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ANNA HICKS

Intracerebral Stem Cell Transplantation In Experimental Stroke Models

Doctoral dissertation

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ABSTRACT

Cerebral ischemia causes severe functional deficits leaving majority of patients with permanent disabilities. Physical rehabilitation remains the only widely accessible therapy that facilitates functional recovery. Stem cells are suggested to possess the ability to replace lost cells and tissue and thus may enhance functional recovery after stroke. Indeed, according to previous studies stem cells moderately facilitated recovery in animal models of stroke.

We used rat models of focal cerebral ischemia and intracerebral transplantation of stem cells. Cells were injected into close proximity of the injured brain tissue one week after the stroke. The animals were tested in sensorimotor tests in various timepoints to quantify functional recovery. We transplanted neurospheres isolated from subventricular zone (SVZ) of mice or human neuronal progenitor cells (hNPC) derived from human embryonic stem cells (hESC). Survival, migration and differentiation of stem cells were examined using immunohistochemistry, immunofluorescence techniques, stereology and confocal microscopy. In addition, the effects of enriched environment (EE) rehabilitation and running exercise combined with stem cell therapy were examined. Animals received immunosuppressive drug to minimize rejection of the transplants.

Results show that the combination therapy of rehabilitation and SVZ stem cell transplantation resulted into enhanced migration of stem cells and better functional outcome within first weeks after the ischemic insult. Longterm studies of SVZ cells revealed that the majority of transplanted cells disappeared after the first month and the remaining cells differentiated into glial cells within two and three months after the transplantation. Transplantation of hESC-derived hNPCs resulted into modest functional recovery. Again the survival of cells was minimal. Majority of hNPCs expressed precursor marker nestin, however some of the cells had neuronal phenotypes two months after the transplantation. In longterm studies the EE rehabilitation had no effect on the transplanted SVZ/hNPC cells. We found sustained activation of immune cells in the brains that had received cell transplantation.

In conclusion, stem cell therapy supports endogenous brain repair mechanisms during the first weeks after transplantation, and EE rehabilitation supports the beneficial effects of stem cells. However, the cells fail to survive for protracted time periods after intracerebral transplantation. It is crucial to identify the factors restricting stem cell survival and migration in stroke injured cerebral microenvironment before the true effects of transplantation therapy can be measured.

National Library of Medicine Classification: WL 355; WL 354; QU 325; QU 328; QY 58; QY 60.R6
Medical Subject Headings: Brain; Brain Ischemia; Stroke; Stem Cells; Embryonic Stem Cells; Rehabilitation; Running; Recovery of Function; Disease Models, Animal; Rats; Mice, Transgenic; Cell Transplantation; Stem Cell Transplantation; Cell Survival; Cell Movement; Cell Differentiation; Cell Death; Graft Rejection; Cerebral Ventricles; Green Fluorescent Proteins; Nerve Tissue Proteins; Immunohistochemistry; Fluorescent Antibody Technique; Microscopy, Confocal

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St John's, December 2008

Anna Hicks

ABBREVIATIONS

ANOVA	analysis of variance
AP	anterior-posterior
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
DAB	diaminobenzidine
DCX	doublecortin
dMCAO	distal middle cerebral artery occlusion
DMEM	Dulbecco's modified eagle medium
DV	dorso-ventral
EE	enriched environment
EGF	epidermal growth factor
ESC	embryonic stem cell
ET-1	endothelin-1
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
hESC	human embryonic stem cell
hNPC	human neural precursor cell
HUCB	human umbilical cord blood cell
HuNu	human nuclei marker
i.p.	intraperitoneal
i.v.	intravenous
ML	medio-lateral
MAP-2	microtubule associated protein-2
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MSC	mesenchymal stem cell

NDS	normal donkey serum
NF	neurofilament
NPC	neural precursor cell
NGF	nerve growth factor
NSS	neurological severity score
OB	olfactory bulb
PFA	paraformaldehyde
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RMS	rostral migratory stream
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
SC	stem cell
ST	standard housing
SVZ	subventricular zone
TBI	traumatic brain injury
VEGF	vascular endothelial growth factor
Veh	vehicle

LIST OF ORIGINAL PUBLICATIONS

This work is based on four original articles which are referred to by Roman numerals I-IV in the following text.

I **Hicks AU**, Hewlett K, Windle V, Chernenko G, Ploughman M, Jolkkonen J, Weiss S, Corbett D. Enriched environment enhances SVZ stem cell survival, migration and functional recovery after stroke. Neuroscience 2007;146:31-40.

II **Hicks AU**, MacLellan CL, Chernenko GA, Corbett DR. Long-term assessment of enriched housing and subventricular zone cell transplantation after focal ischemia in rats. Brain Research 2008;1231:103-112.

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

IV **Hicks AU**, Schallert T, Jolkkonen J. Cell based therapies in animal models of stroke-focus on functional recovery. Submitted

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1. INTRODUCTION

During ischemia lack of blood flow, oxygen and nutrients, and accumulation of toxic metabolites in brain tissue lead acutely to brain odoema, and cellular stress and eventually to cell death and tissue loss. Cerebral ischemia can be caused by a blood vessel occlusion or by an eruption of blood vessel (hemorrhagic stroke) in brain. In this study we focus on focal occlusion of middle cerebral artery (MCA), which is the most common form of stroke, and has been successfully mimicked in animal models.

Unfortunately, there is no cure for stroke after the incident, despite extensive research to discover compounds to prevent cell loss. Tissue-plasminogen activator can be administered to some patients within three-hour time window after the onset of stroke symptoms (Hoyte and Kaur, 2004; Meairs et al., 2006). Physical therapy remains the only widely accessible therapy that promotes functional recovery in stroke patients.

In search of therapies for stroke, stem cells have been proposed to be able to restore function by replacing lost cells and tissue (Gage, 2000; Emsley et al., 2005; Haas et al., 2005; Steindler, 2007). Indeed, according to some studies stem cells do provide moderate functional benefits after cerebral ischemia (Haas et al., 2005; Chang et al., 2007). Specifically, bone derived mesenchymal stem (MSC) cells (Chen et al., 2001; Zhao et al., 2002; Shen et al., 2007), human neural precursor (hNPC) cells (Borlongan et al., 1998; Bliss et al., 2006; Daadi et al., 2008) and human umbilical cord blood (HUCB) cells (Chen et al., 2001; Borlongan et al., 2004; Vendrame et al., 2004) have been shown to improve simple sensorimotor functions after cerebral ischemia in rats. It is still questionable whether or not stem cells can replace lost cells since increasing amount of experimental data suggest that transplanted cells actually support the endogenous neuroplasticity mechanisms, such as angiogenesis and local trophic factor release (Chen et al., 2005), promotion of endogenous precursor cells (Chang et al., 2007) and neuroprotection by suppressed immune response (Vendrame et al., 2005).

Brain self-repair has garned a great deal of interest in experimental stroke research. These so called neuroplastic repair mechanisms in brain contribute to functional recovery and can be enhanced by enriched environment (EE) and rehabilitation (Biernaskie and Corbett, 2001; Johansson and Belichenko, 2002). Enriched environment

produces several beneficial effects among others increased dendritic arborisation (Biernaskie and Corbett, 2001) and increased levels of growth factors in the injured brain (Dahlqvist et al., 1999; Ickes et al., 2000; Gobbo and O'Mara, 2004). When EE is combined with rehabilitation of stroke affected limb (Biernaskie and Corbett, 2001; Ploughman et al., 2007a) or with running exercise (Ploughman et al., 2005), it is possible to achieve more pronounced functional recovery in rats. In future it may be possible that stroke patients will receive a combination of intensive rehabilitative therapies and task-specific rehabilitation with administration of for instance stem cells and trophic factors known to induce neuroplasticity. Thus, the current study focuses on the combination of stem cell transplantation and rehabilitation in experimental stroke models. We aimed to reveal whether or not transplanted cells survive, migrate and differentiate in the rat brain after cerebral ischemia and if EE can enhance cell therapy success. Furthermore, behavior tests were performed to study the possible effects on functional recovery of the stroke-affected forelimb.

2. REVIEW OF THE LITERATURE

2.1 Functional recovery after stroke

Stroke affects multiple brain areas causing several dysfunctions such as motor deficits of the limbs, muscle weakness, speech problems and somatosensory and cognitive deficits. The recovery of each system function is different and responses to rehabilitation vary depending on the location of the lesions in the brain (Cramer and Riley, 2008). The brain infarct may include parts of the thalamus, hippocampus and different cortical areas. All cell types are lost by ischemic insult; i.e., neurons, astrocytes, oligodendrocytes and endothelial cells (Savitz et al., 2004). Thus, it is not surprising that the recovery seen within weeks and months after stroke is incomplete. So far in clinical settings physical therapy for improvement of gait by treadmill walking (Peurala et al., 2008) and constraint-induced therapy of the affected hand (Ploughman and Corbett, 2004) have been proved beneficial for the recovery of function. In experimental settings various forms of rehabilitation, such as EE and running exercise (Biernaskie and Corbett, 2001; Ploughman et al., 2005), intensive motor rehabilitation (Jones et al., 1999) and molecular therapies such as administration of erythropoietin and epidermal growth factor (Kolb et al., 2007) have been shown to enhance functional recovery and brain self-repair.

2. 2 Enriched environment and running exercise after stroke

Effects of the experimental rehabilitation paradigm, EE, have been widely studied (Johansson and Belichenko, 2002, Mohammed et al., 2002). Beneficial effects include among others upregulation of endogenous plasticity related growth factors, synaptic plasticity, increased dendritic branching and enhanced neurogenesis (Falkenberg et al., 1992; Torasdotter et al., 1998; Dahlqvist et al., 1999; Pham et al., 1999; Ickes et al., 2000; Biernaskie and Corbett, 2001; Briones et al., 2004; Dobrossy et al., 2004; Gobbo and O'Mara, 2004; Komitova et al., 2005a). Importantly, enriched environment has positive effects on endogenous stem cells (Komitova et al., 2005a) which resulted in enhanced functional recovery. In addition, neural grafting resulted in better functional

outcome in ischemic rats housed in EE (Mattsson et al., 1997). Recently neurogenesis has been reported to occur in ischemic human brain (Jin et al., 2006; Curtis et al., 2007), which is encouraging for clinical stroke rehabilitation and might create more interest in rehabilitation that induces neurogenesis.

Rehabilitation of the stroke impaired forelimb by reaching rehabilitation in rat, alone (Ramanathan et al., 2006) or combined either with EE (Biernaskie and Corbett, 2001) or running exercise (Ploughman et al., 2007a) leads to even more effective recovery and enhanced brain plasticity. Reaching rehabilitation combined with running exercise leads to elevated levels of plasticity related growth factors, and better functional recovery (Ploughman et al., 2007a,b). Voluntary exercise is also used in rehabilitation after cerebral ischemia and has been shown to have several beneficial effects, such as increased levels of growth factors and increased neurogenesis (Neeper et al., 1996; van Praag et al., 1999; Vaynman et al., 2003; Griesbach et al., 2004; Ploughman et al., 2005; Ying et al., 2005). Interestingly, running exercise increases cerebral blood flow and neurogenesis in hippocampus both in mice and in humans (Pereira et al., 2007). In addition intensive rehabilitation of motor skills (acrobatic learning) appears to result in enhanced synaptogenesis and forelimb use after forelimb cortex damage (Jones et al., 1999; Chu and Jones, 2000).

These encouraging results of positive changes in the microenvironment of the brain due to rehabilitation and regeneration of tissue due to growth factors (Kolb et al., 2007) might benefit stroke patient in future especially when combined with stem cell therapy.

