RIIKKA ÄÄNISMAA

Human Embryonic Stem Cell-Derived Neural and Neuronal Cells *in vitro* and *in vivo*

Treatment of experimental cerebral ischemia

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Small Auditorium of Building B,
Medical School of the University of Tampere,
on March 26th, 2010, at 12 o'clock.

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Abstract

Human pluripotent stem cells and their neural derivatives are considered potential regenerative material for treating central nervous system deficits resulting from traumatic injury (e.g. spinal cord injury) or neurodegenerative disease (e.g. ischemic stroke, multiple sclerosis, Parkinson's disease). Although several studies have examined stem cell transplantation as a treatment for these conditions, the results have been highly variable and much more work is needed to address the many remaining questions. Clinical applications for neural cell transplants are currently being designed to treat brain injuries resulting from stroke and spinal cord injury.

This thesis describes efforts towards the generation of an efficient and simple protocol to differentiate human embryonic stem cells (hESCs) into neural progenitors and young neuronal cells. Additionally, a neuron-specific culturing matrix has been designed to improve the maintanence and differentiation of neural progenitors. The electrophysiologic properties of neuronal networks were also investigated *in vitro*. In addition, neural progenitor cell transplantation was performed in animal models of stroke and evaluated with regard to the optimal transplantation route and their effects on functional recovery of animals in combination with rehabilitation, i.e. housing in an enriched environment.

Neural differentiation of hESCs was achieved with a relatively simple differentiation protocol that was assessed using molecular biological methods. A hESC line-dependent variation in differentiation efficacy was observed. Regardless of the hESC line used, neuronal cells that were produced formed functional electrically active networks *in vitro*. Thus, the method developed in this thesis clearly produces functional neuronal cells. Moreover, neural adhesion molecule antibodies effectively produced a specific surface matrix for the selection of neuronal cultures.

In animal studies, the optimal delivery route to induce the accumulation of transplanted neural progenitor cells into damaged brain tissue was evaluated. The non-invasiveness of intravenous administration of cell grafts would be optimal for a clinical setting. Based on our findings that grafted neural progenitor cells accumulated mainly in the liver, kidneys, and spleen following intravenous administration, this method appears to be not effective. We also attempted intracerebral transplantation of the neural progenitor cells into rats with experimentally induced stroke that were housed in either an enriched environment or standard cages. Regardless of the type of housing, rats with neural progenitor cell transplants showed significant improvement in a postural support task during the first month after treatment when compared to vehicle-treated animals. Neither group of rats showed any improvement in a reaching task. *In vivo* cell survival was minimal.

In conclusion, hESCs can be efficiently differentiated into neural progenitors and neuronal cells, but hESC line-dependent variations in differentiation potential must be considered, especially when planning and designing clinical applications. In addition, the electrophysiologic properties of the produced neuronal cells and networks should be carefully studied *in vitro* to ensure the functionality of the neurons. Neuron-specific antibodies can be used as a selective culturing matrix for neuronal cells. Intravenous transplantation of the cell grafts into the ischemic brain is currently not feasible and more work is needed to enhance the efficacy of intracerebrally transplanted cells.

Tiivistelmä

Ihmisen monikykyiset kantasolut ja niistä erilaistetut hermosolut ovat olleet kiihkeän tutkimuksen kohteena kymmenisen vuotta, sillä ne vaikuttavat erittäin lupaavilta kudosteknologisten sovellusten kannalta. Erityisesti monia keskushermoston sairauksia ja vammoja, kuten aivohalvaus, selkäydinvaurio, multippeli skleroosi, sekä Parkinsonin tauti, toivotaan tulevaisuudessa voitavan hoitaa solusiirteillä, jotka korvaisivat tuhoutuneen kudoksen ja palauttaisivat aivojen tai selkäytimen normaalin toiminnan. Tutkimustyötä on tehty paljon, mutta saadut tulokset eroavat toisistaan paikoin huomattavasti ja monia kysymyksiä on vielä vailla vastauksia. Siitä huolimatta ensimmäiset kliiniset solusiirrekokeet aivohalvaus- ja selkäydinvammapotilailla tullaan aloittamaan lähitulevaisuudessa.

Tässä väitöskirjassa pyrittiin kehittämään tehokas erilaistamismenetelmä esiasteellisten hermosolujen tuottoon ihmisalkion kantasoluista. Tuotetuille hermosoluille etsittiin spesifistä kasvatusalustaa, ja erityisesti hermosolujen muodostamien verkostojen sähköistä aktiivisuutta ja toiminnallisuutta tutkittiin. Lisäksi hermosoluja testattiin aivoiskemia-eläinmalleilla optimaalisen siirtotavan selvittämiseksi sekä tutkittiin solujen ja rikastetun ympäristön vaikutuksia eläinten toiminnalliseen kuntoutumiseen.

Kehitetyllä erilaistamismenetelmällä saatiin tuotettua tehokkaasti puhtaita hermosolupopulaatioita, mikä osoitettiin monin molekyylibiologisin menetelmin. Hermosoluille spesifinen vasta-aine osoittautui toimivaksi ja lupaavaksi sovellukseksi hermosolujen kasvatusalustana. Ihmisalkion kantasolulinjojen välillä havaittiin merkittäviä eroja erilaistumistehokkuudessa mutta jokainen kantasolulinja pystyi tuottamaan sähköisesti aktiivisia, toiminnallisia hermosoluja.

Eläinkokeissa havaittiin, että vaikka suonensisäinen pistos olisi kliinisissä sovelluksissa helpoin tapa siirtää solusiirteet potilaaseen, se ei käytännössä ole toimiva menetelmä. Suuri osa näin injektoiduista soluista kerääntyi maksaan, munuaisiin ja haimaan eikä kohdekudokseen eli aivoihin. Toisessa kokeessa hermosolut istutettiin suoraan rottien halvaantuneeseen aivokudokseen, ja niiden sekä rikastetun ympäristön vaikutusta toiminnalliseen kuntoutumiseen seurattiin kahden kuukauden ajan. Ympäristöstä riippumatta solusiirteen saaneet rotat toipuivat huomattavasti nopeammin tassun käyttöä mittaavassa sylinteritestissä ensimmäisen kuukauden aikana, mutta hienomotoriikkaa vaativassa kurotustestissä eroja rottien välillä ei havaittu. Solujen selviäminen aivokudoksessa oli vähäistä.

Johtopäätöksenä voidaan todeta, että ihmisalkion kantasolujen erilaistaminen hermosoluiksi onnistuu kehitetyllä menetelmällä, mutta solulinjojen välillä olevat erot erilaistumistehokkuudessa on otettava huomioon. Lisäksi tuotettujen hermosolujen elektrofysiologisia ominaisuuksia tulisi tutkia rutiininomaisesti, jotta varmistettaisiin solujen sähköinen toiminnallisuus. Vasta-ainepintojen käyttö voisi

mahdollisesti olla hyvä keino hermosolujen kasvatukselle ja niiden kypsymiselle edelleen. Käytännössä vaikuttaa siltä, että solusiirteitä ei voida toimittaa aivoihin suonensisäisin injektioin ja vaikka siirretyillä hermosoluilla saavutetaan toiminnallista kuntoutumista, on vielä tehtävä lisää työtä sen eteen, että solut selviytyisivät paremmin kohdekudoksessa.

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Tampere, January 2010

List of abbreviations

AA	ascorbic acid
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BLBP	brain lipid binding protein
BMP	bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
CCD	charge-coupled device
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CT	computed tomography
D-AP5	D(-)-2-amino-5-phosphono-pentanoic acid
DCX	doublecortin
DMEM	Dulbecco's Modified Eagle Medium
EB	embryoid body
ECM	extra cellular matrix
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GABA	gamma-aminobutyric acid
GDNF	glial cell derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLAST	glutamate transporter
GMP	good manufacturing practice
hESC	human embryonic stem cell
hNT/NT2N	human teratocarcinoma cells
HuNu	human nuclei
iPS cell	induced pluripotent stem cell
IVF	*in vitro* fertilization
Ko-SR	knock-out serum replacement
MAP-2	microtubule associated protein
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MEA	microelectrode array
MEF	mouse embryonic fibroblast
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
NCAM	neural cell adhesion molecule
NDM	neural differentiation medium
NF	neurofilament
NSE	neuron specific enolase
PBS	phosphate buffered saline
PEI	polyethyleneimine

PFA	paraformaldehyde
pTHMMAA	*N*-[tris(hydroxymethyl)methyl]-acrylamide
RA	retinoic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SHH	sonic hedgehog
SPECT	single photon emission computed tomography
TH	tyrosine hydroxylase
USPIO	ultrasmall superparamagnetic iron oxide

List of original publications

The present thesis is based on the following original publications/studies which are referred to by their Roman numerals (**I-V**).

I. **Lappalainen RS**, Salomäki M, Ylä-Outinen L, Heikkilä TJ, Hyttinen JAK, Pihlajamäki H, Suuronen R, Skottman H, Hovatta O, Narkilahti S. Similarly derived and cultured hESC lines show variation in their developmental potential towards neuronal cells in long-time culture. *Submitted to Regenerative Medicine*.

II. Auer S and **Lappalainen RS**, Skottman H, Suuronen R, Narkilahti S, Vikholm-Lundin I. An antibody surface for selective neuronal cell attachment. Journal of Neuroscience Methods, *in press*.

III. Heikkilä TJ, Ylä-Outinen L, Tanskanen JMA, **Lappalainen RS**, Skottman H, Suuronen R, Mikkonen JE, Hyttinen JAK, Narkilahti S. Human embryonic stem cell-derived neuronal cells form spontaneously active neuronal networks *in vitro*. Experimental Neurology 2009, 218:109-116.

IV. **Lappalainen RS**, Narkilahti S, Huhtala T, Liimatainen T, Suuronen T, Närvänen A, Suuronen R, Hovatta O, Jolkkonen J. The SPECT imaging shows the accumulation of neural progenitor cells into internal organs after systemic administration in middle cerebral artery occlusion rats. Neuroscience Letters 2008, 440:246-250.

V. Hicks AU and **Lappalainen RS**, Narkilahti S, Suuronen R, Corbett D, Sivenius J, Hovatta O, Jolkkonen J. Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. European Journal of Neuroscience 2009, 29:562-574.

Table of contents

1. Introduction

Almost 10 years after the "Decade of the Brain", proper preventive and curative treatments are still lacking for many central nervous system (CNS) deficits. Human pluripotent stem cells and their neural derivatives have potential in regenerative medical applications for the treatment of CNS disorders and traumatic injuries (Lindvall and Kokaia 2006, Daadi and Steinberg 2009, Lee et al. 2009). Although several studies have examined the effects of such treatment, the results have been quite heterogeneous due to the variety of cell types and disease models evaluated.

Human embryonic stem cells (hESCs) were first described a decade ago (Thomson et al. 1998) and their neural differentiation was described shortly thereafter (Carpenter et al. 2001, Reubinoff et al. 2001, Zhang et al. 2001). Today various protocols exist for neural differentiation of hESCs and studies are currently aimed towards xeno-free culture systems and clinical applications for treatment of traumatic injuries such as spinal cord injury (Geron Corporation, USA).

Neural progenitor and neuronal cells derived from hESCs can be successfully cultured on extra cellular matrix (ECM) proteins (Cooke et al. 2009), but the development of more neuron-specific surface matrices is needed to support the attachment, growth, neurite extension, and maturation of neuronal cells, while at the same time preventing the attachment and growth of non-neural cells.

Neurons produced and designed for clinical applications toward the treatment of CNS disorders should essentially be functional, i.e. electrically active and capable of connecting with the host brain or spinal cord (Srivastava et al. 2008). This aspect has not yet been extensively studied with hESC-derived neurons.

For clinical application in an effort to treat patients using cell transplants, methods of cell delivery and assessment of functional recovery must be standardized. Ideally, cells could be transplanted via intravenous injection in a clinical setting without the need for specialized doctors and complicated procedures. In addition, the transplanted cell grafts should lead to functional recovery, possibly by replacing the lost tissue and regenerating a functional neural network with the host tissue.

In the work for this thesis, an efficient, yet simple protocol for the differentiation of hESCs into neurons was developed and tested using several different hESC lines. The hESC lines that were evaluated demonstrated a large difference in their potential for neural differentiation, as some lines efficiently produced nearly pure populations of neuronal cells, while others did not. The electrophysiologic properties of the produced neuronal networks were evaluated. In addition, neural cell adhesion molecule (NCAM) was tested as a neuron-specific cell culture surface matrix. Further, the produced neural progenitor cells were tested in animal models of stroke to evaluate the optimal transplantation route and the effects on functional recovery together with rehabilitation i.e. housing in an enriched environment.

2. Review of the literature

2.1 Stem cells

Stem cells are classified as undifferentiated cells capable of self-renewal and differentiation. Only totipotent stem cells in the embryo are capable of producing a new individual upon implantation. Next, depending on the origin of stem cells, they are defined as pluripotent (i.e. embryonic) or multipotential (i.e. fetal and adult) stem cells (Figure 1). This review of the literature introduces human embryonic stem cells (hESCs) and their neural applications for ischemic stroke.

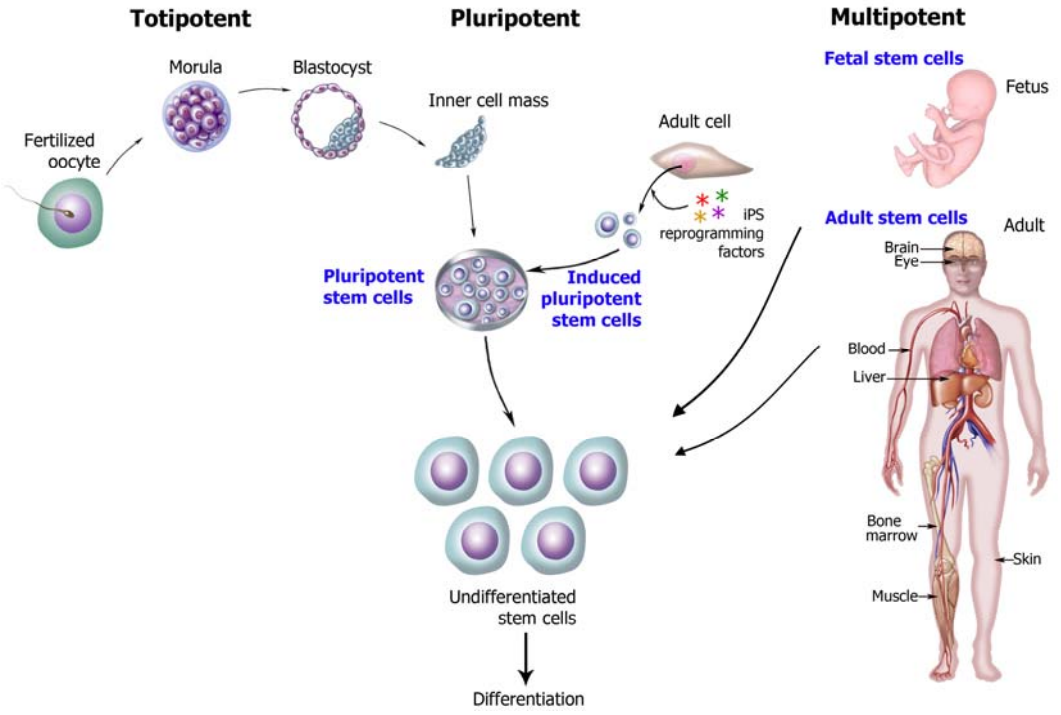

Figure 1. Stem cells. Stem cells can be divided in groups in accordance to their differentiation capacity. Embryos in zygote and morula stages are defined as totipotent. In blastocyst stage the inner cell mass is capable of producing the three germ layers and primordial germ cells, thus defined as pluripotent embryonic stem cells (ESCs). Adult cells can be re-programmed to produce embryonic stem cell-like pluripotent cells (iPS cells). Multipotent stem cells exist in fetal tissues during development and also retain in adult tissues. Figure modified from Bettina Lindroos, original images prepared by Cathrine Twomey from the National Academies *Understanding stem cells: An Overview of the Science and Issues*, http://www.nationalacademies.org/stemcells.

