From the Department of Clinical Science, Intervention and Technology
Division of ENT Diseases
Karolinska Institutet, Stockholm, Sweden

# Regeneration of the auditory nerve - a cell transplantation study

Björn Palmgren

Karolinska
Institutet

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*"The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!) but rather, "hmm.... that's funny...."*

*- Isaac Asimov*

# ABSTRACT

Since in mammals, the hair cells or the spiral ganglion neurons (SGNs) in the inner ear do not regenerate, damage to these cells is an irreversible process. Presently the only aid for patients with severe to profound hearing impairment due to damaged hair cells is a cochlear implant (CI). A CI converts sound to electrical signals that stimulate the SGNs via an electrodes that is implanted into the cochlea. Hence, for a successful outcome the CI is dependant on the activation of the auditory nerve. There are several conditions, diseases or even traumatic events that primarily may impair the function of the SGNs in the auditory nerve. It is also known that in the absence of nerve stimuli due to hair cell damage, the SGNs will eventually degenerate. Lately there has been an increasing interest in regenerative medicine and bioengineering. This thesis presents results from in vivo experiments aiming to replace or repair the injured SGNs with the use of transplanted stem cells or neuronal tissue. All transplanted cells were labeled with a green fluorescent protein facilitating identification in the host animal.

**Paper I** presents a new animal model of selective auditory nerve injury with preserved hair cells. The lesion was induced in rats by the application of β-bungarotoxin to the round window niche. Immunohistochemical straining confirmed the loss of SGN while the hair cells were kept intact. The induced hearing impairment was verified by auditory brain stem response (ABR).

**Paper II** presents a surgical approach for the injection of stem cells to the auditory nerve by the internal auditory meatus (IAM). It was shown that this approach does not significantly affect the hearing as verified by ABR. Further, neuronal tract tracing with the enzyme horseradish peroxidase illustrated that injection of selected substances may be distributed by intra-axonal transportation centrally to the brain stem as well as peripherally to the cochlea. Furthermore it was illustrated that statoacoustic ganglions transplanted by the IAM survived for up to five weeks, though in low numbers. No cells had migrated through the Schwann-glia transitional zone into the cochlea.

**Paper III** presents an assessment of mouse tau-GFP embryonic stem cells transplantated to the auditory nerve trunk by the IAM or into the modiolus in previously deafened rats. It was shown that supplementary treatment of BDNF in a bioactive peptide amphiphile (PA) nanogel increased

survival and neuronal differentiation of the transplanted cells. It was also demonstrated that supplement of the enzyme chondroitinase ABC in PA gel facilitated migration of transplanted cells through the transitional zone.

**Paper IV** presents the use of human neural progenitor cells for transplantation to the auditory nerve by the IAM. We further assessed supplement of BDNF in the PA gel. After three weeks, survival and differentiation of the transplanted cells were observed. After six weeks of survival the majority of the surviving cells had differentiated into neurons. The addition of BDNF in PA gel significantly increased both survival and differentiation. The transplanted cells migrated to the brain stem and formed neuronal profiles including extensive arborisation of nerve fibers in the vicinity of the cochlear nucleus.

In conclusion, this thesis presents a new animal model for a selective lesion of the auditory nerve. Further, promising results were demonstrated regarding the possibility of replacing auditory SGNs including increased rates of survival and neuronal differentiation of the transplanted cells in the presences of BDNF. These results suggest for further studies on auditory nerve replacement but also for functional assessment of the transplanted cells.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I.    **Palmgren B**, Jin Z, Ma H, Jiao Y, Olivius P. β-Bungarotoxin application to the round window: an in vivo deafferentation model of the inner ear. Hearing Research 2010 Jun 14;265(1-2):70-6. Epub 2010 Feb 23

II.   **Palmgren B**, Jin Z, Jiao Y, Kostyszyn B, Olivius P. Horseradish peroxidase dye tracing and embryonic statoacoustic ganglion cell transplantation in the rat auditory nerve trunk.
Brain Res. 2011 Mar 4;1377:41-9. Epub 2011 Jan 6.

III.  **Palmgren B**\*, Jiao Y\*, Novozhilova E, Stupp S, Olivius P. Survival, migration and differentiation of mouse tau-GFP embryonic stem cells transplanted into the rat auditory nerve. Unpublished data - submitted

IV.   Jiao Y\*, **Palmgren B**\*, Novozhilova E, Spieles-Engemann A, Englund-Johansson U, Stupp S, Olivius P. Survival, differentiation and migration of human neural progenitor cells transplanted to the auditory nerve in a rat model of neuronal damage. Unpublished data - submitted

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABR | auditory brainstem response |
| ABI | auditory brainstem implant |
| AN | auditory nerve |
| β-BuTx | β-bungarotoxin |
| BDNF | brain derived neurotrophic factor |
| BS | brain stem |
| ChABC | chondroitinase ABC |
| CI | cochlear implant |
| CN | cochlear nucleus |
| CNS | central nervous system |
| DAPI | 4',6-diamidino-2-phenylindole |
| E13 | embryonic day 13 |
| EDTA | ethylenediaminetetraacetic acid |
| ESC | embryonic stem cell |
| GAG | glycosaminoglycan |
| GFP | green fluorescent proteine |
| HRP | horseradish peroxidase |
| IAM | internal auditory meatus |
| IKVAV | isoleucine-lysine-valine-alanine-valine |
| NT-3 | neurotrophin 3 |
| PA | peptide amphiphile |
| PBS | paraformaldehyde |
| PNS | peripheral nervous system |
| ROI | region of interest |
| SGN | spiral ganglion neuron |
| ST | scala tympani |
| TUJ1 | β tubulin antibody |
| TZ | transitional zone |

# INTRODUCTION

## The ear and hearing

The ear is a sensory organ residing both the auditory system, responsible for sound detection, and the vestibular system being the basis for our maintenance of balance. Anatomically, the ear can be divided into the external -, the middle - and the inner ear.

### *The external ear*

The external ear is composed of the auricle that is the most external portion of the ear, the ear canal and the outer part of the eardrum. The external ear acts as a reflector that captures and compresses sound waves transferring them to the middle ear.
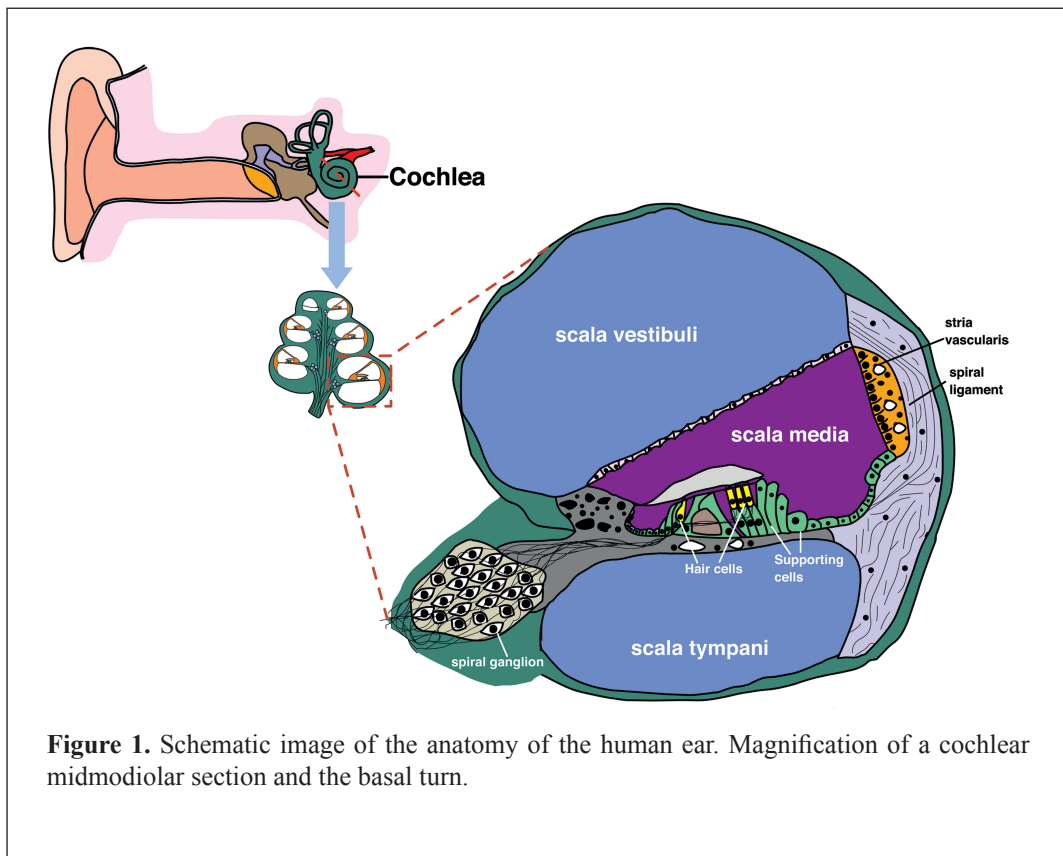


**Figure 1.** Schematic image of the anatomy of the human ear. Magnification of a cochlear midmodiolar section and the basal turn.

### *The middle ear*

This is an air filled cavity containing the middle ear ossicles, the malleus, the incus and the stapes. The main purpose of the middle ear is to conduct, amplify and trans-

form sound pressure on the tympanic membrane to mechanical energy. The malleus is attached to the eardrum conducting the energy and the frequencies of the sound waves along the chain of ossicles, via the stapes, to the inner ear. As the stapes is connected to the oval window, the oscillating energy is transduced to generate waves in the fluids of the inner ear cochlea.

*The inner ear*
The inner ear includes both the organ of hearing, the cochlea, and the vestibular organ dedicated to the maintenance of balance and motion (**Fig. 1**). The inner ear is embedded deep into the scull within the temporal bone. The cochlea is a spiral formed conical structure with three fluid filled compartments; scala tympani (ST), scala medium (SM) and scala vestibuli (SV). These compartments are separated from each other by the Reissner's membrane (between SM and SV) and the basilar membrane (between SM and ST). On the basilar membrane lies the true hearing organ, the Organ of Corti. Here the hair cells, organized in one row of inner hair cells (IHC) and three rows of outer hair cells (OHC), convey the mechanical signals from the fluid in the scala tympani into electrical signals. In humans, there are about 15 000 hair cells (Wright, Davis et al. 1987) while in rats there are about 5000 (Keithley and Feldman 1982). As the waves in the cochlear fluids reach the hair cells, the stereocilia of the IHC release neurotransmittors that trigger afferent action potentials in the nerve endings of the spiral ganglion neurons (SGNs). The OHC on the other hand have afferent and efferent innervation and are believed to provide mechanical feedback amplification resulting in better frequency selectivity and hearing sensitivity (Dallos and Corey 1991). The SGNs are a group of nerve cells that can be found in the conical central axis of the cochlea, the modiolus. Together they form the auditory nerve, also named the cochlear nerve or the auditory portion of the vestibulo-cochlear nerve (cranial nerve VIII). The number of SGNs in humans is estimated to be around 35 000-50 000 (Otte, Schunknecht et al. 1978; Bear, Connors et al. 2006) and they are divided into two types. Type I SGNs innervate the IHCs and in the humans they comprise about 88 % of the total numbers of SGNs (Webster and Fay 1992). The type I SGNs are myelinated and bipolar neurons, while the type II neurons are unmyelinated unipolar neurons innervating the OHCs (Spoendlin 1972). The afferent signals triggered by synapses from the hair cells are conducted through the SGNs, initially to the cochlear nuclei in the brain stem and then via further relay stations to the final destination, the auditory cortex in the temporal lobe. Here, the brain translates electrical impulses into an audible sound.