2. 3 Stem cells

Stem cells possess two unique properties compared to other cells; firstly, they can proliferate indefinitely and secondly, they can give rise to progenitor cells that differentiate into specialized cells performing specific functions. Two main types of stem cells used in research are embryonic stem cells (ESC) and adult stem cells. Embryonic stem cells in a blastocyst are pluripotent; they give rise to all cell types in developing tissues, ectoderm, mesoderm and endoderm (Fig. 1). These specialized tissues have then

their own population of stem cells, the multipotent adult stem cells that can be used for repair and replacement, such as neural stem cells found in brain (Bethesda, 2006). Neural stem cells (Fig. 2) can give rise to neuronal (progenitors producing only neurons) or glial progenitors (producing astrocytes or oligodendrocytes). Importantly, ESCs and adult stem cells can be grown in culture, manipulated and then transplanted in injured tissue in hope of finding cure for several diseases (Gage, 2000; Lindvall, 2004; Bethesda, 2006).

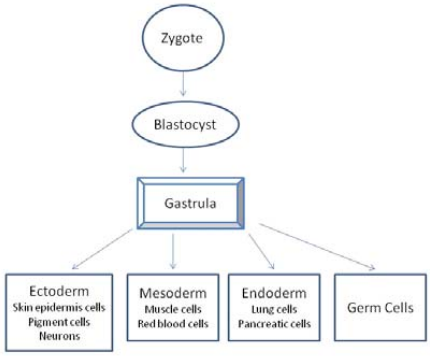

Figure1. Differentiation of embryonic stem cells in a developing fetus.

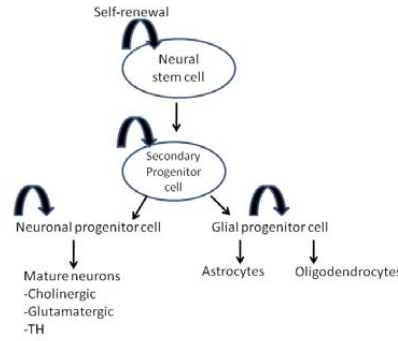

Figure 2. Differentiation of neural stem cells after isolation from stem cell containing tissue (e.g. embryonic tissue, brain ventricle walls).

2.3.1. Subventricular zone stem cells

There is a continuous production of stem cells in adult mammal brain in two neurogenic regions, the subgranular zone of the hippocampus and the subventricular zone (SVZ) surrounding the lateral ventricles (Altman, 1962; Doetsch et al., 1997; Reynolds and Weiss, 1992; Curtis et al., 2007). These stem cells and their progeny migrate long distances in an intact brain via rostral migratory stream (Fig. 3) to the olfactory bulbs where they differentiate into functional granule or periglomerular neurons (Lois and

Alvarez-Buylla, 1994). However, after a brain insult the direction of migration of SVZ stem cells changes and they migrate towards damaged brain areas (Arvidsson et al., 2002; Parent et al., 2002; Goings et al., 2004; Collin et al., 2005; Yamashita et al., 2006). Proliferating cells originating from SVZ were reported to be able to differentiate into striatal spiny neurons and interneurons after excitoxic lesion in rat striatum (Collin et al., 2005) six weeks after the injury. In addition, three months after middle cerebral artery occlusion (MCAO) in mice SVZ originated cells had differentiated into neurons that formed synapses with neighboring striatal cells (Yamashita et al., 2006). In animal models of stroke increased levels of SVZ neurogenesis are reported after infusion of epidermal growth factor (EGF) (Kolb et al., 2007), and after housing in enriched environment (Komitova et al., 2005a). Importantly, SVZ neurospheres can be isolated from brain, cultured and manipulated *in vitro* (Reynolds and Weiss, 1992; Palmer et al., 1999; Shimazaki et al., 2001; Zhang et al., 2003; Zhang et al., 2005; Zhang et al., 2006).

When these cells are injected into the striatum, cortex or olfactory bulb, only poor survival is reported with or without excitotoxic lesion (Herrera et al., 1999). Also poor migration of transplanted SVZ cells was reported in the intact rodent brain (Soares and Sotello, 2004), but after incubation in basic fibroblast growth factor (bFGF) migration and neuronal differentiation of SVZ cells were observed after transplantation into the intact rat striatum (Zhang et al., 2003). Interestingly, brain injuries such as stroke (Zhang et al., 2005; Liu et al., 2006; Zhang et al., 2006) induce molecular changes in SVZ cells that differ from SVZ cells in the intact brain. SVZ cells response to injury by recapturing embryonic molecular signals (Liu et al., 2006). This might explain why endogenous replacement might be more efficacious than the transplantation of SVZ cells. Human neurogenesis has been reported recently in a normal brain (Curtis et al., 2007a; Curtis et al., 2007b), and after stroke (Jin et al., 2006). Conduction of neurogenesis studies in humans is challenging and it remains to be shown whether or not endogenous neuronal replacement takes place after brain insults leading to functional recovery.

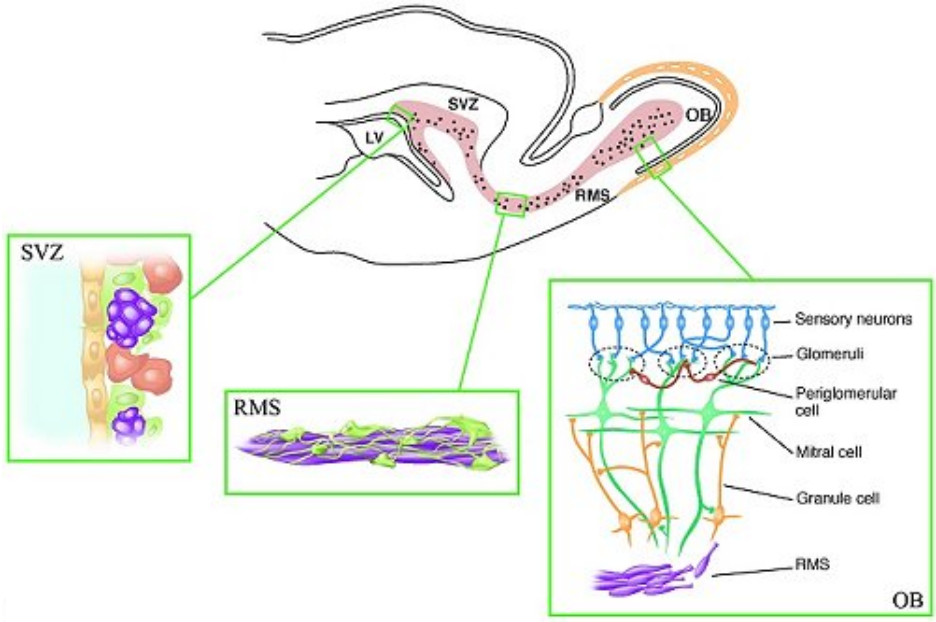

Figure 3. SVZ stem cells and their progeny migrate long distances in an intact brain via rostral migratory stream (RMS) to the olfactory bulbs (OB) where they differentiate into functional granule or periglomerular neurons (Reprinted with permission from Sage Publication, Lledo et al., 2004)

2.3.2 Embryonic stem cell derived neural precursor cells

Embryonic stem (ESC) cell lines can be derived from the inner cell mass of human blastocysts (Thomson et al., 1998; Reubinoff et al., 2000). These ESCs are pluripotent and can differentiate into any type of cells in the body (Evans and Kaufman, 1981; Martin, 1981). Cell lines of human ESCs (hESC) have been maintained as long as one year without losing the ability to divide indefinitely and to differentiate into mesodermal, ectodermal or endodermal cells (Amit et al., 2000; Itskovitz-Eldor et al., 2000) in the presence of growth factors. If ESCs aggregate into embryoid bodies they can start differentiation process into progenitor cells automatically. Cell progenitors of interest can be identified by markers, expression of reporter genes, and characteristic morphology and in case of neural precursors can be cultured as neurospheres. The

progenitor cells in neurospheres can be enriched into progenitor types (Fig. 2) and further cultured to more mature cell types (Trounson, 2006).

Neural precursor cells (NPC) have been derived from hESC lines that express both immature and mature neuronal proteins (Schuldiner et al., 2001) and contain voltage-dependent channels that can be triggered to fire action potentials (Carpenter et al., 2001). These *in vitro* differentiated NPCs survive, migrate and express neuronal, astrocytic and oligodendrocytic proteins when transplanted into lateral ventricles of neonatal mouse brain (Reubinoff et al., 2001; Zhang et al, 2001) or into the ventricles of neonatal immune-deficient mice (Guillaume et al., 2006).

2.4 Stem cell studies in animal models of stroke

2.4.1 Endogenous stem cells

Cerebral ischemia causes endogenous stem cells to proliferate and migrate towards injured brain tissue (Garcia-Verdugo et al., 1998; Arvidsson et al., 2002; Goings et al., 2004; Emsley et al., 2005; Hagg, 2005; Zhang et al., 2005; Yamashita et al., 2006). In addition, stem cells from brain can be isolated, differentiated *in vitro*, and transplanted into ischemic brain (Gage, 2000; Zhang et al., 2003; Emsley et al., 2005; Haas et al., 2005). Stem cells respond to molecular cues and growth factors in the cerebral microenvironment (Imitola et al., 2004; Hagg, 2005; Komitova et al., 2005a; Wang et al., 2006) and *in vitro* (Aarum et al., 2003; Hagg, 2005; Zhang et al., 2006). During brain development specific genes and proteins are turned on to encourage cell genesis, proliferation, cell survival, migration and finally differentiation in the target area in the brain (Gage, 2000; Hagg, 2005; Komitova et al., 2006b). Some of these proteins, such as nestin, brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) are expressed adjacent to injured tissue (Carmichael, 2005a) after cerebral ischemia. It has been suggested that these proteins also play a role in repair mechanisms, such as neurogenesis (Cramer and Chopp, 2000) and might promote survival of transplanted cells. Despite upregulation of several molecules supportive of survival and migration there are also restricting factors such as strong astroglial response that limits

stem cell survival. Repulsive molecular cues in peri-lesional areas (Garcia-Verdugo et al., 1998; Nguyen-ba-Charvet et al., 2004; Schwab et al., 2005), such as Ephrins, slits and Nogo-A also can restrict stem cell/transplant survival and migration of stem cells. So far only minimal survival and neuronal differentiation have been reported following endogenous and exogenous stem cell therapies after cerebral ischemia. This creates urgency for more supportive combination therapies where stem cell survival and differentiation are encouraged by growth factors and other supportive molecules in the cerebral microenvironment.

The mechanisms underlying stem cell induced brain plasticity might include angiogenesis (Chen et al., 2005; Wang et al., 2006), local elevation of growth factors (Zhang et al., 2005; Komitova et al., 2006b; Zhang et al., 2006) and synergy between immune cells and stem cells (Aarum et al., 2003; Imitola et al., 2004). Interestingly, brain microglial cells have been shown to promote migration and differentiation of stem cells *in vitro* (Aarum et al., 2003). Taken together, factors that might underlie beneficial effects seen after stem cell therapy are complicated and more studies are needed to unravel these mechanisms.

2.4.2 Transplanted stem cells

Several studies have used stem cells to treat cerebral ischemia in animal models (Chen et al., 2001; Englund et al., 2002; Zhao et al., 2002; Kelly et al., 2004; Kurozumi et al., 2004; Vendrame et al., 2004; Haas et al., 2005; Kurozumi et al., 2005; Bliss et al., 2006; Daadi et al., 2008). Eventhough a great number of studies have been published in recent years most of them do not report behavioral outcome after stem cell therapy. Only few studies show modest improvements in sensorimotor functions. For instance, intravenous injection of MSCs 24 hours after transient MCAO in rats resulted in improved sensorimotor function (Chen et al., 2001; Shen et al., 2007) in tape removal test, neurological severity score (NSS) and rotarod test, which all measure simple sensorimotor functions. Stem cells isolated from HUCB were introduced to rats intravenously (i.v.) 24 hours (Chen et al., 2001; Borlongan et al., 2004; Vendrame et al., 2004) after transient MCAO. Improved behavioral recovery was seen in spontaneous

activity, elevated body swing, step tests, passive avoidance, rotarod and NSS tests. Intracerebral transplantation of neuronal stem cells derived from human teratocarcinoma cell line were shown to improve motor functions in a beam walking test, but not in three other behavioral tests one month after permanent MCAO in rat (Bliss et al., 2006). Transplanted human NPC cells derived from hESCs improved function in a cylinder test after MCAO in rats (Daadi et al., 2008) but showed no benefits in another study when combined with running exercise (Kim et al., 2007).