2.1.1 Human embryonic stem cells

Short-term *in vitro* culture of the inner cell mass of human blastocysts was first reported in 1994 (Bongso et al. 1994) whereas the isolation, successful culturing, and proper characterization of undifferentiated hESCs was first reported just over a decade ago in 1998 (Thomson et al. 1998). The hESC lines derived in that study (H1, H7, and H9) are still used in many laboratories today (Geron, Erceg et al. 2008, Li et al. 2008). Surplus embryos for hESC line derivation are commonly donated with informed consent by couples undergoing *in vitro* fertilization (IVF) treatments. The hESC populations growing in colonies have the following characteristics: 1) expression of transcription factors Nanog, Oct-3/4, and Sox-2; cell surface markers SSEA-3 and -4; and the keratan sulphate-related antigen markers Tra-1-60, and Tra-1-81; 2) having the developmental potential to form all three primary germ layers (ecto-, endo-, and mesoderm); and 3) formation of teratomas when transplanted into immunodeficient mice (Thomson et al. 1998, Adewumi et al. 2007, Skottman et al. 2007). These are required characteristics of all newly derived hESC lines (Skottman et al. 2007).

Culturing of hESCs was originally performed using mitotically inactivated mouse embryonic fibroblasts (MEFs) as a feeder cell layer and fetal bovine serum (FBS) in the culture medium (Thomson et al. 1998) as with mouse ESCs (Bibel et al. 2004). FBS was found to have a negative effect on hESCs, however, as colonies underwent excessive differentiation in FBS-supplemented medium (Amit et al. 2000, Amit and Itskovitz-Eldor 2002). A few years later, the first article was published describing serum-free culture conditions for hESCs using a commercial serum replacement (KnockOut Serum Replacement, Ko-SR, Invitrogen) in the medium instead of FBS at a 20 % concentration (Amit et al. 2000, Koivisto et al. 2004). Since then ko-SR has commonly been used in hESC culture medium. In addition to MEFs, other feeder cell types have also been used, such as commercially available human foreskin fibroblasts (Hovatta et al. 2003, Inzunza et al. 2005). Feeder-free systems, e.g., Matrigel have also been employed (Gerrard et al. 2005, Benzing et al. 2006, Hakala et al. 2009). There are clear indications, however, that hESCs cultured without feeder cells exhibit more abnormalities caused by suboptimal culture conditions and enzymatic passaging in long-term cultures (Mitalipova et al. 2005, Imreh et al. 2006). Thus, even though the culture conditions of hESCs have been systematically improved towards containing only human or synthetic components, many substances still include animal components, e.g., Ko-SR includes bovine serum albumin and Matrigel is derived from mouse tumor cells.

The diverse derivation and culture conditions influence gene expression and thus many other properties of hESCs (Skottman et al. 2006). Thus, hESC banks and standardized differentiation methods for various types of cells intended for clinical treatments are needed. For the clinical-grade production of hESCs (i.e. the cells appropriate for human use), the culture system should be totally xeno-free and at the level of good manufacturing practice (GMP). GMP guidelines are have been legislated compulsory with pharmaceutical products in many countries and include procedures, such as control and validation of manufacturing processes, clear instructions and procedures, training of operators, recording of manufacture, error management, standard operating procedures, quality control and auditing, and standard facilities and equipments (De Sousa et al. 2006). Recently, much effort has

been devoted to developing totally animal component-free and GMP level compatible culture conditions for hESCs (Ellerstrom et al. 2006, Ludwig et al. 2006, Ellerström et al. 2007, Rajala and Skottman 2008).

2.1.2 Human fetal stem cells

Fetal stem cells can be isolated from various structures of aborted human fetuses, especially from developing brain regions (Uchida et al. 2000, Caldwell et al. 2001, Kelly et al. 2004, Kallur et al. 2006, Darsalia et al. 2007, Nelson et al. 2008). The proliferation and differentiation of neural stem cells from human fetuses was described 1995 (Buc-Caron 1995). Since then, cortical, striatal, and spinal cord human neural stem cells have been isolated, cultured, and tested in experimental models such as ischemic stroke (Jeong et al. 2003, Chu et al. 2004, Ishibashi et al. 2004, Kelly et al. 2004, Darsalia et al. 2007), intracerebral hemorrhage (Lee et al. 2007), and spinal cord injury (Akesson et al. 2007, Emgard et al. 2009, Hwang et al. 2009). Further, fetal stem cells have been tested clinically, e.g., in Parkinson patients, but the published results are equivocal (Lindvall and Kokaia 2006).

2.1.3 Human mesenchymal stem cells

Human mesenchymal stem cells (MSCs) can be isolated from various adult tissues such as bone marrow, adipose tissue, cartilage, placenta, and cord blood (Ashammakhi et al. 2004). These cells characteristically have a limited potential for self-renewal and possess a differentiation capacity mostly restricted to the cell types from their own germ layer (Choumerianou et al. 2008). The most common applications for MSCs are the production of bone, cartilage, muscle, tendon, adipose tissue, and other connective tissues (Pittenger et al. 1999). Many attempts to produce neural progenitor and neuronal cells from MSCs have been published (Pittenger et al. 1999, Hermann et al. 2006). Functional studies of the electrophysiologic properties of the produced neural-like cells are not yet sufficient and it remains as an open question whether neural cells can be produced from cells originating from the mesodermal germ layer. Some clinical trials have studied the use of MSCs for the treatment of neurologic diseases. Few studies were recently published reporting the use of bone marrow mononuclear cells in chronic stroke (Barbosa da Fonseca et al. 2009a, Barbosa da Fonseca et al. 2009b). Also, a recent study investigating the use of adipose stem cells in multiple sclerosis (Riordan et al. 2009) described good and promising results as all three patients reported improvement in cognition, balance, and coordination. Other clinical trials can be found at http://clinicaltrials.gov. For example, at Cairo University in Egypt, a study was conducted at the end of 2008 to treat chronic spinal cord injury patients with autologous bone marrow transplants. Imperial College London is currently recruiting stroke patients to be treated with CD34+ autologous stem cells, and at the Grenoble University Hospital in France a study on autologous mesenchymal stem cells for treating ischemic stroke is scheduled to begin soon.

2.1.4 Induced pluripotent stem cells

One of the newest, most remarkable developments in stem cell research has been the reprogramming of lineage-restricted cells into pluripotent-like cells by the ectopic expression of defined transcription factors (Amabile and Meissner 2009). Briefly, a set of transcription factor genes is delivered to e.g. fibroblast cells with retrovirus-mediated transfection. If successful, in ~20 days hESC-like colonies can be detected, eventhough the efficacy is rather low. This was first described 2 years ago by two groups using four factors: Oct-3/4, Sox-2, Klf-4, and c-Myc, commonly named the Yamanaka factors (Takahashi et al. 2007) or with Oct-3/4, Sox-2, Nanog, and Lin28 (Yu et al. 2007). Both methods resulted in fibroblasts turning into growing colonies similar to hESC cultures that were positive for pluripotency markers such as SSEA-4, Tra-1-60, and Tra-1-81. In addition, the karyotype of induced pluripotent cells was normal and the cells maintained the potential to develop into all three germ layers. Since then, development in this field has been rapid due to the vast number of possibilities of the use of patient-specific cells in regenerative medicine. The induced pluripotent cells, however, are still far from being used clinically due to the fact that methods for producing these cells do not currently meet GMP standards (Aalto-Setala et al. 2009). In addition, these cells are difficult to produce in a short enough time for sub-acute settings.

2.2 Neural differentiation of human embryonic stem cells *in vitro*

Schematic presentation on neural differentiation of hESCs is presented in Figure 2.

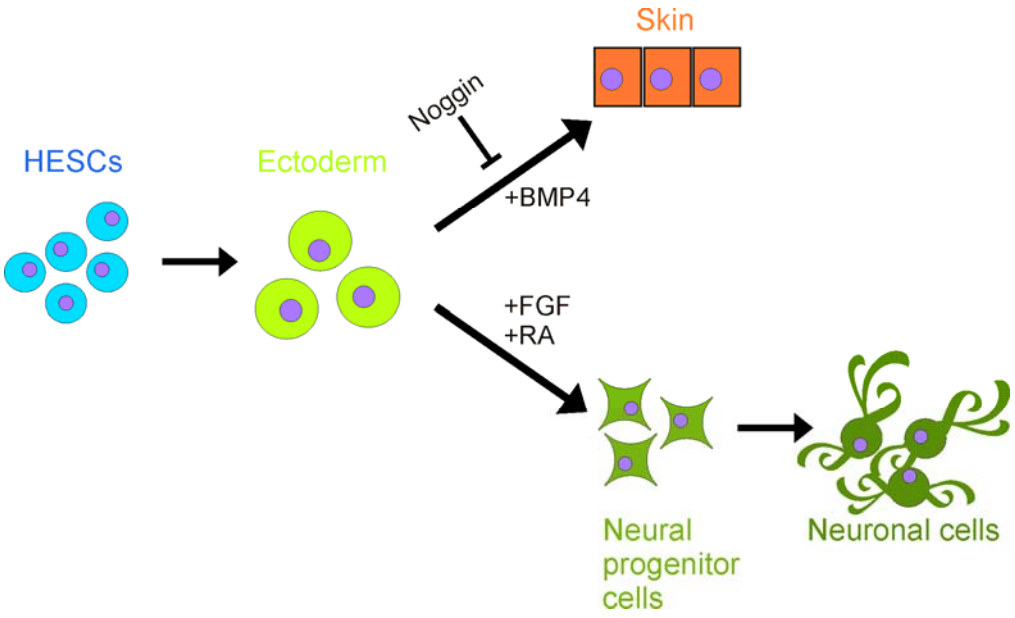

Figure 2. HESCs differentiate towards ectoderm and further into epidermal structures (skin) or into neural progenitor and neuronal cells. Bone morphogenetic protein 4 (BMP4) drives the ectodermal cells toward skin and this is blocked by noggin. Fibroblast growth factor (FGF) and retinoic acid (RA) are influencing neural differentiation. Figure modified from Murry and Keller 2008.

The first articles on neural differentiation of hESCs were published in 2001 (Carpenter et al. 2001, Reubinoff et al. 2001, Zhang et al. 2001). Both Carpenter and collaborators (2001) and Zhang and co-workers (2001) used the first hESC lines, H1, H7, and H9, derived by Thomson (1998); and Reubinoff and co-workers (2001) used the HES-1 line, derived in their own laboratory (Reubinoff et al. 2000). All of the lines were cultured on MEFs. Each of these protocols relied on embryoid body (EB) formation and further replating of the cells on appropriately coated surfaces in neural medium. Regardless of the differentiation methods used, these groups all showed that neural progenitors, specific neuronal cells, astrocytes, and, to a lesser extent, oligodendrocytes were produced. These studies have opened up the field for research on neural applications of hESCs.

Several methods and protocols to induce neural differentiation of hESCs have been published. Many studies report differentiation using co-cultures with other cell types such as PA-6 stromal cells (Pomp et al. 2005, Aberdam et al. 2008, Pomp et al. 2008, Vazin et al. 2008), MS5 stromal cells (Perrier et al. 2004, Sonntag et al. 2006, Lee et al. 2007), or conditioned medium from, for example, human hepatocarcinoma cells (Schulz et al. 2003, Shin et al. 2005). These protocols create challenges for human treatments due to the use of animal cells and xenoantigen contamination of hESCs (Heiskanen et al. 2007) or the unidentified factors in conditioned media (Mallon et al. 2006, Skottman et al. 2007, McDevitt and Palecek 2008). In general, the media used for neural cell differentiation and culturing consist of commercial neurobasal media, neural supplements (e.g. B27, N2), and glutamine (Nat et al. 2007). A few studies have been performed using chemically defined culture conditions with minimal amounts or completely without animal-derived components (Yao et al. 2006, Erceg et al. 2008). These defined protocols could be modified to achieve GMP standards for clinical applications.

Methodologically hESCs can be differentiated towards neural lineages using adherent and suspension culture systems or their various combinations. Gerrard and co-workers achieved differentiation of hESCs into neural progenitor cells in adherent culture by changing the medium composition between passages (Gerrard et al. 2005). The bone morphogenetic protein (BMP) signaling blocker noggin was used to induce the neural progenitor differentiation. Further specification of the produced neuronal cells was induced by various growth factors and supplements [sonic hedgehog (SHH), fibroblast growth factor (FGF)-8, ascorbic acid (AA), brain derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF)]. In approximately 30 days noggin increased the number of neural progenitor cells as indicated by the expression of musashi, nestin, and polysialated-NCAM, and some cells were positive for microtubule associated protein 2 (MAP-2) and β-tubulin$_{III}$. Glial fibrillary acidic protein (GFAP)-positive glial cells were not detected until after approximately 80 days of culturing and oligodendrocytes were not detected at all (Gerrard et al. 2005). Similarly, Baharvand and co-workers induced neural progenitor differentiation with an adherent culture system in which the composition of the medium was changed and finally morphologically neural tube-like structures (also called rosettes) were isolated and plated for further culturing (Baharvand et al. 2007). This protocol also resulted in MAP-2 positive neuronal cells. The key factor for differentiation was the use of retinoic acid (RA) instead of the BMP blocker noggin (Baharvand et al. 2007). Also, Erceg and collaborators reported efficient differentiation using an adherent culture (Erceg et al. 2008). Their protocol resulted

in musashi-positive neural progenitors that further differentiated into rostral e.g. forebrain neural lineage (positive for e.g. Gbx2 and Otx2) and more caudal (positive for e.g. HB9 and Chat) neuronal phenotypes with the aid of FGF. In this work, RA was used to specifically suppress rostral differentiation to produce neuronal cells with a spinal positional identity e.g. motorneurons (Erceg et al. 2008).

Differentiation of hESCs has also been described using a suspension culture system (Itsykson et al. 2005, Li et al. 2008). Itsykson and co-workers (2005) used a relatively simple system by differentiating hESCs in spheres with neural progenitor medium under the influence of noggin, as in the adherent system (Gerrard et al. 2005). They reported excessive cell death during days 2 and 3 of culturing, but an increase in the aggregate size over the 3 weeks follow-up period. At the end of 3 weeks there was a clear difference between the groups: the spheres cultured without noggin formed significantly more cystic structures than the spheres cultured with noggin. The cells in the spheres, regardless of the use of noggin, were primitive anterior neuroectodermal cells, which further differentiated into subpopulations of GABA-, glutamate-, serotonin-, and tyrosine hydroxylase-positive neurons (Itsykson et al. 2005). In contrast, Li and co-workers used RA and SHH in their suspension culture system (Li et al. 2008). HESC-derived neuroepithelial cells were produced as previously described (Zhang et al. 2001) and these cells were then treated with RA and sequentially with RA and SHH. The end result was ventral spinal progenitors and motor neurons as determined on the basis of immunocytochemical stainings and RT-PCR.

Nat and co-workers compared the differentiation potential of suspension vs. adherent culture systems for up to 6 weeks (Nat et al. 2007). No significant differences were detected between the differentiation systems; both systems produced the same amounts of nestin-positive cells from day 7 onwards, brain lipid binding protein- and glutamate transporter-positive radial glial cells from day 14 onwards, β-tubulin$_{III}$- and MAP-2-positive neuronal cells from day 7 onwards, and mostly GFAP-positive astrocytes on day 42. This study shows that neural progenitor and neuronal cells can be produced from hESC with similar efficacies, regardless of the culture system used, at least in short-term culture systems.

Adherent and suspension systems have also been combined for neural differentiation. Benzing and co-workers describe an efficient protocol in which hESCs were differentiated in adherent conditions on Matrigel with FGF to propagate neural cluster formation (Benzing et al. 2006). These neural clusters were detached and further cultured as neurospheres. The cells were then replated and the forming outgrowing population was considered as passage 1 neural progenitor cells because the cells stained positive for markers such as nestin, polysialated-NCAM, and β-tubulin$_{III}$. Cho and co-workers, on the other hand, used an EB formation step before plating the cells on Matrigel (Cho et al. 2008). Formed neural tube-like structures were mechanically detached and cultured as spherical neural masses resembling neurospheres in suspension. These masses were cultured for approximately 4 weeks after which they were plated on Matrigel and in a few days β-tubulin$_{III}$-positive cells were detected migrating from the clusters. Further on, SHH, FGF-8, and ascorbic acid were used to mature the neuronal cells into tyrosine hydroxylase-positive neurons.

