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.

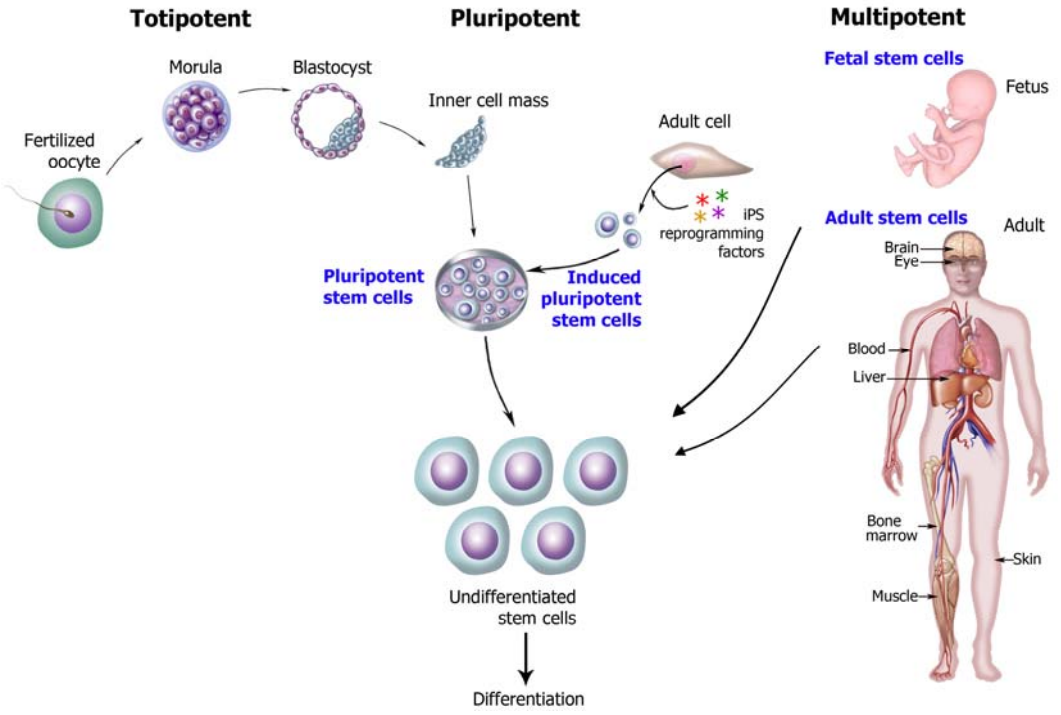

Figure 1. Stem cells. Stem cells can be divided in groups in accordance to their differentiation capacity. Embryos in zygote and morula stages are defined as totipotent. In blastocyst stage the inner cell mass is capable of producing the three germ layers and primordial germ cells, thus defined as pluripotent 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.

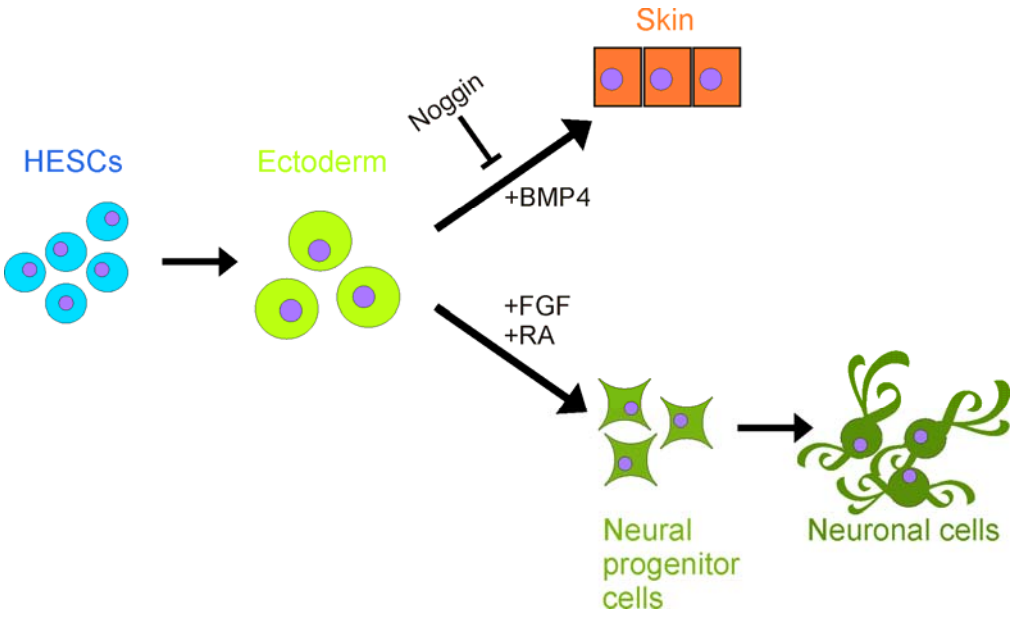

Figure 2. HESCs differentiate towards ectoderm and further into epidermal structures (skin) or into neural progenitor and neuronal cells. Bone morphogenetic protein 4 (BMP4) drives the ectodermal cells toward skin and this is blocked by noggin. Fibroblast growth factor (FGF) and retinoic acid (RA) are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

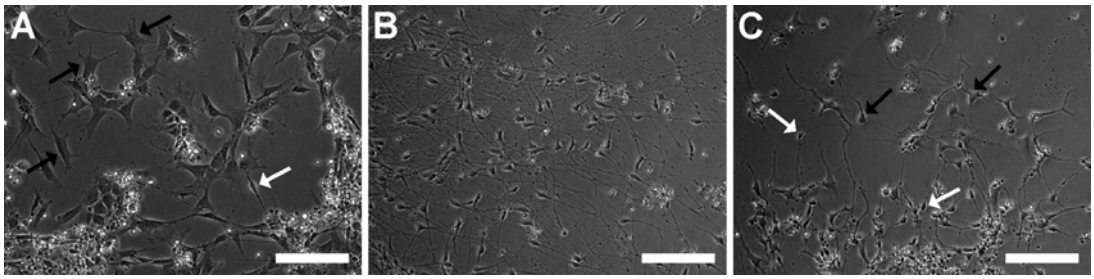

Figure 3. Representative cell cultures from hESC-derived neurospheres differentiated for 3-15 weeks. A) Neurospheres differentiated for 1-3 weeks produce neuronal cells (white arrows) but also flat epithelial-like cells (black arrows). B) Neurospheres differentiated for 6-9 weeks produce solely neuronal 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.

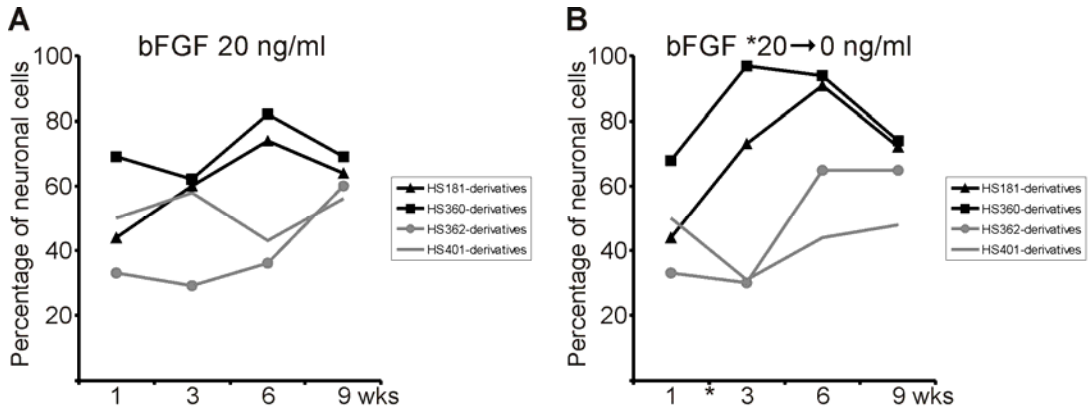

Figure 4. Differentiation of four hESC lines into neuronal cells in relation to bFGF concentration. A) HS181- and HS360-derived neurospheres produced ~80 % of neuronal cells after 6 weeks of differentiation with constant 20 ng/ml bFGF. HS362- and HS401-derived neurospheres differentiated slower and produced < 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.

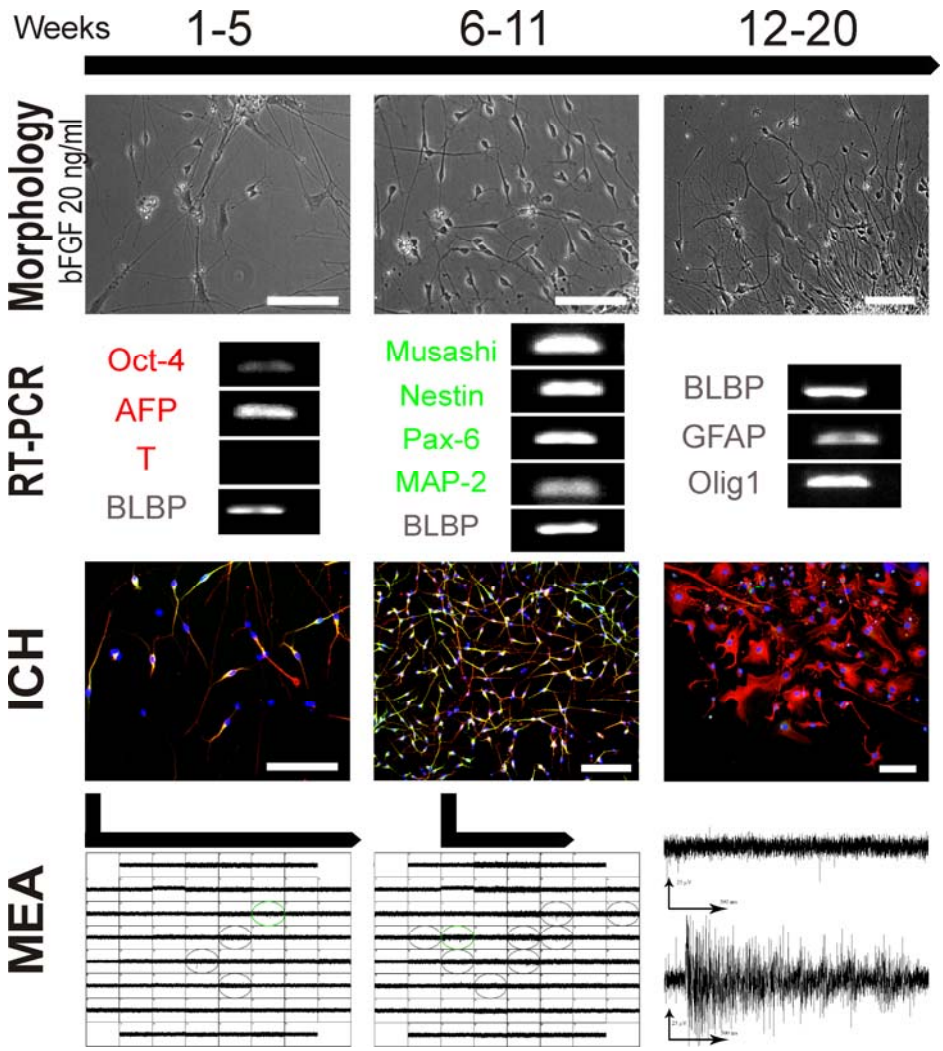

Figure 5. Neural differentiation of hESCs. Morphology: in constant 20 ng/ml bFGF, in 1-5 weeks the HS360-derived neurospheres produced neuronal and flat epithelial-like cells. At 6-11 weeks mostly neuronal cells were produced, and at 12-20 weeks production of astrocytes along with neuronal cells occurred. 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.

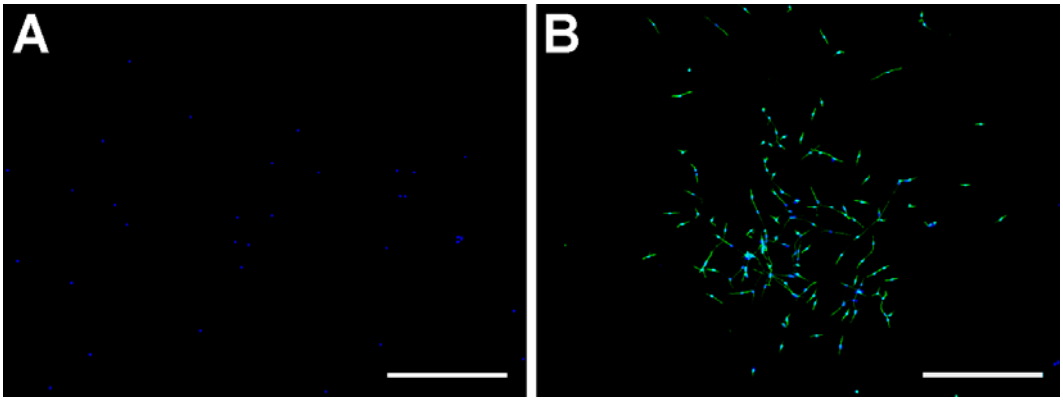

Figure 6. Neurospheres plated on NCAM-surfaces. A) In wells coated with 0 µg/ml NCAM and 200 µg/ml pTHMMAA polymer (control) some cells negative for neuronal marker MAP-2 attached. B) Wells coated with 50 µg/ml NCAM and 200 µg/ml pTHMMAA polymer contained only MAP-2 positive neuronal cells. Blue = nuclear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

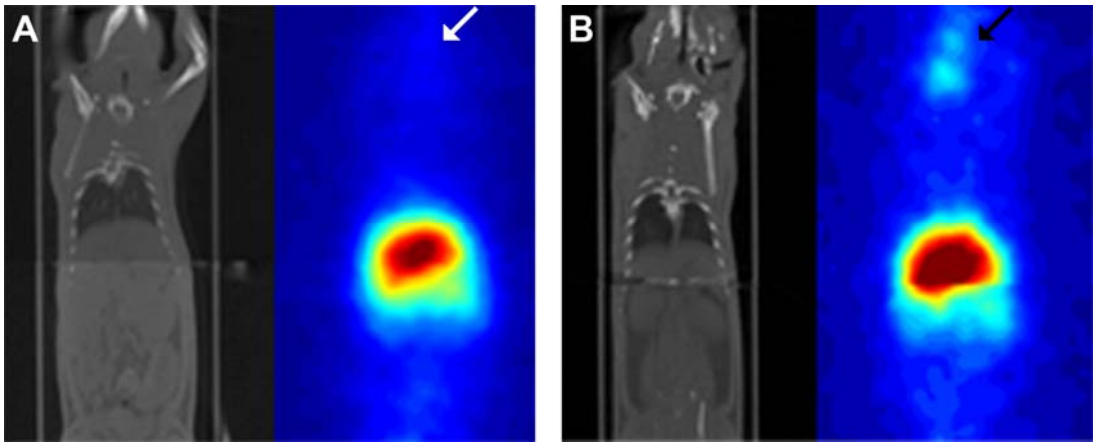

Figure 7. ^{111}In-oxine labeled hESC-derived neural progenitor cells 24 h post-transplantation in stroke rats. A) Nearly all cells accumulated into the internal organs (mainly liver) after intravenous delivery. No signal was detected from the head area (white arrow). B) After intra-arterial delivery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

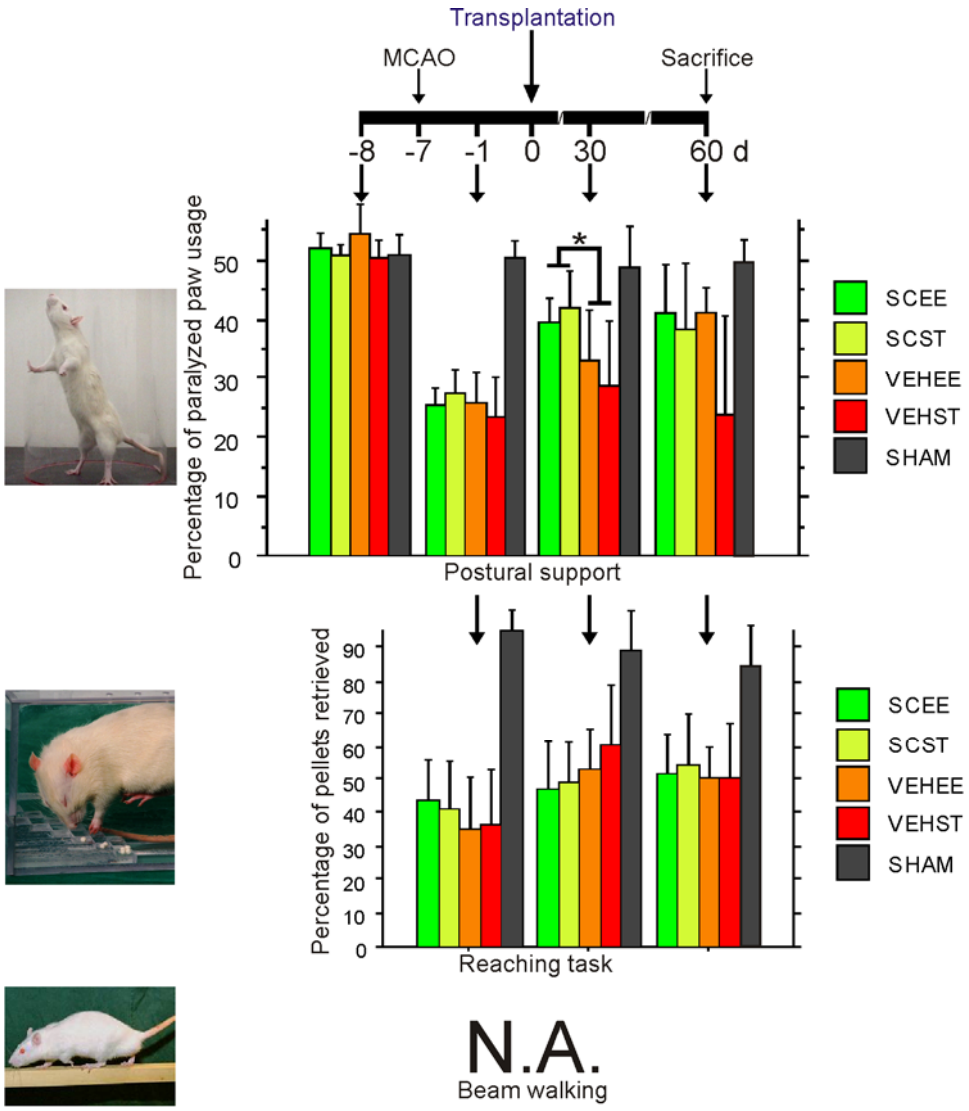

Figure 8. The outline and behavioral results of **study V**. Transplantation of hESC-derived neural progenitor cells was carried out 7 days after MCAO. The animals were divided in stem cell (SC) and vehicle (VEH) groups and further into enriched environment (EE) and standard housing (ST). Regardless 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*.

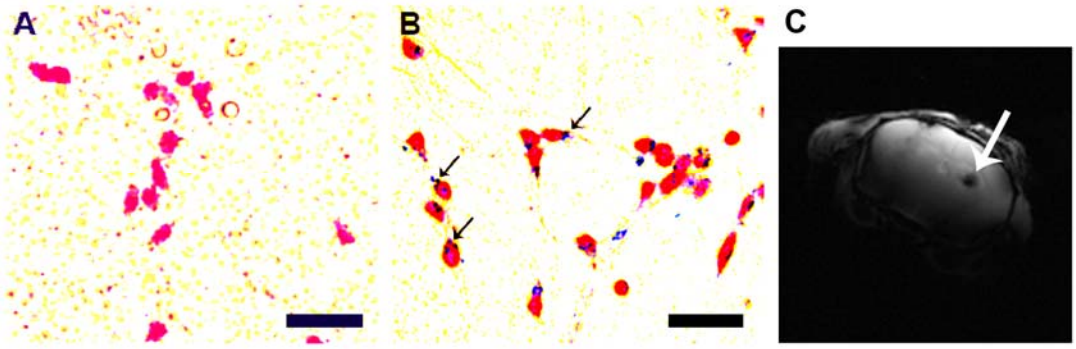

Figure 9. USPIO-labeled hESC-derived neuronal cells. A) Prussian blue stained unlabeled neuronal cells (control). B) Prussian blue stained neuronal cells labeled for 24 h with 200 µg/ml USPIO and 375 ng/ml poly-L-lysine. Iron particles pointed with black arrows. Red = nuclear fast red staining. C) Representative 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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Short communication

An antibody surface for selective neuronal cell attachment

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ABSTRACT

An optimal surface for culturing human embryonic stem cell (hESC)-derived neuronal cells is of high interest. In this study, a specific antibody to a neural cell adhesion molecule (NCAM) was immobilised on a solid surface of polystyrene and used as a selective matrix for culturing of hESC-derived neuronal cells. Thereafter, hESC-derived neurospheres were seeded on the matrix. The neurospheres did not attach to the NCAM antibody containing matrix whereas individual neuronal cells did. The neuronal cell attachment was depended on the NCAM antibody concentration. The neuronal cells were viable on the NCAM antibody containing matrix during an 8 day follow-up and exhibited typical bipolar morphology of immature neurons. Specific binding of the NCAM antigen to an immunoglobulin-polymer coated surface was verified by surface plasmon resonance (SPR) measurements. This study is to our knowledge the first demonstrating the use of an antibody layer as a selective surface for hESC-derived neuronal cells.

