**ABSTRACT**

Deafness is one of the most common health conditions in the developed countries, and worldwide, an estimated 70 million people are deaf. For people with severe to profound hearing loss, a cochlear implant is the only treatment today. The most common forms of severe hearing loss and deafness are related to morphological changes in the cochlea.

**Aim:** The aim of these studies was to investigate several therapeutic compounds, including nucleosides and nucleotides, two types of neurotrophic factors, and two oxysterols, to determine if they could preserve spiral ganglion neuron (SGN) survival and maintain SGN electrical responsiveness, as determined by measuring electrically-evoked auditory brainstem response (eABR) in deafened guinea pigs. It was also important to investigate the compounds’ effectiveness when delivered into the inner ear several weeks after deafening (“Delayed treatment”). In some experiments, the animals stayed in the study for several weeks after cessation of treatment to determine if eABR threshold remained and the extent of SGN survival after treatment cessation.

**Methods:** All animals in these studies were deafened with the ototoxic compound neomycin sulfate by intracochlear or transtympanic infusion. They received a cochlear implant and an osmotic pump for inner ear drug delivery. To determine any changes in hearing (i.e., SGN electrical responsiveness), electrically-evoked brainstem response was measured weekly. After the last measurement cochleae were collected for morphological analysis.

**Results:** We found that nucleosides and nucleotides seem to have a trophic effect on spiral ganglion neurons, showing low eABR thresholds and a statistically significant (p<0.001) SGN survival compared with the control group. Results from the study with glial cell line-derived neurotrophic factor (GDNF) showed that delayed GDNF treatment helped to prevent loss of electrical responsiveness and auditory nerve cell death up to four weeks after GDNF cessation. Comentin, a new neurotrophic factor, showed low eABR thresholds but with fewer surviving SGNs. The oxysterols study showed a different pattern compared to all our previous studies. In the acute study both oxysterols showed low eABR thresholds compared to the control group, but SGN survival was equal to the control group that did not received any treatment. In the delayed treatment study only one of the oxysterols showed lower eABR thresholds during the whole experiment compared to the control group. Despite that, SGN survival was equally low in the oxysterol groups and the control group.

Of the therapeutic agents tested in this study GDNF was most the promising compound.
LIST OF PUBLICATIONS


IV. Fransson A., Silvente-Poirot S., Poirot M. and Ulfendahl M. “Dendrogenin A and B, two new alkylaminooxysterols increasing neural responsiveness in an animal model”. (manuscript)

V. Fransson A. and Ulfendahl M. “Morphological changes in the inner ear of the experimentally deafened guinea pig over time”. (manuscript)
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<td>Spiral ganglion neuron</td>
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1 INTRODUCTION

Hearing loss affects over 360 million people worldwide (World Health Organization 2012) and has significant detrimental impacts economically and emotionally on individuals and on society (de Graaf and Bijl 2002). It is estimated that of these approximately 70 million people worldwide are deaf (Hoffmann and Strasnick 2004). According to WHO, disabling hearing loss refers to loss greater than 40 dB in the better hearing ear in adults and a hearing loss greater than 30 dB in children. “Deaf” refers to people with a profound hearing loss which implies very little or no hearing.

For patients with a severe to profound hearing loss a cochlear implant is the only treatment today. Treatment with a cochlear implant can dramatically improve speech perception and production as well as quality of life.

1.1 The hearing organ

The ear can be divided anatomically into three major parts: outer, middle and inner ear. The outer ear consists of the pinna and the external auditory canal leading to the tympanic membrane. The pinna acts as a reflector to capture sound and focus it into the ear canal (Figure 1).

![Figure 1.1](www.medicalook.com)

Schematic drawing illustrating the human ear.
Modified from: www.medicalook.com

The middle ear is an air-filled cavity. It includes the tympanic membrane and the auditory ossicles, malleus, incus and stapes, connected as a chain. When the tympanic membrane vibrates as a result of sound waves traveling in the ear canal, the vibration is passed along the ossicular chain.

The inner ear, located in the temporal bone, consists of two parts: the cochlea, the sensory organ for hearing, and the vestibular system, which is the organ of balance. The spiral-
shaped cochlea has three fluid filled compartments (Figure 2). The fluid in scala tympani and scala vestibuli is called perilymph and has a high Na\(^+\) concentration. Scala media contains its own fluid (endolymph) and is a closed compartment between scala vestibuli and scala tympani, bounded by Reissner’s membrane and the basilar membrane. The organ of Corti, a series of receptor cells (hair cells) for hearing and their supporting structures and the stria vascularis lie within scala media. Stria vascularis is a vascularized epithelium with 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. Vibrations passed along the ossicular chain enter the cochlea at the oval window, creating pressure waves in the cochlear fluid, causing the basilar membrane to vibrate. The auditory sensory cells sense the mechanical vibration, their stereocilia move, and mechanically gated ion channels open. K\(^+\) from the endolymph crosses the hair cell membrane, depolarizing the cells and opening Ca\(^{2+}\) channels. A transmitter substance is released, exciting afferent nerve fibers of the SGN. The signals are delivered by the auditory nerve to the brainstem and the central nervous system where the signals are interpreted as sound.

