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Akademisk avhandling

**Human Neural Stem Cells**
region-specific properties and prospects for cell therapy

av

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**Title and subtitle**
Human neural stem cells: region-specific properties and prospects for cell therapy

**Abstract**

Cell replacement by neural transplantation can, in animal models of neurodegenerative diseases, reconstruct damaged brain circuitry. In the clinical situation, the graft material used for cell therapy will most likely need to be of human origin. The human fetal brain is one potential source of neural stem cells (NSCs) for cell replacement therapy in neurodegenerative disorders such as stroke. Stroke is the leading cause of disability in adult humans, and beneficial treatments for efficient recovery are today lacking. In the most common form of human stroke, caused by occlusion of the middle cerebral artery, mainly neurons in the cortex and striatum die. Therefore, we wanted to generate NSCs lines derived from the human fetal cortex and striatum and explore whether they maintain an intrinsic cellular identity in culture, consistent with their region of origin. Moreover, we wanted to investigate their capacity and neurogenic potential after transplantation into the striatum of intact newborn and stroke-lesioned adult rats. Furthermore, we wanted to determine whether we could drive the NSCs towards a neuronal fate by overexpressing the transcription factor Pax6. We found that the cortical and striatal NSCs have similar properties during expansion as neurospheres. However, upon long-term differentiation *in vitro*, the cortical and striatal NSCs generated region-specific neuronal subtypes. After transplantation into the neonatal rat striatum, both cortical and striatal NSCs survived well and migrated similar distances, and had the capacity to differentiate into astrocytes, oligodendrocytes, and mature neurons. When the NSCs were grafted into the striatum of rats subjected to stroke, both cortical and striatal NSCs survived and migrated to the same extent, and almost exclusively generated neurons outside the graft core. However, the striatal NSCs occupied a larger volume of the striatum and generated a higher proportion of neurons with the molecular identity of striatal neurons. Upon overexpression of Pax6, the generation of region-specific neurons *in vitro* was increased in the striatal NSCs. When striatal NSCs overexpressing Pax6 were implanted into the neonatal rat, there was an increased generation of neuroblasts compared to control. Taken together, we consider cortical and striatal NSCs derived from the human fetus as a safe cell source possessing a very strong neurogenic capacity. Thus, these cells may be promising candidates for cell replacement therapy. However, before any clinical application of cell replacement therapy can be considered, there are several key points to address: the selection of established and guaranteed safe cell sources with fully controllable differentiation potential, the complete knowledge of disease mechanisms and progression, the optimized number of cells and time for transplantation, and the careful selection of patients with best prognosis to benefit from cell therapy.

**Key words:** neural stem cells, human, fetal cortex and striatum, transplantation, stroke, neurospheres, region-specific properties, overexpression, Pax6

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Human Neural Stem Cells
region-specific properties and prospects for cell therapy

by

Thérèse Kallur

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Division of Neurology
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Lund 2008
Cover
Human neural stem cells derived from the fetal striatum were transplanted into neonatal rat brains. After one month, the human neural stem cells differentiated into cells with neuronal morphology. Cell of human origin in the rat brain is co-expressing human nuclei (red) and green fluorescent protein (dark blue processes).

Cover artwork by Bengt Mattsson and Therése Kallur
Till Pappa
(1943-1988)

Där hänger på boklädfönstret
En tunnklädd liten bok.
Det är ett urtaget hjärta
Som dinglar där på sin krok.

August Strindberg
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CELL FATE DETERMINATION

CELL REPLACEMENT THERAPY IN THE CLINIC

ACKNOWLEDGEMENTS

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  Paper II  Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged striatum
  Paper III  Pax6 promotes neurogenesis in human neural stem cells

COLOUR PLATES
ORIGINAL PAPERS AND MANUSCRIPTS

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

I. Human fetal cortical and striatal neural stem cells generate region-specific neurons \textit{in vitro} and differentiate extensively to neurons after intrastriatal transplantation in neonatal rats (2006)
   \textbf{Kallur T}, Darsalia V, Lindvall O and Kokaia Z
   \textit{Journal of Neuroscience Research}, Dec 84(8): 1630-44

II. Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged striatum (2007)
    Darsalia V, \textbf{Kallur T} and Kokaia Z
    \textit{European Journal of Neuroscience}, Aug 26(3): 605-14

III. Pax6 promotes neurogenesis in human neural stem cells (2008)
    \textbf{Kallur T}, Gisler R, Lindvall O and Kokaia Z
    \textit{Molecular and Cellular Neuroscience, In Press}
SUMMARY

Cell replacement by neural transplantation can, in animal models of neurodegenerative diseases, reconstruct damaged brain circuitry. In the clinical situation, the graft material used for cell therapy will most likely need to be of human origin. The human fetal brain is one potential source of neural stem cells (NSCs) for cell replacement therapy in neurodegenerative disorders such as stroke. Stroke is the leading cause of disability in adult humans, and beneficial treatments for efficient recovery are today lacking. In the most common form of human stroke, caused by occlusion of the middle cerebral artery, mainly neurons in the cortex and striatum die. Therefore, we wanted to generate NSCs lines derived from the human fetal cortex and striatum and explore whether they maintain an intrinsic cellular identity in culture, consistent with their region of origin. Moreover, we wanted to investigate their capacity and neurogenic potential after transplantation into the striatum of intact newborn and stroke-lesioned adult rats. Furthermore, we wanted to determine whether we could drive the NSCs towards a neuronal fate by overexpressing the transcription factor Pax6. We found that the cortical and striatal NSCs have similar properties during expansion as neurospheres. However, upon long-term differentiation in vitro, the cortical and striatal NSCs generated region-specific neuronal subtypes. After transplantation into the neonatal rat striatum, both cortical and striatal NSCs survived well and migrated similar distances, and had the capacity to differentiate into astrocytes, oligodendrocytes, and mature neurons. When the NSCs were grafted into the striatum of rats subjected to stroke, both cortical and striatal NSCs survived and migrated to the same extent, and almost exclusively generated neurons outside the graft core. However, the striatal NSCs occupied a larger volume of the striatum and generated a higher proportion of neurons with the molecular identity of striatal neurons. Upon overexpression of Pax6, the generation of region-specific neurons in vitro was increased in the striatal NSCs. When striatal NSCs overexpressing Pax6 were implanted into the neonatal rat, there was an increased generation of neuroblasts compared to control. Taken together, we consider cortical and striatal NSCs derived from the human fetus as a safe cell source possessing a very strong neurogenic capacity. Thus, these cells may be promising candidates for cell replacement therapy. However, before any clinical application of cell replacement therapy can be considered, there are several key points to address; the selection of established and guaranteed safe cell sources with fully controllable differentiation potential, the complete knowledge of disease mechanisms and progression, the optimized number of cells and time for transplantation, and the careful selection of patients with best prognosis to benefit from cell therapy.
SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING


Vid en stroke dör främst nervceller i två regioner av hjärnan, kortex och striatum. Därför genererade vi kortikala och striatala stamcellslinjer, där vävnad initialt togs från aborterade foster. Vi ville undersöka skillnaden mellan kortikala och striatala stamceller som odlats och expanderats under lång tid i cellkulturer. Ett mål var att ta reda på om stamcellerna fortfarande kunde ge upphov till de nervceller som är typiska för respektive region av hjärnan som de ursprungligen kom ifrån. Vi kom fram till att så är fallet – de kortikala och striatala stamcellerna blev kortex- respektive striatumspedicifiska nervceller. Nästa mål var att transplantera stamcellerna till rått hjärnor, både intakta och strokeskadade, för att utröna vilken påverkan miljön i hjärnan hade på cellerna samt deras kapacitet att överleva, mogna ut och i så fall till vilka typer av celler. Vi fann att både de kortikala och de striatala stamcellerna överlevde, integrerade med värdhjärnan och utvecklades till mogna nervceller. Det tredje målet var att försöka styra stamcellerna till att producera fler nervceller, då antalet nervceller brukar vara tämligen lågt i förhållande till andra typer av celler. Vi modifierade cellerna genom att införa en gen, Pax6, som tidigare, från studier på stamceller från möss, har visats öka antalet nybildade nervceller. Vi kunde visa att Pax6 ökade antalet nervceller som de striatala stamcellerna genererade och att de nya nervcellerna fortfarande var striatumspedicifika. Dessutom transplanterade vi de Pax6-överuttryckande stamcellerna till intakt rått hjärna och såg att de även i hjärnan kunde ge upphov till fler neuronala celler jämfört med celler utan överuttryckt Pax6.
De resultat som redovisas in den här avhandlingen gör att vi kan säga att kortikala och striata neurala stamceller från mänskliga foster är en möjlig reservoar av celler för transplantation. Vidare innehar de en hög kapacitet att kunna generera nervceller, både i odling och efter transplantation till råthjärna. Innan vi kan använda den här typen av behandling på patienter återstår dock mycket forskning kring cellernas förmåga att funktionellt integrera med redan existerande nervceller i hjärnan, att förstå de sjukdomsmekanismer som äger rum vid stroke, att bestämma vilka patienter som är bäst lämpade för cellterapi och att ta reda på hur många celler som ska placeras var i hjärnan samt när, det vill säga vid vilken tidpunkt efter stroke.
### THESIS GLOSSARY

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apoptosis</strong></td>
<td>Programmed cell death</td>
</tr>
<tr>
<td><strong>Astrocyte</strong></td>
<td>Type of large neuroglia cell in the central nervous system</td>
</tr>
<tr>
<td><strong>Axon</strong></td>
<td>Extension of a nerve cell, only one per cell, which transmits stimuli to primarily dendrites on other nerve cells</td>
</tr>
<tr>
<td><strong>Clonal</strong></td>
<td>Indicating a line of cells originating from one single cell</td>
</tr>
<tr>
<td><strong>Commitment</strong></td>
<td>Cell entering a specific path, which will eventually lead to differentiation</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td>A term collectively used for a large variety of proteins produced and secreted by cells and used to communicate with other cells</td>
</tr>
<tr>
<td><strong>Cytoplasm</strong></td>
<td>The contents of a cell other than the nucleus</td>
</tr>
<tr>
<td><strong>Dendrite</strong></td>
<td>Extension of a nerve cell, typically short and branched, that receives stimuli from other nerve cells</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
<td>The process whereby a cell acquires the features of a specialized cell</td>
</tr>
<tr>
<td><strong>Growth factor</strong></td>
<td>Protein that stimulates cell proliferation and cell survival</td>
</tr>
<tr>
<td><strong>Heterogenous</strong></td>
<td>Mixed composition</td>
</tr>
<tr>
<td><strong>Homogenous</strong></td>
<td>Unanimous composition</td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td>In a laboratory dish, flask or test tube; literally ‘in glass’</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td>In the living subject/organism</td>
</tr>
<tr>
<td><strong>Medium</strong></td>
<td>Solution that contains salts, hormones and growth factors sustaining cell growth</td>
</tr>
<tr>
<td><strong>Morphology</strong></td>
<td>The shape and structural features of a cell and/or tissue</td>
</tr>
<tr>
<td><strong>Necrosis</strong></td>
<td>Uncontrolled cell death</td>
</tr>
<tr>
<td><strong>Neuron</strong></td>
<td>A nerve cell; functional unit of the central nervous system</td>
</tr>
<tr>
<td><strong>Oligodendrocyte</strong></td>
<td>A cell that provides insulation of nerve cell axons by forming myelin sheaths</td>
</tr>
<tr>
<td><strong>Passage</strong></td>
<td>A round of cell growth and proliferation in culture, allowing cell culture expansion</td>
</tr>
<tr>
<td><strong>Plasticity</strong></td>
<td>Tissue stem cells and cells broaden potency in response to physiological demands, insults, or other stimuli. Also: changes in the circuitry/synapses/cell signaling etc.</td>
</tr>
<tr>
<td><strong>Proliferation</strong></td>
<td>Cell division, which generates new cells and thereby expands cell population</td>
</tr>
<tr>
<td><strong>Transcription factor</strong></td>
<td>Protein that binds to DNA, or to other proteins, and regulates gene activity</td>
</tr>
<tr>
<td><strong>Transit-amplifying cell</strong></td>
<td>Stem cell progeny that is still proliferating and is fated for differentiation although it may retain self-renewal</td>
</tr>
<tr>
<td><strong>Xenografting</strong></td>
<td>To transplant from one species to another</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

bFGF  basic fibroblast growth factor
Rlbp  retinol-binding protein
cDNA  complementary DNA
CMV   cytomegalovirus
CNS   central nervous system
DARPP-32 dopamine and cAMP-regulated phosphoprotein-32
DCX   doublecortin
DNA   deoxyribonucleic acid
EGF   epidermal growth factor
ES    embryonic stem
FACS  fluorescence activated cell sorter
GABA  γ-aminobutyric acid
GE    ganglionic eminences
GFAP  glial fibrillary acidic protein
GFP   green fluorescent protein
GW    gestational week
HBSS  Hank's balanced salt solution
iPS   inducible pluripotent stem
KPBS  potassium phosphate buffered saline
LIF   leukemia inhibitory factor
LGE   lateral ganglionic eminences
LTR   long terminal repeat
MCAO  middle cerebral artery occlusion
MGE   medial ganglionic eminences
MOI   multiplicity of infection
mRNA  messenger RNA
NeuN  neuron specific nuclear protein
NS    neural stem
NSC   neural stem cell
PCR   polymerase chain reaction
pH3   phosphorylated histone 3
PFA   paraformaldehyde
PLL   poly-L-lysine
Q-PCR quantitative PCR
PGK   phophoglycerate kinase
PSA-NCAM polysialylated neural cell adhesion molecule
RA    retinoic acid
RMS   rostral migratory stream
RNA   ribonucleic acid
RT-PCR reverse transcriptase PCR
SVZ   subventricular zone
TH    tyrosine hydroxylase
TU    transfecting units
VMAT-2 vesicular monoamine transporter 2
VSV-G vesicular stomatitis virus G protein
VZ    ventricular zone
Introduction
INTRODUCTION

NEURAL STEM CELLS

Neural stem cells (NSCs) have attracted major research and public interest during recent years. One explanation is the potential use of NSCs in treating or reducing the impairment for patients suffering from various neurodegenerative diseases such as Parkinson’s disease, Huntington’s disease, Alzheimer’s disease, and stroke (Lindvall and Kokaia, 2006; Lindvall et al., 2004). The majority of the cells dying following stroke are neurons located in the cortex and striatum, and when considering cell replacement therapy in patients, human cells are the preferable choice of cell source. There are different sources of human NSCs and the most suitable for every specific application must be selected. In order to do that, first, the optimal conditions for the cells’ expansion and differentiation must be determined. Second, the inert potential for proliferation and differentiation of the NSCs must be investigated, and third, the potential the NSCs can acquire through manipulation must be explored.

