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Academic Dissertation

Neural Stem Cells
interaction with the brain environment and prospects
for cell replacement therapy for Stroke

by

Emanuela Monni

With the approval of the Faculty of Medicine, Lund University, Sweden,
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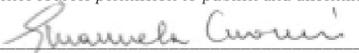


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Abstract <p>Stroke is an acute neurological condition and the leading cause of disability in adult humans. Treatments for efficient recovery are not available. The most common form of stroke results from the occlusion of the middle cerebral artery, which causes loss of brain parenchyma and many types of neurons as well as astrocytes and oligodendrocytes. Neural stem cells (NSCs) could potentially be used to develop novel therapies to restore loss of function after stroke.</p> <p>We generated a NSC line derived as monolayer cultures from the human fetal striatum, termed NS, and described the <i>in vitro</i> and <i>in vivo</i> potential of these cells. The derived hNS are very stable during expansion and efficiently generate neurons <i>in vitro</i> and <i>in vivo</i> upon transplantation into the rat neonatal brain.</p> <p>In order to establish successful NSCs-based therapies, factors such as the number of cells and the appropriate time of transplantation play an important role. We found that when transplantation of NSCs into the stroke-damaged striatum was performed at 48 hours after stroke, it resulted in better cell survival than did transplantation at 6 weeks. Increasing the number of grafted NSCs beyond a certain number did not result in a greater number of surviving cells or increased neuronal differentiation. Transplantation at 48 hours exposed the cells to a less hostile environment compared to 6 weeks following stroke. In the present thesis we reported that ES-derived NS cells fuse with microglia and cortical neurons both <i>in vitro</i> and <i>in vivo</i>. We found that microglia, the resident immune cells of the brain are important players in the fusion process. However further investigation is needed to understand the dynamics and the physiological relevance of this phenomenon.</p> <p>In summary, we have reported here previously undescribed characteristics of NS cells, which are relevant to better understanding of NSCs and their interaction with the brain environment after transplantation. Fusion could be of potential interest in the regenerative medicine due to the nuclear reprogramming implications but needs extensive investigation before it can be considered in the clinical setting. A relevant aspect of the cell-based therapy approach is that it could extend the therapeutic time window of intervention for ischemic stroke, which is now limited, thus benefiting a larger number of stroke-patients. Therefore, the findings described in this thesis have direct clinical implications.</p>		
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Academic Dissertation

Neural Stem Cells
interaction with the brain environment and prospects
for cell replacement therapy for Stroke

by

Emanuela Monni

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Lund 2015



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TABLE OF CONTENTS

ORIGINAL PAPERS	11
SUMMARY	15
SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING	16
ABBREVIATIONS	19
INTRODUCTION	23
STROKE	23
NEURAL STEM CELLS (NSCS)	24
<i>In vitro</i> expansion of human NSCs	25
Neurospheres	25
Monolayer cultures	25
TRANSPLANTATION OF STEM CELLS IN STROKE	25
Transplantation of NSCs	26
Transplantation of EScs	26
Transplantation of iPSCc	27
Other sources of non-neuronal origin	28
CELL FUSION	29
Physiological cell fusion	29
AIMS OF THE THESIS	31
MATERIALS AND METHODS	35
Human fetus-derived neural tissue isolation	35
Generation of monolayer cultures of NSC and derivation of NS cells	35
Generation and expansion of neurosphere cultures	35
Culture media for expansion of human NSCs	36
<i>In vitro</i> differentiation of human NS cells	36
<i>In vitro</i> differentiation of human neurospheres	37
Mouse NS and Fused NS/Microglia cells expansion	37
<i>In vitro</i> neuronal differentiation of mouse NS cells and Fused NS/ Microglia cells	38
Mouse and Rat Microglia culture	39
Mouse and Rat Primary Cortical Cell Culture	39
Human fetus-derived cortical cultures	40
COCULTURES	40
Primary Cells and NS cells coculture	40
Microglia and NS cells coculture	40
Primary cells and Microglia coculture	40
Mouse fetus-derived cortical neurospheres and Microglia	41

Mouse-derived cortical cells and Fused NS/Microglia cells	41
Human-derived cells and Microglia	41
Human-derived Cells and Fused NS/Microglia cells	41
ANIMAL PROCEDURES	42
Transplantation in rat and mouse pups	42
Transplantation of hNS into the striatum	42
Transplantation of mouse NS into the cortex	42
Lumafluor Injection	43
Middle Cerebral Artery Occlusion (MCAO)	43
Transplantation to adult stroke-damaged rats	43
FLUORESCENCE-ACTIVATED CELL SORTING	44
IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY	45
LIST OF PRIMARY ANTIBODIES	46
MICROSCOPY AND QUANTITATIVE ANALYSIS	47
ELECTROPHYSIOLOGY	47
RESULTS AND COMMENTS	51
CHARACTERISTICS OF A NEW HUMAN FETAL TISSUE-DERIVED NSC LINE	51
<i>In vitro</i> characteristics of proliferating human fetus-derived NS cells	51
<i>In vitro</i> differentiation potential of hNS cells	52
Differentiation potential of hNS cells after implantation into rat newborn striatum	52
FACTORS INFLUENCING SURVIVAL AND DIFFERENTIATION OF NSCs	53
GRAFTED IN STROKE-INJURED BRAIN	53
Effect of post-stroke delay and cell numbers on survival and migration of grafted cells	53
Effect of post-stroke delay and cell numbers on proliferation and differentiation of grafted cells	54
Characterization of the environment surrounding grafted Cells	54
OCCURRENCE AND MECHANISMS OF FUSION BETWEEN NS CELLS AND CORTICAL NEURONS	55
Fusion of NS cells with primary cortical neurons <i>in vitro</i> is mediated by microglia	55
Double-labeled cells result from fusion and not from transfer of fluorescent proteins between cells	55
Fused NS/Microglia cells display properties of NS cells	56
Neuronal differentiation potential of Fused NS/Microglia cells	56
Fused NS/Microglia cells express functional properties of microglia	56
Fusion of NS cells and microglia involves a phosphatidylserine-dependent mechanism	57

Transplanted NS cells fuse with mature neurons <i>in vivo</i>	58
GENERAL DISCUSSION	61
CHARACTERIZATION OF HUMAN FETAL STRIATAL-DERIVED NS CELL LINE GENERATED FROM NSCS	61
SURVIVAL AND DIFFERENTIATION OF GRAFTED NSCS IN STROKE- INJURED BRAIN	64
FUSION BETWEEN NS CELLS AND CORTICAL NEURONS	66
CONCLUDING REMARKS	71
ACKNOWLEDGMENTS	75
REFERENCES	83
APPENDIX	89
PAPER I	93
PAPER II	105
PAPER III	115

ORIGINAL PAPERS

The present thesis is based on the following papers:

- I. Fetal striatum-derived neural stem (NS) cells differentiate to mature neurons *in vitro* and *in vivo* (2014)
Current Stem Cell Research & Therapy 9(4): 338-346.
Monni, E., Cusulin, C., Cavallaro, M., Lindvall, O. and Kokaia, Z.
- II. Cell number and timing of transplantation determine survival of human neural stem cell grafts in stroke-damaged rat brain (2011)
Journal of Cerebral Blood Flow and Metabolism 31(1): 235-242.
Darsalia, V., Allison, S. J., Cusulin, C., **Monni, E.**, Kuzdas, D., Kallur, T., Lindvall, O. and Kokaia, Z.
- III. Embryonic stem cell-derived neural stem cells fuse with microglia and mature neurons (2012)
Stem Cells 30(12): 2657-2671.
Cusulin, C.*, **Monni, E.***, Ahlenius, H., Wood, J., Brune, J. C., Lindvall, O. and Kokaia, Z.

* Equal contribution

Summaries

SUMMARY

Stroke is an acute neurological condition and the leading cause of disability in adult humans. Treatments for efficient recovery are not available. The most common form of stroke results from the occlusion of the middle cerebral artery, which causes loss of brain parenchyma and many types of neurons as well as astrocytes and oligodendrocytes. Neural stem cells (NSCs) could potentially be used to develop novel therapies to restore loss of function after stroke.

We generated a NSC line derived as monolayer cultures from the human fetal striatum, termed NS, and described the *in vitro* and *in vivo* potential of these cells. The derived hNS are very stable during expansion and efficiently generate neurons *in vitro* and *in vivo* upon transplantation into the rat neonatal brain.

In order to establish successful NSCs-based therapies, factors such as the number of cells and the appropriate time of transplantation play an important role. We found that when transplantation of NSCs into the stroke-damaged striatum was performed at 48 hours after stroke, it resulted in better cell survival than did transplantation at 6 weeks. Increasing the number of grafted NSCs beyond a certain number did not result in a greater number of surviving cells or increased neuronal differentiation. Transplantation at 48 hours exposed the cells to a less hostile environment compared to 6 weeks following stroke.

In the present thesis we reported that ES-derived NS cells fuse with microglia and cortical neurons both *in vitro* and *in vivo*. We found that microglia, the resident immune cells of the brain are important players in the fusion process. However further investigation is needed to understand the dynamics and the physiological relevance of this phenomenon.

In summary, we have reported here previously undescribed characteristics of NS cells, which are relevant to better understanding of NSCs and their interaction with the brain environment after transplantation. Fusion could be of potential interest in the regenerative medicine due to the nuclear reprogramming implications but needs extensive investigation before it can be considered in the clinical setting. A relevant aspect of the cell-based therapy approach is that it could extend the therapeutic time window of intervention for ischemic stroke, which is now limited, thus benefiting a larger number of stroke-patients. Therefore, the findings described in this thesis have direct clinical implications.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Stroke är ett akut neurologiskt tillstånd och den främsta anledningen till funktionsnedsättning hos vuxna. Det finns ingen behandling för effektiv återhämtning. Den vanligaste formen av stroke orsakas av att mellersta storhjärnsartären blockeras vilket gör att man förlorar hjärnceller, till exempel neuroner.

Neurala stamceller (NSCs) skulle kunna användas för att återställa hjärnfunktionen efter stroke. Vi har därför skapat en neural stamcellslinje av celler från striatum, en del av hjärnan, hos mänskliga foster och beskrivit dessa cellers potential *in vitro* och *in vivo*. De är mycket stabila när man odlar dem och bildar effektivt neuroner *in vitro* och *in vivo* om de transplanteras in i hjärnan på nyfödda råttor. För att skapa en fungerande NSC-baserad behandling spelar också andra faktorer, som antalet transplanterade celler och tidpunkten för transplantationen, en avgörande roll.

I denna avhandling har vi undersökt hur de olika stadierna av neurogenesen, nybildandet av neuroner, påverkas av att mänskliga NSCs transplanteras in i striatum efter en stroke. Vi upptäckte att NSCs som transplanteras som neurosfärer överlevde i ett strokeskadat striatum, migrerade från transplantationsstället och bildade neuroner. NSCs som transplanterades in 48 timmar efter en stroke överlevde bättre än NSCs som transplanterades sex veckor efter stroke. Senarelagd transplantation påverkade inte migrationens omfattning, bildandet av neuroner eller celldelningen i transplantationsstället. Intressant nog upptäckte vi att man inte kunde öka antalet överlevande celler eller antalet bildade neuroner genom att transplantera fler NSCs.

Vi ville också undersöka den miljö som de neurala stamcellerna transplanterats till. Vi upptäckte en ansenlig mängd aktiverade mikroglia, de naturliga immuncellerna i hjärnan, 48 timmar efter skadan i striatum. Antalet mikroglia var dock som störst efter en till sex veckor, vilket visar att en senarelagd transplantation eventuellt hade exponerat cellerna för en skadlig miljö.

Dessa resultat är av stor klinisk relevans då antalet transplanterade celler och den optimala tidpunkten för transplantation kommer att bli viktiga parametrar när NSC-transplantation ska testas på strokepatienter i kliniken.