There are four rows of sensory cells in the organ of Corti: one row of inner hair cells and three rows of outer hair cells resting on the basilar membrane. In the human cochlea the basilar membrane is approximately 31mm long and contains 3500 inner hair cells and 12000 outer hair cells (Ulehlova et al. 1987), the basilar membrane of the guinea pig is approximately 19 mm long and has approximately 1900 inner hair cells and 6500 outer hair cells. When sensory cells die or are damaged, they cannot regenerate and consequently there will be a secondary spiral ganglion neuron (SGN) degeneration.
1.2 Spiral Ganglion Neurons

The SGNs are found in the coiled channel called Rosenthal’s canal, located in the central bony core (modiulus) of the cochlea. This canal spirals from the base to the apex. The neurons are bipolar with one peripheral process projecting towards the organ of Corti also known as the “peripheral axon” and one projecting towards the cochlear nucleus known as the “central axon”. The peripheral process is sometimes referred to as a dendrite, although this term is somewhat inaccurate. Unlike the typical dendrite the peripheral process generates and conducts action potentials which then jump across the cell body and continue to propagate along the central axon. Both the peripheral process and the central axon are myelinated. The axon from the cochlear nerve terminates in the cochlear nucleus and the auditory pathway up to primary auditory cortex in the brain.

Early studies in the cat (Spoendlin 1973) and guinea pig (Friede 1984; Morrison et al. 1975) showed that 90-95% of the fibers in the auditory nerve contact only the inner hair cells. Every inner hair cell is connected to approximately 20 afferent neurons of unbranched fibers (Spoendlin and Schrott 1988). A few smaller afferent axons connect to the outer hair cells where they innervate approximately 10 cells by extensive terminal branching (Spoendlin, 1973). The efferent neurons can be divided into two systems; medial efferents which innervate outer hair cells and lateral efferents which synapse on the inner hair cells afferents. The main neurotransmitter in the synapse between the afferent neuron and the inner hair cell is glutamate (Nordang et al. 2000), although other neurotransmitters are involved.

There are two kinds of SGNs, type I and type II. Type I neurons comprise 90-95% of the SGN. There are small differences depending on species. Also depending on species, type I perikarya can be myelinated with 4-25 lamellae of Schwann cells. A typical type I SGN is bipolar and has a large round nucleus, many large mitochondria, Golgi apparatus, ribosomes, some neurofilaments, and rough and smooth endoplasmic reticulum. Most human SGNs lack a myelinated coat but are surrounded by a thin rim of Schwann cell cytoplasm (Tylstedt et al. 1997). SGN type II ganglion cells (5-10% of all SGNs) are smaller than type I, have a rather large nucleus, and a cytoplasm rich in neurofilaments. They are pseudounipolar, unmyelinated or thinly myelinated, covered by a simple sheet of Schwann cells, and surrounded by a basement membrane. Type II cells contact mainly the outer hair cells (Rosenbluth 1962) but also the afferent fiber terminals under the inner hair cells.

The fibers of the VIIIth nerve transmit sensory information directly to the central nervous system. The time for degeneration of SGNs after trauma varies among different species. Here guinea pigs have been studied and reports regarding the time-line of SGN degeneration are presented in Paper II & V.
1.3 Ototoxic drugs

Hearing loss can arise from several factors, the most common being noise exposure, but some therapeutic drugs, e.g., aminoglycoside antibiotics (neomycin, genatamycin, kanamycin among others), the loop diuretic ethacrynic acid, and chemotherapeutic agents such as cisplatin can also be damaging to the sensory organ. Today their use is limited in the industrial world due to often significant side-effects. In third world countries, this drugs are still frequently used since they are cheap and easy to administer. Aminoglycosides are both neurotoxic and nephrotoxic (Forge and Schacht 2000).

Noise exposure and several of the drugs mentioned above are used in basic research to induce hearing loss in experimental animals. In our lab (and others), the preferred agent is neomycin sulfate (Fransson et al. 2010; Miller et al. 2000). It has the advantage of being administered by different routes: bilaterally, unilaterally, allowing the contralateral ear to be a control; transtympanically or intracochlearly. Other groups use other methods, such as intravenous injection of the loop diuretic furosamide followed by subcutaneous injection of gentamycin (McGuinness and Shepherd 2005). Another common method is subcutaneous kanamycin followed by intravenous injection of ethacrynic acid (Coco et al. 2007). The last two methods induce a bilateral deafness.

Figure 1.3. Cross-section of the Rosenthal’s canal.

Normal guinea pig. Guinea pig, neomycin deafened for 11 weeks

1.4 Neurotrophic factors

Neurotrophic factors (also called growth factors) are secreted proteins responsible for migration, growth, and survival of neurons during development and for the maintenance and plasticity of adult neurons.

Neurotrophic factors are divided into different families, one of the most investigated families is neurotrophins. There are two classes of neurotrophin receptors: the tyrosine kinase (Trk) receptors and the p75 receptor. Trk A is the receptor for nerve growth factor (NGF), Trk B is the receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), and Trk C is the receptor for neurotrophin-3 (NT-3). NT-3 can under some conditions also bind to Trk B. All neurotrophins can activate the low-affinity receptor p75.
There are other members of the growth factor families with neurotrophic activity, such as fibroblast growth factor (FGF), the neurokine family (e.g., ciliary neurotrophic factor), the neurotrophic factor GDNF (see Paper II and IV). New neurotrophic factors have recently been discovered, such as Cometin (see Paper III).

