NEURAL STEM/PROGENITOR CELLS IN THE POST-ISCHEMIC ENVIRONMENT

Proliferation, Differentiation and Neuroprotection

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Cover illustration: (Top left) Brightfield microphotograph of neural stem/progenitor cells *in vitro*. (Top right) Immunofluorescent microphotograph of cultured neural stem/progenitor cells expressing the astrocytic protein glial fibrillary acidic protein (red). Cell nuclei were visualised with Hoechst 33258 (blue). (Bottom left) Microphotograph showing a hippocampal slice culture after NMDA-exposure. The slice was stained with a marker of cell death, propidium iodide (red). (Bottom right) Microphotograph of a coronal section of a rat brain after focal ischemia (occlusion of the middle cerebral artery). The section was stained with 2,3,5-triphenyl-2H-tetrazolium chloride, a marker of non-damaged tissue (grey).

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NEURAL STEM/PROGENITOR CELLS IN THE POST-ISCHEMIC ENVIRONMENT: Proliferation, Differentiation and Neuroprotection

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Abstract
Stroke is one of the leading causes of chronic disability and death in the Western world. Today, no treatment can repair the cellular loss associated with an ischemic lesion. However, the discovery and dynamic regulation of neural stem/progenitor cells in the adult mammalian brain has resulted in exciting possibilities for future therapeutic interventions. Endogenous or grafted neural stem/progenitor cells are activated following an ischemic insult. These cells undergo directed migration towards infarcted areas, and differentiate in response to the insult. Unfortunately, the results of this regenerative effort are limited compared to the amount of tissue loss. This could be due to low survival of the recruited cells, but could also be explained by insufficient activation or dysfunctional lineage selection. Whether the lineage selection of neural stem/progenitor cells is altered following a lesion in the brain, what signals that are responsible for their activation or whether these cells can participate in post-lesion regeneration, astrogliosis or neuroprotection have yet to become clear. A greater understanding of these processes is necessary for finding ways to improve the endogenous regenerative capacity.
We found that reactive astrocytes, a prominent part of the post-ischemic environment, induced astroglial differentiation of adult neural stem/progenitor cells *in vitro*. Moreover, astrocytes derived from these cells were shown to participate in glial scar formation *in vitro*.
After studying gene expression in the peri-infarct region following focal ischemia, the expression of several genes was induced. We chose to focus our attention on one of these genes and its product, thyrotropin-releasing hormone (TRH). Immunoreactivity for TRH was found in several areas in both lesioned and intact brain regions, including in microglia present in the areas surrounding the lesion. Furthermore, TRH receptors were expressed on cultured neural stem/progenitor cells and TRH potently induced the proliferation of these cells. TRH is an interesting target for stroke treatment, but it also has many central effects in the brain and systemic administration may prove problematic. An interesting protocol for local delivery of TRH would be by grafting stem/progenitor cells, genetically engineered to secrete the peptide. In order to create a foundation for neuroprotective gene therapy, we developed efficient methods for non-viral transfection of neural stem/progenitor cells.
Since neural stem/progenitor cells migrate towards the ischemic area we wanted to investigate whether these cells secreted factors that could protect neurons against excitotoxicity, the main inducer of cell death following a stroke. Mass spectrometric analysis of factors secreted from cultured neural stem/progenitor cells led to the identification of a novel neuroprotective peptide, which we termed pentinin. This peptide potently reduced excitotoxicity in both mature and immature neurons in an *ex vivo* hippocampal slice model.
The results presented in this thesis show that the proliferation and differentiation of neural stem/progenitor cells can be dramatically affected by factors in the post-ischemic environment. Furthermore, the results suggest that neural stem/progenitor cells can participate in both glial scar formation and neuroprotection after an ischemic lesion. Finally, a novel neuroprotective peptide was identified. This peptide may be important for the protection of endogenous cells following insults in the brain and may represent an effective novel target for the treatment of stroke.

Keywords: Neural stem cells, neural progenitor cells, stroke, ischemia, reactive astrocytes, astrogliosis, proliferation, differentiation, neurogenesis, excitotoxicity, transfection, neuroprotection

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Idag finns det ingen behandling som kan reparera den cellförlust som t ex en stroke (blodpropp eller blödning) orsakar i hjärnan. Stroke är den ledande orsaken till bestående handikapp samt den tredje vanligaste dödsorsaken i västvärlden och mycket ansträngningar har lagts på att hitta nya behandlingsmetoder. En intressant möjlighet för framtida behandling innefattar aktivering av neurala stam/progenitor celler (NSPC). Dessa omogna celler finns i specifika områden i den vuxna hjärnan och definieras av att de kan byta skepnad och bli till nervceller eller stödjeceller. Det är känt att NSPC aktiveras efter en syrebristskada i hjärnan och vandrar mot skadeområdet. Det är dock inte känt hur dessa celler påverkas efter en stroke eller om NSPC är inblandade i återhämtningen efter den här typen av skada. Mitt avhandlingsarbete har syftat till att förstå mer om de här processerna och har belyst olika interaktioner mellan NSPC och viktiga faktorer i miljön i vävnaden omkring en syrebristskada i hjärnan. Ökad förståelse inom detta område skulle förhoppningsvis kunna användas till att förbättra hjärnans förmåga att återhämta sig efter en skada.

Astrocyter är den vanligaste celltypen i hjärnan och räknas som den viktigaste stödjecellen i centrala nervsystemet. Utöver sin funktion som stöd för nervceller har astrocyter en mycket viktig roll vid en skada i hjärnan då de aktiveras och skapar en avgränsning mellan frisk och sjuk vävnad. Tack vare bildningen av detta astrocyt-ärr minskas spridningen av skadan. Aktiverade astrocyter finns i stort antal i de områden dit NSPC vandrar efter en stroke. Samspelet mellan dessa två celltyper har emellertid inte undersökts tidigare. För att kunna studera detta samspel utan inblandning av andra faktorer använde vi ett modellsystem baserat på cellodling. Våra resultat visar att aktiverade astrocyter utsöndrar faktorer som påverkar NSPC att mogna till astrocyter samt att dessa nybildade astrocyter kan delta i bildningen av astrocyt-ärret.

För att få ännu mer kunskap om området dit NSPC rekryteras undersökte vi hur 1200 gener påverkas efter en experimentellt inducerad stroke i vuxna råttor. I detta arbete identifierades en rad intressanta faktorer. Vi var särskilt intresserade av utsöndrade faktorer och såg att en intressant peptid, thyrotropin-releasing hormone (TRH), var uppreglerad efter stroke. Detta fynd följdes upp och vi upptäckte att TRH, på ett kraftfullt sätt, stimulerade celldelning i NSPC. Det är möjligt att TRH har betydelse för aktiveringen av NSPC efter stroke. Då TRH tidigare har visat sig kunna skydda nervceller vid skada gör kombinationen med effekter på regeneration peptiden mycket intressant för behandling av skador i hjärnan. Tyvärr ger TRH många centrala biverkningar och vi utvecklade därför en ny administreringsmetod för den här typen av substanser. Denna metod baserar sig på icke-viral transfektion av NSPC och lämpar sig för lokal administrering av TRH.

En viktig roll för NSPC efter stroke skulle kunna vara produktion och utsöndring av skyddande faktorer med syfte att öka överlevnaden hos skadade celler. Vi undersökte därför om NSPC utsöndrar faktorer som skyddar mot excitotoxicitet, den viktigaste mediatorn för celldöd vid stroke, och fann att så var fallet. Vidare lyckades vi identifiera en ny peptid, vilken vi döpte till pentinin, som uppvisade potenta nervcells-skyddande egenskaper.

Sammanfattningsvis har vi visat att linjevalet hos NSPC (mognad eller celldelning) påverkas av viktiga faktorer i miljön omkring en stroke. Dessutom visar vi helt nya funktioner hos två peptider, TRH och pentinin. För att kunna använda den här typen av peptider i kombination med NSPC i experimentell behandling av stroke utvecklade vi en effektiv metod för icke-viral transfektion av dessa celler. Avslutningsvis anser vi att både TRH och pentinin är intressanta kandidater för utveckling av nya behandlingsstrategier för patienter med stroke.

PAPERS INCLUDED IN THE THESIS

Paper I. Faijerson J., Tinsley R.B., Apricó K., Thorsell A., Nodin C., Nilsson M., Blomstrand F., and Eriksson P.S.
Reactive astrogliosis induces astrocytic differentiation of adult neural stem/progenitor cells *in vitro*.
Journal of Neuroscience Research (2006) 84:1415-1424.

Paper II. Faijerson J., Anderson M.F., Apricó K., Nilsson M., Eriksson P.S. and Komitova M.
Gene expression profiling in the perifocal neocortex after experimental stroke in rats: TRH up-regulation and effects on adult neural stem/progenitor cells.
In manuscript.

Paper III. Tinsley R.B.*, Faijerson J.* and Eriksson P.S.
Efficient non-viral transfection of adult neural stem/progenitor cells, without affecting viability, proliferation or differentiation.
Journal of Gene Medicine (2006) 8:72-81.

Paper IV. Faijerson J.*, Tinsley R.B.*, Thorsell A., Strandberg J., Hanse E., Sandberg M. and Eriksson P.S.
Adult neural stem/progenitor cells reduce excitotoxicity via pentinin, a novel neuroprotective peptide.
In manuscript.