The work in this thesis focuses on NSCs derived from human fetal brain. NSCs from two forebrain structures, cortex and striatum, have been isolated and compared, both in vitro and in vivo, in order to assess the influence of the cells’ region of origin after culturing long-term in vitro and to investigate the influence of the in vivo environment following grafting. Furthermore, the cells have been genetically manipulated to overexpress Pax6 to investigate the influence of forced Pax6 expression on their neurogenic potential both in vitro and in vivo.

General definitions

A stem cell is a non-specialized cell with the capacity to give rise to more stem cells, self-renewal, for an extensive period of time and produce progeny that in the end will terminally differentiate into the major cell types of the tissue of origin, multipotency (Gage, 2000; Seaberg and van der Kooy, 2003). NSCs generally refer to stem cells derived from the central nervous system (CNS) or from the inner cell mass of the blastocyst, that maintain the capacity for self-renewal, and can generate neurons, astrocytes, and oligodendrocytes (Gage, 2000; Temple, 2001).

Stem cells can be arranged in a hierarchy depending on their self-renewal and differentiation capacity (see Figure 1). Totipotent stem cells can give rise to all cells that will make up an embryo including supporting the development of the embryo, for example the fertilized egg. Pluripotent stem cells can be found in the inner cell mass of the blastocyst and can generate all cells of the three germ cell layers which will give rise to all tissues in the body. Stem cells obtained from this source are called embryonic stem (ES) cells. Multipotent stem cells can give rise to all major mature cell types in the tissue from which they were originally obtained (Gage, 2000; McKay, 1997).
Stem cells divide, symmetrically, producing daughter cells that either are two identical stem cells, or two progenitor cells, or asymmetrically, producing one stem cell and one progenitor cell. The progenitor cell is frequently defined to be more lineage restricted, and can be bipotent or even unipotent. It has less self-renewal capacity than the stem cell. The progenitor cells will eventually generate more specialized cells that are committed towards a particular lineage, and, in the case for neural progenitor cells, eventually give rise to neurons and glia. *Precursor* cells is a term collectively used for both stem and progenitor cells and defines unspecifically a cell earlier in development than the progeny it gives rise to (McKay, 1997).
In this thesis the term ‘neural stem cells’ consequently will be used. Even though the neurosphere expanded human cortical and striatal cell cultures most likely contain both neural stem and progenitor cells, the expanded cells long-term capacity for self-renewal, multipotency upon differentiation, and ability to give rise to a wide range of neuronal phenotypes both in vitro and in vivo are, according to the less stringent definition (Temple, 2001), characteristics of NSCs.

**Neural stem cells in the developing brain**

The mammalian CNS is generated from one of the three germ layers, the ectoderm, formed during gastrulation. Initially, the neuroectoderm forms the neural plate on the dorsal surface of the emerging embryo. Later in embryogenesis the neural plate folds during the process of neurulation, forming the neural tube (Gilbert, 1997), and at around 4 gestational weeks (GW) in humans, the neural tube starts to close along the dorsal midline (O’Rahilly, 1999; O’Rahilly and Muller, 1999). The neuroepithelial cells located in a single layer closest to the ventricular space called the ventricular zone (VZ) are often called neural stem cells. Before the onset of neurogenesis, the proliferative cells in the VZ have radial processes and divide symmetrically, through a process termed interkinetic nuclear movement, in which the nuclei migrate up and down in the VZ during the cell cycle, dividing when the nuclei are close to the ventricle (Bystron et al., 2008; Gotz and Huttner, 2005).

At the onset of neurogenesis, at around 5GW in humans, the neuroepithelial cells, now forming several cell layers, change their mode of division to also include asymmetrical division (Bystron et al., 2008), and their epithelial character changes to the more fate-restricted phenotype of radial glial cells (Anthony et al., 2004; Gotz and Huttner, 2005; Guillemot, 2007). Radial glial cells express a combination of markers, including nestin, vimentin, GLAST, GFAP, CD133/prominin-1 and BLBP (Gotz et al., 2002; Hartfuss et al., 2001; Noctor et al., 2002), divide both symmetrically and asymmetrically, and are also bipolar; extending one short process with a large endfoot to the ventricular surface and one long radial process to the pial surface (Fishell and Kriegstein, 2003; Gotz et al., 2002; Noctor et al., 2002). The processes are thought to act as guides for neuroblasts migrating to their final destinations. Another neuronal progenitor appearing at the onset of neurogenesis is the basal progenitor, which originates from the asymmetrical division of radial glial cells (Alvarez-Buylla et al., 2001; Bystron et al., 2008; Miyata et al., 2004). The basal progenitors will, at around 7GW in humans, create the second proliferative zone, the subventricular zone (SVZ), above the VZ (Zecevic, 2004; Zecevic et al., 2005). The progenitors in the SVZ are mainly neurogenic and divide symmetrically, generating two neuronal daughter cells (Haubensak et al., 2004; Noctor et al., 2004; Zecevic et al., 2005). Just prior the cessation of the major phase of neurogenesis, by 25GW, the human VZ is reduced in size to a one cell layer thick ependymal layer (Zecevic et al., 2005), and the SVZ becomes the principal source of neurons, with continued proliferation until 40GW (Bystron et al., 2008).
Neural stem cells in the adult brain

In the adult mammalian brain there are two main neurogenic areas where neurogenesis persists throughout the life-span of the organism; the SVZ lining the walls of the lateral ventricle (Alvarez-Buylla and Garcia-Verdugo, 2002), and the subgranular zone of the dentate gyrus in the hippocampus (Gage, 2002; Kempermann, 2002). Neural stem cells and more restricted precursor cells can also be obtained from other areas of the adult brain, such as the striatum, cerebral cortex and spinal cord (Palmer et al., 1999; Palmer et al., 1995; Reynolds et al., 1992; Weiss et al., 1996), although the *in vivo* significance of these populations currently is under debate (Cameron and Dayer, 2007). In the adult human brain neurogenesis has been demonstrated in the SVZ and the subgranular zone (Eriksson et al., 1998; Kukekov et al., 1999), and there is evidence for a remarkable similarity between human and rodent olfactory systems, in which progenitors born in the SVZ migrate to the olfactory bulb and generate new neurons (Curtis et al., 2007).

HUMAN NEURAL STEM CELLS: EXPANSION IN VITRO

There are three main sources of human NSCs for *in vitro* culture expansion. Pluripotent ES cells derived from the inner cell mass of the blastocyst, and multipotent somatic stem cells that can be generated either from the developing fetal or mature adult CNS.

In this thesis, human cortical and striatal fetal tissue was dissociated into single cells and plated in culture flasks with DMEM/F12 medium supplemented with N2, a mixture of salts and hormones that support the growth of neural cells, and with the mitogens epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF). These factors stimulate the cells to divide, symmetrically and/or asymmetrically (self-renew), and upon mitogen withdrawal, the cells exit mitosis and begin to differentiate, generating the three cardinal cells of CNS (demonstrating multipotency).

When NSCs are grown only under the stimulation of external growth factors, they are said to be propagated epigenetically. NSCs can also be genetically perpetuated, by immortalizing cells, by inserting an immortalizing protein such as *v-myb*. However, in some cases, the genetically immortalized cells retain their dependency upon mitogenic stimulation for continued cell proliferation, since upon mitogen withdrawal the cells start to differentiate (Martinez-Serrano et al., 2001).

Neurosphere cultures

The most common way to expand human neural stem cell cultures is as neurospheres. A neurosphere is a free-floating, spherical cell aggregate potentially generated from one single cell responsive to EGF and/or bFGF that is stimulated to divide, generating daughter cells that are also responsive to these mitogens, forming a sphere (Reynolds et al., 1992; Reynolds and Weiss, 1992, 1996) (see Figure 2A). One must bear in mind that neurospheres are consti-
tuted of cells at different levels of maturity; thus, neurosphere cultures are considerably heterogeneous by nature. Culture conditions such as cell density, growth factor addition, medium supplementation, passaging technique and timing are therefore of utter importance. Any small change in any of these factors in cultures of such heterogeneous cell populations, can change the cells’ potential and possibly select for subpopulations of cells exhibiting similar properties to each other (Jensen and Parmar, 2006; Reynolds and Rietze, 2005; Whittemore et al., 1999).

The neurosphere assay can be used to assess the stem cell characteristics of self-renewal and multipotency (Seaberg and van der Kooy, 2003). To test for self-renewal, clonally derived neurospheres are dissociated and then replated at clonal density, in order to determine the cells’ capacity to form new spheres, so called secondary sphere formation. To test for multipotency, clonally derived neurospheres are cultured under differentiating conditions, in order to monitor the ability of these cells to generate the three main cell types of the CNS (Reynolds and Weiss, 1996). However, the accuracy and stringency of the neurosphere assay has recently been debated, when not the one-cell-per-well assay has been utilized, as originally, for obtaining clonally derived neurospheres. In fact, neurospheres from cells grown at so called clonal density do quite frequently merge, questioning the reliability of assuming that one sphere is generated from one stem cell (Reynolds and Rietze, 2005; Singec et al., 2006). The phenomenon of sphere merging can be partially attributed to the cilia-like structures, or microspikes, on the surface of cells located outermost of the sphere, making movement towards nutrition or chemoattractants possible (see Figure 2B). Merging events occur even when cells are grown at lower than clonal density, and when spheres are grown attached in a three-dimensional structure called matrigel (Singec et al., 2006). Furthermore, among the heterogeneous neurosphere cell population there are progenitor cells with proliferative capacity, although limited, but because of this have sphere-forming ability. Therefore, one must be careful in assuming that a one-to-one relationship between sphere and stem cell is always true (Reynolds and Rietze, 2005). Despite the possible drawbacks of the neurosphere assay, it is the most used in vitro assay, but one must take care in designing experiments and interpreting results obtained.

As mentioned before, to induce differentiation of the cells grown as neurospheres (see Figure 2C), the mitogens are removed from medium and the spheres are plated on a surface permissive for their attachment, often in the presence of serum.

**Monolayer cultures**

Human NSCs can also be expanded as attached monolayer cultures (Buc-Caron, 1995; Palmer et al., 1997; Skogh et al., 2001). Clonogenic assays, to establish stem cell properties, are more difficult in attached monolayer cultures than for neurosphere cultures, but has been achieved by tagging individual cells with retroviral vectors (Palmer et al., 1997). Monolayer cultures have until recently not been very successful for long-term culturing of human NSCs, unless the cells were immortalized. However, the addition of the mitogens EGF and FGF-2
to the defined and refined medium seems to reduce the rate of apoptosis and sustain the proliferative capacity of these cells long-term (Conti et al., 2005). These monolayer cultures of neural stem (NS) cells, whether derived originally from ES cells, fetal (Conti et al., 2005), or adult NSCs (Pollard et al., 2006), generate a quite homogenous population as assessed by both molecular and morphological methods. Due to the homogenous molecular expression profile typical of radial glial cells within the NS cultures, it has been suggested that radial glial cells are equivalent to the neurosphere-forming cells in neurosphere cultures (and thus being bona fide stem cells).

**CELL FATE SPECIFICATION AND FOREBRAIN PATTERNING**

During development in the mammalian forebrain, transcription factors are important for regulating both cell fate and cell differentiation. The combinatorial action of several proteins are involved in each step of the cells’ fate specification and subsequent differentiation (Guillemot, 2005; Schuurmans and Guillemot, 2002).