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 preserve the functional responsiveness of the auditory system (Agterberg et al. 2009; Maruyama et al. 2008; Shinohara et al. 2002; Yamagata et al. 2004). The work presented here is mainly focused on GDNF and its effect on the deafened guinea pig inner ear.

1.5 Glial cell line-derived neurotrophic factor (GDNF)

GDNF was isolated and cloned by Lin and colleagues (Lin et al. 1993; Lin et al. 1994) as a part of the identification of new neurotrophic factors for midbrain dopamine neurons (Cass et al. 1998). Since then research has shown that GDNF plays an intricate role in the survival and development on neuronal and nonneural cells.

The GDNF family consists of four neurotrophic factors: GDNF, neurturi, artemin, and persephin. GDNF initially binds to a glycosed phosphatidyl inositol-linked receptor GFRα. Once bound, the complex binds to the transmembrane receptor tyrosine kinase Ret. The most prominent feature of GDNF is its ability to support the survival of dopaminergic and motor neurons. These neural populations die in the course of Parkinson’s disease and amyotrophic lateral sclerosis (ALS). GDNF is often used in research on these and other degenerative diseases.

1.6 Nucleotides and Nucleosides

Nucleotides and nucleosides are known as neurotransmitters and neuromodulators and it has been suggested that they can also act as trophic factors in the central and peripheral nervous systems (Neary et al. 1996). Nucleotides and nucleosides acts via purinoreceptors P1 and P2 receptors respectively (Burnstock 2006). P1 receptor are expressed at several locations in the inner ear e.g. the organ of Corti and the SGNs (Vlajkovic et al. 2007). P2 receptors are divided into two groups, ionotropic P2X receptors and metabotropic P2Y receptor each group have several sup-types. P2 receptors have been found in the SGN region (Housley 2000). According to Housley (2000), nucleotides are released from the hair cells following trauma and act post-synaptically on SGN.

In paper I we investigated the effect of nucleotides and nucleosides, uridine triphosphate (UTP) and uridine. Deafened guinea pigs received a cochlear implant and an osmotic pump for intracochlear infusion of UTP and uridine. The SGNs electrical responsiveness was elicited by eABR measurements and morphological analysis was performed.
1.7 Antioxidants

It has previously been shown that antioxidants can prevent SGN degeneration given systemically in deafened guinea pigs (Maruyama et al. 2007). In Paper II, we investigated if the antioxidants Trolox (a water-soluble vitamin E analogue) and ascorbic acid could effect the SGNs electrical responsiveness and survival when given as a subsequent treatment in deafened GDNF treated guinea pigs.

1.8 Treatment for the damaged inner ear

Sensorineural hearing loss as a result of damage to or destruction of sensory cells results in a secondary degeneration of SGNs due to i.e. loss of neurotrophic support. In the last 25 years the main focus for finding a drug for treatment of inner ear injury has been on neurotrophic factors (Gillespie et al. 2003; Gillespie et al. 2004; Maruyama et al. 2008; Shinohara et al. 2002). But nucleosides, nucleotides (Housley 2000), steroids (Li et al. 2013), and antioxidants (Ewert et al. 2012; Maruyama et al. 2007) have also been investigated. Electrical stimulation alone (Leake et al. 1999); Miller et.al., 2000) and together with neurotrophic factors (Kanzaki et al. 2002; Coco et al., 2007) have also been tested and, except for electrical stimulation alone which had very little effect, shown to be successful treatments in varying degree in the prevention of degeneration of SGNs.

Extra cellular nucleotides and nucleosides are known to function as neurotransmitters and neuromodulators (Burnstock 2006; Neary et al. 1996). In paper I we hypothesized that nucleosides and nucleotides have a trophic effect on SGN. Guinea pigs were deafened and treated with intracochlear UTP and uridine for 23 days and electrically-evoked auditory brainstem response were measured weekly and the cochleae collected for morphological analysis.

In paper II we investigated the well-known neurotrophic factor GDNF and the result of delayed onset of GDNF treatment in deafened guinea pigs, the effects of cessation of GDNF treatment, and the effects of subsequent antioxidants on electrical responsiveness and survival of the SGNs. EABRs were measured weekly for eight weeks. The cochleae were collected for structure analysis.

In Paper III a novel neurotropic factor, Cometin, was tested for the first time as treatment for inner ear damage. Deafened guinea pigs received a cochlear implant and intracochlear infusion of Cometin via an osmotic pump for two weeks. After two weeks the pump was removed and the animals stayed in the study for an additional two weeks to investigate if the effect of Cometin remained. The effect was measured by elicit eABR. EABRs were measured weekly and the cochleae collected for morphological analysis.

In Paper IV we investigated two new oxysterols, Dendrogenin A and B (de Medina et al. 2009). In vitro, Dendrogenin A has been shown to induce growth control, differentiation, and the death of tumor cell lines. Dendrogenin B induces neurite outgrowth in various cell lines and neuronal differentiation in pluripotent cells. Guinea pigs were deafened and
received a cochlear implant and an osmotic pump for intracochlear infusion. EABRs were measured weekly and the cochleae were collected for morphological analysis.