Therefore, the production of neural progenitors and specific neuronal phenotypes from hESCs appears to be possible with many methods, growth factors, and inducing agents. The efficiency of the method or the functionality of the produced neuronal cells has not, however, been taken into consideration on a large scale. The next step will be comparing the efficiency and characteristics of produced cells to see if some methods are superior to others. Especially for the large-scale GMP production of neural progenitor cells for clinical applications, the production of these cells must be standardized to ensure similar efficacy for all patients.

2.3 Culture surface for neuronal cells

The interaction of neural cells with the ECM is essential during *in vivo* development. Also, *in vitro* ESC-derived neural progenitors and neuronal cells require an appropriate surface to attach, proliferate, extend neurites, and mature (Pierret et al. 2007). Indeed, many ECM proteins and mixtures have been tested and used with different kinds of cells (Kleinman et al. 1987, Aota et al. 1994, Whittemore et al. 1999, Nakaoka et al. 2003, Feng and Mrksich 2004, Flanagan et al. 2006). A more specific comparison of ECM peptides fibronectin, collagen I, collagen IV, and laminin in neural cell culturing was recently published (Cooke et al. 2009) and the results indicated that all the peptides supported cell attachment and neurite growth equally well. In addition to ECM proteins and peptides, other factors, such as epidermal growth factor (Nakaji-Hirabayashi et al. 2007), have been successfully tested as neural cell culturing surfaces. These factors create a 2-D platform for the cells, but more complex scaffolds are needed for 3-D culture systems. Also, more complex neural cell grafts are developed when the supporting scaffold is functionalized using various peptides (Beckstead et al. 2006, Place et al. 2009). Other studies have been conducted with hydrogels (Nisbet et al. 2008) or microparticles (Bible et al 2009), which are potentially applicable for transplantation.

2.4 Electrophysiologic properties of neuronal cells

Successful production of hESC-derived neural progenitor and neuronal cells is relatively straightforward and easy to achieve with various methods. It is important, however, that the produced cells are characterized. In particular, the electrical properties and functionality of the cells must be confirmed prior to the use of these cells in disease models, drug screening platforms, or regenerative medicine applications (Hess and Borlongan 2008). The regenerative potential of neural progenitor and neuronal cells in the central or peripheral nervous system is dependent on their ability to process and transmit electrical signals received from the host tissue. These aspects have been studied to a small extent with neuronal derivatives of hESCs. The electrical properties of hESC-derived neuronal cells were first described in one of the first articles on neural differentiation of hESCs (Carpenter et al. 2001). Using a patch clamp system Carpenter and co-workers demonstrated that hESC-derived neuronal cells expressed voltage-gated ionic

currents and produced action potentials. Since then, many other studies have also used the patch clamp approach to evaluate the electrophysiologic functionality of hESC-derived neuronal cells (Perrier et al. 2004, Schulz et al. 2004, Itsykson et al. 2005, Li et al. 2005, Johnson et al. 2007, Lee et al. 2007, Wu et al. 2007, Cho et al. 2008, Erceg et al. 2008). Patch clamp recording is a useful method of assessing the electrophysiological properties of individual cells, but it has become clear that the electrical functionality at the neuronal network level *in vitro* is also critically important (Tateno et al. 2005, Illes et al. 2007).

A microelectrode array (MEA) system was described almost 3 decades ago (Gross et al. 1977, Pine 1980). In this system, neural progenitor or neuronal cells grow on an appropriate surface on top of a number of small electrodes. When neuronal cells connect with each other, a neural network is formed and with sensitive electrodes beneath them, the spatial and temporal action potentials, i.e., electrical signals across the network, can be detected and measured. Even though most of these are planar 2D systems, they reveal general information of the electrophysiologic properties, activity patterns and changes, and possible network properties required for learning in the nervous system (Maeda et al. 1995, Ben-Ari 2001, Wagenaar et al. 2006, Madhavan et al. 2007). The use of commercial MEA with mouse ESC-derived neuronal cells was first described by Illes and co-workers (Illes et al. 2007). Mouse ESC-derived neuronal cells first exhibited spontaneous activity as single spikes, but as the network matured, the functionality developed spike trains and more synchronous bursts, similar to that described in neurons cultured from rat embryos (Wagenaar et al. 2006). In addition to the expected electrical properties of the neural network, mouse ESC-derived neuronal cells respond to pharmacologic stimulation based on neurons derived from the rodent brain (Wagenaar et al. 2006, Illes et al. 2007). In the future, it would be very useful to combine MEA and patch clamp techniques to gain more specific knowledge at the neuronal network level (e.g. startpoint of the signaling), as well as at the individual cell level (e.g. neuron subtypes).

2.5 Experimental cerebral ischemia

Cerebral ischemia (e.g. stroke) is one of the most substantial health-related challenges in Western countries. Annually, 15 million people suffer a stroke worldwide (World Health Organization 2010) and the number in Finland is 14 000 annually, which means that 38 new stroke patients are admitted every day (Aivohalvaus- ja dysfasialiitto 2009). As the population ages, the number of these cases is estimated to rise exponentially. Two-thirds of the patients survive and approximately half of them are left with permanent deficits despite thrombolytic therapy and rehabilitation. This makes stroke a growing social and economical burden. Research related to cerebral ischemia is widely conducted and many experimental models of stroke have been reported.

The most common form of stroke is the focal occlusion of the middle cerebral artery (MCA). It is therefore not surprising that the most frequently used stroke models are permanent and, to a lesser extent, transient models. In permanent focal ischemia, the distal MCA of the animal is permanently occluded together with temporal occlusion

of both common carotid arteries (Chen et al. 1986), whereas in transient ischemia the artery is occluded by inserting a thin filament into the artery and removing it after a certain period of time (Longa et al. 1989) or by injecting a vasoconstrictive peptide into the MCA (Biernaskie et al. 2001, Hicks et al. 2007). The transient model in principle more closely relates to the cerebral ischemia that occurs in patients, but it is experimentally more difficult to master. There is a large variation between animals and thus a large number of animals is needed for each experiment. In addition, the damaged area is relatively large in relation to human ischemia, including both cortical and striatal tissue. Permanent ischemia, on the other hand, results in a well-defined cortical injury and clear functional arrest of the contralateral forepaw in rats. Thus, the functional recovery of the animals after any treatment is easier to detect, follow, and analyze. These types of models are commonly used when studying cell-based transplantation therapies for stroke.

2.6 Stem cell-based treatments for stroke

Different types of stem cells have been used for various injuries and diseases of the CNS experimentally (Burns et al. 2009, Kriegstein and Pitkänen 2009, Lindvall and Kokaia 2009) as well as in clinical settings, e.g., bone marrow-derived stem cells for multiple sclerosis (Narkilahti et al. 2009). This section mainly considers the use of human neural progenitor cells for the treatment of stroke.

Reubinoff (Reubinoff et al. 2001) and Zhang (Zhang et al. 2001) transplanted the hESC-derived neural progenitor cells into healthy neonatal mice brain and showed that the cells survived and migrated into the brain parenchyma. The healthy brain is not inflamed and thus cell survival was expected even though Reubinoff and co-workers reported large variations in cell survival between transplanted animals (Reubinoff et al. 2001). Many studies have since been conducted with hESC-derived neural progenitor and neuronal cells transplanted into injured CNS, including experimental cerebral ischemia (Daadi et al. 2008, Daadi et al. 2009, Daadi and Steinberg 2009), spinal cord injury (Lee et al. 2007, Hatami et al. 2009), Parkinson's disease (Ben-Hur et al. 2004, Schulz et al. 2004, Cho et al. 2008, Geeta et al. 2008), and multiple sclerosis (Ben-Hur et al. 2007, Aharonowiz et al. 2008).

In experimental stroke studies, many cell types have been tested as potential grafts such as human fetal neural stem cells (Darsalia et al. 2007, Bacigaluppi et al. 2009), human NT2N teratocarcinoma-derived neural progenitor cells (Borlongan et al. 1998, Bliss et al. 2006), neural progenitor cells derived from bone marrow stromal cells (Hayase et al. 2009), and human MSCs (Detante et al. 2009). Bliss and co-workers reported a stroke study conducted with human NT cells that were derived from teratocarcinoma (Bliss et al. 2006). In this study, they used a permanent occlusion stroke model and transplanted the cells as a single cell suspension 7 days after the injury with a follow-up time of 35 days. They reported robust survival and neuronal differentiation of the cells as approximately 40 % of the human cells were detected histologically in the brain slices. Significant migration of the cells was not detected, thus the cells mainly remained at the transplantation site. Only a few of these types of studies have been reported with hESC-derived neural progenitor cells (Daadi et al. 2008, Daadi et al. 2009). Daadi and co-workers reported nearly 40 %

survival of hESC-derived neural progenitor cells in rats with experimentally induced stroke 2 months after transplantation (Daadi et al. 2008). On the other hand, some studies report minimal survival of transplanted mouse or human ESC-derived neural progenitor cells (Bühnemann et al. 2006, Kim et al. 2007). The different origin of cells and variations in the differentiation protocol and transplantation techniques used may affect cell survival *in vivo*. In addition, precise stereologic cell counting is critical, but not properly described in many articles. Thus, directly comparing the results of the studies is somewhat challenging.

Almost all published articles on experimental stroke and cell transplantation have reported some functional recovery. The number of functional tests used varies from one (Daadi et al. 2008) to several (Bliss et al. 2006). Some tests, like the cylinder task (Woodlee et al. 2005), measure the general behavior of the animals and some tests measure specific sensory-motor recovery (Montoya's reaching task) (Montoya et al. 1991). Functional recovery should be examined with several different behavioral tests to adequately measure various aspects of brain function and to more thoroughly investigate the effects of the cell transplants.

Regardless of the variable results from experimental animal work, some stroke patients have been treated with cell transplants (Kondziolka et al. 2000, Bang et al. 2005, Savitz et al. 2005). In these studies, the origin of cells has been diverse and since adverse effects were noted e.g. with porcine cells, further clinical studies on stroke and cell therapies are required. Although the research in this field is progressing rapidly, much more work is required before cell-based therapies can be routinely offered to human patients with stroke. Currently, one company, StemCells, Inc., just finished a Phase I clinical trial with human fetal neural stem cells to treat neuronal ceroid lipofuscinosis i.e. Batten diseases (www. stemcellsinc.com) and two companies are in the process of designing cell-based therapies for patients. ReNeuron Group plc is concentrating on treating stroke patients with human neural stem cells (www.reneuron.com) and the Geron Corporation is focused on treating spinal cord injury patients with hESC-derived oligodendrocyte progenitors (www.geron.com).

Thus, our focus here has been to differentiate hESCs into neural progenitors and neuronal cells using various methods and supplements, but a simple and efficient protocol which results in the yield of 90-100 % neural progenitors and is GMP-convertible is needed. The electrical properties and functionality of neuronal cells require close inspection *in vitro* prior to transplantation. In addition, the optimal route of delivery to induce accumulation of the cells into ischemic host brain tissue remains unknown. Finally, the effects of hESC-derived neural progenitor cells in an animal model of stroke have not yet been widely reported and more information is needed on this area.

3. Aims of the study

The aims of this project were to generate, set up, and test an efficient but simple neural differentiation protocol for hESCs. An appropriate neuron-specific cell culture surface matrix was tested, and the electrophysiologic properties of the produced neuronal cell-derived networks were extensively studied. Finally, the hESC-derived neural progenitors were tested in animal models of stroke to evaluate the optimal transplantation route and possible improvements in functional recovery in combination with enriched environment housing.

These studies were divided into 5 specific aims:

1) Produce a simple, efficient protocol for the neural progenitor and neuronal differentiation of hESCs (**study I**).
2) Test a novel antibody-based surface for neuronal cell attachment (**study II**).
3) Investigate the functionality, i.e., electrical properties of produced neuronal cells (**study III**).
4) Test delivery routes for accumulating the transplanted neural progenitor cells into the damaged brain tissue (**study IV**).
5) Test if neural progenitor cell transplants can induce functional recovery in animals in combination with an enriched housing environment (**study V**).

4. Materials and methods

4.1 Cell cultures

4.1.1 Human embryonic stem cells

The hESC lines used in these studies were derived either at the Karolinska Institutet (HS lines, Hovatta et al. 2003, Inzunza et al. 2005) or at Regea – Institute for Regenerative Medicine (Regea lines, Skottman 2009, European Human Embryonic Stem Cell Registry). Poor quality or surplus embryos that could not be used for fertility treatments were voluntarily donated for the purpose of deriving hESC lines by couples going through *in vitro* fertilization procedures. The Karolinska Institute has the approval of the Ethics Committee of the Karolinska Institute to derive and culture hESCs. Regea has the approval of the National Authority for Medicolegal Affairs to do research with human embryos (Dnro 1426/32/300/05). Ethics Committee of Pirkanmaa Hospital District provided the permission to culture, characterize, and differentiate hESCs derived at the Karolinska Institute (Skottman R05051) and to derive, culture, characterize, and differentiate new hESC lines (Skottman R05116). Donors did not receive financial compensation for donating the embryos and researchers did not know the origin of the embryos. Derivation, culturing, and characterization of the undifferentiated hESCs were performed similarly for all lines evaluated. Human ESCs were derived from morula- or blastocyst-stage embryos by mechanical derivation using specially made flexible metal needles (Hunter Scientific, Essex, UK) and surgical knives. The isolated inner cell mass was transferred to and further cultured on top of a human foreskin fibroblast feeder cell layer (CRL-2429, cells purchased from American Type Tissue Collection, Manassas, VA). The medium for maintaining hESCs in an undifferentiated stage (hES medium) comprised Knockout Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20 % Ko-SR, 2 mM GlutaMax, 0.1 mM 2-mercaptoethanol (all from Gibco Invitrogen, Carlsbad, CA), 1 % non-essential amino acids (Cambrex Bio Science, East Rutherford, NJ), 50 U/ml penicillin/streptomycin (Lonza Group Ltd, Switzerland), and 8 ng/ml basic FGF (bFGF, R&D Systems, Minneapolis, MN). The hESCs were passaged every 5 to 7 days. The passaging was performed manually using scalpels and needles, after which the dissected undifferentiated small colony areas were transferred on top of a fresh feeder cell layer. The medium for hESCs was changed 6× per week and the cells were kept in humidified incubators (+37 °C, 5 % CO_2). The passage number of hESCs differentiated into neural progenitors and neuronal cells used in **studies I-V** never exceeded 90 (range p25-90 in study I, range p35-49 in study II, range p40-50 in study IV, p59 in study V).

Morphology of the hESC colonies was assessed daily and EB studies, reverse transcriptase polymerase chain reaction (RT-PCR), and immunocytochemical staining with undifferentiated stage markers, such as Oct-3/4, SSEA-4, and Tra-181, were performed approximately once every 2 months. In addition, the cultures were routinely tested and found to be free of mycoplasma contamination.

4.1.2 Neural differentiation of human embryonic stem cells

Neural differentiation of hESCs was performed using two methods. Regardless of the method, the basic formula of the neural differentiation medium (NDM) was: 1:1 DMEM/F12:Neurobasal media supplemented with 2 mM GlutaMax, 1×B27, 1×N2 (all purchased from Gibco Invitrogen), and 25 µg/ml penicillin/streptomycin (Lonza Group Ltd.). In addition, bFGF was the only growth factor used.

First, the neural differentiation of hESCs was performed in adherent culture. The undifferentiated hESC colonies were manually cut into small colony areas and replated on 6-well CellBIND plates (Corning Inc, Corning, NY) in NDM supplemented with 20 ng/ml bFGF. Cell attachment was analyzed after 2 days. After 7 to 14 days the centers of the populations began to develop neural tube-like structures, called rosettes (Zhang et al. 2001, Elkabetz et al. 2008). Rosettes contain neural progenitor cells that stain positive for neural progenitor markers such as Musashi, Nestin, and Pax-6 (Elkabetz et al. 2008). The rosettes can be manually dissected and transferred for further studies and/or applications, as in **study IV**.

