	0.003	0.009	0.79
	normal–6wdu	0.008	0.003	0.04
	normal–6wdBDNF	0.05	0.45	0.89
	2wdu–6wdBDNF	0.004	0.17	0.17
Paired	*6wdu–6wdBDNF*	0.02	0.02	0.06

Fig. 7. Quantitative data for perikaryal area, cell circularity and electron density of type-I SGCs with an obvious nucleus at location B2. Data shown were obtained by averaging all individual measurements within one spiral ganglion, followed by averaging over cochleae. *P* values resulting from the *t*-test analyses are presented in the table. (A) Perikaryal area. (B) Cell circularity. (C) Electron density. N: the total number of SGCs measured in each group. Error bars: SEM.

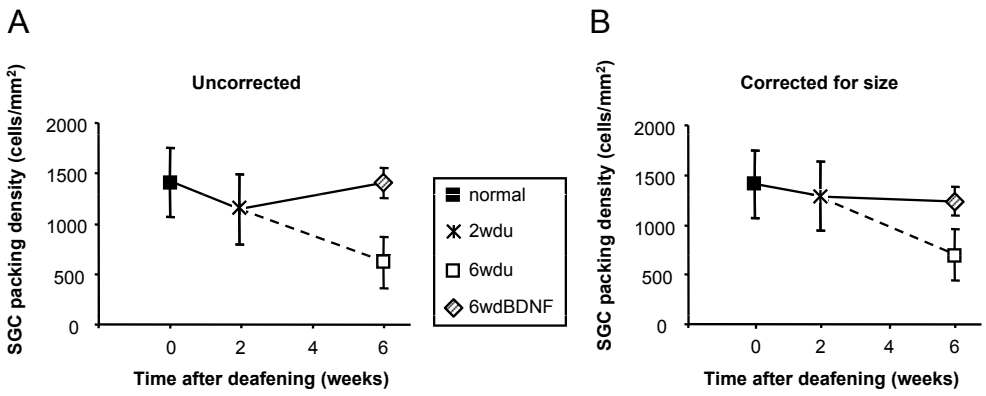


Fig. 8. (A) Comparison of SGC packing densities at location B2 for all groups without correction for SGC size. (B) Comparison of the SGC packing densities at location B2 in the experimental groups *2wdu, 6wdu,* and *6wdBDNF* after correction for SGC size.

4. Discussion

This study examined the morphology of SGCs in hair-cell deprived cochleae rescued with BDNF. Application of BDNF in the cochleae of deafened guinea pigs, starting two weeks after deafening, had the following effects on SGC packing density and morphology. First, it prevented degeneration of SGCs after deafening. Second, the perikaryal area of SGCs treated with BDNF became larger, circularity was restored, electron density was unchanged and the myelin layers were reduced as compared to SGCs in normal-hearing guinea pigs.

4.1. SGC packing densities

Application of BDNF to the right cochlea, starting two weeks after deafening and lasting for four weeks, resulted in higher SGC packing densities than in the untreated left cochleae. This enhanced survival of SGCs after delayed neurotrophic treatment (at least two weeks after deafening the guinea pigs) has also been reported by others (Gillespie et al., 2004; Yamagata et al., 2004; Richardson et al., 2005; Wise et al., 2005; Miller et al., 2007). In these studies various neurotrophins have been used (BDNF, NT-3, NT-4/5, NGF, BDNF/CNTF) in dosages in the range of 50 to 100 µg/ml. In addition, when treatment was started 6 weeks after deafening, a clear protective effect was observed (Yamagata et al., 2004; Miller et al., 2007). Overall, including in our study, the packing density after treatment is found to be similar to that at the start of treatment. Remarkably, Miller et al. (2007) have reported higher packing densities following treatment with a cocktail of BDNF and fibroblast growth factor than at the start of treatment.

We found that SGCs were predominantly prevented from degeneration in the basal part (location B1-M1) of the cochlea. Similar results have been reported in other studies (Shah et al., 1995; Shepherd et al., 2005; Wise et al., 2005; Rejali et al., 2007). However, a significant effect across the entire cochlea has been reported too (Kanzaki et al., 2002; Yamagata et al., 2004). The methods differ with respect to delivery site (round window or cochleostomy), use of electrically evoked auditory brainstem responses, and type of neurotrophins, but no systematic pattern is evident when comparing the different studies. A simple explanation for the relatively high susceptibility to BDNF at the base of the cochlea may involve the location of delivery. Indeed, Richardson et al. (2004) have reported a higher concentration of neurotrophins at the base after a single injection through a cochleostomy near the round window. On the other

hand, Miller et al. (1997) have reported that delivery of BDNF at a concentration of 50 ng/ml suffices for enhanced survival of SGCs at the base of the cochlea. Thus, SGCs need only very small amounts of BDNF for rescue, which in our study should be reached at the apex with the delivery of BDNF at the basal cochlear turn (location B1) at a dose of 100 µg/ml. Alternatively, the predominance of the BDNF effect in the basal part of the cochlea might be related to regional variations in expression patterns of endogenous BDNF within the cochlea. It has been demonstrated that the expression of neurotrophins and their receptors in the basal turns of the cochlea are higher than at the apex (Ylikoski et al., 1993; Davis, 2003).

4.2. Morphology of BDNF-treated SGCs

In our study perikaryal area obtained with electron microscopy was analyzed in the basal part (location B2) of the cochlea. Our results on SGC perikaryal area (Fig. 7) corroborate the results obtained with light microscopy (Shepherd et al., 2005; Richardson et al., 2005). These studies reported that SGCs in the basal turn of BDNF-treated cochleae were larger (30-70%) than SGCs in cochleae of normal-hearing animals. They report no effects on SGC perikaryal area after infusion of Ringer's solution in deafened guinea pigs. In our study, additional measurements (data not shown) obtained with light microscopy showed no effect on perikaryal area in a part of the cochlea where BDNF did not significantly affect SGC density (location M2 in Fig. 3). This is consistent with data obtained by Shepherd et al. (2005). They reported that the perikaryal area of SGCs at a more apical location (cochlear turns t3 and t4 in their Fig. 7) approached the perikaryal area of SGCs in the deafened, untreated controls.

A decrease in the size of SGCs after deafening, and without neurotrophic treatment, has frequently been reported (Staecker et al., 1996; Leake et al., 1999; Shepherd et al., 2005; Richardson et al., 2005). Our data indicate that the main effect of hair cell loss upon SGCs is an initial decrease in cell size followed by a loss of SGCs.

Our quantitative data with respect to SGC circularity (Fig. 7) confirm qualitative descriptions of SGCs after neurotrophic treatment (Shah et al., 1995; Gillespie et al., 2004; Miller et al., 2007). In these studies, SGCs have been described as more circular in BDNF-treated cochleae. In addition, our data demonstrate that BDNF-treated SGCs have a normal circularity, whereas untreated cells, already after two weeks, are less circular. This decrease in circularity reflects a more elongated or dendritic shape of the cells. The return to a normal circularity after BDNF treatment can be thought of as a direct consequence of the increase in size.

The ultrastructural appearance of BDNF-treated SGCs is normal, especially with respect to the cytoplasmic content, the degree of electron density and the mitochondrial size. After deafening various SGC features undergo time-related changes. Initially, after two weeks, the SGCs decrease in size and in circularity. Subsequently, after six weeks, SGC electron density increased, mitochondria became smaller, and packing density decreased. This increased electron density is a characteristic feature of cell death (Dodson, 1997), whereas reduction in size of the mitochondria is a frequently observed abnormality in cells during apoptosis (Desagher and Martinou, 2000). After BDNF treatment, packing density, electron density, and mitochondrial area do not change, and circularity returns to normal values. These findings would indicate that the physiological condition of BDNF-treated SGCs — in contrast to the untreated SGCs, which are smaller, have lost their ovoid shape, contain smaller mitochondria and have an increased electron density — is normal and healthy, which is supported by qualitative observations (Shah et al., 1995; Staecker et al., 1996; Gillespie et al., 2004; Miller et al., 2007). The cellular feature that differs from the SGCs in the cochleae of normal-hearing guinea pigs is the perikaryal area. We also observed a reduction in the number of layers of the myelin sheath in BDNF-treated SGCs, which is consistent with findings of Glueckert et al. (2008). Thus, BDNF does not prevent demyelinisation. The question arises whether or not the larger than normal size and the reduced myelin sheath reflects a suboptimal or unstable condition. The SGCs might be in a vulnerable condition and this could lead to an abnormally fast degeneration of SGCs after cessation of neurotrophic treatment as has been reported by Gillespie et al. (2003). This is reminiscent of the findings by Dodson and Mohuiddin (2000), who reported that SGCs become larger after deafening.

4.3. Clinical implications

This study shows that neurotrophic treatment in a hair-cell deprived cochlea prevents SGC degeneration, in agreement with several other studies (for overviews, see Marzella and Clark, 1999; Miller et al., 2002; Gillespie et al., 2005; Pettingill et al., 2007). Potentially, neurotrophic treatment of SGCs might be clinically relevant for human cochlear implant candidates. However, there are three major concerns when considering clinical treatment along this line.

First, there is the issue of the safety of the technique. Application of neurotrophins by infusion via an osmotic pump involves a high risk of infection. An effective technique with less risk of infection is coating the electrode with cells releasing neurotrophins (Rejali et al., 2007). Alternatively, application via a biodegradable hydrogel or alginate beads on the round

window, which provides a slow release of neurotrophins, seems more appropriate clinically. These techniques have also proven to be effective (Ito et al., 2005; Noushi et al., 2005).

Second, there is still limited evidence that more SGCs will result in better speech perception in cochlear implant users. Despite the seeming advantage of a higher population of SGCs, it is possible that changes in the central auditory system dominate speech perception in a negative way (Nadol et al., 2001; Miller, 2001; Fallon et al., 2007).

The third issue that should be investigated before considering clinical application is the time course of the effect. Gillespie et al. (2003) reported an accelerated degeneration after cessation of BDNF treatment. This would imply the necessity for lifelong treatment in patients. Alternatively, neuronal activity may prevent the neuron from degenerating. It might be that electrical stimulation following neurotrophic treatment has a long-term protective effect. Shepherd et al. (2008) investigated whether chronic electrical stimulation could maintain SGC survival after cessation of BDNF delivery. They reported that electrical stimulation significantly reduced the rate of SGC loss although this effect was restricted to only the region of the cochlea proximal to the electrode array.

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Enhanced survival of spiral ganglion cells after cessation of treatment with brain-derived neurotrophic factor in deafened guinea pigs

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Abstract

Exogenous delivery of neurotrophic factors into the cochlea of deafened animals rescues spiral ganglion cells (SGCs) from degeneration. To be clinically relevant for human cochlear implant candidates, the protective effect of neurotrophins should persist after cessation of treatment and the treated SGCs should remain functional. In this study the survival and functionality of SGCs were investigated after temporary treatment with brain-derived neurotrophic factor (BDNF). Guinea pigs in the experimental group were deafened and two weeks later the right cochleae were implanted with an electrode array and drug-delivery cannula. BDNF was administered to the implanted cochleae during a four week period via a mini-osmotic pump. After completion of the treatment the osmotic pumps were removed. Two weeks later the animals were killed and the survival of SGCs was analyzed. To monitor the functionality of the auditory nerve, electrically evoked auditory brainstem responses (eABRs) were recorded in awake animals throughout the experiment. BDNF treatment resulted in enhanced survival of SGCs two weeks after cessation of the treatment and prevented the decreases in size and circularity that are seen in the untreated contralateral cochleae. The amplitude of the suprathreshold eABR response in BDNF-treated animals was significantly larger than in deafened control animals and comparable to that in normal-hearing control animals. The amplitude in the BDNF-treated group did not decrease significantly after cessation of treatment. The eABR latency in BDNF-treated animals was longer than normal and comparable to that in deafened control animals. These morphological and functional findings demonstrate that neurotrophic intervention had a lasting effect, which is promising for future clinical application of neurotrophic factors in implanted human cochleae.

Keywords: auditory nerve, cochlear implant, deafness, degeneration, electrically evoked auditory brainstem response, neurotrophic factors

1. Introduction

A progressive loss of spiral ganglion cells (SGCs) after loss of inner hair cells is presented in several animal models (Spoendlin 1975; Webster and Webster 1981). In these models electrical stimulation (ES; Lousteau et al. 1987; Hartshorn et al. 1991; Leake et al. 1991) and administration of neurotrophins (Staecker et al. 1996; Miller et al. 1997; Gillespie and Shepherd 2005; Pettingill et al. 2007) via a cochlear implant are effective in preventing SGCs from degeneration after induced deafness. The preservation of SGCs might be important since the success of the cochlear implant is thought to be related to the number of excitable SGCs. In the majority of studies neurotrophic treatement was started within the first two weeks after deafening (see review Gillespie and Shepherd 2005). It has been shown that neurotrophic treatment is also effective after degeneration has set in, a condition that mimics the clinical situation (Gillespie et al. 2004; Yamagata et al. 2004; Wise et al. 2005; Miller et al. 2007; Agterberg et al. 2008; Glueckert et al. 2008). In these studies intracochlear infusion of neurotrophins, by means of an osmotic pump system, was started 2-6 weeks after deafening. Neurotrophic treatment prevented SGC degeneration, but SGCs treated with BDNF were larger than SGCs in normal cochleae and their myelin layers were reduced (Richardson et al. 2005; Shepherd et al. 2005, 2008; Agterberg et al. 2008; Glueckert et al. 2008). These morphological changes might reflect a suboptimal condition. Nevertheless, SGCs treated with neurotrophins remained electrically excitable and animals treated with neurotrophins showed reduced thresholds of electrically evoked auditory brainstem responses (eABRs) as compared to animals in deafened control groups infused with artificial perilymph (Shinohara et al. 2002; Yamagata et al. 2004; Shepherd et al. 2005; Maruyama et al. 2008).

An important question with respect to clinical application of neurotrophins is whether continuous treatment is necessary for lasting protective effects. According to the neurotrophin hypothesis and based upon findings in other sensory systems (Montero and Hefti, 1988; Mansour-Robaey et al. 1994), it is predicted that cessation of neurotrophic treatment would result in degeneration of SGCs. This prediction has been addressed in recent studies, which yielded contradictory results. Gillespie et al. (2003) and Shepherd et al. (2008) reported an abnormally rapid degeneration of SGCs after cessation of BDNF treatment. Two weeks after cessation the number of SGCs was not significantly different from that in deafened, untreated cochleae. In addition, Shepherd et al. (2008) found that chronic electrical stimulation partially reduced the rate of SGC loss in the basal cochlear turn, i.e., the area adjacent to the electrode

array. In contrast to these findings with BDNF, Maruyama et al. (2008) demonstrated that SGC survival and electrical responsiveness were well preserved after cessation of intracochlear infusion with glial cell line-derived neurotrophic factor (GDNF).

In the present study we investigated the effects of cessation of BDNF treatment. Treatment was started two weeks after deafening, when degeneration had set in. As Gillespie et al. (2003) we used BDNF as neurotrophic factor, and similar to Maruyama et al. (2008) we recorded eABRs to monitor the excitability of the SGCs. Light microscopy was used to determine the SGC packing density and to examine morphological features, such as cell size and shape (circularity). The functionality of the SGCs was assessed with the suprathreshold amplitude (Hall, 1990) and latency of the first negative eABR peak.

2. Methods

2.1 Animals and experimental design

Twenty albino female guinea pigs (strain: Dunkin Hartley; weighing 250-350 g) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the animal care facility of Utrecht University. All animals had free access to both food and water and were kept under standard laboratory conditions. Lights were on between 7:00 am and 7:00 pm. Temperature (21 °C) and humidity (60%) were kept constant.

Six guinea pigs were bilaterally deafened and two weeks thereafter implanted in the right cochlea with an electrode array and cannula. Consecutively, they received BDNF during a period of four weeks. After completion of BDNF treatment, the osmotic pumps were removed. Two weeks after BDNF treatment (i.e., eight weeks after deafening) these animals were killed and processed for histology. For histological analysis, a comparison was made between BNDF-treated and untreated ears in the same animals; furthermore, the histological data of BDNF-treated ears were compared to data from normal cochleae (n = 4 animals) presented in a previous paper (Agterberg et al. 2008).

After implantation, eABRs in BDNF-treated animals were recorded weekly to assess auditory function in relation to electrical stimulation of the treated cochlea. This paradigm is schematically explained in Fig. 1A. The eABR data of the treated animals were compared to eABRs in both normal-hearing and deafened untreated animals. For this we used three groups of control animals (Fig. 1B-D). A group of two animals were deafened and implanted in the right cochlea with a regular electrode array (without drug-delivery cannula) two weeks

thereafter, and eABRs were recorded up to more than eight weeks after deafening (Fig. 1B). A second group of eight normal-hearing control animals were implanted in the right cochlea with a regular electrode array and eABRs were recorded weekly (Fig. 1C). In order to examine the effect of deafening on the eABRs in the same animals, these animals were deafened after four weeks and killed another six weeks later (in one of these animals eABRs could not be recorded anymore in later sessions due to electrode failure). A third group of four animals were implanted as the second group but killed after four weeks while in normal-hearing condition (Fig. 1D).

All surgical and experimental procedures were approved by the Animal Care and Use Committee of Utrecht University (DEC-UMC # 03.04.036).

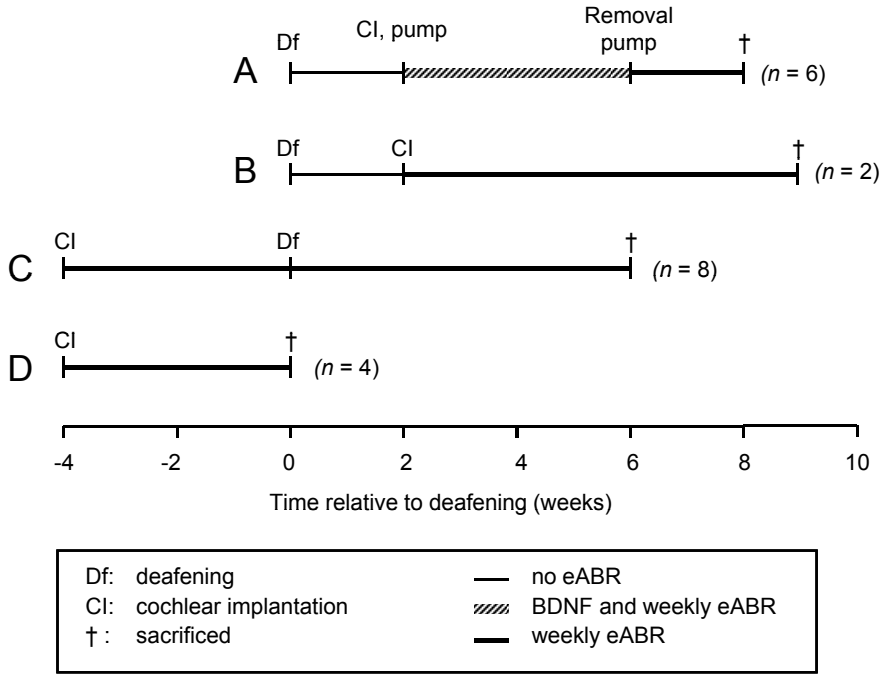


Fig. 1. Treatment schedule of four different animal cohorts (A-D). A: deafened and two weeks later implanted and treated with BDNF; B: deafened and two weeks later implanted; C: first implanted and four weeks later deafened; D: only implanted. Deafening was performed systemically affecting both ears. Cochlear implantation (A-D) and BDNF treatment (A) was applied to the right ear. After implantation eABRs were regularly recorded in each group (by electrically stimulating the implanted right ear). For electrophysiological analysis, eABRs of the BDNF-treated animals (A) were compared to eABRs of normal-hearing animals (C *before deafening*, D) and to eABRs of deafened animals (B, C *after deafening*). Note that data in normal-hearing and deafened conditions were obtained in the same animals (C). For histological analysis, the main comparison was made within the animals treated with BDNF (A): BNDF-treated right ears were compared to untreated left ears.