1. Introduction

The growth and maturation of human embryonic stem cell (hESC)-derived neuronal cells requires a supporting matrix to which cells can adhere. The most used matrixes for this purpose are extracellular proteins like laminin, collagen, or fibronectin (Flanagan et al., 2006; Whittemore et al., 1999; Rappa et al., 2004). HESCs are pluripotent cells typically derived from poor quality embryos donated by couples undergoing *in vitro* fertilization treatments. In theory, hESCs can be differentiated into all cell types of the human body including neuronal cells (Skottman et al., 2007). Indeed, hESCs have been differentiated into neural precursor cells and further into different neuronal subtypes and glial cells (Guillaume and Zhang, 2008). Since current differentiation protocols produce not entirely homogenous populations containing both neural and non-neural cells, a selective surface supporting only neural cell attachment, growth and maturation is highly desirable.

Examples of more selective growth matrices for cells can be found: oriented surfaces of an epidermal growth factor have been used with rat fetal neural stem cells (Nakaji-Hirabayashi et al., 2007) and specific terminal peptide sequences of laminin (pentamer IKVAV) or fibronectin (tetramer RGDS) have also been utilized in recent publications in culturing of among others human umbilical vein endothelial cells (Jung et al., 2009). Antibodies for defined cell-surface targets would offer an even more selective attachment and growth surface. The neural cell adhesion molecule (NCAM) is a binding glycoprotein expressed on the surface of neurons, glia, skeletal muscle, and natural killer cells. NCAM has been implicated in having a role in cell–cell adhesion and neurite outgrowth (Ditlevsen et al., 2008). Thus, an NCAM specific antibody might be used as a supportive and selective matrix for binding of neuronal cells.

Our aim in this study has been to clarify if surface immobilised antibodies to a defined target on the cell surface can be used to alleviate cell attachment. We have previously immobilised antibody Fab′-fragments site-directly onto gold through the free thiol groups and included hydrophilic polymers in between the proteins to hinder non-specific binding (Vikholm-Lundin and Albers, 2006; Vikholm-Lundin et al., 2007). The role of the polymer on the surface is to provide a hydrophilic surrounding for the antibodies and to preserve the native-like water-surrounded environment. First, we studied the interaction of NCAM antigen with binary monolayers composed of anti-NCAM antibodies physisorbed on gold and post-treated with a non-ionic hydrophilic polymer *N*-[tris(hydroxymethyl)methyl]-acrylamide (pTHMMAA). Secondly, neuronal cells derived from hESCs were allowed to attach on layers composed of only the polymer, or anti-NCAM antibodies and the polymer on polystyrene, which is the surface normally used for culturing of cells. To our knowledge this is the first study demonstrating the use of a selective antibody surface for attachment of hESC-derived neuronal cells and could be a very useful technique also for other researchers working in this field.

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2. Materials and methods

2.1. Materials for surface construction and SPR measurements

The anti-NCAM antibodies developed by P.W. Andrews (Andrews et al., 1990) were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, IA). The NCAM antigen was purchased from Abcam (Cambridge, UK) and was specified as "Recombinant fragment, corresponding to amino acids 20–220 of Human NCAM", which covers the first two extracellular N-terminal Ig-like domains of the protein. Buffers used were 10 mM HEPES-buffer containing 150 mM NaCl, pH 6.8 and phosphate-buffered saline (PBS) composed of 50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.5. HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]) (minimum 99.5%) was purchased from Sigma–Aldrich (Steinheim, Germany), Na_2HPO_4 was purchased from Merck, NaH_2PO_4 and NaCl from J.T. Baker. The polymer of *N*-[tris(hydroxymethyl)methyl]-acrylamide (pTHMMAA) (Fig. 1A) was prepared as previously described (Vikholm-Lundin et al., 2007). Polystyrene well plates were purchased from Nunc, Thermo Fisher Scientific, Rochester, NY. Hydrogen-peroxide (30%) was bought from Merck KGaA and ammonium hydroxide (28–30% NH3) and bovine serum albumin (BSA, minimum 98% purity) from Sigma–Aldrich.

2.2. Antibody–polymer layers on gold for SPR measurements

Studies on binary monolayer formation of antibodies and polymer on gold were carried out on gold *in situ* with surface plasmon resonance (SPR) (Biacore 3000, GE Healthcare). Thin glass slides were coated with a 50 nm thin gold layer in-house by RF magnetron sputtering. The gold surfaces were always cleaned in a boiling solution of hydrogen-peroxide–ammonia in water (1:1:5) and rinsed with water prior to surface assembling. Instantly after the cleaning step, the slides were mounted in a plastic chip cassette by double-sided tape and inserted into the Biacore 3000 SPR instrument. First, the antibody was allowed to physisorb on the pre-cleaned gold surface for 15 min, followed typically by 10 min wash with PBS buffer. Next, the pTHMMAA polymer at a concentration of 200 μg/mL was post-adsorbed on the surface. Non-specific binding was measured by running BSA at a concentration of 500 μg/mL on the surface. The NCAM antigen binding was measured in HEPES-buffer by injecting increasing concentrations of antigen (0.0001–10 μg/mL) over the surface and rinsing with HEPES-buffer.

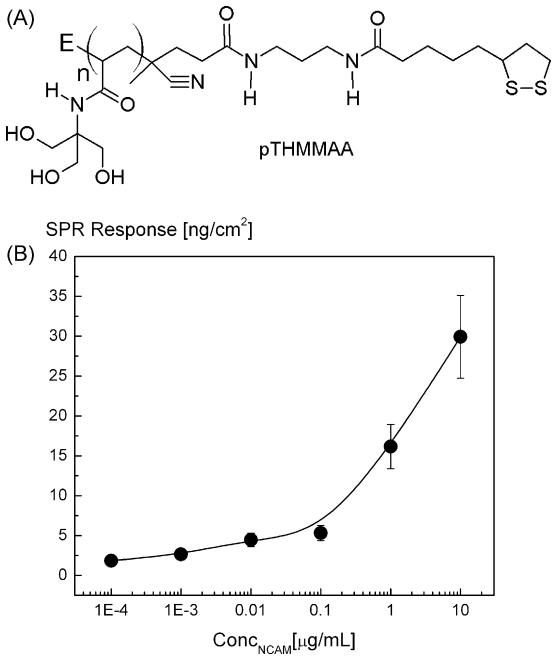

Fig. 1. (A) The structure of the pTHMMAA–polymer. (B) SPR standard curve showing NCAM antigen binding to a layer composed of anti-NCAM antibodies and pTHMMAA–polymer spread from concentration of 50 and 200 μg/mL, respectively.

2.3. Human embryonic stem cells and neural differentiation

The hESC lines used were Regea 06/040 or 08/023, derived and characterized at Regea - Institute for Regenerative Medicine, University of Tampere, Finland (Skottman, in press; European Human Embryonic Stem Cell Registry, www.hescreg.eu). Regea has the approval from the Ethics Committee of the Pirkanmaa Hospital district in Finland to derivate, culture, and differentiate new hESC lines from surplus embryos after obtaining signed informed consent from donating couples undergoing *in vitro* fertilization treatment. Briefly, hESCs were cultured in an undifferentiated stage in Knockout Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% Knockout serum replacement, 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 fibroblast growth factor (bFGF R&D Systems, Minneapolis, MN) on top of a human feeder cell layer (CRL-2429, ATCC, Manassas, CA). The undifferentiated stage of hESCs was assessed daily by morphologic analysis and periodic immunostaining for hESC-markers Nanog, OCT-3/4, SSEA-4, and Tra-1-60. In addition, karyotyping was performed and indicated that the hESC lines maintained normal karyotype. All cultures were tested mycoplasma-free.

The differentiation protocol (Hicks et al., 2009) was further developed from Nat et al. (2007). For neural differentiation the hESC colonies were manually dissected into small clusters containing ~3000 cells which were transferred into 6-well ultra low attachment plates (Nunc), and cultured as floating aggregates, hereafter called neurospheres, for 6 weeks prior to plating on the NCAM antibody matrix. The neural differentiation medium consisted of 1:1 DMEM/F12:Neurobasal media supplemented with 2 mM GlutaMax, 1 × B27, 1 × N2 (all from Gibco Invitrogen), 25 U/mL penicillin and streptomycin (Lonza Group Ltd.) and 20 ng/mL bFGF (R&D Systems). The neurospheres were manually dissected into neural aggregates approximately 300 μm in diameter when they were seeded on the NCAM antibody–pTHMMAA polymer matrix. At the time of seeding and for the follow-up period bFGF was withdrawn from the medium to further induce the neuronal differentiation of neural aggregate cells.

2.4. Construction of antibody–polymer layers onto polystyrene for cell attachment

Anti-NCAM antibodies were allowed to physisorb on polystyrene by applying concentrations of 0, 25, 50, 75, or 100 μg/mL onto the well plates for 15 min. The wells were rinsed with PBS buffer and post-treated with the pTHMMAA polymer (200 μg/mL) for an additional time of 15 min. The wells were thereafter rinsed again with PBS buffer. An improvement of the non-fouling properties of binary monolayers composed of antibodies and polymer have previously been noticed if the layers are allowed to stand for a few days (Vikholm-Lundin and Albers, 2006). Thus, the treated well plates were let to stabilize in the buffer for 2 days at +4 °C before cell seeding. Next, the wells were rinsed once with neural differentiation medium without bFGF after which 800 μL of the same medium was added to each well. The well plate and the medium were pre-warmed at +37 °C

and then the neural aggregates were applied on the surface for attachment. The cells were cultured on the matrix for 8 days and half of the medium without growth factors was changed every second day. At days 3 and 8 the cells were imaged using an Olympus microscope (IX51, Olympus, Finland) to assess the cell types attached and the cell growth. Thereafter, the cells were fixed for immunocytochemical analysis using 4% paraformaldehyde for 20 min at room temperature. Altogether two parallel wells of each NCAM concentration for both hESC line-derived neural cells were prepared.

2.5. Staining of the cells for imaging and analysis

The fixed cells were stained with polyclonal rabbit anti-microtubule associated protein (MAP-2, 1:400, Chemicon, Temecula, CA) for neuronal cells or for mouse anti-human OCT-3/4 (Millipore, Billerica, MA) or monoclonal mouse anti-Tra-1-60 (Chemicon) for undifferentiated hESCs. Briefly, the cells were blocked against non-specific antigen binding with 10% normal donkey serum, 0.1% Triton X-100, and 1% BSA in PBS for 45 min and washed with 1% normal donkey serum, 0.1% Triton X-100, and 1% BSA in PBS. The primary antibody was diluted with the washing solution, added to the cells, and incubated overnight at +4 °C. The next day, the cells were washed with 1% BSA in PBS and incubated for 1 h at RT with the same solution containing Alexa Fluor-488 or -568 (1:400, Invitrogen) conjugated anti-rabbit or anti-mouse secondary antibody. Thereafter, cells were sequentially washed with PBS and phosphate buffer, mounted with Vectashield with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Peterborough, UK), and cover-slipped. When primary antibodies were omitted (negative control), no positive labelling was detected. Stained neuronal cells were imaged and counted using an Olympus microscope (Olympus) equipped with a fluorescence unit and camera (DP30BW, Olympus). For statistical analysis, neuronal cell samples derived from two hESC lines were pooled together, thus the number of samples was 4 for each NCAM antibody concentration. A non-parametric Mann–Whitney *U*-test and an SPSS 17.0 statistical software package (SPSS Inc., Chicago, IL) were used for statistical analysis. A *p*-value less than 0.05 was considered significant.

3. Results and discussion

3.1. Binding of NCAM antigen to antibody–polymer layers immobilised on gold

The binding of NCAM antigen to a mixed NCAM antibody and pTHMMAA monolayer was first evaluated with SPR. A fast increase in response was observed when antibodies at a concentration of 50 μg/mL were physisorbed on the gold surface (data not shown). The antibody layer formation showed a response of 1900 ± 200 RU corresponding roughly to 190 ng of antibodies/cm^2 (Vikholm-Lundin and Albers, 2006). Next, the pTHMMAA polymer at a concentration of 200 μg/mL was post-adsorbed on the surface with a response of 200 ± 40 RU. The polymer intercalates on the surface to sites not coated by antibodies and has previously been used in immunoassays for reducing the non-specific binding of interfering molecules (Vikholm-Lundin and Albers, 2006). The non-specific binding was measured by running BSA at a concentration of 500 μg/mL on the surface. Non-specific binding of BSA was 60 ± 40 RU corresponding only to 6 ± 4 ng/cm^2, which suggests that the polymer is intercalated between the antibodies and effectively shielding them. Non-specific binding has otherwise been noticed to take place in the vicinity of the antibodies (Vikholm et al., 1999).

Next, the NCAM antigen binding to the layer was studied (Fig. 1B). The antigen binding to the layer increased with concentration, giving a response of 250–350 RU, when the monolayer was spread from an anti-NCAM concentration of 50 μg/mL. The same surface was also constructed with a lower antibody concentration (25 μg/mL; data not shown) resulting in a 60 units lower response at the highest antigen concentration of 10 μg/mL. These results are in agreement with previous studies when binding antigen to binary layers of antibodies and the pTHMMAA polymer (Vikholm-Lundin and Albers, 2006). Next, the cell-growth studies were carried out encouraged by the good characterization results obtained by SPR.

3.2. Binding of neuronal cells to antibody–polymer layers immobilised on polystyrene

First, the anti-NCAM antibodies at concentrations from 0 to 100 μg/mL were physisorbed on polystyrene and then post-treated with the pTHMMAA polymer (200 μg/mL) to produce a binary monolayer. Neurospheres differentiated for 6 weeks are a heterogeneous cell population containing mostly neural precursor cells. If plated on laminin some non-neural cells can occur (unpublished). In order to verify cell attachment on the antibody/pTHMMAA layer the cells were imaged during culturing and fixed and stained after 8 days on either polystyrene coated with only the polymer or with the polymer (200 μg/mL) and antibodies (25–100 μg/mL). The staining of the cells after fixation verified the phenotypes of attached cells. HESC-derived neuronal cells do not normally adhere on plain polystyrene and this was also observed when only the pTHMMAA polymer was applied on the surface as only MAP-2 negative, non-neuronal cells attached to the wells (Fig. 2C). If NCAM antibodies, on the other hand, were immobilised on the surface, attachment of MAP-2 positive neuronal cells could be observed (Fig. 2D and E). In fact, all the attached cells on NCAM antibody matrices of 25 or 50 μg/mL were MAP-2 positive neurons as 100% co-localization of MAP-2 and nuclear stain DAPI was observed. The cell counts revealed that significantly higher amounts of neuronal cells were attached when the concentration of the NCAM antibody was increased from 25 to 50 μg/mL on the surface ($p < 0.05$, 52 ± 31 cells vs. 206 ± 69 cells, respectively, Fig. 2F). This suggests a specific cellular NCAM protein binding to the immobilised NCAM antibodies on the well plate surface. The polymer seems to hinder quite efficiently non-neural cell attachment.

At an NCAM antibody concentration of 75 or 100 μg/mL, few MAP-2 negative cells were also observed besides neuronal cells (data not shown). Thus, these concentrations were not considered optimal for neuronal cell attachment and not studied further. This suggests that at high NCAM antibody concentrations the binding selectivity starts to diminish due to a steric hindrance with increased non-epitopic binding sites of antibodies available on the surface for non-neuronal cell attachment. Corresponding binding characteristics have been observed for antigen binding to monolayers developed for immunoassays (Vikholm-Lundin and Albers, 2006). The optimum amount of antibodies in the layer is dependent on the size of the antigen. A higher antigen binding and an improved neuronal cell growth could be expected if the antibodies were further site-directly immobilised on polystyrene.

During culturing no significant neuronal cell proliferation or extensive neurite extension was observed (data not shown). Most likely NCAM antibody sites are occupied by attached neuronal cells and thus the pTHMMAA polymer does not support cell proliferation. Also, there was no support for the neurite extension and the cells remained as bipolar immature neuronal cells. Thus, this NCAM antibody–pTHMMAA polymer surface may be used as a selective matrix for immature neuronal cells. It remains to be studied whether adding of different antibodies or patterning of the NCAM antibodies to the matrix would enhance neuronal cell maturation.

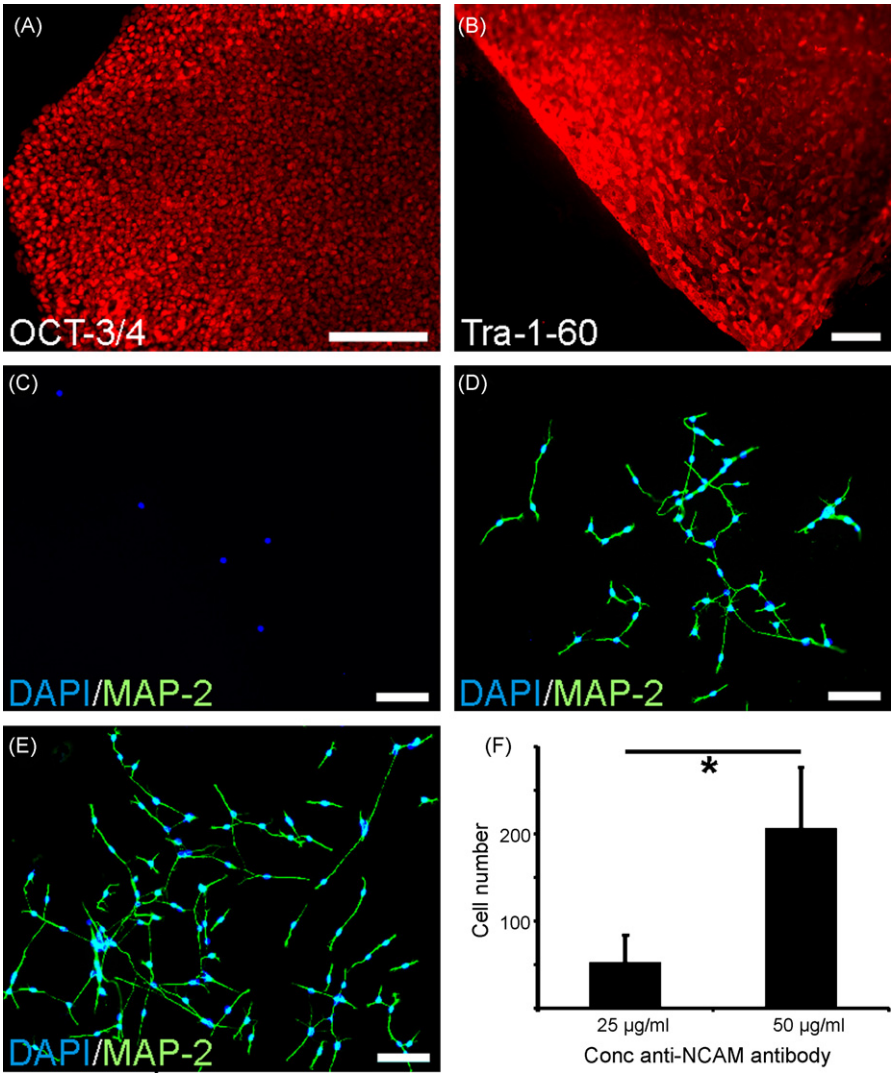

Fig. 2. Human embryonic stem cell (hESC) lines used routinely stained positive for pluripotent markers (A) OCT-3/4 (Regea 06/040) and (B) Tra-1-60 (Regea 08/023). (C) MAP-2 positive hESC-derived neuronal cells did not attach on plain polystyrene surface with pTHMMAA polymer (200 μg/mL) whereas there was concentration dependent attachment on surfaces coated with (D) 25 μg/mL or (E) 50 μg/mL of anti-NCAM antibodies and 200 μg/mL of pTHMMAA. (F) The attachment of MAP-2 positive neuronal cells was significantly higher to surfaces prepared from 50 μg/mL anti-NCAM antibodies compared to surfaces prepared from 25 μg/mL anti-NCAM antibodies ($p < 0.05$). Results represented as mean ± standard error of mean (SEM). Scale bar = 200 μm.

4. Conclusions

In this paper we have shown that hESC-derived neuronal cells can be attached selectively on polystyrene with the aid of NCAM antibodies embedded in a monolayer of hydrophilic pTHMMAA polymer molecules. The amount of neuronal cells on the surface significantly increased in relation to the increased amount of the antibodies in the monolayer. Plain polystyrene with pTHMMAA polymer alone did not present adhering of the neuronal cells. In the future our aim is to increase the amount of the functional antibodies in the layer by using site-directed immobilisation of the antibodies in order to improve the selective neuronal cell attachment and maturation.

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Human embryonic stem cell-derived neuronal cells form spontaneously active neuronal networks *in vitro*

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ABSTRACT

The production of functional human embryonic stem cell (hESC)-derived neuronal cells is critical for the application of hESCs in treating neurodegenerative disorders. To study the potential functionality of hESC-derived neurons, we cultured and monitored the development of hESC-derived neuronal networks on microelectrode arrays. Immunocytochemical studies revealed that these networks were positive for the neuronal marker proteins β-tubulin$_{III}$ and microtubule-associated protein 2 (MAP-2). The hESC-derived neuronal networks were spontaneously active and exhibited a multitude of electrical impulse firing patterns. Synchronous bursts of electrical activity similar to those reported for hippocampal neurons and rodent embryonic stem cell-derived neuronal networks were recorded from the differentiated cultures until up to 4 months. The dependence of the observed neuronal network activity on sodium ion channels was examined using tetrodotoxin (TTX). Antagonists for the glutamate receptors NMDA [D(−)-2-amino-5-phosphonopentanoic acid] and AMPA/kainate [6-cyano-7-nitroquinoxaline-2,3-dione], and for GABA$_A$ receptors [(−)-bicuculline methiodide] modulated the spontaneous electrical activity, indicating that pharmacologically susceptible neuronal networks with functional synapses had been generated. The findings indicate that hESC-derived neuronal cells can generate spontaneously active networks with synchronous communication *in vitro*, and are therefore suitable for use in developmental and drug screening studies, as well as for regenerative medicine.