1.9 Cochlear implant

In most cases of hearing loss, the sensory cells (hair cells) are damaged or missing. This results in a gradual secondary degeneration of SGNs. With a cochlear implant, the malfunctioning sensory cells are bypassed and the subsequent functional level of the auditory system is stimulated directly. The success of a cochlear implant depends in part on the survival and electrical responsiveness of the SGNs, the target of the implant. Extensive research has been done in animal models to develop a more functional electrode (multichannel) (Richardson et al. 2009; Shepherd and Xu 2002) and different methods of drug delivery together with the electrode. Paper II presents a study using a combined electrode and cannula for drug delivery into the inner ear.

1.10 Auditory Brainstem Response (ABR)

Auditory brainstem response (ABR) is a noninvasive way of measuring hearing in animal models and in humans. In animals the auditory nerve is stimulated with a click-stimuli or a frequency specific sound. Subdermal needle electrodes register the neurological responses which are displayed as waveforms. Hearing threshold is defined as the lowest intensity stimulus (dB SPL) that gives a visually detectable response and is reproducible.

1.11 Electrically-evoked Auditory Brainstem Response (eABR)

EABR is used for measuring the function of a cochlear implant. In most cases of sensorineural hearing loss the sensory cells are damaged or missing and as a result sound perception is severely impaired. With a cochlear implant, the damaged cells are bypassed and the implanted electrode stimulates the auditory nerve directly. The neurological response is registered with an epidural screw at the vertex of the skull, a subdermal electrode above the bulla, and a grounding electrode in the hind leg. Threshold is defined as the lowest intensity (µA) that gives a response and is reproducible. The eABR is described in detail in the Materials and Methods section.
Figure 1.4. Average electrophysiological wave forms elicited from varied intensity of stimulation (µA). To avoid contamination by electrical artifact, thresholds were defined on the basis of P III.
1.12 The aim of this study

The general aim of the study presented here was to investigate therapeutic compounds that could be used as treatment for inner ear injury. The investigation included their effect on SGN survival, electrical responsiveness and morphological changes in the deafened and treated guinea pig.

The specific aims were to

- Investigate if nucleosides and nucleotides have a trophic effect on the SGNs in the acutely damaged inner ear
- Assess delayed GDNF treatment after deafening, the effect of cessation of GDNF treatment, and the effects of subsequent antioxidants on electrical responsiveness and survival of the SGN
- Determine if the novel neurotropic factor Cometin, has a therapeutic potential in the damaged inner ear
- Evaluate two new oxysterol compounds and their therapeutic potential for the damaged inner ear
- Characterize degenerative changes of the SGN in the guinea pig after experimental deafening over time
2 MATERIAL AND METHODS

2.1 Subjects

All animal procedures were performed in accordance with the ethical standards at Karolinska Institutet and consistent with national regulation for care and use of laboratory animals. All guinea pigs were tested for normal hearing before the start of the experiment. Ethical permission numbers N 113/01, N 468/03, N 35/07, and N 14/10 were used for the studies presented in this thesis.

2.2 Anesthetic

In all studies presented here, ketamine (40mg/kg i.m.) and zylazine (10 mg/kg i.m.) were used as anesthet. For local anesthesia, lidocaine was used in papers I, II, and IV; in papers III and V, the local anesthesia marcain was used.

2.3 Neomycin deafening

In all studies, the animals had been deafened by the ototoxic compound neomycin sulfate, an aminoglycoside antibiotic. In papers I, III, and IV, neomycin was administered by intracochlear infusion for forty-eight hours, using a pre-loaded cannula inserted into scala tympani and connected to a mini-osmotic pump (Brown et al. 1993). In papers II and V, neomycin was administered transtympanically by which the middle ear cavity was filled with neomycin solution using a syringe and 30G needle inserted through the tympanic membrane. The neomycin solution penetrates the round window and into scala tympani (Zappia and Altschuler 1989), where degeneration of the sensory cells starts immediately. Within forty-eight hours, most hair cells (sensory cells) are lost. The extent of the resulting hearing loss depends on neomycin concentration and varied among individuals even within the same concentration groups.

2.4 Cannulas

Cannulas used in papers I, III, IV, and V were made in the laboratory of vinyl tubing (inner diameter 0.72 mm; Scientific Commodities Inc., AZ, USA). A small piece of polyamide tubing (Microlumen Inc., FL, USA) was inserted in one end and secured in place with silicone glue (MDX-4-4210, Dow Corning Corp., MI, USA). A small silicone ball was made 0.5 mm from the tip of the polyamide tube to prevent the tube from being inserted too deeply into scala tympani. In paper II, a custom-made combined electrode/cannula device was used with a combined electrode/cannula (MedEl GmbH, Innsbruck, Austria).
2.5 Electrodes

Laboratory-made electrodes (single channel) were made of two Teflon-coated platinum 90/iridium 10 wires (Advent Research Material Ltd., Oxford, England): a stimulus electrode (0.075 mm diameter) and a ground electrode (0.125 mm diameter). At the end of the stimulus electrode a small ball was formed as the Teflon coating was removed in a flame, thus increasing the surface area of the electrode stimulating the auditory nerve. The other end was soldered to a percutaneous connector together with the ground wire.