The embryonic telencephalon is classically divided into a dorsal pallium and a ventral subpallium, that primarily give rise to the mammalian cerebral cortex and basal ganglia, striatum and pallidum, respectively. The dorsal telencephalon can further be subdivided into the medial, dorsal, lateral, and ventral pallium, while the ventral telencephalon consists of two major progenitor domains; the medial (MGE) and the lateral (LGE) ganglionic eminences,
Progenitors in the dorsal telencephalon give rise to glutamatergic projection neurons, and in the human brain, also local cortical interneurons, whereas progenitors in the ventral telencephalon generate local basal ganglia GABAergic neurons, olfactory bulb interneurons, and interneurons migrating tangentially to the cortex (Guillemot, 2005; Letinic et al., 2002). In addition to these regional differences, each subdivision produces a vast amount of diverse neuronal subtypes, which differ greatly in their molecular profiles, morphology, connectivity, and physiological properties (Campbell, 2005; Flames and Marin, 2005). This generation of diverse cell types involves many developmental mechanisms, such as positional and temporal specification, and the formation of different progenitor populations.

**The role of Pax6 during development**

Members of the Pax gene family are known to function as master regulators in several organs where they influence cell proliferation and cell fate (Chi and Epstein, 2002). Pax6 is one of the family members encoding a transcription factor, that is crucial in the development of the eye, pancreas, and brain (Simpson and Price, 2002; St-Onge et al., 1997). The sequence and function of the Pax6 protein is highly conserved across species (Halder et al., 1995; Onuma et al., 2002).
The role of Pax6 during embryonic development has primarily been studied in mice. In the developing mouse brain, Pax6 is expressed in a gradient from caudio-medial low to rostro-lateral high in the pallium, and, additionally, in the pallio-subpallial boundary, in a stream through striatum, in the basal part of the subpallium, and at low levels in the LGE (see Figure 3) (Hallonet et al., 1998; Manuel and Price, 2005; Stoykova et al., 2000). Similarly, during human fetal forebrain development, Pax6 is expressed in cortical VZ and SVZ. However, unlike the situation in the developing mouse forebrain, in the developing human forebrain Pax6 is expressed equally strong in the LGE and caudal GE compared to cortex (Lindsay et al., 2005; Mo and Zecevic, 2007). This suggests that the regulation of development by transcription factors, including Pax6, may differ in humans and other mammals.

Homozygous Small eye (Sey) mutant mice, without functional Pax6 protein, lack eyes and nasal structures completely, have severe brain abnormalities, and die at birth (Manuel and Price, 2005; Schmahl et al., 1993). The absence of Pax6, as in Small eye (Sey) mutant mice, results in ventralization of the dorsal telencephalon, due to the ectopic expression of subpallial markers such as Mash1, Gsh2, and Dlx2, and, dorsal retraction of dorsal markers such as Ems1, Ngn2, and Tbr1 (see Figure 3) (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001).

During cortical neurogenesis in mouse development, Pax6 expression is confined to radial glial cells (Gotz et al., 1998), and in its absence, radial glial cells change cell-autonomously in morphology, numbers, and cell cycle kinetics. The number of radial glial cells and neurons are reduced in Sey/Sey cortex and therefore the cerebral cortex of mutant mice is much thinner compared to that of wild-type (Schmahl et al., 1993). However, there is an increase in precursor proliferation in the mutant mouse cortex, which is also observed in isolated cells from the cortex of mice (Estivill-Torrus et al., 2002; Heins et al., 2002). Therefore, the reduction in numbers of neurons can be explained by a reduced neurogenic potential of radial glial cells and an increase in the number of multipotent precursors that are not yet restricted to the neuronal fate (Heins et al., 2002).

**Forced Pax6 expression**

The majority of reports regarding Pax6 and its role in cortical neurogenesis have primarily been loss-of-function studies in rodents. However, gain-of-function experiments are adding further evidence and confirming previous results implying Pax6 as a key factor regulating neuronal fate in radial glial cells (Gotz, 2003).

One recent study investigating the effects of Pax6 gain-of-function on corticogenesis, demonstrated that conditional activation of two Pax6 isoforms in transgenic mice, resulted in an inhibition of cortical progenitor proliferation and progenitor pool apoptosis (Berger et al., 2007). In another study, when primary precursor cells from the embryonic mouse cortex were infected with a retroviral vector carrying Pax6, increased neurogenesis and concomitant reduction of proliferation was observed (Heins et al., 2002). Furthermore, overexpression of
Pax6 in adult mouse SVZ and embryonic mouse cortical and striatal cells expanded as neurospheres resulted in a dramatic increase in the number of neuroblasts and neurons generated (Hack et al., 2005; Hack et al., 2004). Most notably however, is that Pax6 is potent enough to instruct neurogenesis in astrocytes isolated from the postnatal mouse cortex several weeks following cessation of neurogenesis (Heins et al., 2002).

**STROKE**

Stroke is the leading cause of disability, and third leading cause of death, in the industrialized world after cardiovascular disease and cancer (Murray and Lopez, 1997). Stroke can be divided into two major types depending on the cause: ischemic and hemorrhagic. Hemorrhagic stroke results from intracerebral bleeding caused by a rupture of a vessel in the brain, which can cause physical damage to the brain due to the build-up of pressure. Ischemic stroke can in turn further be subdivided into embolic and thrombotic. Thrombotic stroke is due to a clot gradually forming within a vessel, while embolic stroke results from an embolus formed somewhere in the body that travels through the blood stream, and blocks a vessel within the brain.

The brain has the highest demand of glucose and oxygen in the body and is therefore particularly sensitive to reduced blood flow. Due to the deprivation of glucose and oxygen caused by the stroke, a chain of detrimental events, including cell respiration failure, uncontrolled glutamate release, cellular edema, accumulation of free radical species, takes place, leading to cell death (Lipton, 1999). Within the ischemic core, cells quickly die through necrosis, which does not require any energy, is uncontrolled and usually involves several cells simultaneously. In the tissue peripherally surrounding the ischemic core, the ischemic penumbra, cells gradually die through apoptosis, which is an individual cell’s execution of an internal suicide program (Yuan et al., 2003). Apoptosis is either intrinsically or extrinsically activated and is ongoing for at least several days after the insult in parallel to inflammation (Dirnagl et al., 1999).

Apart from causing personal and familial tragedies, stroke and stroke-related rehabilitation places a heavy economical burden upon society (Meairs et al., 2006). Depending on the size and the area of the brain affected, various common symptoms of stroke are sensorymotor and somatosensory dysfunction, paralysis, aphasia, nausea, and headache (Fatahzadeh and Glick, 2006). Current treatments for stroke are very limited, focusing on removal of the clot in the acute phase, either by thrombolysis alone or in combination with mechanical removal of the clot (Smith et al., 2005).

In order to study stroke, several animal models have been developed. The most commonly used model involves unilateral occlusion of the middle cerebral artery (MCAO) in the rat or mouse, which can be induced in several ways. In this thesis (Paper II) the suture
model of MCAO was used (Koizumi, 1986). In this model, a nylon filament with a rounded tip with size adjusted to the distal part of middle cerebral artery is inserted via an incision in the common carotid artery and advanced through the internal carotid artery into the circle of Willis (see Figure 5, Materials and Methods). Reperfusion of the tissue by withdrawal of the filament is possible at any time during the surgery, and therefore the length of ischemia, and thus, extent of neuronal damage, can be controlled.

TRANSPLANTATION OF NEURAL STEM CELLS

The brain has regenerative capacity after an injury such as stroke (Arvidsson et al., 2002; Parent et al., 2002), and the neurogenic response in the stroke-damaged rat brain persists for up to four months after initiation of stroke (Thored et al., 2006). However, the number of new neurons surviving is low, and hence, the number of newly formed DARPP-32 positive striatal projection neurons is even lower (Arvidsson et al., 2002; Lindvall et al., 2004). Therefore, it is possible that the endogenous NSCs could be stimulated in combination with cell transplantation, in order to achieve maximal functional recovery. The objective for the grafted NSCs is either to stimulate and/or support the proliferation, survival, migration, and differentiation of endogenous cells, or, to replace the dying or dead endogenous cells. In the prospect of cell replacement therapy, the implanted NSCs must be able to survive and generate new neurons of the appropriate types that functionally integrate into the damaged host brain circuitry.

Transplantation into the intact neonatal and adult brain

Transplantation of NSCs into the intact neonatal brain serves as an excellent initial tool to evaluate the survival and differentiation potential, as well as migratory capacity of human NSCs, since the neonatal brain is rich in developmental instructive signals. Therefore, neonatal implantation of NSCs is often used as an intermediate step between the controlled, artificial in vitro environment, and transplantation into the less plastic adult, intact or damaged, brain, in regards of estimating the properties of the cells. Previously, several groups have reported the implantation of human fetal NSCs either isolated from the whole fetal forebrain (Englund et al., 2002a; Englund et al., 2002b; Fricker et al., 1999; Rubio et al., 2000; Uchida et al., 2000) the LGE (Parmar et al., 2003), or the fetal cortex (Burnstein et al., 2004; Caldwell et al., 2001; Le Belle et al., 2004; Ostenfeld et al., 2000) into different sites of the intact embryonic, neonatal, and adult rodent brain.

Beginning with the earliest graft recipient age, human fetal NSCs were obtained and transplanted either directly as fresh tissue or as expanded monolayer or neurosphere cultures. Human cells were implanted in utero into the ventricular system of the embryonic rat brain and subsequent investigation found human cells throughout the brain, generating progeny of all CNS lineages but preserving the cytoarchitecture of the developing recipient brain. However, cells that had been expanded as neurosphere cultures migrated more extensively than fresh
tissue implants (Brustle et al., 1998). Human cell transplantation experiments have also been described in the neonatal mouse (Flax et al., 1998; Tamaki et al., 2002; Uchida et al., 2000) and rat (Englund et al., 2002b; Parmar et al., 2003; Rosser et al., 2000) brain (Svendsen and Caldwell, 2000). These reports describe that the human cells survive, migrate long distances, and differentiate into neurons and glial cells irrespective of graft placement in neurogenic (SVZ and hippocampus) and non-neurogenic zones (striatum and neocortex) in the brain. Moreover, cells grafted into the SVZ were found migrating in the rostral migratory stream (RMS), indicating that the transplanted cells displayed similar properties to the endogenous brain cells (Englund et al., 2002a; Parmar et al., 2003).

In studies implanting human cells into the intact adult rat brain at multiple sites (SVZ, striatum and hippocampus), it has been reported that the transplanted cells differentiate into neurons and glia when placed in neurogenic areas, and that the undifferentiated portion of the grafted cells migrated widely in the brain (Englund et al., 2002a; Fricker et al., 1999; Le Belle et al., 2004; Rubio et al., 2000). However, when the human cells were grafted into the striatum, the number of grafted cells that differentiated into neurons was reduced and the majority of cells were instead GFAP positive astrocytes (Englund et al., 2002a; Fricker et al., 1999).

Transplantation into the damaged brain

In order to more closely mimic the clinical situation and to evaluate possible therapeutic avenues for patients suffering from neurodegenerative diseases, transplantation of human NSCs can be performed in adult animals with lesioned brains.

Human fetal-derived NSCs have been grafted into the dopamine-denervated striatum (modeling Parkinson’s disease) (Burnstein et al., 2004; Caldwell et al., 2001; Ostenfeld et al., 2000; Svendsen et al., 1997; Vescovi et al., 1999), the excitotoxic lesioned striatum (modeling Huntington’s disease) (Armstrong et al., 2000; Svendsen et al., 1996), and the stroke damaged brain (Ishibashi et al., 2004; Kelly et al., 2004; Lee et al., 2007a; Lee et al., 2007b). Several observations are common to a number of studies: the grafted cells integrated poorly, as judged by the compact cell mass found at or adjacent to the implantation site, and neurons were only encountered at the main deposit site, whereas the only migrating cells were GFAP positive (Caldwell et al., 2001; Ostenfeld et al., 2000; Svendsen et al., 1997; Svendsen et al., 1996; Vescovi et al., 1999). However, the non-migratory neurons innervated the host brain extensively, with long axonal projections found in the contralateral corpus callosum and ventral mesencephalon (Ostenfeld et al., 2000). Furthermore, a very low fraction of the grafted human cells differentiated into neurons, and even fewer expressed the phenotypical marker characteristic for striatal projection neurons, DARPP-32 (Armstrong et al., 2000; Svendsen et al., 1996), although the proportion of neurons could be increased by “predifferentiating” the human NSCs prior to grafting (Burnstein et al., 2004). Human NSCs isolated from the whole fetal forebrain have been used for transplantation in various models of stroke in rat (Kelly et al., 2004), mongolian gerbils (Ishibashi et al., 2004), and mouse (Lee et al., 2007a; Lee
et al., 2007b). Results from these studies suggest that the grafted human cells survive rather poorly (Ishibashi et al., 2004), however, survival was influenced by proximity of the graft to the stroke lesion (Kelly et al., 2004). Grafted cells differentiated primarily into neurons and astrocytes, and migrated towards the lesion (Kelly et al., 2004; Lee et al., 2007a), and could promote some behavioral improvement (Ishibashi et al., 2004; Lee et al., 2007a; Lee et al., 2007b).