The second protocol used for neural differentiation was a simple suspension method. The hESC aggregates were transferred to low attachment 6-well plates (Nunc, Thermo Fisher Scientific, Rochester, NY) containing NDM. In the suspension cultures, variable concentrations of bFGF were tested: constant 20 ng/ml, constant 4 ng/ml, or 20 ng/ml for 2 weeks after which the bFGF was withdrawn. Regardless of the concentration of bFGF, within approximately 5 days the cell aggregates began to form round constant spheres that are hereafter called neurospheres. Basically all cell aggregates formed these neurospheres, thus the starting number of neurospheres depended on the number of colonies/cell aggregates used. The size of the neurospheres did not increase during the first 3 weeks of suspension culture, but thereafter the spheres had to be cut manually into 4 to 8 new spheres each week. It was important that the spheres were kept small enough (maximum: ∅ ~500 µm) to keep most of the cells exposed to NDM. The suspension cultures could be maintained for up to several months.

For *in vitro* differentiation the neurospheres were collected and either enzymatically dissociated into single cell suspension or mechanically cut into small aggregates, and replated on 10 µg/ml human laminin (Sigma-Aldrich, St. Louis, MO) coated 12- or 24-well plates in NDM without bFGF. For single cell suspensions, the neurospheres were incubated with 1× trypsin at + 37°C for 5 minutes and was then inactivated by adding 5 % human serum (PAA Laboratories, Austria) in sterile phosphate buffered saline (PBS, Lonza). After centrifugation, the cell pellet was resuspended in NDM without bFGF. Mechanically cut small aggregates were directly replated.

4.2 Characterization of the neural progenitor and neuronal cells

4.2.1 Morphology

Neurosphere morphology was analyzed using a stereomicroscope (Nikon SMZ800) or a phase-contrast microscope (Nikon T2000S). The *in vitro* differentiated cells were analyzed using phase-contrast microscopy. The cells could be categorized according to their morphology. Groups included non-neural flat epithelial-like cells (large flat cells), neuronal cells (tight soma and variable number of neurites), and glial cells (medium sized soma and neurites) (Figure 3).

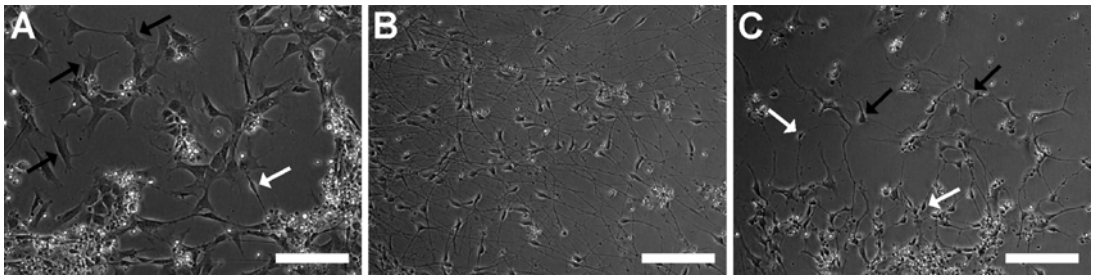

Figure 3. Representative cell cultures from hESC-derived neurospheres differentiated for 3-15 weeks. A) Neurospheres differentiated for 1-3 weeks produce neuronal cells (white arrows) but also flat epithelial-like cells (black arrows). B) Neurospheres differentiated for 6-9 weeks produce solely neuronal cells. C) Spheres differentiated for 12-20 weeks are shifting into astrospheres as they produce astrocytes (black arrows) along with neuronal cells (white arrows). Scale 100 µm.

4.2.2 Time-lapse imaging

The cells were imaged using a time-lapse imaging system Cell-IQ® (Chip-man Technologies Ltd, Tampere, Finland). The system consisted of a thermal chamber (+36.5°C) in which two culture plates, ranging in size from 6- to 96-wells, could be set into an integrated plate holder with gases (5 % CO_2) directly transferred into the plates. The chamber includes a green LED light source below and microscopic phase-contrast optics (10×) above the culture plates. A charge-coupled device camera was used to acquire the images. The system was connected to a computer through which the imaging was processed using Cell-IQ® Imagen software. The plate holder could be moved in the xy-axis (± 1 µm), which enabled controlled, precise movement of the plates. Briefly, the areas of interest were selectively chosen and imaged in each well in single squares (500 × 670 µm) or in stitched grid squares of 2 × 2 up to 7 × 7 (sizing from 1000 × 1340 µm to 3500 × 4690 µm of imaged areas). The system utilized a motorized z stage (± 0.4 µm precision) and a dynamic Z-stack (user defined) system resulting in all-in-focus images. Single captured images were stored in separate folders in the JPEG-format. These images could be opened and converted into a movie format for further analysis. For example, user-defined cell recognition programs utilizing machine vision technology could be built with the analysis software. This enabled the analysis of cell types, neurite

outgrowth, cell division, and other events from the captured images (Narkilahti et al. 2007).

4.2.3 RT-PCR

Gene expression of the produced neural progenitor cells was evaluated at the mRNA level. Neurospheres were collected into lysis buffer, then the total RNA was extracted according to manufacturer's instructions using RNeasy®Micro or Mini kits (both from Qiagen, Hilden, Germany) or NucleoSpin® RNA II kit (Machery-Nagel GmbH & Co, Düren, Germany). The purity and quality of the isolated mRNA was evaluated using NanoDrop (Thermo Fisher Scientific). Total RNA was then reverse-transcribed into single-strand complementary DNA (cDNA) using oligo-dT-primers and Sensiscript Reverse Transcriptase kit (Qiagen) or High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The cDNA was then added to a PCR buffer (–MgCl, +KCl) supplemented with $MgCl_2$, dNTP mix, Taq DNA polymerase (all from Qiagen), and both forward and reverse primers (biomers.net, Germany). The primers were specific for *Oct-4* for undifferentiated hESCs; *α-fetoprotein* for endodermal cells; *Brachyury/T* for mesodermal cells; *Musashi*, *Nestin*, and *Pax-6* for neural progenitors; *MAP-2*, and *neurofilament-68* for neuronal cells; *brain lipid binding protein* for radial glial cells; *GFAP* for astrocytes; and *Olig 1* for oligodendrocytes. The cDNA was amplified and the PCR end products were separated electrophoretically on agarose gels containing ethidium bromide and visualized under UV-light.

4.2.4 Measuring of proliferation

The proliferation of produced neurospheres was measured in **study I** using a colorimetric enzyme-linked immunosorbent assay for 5-bromo-2'-deoxyuridine (BrdU kit; Roche, Basel, Switzerland). The manufacturer's instructions were followed with some modifications. Briefly, the neurospheres were manually cut into smaller spheres containing approximately 10^4 cells one day before starting the assay. The spheres were incubated with BrdU labeling solution for 15 h after which the spheres were enzymatically dissociated into single cell suspension and replated into 96-well plates. The culture plates were centrifuged at room temperature for 10 minutes after which the cells were dried and fixed with FixDenat. The anti-BrdU monoclonal antibody conjugated with peroxidase was then added. The cells were washed with PBS, substrate solution was added, and the reaction was stopped with H_2SO_4. Absorbance was measured using a Viktor^2 1420 Multilabel Counter (PerkinElmer-Wallac, Waltham, MA) at a wavelength of 450 nm. Background absorbance was measured from negative controls and subtracted from the measured sample absorbances.

4.2.5 Microelectrode array system

Electrical activities of the produced neuronal networks were measured using microelectrode array (MEA) dishes with 59 substrate-embedded titanium nitride microelectrodes of 30 µm in diameter with a 200 µm distance between the electrodes (200/30iR-Ti-gr, Multi Channel Systems MCS GmbH, Reutlingen, Germany). For cell attachment, the MEA dishes were coated with 0.1 % polyethyleneimine and 20 µg/ml human laminin. Polyethyleneimine was incubated on dishes overnight at +4°C, dishes were rinsed with PBS, dryed, and a drop of laminin was added onto the electrodes and incubated for 2 to 5 h at +37°C. The cells were then seeded on the electrode area of MEA dishes either in small aggregates (10-15 pieces, ∅ 50-300 µm, containing $2x10^3$-10^4 cells) or in single cell suspension ($2x10^5$ cells) in NDM without bFGF to induce *in vitro* differentiation. One week later bFGF (4 ng/ml) and BDNF (5 ng/ml, Gibco Invitrogen) were added to the medium to support neuronal cell growth and maturation. This medium was changed 3× per week. The MEA dishes were maintained in Petri dishes in a humidified incubator at +37°C and 5 % CO_2 atmosphere.

The cultures were kept sterile during measurement by sealing the MEA dishes in a laminar flow hood with a semi-permeable membrane (ALA MEA-MEM, ALA Scientific Instruments Inc., Westbury, NY, USA) that is selectively permeable to gases (O_2, CO_2), as described previously (Potter 2001). For measurements the sealed MEA dish was carefully placed into the MEA amplifier (MEA-1060BC, MCS) and recordings were started after waiting for 5 minutes. The amplifier was placed on top of a phase-contrast microscope (IX51, Olympus) to visually inspect the cells during measurement. All cultures were imaged before or during the recording using an iXon 885 camera (Andor Technology, Belfast, UK) connected with TILLVisION software (TILL Photonics GmbH, Gräfelfing, Germany). A MEA gain of 1100 and bandwidth of 1 to 10 kHz were utilized. Signals were sampled at 20 or 50 kHz using a data acquisition card controlled through MC_Rack software (MCS). The temperature was maintained at +37 °C using a TC02 temperature controller (MCS). All recordings were stored on the computer and visually inspected for artifacts. A high-pass filter (2^{nd} order Butterworth filter) with a bandpass cut-off frequency set to 200 Hz was used to remove baseline fluctuations. Spike detection using MC_Rack software was performed with a threshold of 5.5× the standard deviation of the noise level. NeuroExplorer (Nex Technologies, Littleton, MA, USA) or Matlab (MathWorks, Natick, MA) were used to visualize the processed spike data.

The recording time for each MEA dish was 5 to 15 minutes in **studies I** and **III**. All the cultures were measured 2 × per week starting one week after plating the cells. MEA cultures were discarded if the cells did not attach, became detached, or the electrophysiologic signals could not be detected during the first 2 weeks.

Pharmacologic modulation was performed to investigate the properties of neuronal networks. Pharmacologic substances were mixed with fresh medium that was then added to cells. The recordings were started after 5 minutes of incubation. After 5 minutes of recording, the cells were washed and incubated with fresh medium for 15 minutes before adding the next pharmacologic substance. The substances used were an AMPA/kainate antagonist (6-cyano-7-nitroquinoxaline-2,3-dione, CNQX), a NMDA antagonist [D(-)-2-amino-5-phosphono-pentanoic acid, D-AP5], a gamma-

aminobutyric acid (GABA), and a $GABA_A$ antagonist (-)-bicuculline methiodide, bicuculline (all from Sigma-Aldrich). Briefly, baseline activity was measured first, then the effects of CNQX alone and CNQX together with D-AP5 were investigated. Next, a washout was performed and the effects of GABA and bicuculline were tested.

4.2.6 Immunocytochemical staining

For immunocytochemical staining in **studies I, II, IV,** and **V**, the neural progenitor and neuronal cells were fixed with cold 4 % paraformaldehyde (PFA) for 20 minutes at room temperature and rinsed with PBS prior to proceeding with the staining protocol. Nonspecific labeling was reduced by blocking the cells for 45 minutes at room temperature with 10 % normal donkey serum, 0.1 % Triton X-100, and bovine serum albumin in PBS. The primary antibodies were diluted in a solution of 1 % normal donkey serum, 0.1 % Triton X-100, and bovine serum albumin in PBS, and added to the wells for overnight incubation at +4°C. The following day, the cells were washed and secondary antibodies diluted in a solution of 1 % bovine serum albumin in PBS were incubated with the cells for 1 h at room temperature in the dark. Thereafter, the cells were sequentially washed with PBS and phosphate buffer, lightly dried, mounted with Vectashield with 4'6-diamidino-2-phenylindole (Vector Laboratories, Peterborough, UK), and cover-slipped for imaging. The primary antibodies used were: for neural progenitor cells NCAM, nestin, and Pax-6; for neuronal cells β-$tubulin_{III}$, doublecortin (DCX), MAP-2, and neuron-specific enolase (NSE); for radial glial cells brain lipid binding protein (BLBP); for astrocytes GFAP; for oligodendrocytes galactocerebroside (GalC); and for proliferating cells Ki-67 from Chemicon (Temecula, CA), Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA), Sigma-Aldrich, Santa Cruz Biotechnology (Santa Cruz, CA), NovoCastra (Newcastle, UK), or R&D Systems as presented in Table 1.

Table 1. Antibodies and their concentrations used in studies I, II, IV, and V.

Cell type	Antibody	Concentration	Supplier	Catalog code
Neural progenitors	NCAM	1:800	Chemicon	ab5032
	Nestin	1:100	Chemicon	mab5326
	Pax-6	1:100	DSHB	PAX6
Neuronal cells	ß-$tubulin_{III}$	1:1000	Sigma-Aldrich	T8660
	DCX	1:200	Santa Cruz	sc8066
	MAP-2	1:400-600	Chemicon	ab5622
	NSE	1:100	NovoCastra	ncl-nse-435
Radial glial cells	BLBP	1:800	Chemicon	ab9558
Astrocytes	GFAP	1:600	R&D Systems	af2594
Oligodendrocytes	GalC	1:200	Chemicon	mab342
Proliferating cells	Ki-67	1:800	Chemicon	ab9260

The secondary antibodies were AlexaFluor-488 or -568 conjugated to anti-goat, anti-mouse, anti-rabbit, or anti-sheep secondary antibodies (1:400), all purchased from Invitrogen.

4.3 Neural cell adhesion molecule surface for neuronal cells

Neuronal cell attachment to polystyrene wells was investigated and tested with specific NCAM. In **study II,** neural cell-specific NCAM antibodies at concentrations of 0, 25, 50, 75, or 100 µg/ml were bound to a polystyrene surface using a non-ionic hydrophilic polymer *N*-[tris(hydroxymethyl)methyl]-acrylamide (pTHMMAA) (Vikholm-Lundin and Albers 2006) at a concentration of 200 µg/ml. The antibodies were allowed to physisorb for 15 minutes after which the wells were rinsed with PBS and post-treated with the pTHMMAA polymer for an additional 15 minutes. After rinsing, the wells were filled with PBS and allowed to stabilize for 2 days at +4°C before cell seeding. The neurospheres were seeded on the wells without growth factor and cultured for 8 days before fixation.

4.4 Neural progenitor cell labeling

For the *in vivo* monitoring of the transplanted cells, an appropriate, long-lasting, and efficient label is needed on the cell surface or inside the cells. Two labels were tested.

4.4.1 ^{111}Indium-oxine

In **study IV**, single neural progenitor cells were prepared from rosettes. Cells (1×10^6) were incubated for 30 minutes at room temperature in Tris buffer containing 2.5, 5, or 7.5 MBq ^{111}Indium-oxine (^{111}In-oxine, specific activity 37MBq/ml, Nycomed Amersham, Piscataway, NJ) for *in vitro* viability measurement or with 4.76-5.65 MBq ^{111}In-oxine for transplantation. Control cells went through a similar manipulation without ^{111}In-oxine. After labeling, the cells were washed once to remove unbound tracer and then resuspended in NDM (for a viability test) or saline (0.9 % NaCl) (for transplantation).

The viability of labeled cells was examined in **study IV**. ^{111}In-Oxine-labeled cells were tested with trypan blue (Sigma-Aldrich) staining at 2, 6, 8, 20, and 24 h after the labeling to determine the number of live and dead cells. At each time-point the cells were diluted 1:10 in trypan blue and the numbers of dead and live cells were counted.

4.4.2 Ultra-small superparamagnetic iron oxide

Cells were also labeled with ultrasmall superparamagnetic iron oxide particles (USPIO, 10 mg/ml plain particles, 50 nm in diameter, G. Kisker GbR, Steinfurt, Germany). The concentration and additional substances were optimized and the cells were successfully labeled with 200 µg/ml USPIO and 375 ng/ml poly-L-lysine as a labeling agent. Briefly, neuronal cells were plated on human laminin-coated 24-well culture plates and allowed to attach for 2 days. USPIO and poly-L-lysine were added to the medium, and the labeling medium was added to the cells and incubated for 24 h at +37°C, 5 % CO_2. The labeling medium was then replaced with fresh medium. The cells were transplanted or stained with Prussian blue to verify the labeling efficiency.