## Hearing disorders
In 2005, about 278 million people had a moderate to profound hearing impairment (HI). Out of those, 80 % lived in low and middle income countries (WHO 2010).

Currently, less than 10 % of people who need a hearing aid have got one. Except for the inability to interpret sounds resulting in stigmatization and reduced ability to communicate, there may be several other consequences of HI including economic and educational disadvantages as well as social isolation (Mathers, Smith et al. 2000). Deafness refers to a complete hearing loss of both ears. HI refers to people with a complete or partial loss of hearing from one or both the ears and can be divided into conductive or sensorineural HI according to which part of the hearing chain that is affected. Conductive HI means that the cause of the impairment is found in the outer or middle ear. There are several causes for conductive HI including lesions of the tympanic membrane, chronic middle ear infections, cholesteatomas, otosclerosis and lesions of the bony ossicles. Frequently, restorative surgery will considerably improve the hearing ability. Sensorineural HI is caused by a problem within the inner ear, a cochlear HI, or exists as a retro-cochlear HI in the auditory nerve, the brain stem or the cortex of the brain. Sensorineural HI is usually permanent and may require rehabilitation with an external hearing aid or, in very selected cases, the use of a cochlear implant (CI). The causes of sensorineural HI most commonly include loss of hair cells due to age, noise trauma, ototoxic pharmacological agents or as a sequela to an infection.

In this thesis, the main scope has been to investigate a possible future way of treating lesions in the auditory nerve, i.e. a retro-cochlear HI. From several animal models we know that due to lack of neurochemical stimulation, hair cell loss eventually results in SGN degeneration (Webster and Webster 1981; Bichler, Spoendlin et al. 1983). However in humans, following hair cell death, the SGNs seem to survive for a longer period as compared to rodents (Spoendlin 1975; Bichler, Spoendlin et al. 1983). Other reasons for SGN- and auditory nerve lesions include tumors (most commonly caused by neurofibromatosis type 2), surgery, trauma and auditory neuropathy (Starr, Sininger et al. 2000; Odat, Piccirillo et al. 2011). In auditory neuropathy the pathophysiological mechanisms are not yet fully understood but it was shown that the ABR thresholds were significantly elevated, a finding that does not correspond to the relatively normal otoacoustic emissions (Harrison 1998). Hearing impairment in auditory neuropathy was further suggested to occur either in isolation or due to a generalized neuropathic process (Starr, Picton et al. 1996). Along the peripheral auditory pathway the affected locations may vary but they include the outer hair cells, the SGNs and the auditory nerve fibers (Starr, Sininger et al. 2000).

## Neurotrophic support of the hearing

During the embryonic and postnatal developmental stages neurotrophic factors regulate survival and differentiation of neurons throughout the nervous system. They are also important for the maintenance of the synaptic connectivity and plasticity

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in the adult nervous system (Maness, Kastin et al. 1994; Terenghi 1999). There are several neurotrophic factors present in the auditory system including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), neurotrophin 4/5 (NT 4/5) and glial derived neurotrophic factor (GDNF). Here, we focus on the effects of two of the most studied factors: BDNF and NT-3. Since these factors bind to receptors of the Trk family, TrkB and TrkC respectively, that are present in all SGNs (Fritzsch, Silos-Santiago et al. 1997), these neutrotrophins are vital for the SGNs. Studies have shown that mice with homozygous deletions of both NT-3 and BDNF illustrate a 100 % SGN loss, which is also exemplifying the importance of these factors for development of the auditory nerve (Ernfors, Van De Water et al. 1995). Furthermore, mature human SGNs express TrkB and TrkC and are supported by exogenous BDNF and NT-3 (Roehm and Hansen 2005; Liu, Kinnefors et al. 2011) which also emphasizes the importance of these factors throughout life for maintenance of the SGNs (Ylikoski, Pirvola et al. 1993; Wheeler, Bothwell et al. 1994). The organ of corti is the primary source of BDNF and NT-3, but there are also other sources including the supporting cells, the Schwann cells, the cochlear nucleus neurons and the SGNs themselves (Frostick, Yin et al. 1998; Hansen, Vijapurkar et al. 2001; Hansen, Zha et al. 2001; Zha, Bishop et al. 2001; Stankovic, Rio et al. 2004).

Except for the regular maintenance of the inner ear, BDNF and NT-3 have also been shown to have a neuroprotective effect on the auditory pathway. Staecker and co-workers have shown that guinea pigs exposed to an ototoxic combination of an aminoglycoside and a loop diuretic displayed a significantly better SGN survival following an infusion of either BDNF or NT-3 during an eight weeks period (Staecker, Kopke et al. 1996). Similar results have also been shown in other studies illustrating the neuroprotective effect of BDNF and NT-3 (Ernfors, Duan et al. 1996; Lalwani, Han et al. 2002).

## Delivery techniques for administration of substances into the cochlea or the auditory nerve

Except various surgical approaches there are also several different administration options for the delivery of substances into the inner ear. For all the delivery approaches, substances may be distributed by a direct injection, infusion or via application of slow release compounds (i.e. gels or gel foams). There are also other modes of delivery, e.g. neurotrophic factors may be delivered via cell- or gene-based therapies. An example of the latter is that CI electrodes coated with genetically modified BDNF-producing fibroblasts have been shown to preserve significantly more numerous SGNs as compared to control electrodes (Rejali, Lee et al. 2007). Furthermore, in a number of studies transfer of BDNF-, GDNF- and NT-3 genes

into the cochlea have resulted in protection of SGNs (Staecker, Gabaizadeh et al. 1998; Yagi, Kanzaki et al. 2000; Lalwani, Han et al. 2002) showing that the persistence of gene expression may last for months (Li Duan, Bordet et al. 2002). Systemic administration of drugs by oral, intramuscular or intravenous routes is also a method of delivery. Apart from the easy access, these methods have unacceptable side effects, difficulties for many molecules to pass the blood-brain barrier and also, problems in getting sufficient concentrations of agents in the inner ear (Juhn 1988; Ekborn, Laurell et al. 2002). Thus, a local or regional administration appears to be a more attractive option for a substance delivery into the cochlea or the auditory nerve.

*Cochlea*
The cochlear anatomy constitutes several approaches but also challenges for delivery of neurotrophic factors and toxins.

The easiest way of delivery of substances into the inner ear is via an intra-tympanic injection. Intra-tympanic injections of gentamicin is frequently used in patients with Ménière's disease (Ghossaini and Wazen 2006). It is also possible to deliver drugs via a tympanostomy with application of gels or other matrixes. The middle ear is then used as a reservoir for the drug, which will be passively transported into the inner ear preferentially via the round window membrane. These methods may therefore develop a basal to apical concentration gradient in the cochlea. Such a gradient was measured in guinea pigs that received a continuous gentamicin application to the round window membrane resulting in a concentration in the scala tympani base 4000 X higher than that of the apex (Plontke, Mynatt et al. 2007). Round window delivery of antioxidants have proved to be effective for protecting the inner ear from exposure to noise or ototoxins (Chen, Ulfendahl et al. 2004; Wimmer, Mees et al. 2004). Several animal models of hair cell damage have used intratympanic administration of ototoxic drugs as the mode of choice (Wagner, Caye-Thomasen et al. 2005; Hashimoto, Iwasaki et al. 2007). Agents may also be delivered via a cochleostomy or an injection through the round window membrane directly into the fluid-filled compartments of the cochlea. These approaches facilitate for the drugs to reach higher concentrations, even though they are more invasive as compared to middle ear applications. A common way of administering the drugs is by the use of mini-osmotic pumps (Ekborn, Laurell et al. 2003; Hu, Ulfendahl et al. 2005). These devices distribute the drugs over time through a cannula at a predetermined constant rate. In humans, this is not considered a viable technique except for a limited delivery period as the implanted cannula may cause infections such as meningitis and labyrinthitis (Pettingill, Richardson et al. 2007).

*Spiral ganglion neurons and the auditory nerve*
All delivery methods described in the Cochlea section above can also be applied

to a targeted delivery to the SGNs and the auditory nerve. Depending on the nature of the drug or toxin the effect may either be aimed selectively to the hair cells, the SGNs or concomitantly. It is also possible to administer agents into the modiolus even though this involves hazardous surgery where the cochlear homeostasis may be put at risk. In previous experiments, we have also pointed out the possibility of injecting the factors or similar agents by an occipital approach targeting the auditory nerve by the internal auditory meatus (IAM) (Palmgren, Jin et al. 2011) (Palmgren, Jiao et al, submitted). This approach involves intracranial surgery but preserves the cochlear homeostasis.

## Cochlear implants

The CI is a surgically implanted most advanced electronic device that may partially restore the hearing starting from the external ear to the SGNs. The CI is placed behind the ear pinna and consists of external (microphone, speech processor and a transmitter) and internal (receiver, stimulator and electrodes) devices where the latter is placed under the skin with electrodes reaching inside the cochlea. The external part receives the sound waves and converts them into digital signals. The signals are processed and sent to the implant where the receiver again converts the signal to electrical signals that are sent through the electrodes. In the cochlea, the electrodes stimulate the SGNs, thereby probably bypassing all the hair cells. As of December 2010, approximately 219 000 people had received CIs worldwide (FDA 2011). People of all ages with a bilateral severe to profound hearing impairment can benefit from a CI. However, it is important to point out that a minimum number of functioning auditory nerve fibers are needed for a CI to function sufficiently. This and similar requirements may therefore exclude several patients as CI candidates, even though they suffer from a profound hearing impairment or deafness. Still SGN degeneration may not be a major problem in patients with a short history of profound hearing impairment, since, for a successful outcome of a CI implantation, as low numbers as 10 % of the original SGN population has been reported to be sufficient (Linthicum, Fayad et al. 1991; Khan, Handzel et al. 2005). With the ongoing advances in CI technology, however, in the future, SGN degeneration may become an increasingly limiting factor for optimal outcome of CI implantation. E.g. many patients are interested in having the possibility to better appreciate music that at present is not achievable to all CI users. If ever reachable in the clinic, this will certainly require most advanced strategies to utilize the dynamics in the auditory nervous system. However, an increased number of functioning SGNs may also contribute to such a development. Furthermore, future progress of research on hair cell regeneration may require improved innervation for potentially newly generated hair cells. There are also a number of patients that, because of a non-functioning AN, are not suitable for a CI. This can be due to lesions affecting the nerve e.g. neurofibromatosis, AN trauma or aplasia of the AN. Thus, as advances with CI technology

proceed it is crucial that new strategies should be aimed at preserving, regenerating or replacing the SGNs.

