Neural Stem and Progenitor Cells as a Tool for Tissue Regeneration

ULRIKA WALLENQUIST
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Abstract

Neural stem and progenitor cells (NSPC) can differentiate to neurons and glial cells. NSPC are easily propagated in vitro and are therefore an attractive tool for tissue regeneration. Traumatic brain injury (TBI) is a common cause for death and disabilities. A fundamental problem following TBI is tissue loss. Animal studies aiming at cell replacement have encountered difficulties in achieving sufficient graft survival and differentiation. To improve outcome of grafted cells after experimental TBI (controlled cortical impact, CCI) in mice, we compared two transplantation settings. NSPC were transplanted either directly upon CCI to the injured parenchyma, or one week after injury to the contralateral ventricle. Enhanced survival of transplanted cells and differentiation were seen when cells were deposited in the ventricle. To further enhance cell survival, efforts were made to reduce the inflammatory response to TBI by administration of ibuprofen to mice that had been subjected to CCI. Inflammation was reduced, as monitored by a decrease in inflammatory markers. Cell survival as well as differentiation to early neuroblasts seemed to be improved.

To device a 3D system for future transplantation studies, NSPC from different ages were cultured in a hydrogel consisting of hyaluronan and collagen. Cells survived and proliferated in this culturing condition and the greatest neuronal differentiating ability was seen in cells from the newborn mouse brain.

NSPC were also used in a model of peripheral nervous system injury, and xeno-transplanted to rats where the dorsal root ganglion had been removed. Cells survived and differentiated to neurons and glia, furthermore demonstrating their usefulness as a tool for tissue regeneration.

Keywords: traumatic brain injury, neural stem cells, transplantation, CNS, PNS, progenitor cells, inflammation, CCI

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urn:nbn:se:uu:diva-110095 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-110095)
To my family
Cover page: Neuron stained with a beta-III-tubulin antibody
List of Papers

This thesis is based on the following papers:


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## Contents

**Introduction** ............................................................................................................ 11

The Central Nervous System ......................................................................................... 12
  The organization of the Central Nervous System (CNS) ........................................ 12
  Central Nervous System Development ................................................................ 12
  Extracellular Matrix in the Developing and Adult CNS ........................................ 14

Peripheral Nervous System (PNS) .............................................................................. 15
  Dorsal Root Ganglion ................................................................................................. 15

Stem Cells ................................................................................................................... 17
  Stem Cell Niches in the Adult Brain ....................................................................... 17
  Proliferation and Differentiation of Rodent Neural Stem Cells ............................... 19
  Stem Cell Controversies ........................................................................................... 21

Traumatic Brain Injury (TBI) ..................................................................................... 22
  Pathophysiology of TBI ............................................................................................ 22
  Inflammation ............................................................................................................ 23

Cellular Response to Brain Injury .............................................................................. 24
  Neurogenesis after Injury ....................................................................................... 24
  The Role of Astrocytes after Injury ........................................................................ 25
  Microglia .................................................................................................................. 25
  Injury to the PNS ....................................................................................................... 26

Experimental TBI ........................................................................................................ 28
  Controlled Cortical Impact ....................................................................................... 28
  Strategies for Reducing Experimentally Induced Injury .......................................... 28
  Evaluation of Treatment effects after Experimental TBI ........................................ 29
  Antibodies for Histological Evaluation .................................................................. 29
  Cognitive Evaluation ............................................................................................... 31
  NSPC as a Tool for Tissue Regeneration in the Injured Nervous System .............. 31
  Transplantation of Stem Cells after Experimental TBI .......................................... 31
  From the Laboratory to the Clinic ......................................................................... 34

Present Investigation .................................................................................................... 35

Results and Discussion ............................................................................................... 35

(I) Central nervous system stem/progenitor cells form neurons and peripheral glia after transplantation to the dorsal root ganglion ....... 35
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>CCI</td>
<td>Controlled Cortical Impact</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<td>CNTF</td>
<td>Ciliary Neurotrophic Factor</td>
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<td>COX</td>
<td>Cycloxygenase</td>
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<td>DCX</td>
<td>Doublecortin</td>
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<td>DG</td>
<td>Dentate Gyrus</td>
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<td>DRG</td>
<td>Dorsal Root Ganglion</td>
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<tr>
<td>DRTZ</td>
<td>Dorsal Root Transitional Zone</td>
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<td>E</td>
<td>Embryonic day</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>GDNF</td>
<td>Glial Cell Line-derived Neurotrophic Factor</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
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<tr>
<td>ICAM-1</td>
<td>Inter-cellular Adhesion Molecule 1</td>
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<td>IGF-1</td>
<td>Insulin-like Growth Factor</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
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<td>Map2</td>
<td>Microtubule associated protein 2</td>
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<td>MWM</td>
<td>Morris Water Maze</td>
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<td>NGF</td>
<td>Nerve Growth Factor</td>
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<td>NSAID</td>
<td>Non-steroidal Anti-inflammatory Drug</td>
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<td>NSPC</td>
<td>Neural Stem and Progenitor Cells</td>
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<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<td>Peripheral Nervous System</td>
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<td>SVZ</td>
<td>Subventricular Zone</td>
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<td>T3</td>
<td>Triiodothyronine</td>
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<td>TBI</td>
<td>Traumatic Brain Injury</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor-Alpha</td>
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<td>TUNNEL</td>
<td>Terminal deoxynucleotidyl transferase</td>
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Introduction

The total spectrum of human behaviour, spanning from motor function to feelings and conscious thoughts, is governed by the brain. In the deeper parts of the brain basic functions such as sleep and breathing are regulated. The hemispheres are the most external cerebral structures, harbouring the complex cognitive processes. It is not the size of the brain that is interesting but rather the complex pattern of connections between the neurons and other cells. How the formation, the re-formation and maintenance of all these synapses are regulated are not fully understood nor the exact means of communication between them. Disturbing the intricate network of cell-cell interaction in the brain might have devastating consequences, and presently we do not have the knowledge to re-build a damaged brain. However piece-by-piece this great puzzle is being unraveled.

*If the brain were so simple we could understand it, we would be so simple we couldn’t.*

– *Lyall Watson*
The Central Nervous System

The organization of the Central Nervous System (CNS)

The brain and the spinal cord are the two constituents of the central nervous system, the spinal cord being the link between the brain and the peripheral nerve cells. Two major cell types reside in the CNS, neurons and glial cells. Glial cells are divided in macroglia and microglia. In the human brain there are approximately $10^{11}$ neurons, which are subdivided into specific types. Each neuron is interconnected with a number of other neurons via their synapses creating a complex signalling network. While some neurons, such as motorneurons, only have contact with a few other cells, the Purkinje cells in the cerebellum can form contact with thousands of other cells. Astrocytes and oligodendrocytes, although with distinct functions, are classified as macroglial cells. Astrocytes are the most numerous cells in the brain. Due to their star-like appearance with several processes they are in contact with both the microvessels in the brain and the neurons, and can thereby facilitate the transmission of information between the two. By ensheathing the neuronal synapses, astrocytes can regulate the concentration of neurotransmitters and ions, contributing to their homeostasis (Hewett 2009). The number of synapses that one astrocyte ensheathes varies among species as well as within the different regions of the brain (Nimmerjahn 2009). Rapid and accurate neuronal signalling in the CNS is enabled by the oligodendrocytes. They insulate the axons by myelinating sheaths (Fields and Stevens-Graham 2002). Microglia originate from the myeloid linage and are considered immune cells. They have a phagocytic role and are activated in response to injury or inflammation (Streit et al. 2004).

Central Nervous System Development

During early embryogenesis the developing embryo consists of three distinct cell layers: the endoderm that gives rise to the viscera, the mesoderm that will develop in to muscles and vascular tissue and the ectoderm that gives rise to the central and peripheral nervous system and to the skin. The neural plate develops from the ectoderm. Through a process called neurulation the plate will fold and close, forming the neural tube. This tube is an immature form of the central nervous system, where the anterior part will develop into the brain and the posterior part will form the spinal cord (Lacbawan and Muenke 2002). The regional specification of the CNS will develop under the influence of a multitude of factors that instruct cells to a certain fate, such as Sonic Hedgehog and bone morphogenetic protein that induce the neural tube to form a dorsal-ventral axis (Echevarria et al. 2003; Lacbawan and Muenke 2002; Wilson and Houart 2004). During development of the CNS, cells in the neural tube divide rapidly at different time points in different regions. This unevenly distributed proliferation will lead to the formation of three
vesicles in the rostral part of the neural tube. Two of them will subdivide forming a total of five vesicles, which represent the major subdivisions of the mature brain (Lacbawan and Muenke 2002) (Nowakowski and Hayes 1999). The most rostral of them and also the largest, the telencephalon, develop into the cerebral hemispheres. The enclosed vesicles later become the ventricular system. In the developing brain specialized cells are born according to a time schedule where neurons are produced before glial cells. Neurogenesis in mammals has it’s peak around midgestation and astrocytes around birth while oligodendrocytes mainly develop postnatally (Qian et al. 2000; Sauvageot and Stiles 2002). What causes this switch from neuronal to glial production to occur is not known. One explanation might be that the neurogenic potential of the stem cell declines and instead acquires different properties and hence respond to the environment differently (Qian et al. 2000). During the development, cortex is generated in a temporal fashion where the early produced neurons populate the deeper cortical layers and the later-born neurons migrate to the superficial layers, which results in six different cortical layers (Merot et al. 2009).