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ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
Aβ	amyloid β-protein
AD	Alzheimer's disease
AHPs	adult hippocampal stem/progenitor cells
AIF	apoptosis-inducing factor
AMPA	α-amino-3-hydroxy-5-methylisoxazole -4-propionic acid
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cDNA	complementary DNA
CA	cornu ammonis
CM	conditioned medium
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cAMP-responsive element binding protein
D-AP5	D-2-amino-5-phosphono-pentanoate
DCX	doublecortin
DIV	days *in vitro*
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EPSCs	excitatory postsynaptic currents
FACS	fluorescence activated cell sorter
FDG	fluorescein-β-D-galactopyranoside
FITC	fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCL	granule cell layer
G-CSF	granulocyte-colony stimulating factor
GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GS	glutamine synthetase
IDE	insulin degrading enzyme
IGF-1	insulin-like growth factor 1
IRs	insulin receptors
LDH	lactate dehydrogenase
LIF	leukaemia inhibitory factor
MALDI	matrix-assisted laser desorption/ionisation
MAP2ab	microtubule-associated protein 2 (a+b)
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion

MHC	major histocompatibility complex
mRNA	messenger RNA
MS	mass spectrometry
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NMDAR	NMDA receptor
NSPCs	neural stem/progenitor cells
OB	olfactory bulb
OHSCs	organotypic hippocampal slice cultures
PARP-1	poly(ADP-ribose) polymerase-1
PBS	phosphate-buffered saline
pDNA	plasmid DNA
pHH3	phosphorylated histone H3
PI	propidium iodide
Q-PCR	quantitative real-time polymerase chain reaction
RMS	rostral migratory stream
RT	room temperature
SDF-1α	stromal cell-derived factor 1α
SDS	sodium dodecyl sulphate
SGZ	subgranular zone
SVZ	subventricular zone
TGF-β1	transforming growth factor β1
TRH	thyrotropin-releasing hormone
tPA	tissue plasminogen activator
TRH-R1	TRH receptor 1
TRH-R2	TRH receptor 2
VEGF	vascular endothelial growth factor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

BACKGROUND

Brain ischemia

Stroke is defined by the World Health Organisation as "a focal (or at times global) neurological impairment of sudden onset, and lasting more than 24 hours (or leading to death) and of presumed vascular origin". Based on patophysiology, three types of stroke exist: ischemic stroke from a vascular occlusion (approximately 80%), primary intracerebral haemorrhage (approximately 15%) and subarachnoid haemorrhage (approximately 5%)[1]. Ischemic stroke is caused by either thrombosis or embolism. Thrombosis is the formation of a blood clot (thrombus) inside a blood vessel, leading to an obstruction of blood flow. Embolism occurs when an embolus is transported through the circulation, eventually resulting in the occlusion of a blood vessel in another part of the body.

In addition to stroke, there are other pathological conditions that can cause cerebral ischemia. These include e.g. cardiac arrest[2] and complications during surgery[3].

Stroke is the third most common cause of death worldwide after ischemic heart disease and cancer[1]. It is also a major cause of permanent disability and stroke management is associated with a vast economic burden[4]. Due to an increase in the proportion of elderly people and the future effects of smoking patterns in less developed countries, stroke mortality is estimated to double by the year 2020[1].

Following a stroke, patients often suffer from impairments of motor functions and sensory functions of the body contralateral to the site of lesion. Other common symptoms include speech disturbances, perception disorders and cognitive disturbances. Most patients partially recover after a stroke, but complete recovery is seldom achieved.

Today, the only specific treatment for stroke patients is thrombolysis with recombinant tissue plasminogen activator (tPA). However, this treatment can only be used in a small fraction of patients. Despite intensive research, there is no treatment paradigm that can reduce the cellular loss associated with an ischemic lesion and all clinical trials of neuroprotective drugs for the acute treatment of stroke have been unsuccessful. Therefore, the interest in new aspects of recovery, including lesion-induced neural plasticity and regeneration has increased.

Experimental models

Animal models of ischemia are important for studying pathophysiology and endogenous recovery as well as for evaluating the efficacy of new therapeutic agents. The anatomy of the cerebral vasculature is very similar in rodents and higher species including humans. Therefore, most experiments on cerebral ischemia have been performed in rodents. Furthermore, many aspects of pathophysiology and neuroprotection can be studied using cultured cells and tissue slices. In this thesis, different cell and slice culture systems have been employed to investigate important interactions between neural stem/progenitor cells and factors in the post-ischemic environment.

The most common animal models of ischemia can be divided into two types: focal and global ischemia. Focal ischemia primarily produces lesions in striatal and cortical regions, whereas global ischemia primarily affects the hippocampus. However, neuronal damage can also be observed in the cortex and striatum following global ischemia[5].

Focal ischemia

Unilateral occlusion of the middle cerebral artery (MCA) has been associated with up to 80% of ischemic stroke in humans[6-8]. Occlusion of the MCA is therefore considered to be one of the most clinically relevant models of ischemia. The MCA can be occluded, either in proximal or distal parts, by different means including filament insertion, ligation and electrocoagulation. The size and distribution of the infarct volume is affected by the site and duration of the occlusion[9,10]. Distal occlusion of the MCA using the intraluminar suture model is one of the most commonly used experimental models of stroke and was employed in this thesis in the experiments designed to characterise gene expression in the peri-infarct environment. The MCA can in this model be occluded either transiently or permanently. A shorter occlusion time (30-60 minutes) leads predominantly to infarction in the striatum, while a longer occlusion time also involves the adjacent frontal, occipital and parietal cortex.

Pathophysiology

The brain is an organ that has high demands for oxygen and glucose. This makes the brain very sensitive to reduced perfusion. An acute interruption of blood flow during stroke results in rapid energy depletion since both oxygen and glucose are required for the production of ATP. The infarct core extends from the site of the lesion and is defined by low perfusion and high levels of cell death. This region is surrounded by the penumbra, in which some residual blood supply is present due to collateral circulation. Cells in the infarct core are generally considered to be beyond rescue, while many of the cells in the penumbra region can be salvaged if appropriate reperfusion occurs[11,12]. The energy depletion that occurs after an ischemic lesion initiates a cascade of pathophysiological events, including excitotoxicity, peri-infarct depolarisations, inflammation and apoptosis (Fig. 1).

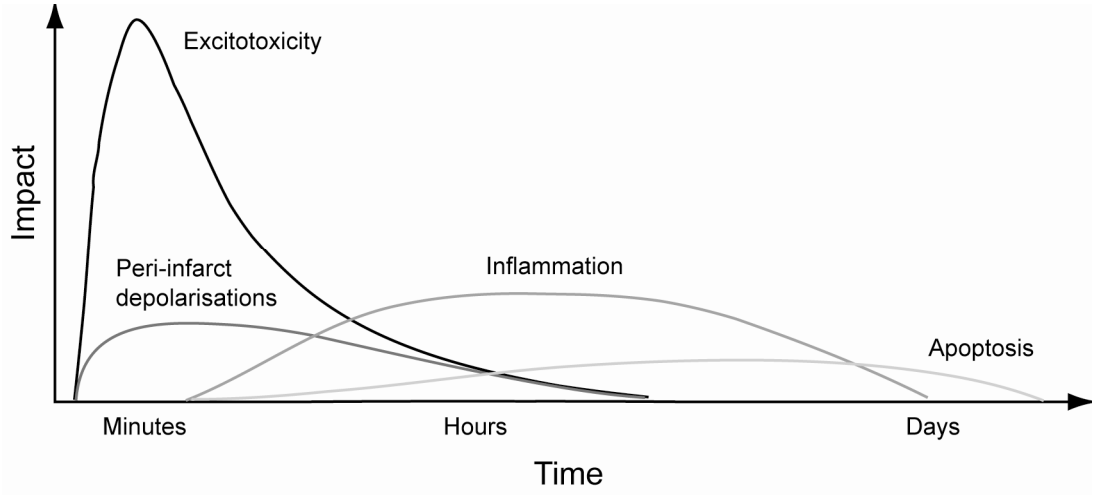

Figure 1. Putative cascade of damaging events in focal cerebral ischemia. The x-axis reflects the evolution of the cascade over time, while the y-axis illustrates the impact of each element of the cascade on final outcome. Adapted from Dirnagl et al 1999[13].

Excitotoxicity

Excitotoxicity is well established as an important trigger and executioner of tissue damage in cerebral ischemia[13]. This process is characterised by high concentrations of excitatory amino acids, in particular glutamate, in the extracellular space. Glutamate is the major excitatory neurotransmitter in the vertebrate brain. The actions of glutamate are mediated by two main types of receptors; ligand-gated cation channels (NMDA, AMPA and kainate receptors) and metabotropic glutamate receptors.

Energy depletion inhibits the activity of ATP-dependent ion pumps, making it difficult for cells to maintain ionic gradients[14]. This results in depolarisation of neurons, leading to synaptic release of glutamate. In addition, transporter-mediated glutamate homeostasis is dramatically impaired after ischemia[15] and glutamate uptake can even be reversed[16], further increasing the concentration of extracellular glutamate. High levels of glutamate result in an abnormal stimulation of NMDA- and AMPA-receptors, which leads to increased influx of Ca^{2+} and Na^{+}. To balance this influx of cations, H_2O and Cl^{-} are transported into the cell. This results in cell swelling and can lead to necrosis if the lesion is severe. The influx of Ca^{2+} also induces neuronal nitric oxide synthase (nNOS), resulting in the formation of reactive oxygen species such as peroxynitrite. These molecules damage DNA which activates poly(ADP-ribose) polymerase-1 (PARP-1). The activation of this enzyme is responsible for the translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus, which, in turn, initiates processes of active cell death[17]. Traditionally, excitotoxicity has been considered to cause a necrotic process. However, recent studies suggest that excitotoxic necrosis and apoptosis can be triggered in parallel in the ischemic brain. The relative contribution of these processes is determined by several factors, including the severity of injury, neuronal maturity, available trophic support and the concentration of intracellular free Ca^{2+}[18].

