

1 ABSTRACT

Hearing impairment is one of the most common health conditions in developed countries. It is estimated that approximately 70 million people worldwide are deaf. For patients with a severe to profound hearing loss a cochlear implant (CI) is the only treatment today.

The function of a CI depends in part of the survival and electrical responsiveness of the spiral ganglion neurons (SGNs), the target of the CI. In order to access the electrical responsiveness in animal models, electrically-evoked auditory brainstem responses (eABRs) were recorded. The survival of SGN was estimated by calculating the density of the SGN in Rosenthal's canal.

Neurotrophic factors due to their neurotrophic support in the cochlea are important in the development and maintenance of the auditory system. Therefore we performed two studies with glial cell line-derived neurotrophic factor (GDNF) treatment, one acute and one with a delayed onset of GDNF treatment. Normal hearing guinea pigs were deafened intracochlear (acute) or by transtympanic injection (delayed) with neomycin. The animals received a CI that consisted of a combined electrode (for eABR measurement) and cannula (for intracochlear infusion). Animals were treated in both studies with GDNF for 4 weeks. The study with delayed onset also received a post-treatment with daily injections (i.p.) of antioxidants or saline. Control animals were deafened and received intracochlear infusion of artificial perilymph.

Nucleosides and nucleotides are known to function as neurotransmitters and neuromodulator and have recently been shown to have a neurotrophic effect on neurons. Here we tested UTP and uridine in an acute study on deafened guinea pigs. Electrical responsiveness was recorded by eABR measurements and the density of the SGN was investigated.

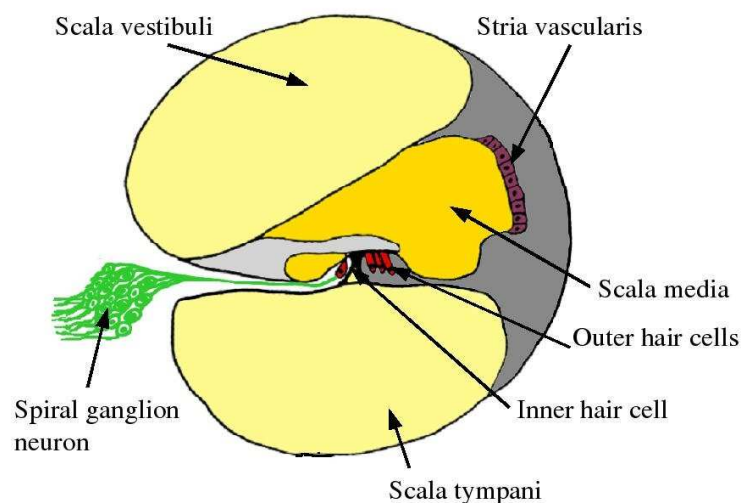
Both studies with GDNF treatment showed a significant difference in eABR thresholds ($p < 0.001$) and SGN density ($p < 0.001$) compared to the control groups. Furthermore, UTP and uridine showed similar results even if not so pronounced.

We concluded that GDNF treatment in deafened animals inhibits the degeneration of the SGN even 4 weeks after end of treatment. It is possible that after a critical time following inner ear trauma endogenous survival factors are activated and able to maintain the surviving SGN population. Nucleotides and nucleosides are novel drugs in inner ear treatment and it is possible that drugs acting on purinoreceptors can be of clinical interest for developing new treatment strategies for the injured inner ear.

2. INTRODUCTION

2.1 *The hearing organ*

The mammalian ear is unique in its way to perceive sound from the environment. The ear is divided into three major parts: outer, middle and inner ear. The outer ear consists of the pinna, the external auditory canal and the tympanic membrane. The pinna helps directing the sound through the external auditory canal to the tympanic membrane. The middle ear is an air-filled cavity behind the tympanic membrane and includes the auditory ossicles; malleus, incus and stapes. The ossicles connect the tympanic membrane to the oval window and their main function is to amplify sound. When a sound wave reaches the tympanic membrane the ossicles moves and the footplate of stapes starts to vibrate the oval window. The subsequent movement of the oval window creates pressure waves in the cochlear fluids and causes the basilar membrane to vibrate. Sensory cells (hair cells) sense the mechanical vibration and their stereocilia moves and mechanically gated ion channels open. K^+ from the endolymph (fluid in scala media) flow into the hair cells. The hair cells depolarize and Ca^{2+} channels opens. Transmitter substance is released and afferent nerve fibers of the spiral ganglion neurons (SGNs) are excited. The signals are delivered by the auditory nerve to the brainstem and the central nervous system where the signals are interpreted as sound.