I denna avhandling har vi också rapporterat förekomsten av fusion mellan neuroner och mikroglia *in vitro* och *in vivo* efter transplantation av NSCs till rätt- och mushjärnor. Fusion kan vara intressant inom regenerativ medicin på grund av omprogrammeringen av cellkärnan, men kräver omfattande utredning innan den kan bli aktuell i kliniken. Vi upptäckte att mikroglia är en viktig del i

fusionsprocessen, men det behövs ytterligare studier för att förstå dynamiken och den fysiologiska betydelsen av fenomenet.

ABBREVIATIONS

BMDC	Bone Marrow Derived Cell
bFGF	basic Fibroblast Growth Factor
BrdU	Bromodeoxyuridine
CNS	Central Nervous System
DCX	Doublecortin
DARPP -32	Dopamine and cAMP-regulated phosphoprotein-32
EGF	Epidermal Growth Factor
ESC	Embryonic Stem Cell
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GABA	γ -amino Butyric Acid
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GLAST	Astrocyte-specific Glutamate Transporter
GW	gestational week
HBSS	Hank's balanced saline solution
KPBS	potassium phosphate buffered saline
IL	Interleukin
iPSC	induced Pluripotent stem cell
LGE	Lateral Ganglionic Eminence
LIF	Leukemia Inhibitory Factor
LPS	Lipopolysaccharide
MAP2	Microtubule Associated Protein 2
MCAO	Middle Cerebral Artery Occlusion
NeuN	neuron specific nuclear protein
NGF	Nerve Growth Factor
NS	Neural Stem
NSA	Neurosphere Assay
NSC	Neural Stem Cell
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PDL	poly-D-lysine
PLL	poly-L-lysine
PSA-NCAM	Polysialylated Neural Cell Adhesion Molecule
q-PCR	quantitative PCR
RFP	Red Fluorescent Protein
RG	Radial Glia
SDF-1	Stromal cell-Derived Factor-1
tPA	tissue Plasminogen Activator

Introduction

INTRODUCTION

Neural stem cell have the potential to provide novel therapies aimed at treating or reducing impairment for individual suffering from various neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and stroke. The ultimate goal of regenerative therapies is to derive sources of cells for replacement of damaged or dead cell in the injured brain of patients. The appropriate sources of stem cells for specific applications must be identified; likely only human-derived cells are candidate for use in the clinical setting. The optimal derivation conditions of neural stem cells (NSCs) lines should be assessed in order to achieve their long-term expansion and consistent reproducible differentiation potential. Ideally these cells must be capable of generating large numbers of the specific kinds damaged or lost under pathological conditions.

In this thesis we explore the influence of the *in vitro* and *in vivo* environment following transplantation of human and mouse NSC lines derived from the fetal striatum and embryonic stem cells, respectively. Furthermore, we assess how the survival of human NSCs grafted in the stroke-damaged rat brain is influenced by the cell number and the time delay of transplantation. We also investigate the potential for fusion of a mouse NSC line, termed NS, with microglial cells or mature neurons *in vivo* after transplantation.

STROKE

Stroke can be divided into two major types depending on the cause: ischemic and hemorrhagic. Hemorrhagic stroke results from intracerebral bleeding caused by rupture of a vessel in the brain. Ischemic stroke results from transient or permanent reduction in cerebral blood flow due to the occlusion of a cerebral artery by an embolus formed somewhere in the body, embolic stroke, or by a clot formed within a vessel, thrombotic stroke. Ischemic stroke is a leading cause of disability and second leading cause of death in the world (World Health Organization, 2012).

The only available treatment for ischemic stroke is tissue plasminogen activator (tPA), which dissolves the clot obstructing the artery in order to restore the brain blood flow, however this treatment is only affective when administered within 3 to 4.5 hours after the onset of stroke. Focal impairment of cerebral blood flow drastically reduces the supply of oxygen and glucose and as a consequence, a series of detrimental events take place such as cell respiration failure, excitotoxicity (augmented release of excitatory amino acids, mostly glutamate), anoxic depolarization, edema, generation and accumulation of free-radical species that

overwhelm endogenous scavenging mechanisms (Dirnagl, Iadecola, & Moskowitz, 1999; Moskowitz, Lo, & Iadecola, 2010). These processes result into massive neuronal and glial cells death by apoptosis and necrosis. The ischemic area is not homogeneously affected by the lack of blood supply. In the core of the ischemic territory, the reduction of the cerebral blood flow is most severe, while in the ischemic penumbra, which lies between the core and the intact normal brain tissue, the energetic metabolism is maintained though functionally compromised. An adequate collateral blood supply can hinder the degeneration of this area. The remaining surviving functionally integrated neurons in the penumbra have a limited synaptic plasticity and their contribution to reinstatement of the disrupted neuronal circuitries is inadequate (Iadecola & Anrather, 2011; Jackman & Iadecola, 2015).

The most commonly occluded vessel in humans is the middle cerebral artery (MCA) that supplies blood to the basal ganglia and cerebral cortex. Much of what is known about the pathobiology of ischemic stroke sprung out of animal research. The MCA occlusion (MCAO) model in animals closely mimics the characteristics and dynamics of the ischemic lesion in humans. In rodent models of MCAO the major site of lesion is the striatum, which represents the largest component of the basal ganglia. The GABA-ergic medium sized striatal projection neurons expressing the typical marker DARPP-32, which comprise the 90-95% of the neurons in the striatum, are the most vulnerable and are profoundly lost after the MCAO, although interneurons are also severely affected (Pulsinelli, 1985). In the prospect of stem cell replacement therapy in stroke, is it necessary to develop safe and reproducible sources of NSCs suitable for transplantation in patients. These NSC lines should be highly neurogenic, able to generate neurons of the appropriate types lost after the ischemic insult and capable of integrating in the damaged host cerebral neuronal network.

NEURAL STEM CELLS (NSCs)

Neurons, astrocytes and oligodendrocytes ultimately derive from a pseudostratified neuroepithelium of ectodermal origin; therefore neuroepithelial (NE) cells are the neural stem cells of the central nervous system (Gotz & Barde, 2005). In general terms, NSCs are the primary progenitors cells at different stages of development that initiate the three fundamental neural lineages leading to the formation of differentiated neurons or glial cells of the CNS (Kriegstein & Alvarez-Buylla, 2009).

***In vitro* expansion of human NSCs**

Neurospheres

Human NSCs and progenitor cells from different fetal and adult brain structures can be maintained and expanded like neurospheres or monolayer cultures *in vitro*. Neurospheres are free-floating spherical aggregates of cells responsive to mitogens such as EGF and/or bFGF. These aggregates are constituted of cells at different stages of maturation, which indicates that they are very heterogeneous. Supposedly only a small percentage of cells within the spheres retain the neurosphere-forming capacity over time and therefore can be considered true NSCs. The sensitivity to variations in the manipulation procedures and culture media and the diminished neurogenic potential after extensive passages (Ostenfeld et al., 2000) has limited the use of neurospheres for the study of biological processes (J. B. Jensen & Parmar, 2006).

Monolayer cultures

An important contribution to the NSCs research field came in 2005 with the development of non-immortalized, stable, long-term expandable, and multipotent NSCs lines termed called NS cells (Conti et al., 2005). NS cells are monolayer cultures of NSCs derived from embryonic stem cells, ESCs, and primary brain tissue. Mouse NS cells are characterized by symmetrical division and are capable of continuous expansion in the presence of mitogenic agents such as EGF and bFGF. Upon growth factors removal, they have the capability to differentiate into neurons and astrocytes *in vitro* and *in vivo* after transplantation into the adult rodent brain (Conti et al., 2005) and oligodendrocytes *in vitro* (Glaser, Pollard, Smith, & Brustle, 2007). NS are not depending on a niche of surrounding cells for their survival and perpetuation (as the case with NSCs grown as neurospheres). Taken together, the characteristics of NS cells make them suitable for transplantation studies in which neuronal replacement is the main objective.

TRANSPLANTATION OF STEM CELLS IN STROKE

Stem cell-based approaches could potentially developed into clinical strategies aimed at promoting the recovery of stroke patients. However, stem cell-based therapies for stroke are not currently available. The clinical trials with cell therapy in patients suffering from Parkinson disease (PD), a focal CNS disorder that leads to progressive loss of the nigrostriatal dopaminergic neurons, have provided proof of principle that the neuronal replacement-strategy is feasible in the human brain

and can induce long-lasting improvement (Lindvall & Bjorklund, 2004). Cell-replacement for treatment of stroke is somehow more challenging to that for PD. The cellular damage following stroke typically involves diverse neuronal and glial cell types and neurotransmitters, therefore it is necessary to identify appropriate cell sources capable of giving rise, upon differentiation, to a wide range of neuronal phenotypes that can functionally integrate into the damaged host brain circuitry.

Transplantation of NSCs

Several studies have demonstrated survival, neuronal differentiation and functional recovery after transplantation of NSCs of human origin in animal models of stroke.

Neurons generated from transplanted human NSCs could potentially replace GABA-ergic striatopallidal medium spiny projection neurons or interneurons that are injured or lost during an ischemic event. Human NSCs expanded *in vitro* as neurospheres, migrate and differentiate into mature neurons after transplantation in the stroke-damaged rat cortex (Kelly et al., 2004) and striatum (Darsalia, Kallur, & Kokaia, 2007) retaining their *in vitro* region-specific differentiation potential (Kallur, Darsalia, Lindvall, & Kokaia, 2006). A report by Andres et al. (Andres et al., 2011) shows enhanced axonal transport, dendritic branching and axonal sprouting of host neurons at 5 weeks after stroke following transplantation of human NSCs, which correlated with the induced behavioral recovery. In addition to cell-replacement mechanisms, intracerebral grafts of NSCs can exert beneficial effects through trophic actions, neuroprotection, modulation of inflammation, promotion of endogenous neurogenesis through enhancement of subventricular zone (SVZ) cells proliferation, thus contributing to a long-term enhancement of several steps of striatal neurogenesis and ameliorating neurological deficits (Mine et al., 2013).

Overall, in the prospect of establishing stem cell-replacement therapies, human NSCs derived from the human fetal brain are promising candidates due to their capacity of differentiating into mature neurons, innervate the regions of interest, and contribute to the restoration of the stroke damaged neuronal circuitry.

Transplantation of ES cells

NSCs with self-renewal capacity and differentiation potential within the neural lineage can be derived from ESCs. Human ES cells can give rise to unlimited numbers of NSCs but are associated with a risk of tumor formation (Seminatore et al., 2010) (Erdo et al., 2003). However, many studies show that if human ES cells are predifferentiated and/or fated *in vitro* towards the neuronal lineage, the formation of tumors after transplantation is virtually abolished. Human ES cells have been

used in recent years for the generation of different types of neurons (Buhnemann et al., 2006; Daadi et al., 2009; Ma et al., 2012) NSCs derived from ES cells implanted into the rat stroke-damaged striatum were able to migrate towards the infarct area, expressed neuronal markers such as Tuj1 and GABA, received synaptic connectivity from host neurons and induced functional improvement (Daadi et al., 2009).

Following transplantation into the rat stroke-injured cortex, the majority of the transplanted human ES-derived NSCs cells were positive for the neural precursor marker nestin and only ~10% of cells expressed the neuronal markers MAP2 at 2 months after transplantation, showing limited survival and differentiation potential (Hicks et al., 2009). Ma et al (Ma et al., 2012) showed that human ES cells could be efficiently differentiated into striatal GABA-ergic neurons by tuning a particular dose of sonic hedgehog or purmorphamin. When these cells were transplanted into the striatum of quinolinic acid-lesioned mice, they generated predominantly DARPP-32+ GABA-ergic neurons. Interestingly, these human ESC-derived GABA medium sized spiny neurons projected to the substantia nigra, received glutamatergic and dopaminergic inputs, and ameliorated impaired motor function. The possibility of generating striatal projection neurons makes NSCs derived from ESCs an attractive source for implantation in the stroke-damaged brain.