## Auditory brain stem implants

The auditory brain stem implant (ABI) may be a good alternative for patients with a sensorineural hearing impairment that are not suitable for a CI due to injury to the cochlea or the AN. The ABI uses a similar technology as the CI but the electrodes stimulate the cochlear nucleus in the brain stem instead of the SGNs. Examples of conditions that may require an ABI are neurofibromatosis type 2, aplasia of the AN and ossification of the cochlea (Colletti, Carner et al. 2005). An acoustic neuroma is often located along the AN and may result in total deafness either due to the tumor itself or to the surgical treatment aimed to have the tumor removed. Today, the surgical procedure for ABIs are considered safe with complications very rarely seen, In a study of 114 patients the complication-rate for surgery on non-tumor patients was as low as for a CI surgery (Colletti, Shannon et al. 2010). Still the possible complications may be more severe than those from CI surgery. As compared to the CI, the ABI is not as efficient since it primarily allows the detection and recognition of environmental sounds, but improves the speech recognition as compared to lip reading only (Schwartz, Otto et al. 2008). Still there are reports of occasional ABI patients, among children as well as adults, which have achieved speech recognition without lip reading (Nevison, Laszig et al. 2002; Eisenberg, Johnson et al. 2008; Skarzynski, Behr et al. 2009).

## Animal models of hearing impairment

In order to efficiently study the results of a SGN cell replacement strategy in vivo, a close-to-clinic animal model is preferred. Since we wanted to examine the possibility to regenerate or repair a selectively damaged rat auditory nerve, we decided to obtain an animal model with a damaged AN but with intact hair cells. By preserving the hair cells the inner ear would also retain an important part of its neurotrophic support. Thus, as compared to utilizing a model with a cochlear damage including destroyed hair cells the present model seemed to provide more appropriate neurotrophical- and electrophysiological conditions for studying an auditory neuronal lesion.

Previous techniques generating iatrogenic damage to the auditory nerve include an intracochlear injection of ototoxic substances (Miller, Chi et al. 1997; Hu, Ulfendahl et al. 2004). Aminoglycosides (McFadden, Ding et al. 2004) and cisplatin (Ding, Wang et al. 1999; Rybak, Whitworth et al. 2007) affect the SGNs but also the hair cells. Others have shown that application of ouabain to the round window leads to a partial to complete loss of the auditory nerve function whereas the hair

cells are kept relatively intact (Schmiedt, Okamura et al. 2002). There are indications that ouabain induces apoptosis in the type I SGNs while most type II neurons survive (Lang, Schulte et al. 2005). Others have shown that ouabain application to the round window leaves the inner hair cells intact but causes degeneration of the outer hair cells and limbal fibrocytes (Hamada and Kimura 1999). Another model of selective nerve damage is compression of the auditory nerve as performed by Sekiya and co-researchers. Here, a suboccipital craniotomy was performed followed by compression of the nerve and the labyrinthine artery between the brainstem and the temporal bone at the internal auditory meatus (Sekiya, Hatayama et al. 2000). In vitro rat cochlear studies also report that sodium salicylate can selectively induce auditory neuronal degeneration (Zheng and Gao 1996).
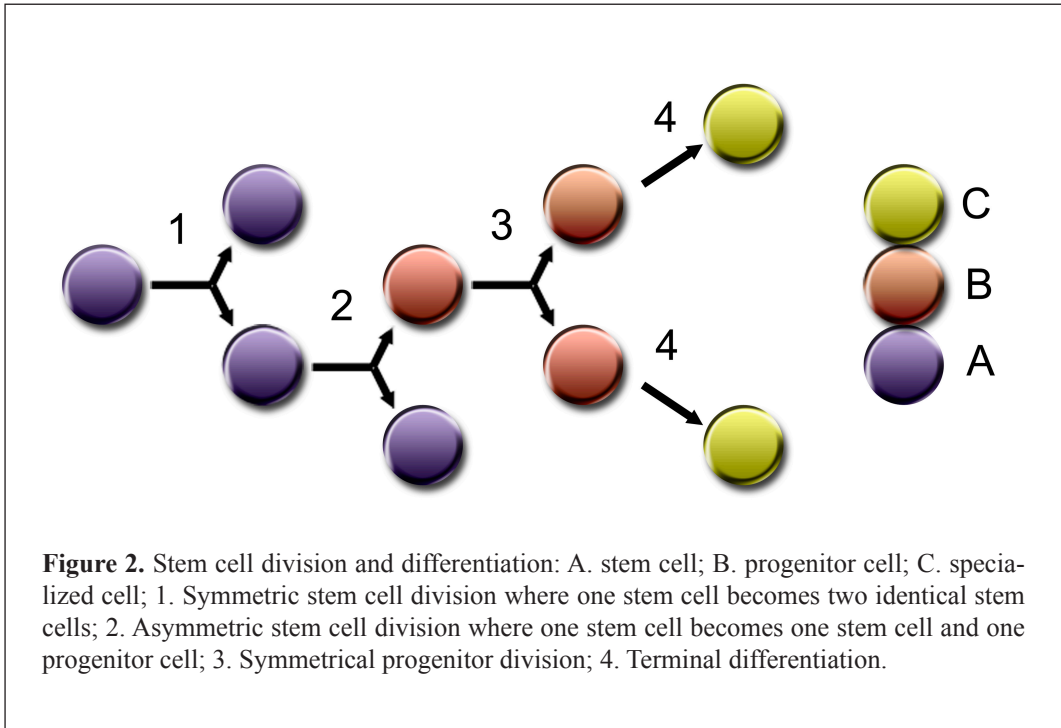
For our purposes the described techniques were not considered to be the most suitable models. Compression of the auditory nerve comprises rather traumatizing surgery as the nerve needs to be compressed by the internal auditory meatus. Several of the described toxins affect the hair cells.

β-bungarotoxin (β-BuTx) is a presynaptic neurotoxin isolated from the venom of the Taiwan banded krait (Bungarus multicinctus). It is composed of two subunits, A and B, where A has a phospholipase A2 activity and B selectively inhibits the voltage-dependent potassium channels (Bostrom, Khalifa et al. 2010). Earlier studies *in vitro* and *in vivo* on chicks have illustrated that the β-BuTx is deafferentating hair cells without traumatizing them (Hirokawa 1977; Martinez-Monedero, Corrales et al. 2006). Therefore, here the β-BuTx was used for the first time to develop a rodent animal model for a selective AN lesion, while sparing the hair cells.

## Stem cells

The definition of a stem cell (SC) is a cell with a capability of self-renewal and a potency to differentiate into diverse specialized cell types (**Fig. 2**). There are two major types of SCs. The embryonic stem cells (ESC) found in the inner cell mass of a blastocyst and the adult SCs found in various types of tissue including the bone marrow. Sometimes multipotent or oligopotent (oligopotency being more limited in the types of cells it can differentiate into) cells, also named progenitor- or precursor cells, are referred to as SCs.

The ESCs are toti- or pluripotent cells that can differentiate into any cell type in the three germ layers ectoderm, mesoderm and endoderm (Burdon, Smith et al. 2002). Assuming that we had the key for the correct differentiation signals, the ESC would be an ideal candidate for clinical cell transplantations. Even though the harvesting of ESC from embryos may raise ethical questions, they can be expanded in vitro for many years after isolation and subsequently differentiated into neural stem- or

**Figure 2.** Stem cell division and differentiation: A. stem cell; B. progenitor cell; C. specialized cell; 1. Symmetric stem cell division where one stem cell becomes two identical stem cells; 2. Asymmetric stem cell division where one stem cell becomes one stem cell and one progenitor cell; 3. Symmetrical progenitor division; 4. Terminal differentiation.

precursor cells or specialized mature cells (Kawasaki, Suemori et al. 2002; Lee, Shamy et al. 2007).

Progenitor cells have the potency of differentiating into a limited number of specialized cells. A SC can replicate an indefinite number of times, whereas progenitor cells can only divide a limited number of times. Progenitor cells often lie dormant in the tissue and possess a low activity but can, when needed, be activated into a proliferative state by various substances such as growth factors or cytokines and thus can be used as repair or rescue cells. Progenitor cells may be derived directly from fetal cells, adult tissues or by a directed differentiation of the ESC via a cell culture manipulation (Wichterle, Lieberam et al. 2002; Gaspard and Vanderhaeghen 2010).

Neural progenital cells (NPCs) are cells that give rise to neuronal- and glial cells in the embryonic, neonatal and adult nervous systems. These cells have been regarded as a potential treatment for a variety of neurological diseases and have been used in several clinical trials. In one study, in two patients, fragments from the adrenal medulla were autotransplanted to the right caudate nucleus. Continuous clinical improvements with diminished tremor and akinesia was observed for up to 10 months after surgery (Madrazo, Drucker-Colin et al. 1987). Later, others have shown positive results with cell transplantation in patients with Parkinson's disease (Freed, Greene et al. 2001). There are also ongoing trials with the NPCs transplanted to pa-

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tients with Huntington's disease (Bachoud-Levi, Gaura et al. 2006). Still there are several problems with NPC transplantation that remains to be solved. First, we need reliable methods to get enough NPCs for an autologous transplantation. Second, we need to regulate the cell neural plasticity of different adult stem cells i.e. how to efficiently incorporate the transplanted cells into the host neural system. Third, we need to learn more about how to regulate the differentiation of NPCs in the nervous system (Hsu, Lee et al. 2007).

New possibilities have emerged with the recent discovery of induced pluripotent stem (IPS) cells. IPS cells are generated from somatic tissues such as fibroblasts and are reprogrammed into ESC-like cells by the addition of transcription factors. Several groups have managed to successfully reprogram fibroblasts using various combinations of genes delivered by vector-, virus-, protein-, or RNA-mediated approaches (Takahashi, Tanabe et al. 2007; Cho, Lee et al. 2010; Yakubov, Rechavi et al. 2010). The discovery of the IPS cells may have huge implications for regenerative medicine since it may render possibilities to avoid hazardous immunological issues including implant rejection. Further, the IPS cells may constitute a reliable source of cells for the transplantation.

One remaining important problem for all kinds of stem cells including that of IPS cells is the risk of tumor formation. More or less all the genes that have shown to promote IPS cell formation are also linked to cancer induction. Indeed, some of these genes are oncogenes. Non-genetic methods have been reported using recombinant proteins for IPS cell production but the efficiency was low compared to the use of gene transfection (Zhou, Wu et al. 2009).