It is also important to consider the route of administration of stem cells after cerebral ischemia. The most common routes to introduce cells are i.v. and intracerebral administration. Mainly HUCBs and MSCs have been systematically administered and moderate benefits are reported after cerebral ischemia in rodents (Chen et al., 2001; Zhao et al., 2002; Kurozumi et al., 2004; Vendrame et al., 2004; Kurozumi et al., 2005). This method of delivery is the most feasible and most likely to be used in clinical settings eventhough contradicting results exist. The cells can either be trapped in inner organs (Lappalainen et al., 2008) or reach the target areas in brain in very small quantities and provide no benefits in demanding tests of functional recovery (Mäkinen et al., 2006). It is known that only a fraction of HUCB cells introduced i.v. reaches brain parenchyma (Borlongan et al., 2004, Mäkinen et al., 2006). Instead of replacement of cells the mechanisms that lead to better functional outcome via i.v. administration might include cytokines and chemokines produced by HUCB cells that might produce anti-inflammatory effects leading to neuroprotection (Vendrame et al., 2005; Mäkinen et al., 2006).

Intracerebral transplantation of neural precursor cells by several groups (Englund et al., 2002; Kelly et al., 2004; Bliss et al., 2006; Darsalia et al., 2007; Kim et al., 2007; Daadi et al., 2008) also report contradicting outcomes. Functional benefits have been mostly modest after cerebral ischemia, and the survival of cells varies from study to study (3%-40%). Overall, the studies have used various stem cell types, differ in behavioral measures, and report contradicting *post-mortem* analysis of the cells. The variability between the studies makes comparison of the results challenging.

2.5 Clinical studies and prospects to future

The task of treating stroke with stem cell transplantation is clearly challenging and we are only at the starting-point of understanding the true potential of stem cells. Brain function is severely impaired after cerebral ischemia, and replacement of different cell types, supporting tissue, brain structures and functional circuitry only by stem cells might be unrealistic. Thus more effort is needed not only in stem cell biology, but also in animal models of brain injury to combine knowledge to discover the next generation of stem cell treatments taking advantage of intrinsic repair mechanisms of the brain and additional treatments. These mechanisms aiming to better survival of transplanted cells might include creating immune-privileged cell lines, administration of growth factors and immune-restricting agents. Also standardized behavioral outcome and histological measurements of cell survival between laboratories would facilitate comparison of results. In addition, cells should be tested in both genders, at different ages and in multiple species. Other important factors (Table 1) that demand studying are: the best delivery route, the timepoint for transplantation after stroke (i.e. acute versus chronic state) and what cell type to use (e.g. neurons versus neural precursors).

Timing of the transplantation	**Cell types**
- Acute- tissue salvage, advantages of endogenous repair mechanisms, rehabilitation - Chronic- cell replacement, rehabilitation	- Endogenous cell activation - Embryonic stem cells - Precursor cells - Differentiated cells - Genetic modification - Growth factor administration
Site of the transplant	**Cell origin**
- Ipsilateral - Contralateral - Intravenous - Intra-arterial - Intraventricle - Multiple sites vs one transplant	- Allogenic - Autogenic - Xenografting - Tissue type (bone marrow, skin, brain) **Others** - Additional medication - Immunosuppression

Table 1. Factors that can affect the success of transplantation and cell therapy after cerebral ischemia.

The goal is that all these factors have to be feasible in clinical settings, and that the cell lines are accepted by authorities such as Food and Drug Administration (FDA) to be used for treating stroke patients.

3. AIMS OF THE STUDY

This study was conducted to examine intracerebral transplantation of stem cells after cerebral ischemia in rats. We wanted to find out if stem cell transplantation can improve recovery of function after stroke. In addition, enriched housing rehabilitation and running exercise were combined with stem cell transplantation to examine the effects of this combination therapy. Both short-term and long-term studies, and an extensive literature review were conducted to answer the following questions:

1. Do transplanted SVZ cells survive, migrate and differentiate in a rat model of transient MCAO (**I, II**)?

2. Do transplanted hNPC cells survive, migrate and differentiate in a rat model of permanent MCAO (**III**)?

3. Does enriched housing rehabilitation have an effect on transplanted stem cell survival, migration and differentiation (**I, II, III**)?

4. Do cell-based therapies with or without enriched housing enhance functional recovery after cerebral ischemia (**I, II, III, IV**)?

4. MATERIAL AND METHODS

4.1 Subjects

*SVZ stem cells short survival study (**I**).* Eighty-two, male, Sprague-Dawley rats (Charles River, Montreal, QC, Canada) weighing 300-350 g (~3 months old) at the time of middle cerebral artery occlusion (MCAO) were used in this study.

*SVZ protracted survival study (**II**).* We used 113 male Sprague-Dawley rats (Charles River, Montreal, QC, Canada) weighing 320-350 g at the time of MCAO. Rats were maintained on a reverse 12-h light-dark cycle (**I-II**).

*Human neural precursor cells (**III**).* Sixty-five male Wistar rats (National Laboratory Animal Centre, Kuopio, Finland) were used in the study weighing 275-300 g at the time of permanent distal MCAO (Chen et al., 1986).

Animals received food and water *ad libitum*, except when food-deprived for behavior training and testing (16 g per day). The rats were housed in single cages or in environmental enrichment cages.

All procedures were in accordance with the guidelines by the European Community Council directives 86/609/EEC and were approved by the Ethics Committee of Kuopio University, Provincial Government of Kuopio, Canadian Council on Animal Care and Memorial University Animal Care Committee.

4.2 Study designs

The rats were tested in behavior tasks before stroke, 5-6 days after stroke i.e 1 day before stem cell transplantation and at different survival time points. Cerebral ischemia was conducted and within one week rats received intracerebral transplantation of stem cells or vehicle injection. Training for skilled reaching in the staircase test started three weeks before dMCAO surgery (**III***)*. At the end of each study the animals were sacrificed to evaluate the infarct volumes and survival, migration and differentiation of the transplanted stem cells.

4.2.1 Assignment of animals into treatment groups

Behavioral scoring was conducted as follows to ensure that experimental groups exhibited similar deficits after ischemia.

*Studies **I-II***. The behavior in a cylinder test after stroke was scored one day before the transplantation procedure and the score was used to ensure all groups had equal deficits. Animals were randomly divided into the following groups: 1) MCAO + stem cell (SC) + enriched environment (EE); 2) MCAO + SC + standard housing (ST); 3) MCAO + vehicle + EE; 4) MCAO + vehicle + ST; 5) Sham + SC + EE and 6) Sham + SC + ST. Each of the MCAO groups was further divided into 7, 14, 30 days and 2 and 3 months (n=6-8 per group) post-transplant survival time. Sham animals (n=24) survived 30 days or 3 months post-transplant. Rats were also tested at 7, 14 days or 1, 2, or 3 months following cell transplant or vehicle injection, depending on group assignment.

*Study **III***. Animals were tested in post-MCAO limb-placement test and cylinder test (see below). A limb-placement task (Rissanen et al., 2006) was performed to distribute animals with low scores (significantly impaired score 4-6 of maximum 14 points) equally to all treatment groups on days 1 and 6 after stroke. Animals were randomly divided into five treatment groups (n=10-11 per group) 1 day before neural precursor cell transplantation as follows: 1) dMCAO + neural precursor cell transplant (NPC) + EE, 2) dMCAO + NPC + standard housing (ST), 3) dMCAO + vehicle injection (Veh) + EE, 4) dMCAO + Veh + ST, and 5) Sham operated + NPC + EE or ST.

4.3 Enriched rehabilitation

*Studies **I-II***. Starting the day after transplantation of stem cells rats were housed, 6 rats per cage, in large wire mesh cages containing a variety of objects (Fig. 4) to stimulate exploratory behavior (e.g. toys, tubes, beams, shelves, rope, ladder) that were changed and rearranged once a week. In addition, the rats were given free access to individual running wheels attached to a cage for 6 h once per week. Food and water were freely available. The number of revolutions run per session were recorded by a

computerized monitoring system. After 6 h rats were returned to the enrichment cages. The rats in standard cages were housed in pairs of two.

*Study **III***. Enriched environment consisted of two large metal cages (61 × 46 × 46 cm) that were connected by a tunnel. The cages (n=8-9 animals per cage) contained objects that encourage sensorimotor activity (toys, ladders, wooden tubes, tunnels, shelves etc) that were changed once a week. The animals in the standard housing group were housed individually in standard cages (53 × 32.5 × 20 cm).

Figure 4. Enriched environment (EE) cage.

4.4 Cerebral ischemia models

4.4.1 Transient middle cerebral artery occlusion (I-II)

Surgical procedures were performed using aseptic technique. Rats were anesthetized with isoflurane (4% induction; 1.5-2.0% maintenance in 30% O_2 and 70%

N_2O) and placed in a stereotaxic frame. Following a midline scalp incision, a hole was drilled at AP: + 0.9 mm, ML: –5.2 mm, and DV: –8.7 mm relative to Bregma. The vasoconstrictive peptide endothelin-1 (ET-1, 1200 pmol in 3 µl sterile H_2O, Calbiochem, La Jolla, CA, USA) was injected adjacent to the right middle cerebral artery over 10 minutes (Windle et al., 2006). Body temperature was maintained between 36.5 and 37.5ºC throughout surgery using a rectal probe and self-regulating heating blanket (Harvard Apparatus, Holliston, MA, USA). Sham surgery included a burr hole but no injection.

4.4.2 Permanent middle cerebral artery occlusion (III)

Focal cerebral ischemia was induced by permanent occlusion of distal middle cerebral artery (dMCAO) and temporary occlusion (60 min) of both common carotid arteries (Chen et al., 1986). In brief, anaesthesia was induced using 4% isofluorane and maintained in 1.5-2.0% in 30% O_2 and 70% N_2O. An incision was made between the left ear and eye and the distal portion of the MCA was exposed through a small burr hole and cauterized just above the rhinal fissure. Another incision was made on the neck and both common carotid arteries were occluded for the period of 60 min. Rectal temperature was monitored and maintained between 36.5-37.5ºC using a self-regulating heating blanket (Harvard Apparatus, Holliston, MA, USA) for the duration of the surgery. Sham-operated animals received the same surgery except the MCA was not cauterized.

4.5 Stem cell preparation, differentiation and characterization

4.5.1 Subventricular zone stem cells (I-II)

Stem cells were harvested from the SVZ of adult actin-green fluorescent protein (GFP) transgenic mice as described (Enwere et al., 2004). Briefly, brains were removed from mice after cervical dislocation and the SVZ tissue was dissected free in PBS. The SVZ tissue was taken from the region surrounding the lateral ventricles, extending from the rostral tip to the crossing point of both ventricles, taking care to avoid any cortex or

hippocampus. After incubation for 20 min at 37ºC in a defined media-hormone mix containing 1.33 mg/ml trypsin, 0.67mg/ml hyaluronidase and 0.2 mg/ml kynureneic acid (all from Sigma, St. Louis MO, USA), the tissue was then triturated (about 20X), incubated for an additional 15 min at 37ºC and triturated again (about 60X) until no small pieces of tissue were left. An equal volume of trypsin inhibitor (Sigma, St. Louis MO, USA) was added and triturated again (about 40X), and then the suspension was spun down at 600 rpm for 5 min. The cells were resuspended and plated in six-well plates at a density of 1000-2000 cells/ml in media-hormone mix containing 20 ng/ml EGF, 20 ng/ml fibroblast growth factor (FGF) and 2 µg/ml Heparan sulphate (Sigma, St. Louis MO, USA). The multipotent neurospheres consisted of ~60% astrocytes, ~10% neurons, ~10% oligodendrocytes, and remaining undifferentiated cells (Shimazaki et al., 2001). Cultures were left to grow for 9 days then harvested and sent to Memorial University by overnight courier from the University of Calgary. Upon arrival the SVZ cells were placed in a 37ºC incubator overnight, and then concentrated for transplant the following morning.