Schematic drawing of the cochlea

The cochlea has three fluid filled compartments. The fluid in scala vestibuli and scala tympani is called perilymph. Between scala vestibuli and scala tympani is a closed compartment called scala media bounded by the Reissner's membrane and the basilar membrane. Scala media contains the organ of Corti and stria vascularis. Stria vascularis is a vascularized epithelium with a high metabolic activity and is responsible for maintaining the high K^+ -concentration and positive endocochlear potential (+80mV) in the scala media. The difference in ionic composition contributes to the magnitude of the receptor potential that occurs in auditory receptor cells. The sensory cells (hair cells) are located in the organ of Corti. They are innervated basally by afferent and efferent nerves originated from the neurons in the spiral ganglion.

2.2 Hair cells

The sensory cells in the Organ of Corti are called hair cells. There are two types of hair cells, one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). The IHCs are connected to the afferent nerve fibers and the OHCs to the efferent nerve fibers. There is a difference in the number of hair cells between species. In humans there are about 3500 IHCs and 12000 OHC (Ulehlova, Voldrich et al. 1987) while in the guinea pig there are about 1900 IHCs and 6600 OHCs in the organ of Corti. Hair cells cannot regenerate and as a consequence of hair cell death there is a secondary degeneration of the SGN due to loss of trophic support.

2.3 Spiral Ganglion Neuron

The spiral ganglion neurons are found in Rosenthal's canal which is located in the central part of the cochlea. This canal spirals from the base to the apex. The neurons are bipolar with one process extending towards the organ of Corti and one projecting towards the brainstem within the auditory nerve.

There are two kind of spiral ganglion neurons (SGN), type I and type II. Early studies in guinea pig (Morrison, Schindler et al. 1975; Friede 1984) showed that SGN type I are 90-95% of the fibers in the auditory nerve and contacts only inner hair cells (IHC).

A healthy type I SGN is bipolar and has a large round nucleus, many large mitochondria, Golgi apparatus, ribosomes, some neurofilament and rough and smooth endoplasmic reticulum. Unlike SGN in mature animals most human SGNs lack a myelin coat, but are surrounded by a thin rim of Schwann cell cytoplasm (Tylstedt, Kinnefors et al. 1997). SGNs type II have thin, unmyelinated fibers and contacts outer hair cells (OHCs) (Rosenbluth 1962). The myelinated fibers vary in size. In guinea pigs the fibers in the apex are thinner compared to the basal fibers (Friede 1984).

The fibers of the VIIIth nerve transmit sensory information directly to the central nervous system (CNS). The time for degeneration of SGN varies between different species. In guinea pigs there is approximate 40% loss three weeks after deafening and approximately 70% loss 11 weeks after deafening (Paper I).

Sensorineural hearing loss as a result of damage or destruction of sensory cells (hair cells) results in a secondary degeneration of SGN due to loss of neurotrophic support. Today it is well known that the degeneration of SGN can be prevented by intracochlear infusion of i.e. neurotrophins (Shinohara, Bredberg et al. 2002; Gillespie, Clark et al. 2003; Gillespie, Clark et al. 2004; Maruyama, Miller et al. 2008). The spiral ganglion neurons are the target for cochlear implants.

2.4 Cochlear Implant

Deafness is one of the most common health conditions in developed countries. It is estimated that approximately 70 million people worldwide are deaf (Gillespie and Shepherd 2005). For patients with a severe to a profound hearing loss a cochlear implant (CI) is the only treatment today. The function of a CI depends in part on the survival and the electrical responsiveness of the spiral ganglion neurons, the target of the CI. In the beginning of the 80s multi-channel CI devices were implanted worldwide. Since then the development of cochlear implant has increased rapidly. The last twenty years of research on cochlear implants (animal models) has focused on SGN treatment, their survival and electrical responsiveness. Different neurotrophic factors administered by long-term intracochlear infusions (Gillespie, Clark et al. 2004;

Yamagata, Miller et al. 2004; Maruyama, Miller et al. 2008), electrical stimulation (ES) alone (Leake, Hradek et al. 1999); Miller, Morris et.al. 2000) and together with neurotrophic factors (Kanzaki, Stover et al. 2002; Coco, Epp et al. 2007) have all shown to be successful treatment to prevent degeneration of the spiral ganglion neurons. The basic external parts of a human cochlear implant device include, a microphone (which picks up sound from the environment), a speech processor (which filters sound and sends the electrical sound signals to the transmitter) and transmitter (held in position by a magnet placed behind the external ear and transmits the processed sound signals to the internal device by electromagnetic induction). The internal parts include a receiver and stimulator secured to the bone beneath the skin (convert the signals into electric impulses and sends them through an internal cable to electrodes), an array of up to 22 electrodes (placed in the cochlea and sends impulses to the nerves and the auditory nerve system).