A key element for proper formation of the CNS is the ability for the newly born neurons to migrate to its corresponding location, integrate in the tissue and form proper synapses. Immature neurons, neuroblasts, can migrate in a radial or a tangential modality (Merot et al. 2009). Radial migration, typically exhibited by projection neurons, is a perpendicular migration from the germinal zone. Radial glia are cells with processes expanding throughout the entire cortex, from the ventricular to the pial surface of the neuroepithelium (Rakic 1972). The neuroblast adheres to this structural support using it as a guide while advancing to its proper location. Tangential migration, when cells move parallel to the ventricle, is often used by inhibitory interneurons to reach their location in the cortex. The differences in migratory pathway between different neuronal classes could be explained by the region from where the cells originate (Merot et al. 2009). Stem cells in the adult brain have maintained their migratory properties and migrate tangentially along the rostral migratory stream to the olfactory bulb (Doetsch et al. 1999). From the centre of the olfactory bulb to the periphery the cells migrate radially along blood vessels, referred to as vasophilic migration (Bovetti et al. 2007).

**Growth Factors Involved in CNS Development**

Many cellular events are induced by external environmental cues such as cell-cell interaction or other ligand receptor interaction that generates an internal signalling cascade. Receptor tyrosine kinase is a large family of receptors involved in various cellular mechanisms such as proliferation and differentiation. The growth factors, fibroblast growth factor (FGF) and epidermal growth factor (EGF), both important mitogens for neural stem cells, belong to the receptor tyrosine kinase family.
Fibroblast growth factor is a family of receptors and contains 22 ligands and 4 receptors. FGFs are implicated to play an important role during development, many of them during the formation of CNS (Mason 2007). The expression pattern of the FGF members differ. Some are expressed exclusively during embryonic development but not in a later stage, others both during embryonic development and in the adult (Ford-Perriss et al. 2001). The expression patterns of FGFs in the developing CNS are concentrated in three regions responsible for patterning of the nervous system, the anterior neural ridge, the isthmus and the rhombomere 4. FGF8 is the most pronounced FGF in these regions and is shown to be essential for the identity of the cells and their survival during development (Mason 2007). As early as the embryonic day (E) 8.5, FGF8 is found to be expressed in the murine developing neural plate (Crossley and Martin 1995). FGF-2 expression starts at E9 in mouse and its expression persists throughout adulthood. It can be found in both neuronal and non-neuronal cells (Ford-Perriss et al. 2001) and is necessary for cell proliferation (Ghosh and Greenberg 1995; Raballo et al. 2000). Total abolishment of FGF signalling by the deletion of the genes encoding the receptors 1 to 3 shows that this signalling is required for a proper formation of telencephalon (Paek et al. 2009) demonstrating the importance of FGF ligands and receptors.

Epidermal growth factor belongs to a family of growth factors including transforming growth factor α. Its receptor (EGFR) is expressed in various regions of the CNS during embryonic and postnatal development. In the adult mammalian brain, EGFR expression is exclusively found in the germinal zone (Cameron et al. 1998). Various biological actions such as differentiation, maturation and survival of neurons can be sustained with EGF (Wong and Guillaud 2004). EGF has been shown in vitro to support the proliferation of neural stem and progenitor cells (Reynolds et al. 1992). In the developing CNS, EGF is also suggested to be involved in precursor migration, since the loss of EGFR expression leads to accumulation of cells in the germinal zones, and an over expression of the receptor contributes to excessive migration (Sobeih and Corfas 2002).

Extracellular Matrix in the Developing and Adult CNS

The extracellular matrix (ECM) is a network of molecules that surround the cells and occupies 20% of the adult brain volume (Nicholson and Sykova 1998). ECM is produced by the cells themselves and also integrates with them abundantly. It is a two-way communication where the integrins on the cell surfaces play a major role. While not only providing physical support, the extracellular matrix additionally modulates the tissue as well as the access and activity of certain growth factors. During brain development the ECM modifies the migration of progenitors, synapse formation and cell proliferation.
(Bellail et al. 2004). The developing central nervous system and the adult brain differ in their extracellular matrix composition. In the immature CNS some ECM components are abundant such as tenascin –C and neurocan, which both are less pronounced in the adult CNS (Rauch 2004). Collagen, a fundamental part of the ECM and an important contributor to axon guidance and synaptogenesis in the developing CNS, is in the adult CNS confined to its margins. However, there are evidence indicating that collagens are involved in maintaining the CNS integrity (Hubert et al. 2009). Hyaluronan is the most abundant ECM molecule in the CNS. It is a linear polysaccharide that serves as a central filament of aggregates with proteoglycans and proteins bound to it (Rauch 2004). CD44 is a cell surface receptor that binds to hyaluronan and by its interaction with the cytoskeleton involved in migration. It is expressed on the majority of immune cells and hence hyaluronan is suggested to play a role in the immune response (Ruffell and Johnson 2008). In brain tumours hyaluronan levels are increased, reaching the levels seen during development (Delpech et al. 1993). Several ECM molecules are involved in neuronal migration such as reelin, laminin etc (Sobeih and Corfas 2002). Other components of the ECM have inhibitory action, such as chondroitin sulphate proteoglycans that inhibit axonal regeneration (Crespo et al. 2007).

Peripheral Nervous System (PNS)

The peripheral nervous system connects the limbs with the central nervous system by transmitting both afferent and efferent signals. As the CNS, it constitutes of both neurons and glia. Schwann cells are glial cells that ensheath all axons in the PNS. Additional glial cells are non-myelinating schwann cells and satellite cells (Jessen 2004). Early in the development neural crest cells are situated at the border between the ectoderm and the neural plate. Between E8.5 and E10 in mouse they begin to migrate away from the neural tube (Marmigere and Ernfors 2007). Neural crest cells are the origin of both neurons and glia in the peripheral nervous system as well as mesodermal derivatives (Le Douarin and Smith 1988).

Dorsal Root Ganglion

During the embryonic development neural crest cells migrate in chain-like structures from the neural plate and form the dorsal root ganglia (DRG), which forms part of the peripheral nervous system (Marmigere and Ernfors 2007). DRG constitutes of afferent sensory neurons. The interface between the PNS and CNS, located in vicinity of the spinal cord, where the axons enter the CNS, is called the dorsal root transitional zone (DRTZ) (Aldskogius and Kozlova 2002). Every rootlet has its own DRTZ that is non-permissive for axonal re-growth (Fraher 1999).
**Stem Cells**

Stem cells are immature cells that fulfill certain prerequisites. Due to difficulties in identifying them owing to their lack of specific markers, stem cells are identified according to certain characteristics. Stem cells have to be able to self-renew and give rise to progeny that is capable of differentiating into all the cell types in the tissue from where it is derived (Gage 2000). This can be achieved by two different modes of division, symmetrical and asymmetrical. Symmetrical division gives rise to two identical daughter cells whereas asymmetrical division generates one cell identical to the mother cell and a progeny that is a bit more differentiated. During early foetal development, stem cells in the CNS undergo symmetric division to expand the stem cell pool before great numbers of differentiated cells are generated. Later during gestation, division of the neural stem cells change to being primarily asymmetric (Morrison and Kimble 2006). The orientation of the cleavage plane of the division is one of the predictors of the fate of daughter cells (Chenn and McConnell 1995).

As an organism develops the stem cell quantity changes. From the beginning there is only one cell, the fertilized egg. It gives rise to all the mature cells in the body as well as the placenta, and can thus be considered a totipotent stem cell. As the stem cell divides the early embryo will form what’s called a blastocyst. In the inner cell mass of the blastocyst, the pluripotent embryonic stem (ES) cells reside. They can form all the cell lineages but not the placenta. Stem cells get progressively more restricted in their plasticity as the development proceeds. Cells that can differentiate to all the cell lineages that constitute the specific tissue of origin are referred to as multipotent stem cell (Gage 2000). During development, stem cells are more prominent but can also be found in adults. Stem cells reside in various adult tissues that are known for their regenerative capacity such as liver, skin, intestine but also in the brain (Lois and Alvarez-Buylla 1993; Reynolds and Weiss 1992).

**Stem Cell Niches in the Adult Brain**

In the embryonic brain as in other tissues, the number of stem cells diminishes as the development continues and the number of more specialized progenitors increase with different responsiveness to growth factors (Kilpatrick et al. 1995; Williams and Price 1995). In the adult the stem cells are restricted to two germinal zones, the subventricular zone (SVZ) and the subgranular layer in the dentate gyrus (DG) of the hippocampus (Gage et al. 1995; Lois and Alvarez-Buylla 1993; Palmer et al. 1997; Reynolds and Weiss 1992; Reynolds and Weiss 1996). This finding is interestingly not restricted to rodents, but could also be found in humans (Eriksson et al. 1998; Johansson et al. 1999). However, neurons in the human neocortex are
not generated in the adulthood in detectable levels (Bhardwaj et al. 2006). The germinal zones, which have a specialized microenvironment, are referred to as stem cell niches. To uphold a favourable milieu, the niches have several responsibilities. They are obliged to maintain a neurogenic setting, keep the stem cells in a quiescent state and govern the proliferation and differentiation when needed (Miller and Gauthier-Fisher 2009). A niche hence has to provide both structural as well as metabolic and biochemical support. Although separate, both the SVZ and the DG contain similarities, such as a mixed population of progenitor and stem cells and a close contact to the ECM and the vasculature (Miller and Gauthier-Fisher 2009).