Introduction

Human embryonic stem cells (hESCs) are pluripotent cells that have nearly unlimited developmental potential. Even after months of growth, hESCs continue to replicate and maintain the ability to differentiate into any human body cell type (Thomson et al., 1998). Their developmental potential makes hESCs a promising and essentially unlimited supply of numerous cell types for basic research and cell transplantation therapies for the treatment of a wide range of degenerative diseases, such as Parkinson's disease (Wang et al., 2007), Alzheimer's disease (Wu et al., 2007), and diabetes (Lock and Tzanakakis, 2007). Large amounts of functional neuronal cells, however, are needed to treat neurodegenerative disorders (Hess and Borlongan, 2008). To date, no reported studies have focused on developing functional neuronal networks of hESC-derived cells that act *in vitro* as they should *in vivo*. Thus, much more *in vitro* study-based information about the characteristics of hESC-derived neuronal cells is required before viable, safe, and functional cell grafts can be developed for successful transplantation and integration into the nervous system. In addition to the potential applications of hESCs in regenerative medicine, hESC-derived cells can be used in other medical, biological, and pharmaceutical applications (Rolletschek et al. 2004; Stummann and Bremer, 2008). Furthermore, more detailed studies of hESCs will contribute valuable information about early human development.

To better understand how single cell activity combines to form network-level functions, it is essential to study how neurons work in concert. Microelectrode array (MEA) technology (Gross et al., 1977; Pine, 1980) allows measuring the electrical activity of a neuronal system at the network-level. The electrical activity of a cell population can be measured while the cells grow *in vitro* on a growth plate with embedded recording electrodes. MEA recordings reveal the spatial and temporal distribution of electrical activity generated by neuronal populations near the microelectrodes. Despite the simplified level of organization of planar cell cultures on MEAs, this system reveals general information about electrophysiological properties, developmental changes in the activity patterns, and basic learning mechanisms of the nervous system (Ben-Ari, 2001; Katz and Shatz, 1996; Madhavan et al., 2007; Maeda et al., 1995; Van Pelt et al., 2005; Wagenaar et al., 2006; Yvert et al., 2004). MEA cultures can be followed for long periods of time over which the populations develop

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from isolated neurons into fully connected neuronal networks, during which they pass through the phases of overproduction of synaptic connections and subsequent synaptic elimination and stabilization (Corner et al., 2002). Spatiotemporal analyses of multi-channel recordings on dissociated rodent cortical or hippocampal slices (Chiappalone et al., 2007; Li et al., 2007; Madhavan et al., 2007; Otto et al., 2003; Van Pelt et al., 2005; Wagenaar et al., 2006) or mouse embryonic stem cell-derived neurons (Ban et al., 2007; Illes et al., 2007) have been performed. Neuronal cell populations derived from hESCs, however, have not been previously studied.

Here, we describe the functional development of hESC-derived neuronal networks cultured for up to 4 months on MEAs. We followed the maturation of the neuronal network activity towards synchronous bursting and observed the responses of the hESC-derived neuronal networks to pharmacologic substances that act on synaptic receptors.

Materials and methods

Briefly, hESCs were differentiated towards neuronal lineage, and the effect of MEA-dish coatings on cell viability and neuronal characteristics were assessed based on live/dead assay and immunocytochemical staining. Neuronal cultures were prepared for MEA dishes and electrophysiological activity of the cultures was recorded with the MEA system. The recordings were used to assess the activity characteristics of the neuronal cells and networks. Similar analyses were conducted using pharmacologic agents that affect cell ionic and synaptic activity.

Neuronal cell differentiation

The hESC lines HS181, HS360, and HS362 derived at Karolinska Institutet (Hospital Huddinge, Stockholm, Sweden), and 06/015 derived at Regea (University of Tampere, Tampere, Finland) were used for neuronal differentiation. The ethics committee of the Karolinska Institutet approved the derivation, characterization, and differentiation of the hESC lines. The ethics committee of the Pirkanmaa Hospital District provided approval for Regea to culture the hESC lines derived at the Karolinska Institutet and to derive and culture new hESC lines. hESC lines were cultured in Knockout Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) with 20% serum replacement, 2 mM GlutaMax (Invitrogen), 1% non-essential amino acids (Cambrex Bio Science, New Jersey, NJ), 50 U/ml penicillin/streptomycin (Lonza Group Ltd., Switzerland), 0.1 mM 2-mercaptoethanol (Invitrogen), and 8 ng/ml basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN) on top of a human feeder cell layer (CRL-2429, ATCC, Manassas, CA). The undifferentiated stage of hESCs was confirmed daily by morphologic analysis and frequently by immunocytochemical stainings for the ESC markers Nanog, Oct-4, SSEA-4, and Tra-1-60.

Neural differentiation of hESCs was performed as previously described (Nat et al., 2007; Sundberg et al., 2009). Briefly, the neural differentiation was induced by dissecting hESC colonies into small clusters of approximately 3000 cells. These clusters were cultured in suspension in low attachment 6-well plates (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) in 1:1 DMEM/F12/Neurobasal media (Gibco/Invitrogen) supplemented with 2 mM GlutaMax (Invitrogen), 1×B27 and 1×N2 (Gibco/Invitrogen), 25 U/ml penicillin/streptomycin and 20 ng/ml bFGF. The clusters formed hESC-derived neural aggregates that were cultured for 4 to 5 weeks with weekly mechanical passaging and medium changes 3 times/week.

Cell viability assay and immunocytochemical characterization

The effects of various coating substrates on cell viability and neuronal characteristics were assessed. Cell culture wells (24-well plates, Nunc, Roskilde, Denmark) were coated with either laminin (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA), 0.1% polyethyleneimine (PEI, Sigma-Aldrich), or PEI + laminin. PEI solution was prepared according to the directions in the MEA manual (Multi Channel Systems, 2005). Neuronal cells were seeded on coated wells (2 parallel samples/coating) and allowed to grow in an incubator (+37 °C, 5% CO_2, 95% air-humidified atmosphere) for 5 days before analysis.

For the cell viability assay, a LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, #L3224, Invitrogen) was used. Briefly, culture medium diluted with calcein AM (0.1 μM) and ethidium homodimer-1 (0.5 μM) was added to the cells. After 30-min incubation at room temperature in a light-protected area, cells were imaged under fluorescence microscopy (IX51, Olympus, Finland), and photographed (DP71 camera, Olympus). Calcein AM stains living cells and emits green light at 515 nm, whereas ethidium homodimer-1 stains dead cells and emits red light at 635 nm. The viability assay was performed also on 3 neuronal cultures grown on PEI + laminin-coated MEA dishes for 6 weeks.

For immunocytochemical staining, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. Thereafter, cells were rinsed in 2× Dulbecco's phosphate-buffered saline (PBS), blocked with 10% normal donkey serum, 0.1% TritonX-100, and 1% bovine serum albumin (BSA) in PBS (all purchased from Sigma-Aldrich) for 45 min at room temperature. After blocking, the cells were washed once with primary antibody solution (1% donkey serum, 0.1% TritonX-100, 1% BSA in PBS) prior to incubation with primary antibodies: β-tubulin$_{III}$ (mouse IgM 1:1000, Sigma-Aldrich) and microtubule-associated protein 2 (MAP-2, rabbit IgG, 1:800, Chemicon International Inc., Temecula, CA) overnight at 4 °C. On the following day, the cells were washed 3×5 min with secondary antibody solution (1% BSA in PBS) and incubated with secondary antibodies donkey anti-rabbit Alexa 488 IgG (1:400) and goat anti-mouse Alexa 568 IgG (1:400, both from Invitrogen) for 1 h at room temperature in a light-protected area. The cells were then washed 3×5 min with PBS and 2×5 min with phosphate buffer (pH 7.0, 0.01 M, Sigma-Aldrich). The cells were mounted with Vectashield Mounting Medium for Fluorescence with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA), covered with a cover slip, and viewed under fluorescence microscopy.

Neuronal culture preparation on MEA dishes

Electrical activities were recorded using MEA dishes with a square array of 59 substrate-embedded titanium nitride microelectrodes (30 μm diameter, 200 μm inter-electrode distance), and an internal embedded reference electrode (200/30iR-Ti-gr, Multi Channel Systems MCS GmbH, Reutlingen, Germany). The MEA dishes were coated with PEI + laminin. Briefly, 1 ml PEI solution (0.1%) was added to the MEA dishes and incubated overnight at 4 °C. On the following day, the MEA dishes were rinsed 4× with PBS before applying a drop of laminin (10 μg/ml) to the electrode area. After 2 h incubation at +37 °C, the laminin was aspirated and dissected pieces of hESC-derived neural aggregates (n = 10–15) containing ~2000 cells each were seeded around the electrode area of the MEA dishes. The dissected neural aggregates were plated on the MEA dishes at the age of 4 to 5 weeks after the onset of differentiation in which stage they expressed only neural cell markers (Sundberg et al., 2009). Altogether, each MEA dish (n = 22) contained 20,000 to 30,000 neuronal cells. In order to enhance the *in vitro* differentiation of the neural cells to neurons, bFGF was withdrawn from the culture medium. 3 days later, the first recordings were performed and the culture medium was changed to 4 ng/ml bFGF and 5 ng/ml of brain derived neurotrophic factor (BDNF, Gibco Invitrogen) containing medium to support the neuronal maturation. Thereafter, medium composition was kept constant and all the following experiments were performed in this medium. The medium was changed 3 times per week or after every recording event.

Between the measurements, the cultures were kept in Petri dishes inside an incubator providing a +37 °C, 5% CO_2, 95% air-humidified atmosphere. At least two neuronal cultures derived from each hESC line were used for the MEA measurements.

The MEA dishes were reused several times. The dishes were thoroughly rinsed with tap water, and a neutral detergent (1% Terg-A-Zyme, Sigma-Aldrich) solution was added for overnight incubation at room temperature. Thereafter, dishes were rinsed with tap water, checked for purity under a microscope, and sterilized with 70% EtOH (15 min) in a laminar hood prior to re-coating.

Recording system

To keep the cultures sterile prior to recordings, the MEA dishes were sealed in a laminar flow hood with a semi-permeable membrane (ALA MEA-MEM, ALA Scientific Instruments Inc., Westbury, NY) that is selectively permeable to gases (O_2, CO_2), as previously described (Potter and DeMarse, 2001). The sealed MEA dishes were carefully placed into the MEA amplifier (MEA-1060BC, Multi Channel Systems) and allowed to equilibrate for 3 to 5 min before starting the recordings. The amplifier itself was placed on top of a phase-contrast microscope (IX51, Olympus) so that the neuronal cultures the electrode area could be viewed during the measurements. Imaging was performed during recording using the microscope's camera (ALTRA 20, Olympus) connected to CellD software (version 2.6, build 1210, Olympus Soft Imaging Solutions GmbH, Munich, Germany). An MEA gain of 1100 and a bandwidth of 1 to 10 kHz were utilized. Signals were sampled at 20 or 50 kHz using a data acquisition card controlled through the MC_Rack software (both from Multi Channel Systems). The culture temperature was maintained at +37 °C using a TC02 temperature controller (Multi Channel Systems). Background noise of less than 10 μV_{rms} was allowed. All the recordings were stored in a computer and visually inspected for artifacts. A high-pass filter (2nd order Butterworth filter) with a bandpass cut-off frequency set to 200 Hz was used to remove baseline fluctuations. Spike detection was performed using MC_Rack (Multi Channel Systems) with a threshold of 5.5 times the standard deviation of the noise level. NeuroExplorer (Nex Technologies, Littleton, MA) was used to visualize the processed spike data.

Cultured neuronal networks

A total of 22 neuronal cultures grown on MEA dishes were included in this study. The maximum culture follow-up time was 130 days ($n = 2$). The cultures on MEAs were measured 1 to 3 times a week, each recording lasting for 5 to 10 min. Cultures were discarded once the cells or coating detached, or upon the cessation of measurable electrical activity.

Pharmacologic testing and electrical stimulation

Each tested pharmacologic substance was first mixed with 1 ml of fresh medium. The whole medium on the MEA dish was changed to the drug containing medium, and measurements were started after 5 min incubation. Recording of 5 min was performed and the culture was washed with 1 ml of fresh medium and allowed to settle down in the incubator for 15 min before the medium containing a new substance was added.

Sodium ion channel blockade with tetrodotoxin (TTX, Sigma-Aldrich) was performed in a separate experiment with 3 cultures on MEAs. First, 5 min of baseline activity was recorded, after which the response to 50 μM TTX was measured for another 5 min. Thereafter, a washout with fresh medium and the third recording was performed.

Pharmacologic modification of post-synaptic responses was performed with 2 cultures by application of the following substances: an AMPA/kainate antagonist (6-cyano-7-nitroquinoxaline-2,3-dione; CNQX, 30 μM), an NMDA antagonist (D(−)-2-amino-5-phosphonopentanoic acid; D-AP5, 20 μM), gamma-aminobutyric acid (GABA, 100 μm), and a $GABA_A$ antagonist [(−)-bicuculline methiodide; bicuculline, 10 μM]. The reagents were purchased from Sigma. The experiment was performed after 23 days of culturing on MEAs. Each culture was photographed during measurement. First, baseline activity was measured after a fresh medium change. Next, we measured the response to CNQX, then the response to combined CNQX and D-AP5. Thereafter, a washout was performed and the culture activity was measured again in fresh medium. Next, we measured the response to GABA, after which we performed a washout as earlier. Finally, the response to the addition of bicuculline was measured.

Electrical stimulation was tested on 2 cultures 25 days after plating on MEAs, using STG2004 stimulus generator driven by the MC_Stimulus software (both from Multi Channel Systems). The stimulation paradigm was a train of 50 biphasic voltage pulses (±800 mV, 400 μsec per phase) at 300 ms intervals. The stimulation train interval varied randomly from 4 to 56 s. Only one electrode was stimulated at a time. The electrode providing the largest spontaneous network activation was selected as the stimulating electrode. The stimulated electrode was blanked for 20 ms after the stimulation in order to reduce the size of the artifacts. Additionally, ±8 ms of the signal around each stimulation time point was removed offline from all the electrodes.

Results

Neuronal cultures on MEA dishes

As a preliminary experiment, we assessed the effects of laminin, PEI, or PEI + laminin coatings on cell viability and attachment. The pH of the medium remained between 7.8 and 8.2 in all cultures regardless of the used coating. The LIVE/DEAD assay results indicated that the coating substrates did not affect cell viability (Supplementary Fig. 1). Based on the visual inspection of β-tubulin$_{III}$ and MAP-2 stainings (Supplementary Fig. 1), neuronal processes grew best on laminin- and PEI + laminin-coated wells. PEI + laminin supported long-term

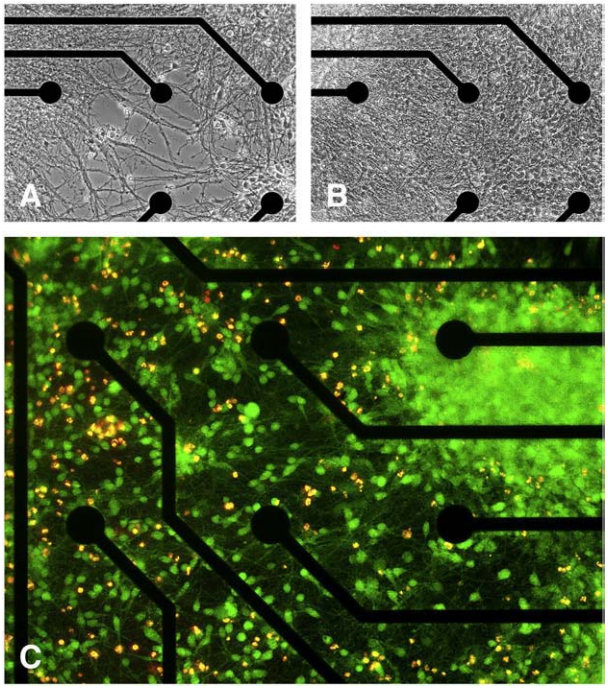

Fig. 1. Maturation of a neuronal network during the 2nd (A) and 3rd (B) weeks of culturing on MEA dish. Neuronal cells were mostly viable (green color) after 6 weeks of culturing (C). Inter-electrode distance = 200 μm.

culturing on MEA dishes. Typically, a growing neuronal network reached confluence after 2–3 weeks of culturing (Figs. 1A, B). Neuronal cells were viable after 6 weeks of culturing on MEA dishes (Fig. 1C). PEI + laminin supported neuronal growth on MEAs for over 4 months. No contamination problems were encountered, even during long culturing periods.

Spontaneous activity of the cultured neuronal networks

Spontaneous electrical activity was observed in 19 of 22 neuronal cultures on MEAs. The 3 of 22 neuronal cultures on MEAs were inactive likely due to the poor cell adhesion or viability as the cultures detached by the 1st week from the MEAs. Activity was detected as

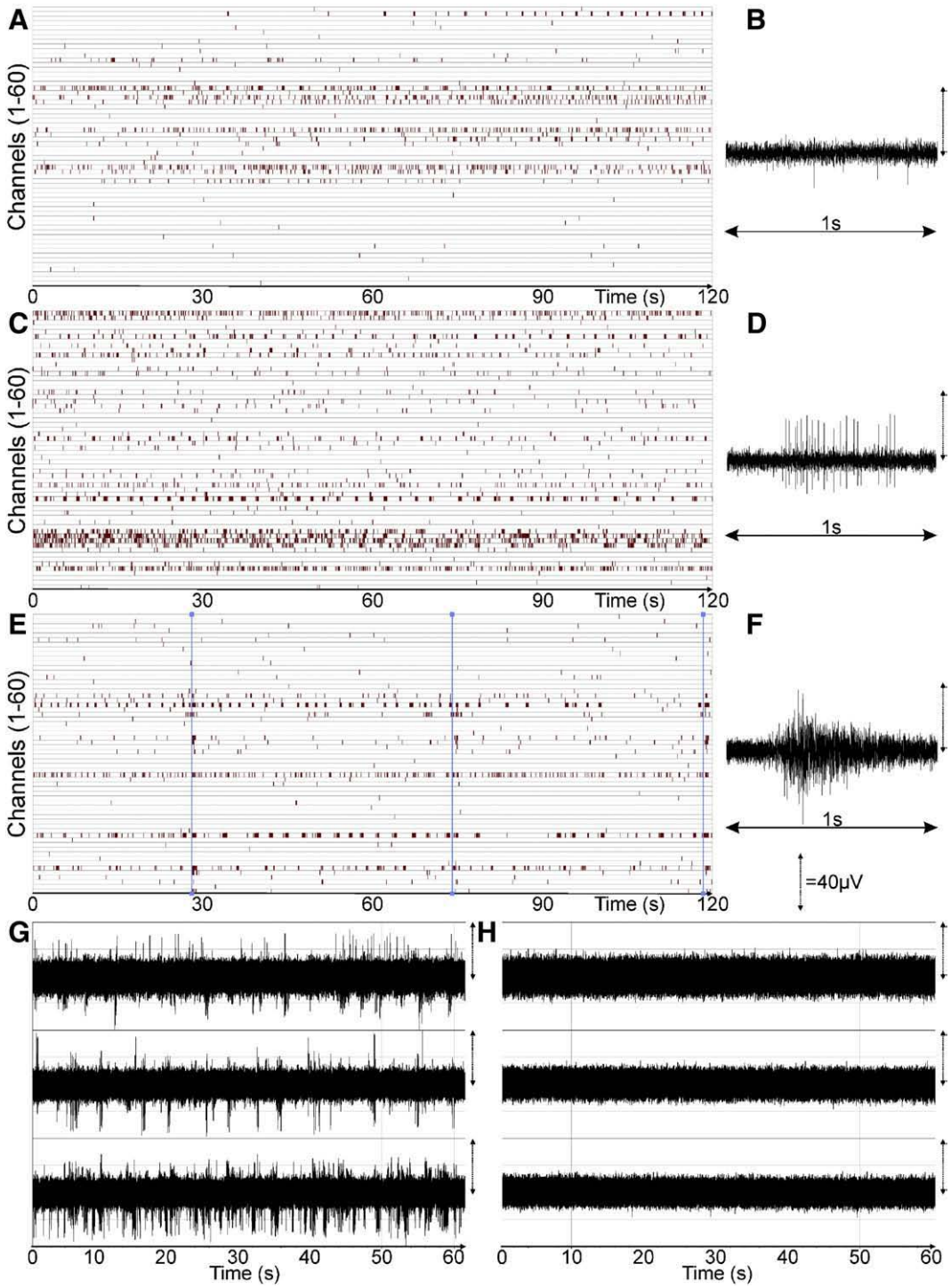

Fig. 2. Development of neuronal signaling over 4 weeks of culturing. At the first stage (1st week on MEA), single spike activity (B) was recorded only by some electrodes (A). At the second stage (weeks 2 to 3), the activity developed to spike trains (D) that were detected at multiple electrodes (C). At the third stage (from 4 weeks onwards), synchronous bursts (F) took over as the dominant kind of activity. The time points of synchronous bursts occurring in 46 s intervals in a 4 week old culture are illustrated with vertical blue lines in the raster plot (E). Neuronal activity (G) was completely inhibited when sodium ion channels were blocked by TTX (H).

early as 3 days after plating the cells on MEA dishes. In general, spontaneous activity appeared within the 1st week of culturing or did not appear at all. Typically, the first signs of electrical activity were random single spikes (Fig. 2B) which later developed to spike trains (Fig. 2D) and bursts (Fig. 2F). Here, based on visual inspection of the signals, we defined a spike train as an event of at least 5 single spikes with regular inter-spike intervals of 20 to 100 ms. Bursts were defined as events of at least 30 spikes during a 500-ms period with inter-spike interval less than 20 ms. Typically, bursts were simultaneously detected at multiple electrodes, albeit burst-like events were recorded also at single electrodes. Multiple electrode-spanning bursts are hereafter referred to as synchronous bursts (Fig. 3B).