4.5 Neural progenitor cell transplants in animal models

4.5.1 Animal models of cerebral ischemia

In all experiments the animals were housed at +21 °C (± 1 °C) with a 12 h:12 h light-dark cycle. Food and water were available ad libitum, except before the Montoya's reaching task (described below). All the procedures were approved by the Committee for the Welfare of Laboratory Animals at the University of Kuopio and by the Provincial Government of Kuopio.

In **study IV**, transient MCAO (intraluminal filament technique) (Longa et al. 1989) was used as the stroke model (n=13 altogether). In **study V,** a permanent MCAO model was used (n=51 altogether). In brief, rats were anesthetized with 1 % to 2 % isofluorane in 30 % O_2 and 70 % N_2O. The body temperature of the animals was maintained at 36.5 to 37.5 °C throughout the surgery using a thermal blanket (Harvard Apparatus, Holliston, MA). Transient MCAO was induced as follows: the common carotid artery was revealed and a thin (∅ 0.25 mm) plain heparinized nylon filament (Kuusamon Uistin Oy) was inserted and advanced into the internal carotid artery to block the blood flow to the MCA territory. The occlusion time was 2 h after which the filament was removed to allow reperfusion and the external carotid artery was closed by electrocoagulation. The sham animals were similarly treated but the filament was not advanced into the internal carotid artery. Lidocaine (2 %, Astra Zeneca) was used for postoperative pain relief. For permanent MCAO, the animals were attached to a stereotactic apparatus, an incision was made between the left ear and eye, and the distal portion of MCA was exposed by drilling a small hole in the skull. The MCA was cauterized just above the rhinal fissure. Another incision was made on the neck and both common carotid arteries were occluded for 60 minutes. Sham-operated animals went through a similar surgery, but the MCA was not cauterized. Temgesic (0.03 mg/ml, Schering-Plough) was used for postoperative pain relief.

4.5.2 Immunosuppression

In **study V,** the animals received a subcutaneous injection of the immunosuppressant Cyclosporin A (SandImmune, Novartis, Basel, Switzerland) one day prior to transplantation to prevent rejection. At the time of transplantation osmotic minipumps (Alzet, Cupertino, CA) filled with Cyclosporin A were inserted under the dorsal skin of rats to continuously deliver the drug for 28 days. At the 1-month timepoint, the osmotic minipumps were replaced with new filled minipumps.

4.5.3 Cell transplants

In **study IV,** neural progenitor cells were transplanted as a single cell suspension directly following the ^{111}In-oxine labeling or without labeling (control cells). In **study V,** the neurospheres were trypsinized into a single cell suspension that was directly transplanted. One batch of prepared cells was used for two rats. Trypan blue was used to assess the viability of the cells prior to transplantation and a few batches of the remaining cell suspension was replated on polystyrene for assessing cell survival and morphology after transplantation. Also, prior to transplantation, a subpopulation of cells was collected for *in vitro* RT-PCR and immunocytologic analyses.

4.5.4 Transplantation routes

4.5.4.1 Intravenous

In **study IV,** single cell suspension of neural progenitor cells, 1×10^6 in 500 µl saline, was injected into the femoral vein of three rats. The rats were lightly anesthetized and the fur at the injection site was shaved before making a small incision at the transplantation site. Post-injection bleeding was prevented by applying pressure to the site and one suture was used to close the incision. Lidocaine (2 %) was used for postoperative pain relief.

4.5.4.2 Intra-arterial

In **study IV,** rats were anesthetized and the fur on the neck area of the animals was shaved. The ipsilateral common carotid artery was revealed and an injection (1×10^6 of neural progenitor cells in 500 µl saline) was made into the external carotid artery. The injection site was electrocoagulated and the skin was sutured. Lidocaine (2 %) was used for postoperative pain relief.

4.5.4.3 Intracerebral

Neural progenitor cells were transplanted intracerebrally into the striatum in **study IV** or into the cortex in **study V**. The coordinates were selected based on a rat brain atlas (Paxinos and Watson 1986). In **study IV,** the coordinates were anterior-posterior (AP) +1.0, mediolateral (ML) –3.0, and dorsoventral (DV) –5.0 mm, and in **study V** i) AP +0.5, ML +1.0, DV –2.0 and –2.5, ii) AP +1.2, ML +1.0, DV –2.0 and –2.5 mm. In **study IV,** a Hamilton syringe was used for transplantations, and in **study V,** a thin glass cannula was attached to the Hamilton's syringe and cells (altogether 800 000 cells in 4 µl/animal) were transplanted through the cannula. Temgesic (0.03 mg/ml) was used for postoperative pain relief.

4.5.5 Single photon emission computed tomography

Single photon emission computed tomography (SPECT) imaging was performed in **study IV**. Rats were monitored under gas anesthesia using a small, rodent-designed SPECT/CT from Gamma Medica Inc. (Northridge, CA, USA) with two gamma cameras, an X-ray source, and a detector enabling imaging of the same coordinates at various time-points. Planar images produced using an imaging time of 240 s and a matrix size of 81 × 81, and 3D images (64 projections, 60 s/projection) of the upper and lower body were combined with computed tomography (CT; voltage 70 kV, imaging matrix size 1024 × 1024) to produce a clear visualization of the biodistribution of the labeled cells. Filtering (6^{th} order Butterworth filter) and interpolation to identical resolution with CT images was performed to smooth the planar SPECT images using Matlab (The MathWorks, Natick, MA, USA). *In vitro* labeled cells were also imaged. For this, the counts of the samples were collected for 600 s with both gamma cameras, the images were processed the same as the *in vivo* images. The obtained images were summed together and average values from the samples were calculated and compared to the noise level from the area of same size to define the detection limit. CT reconstruction (Exxim Computing Corporation, Pleasanton, CA) gave the final 512 x 512 x 512 matrix size with 0.17 mm pixel resolution.

4.5.6 Rehabilitation

The animals in **study V** were not rehabilitated *per se*, but half of them were housed in an enriched environment following neural progenitor cell or vehicle transplantation. The enriched environment comprised two large metal cages (61 × 46 × 46 cm) that were connected by a tunnel. The cages contained objects like ladders, toys, wooden tubes, tunnels, and shelves that engage the rats in sensorimotor-enriched activity. The objects or their locations were changed once a week. A total of 10 animals were housed together in these environments.

4.5.7 Behavioral evaluation

In **study V**, behavioral testing was performed prior to MCAO, prior to transplantation, and 1 and 2 months after the transplantation with four different tasks.

The limb placement task (De Ryck et al. 1989) was performed prior to transplantation to counterbalance the assignment of ischemic animals to the study groups based on limb placement function. The tests consisted of seven limb-placing tasks for assessing the integration of fore- and hindlimb responses to tactile and proprioceptive stimulation. In brief, MCAO rats were placed on the edge of a table nose or side-first, their fore- and hindlimbs were pushed over the edge of the table, and the time required to restore the limb was measured. The test was scored as follows: 0 = the rat did not perform normally, 1 = the rat performed with a delay of more than 2 s, and 2 = the rat performed normally. The final score was the sum of the subtests, the maximum being 14 points.

The forelimb asymmetry test i.e. cylinder test was used to evaluate forepaw use for postural support. Briefly, the rat was placed in a Plexiglas cylinder for at least 4 minutes or until 20 rearings were observed. The session was video-recorded from below and ipsilateral, contralateral, and bilateral forepaw use was evaluated (Woodlee et al. 2005).

The beam walking task was performed to evaluate the fore- and hindlimb use of the animals when walking on a tapered beam. Each animal performed three trials at each test session and each trial was video-recorded. The total numbers of steps taken and the missed steps were counted, and the percentage of successful steps was calculated.

Montoya's reaching task was used to evaluate more specific recovery of forepaw use. The animals were food-deprived for 24 h prior testing. In the test, the animals were placed into a staircase apparatus with 21 food pellets (45 mg, BioServ, Frenchtown, NJ) placed on 7 descending stairs (3 pellets/stair) on the left and right side of the animal. The pellets were accessible with only the ipsilateral forepaw and the dropped pellets could not be retrieved. Prior to MCAO, the animals were trained to pick up at least 15 pellets on both sides.

4.5.8 Immunohistochemical staining

After the follow-up time in **studies IV** and **V** the animals were deeply anesthetized by intraperitoneal injection of a mixture of sodium pentobarbital (9.72 mg/ml) and chloral hydrate (10 mg/ml; 2 ml/kg). The rats were transcardially perfused with saline (0.9 % NaCl) and ice cold 4 % PFA. Post-fixation was performed with 4 % PFA for 90 minutes after which the tissue was kept in 20 % or 30 % sucrose in PBS for 3 additional days. The brains were then frozen in dry ice, and cut into 30- (**study V**) or 35- (**study IV**) μm tissue sections with a cryostat (CM3050S, Leica, Germany) and stored in a cryoprotectant tissue collection solution in a freezer (-20

°C) for immunohistochemical staining. In **study V**, every 8th section was collected for Nissl staining to evaluate the infarct size.

For immunohistochemistry with fluorescence markers the sections were washed with PBS and nonspecific labeling was blocked with 5 % goat or rabbit normal serum and 0.25 % Triton X-100 in PBS for 1 h at room temperature. The primary antibodies were diluted in a solution of Triton X-100 and PBS and incubated with the tissue sections overnight at +4°C. The next day, the sections were washed with PBS, incubated with secondary fluorescent antibody solution (in PBS) for 2 h at room temperature in the dark, washed with PBS, and mounted on slides and coverslipped. The primary antibodies for fluorescence staining were anti-human nuclei (1:1000, Chemicon) for transplanted cells; nestin (1:200, Chemicon) for neural progenitor cells; DCX (1:200, Santa Cruz Biotechnologies), MAP-2 (1:200, Chemicon), and neurofilament 200 (1:100, Chemicon) for neuronal cells; GFAP (1:500, DakoCytomation, Glostrup, Denmark), NG2 (1:150, Chemicon), and S100 (1:50, Sigma-Aldrich) for glial cells; and CD68 (ED-1, 1:500, Chemicon) for macrophages. Secondary antibodies used were AlexaFluor-488 or -633 conjugated to the appropriate secondary antibodies (1:400, anti-rabbit, anti-goat, or anti-mouse), all purchased from Molecular Probes. For light microscopy and stereologic cell counting (human nuclei specific cells) the secondary antibody was biotinylated anti-mouse antibody (1:500, Jackson ImmunoResearch, West Grove, PA) which was coupled with streptavidin-horseradish peroxidase. This was reacted with 3,3'-Diaminobenzidine (DAB).

4.6 Statistics

In **study I,** cell proliferation and Cell-IQ data were analyzed with nonparametric Kruskal-Wallis test followed by a Mann-Whitney U-test (post hoc test). In **study II,** the Mann-Whitney U-test was used to compare two groups. In **study V,** the behavioral data were analyzed by repeated-measures analysis of variance, followed by Scheffe's post hoc test. Cell survival and phenotype were analyzed using an unpaired t-test. A p value of less than 0.05 was considered significant. In the case of multiple comparisons, Bonferroni's correction was performed. All tests were performed with the SPSS (versions 14.0 to 17.0, SPSS Inc., Chicago, IL, USA) statistical software package.

5. Results

5.1 Differentiation of neural progenitor and neuronal cells from human embryonic stem cells

Human embryonic stem cells were differentiated into neural progenitor and neuronal cells using two methods. Differentiation in an adherent culture system resulting in rosette-structures was first used, but there was a large amount of variation in the efficacy using this method. The attachment of the cell clusters to CellBIND plates varied from 20 % to 100 %. Further, 10 % to 100 % of the attached clusters formed rosette-structures, i.e., many of the attached cell clusters grew merely flat epithelial-like cells and never produced neural progenitor cells. In **study IV,** the neural progenitor cell-containing rosette structures (as demonstrated in Figure 1 in **study IV**) were used and the number of rosettes collected was sufficient (72 rosette areas). The method, however, was not efficient or sufficiently reliable for larger scale cell production.

The suspension culture method was modified from a previous method (Nat et al. 2007). The neural differentiation method was designed to keep it as simple as possible. In contrast to the several different media with various supplements used by Nat and collaborators, in **study I** we used only one differentiation medium (composed of two media) including bFGF for differentiation, which was found to be sufficient. In addition, the number of supplements used was kept low (including B27, N2, and GlutaMax) and no transition stages were used. With this relatively simple system, neural progenitors, as well as neuronal and glial cells could be produced after 3 to 20 weeks of suspension culture. The neurospheres and the produced cells expressed the neural progenitor markers throughout the differentiation period, neuronal markers were detected at earlier time-points (3 to 12 weeks), and glial markers at later time-points (15 to 30 weeks). The differentiation followed the developmental process; first neurons then glia.

The only growth factor used in **study I** was bFGF, with various concentrations (see section 4.1.2). With 20 ng/ml bFGF, hESC line-derived neurospheres proliferated similarly (i.e., a decrease in proliferation over time). Altering the bFGF concentrations did not significantly influence the proliferation of neurospheres. The neural differentiation of the hESC lines was, however, affected by bFGF in a concentration-dependent manner. The main goal of the study was to produce pure populations of young neuronal cells. After differentiating the cells in neurospheres for 6 to 8 weeks with constant 20 ng/ml bFGF and then plating the aggregates on laminin-coated polystyrene, 80-100 % of the differentiated cells morphologically resembled neuronal cells, which was verified by immunostaining. It was, however, observed that sometimes the differentiated neurospheres produced pure populations of neuronal cells after 3 weeks and sometimes it took more than 9 weeks, suggesting

that there may be hESC-line dependent variation in neural differentiation capacity. Thus, the neural differentiation potential of hESC lines, derived at the Karolinska Institute or at Regea, was more closely analyzed with methods such as RT-PCR, time-lapse imaging, and immunocytological staining. The results clearly demonstrated that the neural differentiation potential of hESC lines, even though derived at the same laboratory with standard methods, varied. In **study I,** with constant 20 ng/ml bFGF HS181- and HS360-derived neurospheres formed neural progenitor and neuronal cells after 6 weeks of differentiation, whereas HS362- and HS401-derived neurospheres differentiated significantly slower as represented in Figure 4A. The same phenomenon was observed with the Regea hESC lines; Regea 08/023-derived neurospheres were the most effective in differentiating into neural progenitor and neuronal cells while Regea 07/046-derived neurospheres were the most ineffective (unpublished results).With 4 ng/ml bFGF the neurospheres differentiated generally slower than with 20 ng/ml bFGF (Figure 6 in **study I** vs. Supplemental figure 1 in **study I**). If bFGF was withdrawn, three of the hESC lines differentiated faster and into more pure neuronal populations, but one hESC line did not show any response. This was quantified by time-lapse imaging and analysis. Briefly, when bFGF was withdrawn on 2nd week, HS181- and HS360-derived neurospheres produced significantly more neuronal cells already on 3rd week. HS362-derived neurospheres showed a delayed response and produced more neuronal cells on 6th week whereas HS401-derived neurospheres did not show any changes on differentiation regardless of the bFGF condition (Figure 4B).

Figure 4 shows the differentiation of hESC lines in relation to bFGF concentration. Figure 5 shows the general summary of the differentiation protocol and results in relation to the HS360- and HS362-derived neurospheres.