2.2 Deafening procedure

Animals were anesthetized with Domitor® (medetomidine hydrochloride; 10 mg/kg, im) and Ketanest-S® ((S)-ketamine; 40 mg/kg, im). Before the deafening procedure, acoustically evoked ABRs (aABRs) were recorded to check hearing thresholds. Animals were injected subcutaneously with kanamycin (400 mg/kg, sc) followed (15-60 minutes later) by slow intravenous infusion of furosemide (100 mg/kg, iv). This procedure, originally reported by West et al. (1973), has been shown to eliminate almost all cochlear hair cells (Gillespie et al. 2003; Versnel et al. 2007). For the intravenous infusion of furosemide, the left jugular vein was exposed and a catheter was inserted. A successful insertion was confirmed with withdrawal of blood into the catheter.

2.3 Implantation and cochlear infusion

Animals were anesthetized with Domitor® (10 mg/kg, im) and Ketanest-S® (40 mg/kg, im). In the control animals the right cochleae were implanted with an eight-electrode array (Cochlear®, platinum ring electrodes of 0.3 mm width, inter-electrode distance: 0.75 mm). In the experimental animals the right cochleae were implanted with a six-electrode array (same electrode configuration as eight-electrode array) with drug-delivery cannula (~80 mm, ID = 0.8 mm) with a tip of ~20 mm (ID = 0.12 mm; OD = 0.16 mm) designed for guinea pigs (Cochlear®), resembling the array-cannula device described by Shepherd and Xu (2002). The right bulla was exposed retro-auricularly and a small hole was drilled to visualize the cochlea. The array was inserted 3-4 mm through a cochleostomy in the basal turn near the round window. The array cable was fixed onto the bulla with dental cement (Ketac-Cem Aplicap, ESPE dental supplies, Utrecht, The Netherlands) and connected to the skull with a screw (Brown et al. 1993) and dental cement. An Alzet® mini-osmotic pump (model 2004; flow rate 0.25 µl/h; reservoir 200 µl) was attached to the cannula and inserted into a subcutaneous pocket. The incision was closed in two layers with Vicryl®. The cannula and the mini-osmotic pump were filled with BDNF (PeproTech Inc., Rocky Hill, NJ, USA) solution (100 µg/ml). This concentration was chosen because concentrations of neurotrophins in this range (50-100 µg/ml) have proved to be effective in several studies (Gillespie et al. 2003, 2004; Yamagata et al. 2004; Wise et al. 2005; Miller et al. 2007; Agterberg et al. 2008; Shepherd et al. 2008). Bovine serum albumin (BSA, 1%) was added to the BDNF solution. The pumps were incubated in sterile saline for 48 hours at 37°C to guarantee a constant flow rate at implantation.

2.4 Auditory brainstem responses

aABRs and eABRs were measured once or twice a week in awake and freely moving animals. All electrophysiological recordings were performed in a sound-attenuated chamber. The aABRs and eABRs were recorded with three stainless steel screws (8.0x1.2mm) inserted into the skull bone 1 cm posterior to bregma, 2 cm anterior to bregma and 1 cm lateral from bregma, respectively (Mitchell et al. 1997). Stimulus generation and signal acquisition were controlled with custom-written software and a personal computer. The stimuli were synthesized and attenuated using a Tucker-Davis Technologies TDT3 system (modules RP2, PA5 (2x) and SA1). The responses were amplified differentially using a Princeton Applied Research 113 pre-amplifier (amplification: 5,000; band pass filter: 0.1-10 kHz) with the posterior and anterior screws as active and reference electrodes, respectively, and the lateral screw as ground electrode. The amplified signal was digitized by the TDT3 system (module RP2) and made available for off-line analysis.

2.5 aABRs

Broadband click stimuli consisting of monophasic rectangular pulses (width: 20 µs; interstimulus interval: 99 ms) were presented in free field, using a Blaupunkt speaker (PCxb352; 4 Ohm; 30 W) positioned 10 cm above the awake guinea pig. Threshold was defined as the sound level at which the aABR was just visible. The click stimuli were presented from 75 dB above the average threshold of normal-hearing animals (~110 dB peSPL) down to threshold in 10-dB steps. Animals with a threshold shift of > 50 dB, measured 14 days after deafening, were included as ‘deafened’.

2.6 eABRs

After cochlear implantation, eABRs were recorded. Monophasic rectangular pulses generated and attenuated by the TDT3 system were converted to current pulses by a linear stimulus isolator (type A395, World Precision Instruments). The current pulses (width: 20 µs; interstimulus interval: 99 ms) were presented with alternating polarity to the most apical intracochlear electrode using the lateral screw in the skull as return electrode (monopolar eABR recordings). To define the peaks in the eABRs they were compared to the peaks in the aABRs (see Fig. 2). The first positive peak (P_1) in the eABR recordings was often obscured by the electrical artifact. In approximately half of the animals, in at least some of the recordings,

the third positive peak (P_3) was influenced by the digastric muscle response (Hall, 1990). The first negative peak (N_1) and second positive peak (P_2) were always clearly visible and not obscured by the electrical artifact or by the digastric muscle response. Therefore, wave N_1-P_2 was analysed. The N_1-P_2 amplitude, N_1-P_2 threshold, and N_1 latency were determined. The N_1-P_2 amplitude was found to correlate well with SGC density (Hall, 1990), and therefore, it was considered an appropriate parameter to examine excitability of BDNF treated SGCs. Thresholds were defined as the stimulus level that evoked a 2.0 µV (determined with interpolation) reproducible waveform. Stimuli were presented from 400 µA down to threshold with 2-dB steps. Higher current levels would evoke a whisker response. The N_1-P_2 amplitudes for all current levels in the experimental and control animals were analyzed using repeated-measures analysis of variance (rm ANOVA) and *t*-tests were used to analyze measurements at single suprathreshold current levels.

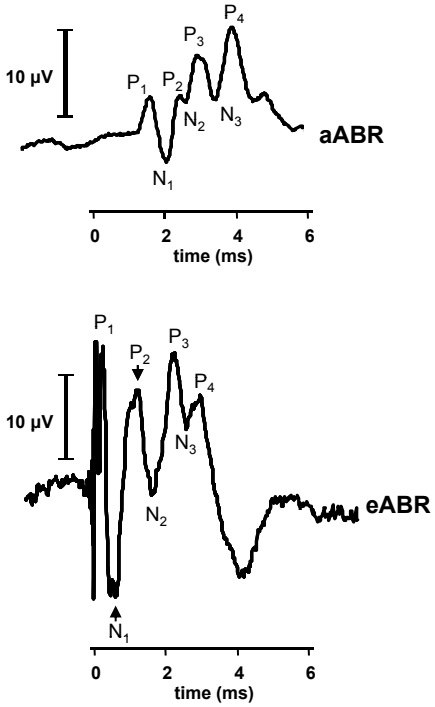


Fig. 2. A typical example of an aABR (*top*) evoked in a normal-hearing animal, with clicks of 45 dB nSL and an eABR (*bottom*) evoked with 400-µA pulses. Stimulus onset was at time 0. Note the large stimulus artifact in the eABR before peak P_1. The peak-to-peak amplitude, N_1-P_2, of the eABR was measured to assess the functional status of the auditory nerve. When the N_1 and/or P_2 consisted of more than one sub peak, the first sub peak on each site of the fast rising part of the eABR complex (indicated with the arrows) is used in the analysis.

2.7 Cessation of BDNF treatment

After four weeks of BDNF treatment the animals were anesthetized with Domitor® (10 mg/kg, im) and Ketanest-S® (40 mg/kg, im). After the connection of the cannula with the osmotic pump was checked, the cannula was cut off at about 40 mm from the cochlea and the osmotic pumps were removed. The open end of the remaining cannula was sutured with Vicryl®. The electrode array was left in place to record eABRs after termination of the BDNF infusion. Two weeks after cessation of BDNF treatment the animals were euthanized for histology.

2.8 Histology

Immediately after the final eABR measurements, the left and right cochleae were fixed by intralabyrinthine perfusion with a fixative consisting of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% DMSO in 0.08 M sodium cacodylate buffer (pH 7.4) followed by immersion in the same fixative for 3h at room temperature. Histological processing of the cochleae was carried out according to our standard protocol (De Groot et al 1987). After decalcification with EDTA, the cochleae were immersed in 1% OsO_4 containing 1% $K_4Ru(CN)_6$ for 2h at 4°C and then rinsed, dehydrated, and embedded *in toto* in Spurr's low-viscosity resin. After polymerization, cochleae were divided into two halves along a standardized midmodiolar plane, and these were re-embedded in fresh resin. Semithin (1 µm) sections stained with methylene blue and azur II were used for light microscopical evaluation and quantitative analyses.

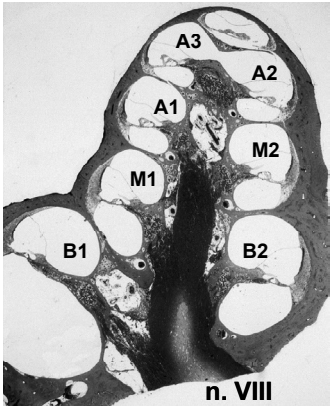
The efficacy of the deafening procedure was assessed, in addition to aABR thresholds, by counting the number of remaining inner hair cells (IHCs) and outer hair cells (OHCs) at seven different locations along the basilar membrane at a half-turn spacing (B1, B2, M1, M2, A1, A2, and A3; Fig. 3).

Determination of SGC packing densities were performed as described previously (Versnel et al. 2007; Agterberg et al. 2008). SGC packing densities were determined using digitized light microscopical images of the spiral ganglia taken from five different cochlear locations (B1, B2, M1, M2, and A1; Fig. 3). Using the image analysis program *NIH Image* (version 1.63; US National Institutes of Health, Bethesda, Maryland), the bony boundaries of Rosenthal's canal were outlined and its cross-sectional area (in mm^2) was calculated. The number of SGC perikarya was counted at each location. The following populations were included in the counts: (1) all perikarya demonstrating the morphological determinants typical of type-I and type-II SGCs (for details, see Romand and Romand 1984); (2) partial and

complete profiles of perikarya; and (3) perikaya with and without evident nucleus or nucleoli. SGC packing density was calculated by dividing the number of SGCs by the cross-sectional area of Rosenthal's canal and expressed as the mean number of SGCs per mm^2.

The cellular features perikaryal area and cell circularity – as a measure of perikaryal cell size and cell shape, respectively – were selected for further quantitative analysis. Because there is a considerable regional variation in SGC perikaryal area within the cochlea (Leake et al 1999), measurements were performed in digitized light microscopical images of the spiral ganglion at one specific location in the basal turn (location B2 in Fig. 3). This location was chosen, because (1) the effect of BDNF on SGC packing densities is most prominent in the basal turn, and (2) it was occasionally not possible to determine SGC packing densities for location B1, which is near to the hook region, due to tangential sectioning of Rosenthal's canal. Only type-I SGCs with an evident nucleus were measured. Perikaryal area was determined by outlining the myelin sheath surrounding the type-I perikaryon. Cell circularity is a feature that can be measured directly in *NIH Image* after delineating the cell's perimeter, i.e. the myelin sheath. It is calculated as follows: $4\pi \cdot A/L2$, where A is area and L is perimeter (circularity is 1 for a perfect circle and less than 1 for an imperfect circle, e.g. 0.78 for a square).

Statistical analyses were performed using SPSS® for Windows (version 15.0.1). SGC packing densities at the different locations (B1-A1) were analyzed using repeated-measures analysis of variance (rm ANOVA). Statistical comparisons of perikaryal area and cell circularity at location B2 were made using paired *t*-tests.



Location	Distance from apex (mm)
B1	16.5
B2	12.5
M1	9.5
M2	7.5
A1	5.5
A2	3.5
A3	1.5

Fig. 3. Light micrograph of a midmodiolar section (1 µm) of a normal guinea-pig cochlea showing the different locations at which SGCs were examined. The distance from the apex of the locations B1 through A3 are specified in the table. N. VIII: cochlear nerve.

3. Results

3.1 Effects of deafening procedure

Two weeks after the deafening procedure all control and experimental animals demonstrated threshold shifts of >60 dB for click-evoked aABRs. This severe hearing loss was microscopically confirmed. All hair cells at cochlear locations B1-A1 were lost, except in two animals (one control animal and one experimental animal). In these animals a few inner hair cells still remained.

3.2 SGC packing densities

Figure 4 shows light micrographs of Rosenthal's canal at cochlear location B2, providing typical examples of SGCs in cochleae of normal-hearing (A) and deafened animals (B-D), and in the left untreated and right BDNF-treated cochlea of an experimental animal (E, F). In the normal cochlea, Rosenthal's canal contained the full complement of SGCs and nerve fibers embedded in a matrix consisting of vascularized connective tissue (Fig. 4A). Two weeks after deafening the cellular distribution within the spiral ganglia was similar to normal (Fig. 4B). Figure 4C illustrates the dramatic loss of SGCs in the left cochlea of a control animal that was examined six weeks after deafening. In the right cochlea, which was electrically stimulated for short periods with the purpose to record eABRs, the loss of SGCs was equally dramatic (Fig. 4D). This indicates that brief electrical stimulation did not prevent degeneration. Eight weeks after deafening a dramatic loss of SGCs was evident in the untreated, contralateral cochlea of a BDNF-treated animal (Fig. 4E). The surviving SGCs had lost their characteristic ovoid shape and, instead had acquired a more elongated or dendritic appearance. In the BDNF-treated cochlea of the same animal, two weeks after cessation of BDNF treatment, no signs of degeneration were observed (Fig. 4F). The amount of SGCs was comparable to that in the normal cochlea, and the SGCs kept their characteristic ovoid shape.

Figure 5 compares SGC packing densities, at cochlear locations from B1 through A1, averaged across animals (n = 6), in BDNF-treated cochleae with those in the untreated contralateral cochleae and with normal data (dashed lines). The data confirm the result shown in Figs. 4E, F. SGC packing densities in BDNF-treated cochleae were near normal at locations B1-M2 and by a factor 3 larger than those in untreated cochleae (rm ANOVA: $F(1, 5) = 65$, $p<0.001$).

It is possible that electrical stimulation during eABR recordings contributed to the enhanced survival of SGCs (cf. Mitchell et al. 1997). Therefore, within untreated deafened animals we compared the SGC packing densities in the stimulated implanted ears (see example in Fig. 4D) to those in the non-stimulated contralateral ears (see Fig. 4C). There were no statistically significant differences in SGC packing density between stimulated implanted ears and non-stimulated contralateral ears (rm ANOVA: $F(1, 5) = 3.2$, $p>0.1$).

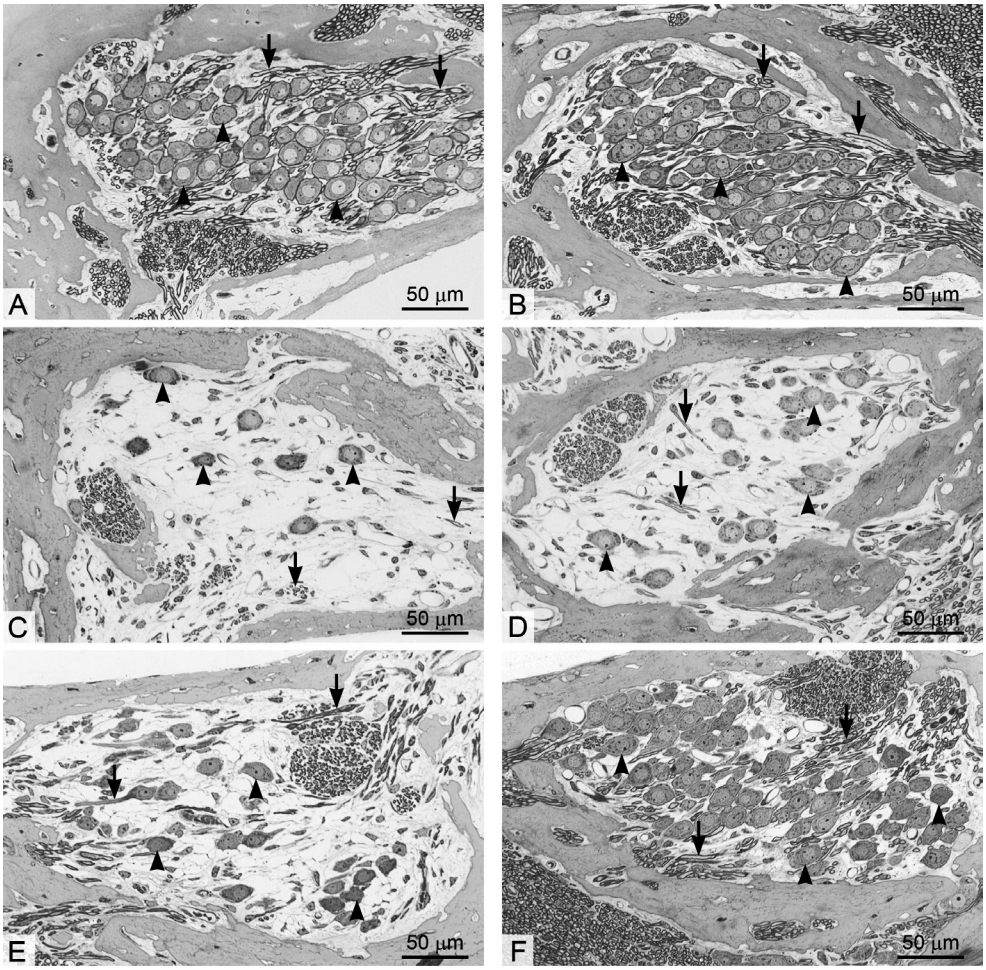


Fig. 4. Light micrographs of Rosenthal's canal at location B2 (upper basal turn) with the distribution of SGCs (arrowheads) and nerve fibers (arrows). SGCs and nerve fibers in the spiral ganglion from (A) a normal cochlea, (B) a cochlea two weeks after deafening, (C) a left cochlea six weeks after deafening, (D) the right implanted cochlea from the same animal as in C, (E) the left cochlea of an animal eight weeks after deafening, and (F) the right implanted cochlea treated with BDNF from the same animal as in E (two weeks after cessation of treatment).