In addition to triggering acute excitotoxicity, extracellular glutamate and K^{+} diffuse from the infarct core and induce repetitive depolarisation in cells in penumbral regions. These peri-infarct depolarisations contribute to the growth of the infarct lesion[19-21].

Inflammation

Increased levels of reactive oxygen species and intracellular Ca^{2+}, as well as hypoxia itself, trigger the expression of pro-inflammatory genes. Consequently, mediators of inflammation are produced by injured brain cells[22]. Inflammatory mediators induce the expression of adhesion molecules, including intercellular adhesion molecule-1, P-selectins and E-selectins, on endothelial cells. Adhesion molecules attract inflammatory cells that cross the vascular wall and enter the brain parenchyma. A myriad of chemokines are produced in the injured brain, guiding the migration of inflammatory cells towards their

target. In addition to blood-borne inflammatory cells, microglia from the parenchyma are also activated and participate in the inflammatory response[13].

Reactive gliosis

Astrocytes are known to play key roles in modulating the pathology and regenerative response to various lesions[23]. The astrocytic response to an ischemic lesion, known as reactive gliosis, is a complex, multistage process and can be triggered by cell death, inflammation or by plasma components entering the brain following injury[24,25]. Reactive gliosis is characterised by hyperplasia, hypertrophy and an increase in immunodetectable glial fibrillary acidic protein (GFAP) in astrocytes[26]. Reactive astrocytes migrate towards the injury and form a glial scar which physically separates the uninjured regions from the lesion. Although this separation can protect the healthy tissue, scar formation can be detrimental for neurite growth into the lesioned area[27-29]. However, it has been suggested that reactive astrocytes could provide a permissive environment for neuritic extension under certain conditions[30].

Interestingly, reactive astrocytes have been shown to secrete a wide range of molecules and growth factors, including leukaemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF)[30-32], suggesting that these cells can influence other cells in the post-ischemic environment.

Lesion-induced plasticity and functional recovery

Many patients exhibit some spontaneous recovery of function following an ischemic brain lesion[33]. Resolution of tissue damage, including edema and inflammation, and spontaneous reperfusion can mediate recovery in the acute phase after a stroke[1,11]. However, studies have shown that reperfusion may worsen the tissue damage in severely damaged areas by inducing edema, reactive oxygen species and accumulation of inflammatory cells[34,35]. Following the acute phase, behavioural compensation and neural plasticity result in some recovery of function[36,37]. The concept of neural plasticity includes not only synaptogenesis and dendritic branching, but also neurogenesis; a relatively novel aspect of structural regeneration[38]. The discovery of ongoing neurogenesis and the

dynamic regulation of multipotent neural stem cells in the adult mammalian brain have opened up new possibilities for therapy in the lesioned brain.

Neural stem/progenitor cells

A neural stem cell is defined as a cell that: 1, can generate neural tissue or is derived from the nervous system; 2, has capacity for long-term self-renewal, and; 3, displays multipotency, i.e. the capacity to generate differentiated progeny of the neuronal, astroglial and oligodendroglial lineages, as well as multipotent stem cells[39] (Fig. 2). Neural stem cells undergo symmetric or asymmetric cell divisions. In a symmetric division, both

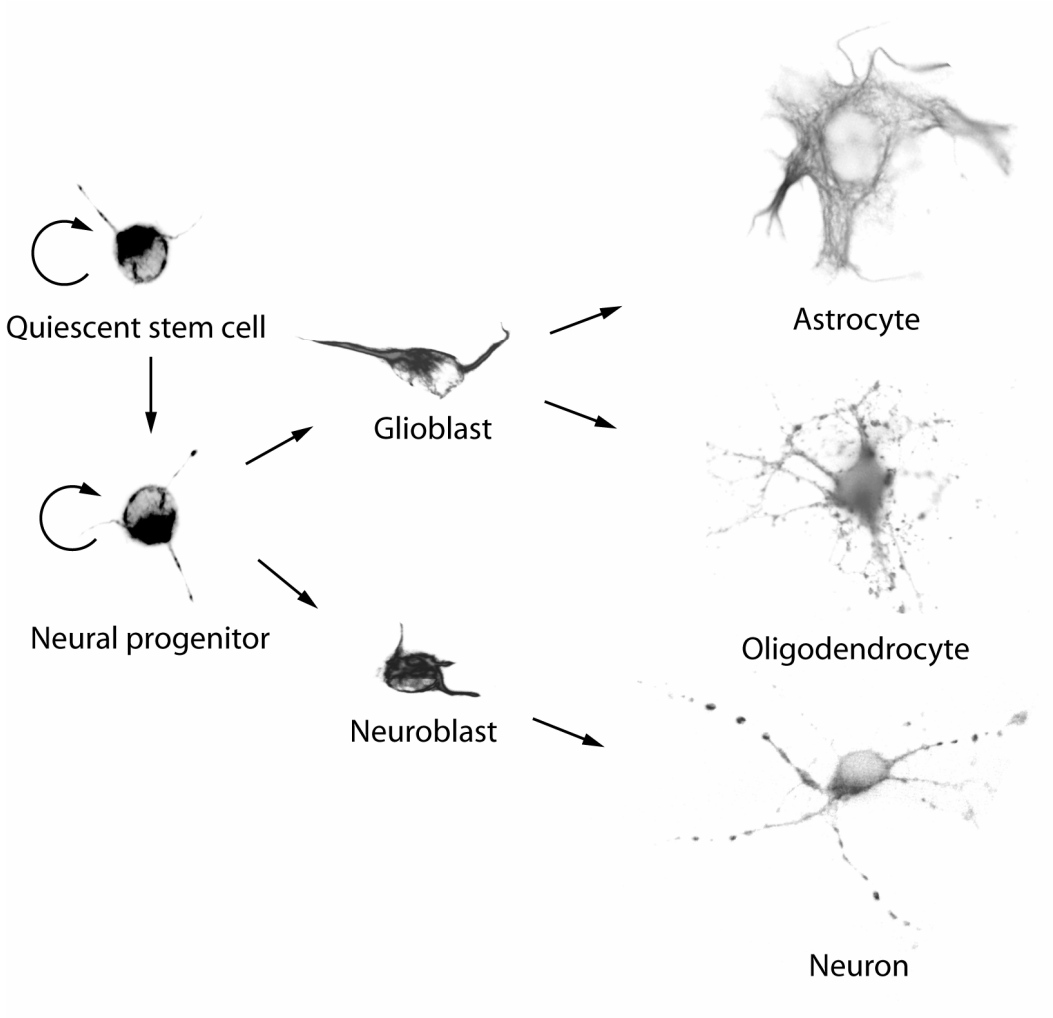

Figure 2. Schematic picture of how quiescent neural stem cells undergo self-renewal as well as give rise to more restricted neural progenitors. These neural progenitors display limited capacity for self-renewal and may differentiate into mature neurons, astrocytes and oligodendrocytes.

progeny will be stem cells. In contrast, an asymmetric division produces one new stem cell that is identical to the mother cell and one cell that is more determined for a certain lineage of differentiation. These daughter cells have less stem cell properties and are termed progenitor cells.

The production of new neurons, neurogenesis, occurs in the adult mammalian central nervous system (CNS) following the migration and differentiation of neural stem/progenitor cells (NSPCs). The majority of these cells reside in one of two germinal zones; the subventricular zone in the wall of the lateral ventricles (SVZ) and the subgranular zone of the hippocampus (SGZ)[40].

Outside of these regions, cell proliferation is common but the result is primarily the production of glial cells[41].

Cell genesis in the hippocampus

Adult hippocampal neurogenesis is conserved across mammalian species, including primates and humans[42-44]. The proliferative cells reside in the SGZ, a germinal zone along the border between the granule cell layer (GCL) and the hilus of the dentate gyrus. Neural stem/progenitor cells divide continuously and mostly give rise to neurons, but also to astrocytes and oligodendrocytes[45,46]. Newborn neuronal precursors migrate into the GCL where they mature and become new dentate gyrus granule cells. These newly generated cells project connections to the CA3 region of the hippocampus and have electrophysiological, morphological and phenotypical characteristics of mature and functional neurons[47]. Furthermore, new granule cells in the dentate gyrus exhibit enhanced synaptic plasticity and are activated during learning[48,49].

Newborn astroglial cells either display characteristics of radial glia cells or post-mitotic astrocytes[50].

Several conditions can influence cell proliferation, fate determination, and survival of hippocampal NSPCs, including physical activity[51], stress[52] and aging[53].

The physiological function of hippocampal neurogenesis has not been fully determined, however it seems to be important for spatial learning and memory[54].

Cell genesis in the subventricular zone

In the intact adult mammalian brain, multipotent stem/progenitor cells in the SVZ predominantly give rise to neuronal precursors. These cells migrate to the olfactory bulb (OB) via the rostral migratory stream (RMS), a tube-like structure consisting of glial cells that form a barrier between the developing neurons and the rest of the brain.