2.5 Neomycin

Neomycin is an aminoglycoside antibiotic. Today the use is limited in the industrial world due to side-effects but in difficult cases like meningitis it is used as a part of the treatment. In third world countries it is more frequently used since it is cheap and easy to administer. Neomycin is very neurotoxic and nephrotoxic (Forge and Schacht 2000). In studies presented here neomycin sulfate was used to deafen normal hearing guinea pigs by intracochlear or transtympanic delivery.

2.6 Electrodes

In the work presented here two different kinds of electrodes and cannulae were used. One manufactured by Med-El, Austria, and one was hand-made (both described in the material and methods part). Lately a variety of different electrodes and cannulae have been developed (Shepherd and Xu 2002) sometimes modified from human cochlear implants (Paasche, Gibson et al. 2003). Today electrodes with at least 8 channels combined with a cannula for drug delivery are available. The cost for these electrodes starts from approximately 4000 SEK each and the more advanced the higher the cost.

2.7 Neurotrophic factors

Neurotrophic factors include the members of the neurotrophin family nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) they also include members of the neurokinin family (e.g. ciliary neurotrophic factor) as well as other growth factors with neurotrophic activity such as fibroblast growth factor (FGF). This is not a complete list of neurotrophic factors although they have all been used in animal models for inner ear research. Neurotrophins bind and activate tropomyosin related kinase (Trk) receptors. The principal but not exclusive binding relationships are NGF to TrkA, BDNF and NT4/5 to TrkB and NT3 to TrkC.

Glial cell line-derived neurotrophic factor (GDNF) was isolated and cloned by Lin and colleagues (Lin et al. 1993, 1994). GDNF initially binds to a glycosyl phosphatidyl inositol-linked receptor GFR α . Once bound the complex binds to the transmembrane receptor tyrosine kinase RET. GDNF was initially identified as a survival factor for midbrain dopamine (DA) neurons (Cass, Kearns et al.1998)

Neurotrophic factors are important in the development and maintenance of the auditory system. A number of studies report that neurotrophic factors (from various families) can prevent SGN degeneration in animal models and that they enhance the functional responsiveness of the auditory system (Shinohara, Bredberg et al. 2002; Yamagata, Miller et al. 2004; Maruyama, Miller et al. 2008). Neurotrophins also regulate adult nervous system plasticity by promoting neuronal survival and stimulation nerve regrowth following injury (Gillespie 2003). BDNF and NT-3 are the most frequently used neurotrophic factors in auditory research. The work presented here is mainly focused on GDNF and its effect on deafened guinea pig inner ear.

2.8 Nucleosides and Nucleotides

A nucleoside consists of a nitrogenous base and sugar. A nucleotide is a nucleoside that has one or more phosphate groups attached. Intracellular nucleotides are among

other things important in the enzyme regulation, energy transformation and also as bricks in the genome. P2-receptors are comprised of P2X (ionotropic) and P2Y (metabotropic, G-protein-coupled). P2-receptors are cell surface receptors activated by extracellular nucleotides for example adenosine triphosphate (ATP) and uridine triphosphate (UTP).

P2-receptors activate through purines and pyrimidines with two or three phosphate groups (Erlinge 1999). Extracellular nucleosides and nucleotides are known to work as neurotransmitters or neuromodulators (Neary, Rathbone et al. 1996; Burnstock 2006) and even as co-transmitters alongside glutamate. Today, P2-receptors are mainly used for treating cardio-vascular diseases. Extracellular nucleotide signaling via nucleotide (P2) receptors is a mechanism that may regulate apoptosis (Burnstock and Williams, 2000). According to (Housley 2000) nucleosides and nucleotides are released from the hair cells following trauma and act post-synaptically on spiral ganglion neurons.

2.9 Aims of the study

This thesis is focused on treatment of the SGN in the deafened guinea pig ear.

1. To find the optimal concentration of GDNF for inner ear treatment.
2. To investigate if there was a difference between immediately and delayed onset of neurotrophic factor treatment to deafened guinea pig ear concerning the electrical responsiveness and the survival of spiral ganglion neurons.
3. To see if the effect of neurotrophic factor treatment remained four weeks after end of treatment.
4. To see if antioxidant had an effect as a post-treatment drug compared to saline.
5. To find out if intracochlear infusion of nucleotides and nucleoside could effect the spiral ganglion neurons electrical responsiveness and their survival after deafening.