## Cell replacement strategies for the SGNs including the auditory nerve

One essential requisite for cell replacement strategies is to be able to deliver the restorative cells to the target with minimal trauma and to maintain the homeostasis of the host by avoiding damage to any residual function. In the inner ear, this is a challenge due to the location and highly specialized nature of the cochlea and the auditory nerve. Previously, the most commonly used approaches have been via a peripheral access to the auditory nerve through the cochlea and possibly through the modiolus. In these approaches, the membranes sealing the peri- and endolymphatic spaces are breached which may result in a disturbed homeostasis of the inner ear fluids. An alternative way to deliver drugs to the cochlea is by diffusion through the round window, however cells are non-diffusible material and will not pass through the membrane (Bianchi and Raz 2004).

Another approach would be to deliver cells to the central portion of the AN. We have used this method to inject cells to the AN by the internal auditory meatus. This was performed via an sub-occipital/intra cranial approach where, by retracting the cerebellum medially, the AN could be visualized being stretched between the brain stem and the cochlea. Here it is possible to inject cells directly into the nerve. Even though this demands for advanced surgery, there are several clinical situations where cell injections can be coordinated with other kinds of surgery where access to the nerve has to be obtained (e.g. surgery on a tumor). In humans, it would probably also be possible to inject cells with the assistance of stereotactical techniques without opening the meningi. Utilizing this central approach would also mean that the injected cells or their nerve fibers in order to reach the cochlea would have to pass the transitional zone, which is separating the central - from the peripheral nervous system in the auditory nerve. The transitional zone barrier appears to be an obstacle for this peripheral outgrowth of fibers and migration of cells (Palmgren, Jin et al. 2011). Other studies have demonstrated that the transitional zone hindered outgrowths of axons from the PNS to the CNS, but did allow axons from the CNS to pass into the periphery (Fraher 2000). In the present work, we have used chondroitinase ABC (ChABC) to facilitate the migration of cells and nerve fibers past the barrier (Papers III). ChABC is an enzyme with an ability to degrade chondroitin sulphate glycosaminoglycan (GAG) chains thereby making the transitional zone more "permeable" to the transplanted cells and their fibers (Grimpe, Pressman et al. 2005; Suzuki, Akimoto et al. 2007; Hyatt, Wang et al. 2010).

Apart from the delivery of exogenous cells, activation of potentially residing endogenous progenitor cells would be an attractive option for regeneration of the AN. Rask-Andersen and co-researchers have found neural progenitor cells in the adult human and guinea pig spiral ganglion cells suggesting that the SGNs have a potential to regenerate (Rask-Andersen, Bostrom et al. 2005). Since we have not identified any spontaneous regeneration of the hair cells or SGNs so far, the question of how to activate these progenitor cells to become functional neurons still remains unresolved.

# AIMS

The general aim for this thesis was to explore the possibilities of developing a novel cell replacement therapy of the auditory nerve including the spiral ganglion neurons. The specific aims for each individual paper were:

I.      To develop an animal model for selective lesions of the auditory nerve. To use this model in following cell transplantation studies.

II.     To develop a feasible surgical approach for cell transplantation to the central portion of the auditory nerve. To assess the distribution of selected substances injected by this approach. To evaluate transplantation of statoacoustic ganglion cells to the auditory nerve.

III.    To, in this pilot study, evaluate the result from tau-GFP ESCs transplanted to the auditory nerve by the internal auditory meatus or via the modiolus. To study the effects of BDNF and/or ChABC applications in PA gel on cell survival, differentiation and migration.

IV.     To evaluate the results from human neural progenitor cells transplanted to the auditory nerve. To assess the promoting effect of BDNF in PA gel on cell survival and neuronal differentiation.

# MATERIAL AND METHODS

## Animals

As the recipients in these *in vivo* experiments we used young female Sprague-Dawley rats (200-270 g). Green fluorescent protein (GFP) positive mice were used as cell donors (Papers II, III and IV). All animal experiments followed the national approved protocol for care and use of animals in Sweden

## Application of β-BuTx to the round window niche (Papers I–IV)

The deafening method developed in Paper I was further used in Papers II–IV.
Here, we applied 5 µl of β-BuTx (0.05 μg/ml, Alexis Biochemicals) absorbed by gel foam to completely fill the round window niche in the middle ear. A piece of fascia was placed to cover the hole in the bulla. In Paper I, evaluating this new animal model of selective damage to the primary auditory neurons, the survival period ranged from 3 to 21 days after which the rats were sacrificed. All animals in the study withstood the surgery and the toxin application well. In Papers II-IV no further surgery was performed until day 21.

## Auditory brainstem response measurements (Papers I and II)

To evaluate the electrophysiological hearing thresholds auditory brainstem response (ABR) measurements were performed. The ABR measurements were conducted under general anaesthesia. In a soundproof booth needle electrodes were placed on the vertex and below the recorded ear with the ground electrode placed on the hind leg. The initial intensity of the stimulus was 90 dB peak sound pressure level that was decreased in 5 dB steps until the ABR-curves disappeared. The ABR threshold was defined as the lowest intensity at which a visible ABR wave was observed in two averaged runs. In Paper I the ABR measurements were performed postoperatively on days 3, 7 and 21 and in Paper II immediately before and two weeks after surgery.

## Histology and immunohistochemistry (Papers I-IV)

Following perfusion, trimming of the bone and decalcification, all specimens were embedded and frozen in the OCT Compound. The specimens were orientated in the compound so that mid-modiolar sections would contain the cochlea, auditory nerve and brain stem (BS) as a continuum. 12 μm, mid-modiolar cryosections were made and mounted on glass slides.

The primary anti bodies used in these studies included:
1. Anti-neuronal class III b tubulin (TUJ1) antibody (1:200, Covance Research Products, Berkeley, CA, USA).
2. Rabbit polyclonal anti myosin VIIa (MYO7A) antibody (1:1000).
3. Conjugated Goat polyclonal to GFP (FITC conjugated) antibody (1:200; Abcam, Cambridge, UK).
4. Chicken polyclonal antibody to laminin (1:200 dilution; Abcam, Cambridge, UK).
5. Mouse anti-chondroitin sulfate (CS-56) antibody (1:200 dilution; Sigma -Aldrich).

Secondary antibodies conjugated with fluorescein isothiocyanate, goat-anti rabbit Cyanine 3 Alexa 488 or IgY (all from Jackson Immunoresearch Europe, Newmarket, Suffolk, UK) were used. Omission of the primary antibody served as negative control. Cell nuclei were stained with 4.6-diamidino-2-phenylindole (DAPI).

## TUNEL staining (Paper I)

The cochlear cryosections used for the TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling) staining were prepared as described for the immunohistochemistry above. The TUNEL assay was performed using an ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon (Millipore), Solna, Sweden) following the manufacturer's instruction. Substitution of the TdT enzyme with distilled water was used as a negative control.

## Cochlear surface preparation (Paper I)

The cochleas were dissected out from the temporal bone and fixed. After washing with PBS, the bony capsule surrounding the cochlea, the cochlear lateral wall and Reissner's membrane were removed. The remaining part of the cochlea was stained with TRITC-phalloidin (1:200 Sigma) for 45 minutes where after the basilar membrane containing the organ of Corti was dissected into half-turns. Each piece of the basilar membrane was placed in an 8-well microscopic slide to be examined under a fluorescence microscope (Zeiss) and documented with a digital camera (Nikon Coolpix 990). The contralateral cochleas served as the controls.

## HRP injections and HRP histochemical staining (Paper II)

For the HRP injections we used the occipital surgical approach as described above. A 10 μl Hamilton syringe attached with a 33-gauge needle was filled with 30 %

HRP (Type VI-A, Sigma) and was mounted in the clamping device of the stereotactic frame. By using a micromanipulator, a total volume of 4 µl of the HRP solution was injected into the nerve root. Two days following injections, the HRP animals were sacrificed and the left temporal bone, the auditory nerve and the adjacent brain stem were carefully excised in a single tissue block. After the decalcification and embedding, 12 µm serial cryostat sections were made. The cryostat sections were processed for HRP using the tetramethylbenzidine (TMB) as the chromagen and sodium tungstate (ST) as the stabilizer (Gu, Chen et al. 1992). Negative controls were made by omission of the TMB in the sections.

## Administration of antibiotics and immunosupressants (Papers II-IV)

To prevent postoperative infections and immune response rejections all transplanted animals received daily doses of tetracycline (1.8 mg/ml, i.p.) and cyclosporine (4.2 mg/ml, i.p.).

## Cell transplantation procedures (Papers II-IV)

*Transplantation of statoacoustic ganglion (SAG) explants to the rat auditory nerve (Paper II)*

The SAG explants dissections were performed on the embryonic day 13 (E13) on GFP-positive BalbC mice. Whole SAG explants were placed into 4-well cell culture plates coated with poly-l-lysine and laminin, and were cultured overnight in culture media. The explants were removed with a needle from the cell culture plates and immediately used for implantation. Surgery on the host rats was carried out by using the sub-occipital approach described above having the rats fixed on a stereotactic frame. Using a syringe clamping device, the SAG explants were injected together with 4 µl of medium into the auditory nerve by the IAM. The needle was kept in place for 10 minutes after injection and then the wound was closed as above. Sham operated animals were injected with 5 µl of the culture medium.

*Transplantation of tau-GFP embryonic stem cells into the auditory nerve by the IAM or into the modiolus (Paper III)*

The mouse tau-GFP ESCs used in this study were generously provided by Dr John Mason, University of Edinburgh, Edinburgh, UK. These cells express GFP after differentiation allowing identification within the host environment (Pratt, Sharp et al. 2000). Also, because the GFP is coupled to the tau protein, being an important component of the microtubules in the axons, it allows the cells and their axons to be visualized in detail without the risk of extracellular diffusion or background stain-

ing in the immunohistochemical preparations (Pratt, Sharp et al. 2000). In Paper III, we injected these cells with the technique described previously, either by the IAM or into the modiolus.

*Transplantations of human neural precursor cells into the auditory nerve by the IAM (Paper IV)*

The cell line was originally from StemCells, Inc. (San Fransisco/ Sunnyvale, CA) and was generously provided by Dr Englund-Johansson (Lund University, Sweden). Forebrain tissue was obtained from a nine-week old (post conception) human embryo and isolated under compliance with the National Institute of Health guidelines, Swedish Government guidelines, and the local ethics committee. The HNPCs were cultured in a medium with the growth factors, human basic fibroblast growth factor (hbFGF, 20 ng/ml; R&D Systems), human epidermal growth factor (hEGF, 20 ng/ml; Invitrogen) and human leukemia inhibitory factor (hLIF, 20 ng/ml; Sigma) added every 3-5 days to the culture. The HNPCs expressed the GFP reporter gene, previously transduced to the cells using a lentiviral infection (for details on lentiviral infection see (Englund, Ericson et al. 2000). As described before, using the IAM approach, cells were injected to the AN.