From the subventricular zone the new-born cells migrate to the olfactory bulb via the rostral migratory stream. This migration pathway was former believed only to exist in rodents but has lately been found also in humans (Curtis et al. 2007). Neuroblasts in the hippocampus however have not shown such an extensive migratory pattern, rather they migrate to the adjacent granule cell layer where they mature and extend processes to integrate into the functional circuitry of the hippocampus (van Praag et al. 2002). Additionally, the nomenclature differs between the cells that reside in the SVZ and in the hippocampus. In the SVZ the stem cells and subsequent progeny have been labelled A, B and C cells. The stem cell, denominated a B cell is a slowly dividing cell that expresses the glial fibrillary acidic protein (GFAP). B cells generate rapidly dividing transit amplifying C cells that lack GFAP expression. Migrating neuroblasts, A cells, are the progeny of the C cells (Doetsch et al. 1999). In the dentate gyrus there are two populations of neural stem and progenitor cells. As in the SVZ there is one slowly dividing cell, type 1 progenitor, that expresses GFAP and another readily proliferating cell that is GFAP negative, type 2 (a and b). A third cell denominated type-3 is expressing doublecortin and can hence be considered a neuroblast (Kempermann et al. 2004). The origin of the stem cells in the adult brain has been debated, but emerging evidence indicate that the transition from the neuroepithelial cell to the glial like stem cell in the adult brain occurs via the radial glial cells. These cells appear before the neurons during gestation and while traditionally seen as cells that generate astrocytes, they have been found to additionally produce neurons (Gaiano et al. 2000; Malatesta et al. 2000; Merkle et al. 2004). Proliferation in the SVZ in the adult brain can be altered by intraventricular infusion of growth factors. FGF-2 was found to enhance neurogenesis in rat and EGF, although increasing the proliferation in the SVZ, reduced the number of new-born neurons (Kuhn et al. 1997).

Oligodendrocyte percursor cells are generated in distinct multiple areas during the embryonic development. Oligodendrocytes from these progenitors migrate out and populate specific regions in the CNS (Nicolay et al. 2007). In the adult, oligodendrocyte precursors persist, located both in white and
gray matter. *In vitro* cultivation of these progenitors gives rise to both astrocytes and oligodendrocytes (McTigue and Tripathi 2008).

**Proliferation and Differentiation of Rodent Neural Stem Cells *in vitro***

Neural stem cells can be isolated and cultured from both the embryonic and the adult brain. In culture, cells can grow as a monolayer attached to a coated surface or they can create free-floating cell aggregates, neurospheres, in non-adherent cultures. The stem cell culture consists of a heterogenous population of cells with different maturity, some which still are stem cells and others that are more differentiated. (Suslov et al. 2002). In fact neurospheres contain approximately no more than 2.4 % *bona fide* neural stem cells (Reynolds and Rietze 2005). Due to this heterogeneity of the culture these cells will be referred to as neural stem and progenitor cells (NSPC). Neurospheres are believed to arise from a single cell, but since cells are prone to merge with other cells or spheres, care must be taken when performing clonal studies (Singec et al. 2006). By addition of epidermal growth factor and/or fibroblast growth factor -2, NSPC can be kept as immature and dividing cells in culture (Gage et al. 1995; McKay 1997; Reynolds et al. 1992; Santa-Olalla and Covarrubias 1995). Sonic hedgehog signalling is involved in early CNS development and is vital for neural development. *In vitro* Sonic hedgehog has been shown to sustain NSPC proliferation (Lai et al. 2003; Rowitch et al. 1999). Addition of Platelet Derived Growth Factor (PDGF) to NSPC cultures maintain the cells proliferating and with an immature morphology. PDGF can however not replace other mitogens such as FGF-2. Without additional stimuli only a partial differentiation to neurons and glia is induced when PDGF is present (Erlandsson et al. 2006; Erlandsson et al. 2001). In this thesis cortices from mouse E13.5 and E14.5 were dissected and cultured as neurospheres with the addition of EGF and FGF-2 in a defined medium.

When the growth factors are withdrawn, cells start to differentiate into neurons, astrocytes, and oligodendrocytes. The differentiation of immature cells to mature cell types is a complex event to date not fully understood. Different extrinsic molecules for example growth factors or hormones but also cell-cell contact lead to internal signalling that affect the differentiation.

Culturing cells under a three dimensional condition that mimics real life situation might have an advantage to the traditional culture systems. Ma et al
Figure 1 Neural stem cells are able to self-renew or differentiate into neurons and glial cells. When cultured in vitro the fate of the stem cells can be directed by different factors.

demonstrated the ability of NSPC from rat to form functional neurons when cultured on collagen gels (Ma et al. 2004). Human ES cells were found to preferentially differentiate into neurons when cultured on a polyurethane scaffold compared to control conditions (Carlberg et al. 2009). The importance of the mechanical microenvironment such as tissue elasticity in cell lineage specification was recently demonstrated. For instance, mesenchymal stem cells developed into mature cells of different lineage depending on the stiffness of the matrix in which they were cultured (Engler et al. 2006). Neural stem cells cultured on substrate that resembles the brain elasticity promotes neuronal differentiation (Leipzig and Shoichet 2009) or neuronal maturation (Teixeira et al. 2009). A great deal of research is aimed at revealing the exact components of differentiation and a number of molecules that promote specific stem cell lineage differentiation have been found. Ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) were early found to shift the cell differentiation into an astrocytic fate (Bonni et al. 1997; Johe et al. 1996), whereas addition of triiodothyronine (T3) or insulin-like growth factor 1 (IGF-1) increase the number of oligodendrocytes in culture (Johe et al. 1996) (McMorris and Dubois-Dalcq 1988).

Neuronal specific induction is more difficult to achieve and at present no single efficient extrinsic factor has been identified that accomplishes this. Activation of the Wnt pathway however, has been shown to increase the neuronal population in an instructive manner (Hirabayashi and Gotoh 2005).
Intrinsic regulators such as, the homeodomain Lmx1a, trigger embryonic stem cells to adopt a dopaminergic cell fate (Andersson et al. 2006).

Stem Cell Controversies

The neural stem cells research field has despite the fact that it is relatively new, or possibly because of it, experienced some controversy. Two formerly debated issues have been put in the limelight again, the actual identity of stem cells in the ventricular area, and the ability of a committed cell to become a cell of a different lineage.

Identity of Adult Neural Stem Cells

In 1999 a study by Johanson and colleagues demonstrated that the ependymal cells surrounding the lateral ventricles showed proliferative and migratory properties of stem cells (Johansson et al. 1999). Though other studies failed to replicate this result (Chiasson et al. 1999; Doetsch et al. 1999), whether ependymal cells actually are stem cells has since remained a controversy. A recent study from the same group that earlier reported the ependymal finding, demonstrated that ependymal cells generate neuroblasts and astrocytes in response to injury but did not self renew in an extent that enable maintenance of their population (Carlen et al. 2009). Contradictory to these findings, CD133+ ependymal cells from the SVZ were found to proliferate both in vivo and in vitro and furthermore to generate olfactory bulb neurons (Coskun et al. 2008). Another group found that B1 cells in the SVZ had epithelial properties with an apical ending that was in contact with the lateral ventricle (Mirzadeh et al. 2008) thus resembling ependymal cells. Taken together, these papers illustrate that the opinions regarding the ependymal cells and ventricular stem cell niche might not be as opposing as it previously has been and that there possibly exists several sources of neural stem cells in the adult ventricular zone.

Transdifferentiation, an Actual Possibility?

Around the millennium shift there was a multitude of reports indicating the ability of a differentiated cell to become a cell of another lineage, so called transdifferentiation. It was suggested that the differentiation ability of a cell was due not so much to intrinsic factors as to external ones, making the environment the determinant of a cells fate (Brustle et al. 1997). Developmentally related cells i.e. arsing from the same germ layer were reported to change cell fate in vitro for example: bone marrow to muscle (Ferrari et al. 1998), skin to neurons (Toma et al. 2001), but also across the germ layers, bone marrow to neural cells (Brazelton et al. 2000; Kopen et al. 1999; Mezey et al. 2000) or neurons to hematopoietic cells (Bjornson et al. 1999). However, other papers suggested the cell lineage changes to occur as a result of cell fusion (Terada et al. 2002; Ying et al. 2002). Discussions of the abil-
ity of a differentiated cell to change fate has then declined. However, in 2006 Takahashi et al reported a groundbreaking result where they had dedifferentiated mouse fibroblast into ES cells by culturing them with four factors Oct3/4, Sox-2, c-Myc and Klf4. When injected into a blastocyst, these cells, denoted induced pluripotent stem (iPS) cells, contributed to the formation of the embryo (Takahashi and Yamanaka 2006). The same group also demonstrated the reprogramming of human fibroblasts using the same factors (Takahashi et al. 2007). Since then, several reports have demonstrated the ability to de-differentiate diverse somatic cells, using the same set of factors or excluding one or several of them (Hester et al. 2009; Kim et al. 2008; Maherali et al. 2007; Wernig et al. 2008; Wernig et al. 2007). The possibility of reprogramming a somatic cell hence seems to be possible even though with the constraint of going back in development and then forward instead of just leaping from one differentiated cell type to another. The prospect of using iPS cells holds promise for transplantation therapies. By utilizing endogenous cells, complication of immune responses within the patient could be avoided. Additionally, iPS cells have the advantage of being easily propagated in vitro. Although iPS cells appear to resemble ES cells (Hester et al. 2009) it is not known to what extent they actually differ. Though iPS cells arise by reversing a differentiated state it is feasible that epigenetics is a factor that might influence their feature.