In the OB, a large proportion of the arriving cells die while the surviving neuroblasts mature into GABAergic interneurons and are functionally incorporated into the OB synaptic circuitry[55]. Most of these cells are found in the GCL, but approximately 5% of the newly formed interneurons reside in the periglomerular layer. A small proportion of these GABAergic periglomerular cells also display a dopaminergic phenotype[56-58]. Neurogenesis in the OB can be modulated by olfactory stimuli and seems to be associated with olfactory memory[59,60].

The RMS seems to play a vital role for neurogenesis in the OB in rodents. Surprisingly, although stem cells have been found in the adult human SVZ, the absence of a similar structure for cell migration in the human brain has been reported[61]. However, a recent study by Curtis *et al* demonstrated that humans indeed have a RMS and that this structure contains migratory progenitor cells that differentiate into mature neurons in the OB[62].

Ischemia-induced responses of NSPCs

Cell proliferation in the SVZ and SGZ has been shown to be robustly increased following both focal and global ischemia in experimental animal models[63-70]. Focal ischemia induces stem/progenitor cells to migrate from the SVZ to the cortex and striatum, hereby changing the default pathway of some of these cells[65,71]. Recent studies have demonstrated that, following focal ischemia, SVZ stem/progenitor cells can migrate into the striatum and replace a fraction of the lost striatal interneurons[63,72]. Interestingly, the activation of NSPCs extends well beyond the acute phase and striatal neurogenesis persists for at least four months after stroke in adult rats[73].

Post-ischemic cortical neurogenesis, originating from SVZ stem/progenitor cells, has been demonstrated in rodents and primates[70,74-76], but these results have been contradicted in other studies that failed to find new neurons in or around the ischemic cortex[63,72,77]. However, evidence for stroke-induced neurogenesis in the human cortex has

recently been presented[78]. Newborn immature neurons were found in the ischemic penumbra surrounding cerebral cortical infarcts and some of these cells displayed a migratory phenotype.

Moreover, neuronal replacement in the cortex after injury was demonstrated in adult mice by Magavi *et al* in a pioneering study in 2000[79]. In this study, corticothalamic neurons in layer VI of the anterior cortex were selectively killed by photolysis. This resulted in migration of stem/progenitor cells towards the lesion site and these cells differentiated into mature neurons[79].

In 2002, Nakatomi *et al* showed that NSPCs migrated from the SVZ into the hippocampus following an ischemic lesion that damaged the hippocampal CA1 region. The ischemic stimulus in combination with high doses of intraventricular infusions of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) resulted in a significant structural reconstitution of the CA1 region[80]. Furthermore, a recent study by Kolb *et al* demonstrated that intraventricular infusions of EGF and erythropoietin stimulated tissue re-growth and recovery of motor function after a focal stroke in the motor cortex[81].

In the absence of growth-promoting treatment, the results of the endogenous regenerative effort following ischemia are limited compared to the amount of tissue loss. This could be due to low survival of the recruited cells, but could also be explained by insufficient activation or dysfunctional lineage selection.

Whether this lesion-induced activation of endogenous NSPCs contribute to functional recovery after stroke is not currently known. However, attenuation of neurogenesis by irradiation has been shown to exacerbate ischemia-induced deficits, suggesting that the production of new cells contribute to functional recovery[82]. A greater understanding of these processes is necessary for finding ways to improve the endogenous regenerative capacity.

Isolation of multipotent stem/progenitor cells

Adult NSPCs can be isolated by dissecting out a brain region containing stem cells, e.g. the hippocampal dentate gyrus or the SVZ of the lateral ventricles. The tissue is disaggregated and cells are cultured with high concentrations of mitogens such as

bFGF[83,84] or EGF[85,86]. bFGF-expanded multipotent stem/progenitor cells derived from the adult rat dentate gyrus (adult hippocampal progenitors; AHPs) are capable of differentiating into neurons, astrocytes and oligodendrocytes *in vitro*, as well as *in vivo* after being transplanted into the adult brain[87-89]. Interestingly, neural progenitor cells with similar characteristics as AHPs can be isolated from many brain regions, including regions that appear to lack neurogenic permissiveness such as the neocortex, striatum and optic nerve[41,90]. Whether this is an artefact due to the isolation and culturing methods, or if these cells can be activated following a lesion, and thereafter behave as stem/progenitor cells is not currently known.

Neuroprotection mediated by NSPCs

Several studies have shown that transplantation of NSPCs is associated with reductions in damage or impairment following various pathological events, including stroke[91,92]. Although neuronal replacement may be a factor in these instances, data suggest that this may be a minor contribution. Thus, it seems that NSPCs can influence the outcomes of pathological events by other means, e.g. by modulating the cellular environment. Indeed, NSPCs have been reported to protect injured neurons by secreting various trophic factors, including glial-derived neurotrophic factor (GDNF), nerve growth factor, and stem cell factor[93,94]. Furthermore, grafted NSPCs can mediate neuroprotection by affecting the immune system[95] or by rectifying gene expression in imperilled neurons[96]. Modulation of the environment around a lesion may be a crucial function for recruited endogenous NSPCs, as well as an important feature of cells grafted into areas of damage.

Gene therapy in CNS repair

Gene therapy is a promising paradigm for treating certain pathologies in the CNS and can be designed to treat both inherited and acquired disease. The former usually involves replacement of a pathogenic gene with a functional homologue. This is a complex goal since extensive and long-term expression of the transgene often is required to correct the genetic flaw. Strategies aiming at treating acquired disease are less complex and can, for

example, be aimed at augmenting the survival of cells following an ischemic lesion by enhancing or altering gene expression.

Several studies have reported positive effects of gene therapy in animal models of focal and global ischemia. Therapeutic transgenes include growth factors and anti-apoptotic factors[97-100].

Gene therapy strategies can be divided into two main classes, direct and *ex vivo* gene transfer[101]. In direct gene therapy, the vector carrying the transgene is delivered directly to endogenous cells *in vivo* (Fig. 3). This method is common, but may be problematic due to toxicity, limited distribution of the vector and difficulties in characterising transfected cells to determine the level of transgene expression. Many of these problems can be overcome using *ex vivo* gene therapy, where target cells are transfected *in vitro* before being

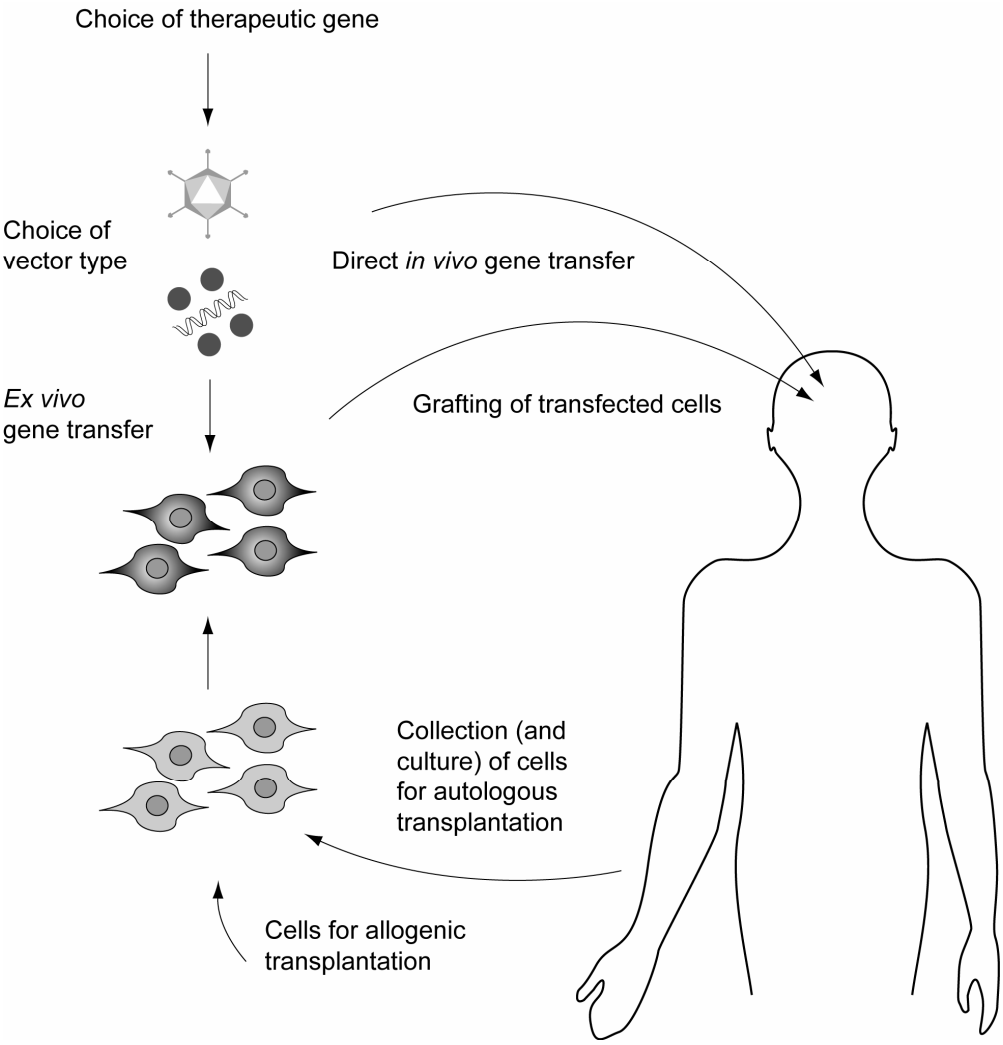

Figure 3. Potential strategies for the application of gene therapy in CNS repair. Adapted from Tinsley *et al* 2004[101].

transplanted into the CNS (Fig. 3). The greatest drawback of *ex vivo* gene therapy is that of obtaining cells suitable for autologous or allogenic transplantation.

