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Auditory Organotypic Cultures and Progenitor Cell Implantation

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Auditory Organotypic Cultures and Progenitor Cell Implantation

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ABSTRACT

According to the WHO, about 5 % of the world's population suffers from disabling hearing loss. In people above the age of 65 the number is more than 30 %. Common effects of this condition are poor speech discrimination and impaired communication abilities. Patient surveys show that hearing loss often leads to a diminished quality of life.

The work presented in this thesis aims to evaluate and improve the performance of transplanted cells *in vitro*. Ultimately, we want to facilitate new connections between neurons of the brainstem (BS) and inner ear structures, or a hearing aid, with the help of transplanted cells. We predict that this will prove to be a feasible path to enhance the rehabilitation results for a selected group of patients with severe hearing disability.

We have developed an organotypic auditory cell culture model for the study of cell performance *in vitro* to help enhance cell transplantation performance *in vivo*. This model utilizes a BS slice including the cochlear nucleus to simulate an auditory target organ for transplanted cells. Here, we use this model to investigate new ways to improve existing transplantation protocols.

Paper I reports the effects of enriching a mouse boundary cap (BC) progenitor cell culture with the trophic factors inherent to a rat auditory BS slice conditioned medium (CM). The BS CM proved to have positive effects on the survival and differentiation of mouse BC cells in culture. We also report the specific brain derived neurotrophic factor (BDNF) and glial-cell derived neurotrophic factor (GDNF) contents of BS CM and how this content fluctuates over time.

Paper II reports the effects on human neural progenitor cells (HNPCs) from the addition of a surface substrate presenting bioactive molecules and a specific neurotrophin content. The commercially available Corning® Matrigel® tested here, proved to enhance the survival of seeded dissociated HNPCs when evaluated after three weeks in culture. We also report on the differentiation characteristics of HNPCs and their axon forming capacity.

In **Paper III** we investigate the survival rate *in vitro* of progenitor cells transplanted at different differentiation time points. Here, neuroepithelial-like stem (NES) cells derived from human induced pluripotent stem cells (hiPSCs) was cultured in three groups, with cells at separate differentiation states (NES cells, neuroblasts and neurons of various maturity (NVM)). These specimens were cultured as a monoculture or in co-culture with a mouse auditory BS slice. Cells seeded as a monoculture, without a BS slice, displayed a considerable lower survival rate as compared to the co-cultured cells. We conclude that the seeded NES were quite dependent of the trophic support from the co-cultured BS slice. We speculate that NES cells are quite vulnerable to the trauma and stress involved in the seeding process, and particularly so when cultured in a more differentiated state. We also report on the differentiation characteristics of cells after culture as revealed by immunocytochemical staining, as well as their tendency to migrate towards the BS slice in co-culture.

In **Paper IV**, we present the evaluation of a method to perform an electrophysiological activity assay of tissue specimens including the cochlear nucleus, cultured ad modum Stoppini. Here, we utilize the microelectrode array (MEA) platform with a modified protocol, to successfully record the endogenous neural activity of auditory BS slices. Our results concur with the basic characteristics of previously reported electrical activity in brain tissue.

In conclusion, these studies report novel information that could not practically have been gathered *in vivo* without utilizing a significantly larger number of experimental animals. We present promising results on the utilization of CM and cell matrix additives, the possible improvement of time schemes for cell transplantation, extensive characterization of cells in differentiation and a new model for future functional evaluation of cells in interface-culture. These results help us to better comprehend the innate obstacles encountered when searching for a successful *in vivo* cell transplantation paradigm to the impaired auditory nervous system.

LIST OF PUBLICATIONS

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

I. Kaiser A, Kale A, Novozhilova E, Siratirakun P, Aquino JB, Thonabulsombat C, Ernfors P, Olivius P. 2014. Brain stem slice conditioned medium contains endogenous BDNF and GDNF that affect neural crest boundary cap cells in co-culture. Brain Res 1566:12-23.

II. Kaiser A, Kale A, Novozhilova E, Olivius P. The effects of Matrigel® on the survival and differentiation of a human neural progenitor dissociated sphere culture. The Anatomical Record. Accepted for publication.

III. Novozhilova E, Kaiser A, Falk A, Olivius P. 2018. The differentiation potential of human induced pluripotent cell-derived neuroepithelial-like stem cells in a mouse auditory brainstem milieu. Submitted.

IV. Novozhilova E, Kaiser A, Olivius P. 2018. The recordings of electrophysiological activity in an interface-cultured organotypic mouse brainstem slice. Manuscript.

LIST OF ABBREVIATIONS

AI	artificial intelligence
BC	boundary cap
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BS	brainstem
BS CM	brainstem conditioned medium
CI	cochlear implant
CM	conditioned medium
CN	cochlear nucleus
CNS	central nervous system
DAPI	4’,6-diamindino-2-phenylindole
DCS	dissociated sphere culture
DCX	doublecortin
DMEM	Dulbecco’s Modified Eagle’s medium
DRG	dorsal root ganglion
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ESC	embryonic stem cell
GDNF	glial cell-derived neurotrophic factor
GFP	green fluorescent protein
HBSS	Hank’s Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hiPSC	human induced pluripotent stem cell
HNPC	human neural progenitor cell
iPSC	induced pluripotent stem cell
MEA	microelectrode array
MG	Matrigel®
NES	neuroepithelial-like stem
NF	neurotrophic factor
NGF	nerve growth factor
NT	neurotrophin
NT3	Neurotrophin 3
NT4/5	Neurotrophin 4/5
NVM	neurons of various maturity
OCT2	octamer-binding transcription factor-2
PBS	phosphate-buffered saline
PDL	poly-D-Lysine
PFA	paraformaldehyde
PNS	peripheral nerve system
SC	stem cell
TUJ1	Neuron-specific β-III tubulin marker

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INTRODUCTION

With the combined work presented here, we bring recent advances in cell transplantation research closer to benefitting the hearing-impaired patient. We study and evaluate the *in vitro* performance of cells in culture and the methods by which these evaluations are performed, resulting in novel information that can be utilized in future hearing rehabilitation research.

BACKGROUND

Hearing

In normal hearing, sound begins as pressure variations in air which are funneled through the outer ear canal to its bottom, where the tympanic membrane resides. As these sound waves bounce off, the generated vibrations are transplanted via the middle ear ossicles into the inner ear. Here, the mechanical stimuli affect the cochlear hair cells and is converted into neuronal signals. Now as electrophysiological signals, the sound information is relayed to the neuronal cells of the spiral ganglion in the center of the cochlea. From here they are sent via the auditory nerve to the second order neurons in the cochlear nucleus (CN) in the brainstem (BS) (Figure 1). From the BS, the sound information is forwarded to the central nervous system (CNS) where the electrophysiological signals translate into sound which is interpreted in the auditory cortex.

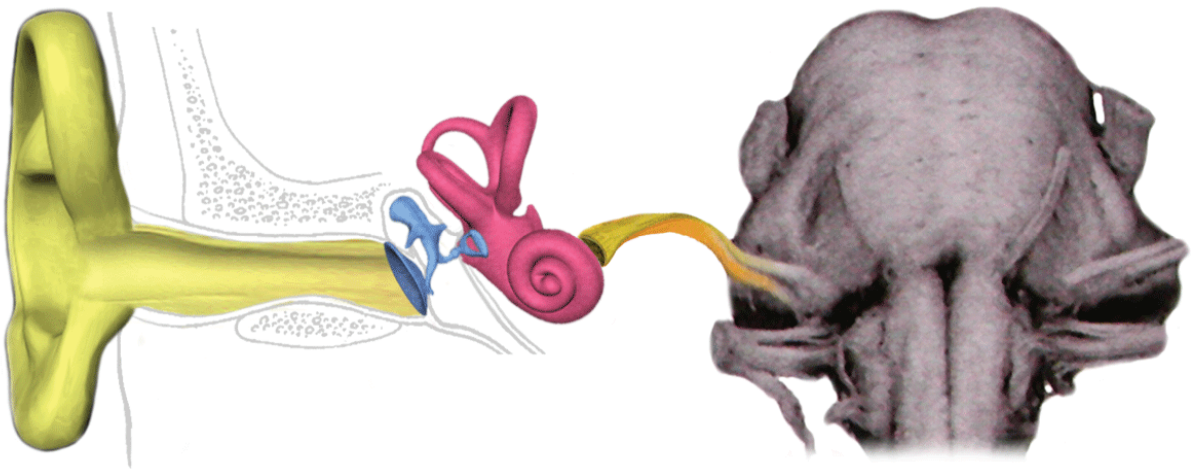

Figure 1. The human auditory system. Via the auricle, through the ear canal, sound funnels to the eardrum which anatomically separates the outer ear (yellow) from the middle ear (blue). Vibrations generated on to the eardrum are transplanted through the ossicles of the middle ear to the fluids of the inner ear (red). Here, these vibrations are transformed into electrical signals by hair-cells. Signals that are passed on to the spiral ganglion neurons and further through the cochlear nerve (orange) to the brainstem (grey). From here, the signals are further relayed to the central nervous system (CNS).

More than 5 % of the human population of the world, and 30 % of the people over 65 years of age, display a disabling hearing loss (WHO, 2018). This impairment is a result of qualitative and quantitative deterioration of different parts of the auditory system, and approximately half of the hearing-impaired patients need a hearing aid. Without aids, many patients will suffer from poor speech discrimination and impaired communication abilities. Patient surveys show that hearing loss often leads to a diminished quality of life, including extreme daytime fatigue, isolation, reduced social activity, sense of alienation and increased incidence of depression symptoms.

In vitro research and hearing

For many patients, a modern hearing aid is a very practical device to accomplish a sufficient hearing rehabilitation. Unfortunately, less than 50 % of the patients in need of a hearing aid receive one. And for some patients, depending on their type of disability, other ways to rehabilitation is needed. If the middle ear structures are damaged from infection, trauma or genetic disorders, surgery may be a feasible way to ameliorate the symptoms. If the inner ear organs are damaged from disease, medication or genetic disorders, the hearing can today sometimes be partly restored with the help of modern prosthetics (i.e. a cochlear implant). It is not uncommon that a combination of surgery and hearing aid/ prosthesis is needed. But, even with modern advances in medicine and hearing aid technology, many patients suffer from inadequate hearing function due to the limitations of available methods, or simply because their disabilities are not accessible for rehabilitation. Investigations into how we can possibly aid these patients are now reaching into the areas of cell transplantation.

Since cell transplantation into patients always are precluded by thorough preclinical trials, all ethical protocols include mandatory studies in animals and studies *in vitro*, prior to clinical use. It is today, for obvious ethical reasons, required to utilize cell cultures when possible to minimize the use of experimental animals. It is also evident that there are many detailed investigations of cell performance that are not only feasible, but also much more efficient in a controlled laboratory environment than within an *in vivo* framework.

Organotypic auditory cell culture model

Our group has extensive experience of cell culture work from many years of research. We have published results from both *in vitro* and *in vivo* experiments using a variety of different cells and experimental setups (Glavaski-Joksimovic et al., 2008; Glavaski-Joksimovic et al., 2009; Novozhilova, Olivius, Siratirakun, Lundberg, & Englund-Johansson, 2013; Palmgren, Jiao, Novozhilova, Stupp, & Olivius, 2012; Thonabulsombat, Johansson, Spenger, Ulfendahl, & Olivius, 2007).

To approach the auditory system *in vitro*, we have developed an organotypic auditory model using an adult mouse or rat donor. Our basic setting is comprised of a 300 µm thick BS slice including the CN and the proximal part of the auditory nerve. This is propagated as an interface-culture ad modum Stoppini on a semipermeable membrane (Stoppini, Buchs, & Muller, 1991), as a model of the host organ (i.e. target organ). A culture like this can be propagated for weeks and constitute a stable experimental platform. Our group has utilized this setting extensively to examine the performance of cells in mono- and co-culture.

Cell culture settings

The basic physiochemical requirements of cell culture (i.e. temperature, pH, osmotic pressure, O_2 and CO_2 tension) do not vary much between different setups. The physiological environment though, consisting of mediums to supply cells in culture with nutrients, hormones and growth factors, often differ between established protocols. The natural reason for this is that the mechanisms involved are not fully understood and there are no consent on what constitutes an optimal medium for a specific purpose. With recent advances, it has also become obvious that the composition of supporting trophic molecules in the culture medium is crucial for cell performance, particularly when it comes to the delicate culturing of stem and progenitor cells.

Also the physical milieu has been subjected to alteration and evaluation. There are several products available designed to mimic the physical milieu at the transplantation site. Some of these products are designed for 2D surface culture applications, while others provide a more complicated 3D structure. Furthermore, the designs of bioactive molecules to modulate culture performance have been introduced as a variable. Altogether, these manipulations aim to construct an *in vitro* environment that has a greater chance of generating results that can be readily translated into similar results *in vivo*.

The microenvironment of cultured cells is determined by cell-cell interactions, matrix interactions and gas exchanges. The cells possibilities to interact in these capacities in culture is greatly influenced by the way the cells are seeded. There are three major preparations in which this can be performed. Either as a tissue specimen (e.g. slice of tissue), which commonly is 200-400 µm thick, as a sphere, which is the free-floating cell aggregate developed by suspended cells in a culture flask, or as a single cell preparation of dissociated cells.

Neurotrophins

Since researchers have started to look for the molecules responsible for the observed growth stimulation of cells, the knowledge concerning these molecules has expanded and the efforts have been organized as new fields of biological science.