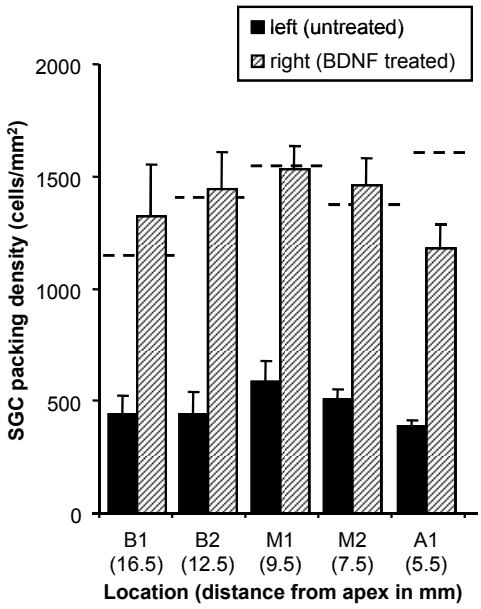


Fig. 5. Mean SGC packing densities at cochlear locations B1, B2, M1, M2, and A1 in the left (untreated) and right (BDNF-treated) cochlea of deafened animals (n = 6). Dashed lines represent SGC densities at locations B1-A1 in normal cochleae. Error bars: SEM.

3.3 Perikaryal area and circularity

Figure 6 shows the mean values of the perikaryal area (Fig. 6A) and circularity (Fig. 6B) of SGCs at cochlear location B2. The perikaryal area of BDNF-treated SGCs was larger than that of SGCs in the untreated contralateral cochleae with a difference of 25% (paired *t*-test, $p<0.05$). Circularity of BDNF-treated SGCs was larger than that of SGCs in the contralateral cochleae with a difference of 8% (paired t-test, $p<0.05$). Perikaryal area and circularity of BDNF-treated SGCs were not statistically different from perikaryal area and circularity of SGCs in normal-hearing guinea pigs. SGCs in the implanted cochleae that were electrically stimulated during eABR recordings were larger than those in the unstimulated contralateral ears (data not shown, 12%, paired *t*-test, $p<0.05$). There was no significant difference in circularity of SGCs in the implanted ears as compared to the unstimulated contralateral ears (paired *t*-test, $p>0.2$).

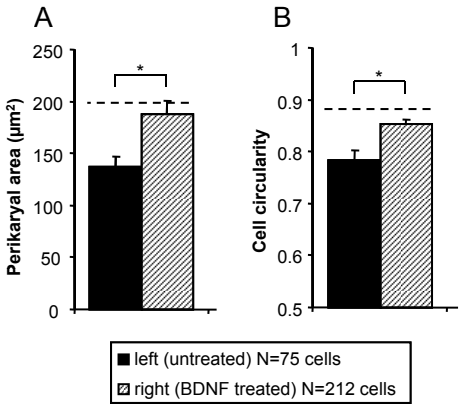


Fig. 6. Perikaryal area (A) and cell circularity (B) of SGCs in left untreated versus BDNF- treated cochleae. Only SGCs with an obvious nucleus in basal location B2 were included. Data were obtained by averaging all individual SGC measurements within one spiral ganglion, followed by averaging across cochleae. Dashed lines represent measurements in normal-hearing animals. n: the number of SGCs measured. Error bars: SEM. *: $p < 0.05$.

3.4 eABRs

Figure 7 shows eABR recordings in two animals (*top* and *middle* row) in the normal (A, C) and deafened (B, D) condition. The decrease in N_1-P_2 amplitude after deafening was prominent in the recordings after deafening for current levels of 318 and 400 µA. The thresholds in these examples did not change after deafening. The P_3 of the eABR recordings in *gp-sp02* (A, B) at stimulus intensities of 400 µA and 318 µA was influenced by the digastric muscle response (arrow). Figures 7E, F depict eABR recordings in *gp-mr02* after four weeks of BDNF treatment (i.e., 6 weeks after deafening) and two weeks after cessation of BDNF-treatment (i.e., 8 weeks after deafening). The N_1-P_2 amplitude did not decrease after cessation of BDNF treatment, and the threshold did not change.

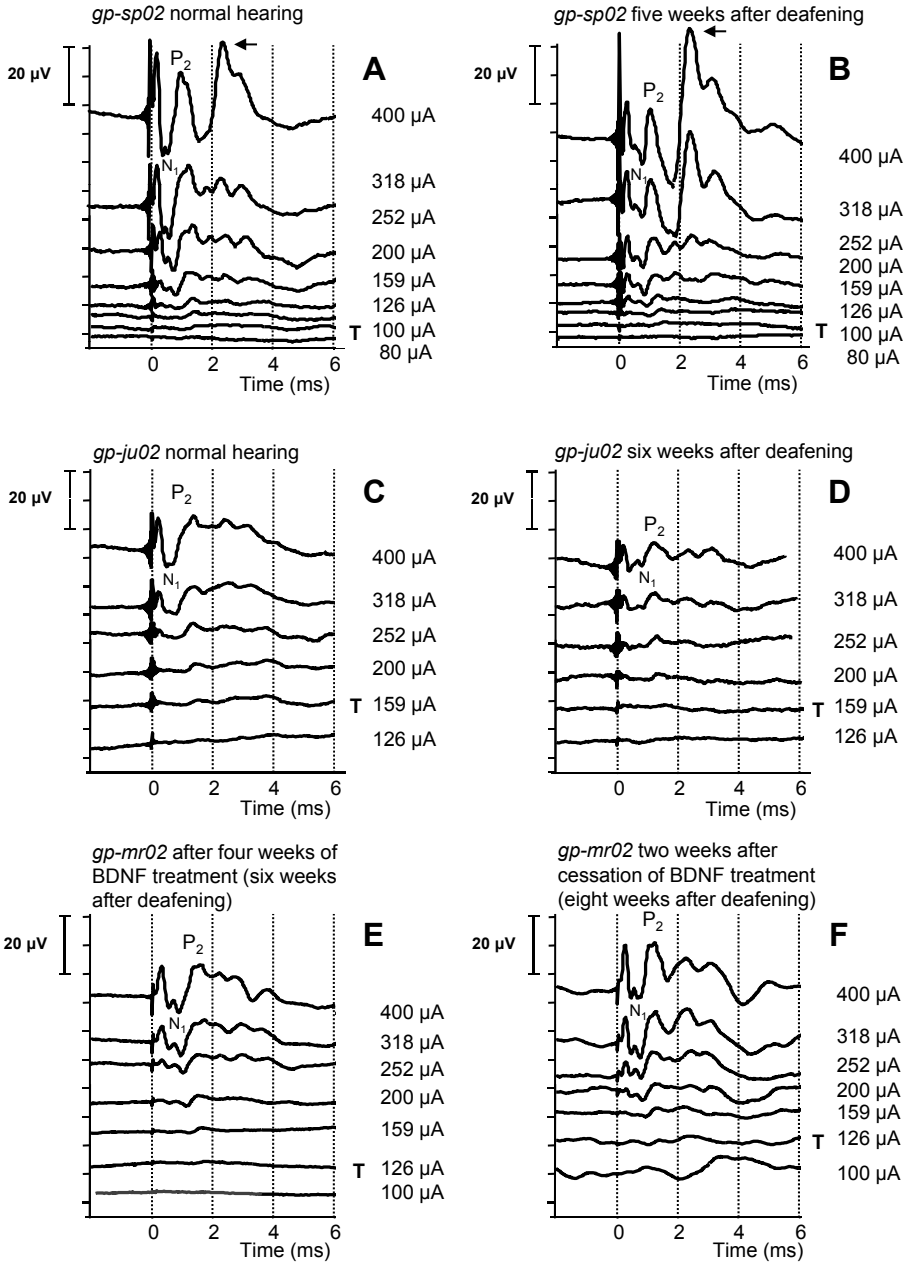


Fig. 7. Recordings of representative eABRs evoked with current pulses of 400 µA down to below threshold in two control animals (gp-sp02 and gp-ju02) and one experimental animal (gp-mr02). Recordings of two control animals are depicted to illustrate the inter-animal variability. A and C are recorded in normal-hearing condition, prior to deafening, B and D are recorded five and six weeks after deafening, respectively. E is recorded after four weeks of BDNF-treatment and F two weeks after cessation of the treatment. T indicates the threshold of wave N1-P2. The arrow indicates the peak that probably reflects the digastric muscle response.

3.5 eABR amplitude

Figure 8 shows the input-output curves of eABRs in normal-hearing animals, animals six weeks after deafening, animals after four weeks of BDNF treatment (i.e., six weeks after deafening), and animals two weeks after cessation of BDNF treatment. The amplitude of the N_1-P_2 complex was decreased in deafened animals for stimulus intensities of 252 µA (unpaired *t*-test, $p<0.05$), 318 µA (unpaired *t*-test, $p<0.01$) and 400 µA (unpaired *t*-test, $p<0.001$) as compared to the amplitude in normal-hearing animals. At lower stimulus currents (100-200 µA) the amplitude differences were smaller and not significant ($p>0.2$). The amplitudes in BDNF-treated animals were near the amplitudes found in normal-hearing animals (unpaired *t*-test, $p>0.4$), and significantly larger than in the deafened animals for stimulus intensities of 318 µA (unpaired *t*-test, $p<0.05$) and 400 µA (unpaired *t*-test, $p<0.01$). There was no significant change in amplitude at any current level after cessation of treatment (paired *t*-test, $p>0.1$).

Figure 9 shows the mean time course of eABR amplitudes elicited with 400 µA pulses for the BDNF-treated animals and the control animals. In both groups amplitudes were increasing during the first two-three weeks after implantation. The control animals showed a gradual and significant decrease in amplitude after deafening. Six weeks after deafening the amplitude was at 60% of the original value. The amplitude did not change significantly after cessation of BDNF treatment ($p>0.6$).

The eABR recordings in these animals also yielded threshold and latency data which will be described in the following sections.

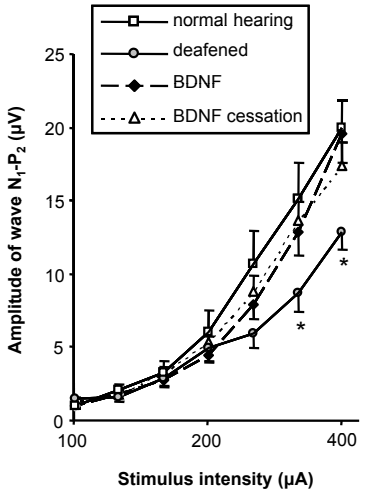


Fig. 8. Mean input-output functions of eABRs recorded in control animals before deafening (normal hearing, n=12) and six weeks after deafening (n=9), and in the experimental animals immediately after four weeks of BDNF treatment (n=5) and two weeks after cessation of BDNF treatment (n=5). Error bars: SEM. *: Indicates statistical significant difference (p<0.05) between the untreated animals six weeks after deafening and each of the other data points.

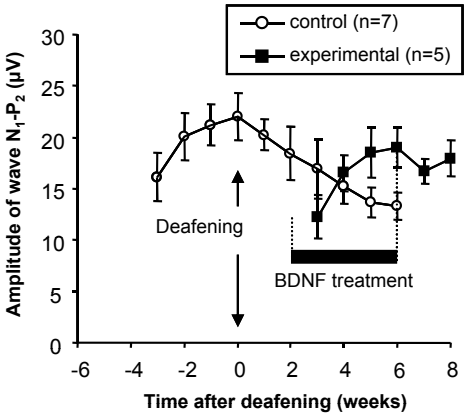


Fig. 9. Mean N_1-P_2 amplitudes of eABRs recorded to 400-μA pulses in control (n=7) and experimental (n=5) animals. The control animals were implanted at –4 weeks and deafened at 0 weeks. The experimental animals were deafened at 0 weeks and implanted in the right cochlea at 2 weeks. Following implantation the experimental animals received BDNF for a 4-week period (indicated with horizontal bold bar). Error bars: SEM.

3.6 eABR threshold

Figure 10 shows a moderate decrease in eABR threshold in the control group during the first weeks after implantation (rm ANOVA: $p<0.05$). The threshold in these animals did not change significantly during the six weeks after deafening (rm ANOVA: $p>0.5$). The eABR threshold significantly decreased over time in the group temporarily treated with BDNF (rm ANOVA: $p<0.01$). After cessation of BDNF treatment the eABR thresholds did not demonstrate any significant changes.

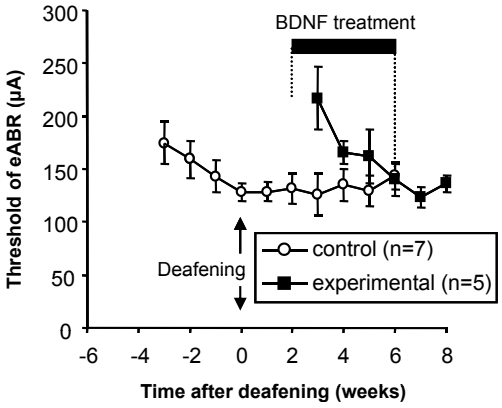


Fig. 10. Mean eABR thresholds observed for wave N1-P2 in control (n = 7) and experimental (n = 5) animals. The experimental animals were deafened at 0 weeks and implanted in the right cochlea at 2 weeks. Following implantation the experimental animals received BDNF for a 4-week period (indicated with horizontal bold bar). Threshold criterion: 2 μV; Error bars: SEM.

3.7 eABR latency

Figure 11 shows the time course of N_1 latencies of eABRs to 400-μA pulses for both the BDNF-treated and control animals. Deafening caused a prominent latency change, which occurred mostly in the first two weeks: first a decrease and then an increase. In the deafened condition, latencies were longer by about 0.15 ms than in the normal condition (paired *t*-test 6 weeks after deafening *versus* normal, $p<0.001$). In general, animals treated with BDNF demonstrated latencies longer than normal. During the treatment period the variance among animals was large, with some animals having longer latencies (as compared to the deafened controls) and others having shorter latencies (as compared to controls one week after deafening). After cessation of treatment all animals had longer latencies, very similar to deafened controls and significantly longer than normal-hearing controls (unpaired *t*-test, $p<0.01$).

Statistical analysis of the latencies performed on all animals (as in Fig. 8) showed similar outcomes.

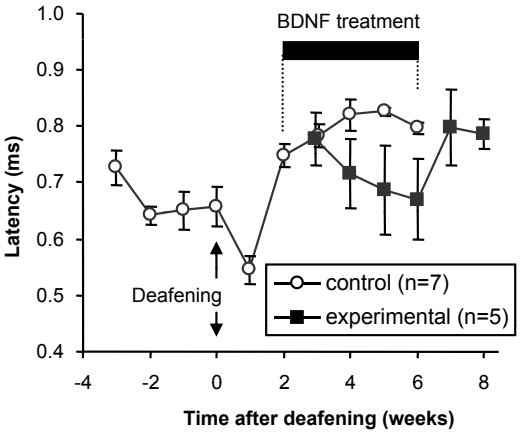


Fig. 11. Mean N1 latencies of eABRs evoked with 400-µA pulses, observed in control (n=7) and experimental (n=5) animals. The experimental animals were deafened at 0 weeks and implanted in the right cochlea at 2 weeks. Following implantation the experimental animals received BDNF for a 4-week period (indicated with horizontal bold bar). Statistical analyses within each group are not shown, but are described in the text. Error bars: SEM.

4. Discussion

The present study showed that temporary treatment with BDNF prevented degeneration of SGCs, which is commonly observed after inner hair cell loss in ototoxically deafened guinea pigs (Ylikoski et al. 1974; Webster and Webster, 1981). Two weeks after cessation of BDNF treatment, SGC packing densities were as in cochleae of normal-hearing guinea pigs, and 3 times greater than in the untreated contralateral cochleae (Fig. 5). Amplitudes of eABR wave N_1-P_2 in BDNF-treated animals were comparable to those in normal-hearing animals and larger than in untreated deafened animals.

4.1 Preservation of SGCs after cessation of treatment

In contrast to our findings, Gillespie et al. (2003) and Shepherd et al. (2008) reported that the number of SGCs was similar to that in untreated, contralateral cochleae, already two weeks after withdrawal of BDNF. In addition, Shepherd et al. (2008) reported that electrical stimulation

after BDNF treatment only partially prevented degeneration of SGCs in the basal turn. The differences between *their studies* and our study that may contribute to the contrasting results are: (1) recordings of the eABRs, (2) concentration of BDNF, (3) period of deafness before the start of the neurotrophic treatment, and (4) method of cessation of BDNF treatment.

(1) We recorded eABRs, while Gillespie et al. (2003) did not. The brief (~20 minutes) stimulation at 10 pulses/s twice per week might have provided some neurotrophic support enhancing the survival of SGCs after cessation of BDNF treatment. Prevention of SGC degeneration in deafened animals, solely due to eABR recordings, has been previously reported (Miller and Altschuler 1995; Mitchell et al. 1997). Chikar et al. (2008) found enhanced SGC survival 12 weeks after a single inoculation with an adenovirus suspension encoding for BDNF (Ad.BDNF), and they argued that eABR recordings contributed to this. Maruyama et al. (2008) demonstrated enhanced survival of SGCs after cessation of intracochlear infusion of GDNF. Similar to our study, brief electrical stimulation during eABR recordings was provided once a week. A synergistic effect of BDNF treatment in combination with chronic electrical stimulation has also been reported (Kanzaki et al. 2002; Shepherd et al. 2005; Song et al. 2009), indicating that synergy of eABR recordings and BDNF treatment might be present. Finally, we found that eABRs prevented decrease of SGC size, which might indicate some trophic effect (Richardson et al. 2005). Based on the data described above, one would argue that eABR recordings explain the discrepancy between our study and *their studies*. However, the following arguments point against this effect of eABRs. First, our results demonstrated that eABR recordings alone did not affect the SGC packing density. Second, Shepherd et al. (2008) reported that electrical stimulation only prevented SGC degeneration in the basal cochlear turn, whereas we found persistent survival in all cochlear locations examined (2.5 cochlear turns).

(2) In our study a BDNF concentration of 100 µg/ml was chosen, and in their studies 62.5 µg/ml. Because concentrations in the range of 50-100 µg/ml are equally effective, as judged immediately after treatment (Gillespie et al. 2004; Yamagata et al. 2004; Wise et al. 2005; Miller et al. 2007; Agterberg et al. 2008), and even lower concentrations have been reported to be effective (50 ng/ml; Miller et al. 1997), it is unlikely that differences in concentration may explain the conflicting results.