Interestingly, NSPCs have many characteristics which make them suitable for cell-based gene therapy in the CNS, including the ability to survive, migrate and integrate in the injured brain[231]. In addition, grafted NSPCs could potentially participate directly in neural repair.

Non-viral transfection of NSPCs

DNA can be introduced into cells using either viral or non-viral gene-delivery protocols. Several types of viruses including retrovirus, adenovirus, adeno-associated virus and herpes simplex virus, have been modified for use as viral vectors *in vitro* and in gene therapy[102,103].

Non-viral vectors are generally based on cationic lipids or polymers. When the carrier molecules interact with plasmid DNA, lipoplexes or polyplexes are formed. These structures are stabilized by electrostatic interactions between the cationic vector and the negatively charged DNA. Transfection of lipoplexes occurs via non-specific endocytosis[104,105], following electrostatic binding to the cell surface[106]. Transfection of polyplexes normally occurs via non-specific endocytosis, however it has been shown that the transfection efficiency can be markedly improved by linking targeting ligands to polymers, enabling receptor-mediated endocytosis[107].

A lot of effort has been put into the development of new viral and non-viral vectors for gene delivery. Each vector has been devised with certain goals in mind, and hence no vector is ideal[101,108]. Rather, it is a case of finding the most suitable vector for a particular application.

Viral vectors generally display higher transfection efficiency than their non-viral counterparts, but transfection using viral vectors can have deleterious effects on transduced cells and can be accompanied with safety risks when used in gene therapy[102]. Viral vectors can also greatly induce differentiation in NSPCs. For instance, adenoviral transduction of neural progenitors induced predominant astrocytic differentiation (97% of cells) and blocked neurogenesis[109].

The advantages of non-viral vectors are that they have a better safety profile and that they can be used in either transient or stable transfections, in a relatively straightforward manner. Unfortunately, non-viral transfection of neural progenitors is generally inefficient and can be cytotoxic[110].

Genetic manipulation can be used to alter both intracellular (e.g. expression of a transcription factor) and autocrine/paracrine signalling (e.g. expression of a secreted factor). These two approaches can be used, either separately or in combination, to alter lineage selection or induce expression of secreted growth factors in NSPCs.

Thyrotropin-releasing hormone

Thyrotropin-releasing hormone (TRH) is produced in the paraventricular nucleus of the hypothalamus[111] and stimulates the secretion of thyroid-secreting hormone (TSH) from the anterior pituitary[112,113]. TSH in turn regulates the biosynthesis and release of thyroid hormone[114]. TRH is central in regulating the hypothalamic-pituitary-thyroid axis. However, TRH is also present in many brain loci outside of the hypothalamus and has been suggested to be a neuromodulator or neurotransmitter in these regions[115,116].

The TRH peptide is generated by enzymatic cleavage of a precursor, proTRH. Cleavage of proTRH results in 5 TRH peptide fragments. Effects of TRH are mediated by the TRH receptors, TRH-R1 and TRH-R2, which belong to the seven-transmembrane-domain G protein-coupled receptor superfamily[117,118]. Both isoforms of the TRH receptor have been found in various brain regions in rodents. However, only the expression of TRH-R1 has been demonstrated in humans[118]. In rodents, the central effects of TRH are mediated by TRH-R1, whereas neuromodulatory effects of the peptide have been attributed to signalling through TRH-R2[118].

Interestingly, therapeutic effects of TRH have been demonstrated in rodent models of ischemia[119-121]. Moreover, a recent study has shown that the TRH peptide is present in adult hippocampal NSPCs[122], suggesting that TRH may affect these cells in a paracrine or autocrine manner.

Insulin in the brain

Insulin is present in the CNS[123,124] and insulin receptors (IRs) are widely distributed in the brain. The highest expression of IRs is found in the olfactory bulb, cerebral cortex, hypothalamus, cerebellum and hippocampus[124,125]. In general, insulin and insulin receptors are primarily located in gray matter[126]. In addition to interacting with IRs, insulin can, at high concentrations, bind to the insulin-like growth factor (IGF) 1 receptor[126,127]. Local production and release of insulin in the CNS has been suggested[128], but it seems that the insulin found in the brain largely is produced by beta-cells in the pancreas and enters the brain across the blood brain barrier (BBB)[126,129,130].

Insulin regulates the glucose uptake and usage in most cell types of the body. However, insulin is not required for glucose utilisation in the CNS[131]. Instead, brain cells are permeable to glucose and can use glucose without the intermediation of insulin. This indicates that insulin has functions other than simply the transport of glucose in the brain. Indeed, recent studies have demonstrated that insulin regulates several processes in the brain, including food intake, energy homeostasis, reproductive endocrinology, synaptic plasticity and neuronal survival[132-135]. Moreover, systemic infusion of insulin in healthy humans promotes learning and memory[136]. Interestingly, individuals suffering from Alzheimer's disease have decreased insulin concentrations in the cerebrospinal fluid[137] and administration of insulin to Alzheimer's disease patients improves their memory[138].

AIMS OF THE STUDIES

The cues involved in the activation of NSPCs, the effects on NSPC lineage selection, and whether NSPCs participate in post-lesion regeneration, astrogliosis or neuroprotection following an ischemic lesion in the brain have yet to become clear. Therefore, the general aim of this thesis was to examine the interactions between NSPCs and important factors in the post-ischemic environment.

Specific aims:

I. To determine whether reactive astrocytes influence the lineage selection of NSPCs *in vitro*, using a mechanical lesion model of reactive astrogliosis.

II. To establish whether NSPC-derived astrocytes could participate in glial scar formation in an *in vitro* model of reactive astrogliosis.

III. To identify differentially expressed genes, coding for cell signalling molecules, in the peri-infarct neocortex after stroke using cDNA array technology.

IV. To investigate whether thyrotropin-releasing hormone affects the dynamics of adult NSPCs.

V. To develop an efficient method of non-viral transfection in adult NSPCs that does not affect viability, proliferation or differentiation of the cells, in order to create a foundation for future gene therapy experiments designed to increase cell survival and facilitate regeneration after an ischemic lesion.

VI. To analyse whether NSPCs secrete factors that can protect neurons against excitotoxicity.

MATERIALS AND METHODS

Cell culturing of NSPCs [I-IV]

The isolation of NSPCs from the adult rat hippocampus (AHP cells) has previously been described[87,89]. Clonally-derived cells were received at passage 4 as a gift from Prof. Fred Gage (Laboratory of Genetics, The Salk Institute, La Jolla, CA, USA). Cells were cultured in N2 medium (Dulbecco's modified Eagle's medium/Nut Mix F12 (1:1), 2mM L-glutamine and 1% N2 supplement; Life Technologies, Täby, Sweden), supplemented with 20 ng/ml human recombinant bFGF (PeproTech, London, England). bFGF is used to keep the cells in a proliferating and undifferentiated state. In these studies, cells were used between passage 5 and 20 postcloning.

Comments: Long-term culturing may transform primary cultures into immortalised cell lines displaying loss of growth control, changes in morphology and alterations in karyotype[139,140]. Karyotyping studies of the NSPCs used in this study have shown that most cells retain a normal diploid karyotype after 35 population doublings, which corresponds to approximately 15 passages[89].

The cells were isolated in the presence of bFGF and it is possible that this procedure selects for a certain population of stem/progenitor cells and changes the pattern of gene and protein expression in the isolated cells. These potential disadvantages might be overcome by different isolation procedures such as fluorescence activated cell sorting (FACS) to select cells on the basis of membrane protein expression or cell density based centrifugation protocols.

A key characteristic of NSPCs is their ability to differentiate into the three neural lineages (neuronal, astrocytic and oligodendrocytic). The NSPCs used for the thesis studies are capable of differentiating into neurons, astrocytes and oligodendrocytes *in vitro*, as well as *in vivo* after grafting into the adult brain[87-89]. In addition, clonally-derived cells have a stable phenotype in long-term culture, retaining identical immunocytological characteristics for more than 30 passages[87].

Primary astroglial cultures [I]

Primary astroglial cultures were prepared from Sprague-Dawley rats (P1-2). Rat pups were decapitated and the hippocampi and cerebral cortices were dissected and mechanically dissociated through 80-μm nylon meshes into Eagle's minimum essential medium (Life Technologies) with 20% foetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 1.6 mM L-glutamine and 1% penicillin/streptomycin (Life Technologies). The medium had extra substances added to the following composition: 1.6 times the concentration of amino acids and 3.2 times the concentration of vitamins (Life Technologies), 48.5 mM $NaHCO_3$ and 7.15 mM glucose (Merck, Darmstadt, Germany). Cells were grown in six-well plates or 35 mm-dishes to confluence (14-21 days). The medium was changed after three days in culture and thereafter three times a week. The experimental procedures were approved by the Ethics Committee of Göteborg University.