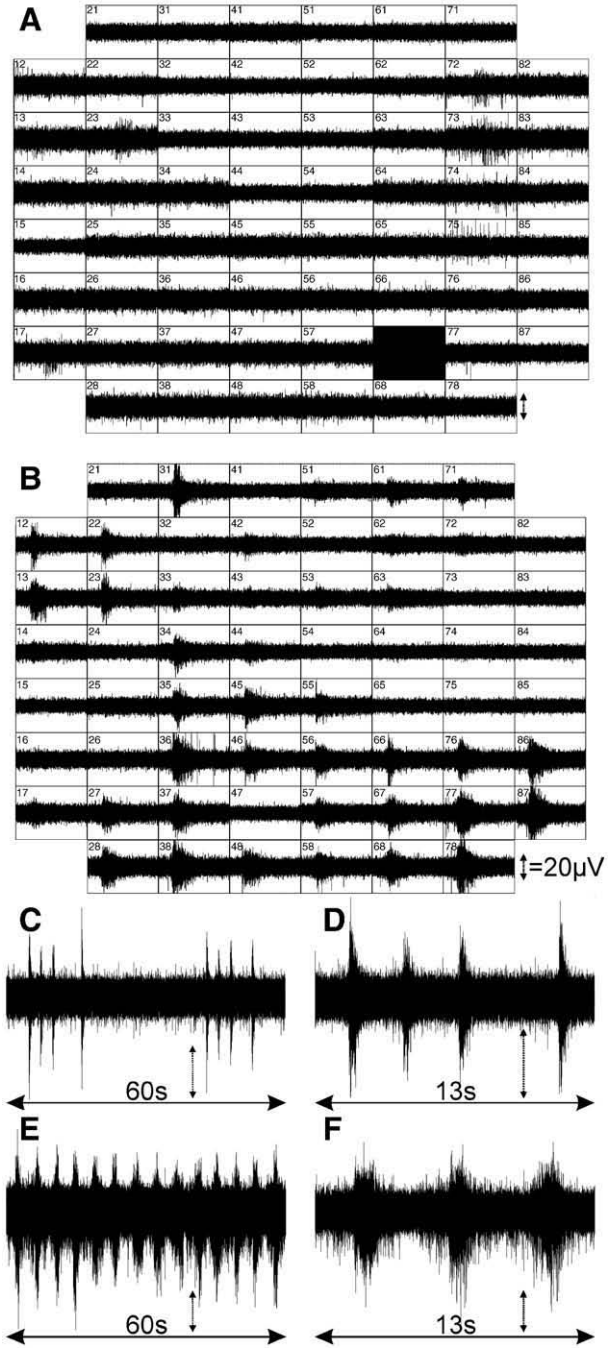

Fig. 3. A wide range of spontaneous synchronous bursts was detected. Typically, the first bursting activity occurred at only 1 to 2 electrodes after 3 to 4 weeks of culturing (A). After 4 to 6 weeks of culturing, as the culture grew denser, the bursting activity propagated over the colony and the burst profile became more bell-shaped (B). The frequency of synchronous bursts varied on different cultures. A recurring series of four bursts separated by longer rest intervals (C) and the set of four burst with closer zoom (D). Constant bursting activity (E) with closer zoom (F).

Both positive and negative monophasic, as well as biphasic spikes, were detected from the same culture. The typical spike amplitude was 20 to 40 μV, although some spike trains as high as 180 μV were recorded. Most of the activity was detected by electrodes located under dense cell layer (Fig. 1B), but some spike trains were recorded also from low-density areas containing mainly neuronal processes (Fig. 1A).

The electrical activity in cultures was confirmed to be sodium ion channel-dependent since the blockade of these ion channels using TTX completely inhibited all activity (Figs. 2G, H). The activity reappeared after washout, indicating that the inhibition was reversible (data not shown).

In 14 of the 19 MEA cultures displaying single spikes (Fig. 2A), the activity evolved into spike trains on multiple electrodes (Fig. 2C) within the first 2 weeks of culturing. Both discontinuous and constantly firing signal trains, as well as synchronous and asynchronous spikes and trains, were observed. Moreover, asynchronous signals in addition to synchronous activity could be detected from the same electrodes. Synchronous bursts (Fig. 2E, Figs. 3A–F) were detected from 6 cultures, and typically appeared after 1 month of culturing. The occurrence of synchronous bursts increased over time.

The incidence of synchronous bursts varied greatly between cultures and measurement days. The burst-envelope curve varied from indistinctly shaped (Fig. 3A) to bell-shaped (Fig. 3B). Usually, cultures with synchronous bursts also contained single spikes and spike train-type activity, but some cultures exhibited only synchronous bursts. The electrodes registering synchronous bursts located typically next to each other (Fig. 3B). The longest distance formed by adjacent burst-detecting electrodes was approximately 3.6 mm (propagation near 18 electrodes with 200 μm spacing). Recurring synchronous bursts were simultaneously detected at a maximum by 41 electrodes (41/59). Inter-burst intervals ranged from seconds to minutes. We sometimes observed rapidly repeating bursts, as previously described as superbursts by Wagenaar et al. (2006) that consisted of 3 to 4 synchronous culture-wide bursts that closely followed each other (Figs. 3C, D). In one culture, constant periodic synchronous bursts occurred at a frequency of 0.25 Hz (Figs. 3E, F).

Pharmacologic testing and responsiveness to electrical stimulation

Baseline activity is shown in Fig. 4A. Application of the AMPA/kainate antagonist CNQX (20 μM) suppressed the activity (Fig. 4B) and abolished synchronous bursts. The NMDA antagonist D-AP5 (30 μM) blocked the remaining activity (Fig. 4C), which did not return during the measurement. After a washout, the electrical activity reappeared (Fig. 4D). GABA suppressed the signaling in a dose-dependent manner (data not shown) and the addition of 100 μM of GABA completely inhibited the activity (Fig. 4E), which did not recover after a washout (Fig. 4F). Application of the $GABA_A$ receptor antagonist bicuculline (10 μM), however, induced the activity to return (Fig. 4G). Additionally, bicuculline treatment increased activity over the baseline level and induced synchronous bursting (visible as dark vertical lines in the raster plot Fig. 4H). Bicuculline treatment had similar effects on non-modulated cultures (data not shown).

In an additional experiment, we tested whether the neuronal activity patterns in hESC-derived neuronal cultures could be influenced by electrical stimulation similarly as described earlier in *in vitro* works (Ban et al., 2007; Chiappalone et al., 2007; Madhavan et al., 2007; Wagenaar et al., 2006). We used the same stimulation paradigm presented earlier by Wagenaar et al. (2006) but altered the interval between stimulations randomly (Supplementary Figs. 2A and B). Stimulation evoked synchronous bursts mainly on electrodes that already displayed spontaneous bursts before stimulations, but spikes were evoked also on other electrodes. hESC-derived neuronal

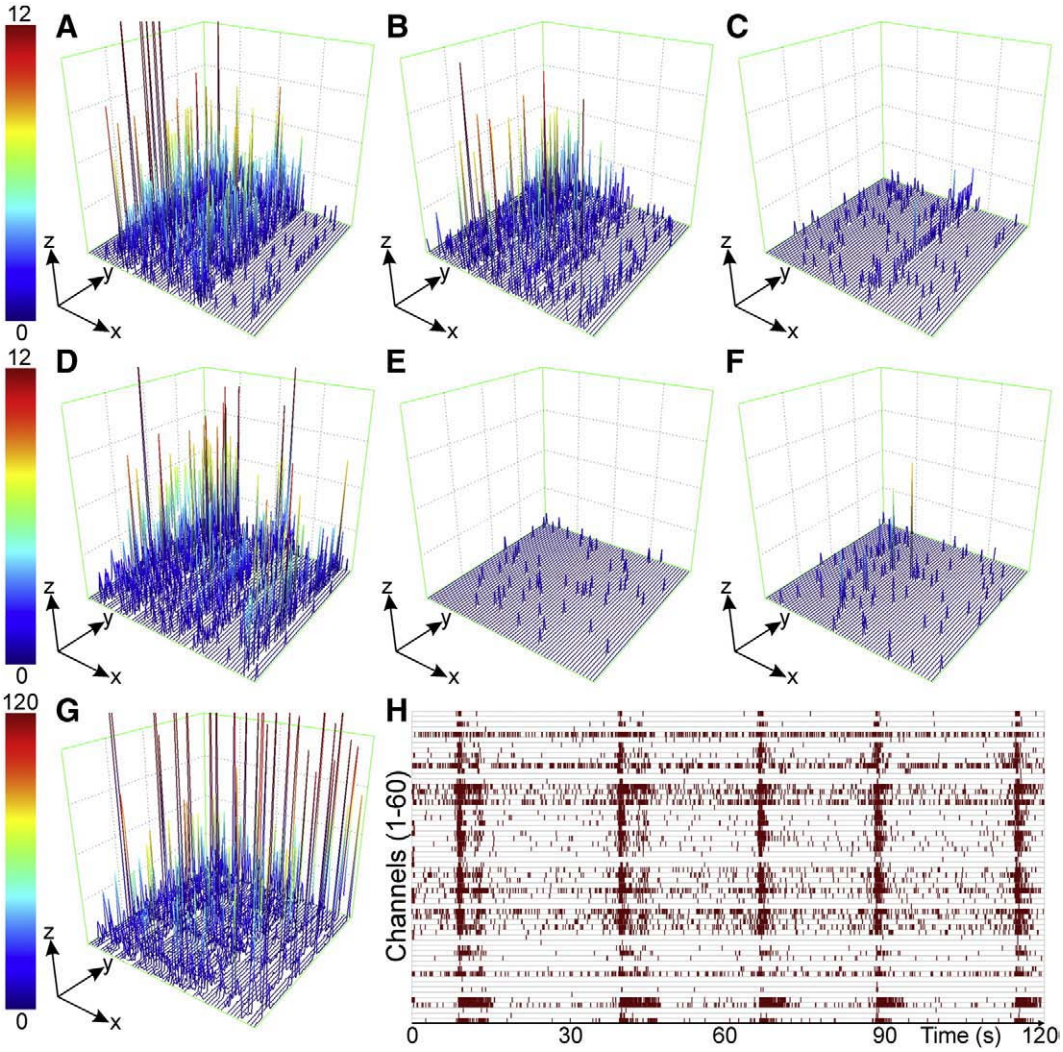

Fig. 4. Pharmacologic modulation of hESC-derived neuronal network activity. 3D-histograms (A–G) display recorded channels 1–60 along the *x*-axis. Time (in seconds; max 120) is represented by the *y*-axis. The spike count (1 s/bin) is displayed on the *z*-axis. Addition of the pharmaceuticals and recording of the network activities were performed in the following order. Baseline activity (A). The activity was partly suppressed by CNQX (B). CNQX and D-AP5 together blocked all activity (C). After a washout, activity reappeared (D). GABA inhibited all activity (E), and the activity did not return after a washout (F). The addition of bicuculline restored the activity (G) to a higher level than at the baseline (A). Note the different *z*-axis scale in G. Raster plot of the bicuculline-induced synchronous activity (H).

cultures responded to external stimulation with regularly evoked spikes. Stimulation at different electrodes produced changes in the response patterns (Supplementary Figs. 2A and B) and times (Supplementary Figs. 2C and D). For example, stimulus-induced activity, inhibited spontaneous activity, or no changes in spontaneous activity were observed on different parts of the culture.

Discussion

In this study, we provide the first evidence that hESC-derived neuronal cells can generate functional networks *in vitro*. After 5 weeks of differentiation, the functional neuronal network activity developed from single spikes to spontaneous bursts in time frame of 1 month on MEA dishes. Active neuronal cultures were followed on MEA dishes for over 4 months without any contamination.

Currently, there are many published protocols for neural differentiation of hESCs using adherent or suspension culturing, co-cultures, and various substances (retinoid acid, growth factors, noggin, etc.) that all result in at least partially pure neural populations (Hoffman and Carpenter, 2005; Nat et al., 2007). Here, we used a simple protocol for neuronal differentiation of hESCs using the suspension method as described earlier (Nat et al., 2007; Sundberg et al., 2009). The differentiated hESC-derived neural aggregates used for MEA cultures contained only neural cells at the time of plating as shown previously (Sundberg et al., 2009) and differentiated *in vitro* into MAP-2 positive neurons. The neuronal origin of the recorded activity was confirmed by the pharmacological experiments.

Previously, functionality of hESC-derived neurons has been described on the single cell level *in vitro* (Carpenter et al., 2001; Erceg et al., 2008; Johnson et al., 2007) and after transplantation *in vivo* (Reubinoff et al., 2001) using the patch clamp technique. None of the reported studies have, however, described the formation of functional networks of neuronal cells derived from hESCs as we showed here. Using the patch clamp technique, evoked action potentials of single neurons have been measurable after 4 weeks of neuronal differentiation of hESCs (Erceg et al., 2008; Johnson et al., 2007). Here, we extended these findings by showing that hESC-derived neuronal cultures start to exhibit spontaneous spikes already after 5 weeks of differentiation. Similarly, as the single hESC-derived neurons have been shown to fire spontaneously after 7 weeks and fire evoked trains of action potentials after 10 weeks of differentiation (Johnson et al., 2007), the functionality of the hESC-derived neuronal network develops from spontaneous single spikes into trains of spikes after 6 weeks and into bursting activity after 8 to 10 weeks of

differentiation. In the future, it would be very beneficial to combine the patch clamp and MEA measurements for more detailed characterization of network functionality of hESC-derived neurons.

Spontaneous neuronal activity has an important role in many aspects of neural development, including neuronal migration, differentiation, and connection patterning (Ben-Ari, 2001; Katz and Shatz, 1996; Yvert et al., 2004; Van Pelt et al., 2005). Previously, development of functional networks of mouse ESC-derived neuronal cultures has been described in a quite similar setting to that presented here (Illes et al., 2007). Interestingly, while the mouse-derived neuronal networks grown for 21 days on MEA dishes after 3 weeks of differentiation started to exhibit "bursts" of 5 ± 2 spikes within a 300-ms time period (Illes et al., 2007; Lee et al., 2000), the hESC-derived neuronal cultures expressed this kind of activity already after 7 days on MEAs after 4 to 5 weeks of differentiation. It has, however, been shown that mouse ESC (line BLC6)-derived neuronal cells can form spontaneous or evoked electrical activity 7 days after culturing *in vitro* after 4 days of differentiation (Strübing et al., 1995). The development of *in vitro* neuronal network is strongly influenced by plating density of the cells (Wagenaar et al., 2006) which may explain the differences in the maturation of functionality between different experiments. Here, we used small hESC-derived neural aggregates plated around the MEA electrode areas, which in our experience create a confluent culture faster compared to plating the cells as dissociated single cell suspension. Thus, it is very important to develop standardized culturing methods for production of structured, controlled neuronal networks for MEA environment that would enable proper comparisons between different studies and also the production of standardized testing platforms for drug screening and neurotoxicological testing.

A general characteristic of developing neuronal networks is their tendency to spontaneously discharge repetitive synchronous bursts (Corner et al., 2002; Gross et al., 1977; Kamioka et al., 1996; Maeda et al., 1995; Potter, 2008; Van Pelt et al., 2005). We recorded synchronous bursts from hESC-derived neuronal networks similar to those that typically appeared 1 month after culturing in mouse ESC-neuronal networks (Illes et al., 2007).The abundance of bursts detected resembled those observed in developing dissociated rat cortical cultures (Wagenaar et al., 2006). Burst patterns such as a recurring series of synchronous bursts separated by longer rest intervals, as well as constant bursts, were recorded.

Using reversible TTX-induced blockade of the sodium ion channels, we demonstrated that the hESC-derived neuronal network activity is dependent on voltage-gated sodium currents. The application of CNQX suppressed local spike activity compared to baseline, but not completely, in line with results in rat cortical cultures (Li et al., 2007). Interestingly, we observed also the disappearance of synchronous bursts after CNQX application. Additional blockade of NMDA receptors with co-application of D-AP5 suppressed all activity, which is consistent with findings in mouse ESC-derived (Ban et al., 2007) and rat hippocampal (Sokal et al., 2000;Ban et al., 2007) neuronal networks. The effect of CNQX + D-AP5 was reversible. The addition of GABA (100 μM) inhibited the neuronal activity similarly to mouse ESC neuronal networks (Illes et al., 2007). Interestingly, the effect was irreversible by a washout, and blunted recovery was also observed after multiple washouts (data not shown). The exogenous GABA addition can be likened to a strong culture-wide extrasynaptic 'spillover' (Trigo et al. 2008; White et al., 2000). The mechanism behind the observation remains to be clarified in future studies with receptor subtype specific agonists. Blocking of $GABA_A$ receptors with bicuculline after GABA addition resulted in a pronounced reappearance of the activity at a higher level than baseline. Bicuculline induced synchronous bursts similarly to those reported for mouse ESC-derived (Illes et al., 2007) and rat hippocampal (Sokal et al., 2000) neuronal networks. Furthermore, we observed that the amplitude of pre-existing synchronous bursts increased and periodic activity was unmasked following the addition of bicuculline to pharmacologically-naive cultures (data not shown). No morphologic changes were observed following the pharmacologic treatments. Altogether, these results indicate that the principal excitatory and inhibitory pathways of the human central nervous system are involved in the functional activity of hESC-derived neuronal networks.

Accordingly, electrical stimulation initiated or inhibited activity demonstrating that hESC-derived neuronal networks were capable of responding to external stimuli. The unresponsiveness to the stimulation directly after a spontaneous burst may result from refractoriness of the network (Maeda et al., 1995). Previous stimulation has been shown to either enhance or reduce next the stimulus response (Tateno and Jimbo, 1999). This could explain the variability in the response to our slow tetanic stimulation. More experiments are required to evaluate the role of the refractoriness in the network and the variability in the responses to the stimulation.

The findings of the present study demonstrated that the MEA measurement system is a useful tool for measuring electrical activity of hESC-derived neuronal cells. Studying the development of neuronal cell networks *in vitro* offers many advantages over *in vivo* approaches. *In vitro* systems are more accessible to microscopic imaging and pharmacologic manipulation than the human brain. Our results demonstrate that hESC-derived neuronal cells generate spontaneously active functional networks with complex patterns of activity and synchronous communication *in vitro*. Thus, these cells are suitable for use in developmental and drug screening studies and suggest a high therapeutic potential for regenerative medicine, as well.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2009.04.011.

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The SPECT imaging shows the accumulation of neural progenitor cells into internal organs after systemic administration in middle cerebral artery occlusion rats

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ABSTRACT

The regenerative potential of stem cells from various sources has been under intense investigation in the experimental models of cerebral ischemia. To end up with a restorative therapeutic treatment, it is crucial to get the cell transplants to the site of injury. Here, we evaluated the feasibility of small animal SPECT/CT in assessing the definite accumulation of ^{111}In-oxine-labeled human embryonic stem (ES) cell-derived neural progenitors and rat hippocampal progenitors after intravenous or intra-arterial administration (femoral vein vs. common carotid artery) in middle cerebral artery occlusion (MCAO) and sham-operated rats. Cell detection was carried out immediately and 24 h after the infusion using a SPECT/CT device. The results showed that after intravenous injections both cell types accumulated primarily into internal organs, instead of brain. In contrast, after intra-arterial injection, a weak signal was detected in the ischemic hemisphere. Additional studies showed that the detection sensitivity of SPECT/CT device was approximately 1000 ^{111}In-oxine-labeled cells and labeling did not affect the cell viability. In conclusion, a small animal SPECT is powerful technique to study the whole body biodistribution of cell-based therapies. Our data showed that intravenous administration is not an optimal route to deliver neural progenitor cell-containing transplants into the brain after MCAO in rats.

Stem cells derived from different origins are of interest due to their regenerative potential in various neurodegenerative diseases including cerebral stroke. Extensive number of studies have been conducted with embryonic stem (ES), fetal neural stem (NS), human umbilical cord blood (HUCB), and mesenchymal stem (MS) cells in rats following focal cerebral ischemia [16,24]. The survival rate, fate, and migration of transplanted cells have, however, been varying which may be due to diverse delivery routes used causing inconsistent localization of the cells. For example, Jin and co-workers compared different transplantation routes in rats subjected to middle cerebral artery occlusion (MCAO), and concluded that mouse cerebral cortical cells, administered via intrastriatal, intraventricular, or intravenous injection reach the brain, although the exact numbers of cells found in the brain were not given [12]. Chen et al. showed that after intravenous administration of HUCB cells in MCAO rats, only 1% of injected cells were detected in the brain [4]. In naïve rats after intravenous and intra-arterial infusion of radioactively labeled rat MS cells, the cells have localized into internal organs, primarily into the lungs [8]. Recently, we demonstrated that after intravenous injection in MCAO rats ^{111}In-oxine-labeled HUCB cells accumulate into the lungs and liver [18].