4.5.2. Human embryonic derived neural precursor cells (III)

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

analyzed in passages 44 and 77. The cultures were mycoplasma free throughout the experiment.

For neural differentiation, the hESC colonies were dissected mechanically into small clusters containing approximately 3000 cells. These clusters were then cultured as floating aggregates for six weeks in 1:1 DMEM/F-12 / Neurobasal media (Gibco/Invitrogen) supplemented with 1×B27 and 1×N2 (Gibco/Invitrogen), 50 U penicillin/ml-50 µg streptomycin/ml (Cambrex Bio Science), 2 mM GlutaMax (Invitrogen), and 20 ng/ml bFGF (R&D systems) in low attachment 12-well plates (Nunc, Thermo Fisher Scientific, Rochester, NY, USA). Throughout the differentiation period of six weeks, the growing neurospheres were dissected once a week and medium was changed three times a week.

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

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

Automated monitoring of in vitro differentiating neuronal cells. After 6 weeks of neural differentiation, some hNPC clusters were *in vitro* differentiated by withdrawal of

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

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

4.6 Stem cell transplantation

*Studies **I-II***. The SVZ cells were gently pelleted, resuspended in 0.25% Trypsin-EDTA for 10 min, washed with DMEM and then resuspended at a concentration of 200 000 cells/µl in DMEM containing 0.5 mg DNase/ml. Using a glass cannula attached to a 30 gauge 5.0 µl Hamilton syringe, 4 X 1.0 µl deposits of this cell suspension were placed in the ipsilateral sensory-motor cortex and striatum immediately above and below the corpus callosum 7 days after stroke (4 deposits = 800 000 cells/rat) at each of the following coordinates relative to bregma: 1) AP +0.7 mm, ML –2.0 mm, DV –2.5 mm and –3.5 mm; 2) AP –0.9 mm, ML –2.5 mm, DV –2.5 mm and –3.5 mm. These placements were selected so as to facilitate migration along the corpus callosum. Vehicle animals received identical injections of DMEM only. All rats received daily intraperitoneal injections (i.p) of the immunosuppressant cyclosporine (SandImmune, 10 mg/kg, Novartis) starting one day prior to the transplant and continuing to the end of the study.

*Study **III***. Prior to transplantation, hESC-derived neural precursor cell clusters were trypsinized (Cambrex) for 5 minutes to produce a single cell suspension. Trypsin was inactivated with 5 % human serum (Sigma-Aldrich) in PBS and the cells were then washed once with PBS before resuspending them into a volume of 20 µl PBS containing 200 000 cells/µl.

Briefly, using a glass cannula attached to a Hamilton syringe, 4 × 1.0 µl deposits of cell suspension were injected in the ipsilateral sensorimotor cortex (the intact sensorimotor cortex on lesion side) 7 days after stroke (4 deposits = 800 000 cells/rat) at each of the following coordinates relative to bregma: 1) AP +0.5 mm, ML +1.0 mm, DV –2.0 mm, and –2.5 mm; 2) AP +1.2 mm, ML +1.0 mm, DV –2.0 mm, and –2.5 mm. Vehicle animals received identical injections of PBS. All rats received one intraperitoneal (i.p.) injection of the immunosuppressant cyclosporine A (SandImmune, 5 mg/kg, Novartis) one day prior to the transplant. Osmotic minipumps (Model 2ML4, Alzet, Cupertino, CA, USA) filled with cyclosporine (LC Laboratories, Woburn, MA, USA) in polyethyleneglycol-400 (PEG-400; Sigma-Aldrich, Steinheim, Germany) were inserted under the skin during the transplantation surgery. The concentration of cyclosporine in

each pump was calculated so that the release of the drug was 5 mg/kg/24 hours. Vehicle animals without transplants received PEG-400 only in osmotic pumps placed under the skin. Body weight was monitored throughout the study. To control that the drug was delivered the remaining liquid was drawn out of the pump at the end of the delivery period.

4.7 Behavioral tests

4.7.1 Forelimb asymmetry

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

4.7.2 Reaching task

Animals were food-deprived 24 hours before initiation of first training or testing session. After that animals received 16 g of food per day when trained or tested, and they maintained about 85% of their pre-testing body weight (pre-operative weight). The staircase apparatus has 21 food pellets (45 mg, BioServ, Frenchtown, NJ, USA) on each of two staircases, divided on seven descending stairs, three pellets on each step. The apparatus (Montoya et al., 1991) is designed so that the pellets on each staircase are accessible to only the ipsilateral forepaw and dropped pellets cannot be retrieved. Animals were trained in a once daily 10 min trial for 16-21 days prior to dMCAO surgery. Pre-surgery criteria required that animals retrieve a minimum of 15 pellets per side on the last eight trials, with a standard deviation of less than ±2 pellets. Baseline score for skilled reaching was calculated as mean score of the last four sessions. Similarly post-surgery performance was based on four trials performed during two consecutive

testing days. After the training and testing periods animals were given free access to food (Windle et al., 2005).

4.7.3 Limb-placement test

The limb-placing test (De Ryck et al., 1989) was used for assigning ischemic animals to groups. This test had seven limb-placing tasks to assess the integration of forelimb and hindlimb responses to tactile and proprioceptive stimulation that are described in detail elsewhere (Rissanen et al., 2006). Briefly, the rat was placed on the edge of a table and its fore- and hindlimbs were pushed over the edge to measure how quickly the rat was able to retrieve his limbs. These tasks were performed from the side and in front of the rat, with and without the help of stimuli provided by whiskers (head lifted in an angle). In addition, forepaw retraction was evaluated when the rat was lifted in the air from its tail, and the forepaw resistance when pushed on the table surface. The tasks were scored as follows: 2 = the rat performed normally; 1 = the rat performed with a delay of more than 2 s; and 0 = the rat did not perform normally. The final score was derived as a sum of all the subtests, a sham-operated animal would reach a maximum of 14 points. Both sides of the body were tested to assess forelimb and hindlimb function.

4.8 Histology and immunohistochemistry

4.8.1 Infarct volumes

*Studies **I-II.*** At 7, 14, 30 days or 2 and 3 months following stem cell transplant or vehicle injection, rats were deeply anesthetized and transcardially perfused with ice-cold heparinized saline followed by 4% paraformaldehyde (PFA). Brains were extracted, fixed in PFA for 90 minutes, and then immersed in 20% sucrose in phosphate buffered saline (PBS) for 3 days. Brains were frozen and 40 µm (**I**) or 30 µm (**II**) coronal sections were taken with a cryostat every 360 µm and stained with Cresyl Violet. Additional consecutive sections were taken and stored in cryoprotectant at -20^{o}C until

immunohistochemistry was performed (see below). The volume of tissue lost was assessed using ImageJ software (Scion Corporation, Frederick, MN):

Tissue loss = Volume of tissue remaining in contralateral hemisphere – volume of tissue remaining in injured hemisphere.

Volume of hemisphere = average area of remaining tissue in hemisphere × section interval × number of sections.

*Study **III.*** Animals were deeply anesthetized with a mixture of sodium pentobarbital (9.72 mg/ml) and chloral hydrate (10 mg/ml) administered by an intraperitoneal injection (2 ml/kg) two months after the transplant or vehicle injection and transcardially perfused with saline followed by 4% PFA. The brains were post-fixed in PFA for 90 minutes and then kept in 20% sucrose in PBS for 3 days, frozen with dry ice and 30 µm tissue sections were cut with a cryostat (CM 3050 S, Leica, Germany). Every eighth section was collected for Nissl staining (thionine) and the remaining sections were stored in a cryoprotectant at -20^{o}C for immunohistochemistry. Every second Nissl stained section (i.e. every sixteenth section) was analyzed with ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health) and the total infarction volume was calculated by subtracting the area of the remaining cortex in the ischemic hemisphere from the area of the intact contralateral cortex of each section. The mean area of tissue damage between sequential two sections was multiplied with the distance of the two sections (480 µm), and these values (mm^2) between all sections were summed and multiplied with the total distance between the first and last section for the final infarct volume (mm^3).

4.8.2 Staining for cell counts

Every eighth (**I, III)** or 12th (**II**) brain section of all animals that received transplants was stained for anti-GFP (**II**) or anti-human nuclei (**III**) and were used for quantification of cell survival. The sections were washed in 3% H_20_2, washed in PBS and blocked in normal goat serum, and then incubated with primary antibody at 4^{o}C with PBS and Triton-X overnight. The following day the sections were washed in PBS, incubated with the secondary antibody for 1 hour at RT, washed in PBS, incubated with 10 µg/ml

Extravadin (Sigma, St Louis, MO, USA) for 1 hour at RT and washed in PBS. Finally, sections were stained with diaminobenzidine (DAB) for 3 min, washed in PBS, mounted and coverslipped. Negative controls were processed for every animal in the same way, except the primary antibody was omitted. For light microscopy, DAB staining was used to label GFP (anti-GFP, 1:1000, Chemicon, Temecula, CA, USA) positive cells (**II),** ED-1 (anti-CD68 for microglia; 1:500, Serotec, Oxford, UK) positive microglia (**II,III**) and human nuclei (anti-HuNu, 1:1000, Chemicon) positive cells (**III**).

4.8.3 Fluorescence immunohistochemistry

Phenotypic characterization of cells was done using immunofluorescence. For immunofluorescence analysis with confocal microscopy the sections were washed in PBS at RT, then blocked in 5% normal goat or rabbit serum for 1 hour (Jackson ImmunoResearch, West Grove, PA, USA) in PBS with Triton-X at RT, and incubated overnight with primary antibodies at 4^{o}C with PBS and Triton-X. The next day the sections were washed in PBS and stained with secondary fluorescence antibodies for 2 hours at RT in the dark, and then washed in PBS, mounted on slides and coverslipped. Coronal sections (from 3-5 rats per group at each survival time) were incubated in the following primary antibodies: chicken anti-green fluorescent protein (GFP, 1:1000, Chemicon, Temecula, CA, USA) for GFP-expressing cells, mouse anti-human nuclei (HuNu, 1:1000, Chemicon), rabbit anti-neurofilament 200 (NF-200 , 1:100, Chemicon), rabbit astrocytic marker anti- S100 (S100, 1:50, Sigma), mouse anti-human nestin (1:200, Chemicon), rabbit anti-microtubule associated protein-2 (MAP-2, 1:200, Chemicon), the astrocytic marker rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, DakoCytomation, Glostrup, Denmark), the neuronal marker mouse anti-neuronal-specific nuclear protein (NeuN, 1:200, Chemicon), the glial progenitor marker NG-2 (NG-2, 1:150, Chemicon), and the neuroblast marker goat anti-doublecortin (DCX, 1:200 Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibodies against GFP, HuNu, NF-200, nestin, S-100, MAP-2, GFAP, NeuN, NG-2 and DCX were detected with secondary antibodies goat anti-chicken/mouse IgG conjugated with Alexa 488, goat anti-rabbit IgG Alexa 633, goat anti-mouse IgG Alexa 633, and rabbit anti-goat IgG Alexa 633,

respectively (all 1:500, Molecular Probes, Eugene, OR, USA). Negative control staining was performed for each animal by omitting the primary antibody.