A great deal of research is however required in order to better understand how to instruct human NSCs to differentiate and adopt the desired molecular phenotypes, innervate the target regions of interest, and avoid uncontrolled innervation of remote brain areas.
AIMS OF THIS THESIS

The main purposes of this thesis were to generate stable human fetal-derived neural stem cell lines from different brain regions and compare their potential both in vitro and in vivo. More specifically the aims and questions have been to:

1. Characterize the potential and properties of two NSC lines derived from the human fetal cortex and striatum in vitro

2. Compare the properties and neurogenic capacity of cortical and striatal NSCs after intrastriatal transplantation into the intact neonatal rat brain and into the stroke-damaged adult rat brain

3. Promote neurogenesis in cortical and striatal NSCs both in vitro and after grafting into the striatum of neonates by means of Pax6 overexpression
Materials and methods
MATERIALS AND METHODS

HUMAN FETAL TISSUE ISOLATION AND CELL CULTURING

The human tissue was obtained from human aborted fetuses aged 6 to 9 weeks postconception in accordance with guidelines approved by the Lund/Malmö Ethical Committee.

Dissection from the human fetal brain

The dissection was conducted under a microscope (Leica, Germany) with the fetus placed in a petri dish with hybernation media (Apoteksbolaget AB, Sweden). The CNS was removed and cleaned from surrounding tissue of mesenchymal origin. The cortex was cut open along and close to the dorsal midline (medial parasagittal), and ganglionic eminences (anlage to striatum) were carefully dissected out followed by the removal of cortex (see Figure 4).

Generation of neurosphere cultures

The tissue pieces were incubated for 45 minutes at 37°C in expansion medium followed by mechanical dissociation. The number of cells was thereafter counted using the Trypan Blue dye exclusion method and then plated at appropriate density in uncoated culture flasks with expansion medium. Cells were maintained at 37°C in a humified atmosphere with 5% CO₂.

After several weeks, neurospheres had formed and before the core of the spheres turned dark, due to lack of nutritional access and subsequent cell death, the neurosphere culture was passaged. Briefly, cells were pelleted by centrifugation and the enzyme Accutase (PAA Laboratories AB, Linz, Austria) was added. After 10 minutes of incubation the cell pellet was rinsed once with basic medium and the neurospheres were dissociated mechanically by trituration until a single cell suspension was acquired. Cells were counted and replated at the required density in flasks with expansion medium and filtered conditioned medium.

Figure 4. Photomicrographs illustrating the dissection of human fetal forebrain, in which the developing cortex and striatum are obtained. From the human fetus (A) the CNS is cleaned from surrounding tissue (B), each hemisphere is opened, and cortex and striatum are taken for subsequent culturing (C).
Cytogenetic analysis

To explore whether the fetal-derived striatal NSCs expanded as neuospheres for an extended time in vitro (over 15 passages; approximately 2 years), exhibited any gross chromosomal abnormalities, karyotyping was performed. Briefly, cells were arrested in metaphase using Colcemid (0.02 µg/ml for 3 hours). In situ preparations were made after hypotonic shock and fixation in methanol:acetic acid (3:1) and G-banding was obtained with Wright’s stain. Nineteen cells in metaphase were analyzed.

Differentiation of human neural stem cells

Small neurospheres, 3 to 4 days after passage, were plated in PLL-coated chamber slides with expansion medium without heparin. The spheres were allowed to attach for 24 hours after which the expansion medium was replaced by differentiation medium. During the time of cell differentiation the medium was replenished every third day until the cells were fixated at the end of differentiation.

List of cell culture media

All media used were constituted of the following basic medium:

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<th>Basic medium</th>
<th>Supplier</th>
<th>Concentration</th>
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Expansion medium

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<td>LIF</td>
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Differentiation medium

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<td>Fetal bovine serum</td>
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</table>
TRANSPLANTATION PROCEDURES AND MCAO

All animal work was performed according to local ethical guidelines and approved by the Swedish National Board for Laboratory Animals. Pregnant Sprague-Dawley rats and adult male Wistar rats were obtained from Scanbur-BK (Sweden). Neonatal rats were kept with their mother until weaning and all rats were housed under 12 hour light/dark cycle and had *ad libitum* access to food and water.

**Preparation of cells for transplantation**

On the day of transplantation, small neurospheres (less than 100 µm in diameter) were collected by centrifugation and resuspended in Hank’s Balanced Salt Solution (HBSS) to reach a final cell density of approximately 100 000 cells/µl. The neurosphere suspensions were kept on ice during all transplantation procedures and the same vial of neurosphere suspension never longer than 5 hours. After every transplantation session, the neurosphere suspensions were replated in order to assess cell viability.

![Figure 5. Schematic figure of the MCAO model in rat. The occluding filament is inserted into the common carotid artery and further advanced in the internal carotid artery until it occludes the origin of the middle cerebral artery. The black area in the figure represents the region supplied by the middle cerebral artery, and thus, being ischemic during MCAO surgery (see page 147, colour plates).](image)
Transplantation to newborn rats

Neonatal (postnatal days 3 to 5) rat pups were deeply anaesthetized by hypothermia before stereotaxic surgery was performed. The skull was aligned in the horizontal plane and 1 to 1.5 µl neurosphere suspension was injected using a Hamilton syringe into the striatum of each animal. Coordinates were 0.5 mm rostral and 2.0 mm lateral from bregma, and 3.0 mm ventral from dura. The needle was kept in place for 5 minutes before it was removed, the wound was closed with suture and animal was resuscitated.

Middle cerebral artery occlusion

Before MCAO was induced by the intraluminal filament technique (Koizumi, 1986; Zhao et al., 1994) the rats were fastened overnight and then anaesthetized by inhalation of isoflurane and a mixture of O₂ and N₂O. The right common carotid artery was isolated and cleaned from surrounding tissue and ligated proximally together with the external carotid artery. The internal carotid artery was temporarily closed with a microvascular clip and a nylon monofilament with a rounded tip was inserted through the common carotid artery, into the internal carotid artery until resistance was felt, and thus, had passed the origin of the middle cerebral artery (see Figure 5). The rats were then allowed to wake up from surgery. After 30 minutes of occlusion the animals were re-anaesthetized and the filament was permanently withdrawn. The body temperature was regulated during the whole surgical procedure and for 2 hours thereafter.

Transplantation to adult stroke-damaged rats

About 2 weeks after MCAO surgery the adult rats received human cell transplantations. Animals were anaesthetized with isoflurane and placed in the stereotaxic frame where the skull was opened with a drill. The animals were injected with neurosphere suspension at 2 sites ipsilateral of the damage at 0.5 mm rostral and 0.5 mm caudal, and 3.0 mm lateral from bregma. Depth of the deposits was set from dura: ventral 5.0 mm. At each site 1.5 µl neurosphere suspension was delivered. The animals were immunosuppressed by injections of cyclosporin (10 mg/kg) every other day during the experiment until they were sacrificed one month later.

IMMUNOCYTOCHEMISTRY

Fixation and post-fixation procedures

Cells were fixed with 4% PFA (including 0.2% glutaraldehyde for GABA and glutamate stainings) for 15 minutes and then rinsed 3 times with KPBS. Neurospheres for cryosectioning were pelleted and fixed with 4% PFA for 15 minutes, then rinsed 3 times in KPBS, and then mounted in Tissue-Tek and cut in 9 µm thick sections on a cryostat. Animals were deeply anaesthetized with sodium pentobarbital and transcardially perfused with 4% ice-cold PFA. Brains were post-fixed over night and then placed in 20% sucrose until they sunk. Coronal 30
μm thick sections were cut on a freezing microtome and sections were then stored in cryoprotective solution at -20°C until they were processed for immunocytochemistry.

### Staining procedures

Staining was performed on cells attached to permanox chamber slides, on cryostat sections attached to glass slides, or on free-floating rat brain sections in glass vials. Sections and cultures were rinsed in KPBS, and preincubated at room temperature for 45 to 60 minutes in 5% normal serum, and 0.025% triton for cell cultures or 0.25% triton for sections, respectively.

### Table of antibodies

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</tr>
<tr>
<td>Pax6</td>
<td>rabbit</td>
<td>1:500</td>
<td>NordicBiosite</td>
</tr>
<tr>
<td>p-H3</td>
<td>rabbit</td>
<td>1:400</td>
<td>Upstate</td>
</tr>
<tr>
<td>S100β</td>
<td>mouse</td>
<td>1:5000</td>
<td>Sigma</td>
</tr>
<tr>
<td>SC121</td>
<td>mouse</td>
<td>1:3000</td>
<td>Dr. Uchida</td>
</tr>
<tr>
<td>TH</td>
<td>mouse</td>
<td>1:200</td>
<td>Chemicon</td>
</tr>
<tr>
<td>TH</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Vimentin</td>
<td>mouse</td>
<td>1:50</td>
<td>Dako</td>
</tr>
<tr>
<td>VMAT2</td>
<td>rabbit</td>
<td>1:500</td>
<td>Chemicon</td>
</tr>
</tbody>
</table>
Primary antibodies were diluted in the preincubation solution, and the cells or sections were incubated in primary antisera on a shaker either at room temperature or at 4°C overnight. Detection of primary antibodies was carried out at room temperature for 2 hours using both fluorescent and/or biotin-conjugated secondary antibodies diluted 1:200 in preincubation solution. For detection of biotin-conjugated antibodies, Alexa 488-conjugated streptavidin (1:200) was used. In order to double-label cells despite using two primary antibodies from the same animal species, two sequential stainings were performed to avoid cross-reactivity. When staining for HuD, a tyramide amplification procedure was included before the avidin step. To stain cell nuclei from all cells to indicate total cell number, the nuclear staining Hoechst (1:1000) was added during the final incubation.

Free-floating sections were mounted on gelatin-coated slides and all stained material was coverslipped using PVA-DABCO mounting medium for fluorescence microscopy.

**GENE EXPRESSION ANALYSIS**

**RNA isolation**

Total RNA was isolated from naïve and sorted retrovirally-transfected cortical and striatal NSC lines using RNAeasy Mini Kit (Qiagen) according to the manufacturer’s guidelines. RNA isolation was followed by DNaseI treatment (Ambion) to rule out possible DNA contamination.

**RT-PCR and Q-PCR**

For semi-quantitative PCR, total cellular RNA (1 µg) was reverse transcribed using oligo-(dT) primers and SuperScript-II Reverse Transcriptase (RT) (Invitrogen). In some samples RT was omitted in cDNA synthesis and an RT-negative control was included for each sample in the PCR reactions. None of the RT-negative controls ever resulted in any amplification product. Semi-quantitative PCR was performed using primers for GAPDH and Pax6 (see list of primers) and products were visualized by ethidium bromide gel electrophoresis.

For quantitative PCR (Q-PCR), total cellular RNA (0.5 µg) was annealed to oligo (dT) primers in the presence of dNTP mixture (both from Takahara Bio inc.). Reverse transcription reactions were performed with PrimeScript TM RTase in the supplied buffer containing RNase inhibitor (Takahara Bio inc.). Quantitative PCR was performed either using TaqMan Universal Master Mix and TaqMan Gene expression assays (100 ng cDNA/reaction) (Applied Biosystems, AB), or using the Power SYBR Green PCR Master Mix (AB) and the IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). All samples were run in triplicate for each investigated transcript and cDNA input was normalized to ACTB and relative gene expression was calculated using the ∆∆CT method. ACTB and target genes were amplified by 45 cycles at a Tm of 60°C.
List of PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence/Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>Hs03023880_g1</td>
</tr>
<tr>
<td>ACTB</td>
<td>5'- AAAGACCTGTACGCCAACCAC -3'</td>
</tr>
<tr>
<td></td>
<td>5'- GTCATACTCCTGCTTGTGTGAT -3'</td>
</tr>
<tr>
<td>AP-2γ</td>
<td>5'- AACCCCTGGAGACCAGAGTCC -3'</td>
</tr>
<tr>
<td></td>
<td>5'- GTTCTGTTCCTCTCCCTA -3'</td>
</tr>
<tr>
<td>Bhlhb5</td>
<td>5'- AACTTTGAGGAGCGAAGAGA -3'</td>
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<tr>
<td></td>
<td>5'- TGCCATTCAAACACTCTCCA -3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'- CCACAGTCCTAGGCGACAC -3'</td>
</tr>
<tr>
<td></td>
<td>5'- TCCACACCCGTTTGCTGTA -3'</td>
</tr>
<tr>
<td>HuD</td>
<td>5'- TGACAGCAAACACACACCTCA -3'</td>
</tr>
<tr>
<td></td>
<td>5'- CTCCCCAAGAGACCTTGCAGAA -3'</td>
</tr>
<tr>
<td>Ncam1</td>
<td>5'- ATGAGCTGGACAAAGAGATGG -3'</td>
</tr>
<tr>
<td></td>
<td>5'- GGGAACTATCGTGCTGCAGAAG -3'</td>
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<tr>
<td>NeuroD6</td>
<td>5'- GCAGAATGAGGTCGCGCAAAGA -3'</td>
</tr>
<tr>
<td></td>
<td>5'- ACATTGATGCGGCAACTG -3'</td>
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<tr>
<td></td>
<td>5'- CATTCTGGGCTCCACCTCATT -3'</td>
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<tr>
<td>Ngn2</td>
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<td>5'- CAGGGGTTTTCTGTGCAGCA -3'</td>
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<td>Olig2</td>
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<td></td>
<td>5'- CGGCTCTGTGCTATCTCTCTT -3'</td>
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<td>Pax6 (mouse)</td>
<td>Mm00443072_m1</td>
</tr>
<tr>
<td>Pax6</td>
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</tr>
<tr>
<td></td>
<td>5'- TGTCTCGGATTTTCCCGAGA -3'</td>
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<td>Rlbp1</td>
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<td></td>
<td>5'- AGAAGGGCTGAGCACACATTG -3'</td>
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<td>Sox3</td>
<td>5'- CTGTTGCTTTGTACCGATGA -3'</td>
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<tr>
<td></td>
<td>5'- CATTTTGGCTGCCTGCAGC -3'</td>
</tr>
</tbody>
</table>

All primer sequences are human specific unless other is stated and order is sense: upper; antisense: lower.