Hence, the delivery route, origin of the cells, as well as the species of the donor and recipient all influence the biodistribution of transplanted cells following ischemic insults. Here, we evaluated the use of a small animal SPECT to study the biodistribution of ^{111}In-oxine-labeled human ES cell-derived neural progenitor cells and rat hippocampal progenitor cells after intravenous or intra-arterial injections in MCAO rats. Histological analysis of the biodistribution of unlabeled cells was performed for cross-validation of the results.

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The human ES cell line used in this study was HS181. The derivation, characterization, and culturing conditions for this line have been described earlier [11,22]. HS181 has been derived at Karolinska Institutet, Stockholm, Sweden. Karolinska Institutet has the approval of the Ethics Committee of the Karolinska Institutet for derivation, characterization, and differentiation of human ES cell lines. Regea, Institute for Regenerative Medicine, University of Tampere, Finland has the approval of the Ethical Committee of Pirkanmaa Hospital District to culture human ES cell lines derived from the Karolinska Institutet. For neural differentiation, human ES cell colonies were manually dissected and replated in CellBIND® 6 well plates (Corning Inc., Corning, NY) in medium containing 1:1 DMEM/F12:Neurobasal media (Invitrogen Corporation, Carlsbad, CA), basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN), and additional supplements as described by Nat et al. [19]. In 1–2 weeks, differentiating colonies formed rosette-like structures. Neural progenitor phenotype of these cells was confirmed with mouse anti-Pax-6 (1:100, DSHB, Iowa, IA) and rabbit anti-Ki-67 (1:800, Chemicon International, Temecula, CA) immunolabeling as described previously [14]. Briefly, the 4% PFA fixed cells were blocked with 10% normal donkey serum, washed once, and primary antibodies were incubated overnight at 4 °C. The next day the cells were incubated with secondary antibodies AlexaFluor-488 and −568 conjugated to mouse and rabbit antibodies (Invitrogen Corporation). Finally, cells were washed and mounted with Vectashield with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Peterborough, UK) and imaged with a microscope (IX51S8F-2, Olympus) equipped with a fluorescence unit and camera (DP71, Olympus).

Rat hippocampal cells were dissected from the Wistar rat embryos on embryonic day 18 as described earlier [3]. Isolated progenitor cells were cultured in DMEM/F12 medium containing penicillin, streptomycin, glutamine (Biowhittaker Inc., Walkersville, MD), B27 (Invitrogen corporation), and bFGF (Roche, Basel, CH).

For labeling and transplantation, both human and rat neural progenitor cells were trypsinized into single cell suspension. After that, cells were labeled with ^{111}In-oxine (specific activity 37 MBq/ml, Nycomed Amersham, Piscataway, NJ). Within the cell, ^{111}In becomes firmly attached to cytoplasmic components and provides a stable label. Briefly, the cells (1×10^6) were incubated with 4.76–5.65 MBq ^{111}In-oxine in Tris–buffer for 30 min, after which the cells were washed once to remove free tracer and then resuspended in saline for immediate transplantation to the animals. For *in vitro* viability assays, human neural progenitor cells (1×10^6) were labeled with 2.5, 5, or 7.5 MBq ^{111}In-oxine. Control cells were treated similarly without ^{111}In-oxine. Next, cells were resuspended in neural differentiation medium and stained with trypan blue 2, 6, 8, 20, and 24 h later to determine the amount of live and dead cells.

Thirteen adult male Wistar rats (National Laboratory Animal Center, Kuopio, Finland) were used in the study. The animals were housed in a temperature (20 ± 1 °C) and light-controlled (12 h:12 h) environment. Food and water were available *ad libitum*. The experimental procedure has been approved by the Committee for the Welfare of Laboratory Animals at the University of Kuopio and by the Provincial Government of Kuopio. MCAO was induced using the intraluminal filament technique [17]. Briefly, a heparinized nylon filament (ø 0.25 mm) was inserted into the common carotid artery and advanced to the internal carotid artery to block the blood flow to the MCA territory. The filament was removed 120 min later to allow reperfusion and the external carotid artery was closed by electrocoagulation. Successful MCAO was verified by assessing sensorimotor impairment before transplantation. Sham-operated rats were treated in a similar manner except that the filament was not introduced into the internal common carotid artery.

Twenty-four hours later, labeled human neural progenitor cells (1×10^6) were injected in 500 μl saline into the femoral vein or ipsilateral common carotid artery of MCAO and sham-operated rats. Labeled rat hippocampal progenitor cells (1×10^6) were administered into the femoral vein of MCAO and sham-operated rats. In addition, stereotactic injection of labeled human neural progenitor cells (100 000 cells) into the striatum was performed (AP: +1.0 mm; L: −3.0 mm, V: −5.0 mm [21]).

SPECT imaging of the brain and abdomen was performed with a small animal SPECT/CT (Gamma Medica Inc., Northridge, CA) consisting of two gamma cameras fastened into the same gantry, as X-ray source and detector enabling imaging at the same coordinates immediately after injection and 24 h thereafter. Planar (imaging time 240 s, matrix size 81 × 81) and 3D images (64 projections, 60 s/projection) of the brain and abdomen combined with CT (voltage 70 kV, imaging matrix size 1024 × 1024) were produced to visualize biodistribution of labeled cells. Butterworth (6th order with cut-off 0.3) filtering and interpolation to identical resolution with CT images was performed for smoothing of SPECT planar images using Matlab™ routines (MathWorks, Natick, MA). To study detected counts from sample with known cell number, the counts were collected 600 s with both gamma cameras. The obtained images were summed together after identical treatment which was performed for *in vivo* planar SPECT images. Finally average values from the sample areas were calculated and compared to noise level from the area of same size to define detection limit. CT reconstruction (Exxim computing Corporation, Pleasanton, CA) was led to final 512 × 512 × 512 matrix size with pixel resolution of 0.17 mm. After the last imaging session weighted tissue samples from the brain, kidneys, liver, lungs, and spleen were collected for radioac-

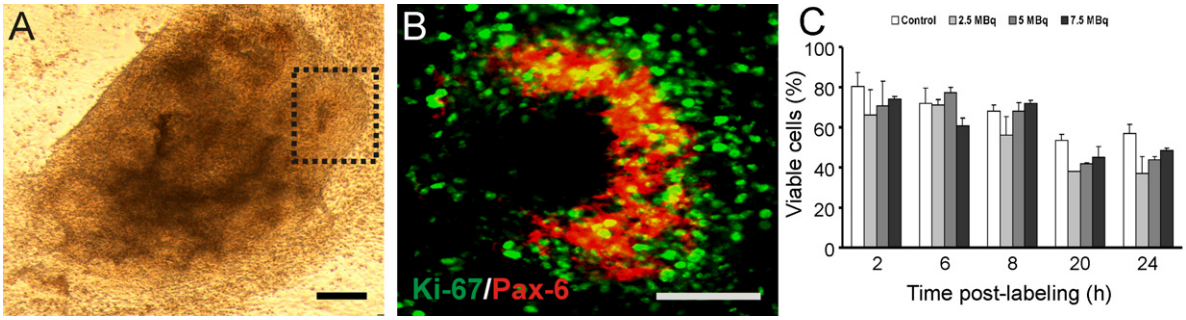

Fig. 1. Human embryonic stem cells formed rosette-like structures, 1–2 weeks after the onset of neural differentiation (A). Cells in rosettes (dotted area in A) labeled positive for the neural progenitor marker Pax-6 and proliferation marker Ki-67 (B). Viability of these human ES cell-derived neural cells after ^{111}In-oxine labeling *in vitro* was similar as the viability of unlabeled cells (C). Percentage of live cells is shown as mean ± standard deviation. There were no significant differences in viability of the cells between different groups, $p > 0.05$, ANOVA with Bonferroni correction. Scale bar 100 μm.

tivity measurement (Wallac Gammacounter). The average weights of total organs [23] were used to calculate the total radioactivity per organ.

To exclude the possibility that the labeling process would have affected the biodistribution of cells, unlabeled human neural progenitor cells (1×10^6) were injected into the common carotid artery of MCAO rats. After 24 h, rats were perfused transcardially with 0.9% NaCl followed by 4% PFA in 0.1 M phosphate buffer, pH 7.4. The brain were removed from the skulls, postfixed in the same fixative, and cryoprotected in phosphate-buffered 30% sucrose. Frozen sections (35 μm) were cut with a sliding microtome and stored in a cryoprotectant tissue collection solution at −20 °C. For immunohistochemistry, sections were rinsed in 0.1 M phosphate-buffered saline (PBS) and endogenous peroxide was removed with

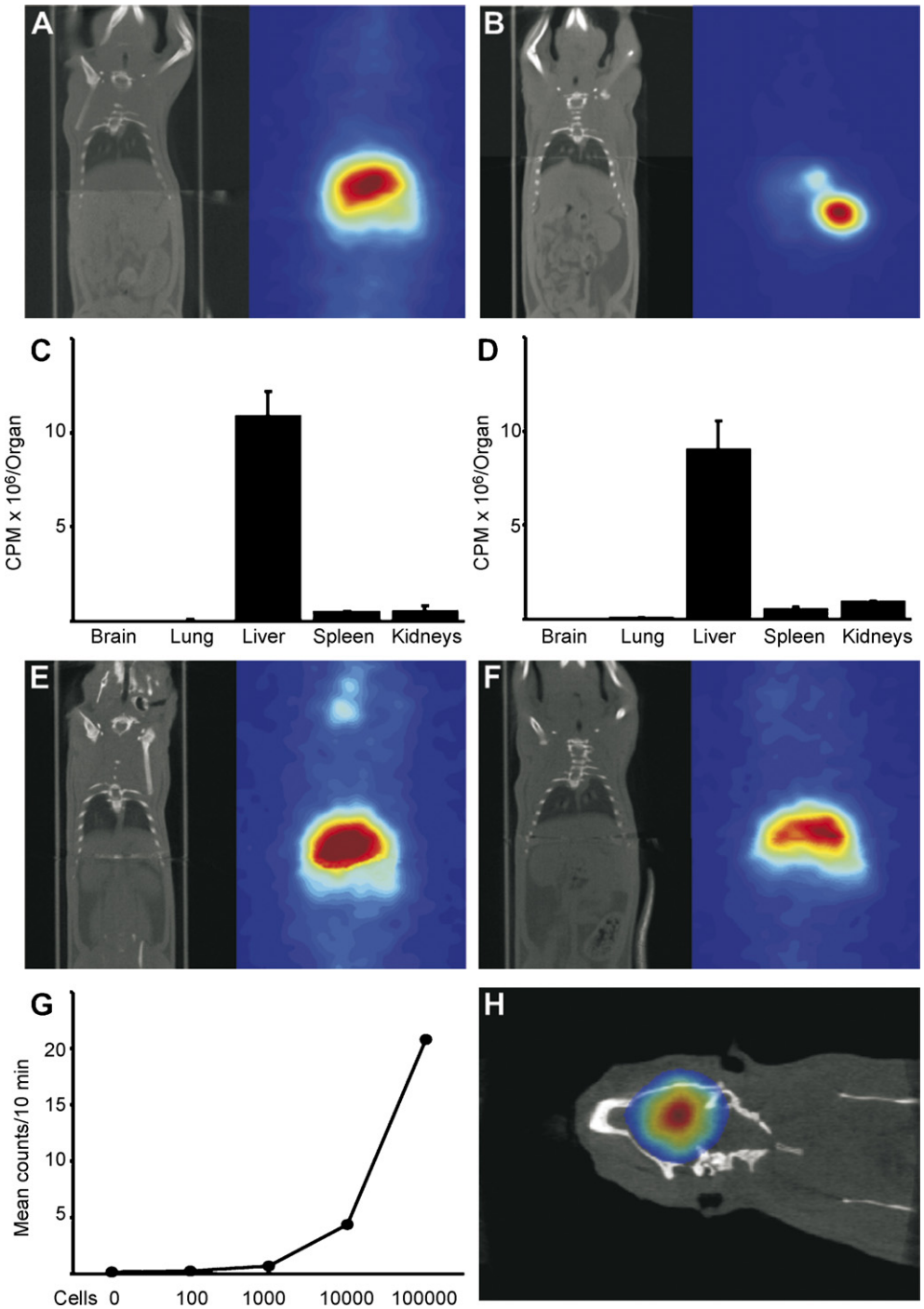

Fig. 2. The accumulation of ^{111}In-oxine-labeled cells 24 h post-transplantation. Human ES cell-derived neural cells (A) accumulated into the internal organs in a similar manner compared to rat hippocampal cells (B) after femoral vein infusion in MCAO rats. Both ^{111}In-oxine-labeled human ES cell-derived neural cells (C) and rat hippocampal cells (D) accumulated mainly to the liver after femoral vein infusion in MCAO rats whereas a minor radioactivity was detected in the spleen and kidneys. After intra-arterial infusion, human ES cell-derived neural cells were detected in the internal organs as well as in the affected brain area in MCAO treated rats (E) whereas in sham-operated rats the cells were only detected in the internal organs (F). A standard curve presenting the sensitivity of the SPECT device for the detection of ^{111}In-oxine-labeled cells from the sample with known number of the cells (G). A SPECT image of 100 000 ^{111}In-oxine-labeled cells in the rat striatum (AP: +1.0 mm, L: −3.0 mm, and DV: −5.0 mm relative to bregma [21]) (H). CPM refers to counts per minute.

10% hydrogen peroxide and 10% methanol in 0.1 M PBS. Nonspecific binding was blocked with 5% normal goat serum, prior to incubating with a 1:1000 diluted monoclonal antibody against human nuclei (MAB1281, Chemicon International), for 4 days at 4 °C. Sections were rinsed in PBS and then incubated in 1:500 diluted horseradish peroxidase (HRP)-conjugated secondary antibody (AP181P, Chemicon International) for 1 h. Triton X-100 (0.25%) was used to increase tissue antibody penetration. After washing the secondary antibody TrueBlue peroxidase substrate (KPL) was used to visualize MAB1281-positive cells. Slides were dried overnight at 37 °C, cleared in xylene, and mounted. Positive (human post-mortem cortex) and negative (rat brain sections without primary antibody) controls were included in all staining series. All sections were examined under a light microscope.

Human ES cell-derived rosette cells (Fig. 1A) were positive for neuroectodermal and neural progenitor marker Pax-6 and cell proliferation marker Ki-67 (Fig. 1B). In RT-PCR, these cells expressed Pax-6, Nestin, and Otx-2 (data not shown). ^{111}In-oxine labeling did not affect the viability of these neural progenitors compared to non-labeled controls ($p > 0.05$, Fig. 1C).

SPECT analysis showed that both ^{111}In-oxine-labeled human and rat neural progenitor cells accumulated in a similar manner into internal organs after femoral vein infusion. Accumulation into internal organs occurred immediately after the infusion and the cells could still be detected there at 24 h post-injection (Fig. 2A and B). The biodistribution of injected cells was similar in MCAO and sham-operated rats. The analysis of radioactivity per organ revealed that most of the radioactivity accumulated into the liver, spleen, and kidneys, respectively (Fig. 2C and D). Organ analysis showed no signal in the brain, in accordance with the SPECT results. Further testing showed that, after the common carotid artery injection, ^{111}In-oxine-labeled human neural progenitor cells accumulated mainly into internal organs, although a weak signal was detected in the brain (Fig. 2A vs. C). The detection threshold of the SPECT imaging used was estimated to be approximately 1000 ^{111}In-oxine-labeled cells in the sample with known number of the cells (Fig. 2G). Additionally, the stereotactic injection of labeled human cells into the striatum supported the sensitivity of SPECT (Fig. 2F). No MAB1281-positive cells were found in the ischemic or contralateral hemisphere 24 h after the infusion of unlabeled human neural progenitor cells in MCAO rats.

The present SPECT data shows that intravenously injected neural progenitor cells of human or rat origin accumulate into internal organs and do not enter the ischemic rat brain. Moreover, the origin of stem cells or administration route did not affect the biodistribution of transplanted cells.

Previous histological studies have shown inconsistent results on whether intravenously transplanted human stem cells migrate into the ischemic brain in animal models (for review see [9]). Although the interpretation of published results is difficult due to the lack of proper cell counts, it seems that homing capability of intravenously injected cells into the brain increases from human neural progenitor (NP) cells, to HUCB cells and further to human MS cells, respectively [2,4,5,9,10,15,25]. Accordingly, our imaging and histological analysis revealed that neither human nor rat neural progenitor cells accumulate into the brain in MCAO rats.

The *in vivo* SPECT data presented here indicates that some human NP cells may accumulate into ischemic hemisphere after intra-arterial injection. This is consistent with the recent *in vivo* MRI study showing intracerebral localization, although variable, after intra-arterial but not intravenous injection of rat MS cells in MCAO rats [25]. Our histological analysis of perfused brain demonstrated, however, that no human nuclei positive cells entered the brain. Thus, it is possible that the labeled cells were trapped within the cerebral microvessels and washed out during perfusion. If this is the case, the neural progenitor cells attached to the endothelial cells of the vessels would potentially be able to migrate into the brain parenchyma during a longer period of time [7]. On the other hand, the weak SPECT signal suggests that the cell population potentially entering into the brain from the vessels may not be large enough for beneficial outcome.

The labeling itself does not explain the poor cell homing to the brain, since transplanted unlabeled human NP cells were not detected in the brain either. Furthermore, in accordance with other studies [1] the viability of human NP cells was not affected by the quantity of radioactivity used (5 Bq/cell). Cell size has also been suggested to affect the biodistribution of transplanted cells. Radioactively labeled rat MS cells (Ø = 20–24 μm) and HUCB cells (Ø = 8–20 μm) have shown to get trapped in the lungs, liver, and other organs, respectively after intravenous injections in naïve and MCAO rats [8,18]. Despite their smaller size (Ø = 5–10 μm), human and rat neural progenitor cells accumulated similarly into internal organs. Relocation of these neural progenitor cells from internal organs is unlikely and the cells entrapped in the liver and spleen will most likely not survive. This hypothesis is supported by the fact that we were not able to amplify human genomic DNA by PCR from any studied rat tissue 25 days after infusion of HUCB cells in MCAO rats [20]. However, it cannot be excluded that transplanted cells can survive for some time in internal organs and relieve the symptoms via a trophic factor effect [2,5,6,13].

Two transplantation approaches are used for treatment of stroke: systemic administration and direct focal injections into brain parenchyma. The positive effect may be caused by cellular replacement or secretion of trophic factors. Here we showed that a small animal SPECT/CT is a powerful technique for whole body imaging and analysis of proportional organ distribution of ^{111}In-oxine-labeled cells to detect the transplantation efficiency. Although intravenous cell administration may have the most immediate access to clinical applications in stroke patients, it seems not to be a practical cell delivery route into ischemic brain parenchyma. Nevertheless, it should be possible to improve the homing capability of transplanted cells to enable intravenous delivery of therapeutic cells to the injured brain.

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NEUROSYSTEMS

Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery

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Keywords: cerebral ischemia, cylinder test, environmental enrichment, neural stem cells, rehabilitation, staircase test

Abstract

Cortical stem cell transplantation may help replace lost brain cells after stroke and improve the functional outcome. In this study, we transplanted human embryonic stem cell (hESC)-derived neural precursor cells (hNPCs) or vehicle into the cortex of rats after permanent distal middle cerebral artery occlusion (dMCAO) or sham-operation, and followed functional recovery in the cylinder and staircase tests. The hNPCs were examined prior to transplantation, and they expressed neuroectodermal markers but not markers for undifferentiated hESCs or non-neural cells. The rats were housed in either enriched environment or standard cages to examine the effects of additive rehabilitative therapy. In the behavioral tests dMCAO groups showed significant impairments compared with sham group before transplantation. Vehicle groups remained significantly impaired in the cylinder test 1 and 2 months after vehicle injection, whereas hNPC transplanted groups did not differ from the sham group. Rehabilitation or hNPC transplantation had no effect on reaching ability measured in the staircase test, and no differences were found in the cortical infarct volumes. After 2 months we measured cell survival and differentiation *in vivo* using stereology and confocal microscopy. Housing had no effect on cell survival or differentiation. The majority of the transplanted hNPCs were positive for the neural precursor marker nestin. A portion of transplanted cells expressed neuronal markers 2 months after transplantation, whereas only a few cells co-localized with astroglial or oligodendrocyte markers. In conclusion, hESC-derived neural precursor transplants provided some improvement in sensorimotor function after dMCAO, but did not restore more complicated sensorimotor functions.

Introduction

Stroke is a devastating disorder leaving patients with life-long functional impairments due to loss of neuronal circuitry in the brain. Physical therapy is used to promote functional recovery in stroke patients, but recovery is often incomplete. Consequently there is great interest in using stem cells to restore lost functions by replacing dead cells and tissue after brain injury (Gage, 2000; Emsley *et al.*, 2005; Haas *et al.*, 2005). For example, human embryonic stem cells (hESCs) are pluripotent cells that have been differentiated successfully into neural progenitors, and specific neuronal and glial subtypes (Carpenter *et al.*, 2001; Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Perrier *et al.*, 2004; Gerrard *et al.*, 2005; Itsykson *et al.*, 2005; Trounson, 2006; Lee *et al.*, 2007; Nat *et al.*, 2007; Cho *et al.*, 2008). The neural derivatives of hESCs represent a potential therapy for several disorders, such as stroke, Parkinson's disease, amyotrophic lateral sclerosis and spinal cord injury (Lindvall & Kokaia, 2006).