(3) We cut off the cannula to stop the BDNF flow exactly four weeks after the start of the treatment. It cannot be excluded that residual BDNF in the cannula (~10 µl) could diffuse in the cochlea resulting in a lasting effect on SGC survival. This seems to be unlikely since the tip of the cannula (0.12 mm) was very small and we observed that the tip was obstructed when we killed the animals for histology (two weeks after the flow rate stopped). In *their studies* the mini-osmotic pumps were not removed after cessation of BDNF treatment. Delivery of BDNF stopped four weeks after implantation because the lifetime of the pump was limited to four weeks. However, an empty reservoir of the osmotic pump will result in an increased osmotic pressure, which might eventually cause damage to pump and cannula. The effect on the cochlea is hard to assess, but one cannot exclude for instance withdrawal or contamination of perilymph jeopardizing the condition of the SGCs.

(4) In *their studies* BDNF treatment started five days after deafening, which is before loss of SGCs whereas in our study it started after two weeks when SGC degeneration has started (Versnel et al., 2007).

Considering the four issues addressed above we propose eABR recordings as the most likely candidate to explain the discrepancy between our data and those of Gillespie et al. (2003). However, we cannot exclude any of the other three arguments.

Although the lasting effect of the neurotrophic treatment is evident in the present study (Fig. 6), this effect is only evaluated two weeks after cessation. Degeneration after deafening is not pronounced after two weeks (~80% of normal density, Versnel et al., 2007). Also the decline in eABR amplitude after deafening is rather slow (Fig. 10). Therefore, to investigate, both histologically and functionally, whether degeneration after cessation of BDNF treatment is slower than degeneration after deafening without intervention, it is important to apply longer periods of survival.

4.2 Morphology of SGCs after cessation of BDNF treatment

Immediately after BDNF treatment SGCs are larger than SGCs in normal-hearing animals (Shepherd et al. 2005; Richardson et al. 2005; Agterberg et al. 2008). We found that two weeks after cessation SGCs had a normal size (while larger than in the untreated contralateral cochlea). This implies that the perikaryal area decreased after cessation from larger-than-normal, to normal values. The question remains if the back-to-normal area is an indication of a normal condition. On the other hand, the decrease of cell size following cessation may be

an early indication for initiation of a degenerative process (Staecker et al. 1996; Leake et al. 1999; Agterberg et al. 2008). To answer this last question the survival of SGCs needs to be investigated longer periods after cessation of the neurotrophic treatment.

4.3 eABR amplitudes

We applied suprathreshold amplitudes as a measure of excitability of the auditory nerve, since the amplitude reflects the summed neural firings of a large population of SGCs, whereas the more commonly used parameter threshold, reflects the function of only the most sensitive auditory nerve fibers. Indeed, at least in case of monopolar stimulation, the amplitude of early eABR waves has been found to correlate well with the number of SGCs (Smith and Simmons 1983; Hall 1990) whereas correlations between eABR threshold and nerve survival were weak (Smith and Simmons 1983). Note that for bipolar stimulation a better correlation was found for threshold measures than for amplitude measures (Miller et al. 1994).

The normal eABR amplitude in BDNF-treated animals indicates that treated SGCs responded as normal to electric stimuli. It should be noted that the amplitude after deafening was smaller, probably because less SGCs were present, thus implicating that the remaining untreated cells responded normally. Our data agree with the results of Maruyama et al. (2008) who reported that amplitudes in neurotrophically treated animals were larger than in untreated animals and were stable after cessation of the treatment.

A decrease of eABR threshold was found the first weeks after implantation in the normal-hearing condition. This might be due to mechanisms as tissue growth around the electrodes and recovery from insertion-induced trauma (Su et al. 2008). We assume the threshold decrease found in the animals treated with BDNF was rather due to these mechanisms than to the preservation of SGCs mediated by the BDNF.

Our threshold data do not agree with other investigators who reported lower thresholds (by 4-9 dB) in deafened guinea pigs treated with neurotrophins as compared to deafened and untreated guinea pigs (Shinohara et al. 2002; Yamagata et al. 2004; Shepherd et al. 2005; Chikar et al. 2008; Maruyama et al. 2008). In these studies, the decrease in eABR threshold corresponded with enhanced survival of SGCs after neurotrophic treatment. Contrary to these studies, in which eABR thresholds were often assessed on the basis of visual inspection of peak 3 and recordings were performed in anesthetized animals, we assessed the eABR threshold on the basis of interpolation of N_1-P_2 amplitudes and we recorded in awake animals.

Possibly, the difference in peaks analysed explains the different threshold data as Mitchell et al. (1997) reported that the threshold of the late eABR peaks in deafened animals decreased over time, while early peak thresholds did not significantly change. Further, the state of the animal plays a role in that the threshold increases with anesthesia as was found for aABRs in mice by Van Looij et al. (2004). The effects of anesthesia may be nonlinear and differ between various experimental conditions.

4.4 eABR latencies

The eABR N_1 latencies were longer in deafened and BDNF-treated animals than in normal-hearing animals (Fig. 11). The initial latency decrease after deafening may be caused by degeneration of dendrites and a loss of spontaneous activity, and the subsequent increase can be attributed to a reduction of the number of myelin layers, which follows dendritic degeneration (Leake and Hradek, 1988). The BDNF-treated nerve cells have a similar degree of myelination as untreated cells (Agterberg et al. 2008; Glueckert et al. 2008), and this might explain the similar latencies of BDNF-treated and untreated animals.

4.5 Clinical implications

Our results demonstrate that SGC survival in deafened animals, in both morphological and functional sense, is not reduced after cessation of intracochlear application of BDNF. The enhanced survival of SGCs is a promising finding for cochlear implant candidates since there are currently no potential methods available to provide neurotrophins for periods that extend the order of months. Lifelong neurotrophic treatment via an osmotic pump is not clinically preferable, because of repetitive invasive manipulations needed to replace the pump and high infection risks.

Before neurotrophic treatment can be considered a clinical option, it is important to investigate in animal studies whether neurotrophic treatment does actually improve the sound perception performance with a cochlear implant. A major topic that need to be further explored is the assumption that more and better functioning SGCs would result in a better performance of cochlear implant users. Alternatively to this hypothesis it might be that a minimum number of SGCs is required for electrical hearing, and that an increase above this minimum would have little functional impact (Blamey 1997). Clinical data do not support the assumption that more SGCs would lead to a better behavioral performance (Nadol et al. 2001;

Khan et al. 2005; Fayad and Linthicum 2006; Nadol and Eddington 2006). These data indicate that abnormalities in the central auditory pathways are at least as important as SGC survival in limiting the performance of cochlear implant users (Nadol et al. 2001). Furthermore, the overall performance of cochlear implant users improves markedly within weeks as a result of implant use, confirming the role of the central auditory system and its plasticity (Kral and Eggermont 2007). Still, we, and others, think that it is unlikely that SGC cell loss does not affect the performance with a cochlear implant and that the human data sets are still too limited to conclude that the extent of SGC survival does not influence cochlear implant performance (Khan et al. 2005; Leake et al. 2008). Moreover, it should be considered that with improved cochlear implant technology the number of SGCs might become more important (Green et al. 2008).

Acknowledgments

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Delivery of brain-derived neurotrophic factor to the guinea pig cochlea through placement of absorbable gelatin sponge onto the round window membrane: A pilot study

6

Abstract

When guinea pigs are deafened with ototoxic drugs, spiral ganglion cells (SGCs) degenerate progressively. Application of exogenous neurotrophins can prevent this degeneration. Most studies used mini-osmotic pumps for the application of these neurotrophins. In the present study a less invasive method, in which neurotrophins are placed on the round window membrane, was developed. Guinea pigs were deafened and two weeks later absorbable gelatin sponge soaked in brain-derived neurotrophic factor (BDNF) was positioned on the round window membrane. SGC packing densities in the basal part of the cochlea were analysed two weeks after placement of the gelatin sponge. Histological examination demonstrated enhanced survival in the basal part of the BDNF-treated right cochlea, as compared to the untreated left cochlea of deafened guinea pigs. This is a promising finding for cochlear implant candidates, because this mode of application of neurotrophins is clinically safer than intracochlear application.

Keywords: absorbable gelatin sponge; brain-derived neurotrophic factor; guinea pig; round window membrane; spiral ganglion cells

1. Introduction

Many factors like period of deafness, integrity of the auditory nerve, proximity of the electrodes to the neurons, stimulus waveform, the extent of fibrous tissue in the scala tympani, damage during electrode implantation and plasticity in the central auditory system influence the perceptual performance of cochlear implant users (Blamey et al., 1992; Incesulu and Nadol, 1998; Kawano et al., 1998; Van Dijk et al., 1999; Kral and Tillein, 2006). An important factor is thought to be the number of excitable spiral ganglion cells (SGCs). Animal studies have shown that as a result of destruction of inner hair cells (IHCs) the SGCs lose their neurotrophic support and degenerate progressively (Ylikoski et al., 1974; Webster and Webster, 1981; Lefebvre et al., 1992; Versnel et al., 2007). Infusion of neurotrophic factors, such as neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF), directly into the cochlea of deafened guinea pigs, has been shown to preserve SGCs (Ernfors et al., 1996; Staecker et al., 1996; Miller et al., 1997; Gillespie et al., 2003; Agterberg et al., 2008). In most of these studies the

neurotrophins are applied by means of a cannula which is attached to a mini-osmotic pump and is inserted into the cochlea. This method with a high surgical complexity increases the risk of infections in a clinical setting and the repeated invasive manipulations to replace the osmotic pump are undesirable for cochlear implant recipients.

There are several other application methods with less risk of infection. Staecker et al. (1998) reported long lasting synthesis of BDNF in the cochleae of mice after gene transfer with use of the herpes simplex virus as vector and Rejali et al. (2007) inserted an electrode coated with cells releasing neurotrophins into the cochlea and reported reduced degeneration of SGCs in the basal cochlear turn. However, these methods still include the opening of the cochlea. Alternatively, neurotrophins can be delivered through the round window membrane. Ito et al. (2005) used a biodegradable hydrogel and Noushi et al. (2005) used hydrogel in the form of beads to provide a slow release of neurotrophins via the round window. Both studies reported that their methods were effective in protection against SGC loss after deafening. These less invasive methods seem more appropriate clinically since they may allow to treat SGCs in deaf human subjects without inner ear surgery (Richardson et al., 2006).

The aim of our study was to assess the SGC packing density after placement of an absorbable gelatin sponge, soaked in a solution of brain-derived neurotrophic factor (BDNF), on the round window. The treatment was started two weeks after deafening, when the degeneration of SGCs had set in (Gillespie et al., 2004; Agterberg et al., 2008).

2. Materials and methods

2.1. Animals and experimental design

Six albino female guinea pigs (strain: Dunkin Hartley; weighing 250-350 g) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the animal care facility of the Rudolf Magnus Institute of Neuroscience. All animals had free access to both food and water and were kept under standard laboratory conditions. Lights were on between 7:00 am and 7:00 pm. Temperature and humidity were kept constant at 21°C and 60%, respectively.

All animals were deafened with the procedure described below. Absorbable gelatin sponge, soaked in BDNF solution, was positioned on the round window of the right cochleae two weeks after the deafening procedure. Subsequently, a gold-ball electrode was placed on the round window to record electrically evoked auditory brainstem responses (eABRs).

Degeneration was examined two weeks after delivery of the gelatin sponge (i.e, four weeks after deafening). Both the left and right cochleae were processed for light microscopical examination and analysis. Data of BDNF-treated ears were compared to data from normal cochleae presented in a previous paper (Agterberg et al., 2008). All experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC # 03.04.036).

2.2. Deafening procedure and ABRs

Animals were anesthetized with xylazine (Sedamun®, i.m. 10 mg/kg) and ketamine (Ketanest®, i.m. 40 mg/kg). Before the deafening procedure, three stainless steel screws (8.0x1.2mm) were inserted into the skull bone to record auditory brainstem responses (ABRs). The screws were inserted 1 cm posterior to bregma, 2 cm anterior to bregma, and 1 cm lateral from bregma (Mitchell et al., 1997). Before, during and after deafening we recorded ABRs to monitor hearing thresholds. Measurements were performed in a sound-attenuated chamber. Broadband click stimuli consisting of biphasic rectangular pulses (100 μs/phase) were presented in free field using a tweeter (Fane J-104) positioned 10 cm above the unanesthetized guinea pig. Stimulus generation and signal acquisition were controlled with custom-written software and a personal computer (for details, see Versnel et al., 2007). Stimuli were presented from 86 dB above threshold in normal-hearing animals down to threshold in 10-dB steps. Threshold was defined as the sound level at which the ABR was just noticeable upon visual inspection of the response.

When initially normal hearing thresholds were confirmed, kanamycin (400 mg/kg, sc) was injected subcutaneously followed (15-60 minutes later) by slow intravenous infusion of furosemide (100 mg/kg, iv). This procedure, originally reported by West et al. (1973), has been shown to eliminate almost all cochlear hair cells (Gillespie et al. 2003; Versnel et al. 2007). For the intravenous infusion of furosemide, the left jugular vein was exposed and a catheter was inserted. A successful insertion was confirmed with withdrawal of blood into the catheter. The control animals received isotonic saline (subcutaneously and intravenously), instead of kanamycin and furosemide. After deafening, the ABRs were measured on days 1, 7, and 14 to assess the extent of hearing loss. All animals included in this study demonstrated a threshold shift of >50 dB, measured 14 days after deafening.

2.3. Absorbable gelatin sponge placement and BDNF treatment

Two weeks after the deafening procedure the animals were anesthetized with xylazine and ketamine after assessment of the extent of hearing loss. The right bulla was exposed retro-auricularly and a small hole was drilled to visualize the cochlea. Cylinders (1 mm^3) of absorbable gelatin sponge (Gelitaspon®, Spongostan®) were soaked in BDNF (PeproTech Inc., Rocky Hill, New Jersey, USA.) solution (1 mg/ml) and positioned near the round window membrane. This volume of sponge absorbs between 3-6 µl of solution, i.e., 3-6 µg BDNF. An electrode, consisting of a stainless steel wire with a gold-ball tip, was used to manipulate the gelatin sponge on the round window and the gold-ball tip was positioned in the round window niche (for details, see Klis et al., 2000). The stainless steel wire was fixed onto the bulla with dental cement (Ketac-Cem Aplicap; ESPE Dental Supplies, Utrecht, the Netherlands). The wire was placed in a connector and fixed with dental cement on the skull. The wound was closed in two layers with Vicryl®.

2.4. Determination of SGC packing densities

The left and right cochleae were fixed by intralabyrinthine perfusion with a fixative consisting of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% DMSO in 0.08 M sodium cacodylate buffer (pH 7.4), followed by immersion in the same fixative for 3 h at room temperature and subsequent histological processing (for further details, see De Groot et al., 1987). Light microscopical assessment and quantitative analyses (hair cell counts and SGC packing densities) were performed using semithin (1 µm) midmodiolar sections stained with methylene blue and azur II in sodium tetraborate. The number of IHCs and outer hair cells (OHCs) present in one midmodiolar plane was counted at five different locations along the basilar membrane at a half-turn spacing (B1, B2, M1, M2 and A1; Fig. 2) to get an indication of the efficacy of the deafening procedure.

Mean SGC packing densities were determined as described previously (Van Ruijven et al., 2004). Digitized light microscopical images of the spiral ganglia taken from the three most basal locations in the cochlea (B1, B2, and M1; Fig. 2) were imported in NIH Image (Version 1.63; US National Institutes of Health, Bethesda, Maryland). The bony boundaries of Rosenthal's canal were outlined and its cross-sectional area (in mm^2) was calculated. The number of perikarya of the SGCs was counted at each location. SGC packing density was calculated by dividing the number of SGCs by the cross-sectional area of Rosenthal's canal and expressed as the mean number of SGCs per mm^2.

3. Results

3.1. ABR threshold shifts

Two weeks after the deafening procedure all animals demonstrated threshold shifts of more than 65 dB for click-evoked ABRs. Microscopical inspections of IHCs and OHCs demonstrated that these threshold shifts were accompanied by a complete elimination of all hair cells at cochlear locations B1, B2, M1 and M2, and an almost total loss of hair cells at cochlear location A1. All implanted animals included in this study were without any trace of otitis media.

3.2. SGC packing densities

Figure 1 shows light micrographs of Rosenthal's canal in the basal turn (B1), providing examples of four different cohorts of cochleae (normal cochlea, two weeks deaf, four weeks deaf, and four weeks deaf treated with BDNF). The examples of a normal cochlea and a cochlea two weeks after deafening were selected from a previous study (Agterberg et al., 2008). In the normal cochlea, Rosenthal's canal contained the full complement of SGCs and nerve fibers embedded in a matrix consisting of vascularized connective tissue (Fig. 1A). Two weeks after deafening the cellular distribution within the spiral ganglia appeared unchanged (Fig. 1B). Four weeks after deafening, a considerable loss of SGCs was demonstrated (Fig. 1C). Most remnants of SGCs had lost their characteristic ovoid shape and, instead, had acquired a more elongated or dendritic appearance. The SGCs seemed to be less tightly packed than the SGCs in the cochlea two weeks after deafening. Figure 1D provides an example of a cochlea treated with BDNF. The amount of SGCs appeared more or less unchanged as compared to the amount of SGCs in the cochlea two weeks after deafening, and the SGCs retained their ovoid shape. In the selected example the SGC packing density was obviously higher than the SGC packing density in untreated cochlea four weeks after deafening (compare Fig. 1D with 1C).

Figure 2 shows the mean SGC packing densities in the left untreated cochleae four weeks after deafening and the right cochleae four weeks after deafening and treatment with BDNF for two weeks. The SGC packing densities in the BDNF-treated cochleae were significantly higher than those in untreated cochleae (RM ANOVA; main effect BDNF treatment across locations (B1-M1): $p = 0.05$). After establishing this significant effect of BDNF treatment, we performed *t*-tests for each location separately. At locations B1 (paired *t*-test, $p < 0.05$) and

B2 (paired *t*-test, $p < 0.01$). At cochlear location M1 this effect of BDNF was not present (paired *t*-test, $p > 0.7$). SGC packing densities in cochleae treated with BDNF at locations B1 and B2, were decreased with a factor 2 as compared to densities at cochlear location B1 and B2 in normal cochleae (dashed lines).