Traumatic Brain Injury (TBI)

Traumatic brain injury is a condition caused by a mechanical insult to the brain. The consequences of TBI are far reaching due to the long-term difficulties the patient suffers. Depression, motor insufficiency, cognitive impairments or even death are examples of common outcomes after TBI, affecting not only the patients but also the relatives. TBI can be caused by a number of events, in the younger population it is usually due to motor vehicle accidents and in the elderly due to fall (Thurman and Guerrero 1999), (Masson et al. 2001), but there are other causes such as sport accidents and warfare. In the clinic 200-500/100 000 inhabitants in Scandinavia are diagnosed with TBI each year (Andersson et al. 2003; Romner et al. 2000) thus making it a socioeconomic problem of big magnitude. Even though modern neurointensive care has reduced mortality and improved functional outcomes (Elf et al. 2002), TBI remains a major public health problem.

Pathophysiology of TBI

The course of injury process after TBI is bi-phasic. The initial injury consists of tissue loss due to acute cell death. The secondary injury mechanisms, which evolves minutes to hours after the insult, are the result of complex
neurochemical cascades. These events such as the alterations of the blood brain barrier (Dietrich et al. 1994) and metabolism, the formation of reactive oxygen species, and inflammation lead to additional cell death (Lenzlinger et al. 2001). While the primary injury is impossible to treat in the clinic, the secondary events however, are feasible targets for neuroprotective therapies and therefore attract research to possible interventional strategies. Despite promising experimental results, clinical trials of neuroprotective drugs have not demonstrated enhanced patient recoveries thus far (Marklund et al. 2006; Narayan et al. 2002; Royo et al. 2003). The multifactorial injury mechanisms and the complexity of the brain indicate that a combinatorial restorative approach might be required.

Inflammation

The brain is protected from infiltration of molecules and immune cells by the blood brain barrier (BBB); moreover it lacks a lymphatic system. This has led to the long-lived belief that the brain is an “immune privileged” organ (Morganti-Kossmann et al. 2007). Presently, neuroinflammation is an accepted concept that generates much attention due to its potential role in a variety of CNS pathologies. Inflammation in the CNS consists of a variety of cardinal features such as glial activation, oedema formation, synthesis of inflammatory molecules, expression of adhesion molecules and invasion of immune cells etc (Lucas et al. 2006), the latter being correlated to the BBB dysfunction that appears after TBI (Holmin et al. 1995). Marked changes in cerebral gene expression chiefly involving genes regulating inflammatory processes and chemokine signalling was recently demonstrated following TBI in mice (Israelsson et al. 2008). Neuroinflammation after TBI has opposing facets being both detrimental and beneficial (Morganti-Kossmann et al. 2007). Cytokines have been extensively studied in an attempt to decipher this mystery (Lucas et al. 2006), a few which will be mentioned below.

Interleukin-1 (IL-1) is a pro-inflammatory molecule that is expressed in the healthy CNS at low levels (Rothwell and Luheshi 2000) but is found to be upregulated after brain injury (Fan et al. 1995). IL-1 has a number of diverse actions, including production of prostaglandins and other cytokines (Lucas et al. 2006). The release of IL-1 per se is not lethal to neurons but rather its synergistic actions with other pro-inflammatory molecules such as cyclooxygenase-2 (cox-2). IL-1 also exhibits neuroprotective effects accordingly to the duality concept (Clausen et al. 2009). IL-6 is another interleukin with a dual role in inflammation, being viewed as essentially neuroprotective by stimulating the production of molecules that defend against oxidative stress and contribute to revascularisation in addition to inhibit pro-inflammatory molecules (Morganti-Kossmann et al. 2007). Conversely it was demonstrated that IL-6 knockout mice exhibited a greater neuronal loss...
after brain injury than wild type mice (Penkowa et al. 2000). Furthermore it also displays a role in glial scar formation and pro-inflammatory cytokine release (Morganti-Kossmann et al. 2007). Expression of tumour necrosis factor alpha (TNF-α) has been associated with neurological dysfunction after experimental TBI (Knoblach et al. 1999; Shohami et al. 1996). In a study with TNF deficient mice it appears that in the initial stages after TBI TNF may be disadvantageous, but in the long-term recovery process may act beneficially. These intricate effects of several of the inflammatory actors make the attempt to decimate the inflammation a delicate assignment. The temporal, spatial and functional modes of action are not obvious. Rather than annihilate the inflammation a transient attenuation might be more efficient.

**Cellular Response to Brain Injury**

**Neurogenesis after Injury**

Neurogenesis is the process where newly generated cells differentiate into neurons in vivo. After cortical injury (Ramaswamy et al. 2005; Rice AC et al. 2003; Sundholm-Peters et al. 2005) or ischemia (Arvidsson et al. 2002; Jin et al. 2003) newborn cells have been found in the SVZ. Some of them differentiate into neurons. In addition, precursor cells also migrate towards the injury site (Arvidsson et al. 2002; Ramaswamy et al. 2005; Sundholm-Peters et al. 2005). Increased proliferation of cells in the SVZ after TBI can persist up to one year and the resulting progeny differentiate into both neurons and glia (Chen XH et al. 2003). Cell proliferation has also been detected in the hippocampus in response to trauma (Braun H et al. 2002; Chirumamilla et al. 2002; Dash et al. 2001; Kernie et al. 2001; Yoshimura S et al. 2001). Neuroinflammation has the ability to influence adult neurogenesis in a dual fashion. Monje et al recognized a reduction in proliferation of stem cells from the hippocampus when exposed to IL-6 and TNF-α (Monje et al. 2003). Two factors secreted from both microglial cells as well as astrocytes. Another group found that chronic activation of microglia diminished the expression of IL-1, IL-6 and TNF-α in favour of other factors that proved to promote cell survival and neuronal differentiation (Cacci et al. 2008). Furthermore, inflammation regulates the integration of neurons in the dentate gyrus (Jakubs et al. 2008).

In response to injury, endogenous stem cells migrate to the lesioned area a migration that seems to be specific for the newly generated SVZ neurons and not for the hippocampal ones (Jin et al. 2003; Picard-Riera et al. 2004). This might reflect the normal situation in rodents since the cells from the SVZ are more prone to migrate over larger distances. Cells that have been grafted to the brain post injury are also found to migrate to the lesion (Kelly et al.
2004; Ramaswamy et al. 2005; Wennersten et al. 2004). Additionally, cells that were grafted to the brain and integrated in the parenchyma prior to injury also migrated to the lesion site (Park et al. 2006). By magnetic resonance imaging grafted cells were monitored and shown to migrate via the white tract of corpus callosum (Hoehn et al. 2002; Modo et al. 2004). Neural stem cells have also been shown to track tumour cells in the CNS after intravascular transplantation (Aboody et al. 2000). This migratory capability of stem cells and their capacity to “home in on” the injury is an intriguing ability. It might partly be explained by the fact that cells in the injured area release chemoattractants. Astrocytes release stromal cell derived factor 1α, which induce the migration of the newborn neural stem cells that express the CXC4 receptor (Imitola et al. 2004). Stem cell factor is another factor released from the injury site that is proven to induce stem cell migration both in vitro (Erlandsson et al. 2004) and in vivo (Sun et al. 2004). Stem cells in the CNS can furthermore be recruited by other factors involved in the immune response (Aarum et al. 2003; Ziv et al. 2006).

The Role of Astrocytes after Injury

Upon brain trauma, astrocytes respond by increased proliferation and changes in gene expression. The astrocytic response is correlated to the severity of the injury. Due to their diverse functions, the role of these reactive astrocytes might be both harmful and beneficial (Pekny and Nilsson 2005). A few days after injury, astrocytes have formed a glial scar around the wounded tissue. This scar tissue prevents the axonal outgrowth and hinders cell-repopulation of the injury area. Moreover, astrocytes release pro-inflammatory cytokines that might contribute to injury progression (Lloyd et al. 2008). Contradictory, favourable actions of reactive astrocytes have also been shown. Neuronal synapses were protected by astrocytes in an acute stage after injury (Wilhelmsson et al. 2004). After moderate CCI, reactive astrocytes restrict inflammation and contribute to preserving neural tissue (Myer et al. 2006). Oligodendrocytes might also contribute to the increased number of astrocytes after injury. They have been found to be able to convert into astrocytes in vivo after brain ischemic injury (Kohyama et al. 2008).

Microglia

Microglial cells are phagocytic cells that are resident in the CNS (Ladeby et al. 2005b). In response to trauma they start to proliferate and accumulate at the site of the injury. Two alternative models of explanation to this microglial expansion have been proposed. One model states that additionally to the recruitment of cells from the blood stream, microgliosis occurs within the CNS (Ladeby et al. 2005a). The other model proposes that augmentation of microglia after injury is exclusively due to the self-renewal of resident
cells and consequently their preservation continues throughout a life time (Ajami et al. 2007).