The brain has an intrinsic capacity to repair itself (Arvidsson *et al.*, 2002; Dancause *et al.*, 2005), and these so-called neuroplastic mechanisms leading to functional recovery can be enhanced by enriched environment (EE) and rehabilitation (Biernaskie & Corbett, 2001; Johansson & Belichenko, 2002). EE induces beneficial effects after brain injury, such as increased dendritic arborization (Biernaskie & Corbett, 2001), enhanced neurogenesis (Komitova *et al.*, 2005) and increased levels of growth factors (Dahlqvist *et al.*, 1999; Ickes *et al.*,

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2000; Gobbo & O'Mara, 2004). Because the majority of stroke patients receive physiotherapy, it is important to study the dual effects of stem cell transplantation and rehabilitation. Interestingly, EE combined with running exercise enhances the beneficial effects of subventricular zone (SVZ) mouse stem cells transplanted after middle cerebral artery occlusion (MCAO) in rats (Hicks *et al.*, 2007).

In the present study we transplanted human neural precursor cells (hNPCs) derived from hESCs into the rat cortex after permanent occlusion of the distal middle cerebral artery (dMCAO) or sham surgery. The rats were housed in EE or standard (ST) cages after cell transplantation or vehicle injection. The hNPCs and *in vitro* differentiated neuronal cells were characterized before transplantation. We followed the sensorimotor recovery in the staircase and cylinder tests. The survival of hNPCs was investigated using stereology, and cell differentiation was evaluated by confocal microscopy 2 months after transplantation.

Materials and methods

hESCs and neural differentiation

The hESC line used in this study was HS181, passage 59. HS181 has been derived at the Fertility Unit of Karolinska University Hospital Huddinge, Karolinska Institutet, Sweden (Hovatta *et al.*, 2003). Regea, Institute for Regenerative Medicine, University of Tampere and Tampere University Hospital, Finland have the approval of the Ethical Committee of Pirkanmaa Hospital District to culture hESC lines derived at Karolinska Institutet. hESCs were cultured in knockout Dulbecco's modified eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 20% serum replacement (Invitrogen), 2 mM GlutaMax (Invitrogen), 1% non-essential amino acids (Cambrex Bio Science, New Jersey, NJ, USA), 50 U/mL penicillin/streptomycin (Cambrex Bio Science), 0.1 mM 2-mercaptoethanol (Invitrogen) and 8 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA) in the top of human feeder cell layer (CRL-2429; ATCC, Manassas, CA, USA). The undifferentiated stage of hESCs was confirmed daily by morphological analysis and occasionally by immunocytochemistry with ESC-markers Nanog, Oct-4, SSEA-4 and Tra-1-60. The karyotype of HS181 was normal, 46XX, as analysed in passages 44 and 77. The cultures were mycoplasma free throughout the experiment.

For neural differentiation, the hESC colonies were dissected mechanically into small clusters containing approximately 3000 cells. These clusters were then cultured as floating aggregates for 6 weeks in 1 : 1 DMEM/F-12/Neurobasal media (Gibco/Invitrogen) supplemented with 1× B27 and 1× N2 (Gibco/Invitrogen), 25 U/mL penicillin streptomycin (Cambrex Bio Science), 2 mM GlutaMax (Invitrogen) and 20 ng/mL bFGF (R&D systems) in low attachment 12-well plates (Nunc, Thermo Fisher Scientific, Rochester, NY, USA). Throughout the differentiation period of 6 weeks, the growing neurospheres were dissected once a week and medium was changed three times a week.

Characterization of hESC-derived neural precursor cells prior to transplantation

A subpopulation of hESC-derived cells was characterized prior to transplantation to ensure their neural phenotype.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

After 6 weeks of neural differentiation, cell clusters were collected into lysis buffer for RT-PCR analysis, and total RNA was isolated according to the manufacturer's instructions using RNeasy®Micro kit (Qiagen, Hilden, Germany); then 50 ng of total RNA was reverse-transcribed (1 h at 37°C) into first-strand cDNA using oligo-dT primers in a reaction volume of 20 μL with Sensiscript Reverse Transcriptase (Qiagen). An aliquot of cDNA (1 μL) was used in PCR containing 0.2 mM both forward and reverse primers, 1× PCR buffer (−MgCl, +KCl), 1.5 mM $MgCl_2$, 0.1 mM dNTP mix and Taq DNA polymerase (Qiagen). The cDNA was amplified using 35 PCR cycles with an initializing step of 3 min at 95°C, DNA denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min. RT-PCR samples were then separated electrophoretically on 1.5% agarose gel containing ethidium bromide and visualized under UV-light. The primers used for the RT-PCR were: for undifferentiated hESCs – Oct-4; for endodermal phenotypes – α-fetoprotein; for mesodermal phenotypes – brachyury/T; and for neuroectodermal phenotypes – Mash-1, Musashi, Nestin and Pax-6 (for a complete primer list see Supporting information, Table S1).

Automated monitoring of in vitro differentiating neuronal cells

After 6 weeks of neural differentiation, some hNPC clusters were *in vitro* differentiated by withdrawal of bFGF in human laminin (10 μg/mL; Sigma-Aldrich, Steinheim, Germany)-coated wells. The *in vitro* neuronal differentiation was continuously monitored in Cell-IQ® cell culturing platform (Cell IQ, Chip-man Technologies, Tampere, Finland), as described earlier (Narkilahti *et al.*, 2007; Nat *et al.*, 2007), 1 day later for the next 48 h. After monitoring, cells were fixed with 4% paraformaldehyde (PFA) at room temperature (RT) for immunocytochemical analysis.

Immunocytochemical characterization of in vitro differentiated neuronal cells

Antibodies used were: polyclonal goat anti-doublecortin (DCX; 1 : 200, sc-8066; SantaCruz Biotechnologies, Santa Cruz, CA, USA), polyclonal sheep anti-glial fibrillary protein (GFAP; 1 : 600, AF2594; R&D Systems) and polyclonal rabbit anti-microtubule-associated protein-2 (MAP-2; 1 : 400, AB5622; Chemicon, Temecula, CA, USA). Briefly, after fixation cells were blocked with 10% normal donkey serum (NDS) in phosphate-buffered saline (PBS) with 0.1% Triton X-100 and 1% bovine serum albumin (BSA) for 45 min, and washed once with 1% NDS, 0.1% Triton X-100 and 1% BSA in PBS. Cells were incubated in the same solution overnight at +4°C with primary antibodies. The next day cells were washed with 1% BSA in PBS and incubated in the same solution with secondary antibodies AlexaFluor-488 or AlexaFluor-568 (1 : 400; Molecular Probes, Eugene, OR, USA) conjugated to goat, rabbit or sheep antibodies. Finally, cells were washed with PBS and phosphate buffer, mounted with Vectashield with 4′6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Peterborough, UK) and coverslipped. For double-labeling, two primary antibodies and two secondary antibodies were applied to the same cells. For control cells, primary antibodies were omitted from the staining protocol, and that resulted in disappearance of all positive labeling. The imaging of cells was performed with Olympus microscope (10× magnification, NA 0.3, Olympus, IX51S8F-2) equipped with a fluorescence unit and camera (Olympus, DP71). The fluorescent images were processed with Adobe Photoshop CS2 (Abode Systems, San Jose, CA, USA). The possible background noise was reduced, and the objects were sharpened and brightened by changing the input levels of the particular RGB channels. No other digital manipulation was performed.

Subjects

Fifty-one male Wistar rats (National Laboratory Animal Centre, Kuopio, Finland) weighing 275–300 g at the time of permanent dMCAO (Chen *et al.*, 1986) were used in the study. Animals received food and water *ad libitum*, except when food deprived for behavioral training and testing (16 g per day). The rats were housed in single cages or in EE cages (see below). All procedures were in accordance with the guidelines by the European Community Council directives 86/609/EEC, and the study was approved by the Ethics Committee of Kuopio University and Provincial Government of Kuopio.

Altogether 66 rats were originally included in the study. The following exclusion criteria were used: (i) animals that did not learn the staircase task after 21 trials (left more than 15 pellets uneaten in the last eight trials, see below); (ii) animals that had no obvious impairment 6 days after dMCAO (more than 40% reliance on the effected forepaw in the cylinder test, ate more than 80% of pellets in the staircase test and got more than 10 points in the limb-placing task); (iii) animals that had too severe impairment (less than 4 points in the limb-placing task, no rearing and no reaching). Altogether three rats were excluded because they did not learn the staircase task, six rats died after dMCAO surgery, and an additional six rats were excluded because they showed minor or too large deficits in behavior tests after dMCAO. Thus, 51 out of 66 rats were included in the final analysis after 2 months follow-up.

Animals were divided into five treatment groups (n = 10–11 per group) before hNPC transplantation as follows: (i) dMCAO + hNPC transplant (hNPC) + EE; (ii) dMCAO + hNPC + ST; (iii) dMCAO + vehicle injection (Veh) + EE; (iv) dMCAO + Veh + ST; and (v) sham-operated + hNPC + EE or ST. The experimental study design is presented in Fig. 1. Animals were assigned to treatment groups (see below) based on scores in post-MCAO limb-placement test and cylinder scores (6 days after dMCAO, 1 day prior to hNPC transplantation) to ensure that all groups exhibited similar deficits in forelimb function.

Permanent distal MCA occlusion

We decided not to use transient MCAO models as they result in both cortical and striatal injury, and chose to focus on cortical injury. Focal cerebral ischemia was induced by permanent occlusion of the MCA and temporary occlusion (60 min) of both common carotid arteries as described earlier (Chen *et al.*, 1986). In brief, anesthesia was induced using 1.5–2.0% isofluorane in 30% O_2 and 70% N_2O. An incision was made between the left ear and eye, and the distal portion of the MCA was exposed through a small burr hole and cauterized just above the rhinal fissure. Another incision was made on the neck, and both common carotid arteries were occluded for a period of 60 min. Rectal temperature was monitored and maintained between 36.5 and 37.5°C using a self-regulating heating blanket (Harvard Apparatus, Holliston, MA, USA) for the duration of the surgery. Sham-operated animals received the same surgery except the MCA was not cauterized. Temgesic (0.03 mg/kg) was used for post-operative pain relief.

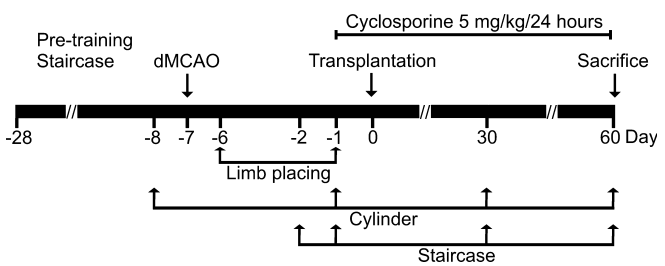

FIG. 1. Experimental design. Animals were trained for 21 days in the Montoya staircase test prior to dMCAO surgery. The limb-placement task was performed twice before transplantation to balance the groups according to the magnitude of behavioral impairment. The forelimb asymmetry test (cylinder) was performed as single trials on Day –8 (before dMCAO), on Day –1 (before transplantation), and on Days 30 and 60 (1 and 2 months after transplantation). The staircase test was performed as two daily trials during two consecutive days at the same time points. All animals received a single i.p. injection of immunosuppressant cyclosporine A 1 day prior to transplantation. During transplantation surgery osmotic minipumps filled with cyclosporine A in PEG-400 were inserted under the skin of all animals receiving cell transplants. dMCAO, distal middle cerebral artery occlusion; i.p., intraperitoneal.

Behavioral evaluation

All animals were tested in the Montoya's staircase reaching task (Montoya *et al.*, 1991) and the cylinder forelimb asymmetry task (Woodlee *et al.*, 2005). In addition, a limb-placement task (Rissanen *et al.*, 2006) was performed on Days 1 and 6 post-MCAO to distribute animals with low scores (impaired score 4–6 of maximum 14 points) equally to all treatment groups. Training for skilled reaching in the staircase test started 3 weeks before dMCAO surgery. Measurement of baseline forelimb use in the cylinder test was recorded on a single trial 1 day before dMCAO. All animals were tested in the Montoya staircase and cylinder tests before dMCAO, 6 days after dMCAO, 1 and 2 months after the hNPC transplantation.

Montoya's staircase test

Animals were food deprived 24 h before initiation of the first training or testing session. After that animals received 16 g of food per day when trained or tested, and they maintained about 85% of their pre-testing body weight. The staircase apparatus has 21 food pellets (45 mg; BioServ, Frenchtown, NJ, USA) on each of two staircases, divided on seven descending stairs, three pellets on each step. The apparatus is designed so that the pellets on each staircase are accessible to only the ipsilateral forepaw and dropped pellets cannot be retrieved. Animals were trained in a daily 10-min trial for 16–21 days prior to dMCAO surgery. Pre-surgery criteria required that animals retrieve a minimum of 15 pellets per side on the last eight trials, with a standard deviation of less than ± 2 pellets. The baseline score for skilled reaching was calculated as the mean score of the last four sessions. Similarly, post-surgery performance was based on four trials performed during two testing days. After the training and testing period animals were given free access to food.

Forelimb asymmetry test

The rats were placed in a Plexiglas cylinder (∅ 20 cm) to assess forepaw use for postural support. Animals were in the cylinder for at least 4 min or until a minimum of 20 rears was observed. Sessions were video-recorded from below to determine ipsilateral, contralateral and bilateral limb contacts. The asymmetry score (%) was calculated as: 100× (contralateral contacts + 1/2 bilateral contacts)/(total contacts) (Woodlee *et al.*, 2005).

Limb-placement test

The limb-placing test (De Ryck *et al.*, 1989) was used for assigning ischemic animals to groups with equivalent behavioral deficit. This test had seven limb-placing tasks to assess the integration of forelimb and hindlimb responses to tactile and proprioceptive stimulation that

are described in detail elsewhere (Rissanen *et al.*, 2006). Briefly, the dMCAO rats were placed on the edge of a table, and their fore- and hindlimbs were pushed over the edge to measure how quickly the rats were able to retrieve their limbs. These tasks were performed from the side and in front of the rat, with and without the help of stimuli provided by whiskers (head lifted in an angle). In addition, forepaw retraction was evaluated when the rat was lifted in the air by its tail, and finally forepaw resistance was noted when the rat was pushed on the table surface. The tasks were scored as follows: 2 = the rat performed normally; 1 = the rat performed with a delay of more than 2 s; and 0 = the rat did not perform normally. The final score was derived as a sum of all the subtests, a sham-operated animal would attain a maximum of 14 points. Both sides of the body were tested to assess forelimb and hindlimb function.

EE

EE consisted of two large metal cages (61 × 46 × 46 cm) that were connected by a tunnel. The cages (n = 8–9 animals per cage) contained objects that encourage sensorimotor activity (i.e. toys, ladders, wooden tubes, tunnels, shelves) that were changed once a week. The animals in the ST group were housed individually in standard cages (53 × 32.5 × 20 cm).

hNPC transplantation

We chose a cortical transplantation site to study the effects of possible cortical regrowth on functional recovery. Prior to transplantation, hNPC clusters were trypsinized (Cambrex) into a single cell suspension. Trypsin was inactivated with 5% human serum (Sigma-Aldrich) in PBS, and the cells were then washed once with PBS before resuspending them into a volume of 20 μL PBS containing 200 000 cells/μL. The transplantation technique is described in detail elsewhere (Hicks *et al.*, 2007). Animals were anaesthetized with isoflurane as described in the dMCAO (distal MCA occlusion) section. Briefly, using a glass cannula attached to a Hamilton syringe, 4 × 1.0 μL deposits of cell suspension were injected into the ipsilateral sensorimotor cortex 7 days after stroke (4 deposits = 800 000 cells/rat) at each of the following coordinates relative to bregma: (i) AP +0.5 mm, ML +1.0 mm, DV −2.0 mm and −2.5 mm; (ii) AP +1.2 mm, ML +1.0 mm, DV −2.0 mm and −2.5 mm (Paxinos & Watson, 1997). Vehicle animals received identical injections of PBS. All rats received one intraperitoneal (i.p.) injection of the immunosuppressant cyclosporine A (SandImmune, 5 mg/kg, Novartis) 1 day prior to the transplantation. Osmotic minipumps (Model 2ML4, Alzet, Cupertino, CA, USA) filled with cyclosporine (LC Laboratories, Woburn, MA, USA) in polyethyleneglycol-400 (PEG-400; Sigma-Aldrich, Steinheim, Germany) were inserted under the skin during the transplantation surgery. The concentration of cyclosporine in each pump was calculated so that the release of the drug was 5 mg/kg/24 h. Vehicle animals without hNPC transplants received PEG-400 via osmotic pumps (Model 2002, Alzet). After the osmotic pumps were removed, the volume of the remaining solution was measured. Body weights of the animals were monitored throughout the study.

Infarct volume assessment

Animals were deeply anesthetized with a mixture of sodium pentobarbital (9.72 mg/mL) and chloral hydrate (10 mg/mL) administered by an i.p. injection (2 mL/kg) 2 months after the hNPC or vehicle injection, and transcardially perfused with saline followed by 4% PFA. The brains were post-fixed in PFA for 90 min, and then kept in 20% sucrose in PBS for 3 days, frozen with dry ice and cut into 30-μm tissue sections with a cryostat (CM 3050S, Leica, Germany) throughout the entire ischemic damaged brain. Every eighth section was collected for Nissl staining (thionine) and the remaining sections were stored in a cryoprotectant at −20°C until immunohistochemistry was performed. Every second Nissl-stained section (i.e. every 16th section) was analysed with ImageJ (Rasband, 2008), and the total infarct volume was calculated by subtracting the area of the remaining cortex in the ischemic hemisphere from the area of the intact contralateral cortex of each section. The mean area of tissue damage between two sequential sections was multiplied by the distance between the two sections (480 μm), and these values (mm^2) between all sections were summed and multiplied with the total distance between the first and last section for the final infarct volume (mm^3).

Immunohistochemistry

The adjacent sections were stained with mouse anti-human nuclei marker (HuNu; 1 : 1000, MAB1281; Chemicon), goat anti-DCX (1 : 200, sc-8066; SantaCruz Biotechnologies), rabbit anti-GFAP (1 : 500, Z0334; DakoCytomation, Glostrup, Denmark), rabbit anti-MAP-2 (1 : 200, AB5622; Chemicon), mouse anti-human nestin (1 : 200, MAB5326; Chemicon), rabbit anti-neurofilament 200 (1 : 100, AB1982; Chemicon), rabbit anti-NG2 (1 : 150, AB5320; Chemicon), rabbit anti-S100 (1 : 50, S2644; Sigma) and mouse anti-CD-68 (anti-ED-1, 1 : 500, MAB1435; Chemicon). The secondary antibody for light microscopy was biotinylated goat anti-mouse (1 : 500, 115-065-003; Jackson ImmunoResearch, West Grove, PA, USA) used for the detection of HuNu or mouse anti CD-68. Secondary antibodies for confocal microscope analysis were goat anti-rabbit AlexaFluor-633 (1 : 500, A-21070), rabbit anti-goat AlexaFluor-633 (1 : 500, A-21086), goat or rabbit anti-mouse AlexaFluor-488 (1 : 500, A-11001, A-11059), all purchased from Molecular Probes. For immunofluorescence analysis with confocal microscopy the sections were washed in PBS at RT, then blocked in 5% normal goat or rabbit serum for 1 h (Jackson ImmunoResearch, West Grove, PA, USA) in PBS with Triton-X at RT, and incubated overnight with primary antibodies at 4°C with PBS and Triton-X. The next day the sections were washed in PBS and stained with secondary fluorescence antibodies for 2 h at RT in the dark, and then washed in PBS, mounted on slides and coverslipped. For diaminobenzidine (DAB) staining the sections were washed in 3% H_2O_2, washed in PBS and blocked in normal goat serum, and then incubated with primary antibody at 4°C with PBS and Triton-X overnight. The following day the sections were washed in PBS, incubated with the secondary antibody for 1 h at RT, washed in PBS, incubated with 10 μg/mL Extravadin (Sigma, St Louis, MO, USA) for 1 h at RT and washed in PBS. Finally, sections were stained with DAB for 3 min, washed in PBS, mounted and coverslipped. Negative controls were processed for every animal in the same way, except the primary antibody was omitted.

Every eighth brain section of all animals that received hNPC transplants was stained for anti-human nuclei or anti-CD68 with DAB, and was used for quantification of cell survival and ED-1-positive microglia detection. For phenotypic characterization using immunofluorescence, subgroups of animals were chosen randomly (n = 3 per treatment group, total n = 12).

Microscope analysis

Quantification of survival of transplanted cells

Quantification of transplanted hNPCs survival was performed on all animals (n = 31) as described earlier (Hicks *et al.*, 2007). The number of human nuclei-positive cells was counted with the optical fractionator method using Stereo Investigator (MBF Bioscience, Williston VT, USA). Briefly, the number of cells expressing human nuclei in every eighth section was calculated using a light microscope (20× magnification, NA 0.5, Leica, DMRXE) with randomly generated boxes superimposed on the areas of interest where surviving cells were located.