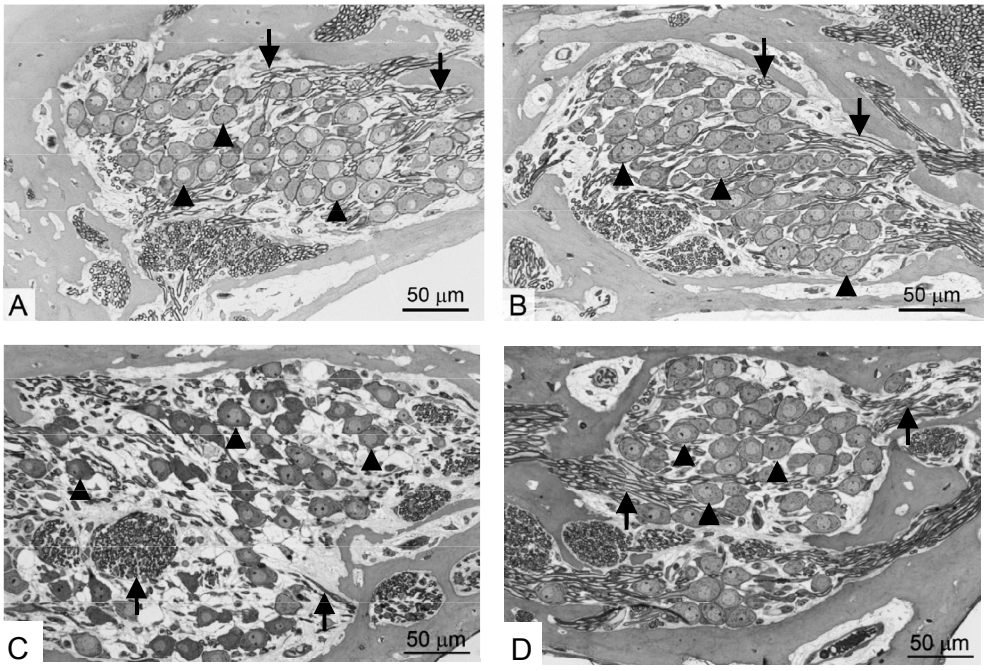

Fig. 1. Light micrographs of Rosenthal's canal at location B1 (C and D) or B2 (A and B) in four cochleae. (A) SGCs (arrowheads) and nerve fibers (arrows) in the spiral ganglion from a normal cochlea. (B) Two weeks after deafening. (C) Four weeks after deafening. (D) Two weeks after positioning of the gelatin sponge soaked in BDNF (four weeks after deafening).

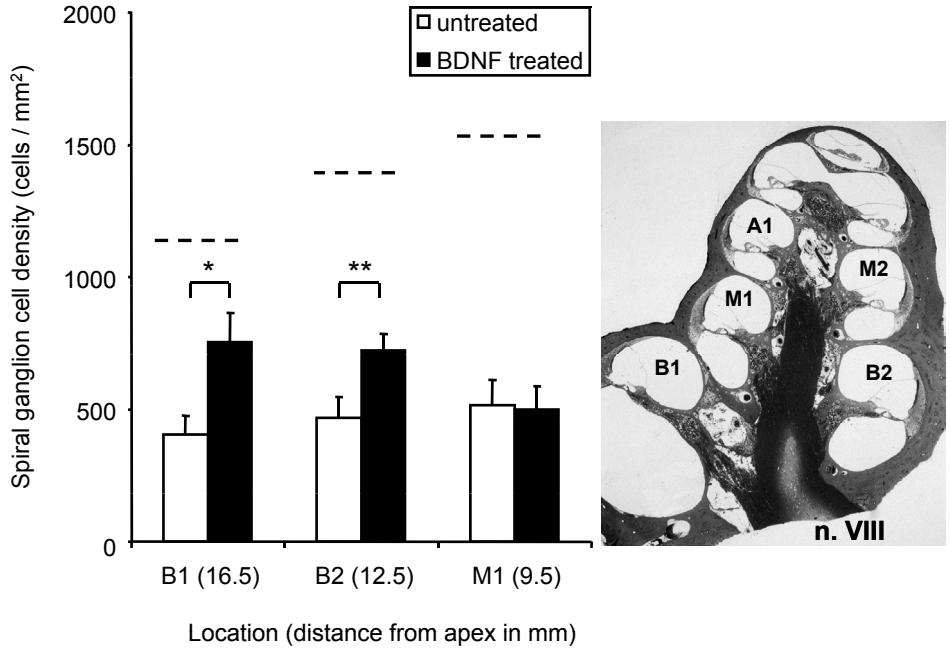

Fig. 2. Mean SGC packing densities at locations B1, B2 and M1 in the left (untreated) and right (treated) cochleae four weeks after deafening. *:*p*<0.05; **:*p*<0.01. Dashed lines represent SGC densities at locations B1-M1 in normal cochleae. Error bars: SEM. The light micrograph shows a midmodiolar section (1 µm) of a normal guinea pig cochlea showing the different locations (B1-M1) at which SGCs were examined. N. VIII: cochlear nerve.

4. Discussion

4.1. SGC packing densities

This study examined the effect of BDNF application with absorbable gelatin sponge on SGC packing density in hair-cell-deprived cochleae. Gelatin sponge, soaked in BDNF and positioned on the round window membrane two weeks after deafening, reduced degeneration of SGCs in the basal turn of the cochlea. Enhanced survival of SGCs after delayed neurotrophic treatment (treatment started 2-4 weeks after deafening) has also been reported after intracochlear infusion of BDNF by means of an osmotic pump system (Gillespie et al., 2004; Wise et al., 2005; Agterberg et al., 2008). Often with mini-osmotic pump delivery of neurotrophic factors, more effect is found in the basal than in the apical part of the cochlea (Shah et al., 1995; Shepherd et al., 2005; Wise et al., 2005; Agterberg et al., 2008). In these studies, SGC packing densities in treated cochleae were comparable to the SGC packing density in normal-hearing

cochleae. In our study, SGC packing densities in treated cochleae at cochlear locations B1 and B2 were substantial lower than in normal-hearing cochleae. Thus, BDNF treatment applied as described here is less effective than applied with an osmotic pump. Nevertheless, this is the first study that demonstrated an increased survival of SGCs after delivery of BDNF with gelatin sponge positioned on the round window. Our results are not in agreement with other studies that reported that neurotrophins absorbed in gelatin sponge did not affect the SGC density (Richardson et al., 2005, 2006).

We found that BDNF prevented SGC degeneration in the basal turn (locations B1-B2) of the cochlea. In this pilot study, SGC packing densities were only determined at the cochlear locations B1-M1, and not at the more apical locations. However, it is unlikely that we missed an effect at the more apical turns. Tracer studies have shown that concentrations of the markers horseradish peroxidase and trimethylphenylammonium, irrigated on the round window membrane, remained high in the basal turn near the round window while at more apical regions the markers were undetectable (Saijo and Kimura, 1984; Salt and Ma, 2001).

4.2. Size of BDNF-treated SGCs

Several studies reported that SGCs in the basal turn of BDNF-treated cochleae were larger (30-70%) than SGCs in cochleae of normal-hearing animals (Shepherd et al., 2005; Richardson et al., 2005; Agterberg et al., 2008). Agterberg et al. (2008) discuss the effect of size in relation to the determination of SGC packing densities. When treated SGCs are larger than untreated SGCs, the increased size leads to overestimation of the SGC packing density (Coggeshall and Lekan, 1996; Leake et al., 1999). In this pilot study, size of the SGCs was not measured. However, even though our results on SGC packing densities may be biased by an possible increase in size of the SGCs (compare Fig. 1C to D for size of the remaining SGCs), the fact that the SGCs in BDNF-treated ears are larger and closer to normal with respect to size, is in itself evidence of the trophic effect of BDNF and demonstrates that BDNF has reached the cells (Richardson et al., 2005).

4.3. Clinical implications

Neurotrophic treatment of SGCs might be clinically relevant for human cochlear implant candidates and this study demonstrates that a potentially safe technique, i.e., potentially safer than intracochlear techniques, can enhance the survival in the basal part of the cochlea of

deafened guinea pigs. Our results show an effect which is much smaller than with intracochlear delivery (Staecker et al., 1996; Miller et al., 1997; Agterberg et al., 2008). However, there are possibilities for further optimalisation, for instance by increasing the amount of gelfoam or by increasing the amount of BDNF in the sponge. Also, the addition of additional compounds such as a combination of neurotrophins, might increase the effectiveness.

A point of concern may be the permeability of the round window membrane. In guinea pigs, the round window membrane is thinner than in humans (Richardson et al., 2006). This may be a decisive factor for the amount of BDNF that actually diffuses into the cochlea and this may hamper translation of this technique from the laboratory to the clinical setting. Nevertheless, our results indicate that it might be possible to deliver neurotrophins to the inner ear of candidates for a cochlear implant without opening the cochlea.

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Proposal for a fast behavioral task: Detection of electrical stimulation with a cochlear implant in deafened guinea pigs

7

Abstract

The main purpose of this study was to develop a behavioral task that can be used to determine the ability of deaf guinea pigs to detect electric pulse trains delivered with a cochlear implant. A secondary purpose was to investigate the effect of chronic intracochlear electrical stimulation (CES; 4 h/day, 5 days/week) on the behavioral performance. Normal-hearing guinea pigs were implanted with an electrode array in the right cochlea and trained to avoid a stream of air in a modified shuttle-box. Training sessions were short (~10 minutes) and consisted of 20 trials. For the initial training, the target stimulus was a narrow-band noise of fixed sound pressure level (78 dB SPL). Subsequently, they were tested in the shuttle box with the noise stimulus presented at 4 different sound pressure levels (58-88 SPL). Four weeks after implantation the animals were deafened and divided over a group to receive CES (*CES* group; n=5) and a group not to receive CES (*noCES* group; n=5). Five weeks after deafening the animals were tested in the shuttle-box with electric pulse trains delivered via the electrode array at different current levels (142-400 μA). In the first five training sessions guinea pigs learned to respond well to the narrow-band noise of 78 dB SPL and, in subsequent sessions, the animals showed a gradual increase of response rates with noise level. After deafening, the animals responded well in the first session to the electric pulse trains and their performance improved in further sessions. Similarly to the responses to the acoustic stimuli, the correct responses to the electrical stimulation increased with current level over a wide range. No significant difference was found between the two groups (*CES vs noCES*). The presented behavioral model indicates that bilaterally deafened guinea pigs, which had lost ~50% of their SGCs, can differentiate current levels. Overall the data suggest that the animals experience an auditory sensation when they are stimulated via the cochlear implant. Furthermore, the data demonstrate that it is possible to train animals within only 5 sessions of 20 trials.

1. Introduction

To date, a cochlear implant is the most successful sensorineural prosthesis. World wide more than 110.000 people have been implanted with success (Fallon et al., 2008). However there is a wide variability in the perceptual performance of cochlear implant users. There are several variables which may influence their performance (Blamey et al., 1992, 1996;

Knutson et al., 1991; Van Dijk et al., 1999). An important factor might be the degeneration of the spiral ganglion cells (SGCs; Spoendlin, 1975; Webster and Webster, 1981; Leake and Hradek, 1988; Shepherd and Hardie, 2001). Many animal studies have been performed to prevent the degeneration of SGCs (for overviews, see Miller, 2001; Pettingill et al., 2007) that occurs after the loss of inner hair cells (Ylikoski et al., 1974; Webster and Webster, 1981). Post-deafening treatments like chronic electrical stimulation (CES; Lousteau, 1987; Chapter 3) and intracochlear infusion of neurotrophins (Ernfors et al., 1996; Agterberg et al., 2008; Chapter 5), have proven to be successful in preventing degeneration of SGCs, with CES being less effective than neurotrophic treatment (Miller, 2001; Leake et al., 2008). The studies described above all focus on morphological and/or physiological measurements. However, these measures are not more than a rough indicator of the animal's ability to perceive and process the electrical stimulation applied with the cochlear implant. A behavioral task, in which the ability of deaf guinea pigs to detect electrical stimulation applied with the cochlear implant is tested, would be more appropriate and it might be able to demonstrate the relevance of preserving SGCs from degeneration in deafened guinea pigs with such a task. Ultimately we want to know whether more and better functioning SGCs do result in a better perception of the stimuli.

Compared to other species, guinea pigs are hard to train on a behavioral task because of their erratic behavior (Philippens et al., 1992). In the group of Pfingst, Miller et al. (1995) successfully performed behavioral studies with deafened guinea pigs equipped with a cochlear implant. They used a positive reinforcement procedure which, however, is generally time consuming (3-6 months with a 90-minute training session per day, for 5 days/week, to obtain stable detection thresholds). As we were aiming for a relatively fast method, we used a negative reinforcement. To reduce the stress that such a procedure might induce, we used a stream of air which was thought to be milder than the conventionally used electric foot shocks (Philippens et al., 1992).

Pfingst's method was used by his group to investigate whether a post-deafening neurotrophic treatment affected the psychophysical detection of electrical stimulation, presumably of the auditory nerve, with a cochlear implant (Chikar et al., 2008). In this study a single inoculation of an adenovirus suspension, coding for brain-derived neurotrophic factor (BDNF), was applied in the scala tympani. Chikar et al. (2008) reported lower psychophysical thresholds, which are an indication of a better cochlear implant performance, as well as

higher survival rates of SGCs in animals treated with BDNF than in untreated animals. This is a promising result, indicating that prevention of SGC degeneration might be beneficial for cochlear implant recipients. However, this experiment was performed in unilaterally deafened guinea pigs. We wanted to apply our behavioral model to bilaterally deafened guinea pigs, because that mimics the clinical situation of cochlear implant patients. Furthermore, the effect of a neurotrophic treatment is usually analyzed in bilaterally deafened guinea pigs (Chapters 3-5). In this study we were interested in the detection of suprathreshold stimuli since it has been reported that correlations between the thresholds of electrically evoked auditory brainstem responses (eABRs) and nerve survival were weak, while amplitudes of suprathreshold eABRs correlated well with the number of SGCs (Dobie and Kimm, 1980; Miller et al., 1983; Smith and Simmons, 1983; Hall, 1990).

The model was applied in an experiment in which deafened animals were treated with chronic electrical stimulation (CES), for which results of SGC histology and electrically evoked auditory brainstem responses (eABRs) are described in Chapter 3. Since those results did not show an effect on SGC survival or eABR response, we do not focus in this chapter on the CES treatment. Our prime interest is development of a fast behavioral paradigm that enables to examine the animal's perception of electrical stimuli delivered through a cochlear implant.

2. Materials & Methods

2.1 Animals and experimental design

Ten female albino guinea pigs (strain: Dunkin Hartley; weighing 250-350 g) were purchased from Harlan Laboratories (Horst, the Netherlands) and housed in the animal care facility of the Rudolf Magnus Institute of Neuroscience. All animals had free access to food and water, and were kept under standard laboratory conditions. Lights were on between 7:00 am and 7:00 pm. Temperature and humidity were kept constant at 21°C and 60%, respectively. The animals that were used in this behavioral experiment were also used to investigate the effect of CES on SGC survival (see Chapter 3 of this thesis).

Animals were accustomed to the laboratory conditions for a week. During this week the animals were handled daily by transporting them to the sound-attenuated room in which the behavioral tests were performed. The experimental design, with a total duration of 12 weeks, is illustrated in Fig. 1. Animals were first trained to respond to an acoustic stimulus

in a shuttle-box (week -6 to -4). After the training, the animals were implanted in the right cochlea with an eight-electrode array (Cochlear®, Mechelen, Belgium). Following implantation, electrically evoked auditory brainstem responses (eABRs) were measured once or twice a week in freely moving animals. Four weeks after implantation the animals were deafened, and divided in a control (noCES, n=5) and an experimental (CES, n=5) group. Animals in the experimental group received 4 hours of chronic electrical stimulation (CES) per day, 5 days/ week, for a period of six weeks. The control (noCES) animals were placed in the same cages for the same amount of time as the animals in the experimental group, but without receiving CES. CES started two days after deafening. In the last week (week 6) of the experiment the guinea pigs were trained to respond to electrical stimulation delivered via the cochlear implant. The animals were killed for histology immediately after finishing the last training. Effects of CES on the eABR recordings and on the survival and morphology of spiral ganglion cells are described in chapter 3 of this thesis. All experimental procedures were approved by the University's Committee on Animal Research (DEC-UMC # 03.04.036).

2.2 Shuttle-box study design

The behavioral test procedure was adapted from Philippens et al. (1992). The shuttle-box consisted of two equal compartments (23x23x23 cm), connected by a passage. The passage was a narrowing of the cage with plastic frames at both sides in the middle of the shuttle-box walls. The passage was guarded by an infrared beam on each side. First we investigated the functional psychophysical response to an acoustic stimulus (sessions 1-12). Subsequently, after deafening, we investigated the responses to electric pulse trains applied with a cochlear implant (sessions 15-20). The guinea pigs were trained to avoid an unpleasant stream of air (6,250 cm^3/s, air tube diameter 1 cm, duration 5 s) by moving into the other compartment within 15 s after the stimulus had been turned on. The acoustic stimulus was a narrow-band noise centered around 10 kHz (width at -6 dB: 1 octave). The animals were trained during 10 daily consecutive training sessions (sessions 1-10 in week -6 and -5, see Fig. 1). During the first two training sessions the stimulus was a narrow-band noise of a fixed level of 78 dB SPL, and the animals received 10 trials per session. On training sessions 3 to 5, 20 trials at 78 dB SPL were presented. During training sessions 6-14 the narrow band noise was presented at four sound levels (58, 68, 78 and 88 dB SPL), with on each session 5 trials per sound level. One session took 10 to 12 minutes.