Injury to the PNS

Unlike injuries in the CNS, the lesioned PNS has some capability to self-repair and the number of neurons return to pre-injury levels within a few months (Li et al. 2007). However, growing axons reaching the DRTZ do not extend into the CNS. Different approaches to overcome this predicament have been used; suppressing the inhibiting factors such as certain proteoglycans, diminish the glial cell response, replacing the mature DRG cells with embryonic cells etc (Aldskogius and Kozlova 2002). In the adult, stem cells have been found in the DRG (Namaka et al. 2001), and in an attempt to characterize their in vivo identity, a sub population of DRG cells were found to proliferate and give rise to neurons and cells of glial origin (Li et al. 2007). These progenitors might be involved in the neurogenesis seen after injury.
Coronal sections of mouse brain
five weeks after experimental brain injury
Experimental TBI

A condition as heterogeneous as TBI is difficult to model due to the diversity of variables involved that influence the outcome. Standardized in vivo models are being used to address different aspects of the clinical disease, mostly in rodents. Some models produce focal injuries: weight drop (Feeney et al. 1981) midline fluid percussion (Dixon et al. 1987), and controlled cortical impact (CCI) (Dixon et al. 1991; Smith et al. 1995). Other models produce diffuse injury such as acceleration models (Marmarou et al. 1994), and some create both a focal and a diffuse injury as in lateral fluid percussion (Morales et al. 2005). Taken together these models have provided much information about the pathophysiology of the TBI, which can be translated to the clinical setting. Injury severity is often related to the post-trauma outcome (Yu et al. 2009).

Controlled Cortical Impact

In the controlled cortical impact model, a pneumatically driven piston impacts the exposed dura and compresses the parenchyma of the deeply anaesthetized animal. The velocity and impact depth are predetermined. The ability of controlling the severity of the injury in addition to its reproducibility are some of the model’s advantages. CCI gives a focal injury with cortical cell loss but also affects the underlying structures such as the hippocampus (Smith et al. 1995). Besides cell death, CCI also causes oedema formation, disruption of the BBB, alters the cerebral blood flow, and produces inflammation. Motor as well as cognitive impairments are seen after CCI injury (Dixon et al. 1999; Smith et al. 1995). However the motor deficits are often transient with a spontaneous recovery (Morales et al. 2005).

Strategies for Reducing Experimentally Induced Injury

The convolution of secondary events upon trauma presents a “smorgasbord” of possible intervention options, such as providing neurotrophic support, modulating the immunorespons, inhibiting specific cellular mechanisms that lead to cell death, scavenging of reactive oxygen species etc (Jain 2008). All of these treatment strategies have to some extent proven successful in an experimental setting (Marklund et al. 2006). Specifically, apoptotic cell death occurs after TBI in different species and in several cell types, both in neurons and glial cells (Fox et al. 1998; Newcomb et al. 1999). Attempts to inhibit caspases, a family of proteases involved in the apoptotic pathway, have been demonstrated in a number of studies to enhance outcome (Fink et al. 1999; Yakovlev et al. 1997). Cell cycle inhibition after TBI showed tissue sparing in addition to improved motor and cognitive function (Di Giovanni et al. 2005). Selectively targeting a pro-inflammatory agent such as IL-1β (Clausen et al. 2009; Li et al. 2009) or to reduce expression of additional
pro-inflammatory molecules (Lloyd et al. 2008) diminish the inflammation and improve the outcome. Hypothermia, a clinically used method however debated, showed to reduce the BBB permeability after TBI (Lotocki et al. 2009) and attenuated the hippocampal cell loss (Jia et al. 2009).

Evaluation of Treatment effects after Experimental TBI

Outcome after experimental TBI and the potential treatment effect can be evaluated according to genetic, morphological/histological, cognitive and motor criteria (Fujimoto et al. 2004). Improvement in the behaviour of the animal such as ameliorated motorical and cognitive function is the utmost aim of any given therapy. A battery of different test to assess behaviour is currently used. To chose the correct test several aspects has to be taken into account; validity, (whether the test measure the disease related mechanisms); reliability, (however the test produces the same repeated result); utility (regarding the efficiency both economical and time-wise and objectivity of the measuring criteria); and sensitivity (Brooks and Dunnett 2009). Histological evaluation of the tissue can provide detailed information on the molecular level such as interaction between receptor ligand, up or down regulation of gene expression such as mRNA levels and so forth.

Antibodies for Histological Evaluation

Histological outcome is often analysed by immunohistochemistry. Antibodies directed against a specific protein are used to visualize its localization within the tissue. Often more than only one antibody can detect a specific cell type. A cell early in development usually has a different expression pattern of markers than the same cell type in a later developmental stage. Cell specific antibodies thus can not only identify a certain cell type but also the maturity state of that cell. Analysing stem cells however has proven to be difficult, due to the fact that immature cells express few cell specific markers. Nevertheless several markers are used to detect stem cells. Nestin, an intermediate filament (Lendahl et al. 1990) is perhaps the most used marker when studying the stem cells in the CNS.
<table>
<thead>
<tr>
<th>Cell target</th>
<th>Markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem and progenitor cells</td>
<td>Nestin, Sox1 and 2, CD133, GFAP (adult)</td>
<td>(Lendahl et al. 1990), (Pevny et al. 1998), (Sasai 2001), (Uchida et al. 2000), (Doetsch et al. 1999), (Seri et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>CD133, Sox1 and 2, GFAP (adult)</td>
<td></td>
</tr>
<tr>
<td>Immature neurons</td>
<td>DCX, PSA-NCAM</td>
<td>(Couillard-Despres et al. 2005), (Bonfanti 2006)</td>
</tr>
<tr>
<td>Immature astrocytes</td>
<td>CD44</td>
<td>(Liu et al. 2004)</td>
</tr>
<tr>
<td>Immature oligodendrocytes</td>
<td>PDGFRα, NG2, Olig2</td>
<td>(Ellison and de Vellis 1994), (Nishiyama et al. 1999), (Takebayashi et al. 2000)</td>
</tr>
<tr>
<td>Neurons</td>
<td>Map2, Beta-III-tubulin, NeuN</td>
<td>(Garner et al. 1988), (Matus 1988), (Caccamo et al. 1989)</td>
</tr>
<tr>
<td>Mature Neurons</td>
<td>Synaptophysin, GABA, TH</td>
<td>(Wiedenmann and Franke 1985), (Kisvarday et al. 1990), (Pickel et al. 1975)</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>GFAP</td>
<td>(Eng et al. 2000)</td>
</tr>
<tr>
<td>Oligodendrocyte</td>
<td>CNPase, PLP</td>
<td>(Trapp et al. 1988), (Hartman et al. 1982)</td>
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</table>
Cognitive Evaluation

Improvement of cognitive outcome after experimental brain injury is often measured as the ability to form new memories. After CCI this is a valid parameter as the hippocampus, the structure virtually responsible for the establishment of new memories, is affected by the impact. A variety of different maze-tests are used, the Morris water maze (MWM) being the most widely employed (Fujimoto et al. 2004). The animals’ ability to find a submerged escape platform in a pool, by guidance of external visual cues, is measured (Morris 1984). The rodent is placed in the pool facing the wall at four different locations. If the rodent is unable to detect the platform by a predetermined time (60 seconds for mice), it is guided there by the investigator. MWM can be used to measure memory retention additional to acquisition depending on its set-up. Besides escape latency other factors such as swim speed and path length can be evaluated. Animal strain and maze characteristic may influence the outcome and hence should be taken into account when designing an experiment (Van Dam et al. 2006).

NSPC as a Tool for Tissue Regeneration in the Injured Nervous System

Even though new cells are generated in the adult brain in response to injury, the capacity for CNS self-repair is limited. However, grafting extrinsic stem cells as a mean for improving outcome is an alluring and hence well-studied option. The rationale for transplantation to the injured nervous system is not only the obvious replacement of lost tissue or a lost function by the graft itself; but also the positive effect the grafted cells might have on its environment, such as the release of trophic factors (Jain 2008). This might improve the survival of the inherent tissue and also endogenous proliferation. Many disease models are exploited for stem cell grafting, stroke (Burns et al. 2009) and TBI being two. Although some experimental results indicate improved outcome, there are many concerns that need to be addressed before translating the results into actual clinical settings, for example efficacy and safety issues (Borlongan 2009).

Transplantation of Stem Cells after Experimental TBI

Studies of transplantation to the traumatically injured rodent brain offers a diverse set-up regarding injury model, cell sources, site of deposition and time point after injury. It is therefore difficult to compare the results between these studies. Nevertheless a general consent seems to be that beneficial effects due to grafting often are found (Schouten et al. 2004). The rodent cell sources comprise cell lines and primary progenitor cells, both embryonic and adult (bone marrow stromal cells). As human cell sources, both primary cells
and cell lines have been utilized. Transplanted cells from different origin such as human donors (Wennersten et al. 2004), mouse ES-derived neuronal precursors (Hoane et al. 2004) and immortalized mouse progenitor cells (Riess et al. 2002) all expressed markers of neurons and astrocytes after grafting. The functional outcome varies between different studies (Schouten et al. 2004) but several groups have found augmented motor function after transplantation to the injured brain (Hoane et al. 2004; Lu et al. 2002a; Mahmood et al. 2001) or the spinal cord (Cummings et al. 2005; Ogawa et al. 2002) and some groups report improved cognitive function (Longhi et al. 2004; Watson et al. 2003). Stem cells originating from outside the CNS have demonstrated ability to migrate into the injured parenchyma, and improve functional outcome when administered intravenously as seen with bone marrow derived stromal cells (Qu et al. 2008) and human umbilical cord blood (Lu et al. 2002b). Cells from the latter were additionally found to express neuronal markers. The effect of the endogenous neurogenesis was investigated by a comparison between grafting of marrow stromal cells via intravenous and intracerebral administration. It showed that both routes of delivery gave endogenous proliferation. While the intracerebrally grafted cells induced proliferation in the SVZ and in the boundary zone the intravasally grafted cells induced additional proliferation in the hippocampus compared to the control group. Functional amelioration was seen in both treated groups (Mahmood et al. 2004). Long-term functional improvement was found up to 14 months post transplantation where surviving transplanted cells were found throughout the injured hippocampus (Shear et al. 2004).