Confocal analysis

A laser-scanning confocal microscope (20× magnification, NA 0.7, Olympus, BX6DWI) was used to identify the phenotypes of the transplanted human nuclei-expressing cells. Two randomized areas within the sensorimotor cortex that had HuNu-positive cells were chosen, and at least 50 human-positive cells were analysed per area (n = 3 animals per group, total n = 12). The co-localization of the fluorescent dyes (excitation wavelengths of 488 and 633 nm) was confirmed by z-axis analysis in a series of stacks of 1-μm-thick sections using Fluoview FV300 software. The percentage of cells exhibiting co-localized HuNu and a phenotypic specific marker was calculated. The human nestin-positive cells were analysed semi-quantitatively as both HuNu and nestin antibodies were of mouse origin. The fluorescent images were processed with Adobe Photoshop 7.0.1 (Abode Systems, San Jose, CA, USA). The possible background noise was reduced, and the objects were sharpened and brightened by changing the input levels of the particular RGB channels. No other digital manipulation was performed.

Analysis of host tissue immune response

Tissue sections were stained for ED-1-positive microglial cells and host immune response was analysed: (i) around the cell deposit sites in the cortex; (ii) in the corpus callosum on both hemispheres; (iii) in adjacent tissue to the cortical infarct; and (iv) on the healthy contralateral cortex.

Statistical analysis

All values presented are mean ± standard error of the mean (SEM). Behavioral data (cylinder and staircase) were analysed by repeated-measures for ANOVA followed by Scheffe's *post hoc* tests to compare differences between treatment groups. Unpaired t-tests were used to analyse survival and phenotype data of transplanted neural cells. Differences at $P < 0.05$ were considered significant.

Results

hNPCs express neural markers in vitro *prior to transplantation*

RT-PCR analysis revealed that after 6 weeks of differentiation *in vitro*, Oct-4, a marker for hESCs, was absent in hNPCs used for transplantation. In addition, there was no expression of endodermal or mesodermal markers. Instead, the cells expressed Musashi, Nestin and Pax-6, markers typically associated with neural precursor cells (Fig. 2B).

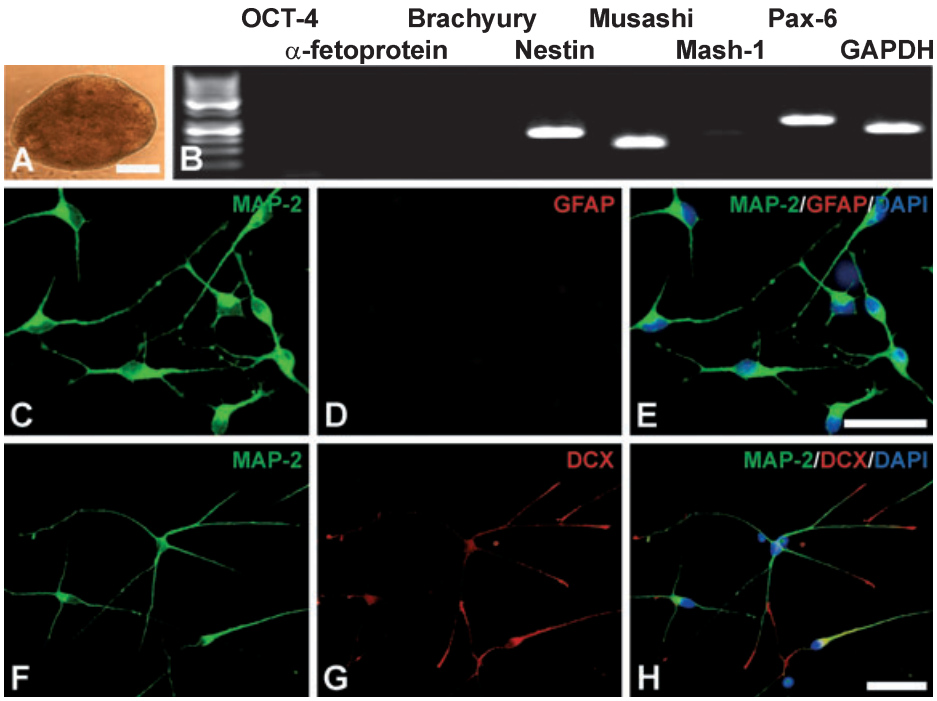

FIG. 2. Characterization of hESC-derived cells after 6 weeks of neural differentiation indicated the neural phenotype of the cells. (A) hESC-derived cells were differentiated in suspension culture as floating neurospheres. Scale bar: 200 μm. (B) RT-PCR analysis of hNPC showed the expression of neural precursor markers Musashi, Nestin and Pax-6, whereas there was no expression of markers of undifferentiated stem cells (Oct-4), endodermal (α-fetoprotein) or mesodermal lineages (Brachyury = T) detected. For immunocytochemical analysis differentiating cells were mechanically dissected and replated in laminin-coated wells. (C–E) The cells migrating out of the attached cell clusters were positive for neuronal microtubule-associated protein-2 (MAP-2) but not for glial fibrillary protein (GFAP). (F–H) The migrating neuroblast protein doublecortin (DCX) co-localized with MAP-2-positive cells mainly in the tips of the neurites and was detected in cells that migrated out of the cluster. Scale bar: 50 μm. DAPI, 4′6-diamidino-2-phenylindole; GAPDH, glyceralaldehyde-3-phosphate-dehydrogenase.

The Cell-IQ video showed *in vitro* neuronal differentiation of attached hNPC clusters (supporting Video S1). Differentiating cells were highly viable and migrated efficiently.

Immunocytochemical analysis showed that the vast majority of the *in vitro* differentiated hNPCs expressed neuronal protein MAP-2 (Fig. 2C), and none or very few cells expressed glial protein GFAP (Fig 2D). The MAP-2-positive cells that had migrated out of the attached cell clusters expressed DCX in the tips of the neurites (Fig. 2F–H).

Infarct volumes

The permanent dMCAO resulted in cortical infarction in all animals and typically included most of the parietal sensorimotor cortex (Fig. 3). There were no significant differences in the infarct volumes between the experimental dMCAO groups (Table 1), and thus no preservation or replacement of tissue was observed due to stem cells transplantation or housing conditions. The mean cortical infarct volume averaged across groups was 68.7 ± 5.1 mm^3 (Fig. 3).

TABLE 1. Cortical infarct volumes

Group	(*n*)	Volume (mm^3)
hNPCs + EE	(10)	71.2 ± 1.7
hNPCs + ST	(10)	69.3 ± 0.8
Veh + EE	(10)	72.0 ± 1.6
Veh + ST	(10)	62.1 ± 2.3
Sham + EE	(6)	0
Sham + ST	(5)	0

Values are mean ± SEM. *n* = number of rats. There were no significant differences in infarct volumes between ischemic groups. EE, environmental enrichment housing; hNPC, human neural precursor cell; ST, standard housing; Veh, vehicle.

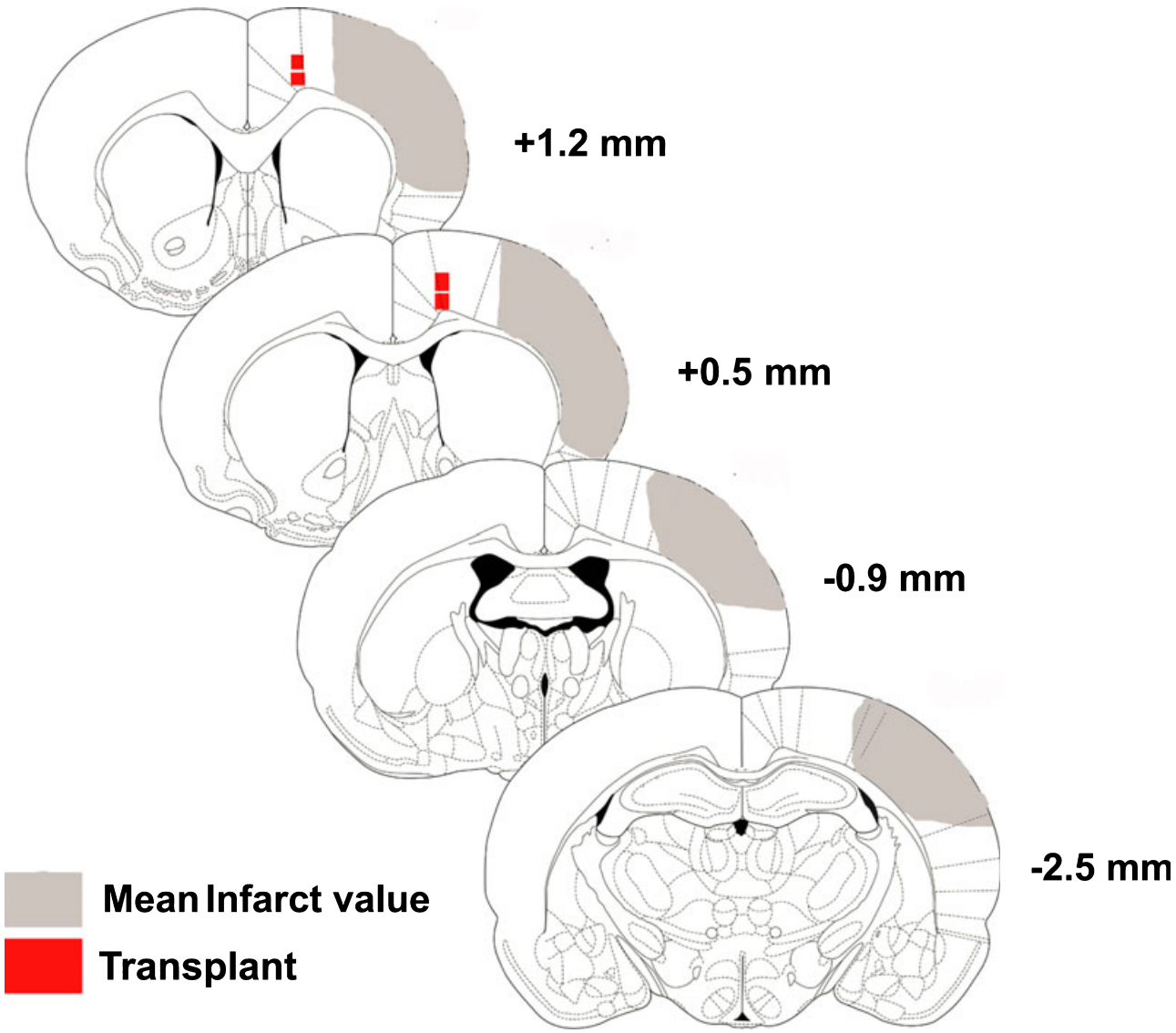

FIG. 3. Permanent occlusion of the dMCAO caused significant damage to most of the parietal sensorimotor cortex. Mean value of cortical infarct volume is illustrated in light gray. Transplantation sites of hESC-derived hNPCs are illustrated by dark gray rectangles. Distances in mm measured from Bregma (Paxinos & Watson, 1997).

hNPC transplants alleviate functional deficits in a forelimb asymmetry task but not in a reaching task

Repeated-measures ANOVA revealed that in the cylinder task there was an overall group effect ($F_{3,46} = 6.2$, $P < 0.0001$) in the use of the contralateral forepaw (Fig. 4A). ANOVA and *post hoc* tests revealed that all ischemic groups were impaired compared with the sham group 6 days after dMCAO ($P < 0.001$, i.e. 1 day prior to hNPC transplantation or vehicle injection). There were no differences between the groups before induction of dMCAO ($P > 0.05$). The sham-operated animals were pooled into one group regardless of the housing used. One month after transplantation or vehicle operation both the Veh + EE ($P = 0.004$) and Veh + ST ($P = 0.02$) groups remained significantly impaired compared with the sham group. Both neural precursor cell transplantation groups (hNPC + EE, hNPC + ST) exhibited recovery of function, and were not significantly different from the sham group ($P > 0.05$). Two months after the treatment Veh + ST remained significantly impaired compared with the sham group ($P = 0.03$), whereas the hNPC transplantation groups and the Veh + EE group did not differ significantly from the sham group ($P > 0.05$).

In the staircase test all groups were significantly impaired before the transplantation or vehicle operation compared with the sham group ($F_{3,46} = 6.2$, $P < 0.0001$), and maintained similar levels of reaching impairment at 1 month ($P < 0.01$) and 2 months ($P < 0.001$) after the hNPC transplantation or vehicle injection (Fig. 4B).

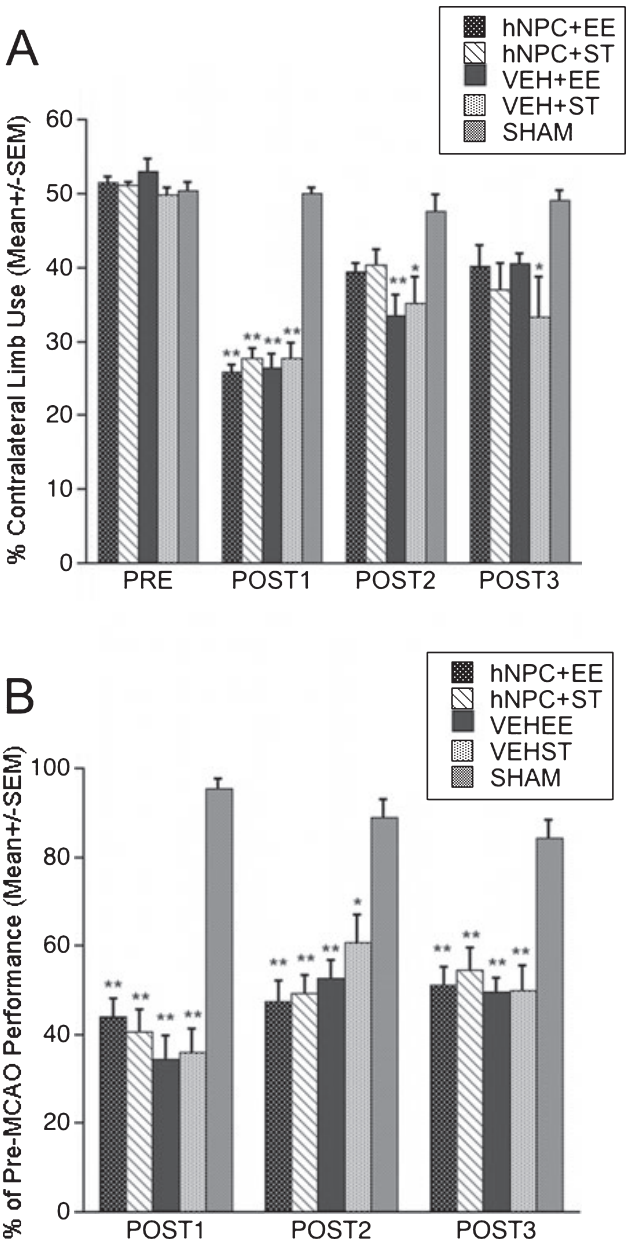

FIG. 4. (A) Repeated-measures ANOVA revealed that in the cylinder test there was a main group effect. ANOVA and *post hoc* test showed that all the groups exhibited significant impairments in the use of the contralateral forepaw 6 days after distal middle cerebral artery occlusion (dMCAO). One month after hNPC transplantation two groups that received transplants (hNPCs + EE and hNPCs + ST) were not significantly different from the sham group, whereas both vehicle control groups remained significantly impaired. Two months after transplantation, only the Veh + ST group remained significantly impaired compared with the sham group. (B) In the staircase test all stroked groups were significantly impaired before transplantation or vehicle operation compared with the sham group, and maintained impairment throughout the study. $^{**}P < 0.01$, $^{*}P < 0.05$. EE, enriched housing; hNPC, neural pre-cursor cell-transplanted group; POST1, testing 6 days after dMCAO, 1 day before hNPC transplantation; POST2, 1 month after transplantation; POST3, 2 months after transplantation; PRE, behavioral testing before dMCAO; SHAM, sham-operated group; ST, standard housing; Veh, vehicle-injected group.

Microscopical analysis

Survival of the hNPC cells

Altogether, HuNu-positive cells were found in 15 out of 31 animals that received cell transplants, that is, in 5/10 animals in hNPCs + EE, 5/10 in hNPCs + ST and 5/11 in sham + hNPCs groups. HuNu-positive cells were located in the sensorimotor cortex, and did not show significant migration away from the transplantation sites (Fig. 5A). There were no significant differences in the number of surviving cells (Fig. 5B) between hNPC + EE (8343 ± 7470), hNPC + ST (2617 ± 1920) or sham + hNPC (4587 ± 2566) groups. The survival rate of the transplanted cells was approximately 1%.

Confocal analysis

Confocal analysis of transplanted cells showed that a portion of cells differentiated into neuronal cells in the ischemic and sham-operated rat brain. There was no effect of housing on cell differentiation, and the ischemic and sham-operated groups were pooled for the final differentiation analysis. The majority of the cells were human-nestin positive (Fig. 6A). Transplanted HuNu-positive cells (10.4%) expressed MAP-2 that mainly localized into dendrites (Fig. 7A). Interestingly, the deposit sites were enriched with neurofilaments compared with the surrounding cortical areas (Fig. 6B). Even though no significant migration of cells was observed, 10.8% of HuNu cells expressed DCX (Fig. 7B). Only a few HuNu cells (less than 2%) co-localized either with GFAP, S-100 or NG2. Hence, there was no significant differentiation into glial cells. Moreover, there were no correlations found between the behavioral data and cell survival rate/differentiation.

Host tissue responses

Cortical tissue surrounding cell transplants stained strongly for GFAP-positive endogenous astrocytes (Fig. 8). In addition, ED-1-expressing microglial cells were surrounding the deposit site, corpus callosum and the cortical infarct areas (data not shown), but were absent in corresponding areas on the other side of the brain.

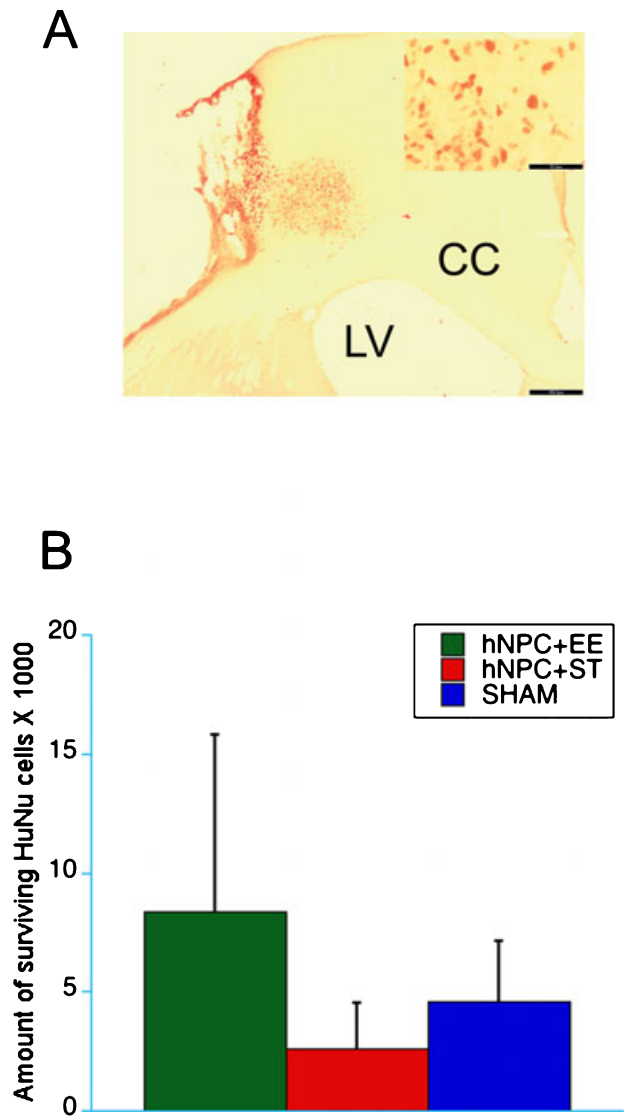

FIG. 5. (A) Human nuclei-stained neural cells show no migration. HuNu-stained cells (brown) remained at the site of injection in all groups that received transplants. The insert illustrates HuNu cells in higher magnification. Scale bars: 400 μm and 50 μm. (B) A graph of the number of surviving HuNu-labeled cells. There were no significant differences in the number of surviving cells between groups. CC, corpus callosum; EE, enriched housing; hNPC, neural pre-cursor cell-transplanted group; LV, lateral ventricle; SHAM, sham-operated group; ST, standard housing.

Discussion

Stem cell transplantation holds a promise for stroke patients in replacing lost sensorimotor functions. It is, however, imperative before proceeding to clinical trials to study the long-term effects of this treatment in animal models of stroke. In this study, transplantation of hESC-derived neural progenitor cells (hNPCs) facilitated recovery of forelimb function in the cylinder task but not in the staircase task. Stereology and confocal analysis showed minimal survival of the grafted cells and differentiation into neuronal phenotype 2 months after the cortical transplantation.

Phenotype of hNPC prior to transplantation

The source and quality of cells to be grafted are considered notable challenges when designing transplantation protocols (Kondziolka *et al.*, 2002). After stroke, multiple types of brain cells are lost, which makes it necessary to transplant neural progenitors that are able to differentiate both into neurons and glial cells. Indeed, many studies report the use of ESC-derived neural stem/progenitor cells in animal models of stroke (Bühnemann *et al.*, 2006; Hayashi *et al.*, 2006; Kim *et al.*, 2007; Daadi *et al.*, 2008). In the present study, we used hESC-derived neural precursor cells that were differentiated as neurospheres, as described previously (Nat *et al.*, 2007; Sundberg *et al.*, 2008). The hNPC neurospheres used for transplantation were positive for Musashi, Nestin and Pax-6, confirming that they were at the neural precursor stage. The *in vitro* differentiation test showed that these cells differentiated into neurons rather than astrocytes, as shown by continuous time-lapse imaging and immunocytochemistry. The cell grafts used did not contain undifferentiated hESCs as confirmed by the absence of Oct-4 expression in RT-PCR, and no tumor formation was detected 2 months after transplantation.