Transplantation of iPSCs

The seminal work by Takahashi and Yamanaka demonstrated that it is possible to reprogram fibroblasts into pluripotent stem cells, iPSCs, by introduction of transcription factors (Takahashi & Yamanaka, 2006). The iPSCs possess the capacity to differentiate into specific neuron types such as dopaminergic neurons (Hargus et al., 2010; Wernig et al., 2008) and motor neurons (Dimos et al., 2008). The iPSCs technology allows for the derivation of patient-specific cell lines, which would eliminate the concern of immunorejection. Another benefit of iPSCs is that the ethical issues associated with the use of human ES and fetus-derived cells are avoided. Human fibroblast-derived iPSCs grafted into the striatum of stroke-damaged rats migrate to the infarct areas and improve sensorimotor recovery at 4–16 days following transplantation, however it is not clear whether these cells differentiated into neurons (Jiang et al., 2011). Another study did not detect any effect of human fibroblast-derived iPSCs on stroke-induced behavioral although about 80% of the differentiated cells expressed the neuronal markers MAP2 and β III-tubulin (M. B. Jensen, Yan, Krishnaney-Davison, Al Sawaf, & Zhang, 2013). Motor function was restored by transplanting human iPSC-derived NSCs into the marmoset spinal cord injury model, and long-term motor function was recovered without observable tumor formation (Kobayashi et al., 2012).

Long-term expandable neuroepithelial-like stem (lt-NES) cells generated from iPSCs, exhibit extensive self-renewal, clonogenicity, and stable neurogenesis (Falk et al., 2012). The human iPSC-derived lt-NES cells transplanted into the stroke-damaged mouse and rat brain generated neurons with mature morphological properties and electrophysiological characteristics of functional neurons *in vivo*. Intrastratial transplantation of lt-NES cells into the mouse stroke-damaged brain improved motor recovery (Oki et al., 2012). When lt-NES cells were fate to the cortical phenotype *in vitro* they showed efficient conversion to mature electrophysiologically functional cortical neurons following transplantation into the rat stroke-damaged cortex (Tornerio et al., 2013). Therefore, lt-NES cells show a high degree of lineage plasticity and neurogenic potential that makes these cells suitable for cell-replacement approaches.

Other sources of non-neuronal origin

Stem cells of non-neural have been transplanted in animal models of stroke and neurodegenerative disease. Stem cells from umbilical cord blood, bone marrow-derived hematopoietic stem cells (BMDCs) and mesenchymal stem cells (Vu, Xie, Eckert, Zhao, & Cramer, 2014) have been shown to influence and reduce stroke-induced functional impairments in rodents through mechanisms other than neuronal replacement (Park et al., 2009). Mesenchymal stem cells can promote angiogenesis in the stroke subjected mouse brain (Zhu et al., 2015). Overexpression of VEGF in mesenchymal cells enhanced angiogenesis and improved functional recovery in rodents (H. J. Lee, Kim, Park, & Kim, 2007; Onda et al., 2008). Genetically modified mesenchymal stem cells expressing glial cell line-derived neurotrophic factor (GDNF) provided neuroprotection against injury in a cerebral ischemia model in the adult rat (Horita et al., 2006) and ameliorated impaired neurological function (Nomura et al., 2005).

In a long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke, clinical improvement in the group of patients that received the graft was associated with serum levels of stromal cell-derived factor-1(SDF-1) α at the time of MSC treatment (J. S. Lee et al., 2010), however this study was not double-blinded and therefore placebo effects could not be excluded.

CELL FUSION

Cell fusion is a highly specialized process that has an important biological role in the development, physiology and disease of multicellular organisms by which two cells merge to give rise to a single cell. When fusion occurs between cells of the same type the process is called a homotypic cell fusion. The process is referred to as heterotypic cell fusion if two different kinds of cells are involved. The cell resulting from fusion can bear one nucleus, synkaryon, if the nuclei themselves fuse, or two nuclei, heterokaryon.

Physiological cell fusion

Cell fusion is essential for the normal development of organisms. A good example of cell fusion naturally occurring is in higher organisms is the fusion between the oocyte and the sperm, which gives rise to the zygote (heterotypic fusion). In the developing organism, myoblasts fuse to form multinucleated skeletal muscle fibers, while cells of monocytic origin fuse to form osteoclasts, which participate in bone sculpturing. Cell fusion does not only play a role in the development, but is also indispensable in plenty of processes such as tissue repair and homeostasis maintenance. As an example, macrophages can fuse to give rise to multinucleated giant cells with improved phagocytic capabilities, osteoclasts and giant cells, which are involved in bone resorption and immune response, respectively (Chen & Olson, 2005; Dittmar & Zanker, 2011; Larsson, Bjerregaard, & Talts, 2008).

Very few reports have dealt with fusion of NSCs under normal and pathological condition and in the context of inflammation. Therefore, the fusogenic capacity *in vitro* and *in vivo* after transplantation of NSCs is largely unknown and needs to be investigated.

AIMS OF THE THESIS

- Characterize a new human NSC line, so-called NS cells, isolated from the fetal striatum, *in vitro* under proliferation and differentiation conditions and *in vivo* after transplantation into the neonatal rat brain
- Determine the optimal number and timing of transplantation for survival of human fetal-derived NSCs grafted in the stroke-damaged rat striatum
- Investigate the occurrence and mechanism of fusion between mouse NS cells and primary cortical cells *in vitro* and *in vivo* following implantation into the neonatal rat or mouse brain

Materials and Methods

MATERIAL AND METHODS

Human fetus-derived neural tissue isolation

Neural tissue from dead, aborted human fetuses, aged 7-9 weeks post-conception, was obtained from Lund and Malmö University Hospitals according to guidelines approved by the Lund/Malmö Ethical Committee.

The dissection was conducted under a stereomicroscope (Leica, Germany) in hibernation media (Apoteksbolaget AB, Sweden). The CNS was removed and cleaned from surrounding tissue. The cortex was cut open along and close to the dorsal midline and the striatum was dissected out followed by the removal of the cortex.

Generation of monolayer cultures of NSC and derivation of NS cells

The fetus-derived cortex and striatum were incubated for 20-30 min at +37°C in Euromed-N (Euroclone), and mechanically dissociated until a single cell suspension was obtained. The cells were plated at a density of 10^5 cells/mL in expansion medium (see Table 1). After 18-24 hours neurospheres had formed. One week after plating, neurospheres were dissociated with the enzyme Accutase (PAA Laboratories), mechanically triturated until a single cell suspension was obtained and finally plated at clonal density. After 2-3 days, these cells formed secondary neurospheres, which were plated on laminin-(10 μ g/mL, Sigma-Aldrich) coated dishes, giving rise to monolayer culture of hNS cells. The hNS cells were routinely passaged 1:3/1:5 every 5 to 7 days by Accutase treatment to detach and replated on laminin-coated culture dishes.

Generation and expansion of neurosphere cultures

The fetus-derived striatal tissue was incubated for 20-40 min at +37°C in expansion medium (see Table 2) followed by mechanical dissociation. The cells were counted using the Trypan blue exclusion method and then plated at a density of 10^5 cells/mL in expansion medium for neurospheres as in Table 1. Primary neurospheres were visible after about 24 hours. When the core of the spheres had turned dark, which would happen around two weeks after the initial plating of the cells, the neurospheres were passaged. To do that, neurospheres were pelleted by centrifugation and exposed to Accutase for 10 minutes at room temperature. Subsequently, Accutase was removed and the neurospheres were dissociated mechanically in expansion medium by trituration until a single cell suspension was obtained. Cells were counted and replated at 10^5 cells/mL in expansion medium.

Culture media for expansion of human NSCs

Table 1. Expansion medium for NS cells

Components	Supplier	Concentration
Euromed-N	Euroclone	1:1
N2 supplement	Gibco, Lifetechnologies	1%
B27 supplement	Gibco, Lifetechnologies	2%
EGF	R&D Systems	20ng/mL
bFGF	R&D Systems	20ng/mL

Table 2. Expansion medium for neurospheres

Components	Supplier	Concentration
DMEM/F12	Gibco, Lifetechnologies	1:1
L-glutamine	Gibco, Lifetechnologies	2mM
Hepes	Gibco, Lifetechnologies	15mM
NaHCO3	Sigma-Aldrich	7.5%
Glucose	Sigma-Aldrich	0.6%
Heparin	Sigma-Aldrich	2µg/mL
N2 supplement	Gibco, Lifetechnologies	1%
EGF	R&D Systems	20ng/mL
bFGF	R&D Systems	10ng/mL
LIF	Sigma-Aldrich	10ng/mL

In vitro differentiation of human NS cells

Prior to inducing differentiation, the hNS cells were plated onto poly-L-ornithine/laminin-coated (both 10µg/mL, Sigma-Aldrich) chamber slides (Labtek, Nunc) or glass coverslips and maintained in expansion medium for 2 days. The cells were then shifted to a differentiation protocol. Euromed-N (Euroclone, Italy) containing 0.5% N2, 1% B27 and 20ng/mL bFGF was used for 1 week to initiate the neuronal induction. The cells were cultured for another week in the same expansion medium containing half the concentration of bFGF (10ng/mL). During the third week of differentiation, the medium was composed of a 1:1 mixture of Euromed-N and Neurobasal (Gibco), with 2% of B27, 5ng/mL bFGF and 5ng/mL BDNF (Lifetechnologies). Subsequently, the cells were cultured one more week

in Neurobasal medium containing 2% of B27 and 10ng/mL BDNF. At this stage, the differentiation was considered complete and the slides were processed for immunocytochemical analysis.

***In vitro* differentiation of human neurospheres**

Three to four days after passage, small neurospheres were plated onto poly-L-Lysine(PLL)- coated chamber slides with expansion medium devoid of heparin. The spheres were allowed to attach for 24 hours and then the medium was replaced by the differentiation medium, which consisted of proliferation medium without growth factors.

Mouse NS and Fused NS/Microglia cells expansion

Mouse ES-derived NS cells (LC1) (Conti et al., 2005) and mouse Fused NS/Microglia sorted GFP+/RFP+ cells were maintained, on uncoated and poly-L-ornithine/laminin-coated flasks, respectively in expansion medium, NS-med as reported in Table 3. Unless otherwise stated, we will refer to ES-derived LC1 cells as mouse NS cells. Mouse fetal cortex-derived NS cells (Cor1) (Conti et al., 2005) were grown on laminin-coated flasks (3µg/ml, Lifetechnologies) in the same mouse NS medium.

Table 3. NS-med expansion medium for mouse NS and Fused NS/Microglia cells

Components	Concentration
Euromed-N	1:1
N2 supplement	1%
EGF	20ng/mL
bFGF	20ng/mL

***In vitro* neuronal differentiation of mouse NS cells and Fused NS/Microglia cells**

Mouse NS cells and Fused NS/Microglia cells were plated onto laminin-coated glass coverslips (3µg/mL) and differentiated following a three-step protocol according to Spiliotopoulos et al., (Spiliotopoulos et al., 2009) with minor modifications as in Table 4.