3. MATERIAL AND METHODS

3.1 Subjects

All animal procedures were performed in accordance with the ethical standards at Karolinska Institutet and consistent with national regulations for care and use of animals. All animals were housed at the animal department at the Karolinska University Hospital (KUS), Solna. Surgery and electrophysiology measurements were performed at the Gustav V Research Institute at KUS, Solna. In study I (ethical approval no N113/01, N468/03) we used 57 pigmented guinea pigs of both sexes weighing 200-370g (BioJet, Uppsala, Sweden). In this study all animals were tested for normal hearing using click-evoked auditory brainstem response (ABR) prior to the experiment. In Study II (ethical approval no N 113/01) a total of 44 pigmented guinea pigs weighing 280-440g of both sexes (Lidköpings Kaninfarm HB, Lidköping, Sweden) were used. Prior to surgery the guinea pigs were tested for the Preyer reflex to assure normal auditory function.

3.2 Experimental design

Paper I consisted of two studies, one regarding long-term treatment effects and one for acute treatment. In the long-term treatment study, pigmented guinea pigs were tested for normal hearing by measuring click-evoked auditory brainstem responses (ABRs). All normal hearing animals were deafened by transtympanic injection of a 15% neomycin solution. Click ABRs were measured weekly and only subjects demonstrating a threshold shift of 40-60 dB three weeks after neomycin treatment were used in the study. Three weeks after deafening one group of animals (n=6) was sacrificed for morphological studies. The remaining animals received a combined electrode-cannula connected to a mini-osmotic pump and treated with intracochlear infusion of 1µg/ml GDNF or artificial perilymph for four weeks. After four weeks of treatment one group (n=5) was sacrificed for morphological studies. The GDNF treated animals (n=16) were divided into two groups for post-treatment and injected

daily (i.p.) with antioxidants or saline. The control group (n=7) treated with artificial perilymph received daily injections with saline i.p. During these eight weeks of treatment and post-treatment, the auditory function was measured weekly by recording electrically-evoked ABRs (eABRs). After the final eABR measurement the animal was sacrificed and the cochlea processed for morphological analysis and SGN counting.

In the second study in Paper I, pigmented guinea pigs were tested for normal hearing using click-evoked auditory brainstem response (ABR) 5 days prior to implant surgery. For electrical stimulation and intracochlear drug delivery, a combined electrode and cannula (Med-El, Austria) was used. The cannula was preloaded with neomycin in order to deliver neomycin for forty-eight hours followed by 1µg/ml GDNF, 0.1µg/ml GDNF or artificial perilymph for four weeks (one pump change). After four weeks the pump was removed and the animal stayed in the experiment for another two weeks. During these six weeks eABRs were measured weekly. After the final measurement the animal was sacrificed and the cochleae collected and processed for morphological analysis and SGN counting.

In Paper II, 44 pigmented guinea pigs were tested for the Preyer reflex to assure normal hearing. The guinea pigs received a cochlear implant and a micro-cannula connected to a mini-osmotic pump for drug delivery. All animals were deafened by intracochlear infusion with the ototoxic agent neomycin for forty-eight hours followed by 23 days of nucleosides, nucleotides or artificial perilymph. The electrical responsiveness of the spiral ganglion neurons (SGNs) was monitored weekly during the experiment by recording the electrically-evoked auditory brainstem response (eABR). After the final measurement, the cochleae were harvested and processed for morphological analysis and SGN counting.

3.3 Deafening

Two different deafening designs were used. When deafening with transtympanic injection of neomycin (Paper I) the middle ear cavity was filled with 15% neomycin

sulfate solution through the upper left quadrant of the tympanic membrane. The neomycin penetrates through the round window into scala tympani and causes the death of the sensory cells in the organ of Corti. This results (Zappia and Altschuler 1989) in a secondary degeneration of the spiral ganglion neurons. For intracochlear injections with neomycin, a cannula was preloaded with 10% neomycin, which was administered for 48 hours into the inner ear.

3.4 Mini-osmotic pumps

A mini-osmotic pump (ALZET 2002, DURECT Corp., CA, USA) connected to a micro-cannula makes a continuous drug delivery to the cochlea possible. The pump consists of two parts, a pump and a flow moderator. It works due to an osmotic pressure difference between a compartment within the pump, called the salt sleeve and the tissue environment in which the pump is implanted. The rate of the delivery is controlled by the water permeability of the pump's outer membrane. The pumps compressed reservoir cannot be refilled, which makes it single use only. There is a variety of pump sizes and flow rates to choose from but for inner ear delivery it is necessary to use a slow delivery rate, hence 0.5 μ l/hour was used in all studies presented here.