Enhancing graft survival and improving lost function after transplantation by genetic modification of the transplanted cells is an appealing idea. To increase the effect of transplantation, cell lines have, prior to grafting, been engineered to secrete growth factors such as nerve growth factor (NGF) (Longhi et al. 2004; Philips et al. 2001; Watson et al. 2003), glial cell line-derived neurotrophic factor (GDNF) (Bakshi et al. 2005), or to constitutively express an activated form of epidermal growth factor receptor (Boockvar et al. 2003). The results have been encouraging, showing improved cognitive function with both NGF and GDNF.

To decide how and when to perform a cellular transplantation it might be helpful to consider several aspects such as cell source and the time line of the endogenous response to trauma. For example using un-differentiated human ES cells might cause tumour formation (Riess et al. 2007). Moreover the time aspect could be important since the inflammatory reactions to the trauma may have adverse effects on the grafted cells.
Figure 2. Schematic illustration of experimental set-up. NSPC from embryonic mice are cultured before transplanted to the traumatically injured brain. Differentiation and proliferation of cultured cells can be evaluated by immunocytochemistry. Outcome after CCI and transplantation can be evaluated both by cognitive testing and immunohistochemistry.

**Transplantation with Scaffolds**

Providing cells with a suitable frame to grow on, when transplanting them to an injured brain, thereby imitating the ECM may facilitate the proliferation and enhance the survival of the cells as is seen in vitro. In a hypoxic injury, a synthetic polymer scaffold seeded with stem cells reduced the parenchyma loss and both endogenous and transplanted neurites could be found in the scaffold (Park et al. 2002). Using scaffolds that are biologically derived could be beneficial since they better mimic the cells natural environment and are less likely cytotoxic to the host (Jansen et al. 2004). Matrices with naturally occurring substrates have been tested. Hyaluronan hydrogels, modified with laminin resulted in cell infiltration, neurite extension and angiogenesis in vivo (Hou et al. 2005). Attempts with a collagen/fibronectin gel containing NSPC grafted to the cavity after TBI showed increased survival and migration compared to cells injected to the cavity alone (Tate et al. 2002). A collagen scaffold seeded with human stromal cells before placed in the cavity formed by TBI showed reduced tissue loss and improved functional outcome (Lu et al. 2007). To keep the transplantation invasiveness to a minimum, injectable scaffolds might be preferable. This might be a contradictory problem to the ambition of having the possibility to pre-differentiate the cells in
or having them interact with the scaffold prior to grafting. Bible and associates elegantly provided a solution by creating scaffold particles and coating them to facilitate cell adhesion. Cells were able to be cultured on their scaffolds before being intracerebrally injected into an injured brain (Bible et al. 2009a; Bible et al. 2009b).

From the Laboratory to the Clinic

Although still in an experimental stage, cell transplantation has been tested in the clinic in some neuropathological conditions. Transplantation of foetal mesencephalic tissue to Parkinson’s disease have rendered partly beneficial results. Some patients suffering from Parkinson’s disease showed neurological improvement after receiving grafts (Lindvall and Bjorklund 2004; Lindvall et al. 1990). Long-term graft survival was seen in several patients (Mendez et al. 2008) although some groups reported that the grafted cells had acquired traits specific for the disease (Kordower et al. 2008; Li et al. 2008). Multiple sclerosis is an autoimmune disease affecting the central nervous system. Due to promising results in animal models the cell transplantation therapy now has been transferred to the clinic (Fassas and Kimiskidis 2004). Transplanting autologous haematopoietic stem cells to a malignant form of the disease has rendered encouraging results (Fagius et al. 2009).

Using allografted cells have obvious complications such as the need for immunosurveillance. Autologously retrieved cells serve as a more convenient source of cells, however the accessibility guides the choice of possible cells. The bone marrow offers cells quite easy to retract; hence the research around mesenchymal stem cells might provide useful knowledge. In fact there is an ongoing phase I study that utilize autologous bone marrow derived stem cells for transplantation after TBI in children (Jain 2008).
Present Investigation

The aim of this thesis was to use neural stem and progenitor cells as an instrument for improving outcome after injury to the nervous systems. To this end different strategies were developed to enhance the ability of the cells to survive in the hostile environment of the injured brain. The specific objectives for the included papers were:

I Investigate whether NSPC from the CNS would be a plausible implement to promote functional recovery after injury to the dorsal root.

II Evaluate the migration, differentiation and survival of grafted NSPC following traumatic brain injury

III Study the effect of anti-inflammatory treatment on NSPC grafting after traumatic brain injury

IV Identify cellular fate such as proliferation and differentiation in a three-dimensional hydrogel

Results and Discussion

(I) Central Nervous System Stem/Progenitor Cells Form Neurons and Peripheral Glia after Transplantation to the Dorsal Root Ganglion

To be able to discriminate donor cells from host cells after transplantation donor cells have to differ in some recognizable aspect. For example animals of different species could be used (xenotransplantation) or donor and host could come from different sexes. Donor cells could also express a reporter gene. We used neural stem and progenitor cells from mice that express enhanced green fluorescent protein (EGFP) (Okabe et al. 1997). NSPC were dissected from the embryonic cortex and cultured in vitro with the addition of the growth factors EGF and FGF-2. First we verified that the proportions between the mature neural cell types, neurons, astrocytes and oligodendrocytes corresponded to the distribution acquired from wild type mice. To this
end NSPC cells were differentiated and analysed. The injury model is built on removing the dorsal root ganglia, which creates a cavity. This procedure was performed on deeply anesthetized rats and EGFP expressing mouse neurospheres were transplanted into the cavity. The rats were immunosuppressed to avoid rejection of the graft. After one and three months, the rats were sacrificed and the graft analysed. Surviving EGFP positive cells were found at both time points and markers for differentiated cells could be confirmed in grafted cells. After one month, cells expressed markers for neurons (β-III-tubulin) and astrocytes (Glial Fibrillary Acidic Protein; GFAP). At three months GFAP positive astrocytes were still found, but no β-III-tubulin neurons. Instead Brn3a cells appeared, indicating an adaption of the cells to the peripheral nervous system. Additionally, single cells were transplanted in the intact dorsal root ganglion and analysed after three months. Approximately 5% of the transplanted cells survived. They were found surrounding DRG cells and expressed markers typical for peripheral glia.

We show that NSPC from the CNS can be used as cell source for transplantation into the peripheral nervous system. Although only a portion of the transplanted cells survived for the longer period of three months they obtained peripheral glial identity thus demonstrating an ability to respond to instructive signals from the environment.

(II) Grafted Neural Progenitors Migrate and Form Neurons after Experimental Traumatic Brain Injury

Traumatic brain injury (TBI) is a devastating condition afflicting numerous of persons every year (Andersson et al. 2003). In this paper we investigated whether NSPC from EGFP transgenic mice integrate into an adult mouse brain that has been subjected to experimental TBI. Two time points and two deposition sites were used to evaluate the most favourable conditions for the grafted cells considering survival and differentiation. NSPC were grafted either directly into the injured parenchyma immediately after injury or into the contralateral ventricle one week post trauma. As a control, cells were transplanted to uninjured mice. Under deep anesthesia, the mice were subjected to controlled cortical impact to the right fronto-parietal cortex. Approximately 100,000 EGFP expressing NSPC in a single cell solution were stereotactically injected using a syringe. The mice were sacrificed 1, 4, or 12 weeks after the transplantation. When analysing the brains from mice with an intra-parenchymal transplantation we found that there were few brains with viable transplanted cells compared to the intraventricular transplantation (20% versus 90% in the injured category and 20% versus 70% between the un-injured animals). This indicates that the ventricle is a more permissive region for deposition of NSPC. Transplanted NSPC were found to survive
up to 12 weeks after transplantation when transplanted in the lateral ventricle. EGFP positive cells migrated to the injury site, as found by others (Modo et al. 2004; Wennersten et al. 2004), and were present at all time points investigated. The ability of stem cells to “home in on” an injury (Aboody et al. 2000), might be due to chemoattractants released by the injured parenchyma (Aarum et al. 2003; Imitola et al. 2004; Sun et al. 2004; Ziv et al. 2006). Transplanted cells were also localized to the ventricular linings, the corpus callosum, and the needle tract. On the contrary, cells transplanted in the parenchyma were exclusively found in the vicinity of the deposition site. This gives further indication that the lesioned parenchyma might provide migratory cues.

Using different antibodies specific for CNS cell types, differentiation of the transplanted cells could be visualized. One week after transplantation EGFP positive cells from the intraventricular regimen found in the perilesion area mainly expressed the immature cell marker nestin and the neuronal marker microtubule-associated protein 2 (Map2). At later time points few cells in the peri-lesion area were found that expressed those antigens. GFAP positive astrocytes were found in the lateral ventricles but were otherwise sparse. In the ventricular wall and the corpus callosum, the majority of the transplanted cells looked viable, while in and around the peri-leisonal area the cell morphology indicated that many of the transplanted cells were dead, or dying, as shown by staining for cleaved caspase3. Transplanted cells in the needle tract and in the corpus callosum stained positive for the calcium binding protein S100A4 (Mts-1), a marker for white matter astrocytes. In the corpus callosum, EGFP positive cells extended long processes and had a morphology that resembled mature oligodendrocytes (Jackson et al. 2006), but these cells did not stain for mature oligondendrocytic markers. However, some of them expressed olig2, a marker for oligodendrocyte precursors, indicating an initial differentiation towards the glial linage. The absence of mature markers might imply that these cells somehow are impeded in their differentiation process. Non neurogenic regions favour astrocyte formation over neuronal as seen within the brain as well as in the spinal cord (Brannvall et al. 2006; Shihabuddin et al. 2000). The SVZ is a location of adult neural stem cells. Therefore the deposition of NSPC in a neurogenic environment might explain why differentiated NSPC expressed neuronal markers. This region might also be more permissive for the survival of the transplanted cells as compared to the injured cortex.