The group of molecules displaying a trophic effect on neurons are called neurotrophins. These include factors with different gene ancestors. One family of proteins that share a common gene ancestor (Leibrock et al., 1989) and are essential to mammals is named Neurotrophins (NTs). These were the first neurotrophic factors described and they all play a vital role during neuronal development. It has been shown that NTs are instrumental in the regulation of neurons both centrally and peripherally. This group consist of nerve growth factor (NGF) (Cohen, Levi-Montalcini, & Hamburger, 1954; Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde, Edgar, & Thoenen, 1982), Neurotrophin 3 (NT-3) and Neurotrophin 4/5 (NT-4/5) (Lewin & Barde, 1996). They collectively ensure a suitable match of the neuronal density in their target organs (Lewin & Barde, 1996) and control cell fate, axonal growth and guidance, dendrite structure and pruning, synapse formation and synaptic plasticity (Huang et al., 2001; Kaplan & Miller, 2000; Lewin & Barde, 1996; Poo, 2001).

In addition to the NT's, glial cell-derived neurotrophic factor (GDNF) is shown to have trophic effects on developing (Lin, Doherty, Lile, Bektesh, & Collins, 1993) and adult midbrain dopaminergic neurons (Lapchak, 1996). GDNF has also been reported to increase the survival and neurite growth of spiral ganglion neurons and thus play a vital role in development of the central auditory pathways and the inner ear (Euteneuer et al., 2013).

Conditioned medium

During the early development of the research field into neurotrophic factors, the concept of conditioned medium (CM) comprised a powerful tool to harvest substantial amounts of cell specific trophic molecules in. In short, the CM consists of culture medium collected after it has been used in a cell culture for 24-48 hours. Some nutrients in the medium are consumed during culture, but the medium is also enriched (conditioned) with the excreted secretome of the specific cell type in culture. This secretome contain serum proteins, extracellular matrix proteins, digestive enzymes and a low concentration of highly bioactive proteins. These bioactive proteins have been found to include growth factors, hormones, cytokines and extracellular matrix processing proteases. All of these are now documented to play key roles in regulation of cell differentiation and renewal (Skalnikova, Motlik, Gadher, & Kovarova, 2011).

The CM was found to have trophic effect on cells in culture. Early on it was a common practice to supplement cell culture mediums with CM. Today, when we have gathered more detailed knowledge on what is required to successfully culture cells *in vitro*, we still find the use of CM to be an efficient supplement, even to modern culture mediums.

Few CMs have previously been analyzed in detail. A CM from our utilized BS slice with CN is not one of them. As we have previously reported positive results from experimental cultures using BS CM, we predict that elucidating its contents and concentrations of trophic factors can lead to a better understanding of the mechanisms behind the results achieved utilizing CMs.

Alteration of physical milieu

Much efforts have been directed towards improving the physical milieu of cells *in vitro*. There are a number of conditions that are subject to recent advances and they show that small modulations to the culture environment can have large effects on cell performance.

The *in vitro* milieu into which we transplant cells, needs to have similarities with that of the future transplantation site for comparative reasons. The culture milieu will naturally be drastically different in many ways to a transplantation site, but in some areas we can create a resemblance with the environment that the cells were intended for.

In standard cell culture protocols the specimens are either seeded in a fluid, or onto a glass or plastic surface. Since it is difficult for cells to develop in a suspension in general (with the exception of hematopoetic cells), the culture of free-floating cells are mostly used for propagation and bulk production of cells for later harvest and usage. The solid surface of culture vessels and cover slips is in many ways a more physiological option as it provides the mechanical support needed for all anchorage-dependent cells to thrive. To assist survival and migration, these surfaces are typically coated with attachment and adhesion proteins, such as collagen, laminin and fibronectin, or synthetic polymers as in Poly-D-Lysine.

In 1991, the already established interface-culture method was successfully introduced as a alternative to the existing methods for culture of nervous tissue explants (Stoppini et al., 1991). Compared to the avaible methods this greatly simplified protocols. The method utilize a transparent porous membrane for culture surface and does not require the use of a plasma clot to attach cell specimens. Cells are cultured in the interface between air and culture medium, and yield specimens that remain 1-4 layers thick charaterized by a well preserved organotypic organisation. This also resolved much of the glial scarring problems that was commonly noticed in cultures on solid surfaces as a result of nutrient and oxygen deprivation in the areas furthest away from the medium (i.e. cell-vessel contact area).

Further, the development of surface coatings with molecules supplying a connective tissue like environment has advanced. The products that are available now also include specific bioactive domains that can be utilized to improve cell survival and differentiation. These products can be coated as thin layers (2D) or prepared as gels of different thicknesses supplying a 3D milieu. Cells seeded in 3D can benefit from the provived mechanical scaffold in their arrangement of molecules and such *in vivo*-like structural organization has been shown to enhance the survival of cells in culture

(Webber et al., 2010). It can also improve their differentiation, morphological profiles and general functions. These products are often combined with different contents of trophic factors, providing several cell culture options to be utilized in experimental settings.

One example of scaffolding (utilized in Paper II) is the Corning® Matrigel®. This is a preparation extracted from a mouse sarcoma which is rich in extracellular matrix proteins. Due to the innate capacity of these cells to stimulate teratomas (Vaillant, Lindeman, & Visvader, 2011) they are not likely to be considered a preferred substances for clinical use. However, as a research tool these products can be very useful. They are often rich of substances that are well documented to benefit cells in culture. The Matrigel® (used here), contain laminin, collagen, entactin and a naturally provides a wide range of growth factors.

Cell transplantation

It is delicate work to identify a suitable donor cell type to serve as a substrate in a cell transplantation paradigm, much due to the fact that the specifics of a working cell transplantation protocol is not yet defined. And prioritizing research efforts is increasingly complicated as there is an ever growing number of potential donor cells available through commercial suppliers, as well as through research collaborations.

The autologous transplantation of cultured cells have been a tantalizing paradigm on the mind of many researchers ever since the discovery of adult neurogenesis (Altman & Das, 1965) as a product of the budding field of stem cell (SC) research (Becker, Mc, & Till, 1963). The SC was subsequently defined as a cell with intact capability to differentiate into cells of all three germ layers and to renew themselves through cell division. To a researcher involved in the field of cell transplantation, this constitutes a very plausible substrate for the regeneration or replacement of an impaired organ.

Three types of stem cells

The embryonic stem cells (ESCs) are the ‘‘primitive (undifferentiated) cells derived from the early embryo that have the potential to become a wide variety of specialized cell types’’ (IOM, 2008). These cells can be found in humans (and most other mammals) during the first 4 cell divisions after conception (eventually a 16-cell morula)(Svendsen, 2008). After this, the cells of the budding embryo (blastocyst) start to differentiate down a regulatory pathway. This pathway is one-way and eventually render the cells specialized with less capacity to differentiate into other cells. It has been shown that the ESCs are very good candidates for usage in regenerative medicine (Nury, Neri, & Puceat, 2009).

The adult SC is “an undifferentiated cell found in a differentiated tissue that can renew itself and (with limitation) differentiate to yield the specialized cell types of the tissue from which it originated” (IOM, 2008). The existance of adult SCs were first documented in the spleen in 1963 (Becker et al., 1963), and has later been documented in many organs, e.g. brain (Lewis, 1968), bone marrow (Trentin, 1989) and skin (Toma, McKenzie, Bagli, & Miller, 2005). Adult SCs can be very useful for transplantation in degenerative diseases (Gogel, Gubernator, & Minger, 2011). These cells can quite easily be incorporated into host tissue, develop highly specialized functions and improve organ function (Lumelsky et al., 2001; Studer, Tabar, & McKay, 1998).

The induced pluripotent stem cell (iPSC) is derived from an adult cell by reprogramming and was first established in 2006 (Takahashi & Yamanaka, 2006). They are generated via the introduction of retroviral transcription of factors. Human iPSCs (hiPSCs) were first produced from skin fibroblasts and rendered pluripotent cells with similar features as ESCs (Takahashi et al., 2007). They do not raise ethical problems like the ESCs since hiPSC research does not destroy blastocysts or embryos. However, there are concerns that the introduction of retroviruses may cause cancer or deleterious mutations in the host (Hyun, Hochedlinger, Jaenisch, & Yamanaka, 2007).

Progenitor cells

The progenitor cells are more differentiated cells as compared to the SCs. These cells are still able to divide multiple times but have started their differentiation downstream (Petersen, Zou, Krauss, & Zhong, 2004). Studies have shown promising use of progenitor cells in myocardial neovascularization treatment of ischemic disease (Kawamoto et al., 2001), CNS injury treatment (Nandoe Tewarie, Hurtado, Bartels, Grotenhuis, & Oudega, 2009), as well as in liver and acute kidney injury repair (Becherucci et al., 2009; Zhao, Ren, Zhu, & Han, 2009). Transplantation of neuronal precursor cells has led to replacement of damaged neurons in the adult CNS (Bjorklund & Lindvall, 2000). It has also been shown that transplanted progenitor cells can reduce the inhibitory gliosis and scarring that occurs as part of the healing process in the CNS following injury (Palmgren et al., 2012).

The BC cells (utilized in paper I) comprise a source of multipotent sensory specified SCs that has been shown to differentiate spontaneously into functionally diverse types of sensory neuronal (Hjerling-Leffler et al., 2005), and non-neuronal cell types (Aquino et al., 2006). They normally constitute a source of neuronal and glial cells to the peripheral nervous system (Maro et al., 2004).

Stem- and progenitor cell limitations

There are a few safety considerations inherent to the concept of using immature cells when generating cells for transplantation *in vivo*. Since both SCs and pluripotent cells have the ability to differentiate into various cell types, an *in vivo* transplantation will induce a potential risk of local teratoma formation or unwanted over-proliferation of cells resisting final differentiation. One way to reduce this risk is to efficiently pre-differentiatiate cells into the desired fate. Other ways to address this is to expose the cells to extrinsic cues (Chambers et al., 2009) or to intrinsic factors (Zhang et al., 2013). However, with increasing maturity at the time of transplant, the capabilities for a successful engraftment and integration of the transplanted cells may decline. To ensure cell type specificity and integration many replacement paradigms suggest transplantation at the stage of cell cycle exit (i.e. neuroblasts) as a reasonable compromise (Kriks et al., 2011; Liu et al., 2013). This will also reduce the risk of tumor formation even though an oncogenic genetic alteration can occur later in any cultured cell type.

Evaluation of cell performance in culture

It is possible to perform a palette of qualitative and quantitative evaluations of cell performance *in vitro*. Modalities that are routinely evaluated when studying cell performance include survival, differentiation and integration. There are a multitude of technical methods available to facilitate these evaluations and a selection of them are commonly included in the basic inventory of any laboratory.

To improve the performance of transplanted cells *in vitro*, we found it necessary to further evaluate the functional properties of neuronal cells in interface-culture. There are many established techniques for the purpose of evaluating the electrical activity in cultured cells, but none of them suit the evaluation of cells cultured ad modum Stoppini.

The first reported method to facilitate evaluation of electrophysiological activity in cells was the voltage clamp technique (Hodgkin & Huxley, 1952). This later evolved into the still frequently utilized patch clamp technique (Neher and Sakmann, 1976; Sakmann and Neher, 1984). The use of these techniques has greatly benefitted the research community in ways of detecting electrical activity over cell membranes (subthreshold synaptic potentials, membrane oscillations and action potentials) and on whole complex cell networks (e.g. brain specimens).

MEA

During the 1980s to the early 1990s, the microelectrode array (MEA) technique was introduced (Novak & Wheeler, 1986). It is a technique that while still undergoing rapid development has matured in many ways into a standard laboratory assay to perform electrophysiological recordings on dissociated cells in culture, and on tissue specimens.

The MEA technique is a powerful complimentary method to the earlier techniques, as it enables the user to measure extracellular field potentials, generated by actions potentials, over large areas determined by a fix pattern of electrodes (Panuccio, Colombi, & Chiappalone, 2018). These electrodes are mounted on plates that can serve as either a culture surface for dissociated cell cultures, or as a measurement fixture for acute recordings on tissue specimens. The known inter-electrode distance is crucial for advanced detailed analyses, as it facilitates an accurate pinpointing of any neuronal network interactions. Today, powerful algorithms are available to analyse recorded signals from MEA plates with the help of computational tools. This has led to many publications of advances into the understanding of brain development, the effects of pharmaceuticals and intricate mechanisms of neuronal disease (Ben-Ari, 2001; Katz & Shatz, 1996; Madhavan, Chao, & Potter, 2007; Maeda, Robinson, & Kawana, 1995; van Pelt, Vajda, Wolters, Corner, & Ramakers, 2005; Wagenaar, Pine, & Potter, 2006; Yvert, Branchereau, & Meyrand, 2004). The advances in MEA-technology with refinement of the initial perforated MEA-plates (Boppart et al 1992), the advent of carbon nanotechnology (Greenbaum et al., 2009) and hyper-dense CMOS sensors (Jackel, Frey, Fiscella, Franke, & Hierlemann, 2012), are continously increasing the quality of the recorded data.

The present MEA technique protocols do not facilitate analyses of established interface-cultures ad modum Stoppini. These specimens will not survive when submerged in the recording media as commonly specified in experiment protocols. It is though, possible to perform acute recordings on similar tissue samples for short periods of time, but there has been no reports on the evaluation of electrophysiological activity in an auditory BS slice.

AIMS

The aim of this thesis is to investigate a set of auditory neuronal experiments to aid the evaluation of transplanted cells in vitro. We specifically:

Paper I. Evaluate the effects of BS CM on BC cell performance in culture, and if these effects possibly correlate with medium BDNF and GDNF content.