3.5 Micro-cannula

In Paper I a combined electrode-cannula (Med-El, Austria) was used. In this device the stimulus electrode is a part of the cannula. The stimulus electrode is placed inside the cannula. At the end of the cone shaped cannula (going in to scala tympani through the round window) there is an opening where the ball on the electrode is located, which makes it possible for the electrode to stimulate the auditory nerve. The drug is delivered into the cochlea from another opening. The ground electrode is placed inside the middle ear cavity touching the wall.

In Paper II a hand-made cannula was used. A 7.5 cm vinyl tubing (inner diameter 0.72 mm; Scientific Commodities Inc, AZ) was used as a cannula. A 7 mm piece of a polyamide tubing (Microlumen Inc. FL, USA) was placed into the end of the vinyl tubing and a two component silicone glue (MDX 4-4210, Dow Corning Corp., MI, USA) 10 parts base/1 part of curing agent was mixed thoroughly. With a fine probe the silicone is put at the opening and allowed to flow into the vinyl tube. Approximately 3.75 mm of the polyamide should be sticking out of vinyl tube. On the part outside the polyamide tubing (3.75 mm), a silicone ball is placed leaving a 0.5 mm tip on the micro-cannula to be inserted to the scala tympani. Twenty-four hours later it is checked that the glue is hard and the micro-cannula is easy to flush.

3.6 Electrodes

Home-made electrodes containing two teflon-coated platinum⁹⁰/iridium¹⁰ wires (Advent Research Materials Ltd., Oxford, England) were used in study I (Brown, Miller et al. 1993). The stimulus electrode had a diameter of 0.075 mm and the grounding electrode 0.125 mm diameter. On the stimulus electrode a small ball (~0.25 mm) is burned (using an oxygen tank) in one end to increase the surface that will stimulate the auditory nerve. At the other end the insulation is removed approximately 15 mm. On the ground electrode the insulation is removed approximately 15 mm at both ends. The electrodes terminate at a connector where they are soldered.

3.7 Surgery

All animals were deeply anaesthetized (ketamine 40 mg/kg i.m. and xylazine 10 mg/kg i.m.). The animals received antibiotics prior to surgery and the following day. Before surgery the animals were weighed, shaved on the head, behind the ear and on the neck and the area of surgery and thoroughly washed with iodine and 70% alcohol. Ophthalmic ointment was applied to prevent corneal ulcers due to the disappearance of the blink reflex. The animal was placed on a heating pad and covered with a sterile

foliodrape. A local anaesthetic, xylocain was injected subcutaneously on the head, neck, behind the ear and on the back. A skin incision was performed starting posterior to bregma and ending at the base of the pinna. A pocket under the skin on the animals back was made for hosting the pump. After removing the periost a hole was drilled through the skull ~10 mm posterior to bregma to allow a stainless screw to contact the dura of the brain. The screw served as a recording electrode for eABR measurements. The screw was secured with dental cement (Dentalon plus Heraeus Kulzer, Inc. NY, USA). Muscles covering the temporal bone were separated and a hole made in the bone to visualize the middle ear cavity and the cochlea.

In Paper I a combined electrode-cannula was used. The combined electrode-cannula was inserted through the round window. On the cannula a black mark 3 mm from the tip had been made by the manufacturer (Mel-El, Austria). The cannula was inserted into scala tympani until the black mark was even with the round window edge. The cannula was temporarily secured with histoacryle and the connector secured on the skull with dental cement. The ground electrode was placed inside the middle ear cavity touching the wall. The hole in the bulla was covered with dental cement which at the same time secured the cannula and the ground electrode. The mini-osmotic pump was filled with drug/artificial perilymph and connected to the cannula and placed in the pocket under the skin on the back of the animal. Subcutalar closure and finally the skin was sutured.

After surgery the animals received 5 ml body warm saline subcutaneously and long time analgesia. Body weight was checked daily at least three days after surgery or until the animals started to gain weight. Analgesia and saline were given subcutaneously on the second day after surgery.

In Paper II the home-made stimulus electrode was inserted through the round window (~2 mm) and temporarily secured with histoacryle. The connector was secured on the skull with dental cement. A soft bend was made on the ground electrode and placed inside the middle ear cavity touching the wall. A small hole was drilled in the basal turn ~2 mm above the round window and the tip of the preloaded cannula was inserted

and secured at the temporal bone with histoacryle. The remaining procedures followed paper II including post-operative care.