4.9 Microscopic analysis

4.9.1 Stereological cell counts and cell migration

*Studies **I-II.*** Quantification of stem cell survival and migration was performed on all animals included in the study. Survival of GFP-labeled SVZ cells was measured with the optical fractionator method using Stereo Investigator® (MBF Bioscience, Williston VT, USA). Briefly, the number of GFP-expressing cells in every eighth (**I**) or 12^{th} (**II**) section was calculated using a fluorescence/light microscope (Leica DMRXE) with randomly generated boxes superimposed on the areas of interest where surviving cells were located.

For stem cell migration, the distance from the transplant site (still visible) to the cell cluster nearest the ischemic area was considered to reflect the migratory distance. The maximal distance (μm) that SVZ cells migrated from each transplant site toward the ischemic areas was measured and the mean value calculated. Migration towards the midline of the brain (i.e. away from the injury, other than injury related migration) was considered migration away from the transplantation site.

*Study **(III)**.* Quantification of transplanted cell survival (HuNu positive) was performed on all animals as described above.

4.9.2 Optical densities

*Study **I.*** Doublecortin stained tissue from transplanted animals was examined with fluorescence microscopy. Counting boxes (410 X 260 μm) were randomly superimposed on the two regions of interest (AP + 0.7 mm and AP - 0.9 mm from bregma) in sensorimotor cortex and from homotopic cortical regions from the contralateral hemisphere at 20X magnification. Since DCX stained cells were so numerous in the SVZ the DCX fluorescence was semi-quantitatively determined (i.e. using relative optical

density) using Stereo Investigator from two sections (AP +0.7 mm and AP -0.9 mm from bregma) from both hemispheres. The value for each DCX sample was expressed as a percentage of background fluorescence intensity measured in the corpus callosum.

*Study **II***. ED-1 positive cells in two coronal sections corresponding to the stem cell transplant sites (AP: +0.7 mm and -0.9 mm relative to Bregma). Six boxes (400 μm x 400 μm) were superimposed onto the following areas of interest: 1) transplant site above the corpus callosum, 2) transplant site below the corpus callosum 3) corpus callosum between the transplant site and ischemic injury, 4) cortex bordering ischemic injury, 5) striatum bordering ischemic injury, and 6) contralateral corpus callosum. Optical density (OD; using ImageJ) was determined in each region of interest in all animals that had received stem cell transplants and expressed relative to optical density in the contralateral corpus callosum. Mean values for each region of interest were calculated from the two coronal sections assessed.

4.9.3 Confocal microscopy

A laser scanning confocal microscope (Olympus BX6DWI) was used to identify the phenotypes of the transplanted human nuclei/GFP-expressing cells. Two randomized areas (20X magnification) within the sensorimotor cortex that had GFP (**I-II)** or HuNu (**III**) positive cells were chosen, and at least 50 human/GFP positive cells were analyzed per area. The co-localization of the fluorescent dyes (excitation wavelengths of 488 and 633 nm) was confirmed by z-axis analysis in a series of stacks of 1 μm thick sections using Fluoview FV300 software. The percentage of cells exhibiting co-localization was calculated.

4.10 Statistical Analyses

Studies **I, III**. All values are mean ± standard error of the mean (SEM). Behavioral data were analyzed by ANOVA followed by Fisher's or Scheffe's post-hoc tests to compare differences between treatment groups. Unpaired t-tests were used to

analyze survival, migration and phenotype data of transplanted stem cells as well as endogenous neuroblast density. Differences at the $P<0.05$ were considered significant

*Study **II***. Analyses were performed by experimenters blind to group identity. Data were analyzed with SPSS (v. 14.0) and values are presented as mean ± SEM. Infarct volume was analyzed using a 3-factor (transplant, housing, time) ANOVA. Survival, migration and differentiation of SVZ cells, as well as microglia activity were analyzed with 2-factor (housing, time) ANOVA. Behavioral data were analyzed by one-way ANOVA.

5. RESULTS

5.1 Mortality and exclusions

Animals with minimal injury were excluded if they exhibited a forelimb asymmetry score of more than 40% reliance on the affected forelimb on day 6 after stroke (**I-III**) and score of more than 10 points in limb-placement test (**III),** and no visible infarct in cresyl violet staining (**I-II**). Accordingly, 17 (**I),** 11 (**II)** and 9 (**III**) rats were excluded. Mortality rate was three (**I),** six (**II**) and six (**III**) rats during two days after MCAO. The final number of animals used in the data analysis were n=62 (**I**), n=96 (**II**) and n=51 (**III**).

5.2 Characterization of human neural precursor cells *in vitro*

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

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

Immunocytochemical analysis showed that the vast majority of the *in vitro* differentiated hNPCs expressed neuronal protein MAP-2 and none or very few cells expressed glial protein GFAP. The MAP-2 positive cells that had migrated out of the attached cell clusters expressed doublecortin in the tips of the neurites.

5.3 Infarct volumes

5.3.1 Transient middle cerebral artery occlusion

Endothelin-1 caused significant cortical and striatal damage. Typically the sensory-motor cortex dorsal and ventral to the rhinal fissure and the dorsolateral striatum

were infarcted. Except in the most severely injured rats, forelimb motor cortex was largely spared, as was the medial striatum. For the most part, the transplant sites were localized to areas of intact tissue or minimal injury in cortex and dorsal striatum. There was no difference in infarct volumes over time (i.e. 7, 14, 30 days, 2 and 3 months). There were no significant main effects of cell, housing, or time, and no interactions, indicating that infarct volume did not significantly differ among groups.

5.3.2 Permanent middle cerebral artery occlusion

The permanent dMCAO resulted in cortical infarction in all animals and typically included most of the parietal sensorimotor cortex. There were no significant differences in the infarct volumes between the experimental dMCAO groups, thus no preservation or replacement of tissue was observed due to stem cells transplantation or housing conditions.

5.4 Behavioral deficits and recovery

5.4.1 Forelimb asymmetry

SVZ short survival (I). Prior to stroke all groups performed similarly showing no limb preference ($p>0.05$) in the cylinder test. On day 6 after the stroke (i.e. 1 day before transplant) all ischemic groups (SC+EE, SC+ST, Veh+EE, Veh+ST) showed a marked reduction ($F_{(3, 58)}=16.79$, $p<0.0001$) in use of the contralateral limb compared to the Sham animals. Since the two ischemic vehicle and the Sham stem cell groups did not differ significantly from one another they were combined into single vehicle and Sham groups. There was a significant group effect ($F_{(3, 58)}=4.07$, $p=0.01$) 7 days after stem cell transplantation. Fisher's post-hoc tests revealed that the Veh group ($p=0.006$) continued to show significant reductions in contralateral limb use 7 days after transplantation. In contrast, by day 7 the SC+EE group exhibited marked recovery compared to the Veh group ($p=0.006$) and did not differ from the Sham group. The SC+ST group showed much more limited recovery but did not differ from Sham animals. On subsequent test

days, the SC+ST and Veh groups continued to show decreased reliance on the contralateral limb relative to Sham and SC+EE animals but these differences were not significant.

SVZ protracted survival study (II). All groups performed similarly prior to stroke and used their forelimbs equally while exploring in the cylinder task (overall mean = 49.7±0.7 %; p=0.883). As expected, all ischemic groups were significantly impaired (i.e., relied less on the affected limb, 30.7±1.7% use of affected limb; p=0.001) relative to Sham animals when tested 6 days after stroke (1 day before stem cell transplant or vehicle injection). There were no significant differences among groups at any time after SVZ cell transplantation or vehicle injection, indicating that all ischemic groups had recovered to Sham performance levels, and that enriched housing did not improve functional recovery at 1 month (overall mean = 43.3±2.6%; p=0.180) 2 months (overall mean = 45.8±2.3; p=0.281) or 3 months (46.8±2.6%; p=0.942).

Human NPC cell study (III). Repeated measures ANOVA revealed that in the cylinder task there was an overall group effect ($F_{3,46}$=6.2, p<0.0001) in the use of the contralateral forepaw. ANOVA and post-hoc tests revealed that all ischemic groups were impaired compared to the Sham group six days after dMCAO (p<0.001, i.e. one day prior to hNPC transplantation or vehicle injection). There were no differences between the groups before induction of dMCAO (p>0.05). One month after transplantation or vehicle operation both the Veh+EE (p=0.004) and Veh+ST (p=0.02) groups remained significantly impaired compared to the Sham group. Both neural precursor cell transplantation groups (hNPCs+EE, hNPCs+ST) exhibited recovery of function, and were not significantly different from the Sham group (p>0.05). Two months after the treatment Veh+ST remained significantly impaired compared to the Sham group (p=0.03), whereas the hNPC transplantation groups and the Veh+EE group did not differ significantly from the Sham group (p>0.05).

5.4.2 Reaching task

Human NPC cell study (III). In the staircase test all groups were significantly impaired before the transplantation or vehicle operation compared to the Sham group ($F_{3,46}$=6.2, $p<0.0001$), and maintained similar levels of reaching impairment at one month ($p<0.01$) and two months ($p<0.001$) after the hNPC transplantation or vehicle injection. There were no correlations found between the behavioral data and cell survival rate/differentiation.

5.5 Immunohistochemistry

5.5.1 Cell count and cell migration

SVZ short survival (I). *Cell survival.* There was a trend for greater survival of GFP-expressing transplanted cells in the EE groups than in ST groups at all time-points. There were also more animals in the EE group that displayed greater than 20000 cells surviving than the standard housed animals. There were no statistical differences between the groups with respect to survival time so they were pooled over the time-points (i.e. 7, 14 and 30 days).

SVZ short survival (I). *Cell migration*. The transplanted cells in the EE groups showed a tendency to migrate closer towards the ischemic areas compared to ST groups. At 30 days post-transplant the cells in EE groups reached the infarct site whereas cells in the ST-reared animals migrated much shorter distances. Migration of SVZ cells in the EE pooled group (1682.7±399.2 μm) was significantly greater ($t_{(14)}$ =2.48, $p<0.05$) than the ST-pooled group (398.6±470.1 μm), showing that the EE housing induced migration of the transplanted stem cells.

SVZ protracted survival study (II). *Cell survival.* Enriched housing did not enhance SVZ cell survival compared to rats in the standard housing condition (2-factor ANOVA, main effect of housing, p=0.906). However, there was a significant main effect of survival time (p=0.021). Housing conditions were therefore combined for further

analyses, which revealed that a greater number of transplanted SVZ cells survived at 1 month (15175±3401 cells) compared with 2 months (5250±3401 cells) or 3 months (1671±3301 cells; p=0.036), which were not significantly different (2 vs. 3 months, p>0.05). A trend for a significant housing × survival time interaction suggested that cell survival was enhanced by EE at 1 month. However, this did not reach statistical significance (p=0.086).

SVZ protracted survival study (II). *Cell migration*. A two-factor (housing, survival time) ANOVA was used to analyze SVZ cell migration towards the ischemic area. There was no significant effect of housing, indicating that enriched housing (overall mean = 427.7±158.5 μm) did not enhance the distance cells migrated towards the infarct compared with standard housing (overall mean = 415.1±174.9 μm; p=0.958). Similarly, there was no main effect of survival time (p=0.798) and no housing × survival time interaction (p=0.163). Analysis of the distance SVZ cells migrated towards the midline (i.e., away from the ischemic area) revealed no significant effect of housing (p=0.223). While it was not significant, a trend (p=0.054) for an effect of survival time suggested that SVZ cells migrated the greatest distance within the first month (overall mean = 557.2±135.7 μm), whereas there was little migration at 2 months (overall mean = 178.7±135.7 μm) or 3 months (overall mean = 111.3±131.7 μm). The housing × survival time interaction was not significant (p=0.606).

Human NPC cell study (III). *Cell survival.* Altogether, HuNu positive cells were found in 15 out of 31 animals that received cell transplants, that is, in 5/10 animals in hNPCs+EE, 5/10 in hNPCs+ST, and 5/11 in Sham + hNPCs groups. There were no significant differences in the number of surviving cells between hNPCs+EE (8343±7470), hNPCs+ST (2617±1920) or Sham+hNPC (4587±2566) groups. Survival rate was approximately 1% of the transplanted cells.