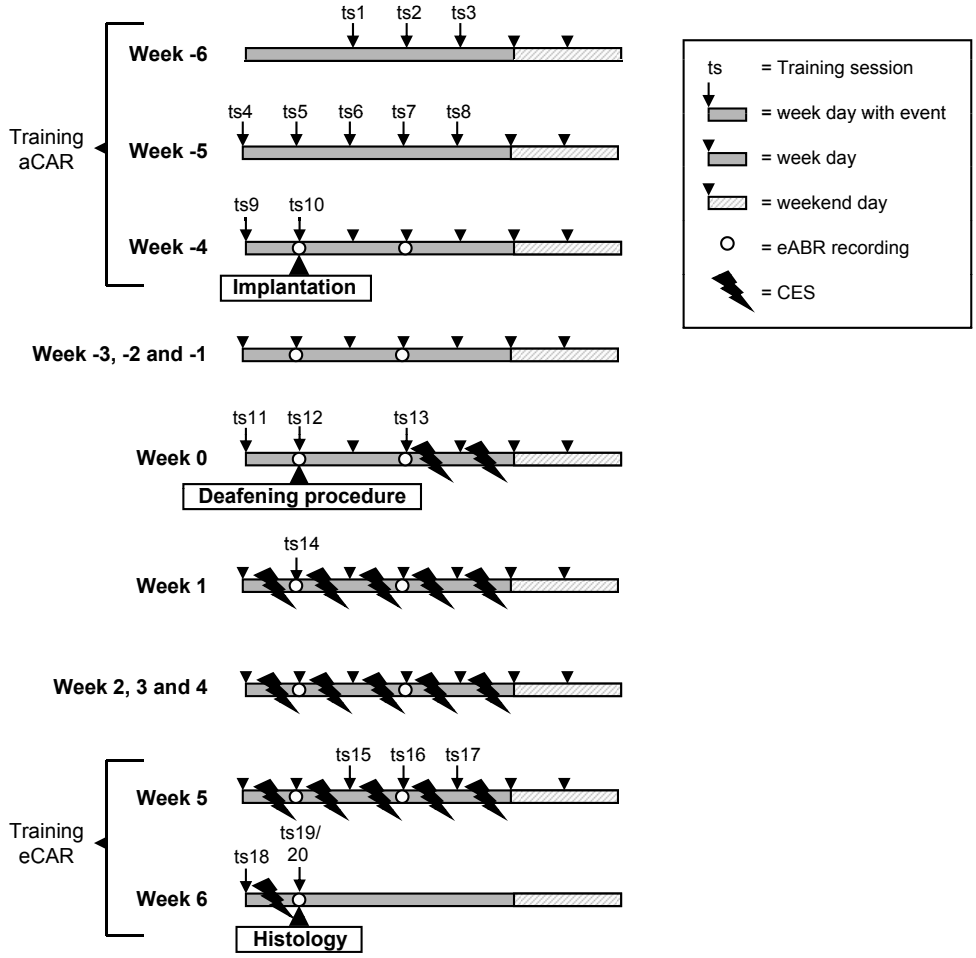

Fig. 1. Graphical presentation of the experimental design for the animals receiving chronic electrical stimulation (CES, n=5). The animals in the control group received no chronic electrical stimulation (noCES, n=5). The training sessions (ts) are indicated. In week -4 the electrode arrays were implanted in the cochlea (*implantation*). In week 0 the animals were deafened (*deafening procedure*). In week 6 the animals were killed for histological evaluation (*histology*). Throughout the experimental protocol, electrically evoked auditory brainstem responses (eABRs) were obtained (○).

During sessions 15-19 (week 5 and 6) the stimulus was an electric pulse train (monophasic pulses of 23 µs with alternating polarity, 111 pulses/s) varying randomly at four different current levels (142, 200, 285 and 400 µA), with on each session 5 trials per current level. These suprathreshold current levels were chosen, based on the eABR recordings to these levels

in a previously performed experiment (see Chapter 5). Immediately after training session 19 the animals were tested in an additional session (session 20), in which current levels of 71 and 100 μA were applied. Because these current levels might be below threshold, the stream of air during session 20 was turned off. The pulse trains were delivered to the cochlear implant's most apical electrode (which was located in the basal cochlear turn), and a skull screw ipsilateral to the cochlear implant, 1 cm lateral to bregma, as return electrode. Stimulus presentation and data collection were performed with custom-written software and a personal computer.

A shuttle response to the stimulus was classified as a correct avoidance response (CAR; aCAR in case of an acoustic stimulus, and eCAR in case of an electrical stimulus). Responding to the stream of air was classified as an escape response. A CAR or an escape response terminated the stimulus or stream of air, and initiated an inter-trial interval. The inter-trial interval was 20 s plus a period randomly varying between 0 and 10 s. When the animal did not cross to the other compartment during the stimulus or stream of air, this was classified as a "no response". A shuttle response before the first trial or during an inter-trial interval, was classified as a spontaneous response. The aCAR and eCAR are expressed in percentage correct responses (CAR% = number of CARs * 100 / number of stimulus presentations).

2.3 Implantation and eABR recordings

Animals were anesthetized with Domitor® (medetomidine hydrochloride; 10 mg/kg, im) and Ketanest-S® ((S)-ketamine; 40 mg/kg, im). Three stainless steel screws (8.0x1.2 mm) were inserted into the skull bone (Mitchell et al., 1997) to record (e)ABRs. The screw ipsilateral to the cochlear implant and the posterior screw were used as return electrodes during CES. The right bulla was exposed retro-auricularly and a small hole was drilled to visualize the cochlea. In both groups of animals the right cochleae were implanted with an eight-electrode array designed for guinea pigs (Cochlear®, Mechelen, Belgium). Each Pt ring electrode was 0.3 mm in width and the inter-electrode distance was 0.45 mm. This electrode array resembles the array-cannula device described by Shepherd and Xu (2002). The array was inserted 3-4 mm through the cochleostomy in the basal turn near the round window. The array was fixed on the bulla with dental cement (Ketac-Cem Aplicap, ESPE dental supplies, Utrecht, The Netherlands). The array was connected to the skull with a screw (Brown et al., 1993) and again fixed with dental cement (Polyfast®, Dental Union, The Netherlands). The incision was closed in two layers with Vicryl®.

After cochlear implantation, eABRs were recorded. For a detailed description of the eABR recordings and for the definition of the peaks, see the Methods section of Chapter 5 in this thesis. Briefly, monophasic rectangular current pulses (width: 20 μs; interstimulus interval: 99 ms) were presented with alternating polarity to the most apical intracochlear electrode using the lateral screw in the skull as return electrode (monopolar eABR recordings). Thresholds were defined as the stimulus level that evoked a 2.0 μV reproducible waveform. The highest current level was 400 μA, as the maximum level in the behavioral experiment.

2.4 Deafening procedure

Before the deafening procedure, acoustically evoked ABRs (aABRs) were recorded to confirm normal hearing. Then, animals were anesthetized with Domitor® (10 mg/kg, im) and Ketanest-S® (40 mg/kg, im), and injected subcutaneously with kanamycin (400 mg/kg, sc) followed (15-60 minutes later) by slow intravenous infusion of furosemide (100 mg/kg, iv) as a loop diuretic. This procedure, originally reported by West et al. (1973), has been shown to eliminate almost all the hair cells (Gillespie et al., 2003; Versnel et al., 2007). For the intravenous infusion of furosemide, the left jugular vein was exposed and a catheter was inserted. A successful insertion was confirmed with withdrawal of blood into the catheter. Animals with a threshold shift of > 50 dB, 14 days after deafening, were included in the study.

2.5 Statistics

Statistical analyses were performed using SPSS® for Windows (version 15.0.1). Repeated-measures analysis of variance (rm ANOVA) was used to test whether the CARs at the different sound levels and current levels varied between the training sessions.

3. Results

After deafening all animals demonstrated threshold shifts with more than 60 dB for click-evoked aABRs. This severe hearing loss was microscopically confirmed. All inner hair cells and outer hair cells were lost at all cochlear locations (except a few inner hair cells in the right cochlea of one control and one experimental animal). SGC packing density decreased progressively, with more than 50% loss as compared to SGC packing densities in normal-hearing guinea pigs (see the Results section of Chapter 3). This progressive degeneration had been previously reported in our group by Versnel et al. (2007) and Agterberg et al. (2008).

Figure 2 shows eABR recordings four weeks after deafening (8 weeks after implantation). These representative recordings indicate that the lowest stimulus level in the behavioral task, a current of 142 µA, was above threshold.

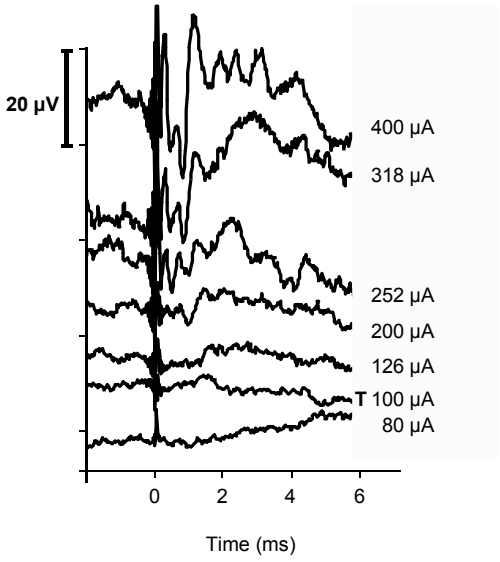

Fig. 2. Representative example of eABR-recordings, recorded 8 weeks after implantation and evoked with current pulses of 400 µA down to below threshold. The eABR example is of a deafened guinea pig (*okt2*) treated with CES. T indicates the eABR threshold.

3.1 Responses to acoustic stimuli

Figure 3 shows the mean percentage of aCARs. During the first 5 training sessions, in which the stimulus had a sound level of 78 dB SPL, the percentage of CARs increased significantly over time (rm ANOVA; F = 17.1, df = 4, p<0.001). The data show that there is an obvious increase in the percentage of CARs in session 4 as compared to the score in session 3. During training sessions 6-10 the sound pressure level varied randomly between 58, 68, 78 and 88 dB, and the percentage aCARs increased with sound level (rm ANOVA; F = 60.9, df = 3, 27, p<0.001). After session 10 the animals were not trained for a period of 4 weeks, during which time they recovered from the implantation (see Fig. 1). After this break, there was no extinction of the animal's performance. At session 11 and 12 their scores were as high as at session 10 (not shown). This also indicates that the performance was not negatively affected

by implantation of the electrode array. After deafening, their performance dropped to a level not different from spontaneous activity. Thus, as expected, they did not perceive the noise stimulus.

The number of trials scored as “no response” was zero, which means that the animals always responded with an escape to the other compartment when they were exposed to the stream of air.

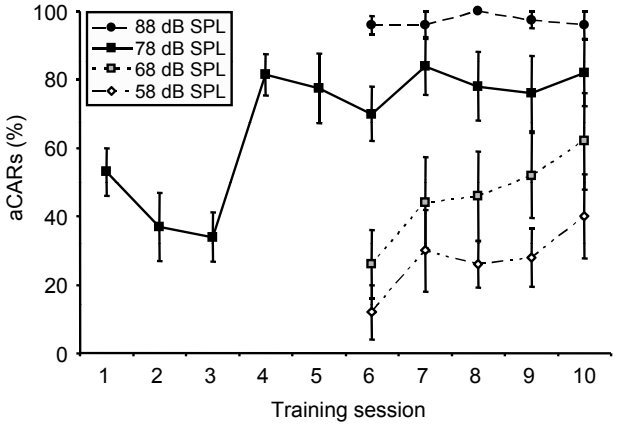

Fig. 3. Mean (± SEM) percentage of aCARs of guinea pigs (n=10) during the first ten training sessions. The acoustic noise stimulus was presented at 78 dB SPL during sessions 1-5, and at levels of 58, 68, 78, and 88 dB SPL at training sessions 6-10.

3.2 Psychophysical detection of eCARs

Figure 4 demonstrates that deafened animals were able to behaviorally respond to electric pulse trains delivered with the cochlear implant. The percentages of eCARs at 400 µA were high (80% for CES and 100% for noCES) immediately at the first session (session 15) with electrical stimulation. As with the response to acoustic stimuli (Fig. 3), the response to lower current levels was lower. ANOVA on the data presented in Fig.4 revealed the following. The performance significantly increased with training sessions ($F = 4.3$, $df = 4, 32$, $p<0.01$), and the increase of eCARs with current level was highly significant ($F = 32.8$, $df = 3, 24$, $p<0.001$). There was no significant difference in performance between the CES treated and untreated animals.

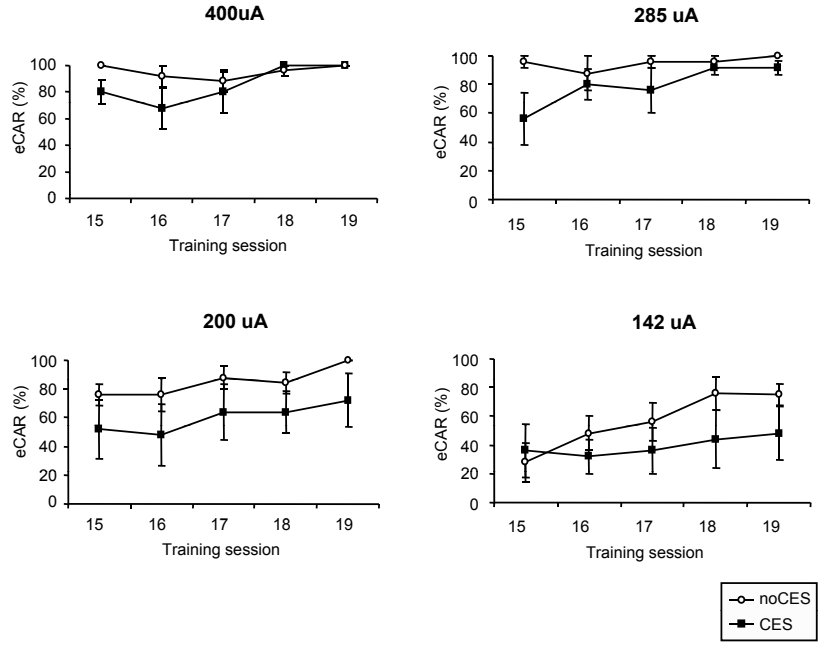

Fig. 4. Mean (± SEM) percentage of eCARs for both groups of animals (noCES, n=5; CES, n=5), for the first five training sessions to electrical pulse trains (111 pulses/s). Each plot shows the eCARs at a specific current level.

Figure 5 shows the mean percentage of eCARs during an additional session in which also two lower current levels (71, 100 μA) were tested. The negative reinforcement stimulus was turned off because these current levels were below eABR threshold for most animals (see the eABR recordings in Fig. 2). Indeed, eCAR-scores were very low at these current levels and similar to those due to spontaneous activity in the shuttle box.

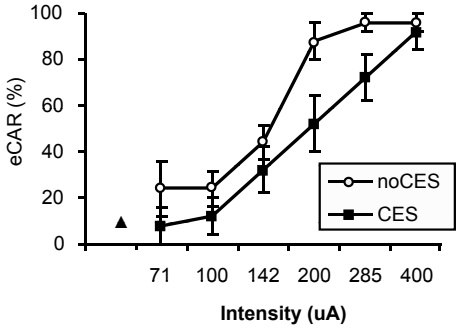

Fig. 5. Mean (± SEM) percentage of eCARs for both groups of animals (noCES, n=5; CES, n=5) during session 20, in which the stream of air was turned off. ▲: represents the percentage of spontaneous responses during the trials at 71 and 100 μA derived from the inter-trial shuttle events.

3.3 Latencies

Figure 6 shows the mean response latency for each sound level (A) and for each current level (B). The shuttle responses were relatively slow (2-7 s) both for acoustic and electric stimuli. Often the shuttle responses were preceded by head and/or body movements that could occur within a second after stimulus onset possibly indicating awareness of the stimulus. The latencies increased significantly with decreasing sound level (rm ANOVA; F = 51.1, df = 3, 21, p<0.001) and current level (rm ANOVA; F = 21.8, df = 3, 27, p<0.001).

The mean latency of the escape response elicited with the stream of air was 0.6 s in hearing condition and 0.9 s in the deafened condition.

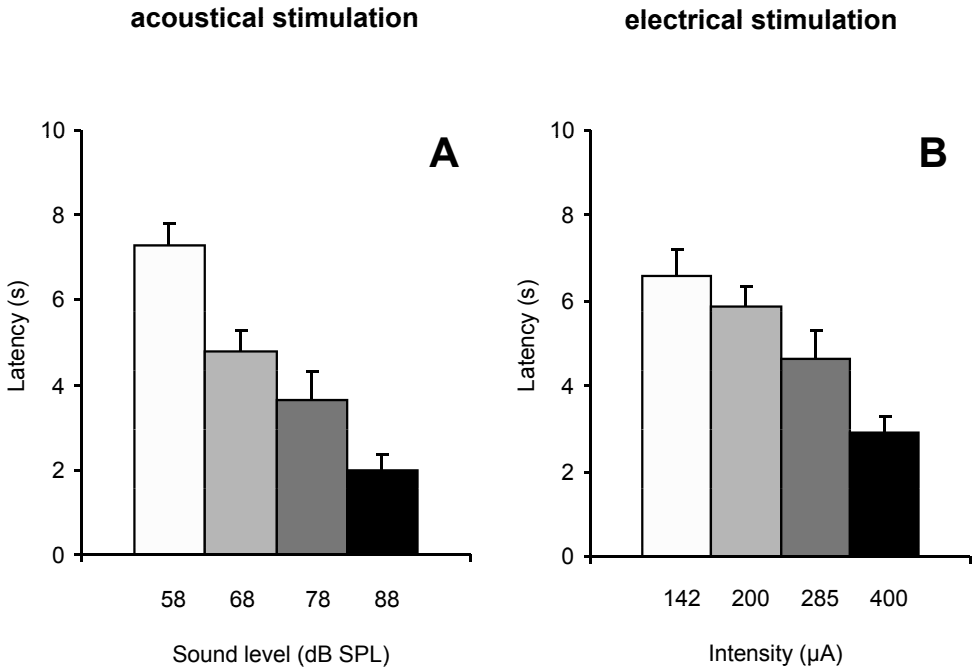

Fig. 6. Mean (+ SEM) latencies of the aCARs (A) and eCARs (B) for each sound level and current level. The latencies were averaged across five training sessions (6-10 for A; 15-19 for B), and then averaged across animals.

4. Discussion

4.1. Psychophysical detection of the acoustic and electric stimulus

In the present study we examined the psychophysical detection of electric pulse trains provided with a cochlear implant in deafened guinea pigs. After the animals had learned to respond to narrow-band noise of different sound levels (58-88 dB SPL), the animals learned rapidly, within 5 training sessions of roughly 10 minutes, to avoid an unpleasant stream of air when the conditioned stimuli were electric pulse trains of different current levels.

The consistent high response rate (> 80%) on the current level of 400 μA (Fig. 4) was probably the result of the acoustic training. An acoustic stimulus of a high sound level (78-88 dB SPL) produced a robust high behavioral response rate (80-100%). Assuming that strong electrical stimulation of the cochlea results in strong activation of the auditory nerve and subsequently to an intense auditory sensation, a high response rate to the electrical stimulus would be expected indeed. There was a long interval between the last acoustic training session and the first electrical training session (5 weeks, see Fig. 1). We assume that the animals were still able to perform the task because the earlier long interval of several weeks without training between two subsequent acoustic sessions (session 10 and 11, see Fig. 1), did not worsen the behavioral performance.

It is not very likely that the intense acoustic and electric stimuli evoked an aversive reaction since the responses to these stimuli (2-3 s) were substantially slower than the escape response elicited by the stream of air (0.9 s).

A point of concern is the whisker responses which were sometimes seen at high current levels (400 μA). These might indicate spread of the electrical stimulus to areas beyond the cochlea and imply that we can not exclude that other sensory modalities play a role in the detection of the stimulus. An argument against this proposition is that the animals responded to currents that did not evoke whisker activity (200 μA). Finally, it should be noted that strong acoustic stimuli can also lead to whisker activity (Agterberg, unpublished observations).

Whereas we suggest that the acoustic training facilitated the behavioral detection of an electrical stimulus, Kadner and Scheich (2000) reported that acoustic training negatively affected an electrical discrimination task. They showed that over a 6-day training period, gerbils with previous acoustic training experience achieved no significant discrimination performance

while animals without previous acoustic training demonstrated an increased discrimination performance. The disagreement between their work and ours might be related to the fact that we performed a detection task while Kadner and Scheich (2000) performed a discrimination task where the animals learned to respond to a specific stimulus and ignore others.