Comments: Primary cultures have been widely used as a model system for studying astroglial properties for more than 30 years[141]. Due to the difficulty of establishing controlled experimental studies of astrocytic functions exclusively, *in vivo* or *in situ* primary astrocytic cultures are valuable. Primary cultures are prepared from tissue taken directly from the organism and are regarded as primary cultures until subcultivated. The cells are derived from immature rats and are cultivated in an artificial milieu, without the extracellular environment and cytoarchitecture found *in vivo*. Cell cultures should be considered a model system and direct comparisons between the *in vivo* and *in vitro* situation should be made with caution.

Scratch injury model of astrogliosis [I]

Methods for studying astrogliosis *in vitro* have been developed[26,142]. Briefly, confluent astroglial cultures were washed twice and transferred to a defined serum-free medium (N2 medium) supplemented with 1% penicillin/streptomycin. After four hours of equilibration in the serum-free medium, confluent cultures were mechanically lesioned using a pipette tip in a 5 mm-grid frame. Conditioned medium (CM) was collected 48h after the injury, filtered at 0.22 μm (Pall Corporation, East Hills, NY) and immediately frozen at -20°C. After thawing, CM was diluted in fresh medium (N2 medium, 1:1).

For co-culture experiments, confluent hippocampal or cortical astrocytes were transferred to the serum-free medium before being mechanically lesioned. After the lesions were induced, NSPCs expressing green fluorescent protein (GFP) were added to the cultures at a density of approximately 2.0 x 10^3 cells/cm^2. These GFP^+ NSPCs have previously been used in co-culture paradigms with primary astrocytes[143]. For differentiation experiments, GFP^+ cells were co-cultured with the astrocytes for six days before lineage selection was assayed. In proliferation experiments, the cells were assayed 48 h after seeding.

Comments: Astrocytic cultures provide a manageable and convenient method for the study of reactive astrogliosis, and many culture systems have been established for this purpose. These include chemical treatment of astrocytes with inducers known to promote astrogliosis, such as various cytokines and endothelin-1, which result in morphological and biochemical changes in activated astrocytes[142,144-146]. Another alternative is to mechanically lesion astrocytes, either by scratching/scraping or stretching a confluent astrocytic monolayer. This results in the characteristic changes seen in reactive astrocytes and has been used to mimic the astrocytic reactions following a stab wound or an ischemic injury *in vivo*[26,147]. One of the disadvantages of using a chemical treatment to induce astrogliosis in this study was the risk of direct effects of the agent on the NSPCs. Therefore, the mechanical scratch injury model of astrogliosis was chosen. In the present studies, mechanically lesioned astrocytes changed to a polarised morphology[148] and the immunoreactivity of GFAP, vimentin, nestin and fibronectin increased in these cells. This demonstrates that the astrocytes displayed several characteristics of reactive astrocytes, and presumably these cells also secrete factors associated with reactive astrogliosis. Interestingly, astrocytes in this lesion model also produce and release a similar array of cytokines as astrocytes exposed to ischemic conditions *in vitro*[149]. The main disadvantage with the scratch injury model is the fact that only a fraction of the cells in a culture are directly affected by the lesion. Many cells could be affected by the lesion through gap junction signalling, but only the cells bordering the lesion display the typical characteristics of reactive astrocytes.

Immunocytochemistry [I-IV]

NSPCs were seeded onto polyornithine/laminin coated glass coverslips, at a density of approximately 1.0 or 1.5 x 10^3 cells/cm^2. Cells were seeded in N2 medium and the treatments (CM or transfections) were initiated on the following day. Culture medium was replaced every two days throughout the experiment. Ten days after the treatment was initiated, NSPCs were fixed (4% paraformaldehyde in phosphate-buffered saline (PBS), 4°C, 10 min). For immunocytochemical analysis of the TRH-receptors and insulin degrading enzyme, NSPCs were cultured in N2 medium supplemented with bFGF.

After fixation, cells were pre-incubated for 30 min with PBS containing 3% bovine serum albumin (BSA) and 0.05% saponin (Sigma-Aldrich, St Louis, MO, USA) at room temperature (RT). Cells were then incubated with primary antibodies for 1 h at RT. Antibodies were diluted in PBS containing 1% BSA and 0.05% saponin. Following three washes in PBS, cells were incubated for 1 h at RT with appropriate secondary antibodies and the nuclear dye bisbenzimide from a stock at 5 μg/ml (1:80, Hoechst 33258, Sigma-Aldrich). In double-labelling experiments of transgene-expressing cells with lineage specific markers, cells were also incubated with the β-galactosidase substrate fluorescein-β-D-galactopyranoside (FDG, 0.5 mM, Marker Gene Technologies Inc., Eugene, OR, USA), for 1 h at 37°C.

For co-culture experiments and validation of the astrogliosis model, cells were blocked and immunocytochemically stained with primary and secondary antisera in PBS containing 3% donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and 0.05% saponin. The primary and secondary antibodies used in this thesis are listed in **Table 1**.

Cells from at least ten non-overlapping fields were counted to quantify effects on differentiation. Results were obtained from 4-6 independent experiments. All counts were performed with the observer blind to the groups.

Comments: Immunocytochemistry is a widely used method to demonstrate the presence and cellular distribution of different antigens. However, there is always a risk of unspecific staining, especially when uncharacterised antibodies are used. The antibodies used against cell-type specific markers in this thesis have been widely used, tested and thoroughly characterised in previous studies. Consequently, the specificity of these markers was based

ANTIBODY	SOURCE	IMMUNOGEN	APPLICATION	DILUTION	COMPANY
Primary					
Caspase 3A	rabbit	activated human caspase 3	IHC	1:250	Cell Signalling Technology
CNTF	goat	rat ciliary neurotrophic factor	WB	1:500	R&D Systems
Doublecortin	goat	human doublecortin	IHC	1:400	Santa Cruz
Fibronectin	rabbit	human fibronectin	ICC	1:250	Sigma-Aldrich
GFAP	mouse	GFAP from pig spinal cord	IHC	1:200	Sigma-Aldrich
GFAP	rabbit	GFAP from cow spinal cord	ICC, IHC	1:500	DAKO
GS	mouse	sheep glutamine synthetase	ICC	1:250	Chemicon
Iba1	goat	human Iba1	IHC	1:500	Abcam
IDE	mouse	human insulin degrading enzyme	ICC	1:250	Covance Research Products
LIF	goat	human leukaemia inhibitory factor	WB	1:200	Santa Cruz
MAP2ab	mouse	bovine microtubule associated protein 2ab	ICC, IHC	1:100	Sigma-Aldrich
Musashi	rabbit	human musashi	ICC	1:250	Chemicon
Nestin	mouse	rat nestin	ICC	1:300	BD Pharmingen
NeuN	mouse	purified cell nuclei from mouse brain	IHC	1:500	Chemicon
pHH3	rabbit	human phosphorylated histone H3	ICC	1:200	Upstate Biotechnology
proTRH	rabbit	rat proTRH	IHC	1:150	Gift from E. Nillni, Brown University
RIP	mouse	rat olfactory bulb	ICC, IHC	1:50	Dev. Studies Hybrid. Bank, Univ. of Iowa
TRH receptor-1	goat	rat TRH receptor-1	ICC	1:100	Santa Cruz
TRH receptor-2	goat	rat TRH receptor-2	ICC	1:100	Santa Cruz
Vimentin	mouse	porcine vimentin	ICC	1:200	DAKO
Secondary					
Alexa 488	donkey	goat, mouse, rabbit IgG	ICC, IHC	1:800-1:2000	Molecular Probes
Alexa 488	goat	mouse IgG	ICC	1:2000-1:4000	Molecular Probes
Alexa 555	donkey	mouse, rabbit IgG	ICC, IHC	1:800-1:2000	Molecular Probes
Alexa 594	goat	rabbit IgG	ICC	1:3000	Molecular Probes
Alexa 633	donkey	goat IgG	IHC	1:800	Molecular Probes
Alexa 647	donkey	mouse, rabbit IgG	IHC	1:800	Molecular Probes
Biotin	horse	goat IgG	WB	1:10000	Vector Laboratories
FITC	donkey	mouse IgG	ICC	1:150	Jackson Immuno-research
Texas Red	donkey	goat IgG	ICC	1:150	Jackson Immuno-research

Table 1. List of primary and secondary antibodies used in the thesis studies (ICC-immunocytochemistry, IHC-immunohistochemistry, WB-Western blot).

on the morphology of the cells and the cellular distribution of the immunoreactivity. In addition, negative controls where primary antisera were omitted were performed for all secondary antibodies.

Immunohistochemistry [II]

In paper II, characterisation of TRH localisation was performed with immunohistochemistry. Briefly, brains were fixed in 4% paraformaldehyde and dehydrated with graded ethanol and xylene before paraffin-embedding. Subsequently, the brains were cut into 5 μm coronal sections. Sections were deparaffinised in xylene and rehydrated in graded ethanol before staining. Antigen retrieval was performed by heating the sections in sodium citrate buffer (pH 6.0, 95°C) for 10 min. Non-specific binding was blocked by incubating the sections for 30 min in PBS with 4% donkey serum and 0.1% Triton-X.

Sections were then incubated with primary antibodies overnight at 4°C. Both primary and secondary antibodies were diluted in PBS containing 4% donkey serum and 0.1% Triton-X. Following several rinses in PBS, sections were incubated with secondary antibodies for 2 h at RT. Sections were rinsed in PBS and mounted with ProLong Gold containing DAPI (Molecular Probes, Eugene, OR, USA).