Paper II. Evaluate the effects on dissociated HNPC survival and differentiation in culture by the addition of a neurotrophic factor rich bioactive matrix coating to the culture surface.

Paper III. Characterize the performance of neuroepithelial-like stem cells seeded to culture at different states of differentiation.

Paper IV. Assess if the MEA technique can be utilized to perform evaluation of electrophysiological activity in an auditory BS slice interface-culture.

Here we present the overall layout of the included experiments (Figure 2).

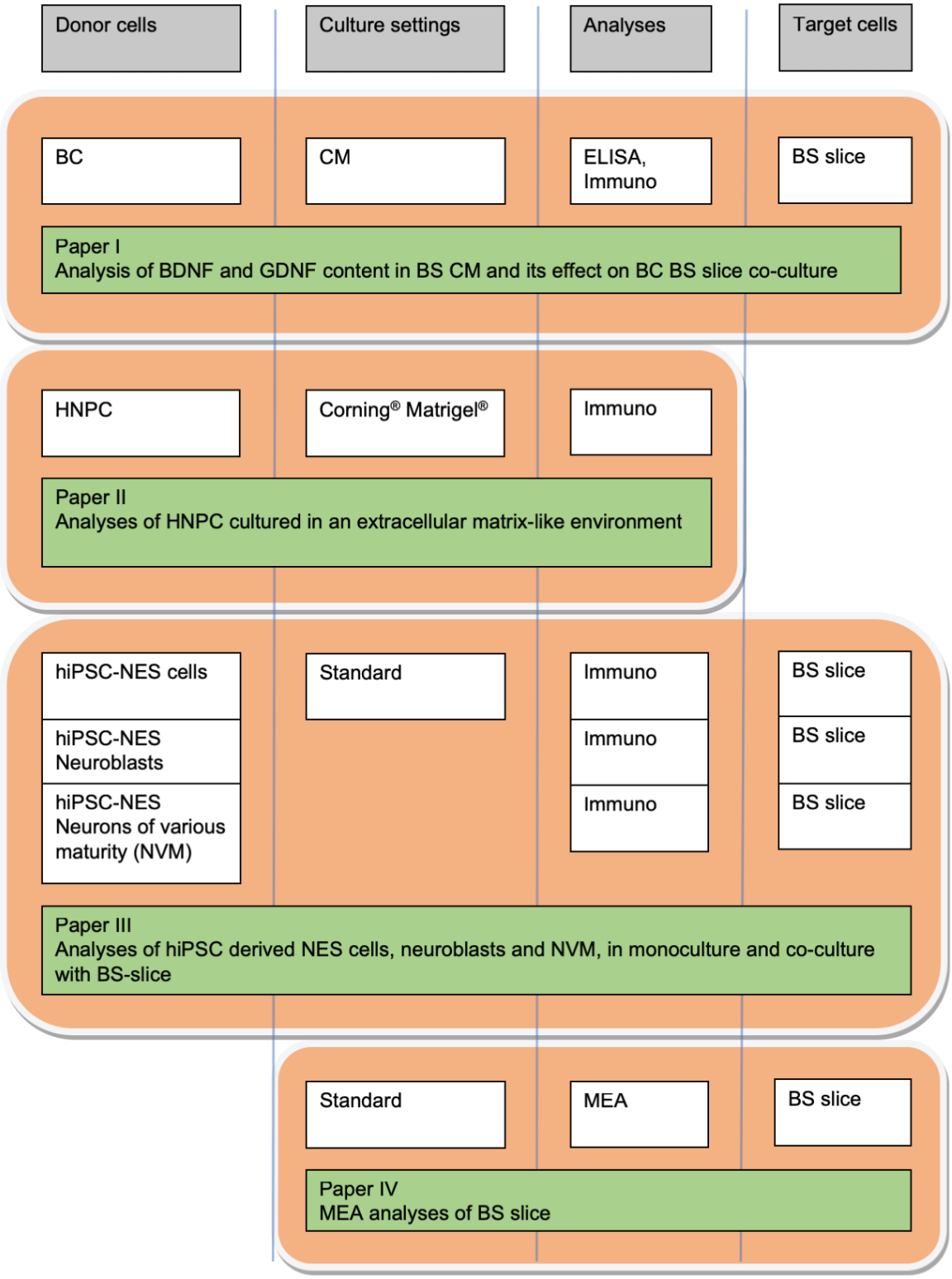

Figure 2. Schematics of experiment layout in papers I-IV. Four papers are included in this thesis. The papers address the choice of donor cells, aspects of culture settings, ways to analyze results and the effect from co-culture with target cells (brainstem slice with cochlear nucleus). BC = boundary cap; CM = conditioned medium; BS = brainstem slice; HNPC = human neural progenitor cell; hiPSC = human induced pluripotent stem cell; NES = neuroepithelial-like stem (cell); NVM = neurons of various maturity; MEA = microelectrode array.

MATERIAL AND METHODS

Animals (Papers I, III and IV)

Pregnant Sprague-Dawley rats (Scanbur) were obtained and their pups (P12-P14) were used for preparing BS slices (Paper I).
GFP-Tau mouse (Charles River Lab, Sulzfeld, Germany) embryos were used as donors for BC cell preparations (E11.5)(Paper I).
P9 neonatal C57BL/6 mice were used for preparing organotypic BS slices (Papers III and IV).

All work was done in accordance with local ethical guidelines and approved animal care protocols N329/07, N3/11, N4/11 (Stockholms Norra Djurförsöksetiska nämnd)(Paper I) and C100115/15 (Uppsala Djurförsöksetiska nämnd)(Papers III and IV).

Brainstem slices (Papers I, III and IV)

Animals were sacrificed by decapitation. Transverse sections (300 µm thick) of the excised brains were obtained using a McIlwain tissue chopper. Sections where chosen encompassing the proximal part of the cochlear nerve and the cochlear nucleus (CN) in the BS according to local anatomical landmarks (Herlenius et al., 2012; Thonabulsombat et al., 2007)(Figure 3). The size of each BS slice and amount of CN were approximated to be equal for all used slices. Slices were propagated as interface-cultures (Stoppini et al., 1991) on polyester membranes coated with 5 µg/ml poly-D-lysine hydrobromide and 10 µg/ml laminin. The culture medium was changed on the day after preparation of slices, followed by every other day thru the culture period.

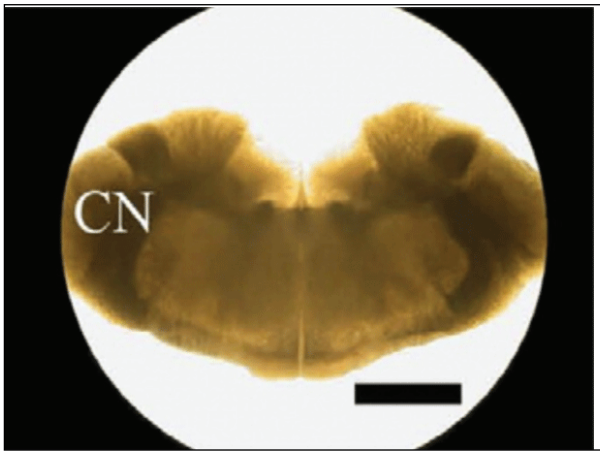

Figure 3. Bright-field picture of a brainstem slice. CN = cochlear nucleus. Scale bar = 500µm.

Transplanted cells (Papers I-III)

Boundary Cap (BC) cells were isolated as previously described (Hjerling-Leffler et al., 2005). Briefly, GFP-Tau mouse (E11.5) dorsal root ganglions (DRGs) were dissected. Following dissociation using 1 mg/ml Collagenase/Dispase the cells were plated. From the DRG cell culture BC sphere formations could be observed within two weeks of culture. BC spheres expressing GFP were selected after 2–3 passages for use in protocols (Paper I).

The HNPCs used were originally established by L Wahlberg, Å Seiger, and now kindly provided by by Prof. A. Björklund. The cell line was derived from human embryonic 9-week post conception forebrain and perpetually continued as sphere culture. Spheres were seeded following dissociation (Paper II).

The long-term self-renewing NES cells used here were derived from a hiPSC line established by standard retroviral transduction (Falk et al., 2012). The continual propagation of stem/progenitor cells were maintained as monolayers in poly-L-ornithine and laminin coated flasks. The density of seeded cells were 40 000 cells/cm^2 and split every 2-3 days 1:3 to maintain a high density. With the planned medium changes adjusted to be performed every other day, along with an increased laminin concentration when coating, the NES cells begin to differentiate into subtypes of cells further down the neuronal lineage. This starts 4-5 days' after bFGF/EGF growth factor withdrawal and render neuroblasts after 5-7 days, and a group of neurons of various maturity (NVM) after 2 weeks of culture (Paper III).

Mediums utilized (Papers I-IV)

A wide range of mediums were utilized in the preparation of cultures, experiments and analyses. For reference purposes, we here provide their names, contents and in which paper they were utilized (Table 1).

Conditioned medium (Paper I)

Medium was collected from rat BS slice monocultures every two to three days. To produce BS CM this was filtered and mixed with equal amounts of fresh medium (here BC medium) and pooled into one solution which was named BS conditioned culture medium. We included medium collected over a three-week period.

Two groups of CM with different amounts of BS slices were prepared, one with one slice and another with two slices per well.

Three mediums where prepared for the mono- and co-culture experiment groups: BC medium (standard protocol); propagation medium (BC medium with added factors); BC conditioned culture medium (propagation medium with BS CM).

Matrigel® (Paper II)

Cover glasses were coated with a thin preparation of Corning® Matrigel® matrix gel according to supplier instructions (50 µl/cm^2) and placed on the bottom of culture wells.

Immunocytochemistry (Papers I, II and III)

The cultures were fixed in 4 % paraformaldehyde (PFA) in PBS (pH 7.4) for 1 hour at room temperature (RT). After 5X wash they were treated with ice-cold 20 % methanol in PBS for 5 minutes. After 3X wash the tissues were treated with 0.5 % Triton-X in PBS overnight at 4 °C for permeabilization. After another 3X wash the specimen were incubated with 20 % BSA in PBS blocking solution for 12 hours at 4 ºC. Then the incubation with primary antibodies started. The specimens were incubated with primary antibodies (Table 2) diluted in 5 % BSA in PBS overnight. After 4X wash the primary antibody-antigen complexes were visualized with secondary antibodies by incubation for 4 hours followed by a 4X wash. Staining specificity was confirmed by omission of the primary antibody. 4´,6-Diamidino-2-phenylindole (DAPI) nuclear stain (10 µg/ml) was introduced before the final 4X wash of specimens.

Table 1. Mediums utilized in cultures.

Medium	*Content*	*Used in paper no.*
Dissection medium	HBSS, glucose 20 %, antibiotic-antimycotic 1 %.	I, III, IV
Boundary cap (BC) medium	Neurobasal medium, N2 1 %, B27 1 %.	I
Propagation medium/ boundary cap (BC) medium	Neurobasal medium, N2 1 %, B27 1 %, epidermal growth factor (EGF) 20 ng/ml, basic fibroblast growth factor (bFGF) 20 ng/ml.	I
Brainstem (BS) medium/ brainstem (BS) culture medium	High glucose DMEM, HBSS 30 %, glucose 6.5 g/l, 25 mM HEPES, fetal bovine serum (FBS) 10 %, antibiotic-antimycotic 1 %.	I, III, IV
Brainstem (BS) conditioned culture medium	Medium collected from brainstem (BS) culture 50 %, propagation medium (Neurobasal medium, N2 1 %, B27 1 %, epidermal growth factor (EGF) 20 ng/ml, basic fibroblast growth factor (bFGF) 20 ng/ml) 50 %.	I
Brainstem conditioned medium (BS CM)	Medium collected from brainstem (BS) culture.	I
Culture medium	DMEM-F12, L-Glutamine 2.0 mM, glucose 6.5 g/l, N2 1 %, heparin 2.0 µg/ml, human basic fibroblast growth factor (hbFGF) 20 ng/ml, human epidermal growth factor (hEGF) 20 ng/ml, human leukaemia inhibitory factor (hLIF) 20 ng/ml.	II
Differentiation medium (DM)	DMEM-F12, L-Glutamine 2.0 mM, glucose 6.5 g/l, N2 1 %, heparin 2.0 µg/ml, human basic fibroblast growth factor (hbFGF) 20 ng/ml, human epidermal growth factor (hEGF) 20 ng/ml, human leukaemia inhibitory factor (hLIF) 20 ng/ml, fetal bovine serum (FBS) 10 %.	II
Culture medium/NES medium	DMEM/F12-GlutaMAX medium, N2 1 %, B27 0.1 %, penicillin-streptomycin 1 %, basic fibroblast growth factor (bFGF) 10 ng/ml, epidermal growth factor (EGF) 10 ng/ml.	III
Neuroepithelial-like stem cell (NES) differentiation medium	DMEM/F12-GlutaMAX medium, N2 1 %, B27 1 %, penicillin-streptomycin 1 %.	III

Table 2. Primary antibodies used in Papers I-IV.