3.8 Electrically evoked auditory brainstem response eABR

For measuring the electrical responsiveness of the spiral ganglion neurons, eABR were recorded. Animals were anesthetized (ketamin 40 mg/kg i.m. and zylazine 10 mg/kg i.m.) and placed in a sound proof box. The recordings have been described in detail by Hall (1990). Briefly, responses to monophasic current pulses (50- μ s long: presented at 55 pps) with alternative polarity were recorded between a permanent electrode (active) placed at the vertex and a subdermal needle (reference) placed above the bulla. A needle inserted subcutaneously in the hind leg served as a ground electrode. High-and low-pass filters were set at 5 Hz and 15 kHz respectively. The responses were amplified using a preamplifier (HS4, Tucker-Davies Technologies Inc., USA) and sampled by an analogue to digital converter (Tucker-Davies Technologies Inc., USA). The eABR threshold was defined as the lowest stimulus level (in μ A) that elicit 0.3 μ V reproducible wave form.

3.9 Histology

After the final measurement the animals were deeply anaesthetized with sodium pentobarbital sodium (25mg/kg, i.p.) and transcardiacly perfused with saline (37° C) followed by cold 2.5% glutaraldehyde. The cochleae were collected and decalcified in 0.1M EDTA in phosphate buffer, dehydrated and embedded in JB-4 plastic (Polyscience Inc., Warrington, PA, USA) and sectioned in 4 μ m sections (Paper I) or 3 μ m sections (paper II). When reaching the mid-modiolar plane i.e., when all six cross-sections of Rosenthal's canal can be seen, every third section (Paper I) or every fourth section (Paper II) was collected and mounted on glass slides. All mounted sections were stained with Paragon and six consecutive sections were collected for spiral ganglion neuron counting. The criteria for a guinea pig spiral ganglion neuron profile

(Type I) were a cell diameter of 14-20 microns and a nucleus with diameter in range of 7-10 microns. Collecting every fourth section was to make sure that each SGN was only counted once. In all animals a total of 36 cross-sections of Rosenthal's canal were analyzed. The Rosenthal's canal profile was traced (using Sigma ScanPro 4 software) to estimate the area. The SGN profile density was den expressed as the number of SGN profiles per 10 000 μm^2 .

3.10 Glial Cell Line-Derived Neurotrophic Factor (GDNF)

Previous studies (Shinohara, Bredberg et al. 2002; Yamagata, Miller et al. 2004; Maruyama, Miller et al. 2008) have demonstrated that exogenous neurotrophins not only maintain SGN survival in deafened animals but also enhance the electrical responsiveness. In these studies we investigated a delayed onset of GDNF treatment and its long-term effects on deafened guinea pigs.

In the second part of the study two different concentrations (1 $\mu\text{g}/\text{ml}$ and 0.1 $\mu\text{g}/\text{ml}$) of GDNF were investigated when delivered immediately after deafening.

3.11 Nucleosides/nucleotides

In an initial study different nucleosides and nucleotides were selected based on their relative receptor subtype selectivity and on the presence of such receptor subtypes in cochlear tissues. The compounds included a) UTP (100 μM ; Sigma-Aldrich; n=4), acting mainly on P2Y2 and P2Y4 receptors; b) alpha-, beta-methylene adenosine 5'-triphosphate ($\alpha\beta\text{meATP}$, 100 μM ; Sigma-Aldrich; n=4) acting mainly at P2X₁ and P2X₃ receptors; c) N⁶-cyclopentyladenosine (CPA 100 nM; Sigma-Aldrich n=3) active at adenosine A1 receptors; and d) 2methylthio-ATP (2MeSATP, 100 μM ; Research Biochemicals, Inc., n=4) acting primarily at P2Y₁ and P2X₁ and P2X₂ receptors.

3.12 Antioxidants

It has previously been shown that antioxidants (Maruyama, Yamagata et al. 2007) have an effect when given i.p. together with intracochlear infusion of GDNF on deafened guinea pigs. The antioxidants used in the GDNF study were a cocktail containing Trolox (10mg/kg) and Ascorbic acid (200mg/kg).

3.13 Statistical analysis

For the statistical analysis in the eABR study and for the calculation of the spiral ganglion neuron density Two-way Anova, was used. As post hoc test the Tukey test was used. Data are presented as mean \pm sem.

4. RESULTS AND DISCUSSION

4.1 Paper I

When GDNF was delivered with delayed onset there was no difference in the eABR thresholds between the GDNF treated groups given saline or antioxidants as post-treatment. From week two after treatment started there was a significant difference ($p < 0.05$) between the group receiving AP (CTRL saline) and the groups given GDNF. At week three the difference had been even more pronounced ($p < 0.001$). Six weeks after the treatment started the eABR threshold for the CTRL saline group reached the limitation of the equipment.