## IKVAV peptide amphofile nanofiber gel (Papers III and IV)

BDNF and ChABC were administered in a bioactive nanofiber gel (Silva, Czeisler et al. 2004; Stendahl, Wang et al. 2008; Tysseling, Sahni et al. 2010). This gel consist of self-assembling peptide amphiphile (PA) molecules designed to present to cells the neurite-promoting laminin epitope isoleucine-lysine-valine-alanine-valine (IKVAV). The gel has been shown to promote the differentiation of neural progenitor cells into neurons and prevent the development of astrocytes (Silva, Czeisler et al. 2004). This astrocyte proliferation inhibition might be important in the prevention of the development of glia scars occurring after the cell injections. Still, our experimental setup may not have taken full advantage of the bioactive ability of the gel since we applied the PA gel over the ESC-injection site and not inside the auditory nerve. In Papers III and IV we mixed the PA gel with BDNF/ChABC in order to possibly provide prolonged trophic support for the cells.

## Supplement of brain derived neurotrophic factor and chondroitinase ABC (Papers III and IV)

In Paper III, some of the animal groups received a supplementary treatment of locally administered BDNF or ChABC (solely or in combination). These agents were mixed with the PA gel, where after ten microliters of the PA solution containing BDNF/ChABC were applied over the nerve by the IAM or in the scala tympani (ST). For the IAM approach the PA gel was either applied over the peripheral nerve

at the transplant injection site or applied separately at a distance from the injection site to fill the opened ST. In the modiolar cell transplantation approach, the PA gel was either applied to the opened ST or separately over the nerve by the IAM.

In Paper IV, we only used BDNF mixed with PA gel. In this experiment all cell injections and all BDNF/PA gel applications were made by the IAM.

## Cell and branch point quantifications (Papers I, III and IV)

Paper I: To verify the effect of β-BuTx on the SGNs, the SGN survival was quantified by measuring the neural density in the cochlear sections. The sections were put in 1 % toluidine for 1 minute and then rinsed in distilled water for 5 minutes. After drying, the sections were placed and examined under a microscope. For quantification of the number of surviving SGNs and measurement of the Rosenthal's canal area the imaging software programme cell-B (Olympus Life and Material Science Europe, Hamburg, Germany) was used.

Paper III: At a 40X magnification the number of transplanted cells was defined as the number of cell profiles with expression of GFP and DAPI and differentiated cells as double stained with GFP and TUJ1. Each section was divided into four specimen regions of interest (ROI): the ST, the modiolus, the auditory nerve and the BS. To avoid double counting, cells were quantified in every third section from a total of 20-30 sections in all the specimens.

Paper IV: Cells were quantified with the same technique as in Paper III. Further, we quantified cells with an outgrowth of neurites and also the number of branch points in each ROI.

## Statistics (Papers I-IV)

Kruskal-Wallis ANOVA on Ranks test (Paper I) and Student's t test (Papers III and IV) were used for statistical analyses of cell densities and cell numbers.
Statistical analysis of mean values from ABR was performed by Student's two-tailed t test (Paper II).

# RESULTS

## β-bungarotoxin animal model (Papers I-IV)

Animals treated with β-bungarotoxin application to the middle ear displayed significantly elevated ABR thresholds in all tested frequencies from day 3. This elevation was observed until the postoperative day 21, when the animals were sacrificed. SGNs showed a well-preserved morphology until day 14. At day 21, almost all SGNs had degenerated (**Fig. 3**). TUNEL-staining confirmed an apoptotic cell death at day 17. No loss of inner or outer hair cells was detected at day 21. Vestibular ganglion neurons were intact but displayed a swelling of the soma at day 21.
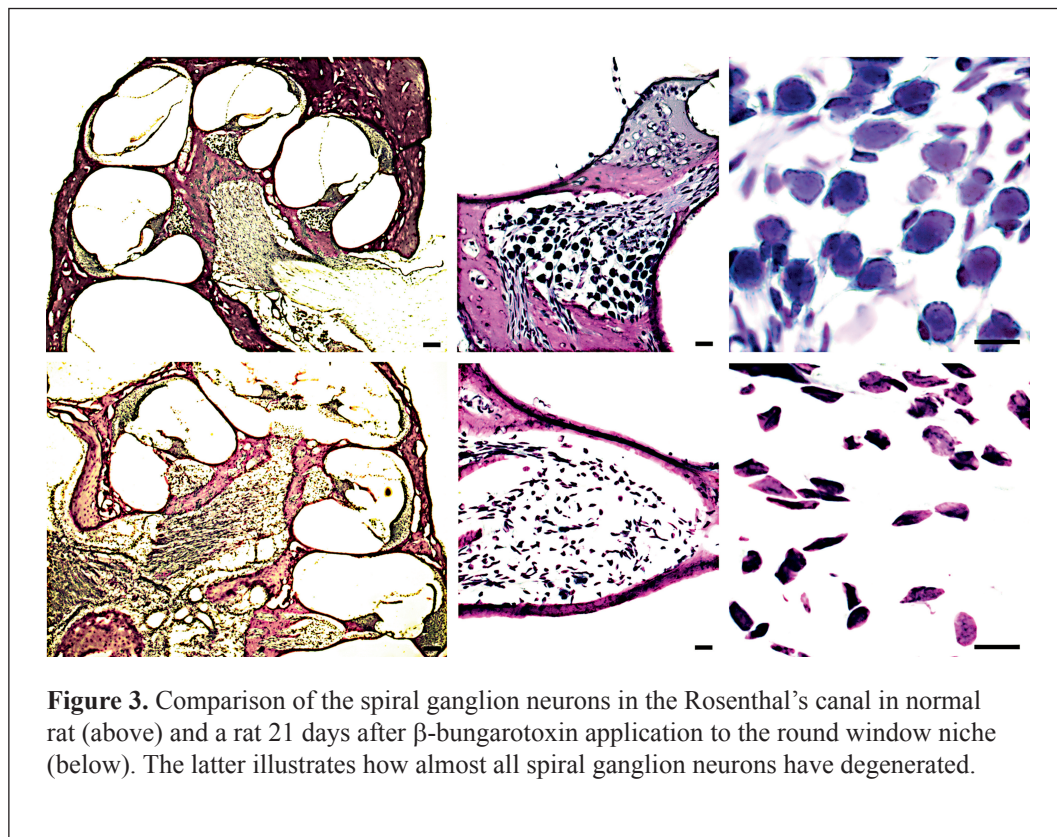


**Figure 3.** Comparison of the spiral ganglion neurons in the Rosenthal's canal in normal rat (above) and a rat 21 days after β-bungarotoxin application to the round window niche (below). The latter illustrates how almost all spiral ganglion neurons have degenerated.

## HRP dye tracing (Paper II)

The accumulation of the tracer around the injection-site was easily identified. From the injection-site the tracer was also transported centrally to the BS as well as peripherally to the cochlea and spiral ganglion neurons.

## Assessment of auditory function (Paper II)

No statistically significant differences in ABR thresholds were measured between pre- and post surgery with injections of HRP to the auditory nerve trunk by the IAM. The result are shown in **Figure 4.**
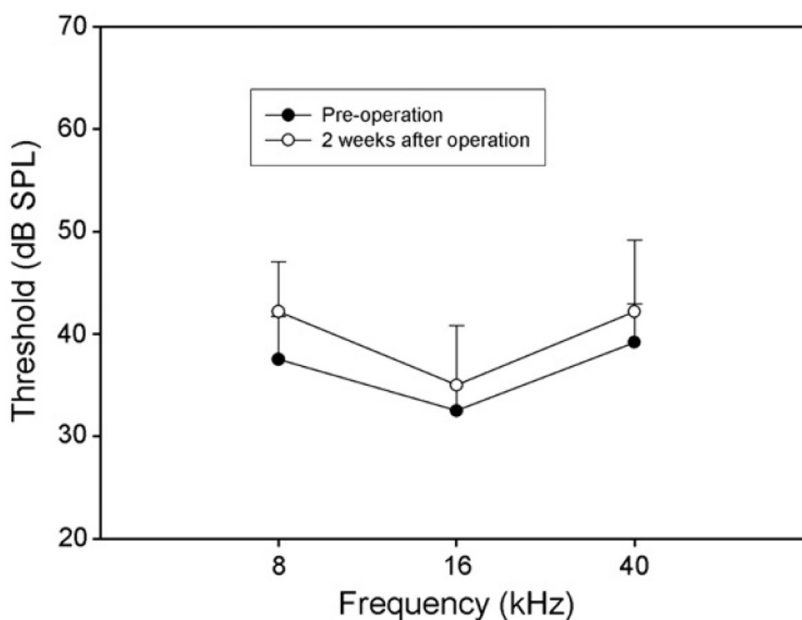


**Figure 4.** ABR threshold pre- and 2 weeks post surgery with injections of HRP to the auditory nerve trunk by the IAM. No statistically significant differences were observed.

## Survival of transplanted cells (Papers II-IV)

*Statoacoustic ganglion cells (Papers I and II)*
Survival of the implanted GFP positive SAGs was observed in both the two- and five weeks survival groups. In the deafened groups we found cell profiles in five out of eight animals whereas among the non-deafened animals, cell profiles were found in one out of nine animals. In some animals we observed GFP positive tissue without any visible DAPI positive cell profiles. In four animals we could not detect any GFP positive profiles at all.

*Tau-GFP embryonic stem cells (Paper III)*
The tau-GFP cells were injected directly to the auditory nerve by the internal auditory meatus or through the modiolus in the cochlea. Some groups received additional treatment with BDNF, ChABC or a combination of both in PA gel either

over the injection site or at the opposite side of the transitional zone. After three weeks the animals were sacrificed. Substantial survival of the transplanted cells was observed in all groups treated with BDNF in PA gel with the best average group survival rate at 29.6 % and the best individual survival rate at 44.6 %. Significantly lower survival was observed in animals injected with cells without BDNF treatment. Further, in this study we did not show any significant effect on cell survival by using the bioactive PA gel alone.

*Human neural progenitor cells (Paper IV)*
Surviving human neural progenitor cells were quantified after 3 and 6 weeks in the different sets of groups (**Fig. 5a**). One group (3 weeks survival) did not receive any additional BDNF in the PA gel while the other two groups (3 and 6 weeks survival) received additional BDNF. All groups displayed surviving transplanted cells but the survival was significantly higher in the groups with BDNF application. The best average survival rate was found after 3 weeks (19 %) which was not significantly better than 6 weeks (17 %). This can be compared to the 3 weeks survival, non-BDNF, group that had a survival rate of 3.4 %.