4.2 Implications

In chapter 3 of this thesis we showed that CES did not affect the SGC packing density and the functionality of the SGCs. For this reason it is not surprising that CES did not affect the behavioral performance either. Still, the present results indicate that the proposed psychophysical task can be used to assess the effect of post-deafening treatments, like CES and intracochlear infusion of neurotrophins. As presented in Chapter 5 of this thesis the amplitude of the eABR response is higher in animals with more SGCs, e.g., in normal-hearing animals and in animals treated with BDNF (see Fig. 8 in Chapter 5). A question of interest (which could not be answered in the current study), is whether in these animals the ability to behaviorally detect electrical stimulation of the cochlea would be better than in animals with significant SGC degeneration.

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Summary, discussion and concluding remarks

8

8 Summary, discussion and concluding remarks

8.1 Cochlear implants

When the first profoundly hearing impaired people received cochlear implants in the late 1970s, many scientists and surgeons were skeptical that it would be possible to help these people to understand speech with a small number of electrodes implanted in the cochlea (Clark, 2003). Furthermore, many thought that the risks of the surgery would be too high. To date, the cochlear implant is the most successful sensorineural prosthesis. More than 110,000 cochlear implants have been implanted worldwide (Fallon et al., 2007), and many patients received great benefits from these devices (Fu and Galvin III, 2008). Some people with an implant are even able to use the telephone. Unfortunately, others hardly benefit from their cochlear implant. The large variability in the performance of cochlear implant recipients is an important motive for the research described in this thesis. A factor explaining the variability in the perceptual performance might be the degeneration of the auditory nerve that often occurs following the destruction of hair cells (Spoendlin, 1975; Webster and Webster, 1981; Leake and Hradek, 1988; Nadol, 1997; Shepherd et al., 2001). The principal aim of this thesis was to investigate the time course of degeneration of the auditory nerve in an animal model for deafness, and to explore strategies that can prevent this degeneration.

8.2 Summary of this thesis

Chapter 1 introduces the cochlear implant and gives an overview of this thesis. Furthermore, it introduces the auditory nerve and the objective of this thesis. The objective of this thesis is the characterization of effects of neurotrophic treatments that may have the potential to prevent or reduce the degeneration of SGCs.

The aim of the study described in **Chapter 2** was to investigate the loss of cochlear hair cells and the secondary degeneration of spiral ganglion cells (SGCs) in a guinea pig model for deafness. Guinea pigs were deafened with a subcutaneous injection of kanamycin followed by slow intravenous infusion of furosemide as a loop diuretic. Deafening by this procedure resulted in severe hair cell loss across the entire cochlea, corresponding hearing loss of >55 dB for clicks, and a progressive degeneration of SGCs. Eight weeks after deafening more than 60% of the SGCs were lost. The ototoxic treatment was appropriate as a deafness model and was used in the experiments that are described in the subsequent chapters. The time course of SGC degeneration demonstrated that degeneration had set in two weeks after

deafening. Because in patients degeneration of the auditory nerve is probably started before they receive their cochlear implant, we decided to start the treatment with neurotrophins in our animal model two weeks after deafening.

Chapter 3 describes an experiment in which the effect of chronic electrical stimulation (CES) on SGC survival was evaluated. We applied amplitude modulated, high pulse rate electrical stimulation to the cochlea of deafened guinea pigs. CES in this form did not affect the SGC density, morphology or functionality. This result indicates that frequent depolarization of SGCs as a result of electrical stimulation, does not automatically prevent degeneration of SGCs. This is not surprising since previous animal studies demonstrated that it is not a consistent finding across laboratories that CES affected the survival of SGCs (Lousteau et al., 1987; Li et al., 1999; Miller, 2001; Shepherd et al., 2005). A secondary finding in this study is that we found small but significant alterations in the morphology of the SGCs in the implanted (right) ears compared to their contralateral nonimplanted (left) ears. The cells were larger on the implanted side. However, we found these alterations in both groups of implanted ears, the ones who received CES and the ones which did not. We suggest that these alterations are caused by the electrical stimulation in these ears which was applied to record eABRs and to register behavioral responses.

The aim of the experiment described in **Chapter 4** was to investigate the effect of continuous intracochlear infusion of brain derived neurotrophic factor (BDNF) through an osmotic pump and cannula. The BDNF treatment was started two weeks after deafening, when degeneration of SGCs had set in. The SGC packing densities in the BDNF-treated cochleae were significantly larger than those in untreated cochleae. In concert with others (Gillespie et al., 2004; Yamagata et al., 2004; McGuinness and Shepherd, 2005), this experiment demonstrated that delayed intracochlear infusion of BDNF is effective in preventing SGCs from degeneration after ototoxic deafening. However, there were some morphological differences between BDNF-treated SGCs as compared to SGCs in cochleae of normal-hearing guinea pigs. BDNF-treated SGCs were larger and furthermore, the thickness of their myelin sheath was reduced, i.e., the treatment did not prevent demyelinisation of the SGCs. It might be that these differences reflect a suboptimal condition of the cells. In this case BDNF treatment prevented degeneration of SGCs but the rescued SGCs are not as healthy as in cochleae of normal-hearing guinea pigs. An unhealthy state of the SGCs could lead to a rapid degeneration of SGCs after cessation of BDNF treatment, as reported by Gillespie et al. (2003). Their data are shown in Fig. 1A. The effects of cessation of BDNF treatment is addressed in the next chapter.

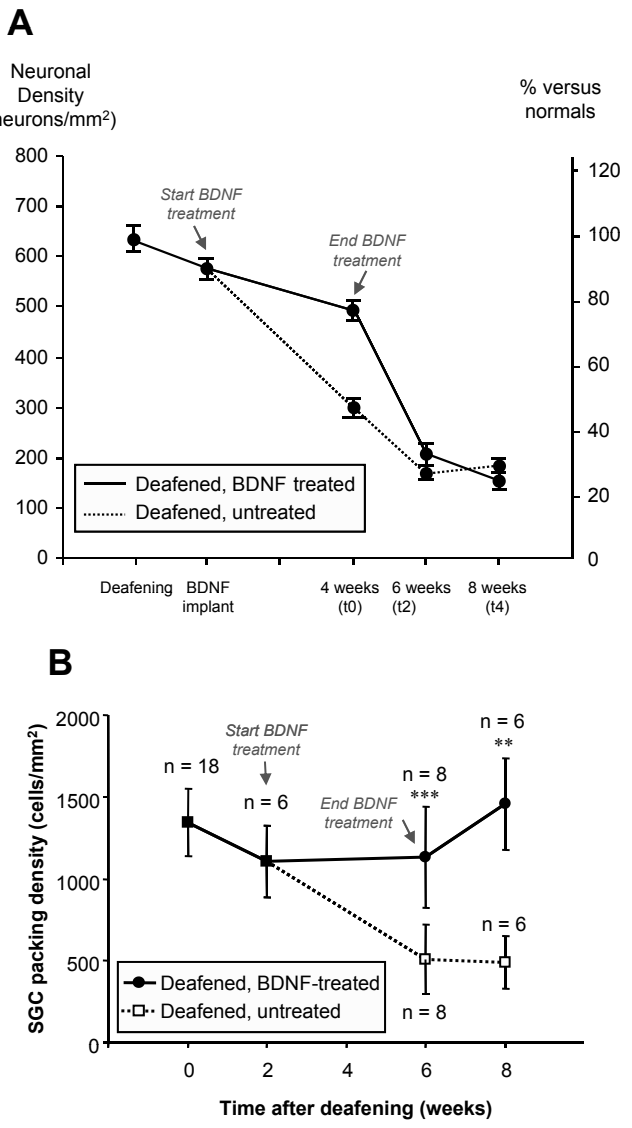

Fig. 1. The longevity of BDNF survival effects in (A) an experiment performed by Gillespie et al. (2003), and (B) the experiment presented in Chapter 5. (A) The survival promoting effects of BDNF on SGCs following deafening are rapidly lost following cessation of the treatment. The results are expressed as average density of auditory neurons per mid-modiolar section. Only neurons with a visible nucleus and nucleolus were counted and used in the determination of the neuronal density. Printed with permission of the first author. Error bars: SEM. *n* = 5 for each treatment group. (B) Maintained survival of SGCs after cessation of the BDNF treatment as presented in Chapter 5. The results are expressed as average SGC density in cochlear locations B1, B2 and M1 (see Fig. 2 in Chapter 1). All cells demonstrating morphological determinants typical of type-I SGCs, and not only cells with a visible nucleolus, were used in the determination of the SGC packing density, which explains the difference in cell count range between the two studies. Error bars: standard deviation. ** *p*< 0.01, * *p*< 0.001.**

Chapter 5 describes an experiment that investigated the survival and functionality of SGCs after cessation of BDNF treatment. The neurotrophin was again, as in Chapter 4, infused by means of an osmotic pump and a cannula. In contrast to the results reported by Gillespie et al. (2003) we demonstrated that the protective effect lasted at least two weeks after cessation of the neurotrophic treatment (Fig. 1). In this experiment we assessed the functionality of the SGCs by recording of the electrically evoked auditory brainstem responses (eABRs). The amplitudes of these responses in BDNF-treated animals were comparable to those in normal-hearing animals, indicating that the SGCs remained functional after cessation of the treatment. We propose that the most likely explanation for the contradictory results between our study and the study performed by Gillespie et al. (2003), is that we provided trophic support of the SGCs during the recordings of the eABRs, which we recorded throughout the study, while Gillespie et al. (2003) did not. Possibly, brief electrical stimulation applied during the recordings of the eABRs, in combination with neurotrophic treatment had a different effect than neurotrophic treatment only.

The aim of the study described in **Chapter 6** was to investigate the survival of SGCs in deafened guinea pigs after placement on the round window of an absorbable gelatin sponge soaked in a solution of BDNF. This route of delivery was explored because the cannula that was used in the experiments described in Chapters 4 and 5, creates an open connection between the subcutaneous pump and the cochlea, which bypasses the blood brain barrier and creates a route of access for bacteria. The repeated invasive manipulations that are necessary to replace or refill the osmotic pump would result in a high risk of infections in the clinical setting. We demonstrated that when BDNF was delivered on the round window with an absorbable gelatin sponge, the rate of degeneration of SGCs in the basal turn of the cochlea was reduced (Fig. 2). Our results demonstrate that it is possible to deliver the neurotrophins to the cochlea of guinea pigs through the round window membrane. This result is promising for cochlear implant candidates.

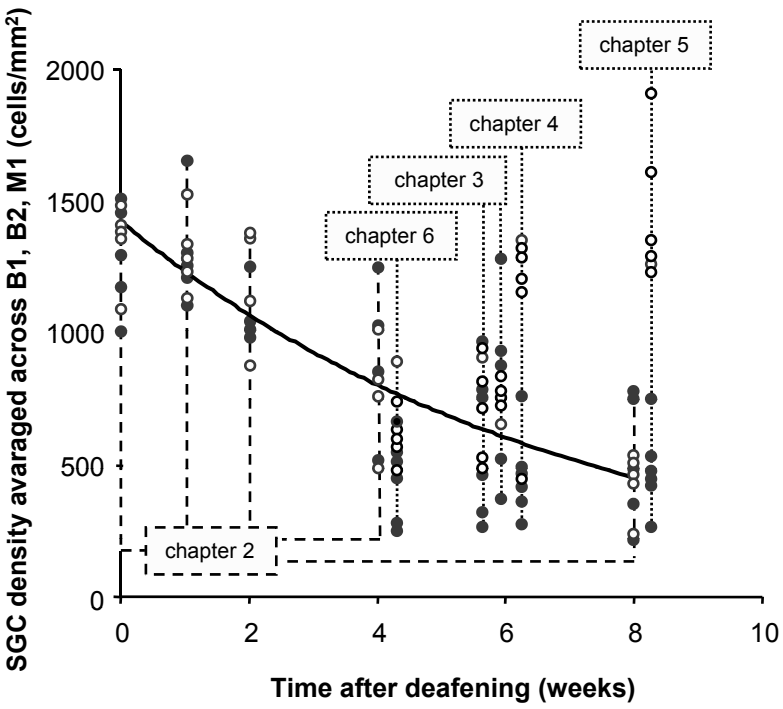

Fig. 2. SGC densities in all cochleae of the animals included in the studies that are presented in this thesis. The text-boxes indicate in which chapter the data are presented. SGC densities are averaged across cochlear locations B1, B2 and M1 (basal part of cochlea, see Fig. 2 in Chapter 1). The SGC packing densities in the left (untreated) cochleae (●) and right (BDNF treated) cochleae (○) are indicated.

The aim of **Chapter 7** was to develop a behavioral model that can be used to investigate whether deafened guinea pigs experience an auditory sensation when electric pulse trains are provided with a cochlear implant. Such a model might be helpful to demonstrate the relevance of preserving SGCs from degeneration. We used a modified shuttle-box, a box in which guinea pigs can shuttle from one compartment to the other in order to avoid a punishment. In this modified shuttle-box, developed by Philippens et al. (1992), a less stressful unconditioned stimulus, in the form of a stream of air, was used instead of foot-shocks. The obtained results demonstrated that deafened guinea pigs were able to detect and discriminate such electric pulse trains. This model might be useful to examine whether more SGCs will result in a better performance with a cochlear implant.

The results, with respect to SGC survival, obtained in the experiments that are described in the various chapters of this thesis are summarized in Figure 2. The presented SGC packing densities were determined by averaging the SGC packing densities in cochlear locations B1, B2 and M1. This Figure clearly demonstrates:

(1) Progressive degeneration of SGCs after deafening (Chapter 2-6).

(2) That CES was not successful in preventing SGCs from degeneration (Chapter 3).

(3) Enhanced survival of SGCs after continuous BDNF-infusion into the cochlea (Chapter 4).

(4) No degeneration two weeks after cessation of the BDNF treatment (Chapter 5).

(5) Enhanced survival of SGCs when BDNF was delivered on the round window with absorbable gelatin sponge (Chapter 6).

8.3 Discussion

8.3.1 Clinical relevance of neurotrophic treatment

There are many topics that need to be further explored before neurotrophic treatment of the auditory nerve will become a clinical reality. An important assumption that motivates the experiments described in this thesis is that when there are more and better functioning SGCs, this would result in a better performance of cochlear implant users. In other words, that cochlear implant recipients with many SGCs hear better than those who have a small number of SGCs. Clearly, complete disappearance of all SGCs will make electrical stimulation in the cochlea useless. Alternatively to the hypothesis that more SGCs result in a better performance with a cochlear implant, it might be that a minimum number of SGCs is required for electrical hearing, and that an increase above this minimum would have little functional impact (Blamey, 1997; Miller, 2001). When this minimum number of SGCs is present because the human auditory nerve does not degenerate completely following deafness (Nadol et al., 2006; Glueckert et al., 2008), protection of SGCs against degeneration, and thus the intracochlear delivery of neurotrophins, would be irrelevant. However, Chikar et al. (2008) reported that in animals, compared to a control group, a BDNF-treated group had lower psychophysical thresholds as well as higher survival of SGCs. Their study is the first animal study presenting promising psychophysical measurements in BDNF-treated animals because these psychophysical thresholds are functional measures of cochlear implant performance. Still, the authors, and others (Leake et al., 2008; Shepherd et al., 2008), mentioned that it is believed instead of

proven, that more SGCs will result in a better performance, and they highlighted the lack of behavioral animal studies supporting this idea (Chikar et al., 2008). Furthermore, there is no evidence available from cadaveric temporal bones of human subjects who underwent cochlear implantation during life, that more SGCs result in a better speech performance (Nadol et al., 2001; Khan et al., 2005; Fayad and Linthicum, 2006; Nadol and Eddington, 2006), emphasizing the importance of abnormalities in the central auditory pathways and brain plasticity in clinical performance (Nadol et al., 2001; Shepherd et al., 2008). However, it is unlikely that SGC cell loss does not affect the performance with a cochlear implant. We, and others, think that the human data sets are still too limited to conclude that the extent of SGC survival does not influence cochlear implant performance (Khan et al., 2005; Leake et al., 2008).

Even when enhanced survival of SGCs turns out to be not important for users of the current types of cochlear implants, it might be that it will become important when the cochlear implant technology is further improved (Green et al., 2008).

8.3.2 Generalization of the reported results from rodents to humans

An important question is whether it is possible to generalize the reported protective effects of BDNF treatment found in guinea pigs (Ernfors et al., 1996; Gillespie et al., 2004; Yamagata et al., 2004; Richardson et al., 2005; Wise et al., 2005; Miller et al., 1997, 2007; Agterberg et al., 2008) to other species. Recently, it has been demonstrated that BDNF treatment is successful in preventing degeneration of SGCs in another species than the guinea pig, namely in the rat (McGuinness and Shepherd, 2005; Song et al., 2008). However, there are differences between SGCs in rodents (mice, rats, guinea pigs) and SGCs in humans. For example, in humans 94% of the SGC population lacks a myelin sheath around the perikaryon (Ota and Kimura, 1980; Glueckert et al., 2005), while in guinea pigs the myelin sheath contains 12-16 layers (Glueckert et al., 2008). Furthermore, SGC degeneration in humans without inner hair cells is slower than in rodents (Leake and Rebscher, 2004).

8.3.3 Safety of BDNF infusion into the cochlea

One goal in current research is the development of a method for the clinically safe application of neurotrophins. The experiments described in this thesis demonstrate improved survival and morphology of SGCs after continuous infusion of neurotrophins with a cannula connected

to an osmotic pump. This method has been proven to be successful in several studies with guinea pigs (for an overview, see Gillespie and Shepherd, 2005). Other methods, like cells and viruses releasing or evoking the release of neurotrophins in the scala tympani (Staecker et al., 1998; Rejali et al., 2007) or a hydrogel releasing neurotrophins via the round window (Ito et al., 2005; Noushi et al., 2005), including the placement of an absorbable gelatine sponge soaked in BDNF on the round window (Chapter 6), may decrease the risk of infections. Still, a point of concern here is that the human round window membrane is thicker than the membrane in guinea pigs, which might decrease effectiveness (Richardson et al., 2006).

8.3.4 Effects of chronic electrical stimulation

The effects of chronic electrical stimulation (CES) without neurotrophic treatment are variable across animal studies. Some studies reported an effect on SGC density (Lousteau et al., 1987; Hartshorn et al., 1991; Leake et al., 1991; Scheper et al., 2008) while other studies reported no effect of CES on SGC density (Li et al., 1999; Shepherd et al., 2005; Chapter 3). Surprisingly, Miller and Altschuler (1995) and Mitchell et al. (1997) reported a preventive effect, i.e. a reduction of SGC degeneration, after brief electrical stimulation during eABR recordings. In human temporal bones of people who used a multichannel cochlear implant during life, SGC survival did not differ between implanted and non-implanted ears (Fayad and Linthicum, 2006), indicating that CES might not affect the SGC density in human cochlear implant users. Several animal studies reported an effect of CES on the morphology of the remaining SGCs (Araki et al., 1998; Coco et al., 2007). In accordance with these animal studies (Araki et al., 1998; Coco et al., 2007), we demonstrate in Chapter 3 that it was probably the brief electrical stimulation during the eABR recordings that affected the size and circularity of SGCs. Considering the apparent contrasting findings, it is not clear which attributes of electrical stimulation are important for SGC survival.