Primary antibody	*Target*	*Dilution*	*Human specific*	*Utilized in paper no.*
β-III tubulin (TUJ1)	A structural protein found almost exclusively in neurons, a major constituent of micro-tubules.	1:200	no	I, II, III
DCX	A microtubule-associated protein expressed in developing neurons.	1:200	no	II, III
GFAP	An intermediate filament protein type III found in astrocytes and other glia.	1:500	yes	II, III
Human cytoplasmic marker	A cytoplasmic protein of human cells.	1:500	yes	III
Human nuclei marker	The Ku80 protein located in the nucleus of human cells.	1:50	yes	III
Ki67	General proliferation markers.	1:200	no	III
MAP2	A microtubule-associated protein involved in microtubule assembly and in determining dendritic shape during the neuronal develop-ment.	1:200	no	II, III
Musashi	A molecule that plays a role in the main-tenance of the stem cell state, selectively expressed in neural progenitor cells, including neural stem cells.	1:200	no	III
Nestin	A type VI intermediate filament expressed in the neuronal precursor and developing cells.	1:500	yes	II, III
NeuN	A protein expressed in post-mitotic maturing neuronal nuclei.	1:200	yes	III
NF heavy chain	Type IV intermediate filament, comprise axoskeleton and functionally maintain neuronal caliber.	1:1000	no	III
OCT2	A marker for undifferentiated stem cells	1:500	no	II
Peripherin	A marker of neuronal development that is expressed concomitantly with axonal growth following axonal differentiation of sensory neurons.	A:500	no	I
SOX2	A pluripotency transcription factor that plays a central role in the maintenance of stemness.	1:200	no	III
SSEA-1	A negative surface marker for human undifferentiated stem cells, upregulated with differentiation.	1:300	yes	III
SV2	A glycoprotein maintaining the readily releasable pool of secretory vesicles in neural and endocrine cells.	1:1000	no	II
Synapsin 1	A phosphoprotein specific for neuronal synapses of the CNS and PNS.	1:1000	no	II

Image analysis (Papers I, II and III)

Photomicrography in combination with immunofluorescence is a powerful method for qualitative assessment and quantification of survival, differentiation and migration. Results are saved as pictures for visual evaluation and objective quantification.

Evaluation of migration (Paper I)
The specimen transplant area was divided into quadrants with the center of the BC cell transplant after fixation as origo. The total number of identified neurons with axonal growth in the absolute vicinity of the transplant was quantified at 100x magnification. Care was taken to blind the group affiliation and orientation of the transplants on the slide. The cells found in the quadrant encompassing the hiatus between the transplanted cells and the BS slice were accounted for as attracted. The data are presented as a percentage of neurons in the attracted quadrant, out of the total number of neurons in the transplant area. This was presented separately for each experimental group.

Evaluation of axonal length (Paper I)
Axonal length was measured with a calibrated freehand line drawn along the axon of every selected cell. This was performed utilizing the drawing tool in ZEN software (blue edition).

Automated 2D analyses (Paper III)
An automated cell identification and quantification process was utilized to collect the vast data available from the recorded material in paper III. Establishing the fully automated cell analysis was done with the kind support from Department of Information Technology at Uppsala University. Here, we report analyses from a total of 412 206 characterized cell profiles, utilizing this method.

In brief, the cell quantification was performed using CellProfiler cell image analysis software (Carpenter et al., 2006) and the scoring of phenotypes by machine learning was done using CellProfiler Analyst software (Jones et al., 2009). A first pipeline, Preprocess, was used on a reference picture set to generate basic data identifying the nuclei and the cytoplasm (Figure 4 and 5). This data was then used in CellProfiler Analyst to generate rules to facilitate the automatized quantification of positive cell profiles. These rules were utilized in the second pipeline, Classifier, to analyze all pictures of a specific staining (Figure 5). To avoid confounders, all parameters in the analysis methods within the pipelines analyzing each group were kept identical.

ELISA (Paper I)

The ELISA method was utilized to characterize the specific contents of BS CM. BDNF and GDNF levels were assayed with ELISA kit reagents (Abnova Corporation). Culture medium samples were added in duplicates in individual pre-coated wells of a 96 well plate (Figure 6) and incubated for 90 minutes. The plates were then emptied and blotted. 100 µl of biotinylated anti-rat antibody working solution was added and incubated for 60 minutes. After washing, 100 µl of ABC working solution was added and incubated for 30 minutes. After another washing, 90 µl of color developing agent was added and the contents were incubated in the dark for about 20 – 25 minutes. This was all done according to protocols provided by the supplier. Evaluation of the optical density were read at 450 nm.

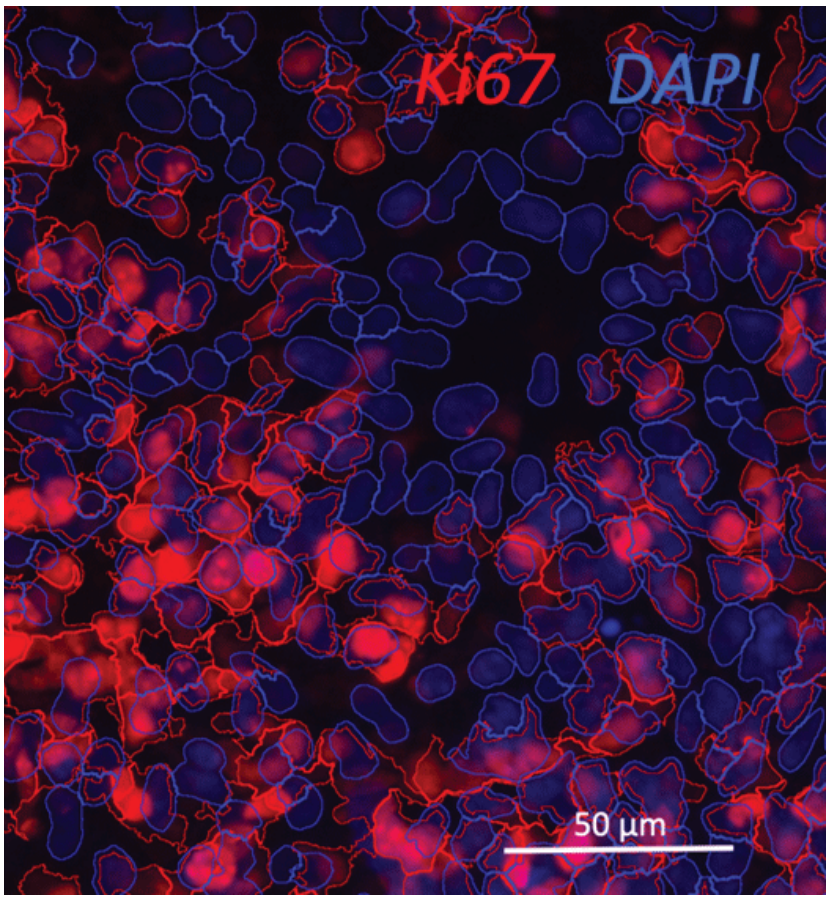

Figure 4. A sample picture rendered by CellProfiler software showing estimated cell marker boundaries. Shown here is an example of how the CellProfiler and Cell-Profiler Analyst software define the nuclei (DAPI, blue profiles with blue borders) and the cytoplasm (Ki67, red profiles with red borders) borders in a sample picture.

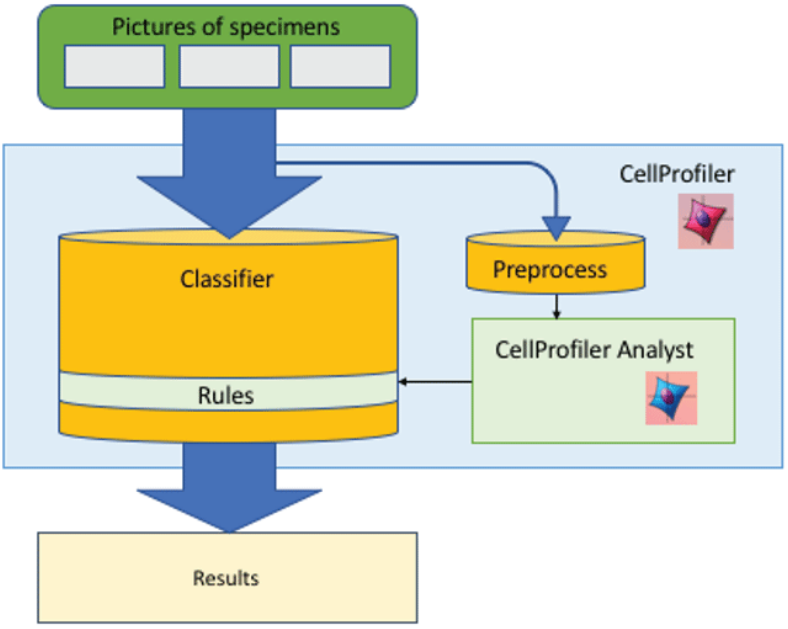

Figure 5. A schematic figure showing the process of automated cell profile evaluation utilizing the CellProfiler and CellProfiler Analyst softwares.

Figure 6. A 96 well ELISA kit plate. BS CM samples were analyzed individually in pre-coated wells.

MEA (Papers IV)

All electrophysiological recordings of spontaneous activity were performed utilizing a MEA 1060 amplifier (Multichannel systems, MCS) on a MEA 60MEA200/30iR plate (200 µm electrode spacing and 30 µm electrode diameter) with plastic ring (Figure 7A).

A membrane insert containing one BS slice specimen was cut out with a scalpel and mounted face-down on the MEA plate. A Harp slice grid (ALA Scientific Instruments) was used to keep specimen in stable contact with the recording area (Figure 7B). The recordings started immediately after the slice had been mounted on the plate (Figure 7C). The data was continuously recorded at a sampling rate of 20 kHz for 30 minutes. Data acquisitions and analyses were performed using either MC_Rack software (V 4.6.2) or Multi Channel Suite software (Multi Channel DataManager V 1.10.1) from Multi Channel Systems (MCS).

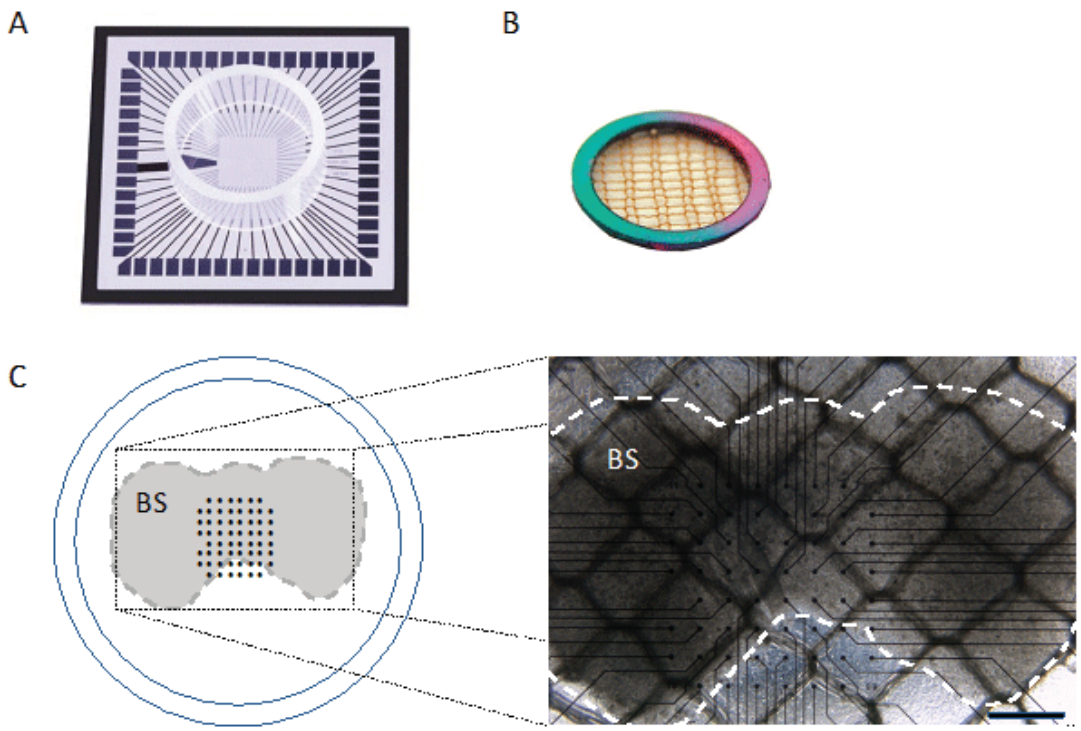

Figure 7. (A) A MEA plate with a plastic ring for the cell culture. (B) A Harp slice grid to hold specimen in place. (ALA scientific instruments). (C) Schematic picture of a mounted BS slice in the well of a 60-electrode MEA plate (left)(• marking electrode positions). Bright-fielded picture of a BS slice after 21 days of culture ad modum Stoppini, cut out and mounted face down on a MEA plate (right, with a Harp slice grid on top). BS = brainstem slice; Scale bar =500 µm.

Statistics (Papers I, II and III)

Where applicable, all results were expressed as mean ± standard error of the mean (SEM). The values in control and experimental groups were compared by one-way ANOVA test followed by post hoc Tukey's multiple comparison test. Differences of $P \leq 0.05$ were considered statistically significant.

RESULTS

Paper I

Here we study the neurotrophin content in BS CM and its effects on BC cells. Two groups of mediums were evaluated for their BDNF and GDNF contents, one with one BS slice per well and another with two BS slices per well. BS medium was used as control. To evaluate the BS CM effects on BC cells we performed monocultures and co-cultures with BS slice, ad modum Stoppini, in three different mediums: BC medium, propagation medium and BS conditioned culture medium.

BDNF and GDNF analyses

BDNF-levels in standard BS medium were on average 37 ± 18 pg/ml. BDNF-levels in BS CM were on average 40 pg/ml in the one slice setting, and 62 pg/ml in the two-slice setting. GDNF-levels in BS medium were on average 79 ± 32 pg/ml. On average, the level of GDNF in BS CM was 66 pg/ml in the one slice setting and 90 pg/ml in the two-slice setting.