Comments: Immunohistochemistry is a powerful method for demonstrating the presence and distribution of different proteins in tissue sections. In this thesis, only thoroughly characterised antibodies were used and the specificity of these markers was based on cell morphology and the cellular distribution of the immunoreactivity (see section on Immunocytochemistry for more information on specificity and negative controls).

[^{3}H]Thymidine incorporation assay [I, III]

DNA synthesis was assayed by detection of [^{3}H]thymidine incorporation using scintillation spectrometry. Cells were cultured in 96-well plates, coated with polyornithine/laminin, at a density of 1 x 10^4 cells/cm^2 in N2 medium (**I**) or 5 x 10^4

cells/cm^2 in N2 medium supplemented with bFGF (**III**). After 24 h, cells were either transfected or different CM were added to the cultures. On day 3 (after 48 h), [^{3}H]thymidine was added to the cells (Amersham Biosciences, Uppsala, Sweden), resulting in a final concentration of 1 μCi/ml. After 72 h, the medium was removed and cells were then washed with PBS and resuspended with 0.4 M sodium hydroxide. The suspension was transferred to a scintillation vial, neutralised with 0.4 M hydrochloric acid and 4 ml of scintillation fluid (Ready Safe, Beckman Coulter Inc., Fullerton, CA, USA) was added. Samples were counted for 2 min using a scintillation counter (Beckman LS 6500, Beckman Coulter Inc., Fullerton, CA, USA). Four counts were collected and averaged for each experiment, and the mean value of 4-8 independent experiments was determined for each treatment.

Comments: Radioactive thymidine can be used to investigate cellular proliferation. Incorporation of [^{3}H]thymidine occurs during the S-phase of the cell cycle and the degree of radioactivity in a cell population is correlated to the number of mitotic events. In the experiments performed in this thesis, [^{3}H]thymidine was added to the cultures 24 h before the analysis. With this protocol the extent of cell divisions during the last day in culture can be measured and compared between different treatments.

DNA content proliferation assay [II]

A DNA quantification assay was used in order to assess possible changes in cellular proliferation when NSPCs were treated with TRH. Cells were cultured in 24-well plates, coated with polyornithine/laminin, at a density of 2 x 10^3 cells/cm^2 in N2 medium supplemented with bFGF. After two days in culture, cells were grown for two days without bFGF in the medium and thereafter the cells were grown for two days either without bFGF (control), with 20 ng/ml bFGF or with different concentrations of TRH (Sigma-Aldrich). Thereafter the medium was aspirated and the cells were washed once in PBS and the plates were frozen at -80°C overnight. Measurement of DNA content was performed using the CyQUANT Cell Proliferation kit (Molecular Probes) according to the instructions of the manufacturer. Briefly, cells were thawed, resuspended in lysis buffer containing ethylenediamine tetraacetic acid (EDTA, 1mM) and DNase-free RNase (1 μg/mL, Sigma-Aldrich) and incubated for 1 h at RT. Lysates were transferred to a 96-

well plate. The DNA-binding fluorescent CyQUANT GR dye was added and incubated for 5 minutes at RT, after which the DNA concentration was measured by fluorescence spectroscopy with a TECAN Genios microplate reader (TECAN Austria GmbH, Grödig/Salzburg, Austria) at 485 and 530 nm excitation and emission wavelengths. The experiment was repeated 4 to 6 times and from each experiment means were calculated from four different wells.

Comments: Cellular proliferation can be analysed using the CyQUANT assay. This assay is based on measurement of cellular DNA content via fluorescent dye binding. Because cellular DNA content is highly regulated, it is closely proportional to cell number. bFGF was used as a positive control for proliferation, since this factor is known to be a potent mitogen for NSPCs[150].

Analysis of proliferation using pHH3 expression [I, II]

To determine whether the fraction of mitotic NSPCs was altered when co-cultured with lesioned astrocytes or treated with TRH, the expression of a mitotic marker, phosphorylated histone H3 (pHH3), was investigated. Cells were fixed 48 h after the treatment was initiated (4% paraformaldehyde in PBS, 4°C, 10 min) and cells were immunocytochemically stained with pHH3 antiserum according to the method described in the immunocytochemistry section. For quantification of mitotic cells, the total number of NSPCs and the number of cells co-labelled with pHH3 from at least 12, randomly selected, non-overlapping fields were counted in each sample. Results were obtained from 6 independent experiments. All counts were performed with the observer blind to the groups.

Comments: Phosphorylated histone H3 is a marker of mitotic cells. The phosphorylation is highly correlated with the G2 to M transition in the cell cycle where histone H3 is phosphorylated at Serine-10[151]. This method gives a snapshot of the number of mitotic cells, at a selected time point, but it gives no indication of the number of cells that have divided during a certain period of time. For these reasons, quantification of mitotic cells is a good complement to analyses of [^{3}H]thymidine incorporation and DNA content.

Western blot [I]

Samples of CM (200 µl) from lesioned and control hippocampal astrocytes were protein precipitated for Western blot analysis by adding ice-cold acetone, three times the volume of the CM. The samples were mixed, stored at -20°C overnight, centrifuged (10 min, 35,000 g), and the supernatant was carefully removed. Protein pellets were dissolved in 12 µl of Nu-PAGE sample buffer (0.14 M Tris base, 0.11 M Tris-HCl, 0.5 mM EDTA, pH 8.5, 10% Glycerol, 75 mM lithium dodecyl sulphate) and separation was performed using Nu-PAGE 10 % Bis-Tris gels (Novex, San Diego, CA, USA) and MOPS running buffer (50 mM MOPS, 50 mM tris, 3.5 mM sodium dodecyl sulphate (SDS), 0.8 mM EDTA) at a constant voltage (200 V, 50 min). Proteins were transferred for 45 min at 1.0 mA/cm^2 onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) under semi-dry conditions (Hoefer Semiphor, Amershan Bioscience, Buckinghamshire, UK) using Nu-PAGE transfer buffer (25 mM Bicine, 25 mM BisTris, 1 mM EDTA, 20 % methanol). Membranes were blocked with 5% milk powder in PBS–Tween (0.05% Tween-20; PBS-T). After blocking, the membranes were incubated overnight with primary antibodies raised against CNTF and LIF (see Table 1 for details), diluted in PBS-T. The membranes were then washed with PBS-T for 3x2 min and incubated for 1 h with a secondary biotinylated antibody. A second PBS-T wash was performed for 3x2 min and then the membranes were incubated for 1 h with streptavidin-biotinylated horseradish peroxidase complex (1:3000, Amersham Pharmacia Biotech, Uppsala, Sweden). Following 3x5 min washes, membranes were developed for 2 min with ECL Advanced solution (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The emitted signal was detected by a CCD camera (Fluor-S Max MultiImager, Bio-Rad, Hercules, CA, USA) and the intensity of protein bands was analysed (ImageJ v1.29x). The expression of CNTF and LIF was investigated in CM from five independently repeated experiments.

Comments: Western blotting was used to analyse the presence of CNTF and LIF in medium conditioned by reactive astrocytes. Total protein concentration was analysed to confirm that the different CM had similar protein contents. No expression of CNTF or LIF was detected when negative control samples (N2 medium) were analysed.

MCA occlusion model of focal ischemia [II]

The experimental procedures were approved by the Ethics Committee of Göteborg University. Male Sprague-Dawley rats (weighing 270-300 g) were supplied by Möllegard Breeding Center (Ejby, Denmark). Animals were weighed and fasted overnight prior to all surgical procedures. Anaesthesia was induced in a perspex box with 5% isoflurane in an O_2/N_2O mix (30/70%). When unconscious, the animal was intubated and mechanically ventilated with 2.0 to 3.0% isoflurane in the same gas mix. Body temperature was monitored with a rectal temperature probe and maintained around 37°C with a thermo-coupled heating pad. Permanent focal ischemia was achieved by occluding the origin of the MCA using the intraluminal thread method[152]. Briefly, a 4-0 monofilament nylon suture with a tip rounded by heating near a flame and coated in 0.1% poly-L-lysine[153] was introduced into the external carotid artery. The suture was advanced into the internal carotid artery 17-18 mm past the external carotid/internal carotid artery bifurcation and secured in the external carotid artery. Although blood pressure and blood gas concentrations are routinely measured during surgery via a femoral or tail arterial catheter, we were concerned that surgical manipulations surrounding the insertion of such a catheter might hinder the animal's ability to freely move and interact with the environment. Instead, blood gas samples were obtained from the external carotid artery immediately prior to insertion of the nylon suture. All samples were analysed using an ABL 5 blood gas system (Radiometer Copenhagen, Denmark). Although blood pressure could not be measured in the present investigation, we know from previous studies that blood pressure is stable under the surgical conditions used in this investigation[154,155]. Anaesthesia was discontinued approximately 15 to 20 min after induction of ischemia. When the rat was able to breathe spontaneously, it was extubated and allowed to recover. Animals were tested at 2 h for tight anti-clockwise circling as an indicator of successful MCA occlusion (MCAO). Rats not meeting the criterion were excluded from the study. Sham-operated animals underwent the same surgical procedures with the exception of insertion of the monofilament nylon suture to occlude the MCA.

After the surgery, animals were housed individually overnight and then transferred to standard cages (2-3 rats per cage). All animals received standard laboratory chow and water *ad libitum* for the entire experimental period. The following groups of animals were used in the study: MCAO rats sacrificed 3 days post-stroke, M3 (n=7); sham-operated rats

sacrificed 3 days post-surgery, S3 (n=7); MCAO rats sacrificed 14 days post-stroke, M14 (n=5); sham-operated rats sacrificed 14 days post-surgery, S14 (n=9).