A total of six groups including a normal group of guinea pig cochleae were analyzed for SGN density. One group of animals was sacrificed three weeks after deafening and showed approximately a 40% loss in SGN density. Animals deafened for 3 weeks before 4 weeks of GDNF treatment followed by 4 weeks post-treatment resulted in approximately 25% loss in SGN density. This should be compared to the CTRL saline group (3 weeks deafened animals with 4 weeks of AP treatment followed by 4 weeks of post-treatment saline). The CTRL saline group showed approximately 70% loss in SGN density. The measurement of the soma size in normal, GDNF saline and CTRL saline showed that the normal SGN soma size was significantly smaller than in both the GDNF saline group ($p < 0.01$) and the CTRL saline group ($p < 0.001$). There was also a difference between the GDNF saline treated and CTRL saline treated groups ($p < 0.05$).

EABR thresholds in the acute study (GDNF 1 μ g/ml and GDNF 0.1 μ g/ml) showed significant lower threshold ($p < 0.001$) in the GDNF-treated animals than in the control animals that received AP. In the SGN density the group treated with 1 μ g/ml GDNF showed a higher difference ($p < 0.001$) compared to the group treated with AP. The difference between the 0.1 μ g/ml GDNF treated group and the CTRL group was $p < 0.01$. Between the two GDNF treated groups there was a significant difference of $p < 0.05$ in favor for 1 μ g/ml GDNF treated group.

The delayed study was designed to imitate a clinical approach. Deafening with neomycin transtympanical injections showed clearly that the degeneration of SGN continues under a long period of time. Three weeks of deafness might not be considered to be a long time from a clinical aspect but a 40% loss of SGN would correspond to a significant hearing loss. The results show that without GDNF intervention the degeneration of the SGN continues probably due to the loss of neurotrophic support (Mattson 1998). Gillespie and co-workers (2003) reports that cessation of neurotrophic treatment (in this case BDNF), lead to a rapid decrease in SGN survival. In the long-term study presented here, the survival of SGN remains at least 4 weeks after cessation of GDNF treatment. This could be due to differences in the action of BDNF and GDNF on the cellular level. In the report from Gillespie and co-workers (2003) another deafening technique was used (i.v. injection of kanamycin and furosamide) which has to be taken into consideration. This could effect the SGN differently than transtympanic injections of neomycin. It is possible that i.v. injection of kanamycin and furosamide damage the SGN so severely that it is not possible to reverse. The Gillespie study did not include measurements of the SGNs electrical function.

4.2 Paper II

The pilot study with nucleosides and nucleotides showed a large variation in eABR thresholds within the groups and it was therefore difficult to interpret the results. The UTP-treated group was the only group where all animals showed good eABR response compared to the control group given artificial perilymph. Based on these results the experiment continued with UTP and its degradation product uridine.

At the end of the experiment (twenty-three days of treatment) both UTP and uridine treated groups showed a significant difference in eABR threshold ($p < 0.001$) compared to the control group.

The spiral ganglion neuron density was significantly lower ($p < 0.001$) in the control group treated with artificial perilymph compared to the UTP and uridine treated groups. No significant difference was found between the UTP treated and uridine treated group.

UTP and its degradation product uridine are novel drugs concerning treatment of inner ear injury. The results from this study demonstrate that UTP and uridine are promising drugs for treatment of the survival and electrical responsiveness of the SGN to increase cochlear implant benefits. It also indicates that nucleotides and nucleosides are involved in the neurotrophic support of in the inner ear.

In this study drug treatment was delivered into the inner ear immediately after deafening but it must be considered that it takes several weeks for the degeneration of SGN, so the onset of drug delivery could be the critical point in this study. UTP and uridine showed very promising results but it would be interesting to see if the results were this good with a “delayed” onset of the UTP and uridine delivery and if the positive effect is preserved after end of treatment.

The difference in soma size indicates that neomycin effect the soma size SGN but treatment with UTP in part inhibit that effect. The reason to changes in soma size is not known.

4.3 General discussion

Deafness, with loss of sensory cells (hair cells) results in a progressive degeneration of SGN due to loss of neurotrophic support. Deafferentation of a neuron is caused by neurotrophin loss and can lead to a change in oxidative state (formation of free radical) and changes in intracellular Ca^{2+} (Miller, Miller et al. 2002). Elevation of Ca^{2+} -and oxidative stress contribute to damage of proteins, lipids, and DNA and finally to cell death (Mattsson 1998). Intervention therapies in animals with neurotrophins can preserve the auditory nerve. There have been numerous reports on how to prevent degeneration of SGN with neurotrophins alone, together with chronic electrical

stimulation (ES) or ES alone. They have all reported an increase of SGN survival in deafened animals compared to their control groups.