The BS CM levels of both BDNF and GDNF showed increments that also varied over time. A biphasic release pattern was noticed (BDNF in days 5 and 15; GDNF days 3 and 12) in the BS CM collected from cultures with two slices (Figure 8).

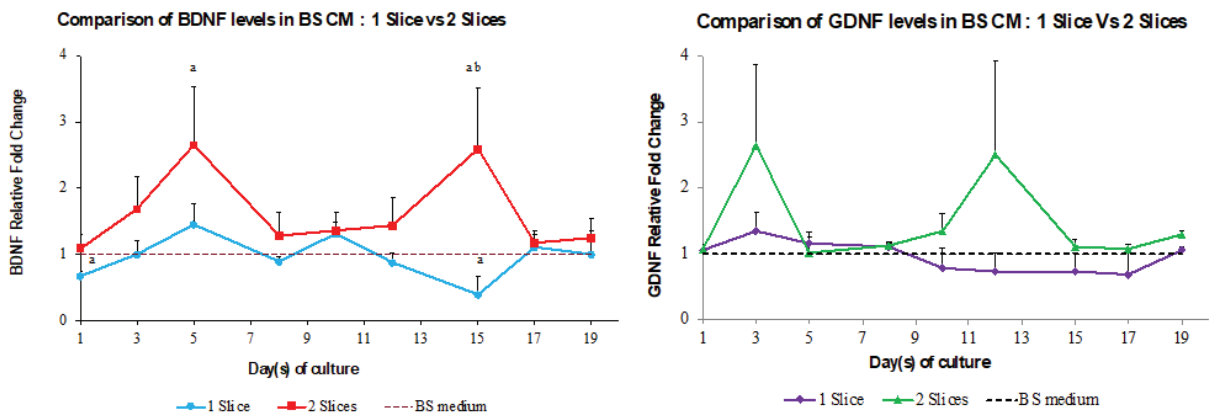

Figure 8. Relative fold of BDNF and GDNF content in brainstem (BS) conditioned medium (CM). Two groups of BS CM preparation from 3 weeks of culture (one slice per well and two slices per well). ELISA analysis is shown as relative fold of BDNF (left) and GDNF (right) content in BS CM as compared to standard BS medium levels (=1). A biphasic pattern over time is noted (BDNF statistically significant). $^{a}P < 0.05$, compared to 1 slice BS medium; $^{b}P < 0.05$, compared to 2 slice BS medium.

Analyses of BC cell monoculture

In BC medium, cells developed peripherin-positive neurite processes, but after six days of culture they already displayed abundant cell death. In propagation and BS conditioned culture medium the cell survival was high (Table 3). In propagation medium, the cells illustrated peripherin-negative cells, indicating lack of differentiation towards a neuronal fate (Table 3). In BS conditioned culture medium, the BC cells illustrated peripherin-positive neurite outgrowths within five days of culture and they maintained their strong peripherin expression during the entire three-weeks (Table 3).

Table 3. Survival and peripherin expression of mouse BC cell monocultures in three different mediums.

BC monoculture	Survival	Peripherin staining
BC medium	<6d	+
Propagation medium	>3w	-
BS conditioned culture medium	>3w	+

Analyses of BC cells in auditory BS slice co-culture

In co-culture with a BS slice, BC cells in BC medium and propagation medium did not survive for more than two weeks, whereas in BS conditioned culture medium BC cells were still propagating at the three weeks' termination point (Table 4).

Signs of differentiation in BS conditioned culture medium was prominent as compared to the co-cultures in BC medium and propagation medium. Directional growth of axons from the differentiated BC cells towards the BS was seen in all three co-culture groups (Table 4), but the unidirectional growth towards the BS co-cultures in propagation medium and BS conditioned culture medium was stronger (Table 4).

Table 4. Survival, peripherin expression and attraction of mouse BC cell co-culture with rat BS slice in three different mediums.

Co-culture	Survival	Peripherin staining	Attraction
BC medium (n=13)	2w	-	53% ±11
Propagation medium (n=12)	2w	-	72% ±11
BS conditioned culture medium (n=3)	>3w	+	66% ±13

Paper II

Here we examine the effect of a hydrogel matrix coating on the survival of cells in surface culture. The experimental setup included two groups. Human neural progenitor cells (HNPCs) seeded as dissociated sphere cultures on Matrigel® coated coverslips, or on Matrigel® uncoated coverslips (standard Laminin coated). Cell differentiation was also studied using immunocytochemistry.

Survival of HNPCs

For quantification of surviving HNPCs in dissociated sphere culture (DSC) intact DAPI positive nuclei profiles were counted after three weeks. The experimental group displayed abundant survival with a high ratio of DAPI positive nuclei to seeded cells (99 % ± 5 %; Figure 9). The control group cultured on Matrigel® uncoated coverslips showed a low survival rate with only few intact nuclei to seeded cells (1,5 % ± 0,2 %; Figure 9).

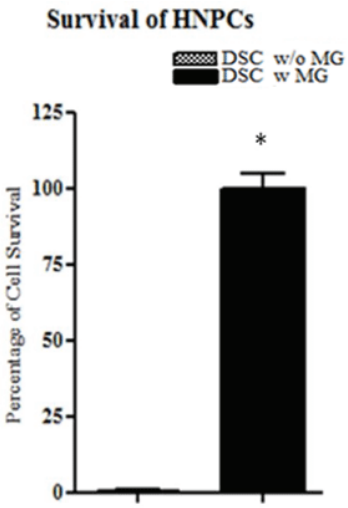

Figure 9. Evaluation of the survival of dissociated sphere cultures (DSC) on Matrigel® (MG) coated coverslips. The number of intact cell profiles in the group cultured on Matrigel® coated coverslips after three weeks in culture were 99 % ± 5 %, and in the control group 1,5 % ± 0,2 %, as compared to the original amount of seeded cells. * indicates $p < 0.05$.

Expression of progenitor markers

To identify cells with immature profiles we did immunocytochemical analyses with nestin, a marker for stem/progenitor cells, and OCT2, a marker for undifferentiated SCs. The results illustrate that despite the high HNPC survival in the experimental group (Figure 9), nestin and OCT2 showed zero positive profiles, illustrating strong cell differentiation. The same results were found in the control group with zero positive profiles from nestin and OCT2 staining.

Expression of neuronal and glial markers

β-III tubulin and GFAP

The fate of the HNPCs in DSC was observed using the neuronal marker β-III tubulin that is almost exclusively found in neurons, and GFAP, a intermediate filament protein type III found in astrocytes and other glia. Most surviving cells grown on Matrigel® expressed β-III tubulin (60 % ± 5 %; Figures 10 and 11) indicating the onset of differentiation down the neuronal lineage. There was also a substantial portion of the cells expressing GFAP (25 % ± 3 %; Figures 10 and 11). A number of cells (15 % ± 3 %) co-expressed β-III tubulin and GFAP (Figures 10-11).

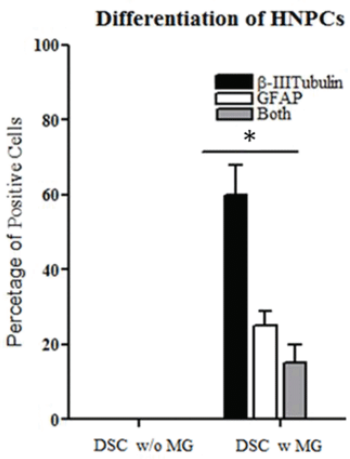

Figure. 10. Percentage of HNPCs in dissociated sphere cultures (DSC) expressing β-III tubulin and GFAP on Matrigel® (MG) coated or Matrigel® uncoated coverslips. HNPCs on Matrigel® coated coverslips showed signs of acquiring a neuronal properties with β-III tubulin positive profiles (60 % ± 5 %). Also, 25 % ± 3 % of the cultured cells expressed GFAP staining illustrating glial properties. A marked co-expression of β-III tubulin and GFAP (15 % ± 3 %) was also noted. The control group, not cultured on Matrigel® coated coverslips, showed no staining (here survival was close to zero). * indicates $p < 0.05$.

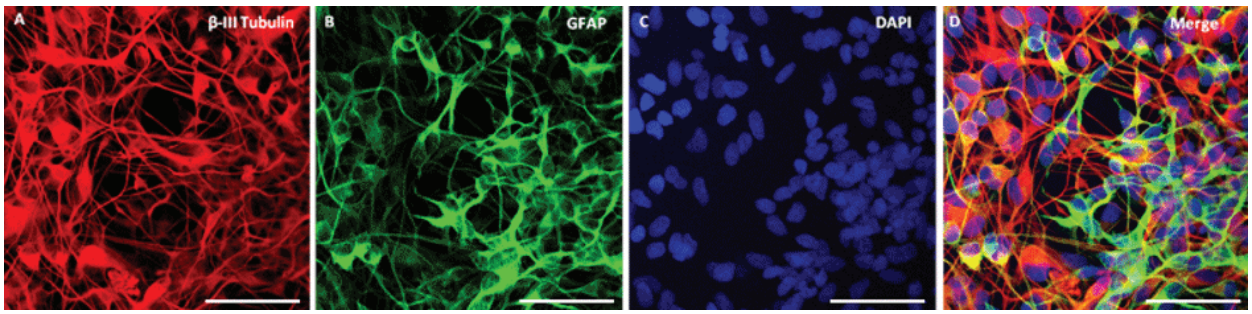

Figure 11. (A-D) Dissociated HNPC sphere culture on a Matrigel® coated coverslip. Shown here are examples of cells expressing β-III tubulin (Red), GFAP (Green), DAPI (Blue) markers. The DAPI-positive profiles indicate an intact nuclei morphology. Scale bar = 100 µm.

MAP2 and DCX

To further characterize the expression of neuronal markers, microtubule-associated protein 2 (MAP2) and doublecortin (DCX) expression was characterized in separate specimens. All MAP2 and DCX positive cells were also positive for β-III tubulin. A high rate of cells were MAP2 positive (60 % ± 7 %; Figure 12) and DCX positive (70 % ± 4 %; Figure 12), indicating ongoing neuronal development (Figure 12). Out of the β-III tubulin positive cells, 60 % ± 5 % (Figure 12) co-expressed MAP2 and 50 % ± 4 % (Figure 12) co-expressed DCX.

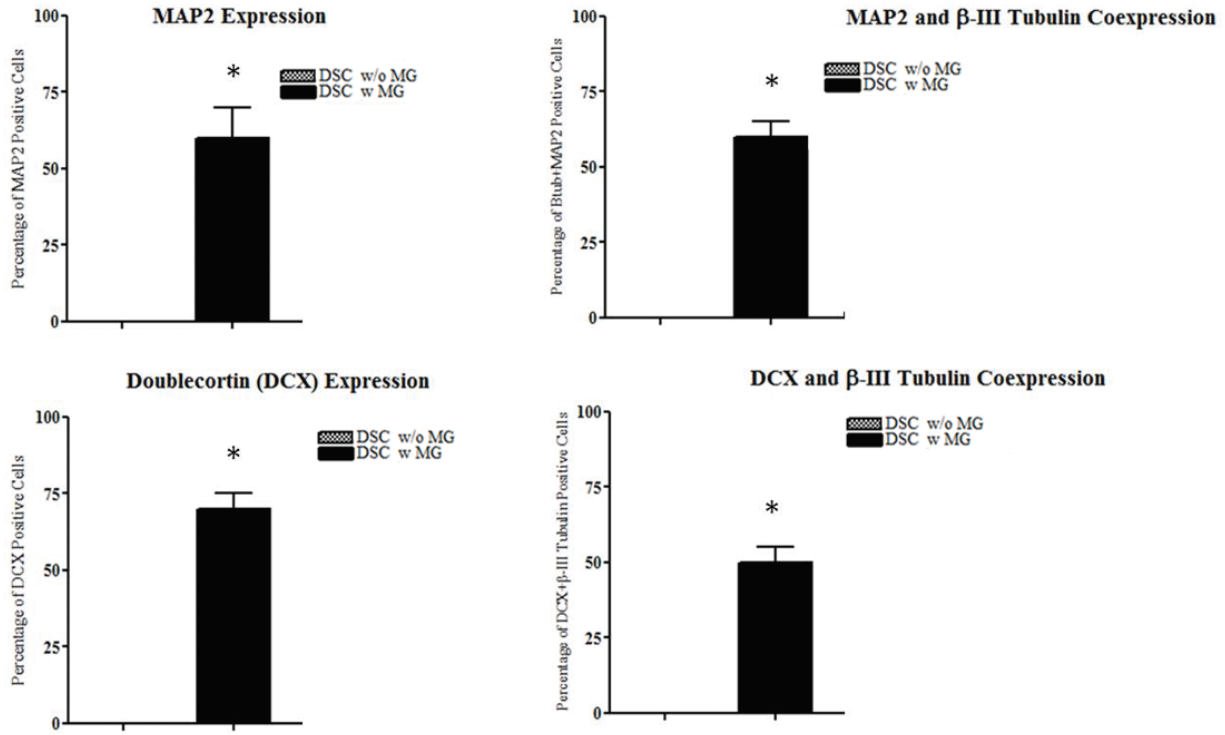

Figure 12. Expression of the neuronal markers MAP2 and DCX, and their co-expression with β-III tubulin in HNPC dissociated sphere cultures (DSC) on Matrigel® (MG) coated coverslips.
Out of the total number of surviving cells, 60 % ± 7 % were MAP2 positive and 70 % ± 4 % expressed DCX. All MAP2 and DCX positive cells were also positive for β-III tubulin. Out of the β-III tubulin positive cells, many co-expressed MAP2 (60% ± 5 %) and DCX (50 % ± 4 %). In the control group (not cultured on Matrigel® coated coverslips) there was no differentiation observed (here survival was close to zero; Figure 9). * indicates $p < 0.05$.