VIRAL TRANSFECTION OF CELLS

Labeling cells with GFP

Before transplantation of human fetal cortical and striatal NSCs into the striatum of rat pups (Paper I), the cells were transfected with recombinant VSV-G pseudotyped retrovirus carrying enhanced green fluorescent protein (GFP). The CMV promoter drives the expression of enhanced GFP (van Praag et al., 2002). The titer (transfecting units, TU/ml) of virus
was 4.5 x 10⁸ TU/ml. Before transplantation of human fetal striatal and cortical NSCs into the stroke-damaged rat striatum (Paper II), the cells were transfected with a lentivirus carrying enhanced GFP under the control of a PGK promoter (kindly provided by Drs. A. Consiglio and F. H. Gage) with a virus titer of 5.4 x 10⁷ TU/ml. The multiplicity of infection (MOI) used for both viruses was 1 (ratio 1 TU/cell).

Viral vectors in Pax6 experiments

In Paper III, human cortical and striatal NSCs were transfected with two different VSV-G pseudotyped retroviruses, one with IRES2-GFP (as a control virus), and one with Pax6-IRES2-GFP (kindly provided by Dr. M. Götz). The titer of IRES2-GFP was 4.0 x 10⁸ TU/ml and the expression was under the control of CMV promoter. The Pax6-IRES2-GFP virus had a titer of 2.0 x 10⁸ TU/ml and the expression was under the control of LTR. For both viruses a MOI of 2 was used.

Transfection procedures

Virus was applied directly to flasks containing small neurospheres (3 days after last passage). Neurospheres were incubated with the viral particles for 24 hours, then the spheres were washed by centrifugation to remove virus excess. The pellet was rinsed with basic medium, and neurospheres were replated for further expansion. After another 24 hours, GFP expression was detectable in individual cells in the neurosphere cultures.

SORTING CELLS BY FACS

Neurosphere cultures were passaged and the resulting single cell suspensions were passed through 70 µm cell strainers and subsequently diluted to 2 x 10⁶ cells/ml. To exclude dead cells, 7-amino-actinomycin-D was added at a concentration of 10 µl/ml. To exclude cell aggregates and debris, an initial gate based on forward and side scatter was set. Gates for GFP<sup>pos</sup> and GFP<sup>neg</sup> were chosen arbitrarily, but gates for main GFP<sup>pos</sup> population were always set at least 1 log higher than for the GFP<sup>neg</sup> population. Sorted cells were collected in expansion medium, collected by centrifugation, and replated in flasks for expansion.

MICROSCOPICAL AND STATISTICAL ANALYSIS

Quantification methods

The method for quantification of cell number was dependent on the size and density of the sample. When high numbers of cells within a small, defined area, or high numbers of cells evenly distributed within an area were to be quantified, a computerized setup for stereology driven by a C.A.S.T. - Grid software (Olympus, Denmark) was used. In all other cases, quantification was performed using an epifluorescent/light microscope (Olympus BX61). Double
labeling of cells was validated using a confocal laser-scanning microscope (Leica, Germany). Cells were considered double labeled when double labeling was seen throughout the entire nucleus for nuclear markers, or when a cytoplasmic marker surrounded the stained nucleus in all 3 cross sections produced by orthogonal reconstruction from z-series.

**Statistical methods**

Group differences were evaluated with one-way analysis of variance and a Bonferroni-Dunn post hoc test. When there only were two groups, Student’s unpaired t-test was used. Differences in all analyses were considered statistically significant at $p < 0.05$. 
Results and discussion
RESULTS AND DISCUSSION

Intrastriatal transplantation of human fetal primary tissue, which is rich in post mitotic neurons and glia cells, has in clinical trials provided proof-of-principle that neuronal replacement can work in the human diseased brain (Bachoud-Levi et al., 2000; Lindvall and Bjorklund, 2004). However, the use of primary fetal tissue is not feasible as a source for cell replacement therapy, due to severe logistical problems in coordinating patient, surgery and tissue material, great variability from fetus to fetus, and limited tissue availability. Therefore, we need to identify reliable sources of easily expandable cells of human origin with the capacity to generate cells with similar properties to the cells that die in the different neurodegenerative disorders.

IN VITRO CHARACTERISTICS OF HUMAN NEURAL STEM CELLS

In ischemic stroke, Huntington's disease, and traumatic brain injury, mainly degeneration of neurons in the cortex and striatum occurs. Therefore, we wanted to determine whether human fetal-derived cells could be maintained as neurospheres long-term in vitro, and whether the cells' region of origin influenced their properties after long-term in vitro expansion as neurospheres. In order to study this, we isolated NSCs from the cortex and striatum of aborted human fetuses, expanded them for over one year, and compared their in vitro properties and differentiation capacity.

Similar properties of cortical and striatal NSCs during neurosphere expansion (Paper I)

We found that human cortical and striatal NSC cultures expanded at the same rate, as determined by the proliferation of these cells from passage 3 to passage 16. The proliferative capacity of these cells is similar to that previously reported for human fetal-derived NSCs originating from the rostral part of the telencephalon when expanded as neurospheres (Carpenter et al., 1999; Horiguchi et al., 2004; Ostenfeld et al., 2002). There was also no difference between the cultures either in the size of the neurospheres generated or in the number of secondary neurospheres derived from primary neurospheres, providing a measure of the cells' self-renewal capacity and the proportion of cells with sphere-forming capacity within the cultures, as determined between passage 11 to 15. Additionally, a similar proportion of cells in cortical and striatal NSC cultures were positive for the mitotic marker Ki67 during proliferation, and these Ki67-positive NSCs were found to be evenly distributed within the neurospheres from both cortical and striatal cultures (see page 147, Colour Plates).

In order to determine the phenotypes of the cells within the cortical and striatal neurospheres, we cryosectioned whole neurospheres and stained them for nestin, vimentin, GFAP, and β-III tubulin (see page 148, Colour Plates). Virtually all of the cells in both cortical and striatal neurospheres were nestin-positive and a majority of them co-expressed vimentin, while many nestin-positive cells were also positive for GFAP. Nestin and vimentin are stem/
progenitor cell markers (Hockfield and McKay, 1985; Lendahl et al., 1990) and astrocytic marker GFAP is also known to be expressed in stem/progenitor cells in the adult SVZ (Doetsch et al., 1999). Interestingly, we detected a few cells in every sphere that already expressed β-III tubulin, and had a somewhat differentiated morphology. This is in line with previous data from rodent-derived neurospheres (Parmar et al., 2002), but contrasts with data from human-derived neurospheres, where Tuj1-positive cells were shown to be absent in neurospheres (Svendsen et al., 1998). Together, this indicates that the majority of cells in the cortical and striatal neurospheres were immature stem/progenitor cells. However, some neuronal differentiation was occurring in the neurospheres, further illustrating their heterogeneous nature. Hence, we have shown that neurospheres, derived from the human fetal cortex and striatum, consist of cells at different levels of commitment, consistent with previous studies (Svendsen and Caldwell, 2000).

Region-specific properties after long-term differentiation (Paper I)

In order to determine whether the cortical and striatal NSCs ceased to proliferate under differentiation conditions, neurospheres were plated for differentiation, and were fixed and subsequently stained for Ki67 at various time points (see page 147, Colour Plates). Initially at one day, approximately 30% of the cells in both cortical and striatal cultures were Ki67-positive. As time under differentiation condition progressed, there was a gradual decrease in the number of Ki67-positive cells in the cortical culture, whereas a sharp decrease at 2 weeks was observed in the striatal culture. After 4 weeks of differentiation the number of Ki67-positive cells in both cultures had decreased to approximately 10% of the total cell number. This demonstrated that the cells were capable to responding to the withdrawal of mitogens, exiting the cell cycle and initiating the process of differentiation.

We next investigated the cell phenotypes obtained from the cortical and striatal cultures following differentiation. One day after plating for differentiation, in both cortical and striatal cultures, the majority of cells were nestin- and GFAP-positive, and only very few cells were positive for β-III tubulin. The β-III tubulin-positive cells did, however, display quite mature neuronal morphology with neurites and predominantly bipolar cell bodies. The quantification of the number of cells with these phenotypes is in agreement with the earlier observations from the cryosectioned neurospheres. Differentiation of cortical and striatal cells for 2 and 4 weeks, resulted in an increased number of β-III tubulin-positive cells generated and a concomitant reduction in the number of nestin- and GFAP-positive cells. The differentiated β-III tubulin-positive cells in cortical and striatal cultures displayed bipolar morphology and covered the surface of the well completely, forming an extensive network of dendrites and axons. The gradual decrease in the number of GFAP-positive cells observed in both cortical and striatal cultures, most likely reflected a decrease in the number of the before-mentioned GFAP immunoreactive cells with stem/progenitor characteristics (Doetsch et al., 1999). The GFAP-positive cells gradually matured during the course of differentiation, from lacking specific morphology initially to acquiring mature astrocytic morphology at 4 weeks of differentiation. A substantial amount of the cortical and striatal cells were still nestin-positive
at 4 weeks of differentiation, probably due to the lack of specific developmental cues under \textit{in vitro} conditions, suggesting that the cells were either unresponsive to the differentiation conditions or that the cells simply lacked fate commitment.

The cerebral cortex contains mainly pyramidal projection neurons that are glutamatergic and the rest, around 25\%, are GABAergic interneurons (Jones, 1993). In the striatum, the majority of neurons are GABAergic medium-sized spiny projection neurons, which are positive for DARPP-32 (Deacon et al., 1994; Olsson et al., 1998), as well as either cholinergic or GABAergic interneurons containing parvalbumin, calretinin, somatostatin, neuropeptide Y or neuronal nitric oxide synthase (Kawaguchi et al., 1995). Interestingly, after 4 weeks of differentiation, the cortical NSCs generated a greater number of glutamate-positive neurons than the striatal NSCs. Conversely, the striatal NSCs had generated more DARPP-32-positive neurons than the cortical NSCs. The majority of the neurons generated in both groups were GABAergic and a substantial number were calretinin-, VMAT-2-, and parvalbumin-positive. There were more calretinin-positive cells in the cortical NSC cultures, probably being cortical GABAergic interneurons (del Rio and DeFelipe, 1996). There were also several fold more cells positive for GABA than DARPP-32 in the striatal NSCs cultures, which may indicate that only a portion of the GABA positive cells were striatal projection neurons or that they were not fully mature and did therefore not express DARPP-32 protein. The remaining GABA positive cells were probably parvalbumin and calretinin positive interneurons. The immunoreactivity to VMAT-2 may suggest the presence of catecholaminergic neurons. Furthermore, the VMAT-2 positive cells generated from cortical NSCs co-expressed TH (see page 148, Colour Plates), which is the rate-limiting enzyme in the conversion of catecholamine into dopamine. It has been shown that during brain development in the mouse, cells in the LGE give rise to cells that migrate in the RMS to the olfactory bulb, where some of the cells differentiate into TH-positive neurons (Wichterle et al., 2001). Moreover, NSCs isolated from the mouse developing striatum can generate TH-positive neurons \textit{in vitro} (Daadi and Weiss, 1999). Although there are few TH-positive cells in the adult human striatum (Cossette et al., 2005), it is not known whether the human fetal striatum also is able to produce TH-positive neurons \textit{in vitro}.

These data indicate that the human fetal cortical and striatal NSCs maintain the capacity to generate many types of neurons \textit{in vitro}, and that they retain an intrinsic fate specification consistent with the region from which they were taken.

\textbf{POTENTIAL OF HUMAN NEURAL STEM CELLS AFTER IMPLANTATION}

The fetal-derived cortical and striatal NSCs expanded for an extended period of time as neurospheres \textit{in vitro} had the capacity to generate multiple types of neurons and maintained some degree of region-specificity upon differentiation. Moreover, even after long periods in culture, the NSCs still proliferated at the same rate, irrespective of region of origin, produc-
ing a vast amount of cells as is required for cell transplantation. The next step, in considering these human NSCs for cell replacement therapy, was to explore how the cells reacted to the \textit{in vivo} environment’s inductive signals and to what extent the region of origin influenced the cells’ properties and differentiation potential. In order to answer these questions, we transplanted the cortical and striatal NSCs into the striatum of neonatal rats and stroke-damaged adult rats and analyzed the survival, migration, and differentiation capacity of the grafted cells.