Table 4. Neuronal differentiation protocol for mouse NS and Fused NS/Microglia

Step	Differentiation medium	Components	Time exposure
1	D1	Euromed 1:1 B27 1% N2 0.5% bFGF 10ng/mL	3 days
2	A	DMEM/F12-Neurobasal 1:3 B27 1% N2 0.5% bFGF 10ng/mL BDNF 20ng/mL (Lifetechnologies)	3 days
3	B	DMEM/F12-Neurobasal 1:3 B27 1% N2 0.5% bFGF 6.7ng/mL BDNF 30ng/mL	3 days
	B terminal differentiation	DMEM/F12-Neurobasal 1:3 B27 1% N2 0.5% bFGF 5ng/mL BDNF 30ng/mL	10-15 days

Mouse and Rat Microglia culture

Primary microglia were isolated from brains of neonatal Wistar rats (Taconic, Bomholt, Denmark) according to Giulian and Baker (Giulian & Baker, 1986) from transgenic mice expressing green fluorescent protein (GFP) under chicken β -actin promoter (Okabe, Ikawa, Kominami, Nakanishi, & Nishimune, 1997). Primary cultures were maintained in microglia medium (Micro-med) consisting of DMEM/F12 supplemented with Glutamax, 10% fetal bovine serum (FBS), and penicillin-streptomycin (all from Gibco). Microglia cells were plated on PLL-coated chamber-slides (Lab-Tek, Nunc) or glass coverslips (20,000 cells per cm^2). These cultures contained $93.1\% \pm 1.4\%$ Iba1+ microglia, and $1.0\% \pm 0.8\%$ glial fibrillary acidic protein (GFAP)+ astrocytes. In case of rat microglia, cells were labeled using PKH26 (Sigma-Aldrich) according to manufacturer's instructions. BV2 mouse microglia cell line was obtained from Interlab Cell Line Collection (Genova, Italy). Cells were grown on uncoated plastic flasks in a medium based on RPMI 1640, containing 10%FBS and 2mM glutamine (all from Gibco, Lifetechnologies). The BV2 cells could be maintained in NS-med, without any noticeable sign of cell death or damage. For experiments, BV2 cells were infected with either CAG-GFP or CAG-RFP retrovirus.

Mouse and Rat Primary Cortical Cell Culture

Cortical tissue from E17 β -actin-GFP mice [18] or E18 Wistar rats (Taconic) was used to isolate primary cells. These cells were plated on poly-D-lysine (PDL)/ laminin-coated slides at 20,000 cells per cm^2 in primary medium, Pri-med (see Table 5).

Table 5. Pri-med medium for mouse and rat primary cortical cultures

Components	Concentration
Neurobasal	1:1
B27	2%
Glutamine	0.5mM
Penicillin-Streptomycin	1%

Human fetus-derived cortical cultures

Primary cortical human fetus-derived cultures from dead aborted human fetuses 8 weeks post-conception were derived as described above. Cortical cells were plated on PDL/laminin-coated glass coverslips (20,000 cells per cm²) and maintained in Pri-med (see Table 4) but without antibiotics. For some experiments human cortical primary cultures were transduced with CAG-GFP retrovirus.

COCULTURES

Primary Cells and NS cells coculture

Seven to twelve days after plating primary cells, NS cells were plated on top (10,000 cells per cm²) in Pri-med or NS-med for 1–3 days. For one set of experiments, rat primary cells were treated with 10nM Mac1-Saporin (Advanced Targeting Systems) or control mouse anti- Mac1 antibody (Advanced Targeting Systems) during the 5 days prior to the coculture, and analyzed 3 days thereafter. Primary cortical cells, isolated from E17 Wistar rats, were labeled with PKH-26 (Sigma) and plated on PDL/laminin- coated glass coverslips (20,000 cells per cm²) in Pri-med. After 12 days of culture, cells were treated with 10nM Mac1-Saporin for 4 days. Primary microglia isolated from P2 Wistar rats were plated on half of the microglia-depleted primary cortical cultures at 20,000 cells per cm² and cocultured for 5 days. A fraction of isolated microglia analyzed before the coculture showed that virtually all cells were immunopositive for the microglia marker Iba1. After 5 days of coculturing, GFP+ NS cells were plated onto the microglia-depleted primary cultures for additional 4 days.

Microglia and NS cells coculture

NS cells were plated on microglial cultures as described above, maintained in NS- med, and analyzed 1–5 days thereafter. To label microglia with bromo-2-deoxyuridine (BrdU), cells were cultured for 5 days with 10 μM BrdU (BD Biosciences, San Diego, CA). For AnnexinV experiments, cocultures were performed in presence of 2.5% AnnexinV (Lifetechnologies).

Primary cells and Microglia coculture

GFP-positive CAG-retrovirus infected microglia were plated on 7–12 days old primary cell cultures at a density of 20,000 cells per cm² in Pri-med and analyzed 1–3 days thereafter.

Mouse fetus-derived cortical neurospheres and Microglia

GFP-CAG-retrovirus infected mouse fetal cortex-derived neurospheres (Conti et al., 2005) were plated onto PLL-coated flasks at about 20,000 cells per cm². After 24 hours, RFP+ microglia were plated on the top of attached neurospheres (20,000 cells per cm²). Cocultures were maintained under adherent proliferation conditions in NS-med for 3–6 days and treated daily with Interleukin(IL)-4 10ng/ml (R&D Systems). After 3–5 days, cocultures were disaggregated to single cells and plated onto poly-L-ornithine/laminin-coated glass coverslips (20,000 cells per cm²). After 1 hour cells were fixed and processed for immunocytochemistry.

Mouse-derived cortical cells and Fused NS/Microglia cells

Primary cortical cultures were derived from postnatal day 1 BALB/c mouse. Cells were labeled with CellVue Claret (Sigma-Aldrich), plated onto PDL/laminin-coated glass coverslips (20,000 cells per cm²) and maintained in Pri-med. After 20 days, fused NS/microglia (GFP+/RFP+) cells (20,000 cells per cm²) were plated on top of the primary cortical cultures in Pri-med and cultured for 5 days, and then processed for fluorescent-activated cell sorting (FACS).

Human-derived cells and Microglia

Human fetal cortex-derived GFP+ cells expanded as neurospheres, human fetal cortex-derived cells expanded as NS cells, and human iPSCs-derived It-NES cells were plated on PDL, PDL laminin- or PLL-coated flasks, respectively, at a density of approximately 20,000 cells per cm². After 24 hours, RFP+ microglia cells were plated on top of the cultures (20,000 cells per cm²). After 3–5 days, cocultures were analyzed for fusion using immunocytochemistry.

Human-derived Cells and Fused NS/Microglia cells

Differentiated human iPSCs-derived It-NES cells labeled with CellVue and human fetal cortex-derived primary cortical cells were plated (20,000 cells per cm²) onto poly-L-ornithine/laminin- and PDL laminin-coated glass coverslips, respectively. After 20 days of culture, GFP+/RFP+ fused NS/microglia cells were plated on top of the primary cultures, cocultured for 4–5 days, and then analyzed thereafter by immunocytochemistry.

ANIMAL PROCEDURES

Experimental procedures were approved by the Lund/Malmö Ethical Committee and conducted in accordance with European Union declaration on the subject of animal rights.

Transplantation in rat and mouse pups

Wistar rats pups (Scanbur BK AB, Sollentuna, Sweden) and C57BL/6 mouse pups (Taconic) were anaesthetized by placing them on ice and lowering body temperature to +1-3°C. Animals were placed in a cooled Cunningham's stereotaxic frame and skull surface was aligned in the horizontal plane. The cell suspension was injected through a glass capillary (inner diameter ~50µm) connected to a Hamilton syringe. Following the transplantation, the needle was kept in place for 5 min. Wounds were closed with surgical suture, and animals were resuscitated by slowly increasing body temperature to normal level. Litters were kept together with mother during weaning period and were housed under 12 h light/12 h dark cycle with ad libitum access to food and water.

Transplantation of hNS into the striatum

Postnatal day 2-3 Wistar rats newborns received GFP-labeled hNS cells or unlabeled cells. The cell suspension (1µl) was injected into the right striatum at the following coordinates:

AP +0.5mm; L -2.0 from midline; V -3.0 mm below brain surface.

Transplantation of mouse NS into the cortex

Two to 3 days old Wistar rat pups were injected with mouse NS cell suspension (1 µl) unilaterally into cortex at the following coordinates:

AP +0.5; L -2.0 from midline; V -0.8 mm from brain surface

Rats were euthanized either at 1 week or 4 weeks after transplantation.

Four to 5 days old C57BL/6 mouse pups (Taconic) were subjected to a similar transplantation procedure at following coordinates:

AP +2.5; L -1.1 from midline; V -0.6 mm from brain surface and processed after 1, 2, 4, 6, or 8 weeks.

Lumafluor Injection

Two days old C57BL/6 mouse pups were injected with 0.4 μ l of Lumafluor RedBeads (Lumafluor Inc) in the left dorsolateral striatum at following coordinates: AP +2.1; L +1.2; V -1.2. Two days later, animals received transplants in the right cortex as described above and were sacrificed 8 weeks thereafter.

Middle Cerebral Artery Occlusion (MCAO)

Prior to inducing MCAO by the intraluminal filament technique (Kokaia et al., 1995; Zhao, Memezawa, Smith, & Siesjo, 1994) adult rats were fastened overnight with ad libitum access to water. Anesthesia was induced by inhalation of isofluorane and a mixture of O₂ and N₂O. The right common carotid artery was isolated and ligated proximally together with the external carotid artery. The internal carotid artery was temporarily closed with a microvascular clip. A nylon filament with a rounded tip was inserted through the common carotid artery into the internal carotid artery until resistance was felt, which indicates that the filament had passed the origin of the middle cerebral artery. Wounds were temporarily closed and animals were allowed to wake up. After 30 minutes of occlusion the animals were re-anesthetized and the filament was then withdrawn permanently. The skin incision was sutured. Temperature was regulated during the surgical procedure and during the following 2 hours.

Transplantation to adult stroke-damaged rats

Forty-eight hours or 6 weeks after the MCAO surgery, rats received human striatal neurospheres into the striatum. The time of grafting and numbers of transplanted cells are summarized in Table 6. Intact rats were also grafted. Animals were anesthetized with isofluorane and placed in the stereotaxic frame where the skull was opened with a drill. The animals were injected with neurospheres suspension that had the concentration of either 100,000 or 250,000 viable cells/ μ L by the use of a Hamilton syringe at a speed of 0.5 μ L/minute. The neurosphere suspension was kept on ice throughout the transplantation procedure.

A volume of 1.5 μ L of neurospheres suspension was delivered to the right striatum of each rat ipsilateral to the site of injury at either two sites at following coordinates:

+ 0.5 mm anterior and 3 mm lateral from bregma and 5 mm from brain surface

0.5mm posterior and 3mm lateral from bregma and 5 mm from brain surface

Or at four sites at the following coordinates:

0.5mm anterior and 3mm lateral from bregma and 4 or 6 mm from brain surface

0.5 mm posterior and 3 mm lateral from bregma and 4 or 6 mm from brain surface.

A total of 300,000, 750,000 or 1,500,000 cells were grafted.

Rats were euthanized for immunohistochemical analyses at 4 hours, 1, or 5 weeks after transplantation. All animals received 10 mg/kg Cyclosporine-A subcutaneously every second day from the day of transplantation.

Table 6. Time of grafting and numbers of transplanted cells

Number of animals	Time of grafting after MCAO	Number of deposits	Density of neurosphere suspension	Number of grafted cells	Time of euthanizing after grafting
3	48 hours	2	100,000 cells/ μ L	300,000	4 hours
4	48 hours	2	100,000 cells/ μ L	300,000	1 week
8	48 hours	2	100,000 cells/ μ L	300,000	5 week
8	48 hours	2	250,000 cells/ μ L	750,000	5 week
5	48 hours	4	250,000 cells/ μ L	1,500,000	5 week
4	6 weeks	2	100,000 cells/ μ L	300,000	4 hours
5	6 weeks	2	100,000 cells/ μ L	300,000	1 week
5	6 weeks	2	100,000 cells/ μ L	300,000	5 week
4	Intact	2	100,000 cells/ μ L	300,000	1 week
6	Intact	2	100,000 cells/ μ L	300,000	5 week
5	Intact	–	–	–	–

FLUORESCENCE-ACTIVATED CELL SORTING

FACS was performed on FACSAria SORP system with FACS Diva v6.0 software (BD Biosciences). Doublets were excluded via gating through both forward scatter-height versus width and side scatter-height versus width. The gate identifying fused (GFP+/RFP+) cells was set according to non-GFP/RFP expressing controls. Gate locations were verified using cocultures of non-GFP/RFP expressing cells. The gate identifying triple GFP+/RFP+/CellVue+ cells was set according to fused GFP+/RFP+ cultures and CellVue-labeled mouse cortical cells. Gate locations were verified using cultures of fused GFP+/RFP+/CellVue+ cells as well as cocultures of microglia (RFP+), NS (GFP+), and cortical (CellVue+) cells and unlabeled cocultures

of microglia, NS, and cortical cells. Correct sorting of the gated double- and triple-positive cell population was confirmed by FACS reanalysis and fluorescence microscopy. Flow cytometric data were analyzed using FlowJo software (Tree star, Ashland, OR).

IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Cell cultures were fixed using 4% paraformaldehyde (PFA) for 20 minutes, with addition of 0.2% glutaraldehyde for GABA staining. Animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with 4% ice-cold PFA. Brains were post-fixed overnight and then cryoprotected in 20% sucrose until they sunk. Coronal 30mm-thick coronal sections were cut on a freezing microtome and sections were stored in cryoprotective solution at -20°C until they were processed for immunohistochemistry.

For both cells and sections, nonspecific binding was blocked using a 5% solution of the appropriate serum in buffer, containing 0.025% or 0.25% Triton, respectively. Incubation with primary antibodies was performed overnight at 4°C. Primary antibodies were detected with appropriate fluorescent or biotinylated secondary antibodies. In the latter case, the reaction was eventually developed with the appropriate fluorophore-conjugated streptavidin Cy3, Cy5 (Jackson Immunolabs) or Alexa488 (Lifetechnologies). Hoechst 33342 (1:1,000, Lifetechnologies) or TO-PRO-3 (1:1,000, Lifetechnologies) were used to label cell nuclei. For AnnexinV staining, cells were incubated with biotin-conjugated AnnexinV (1:50) in binding buffer (10 mM HEPES, pH 7.5, containing 140mM NaCl and 2.5mM CaCl₂) for 10 minutes. After washing, fluorescent streptavidin was used to detect AnnexinV. As negative control, the procedure was performed without Ca²⁺, which is required for binding of AnnexinV.

For chromogenic visualization, biotinylated secondary antibodies, avidin–biotin complex (Elite ABC kit, Vector), and diaminobenzidine were used. Tyramide Signal Amplification (TSA, Perkin-Elmer) was used for HuD staining.

LIST OF PRIMARY ANTIBODIES

Antigen	Raised in	Dilution	Provider
β III tubulin	mouse	1:400	Covance
β III tubulin	rabbit	1:2000	Covance
Calbindin	rabbit	1:500	Sigma
Calretinin	goat	1:1000	Millipore
DARPP-32	mouse	1:200	AbD Serotec, Bio-rad
DCX	goat	1:400	Santa Cruz
ED1 (CD68)	mouse	1:200	AbD Serotec, Bio-rad
GABA	rabbit	1:2000	Sigma-Aldrich
GFAP	rabbit	1:400	Dako
GFAP	mouse	1:500	Sigma-Aldrich
GLAST	guinea pig	1:2000	Millipore
GFP	chicken	1:3000	Millipore
GFP	rabbit	1:10000	Abcam
GFP	mouse	1:10000	Abcam
Iba1	rabbit	1:2000	Wako
Iba1	goat	1:200	Millipore
HuD	rabbit	1:100	Millipore
Human Nestin	rabbit	1:200	Millipore
Human Nuclei (HuNu)	mouse	1:400	Millipore
Ki67	rabbit	1:2000	Leica, Novocastra
Ki67	mouse	1:100	Leica, Novocastra
MAP2	mouse	1:500	Sigma-Aldrich
MBP	mouse	1:200	Millipore
Musashi-1	rat	1:100	prof. H. Okano
M2/M6	rat	1:50	Hybridoma Bank
NeuN	mouse	1:200	Millipore
Olig2	rabbit	1:600	Abcam
RFP	mouse	1:200	Abcam
RFP	rabbit	1:500	Abcam
PSA-NCAM	mouse	1:200	Millipore
SC121	mouse	1:200	provided by Dr N. Uchida
STEM121	mouse	1:2000	StemCell Inc.
V-Glut-1	mouse	1:500	Synaptic Systems
V-Glut-1	rabbit	1:2000	Synaptic Systems

MICROSCOPY AND QUANTITATIVE ANALYSIS

Specimens were analyzed using either fluorescence microscopy (Olympus, Ballerup, Denmark) or laser scanning confocal microscopy (Leica, Wetzlar, Germany). Cell counting of *in vitro* experiments was carried out by a blinded observer using stereology with randomized sampling system (C.A.S.T.- GRID, Olympus) attached to Olympus BX61 epifluorescence/light microscope. Double staining was verified with a laser scanning confocal microscope (Leica Microsystems).

In paper II, the number of HuNu+ cells was quantified using the optical fractionator method. Grafts were displayed live on the computer monitor and delineated at low magnification. Quantifications were performed using 100X oil immersion lens with high numeric aperture. Every section in parallel-cut series that contained grafts in striatum was included. Random sampling was performed using the counting frame, which systematically moved at predefined intervals, so that between 100 and 200 immunoreactive cells were counted. Total number of cells was estimated according to the optical fractionator formula (West, Slomianka, & Gundersen, 1991). The magnitude of migration was assessed as the number of HuNu+ cells outside the graft core. Cell differentiation is presented as the number of HuNu+ cells coexpressing DCX or HuD. In addition, to describe differentiation qualitatively, numbers of DCX/HuNu and HuD/HuNu double-positive cells are presented as percentage of the total number of HuNu+ cells outside the graft core. Statistical analyses were performed using one-way analysis of variance followed by Bonferroni *post hoc* test. Data are given as means \pm SEM and differences significant at $P < 0.05$.

In paper III, at least 200 cells per slide were counted for general characterizations and at least 500 cells were analyzed when quantifying fusion. In Mac1-Saporin experiments, more than 1,500 cells were counted. Two-tailed unpaired *t* test and one-way ANOVA, followed by Dunnett *post hoc* test were used to assess differences between the groups. Data are expressed as means \pm SEM.

ELECTROPHYSIOLOGY

Cells plated on coverslips were constantly perfused with heated (32–34°C) gassed (95% O₂, 5% CO₂) solution (pH 7.2–7.4, 295–300 mOsm) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 25 glucose. Recording pipettes were filled with solution (pH 7.2–7.4, 295–300 mOsm) containing (in mM): 122.5 potassium gluconate, 12.5 KCl, 10.0 KOH-HEPES, 0.2 KOH-EGTA, 2 MgATP, 0.3 Na₃-GTP, and 8 NaCl, resulting in pipette resistances of

3–5 M Ω . Biotin (0.5%; Sigma) was freshly dissolved in the pipette solution before recordings for post hoc identification of recorded cells.

Results and Comments

RESULTS AND COMMENTS

CHARACTERISTICS OF A NEW HUMAN FETAL TISSUE-DERIVED NSC LINE

(Paper I)

Stroke is an acute cerebrovascular disorder causing loss of brain parenchyma and many types of neurons as well as astrocytes and oligodendrocytes. NSCs could potentially be used to develop novel therapies aimed at restoring impaired function after stroke. The main NSC-based strategies currently explored in animal models (Lindvall & Kokaia, 2011) are replacement of lost neurons, through intracerebral transplantation of neuroblasts or neuronal precursors generated from NSCs *in vitro*, stimulation of endogenous neurogenesis and immune-modulation.

Here we describe the *in vitro* and *in vivo* behavior of human NS cells, the generation of which represents a new method of derivation and expansion of NSCs. Human NS cells derived from the human fetal striatum are capable of differentiating into neurons *in vitro* and *in vivo* after transplantation into the rat neonatal striatum, which makes these cells suitable for transplantation studies aimed at replacement of lost and dysfunctional striatal neurons, such as in the case of stroke and Huntington's disease.

***In vitro* characteristics of proliferating human fetus-derived NS cells**

We characterized the properties of human fetus-derived NSCs expanded as monolayer cultures cells *in vitro* and *in vivo* after transplantation into the neonatal rat brain. Human striatal NS, hNS, exhibited homogeneous morphology under proliferative conditions and expanded rapidly. The proliferative capacity of these cells was stable over the passages, indicating that symmetric division prevailed over asymmetric division. Interestingly, the hNS cells expressed the early neuronal marker β III tubulin and the neuroblast marker DCX under proliferative conditions. Often β III tubulin+ cells coexpressed Ki67, a marker of proliferation. This pattern was observed in cells at different number of passages, suggesting that it is a stable property of hNS cells. The expression of β III tubulin in NS cell cultures under proliferative conditions has not been described previously. Onorati et al. (Onorati et al., 2010) observed that under self-renewing conditions of mouse induced pluripotent stem (iPS) cell-derived NS cells few cells (1–2 cells per field) expressed β III tubulin. However, as ES cell-derived NS cells did not express β III tubulin under self-renewal conditions (Garavaglia et al., 2010), it was assumed that the β III tubulin-

positive cells represented committed neuronal progenitors and young neurons that can be removed from the culture by temporarily plating NS cells on gelatin-coated dishes (Sun et al., 2008). We showed that the β III tubulin-positive cells present in our hNS cultures could undergo proliferation and co-express the mitotic marker Ki67. The presence of β III tubulin/Sox2 and β III tubulin/Vimentin double-positive cells provides evidence that they are of NSC/radial glial nature (Pinto et al., 2008). It is known that neuroblasts can proliferate for a limited time period and express β III tubulin and DCX (Brown et al., 2003). However, it is unlikely that the hNS cells are simply dividing neuroblasts. If that would be the case, they should have rapidly lost their proliferative and neurogenic capacities over the series of passages (Palmer, Ray, & Gage, 1995).

***In vitro* differentiation potential of hNS cells**

Human NS cultures exhibited high neurogenic potential upon differentiation. At 2 days of differentiation ~35% of the cells expressed the early neuronal marker β III tubulin. Neurogenesis increased over time, ~55% and 50% of the cells being positive for β III tubulin and the mature neuronal marker MAP2 respectively, after weeks of differentiation. The percentage of β III tubulin-positive cells in our hNS cultures was comparable to that observed by Sun et al. (Sun et al., 2008) in NS cells derived from human fetal cortex (~55 vs. ~45%), but the frequency of neurons expressing more mature and sub-type-specific neuronal markers such as NeuN or GABA, was substantially higher in our NS cultures. At 4 weeks of differentiation, 23% of hNS cells expressed NeuN whereas 39% expressed GABA. This finding raises the possibility that part of the GABA-positive cells in the cultures were not mature neurons but rather post-mitotic precursors expressing GABA prior to their terminal maturation (Achim, Salminen, & Partanen, 2014). The hNS cells showed a high neurogenic potential, which indicates that the derivation and expansion of human fetal striatal cells as adherent monolayer cultures adequately maintained their neurogenic potential.

Differentiation potential of hNS cells after implantation into rat newborn striatum

In order to study the differentiation potential of the hNS cells, we transplanted these cells into the intact striatum of newborn rats. Following implantation, the hNS cells survived and integrated morphologically in the host striatum. The grafted hNS cells decreased their proliferation, as indicated by the presence in the core of the graft of only few Ki67+ cells. No animals showed transplant overgrowth or tumor formation. Although many of the hNS cells remained close to the injection site, a

substantial amount of cells were localized outside the core of the graft, exhibiting bipolar morphology characteristic of migrating neuroblasts. A great number of hNS cells were also observed in white matter tracts, consistent with previous findings that represent a good environment for migration (Tabar V, et al 2005). The vast majority of implanted hNS cells expressed markers for young neurons, DCX and β III tubulin, but not for astrocytes, GFAP. Many cells especially in the core of the graft expressed nestin, suggesting that they were still in early stages of differentiation or had become quiescent, as indicated by the very low number of proliferating cells. In accordance, we found few cells expressing the mature neuronal marker NeuN.