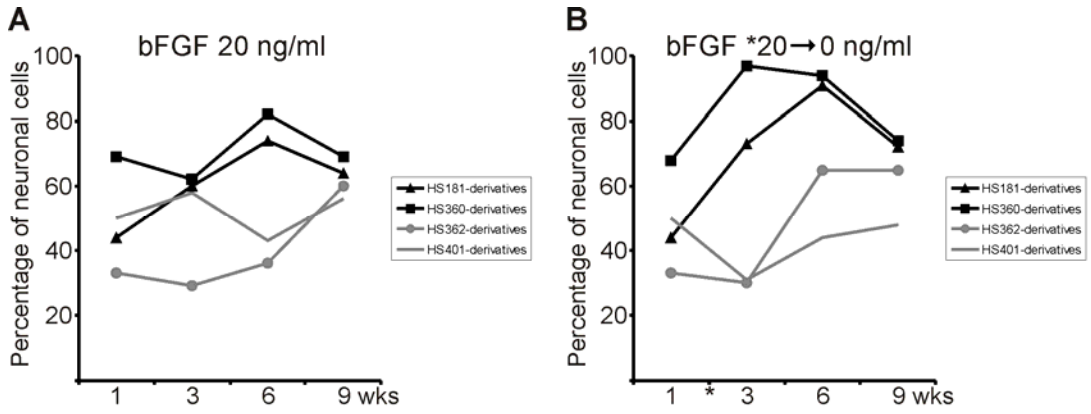

Figure 4. Differentiation of four hESC lines into neuronal cells in relation to bFGF concentration. A) HS181- and HS360-derived neurospheres produced ~80 % of neuronal cells after 6 weeks of differentiation with constant 20 ng/ml bFGF. HS362- and HS401-derived neurospheres differentiated slower and produced < 70 % neuronal cells after 9 weeks of differentiation. B) If bFGF was withdrawn on 2nd week (*), HS360-derived neurospheres produced nearly 100 % of neuronal cells on 3rd week. HS181-derived neurospheres purified towards 6th week. HS362-derived neurospheres showed delayed reaction to bFGF withdrawal, whereas HS401-derived neurospheres did not react at all.

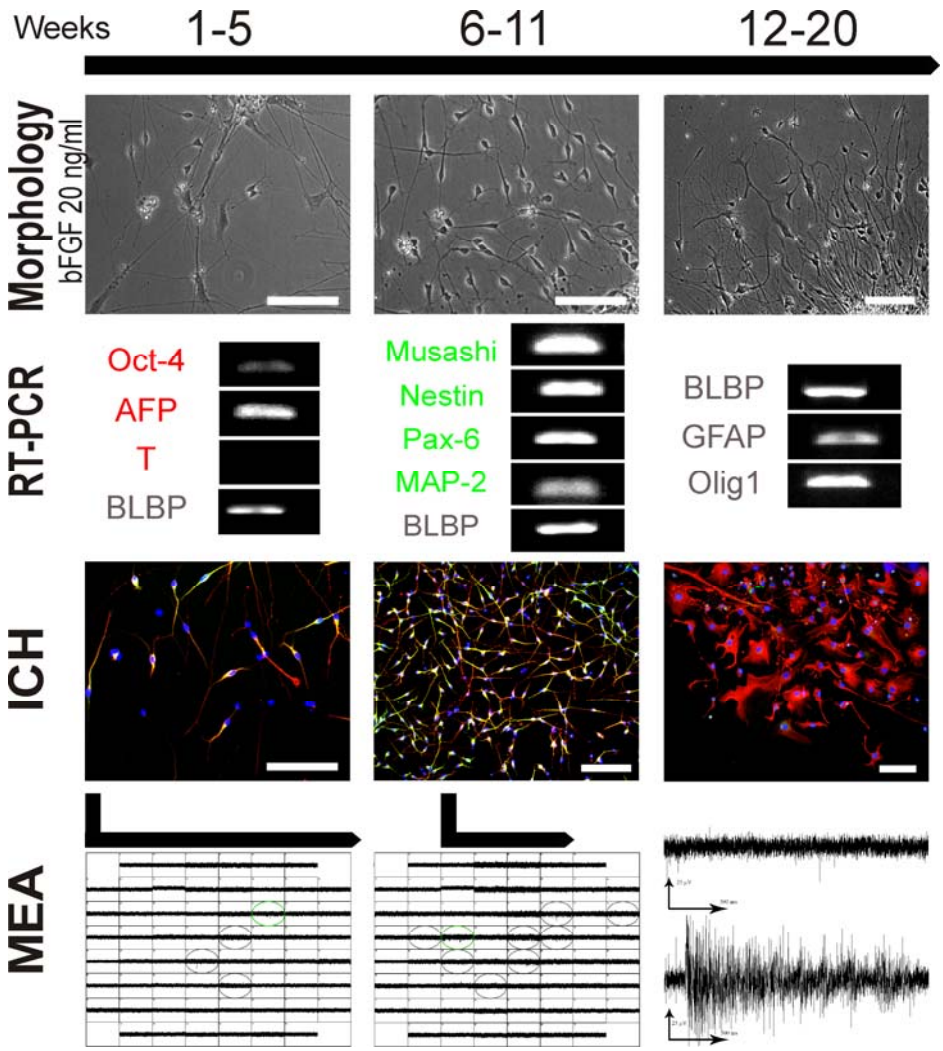

Figure 5. Neural differentiation of hESCs. Morphology: in constant 20 ng/ml bFGF, in 1-5 weeks the HS360-derived neurospheres produced neuronal and flat epithelial-like cells. At 6-11 weeks mostly neuronal cells were produced, and at 12-20 weeks production of astrocytes along with neuronal cells occurred. RT-PCR: At weeks 1 and 3 pluripotency gene Oct-4 and endodermal genes α-fetoprotein (AFP) but not mesodermal gene Brachyury (T) were expressed in HS360-derived neurospheres. The expression of these genes disappeared toward weeks 6 and 9 and the spheres expressed several neural progenitor (Musashi, Nestin, Pax-6) and neuronal (MAP-2) genes. Glial genes were expressed during weeks 12 and 15. ICH: Immunocytochemistry verified the presence of neuronal and astrocytic cells at different time-points. Blue = nuclear marker DAPI, green = MAP-2, red = β-tubulin$_3$ (1-5 and 6-11 weeks) or GFAP (12-20 weeks). MEA: The young 2-week-old HS362-derived neurospheres (not fully differentiated) produced electrically active networks 5 weeks after cell plating. More activity was observed from 9-week-old HS362-derived neurospheres (fully differentiated) 2 weeks after cell plating. Examples of a silent MEA channel and a bursting channel. Scale 100 µm in all figures.

5.2 Optimal surface matrix for neuronal cells

Many substances were tested as coating material on a polystyrene surface for neuronal cell attachment, such as collagen I-IV, fibronectin, laminin, poly-L-lysine, and vitronectin (Jansson, unpublished results). The prominent surface component

was ECM protein laminin with the attachment efficacy of ~100 % for both neuronal and glial cells (astrocytes) if the laminin lot was of good-quality. Non-neuronal cells also attached, grew, and proliferated on laminin as efficiently as neuronal cells. The replated neural progenitor cell population thereby needed to be rather pure from the beginning. The approach of coating the polystyrene wells with neuron-specific NCAM antibodies was tested. The specific non-ionic hydrophilic polymer pTHMMAA was used together with NCAM antibodies. The pTHMMAA polymer alone on polystyrene did not enhance neuronal cell attachment, as only a few non-neuronal cells were attached to these surfaces (Figure 2 in **study II**). If NCAM antibodies were added at concentrations of 25 µg/ml or 50 µg/ml the effect was remarkable: only neuronal cells positive for MAP-2 attached to the plate and no other cell types were observed (Figure 2 in **study II**). Further, 50 µg/ml NCAM antibody significantly ($p<0.05$) increased the number of attached neuronal cells compared to 25 µg/ml NCAM antibody. If more NCAM antibody was physisorbed to the surfaces (75 or 100 µg/ml) non-neuronal cells were detected along with the neuronal cells due to steric hindrance, thus 50 µg/ml was the optimal concentration for NCAM antibody coating. During the 8 days follow-up, no excessive cell proliferation or neurite extension was observed. Figure 6 shows the cells attached to control wells and the cells attached to wells with pTHMMAA polymer and 50 µg/ml NCAM antibodies.

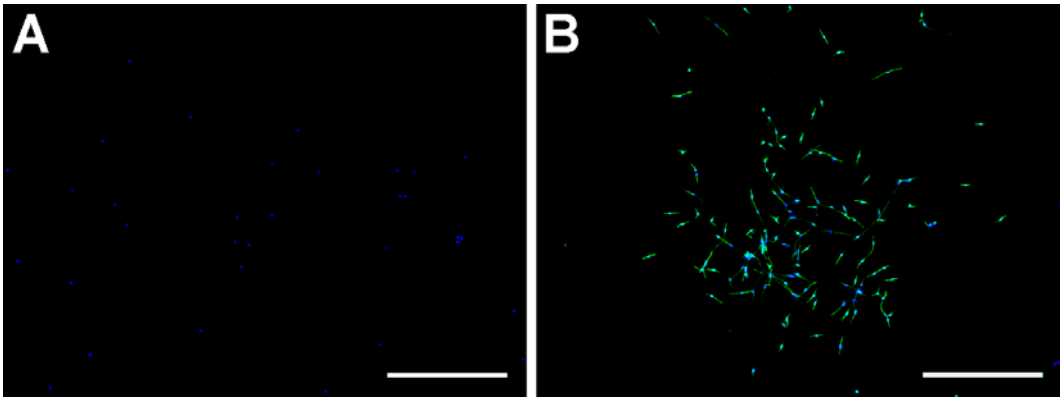

Figure 6. Neurospheres plated on NCAM-surfaces. A) In wells coated with 0 µg/ml NCAM and 200 µg/ml pTHMMAA polymer (control) some cells negative for neuronal marker MAP-2 attached. B) Wells coated with 50 µg/ml NCAM and 200 µg/ml pTHMMAA polymer contained only MAP-2 positive neuronal cells. Blue = nuclear marker DAPI, green = MAP-2. Scale 500 µm.

5.3 The electrophysiologic functionality of the produced neuronal cells

Electrical properties and signaling is a key function of neuronal cells. In **studies I** and **III** these properties of the developing neuronal networks were studied using a refined MEA system. In **study III** it became clear that the produced neuronal cells formed neuronal networks that were spontaneously active and exhibited several electrical firing patterns. The maturation of the networks could be divided into three stages based on the signals detected. During the first week on the MEA, the

networks exhibited random single spike activity (stage 1). During the second and third weeks, the networks produced spike trains (stage 2). After the fourth week, the network produced mostly synchronous bursts (stage 3). These results are presented in Figure 2 in **study III**. In addition, the networks responded as expected to neuropharmacologic substances such as D-AP5 and CNQX (NMDA and AMPA/kainate antagonists, respectively) as well as to GABA and GABA antagonist bicuculline, as presented in Figure 4 in **study III**. Thus, the produced neuronal cells clearly formed functional neuronal networks.

The network's electrical properties were studied in more detail to evaluate if all the hESC lines were capable of producing functional neurons (**study I)**. The hESC lines HS181 and HS362 were differentiated in neurospheres for 2 or 9 weeks and then replated on MEA dishes in parallel to assess their possible differences in forming functional neuronal networks. Neural derivatives of both hESC lines formed spontaneously active networks, regardless of the differentiation time. The neuronal networks that formed from 2-weeks-old neurospheres matured remarkably slower than neuronal networks formed from 9-weeks-old neurospheres. The latter networks exhibited spike trains and bursts after 2 weeks on MEA, whereas the former required 5 weeks to develop the same activity level (Figure 5).

5.4 Accumulation of the neural progenitor cells into the damaged cerebral tissue

In regenerative medicine, the ultimate aim is to deliver cells to the site of injury where regeneration is needed. For this purpose, the most optimal transplantation route for stroke was evaluated in **study IV**. Transient MCAO, which results in severe cortical and striatal injury, was induced in the rats. The neural progenitor cells were labeled with ^{111}indium-oxine, which is nontoxic to the cells, and transplanted either into the femoral vein or carotid artery, or intracerebrally into the brain. SPECT imaging clearly showed that after intravascular transplantation the labeled cells immediately accumulated in the internal organs of the rats and remained there for at least 24 h (Figure 7). Organ-specific analyses verified that most of the cells were trapped in the liver and to some extent in the kidneys and the spleen. A faint signal was detected in the head of the rats after carotid artery transplantation, but no human cells were detected in the brain using immunohistochemical analysis. Thus, intravascular transplantation did not seem effective for delivering cells to the damaged brain area.

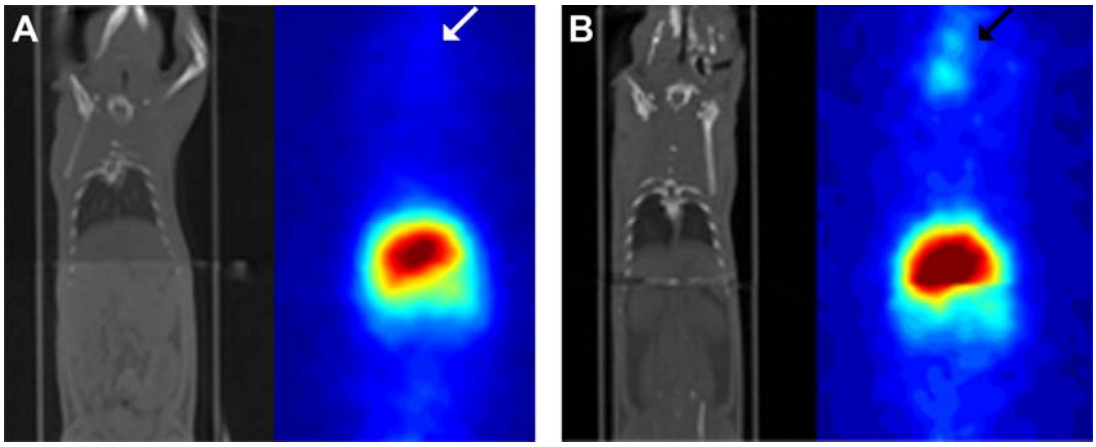

Figure 7. ^{111}In-oxine labeled hESC-derived neural progenitor cells 24 h post-transplantation in stroke rats. A) Nearly all cells accumulated into the internal organs (mainly liver) after intravenous delivery. No signal was detected from the head area (white arrow). B) After intra-arterial delivery a faint signal was detected from the head area of the rats (black arrow) whereas the rest of the cells again accumulated into the internal organs. Figure modified from Lappalainen et al. 2008.

5.5 Neural progenitor cell transplants together with an enriched housing environment

In **study V,** neural progenitor cells were transplanted stereotactically into the brain of rats with permanent MCAO. A subpopulation of the spheres was collected for RT-PCR analysis and *in vitro* differentiation. RT-PCR showed that the cells expressed several neural progenitor markers without the expression of pluripotent or endo- or mesodermal markers, as demonstrated in Figure 2 in **study V**. *In vitro* differentiation resulted in β-tubulin$_{III}$, MAP-2, and DCX positive neuronal cells (Figure 2 in **study V**). No glial differentiation was observed. After transplantation, the animals were housed either alone in standard cages or in groups in an enriched environment. The animals were followed for 2 months during which their behavioral recovery was evaluated once a month based on the beam walking test, cylinder test, and Montoya's reaching task. Unfortunately, because the minipumps used for Cyclosporin A delivery impaired the ability of the rats to walk, the beam walking test was unsuccessful and therefore excluded from the analysis. Further, in animals transplanted with cells, no significant effect was observed in Montoya's reaching task. In the cylinder test, however, animals transplanted with cells recovered significantly faster during the first month compared to vehicle-injected animals (Figure 4 in **study V**). The same effect was not observed during the second month. Immunohistologic analysis confirmed that there was no significant tissue replacement and a minimal number of surviving human cells 2 months after the transplantation. The cells that survived mostly remained nestin positive neural progenitors and approximately 10 % of them were positive for neuronal marker MAP-2 (Figures 6 and 7 in **study V**). The number of glial cells was low. Figure 8 represents the study and the results of behavioral tests.