The last couple of years there have been reports about changes in the soma size when treated with neurotrophic factors. Several studies have shown an increased soma size (Shepherd, Coco et al. 2005; Glueckert, Bitsche et al. 2008) in SGN from deafened animals treated with BDNF, compared to the SGN soma size of deafened control animals. In our studies we found the opposite, SGNs soma size was reduced in both the GDNF study and the UTP study compared to deafened untreated animals. However, in both UTP and GDNF treated animals, SGN soma size was increased compared to normal untreated animals. It is possible that this is due to a different deafening technique and that intravenous administration (kanamycin and ethacrynic acid/ kanamycin and furosamide) affect the SGN in a different way than local infusion of neomycin into scala tympani that is been presented here. At this time there is no report on how an increased soma size affects the electrical function of the SGN.

A single GDNF injection leads to significant increase in midbrain dopamine (DA) neurons in monkeys that expressed Parkinson's disease (Cass, Kearns et al). The improvement lasted for at least 4 weeks (the longest time-point examined). In auditory research there is one report about a single dose of neurotrophin-3 (labeled with ¹²⁵I for tracing NT-3 in the organ of Corti) (Richardson, O'Leary et al. 2005). Neurotrophic-3 was intracochlear infused to deafened animals (kanamycin and frusamide). After a single dose of NT-3 they found increased soma size and an overall healthier appearance in the SGN than in the untreated controls. The result shows that a single injection of NT-3 provides neurotrophic support for at least 1 week. It is possible that this is only a temporary trophic support due to the fact that SGN degeneration continues following withdrawal of neurotrophic treatment (Gillespie, Clark et al. 2003).

If it is possible to provide neurotrophic support for a longer time period with a single injection of neurotrophins there might be an opening for patients receiving CI. In that case, intracochlear injections could be performed at the time of surgery. It would also

be possible to coat electrodes before insertion into scala tympani, which would give longer treatment duration.

Several factors influence the physiology of the denervated SGN and the performance of a cochlear implant. The general opinion is that in the absence of SGNs, electrically-evoked potentials in the ascending auditory pathways cannot be generated with a cochlear implant. However, there have been several reports from studies where no obvious correlation between the outcome of the cochlear implant and the amount of survival SGN (Hall 1990; Incesulu and Nadol 1998). In a study (results not shown) here at the Center for Hearing and Communication Research two types of synthetic steroids were tested with the same design as in the GDNF concentration study. The eABR results did not differ much from the GDNF study but the density of the SGN was similar to that of the AP treated animals. Different concentrations of the steroids were examined and the result showed an increase of SGN density at the highest concentration. This shows the importance of finding the right concentration for intracochlear infusion. The question still remains how the eABR response could be so good with so few SGN. This study will be followed-up with the same design but with focus on the cochlear nucleus.

5. SUMMARY

GDNF is known to have a neurotrophic effect on SGN when delivered into the inner ear. In the present study we show that GDNF is effective for maintaining the electrical responsiveness and the survival of SGN when given three weeks after deafening. In the same study we found that up to four weeks after GDNF-treatment ended, there was still a beneficial effect on cell survival and electrical responsiveness. When different concentrations were tested a difference in SGN survival in favor for 1µg/ml of GDNF compared to 0.1µg/ml of GDNF ($p<0.01$) was shown. This difference was not seen in the measurement of the electrical responsiveness. The morphology clearly showed that the degeneration of SGN in animals deafened for 11 weeks is approximately 70% compared to normal animals. Three weeks after deafening the loss of SGN is approximately 40%. Three weeks after deafening, animals were treated with GDNF for four weeks followed by a four weeks post-treatment with daily injections (i.p.) of antioxidants or saline. This resulted in a SGN loss of approximately 25%. Treatment with GDNF inhibited the degeneration and the loss of SGN even after a delayed onset and four weeks of post-treatment i.p. injection.

Both UTP and uridine showed to be substances of interest for studies in the treatment of the traumatized inner ear. The electrical responsiveness after 23 days of treatment was similar to the effect that is seen in deafened animals treated with neurotrophic factors. The density of the spiral ganglion neurons was not as high as in neurotrophic factor-treated animals, but considerably higher than in untreated animals. The results demonstrate that UTP and uridine rescue auditory neurons and suggests that drugs acting on purinoreceptors could be of clinical importance.