2.6 Osmotic pumps

In all papers, osmotic pumps (ALZET osmotic pump, DURECT Corp., CA, USA) were used for drug delivery into the cochlea. The pump consists of a capsule and a metal tube (flow moderator) to which the cannula was attached. The pump operates because of osmotic pressure difference between a compartment within the pump, called the salt sleeve, and the tissue environment in which the pump is implanted. A variety of flow rates and duration are available, but for intracochlear infusion a slow rate of 0.5µl/hour was used to eliminate any risk of harming the hair cells with too high flow pressure.

2.7 Surgery

Surgical procedures are described in detail in papers I and II. In papers I, III, IV, and V, all animals underwent aseptic surgery to implant intracochlear stimulating electrodes and an epidural recording electrode. The middle ear was exposed by a postauricular approach. A cochleostomy was performed in the basal turn for access to scala
tympani, and a pre-loaded cannula was inserted (Brown et al. 1993; Prieskorn and Miller 2000) ~0.5 mm through a carefully made fenestra in the otic capsule ~2 mm ventral to the round window. The round window membrane was pierced and the stimulus electrode inserted ~1.5-2 mm into scala tympani. The ground electrode was placed in the middle ear cavity in contact with the middle ear wall. The hole in the temporal bone was covered with dental cement to fix the electrode and the cannula in place. The cannula was connected to the osmotic pump and the pump placed under the skin of the back. The wound was closed in layers with internal and external sutures.

In paper II, a custom-made electrode/cannula from MedEl (Innsbruck, Austria) was used so there was no need for the otic capsule fenestra. The combined electrode/cannula was inserted into scala tympani through the round window. Apart from this, all other procedures were as described above.

### 2.8 Auditory Brainstem Response (ABR)

A noninvasive way to measure an animal’s hearing is to use ABR. The animal is anesthetized and placed in a sound proof chamber. The stimulus signal is generated through a signal analyzer (Tucker-Davies Technologies Inc., FL), and controlled by a personal computer and delivered by an earphone within the ear canal. Neurological responses are collected using subdermal needle electrodes placed at the vertex, above the bulla, and a ground electrode (subcutaneous in the thigh). The stimuli are provided at various intensity (5 dB steps) to determine the threshold. Threshold is defined as the lowest intensity that can produce a visually consistent waveform and is reproducible.

### 2.9 Electrically-evoked Auditory Brainstem Response (eABR)

In most cases of hearing loss, the sensory cells (hair cells) are damaged or missing and as a result sound perception is severely impaired. It is however possible to bypass the hair cells and directly stimulate the spiral ganglion neurons in the auditory nerve with an electrode implanted into scala tympani, i.e., a cochlear implant. To measure auditory nerve activity with electrical stimulation, i.e. eABR in anesthetized animals is used (Hall 1990; Maruyama et al. 2008; Yamagata et al. 2004). This method is described in papers I, II, IV, and V. Details of recordings are described in (Hall 1990). Briefly, responses to monophasic current pulses (50-µs duration, presented at 50 pps) with alternating polarity were recorded between an 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. The eABR threshold was defined as the lowest stimulus level (in µA) that elicits a 0.3µV reproducible waveform.
2.10 Histology

In all studies, after the last measurement animals were deeply anaesthetized (sodium pentobarbital 0.25 mg/kg i.p.) and trans-cardiacly perfused with saline (37°C) followed by cold 2.5% glutaraldehyde. Temporal bones were collected and cochleae decalcified in 0.1M EDTA in 0.1M phosphate buffer. Histology methodology is described in papers I, II, and IV. Briefly; after decalcification, the cochleae were dehydrated, embedded in plastic (JB-4), and sectioned. When the midmodiolar plane was reached, every fourth section was mounted on glass slides and stained with Paragon. Six consecutive sections were chosen where all Rosenthal’s canal could be seen. All spiral ganglion neurons considered to be type I within Rosenthal’s canal were counted and the outline of the canal was traced (Sigma ScanPro 4 software) to estimate the area. Density was then expressed as the number of spiral ganglion neurons per 10,000 µm².

In papers III and V, stereology, another method for investigating the spiral ganglion, was used; methodology is described in paper V. Briefly; after decalcification and dehydration, cochleae were embedded in plastic and sectioned consecutively throughout cochlea. The sections were mounted on slides and stained with haematoxylin and eosin. A Zeiss Axioplan microscope with a motorized stage and an electronic microcator for measuring movements in the z-direction was interfaced with a digital camera. The optical fractionater technique (Gundersen et al. 1988; Møller et al. 1990; Watanabe et al. 2010) was applied to estimate the total number of remaining spiral ganglion neurons in Rosenthal’s canal using NEW CAST software from Visiopharm. In paper V, the somas and nucleus volume of the spiral ganglion neurons were estimated with the nucleator technique (Gundersen et al. 1988; Tandrup 1993), also with NEW CAST software. Briefly; a point at the center of the nucleus was marked as the sampling unit. From the sampling unit four lines were randomly placed and the point where the lines crossed the cell wall was manually marked and the software calculated the volume of the cell soma.
2.11 Statistical analysis