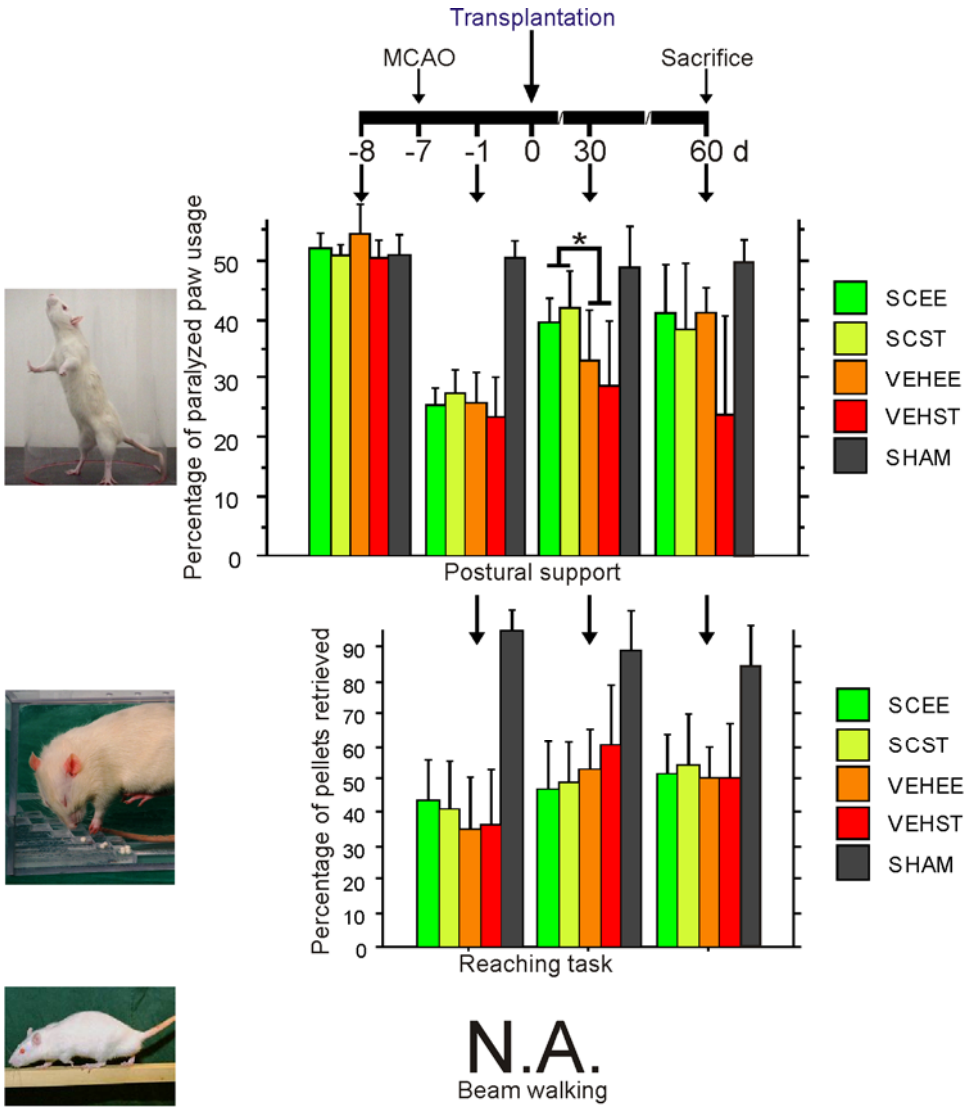

Figure 8. The outline and behavioral results of **study V**. Transplantation of hESC-derived neural progenitor cells was carried out 7 days after MCAO. The animals were divided in stem cell (SC) and vehicle (VEH) groups and further into enriched environment (EE) and standard housing (ST). Regardless of the housing, postural support i.e. the cylinder task showed a significant improved of SC animals at the 1 month time-point if compared to VEH animals. Otherwise significant differences were not detected in cylinder or reaching task i.e. Montoya. Beam walking was not analyzed, N.A. Data is presented as means and standard deviations.

5.6 USPIO-labeled neuronal cells and magnetic resonance imaging

USPIO (at the concentration of 200 µg/ml) successfully labeled 80-100 % of neuronal cells if poly-L-lysine (375 ng/ml) was used as the transfection agent. Prussian blue staining *in vitro* verified that the iron particles were located in the cells (Figure 9A and B). No changes were detected in cell survival, morphology, or activity with visual inspection if compared to control cells. If these cells were transplanted into intact rats or rats with experimentally induced stroke (200 000

cells/2 µl PBS, AP -0.2, ML +2.0, and DV -3.0 mm) the cells could be visualized by magnetic resonance imaging (9.4T; unpublished results). Figure 9C shows a representative MR image of iron-containing hESC-derived neuronal cells *in vivo*.

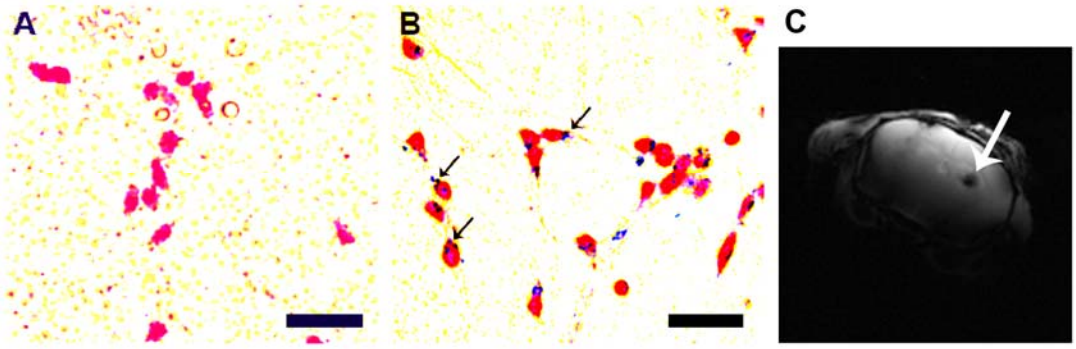

Figure 9. USPIO-labeled hESC-derived neuronal cells. A) Prussian blue stained unlabeled neuronal cells (control). B) Prussian blue stained neuronal cells labeled for 24 h with 200 µg/ml USPIO and 375 ng/ml poly-L-lysine. Iron particles pointed with black arrows. Red = nuclear fast red staining. C) Representative Gradient Echo Multi-slice (GEMS) image of iron-containing hESC-derived neuronal cells *in vivo*. Signal from the cells pointed with white arrow. GEMS picture taken by PhD Anu Muona, A.I.Virtanen Institute, University of Kuopio.

6. Discussion

In this thesis, we developed and tested a simple and efficient neural differentiation protocol for hESCs, and evaluated the electrical properties of produced neuronal networks. NCAM was tested as a neuron-specific cell culture surface matrix. In addition, the neural progenitor cells were tested in animal models of stroke to evaluate the optimal transplantation route and the effects of cells and enriched housing on functional recovery.

6.1 Methodologic consideration

Many methods were used in the experiments performed for this thesis, some of which were newly invented (e.g. NCAM antibodies and pTHMMAA coating) and others that are commonly used in many laboratories (e.g., stroke models). For cell culturing, the derivation and maintenance of undifferentiated hESC lines were performed using previously published methods and commonly used protocols (Hovatta et al. 2003, Inzunza et al. 2005, Skottman et al. 2006, Rajala et al. 2007, Skottman et al. 2007). The karyotypes of the cells were periodically tested and determined to be unchanged. Thus, the cells used in the experiments presented in this thesis were considered normal. The neural differentiation protocol, however, was modified from previous work (Nat et al. 2007) in **study I**. While Nat and co-workers used three medium compositions for their differentiation protocol, in this thesis only one differentiation medium composition (described in section 4.1.2) was used. In addition, the medium consisted of a rather minimal amount of supplements and no extra factors such as RA or noggin were used. Differentiating hESCs in suspension eliminates the need for pre-coated plates, which can be quite expensive due to the coating material (e.g. human laminin). On the other hand, the weekly manual passaging of the spheres is laborious, thus automatic systems for this procedure would be needed. Our method can be developed into a GMP-compliant procedure.

Coating the wells with NCAM antibodies along with the pTHMMAA polymer in **study II** was a novel, successful approach. The attachment of solely neuronal cells was clearly detectable with a microscope and immunocytologic staining. Proliferation and extension of the cells, however, was not detected in an 8 days follow-up period. It would have been useful to extend the follow-up time and stain the cells with other neuron subtype-specific markers to assess whether the cells were maturing on the NCAM-pTHMMAA surface. Also, a layer of gold beneath the antibodies and polymer would have improved the orientation of NCAM antibodies. These issues have been discussed and the experiments will be conducted in the future.

The SPECT/CT imaging study was conducted with hESC-derived neural progenitor cells or with rat neural stem cells in a transient ischemia model. Transient ischemia was used to induce a severe and inflammatory injury that would send signals to guide the cells to the injury site. The cells were transplanted one day after the induction of the ischemia. The follow-up time was 24 h, during which the cells were detected in a similar manner in internal organs regardless of the cell origin or the time-point studied. The follow-up time could have been extended to determine if the cells would eventually migrate into the cerebral tissue, but we hypothesized that once the cells were trapped in the liver, they would not be released into the blood stream again. No immunosuppressants were used in this study due to the relatively short follow-up time and the severity of the injury, which makes the animals quite sick. The need for immunosuppressants is an open question, but in this setup, with the short follow-up time, we assumed it would not influence the results.

Study V was conducted in collaboration with a researcher specializing in stroke models at Kuopio University. Regardless of the extensive planning, however, some problems occurred. The cells were transferred to Kuopio in neurospheres to keep them viable. A subpopulation of all the transplantable cell batches were inspected with trypan blue staining prior to transplantation, and this suggested over 90 % viability with each batch. Also, some of the remaining cells were replated on polystyrene after the transplantation to visually inspect the survival and morphology of the cells. We did not use any exclusion criteria for cells due to the constant high graft viability. This aspect is important to study since it has been reported that transplanting dead cells increases the lesion volume and behavioral deficits (Modo et al. 2003). At the end of the transplantation period, the neurospheres underwent a significant and unexplained change as a single cell suspension could not be produced due to strong cell-to-cell attachments. A new batch of neurospheres was transferred from Tampere to Kuopio to complete the transplantations. RT-PCR and immunocytologic analysis of subpopulations of both cell batches indicated that they were similar. The wellbeing of the animals was assessed and followed daily by the trained animal facility staff and researchers. The immunosuppressant Cyclosporin A was delivered by subcutaneous implantation of Alzet minipumps to avoid repeated and stressful injections of rats during 2 month follow-up. Accumulation of intestinal fluid in the implantation cavity together with rather large physical size of the pump, however, seemed to affect the beam walking behavioral performance of the rats. Thus, delivery of immunosuppressant should be carefully considered depending on the outcome measures applied (i.e. histology vs. behavior).

6.2 Neural differentiation of human embryonic stem cells *in vitro*

ESCs have been differentiated into neural progenitor and specific neuronal types of cells by many methods. The base media for neural cells or their differentiation are fairly similar, containing commercial neural cell mediums, e.g., Neurobasal and DMEM/F:12 (Gibco Invitrogen) as well as neural supplements B27 and/or N2 (Gibco Invitrogen) or components of those supplements (Carpenter et al. 2001, Reubinoff et al. 2001, Zhang et al. 2001, Bibel et al. 2004, Perrier et al. 2004,

Schulz et al. 2004, Gerrard et al. 2005, Itsykson et al. 2005, Benzing et al. 2006, Ferrari et al. 2006, Sonntag et al. 2006, Baharvand et al. 2007, Darsalia et al. 2007, Lee et al. 2007, Lee et al. 2007, Nat et al. 2007, Pankratz et al. 2007, Shim et al. 2007, Erceg et al. 2008, Hong et al. 2008, Li et al. 2008, Metallo et al. 2008, Nelson et al. 2008, Tavakoli et al. 2009). In addition, bFGF is commonly used in these protocols, and also in our **study I** (Lappalainen et al. submitted). Additionally, many other reagents have been used to improve differentiation, such as noggin (a BMP-signal blocker), which prevents differentiation into epidermal structures (Gerrard et al. 2005, Itsykson et al. 2005, Baharvand et al. 2007). On the other hand, RA has been used to initiate or improve neural differentiation (Li et al. 2008, Chatzi et al. 2009). The neural differentiation protocol used in **study I** did not contain any of these substances (Lappalainen et al. submitted). Noggin was tested during the development of the protocol (Salomäki 2009) and was determined to decrease cystic structure formation, as reported earlier (Itsykson et al. 2005), but no other clear benefits were observed. As cystic structures were only detected in some hESC lines, it seemed more efficient to select other hESC lines and remove the expensive noggin from the differentiation protocol. RA is a challenging substance because the appropriate exposure time and the most efficient concentration are not known, even though they have been widely tested with mouse ESC differentiation (Chatzi et al. 2009). In addition, RA is very unstable and requires a daily medium change, which also makes the work more laborious. Further, in some studies, RA has been used to produce specific kinds of neurons (Chatzi et al. 2009) or to guide the neural progenitor cells into caudal phenotypes (Li et al. 2008, Erceg et al. 2009), which was not the purpose in **study I**. The differentiation protocol introduced in **study I** was assessed to be efficient and relatively simple to produce large amounts of neural progenitor cells and further neuronal cells (Lappalainen et al. submitted) that are potentially capable of further specification into, e.g., glutaminergic and GABAergic cells. In addition, it is noteworthy that we did not produce EBs in the beginning of differentiation as hESC aggregates were directly moved to NDM. It does not seem useful to guide cells into forming all three germ layers if only one of them is eventually desired.

BFGF was the only growth factor included in our neural differentiation medium (Lappalainen et al. submitted). We tested the bFGF concentrations of 20, 20→0, or 4 ng/ml and did not see any differences on cell proliferation as the proliferation of neurospheres gradually decreased in time regardless of the bFGF concentration. Itsykson and co-workers, on the contrary, reported that neural progenitor cells cultured with bFGF were mitotically more active than cells without bFGF (Itsykson et al. 2005). In relation to differentiation, however, the withdrawal of bFGF induced three of the hESC lines to differentiate more efficiently compared to constant 20 ng/ml bFGF in **study I** (Lappalainen et al. submitted), whereas Itsykson and co-workers reported similar percentages of neural progenitor cells with or without bFGF (Itsykson et al. 2005). The endogenous FGF expression in each hESC line might explain these differences. In general, bFGF is used in neural differentiation protocols in concentrations ranging from 8 ng/ml (Erceg et al. 2008) and 10 ng/ml (Benzing et al. 2006, Carpenter et al. 2001) to 20 (Gerrard et al. 2005, Itsykson et al. 2005, Lappalainen et al. submitted) or 25 ng/ml (Baharvand et al. 2007).

In **study I,** we detected that while some hESC lines produced nearly pure neuronal cell populations within 3 to 6 weeks, for others it took a longer time and the

populations always included non-neuronal cells along with the neuronal cells (Lappalainen et al. submitted). Indeed, there was a clear variation in the differentiation potential of these lines as HS181 and HS360 were superior in differentiating into neural progenitor and neuronal cells while HS362 was significantly slower and HS401 was the poorest line for producing neural progenitor and neuronal cells as described in **study I**. The same phenomenon was observed with Regea lines (unpublished data) and previously with human neural stem cells (Johansson et al. 2008). This notion is crucial in relation to clinical applications. To date, many neural differentiation protocols have been published that evaluated only the differentiation of the first lines H1 and/or H9 (Daadi et al. 2008, Erceg et al. 2008) or just two hESC lines (Itsykson et al. 2005). In addition, some studies report no differences between the hESC lines used. For example, Shin and co-workers reported similar behaviour of BG01 and BG02 and pooled the data in their differentiation study (Shin et al. 2005), and Nat and co-workers reported six hESC lines that differentiated into neural progenitor cells in a similar manner (Nat et al. 2007). In contrast, a couple of articles describing variations between the differentiation capacities of hESC lines were recently published. Wu and co-workers compared neuronal differentiation in two similarly derived hESC lines and concluded that there was a clear difference between the lines (Wu et al. 2007). Very recently Tavakoli and co-workers came to the same conclusion (Tavakoli et al. 2009). Our protocol is a simplified version of the protocol published by Nat and co-workers (2007) and we detected clear differences in the differentiation capacity of the hESC lines. It is important to note that while Nat and co-workers performed the differentiation for only 42 days, i.e., 6 weeks, we extended the differentiation time to 20 weeks and detected differences mostly between 6 to 12 weeks. Thus, long-term cell culture studies are crucial and this difference may explain the differences in our findings.

Electrical functionality of neurons is essential for the cells and for regenerative medicine applications. Illes and co-workers studied the electrical properties of mouse ESC-derived neural progenitor and neuronal cells at the network level (Illes et al. 2007, Illes et al. 2009). In **study III,** the functional properties of human neuronal networks were studied using hESC-derived neural progenitor and neuronal cells differentiated with the protocol developed in **study I**. The networks first exhibited spontaneous activity with single sporadic spikes, which developed further into more complex signaling of spike trains and bursts, as described with mouse ESC neural derivatives (Illes et al. 2007, Heikkilä et al. 2009). In addition, at the last stage, the electrical activity resembled signals reported from rat embryonic neural cell-derived neuronal networks (Wagenaar et al. 2006). Thus, it is clear that the cells produced using our differentiation method were functional and capable of forming electrically active networks *in vitro*.