## Migration of transplanted cells (Papers II-IV)
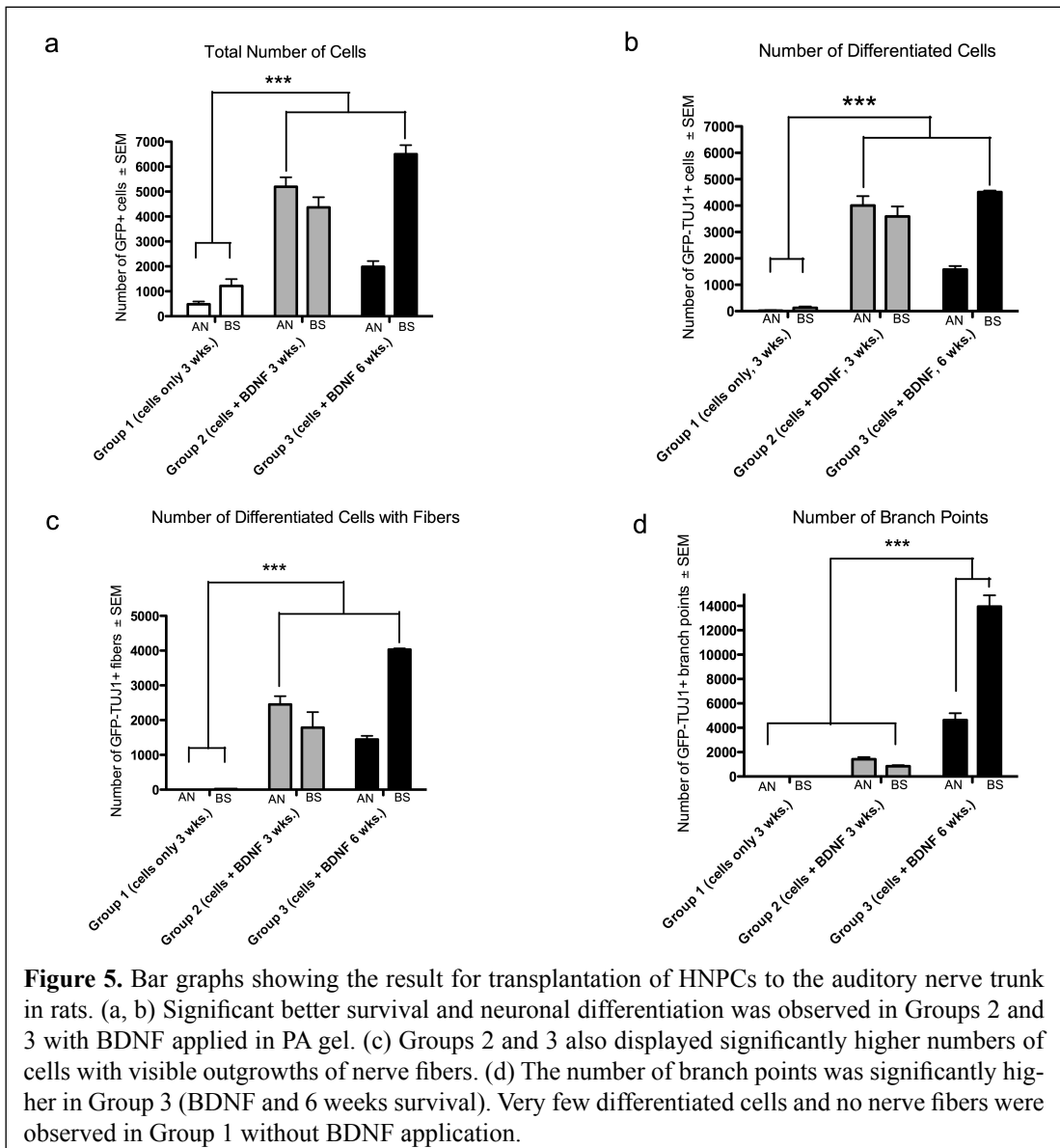
*Statoacoustic ganglion cells (Paper II)*
SAGs cells in all the groups were found at/nearby the injection- site but also along the auditory nerve. Peripherally, GFP positive cell profiles lining the boundary of the Schwann-glial transitional zone were observed but we did not observe any cells passing through this barrier towards the periphery of the auditory nerve. Furthermore, there were no transplanted cells migrating centrally into the cochlear nucleus.

*Tau-GFP embryonic stem cells (Paper III)*
In all groups we observed migration of the transplanted cells. Since the cells were transplanted either to the peripheral or to the central portion of the auditory nerve migration of the cells or growth of their nerve fibers through the transitional zone was considered essential. In order to facilitate migration between the peripheral and central nerve and vice verse, in some groups, the enzyme chondroitinase ABC was used. Only in groups that received ChABC application we observed migration over the transitional zone, confirming the GAG degrading effect of the enzyme. Additionally, in the group with BDNF/ChABC injected into the scala, adjacent to the cell injection in the modiolus we found a significantly higher migration of cells as compared to the groups where ChABC was injected at a distance from the cells indicating the benefits of applying the BDNF in close vicinity of the cells. Independently of the surgical approach used, in all groups with substantial cell-survival the majority of the cells migrated to the brainstem.

## Human neural progenitor cells (Paper IV)

After a 3 week survival time a number of the surviving cells had migrated centrally and were evenly distributed between the AN (54 %) and the BS (46%). However, at the 6 week time-point significantly more cells had migrated into the BS (77 %) as compared to the AN (23 %; **Fig. 5a**). This indicates that it may take time for the cells to reach the BS. Additionally, the number of double-stained differentiated cells and branch points were also significantly higher in the BS than in the AN after 6 weeks. Migrated cells were found in close vicinity to the cochlear nucleus in the brain stem. None of the cells migrated from the injection site and peripherally into the cochlea.



**Figure 5.** Bar graphs showing the result for transplantation of HNPCs to the auditory nerve trunk in rats. (a, b) Significant better survival and neuronal differentiation was observed in Groups 2 and 3 with BDNF applied in PA gel. (c) Groups 2 and 3 also displayed significantly higher numbers of cells with visible outgrowths of nerve fibers. (d) The number of branch points was significantly higher in Group 3 (BDNF and 6 weeks survival). Very few differentiated cells and no nerve fibers were observed in Group 1 without BDNF application.

# Differentiation of transplanted cells (Papers II-IV)

*Statoacoustic ganglion cells (Paper II)*
In two animals we observed low numbers of differentiated, double-labeled GFP-and Tuj1-positive cells with detectable neurites in the nerve, oriented peripherally from the IAM. No differentiated cells were found in the brain stem.

*Tau-GFP embryonic stem cells (Paper III)*
These groups displayed a significant positive correlation between the numbers of differentiated (double labeled GFP/TUJ1 positive) cell profiles and BDNF applications. Thus, all the groups with BDNF applications had differentiated cells (**cf. Fig. 6**). In the groups that did not receive BDNF differentiated cells were not observed. In the best-survival group, 15.7 % of the total number of transplanted cells and 69.5 % of the total number of surviving cells had differentiated as illustrated by their neuronal markers. This further implies that in the present setup BDNF promotes the differentiation of the transplanted tau-GFP ESCs.

*Human neural progenitor cells (Paper IV)*
The 3 weeks survival group without BDNF displayed a very low differentiation rate. On the contrary, all the BDNF application groups illustrated very high differentiation rates of surviving cells in both the 3 weeks (79.3 %) and the 6 weeks survival (71.7 %) groups (**Figs. 5b and 7** ). After 3 weeks the differentiated cells were evenly distributed between the AN and the BS. After 6 weeks a majority of the differentiated cells were found in the brainstem. Therefore, it appears that the BDNF promotes differentiation and that the differentiated cells may survive better in the BS as compared to the AN.

After 6 weeks 90 % of the differentiated cells had visible neuronal outgrowths (**Fig. 5c**).
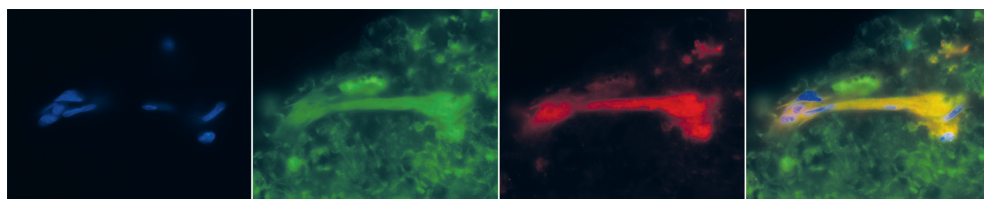


**Figure 6.** Tau-GFP embryonic stem cells transplanted to the auditory nerve trunk with BDNF application. After three weeks survival neuronal differentiation was observed as verified by co-expression of GFP (green) and the neuronal marker TUJ1 (red). Cell nuclei were stained with DAPI. Magnification 60X.

## Branching of nerve fibers in differentiated cells (Paper IV)

In order to analyze the newly formed neurites in the differentiated cells we performed quantifications of the number of branch points in each specimen (**Fig. 5d**). We speculate that the number of branch points might be an indication of the neural activity and plasticity of the transplanted differentiated cells (Tessier-Lavigne and Goodman 1996; Marler, Becker-Barroso et al. 2008; Schmidt and Rathjen 2010). A branch point was defined as a bifurcation of one nerve fiber that illustrated positive staining for both GFP and TUJ1. Extensive arborization of fibers was observed after 6 weeks in the BDNF treated group which was significantly higher than in the 3-week survival/BDNF treated group. In the 3-weeks survival/no BDNF group no branch points were observed. These results suggest that a major part of fiber growth and branching takes place in between 3 and 6 weeks after the HNPC transplantation (**Fig 7**).
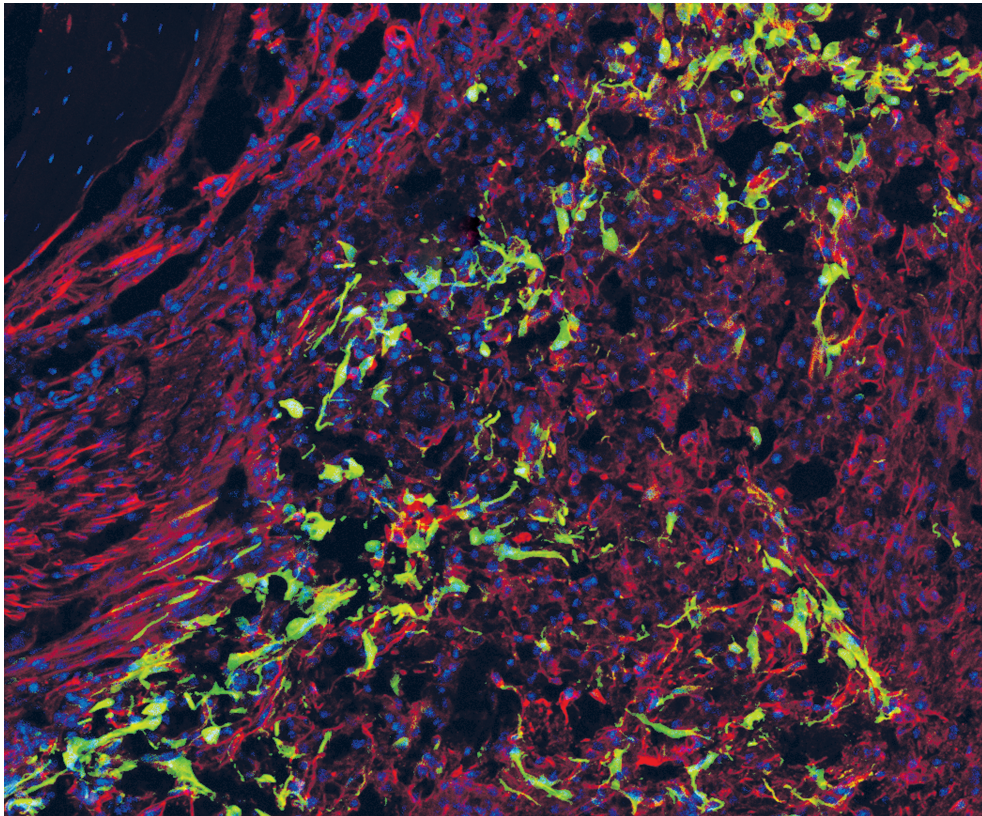


**Figure 7.** Human neural progenitor cells transplanted to the auditory nerve by the internal auditory meatus. Three week after transplantation extensive survival and differentiation can be observed. Many of the differentiated cells also display nerve fibers. Immunohistochemical staining was performed with DAPI for cell nuclei, GFP for transplanted cells, and TUJ1 to verify differentiation.