Human NPC cell study (III). *Cell migration.* Human nuclei stained cells were located in the sensorimotor cortex, and did not show significant migration away from the transplantation sites.

5.5.2 Optical densities

Doublecortin stained endogenous cells (I). Enriched rehabilitation resulted in a significant increase ($t_{(14)}$=2.48, $p<0.05$) in DCX-expressing endogenous progenitor cells in the ipsilateral SVZ compared to ST housed animals.

Microglial activation (III). Relative optical density measurements revealed ED-1 positive cells (i.e., activated microglia) around the stem cell deposit (OD: 1.43 ± 0.07), the ipsilateral corpus callosum (OD: 1.40 ± 0.08), ipsilateral cortex (OD: 1.15 ± 0.07), and ipsilateral striatum (OD: 1.26 ± 0.08). There were no significant effects of housing or survival time in any of the regions sampled. When all animals were included (e.g., collapsed across housing and survival condition), we found significant negative correlations between transplanted cell survival and activated microglia in the stem cell deposit (R= -0.424, p=0.019) and corpus callosum (R= -0.364, p=0.048), but not in the tissue bordering the ischemic cortex (R= -0.215, p=0.250) or striatum (R= -0.162, p=0.393).

5.5.3 Confocal analyses

SVZ cells (I). During the first month immunofluorescent staining for GFAP revealed similar numbers of co-labeled cells between the EE and ST groups indicating that some of the transplanted cells had an astrocytic phenotype (EE = 10%±1.0, ST = 9.1%±1.0) regardless of treatment. GFAP positive transplanted cells were found in all areas studied, including the transplant site, along migrating cells in corpus callosum and in the cortical areas surrounding the infarct. The migrating cells seemed to be intertwined with the processes of GFAP positive host cells in the corpus callosum. MAP-2 labeled cells were evident but only rarely co-localized with GFP. Transplanted SVZ cells migrating in the corpus callosum and in the region close to the infarct from the EE group showed enhanced staining ($t_{(14)}$=5.15, p=0.0001) for DCX relative to the ST group.

SVZ cells (II). Still after two and three months many (overall mean of 28.8±3.1%) GFP-positive cells were co-localized with GFAP, indicating that a large proportion of the transplanted cells had differentiated into astrocytes, mostly located in corpus callosum. There was no effect of housing ($p=0.262$), but a significant main effect of time ($p=0.013$) indicated that there were more GFAP cells at 2 and 3 months compared to the one month survival time. A smaller proportion of cells (10.5±1.9%) expressed NG-2, a marker for progenitor glial cells that were mainly located in areas close to infarct border. Neuronal markers for neuroblasts (DCX) and neurons (NeuN) were expressed in very few cells (overall mean of 1.7±0.6% and 0.7±0.3%, respectively). The proportion of DCX, NeuN and NG2 cells did not depend on housing condition ($p \geq 0.292$) or survival ($p \geq 0.074$). The phenotype of the remaining ~58% of GFP cells was not determined.

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

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

6. DISCUSSION

6.1 Methodological considerations

6.1.1 Injury models and recovery measurements

We used two different models of middle cerebral artery occlusion, transient occlusion caused by vasoconstriction, and permanent occlusion caused by cauterization of the distal branch of MCAO. These models cause injury either to both the striatum and cortex (ET-1 MCAO) or to the cortex (dMCAO). These injury models result into severe deficits in forelimb function that were measured by cylinder test (**I-III**) and reaching task (**III**).

Reaching task is a task specific test of forelimb reaching ability and the animal cannot compensate for the deficit in the reaching apparatus. Thus this test is a reliable measure of true recovery of function of forelimb. In the cylinder test the animal can use compensatory mechanisms for body support, and thus the true recovery may be masked, especially in later time points after ischemia. This might have been the case in study **II** where no differences were found between treatment groups in later time points. We found modest recovery in the cylinder test using ET-1 model of MCAO only during the first week (**I**) after the cell transplantation, and using dMCAO still one month after transplantation (**III**). After tMCAO the animals in all treatment groups were equal in the cylinder test after one month (**I-II)**. Eventhough tMCAO causes larger infarcts animals did show more spontaneous recovery than after dMCAO in the cylinder test. This might be explained by the variability of the injury size and location in ET-1 induced MCAO model. Cortical infarct caused by dMCAO was consistent in all animals, which enhanced the power of the data. There were no correlations between the amount of cell survival and any of the behavioral test results, thus the recovery of function was most likely not hidden by inter-animal variability of cell survival.

6.1.2 Transplantation sites

We used cortical and striatal (**I-II**) or cortical injections (**III**) of stem cells. According to recent studies both cortical and/or striatal injections have good survival rates, or poor survival rates, and migration has been shown to occur from both sites (Kelly et al., 2004; Kim et al., 2007; Darsalia et al., 2007; Daadi et al., 2008). In studies **I-II** we chose transplantation sites above and below corpus callosum to facilitate migration of the cells. In the study **III** since dMCAO causes strictly cortical injury we chose to transplant cells in the cortex to study cortical regeneration.

6.2 Transplanted cells

6.2.1 Survival of SVZ cells

During the first week after cell transplant ischemic animals exposed to an enriched environment and running exercise showed a tendency for greater survival of SVZ cell transplants (**I**). Unfortunately, transplanted SVZ cells did not survive for protracted periods (**II**). Indeed, less than 1% of transplanted cells survived for 3 months after transplantation. Human neural precursors also showed minimal survival (1 %) after two months in the rat brain (**III**).

The trend for increased survival of stem cells seen in the EE group may have been a result of the rehabilitation experience inducing several factors known to promote cell survival and plasticity. Enriched environment increases dendritic branching and spine numbers, neuron size, and induce neurogenesis, synaptogenesis, and up-regulate several growth factors associated with plasticity such as neurotrophin-3, nerve growth factor (NGF) (and its receptors), and BDNF in the hippocampus, basal forebrain and the cortex (Falkenberg et al., 1992; Torasdotter et al., 1998; Dahlqvist et al., 1999; Pham et al., 1999; Ickes et al., 2000; Biernaskie and Corbett, 2001; Briones et al., 2004; Dobrossy et al., 2004; Gobbo and O'Mara, 2004; Komitova et al., 2005a). Specifically, EE has been shown to restore levels of endogenous SVZ cell populations following MCAO (Komitova et al., 2005a) and to increase proliferation in the contralateral SVZ after

cortical infarction in the adult rat (Komitova et al., 2005b). The effects of voluntary exercise on cell survival are less clear with conflicting data (Komitova et al., 2005b; Redila and Christie, 2006), but a recent review suggests that EE and exercise both benefit neurogenesis by different mechanisms; EE through cell survival and exercise through increased cell proliferation (Olson et al., 2006). Voluntary exercise has also been shown to increase NGF and BDNF (Neeper et al., 1996; Griesbach et al., 2004; Ploughman et al., 2005). This evidence suggests that factors, which are increased by EE and voluntary running, may play an important role in stem cell survival.

Some EE rats exhibited 3-5 times the number of surviving SVZ transplanted cells compared to similarly transplanted rats not exposed to enrichment; however, most of the EE housed rats showed comparable levels of SVZ stem cell survival as standard housed animals. The reasons for the marked variability in stem cell survival in the EE housed animals are presently unclear. One possibility is that the MCAO severity was too great and the resulting inflammatory response was detrimental to stem cell survival. In support of this notion, are results showing that the survival of transplanted stem cells is dependent on the severity of TBI (Shindo et al., 2006). Embryonic mouse stem cells survive transplantation in adult mice following mild traumatic brain injury (TBI) but not after severe TBI (Shindo et al., 2006) and interestingly, mRNA levels of NGF and BDNF are only increased following mild TBI (Shindo et al., 2006). Also, there is a negative correlation between survival of transplanted stem cells and the magnitude of the inflammatory response (Kelly et al., 2004) following focal ischemia in the rat. Thus if the transplant sites in some of the EE animals were more distal to the area of infarction than in other EE animals this could account for the increased stem cell survival. Variation in the number of transplanted cells and the precise location of each deposit site relative to the corpus callosum (the preferred conduit for migration) also likely contribute to the observed variability. Nevertheless, in striking contrast to the EE rats, none of the standard housed rats had transplanted stem cell survival numbers in excess of 25000 cells.

Despite the use of enriched housing and exercise, we confirmed that approximately 3% of transplanted SVZ cells survived for one month, but less than 1% of SVZ cells were present at two and three months. Substantial variability in stem cell survival lasted over protracted time periods, especially in rats exposed to enriched

housing and exercise. We hypothesized that variability in lesion size impacted stem cell placement within the brain and may have contributed to variable cell survival. For example, cells deposited more distal to the injury may have survived. However, in the study **II** we produced a relatively smaller volume of injury (possibly due to variations in batches of endothelin-1 or different surgeons), and therefore the SVZ cells were placed more distal to the injury compared to the previous study. Despite this, transplanted stem cell survival was again variable and not improved. Alternatively, it is possible that the ischemic injury produced an immune response detrimental to stem cell survival (Ekdahl et al., 2003). Indeed, others have reported decreased survival of transplanted cells with increased injury (Kelly et al., 2004). Furthermore, poor survival of mouse ESCs after fluid-percussion injury may be related to the observation that reactive astrocytes, microglia and macrophages surrounded the cells, even when they were transplanted into the undamaged hemisphere (Molcanyi et al., 2007). Thus, we measured activated microglia in the brain at one, two and three months to determine whether a sustained immune response related to poor cell survival. Relative optical density of activated microglia was elevated in all areas of the brain evaluated and persisted for three months. Also survival rate negatively correlated with the optical density of activated microglia in transplantation site and in corpus callosum. Future studies are needed to clarify the role of the immune/inflammatory response on stem cell survival and migration.

Inflammation and immune response after cerebral ischemia act for both good and bad in relation to the damaged tissue in the brain. Brain normally lacks the ability to reject foreign tissue, as it is isolated from the systemic inflammatory system by the blood-brain barrier (BBB). Also the antigen-presenting microglia is thought to lack the ability to travel to lymph nodes to activate the adaptive immune response against foreign material, even though microglia cells are activated after a brain injury to cause a local immune response (Nimmerjahn et al., 2005; McCombe and Read, 2008). The BBB leaks after stroke, and this leads to activation of adaptive immune response, leukocytes from the systemic circuit travel to the brain and produce antibodies against brain antigens, such as myelin basic protein (Bornstein et al., 2001; McCombe and Read, 2008; Becker, 2009). This response is found one day after the stroke onset, and the amount of leukocytes and macrophages peak seven days after stroke (Schroeter et al., 1994). In

addition, increased inflammation appears after stroke due to anti-inflammatory cytokines that are activated by ischemic stroke (Chamorro et al., 2006; McCombe and Read, 2008). However, the increased levels of these anti-inflammatory cytokines can lead to a better outcome after stroke (Schroeter and Jander, 2005). Interestingly, activated microglia and astrocytes can have protective role after brain injury, firstly by aiding by phagocytosis of the debris material, and secondly by secreting supportive molecules to the nervous tissue, such as excitatory amino acid transporters (Beschorner et al., 2007). Importantly, it has been shown that some T-cell types deliver protective cytokines and growth factors to the injury site (Schwartz and Kipnis, 2001; Hendrix and Nitsch, 2007; McCombe and Read, 2008). Thus inflammation may also promote neural repair. Ischemic tissue also releases chemokines that mobilize mesenchymal stem cells (MSC), transplanted stem cells, (Paczkowska et al., 2005; Ziv et al., 2006) and T-cells have been shown to promote endogenous stem cell proliferation in the brain (Imitola et al., 2004).