\textbf{Investigation of the survival and migration of grafted human NSCs (Paper I and II)}

Small cortical and striatal neurospheres were transplanted into the striatum of newborn rats and the animals were sacrificed at 1 or 4 months after implantation. To assess the total number of surviving grafted cells, the brain sections were stained with HuNu antibody, which exclusively stains cells of human origin. Quantification of the total number of cells positive for HuNu at 4 months revealed that around 10\% of the grafted cortical and striatal cells had survived. Similarly, cortical and striatal neurospheres were implanted into the striatum of adult rats subjected to stroke. The neurosphere suspension was injected at the border of the intact and stroke-damaged striatal tissue, and animals were sacrificed at one month after transplantation. In contrast to the neonatal brain, approximately 30\% of the grafted cortical and striatal cells in the damaged adult brain survived. Neither transplantation into the neonatal striatum nor transplantation into the damaged adult striatum, seemed to provide any region-specific survival signals, since there was no difference between cortical and striatal NSCs survival after grafting. However, overall graft survival was higher in the stroke-damaged brain, which might be attributable to a higher concentration of cytokines, growth factors, chemokines and neurotrophic factors in the brain tissue as a result of the inflammatory response following ischemic stroke (del Zoppo et al., 2000; Kokaia et al., 1995; Lindvall et al., 1994; Stoll et al., 2002).

The analysis of the migration of the cortical and striatal cells in rostral-caudal direction when grafted in neonatal rats, showed that the cortical and striatal cells migrated similar distances from the implantation site. Furthermore, the majority of cortical and striatal cells were located and dispersed throughout the entire striatum, but a portion of the grafted cells had migrated and were located in the corpus callosum, globus pallidus and cerebral cortex. Thus, despite the ectopic placement of cortical NSCs in the striatum, we did not detect any differences following transplantation of these cells into neonatal rats in regards the cells’ migratory capacity. However, the vast majority (95\%) of the cortical and striatal NSCs grafted into stroke-subjected animals remained within the striatum and migrated predominantly into the lesioned area. It has previously been demonstrated that grafted NSCs are responsive to the chemokines released through the inflammatory response evoked by cerebral ischemia, inducing their migration towards the site of damage (Belmadani et al., 2006; Imitola et al., 2004). In addition, migrating neuroblasts of human origin were identified in the RMS, together with endogenous migrating rat neuronal progenitors at one month after transplantation to adult rats subjected to stroke, indicating some degree of integration with the host brain (see page 52.
In contrast to the observations in neonatal animals (Paper I), the striatal NSCs migrated more extensively within the striatum compared with cortical NSCs, occupying a greater volume of the striatum. Hence, the grafted striatal NSCs were more responsive to the migratory stimuli produced by the injury, than the ectopically grafted cortical NSCs. Additionally, the striatal NSCs migrated more caudally from the implantation sites than the cortical NSCs. The greater migratory capacity of the striatal NSCs in the stroke-damaged brain could either be related to a greater migratory responsiveness of the striatal NSCs to injury in general, or to an inability of the ectopically grafted cortical NSCs to fully respond to the signals in the damaged striatum.

**Differentiation of human NSCs after intrastratial transplantation (Paper I and II)**

In the transplanted neonatal animals, the graft core was undetectable at 1 and 4 months after transplantation, whereas in the grafted adult animals with stroke, a substantial portion of the cortical and striatal cells remained at the injection sites. Almost all of the cells within the transplantation core in the stroke-subjected rats had an undifferentiated morphology and most of them expressed nestin and DCX. Many grafted cells within the core were also positive for GFAP, calretinin, and β-III tubulin. About 30% of HuNu-positive cortical and striatal cells in neonatal rats at one month after transplantation were immunoreactive to human nestin. At 4 months the number of nestin-positive grafted striatal cells had decreased to approximately 20%, whereas the grafted cortical cells remained at the same level observed at 1 month. Similarly, in the stroke-damaged adult animals, a substantial amount (20%) of both cortical and striatal grafted cells were positive for human nestin. These findings are in line with previous studies showing that significant numbers of grafted human cells, isolated from the fetal forebrain, express nestin (Englund et al., 2002b). When considering the use of human NSCs in different transplantation settings, this high number of undifferentiated cells could potentially be troublesome. If the grafted undifferentiated cells continue to proliferate, eventually this might lead to unwanted tumor formation. However, only about 1% of the undifferentiated cells were positive for proliferation markers such as pH3 and Ki67, suggesting that the grafted cells within the core were non-proliferating and quiescent neural stem/progenitor cells (see page 150, Colour Plates). Interestingly, under *in vitro* differentiation conditions, the same cells still have proliferative activity, and after 4 weeks of differentiation approximately 10% of cortical and striatal NSCs are Ki67-positive (Paper I). Altogether, this might indicate that the stroke environment *per se* is suppressive for proliferation of the grafted cells, or that the grafted cells, by some unknown mechanism, stop proliferating when placed into the damaged *in vivo* environment.

Quantitative analysis of the phenotype of the HuNu-positive grafted cortical and striatal cells in neonates revealed that at 1 month after implantation, around 10% of grafted cortical and striatal cells were positive for GFAP and NeuN, and 45% were positive for DCX. At 4 months after transplantation, the number of grafted GFAP-positive cells remained the same as at 1 month. However, the number of grafted cortical and striatal cells positive for DCX had decreased 9-fold, and concomitantly, the number of NeuN-positive grafted cells had
increased 6-fold (see page 149, Colour Plates). This can probably be attributed to the gradual maturation of immature DCX-positive neuroblasts at 1 month into NeuN-positive neurons after 4 months following transplantation. Importantly, there were no significant differences between grafted cortical and striatal cells at either of the two time points with any of the phenotypical markers used. The human cortical and striatal NSCs implanted, ectopically and homotopically, into the neonatal striatum differentiated and matured into neurons to the same extent. Hence, there was no evidence to suggest that the cells’ region of origin influenced their neurogenic capacity after grafting in neonates.

In stroke-damaged adult animals, quantitative phenotypical analysis was performed on the grafted NSCs located outside the core, since it was not possible to accurately count cells within the core due to the high density of cell bodies and fibers. Outside the graft core, around 15% of the grafted cortical and striatal cells were β-III tubulin-positive, 35% DCX-positive, and 35% HuD-positive (see page 151, Colour Plates). There were no grafted cortical or striatal cells outside the graft core that had differentiated into astrocytes as assessed by expression of GFAP and/or S100β, or oligodendrocytes that expressed NG2 and/or CNPase. Immunoreactivity to GFAP was only observed in cells located in the graft core, most likely being undifferentiated stem/progenitor cells (Doetsch et al., 1999). Since the cortical and striatal NSCs differentiate into neurons and glial cells when grafted in the neonatal brain, this finding indicates that the micro-environment in the stroke-damaged brain drives the grafted cells preferentially towards a neuronal, rather than glial cell fate.

In order to further examine the neuronal phenotypes of the grafted cortical and striatal cells in the adult stroke-damaged animals, we stained brain sections for calcium-binding proteins such as calretinin, calbindin, and parvalbumin, which are present in several types of neurons (Fonseca and Soriano, 1995; Kawaguchi et al., 1995). Between 6 to 10% of both cortical and striatal grafted cells located outside the core expressed calretinin. Although calretinin is expressed in interneurons in the adult brain (del Rio and DeFelipe, 1996), it is also present in neuroblasts at various stages of differentiation and subsequent maturation. The majority of the grafted calretinin-positive cells resembled neuroblasts rather than mature interneurons, as indicated by their bipolar morphology with less elaborate processes. Less than 1% of the grafted human cells were positive for calbindin. However, the grafted striatal NSCs generated significantly more calbindin-positive neurons than cortical NSCs. Calbindin is expressed in both interneurons and projection neurons in the striatum, but they can be separated by morphology and level of immunoreactivity. Calbindin-positive striatal interneurons express the protein strongly and have a few processes that are branched sparsely, whereas calbindin expression of striatal projection neurons is weaker, and without clear staining of the processes (Cicchetti et al., 2000). We observed that the grafted human cells exclusively displayed characteristics of the latter cell type, and thus, grafted cortical and striatal cells more closely resembled projection neurons than interneurons.
In both neonatal and ischemia-induced adult rats, the implanted cortical and striatal cells were parvalbumin-positive. Parvalbumin is expressed by a subtype of GABAergic interneurons (Kawaguchi et al., 1995). Morphologically, the grafted parvalbumin-positive neurons resembled the resident host parvalbumin expressing neurons, but with somewhat smaller cell bodies. In the neonatal animals and under in vitro conditions, the number of parvalbumin-positive neurons generated from cortical and striatal NSCs was similar. Furthermore, the number of grafted cortical and striatal NSCs that co-expressed parvalbumin after neonatal implantation, was markedly greater than the number of parvalbumin-positive interneurons normally present in the rat striatum (Luk and Sadikot, 2001). Thus, the phenotype of the xenografted human NSCs after implantation to neonates did not reflect the normal composition of the host striatum. In contrast, in the stroke-damaged animals, the number of grafted parvalbumin-positive cells was not disproportionately high. Interestingly, the grafted striatal NSCs generated significantly more parvalbumin-positive neurons (6%) compared to cortical NSCs (2%) in adult rats with stroke. These data indicate that alterations in the environment, related to the stroke lesion, may have a differential effect on the fate of the grafted cortical and striatal cells.

Thus, morphologically and phenotypically the grafted human NSCs were able to generate neurons. However, whether they can successfully become functionally integrated with the host brain remains unclear. Nevertheless, we encountered grafted human cells migrating in the RMS towards the olfactory bulb together with host neuroblasts, indicating, that the grafted human cells are able to properly respond to internal cues of the host rat brain.

INCORPORATING THE CELLS’ NEUROGENIC POTENTIAL WITH PAX6

For further development of safe, standardized, and unlimited human stem cell sources, requiring a guaranteed high and efficient yield of neurons of the desired type after transplantation, we need to first be able to control the cells’ lineage commitment and differentiation in vitro. Furthermore, for the development of successful protocols for future cell replacement strategies in neurodegenerative diseases, the ability to increase the neurogenic potential of human NSCs is of crucial importance. In order to explore this possibility, we genetically manipulated the cortical and striatal human NSCs by means of retrovirally mediated overexpression of the transcription factor Pax6.

Pax6 increases the generation of neurons in human striatal NSCs (Paper III)

The cortical and striatal NSC cultures were passaged over 15 times prior transfection with retrovirus either carrying GFP alone or GFP together with the mouse sequence encoding Pax6. We determined by RT-PCR and immunocytochemistry that both naïve cortical and striatal NSCs endogenously expressed Pax6. In contrast to the rodent brain, Pax6 is expressed in the human fetal cortex and striatum (Manuel and Price, 2005; Mo and Zecevic, 2007) and this expression is maintained after long-term culturing of NSCs.
Quantification of the number of β-III tubulin-positive cells following Pax6-transfection and subsequent differentiation of the cortical and striatal NSCs, revealed that around 40% of transfected cells were β-III tubulin-positive in both groups at 2 weeks and was significantly greater compared to their respective control group. Two weeks later, at 4 weeks of differentiation, the number of β-III tubulin-positive cells was further increased in both the Pax6- and control-transfected human cortical cells. However at this time point, there was no difference between the cortical cell cultures transfected with Pax6 and GFP control virus. In contrast, when we analyzed the number of β-III tubulin-positive cells generated from the transfected human striatal cells; there was only an increase in the number of β-III tubulin-positive cells in the Pax6-transfected striatal cells. At 4 weeks of differentiation around 70% of the Pax6-transfected striatal cells were β-III tubulin-positive, which was substantially higher compared to the control-transfected striatal cells (see page 152, Colour Plates). These data indicate that, although Pax6-overexpression increased the number of β-III tubulin-positive cells at 2 weeks in both the cortical and striatal cell cultures, only striatal NSCs transfected with Pax6 could generate more neurons for an extended time of in vitro differentiation.

Pax6 is a potent neurogenic determinant for radial glia precursor cells and is sufficiently potent to direct postnatal cortical astrocytes towards a neuronal phenotype (Berninger et al., 2007a; Berninger et al., 2007b; Gotz et al., 1998; Heins et al., 2002). Therefore, we explored whether the increased generation of β-III tubulin-positive neurons after Pax6-overexpression in striatal NSCs also was associated with changes in the generation of glial cells. We found that in the striatal Pax6-overexpressing NSCs, there was a gradual decrease in the generation of GFAP-positive cells over time during differentiation, whereas the same gradual decrease was not observed in the cortical Pax6-overexpressing NSCs. Thus, at 4 weeks of differentiation of the striatal Pax6-overexpressing cells, the number of GFAP-positive cells was significantly decreased compared to control. Indicating that the increased neurogenesis observed occurs partly at the expense of GFAP-positive glia cells (see page 152, Colour Plates). This is in agreement with a recent study where the silencing of Pax6, by means of siRNA in human cultured stem/progenitor cells resulted in decreased neurogenesis and increased generation of GFAP-positive cells (Mo and Zecevic, 2007).