# DISCUSSION

## Hypothesis
In this thesis we posed the question of whether it is possible to replace the SGNs with transplanted immature cells. This would provide a probable treatment for selective lesions of the auditory nerve or a complementary treatment for a combination of lesions where both hair cells and spiral ganglion neurons are affected. Furthermore, it would also be of importance for the treatment of severe injuries to other sensory cranial nerves.

In order to investigate possibilities of developing methods for cell replacement therapies following auditory nerve lesions, we have carefully designed, performed and analyzed a number of in vivo experiments.

## Animal model
β-bungarotoxin (β-BuTx) was never before used for rodent models of selective damage to the SGNs. Here, we have utilized this toxin to develop a safe and easy-accessible method of deafening rats while the hair cells are being preserved. Our results show that β-BuTx has a significant reducing effect on the number of SGNs as well as on the hearing ability. The surgical approach composes no major difficulties since the round window niche in the rat is easily reached by a retro-auricular incision and opening of the temporal bulla. Further, we did not observe any side effects of the toxin, as the rats appeared to withstand the surgery and the application of β-BuTx very well. None of the deafened rats had to be sacrificed due to the humane endpoints set up in the animal protocol. As no dose-effect measurements were performed we could not assess how higher or lower concentrations of β-BuTx that affect the results. Still, since almost all SGNs were destroyed in the treated animals and no hair cell loss was observed, for the intended purpose the concentrations appeared to be appropriate.

The SGNs in rats consist of about 5-10 % of type I and 90-95% of type II neurons (Romand and Romand 1985). In the quantification process we did not differentiate between these two types of neurons. However, as β-BuTx destroyed close to all the SGNs we conclude that both type I and type II neurons were affected by the toxin. The ABR curves were significantly affected already 3 days after β-BuTx treatment whereas the SGN numbers were not reduced until after 14 days. This illustrates that the functional loss of the SGNs preceded that of the apoptosis. We speculate that this delay may be dose dependant.

## Surgical approaches

The auditory nerve is very well protected by the temporal bone and is located at the pontine angle in the brain stem. As a result of such anatomical conditions the surgical access to the nerve constitutes major challenges. The nerve has to be accessed either in its peripheral portion in the cochlea or centrally in the brain stem. Both approaches demand for potentially dangerous surgery, each with various risks for complications. In rats the small diameter of the nerve further complicates the injection of cells.

Access to the cochlear part of the nerve is achieved by a cochleostomy where after a hole is drilled through the cochlear modiolus. With this approach the encapsulated bony shell of the cochlea is breached indicating that the risk for a disturbed cochlear homeostasis is apparent. There is also a risk for disturbed vestibular function and in few animals we experienced some balance disorders. Still, obviously, the size of the rat cochlea is smaller than the human, resulting in more difficult surgery and higher risk of complications (e.g. cochlear fractures) as compared to the similar surgery on humans. One possible benefit of this approach would be the possibility of using the demarcated cochlear fluid-filled cavities as containers for cell-promoting substances (e.g. neurotrophic factors). In one study (Paper III) we used the PA-gel mixed with BDNF and/or ChABC and applied this into the scala tympani.

Access to the central portion of the AN in rats may be achieved by a sub-occipital craniotomy and an incision of the dura targeting the nerve by its entrance to the cochlea through the internal auditory meatus. Here, to be able to visualize the nerve, the cerebellum has to be moved medially. In humans, this central approach is commonly used for surgery of acoustic schwannomas sometimes resulting in a traumatized nerve. If cell replacement therapies in the future will compose a possible treatment of auditory nerve lesions a central cell transplantation approach would have an advantage of being performed in conjunction with the schwannoma surgery. Further, with this approach the cochlea is preserved intact. One important issue with central injections of cells is to find out how to apply the cell-promoting substances. In the pontine angle, unlike in the cochlea, there is no enclosed space that can harbor factors. On the contrary, a continuous circulation of cerebro spinal fluid may dilute and rinse away the applied factors. This obstacle can possibly be overcome by the use of gels or scaffolds containing the substances of choice.

Independently of the choice of surgical approach for cell transplantations to the auditory nerve, a requirement for a successful integration to the host nervous system is the bipolar connections, e.g. centrally to the cochlear nucleus and peripherally to the potentially preserved hair cells. Previous studies have shown that axons growing from the periphery to the central will stop growing when they encounter the astrocytes in the central nervous system, i.e. the transitional zone (Carlstedt 1985; Fraher 2000). Thus, the axons and possibly the cells may be inhibited when trying

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to bypass the transitional zone in the central direction. In this study, however, we have shown that when ChABC was applied the transplanted cells could pass the transitional zone regardless of the approach used.

It may be that the exact location of the transplanted cells is not essential. As long as the cells survive, differentiate and extend neurites that connect the cochlear hair cells with the cochlear nucleus in the brain stem, function may be restored. In that case, other aspects as the surgical accessibility, possibilities of applying the promoting substances and the risk for complications may be more important.

## Choice of cells for transplantation

As there are multiple and different types of cell candidates for the replacement of auditory neurons, the candidacy preference may be difficult. Cells of different origin and different kinds of maturity have been assessed in a variety of both in vitro and in vivo studies (Hu, Ulfendahl et al. 2004; Olivius, Alexandrov et al. 2004; Regala, Duan et al. 2005). In the present thesis, three different types of cells were selected. Assessments regarding their survival, differentiation and migration in the rat auditory nerve were made.

### *Statoacoustic ganglions*

These explants were harvested from the auditory tract in GFP positive mice from embryonic day 13 (E13) embryos. The ganglions contain embryonic progenitor cells responsible for the development of both cochlear and vestibular neurons (Sher 1971). E13 is the time in the embryonic development when the neuronal responses to sound initialize (Uziel, Romand et al. 1981). The explants survived for up to five weeks but in low numbers and with only very few differentiated (TUJ1 positive) cells. None of the transplanted cells had migrated into the brainstem and none had migrated peripherally through the TZ. Since we observed multiple GFP positive cell profiles that were lining up along the TZ, we speculate that the TZ may compose an obstacle for a peripheral axonal sprouting and/or cell migration. It should be noted that in this study, as compared to Papers III and IV, BDNF and ChABC were not used. Therefore, the survival, differentiation and migration cannot be impartially compared to the much more promising results from Papers III and IV. Still, with the set up applied here, SAG cells cannot be considered a feasible candidate for a cell transplantation paradigm.

### *Tau-GFP embryonic stem cells*

Since the ESCs may differentiate into any specialized cell, these cells would be good candidates for an auditory cell replacement model providing that their differentiation can be directed into a neuronal fate with functioning properties. As the

cells have to be harvested from embryos, the ethical aspects of using the ESCs also need to be considered. However, the properties of the stem cells to continuously divide offer opportunities to expand their production, thereby ensuring a stable source of cells for clinical transplantation. Still, maintaining the stem cell division in a culture for longer periods of time while ensuring their differentiation after transplantation requires genetic modifications that may influence the genetic stability and survival of the cells in vivo (Hodges, Pollock et al. 2007). Previous experiments by our group have shown that the tau-GFP ESCs survived for up to 9 weeks following transplantation to the rat auditory nerve in the modiolus (Hu, Ulfendahl et al. 2004). The survival-rate was low, approximately 1-1,5 % but the tau-GFP cells had all the properties of ESCs. Further, since GFP is coupled to the tau-protein it allows the cells and their axons to be visualized in detail and reduces the risk of an extracellular diffusion and a background staining in the immunohistochemical preparations (Pratt, Sharp et al. 2000). The cells continue to express GFP after their differentiation (Pratt, Sharp et al. 2000). In Paper III, we illustrate how the tau-GFP cells survive and differentiate in considerably higher numbers as compared to our previous studies. We showed that the groups that received BDNF in PA-gel illustrated a significantly better survival as compared to the non-BDNF groups and in individual groups of animals we observed average 3-week survival rates for up to 44.6%. In the non BDNF-groups survival rates were low (0.9 %) which is in accordance to our previous experiments (Hu, Ulfendahl et al. 2004). Thus, according to these results BDNF in the PA-gel seems to have a major impact on survival of the transplanted tau-GFP cells. The BDNF/PA-gel combination also seemed to promote differentiation of the cells as the majority of the surviving cells also proved to be TUJ1 positive. The use of ChABC in the PA gel resulted in extensive migration of the implanted cells, including past the TZ. Thus in Paper III we have, apart from managing to ensure good survival of the transplanted tau-GFP cells as well as directing them into a neuronal fate, also facilitated their migration to different locations along the auditory nerve.

*Human neuronal progenitor cells*

Since the neuronal progenitor cells, as compared to the embryonic stem cells, are more directed towards a neuronal fate, it may be logical to assume that these cells would prove to be a better and more easily directed candidate to replace the damaged auditory neurons. Additionally, the use of human cells in animal experiments undeniably brings us closer to the perspective of using the cells in human clinical trials. Indeed, the HNPCs have successfully been used in several animal studies of the neurodegenerative diseases (Fricker, Carpenter et al. 1999; Armstrong, Watts et al. 2000; Englund, Fricker-Gates et al. 2002). It has also been demonstrated that it is possible to culture human spiral ganglion tissue in order to obtain neurons, indicating the presence of HNPCs in the adult human auditory nervous system (Rask-Andersen, Bostrom et al. 2005). In Paper IV, we use the HNPCs for transplantation

into the auditory nerve by the IAM. Corresponding to the transplantation of tau-GFP ESCs in paper III, we used BDNF in the PA gel with the intension to promote the survival and differentiation, and consequently integration of the transplanted cells. The HNPCs have proved to be very vital cell transplantation candidates as they not only survived in high numbers but also differentiated and produced nerve fibers to an extent that we did not observe before. Similar numbers of surviving cells were observed three and six weeks after transplantation indicating that few cells after 3 weeks go through apoptosis. The differentiation-rate of the surviving cells was also high, 79 % and 72 % respectively. As most of the differentiated cells at the six-week time point had migrated to the brain stem, we speculate that the BDNF, applied in the PA-gel by the injection site next to the IAM, mainly promoted cell survival in the first three weeks. After that it is possible that the microenvironment with site-specific cues (e.g. endogenous factors) take over and promote migration and fiber outgrowth to the brain stem. We also observed an extensive arborisation of fibers in the six weeks survival group indicating a high neural activity and plasticity in the differentiated cells.