In papers I, II, and IV, one-way ANOVA statistical analysis was used for eABR studies and for the calculation of spiral ganglion neuron density, followed by Tukey post hoc test. In papers III and V, one-way ANOVA was used for the eABR studies followed by Tukey as post hoc test. For studies of the remaining spiral ganglion neurons and the measurement of the somas and nuclei size, one-way ANOVA was used followed by Holm-Sidak post hoc test. Data are presented as mean ± SEM.
3 RESULTS AND DISCUSSION

3.1 Paper I – In vivo infusion of UTP and uridine to the deafened guinea pig ear: effects on response thresholds and neural survival

It is well known that neurotrophic factors can maintain and protect spiral ganglion neurons against degeneration. This study demonstrates that both UTP and uridine are promising drugs for SGN preservation and maintenance of their electrical responsiveness, thus increasing potential cochlear implant benefits. It also indicates that nucleotides and nucleosides are involved in the neurotropic support in the inner ear. Our hypothesis was that nucleosides and nucleotides can act as trophic factors, protecting the spiral ganglion and preserving auditory function after ototoxic damage. In a pilot study, we tested four different nucleosides and nucleotides. The UTP-treated group was the only group where all animals showed improved eABR thresholds compared to the control group (not treated) that received artificial perilymph (AP). Based on these results the study continued with UTP and its degradation product uridine. Twenty-four animals underwent surgery and were deafened by 48 hours cochlear infusion of the ototoxic compound neomycin sulfate, followed by three weeks of treatment (UTP/uridine/control).

At the end of the experiment both the UTP and uridine treated groups showed a significant difference in eABR thresholds (p<0.001) compared to the control group. Morphological examination showed a significantly higher (p<0.001) SGN density for the UTP and the uridine treated groups than for the control group. In this study drug treatment was delivered into the inner ear immediately after deafening. However, SGN degeneration takes time (Kanzaki et al. 2002; Zappia and Altschuler 1989) starting the first week after the sensory cells death and continues for months. This and SGN soma size could be among important factors affecting this study results. SGN soma size has been reported to increase when treated with BDNF (Glueckert et al. 2008; Shepherd et al. 2005). SGN soma diameter was measured in this study and we found an increase in the group treated with AP. Average soma diameter was 21.5 (SEM ± 1.6) µm compared to the UTP treated group were it was 18.3 (SEM ± 0.8) µm. Average soma diameter in normal animals (measured in another study from this lab) is 14.7 (SEM ± 0.13) µm. This indicates that neomycin affects the SGN soma size but treatment with UTP in part inhibits that effect. The reason for this change in soma size is not known. It has been suggested that increase in soma size after exogenous BDNF delivery in deafened animals is concentration dependent, but the underlying mechanism is unclear (Shepherd et al. 2005).
3.2 Paper II – Post-Treatment effects of local GDNF administration to the inner ears of deafened guinea pigs

In paper II the clinical situation was mimicked with a delayed onset of GDNF-treatment in deafened guinea pigs, followed by an antioxidant (Trolox and ascorbic acid) post-treatment to assess the effects of GDNF treatment regarding the SGNs responsiveness and survival. Guinea pigs were transtympanically deafened with neomycin sulfate and after three weeks six animals were sacrificed and the remaining deafened animals underwent surgery for cochlear implantation and implantation of an osmotic pump for intracochlear infusion. Twenty-one animals received GDNF-treatment and eight animals received AP which served as control. After four weeks, five animals were sacrificed for morphological analysis. For the remaining animals, the pump was removed and the GDNF-treated animals were divided into two groups. One group received an antioxidant as post-treatment; the other group received saline. The control group (AP) received saline. Post-treatments were administered daily by i.p. injection for four weeks.

The eABR showed that two weeks after treatment initiation there was a significant difference (p<0.05) between the GDNF-treated groups and the control group. By week three, the difference was even more pronounced (p<0.001). EABR threshold for animals treated with AP increased throughout the experiment and after six weeks it was not possible to elicit any eABR from the control group using the present equipment. The GDNF-treated group displayed approximately the same eABR threshold throughout the eight experimental weeks, and no significant difference between the GDNF-treated groups was seen throughout the experiment.
The results from the morphological examination of the SGN showed that three weeks after deafness (at the time of treatment onset) there was a 40% loss of SGNs compared to the normal group \((p<0.001)\). At the end of the study the GDNF-treated groups with post-treatment had a 30% SGN loss compared to the normal group. There was no significant difference between the GDNF-treated groups. For the group that had received AP (no treatment) followed by four weeks of saline as post-treatment, there was a 70% SGN loss compared to the group with normal animals.

Three weeks of deafness might not be considered long from a clinical aspect but a 40% loss of SGN would correspond to a significant hearing loss. Results showed that without GDNF intervention SGN degeneration continues, probably due to the loss of neurotrophic support \((Mattson\ 1998)\). Gillespie and co-workers \((2003)\) report that cessation of neurotrophic treatment (in that case, BDNF) lead to a rapid decrease in SGN survival. In this study the survival of SGN remains at least 4 weeks after cessation of GDNF treatment. This could be due to differences in BDNF and GDNF activity at the cellular level or to different deafening techniques \((Gillespie\ used\ i.v.\ injection\ of\ furosemide\ and\ a\ subcutaneous\ dose\ of\ kanamycin\ sulphate)\). It is possible that Gillespie’s deafening technique damaged the SGN so severely that it was not possible to reverse. Also there were no measurements of SGN electrical function in that study.