FACTORS INFLUENCING SURVIVAL AND DIFFERENTIATION OF NSCs GRAFTED IN STROKE-INJURED BRAIN

(Paper II)

In paper II we investigated how various steps of neurogenesis are affected by intrastriatal transplantation of human NSCs at different time points after stroke and with different numbers of cells in each implant.

Effect of post-stroke delay and cell numbers on survival and migration of grafted cells

Human NSCs derived from the fetal striatum and expanded as neurospheres were implanted into intact and stroke-damaged striatum 48 hours or 6 weeks after the insult. Better cell survival was found by transplantation of these cells shortly after stroke, 48 hours, as compared with a later time point, 6 weeks. Increasing the number of grafted NSCs beyond a certain number (300,000) did not result in a greater number of surviving cells. This observation suggests an optimal threshold of cell numbers for graft survival, which, if saturated in the damaged striatum, may result in insufficient amounts of nutrients reaching the grafted cells, resulting in a progressively decreasing survival rate. To assess the migration of the grafted NSCs, we quantified the number of HuNu+ cells located outside the graft core. The NSCs migrated outside the core of the implant to comparable extent regardless of whether they were implanted 48 hours or 6 weeks after stroke; by comparison, the migratory capacity of cells implanted into the intact brain was reduced. Cell migration was not influenced by the number of implanted NSCs. Migration of grafted NSCs did not differ between stroke-subjected groups, but was reduced in intact brain. These findings are consistent with earlier reports showing that the injured brain is more favorable for stem cell migration than is intact tissue (Lu et al, 1991; Coyne et al, 2006).

Effect of post-stroke delay and cell numbers on proliferation and differentiation of grafted cells

The percentage of proliferating cells in the grafts decreased substantially by 5 weeks after transplantation regardless of the post-stroke delay and numbers of NSC that were implanted. Neuronal differentiation of grafted NSCs was also not influenced by these parameters. The grafts in the stroke-subjected groups exhibited a similar percentage of DCX+ and HuD+ cells. Thus, both proliferative activity and neuronal differentiation of grafted NSCs were determined mainly by intrinsic properties rather than by the characteristics of the pathological tissue environment. The highest number of HuD+ mature neurons in the grafts was observed when transplantation was performed 48 hours after stroke. Increasing the number of grafted NSCs did not result in significantly higher numbers of mature neurons. The proportion of cells, which differentiated into HuD+ neurons, was similar in all groups.

Characterization of the environment surrounding grafted cells

Survival and migration of grafted NSCs are markedly influenced by the inflammation associated with stroke (Friling et al, 2009). A substantial number of ED1+ -activated microglia were observed in the damaged striatum 48 hours after stroke. At 1 week, numbers of ED1+ -activated microglia had increased further and remained at this level until 6 weeks and declined at 11 weeks. ED1+ microglia constituted 50% of Iba1+ cells at 1 week and ~80% at 6 and 11 weeks. Thus, NSCs implanted early after stroke (48hours) were exposed to less hostile conditions than those transplanted at 6 weeks, when the NCSs encountered well-established inflammation. The microglia response to graft alone without stroke was much smaller compared with that after stroke. Therefore, the contribution of graft-induced inflammation to the overall inflammatory response after stroke is most likely only marginal.

OCCURRENCE AND MECHANISMS OF FUSION BETWEEN NS CELLS AND CORTICAL NEURONS

(Paper III)

In paper III we describe the occurrence of fusion between murine NS cells and neurons both *in vitro* and after transplantation *in vivo*. We show that microglia are involved in the fusion process. These findings shed a light on the behavior of microglia in response to intracerebral transplantation of mouse NS.

Fusion of NS cells with primary cortical neurons *in vitro* is mediated by microglia

To explore whether mouse ES cell-derived NS cells can fuse with neurons *in vitro*, we first cultured primary cortical cells from b-actin-GFP mouse embryos. After 7–12 days, all cells were GFP+, and 79.2%±3.7% were MAP2+ neurons 5.6%±1.9% were Iba1+ microglia. We then plated RFP+ NS cells onto these cultures and after 3 days of coculture, 5.2%±1.2% of cells expressed both GFP and RFP and 27.9%±6.1% of the GFP+/RFP+ cells had two nuclei, further suggesting that they had fused. Similar results were obtained when mouse or rat primary cortical cells were cocultured with mouse NS cells. The appearance of many cells bearing two markers, indicating the formation of a hybrid cell, occurred regardless of the cell labeling system employed (virus-mediated expression of GFP and GFP or lipophilic membrane dyes). To assess whether microglia could be involved in the fusion between NS cells and primary cortical neurons *in vitro*, we took advantage of the Mac1-saporin, which is a toxin that specifically targets and ablates microglia. Three days after plating NS cells on microglia-depleted primary cortical cultures the number of fused cells was reduced from 3.5%±0.1% in cultures containing microglia to 0.1%±0.1% in microglia-depleted cultures. Reintroduction of microglia restored the decreased level of fusion caused by microglia ablation to the initial level, which strongly indicates that microglia is involved in the process of fusion.

Double-labeled cells result from fusion and not from transfer of fluorescent proteins between cells

To rule out the transfer of fluorescent proteins between cells, we pre-labeled GFP+ microglia with BrdU before coculturing them with RFP+ NS cells. The occurrence of GFP+/RFP+/BrdU+ triple-positive cells provided further evidence that microglia and NS cells had fused. In order to assess that the generation of double-labeled fused cells cannot be attributed to phagocytosis, microglia were cocultured with

NS that had been killed by freezing-thawing cycles. Under this condition, we could not find any double-labeled fused cells. The absence of any fused cells in these cultures indicated that fusion required living NS cells and further excluded the possibility of nonspecific label transfer. To test whether NS fuse with microglia, we cocultured GFP+ NS cells with RFP+ microglia (BV2 cell line). A large portion of the cells became positive for both GFP and RFP after 2 days of coculturing. Similar results were obtained when primary mouse or rat microglia were cocultured with NS cells. Fusion was not restricted to ES-derived NS cells, as fusion has been observed in cocultures of microglia with mouse NS derived from the mouse fetal cortex, Cor1. Therefore, fusion between NS cells and microglia occurs as a result of a specific interaction between these cells. Interestingly fusion does not occur when rodent microglia are cocultured with cells of human origin, thus indicating that this phenomenon is species-sensitive.

Fused NS/Microglia cells display properties of NS cells

Quantitative polymerase chain reaction (PCR) analysis of sorted GFP+/RFP+ cells showed that they expressed the neural markers, nestin, Sox2, and Mash1, similarly to NS cells. Under proliferative conditions, virtually all GFP+/RFP+ cells were immunopositive for the NSC marker nestin. In addition, fuse cells expressed microglial markers CD11b and F4/80 although at much lower levels as compared to non-fused microglia.

Neuronal differentiation potential of Fused NS/Microglia cells

When applying a neuronal differentiation protocol, the GFP+/RFP+ fused cells and the GFP+ NS cells were efficiently differentiated to neurons, expressing MAP2, GABA, β III tubulin, or doublecortin (DCX) while the single-RFP+ microglia fraction did not give rise to any cells positive for neuronal markers. Similarly, using a glial differentiation protocol, the GFP+/RFP+ fused cells and the GFP+ NS fraction, but not the RFP+ microglia fraction, gave rise to cells immunopositive for the astrocytic marker GFAP. Taken together, these results indicate that the fused cells retain features of NS cells, are able to respond to differentiation into the neuronal and astrocytic phenotype when exposed to the appropriate medium.

Fused NS/Microglia cells express functional properties of microglia

We wanted investigate the capacity of double positive fused cells to respond to molecules known to induce activation and polarization in microglia and

macrophages, such as lipopolysaccharide (LPS) and Interleukin(IL)-4/IL-13. Fused cells increased their expression of pro-inflammatory molecules, as inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF) α when activated with LPS and of anti-inflammatory markers such as CD206 and arginase, when stimulated with IL-4/IL-13. No such change in expression of pro inflammatory or anti-inflammatory molecules was detected in NS cells following LPS or IL-4/IL-13 exposure, respectively. The fused cells exhibit partially the phenotype of both classically activated M1 macrophages, which are involved in neurotoxicity, and alternatively activated M2 macrophages, which are neuroprotective.

Fusion between microglia and fetal cortical neurons or NSCs *in vitro* was enhanced by LPS-induced activation of microglia or treatment with IL-4, respectively, indicating that the activated status of microglia can promote their fusion with mature neurons.

Fusion of NS cells and microglia involves a phosphatidylserine-dependent mechanism

Externalization and transient exposure of phosphatidylserine (PS) on the surface occurs during myoblasts (van den Eijnde et al., 2001) macrophage (Helming et al., 2009), fusion and trophoblast formation (Huppertz et al., 2006). We hypothesized that PS could also play a role in the fusion of NS cells and microglia. AnnexinV binds to exposed PS (Andree et al., 1990) and expression of the scavenger receptor CD36 is required on at least one of the fusing cells (Helming et al., 2009). Staining with AnnexinV or CD36 antibody showed that NS cells constitutively expose PS and microglia express CD36 in our coculture system. In order to determine the functional significance of PS exposure on the membrane of NS cells for fusion with microglia, we masked the exposed PS with AnnexinV. In NS cultures treated with AnnexinV, the quantification of GFP+/RFP+ cells revealed a 50% decrease in the occurrence of fused cells which supported an important role of PS in the fusion process. Normally, PS exposure on apoptotic cells is considered as a signal to macrophages for their clearance (Henson et al., 2001). However, our data indicate that NS cells expose PS without undergoing apoptosis and that the fusion of microglia with NS rather than leading to their removal, results into formation of a new chimeric cell that displays properties of both parental cells. These fused cells could be passaged multiple times without losing their dual, neural and microglial features.

Transplanted NS cells fuse with mature neurons *in vivo*

We found that ES cell-derived NS cells fuse with mature host neurons *in vivo* after transplantation. At three weeks after implantation of GFP+ mouse NS cells in the cerebral cortex of newborn rat pups, two distinct populations of GFP+ cells were observed. One population displayed immature morphology and was located in superficial cortical layers. The other group with characteristics of mature cortical pyramidal neurons, representing ~55.0% of all GFP+ cells, was located mostly in cortical layer V, exhibited very long apical dendrites, and expressed the mature neuronal marker NeuN. Similar results were observed when GFP+ or RFP+ NS cells were implanted into cerebral cortex of mouse pups. Virtually all GFP+ and RFP+ cells with mature pyramidal neuron morphology were negative for the mouse-specific neuronal-glia markers M2/M6 in brain sections from rats grafted with mouse ES cell-derived GFP+ NS cells. After transplantation of GFP+ NS cells into cortex where pyramidal neurons had been prelabeled retrogradely with a fluorescent tracer, we detected colabeling of tracer and GFP in some pyramidal neurons, indicating that they were of host origin. Confocal analysis of GFP+ pyramidal neurons in brain sections from rats grafted with NS cells revealed that 17.2% of the cells had two distinct nuclei in their soma. Grafted cells that have fused are often in the vicinity of activated ED1+ microglia. Some research groups have documented fusion of BMCs with and Purkinje neurons in lethally irradiated mice (Johansson et al., 2008) and fusion of myeloid and lymphoid origin in response to injury and inflammation (Nygren et al., 2008) indicating that fusion can occur in the context of microglia activation.

Although we do not have any direct *in vivo* evidence that this activation of microglia is involved in the fusion of grafted NS cells with resident cortical neurons, our *in vitro* data provide supportive evidence that this could indeed be the case. In fact, ablation of microglia from primary cortical neurons prior to coculturing with NS cells dramatically decreased the number of fused cells. Reintroduction of microglia after depletion fully restored the fusion rate, thus indicating that microglia were necessary for the fusion to occur.