In this study we demonstrate that NSPC from embryonic mouse cerebral cortex survive transplantation to the lateral ventricle and migrate to the other hemisphere to the injury site in a mouse model of TBI. Transplanted cells differentiated to both neurons and glia suggesting that embryonic NSPC may serve as a source for neurorepair after experimental TBI.
(III) Ibuprofen Attenuates the Inflammatory Response and Enhances Migration of Grafted Stem Cells Following Traumatic Brain Injury in Mice

The initial insult after TBI is followed by an inflammatory response that is thought to have both beneficial and adverse effects on the secondary brain injury process. This inflammation may produce a hostile environment that might not be beneficial for transplanted cells (Molcanyi et al. 2007). We aimed to test if a reduced inflammatory response would improve cell survival after grafting. The commercially available non-steroidal anti-inflammatory drug (NSAID) ibuprofen was used to decrease inflammation. Ibuprofen promotes its effect by inhibiting cyclooxygenase (cox) enzymes 1 and 2, which are required in the formation of prostaglandins from arachidonic acid. The expression of cox-2 is found to be increased after trauma (Kunz et al. 2002). Ibuprofen improves cell viability in vitro (Casper et al. 2000) and demonstrates beneficial cognitive effects in animal models of Alzheimer (Lim et al. 2001; McKee et al. 2008). Ibuprofen was administered via the drinking water starting at 48 hours prior to transplantation and throughout the study (one and four weeks post transplantation). The control group received vehicle instead of ibuprofen. As in previous studies, 100,000 EGFP expressing NSPC were stereotactically injected to the contralateral ventricle one week after TBI. The effect of ibuprofen on inflammation was illustrated by the reduction of the inflammatory molecules ICAM-1 (intra cellular adhesion molecule 1) and mac-2 after four weeks, hence demonstrating proper uptake of the drug. ICAM-1 is a molecule that shows increased expression after TBI (Carlos et al. 1997) and is associated with the infiltration of leukocytes (Greenwood et al. 2002). Mac-2 is expressed by microglial cells, which are activated following TBI in this model (Clausen et al. 2009). One week after transplantation EGFP expressing cells, detected with an anti-GFP antibody, where found in the peri-lesion zone in animals treated with ibuprofen. In the un-treated group grafted cells were not detected in this area. After four weeks, transplanted cells in the injured cortex could be found both in the treated and in the un-treated group. In the hippocampus, which also is affected by the TBI, EGFP positive cells could only be found in animals treated with ibuprofen. There was an indication that cell survival was improved by anti-inflammatory treatment, however this could not be verified statistically. The majority of the transplanted cells were found integrated in the walls of the ventricle where they were deposited. Some cells however, had formed cell aggregates resembling spheres either attached to or detached from the ventricle wall. Although of different origin, these attached aggregates morphologically resemble the hyperplastic nodules formed by transforming growth factor alpha (TGF α) infusion into 6-OHDA rats (Cooper and Isacson 2004).
To analyse the differentiation fate of the transplanted cells, markers for different cell lineages were used. A marker for migratory neuroblasts, double-cortin, (DCX) indicated that ibuprofen influenced the differentiation of migrating neuroblasts since transplanted cells in the peri-lesion zone expressing DCX were exclusively found in ibuprofen treated animals. Regarding the expression of the astrocytic marker GFAP, ibuprofen treatment showed no difference to untreated animals. The greater part of the EGFP/ GFAP double positive cells were found in the ventricles. Although the identity of the cells in the aggregates in our study has not been clarified some expressed GFAP.

No tissue sparing effect of ibuprofen was found, as observed by others (Browne et al. 2006). Cognitive outcome as assessed by the Morris water maze in the group with longer survival time, did not demonstrate any difference in visuospatial learning. Conversely, Browne et al found an augmentation in cognitive dysfunction after ibuprofen administration when assessed with MWM. The discrepancies in the results can be due to several factors. In the study by Browne, ibuprofen administration continued for a more extended period (four months) versus four weeks in our study. An influence of the transplanted NSPC per se can however not be ruled out, since the Browne study did not include grafting.

Reducing inflammation by ibuprofen administration appears to be beneficial to the transplanted cells in aspect of cell survival even though this effect failed to be proven statistically. Nevertheless, an additional measure needs to be taken to improve tissue sparing and cognitive outcome following TBI. Conceivably a more selective anti-inflammatory drug might be favourable. Another alternative approach could be to promote cell survival by neurotrophic/protective factors.

The development of the technique to genetically modify mice has had an enormous impact on research. Transgenic mice has facilitated basic science and increased our knowledge of many diseases and gene functions. In our investigations we have used a transgenic mouse that express EGFP under the β-actin promotor. These mice express GFP in all cells except erythrocytes (Okabe et al. 1997). Although the most commonly used method to detect GFP may be direct microscopy, an anti-GFP antibody was used in paper III to ensure an accurate estimation of transplanted cells (Swenson et al. 2007). Without the antibody some transplanted cells might go undetected though the GFP expression might diminish. Additionally, traumatized cells can autofluoresce and hence problems of discriminating between endogenous and transplanted cells might occur (Clark et al. 1999; van den Pol 2009). Using the antibody did facilitate detection of transplanted cells in our study. NSPC from these mice have proven to be an efficient tool used for transplantation to the injured nervous system.
(IV) Enhanced Neuronal Differentiation in a Three-Dimensional Collagen-Hyaluronan Matrix

Tissue loss, leading to cortical cavity formation, is a major consequence of TBI. Regeneration of lost brain tissue might possibly be augmented if cells that are transplanted directly into the cavity have a scaffold to grow on (Park et al. 2002). To develop a 3D NSPC system suitable for transplantation after experimental TBI, NSPC were cultured in a hydrogel made of hyaluronan and collagen type I, two naturally occurring components. NSPC from EGFP transgenic mice of different ages (E14.5, P6 and adult) were seeded in the scaffold and their ability to survive, proliferate and differentiate was characterized. The cells were mixed with the hydrogel components at 4°C, at which the hydrogel is in a liquid state. When incubated in 37°C the hydrogel solidifies, thus enclosing the cells. NSPC were grown under proliferating conditions with the addition of EGF and FGF2. By withdrawal of the growth factors, differentiation into neurons and glia was induced, in analogy with regular 2D NSPC culture. The cell containing gels were fixed at different time points and the cell-matrix interaction was visualized by Scanning Electron Microscopy and immunocytochemistry. By addition of the thymidine analogue BrdU prior to fixation, cell proliferation could be evaluated. Cells from the different donors interacted differently with the hydrogel. NSPC from all ages proliferated in the matrix but to a lesser extent compared to the 2D culture system. As might be anticipated, embryonic cells had the highest proliferation rate, 46%, in the hydrogel at day six, and adult NSPC the lowest (27%). All cell types had a proliferation peak between six and nine days in culture. TUNEL staining showed that embryonic NSPC had the highest apoptotic percentage (39%) and P6 the lowest (16%). The relatively high number of apoptotic cells and low number of BrdU positive cells as compared to 2D cultures might be explained by limited diffusion of nutrients into the gel, and that dead cells are trapped within the matrix. After six days of differentiation in the 3D scaffold, NSPC from the postnatal brain had generated as much as 70% neurons, compared to 14% in 2D. Cells from embryonic mice rendered approximately the corresponding amount of neurons as they do when cultured as a monolayer, comparable to those seen by Ma et al (Ma et al. 2004), as did cells from adult. Neurons from P6 cell culture expressed neurotransmitters GABA and glutamate after eleven days of growth factor withdrawal as well as the mature neuronal marker neurofilament H, indicating morphological neuronal maturation. The observation that cells from postnatal donor showed the lowest apoptosis and the highest percentage of neurons, suggests a different interaction with the hydrogel components than embryonic and adult NSPC.

This study shows that NSPC from embryonic, postnatal, and adult brain proliferate and form neurons, astrocytes, and oligodendrocytes in a 3D scaf-
fold containing hyaluronan and collagen type I. Early neural progenitors from postnatal brains are more suitable than progenitors from the embryonic and adult brains for the generation of large quantities of neurons in this particular scaffold. Most likely the postnatal cells express cell surface receptors that enables a favourable interaction with the hydrogel resulting in a high neuronal content. For the purpose of tissue regeneration this hydrogel might provide an attractive option. Inducing neuronal differentiation and achieving a pure neuronal population without genetic manipulation has not yet been shown. Even though it is not a pure population, this model provides an abundant differentiation to morphologically mature neurons. After TBI when a cavity is formed in the cortex, a scaffold that generates a mixed culture might in fact be preferable since it reflects the *in vivo* situation.
NSPC from embryonic EGFP positive mice have shown to be a suitable cell source for transplantation studies in animal models of nervous system injuries, as they are easily detected, survive for long periods, and differentiate into neurons and glial cells. These cells constitute an attractive model since they are effortlessly propagated in vitro and can be used as allografts as well as xenografts.