Expression of synaptic markers

The synaptic markers synapsin and SV2 were used to determine whether the DSCs on Matrigel® coated coverslips also developed a capability to form neuronal connections. Since all HNPCs acquiring a neuronal fate were β-III tubulin positive, the co-expression of the anti β-III antibody and synapsin or SV2 markers were used to further explore their profiles. The results illustrate that 80 % ± 9 % (Figure 13) of the β-III tubulin positive profiles were also positive for synapsin and 62 % ± 4 % (Figure 13) were also positive for SV2.

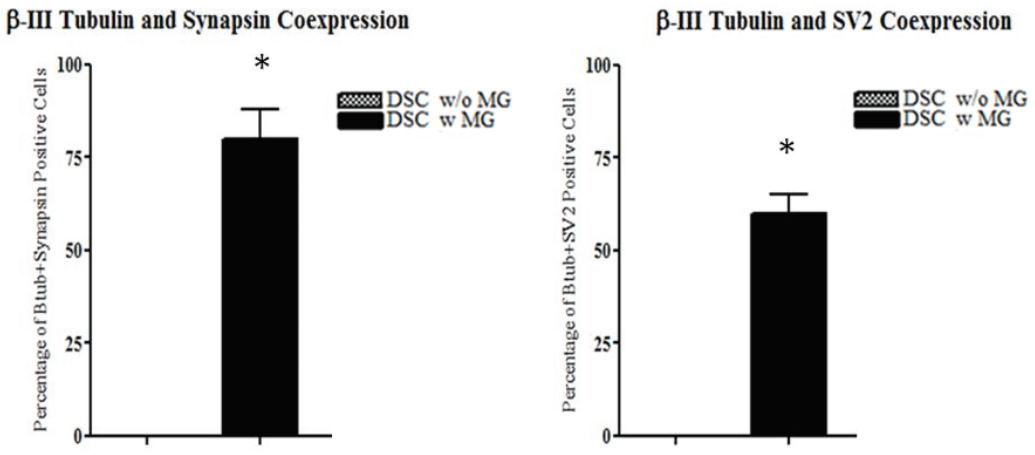

Figure 13. Co-expression of β-III tubulin and synapsin or SV2 in HNPC dissociated sphere cultures (DSC) on Matrigel® (MG) coated coverslips.
To explore developing contact capabilities in HNPCs, co-expression of the neuronal marker β-III tubulin and SV2 or Synapsin was evaluated in DSC on Matrigel® (MG) coated or MG uncoated coverslips. In the control group (that was not cultured on Matrigel® coverslips), there was no positive markers observed (since survival was close to zero; Figure 9). * indicates $p < 0.05$.

Paper III

Here, studies on cell survival after transplantation *in vitro* were performed. NES cells were cultured in a setup with three groups. The first group consisted of NES cells without further manipulation. The second and third groups consisted of NES cells that were left to differentiate into neuroblasts and NVM (Figure 14). The recording and analyses of differentiation characteristics of a large number of cells (> 410 000) as according to immunocytochemical marker stainings were executed after 19 days of culture.

Survival of seeded specimens

Following 19 days of culture, 77 % of NES cell specimens seeded in the control group displayed intact cell profiles (27 out of 35; Figure 14) versus 79 % (38 out of 48; Figure 14) of specimens seeded in the experiment group. In the neuroblast group only 20 % (6/30; Figure 14) persisted in control group, versus 78 % (29/37; Figure 14) in the experiment group. The groups with cultured NVM displayed a severely limited number of cells with intact nuclear profiles. Although, as judged per specimen survival the rate in both groups was 50 % (1/2 in control group; 4/8 in experiment group; Figure 14).

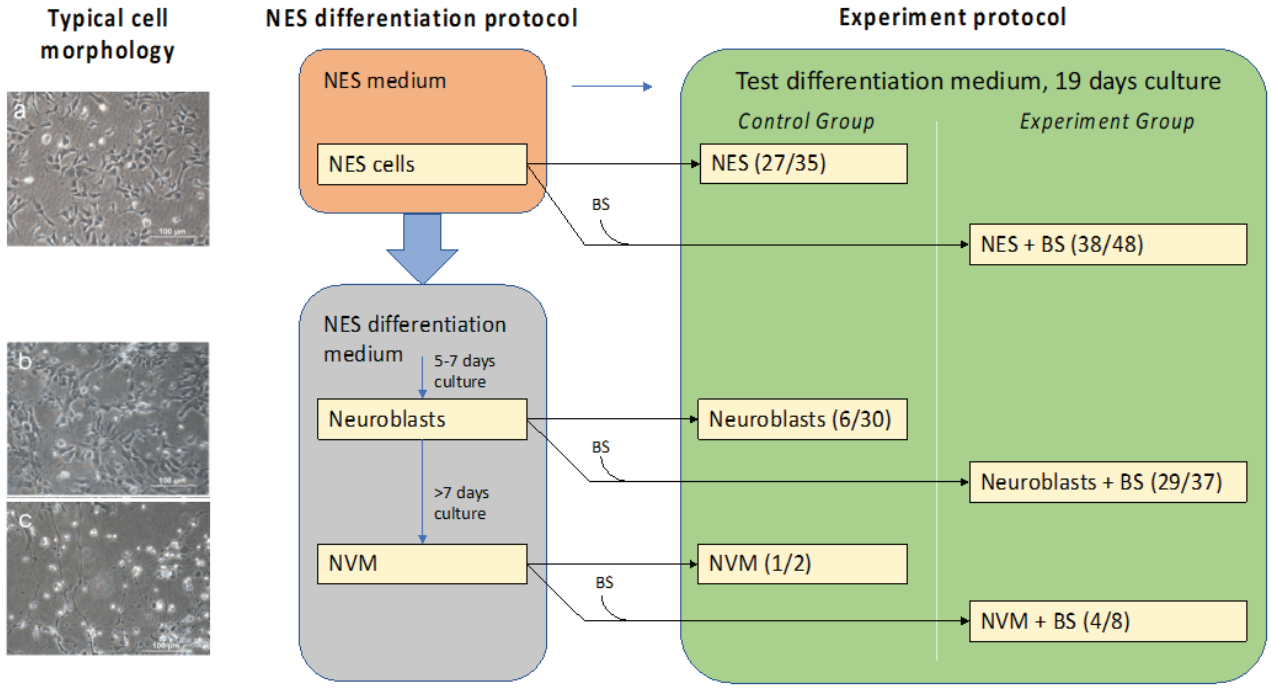

Figure 14. Schematics of experimental setup using neuroepithelial-like stem (NES) cells to evaluate the cell survival rate after transplantation to monoculture and a co-culture setup with BS slice. Three groups of NES cells with different differentiation status was used. Undifferentiated NES cells, Neuroblasts and neurons of various maturity (NVM). The control groups was seeded as monocultures. In the experiment groups the cells were seeded together with a rat auditory brainstem (BS) slice. The number of cultures with surviving cells out the number of seeded cultures is shown for each group (in brackets). Bright-field pictures of cells in culture prior to dissociation are shown on the left (a-c).

Migration

The characteristic profiles of cells displaying elongated nuclei, as a sign of migration, were only observed in the NES experiment group. Here it reflected a targeted attraction toward the BS slice.

Axon morphology

In the NES control group, 55 % (Figure 15) of analyzed cells displayed nestin-expressing processes. These were mostly comprised of primitive, thick, short and irregular branches. In the NES experiment group, the number of cells positive for nestin staining was higher (74 %, Figure 15) revealing long thin processes, branched into large networks. Some of the processes was bearing vesicular dilations and displaying an organized morphology reflecting neuronal integrity. In both NES control and experiment groups, developing neuronal cells positive for TUJ1 (β-III tubulin) had slim, round or spindle-shaped cell bodies with mostly one leading process and many smaller multi-branched processes. These are all features of cells acquiring neuronal competence.

In the neuroblast control group some mature cells displayed long leading processes,
but the NF positive axons detected were sparse and short. In the neuroblast experiment group, NF heavy chain marker staining revealed a complex disorganized network of multi-branched nerve cells in 100 % of the specimen tested. Neurites also displayed vesicular dilations reflecting possible ongoing axonal transport.

The NVM groups were not analyzed in respect to the development of neural competence as revealed by marker staining due to low number of intact cell profiles.

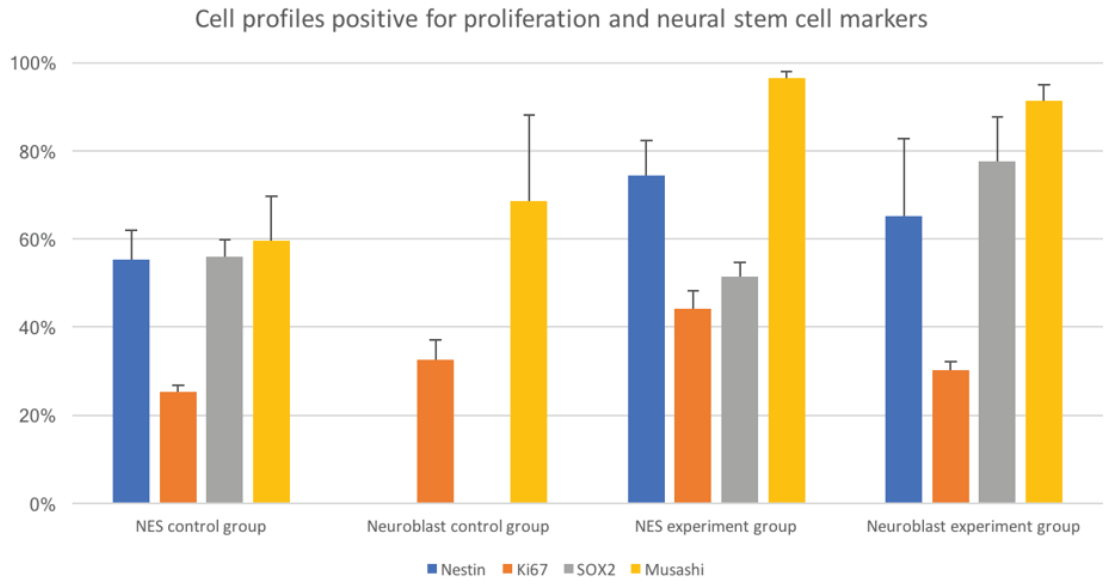

Figure 15. Percentage of cell profiles positive for proliferation and neural stem cell markers in the control and experiment groups. Cell profiles were stained for the markers nestin, Ki67, SOX2 and Musashi. All nuclear profiles were identified with a DAPI/human nuclei marker. Recorded pictures taken from representative areas of the specimens were analyzed using CellProfiler and CellProfiler Analyst software (mean ± S.E.M.).

NES cell differentiation following 19 days of culture

Analyses of both the NES control and experiment groups revealed two distinct populations of differentiating cells. These were either less mature nestin and SOX2 positive cells, or had started to differentiate down a neuronal lineage into more mature cells expressing TUJ1 (β-III tubulin), MAP2 and DCX.

The less mature nestin positive cells were more common in the periphery illustrating limited differentiation. The SOX2 and nestin markers were predominately co-expressed in all cultured cells.

Almost all mature cells were situated in the center of the seeded cell population (control groups) or adjacent to the explant (experiment groups). Double staining with DCX and MAP2 revealed a predominant co-expression in all groups.

The proliferation marker Ki67 showed scattered chromatin bundles in 25 % (Figure 15) of the nuclei in the control group, and in 44 % (Figure 15) of the cells in the experiment group. This indicates that cells were still undergoing division as a sign of persistent immature traits.

The Musashi staining in the control group (60 %; Figure 15) and the experiment group (96 %; Figure 15) was noted to be strong in the cytoplasm of the more primitive cells devoid of neuronal processes. The Musashi expression was stronger in cells located in the middle of the seeded cell specimens, indicating a less differentiated state. However, at the same time it was also noticed that markers for more mature neuronal characteristics, such as MAP2 and DCX, were mostly expressed in the same location. These cells however, were visibly not the same as the Musashi positive cells (Figure 16). This could suggest an ongoing cross talk between cells, indicating that they are depending on signals from neighboring cells to differentiate further.

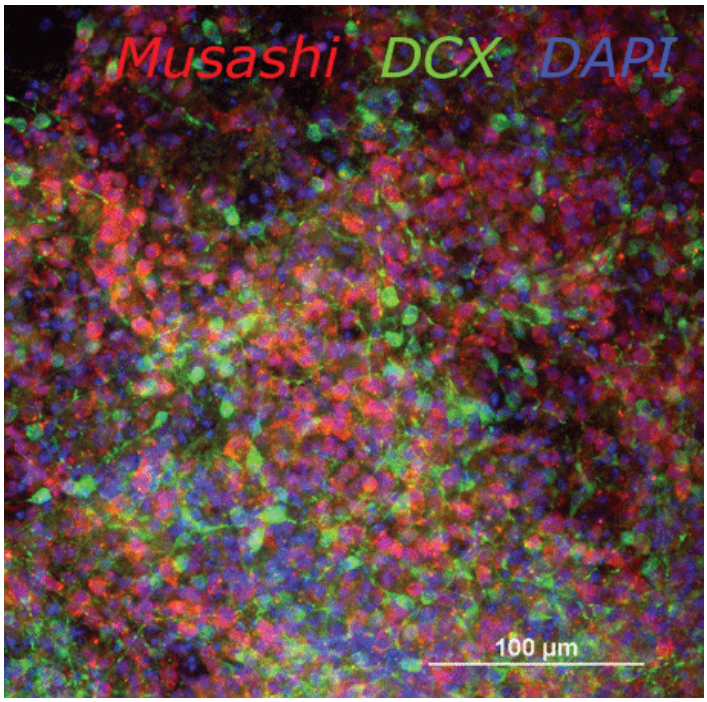

Figure 16. Merged picture of Musashi, DCX and DAPI positive cell profiles in NES experiment group. Illustration of low co-expression by SC markers Musashi and DCX in culture specimen. DAPI positive cell nuclei.