EE

We examined the effects of EE housing rehabilitation on transplanted hNPCs, and the effects of the combination therapy on functional recovery after dMCAO in rats. Interestingly, EE has a number of positive effects on endogenous brain plasticity-related functions, such as increased dendritic branching, increased levels of growth factors in brain and enhanced neurogenesis (Dahlqvist *et al.*, 1999; Biernaskie & Corbett, 2001; Komitova *et al.*, 2005). Importantly, EE increases the pool of endogenous neural stem cells (Komitova *et al.*, 2005) and enhances migration of transplanted mouse neural stem cells (Hicks *et al.*, 2007) after MCAO in rats. In our study, however, housing animals in EE did not increase the survival, migration or differentiation of transplanted cells. Interestingly though, the Veh + EE group showed a delayed modest recovery in forelimb asymmetry during the second month, whereas the animals transplanted with hNPCs (with or without EE) had recovered slightly already during the first month after stroke. Thus, hNPCs may facilitate the process of functional recovery, although we did not detect significant differences between the vehicle groups and the cell-treated groups.

Functional recovery

To our knowledge there is only one study in which hESC-derived neuronal precursor cells were used together with rehabilitation after transient MCAO in rats (Kim *et al.*, 2007). In that study there were no clear benefits reported 3 weeks following transplantation. Contrary, in our study hNPC-transplanted animals, regardless of the housing paradigm, exhibited modest recovery in the forelimb asymmetry task, but no recovery was observed in reaching ability measured in the staircase task. In line with our result, a very recent study also showed improvement in the cylinder test 1 and 2 months after striatal transplantation of hESC-derived neural stem cells (Daadi *et al.*, 2008) after MCAO in rats, but did not, however, report any other behavioral tests for assessing functional recovery. In addition, Bliss and colleagues (Bliss *et al.*, 2006) reported that one out of four tests showed improved functional recovery after transplantation of human neural teratocarcinoma stem cells in MCAO rats. These findings together with our results highlight the importance of the use of multiple tests when assessing behavioral recovery in stem cell-transplanted animals.

The behavioral data did not correlate with the cell survival rate or *in vivo* differentiation, nor did the transplantation prevent tissue loss. Permanent occlusion of dMCAO resulted in well-defined injury in the

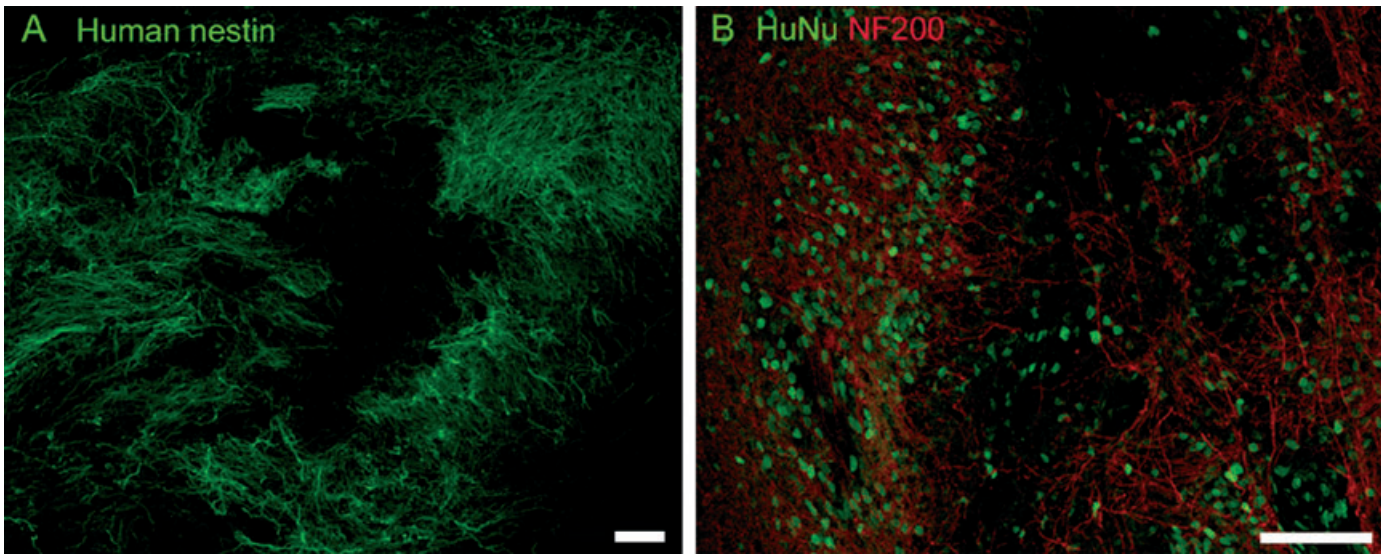

FIG. 6. Confocal micrographs of the hNPC deposits in the rat cortex. (A) The majority of the cells were human nestin-positive (green). (B) The deposit is enriched with NF-200-positive neurites (red) among the human nuclei cells (green) compared with the surrounding adjacent tissue. Scale bar: 100 μm. HuNu, human nuclei marker.

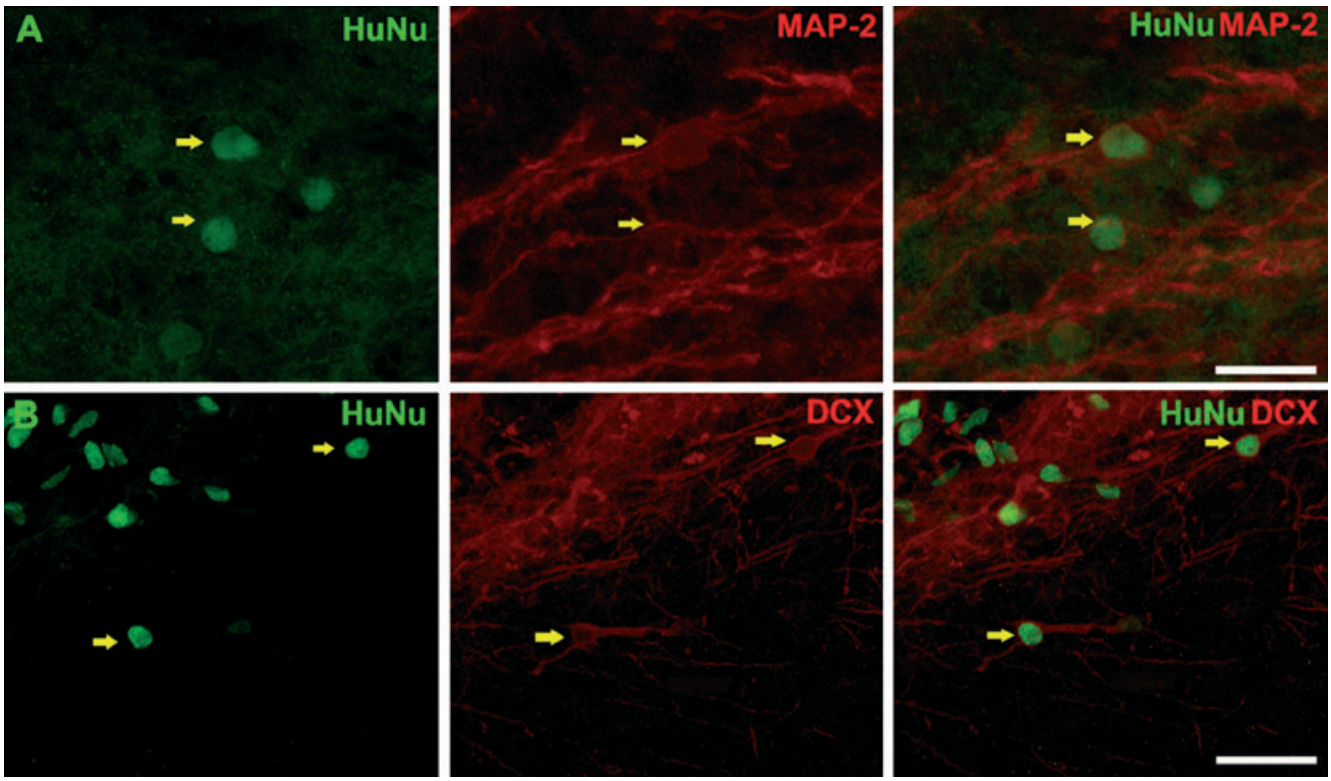

FIG. 7. Confocal analysis of the phenotypes of the transplanted cells. (A) 10.4% of human nuclei-expressing cells (green) co-localize with neuronal marker microtubule-associated protein-2 (MAP-2) in their neurites (red). (B) Doublecortin (DCX) was expressed in 10.8% (red) of the total human nuclei marker (HuNu; green) cells. Scale bar: 20 μm.

sensorimotor cortex, which lead to fairly similar deficits in all animals included in the study. Transient MCAO on the other hand results in both cortical and striatal injury, with variation between animals. Especially in the cylinder task, dMCAO-induced impairment was similar (SD < 5.2) in all groups as all the animals had a similar defect in the forelimb–hindlimb motor cortex. Thus, we chose cortical transplantation site to study possible effects of cortical regeneration on functional recovery.

The dMCAO animals with transplants performed similarly as the sham group, but not significantly better than the vehicle group in the cylinder test 1 and 2 months after transplantation, implicating that

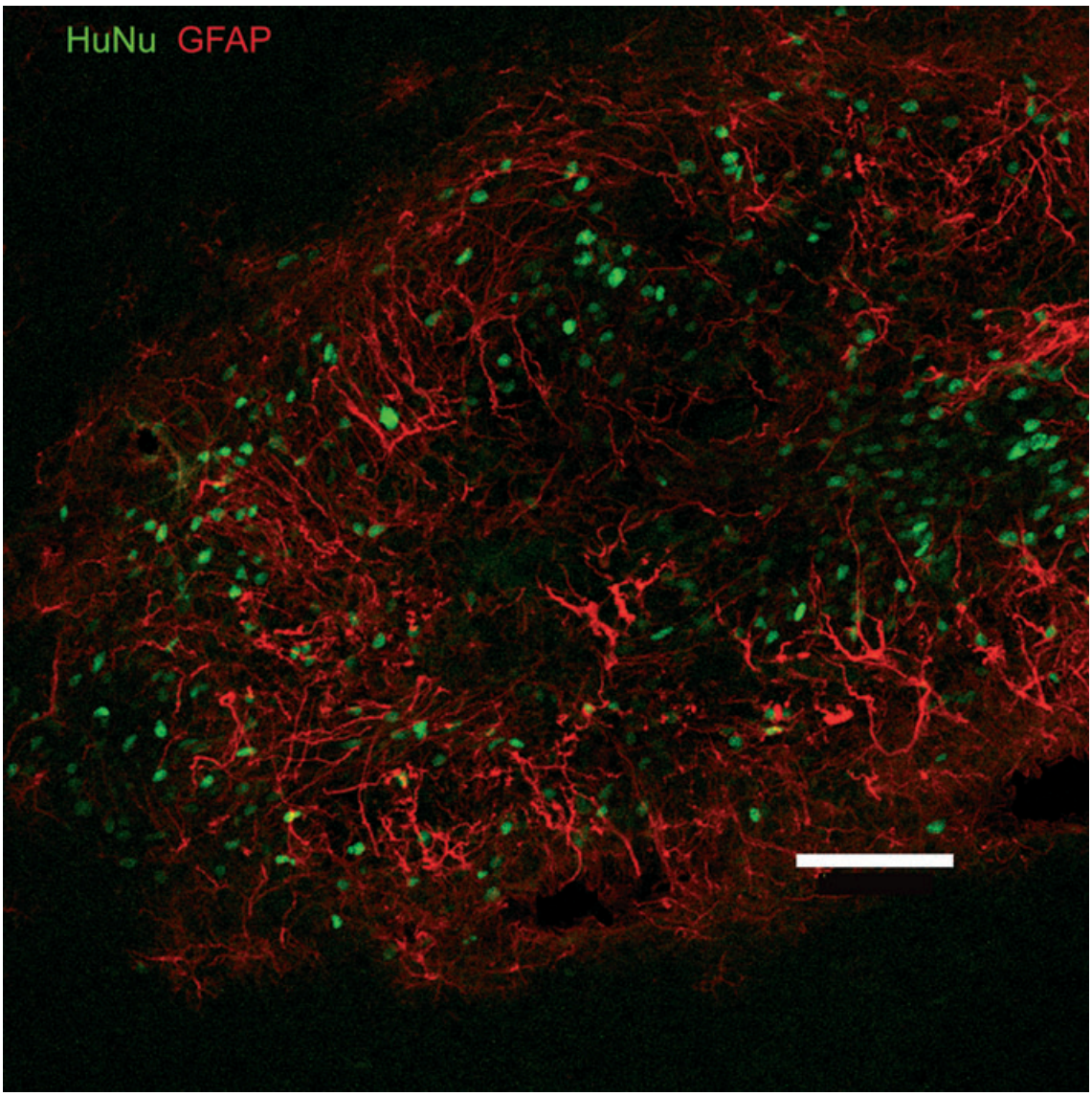

FIG. 8. hNPC transplants were strongly surrounded by host astrocytes in the rat cortex. Human nuclei-stained (green) neural cells are strongly surrounded by glial fibrillary protein (GFAP; red)-stained glial cells compared with adjacent weakly GFAP-stained host tissue in the motor cortex. Scale bar: 100 μm. HuNu, human nuclei marker.

the recovery was modest. Previously, we have shown that in the transient MCAO model no significant benefits were found in the cylinder task after both cortical and striatal transplantation (Hicks *et al.*, 2008). The forelimb asymmetry test measures the ability to use the impaired forepaw for postural support during vertical exploration (Schallert *et al.*, 2000). Postural support includes a variety of corticostriatal and corticospinal pathways (Kandel *et al.*, 2000a). Thus, this behavior tends to involve a wider range of motor circuits compared with reaching abilities. Plasticity mechanisms can spread to wider cortical areas (Kandel *et al.*, 2000b) from these pathways. Hence, it may be possible that instead of structural recovery some other compensatory mechanisms are responsible for the limited functional recovery seen here. The transplanted cells might have had an impact on local cortical microenvironment during post-ischemic cortical reorganization (Carmichael *et al.*, 2005; Carmichael, 2006), or the cells might have contributed to faster recovery by increasing growth factors in areas adjacent to transplant sites. Even though the levels of growth factors were not measured in our study, it is known that embryonic mouse stem cells secrete several cell growth and survival factors that can stimulate the growth and proliferation of other stem cells (Zhang *et al.*, 2006). Interestingly, when human neural stem cells were transplanted in parkinsonian monkey brain, they supported host neurons not by replacement but by promoting homeostatic adjustment of host dopamine neurons, such as normalizing neuron size (Redmond *et al.*, 2007).

The recovery here was restricted to only the cylinder task and not to the staircase reaching task. Recovery of forelimb reaching may require task-specific rehabilitation after cerebral ischemia (Biernaskie & Corbett, 2001) and after motor cortex injury (Ramanathan *et al.*, 2006), and that was not provided to animals in our study.

Survival of transplanted cells

The survival of the stem cells grafted after cerebral ischemia has, for the most part, been reported to be minimal. Kim and co-workers reported a variable graft survival without detailed quantification 3 weeks after hNPC transplantation into basal ganglia after MCAO

in rats (Kim *et al.*, 2007). Bühnemann and colleagues reported poor survival of mouse neural stem cells transplanted into the lesion cavity after 3 months in MCAO rats (Bühnemann *et al.*, 2006). It is quite common not to report quantification of the graft survival, which makes comparison of the studies difficult. Some studies, however, show that nearly 40% of neural stem/progenitor cells transplanted in the striatum or cortex in MCAO models survive (Kelly *et al.*, 2004; Bliss *et al.*, 2006; Darsalia *et al.*, 2007; Daadi *et al.*, 2008). Here, we showed that only about 1% of the transplanted cells survived 2 months after the transplantation, and that half of the transplanted animals had surviving human cells. The trypsination of hNPC neurospheres used in the present study may have impaired the capacity of the cells to integrate into the tissue, even though they continued to grow normally after replating *in vitro* (data not shown). As the cerebral environment after ischemia might not support stem cell survival (e.g. formation of glial scar tissue; Kim *et al.*, 2006; Molcanyi *et al.*, 2007), the poor survival of the grafts is a significant limitation in experimental stroke studies, and more attention should be given to studies trying to improve this aspect. Whether transplantation of small cell clusters instead of single cells would improve cell survival remains to be evaluated.

In our study, the majority of the transplanted hNPCs remained as nestin-positive neural precursors 2 months after the transplantation. Furthermore, ~10% of the surviving cells expressed neuronal marker MAP-2. Even though the deposit sites were heavily stained with the neurofilament marker NF-200, we were not able to specify if they originated from human cells or host cells. Only a minority of cells expressed the astrocytic markers S-100 or GFAP, or oligodendrocyte precursor marker NG2. Previous studies also support these results, as hESC-derived neural stem/precursor cells produce less astrocytes than cells derived from other sources (Bliss *et al.*, 2006; Daadi *et al.*, 2008). Also, a number of the surviving cells expressed DCX, which is often seen in migrating cells. The cells had not, however, migrated significantly from the transplantation sites.

It is also possible that the repulsive signals secreted by the ischemic tissue (Schwab *et al.*, 2005) altered the properties and the survival of the transplanted cells. As shown earlier, cells grafted in close proximity to the ischemic tissue have a poor survival rate (Kelly *et al.*, 2004), which may partially explain the loss of cells in our study. Moreover, the migration could have been prevented by a glial scar, which has been reported to form around the transplant site in ischemic brain (Kim *et al.*, 2006; Molcanyi *et al.*, 2007) and was also evident in our study. The site of transplantation may also play a role, as suggested by the study by Darsalia and colleagues where striatal injection resulted in better survival and migration of the cells (Darsalia *et al.*, 2007). So far, mostly minimal survival and neuronal differentiation have been reported following endogenous and exogenous stem cell therapies after cerebral ischemia (Lindvall & Kokaia, 2006; Kozlowska *et al.*, 2007), which suggests that it is imperative to study in detail the factors that hinder transplant survival and stem cell migration.

The sustained immune response in the brain activated both by the transplant and the existing brain injury hinder transplant survival. Indeed, according to our most recent study, transplantation of SVZ-derived mouse neural stem cells resulted in 1% of cell survival at 2 and 3 months in MCAO rats, and the survival was negatively correlated with microglial activation in the brain (Hicks *et al.*, 2008). Also, in the present study we detected ED-1-positive cells in the cortical areas surrounding the cell deposits, infarcted areas, and even in the corpus callosum adjacent to the infarcted areas. The use of xeno-graft also increases the host immune response, which makes the preclinical evaluation of human cell transplants difficult and may result in biased results. Even mouse-to-mouse transplantation showed that cells survived better when transplanted in the brain of immune-deficient mice than in immune-competent mice (Kim *et al.*, 2006). Also, very poor survival was reported 1 month after transplantation of human cord blood-derived stem cells after cortical stroke (Kozlowska *et al.*, 2007), most likely due to severe host reaction. It may be that transplanted cells are rejected in the long-term studies despite the administration of immunosuppressant drugs (in our study 5 mg/kg/24 h of cyclosporine A for 2 months). Commonly used immunosuppressant cyclosporine A restricts the immune response and facilitates transplant survival, even though it seems that a shorter immunosuppression seems to be as effective as prolonged administration (Wennersten *et al.*, 2006). The limited graft survival creates urgency for more supportive combination therapies with astroglial and immune response-restricting agents.

Conclusions

Transplantation of hESC-derived neural precursor cells into the cerebral cortex resulted in a modest functional recovery after dMCAO in rats. Survival of transplanted cells in the present study was minimal. The majority of the survived cells remained positive for neural precursor marker nestin, but a portion of cells differentiated in neuronal phenotypes during the 2 months follow-up. The poor survival of transplanted cells in the brain after cerebral ischemia, seen also in other studies, is a problem that has to be solved before proceeding in pre-clinical evaluations.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The primers used for the RT-PCR analysis for characterization of a subpopulation of hESC-derived neural precursor cells prior to transplantation.

Video Clip S1. Automated time-lapse imaging of living hESC-derived NPCs.

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Abbreviations

AP, anterior–posterior; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; DAB, diaminobenzidine; DCX, doublecortin; dMCAO, distal middle cerebral artery occlusion; DMEM, Dulbecco's modified eagle medium; DV, dorso-ventral; EE, environmental enrichment housing; GFAP, glial fibrillary protein; hESC, human embryonic stem cell; hNPC, human neural precursor cell; HuNu, human nuclei marker; MAP-2, microtubule-associated protein-2; MCAO, middle cerebral artery occlusion; ML, medio-lateral; NDS, normal donkey serum; NF, neurofilament; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG-400, polyethyleneglycol-400; PFA, paraformaldehyde; RT, room temperature; RT-PCR, reverse transcriptase-polymerase chain reaction; ST, standard housing; SVZ, subventricular zone.

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