General discussion



GENERAL DISCUSSION

In the present thesis we describe the *in vitro* and *in vivo* behavior of human NS cells, derived from NSCs. The human striatal NS cells are highly neurogenic, which makes these cells suitable for transplantation studies aimed at neuronal replacement. In order to establish successful NSCs-based therapies, other factors such as the number of cells and the appropriate time of transplantation after the onset of the injury play a pivotal role. We report how various steps of neurogenesis are affected by intrastriatal transplantation of human NSCs at different time points after stroke and with different numbers of cells in each implant. We also describe the occurrence of fusion of NS cells with mature neurons and microglia *in vitro* and *in vivo* following intracerebral transplantation of murine NS. The physiological relevance of fusion of NS cells is not known and requires careful investigation in order for the fusion to be considered for clinical applications.

CHARACTERIZATION OF HUMAN FETAL STRIATAL-DERIVED NS CELL LINE GENERATED FROM NSCs

Neural stem cells can be maintained and expanded *in vitro* as monolayer or neurospheres, free-floating spherical aggregates of cells at different stages of neural commitment responsive to growth factors such as EGF and/or bFGF. The development of the neurospheres culture system by Reynolds and Weiss (Reynolds & Weiss, 1992) led to the establishment of the neurospheres assay (NSA) that has since become an important tool for assessing the self-renewal and multipotency characteristics of NSCs. Even before the development of the neurosphere assay by Reynolds and Weiss, many research groups had attempted to establish protocols to derive and expand NSCs as monolayers. Despite the use of supplements, EGF, bFGF and nerve growth factor (NGF), these cells had limited expansion capacity, been the asymmetrical division most prominent in these cultures. Overall, the differentiation potential into the neuronal lineage appeared limited and diminished over time (Chenn & McConnell, 1995; Chenn & Walsh, 2002). Viral oncogene-mediated immortalization (such as v-myc, and SV40 largeT) has been used to obtain unlimited expandability of murine and human NSCs, prevent their growth arrest and senescence and maintain the capacity of differentiating into the three lineages when growth factors are omitted from the culture medium. These cell lines maintain many features of the original NSCs they had been derived from and in some cases the oncogene is downregulated after differentiation (Cacci et al., 2007). Immortalized cell lines are extremely valuable for disease modeling studies in neuroscience, but the insertion of oncogenes and the consequent tumorigenic potential raises concerns about their use in a clinical setting.

An important contribution to the NSCs research field came in 2005 with the development of highly proliferative, non-immortalized, stable, long-term expandable and multipotent NSCs lines termed Neural Stem (NS) cells (Conti et al., 2005). NS cells are monolayer cultures of NSCs derived from embryonic stem (ES) cells and primary fetus-derived brain tissue characterized by symmetrical division and continuous expansion in the presence of EGF and bFGF on laminin-coated plates. During proliferation, NS cells uniformly expressed morphological and molecular markers of radial glia, such as nestin, RC2 antigen, BLBP, Sox2, Pax6, GLAST (but not GFAP), lack the expression of markers of pluripotency, such as Oct-4 and Nanog, and mesoderm and endoderm markers. Upon growth factors removal, NS differentiated into neurons and astrocytes *in vitro* and *in vivo* after transplantation into the adult rodent brain (Conti et al., 2005) and oligodendrocytes *in vitro* (Glaser et al., 2007). At 4 weeks of differentiation 40% of the cells expressed neuronal markers such as Tuj1 and MAP2. Differentiated NS cells also expressed GABA and were able to fire action potentials. An optimized strategy increased the yield of neuronal cells with 85% of NS cells expressing Tuj1 at 3 weeks of differentiation (Spiliotopoulos et al., 2009). Most of the differentiated neuronal NS cells expressed GABA, but failed to express any region neuronal subtype markers.

The full characterization of human NS derived from the human fetal cortex and spinal cord was reported by Sun et al. (Sun et al., 2008). Similarly to the murine NS, the human NS were plated onto laminin coated-plates and expressed generic markers of radial glia such as BLBP, 3CB2, GLAST, vimentin and GFAP, the latter being a hallmark of radial glia of human origin (Malatesta, Hartfuss, & Gotz, 2000). Upon removal of growth factors, these cells efficiently generated neurons (43% Tuj1 positive), astrocytes and oligodendrocytes. However, the differentiated neurons did not express any neuronal-subtype marker such as GABA, DARPP-32, calbindin, parvalbumin, NPY, GAD67, Islet-1, ChAT, with the only exception of calretinin that was expressed in 5% of Tuj1+ cells.

NS cells derived from mouse ES and human and mouse CNS regions express notable features that make these cells interesting in the context of cell-replacement therapies. First, NS cells can be grown as monolayers and in contrast to neurospheres they are not depending on a supportive niche for their growth. Second, these cells divide symmetrically and give rise to morphological and molecular homogeneous populations over the passages. Third, upon differentiation, NS cells are consistently highly neurogenic giving rise to electrophysiologically mature neurons. However, further investigation is needed to assess whether it is possible to commit NS to specific neuronal subtypes, which is highly desirable for treatment of pathologies that involve the prevalent loss of defined kinds of neurons. Therefore it is necessary to develop more refined differentiation strategies. Ischemic stroke can lead to striatal damage, which results

in the loss of medium-sized projection neurons and interneurons, astrocytes and oligodendrocytes. Huntington's disease ultimately leads to progressive and irreversible disruption of striatal projection neurons. Therefore, the development of appropriate differentiation protocols aimed at obtaining a high yield of both striatal projection neurons and interneurons is needed. Human and murine NS homogeneously express markers of radial glia, which indicates that the culture conditions allow the preservation of a high degree of plasticity consistently during expansion. According to the original protocol by Conti et al., NSCs were plated onto gelatin-coated plates aiming at removing neuronal committed progenitors and then on laminin-coated substrate. Subsequently, Sun et al. described an alternative method of isolation of hNS cells, which involved the direct plating of the dissociated striatal tissue onto laminin-coated dishes. The role of laminin has been described by Conti et al. (Conti et al., 2005) in relation to the response of mouse NS cells to EGF. Specifically, the authors of this paper state that laminin could preserve cell viability in the absence of EGF and that the combination of laminin plus bFGF primes NS cells for neuroblast commitment following EGF withdrawal. The role of laminin in sustaining a radial glia-type phenotype during expansion of the NS has not been further investigated. In our previous study (Kallur et al., 2006), we found that human fetal striatum-derived neurospheres gave rise to ~25% β III tubulin-positive cells at 4 weeks of differentiation. As both the cell line studied here and the one characterized in Kallur et al. (Kallur et al., 2006) were derived from the striata of 8-week old fetuses, likely the originally isolated primary neural cultures had similar composition of undifferentiated NSCs and committed neuronal and oligodendroglial progenitors. However, the hNS cells had a higher neurogenic potential compared to the hNSC cells expanded as neurospheres, which indicates that the derivation and expansion of human fetal striatal cells as adherent monolayer cultures substantially promoted their neurogenic potential. The astroglial potential was not markedly affected, being the percentage of GFAP-positive cells 7% and 9% in adherent and neurospheres NSCs cultures respectively. Li et al. (Li et al., 2005) described high neurogenic potential of adherent human fetal striatum-derived NSC cultures, giving rise to ~50% β III tubulin and ~20% MAP2-positive cells at three days of differentiation. However, a direct comparison between our study and those of Li et al. is not possible because of major differences between the age of the fetuses (8 weeks vs. 12-20 weeks), the protocol for derivation of the NSCs, and the composition of the expansion and differentiation media.

By mimicking the cellular and molecular principles of development of the human embryonic striatum *in vitro*, conceivably it is possible to instruct NS cells towards a specific regional-differentiation pattern. This approach has been adopted for other sources of cells, like ESCs, by many research groups. Ma and coworkers (Ma et al., 2012) showed that tuning a particular dose of SHH (or purmorphamine) on

neuroepithelial cells derived from hESCs, resulted in an enriched population of lateral ganglionic eminence (LGE)-like progenitors that differentiated predominantly to GABA-ergic DARPP-32-expressing neurons *in vitro* and *in vivo*. A high proportion of our hNS striatal cultures were GABA-ergic following differentiation (39%), indicating that likely these cells retain region-specific properties, which had not been shown before for NS cells. So far, NS cells have not been transplanted in animal models of stroke. This step is necessary to test the full potential of these cells and assess their capacity to replace the damaged or dead cells and contribute to reconstruct disrupted neuronal circuits.

SURVIVAL AND DIFFERENTIATION OF GRAFTED NSCs STROKE-INJURED BRAIN

Stroke-injured rat brain retains neurogenic capacity for up to four months after the onset of stroke (Thored et al., 2006) however the number of new neurons surviving and differentiating into region-specific phenotypes is low (Arvidsson, Collin, Kirik, Kokaia, & Lindvall, 2002; Lindvall, Kokaia, & Martinez-Serrano, 2004) Intracerebral transplantation of NSC has been suggested as a promising therapy for stroke and neurodegenerative diseases. In order to mimic the clinical conditions and explore therapeutic strategies for patients suffering from stroke and degenerative disease, transplantation of NSCs can be performed in the lesioned brain of adult animals. The main NSCs-based strategies currently explored in animal models are:

- 1) replacement of neurons, which occurs when the transplanted NSCs are capable of differentiating into the cell types lost as a consequence of the pathology (depending on specific pathology, one or several cellular types are required),
- 2) reconstruction of injured neural circuitry using either intracerebral transplantation of neuroblasts or neuronal precursors generated from NSCs *in vitro*, or stimulation of neurogenesis from endogenous NSPCs,
- 3) transplanted NSCs can exert beneficial effects through immunomodulation, neuroprotection, and stimulation of angiogenesis in the stroke lesioned tissue (Kokaia, Martino, Schwartz, & Lindvall, 2012; Oki et al., 2012; Tatarishvili et al., 2014; Zhang et al., 2011)

Transplantation of *in vitro* expanded NSCs the intact neonatal brain represents an informative tool to assess the survival, the differentiation potential, and the migratory capacity of human NSCs. The intact neonatal brain has a high degree of plasticity and is a signal-rich developmental instructive environment for the grafted

NSCs. Moreover, the immaturity of the rat and mouse neonatal recipients' immune system favors a better survival, differentiation and integration of xeno-grafts and does not require immunosuppression. The neonatal brain also represents a valuable tool to study the time course differentiation of grafted NSCs and the capacity of the *in vitro* cultured NSCs to retain their fate specification typical of the CNS regions they were originally derived from (Kallur et al., 2006). In the view of developing therapeutic strategies for patients suffering from neurodegenerative disease and stroke, human neural stem cells can be transplanted into the adult lesion animal brain. Transplantation of NSCs into animal models degeneration and stroke should closely mimic the clinical condition of patients allowing the evaluation of relevant aspects such as cell replacement, immune-modulation and functional recovery. The environment that the cells encounter after their implantation in the stroke-damaged striatum changes along with the progression of the pathological condition, and thus affects the survival and differentiation of these cells (Darsalia et al., 2007). Many studies have shown survival, neuronal differentiation and functional improvement after transplantation of human NSCs in the stroke-damaged brain. Human NSCs were isolated by flow-cytometry from the 16 to 20 week old fetuses, expanded as neurospheres and transplanted into the stroke damaged rodent cortex (Kelly et al., 2004). The transplanted cells survived better when implanted further to the lesion, indicating that the non-ischemic tissue is more favorable to survival of the cells. When the cells were grafted in close proximity to the ischemic area, the survival of the cells was severely affected. Likely the ischemic area represents a very hostile inflammatory environment (Ekdahl, Claasen, Bonde, Kokaia, & Lindvall, 2003). Transplanted cells migrated toward the ischemic lesion, expressed the migrating neuroblast marker DCX differentiated and differentiated into β III tubulin positive neurons at the lesion border. Subsequently, the same group showed that the behavioral recovery of the transplanted stroke-subjected animals was mediated by the hVEGF secreted by the grafted cells (Horie et al., 2011). Human NSCs derived from 8 week old fetal striatum and cortex exhibit region-specific differentiation *in vitro* (Kallur et al., 2006). When transplanted into the stroke-damaged striatum of adult rats, both striatal and cortical NSCs exhibited a similar robust survival and migrated throughout the damaged striatum. The grafted cells showed very low proliferation rate and did not form tumors. Interestingly, striatal NSCs migrated further and occupied a larger volume of striatum. While the cells located in the core of the graft exhibited features of immature neural cells, the cells located outside the core displayed mature neuronal morphology and expressed mature neuronal markers such as HuD, calbindin, and parvalbumin (Darsalia et al., 2007). Transplantation of human fetal NSCs into the striatum of stroke-subjected T-deficient rats allows long-term studies concerning the survival and differentiation of the grafted cells and the investigation of the host environment in the absence of cyclosporine induced immunosuppression. At 6 and 14 weeks after transplantation of human