A very small number of NES cells were SSEA-1 or GFAP positive in any group (< 1 %).

The marker NeuN was expressed in 31 % (Figure 17) of cells in both NES control and experiment groups. These cells were also mostly positive for β-III tubulin.

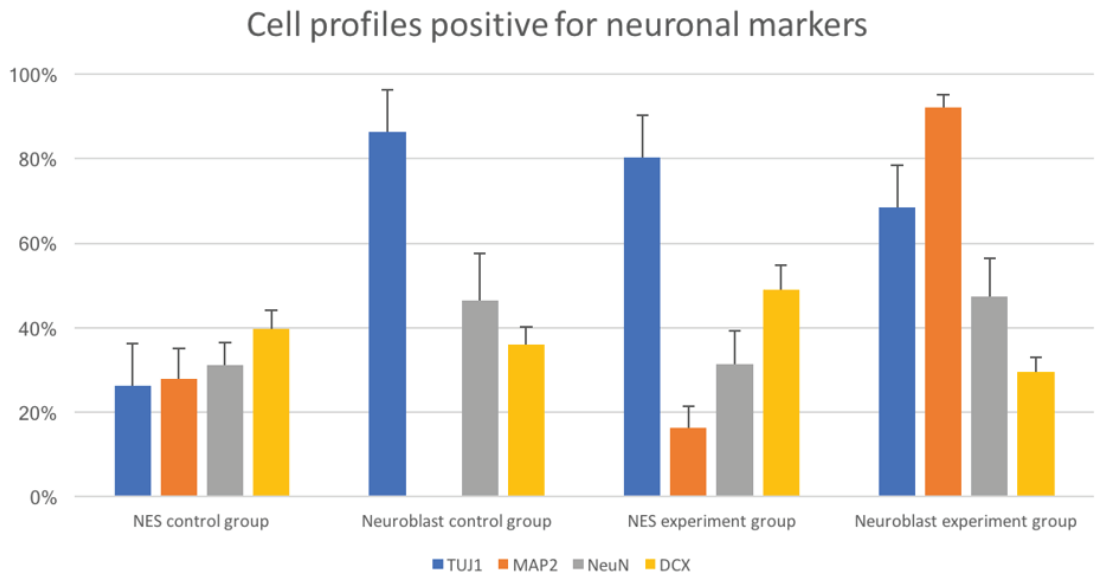

Figure 17. Percentage of cell profiles positive for neuronal markers in the control and experiment groups. Cell profiles were stained for TUJ1 (β-III tubulin), MAP2, NeuN and DCX markers. All nuclear profiles were identified with a DAPI/human nuclei marker. Recorded pictures taken from representative areas of the specimens were analyzed using CellProfiler and CellProfiler Analyst softwares (mean ± S.E.M.).

Neuroblast differentiation following 19 days of culture

The neuroblasts in the control group developed an extensive network of long TUJ1 (β-III tubulin) positive processes (86 %; Figure 17), most of which were double stained for the marker NeuN. No MAP2 staining was observed and few cells were DCX positive (36 %, Figure 17). Staining with DCX and Musashi antibodies revealed two distinct populations of different cell maturity, as previously also noted in the NES culture groups. In this group, as in the NES groups, Ki67 staining was detected in 33 % (Figure 15) of the cells showing ongoing division.

In the neuroblast experiment group cultured with a BS slice, nestin staining (65 %; Figure 15) revealed processes with long fine branches bearing vesicular dilations. Nestin positive processes displayed organized morphological formations reflecting neuronal integrity of the cultured cells. TUJ1 (β-III tubulin) positive cells formed extensive branched networks of long processes. Extensive NeuN expression was detected in 47 % (Figure 17) of the cells and all NeuN positive cells were also TUJ1 (β-III tubulin) positive, whereas some of the TUJ1 (β-III tubulin) positive cells did not express NeuN. Staining with the proliferation marker Ki67 revealed a small population of still dividing cells (30 %; Figure 15). A total of 78 % (Figure 15) of cells were SOX2 positive and 91 % (Figure 15) of the cells were positive for Musashi.

NVM differentiation following 19 days of culture

In the NVM experiment group, only a limited number cells displaying intact nuclear profiles were detected and in the control group there were even less. The produced specimens in the experiment group were analyzed but the results are not included here due to the lack of sufficient controls.

Paper IV

Here, cultured BS slices were subjected to electrophysiological evaluation with MEA technique.

Prior to recording the electrical activity on a MEA plate, the specimens had been cultured for 21 days as interface-cultures on semi-permeable membranes ad modum Stoppini (Stoppini et al., 1991). Out of the 44 cultured and recorded specimens, 34 did not produce any spontaneous activity that could be detected in this experimental setting. 10 specimens (23 %) generated spontaneous activity that was recorded for further analyses. Only basic analyses of the recorded electrical activity were performed within the scope of this paper. Focus were put on identifying spontaneous spikes (extracellular correlate of action potentials), groups of these spikes (bursts) and the inter-spike intervals.

In specimens with detectable spontaneous activity, data were typically recorded from the start of the recording and for another 5 minutes. In some cases, there were still activity to be recorded after 15 minutes. One specimen was still displaying detectable spikes and groups of spikes at the end of the planned recording time of 30 minutes. The detected activity were most often of various amplitude and with a different number of spikes per burst. This also varied between evaluated electrodes and between samples (Figure 18). The overall characteristics though, were in agreement with previously published spontaneous activity-patterns of cerebral cortex (Maeda et al., 1995).

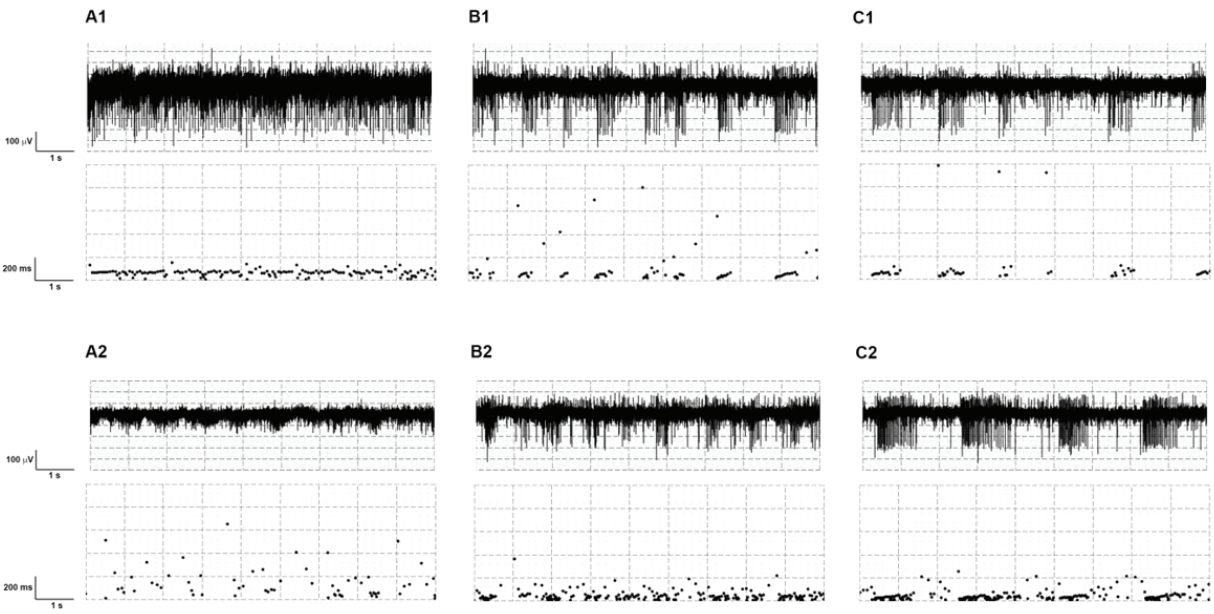

Figure 18. Representative screenshots of recorded spontaneous spiking activity in a neonatal P9 mouse auditory brainstem slice on MEA plate. Recurrent spontaneous spikes and bursts were observed. Here, recordings from three time-intervals of two separate electrodes are illustrated for representative visualization (A: 0:30 min – 0:39 min; B: 1:30 min – 1:39 min and C: 2:30 min – 2:39 min). These electrodes (1: A1-C1, and 2: A2-C2) are located in separate areas of the BS specimen and is presented with traces (top) and a corresponding inter-spike interval chart (bottom). Analysis were done with MC Channel Suite (MCS).

DISCUSSION AND FUTURE PERSPECTIVES

This thesis presents four papers with novel results on cell performance *in vitro*. A selected set of parameters where explored to aid the development of future evaluations into cell transplantation *in vitro.* It contributes to our basic knowledge of how cells behave and how our manipulations affect them in culture.

Trophic support by conditioned medium (Paper I)

The trophic support of neurotrophins is essential to the successful culture of neuronal cells. To provide a trophic support by adding a CM, collected from the same type of cells as used in the experiment, is a well-established strategy. The positive effect of a BS CM on HNPC survival and differentiation reported here is conclusive. Hence, we have good reasons to explore the use of BS CM further in future experimental settings.

This does not conclude that the utilized protocol is optimal. It is quite possible that the concentration of trophic support achieved here is still not high enough, and that an increased concentration is favourable for enhanced cell performance. On the other hand, concentration used could already be a little too high, with no further enhanced performance achievable, or even with undocumented adverse toxic effects already affecting the cells. The results presented here does not provide a dose-response analysis.

We show that the BDNF and GDNF content in BS CM varies with time when sampled consecutively. As the used secretome enriched medium usually is collected over time and then pooled, the concentration variations reported here, are effectively evened out. It is enticing to propose a study to evaluate if the CM collected at a specific time point is actually more favourable in a specific setting, than the pooled collected CM. The analyses of the BS CM of a specific time point that is found to render substantially favourable results could be performed in the same way as we have shown in Paper I. These results could provide clues on how to further improve medium compositions.

Also, analysing the mechanisms responsible for the neurotrophin fluctuations we report, could provide new knowledge regarding cell development that may be useful in future transplantation research.

Trophic support by co-cultured brainstem slice (Papers I, II and III)

A joint conclusion drawn from the results presented here is that the performance of cells is generally enhanced when cultured in the presence of a BS slice. But, since we have not studied the details of the mechanisms behind these results, we can only speculate on the reasons.

Since there is a natural content of BS secretome in a BS co-culture, it is tempting to reason that the effects on cell performance will be similar to that of an added BS CM, at least in character. But, the fluctuations of secretome content reported here, adds a factor to this line of thought. We can only conclude that there could be differences affecting cell performance. This may have implications on future evaluations on how transplanted cells should best be prepared, in co-culture or relying on other means of support.

Another factor that should be taken into consideration is how the distance between the transplanted cells and the target organ affect the cell-cell microenvironment of locally excreted neurotrophins. When the paracrine stimulation of neighbouring cells is the only contributor, the effect of an altered distance could be significant, but probably not so when a BS CM is utilized. The implications may be similar to that of elucidating the concentration levels of optimal trophic support.

Trophic support from Corning® Matrigel® (paper II)

We note similar effects of cell performance enhancement from the use of Matrigel® on HNPC cultures as we do in BC cell and NES cell cultures utilizing BS CM or BS co-cultures (Papers I and III). That is, a marked improvement on cell survival in culture. It is not though, possible to perform an in depth comparison between the presented results since many of the key factors were not shared (e.g. cell substrate and culture setting). There are also large differences in the composition of neurotrophic supporting molecules in the cultures (notably, Matrigel® is devoid of BDNF or GDNF), and the presence of bioactive molecules in Matrigel® coating adds yet another unknown factor. We conclude that, the different cell lines possibly have large dissimilarities in their needs for trophic support and that the provided factors were valid for the specific cell type resulting in enhanced performance. Or, the results indicate that there are other key factors responsible for the effect that are not discerned here.

Seeding of cells (Paper II)

We know that cells need a certain amount of trophic and mechanical support to survive for any longer periods of time *in vitro*. In paper II, we note that there was a marked low rate of surviving cells in the control group despite adequate handling and culture setting. We speculate that the utilized cell line of HNPCs were more sensitive to manipulation overall.

The comparatively high number of passages before culture (14-17), in combination with the omitted serum and trophic support, may have contributed to the poor survival. This even though progenitor cells previously have been shown to be very stable and robust without changes over many passages (Falk et al., 2012).

Attraction (Papers I and III)

We noted signs of cell migration towards the co-cultured BS slice in Papers I and III. We speculate that the observed targeted migration is triggered and guided by a BDNF and/or GDNF gradient excreted by the BS slice. This effect does not seem to be affected by the addition of the BS conditioned culture medium, as we see similar results of migration of cells between the BC specimens and the BS slice in all different experimental groups of Paper I (i.e. with or without BS secretome supplement). This implies that the transplanted cells have a high sensitivity to gradient differences and that these gradients are stable. This could be utilized in further studies into direction guided differentiation of cells candidates for transplantation.