Temporal variations in the formation of functional neuronal networks between the human and mouse cell types were observed and might be due to the cell density and plating method (Illes et al. 2007, Heikkilä et al. 2009). Illes and co-workers plated mouse ESC-derived neuronal cells on MEAs as a single cell suspension (Illes et al 2007) but in **study III** we plated the neural progenitor cells as small cell aggregates and neuronal cells migrating from these aggregates formed the functional networks (Heikkilä et al. 2009). We also evaluated plating a single cell suspension on MEAs, which produces functional networks but at a slower rate than that using the

aggregate approach. If the number of cells is too low (less than 70 000 cells/cm^2, Kreutzer et al. submitted), no functional networks are formed. These cells need to contact each other upon plating. Recently, Illes and co-workers published another article on functional neuronal networks from mouse ESC-derived neural populations (Illes et al. 2009). They described that the formation of functional neuronal networks was dependent on the differentiation protocol. If the mouse ESCs were differentiated in spheres to produce heterogeneous neural cell populations, functionality could be detected at the network level, but functional networks could not be detected with cells produced by adherent differentiation. This is crucial in relation to the way the neural progenitor and neuronal cells are produced and further clarifies the advantages of the neural differentiation method presented in **study I** (Lappalainen et al. submitted). In **study I** we also tested whether a more efficient differentiating hESC line (HS181) would more efficiently produce functional networks than a medium differentiating hESC line (HS362). This is the first time this aspect has been studied. The results indicate that both lines produced functional neuronal networks. After a short differentiation period of the neurospheres (cells partly undifferentiated) electrically active networks could be produced. The networks derived from properly differentiated neurospheres (all cells differentiated), however, developed faster and exhibited stronger signals.

6.3 Proper surface for neuronal cell culturing

For neural progenitor and neuronal cell culture, developmental studies, drug screening platforms, and regenerative medicine, a proper surface matrix for cell attachment, growth, and maturation is essential. Currently, many ECM proteins and their peptides are used, such as collagen I-IV, fibronectin, laminin, poly-L-lysine, poly-L-ornithine, and vitronectin (Cooke et al. 2009). The challenge with these proteins is that many of them are produced from human tissues (e.g., laminin from human placenta). This creates variation and instability between the produced lots and thus problems with attachment and growth in cell culturing platforms. Further, considering GMP production of the cells, these production issues are even more important to sort out.

Poly-L-ornithine combined with laminin is the most often used coating for neural progenitor and neuronal cells (Perrier et al. 2004, Shin et al. 2005, Roy et al. 2006, Sonntag et al. 2006, Baharvand et al. 2007, Lee et al. 2007, Pankratz et al. 2007). Poly-L-lysine is also used with laminin (Gerrard et al. 2005, Itsykson et al. 2005). One group performing experiments with hESCs on top of human feeders and chemically defined medium is using a mixture of collagen IV, vitronectin, and fibronectin (Erceg et al. 2008). In **studies I**, **II**, and **V** as well as in a previous study (Sundberg et al. 2009) only human laminin was used as a coating substrate (Lappalainen et al. 2008, Auer et al. 2009, Hicks et al. 2009). We tested fibronectin and collagens as well as laminin combined with poly-L-lysine or poly-DL-ornithine and evaluated cell attachment and growth (Jansson, unpublished data). Laminin was superior to all other substrates regardless of its combinations with poly-L-lysine or poly-DL-ornithine.

Laminin, however, also allows the attachment and rapid growth of non-neural cells, as do other substrates because they are not neural cell-specific substrates. Other more specific components, such as epidermal growth factor, have been tested as a surface material for neural cell attachment and culturing (Nakaji-Hirabayashi et al. 2007). We tested a novel approach of coating the polystyrene surface with neuron-specific antibodies against NCAM in collaboration with the Technical Research Centre of Finland (VTT, Finland). A specific kind of pTHMMAA polymer was used with the NCAM antibodies. This polymer makes the surface more *in vivo*-like for the cells (Vikholm-Lundin and Albers 2006). We demonstrated that only neuronal cells cultured in wells with surfaces coated with 25 or 50 µg/ml NCAM antibodies and 200 µg/ml of pTHMMAA polymer were attached without any non-neural cells which were detected in the control wells (Auer et al. 2009). Further, wells with 50 µg/ml NCAM antibodies contained significantly more MAP-2 positive neuronal cells than wells with 25 µg/ml NCAM. This study is the first to use antibodies as a neuron specific culturing surface. We did not detect excessive cell proliferation and thus we hypothesize that the created surface could be used as a maturation matrix for neuronal cells.

6.4 Optimal site of transplantation

The major issue for CNS regenerative medical applications is the method of cell delivery to the site of injury, i.e., into the brain or into the spinal cord. Intravascular transplantation would be ideal because it could be administered in normal health center settings. Direct transplantation cannot be performed without the expertise of specialized doctors and equipment and are associated with other complications.

Many cells types have been tested in transplantation studies related to CNS regeneration (Lindvall et al. 2004, Vora et al. 2006). The variation in the results in relation to cell survival and migration might be due to the diverse delivery routes used. One study reported that transplanted mouse embryonic neural cells were detected in the brain after intrastriatal, intraventricular, and intravenous transplantation, but no specific cell numbers were reported (Jin et al. 2005). We transplanted human neural progenitor cells intravenously and intra-arterially in **study IV,** but detected no human cells in the brain (Lappalainen et al. 2008). Recently, a paper reported that labeled human MSCs transplanted intravenously into rats with experimentally induced stroke were detected in the ischemic hemisphere 20 h later (Detante et al. 2009). In contrast, with human umbilical cord blood cells only approximately 1 % of the transplanted cells reached the brain parenchyma (Chen et al. 2001). This cell type accumulates mainly in the lungs and liver (Makinen et al. 2006). Thus, the cell type used might have drastic effects on the final location of the transplanted cells. In addition, the stroke model itself might affect the cells. Interestingly, we as well as Jin and co-workers both used a transient ischemia model (Jin et al. 2005, Lappalainen et al. 2008), where a large cortical and striatal injury is produced with inflammation and thus there is a strong chemotaxis to the transplanted cells. Also Chen and co-workers and Mäkinen and co-workers used the transient stroke model (Chen et al. 2001, Mäkinen et al. 2006). In our study, the cells were first transplanted via the femoral vein. The cells spread out around the body via the aorta and thus not all the cells even passed through the

brain. Therefore, we next transplanted the cells into the carotid arteries to ensure all the cells passed to the brain, but no significant differences were detected in the final location of the cells. In both cases the signals from the cells were detected in the liver, spleen, and kidneys. A faint signal was detected from the brain region of the rats 24 h after intra-arterial transplantation, but when the brains were fixed and stained there were no human cells present in the brain parenchyma. In addition, similar experiments were performed with rat neural stem cells with similar results so the origin of stem cells does not explain the results. (Lappalainen et al. 2008)

The site of delivery and the timing of the cell transplantation after injury remain important issues with no clear answers (Guzman et al. 2008a, Bacigaluppi et al. 2009). It seems clear that the variation in experimental data is due to many aspects, such as the origin of the cells or the time and place of transplantation. Additional preclinical and clinical studies must be performed with standardized cell types and methods to determine the optimal treatments for patients.

6.5 Neural cells for treating stroke

Neural cells of different origin have been used in many stroke studies. While some groups are concentrating on animal cells (Hayashi et al. 2006) or cells derived from human teratocarcinoma (Bliss et al. 2006) other groups are using hESC-derived neural progenitor cells for these studies (Daadi et al. 2008). It is clear that for clinical applications in regenerative medicine the cells should be of human origin to avoid severe graft rejection. Moreover, autologous cells might be the most appropriate cell type for patients, but many issues remain in the area of CNS treatments with patient-specific cells.

We performed one extensive study with rats with experimentally induced stroke treated with hESC-derived neural progenitor cells combined with an enriched housing environment (Hicks et al. 2009). In this study, the permanent MCAO model was used to produce fairly similar cortical injuries in all animals, which resulted in a clear deficient use of the contralateral forepaw. The cells were transplanted into the cortex and the border region of the injury and intact tissue. This is the area in which ischemic tissue is at first intact but is then devastated due to inflammation and secondary damage causing cell death even several weeks after the ischemic insult (Li et al. 1995). Our aim was to increase the survival of this penumbra area by transplanting the neural progenitor cells to that site. Analysis of the brain slices and the size of the destroyed cortical tissue revealed that we did not succeed in restoring the tissue surrounding the actual ischemic area, as the cortex sizes were similar between rats transplanted with cells or vehicle (Hicks et al. 2009). Similar studies have been performed (Bliss et al. 2006, Bühnemann et al. 2006, Shen et al. 2007, Daadi et al. 2008, Guzman et al. 2008b), but none of them reported differences in infarct size between the transplanted and vehicle-treated animals. In fact, to my knowledge only one study describes significantly smaller infarct areas in rats exercised and/or transplanted with hESC-derived neuronal precursor cells when compared to control animals (Kim et al. 2007). This issue requires further attention, especially in regard to regenerative medicine.

Even though, for the most part, tissue survival and reconstruction has not been reported, many studies report high survival of transplanted cells in brain tissue. Bliss and co-workers transplanted hNT cells into ischemic rat brains and found 31 % to 49 % cell survival (Bliss et al. 2006). Daadi and co-workers reported that 21% to 53 % of transplanted hESC-derived neural cells were found in brains 2 months after the transplantation (Daadi et al. 2008). Kelly and co-workers found that ~33 % of transplanted human fetal stem cells survived in ischemic brain 4 weeks post-transplantation (Kelly et al. 2004). In contrast, we found only approximately 1 % of the transplanted hESC-derived neural progenitor cells survived *in vivo* 2 months after transplantation (Hicks et al. 2009). In addition to our results, Bühnemann and co-workers, using mouse ESC-derived neuronal cells, reported only partial survival of cell grafts after a 12 weeks follow-up (Bühnemann et al. 2006). Also, it has been demonstrated that subventricular zone cells do not survive 1 and 2 months after transplanting them into rats with experimentally induced stroke even though enriched housing is used (Hicks et al. 2007, Hicks et al. 2008). Cyclosporin A was used as an immunosuppressant in all of the studies mentioned above, thus the variation in results cannot be explained by a pharmacological difference alone. There are large variations in cell survival in all these studies. It is important to determine why some cells survive robustly after transplantation and others do not. For example, cell counting can be performed by various methods and differences between these methods might provide some explanation for the different results. Furthermore, we did not detect any teratoma formation in the animals (Hicks et al. 2009), possibly due to low cell survival, and others also have not reported teratomas (Bliss et al. 2006, Daadi et al. 2008). Nonetheless, at least one group has reported teratoma formation in the brain of rats with experimentally induced stroke after hESC-derived neural progenitor cell transplantation (Kozubenko et al. 2009) and this is important to remember when interpreting the published results. In the future, supporting scaffolds transplanted with the cells might offer solutions to the aspects of cell survival and teratomas.

Regardless of the tissue loss and variable cell survival, improvement in functional level has been reported in nearly all published articles. We reported that transplants enhanced functional recovery in a cylinder task, but not in Montoya's reaching task, during the first month after transplantation (Hicks et al. 2009). Montoya's reaching task requires a specific fine motoric use of the forepaw, which might be attained with task-specific rehabilitation (Biernaskie and Corbett 2001, Ramanathan et al. 2006) that was not provided to animals in **study V**. The cylinder task, on the other hand, measures the overall use of the forepaws for postural support during vertical exploration (Schallert et al. 2000), and positive results have been demonstrated in other studies (Bliss et al. 2006, Daadi et al. 2008). The results with the cylinder task should not be underestimated, but because stroke can affect multiple regions of the brain and thus performance in a multitude of tasks, other tests besides the cylinder task should be routinely used. Indeed, Bliss and co-workers reported the use of four distinct behavioral tasks after cell transplantation, but showed significant improvement only in one of them (Bliss et al. 2006). Thus, the efficacy of our results are rather modest, although there are technical aspects that will eventually further improve treatment outcomes.

Based on the accumulated data, it seems that the positive results of the previous studies have for the most part been due to trophic factors produced by the

transplanted cells and not due to an actual cell replacement or regeneration. On the other hand, trophic factor production and other possible support offered by the transplanted cells to the host tissue might be sufficient. Further, from this point of view, autologous MSCs from the patient might be sufficient in stroke treatment. This notion remains to be investigated in the future.

Several studies of neural progenitor cell transplants in stroke-induced animals have been performed, but the results still represent variable and contradictory outcomes. It is thus difficult to estimate the true effect of human cell transplants in stroke. In addition, almost all of these studies have been performed in rodents whose bodies reject human cells to some extent, regardless of the immunosuppressant regimen.

6.6 The future of stem cell therapies

If effective stem cell therapies are some day offered to patients suffering from neurologic deficits and diseases, the survival of cell transplants for long period of time, the functional integration of both transplanted and host cells, as well as functional recovery and safety must first be reliably demonstrated in experimental studies. For now, these criteria have not been reliably fulfilled in any study.

Encouraging results were reported by Bühnemann and co-workers who demonstrated that rodent embryonic neural progenitor cells transplanted into rodent ischemic brain survived to some extent, differentiated further, and were able to produce action potentials (Bühnemann et al. 2006). The behavioral testing, however, was not adequate. In other studies, cell survival and functional improvement were reported without functionality data of the transplanted cells *in vitro* or *in vivo*. The STAIR report (Stroke Therapy Academic Industry Roundtable, STAIR, 1999), STEPS initiative (STEPS 2009), and other reviews (Borlongan et al. 2008, Borlongan 2009) are now demanding long-term behavioral testing from the groups reporting good survival of transplanted cells. Additionally, long-term assessment of the potential tumor risk should be rigorously conducted.

GMP should also be considered since it increases the safety of patient applications. In Europe, EU directives and regulations regarding advanced therapy medicinal products (including e.g. tissue engineering products) became operative at the end of 2008. Products manufactured on a non-routine basis and used within the same member state are further regulated on national level (e.g. Fimea in Finland).

A few clinical cell transplant experiments of treatments of patients with neurologic injuries have been reported (Kondziolka et al. 2000, Bang et al. 2005). Recently, Amariglio and co-workers reported a follow-up study of a young patient with ataxia telangiectasia who was treated with human fetal neural stem cell grafts in Russia (Amariglio et al. 2009). The preparation of the cell grafts was poorly described, and the patient developed a donor-derived tumor in the brain at the site of cell transplantation. These kinds of results are very alarming and much more stem cell research is required. ReNeuron Group plc (UK) and Geron Corporation (USA) are companies that are starting clinical studies in the near future based on pre-clinical data (Nistor et al. 2005, Stroemer et al. 2009) of stroke patients treated with

conditionally immortal human neural stem cells and spinal cord injury patients with hESC-derived oligodendrocyte precursor cells, respectively. The science world is waiting for the results with bated breath to decide the direction of future research.

7. Conclusions

In this thesis, five studies are presented regarding neural differentiation of hESCs and the electrical functionality of the produced neuronal cell-derived networks. NCAM antibodies were determined to be appropriate for neuron specific cell attachment. In addition, neural progenitor cell grafts were tested in animal models of stroke. The cells did not accumulate in the injured brain tissue following intravenous injection. Successfully transplanted cells did provide modest support in functional recovery when measured using a postural support task.

Based on these studies, the following conclusions can be drawn:

1) Human ESCs can be efficiently differentiated into neural progenitor and neuronal cells, but there is significant variation in the differentiation potential between hESC lines despite the similar derivation and culturing methods.
2) The NCAM antibody combined with a pTHMMAA polymer seems to be an appropriate neuron specific surface matrix for neuronal cell culturing.
3) The produced neuronal cells and neuronal networks exhibited sufficient electrophysiologic properties as measured using a MEA platform.
4) Intravenously transplanted neural progenitor cells do not accumulate into the ischemic brain tissue; thus, other routes should be considered.
5) Neural progenitor cells facilitate the functional improvement of rats with experimentally induced stroke in a postural support task regardless of their housing environment during the first month. Poor cell graft survival is an issue requiring more attention in future experiments.

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