**Figure 3.2**

A; Recordings of eABR thresholds throughout the experiment

B; SGN density in all groups from the experiment
3.3 Paper III – Cometin is a novel neurotrophic factor that promotes neurite outgrowth and neuroblast migration in vitro and supports survival of spiral ganglion neurons in vivo

Paper III is a collaboration among several research groups investigating the novel secreted neurotrophic factor protein Cometin. Among other techniques, the paper presents sequence analysis, cloning and production, in situ hybridization, and dorsal root ganglion culture. Here for the first time Cometin is tested as treatment for inner ear damage. Guinea pigs were neomycin deafened and treated with Cometin or AP (control group) for 12 days and studied for two additional weeks. EABRs were measured weekly, and after the last measurement the cochleae were collected for morphological analysis. After 12 days treatment a significant difference (p<0.001) was found in the eABR measurements, and the difference increased throughout the experiment. With the use of a stereological method, the morphological examination showed that the remaining spiral ganglion neurons for Cometin treated animals were approximately 40%, that of normal animals. For the control group treated with artificial perilymph, only 17% of the SGN remained.

In Cometin treated animals even after cessation of treatment, the SGN is still functional indicating that Cometin is comparable to other well-known neurotrophic factors such as GDNF and BDNF (Agterberg et al. 2009; Fransson et al. 2010) regarding the preservation and maintenance of the SGNs electrical responsiveness. This suggests that Cometin supports neuronal survival or changes the local milieu, permitting SGN survival despite the loss of trophic support from the sensory cells. Taken together, these studies show that Cometin is a new neurotrophic factor with therapeutic potential.
3.4 Paper IV - Dendrogenin A and B, two new alkylaminooxysterols increasing neural responsiveness in an animal model

The goal of this study was to investigate the therapeutic potential of two new steroids; Dendrogenin A (DA) and Dendrogenin B (DB). Guinea pigs were deafened with a 48-hour intracochlear infusion of neomycin sulfate. All animals received a cochlear implant and an osmotic pump for intracochlear infusion. Two studies were performed. The first was Immediate treatment; 48 hours of neomycin sulfate infusion followed by four weeks of treatment with DA, DB, or GDNF, a well-known neurotrophic factor used for comparison. Control animals were treated with AP. After four weeks treatment the pump was removed and the animals remained in the study for an additional two weeks to investigate if the if the SGN could preserve the electrical responsiveness after cessation of treatment. In the second study, Delayed treatment, the animals received AP for two weeks immediately after deafening, followed by four weeks of DA, DB or AP treatment. Both experiments lasted for six weeks and during this time eABR was measured once a week. After the last eABR measurement the cochleae were collected for morphological analysis.

EABR results from the Immediate treatment group showed a statistically significant difference (p<0.05) for both steroid treated groups compared to the control group from the second week of treatment, and the significant difference increased throughout the entire experiment. In the Delayed treatment study, the DA group showed a different pattern. At week four (after two weeks treatment) both the DA and the DB groups showed statistically (p<0.01) lower eABR thresholds compared to the control group. However, at weeks five and six, eABR thresholds of the DA group had increased so that there was no statistical difference between the DA group and the control group. The statistical difference between the DB and control groups increased throughout the experiment, and from week five there was also a significant difference (p<0.001) between the DA and the DB groups.

Results from the morphological investigation were unexpected. In the Immediate treatment study both the DA and the DB groups showed low eABR thresholds, indicating that the majority of the SGN population remained and retained the ability to respond to electrical stimulation. The control group (AP) showed (as expected) significantly higher eABR thresholds. However, all groups showed very low SGN density, although the SGN density in the DB group was slightly higher (p<0.05) compared to the control group. In the Delayed treatment group, DB showed increased but still reasonably low eABR thresholds throughout the experiment, which would indicate a large population of remaining SGN; however the morphology showed that for all groups there were very few remaining SGNs. These findings show that even with a small SGN population, an eABR response is possible and that it does not always correlate with the SGN population. This phenomenon has also been seen in studies of cochleas in temporal bones from patients (Khan et al. 2005; Nadol and Eddington 2006) assessed by measures such as; audiometry, word recognition, and speech perception, i.e., there is a weak correlation between the SGN population and the outcome of
the cochlear implant. It also suggests that functional efficacy can be modified by drug-based therapies.

3.5 Paper V – Morphological changes in the inner ear of the experimentally deafened guinea pig

Guinea pigs were deafened bilaterally with transtympanically infused neomycin sulfate, and were then divided into five groups; a normal group of animals served as a control group. The animals were sacrificed at different time-points after deafening; (weeks 1, 4, 7, 10, and 12) to investigate morphological changes in the inner ear. In the 12-weeks post-deafening group six animals received a cochlear implant and an osmotic pump containing GDNF (n=3) or AP (n=3). The contralateral ears from these six animals served as 18 weeks deaf comparison. The investigation of the total number of remaining SGNs showed that after 1 week of deafness there was a 15% decrease in the number of SGNs (p<0.01) compared to the normal group. After a 4 week period of deafness, the number of neurons had decreased by 60% (p<0.001) compared to the normal group. For the groups deaf 7, 10, 12 and 18 weeks there was no difference among the groups; all had lost approximately 75% (p<0.001) of the SGN compared to the normal group.