In this study most likely at the time of the transplantation there was a major immune response in the brain, which was at least partly blocked by the cyclosporine treatment. Thus the survival of the transplanted cells might have been compromised by the inflammation/immune response in the post-ischemic brain tissue. In addition, it is known that the acute rejection of foreign tissue takes place during the first week after the transplantation, which might have taken place in our studies, since the beneficial effect was detected only during the first weeks. As shown in study **II** there might have been a chronic rejection response as well, seen in increased levels of microglia in later time points. Interestingly, endothelins (ET-1, ET-2, ET-3) activate glial cells to produce inflammatory mediators, such as prostaglandins and nitric oxide synthase, in the brain parenchyma (Filipovich et al., 2008). We used ET-1 to induce vasoconstriction, and this might have also led to increased inflammation response. Further studies are needed to specify when it is safe to transplant cells after stroke.

The findings of the studies **II** and **III** highlight the importance of using protracted survival times in stem cell studies as has been recommended by the STAIR report (Stroke Therapy Academic Industry Roundtable, 1999) for evaluating neuroprotective agents. Indeed, whereas short-term (e.g. ≤ 1 month) studies may report good stem cell survival, it

is not known whether cells would go on to die or continue to survive at longer study endpoints.

6.2.2 Survival of hNPCs

The survival of the stem cells grafted after cerebral ischemia has, for the most part, been rather minimal. Kim and co-workers reported a variable graft survival without detailed quantification 3 weeks after hNPC transplantation into basal ganglia after MCAO in rats (Kim et al., 2007). Bühnemann and colleagues reported poor survival of mouse neural stem cells transplanted into the lesion cavity after 3 months in MCAO rats (Bühnemann et al., 2006). It is common not to report quantification of the graft survival, which makes comparison of the studies difficult. Some studies however, show that neural stem/progenitor cells transplanted in striatum or cortex in MCAO models can reach nearly 40% survival rates (Kelly et al., 2004; Bliss et al., 2006; Darsalia et al., 2007; Daadi et al., 2008). Here, we showed that only about 1% of the transplanted hNPC cells survived two months after the transplantation, and that half of the transplanted animals had surviving human cells. The trypsination of hNPC neurospheres used in the present study may have impaired the capacity of the cells to integrate into the tissue, even though they continued to grow normally after replating *in vitro* (data not shown). Indeed, our study is the first one to report detailed stereological quantification of the cell survival after transplantation of human cells into rodent brain combined with detailed behavioral testing.

6.2.3 Migration of transplanted cells

Importantly, EE increased the distance transplanted SVZ cells migrated towards the infarct during the first week (**I**). This is consistent with previous findings showing that EE increases migration of endogenous SVZ cells towards a stroke injury (Komitova et al., 2005a), likely due to peri-infarct up-regulation of factors that direct migration. Atorvastatin treatment following MCAO in adult mice increases, among other neurotrophic factors, BDNF expression in the ischemic border, and also increases SVZ

cell migration towards the injury (Chen et al., 2005). Blocking BDNF with antibody treatment significantly attenuates the atorvastatin-induced migration suggesting that BDNF plays a role in stem cell migration (Chen et al., 2005). Immunological factors also modulate stem cell migration. Stromal cell-derived factor 1α enhances proliferation of neural stem cells and migration toward injury whereas blocking the CXC chemokine receptor 4 prevents migration (Imitola et al., 2004). Cytokine treatment following permanent MCAO in the mouse increases SVZ cell migration as well as bone marrow derived stem cell migration, thus improving functional outcome (Kawada et al., 2006). While chemoattractants were not examined in the present study, it is known that both environmental enrichment and exercise can alter immunological factors (Marashi et al., 2003; Ding et al., 2005) suggesting that this may be one way that EE was able to facilitate cell migration. It was also the case that some of the transplanted stem cells migrated medially, away from the infarct, towards the cingulate cortex. It may be that these cells contributed to neuroplasticity and recovery in the present study because the cingulate cortex, among other areas, has been implicated in recovery after stroke (Stroemer et al., 1995; Kolb et al., 2007).

The greatest stem cell migration occurred during the first month (**I**) after transplantation, and relatively little migration occurred at two and three months (**II**). There were non-significant trends for EE to increase migration and survival one month after transplantation in the study **I**. Interestingly, we found relatively greater migration distances within the first month after SVZ cell transplantation in the first (**I**) study (~1700 vs 700 μm in the EE groups) than in **II** study. The natural tendency of SVZ cells to migrate to the olfactory bulb along the rostral migratory stream changes in response to injury such that cells migrate towards the damaged area (Arvidsson et al., 2002; Goings et al., 2004). It is possible that the size of the injury is important in regulating cell migration, as it is for stem cell survival (Kelly et al., 2004). Therefore, we hypothesize that the larger injury produced, and thus possibly greater amounts of inflammatory chemokines encouraging migration (Kelly et al., 2004) in the previous study is at least partially responsible for the greater distance cells migrated towards the injury. This may be due to increased release of molecules such as growth factors and adhesion molecules that regulate stem cell migration, although this hypothesis has yet to be tested. It is also

possible that greatest migration occurred at the earlier time points (7 and 14 days) included in the pooled data in our study **I**, which were not assessed in study **II**. Finally, although it is possible that we lacked sufficient statistical power to detect significant effects, this is unlikely to be the case as we used larger group sizes compared to our previous work.

The human NPCs did not migrate from the transplantation sites. It is possible that the repulsive signals secreted by the ischemic tissue (Schwab et al., 2005) altered the properties and the survival of the transplanted cells. Moreover, the migration could have been prevented by a glial scar, which has been reported to form around the transplant site in ischemic brain (Kim et al., 2007; Molcanyi et al., 2007) and was also evident in our study.

6.2.4 Differentiation

6.2.4.1 Phenotype of transplanted cells prior to transplantation

*SVZ cells (**I-II**).* In this study the SVZ cells were not analyzed but it is known from previous studies that the multipotent neurospheres consisted of ~60% astrocytes, ~10% neurons, ~10% oligodendrocytes, and remaining undifferentiated cells (Shimazaki et al., 2001).

*hNPC cells (**III**).* The source and quality of stem cells to be grafted is considered a notable challenge when designing transplantation protocols (Kondziolka et al., 2002). We used hESC derived NPCs (**III**) that were characterized using three different methods. The results from RT-PCR, continuous time-lapse imaging, and immunocytochemistry verified that the cell population had been differentiated into neuroectodermal lineage, and the cells were neurons rather than astrocytes. Importantly, cell grafts did not contain any undifferentiated hESCs as confirmed with absence of gene expression in RT-PCR. To our knowledge, this is the first study where the transplanted cells were pre-characterized thoroughly before intracerebral transplantation in an animal model of stroke.

6.2.4.2 Differentiation *in vivo*

*SVZ cells (**I-II**)*. Ultimately the goal of stem cell therapy is to replace cells and circuitry destroyed by injury. GFAP expressing stem cells in the SVZ have been suggested to be the main source of endogenous neuroblasts (Garcia et al., 2004) and these cells appear to migrate in the post-ischemic striatum to form mature neurons by day 90 post-ischemia in mice (Yamashita et al., 2006). In our study the transplanted SVZ cells expressed GFAP and very few neuronal phenotypes, but most cells were of an unknown phenotype. Other short term survival studies using the thymidine analogue 5'-bromo-2'-deoxy-uridine to label mitotic cells after MCAO have identified various types of progenitor cells (e.g. glial-like progenitors), but also do not report significant neuronal replacement (Komitova et al., 2005a; Komitova et al., 2006a). In the present study migrating neuroblasts, positive for DCX, were observed in the corpus callosum radiating towards the peri-infarct region in the cortex. Again, EE (compared to ST housing) increased the number of transplanted SVZ cells expressing DCX (**I**). This was also the case among endogenous progenitor cells in the ipsilateral SVZ which showed increased DCX staining as a result of EE (**I**). Because the adult mammalian SVZ has different sub-types of cells (Zhang et al., 2005) this may explain why the population of transplanted SVZ cells behaves so differently. We observed different types of cells that remained around the deposit site; some formed a type of chain migration in the corpus callosum whereas others formed clusters of cells close to the injury as well as individual cells that resided in the cortex and in the striatum. Overall, animals in the EE group exhibited more migrating cells, individual cells and clusters of cells close to the injury and there were only a few cells that remained in the area of the deposit. In accordance with other studies evaluating cell transplantation or the endogenous stem cell response to injury (e.g., Komitova et al., 2006a; Yamashita et al., 2006), we found very little evidence of neuronal differentiation of SVZ cells. This is despite the fact that transplanted cell phenotype was assessed out to three months, which may be necessary to allow undifferentiated cells to acquire a neuronal phenotype. Instead, a large majority of surviving cells expressed glial proteins such as GFAP or NG-2, and this proportion increased over time. When comparing the proportions of the cell phenotypes in post-mortem analysis to *in vitro*

characterization (~60% astrocytes, ~10% neurons, ~10% oligodendrocytes, and remaining undifferentiated cells; Shimazaki et al., 2001) it can be noted that only glial and undefined stem cells survived. Other studies have similarly found that SVZ progenitor cells express GFAP (Garcia et al., 2004) and differentiate into both non-myelinating NG-2 positive glial precursors and myelinating oligodendrocytes (Menn et al., 2006). The phenotype of a large proportion (~58%) of transplanted SVZ cells remains unknown in this study, and it is possible that these cells are undifferentiated stem cells (Cicchetti et al., 2007).

Human NPCs ***(III).*** Majority of the transplanted hNPC cells had maintained the undifferentiated neural precursor phenotype seen as nestin expression. The deposit had numerous neurofilaments, and some of them overlapped with HuNu stained nuclei. Approximately 10% of the surviving cells expressed neuronal marker MAP-2 two months after the transplantation whereas only a minority of cells expressed the astrocytic markers GFAP, S100 or oligodendrocyte precursor NG-2. Our study confirms however, that after sufficient differentiation hESC derived neural cells maintain their neuronal characteristics both *in vitro* and *in vivo.* A substantial number of the surviving cells expressed doublecortin, a marker for migrating neural cells. So far, only minimal survival and neuronal differentiation have been reported following endogenous and exogenous stem cell therapies after cerebral ischemia (Lindvall et al., 2004; Kozlowska et al., 2007), which suggests that it is imperative to study in detail the factors that hinder transplant survival and stem cell migration.

6.3 Functional recovery

6.3.1 Enriched environment

We wanted to examine the effects of enriched housing rehabilitation on transplanted cells, and the effects of the combination therapy on functional recovery after MCAO in rats. Interestingly, EE has been proven to upregulate endogenous plasticity related functions such as growth factors in brain, increased dendritic branching and enhanced neurogenesis (Dahlqvist et al., 1999; Biernaskie and Corbett, 2001; Komitova

et al., 2005a). Importantly, enriched environment has also positive effects on endogenous (Komitova et al., 2005a) SVZ stem cells. However, in our study, housing animals in EE had no effect on cell survival, migration, or differentiation and functional recovery in long term (**II**). The combination of SVZ cell and EE resulted in better functional recovery only during the first few weeks (**I**). Interestingly though, the vehicle group housed in EE showed a delayed modest recovery in forelimb asymmetry (**III**) during the second month but not during the first month, which suggests that hNPCs might have facilitated recovery during the first month.