Immunohistochemical analysis of brains from mice subjected to CCI provides information about graft/host interaction on a cellular level (paper II and III). Although we have provided additional evidence that embryonic NSPC survive, migrate to the injury site, and form neurons and glial cells after experimental TBI, we have no data relating these cells with an actual functional integration into the brain circuitry. Using markers for synaptic maturation might indicate a connection between cells, but to be able to verify a functional incorporation, electrophysiology is needed. The cognitive test did not show any improvement when inflammation was reduced with ibuprofen, however animals subjected to CCI without transplantation were not tested in this paradigm and hence the effect of transplantation per se can not be fully evaluated. Additional behavioral test might also be employed to measure different aspects of cognition such as open field test. “Challenging” the animals by providing external stimulation such as exercise or enriched environment might possibly enhance the functional integration of transplanted cells into the brain. This concept has proven successful with the endogenous neurogenesis (Kempermann et al. 1997; van Praag et al. 1999). More specific anti-inflammatory agents that transiently target an inflammatory molecule might be beneficial for enhancing the survival of the grafted cells as well as an improved overall outcome.

Providing cells with a supporting scaffold when transplanting them into an injured brain provides the opportunity to place the cells directly into the cavity and hence avoiding the need for proper migration. Although migratory cues are provided that guides the cells towards the injured parenchyme (Imitola et al. 2004) (Sun et al. 2004) our own studies indicate that not all cells respond to these cues and hence some potential new-builders are lost. Furthermore the scaffold might also enhance cell survival leading to fewer cells going through apoptosis a prominent complication following transplantation recognized by us and others.
(Bakshi et al. 2005). The hydrogel used in paper IV is in a liquid state at room temperature but solidify at body temperature due to cross-linking, which make it attractive to use in transplantation studies since it can be molded after the cavity. A viscous hydrogel additionally add very little invasive effect as it, together with the cells, can be deposited with a syringe. The high neuronal content of this hydrogel when seeded with P6 NSPC in addition to the glial content suggest that it may be suitable for regeneration of the cortex. In particular it reflects the in vivo situation with a mixture of neurons and glia where neurons are prominent. Attempts to use this approach in our TBI model might provide new insights in how to improve outcome.

Growth factors, neurotrophic factors and anti-inflammatory agents are examples of molecules that might enhance the survival of endogenous and exogenous newborn cells. Attempts using mini-pumps with an intraventricular injection have been made (Clausen et al. 2009; Richardson et al. 2005). However some difficulties might arise with this method such as infection and the size of the mini-pump obstructing the mice. Moreover, a study using intrastriatal infusion of transforming growth factor α have found proliferating nodules at the ventricular wall, indicating a risk for tumor formation (Cooper and Isacson 2004). Avoiding these predicaments could be accomplished by introducing the factors into a scaffold that is injected into the cavity. Factors would be locally distributed while gradually released from the scaffold.

Results from paper IV indicate that NSPC from postnatal day six might be a better source for transplantation material than E14.5 and adult NSPC if transplanted in the hyaluronan/collagen I hydrogel due to the striking difference in neuronal differentiation. This suggests a difference in expression of receptors responsible for cell-matrix interaction. Preliminary studies show a difference in adhesion to the matrix components, which in turn imply different expression patterns of adhesion molecules on the NSPC. In order to elucidate the possible mechanisms behind their interactions we will continue with the in vitro characterization of the cell-matrix system.

The present investigation contains studies of NSPC in vivo and in vitro, aimed at improving the outcome in an experimental setting of TBI. More in vitro data concerning the differentiation processes and possibilities are needed to be able to fully take advantage of the stem cell capacity. TBI unfortunately has an extensive incidence and therefore has rendered much research. All progress and discoveries might provide new insights to resolving the complex clinical picture of TBI, which is vastly needed. The translation of experimental information into the clinical setting remains a great challenge considering the many experimental treatments that fail to render success in the clinic. The first clinical study regarding transplantation to TBI however raises great hope (Jain 2008).
Populärvetenskaplig sammanfattning

Traumatisk hjärnskada (THS) är en skada som uppstår på grund av yttre våld mot huvudet. En vanlig orsak till THS är fall eller trafikolyckor, men även misshandel och sportolyckor kan vara orsaken. I Sverige drabbas över 20.000 personer årligen. Sjukdomsbilden efter THS varierar beroende på vilken del av hjärnan som drabbats och hur svår skadan är. Motorisk funktionsnedsättning, inlärningssvårigheter och depression är exempel på problem som kan drabba en THS patient. Vissa patienter får så omfattande skador att de har kvarstående men, vilket drabbar såväl patienten som anhöriga hårt. THS är en av de vanligaste orsakerna till död och funktionsnedsättning i västvärlden och medför därför allvarliga socioekonomiska konsekvenser även på samhällellig nivå.

Det inledande slaget mot huvudet ger en förlust av hjärnvävnad till följd av att celler dör. Detta följs av en inre kaskad av skademekanismer som ytterligare förvärrar skadan såsom inflammation, ödembildning, uppkomst av fria radikaler etc. Mycket forskning har inriktats på de sekundära skademekanismerna där en terapeutisk intervention är möjlig. Trots många positiva resultat i djurstudier finns idag ännu inget botemedel som kliniskt kunnat påvisa en förbättring hos patienterna.

Omogna celler som kan bilda olika celltyper kallas stamceller. I det vuxna centrala nervsystemet (CNS) kan dessa celler hittas främst i två olika regioner, den subventrikulära zonen vid de laterala ventrikularna samt i hippocampus. När dessa celler mognar kan de bilda de olika typer av celler som en hjärna till största delen består av; nervceller och gliacller. Neurala stam- och progenitor celler (NSPC) kan odlas in vitro med tillsats av tillväxtfaktorer. Potentialen hos dessa celler att kunna bilda nya nervceller gör dem attraktiva för flera olika användningsområden, exempelvis för att ersätta celler som har dött antingen på grund av sjukdomstillstånd som Parkinson eller som svar på ett trauma. Kliniska försök där fetala humana celler har transplanterats till patienter med Parkinson har gett blandade resultat.

För att kunna använda NSPC i dess fulla potential behövs dock ytterligare kunskap. Exempelvis skulle odlingspremisserna kunna optimeras för att styra differentieringen bättre. Hur man ska kunna öka överlevnaden av celler efter en transplantation är ett annat område som behöver utredas mera. Dess-
utom krävs nogranna studier av säkerhetsaspekter för att undvika eventuella biverkningar av stamcellstransplantation för patienten innan transplantations
kan utföras.


Vid stor vävnadsförlust, som är fallet vid experimentell THS, är det troligt att endast tillförsel av nya celler som ska ta över funktionen av de förlorade inte är tillräcklig utan någon sorts förstärkning. Troligtvis behöver dessa transplanterade celler någon form av strukturellt stöd för att kunna integrera och överleva i kaviteten. För att utröna NSPCs förmåga att proliferera och differentiera under tredimensionella odlingspremisser odlades stamceller från tre olika åldrar; embryonala, postnatala dag sex (P6) och adulta, i en matris bestående av hyaluronsyra och kollagen (artikel IV). Hialuronsyra
och kollagen är två molekyler som normalt finns runt cellerna i hjärnan i den s k extracellulära matrisen. NSPC överlevde och mognade i denna matris. Dock uppvisade cellerna från de olika åldrarna skilda beteenden vad gäller proliferation och differentiering. NSPC från de yngre åldrarna prolifererade bättre, vilket är jämförbart med situationen vid vanlig cel lodling på en plast yta. En markant ökning av andelen nervceller kunde ses hos P6-cellerna efter sex dagars differentiering där ca 70% var positiva för nervcellsmakören βIII-tubulin jämfört med 14% vid konventionell 2D odling. Vid de andra åldrarna syntes ingen skillnad vid neuronal differentiering. Detta innebär att en sådan här matris skulle kunna användas vid transplantation efter experimentell THS för att fylla ut kaviteten. De transplanterade cellerna skulle då få strukturellt stöd vilket förhoppningsvis skulle underlätta genererandet av ny nervvävnad.

I artikel I undersökte vi om NSPC kunde användas som ett instrument för att regenerera vävnad även i det perifera nervsystemt (PNS). En avgörande skillnad mellan PNS och CNS är det perifera nervsystemets förmåga att regenerera axonal tilläxt, d.v.s. en större möjlighet till läkning vid nervskada. Denna läkning har dock begränsad effekt vid skada på dorsalrotsganglierna, som är en del av PNS, eftersom nytillväxten upphör i zonen som utmärker gränsen mellan perifert och centralt nervsystem. Genom en skademodell i rätta där dorsalrotsganglion opereras bort, kan de underliggande mekanismerna för denna skillnad studeras. I ett första steg undersöcktes om stamcells transplantation kunde bidra till regeneration av nervvävnad. I den kavitet som uppstår vid avlägsnat dorsalrotsganglion transplanterades NSPC. Överlevande transplanterade celler kunde hittas efter både en och tre månader och markörer för nervceller och astrocyter kunde bekräftas. Dessa celler kunde således även användas i ett annat värddjur och under en annan skadeform.

Neurala stam- och progenitorceller har i denna avhandling visat sig vara ett användbart verktyg för att försöka regenerera vävnad som förlorats på grund av skada i nervsystemet. Innan man kan tillämpa transplantation av dessa celler kliniskt kommer dock ytterligare forskning att behövas.
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