8.4 Concluding remarks

The benefits of cochlear implantation for profoundly and severely hearing impaired people can be enormous. The criteria for candidacy for implantation have been relaxed over the years, and patients with considerable residual low-frequency hearing increasingly become eligible for implantation (Gantz et al., 2005). However, implanted people are still limited in their hearing. These limitations may be related to the SGC survival and alterations in the rest of the auditory

system. There is still a lot we do not know about neurobiological mechanisms underlying the survival of SGCs after deafening and the mechanisms underlying the protective effects of intracochlear electrical stimulation and application of neurotrophic factors. For these reasons it remains important to investigate these issues. With the knowledge to be gained and with the expected continuous technical improvements of cochlear implants, better hearing for implant recipients might be feasible. Not only in relation to their speech perception, but also in their ability to enjoy music and communicate in situations with background noise.

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Samenvatting

Aan het eind van de vorige eeuw zijn de eerste studies uitgevoerd die aantonen dat infusie van groeistoffen in het slakkenhuis van doofgemaakte cavia's voorkomt dat de levensvatbaarheid van de gehoorzenuw achteruitgaat. Verschillende groeistoffen, die neurotrofe factoren worden genoemd, zijn effectief en cocktails van groeistoffen zijn in sommige gevallen nog effectiever gebleken. Het onderzoek naar behoud van de gehoorzenuw is ingegeven door de hypothese dat meer en beter functionerende zenuwcellen in de gehoorzenuw resulteren in een betere waarneming van geluid door dove mensen met een implantaat in het slakkenhuis (de cochlea). Een dergelijk implantaat wordt een cochleair implantaat genoemd. Dove mensen met een cochleair implantaat kunnen horen door directe elektrische stimulatie van de zenuwcellen in de gehoorzenuw, waarbij de beschadigde haarcellen worden overgeslagen. De zenuwcellen in de gehoorzenuw worden spirale ganglion cellen genoemd (SGC). De hypothese dat meer en beter functionerende SGC resulteren in een betere waarneming van geluid, is onder andere gebaseerd op het logische feit dat bij afwezigheid van de gehoorzenuw een cochleair implantaat niet kan functioneren. Immers de zenuw kan in dat geval geen informatie in de vorm van actiepotentialen naar de hersenen overbrengen.

Voordat onderzocht werd of de afname van het aantal SGC voorkomen kon worden door toediening van neurotrofe factoren was al bekend dat de SGC in dove cavia's behouden konden worden door chronische elektrische stimulatie met een cochleair implantaat. Door deze elektrische stimulatie genereren de SGC, die geen input meer ontvangen omdat de haarcellen verdwenen zijn, actiepotentialen. De SGC zijn dus gedurende de periode van elektrische stimulatie actief. Je zou dit kunnen beschouwen als het 'use it or lose it' principe. De effecten van chronische elektrische stimulatie in proefdiermodellen zijn echter niet onomstotelijk. Er zijn namelijk ook onderzoekers die geen effect vinden van chronische elektrische stimulatie terwijl de onderzoeken naar de effecten van toediening van neurotrofe factoren zonder uitzondering wel effectief zijn.

In dit proefschrift wordt voortgebouwd op het onderzoek naar het effect van neurotrofe behandeling op de gehoorzenuw in doofgemaakte cavia's zoals hierboven kort is omschreven. De effecten van neurotrofe behandeling, waaronder wordt verstaan chronische elektrische stimulatie alsmede toediening van neurotrofe factoren, worden gekarakteriseerd met behulp van morfologie, elektrofysiologie en gedrag. De drie belangrijkste parameters

die in dit proefschrift worden beschreven zijn de dichtheid van de SGC (een morfologische parameter), de amplitude van de elektrische opgewekte auditieve hersenstam respons (een elektrofysiologische parameter) en de detectie van akoestische of elektrische stimulatie van de cochlea (een gedrag respons).

Hoofdstuk 2 beschrijft een diermodel voor doofheid. Het doel is om vast te stellen wat de effecten zijn van onderhuidse toediening van een antibioticum (kanamycine) gevolgd door de injectie in een bloedvat van een urineproductie bevorderend middel (furosemide), op de haarcellen in de cochlea en op de dichtheid van de SGC. Een belangrijke bevinding is dat de gehoormeting één dag na doofmaken via deze procedure indicatief is voor het uiteindelijke gehoorverlies. Verder wordt in dit experiment vastgesteld dat het aantal SGC progressief afneemt nadat cavia's zijn doofgemaakt. Acht weken na doofmaken is 60% van de SGC gedegenereerd, en hebben de dieren een ernstig gehoorverlies van meer dan 55 decibel. We concluderen dat het bestudeerde model geschikt is om dieren doof te maken en dit model wordt daarom in de overige experimenten gebruikt.

In **hoofdstuk 3** wordt een experiment beschreven waarin het effect bestudeerd wordt van chronische elektrische stimulatie op de grootte, de vorm en de dichtheid van SGC. In 1986 werd voor de eerste keer aangetoond dat chronische elektrische stimulatie de degeneratie van SGC in de cochlea van dove cavia's kan reduceren. Wij vinden in tegenstelling tot deze eerste studie dat chronische elektrische stimulatie de degeneratie van SGC na doofmaken niet reduceert. We zijn echter niet de enige die geen effect vinden van elektrische stimulatie op de dichtheid van SGC. In hoofdstuk 3 gaan we in op de mogelijke oorzaken van de verschillen tussen de onderzoeksresultaten. Verder beschrijven we dat in geïmplanteerde en elektrisch gestimuleerde cochlea's de SGC groter zijn dan in ongeïmplanteerde cochlea's. We concluderen dat dit effect een gevolg is van de elektrische stimulatie van de SGC.

Hoofdstuk 4 beschrijft de effecten van toediening van brain-derived neurotrophic factor (BDNF) op de SGC dichtheid en morfologie van SGC. Doel van dit onderzoek is om vast te stellen of behandeling met BDNF effectief is ter voorkoming van de degeneratie van SGC en om te bepalen of de behandelde SGC morfologische verschillen vertonen wanneer ze vergeleken worden met SGC in cochlea's van normaal horende dieren. Omdat het waarschijnlijk is dat bij

patiënten de degeneratie van de gehoorzenuw al is begonnen op het moment van implantatie, starten wij onze neurotrofe behandeling twee weken na doofmaken. Na twee weken zijn bij de cavia ongeveer 15% van de SGC gedegenereerd. BDNF wordt locaal toegediend met behulp van een onder de huid geplaatst osmotisch pompje. Via een canule wordt de BDNF oplossing gedurende 4 weken geleidelijk (0.25 µl/uur) in de cochlea geïnfundeerd. In overeenstemming met de literatuur vinden we dat de degeneratie na doofmaken verminderd wordt door toediening van BDNF. De morfologie van de SGC behandeld met BDNF vertoont verschillen met de morfologie van SGC in de cochlea's van normaal horende cavia's. SGC die behandeld zijn met BDNF zijn groter en het aantal myeline lagen in de celmembraan is afgenomen. Deze morfologische veranderingen kondigen mogelijk het begin aan van het degeneratieve proces dat volgens eerdere onderzoeken plaatsvindt nadat de behandeling met BDNF wordt stopgezet. De effecten van het stopzetten van de BDNF behandeling worden beschreven in het volgende hoofdstuk.

In **hoofdstuk 5** onderzoeken we veranderingen in de grootte, de vorm en de dichtheid van de SGC na het stopzetten van de behandeling met BDNF. Daarnaast beschrijven we in dit hoofdstuk de functionaliteit van SGC na het stopzetten van de behandeling. Doel van dit onderzoek is om vast te stellen of de effecten van BDNF behandeling, zoals die zijn beschreven in hoofdstuk 4, behouden blijven of verloren gaan nadat de behandeling is stopgezet. De functionaliteit wordt geschat aan de hand van de amplitude van de elektrisch opgewekte auditieve hersenstamresponsie (eABR). De amplitude van de eABR geeft een indicatie van het aantal SGC dat een actiepotentiaal kan genereren. In dit hoofdstuk wordt beschreven dat twee weken na stopzetten van de behandeling de dichtheid van SGC in behandelde cochlea's groter is dan in de onbehandelde cochlea's. Dit is in tegenstelling tot de resultaten van een eerdere studie gepubliceerd in 2003. In deze publicatie wordt beschreven dat het stopzetten van de behandeling met BDNF resulteert in een zeer snelle degeneratie (in twee weken) van de SGC. In deze publicatie wordt dus beschreven dat de degeneratie van SGC na stopzetten van de behandeling sneller verloopt dan de degeneratie van SGC na doofmaken, en dat het effect van de BDNF behandeling twee weken na stopzetten al volledig verdwenen is. In hoofdstuk 5 wordt ingegaan op een aantal verschillen tussen onze studie en andere studies die de conflicterende onderzoeksresultaten zouden kunnen verklaren. De meest waarschijnlijke verklaring voor de conflicterende onderzoeksresultaten zijn de metingen van

de elektrische opgewekte eABR, die in onze studie wel zijn gemeten maar in sommige andere studies niet. Onze dieren zijn dus kort, minstens ongeveer 15 minuten per week, elektrisch gestimuleerd gedurende het experiment. Naast de conclusie dat SGC na stopzetten van de BDNF behandeling niet degenereren, in ieder geval niet binnen de eerste twee weken na beëindigen van de BDNF behandeling, concluderen we dat de functionaliteit van de SGC, geschat aan de hand van de amplitude van de eABR, niet afneemt.

Hoofdstuk 6 beschrijft een 'pilot' studie waarin we aantonen dat de degeneratie van SGC ook voorkomen kan worden door een absorbeerbaar gelatine sponsje gedrenkt in BDNF te plaatsen op het ronde venster van de cochlea. Doel van dit onderzoek is om een methode te ontwikkelen waarmee neurotrofe factoren eenvoudiger, en met minder risico op infecties, kunnen worden toegediend. Het effect van deze manier van toediening op de dichtheid van de SGC is in ons proefdiermodel kleiner dan het effect van continue intracochleaire toediening van BDNF met behulp van een osmotisch pompje (hoofdstuk 4). In de basale winding van de cochlea is dit effect desalniettemin statistisch significant. Deze methode van BDNF toediening geeft minder risico's op infecties omdat de cochlea niet geopend hoeft te worden en omdat er geen blijvende verbinding is tussen de cochlea en het osmotisch pompje. Wanneer BDNF namelijk toegediend wordt met behulp van een osmotisch pompje, loopt er een canule vanaf het pompje onder de huid naar de cochlea. Via deze canule kan een onderhuidse infectie zich eenvoudig uitbreiden naar de intracochleaire ruimte. De bloed-hersen barrière wordt omzeild en dit brengt waarschijnlijk een onacceptabel hoog risico met zich mee. We concluderen dat de toediening van neurotrofe factoren met behulp van een absorbeerbaar gelatine sponsje een bruikbare en veilige methode is.

In de eerste alinea van deze samenvatting is de hypothese geformuleerd dat meer SGC resulteren in een betere prestatie van de gebruiker van een cochleair implantaat. Hoe logisch deze hypothese ook lijkt, hij wordt niet ondersteund door klinische studies. Post mortem onderzoek wijst uit dat geluidwaarneming met een cochleair implantaat niet beter is in de mensen met meer SGC. Voordat behandeling met neurotrofe factoren in de kliniek overwogen zal worden moet onomstotelijk worden vastgesteld dat meer SGC daadwerkelijk resulteren in een betere waarneming met een cochleair implantaat. Wij hebben een gedragtest ontwikkeld waarmee in de toekomst deze hypothese getoetst kan worden. Deze test wordt beschreven in hoofdstuk 7.

In **hoofdstuk 7** wordt beschreven dat cavia's een psychofysische test snel kunnen aanleren. De dieren leren in eerste instantie te reageren op een auditieve stimulus. De reactie is de verplaatsing naar het andere compartiment van een speciaal voor dit onderzoek aangepaste shuttle-box. De shuttle-box is een kooi met twee compartimenten waarin proefdieren een beloning kunnen verkrijgen of een straf kunnen ontlopen door zich te verplaatsen naar het andere compartiment. We tonen met dit experiment aan dat de auditieve stimulatie wordt waargenomen.

In de traditionele shuttle-box wordt als straf gebruik gemaakt van elektrische schokjes aan de poten. Wij hebben in navolging van een onderzoek uitgevoerd bij TNO, deze schokjes vervangen door een luchtpuf. Dit leidt tot minder stress waardoor de cavia's de taak snel kunnen aanleren. Nadat de cavia's de reactie op de auditieve stimulus hebben aangeleerd worden ze doofgemaakt. De dove cavia's leren vervolgens aan om zich te verplaatsen naar het andere compartiment wanneer de gehoorzenuw elektrisch gestimuleerd wordt met het cochleair implantaat. We tonen aan dat dove cavia's de elektrische stimulatie waarnemen en concluderen dat deze gedragstest bruikbaar is om het effect van neurotrofe behandeling van de gehoorzenuw op de detectie van elektrische stimulatie van de cochlea te onderzoeken.

In de **Discussie** van dit proefschrift worden mogelijke richtingen voor toekomstig onderzoek besproken. Een belangrijke toekomstige onderzoekslijn zou zich moeten richten op de hypothese dat meer SGC resulteren in een betere prestatie met een cochleair implantaat. Door cavia's te behandelen met neurotrofe factoren en vervolgens te testen in de shuttle-box, kan onderzocht worden of cavia's met meer SGC sneller aanleren om intracochleaire elektrische stimulatie van verschillende stroomsterkte waar te nemen. Veel onderzoekers verwachten dat doofgemaakte dieren behandeld met BDNF elektrische stimulatie sneller zullen waarnemen dan doofgemaakte onbehandelde dieren. Er zijn echter ook onderzoekers die veronderstellen dat een minimaal aantal SGC, bijvoorbeeld 10% van de SGC populatie, voldoende is voor een optimale waarneming met een cochleair implantaat. Dat bij sommige mensen de waarneming van geluid met hun cochleair implantaat tegenvalt zou veroorzaakt kunnen worden door veranderingen in het centrale auditieve systeem en niet door degeneratie van de gehoorzenuw. Zo is bijvoorbeeld gevonden dat bij kinderen die meerdere jaren doof zijn de auditieve cortex gebruikt wordt voor de verwerking van visuele informatie en hierdoor is dit hersengebied niet meer responsief voor auditieve input.

Ook wanneer uit toekomstig onderzoek zou blijken dat bij de huidige types cochleaire implantaten het aantal SGC geen invloed heeft op de waarneming van geluid dan zou door de voortdurende technische ontwikkelingen en het gebruik van een nieuwe generatie cochleaire implantaten het behoud van SGC alsnog belangrijk kunnen zijn.

Mensen met een cochleair implantaat zijn nog steeds beperkt in de waarneming van geluid. Het luisteren naar muziek en het voeren van een conversatie in een situatie met achtergrond ruis is in veel gevallen moeilijk. Het hier beschreven onderzoek vergroot het inzicht in één van de factoren die het waarnemen van geluid waarschijnlijk beperkt, namelijk de degeneratie van SGC en geeft de mogelijkheden aan om deze degeneratie te verminderen of zelfs te voorkomen.

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Fig. 1. Saskia Plomp (links), Marloes van den Broek (midden) en Leone Nijman (rechts) met in hun hand ieder een compilatie van het kanaal van Rosenthal. Normal = Spirale ganglioncellen in het kanaal van Rosenthal van een normaal horende cavia. 6wdu = Spirale ganglioncellen in het kanaal van Rosenthal van een cavia zes weken na doofmaken. 6wdBDNF = Spirale ganglioncellen in het kanaal van Rosenthal van een cavia zes weken na doofmaken, waarbij gedurende de laatste vier weken geleidelijk een BDNF oplossing in de cochlea is geïnfundeerd.

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Curriculum Vitae

Martijn Agterberg werd geboren op 8 augustus 1975 te Utrecht. Hij behaalde in 1993 in zijn geboortestad zijn HAVO diploma aan het Sint Gregorius College. In datzelfde jaar werd begonnen aan de hogere laboratorium school (HLO) te Utrecht. Hier behaalde hij in 1998 zijn diploma met als specialisatie Dierkunde. Van 1998 tot en met 2000 werkte hij als analist bij het Rudolf Magnus Instituut voor Neurowetenschappen. Tijdens deze periode behaalde hij een aantal vakken binnen de studie Psychologie aan de Open Universiteit. Hij zag een analistenloopbaan in dienst van de wetenschap niet zitten en begon in 2000 met de studie Psychologie aan de Universiteit Utrecht. In 2001 behaalde hij zijn propedeuse Psychologie en in dit eerste jaar volgde hij ook met succes een aantal vakken bij de faculteit Geneeskunde. Hij koos voor de specialisatie Neuropsychologie en was gedurende twee jaar actief als bestuurslid van Brainwave (de studievereniging Neuropsychologie).

Begin 2003 onderbrak hij zijn studie voor een werkbezoek aan de Universiteit van Californië San Francisco. Onder begeleiding van Prof. dr. M.M. Merzenich deed hij hier onderzoek naar plasticiteit van het auditieve systeem. Aansluitend aan dit werkbezoek werd hij eind 2003 aangesteld als analist bij de afdeling Keel-, Neus en Oorheelkunde (KNO) in het Universitair Medisch Centrum te Utrecht. Hier werd hem door Prof. dr. G.F. Smoorenburg een promotieplek aangeboden op voorwaarde dat hij zijn studie Psychologie zou afronden. Hij ontving zijn doctoraal diploma Psychologie in 2004 en begon met het in dit proefschrift beschreven promotieonderzoek. Na de opstartfase onder begeleiding van Prof. dr. G.F. Smoorenburg had hij het voorrecht om begeleid te worden door Prof. dr. F.W.J. Albers. Helaas is Prof. dr. Frans Albers in 2007 overleden. Prof. dr. V.M. Wiegant heeft toen het promotorschap overgenomen.

2009

Vanaf 1 maart 2009 zette hij zijn wetenschappelijke vorming voort bij de vakgroepen KNO en Biofysica in het Universitair Medisch Centrum St. Radboud te Nijmegen. Hij bekleed hier een postdoctorale positie aan het Donders Institute for Brain, Cognition and Behaviour onder begeleiding van Prof. dr. A.J. van Opstal en Prof. dr. A.F.M. Snik.