It has previously been speculated that Pax6-overexpression can influence cell proliferation (Manuel et al., 2007; Marquardt et al., 2001; Philips et al., 2005) and cell survival (Berger et al., 2007; Nikoletopoulou et al., 2007). However, when we addressed these issues by counting the number of mitotic Ki67-positive cells and apoptotic or dying cells, we did not obtain any data suggesting that Pax6-overexpression in human cortical and striatal NSCs affected cell proliferation and/or survival. Therefore in conclusion, it is likely that the human striatal NSCs overexpressing the transcription factor Pax6 have an enhanced drive towards the neuronal fate, and these changes are likely not a secondary effect due to alterations in cell proliferation and survival.
Pax6 increases the generation of neurons with maintained region-specificity (Paper III)

Since long-term neurogenesis was only promoted by Pax6-overexpression in human striatal NSCs, we sorted the striatal NSCs by FACS in order to further study the effect of Pax6-overexpression on the properties of these cells. After cell sorting, the isolated GFP-positive striatal NSC population was expanded. Firstly, we ensured that the sorted Pax6-transfected striatal NSCs were expressing high levels of Pax6 using QPCR. The sorted GFP-positive Pax6-transfected NSCs had more than a 6-fold higher level of inserted mouse Pax6 compared to the level of the internal control gene β-actin. Furthermore, virtually all of the cells isolated by FACS were GFP-positive and, as determined by immunocytochemistry, many of the GFP-positive cells co-expressed high levels of Pax6 protein. Secondly, in spite of the sorting and subsequent expansion, the sorted Pax6-overexpressing NSCs still generated significantly more β-III tubulin-positive neurons as compared to sorted NSCs transfected only with GFP virus, after 4 weeks of differentiation in vitro (see page 153, Colour Plates).

To determine the phenotype of the neurons generated, we differentiated the sorted Pax6-overexpressing NSCs and control striatal NSCs for 4 weeks and stained for GABA, DARPP-32, and glutamate. Interestingly, quantification revealed that sorted Pax6-overexpressing NSCs generated significantly more (over 50%) of GABAergic neurons as compared to the sorted control NSCs (around 30%). In the control cultures relatively few cells were DARPP-32-positive, whereas several fold more cells differentiated into DARPP-32-positive neurons in the sorted Pax6-overexpressing striatal cultures. In addition, there was no difference in the number of glutamate-positive neurons generated from the sorted Pax6-overexpressing and control NSCs (see page 154, Colour Plates). We have shown previously that expanded human striatal NSCs maintain the specificity of their region of origin and generate mostly GABAergic and DARPP-32-positive neurons following differentiation in vitro (Paper I). In neurosphere cultures, there is a lack of expression or misexpression of region-specific genes (Gabay et al., 2003; Hack et al., 2004; Santa-Olalla et al., 2003), but on the other hand it is known that NSCs expanded as neurospheres partially retain regional specification (Paper I; Hitoshi et al., 2002; Ostenfeld et al., 2002; Parmar et al., 2002; Zappone et al., 2000). It is however conceivable, that the culture conditions can, to some extent, alter the original specification of the neural stem/progenitor cells. Taken together, these results indicate, in contrast to results obtained from mouse cells (Hack et al., 2004), that overexpression of Pax6 in human striatal NSCs increases the generation of neurons with a striatum-specific phenotype.

In order to test whether overexpression of mouse Pax6 in human cells could lead to gene changes similar to observations made in transgenic mice (Holm et al., 2007), we compared the mRNA levels of selected genes in cultured human striatal NSCs with and without Pax6-overexpression during both proliferation and after 4 weeks of differentiation by Q-PCR. Interestingly, we found that several genes implicated to be Pax6-dependent in the mouse brain (Holm et al., 2007), were significantly altered also in the human NSCs overexpressing Pax6. This is in line with the known sequence homology between species in the well-conserved gene Pax6 (Halder et al., 1995; Onuma et al., 2002). However, only the expression level of Rlbp was
strongly increased during proliferation consistent with the previous analysis performed by Holm and coworkers (Holm et al., 2007). In contrast, expression of several early proneurogenic and progliogenic genes such as Nfia, Sox3, Ngn2, NeuroD6, Bhlhb5, and AP2γ, which were positively regulated in mouse cells (Holm et al., 2007), were downregulated upon Pax6-overexpression in human striatal NSCs during proliferation. Furthermore, NCAM and Olig2 were upregulated, whereas HuD was downregulated by Pax6-overexpression in proliferative conditions as compared to control cells. Increasing levels of Pax6 is sufficient to push neural progenitors towards cell cycle exit and neuronal commitment. However, maintained high levels of Pax6 in mouse and chick embryos, make the progenitors unable to continue neuronal maturation (Bel-Vialar et al., 2007). Moreover, high levels of Pax6 counteract the proneuronal activity of Ngn2, and the level of Pax6 is stabilized by autoregulation and by possible negative feedback regulation exerted by Ngn2 (Bel-Vialar et al., 2007; Manuel et al., 2007). It is feasible that Pax6-overexpression drives the NSCs towards a neuronal phenotype already during proliferation as neurospheres, but the neuronal maturation is blocked due to the high expression of Pax6, possibly inhibiting the proneuronal activity by Ngn2. However, at 4 weeks of differentiation, the expression levels of NCAM, Rlpb, and Olig2 were decreased in the Pax6-overexpressing cells compared to the levels present during proliferation. In contrast, Sox3, HuD and AP2γ were increased in the Pax6-overexpressing cells, although their expression remained at lower levels compared to the control cells. This might indicate that the cells subsequently matured from immature neuronal precursors under proliferative conditions to neurons at 4 weeks of differentiation in vitro.

Striatal DARPP-32-positive medium-sized spiny projection neurons and GABAergic interneurons originate during embryonic rodent brain development in the LGE (Olsson et al., 1998; Olsson et al., 1995). In the proliferating striatal neurospheres overexpressing Pax6, we found an upregulation of retinol-binding protein type 1 (Rlpb1) mRNA levels. Rlpb1 is associated with retinoic acid (RA) synthesis and RA signaling has been implicated in neurogenesis and neuronal differentiation (Guan et al., 2001). Moreover, retinoids are important for regulating DARPP-32 expression, and have previously been demonstrated to upregulate levels of DARPP-32 in primary cultures from the rodent striatum (Toresson et al., 1999; Wang and Liu, 2005). It has been suggested that neurosphere cells take on a radial glia-like phenotype (Hack et al., 2004; Hartfuss et al., 2001), and radial glial cells express Rlpb. Interestingly, radial glial cells have been proposed to be the major source of retinoids within the LGE (Toresson et al., 1999), and Pax6 is considered to be the major neurogenic determinant for radial glial cells (Heins et al., 2002; Mo and Zecevic, 2007). Altogether, our findings together with previous published work strongly indicates, that the proneurogenic program initiated by Pax6 in human striatal NSCs involves RA-signaling (Holm et al., 2007).

**Pax6 enhances neuronal differentiation in human striatal NSCs after grafting (Paper III)**

To investigate whether Pax6 also increased the generation of neurons in vivo and whether the properties of the Pax6-overexpressing NSCs were affected by the host brain environment, we grafted the sorted striatal NSCs with and without Pax6 into the striatum of neonates. One
month after transplantation, the majority of transplanted cells were only weakly GFP-positive, probably due to downregulation of GFP expression. Subsequently, the grafted cells were identified based on their immunoreactivity for the human-specific antibody HuNu.

Quantification of the total number of HuNu-positive cells in different forebrain structures revealed that the survival between the two groups, Pax6-overexpressing and control cells, was relatively variable between individual animals (from around 10 to 60%), however, there were no statistically significant differences between the groups. The majority of grafted cells were located in the striatum, with a small portion of cells from both groups also found in the cortex (10%). In contrast, there was a greater number of HuNu-positive cells overexpressing Pax6 located in the corpus callosum as compared to the number of HuNu-positive control cells (45% versus 30%). White matter tracts, such as the corpus callosum, often attract migrating neuroblasts (unpublished observations), and our finding suggests that, although cells were placed in striatum, Pax6-overexpressing cells have a higher drive towards the corpus callosum. Counting HuNu-positive cells at different coronal levels from the implantation site was performed to assess the degree of rostro-caudal migration. There were no differences between Pax6-overexpressing and control cells in the distribution pattern of HuNu-positive cells within the striatum. Additionally, we assessed the proliferation of the grafted cells at one month after implantation by staining for the mitotic marker Ki67. There were significantly fewer HuNu-positive Pax6-overexpressing cells undergoing proliferation than HuNu-positive control cells (around 10% versus 35%). This is in agreement with a decrease in proliferation demonstrated after conditional activation of Pax6 in the embryonic cortex of transgenic mice (Berger et al., 2007; Manuel et al., 2007). Furthermore, previous studies have reported a shortened cell cycle and increased proliferation in the cortex of Pax6 mutant Sey mice and in cells taken from the cortex of Sey mice (Estivill-Torrus et al., 2002).

Since the majority of the grafted cells were found in the striatum, we assessed the number and phenotype of the human striatal NSCs in this structure (see page 155, Colour Plates). At one month after transplantation, many of the grafted HuNu-positive Pax6-overexpressing and control cells still co-expressed nestin (55% versus 40%). Interestingly, there were significantly more Pax6-overexpressing cells that expressed the neuroblast marker DCX (50%), as compared to control cells (20%). Pax6 has been demonstrated to be expressed in PSA-NCAM-positive and DCX-positive neuroblasts in the mouse SVZ (Hack et al., 2005) and RMS (Gritti et al., 2002), and plays an essential role in the neurogenesis and specification of adult olfactory bulb interneurons. Recently, Baer and co-workers (Baer et al., 2007) showed that PSA-NCAM-positive cells in the human post-mortem SVZ are Pax6-positive. This finding indicates that Pax6 could also be an important factor in the human brain in regulating neuronal differentiation and the migration of neuroblasts in the human RMS (Curtis et al., 2007).

In contrast to numbers of DCX-positive grafted cells, only a small proportion of the cells in both groups co-labeled with HuNu and the mature neuronal marker NeuN (3% for Pax6-overexpressing cells and 4% for control cells). Previously, we have shown that from 1
to 4 months after implantation into neonates, the number of grafted cells positive for DCX decrease 9-fold, whereas the number of grafted cells positive for NeuN increase 6-fold (Paper I). This indicates that human cells require at least 4 months of differentiation in the neonatal brain, in order to become mature neurons expressing the marker NeuN. However, another study using transgenic mice demonstrated that premature neurogenesis occurred after conditional activation of Pax6 in the developing cortex (Berger et al., 2007). Therefore, it is conceivable that the greater number of DCX-positive Pax6-overexpressing cells, will with longer survival time of the animals continue to differentiate, and consequently, Pax6-overexpression will lead to an increased number of mature neurons.

In both groups we found similar numbers of grafted cells co-expressing HuNu and β-III tubulin with a minor portion of grafted cells positive for GFAP with astrocyte-like morphology. In small cell clusters we also found low numbers of HuNu-positive cells co-expressing calretinin. However, compared to the control cells, there were significantly fewer Pax6-overexpressing cells co-labeled with the neuronal marker HuD (20% versus 4%). Additionally, as determined by Q-PCR, the HuD mRNA-expression was decreased in the Pax6-overexpressing NSCs under both proliferative conditions and after 4 weeks of differentiation in vitro compared to control cells. HuD has been shown to negatively regulate proliferation of NSCs and to promote the differentiation of postmitotic neurons (Akamatsu et al., 2005), and to induce neuronal differentiation when overexpressed in neuronal progenitors and PC12 cells (Akamatsu et al., 1999; Kasashima et al., 1999). However, despite the lower number of HuD-positive cells in grafted Pax6-overexpressing cells, we detected fewer proliferating cells, and a greater number of DCX-positive cells in animals grafted with Pax6-overexpressing cells (see page 156, Colour Plates). In addition, no difference in the number of grafted NeuN-positive cells was observed between the groups. Taken together, these findings might be the result of the proneuronal and antiproliferative effects exerted by Pax6, which override the potential effects of reduced HuD-levels. Alternatively, the high levels of Pax6 may have prevented further neuronal differentiation and thereby decreased the number of grafted HuD-positive Pax6-overexpressing cells compared to number of HuD-positive control cells (Bel-Vialar et al., 2007).
General discussion
GENERAL DISCUSSION

Today it is no more a far fetched hope, but a realistic goal, to claim that stem cells will be an inexhaustible source of neurons and glia for cell replacement therapies aimed for the treatment of disorders affecting the brain and spinal cord. However, before we can move to the clinic, there are several major issues that need to be resolved before we can safely implement the findings, obtained in cells culture systems and in animal models of disease, to patients.