fetus derived striatal neurospheres, a substantial number of cells in the grafts expressed markers of mature neurons, Fox3, and a fraction of them had established contact with the globus pallidus. This study also showed that increased numbers of activated (ED1+) microglia/macrophages following stroke were suppressed in the striatum of grafted animals at both 6 and 14 weeks after transplantation. Therefore, human NSCs implanted in the stroke-damaged striatum of adult rats altered the environment encountered by the new grafted neurons by modulating inflammation (Mine et al., 2013). The complexity of the reciprocal interaction between the grafted NSCs and the local-damaged environment is key to development of successful cell-based therapies, therefore the time of transplantation of NSCs after the onset of the stroke is of great importance. In the present thesis we showed that better cell survival is achieved by transplantation of the cells shortly after stroke. Indeed, survival was highest, 58%, with transplantation 48 hours after stroke. Only 27% and 31% of transplanted cells survived after implantation at 6 weeks or in the intact striatum, respectively, which is in line with the our previous findings that around 30% of cell survive when the same NSCs were grafted into the rat striatum 1 to 2 weeks after stroke (Darsalia et al., 2007). We found that proliferation migration and differentiation of NSCs are not influenced by the post-delay time of the graft, therefore resulting by intrinsic properties of the cells rather than the encountered environment. Increasing the number of grafted NSCs beyond a certain number did not result in better survival of the cells. These findings suggest an optimal threshold of cell numbers for graft survival, although this parameter likely depends on the specific source of cells implanted. Our findings are the first direct evaluation of how numbers of implanted cells and the timing of transplantation after stroke affect various steps of neurogenesis. Therefore, in the view of cell-based therapies, it will be utmost important to identify the best time window for assuring the survival of the grafted cells in patients affected by stroke.

FUSION BETWEEN NS CELLS AND CORTICAL NEURONS

At only 4 weeks after transplantation into the cortex of newborn rats and mice, mouse GFP-positive ES-derived NS cells had acquired the typical morphological features of mature pyramidal cortical neurons. This observation led us to investigate whether these cells, rather resulting from differentiation, could have originated from the fusion of transplanted NS with the host cortical neurons.

It is been only in recent years that spontaneously *in vivo* occurring fusion between the exogenous transplanted and the recipient organism's cells has been demonstrated and characterized. Many authors expressed criticism when it was reported that NSCs could give rise to hematopoietic cells (Bjornson, Rietze, Reynolds, Magli, &

Vescovi, 1999) and that bone marrow cells (BMDCs) could transdifferentiate into neurons (Mezey, Chandross, Harta, Maki, & McKercher, 2000). Research effort by several authors aimed at understanding this complex phenomenon led to conclude that the differentiation of BMDCs into other lineages (not only neurons, but also cardiac, skeletal, muscle, hepatocytic, intestinal cells) after *in vivo* transplantation, was attributable to cell fusion rather than to transdifferentiation (Terada et al., 2002; Vassilopoulos, Wang, & Russell, 2003; Wang et al., 2003). Alvarez-Dolado and co-workers demonstrated that bone-marrow-derived cells (BMDCs) fuse spontaneously with neural progenitors *in vitro* and that transplanted BMDCs fuse *in vivo* with hepatocytes in liver, Purkinje neurons in the brain and cardiac muscle in the heart, resulting in the formation of multinucleated cells (Alvarez-Dolado et al., 2003). Another research group provided evidence that grafted BMDCs fuse with cerebellar Purkinje neurons in adult mice leading to the formation of stable euploid heterokaryons. The fused BMDC-Purkinje heterokaryons bore two nuclei, of which the nucleus from the BMDC was reprogrammed after fusion as indicated by its expression of a Purkinje-specific promoter (Weimann, Johansson, Trejo, & Blau, 2003). Ackman and coworkers (Ackman, Siddiqi, Walikonis, & LoTurco, 2006) showed for the first time the occurrence of fusion between microglia and mature neurons in the cortex of rat newborns after viral injection. This report showed that injections of a GFP-retrovirus into postnatal rat brains resulted in GFP labeling in layers II/III and V of the prefrontal cortex. The authors meant to investigate cortical neurogenesis, and surprisingly, the GFP-positive neurons did not incorporate BrdU. Inspection of retrovirally GFP-labeled neurons revealed that microglia had fused to the apical dendrites of the labeled pyramidal neurons. The fused cells often bore one supranumerary nucleus located on the apical dendrite that was IB4-positive, revealing its microglial origin. Microglia did not fuse to other glial cell types. *In vitro* cultures not treated with the GFP-retrovirus lacked microglial–neuronal fusion, but activation of microglia by LPS greatly increased the virally induced fusion of microglia to neurons in culture. The authors concluded that fusion occurs only in the presence of the retroviral infection. The first, seminal report that microglia undergoes fusion to form giant cells came from a study centered upon HIV infection and AIDS-related (Price et al., 1988) dementia. Subsequently other papers reported the formation of multinucleated microglia *in vitro* in the presence of cytokines such as IL-3, IL-4, GM-CSF and interferon (IFN) γ (T. T. Lee, Martin, & Merrill, 1993; Suzumura, Tamaru, Yoshikawa, & Takayanagi, 1999).

A recently published report demonstrated that NSCs engrafted in the adult mouse brain fuse with endogenous neurons (Brilli et al., 2013). At 7 days post-grafting, higher frequency of fusion events was detected in the hippocampus when fetal cortical mouse NS GFP-tagged cells, termed Cor-1, were subjected to a three days neuronal priming protocol than self-renewal culture conditions. However,

transplantation of these cells resulted in higher fusion rate in the hippocampus than cortex and striatum. Similar results were obtained when primed ES-derived NS (LC1) GFP-tagged cells were transplanted in the adult mouse hippocampus, however fusion was detectable at 1 month after transplantation. Fused GFP-positive NS/neurons displayed the typical complex morphology, polarity, position, orientation and Prox1 and Calbindin immunoreactivity of endogenous hippocampal granule neurons, with arborized spiny dendrites and axons. In chimeric experiments in which GFP-positive Cor1 cells were transplanted into the hippocampus of the ROSA26 transgenic mice (ubiquitously expressing the β -Galactosidase), GFP immunoreactivity was found to colocalize with β -Gal-positive neurons, strongly indicating that fusion events between transplanted cells and host neurons had occurred. The authors of the above quote paper further confirmed the occurrence of fusion by using a CRE/lox approach. Following transplantation of NSCs derived from CMV-CRE deleter mice in R26RYFP mice, R26-YFP+ cells were observed. Under this condition, YFP expression was detectable only if donor cells had fused with the CRE-carrying host cells as results from the recombination between the 2 genomes. Although the major finding of Brill and coworkers that mouse NS from different sources fuse with the recipient mouse neurons following transplantation are in line with our results, microglia seem not to be involved in the observed phenomenon. While we showed that microglia contributes to fusion of NS and primary cortical neurons and that NS and Cor-1 cells are able to fuse with mouse primary and BV2 microglial cells *in vitro*, Brill and co-workers did not detect fusion events when GFP-positive Cor-1 cells were cocultured with murine RPF+ postnatal microglia. From our observations that microglia were involved in the fusion process, we proceed further to investigate what were the possible cellular mechanisms involved in the process and whether the activation status of microglia could influence the occurrence of fusion. Helming and co-workers (Helming, Winter, & Gordon, 2009) demonstrated that in addition to the role of CD36 in apoptosis, recognition of endogenous lipids by CD36 is involved in fusion of macrophages induced by IL-4. In our study, we found that in NS cultures treated with the PS-binding protein AnnexinV, that the quantification of the NS GFP+/microglia RFP+ cells revealed a 50% decrease in the occurrence of fused cells, which supported an important role of PS in the fusion process. Therefore, we conclude that NS fuse with microglia *in vitro*, at least partly, through interaction between PS exposed on the surface of NS cells and CD36 receptor on microglia. Moreover, we found that fusion between microglia and fetal cortical neurons or NSCs *in vitro* was enhanced by LPS-induced activation of microglia or treatment with IL-4, respectively, which indicates that the activation status of microglia is involved in the occurrence of fusion.

A recent report by Kemp and coworkers has shown the occurrence of binucleated Purkinje cells in the cerebellum of human patients suffering from multiple sclerosis

(Kemp, Gray, Wilkins, & Scolding, 2012). The same study reports the presence of binucleated Purkinje neurons also in control subjects although to a very low extent. The authors of this paper hypothesized that these binucleated cells result from fusion. The observation that inflammation in the CNS promotes migration and infiltration of immune cells to the site of injury in the cerebellum, suggests the possibility that the body targets sites for neural repair or protection. However, Kemp and coworkers did not observe heterokaryon formation occurring preferentially at the lesion sites, suggesting that the formation of heterokaryons relies on systemic inflammatory clues rather than local signals. In line with other authors, Kemp et al. suggest that fusion could be a more efficient evolutionary mechanism compared to cell replacement. The biological relevance of cell fusion and the subsequent nuclear reprogramming is unclear, however fusion might represent a physiological phenomenon to introduce healthy nuclei or functional genes into damaged or degenerated cells (Kemp et al., 2012; Kemp, Wilkins, & Scolding, 2014).

CONCLUDING REMARKS

The present thesis reports new findings on the *in vitro* and *in vivo* behavior of human NS. We show that human striatal cells derived from the fetal striatum possess unique characteristic *in vitro*, giving rise to high number of neurons after differentiation. We also demonstrate that hNS cells are able to survive and differentiate *in vivo* following transplantation in the rat newborn brain, showing a high degree of plasticity. The human NS here described represent a promising source for transplantation studies in animal models of stroke and neurodegeneration.

Understanding the complexity of the reciprocal interaction between the grafted NSCs and the local-damaged environment is key to development of successful cell-based therapies for stroke. One of these variables is the timing of transplantation that is of utmost importance for translating cell therapy research into the clinic. Indeed, one relevant aspect of the cell-based approach is that it could extend the therapeutic time window of intervention for ischemic stroke, which is now limited, thus benefiting a larger number of stroke patients. We have shown how time delay after transplantation and cell number affect the survival of the NSCs grafted in the striatum of stroke-subjected rats. We showed that better survival was achieved when these cells were transplanted 48 hours after the onset of stroke and that increasing the cell number beyond a certain level did not result in better survival. Therefore, these findings have direct clinical implications.

Furthermore, we described the phenomenon of fusion of NS cells with microglia and cortical neurons. This represents a new, not previously described behavior of NS that occurs both *in vitro* and *in vivo*. We showed that at least *in vitro* microglia contributes to the occurrence of fusion through recognition of exposed PS in NS cells and the CD36 receptor in microglia cells. We also characterized the *in vitro*-generated hybrids and showed that they are stable and retain characteristics of the parental lines, indicating that nuclear reprogramming had occurred to a certain extent. Further investigation is required as the fusogenic role of microglia could be even more important after NSCs transplantation into brains affected by stroke and neurodegenerative diseases associated with microglia activation.

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