For the two groups that received a cochlear implant and were treated with either GDNF or AP, no eABR thresholds were detected from either group. When we investigated the amount of remaining SGN, we found however a statistically significant difference (p<0.05) in favor of the GDNF treated group.

The volume of the SGN somata and nuclei were investigated in groups 4, 10, 18 weeks deaf and in the group of normal animals. The soma volume for 4, 10, 18 weeks deaf showed 20
a decrease of 25-30% compared to the normal group, and this was statistically significant p<0.001 in all groups. The volume of the nuclei for the same groups showed a 20% loss compared to the normal group. For the 4 and 18 weeks deaf groups, there was a significant difference (p<0.05) compared to the normal group. For the 10 weeks deaf, the statistical difference was p<0.001 compared to the normal group.

Regarding the soma and nucleus volume in the GDNF and AP treated groups, no significant difference was found between the groups, but both groups showed a statistical difference (p<0.001) for both soma and nucleus volume compared to the normal group.

Figure 3.5 Total number of remaining SGN after neomycin deafening
4 GENERAL DISCUSSION

Deafness, with loss of sensory cells results in a progressive degeneration of SGN due to lack of neurotrophic support. Neuronal deafferentation due to neurotrophin loss can lead to a change in oxidative state (formation of free radicals) and changes in intracellular Ca\(^{2+}\) (Miller et al. 2002). Elevation of Ca\(^{2+}\) and oxidative stress contribute to damage of proteins, lipids, and DNA and finally to cell death (Mattson 1998).

Various mechanisms might play a role in SGN degeneration such as the absence of neurotrophic support, absence of neural activity, and glutamate excitotoxicity.

Intervention therapies with neurotrophic factors in experimental animals help preserve electrical responsiveness and prevent auditory nerve cell death. Here we show that other drugs such as nucleosides, nucleotides, and oxysterols can be used as therapeutic agents. Studies (Gillespie et al. 2003; Richardson et al. 2005) have shown that a single dose of neurotrophic factors (NT-3, BDNF) is not sufficient to prevent SGN loss. However, chronic (at least for several weeks) intracochlear infusion of neurotrophic factors can prevent SGN loss in deafened animals (Agterberg et al. 2009; Miller et al. 1997; Shepherd et al. 2005).

It has generally been assumed that there is a correlation between electrical thresholds and the total number of SGN (Shinohara et al. 2002; Yamagata et al. 2004). During the last decade researchers have received access to human temporal bone from patients who during their lifetime received a cochlear implant. This patients have usually several times pre-mortem executed psychophysical measurements (e.g. speech-reception, single-syllable word test) and now it has become possible to compare the functional outcome with histopathological findings. So far there is no explicit clinical evidence that enhanced SGN survival results in improved performance with a cochlear implant (Incesulu and Nadol 1998; Khan et al. 2005; Nadol et al. 2001).

Similar results were found in the study reported here in paper IV when we investigated two new oxysterols as therapeutic agents for inner ear damage. Deafened guinea pigs treated with oxysterol showed low eABR thresholds, that from our previous experience would indicate a large SGN population. However, the morphology showed that the SGN population was not higher in the treated groups than in the control group. Obviously there is not always necessary with a large SGN population and There is no easy explanation for these findings, but a possible reason is that the functional efficacy can be modified by drug-based therapies.

A major topic that needs to be further explored is the assumption that more and better functioning SGN would result in better implant performance. Another hypothesis is that a minimum number of SGNs is required for electrical hearing and that an increase above this minimum would have little functional impact (Blamey 1997).

Clinical use of osmotic pumps (currently available for use in animal models) for chronic intracochlear delivery is not feasible. Infusion of neurotrophic factors via an osmotic pump could involve a high risk of infection. In the future it may be possible to use electrode coated with a therapeutic agent that would reduce the risk for infection. Biodegradable
hydrogen or alginate beads placed on the round window niche and slowly releasing the neurotrophic factor has been proven to be effective (Ito J et al. 2005; Noushi et al. 2005) and may be clinically feasible.
5 FUTURE PERSPECTIVES

Today’s research that show so good results in animal models with e.g., osmotic pumps used for intracochlear infusion of neurotrophic factor is not a technique that could easily be transferred to the clinic. In the future, research needs to focus on other techniques such as biodegradable hydrogels or alginate beads that can be placed in the round window niche and slowly release e.g., growth factors over a long period of time. These techniques have already been tested in animal models and the results are promising but still needs to improve to match the good results seen after intracochlear infusion. Other options can be a device that could be placed in the inner ear at the same time as the patient receives his or her cochlear implant and then slowly release a therapeutic drug.

Even if there is no explicit clinical evidence that the amount of the surviving SGN correlates with the outcome from the cochlear implant, it should be considered that with improved cochlear implant technology the number of SGN might become more important.

Finally, it is important to develop a method that do not demand repeated operations to refill the supply of the therapeutic drug since according to present studies (Richardson et al. 2005) a single dose does not preserve the SGN.
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7 REFERENCES