CHOICE OF CELL SOURCE AND CULTURE METHODS

Embryonic stem cells and stem cells from the fetal or adult CNS or other tissues might all be suitable for the purpose of cell replacement therapy, since they all have shown they have the capacity to differentiate into multiple cell types of the adult CNS (Conti et al., 2005; Gage et al., 1995; Pollard et al., 2006; Reynolds et al., 1992; Reynolds and Weiss, 1992, 1996; Tropepe et al., 2001; Vescovi et al., 1999). Moreover, the cells from these different sources have been tested in different experimental models (Bjorklund et al., 2002; Kelly et al., 2004; Svendsen et al., 1997; Svendsen et al., 1996), but the data obtained have given somewhat varying results (Zietlow et al., 2008). This might mean that different stem cells, cultured according to stringently controlled and predefined protocols, may be required for each disease, due to the different pathological mechanisms involved in various neurodegenerative diseases.

Embryonic stem cells are the most plastic of all stem cell sources and are pluripotent, i.e. they can give rise to cells from all three germ layers. The potential use of ES cells for brain repair was proven through the transplantation of undifferentiated mouse ES cells into parkinsonian rats. The ES cells differentiated into dopaminergic neurons and restored impaired function to some degree. However, many animals died due to the formation of teratomas (Bjorklund et al., 2002). This experiment highlights the main obstacle of using these cells as a safe cell source from the clinical perspective, namely the uncontrolled growth of undifferentiated ES cells after transplantation. Therefore, one of the main challenges for using ES cells is the fact that they need to be directed towards a defined fate prior transplantation. Protocols for the induction of neural fates (Wichterle et al., 2002), in particular towards a dopaminergic phenotype (Perrier et al., 2004; Yan et al., 2005), are in progress. Nevertheless, the issue related to the risk of undifferentiated ES cells potentially contaminating the grafts needs to be resolved. As an alternative of obtaining ES cells from the inner cell mass of blastocysts, it may be possible to make ES cells from somatic cells taken from the patients themselves. This could be done either using therapeutic cloning techniques, or by the generation of inducible pluripotent stem (iPS) cells by the reprogramming of somatic cells (Meissner et al., 2007; Nakagawa et al., 2008; Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007). In this way, the ethical and immunological issues often raised in regard to the use of ES cells and fetal NSCs would no longer exist, since the cells would be from the patients themselves. However, for patients with neurodegenerative diseases having genetic components, this approach may be
problematic, as the autotransplanted iPS cells also would carry the genetic aberrations causing the disease. Another drawback is that the cells will likely only be produced after the patient becomes ill. Thus, they cannot be used to treat the acute phase of the disease in question. Moreover, quality control of these cells is likely to be difficult and expensive, because each individual patient will require a separate batch of iPS cells to be produced.

Somatic human NSCs can be obtained either from the adult or the fetal CNS. Both adult and fetal human NSCs can be expanded in monolayer and suspension cultures with addition of growth factors (Carpenter et al., 1999; Conti et al., 2005; Horiguchi et al., 2004; Pollard et al., 2006; Tarasenko et al., 2004). Although, for ethical and immunological reasons, adult human NSCs would be optimal, they are rather difficult to obtain. Moreover, despite the success reported by some groups in culturing and transplanting adult human NSCs (Walton et al., 2006; Westerlund et al., 2003), they are notoriously difficult to expand for a long time in vitro (Evans et al., 2003; Nunes et al., 2003), which is required in order to be regarded as a potential source for cell replacement therapy. In regards to the expansion of human fetal NSCs as neurospheres, studies have reported a relatively low yield of neurons with increasing passage number (Anderson et al., 2007; Wright et al., 2006), and after transplantation into rodent recipients (Englund et al., 2002a; Englund et al., 2002b; Fricker et al., 1999; Jain et al., 2003; Svendsen et al., 1997). This poses a problem and limits their usefulness as a source for cell replacement therapy. In contrast, we (Paper I, II and III) and others (Svendsen et al., 1998), have shown that fetal-derived NSCs generate significant numbers of neurons, both in vitro and in vivo, even when cultured and expanded for a considerable amount of time. Monolayer NS cell cultures derived from fetal NSCs have been shown to generate a vast number of neurons in vitro and after transplantation. However, to date the morphology and functional profile of grafted cells has not been described and therefore the potential use of these cells for cell replacement therapy is currently rather inconclusive (Conti et al., 2005).

Human cells are probably needed for application in the clinic and despite the tremendous potential for generating many types of neural cells and neurons, it is necessary to gain significant progress in how to obtain precise control over cell function and cell subtype specification.

**Influence by region of origin on human neural stem cells**

Detailed knowledge of how specific cell types in various areas of the CNS are affected by disease is required in order to fully understand which different cell types are needed in different disorders. It is important to determine whether the cells themselves retain a distinct intrinsic molecular memory of their region of origin, and thus, are able to differentiate into the relevant neuronal subtypes required for replacing dying or dead cells in each particular disorder.

In previous studies, both from our and other groups, it has been demonstrated that human fetal NSCs expanded as neurospheres and as NS cell cultures from mouse developing
brain partially retain regional specification (Hebsgaard, 2008; Horiguchi et al., 2004; Ostenfeld et al., 2002). Differences between cells’ region of origin were shown to exist in growth characteristics of the cell cultures, in regional marker expression during expansion and in the neuronal subtypes generated after differentiation. Additionally, when striatal and cortical human fetal NSCs were transplanted into the striatum of stroke-subjected rats, differences in their migratory capacity and formation of neuronal subtypes were detected (Paper II). However, it has also been reported that NSCs expanded as neurospheres lose some of their region-specific expression of transcription factors (Gabay et al., 2003; Hack et al., 2004; Santa-Olalla et al., 2003). Indicating, that the artificial environment the cells encounter during expansion and differentiation in the culture system, at least to some extent, alters the original specification of the cells. Therefore, we need to better understand the cells’ capacity following isolation from different sub-regions of the brain, how they may be differentially changed under different culture system conditions, and how we can influence their potential for neuronal subtype specification.

**CELL FATE DETERMINATION**

Before we apply stem cell based therapies to patients, we must be able to control their proliferation and be able to direct the differentiation of stem cells into specific, therapeutically valuable cell phenotypes, while preventing tumor formation (Robertson et al., 2008). This can either be achieved by the in vivo manipulation of the endogenous stem cell populations, or by expansion and programming stem cells ex vivo. Cells can be manipulated either through the addition of regulatory proteins/molecules or by incorporating new genetic material encoding, for example, key regulatory transcription factors into the cells’ genome.

There are many known developmentally important regulatory proteins that potentially could control cell function precisely. One of them is RA, a small molecule known to be important for neuronal differentiation during development (Diez del Corral and Storey, 2004; Guan et al., 2001) and has commonly been utilized to induce neuronal differentiation in vitro (Guan et al., 2001; Toresson et al., 1999; Wang and Liu, 2005). However, since these regulatory proteins are highly dose-, and context-dependent, most likely a combined action of factors regulating cell fate will need to be employed. Interestingly, in our Pax6-overexpression experiments, we found that the retinol-binding protein type 1 (Rlbp1) was upregulated in cultures with a Pax6 insert compared to control cultures. Rlbp1 is closely associated with the synthesis of RA, and thus we can speculate that the increased neurogenesis initiated in the striatal NSCs cultures by Pax6-overexpression may involve the RA-signaling pathway (Goncalves et al., 2005; Holm et al., 2007; Mey and McCaffrey, 2004).

The addition of exogenous factors is an approach that requires careful optimization of all parameters such as dose, order of addition, and time of exposure. This is a difficult task given that we are just beginning to elucidate the regulatory factors involved in neuronal differentia-
tion. Additionally, key regulators such as transcription factors, important for mitotic activity, cell fate specification, and differentiation during development of the CNS, have mainly been studied in rodents. Undoubtedly, research on rodents will continue to add to our general understanding of forebrain development. At the same time, it is essential to remember that regulation of development by transcription factors may differ somewhat between humans and rodents (Bystron et al., 2008; Mo and Zecevic, 2007). Therefore, before extrapolating results from animals to the human brain, one should consider that transcription factors may change their expression with species, region of the brain, and stage of development. Moreover, the cell populations that we culture are, for most the part, heterogeneous, which means that the cells will respond differently to added factors. However, by introducing key genes, such as transcription factors, to the cells, or by sorting the cells using antibodies to cell surface markers (Uchida et al., 2000), we can potentially increase the homogeneity of the cells’ response and thereby potentially direct neuronal differentiation. Recently, it was shown that somatic cells could be reprogrammed to become pluripotent stem cells by the overexpression of only four genes (Meissner et al., 2007; Park et al., 2008; Takahashi et al., 2007). Thus, genetic manipulation is a powerful tool in order to generate expandable cells with defined properties. We overexpressed the transcription factor Pax6 in human fetal NSCs and showed that it promoted the production of neurons with region-specific phenotypes (Paper III). However, for the generation of large numbers of transplantable cells with ideal properties, it is conceivable that Pax6 overexpression may need to be combined with other factors in a strictly controlled and ordered fashion. Moreover, before modified cells can be used in patients, it must be proven that genetic manipulation does not lead to any adverse effects such as abnormal function or tumor formation of the grafted cells. The drawback of using integrating retroviruses can be solved with transient conditional gene transfer, that is for example, regulated transgene expression using inducible promoters such as the tetracycline (tet-on/off) system or by choosing promoters facilitating cell-specific expression (Corti et al., 1999; Sinn et al., 2005). An alternative approach to intrinsic modification of the NSCs, is to elucidate the factors that extrinsically will activate appropriate genes and thereby obtain ideal properties of the cells for transplantation.

CELL REPLACEMENT THERAPY IN THE CLINIC

In some disorders, functional improvement can probably only be induced with transplantation of *in vitro* generated stem cells, whereas in other disorders the most beneficial treatment may be to stimulate endogenous CNS stem cells, or a combination of both may be required (Lindvall and Kokaia, 2006). Understanding the mechanisms of stem cell biology is essential to optimize protocols for the development of safe graft material. However, the graft material is one part; the other part is the recipient, which in this case is a patient with a neurological disorder. The pathology in stroke is very dynamic with initial necrosis that gradually is replaced by apoptosis as well as the inflammatory response following the insult. Stroke leads to motor, sensory or cognitive impairments. There are no effective treatments in the
sub-acute phase after a stroke and, therefore, any kind of treatment beneficial for recovery is valuable. Although much is known about the disease mechanisms in stroke, we lack sufficient knowledge of how to promote recovery and the type of cells required to make a stem cell based therapy for stroke safely work in humans. In spite of this, several clinical trials have been conducted with variable results, clearly indicating that these trials were prematurely initiated (Bang et al., 2005; Kondziolka et al., 2005; Kondziolka et al., 2000; Savitz et al., 2005). Some of the questions we need to answer about stroke and disease pathology in order to understand the mechanisms and requirements in the recipient brain, are: firstly, what clinical stage of stroke is the most effective for transplantation, and, secondly, which region of the patient’s brain is the most favorable for transplantation, in terms of both functional integration and patient safety?

Based on our knowledge at this point, the optimal time for transplantation in animal models is at 1-2 weeks after initial insult (Paper II). At this stage the ongoing cell detrimental excitotoxic and inflammatory processes have declined, but substantial amounts of growth factors, cytokines and neurotrophic factors are still present that potentially could aid in the survival and differentiation of the grafted cells. However, the equivalent optimal time point after insult in animals must be translated to the various stroke situations in humans. What is clear though is that the purpose of the therapeutical intervention will determine the optimal time window. For the purpose of promoting rescue of compromised neural cells by neurotrophic activity and stimulating endogenous plasticity, implantation in the early phase after stroke may be most beneficial. In contrast, for the purpose of cell replacement therapy, later phases after stroke may be more favorable since the environment is less hostile, which enhances the grafted cells’ chance of survival.

The site of implantation of course depends, again on, the purpose of treatment and where the ischemic core is located. In more general terms, depositing cells for replacement therapy in the aggressive environment of the core would, likely, be the least optimal place. Since it has been demonstrated that cells migrate in response to stimuli towards the lesion (Belmadani et al., 2006; Imitola et al., 2004), the most favorable placement is probably in the intact tissue just at the border of the penumbra region. Thus, the grafted cells would more easily survive, migrate to the penumbra and differentiate into mature neurons and glia cells. Thereby, the grafted cells could replace dead neurons and possibly supply trophic support to newly formed endogenous cells.

Additionally, we need to determine the optimal number of cells for transplantation and the number of deposits needed for functional recovery. These questions are all related to one another and are issues of transplantation methodology (Zietlow et al., 2008). However, the efficacy of any stem cell based therapy must be demonstrated in animal models resembling the human disease and need to be carefully examined in animal experiments proving their safety and reliability using a set of standardized protocols. Furthermore, to maximize the treatment efficacy, it is essential that we carefully select the patients with the best prognosis of
gaining the most from a cell replacement therapy. Each incidence of stroke in patients is very different, depending on the location of the clot, the size of the damaged area, and the variety of cells and neuronal circuits affected. Moreover, the patients themselves are of different age, sex, physical and psychological status etc. Thus, decision of treatment strategy must be based from characteristics of each individual patient. In the end, it is the ability to provide patients with neurological disorders a safe and long-lasting treatment that substantially improves their quality of life more efficiently than already existing approaches, that determines the usefulness of stem cells in the clinic.
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