The use of automated evaluation of 2D material (Paper III)

The evaluation of cell characteristics with immunocytochemistry is a well-established method and the number of commercially available markers for the detection of specific molecular elements

are ever growing. The quality and effectiveness of the technique makes it easy for any laboratory staff to gather large amounts of data. This can then be analysed with qualitative or quantitative methods. The recorded data from micrography can easily accumulate into a material too vast for practical manual analyses. Fortunately, the development of methods to perform automatized evaluation is advancing and it is now possible to manage advanced systems with only basic knowledge in computer programming. Here, with the support from Department of Information Technology, Uppsala University, we utilized the CellProfiler and CellProfilerAnalyst softwares to analyse cell profiles from 100 specimens. After analyses, it resulted in data from more than 410 000 individual cell profiles. Even if the total computational time for a modern laptop was less than 100 hours, it was still a quite laborious process to produce data of high validity.

It is obvious that the tools used here are powerful and of great help in producing transparent and consistent methods for the evaluation of immunocytochemical results. This is a field that is likely to be enhanced with the development of artificial intelligence (AI) algorithms. In the software used here there are elements of machine learning based on manual analyses of cells to generate rules which are later used as a template for evaluation. A software based on AI will likely execute this type of rule generation more efficiently and accurately in the near future.

MEA (Paper IV)

We here report of a novel application of the MEA technique to measure the endogenous neural activity of cells in interface-culture. This opens up a multitude of new possibilities to study cell performance in cultures ad modum Stoppini.

Understanding the distribution of electrical activity in our cultured specimens is crucial for the evaluation of integration and function of cells in culture. In addition to the basic parameters we report in paper IV, more analyses of first order statistical parameters such as inter-burst interval, mean firing rate and the mean bursting rate (Rieke et al., 1997) will provide a even more detailed baseline for reference.

There are also new techniques available to perform even more detailed studies on the distribution of the electrical activity in specimens. This setting could possibly be expanded to utilize MEA plates with a higher density and larger number of electrodes. The high density CMOS-plates now available, with an excess of 4000 electrodes distributed on a similar surface area as our 64 electrode plate, could present a promising approach to collect much more detailed data for further analyses.

The MEA culture platform is designed for the study of dissociated cells in culture. It would be interesting to culture our organotypic cell culture directly on a MEA plate, but the present settings are not suitable for culture of thick specimens for long periods due to their limitations in providing tissue oxy-genation and nutrition. The introduction of a perforated culture surface with supplementary perfusion from below has proven to facilitate oxygenation enough to admit thicker specimens (<400 µm) to be cultured and recorded, but still only for a few hours (Gonzalez-Sulser et al., 2011). The addition of a rocking culture chamber to increase surface oxygenation has proved effective to enhance tissue oxygenation (Panuccio et al., 2018), but the successful culture time is still very limited. We conclude that it is still not feasible to perform organotypic cultures for our purposes on a MEA plate with the available techniques.

Cell origin and stage suitable for transplantation (Papers I, II, III and IV)

Availability

Cell substrates available to researchers for *in vitro* studies of stem and progenitor cells have multiplied. Historically the source of these cells were embryonic. The discovery of the totipotent and pluripotent cells in the adult mammal increased both the availability and the options for future therapeutic protocols for patients. With the introduction of the hiPSC the prospects expanded even further. It also ameliorates the immunological and ethical challenges innate to previous transplantation research.

During the work on this thesis the availability of cells have caused us to shift cell substrates. Initially, these were harvested from mouse or rat since these were the most relevant cells available. Now focus is entirely on human cells as they have become available. Utilizing human cells is also in line with our efforts to bring our in vitro research closer to the patient.

Cells for transplantation

A cell considered for transplantation in vivo is probably not going to be a SC, but rather a cell that has developed further into a more specialized state. A developed neuron could qualify as a viable candidate in the case of the hearing-impaired patient. Possibly, a neuron that is still differentiating to some degree would also be applicable, as such a cell may have a better chance of adapting to a new environment than the adult specialized neuron. For a two main reasons, the exact differentiational state of a proposed cell for transplantation *in vivo* is recognized as important, i.e. the documented risk of induced teratoma formation and the risk of an uncontrolled proliferation. We further show that, when assessing essential factors for a cell preparation protocol, the vulnerability of cells to the trauma and stress following cell transfer also needs to be considered.

CONCLUSIONS

Paper I

Here we report that the levels of the neurotrophic factors BDNF and GDNF are many times higher in medium after it has been conditioned with an auditory BS slice. We also note that the levels varies depending on the timepoint in culture that was chosen for medium collection. We describe how the BS CM positively affects the survival and differentiation characteristics of BC cells in BS slice co-culture. We note that the BC cells migrate towards the BS slice as a sign of attraction.

Paper II

Here we demonstrate how a Matrigel® coating of the culture surface positively affects the survival rate of dissociated HNPCs. We also report the differentiation characteristics of HNPCs after three weeks in this culture setting.

Paper III

Out of the three explored groups in this report (NES cells, neuroblasts and NVM), the less differentiated cells (i.e. the NES cells) prove to be more resilient to the manipulations involved in the cell transplantation process, as compared to the more differentiated cells. Therefore, based on the overall survival, the less differentiated cells are likely to be the more suitable choice for cell transplantation. The impact and relevance of our extensive evaluation of differentiation characteristics of cells in culture is yet to be determined.

Paper IV

Utilizing a modified protocol, the MEA technique can be used to evaluate the endogenous electrical activity in a BS slice cultured ad modum Stoppini (interface-culture). It is important to evaluate the electrophysical activity of cells *in vitro* to estimate the feasibility of future cell transplant paradigms, especially if this will include the functional integration of the transplanted cells. Here we show that the recorded data patterns of spikes, inter-spike intervals and bursts correspond well with previously published neuronal recordings. We predict that the method presented here will be explored further in future studies.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Hörsel är ett av våra fem sinnen. Då världen idag ställer stora krav på kommunikationsförmåga så är det lätt att förstå att en skadad hörsel resulterar i ett stort lidande. Hörselnedsättning är dessutom ett av de vanligaste handikappen världen över och många studier har även visat att individen upplever sitt lidande som betydande.

Historiskt så är förvärvade hörselnedsättningar ofta en följd av infektioner och skador. Tack vara antibiotika och modern kirurgi kan vi idag hjälpa många av de som är drabbade. Med modern teknik så är det också möjligt att hjälpa många av de som har en gravt nedsatt funktion i själva hörselorganet med hjälp av ett implantat till öronsnäckan (cochlea implantat, CI). Idag har vi således viss möjlighet att hjälpa patienter även i denna grupp, som tidigare varit och förblivit döva.

Det finns dock fortfarande grupper av hörselskadade patienter som trots alla de landvinningar som gjorts in om medicin och teknik, av olika anledningar inte uppnår en god fungerande hörselnivå. Det är bland dessa patienter som vi tänker oss att en transplantation av celler till hörselsystemet skulle kunna bidra till hörselrehabilitering. Kanske kan dessa celler hjälpa kroppen själv att reparera sina skador, eller så kan vi använda dom för att hjälpa till att förbättra kontakten mellan vävnad och implantat, och på så sätt förbättra funktionen av existerande hörhjälpmedel.

I denna avhandling utforskar vi möjligheterna att utnyttja och förbättra våra aktuella cellodlingstekniker för att närma oss en verksam metod att transplantera celler till innerörat.

Vi använder genomgående vår etablerade modell för cellodling i laboratorium för att testa hur olika celler beter sig under specifika förhållanden. Vår modell går ut på att odla en hjärnstamsskiva på ett membran och sedan transplantera cellerna dit. Denna odling pågår under två till tre veckor varefter den stoppas och analyseras. Vi mäter hur cellerna har överlevt under de aktuella förhållandena, hur dom eventuellt har förflyttat sig i odlingen, hur dem har förändrats och utvecklats, samt om dem visar tecken till integration med varandra.

I arbete I så fokuserar vi på att studera den lösning (medium) som cellerna lever i under tiden i odling. För att cellerna ska växa och trivas så krävs det inte bara stabila förhållanden och näring utan även proteiner som stimulerar tillväxt. Dessa proteiner produceras till största del av cellerna själva eller av närliggande celler. Det är komplicerat att producera dessa proteiner varför man gärna samlar in och återanvänder de som utsöndras och ansamlas i mediet under odlingen. Vi har använt återinsamlat medium med tillväxtfaktorer (conditioned medium, CM) tidigare med gott resultat. I artikel I så analyserar vi innehållet i detta medium på ett sätt som inte tidigare är gjort. Vi noterar att tillväxtfaktorerna BDNF och GNDF finns i stor mängd i CM, och att koncentrationen varierar beroende på när vi samlar in mediet.

En del av arbetet går ut på att utforska olika celltypers lämplighet som underlag i en transplantationsmodell. I arbete I, II och III undersöker vi tre olika typer av omogna celler som efter genomgång av aktuellt forskningsläge visat sig varit möjliga transplantationskandidater i detta sammanhang.

Sedan 60-talet har vi känt till att det finns omogna celler även i den vuxna kroppen. Dessa celler har gemensamma egenskaper med de första cellerna som uppstår direkt efter befruktningen. Dessa omogna celler är till en början helt identiska med varandra och har kapacitet att utvecklas till vilken cell som helst i den framtida organismen. Redan i och med den femte celldelningen så påbörjar cellerna dock sin utmognad till olika celltyper i den växande organismen. Ur transplantationssynpunkt så är det lockande att skörda de mest omogna cellerna och sedan förmå dem till att bilda exakt de celltyper som man önskar ersätta. Detta har dock visat sig komplicerat, både tekniskt, på grund av immunförsvarets motvilja att slutligen acceptera okända celler. Dessutom är de viktiga etiska aspekterna nödvändiga att ta med i valet av transplantationskandidater.

Det senaste lovande framstegen vad gäller möjliga kandidater är de inducerade progenitorcellerna (iPS celler). Dessa utgår från vanliga celler, exempelvis från patienten själv, som man sedan styr tillbaka i utvecklingen så att de återfår sin förmåga att likt omogna celler kunna utvecklas till olika celltyper. Under detta arbetes gång så har tillgången till lämpliga celler förändrats. I det första arbetet undersöker vi embryonala celler från mus, medan vi i arbete II och III har gått över till att undersöka humana celler.

De transplanterade cellerna kommer troligen att behöva förbehandlas innan de transplanteras. I arbete I och II visar vi att vår manipulation av den lösning de växer i, samt den justerade behandlingen av odlingsytan är positiv för cellöverlevnad.

Arbete IV är ytterligare metodinriktat. För att värdera hur cellerna integreras så är det värdefullt att göra en bedömning av deras funktioner. Det visar sig att det inte finns någon etablerad modell för att mäta funktionen i den odlingsmodell vi använder. Vi har många gånger värderat förekomsten av viktiga faktorer som krävs för att kunna etablera funktionella kontakter mellan cellerna, men aldrig förekomsten av de elektriska signalerna som överförs. Här modifierar vi en etablerad teknik bestående av små plattor med fixerade elektroder (microelectrode array, MEA) som mäter spänningsskillnaderna och vi och kan på så sätt mäta förekomsten av de elektriska signalerna. Våra resultat visar att det går att mäta signalerna som uppstår spontant i våra odlingar med denna teknik. Detta öppnar ett helt nytt fält av möjliga framtida undersökningar gällande hur väl transplanterade celler integreras med ett konstruerat målorgan i en cellodlingsmodell.

Det är idag inte tydligt hur transplanterade celler bäst kommer att kunna bidra till hörselrehabiliteringen av våra patienter. Det finns två huvudsakliga spår som det forskas mycket kring.

Det ena tar avstamp i kunskapen om cellernas egen förmåga att stimulera reparationen av skadad vävnad då en väl etablerad skada i hörselorganen uppvisar en avstannad läkningsprocess. Man har sett att omogna celler i vissa fall rekryteras lokalt i vävnad, vilken även sker i den vuxna människan, och bidrar till att reparera skadorna. Därför kan man även tänka sig att transplanerade celler framställda från stam- eller progenitorceller till ett skadat område kan bidra till att åter starta en konstruktiv läkningsprocess. Det andra spåret för transplanterade celler är att bidra till att förbättra kontakten mellan ett implanterat hörselhjälpmedel och det avsedda målorganet. Detta skulle ske genom att hjälpa till att etablera bättre kontakter mellan implantatets stimulerande ytor och målorganet. Om detta kommer att ske med hjälp av de transplanterade cellernas förmåga att stimulera cellutveckling lokalt eller genom direkt transplantation av rätt celler till rätt plats är ännu oklart.

Vårt övergripande mål är att bidra med kunskap som förutsäger hur en framtida modell för celltransplantation blir lyckosam. Då mycket fortfarande är oklart har vi lagt mycket tid på att undersöka cellernas karakteristika vad gäller omognad och mognad med hjälp av väl etablerade markörer. Detta materialet kommer bäst att kunna tolkas i relation till ett tydligt behov, exempelvis i form av en viss celltyp som innehar en specifik uppsättning egenskaper. Här bidrar denna doktorsavhandling framför allt med en detaljerad beskrivning av hur cellerna beter sig i den undersökta miljön.

Sammanfattningsvis kan sägas att avhandlingen bidrar med ny information som utvecklar våra möjligheter att undersöka celler i odling och med ny kunskap om hur de olika cellerna överlever och vidareutvecklas. Dessutom ingår vidareutveckling av flera metoder för att analysera dessa transplanterade celler. Slutligen hoppas jag att detta i förlängningen kan bidra till att vi kan hjälpa fler hörselskadade patienter att få en fungerande hörsel.

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