## Promotion of survival, differentiation and migration

As we already discussed, the survival and differentiation of the transplanted cells are essential for the regeneration of any neuronal pathway. Migration of the cells might be of value but we don't know if it is essential, or if the newly formed SGNs instead of migrating to the Rosenthal's canal just as well may reside in the auditory nerve trunk or in the brain stem. Still it is obvious that new connections have to be formed both peripherally and centrally, and hence the nerve fibers have to pass through the TZ. In papers II-IV we observed that the survival rate in the groups that only received cells was rather low. It appears that the transplanted cells, in order to survive in relevant numbers for at least a limited time after injection, are dependant on exogenous factors. There are a number of trophic factors that may influence the survival and differentiation of the cells (cf. in the Introduction). We speculate that a combination supplement of growth factors would benefit the transplant. However, in this thesis, being the only trophic factor supplemented, we have evaluated the effect of BDNF (Papers III and IV). Further, we used a bioactive nanofiber gel that was mixed with BDNF. This gel consists of the self-assembling peptide amphiphile (PA) molecules designed to present a neuron-promoting laminin epitope to the cells. In previous studies, this gel has been shown to promote the differentiation of neural progenitor cells into neurons and to prevent the development of astrocytes (Silva, Czeisler et al. 2004). It should be noted, however, that where this PA-gel is designed to harbor the cells, we instead used it as a container for the BDNF. It is therefore uncertain how much of the cell-promoting effect of the gel that was taken full advantage of. In the groups with the PA-gel alone, the cell survival was signifi-

cantly lower as compared to the groups with the PA-gel and BDNF in combination. Still, we cannot exclude/deny a synergistic cell promoting effect of BDNF and the PA-gel.

In paper III, the double-labeled tau cell profiles were predominantly seen in the cell colonies. We also found survival of the tau-GFP cells dispersed in the nerve and the BS, but with a very few double-labeled cells. This gives us the notion that the cell survival and the differentiation are promoted by the BDNF application as well as by the local support between the transplanted cells (i.e. paracrine signaling). Also, the surviving HNPCs in Paper IV were found grouped together, even though not as tightly as the tau-GFP cells.

In paper III, the ChABC proved to be most efficient in promoting migration of cells and most interestingly in facilitating the migration in between the peripheral and the central part (i.e. the TZ) of the auditory nerve. Previous studies have suggested that the TZ hinders outgrowth of fibers from the PNS to the CNS but allows fibers from the CNS to pass out to the periphery (Fraher 2000). In our ChABC experiments we did not observe any growth of fibers across the TZ while transplanted cells migrated in both directions.

## Tumor formations
One definition of pluripotent stem cells is their ability to form teratomas, i.e. benign tumors where the cells to varying degree differentiate into cells from all three germ layers. To further evaluate the ability of the stem cells to form teratomas, the cells are injected into the immunosuppressed mice. Thus, while immunosuppression may be required for the survival of the transplanted ESC, it is also associated with a risk of tumor formations in the host animal. It has been suggested that the teratoma formation can be prevented by an in vitro pre-differentiation of the cells (Kim, Auerbach et al. 2002; Fukuda, Takahashi et al. 2006). This might therefore support the choice of neural progenitor cells as candidates for a biological implantation model.

## Future perspectives
The results presented in this thesis have confirmed the possibility of transplanting cells into the auditory pathway but also presented a substantial survival and neuronal differentiation of the transplanted cells. Transplanted cells and their nerve fibers were observed to reach relevant target regions centrally in the cochlear nucleus as well as peripherally in the cochlea. The coming step after these morphological results would be an evaluation of the function of the transplant. To validate function of the newly formed neurons further electrophysiological studies to confirm

functional contacts between the transplanted cells and the host cells are needed. In addition, possible improvements of the hearing of the animals need to be confirmed by ABR measurements.

IPS cells may become a very attractive option for cell transplantation in the future. Since these cells can be obtained from the adult somatic cells by inducing expressions of specific genes, it is possible to use such cells for an autologous transplantation and thus avoid a controversial use of embryos and perhaps even immunosuppressants. Since the IPS cells are developed from the patient's own cells, it was believed that the immunogenic responses would be avoided. Still there are reports that this assumption might not be certain (Zhao, Zhang et al. 2011). Also the IPS cells have proved to possess hazardous oncogenic properties (Knoepfler 2009). Consequently, further research on the IPS cells is needed before these will compose a viable alternative for the clinical use in humans.

Neurotrophic factors play an important role in the promotion and maintenance of SGNs, and may also protect the SGNs from further degeneration. Exogenous neurotrophins therefore play an important role in a cell replacement paradigm, at least during a certain time-window. Even though the results presented in this thesis clearly illustrate a cell-promoting capacity of the BDNF, it was not explored whether this would be the optimal supplement for the transplanted cells. Further investigations need to be performed to find the most favorable "cocktail" of neurotrophic factors.

In humans, as compared to in rodents, the auditory nerve has a larger diameter. Therefore, the use of different types of scaffolds or matrixes to harbor and guide the transplanted cells may be of use. Gels in different forms may be one way of linking, for example, two nerve endings to one another. To achieve this is in the human may also technically be less challenging than in the rodent.

# CONCLUSION

**1.**    β-BuTx-application to the round window niche is an efficient way of inflicting a selective damage to the SGN, while sparing the hair cells. The deafening animal model developed in this thesis may be used for laboratory investigations of a number of conditions or diseases such as auditory nerve lesions, auditory neuropathy and degeneration of the auditory nerve.

**2.**    Injection of cells or substances to the auditory nerve trunk by a sub-occipital approach is a feasible approach that does not compromise auditory functions. Selected substances injected with this approach may get access to the entire nerve by an intra axonal transportation.

**3.**    ChABC application in PA-gel by the injection site facilitated migration of the cells to adequate locations within the auditory nerve and the brain stem. With the ChABC treatment cells also traversed the TZ.

**4.**    SAGs implanted to the auditory nerve trunk without any exogenous neurotrophic support survived for up to five weeks but in low numbers.

**5.**    Tau-GFP ESCs and HNPCs, when injected with co-application of the BDNF in a PA gel, displayed excellent survival- and differentiation- rates.

**6.**    HNPCs displayed very neurogenic properties and when injected with co-applications of the BDNF in a PA gel, we observed an extensive outgrowth of nerve fibers. Both the differentiated cells and new nerve fibers migrated and sprouted towards the cochlear nucleus.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

**Celltransplantation i syfte att återskapa en skadad hörselnerv.**
Reservdelsmänniskan är ett begrepp som involverar utbyte eller reparation av allt fler organ i kroppen. Utbyte av hela organ som t ex vid hjärt- eller levertransplantation har utförts sedan många år. Under de senaste åren har forskare sökt metoder för att reparera skadade organ med hjälp av stamceller. Stamceller är en form av basceller med förmåga att dels återskapa sig själva genom delning men även att utvecklas till mer specialiserade celler, t ex nervceller.

Även om grav hörselnedsättning eller dövhet i allmänhet beror på påverkan på sinnescellerna (hårcellerna) i hörselsnäckan, så finns det patienter med hörselnedsättningar som i stället orsakas av en skadad hörselnerv men där hårcellerna är intakta. Skadan kan ha uppstått t ex på grund av en tumör på nerven alternativt som följd av en traumatisk skada eller kirurgi. Om hårcellerna är skadade kan hörselnerven också med tiden, i avsaknad av stimulans, tillbakabildas. Tyvärr självläker inte nerven utan hittills har dessa patienter betraktats som döva för livet. Denna avhandlings första arbete bestod i att ta fram en djurmodell som skulle imitera en sådan selektiv hörselnervsskada (med intakta hårceller). Genom att i mellanörat applicera en typ av ormgift som är skadligt för nerven lyckades vi skapa en bra djurmodell lämplig att använda för senare celltransplantationsförsök. I arbete I visar vi hur toxinet gjort så att hörselnervens celler har förstörts, att hårcellerna inte hade påverkats samt att nervskadan resulterade i dövhet av det berörda örat.

Syftet med denna avhandling har varit att hos råttor ersätta innerörats nervceller och hörselnerv genom att transplantera nervcellsvävnad eller stamceller till innerörat och hörselnerven. Transplantationen utfördes genom att vi med hjälp av en nål sprutade in celler direkt till en skadad hörselnerv.

Samtidigt gav vi vissa grupper av djur en behandling i form av en gel blandad med substanser som vi hoppades skulle främja överlevnad och utveckling av cellerna. Vi sökte även underlätta möjligheterna för cellerna att förflytta sig inom nerven, så kallad migration.

I arbeten II-IV har vi utvärderat resultaten av injektioner med tre olika typer av celler.
1.     Statoakustikus ganglier från mus: En form av embryonal nervvävnad från innerörat som innehåller stamceller som dock har utvecklats något mot att bli mer nervlika. Här utvärderade vi cellerna efter två och fem veckor. Resultaten påvisade överlevande celler efter fem veckor men i lågt antal. Enstaka celler

hade utvecklats till nervceller.

2.  Embryonala stamceller från mus: Här utvärderades cellerna efter tre veckor. Vi såg då en överraskande bra överlevnad av celler samt att en hög andel av cellerna hade utvecklats (differentierats) till nervceller. Grupperna som erhöll behandling med en specifik nervtillväxtfaktor (BDNF) i gel uppvisade en signifikant högre överlevnad av celler jämfört med de grupper där bara cellerna injicerades. Vi såg även att behandling med enzymet ChABC ökade cellernas migration.

3.  Humana neuronala progenitorceller: Dessa är mänskliga stamceller med viss neuronal utveckling. Resultaten utvärderades efter tre och sex veckor. Vi kunde observera betydande överlevnad och även att en stor del av cellerna hade differentierat till nervceller. I anslutning till de transplanterade cellerna såg man vidare utväxt av ett nätverk av nya nervtrådar. Både de nya celler och nervtrådarna sågs lokaliserade precis vid hörselnervens omkopplingsstation i hjärnstammen.

Då vi både observerat överlevnad av celler och nybildning till nervceller blir nästa steg att undersöka om hörselnervens funktion påverkas av de nya cellerna, dvs om de nya cellerna kan ersätta funktionen hos den skadade hörselnerven. Detta görs dels genom att man påvisar nya fungerande kopplingar mellan nya neuron, hårceller och hörselkärnan i hjärnstammen, dels genom hörselmätningar.

Om försöken lyckas skulle detta skapa möjligheter att åtgärda hörselnervskador som idag inte är behandlingsbara. Tekniken skulle kunna användas vid de grava hörselskador som tidigare beskrivits. Den kan även komplettera redan befintliga tekniker, där man opererar in elektroder som ersätter innerörats hårceller, så kallade cochlea implantat. Vid positivt utfall av dessa försök kan man även tänka sig att använda tekniken vid skador på andra viktiga hjärnnerver som t ex synnerven.

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