6.3.2 Partial functional recovery in the cylinder test

We found that the combination of SVZ cell transplantation and EE combined with voluntary wheel running facilitated recovery of the impaired forelimb in the cylinder task. The greatest benefit was seen 7 days post transplantation and therefore was unlikely due to integration of new cells. Similarly, EE has been shown to increase endogenous SVZ cell migration towards an ischemic injury and facilitate rotarod function at an early time point (Komitova et al., 2005a). Although the majority of transplanted SVZ stem cells examined in the present study didn't differentiate into mature neurons, these undifferentiated cells may have contributed to brain plasticity and better functional outcome by secreting growth factors or by enhancing angiogenesis in the region of the infarct. For example, culture medium taken from embryonic mouse stem cells contains several cell growth and survival factors that can stimulate the growth and proliferation of other stem cells (Zhang et al., 2006). Following injury, areas surrounding the lesion often form new connections with other cortical regions as an attempt to restore function (Carmichael et al., 2001; Dancause et al., 2005). The higher number of stem cells found in this region in the EE animals could explain the improved performance in the cylinder task if these cells are supporting the plasticity process although further investigation is required to confirm this. The improved forelimb use in the EE animals was not due to differences in infarct volume since infarct volumes were comparable among the ischemic groups. While the recovery noted here was rather modest it is likely due to the considerable variation in stem cell survival. In order to achieve more complete restoration

of function it will be necessary to attain greater stem cell survival and migration perhaps through augmentation with exogenous growth factors or other chemicals, such as erythropoietin (Tsai et al., 2006; Wang et al., 2006). Indeed, this approach has been successful in increasing mobilization of endogenous SVZ cells to the injury zone and partially restoring sensory-motor function after motor cortex injury (Kolb et al., 2007). According to Kolb et al., the combination of EGF and erythropoietin resulted in regeneration of cortical tissue in the infarcted area and subsequent removal of this newly formed tissue reinstated behavioral deficits. Also, more complete behavioral recovery may require that more cells differentiate into mature neurons to repopulate the injury zone. The most important clinical endpoint for any therapy is improved recovery of function. Thus, it is important that stem cell transplantation studies demonstrate behavioral improvement prior to clinical testing of therapies. Several studies, including present work (**I,II**), report modest reduced behavioral deficits following stem cell infusion either into the brain or into the blood stream (Chen et al., 2001; Zhao et al., 2002; Haas et al., 2005; Bliss et al. 2006). In our review paper (**IV**) we show that even though majority of studies conducted that use cell-based therapy combined with behavior testing do report improvement in functional recovery, the positive results arise from fairly simple sensorimotor tests. In addition, minority of the studies lasted longer than one month, so it is unclear whether or not the positive effects were long-lasting. We found significant improvement in forelimb use asymmetry in the cylinder one week after stem cell transplantation (**I**), but not at later times (**II**). We did not detect differences among groups in the cylinder task at 1, 2 or 3 months following transplantation. The most likely explanation is that complete spontaneous recovery and compensation had occurred by one month regardless of treatment condition, and we were therefore unable to detect deficits or treatment effects. Thus, the use of alternate or additional tests more sensitive to long-term deficits in this model, such as the staircase reaching task (Biernaskie and Corbett, 2001) would have been more useful. It is also possible that our treatments provided early or transient benefit that we did not detect at longer survival times. Our findings highlight the importance of choosing tests sensitive to injury as well as treatment effects in a particular model of brain injury.

Neural cell transplanted animals exhibited (**III**) limited recovery in the forelimb asymmetry task, but no recovery was observed in reaching ability measured in the staircase task. Forelimb asymmetry test measures the ability to use the impaired forepaw for postural support during vertical exploration (Schallert et al., 2000). This behavior tends to involve a wider range of motor circuits compared to reaching abilities. Postural support includes a variety of cortico-striatal and corticospinal pathways (Kandell et al., 2000a). Neuronal plasticity can involve wider cortical areas (Kandell et al., 2000b) from these pathways, thus it may be possible that instead of structural recovery some other compensatory mechanisms are responsible for functional recovery seen here. Forelimb reaching ability is strictly orchestrated by specific cortical motor representations in brain (Ramanathan et al., 2006), and fine motor manipulation in the distal limb like grasping and opening of the paw (Biernaskie et al., 2005; Clarke et al., 2007).

The transplanted hNPC cells might have had an impact on local cortical microenvironment during post-ischemic cortical re-organization (Carmichael et al., 2005a; Carmichael, 2006) that takes place within first few weeks when the brain is "wired" to gain the most benefits of the rehabilitation experiences (Biernaskie et al., 2004) after stroke. Neural cells might have contributed to faster recovery by increasing growth factors in areas adjacent to transplant sites. Even though the levels of growth factors were not measured in our study, it is known that embryonic mouse stem cells secrete several cell growth and survival factors that can stimulate the growth and proliferation of other stem cells (Zhang et al., 2006). In addition, neural stem cells (NSCs) support, when transplanted in the cerebellum of mouse with Purkinje cell deficits, mitochondrial function, dendritic growth, and synaptogenesis leading to the rescue of host cells and restoration of motor coordination (Li et al., 2006). Also secretory products from NSCs can correct a prototypical genetic metabolic defect in neurons and glia *in vitro* (Flax et al., 1998). Interestingly, when human NSCs are transplanted in Parkinsonian monkey brain, they support host neurons not by replacement but by promoting homeostatic adjustment of host dopamine neurons, such as normalizing neuron size (Redmond et al., 2007). It remains to be elucidated in future studies if such supportive mechanisms are responsible for benefits seen after NPC transplantation in animal models of stroke.

The recovery was restricted to only the cylinder task (**I**,**III**) and not to the staircase reaching task. In previous studies recovery of forelimb reaching required task-specific rehabilitation after cerebral ischemia (Biernaskie and Corbett, 2001) and after motor cortex injury (Ramanathan et al., 2006) and that was not provided to animals in our study. Future studies will confirm if stem cell transplantation combined with task-specific rehabilitation may result in improved reaching abilities.

6.4 Future aspects for stem cell studies in experimental stroke research

In order to be effective, stem cell therapies for neurological disease must be optimized such that transplanted cells survive for long periods, integrate into the host circuitry, and promote behavioral recovery. The results in the review paper (**IV**) show that half of the published studies using cell-based therapies and behavioral outcomes after MCAO did not report cell survival quantification. Unfortunately, the studies reporting cell survival clearly show that the vast majority of transplanted stem cells die soon after transplantation and cell survival is variable (Kelly et al., 2004; Lindvall et al., 2004; Bliss et al., 2006; Kozlowska et al., 2007; Molcanyi et al., 2007). For instance, we found that only about 3% of SVZ cells survived for one month after transplantation into ischemic brain tissue (**I**). Furthermore, there was considerable variability in the survival rate among animals. Clearly, more work is needed to identify factors responsible for regulating stem cell migration, survival, and differentiation. In addition, EE must be further optimized in order to significantly and consistently promote stem cell survival and migration. Accordingly, it appears that functional benefit obtained in stem cell studies, including our own (**I, III**), generally cannot be attributed to neuronal replacement. Alternatively, behavioral recovery may be due in part to release of growth factors (e.g., by glial cells). It is clear that further research is needed to provide a better understanding of the role of glial cells in promoting recovery, and how this can be improved such that long-term functional benefit is achieved.

The post-ischemic brain represents a hostile environment for transplanted cells with massive cell death triggering a pronounced and persistent inflammatory response. In order to increase the therapeutic potential of transplanted or endogenous stem cells it will

be necessary to develop strategies to create a climate more supportive of stem cell survival. Our results suggest that environmental factors such as rehabilitation are one way to render the cerebral microenvironment more conducive to cell transplant survival and migration.

The sustained immune response in the brain activated both by the transplant and the existing brain injury hinder transplant survival. Mouse NPCs survived better when transplanted in the brain of immune deficient mice than in immune competent mice (Kim et al., 2006). After fluid-percussion injury intracerebrally transplanted murine ESCs were surrounded by reactive astrocytes, microglia and macrophages, and only few stem cells survived seven weeks after transplantation even when transplanted into the contralateral hemisphere (Molcanyi et al., 2007). Also very poor survival was reported one month after transplantation of HUCB derived stem cells after cortical stroke (Kozlowska et al., 2007) most likely due to severe host reaction. Commonly used immunosuppressant cyclosporine A restricts the immune response and facilitates transplant survival, even though it seems that a shorter immunosuppression seems to be as effective as prolonged administration (Wennersten et al., 2006) suggesting that transplanted cells are rejected in the longterm studies despite the administration of cyclosporine. The limited graft survival creates urgency for more supportive combination therapies combined possibly with immune response restricting agents.

According to Bühnemann and colleagues rodent embryonic neural progenitor cells transplanted into the lesional cavity survive to some degree, differentiate and also fire action potentials (Bühnemann et al., 2006) after MCAO in rats. This study is very promising however more detailed studies should be conducted with behavioral testing by groups reporting such encouraging results. In addition, long-term behavioral testing should be conducted in groups that report good survival and migration of transplanted neural cells (Darsalia et al., 2007) as suggested by STAIR report (Stroke Therapy Academic Industry Roundtable (STAIR), 1999) and STEPS initiative (Borlongan et al., 2008; STEPS, 2009).

7. SUMMARY AND CONCLUSIONS

These studies were conducted to reveal the effects of intracerebral transplantation of stem cells, and in general the effects of cell-based therapies, on functional recovery in animal models of cerebral ischemia. Behavioral measurements were used to quantify functional recovery after cerebral ischemia and stem cell transplantation. In addition, the effects of rehabilitation on survival, migration and differentiation of transplanted stem cells were studied.

The main conclusions are:

1. Longterm studies showed that only minimal amount of transplanted SVZ stem cells survive and that they differentiate into glial cells **(I-II)**.

2. Transplanted human neural precursor cells derived from embryonic stem cells had only minimal effect on functional recovery after cortical stroke. The transplanted cells had poor survival two months after transplantation, but some of the remaining cells expressed neuronal proteins. Majority of the cells had remained undifferentiated neural precursor cells (**III**).

3. Enriched environment rehabilitation combined with running exercise enhances transplanted SVZ stem cell migration and functional recovery during the first few weeks after cerebral ischemia (**I**). Enriched environment had no significant effect on survival, migration, differentiation of transplanted cells, or on functional recovery after the first month (**II, III**).

4. Cell-based therapies lead to a modest improvement of function in animal models of cerebral ischemia (**I, III, IV**). It appears that there are several factors that work in concert in the post-ischemic brain to attract and support progenitor/stem cell survival and migration. Our data indicate that one important factor that can augment the restorative effects of stem cell transplants may be the activation of endogenous

neuroplasticity mechanisms induced by rehabilitation-like experience such as enriched environment (**I**).

Taken together, this study and several others according to the recent literature (**IV**) show that transplanted stem cells support endogenous brain repair mechanisms during the first weeks after the ischemic insult and lead to modest improvement of function. However, a novel finding in this study was that enriched environment rehabilitation has an effect on stem cell migration that leads to a better functional outcome during the first weeks after cerebral ischemia. Unfortunately, the cells are rejected in longterm studies after intracerebral transplantation even with continuous immunosuppressant drug administration, and this seems to be related to microglia expression at the site of the transplants. It is recommended that studies using measures of functional recovery after cell-based therapies in animal models of cerebral ischemia should use protracted survival times and multiple appropriate behavioral tests.

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ORIGINAL PUBLICATIONS I-IV

I

Enriched environment enhances SVZ stem cell survival, migration and functional recovery after stroke

Hicks AU, Hewlett K, Windle V, Chernenko G, Ploughman M, Jolkkonen J, Weiss S, Corbett D.

Neuroscience 2007;146:31-40.

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II

Long-term assessment of enriched housing and subventricular zone cell transplantation after focal ischemia in rats

Hicks AU, MacLellan CL, Chernenko GA, Corbett DR.

Brain Research 2008;1231:103-112.

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III

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

Hicks AU, Lappalainen R, Narkilahti S, Suuronen R, Corbett D, Sivenius J, Hovatta O, Jolkkonen J.

European Journal of Neuroscience 2009;29:562-574.

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IV

Cell based therapies in animal models of stroke - Focus on functional recovery

Hicks AU, Schallert T, Jolkkonen J.

Submitted

PUBLICATIONS
SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

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neuropsychological study with special reference to Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis.

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