In some cases, animals continued to lose weight after the initial post-operative period and these animals were euthanized and not included in the investigation.

Comments: The intraluminal filament model of MCAO is the most widely used experimental stroke model. The method has several advantages, including being anatomically representative of the clinical situation and minimally invasive (not requiring craniotomy). Among the drawbacks are high mortality rate, due to the large volume of the infarct and the accompanying oedema, as well as relatively low reproducibility. However, recent studies have shown that coating of the occluding filament with either silicon[156,157] or poly-L-lysine[153] markedly increases the reproducibility.

Tissue preparation and confirmation of damage after MCAO [II]

After 3 or 14 days post-operation, rats were decapitated, the ipsilateral peri-infarct cortex of each animal quickly dissected out (on ice), placed in liquid nitrogen and stored at -80°C. The remaining brain was sliced into 2 mm sections using a rat brain template and incubated in 3% 2,3,5-triphenyl-2H-tetrazolium chloride (in 0.9g/100 ml NaCl) for 10 min to confirm the presence of an infarct[155]. As the focus of the study was to identify genes up regulated in the peri-infarct cortex in response to damage, only animals that displayed evidence of both striatal and cortical infarction were included in the study.

Comments: The peri-infarct region is easily distinguishable from the infarct region, enabling isolation of the non-injured tissue. This region neighbouring the cortical infarct likely represents an active brain region involved in post-stroke plasticity with suggested positive effects on functional improvement[158,159].

RNA preparation [II]

Total RNA was prepared according to Chomczynski and Sacchi[160], with minor modifications. Briefly, a denaturing buffer containing 4 M guanidiumthiocyanate, 0.1 M β-mercaptoethanol, 25 mM sodium citrate (pH 4.0), 0.5% sarcosyl and H_2O-saturated

phenol was used for homogenisation, followed by the addition of chloroform:isoamyl alcohol (24:1). After centrifugation, the upper phase (containing total RNA) was transferred to a new tube, precipitated using isopropanol and redissolved in RNase free buffer. RNA concentrations were determined using UV-spectrophotometry.

Comments: The Chomczynski method for isolating RNA has been widely used. Brain tissue has high lipid content. Therefore, high amounts of phenol and chloroform (twice the normal amount) are required in the first homogenisation step for total RNA of high purity.

cDNA-array [II]

To characterise genes that might be involved in neuronal plasticity and regeneration in the post-ischemic brain of adult rats, we performed a cDNA array analysis comparing gene expression at different time points (3 and 14 days) after MCAO. This resulted in four groups, from which RNA was isolated. The 'Atlas' pure total RNA labelling system (Becton-Dickinson Biosciences, Clontech Laboratories Inc., Stockholm, Sweden) was used for DNase treatment of total RNA and RNA integrity was evaluated on denaturing gels. Subsequently, poly $(A)^+$ RNA was purified by coupling of biotinylated oligo (dT) and separation using streptavidin-conjugated magnetic beads. Prior to probe synthesis, RNA from individual animals from each treatment group were pooled. cDNA probe synthesis was performed using the primer mix included in the kit, which consists of primers specific only for the later analysed genes. The primer mix was added to the resuspended beads and preheated at 65°C for 2 min after which the temperature was reduced to 50°C and Moloney murine leukemia virus reverse transcriptase was added, followed by addition of a Master Mix including [α-^{32}P]dATP (10 μCi/μl, 3000 Ci/mmol; Amersham Pharmacia Biotech), a deoxynucleotide triphosphate mix, reaction buffer and dithiothreitol. Reagents were incubated for 25 min. Thereafter, the reaction was terminated and the radioactively labelled probes were purified with column chromatography and radioactivity was measured in a gamma counter. Probes had an approximate activity of 12 x 10^6 counts per minute (cpm), which was within the 0.5-20 10^6 cpm range recommended by the manufacturer. Radioactive cDNA probes were hybridised to nylon membranes containing cDNAs for 1176 known genes, 9 housekeeping genes as well as control cDNAs. Each

cDNA fragment attached to the membrane was 200-600 base pairs originally amplified from a region of the gene transcript that lacks the poly(A)$^+$ tail, repetitive elements or sequences highly homologous to other genes in order to minimise cross-hybridization and non-specific binding. A list of all the genes including array coordinates, GenBank and SwissProt accession numbers is available at Clontech's website (http://www.clontech.com/support/tools.asp?product_tool_id=157578&tool_id=157579). The radioactively labelled cDNA probes were mixed with C_ot-1 DNA (added to reduce non-specific binding) and added to the nylon membranes that had been pre-incubated with heat-denatured salmon testes DNA in hybridisation solution (ExpressHyb, Becton-Dickinson Biosciences, Clontech Laboratories Inc.). Hybridisation was performed overnight with continuous agitation at 68°C after which the membranes were washed four times with 2 x SSC (0.3 M NaCl. 30 mM sodium citrate, pH 7.0) and 1% sodium dodecyl sulphate (SDS) solution, followed by one wash with 2 x SSC and 0.1% SDS solution, all performed at 68°C. Finally, membranes were washed with 2 x SSC at RT and exposed to Kodak BioMax MS film (Eastman Kodak Co., Rochester, NY, USA) with a corresponding BioMax MS intensifying screen at -80°C. Films were exposed for 8 h, 24 h, 3 days and 7 days. Data were analysed using the AtlasImage software according to the instructions of the manufacturer. Gene spots that gave a very high signal and bled into other gene spots were excluded. Differential gene expression between the groups was defined as a two-fold or greater difference in the expression of a certain gene. User-defined normalisation was performed using the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Comments: The Atlas cDNA arrays consist of cDNA fragments, selected to have minimal homology with other genes on the membrane. The cloned cDNA fragments are sequenced before amplification and are then quantified and printed on the membrane. In addition, gene specific primers for cDNA labelling are used to avoid cross-hybridisation. The assay is very sensitive and can, according to the manufacturer, detect mRNA that is present at only 10-20 copies per cell (corresponds to approximately 0.0025% of total mRNA in a cell). There are two ways of normalising the signal intensity of different arrays: global normalisation and user-defined normalisation. In global normalisations, the intensity values of all genes on the array are used. This is suitable if few changes in gene expression are expected. On the contrary, user-defined normalisations are best suited for

analyses where many differentially expressed genes are expected. In this study, normalisation was performed by comparing the intensity of each gene spot to a set of housekeeping genes that displayed a stable pattern of expression. The signal threshold was set to be gene-based and signals not visible by eye were disregarded.

In paper II, Atlas cDNA arrays were used, due to their relatively low cost and ease of handling, to find differentially expressed genes in the post-ischemic environment. There are commercial array systems that are based on somewhat different technologies and these may be used for different purposes. Expensive broad screening arrays, such as the Affymetrix platform, generate highly quantitative data comparable to results from northern blots[161]. Alternatively, there are cheaper, semi-quantitative arrays, such as the Atlas cDNA arrays (used in this thesis), highly suitable for screening samples to find potential target genes for future research. These arrays, unlike the Affymetrix platform, are only spotted with known genes and generate easily manageable amounts of data, making them a fast screening tool if not a total quantitative characterisation of the samples is required. In this study, pooled samples were used to decrease costs (four groups consisting of 28 animals were analysed) and biological variation. A recent study by Kendziorski *et al* investigated the utility of pooling biological samples in cDNA array experiments. They concluded that most expression measurements from RNA pools are similar to averages of individuals that comprise the pool and that pooling could dramatically increase accuracy when the number of arrays is limited[162].

Quantitative real-time PCR [II]

Quantitative real-time PCR (Q-PCR), using a LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany) was performed in paper II. cDNA was synthesised from total RNA from individual animals. For each sample, 3 µg total RNA was reverse transcribed for 60 min at 37°C in a 75 µl reaction mixture with 8 U/µl moloney murine leukaemia virus reverse transcriptase, 20 ng/µl random hexamers, 1.6 U/µl RNase inhibitor and 625 µM dNTP mix (Promega, Madison, Wisconsin, USA). Primers for PCR experiments were designed employing the Primer3 software (http://frodo.wi.mit.edu/) and a Basic Local Alignment Search Tool (BLAST) search was performed in order to ensure specificity of the primer pair. The following primer pairs were utilised to assess

levels of TRH mRNA: forward 5'-tgg gaa gaa aaa gag gga ga-3' and reverse 5'-agt gaa ggg aac agg ata ggg-3'; pleiotrophin mRNA: forward 5'-gag agg gga aga aag aaa gca-3' and reverse: 5'-gaa aat caa agc cag gaa gg-3'; TGF-β1 mRNA: forward 5'-cgc aat cta tga caa aac ca-3' and reverse 5'-aca gcc act cag gcg tat c-3'. In the PCR reactions, cDNA was diluted 1:40 and the primers 1:10. For the PCR master mix the LightCycler Fast Start DNA Master SYBR Green I mix was used (Roche Diagnostics). The constitutively expressed housekeeping gene GAPDH was used as an internal standard. The primer for GAPDH was: forward 5'- acc acc atg gag aag gct gg-3' and reverse 5'-ctc agt gta gcc cag gat gc-3'. All primers were purchased from Cybergene (Huddinge, Sweden).

Comments: Quantitative real-time PCR was developed to enable quantification of relatively small differences in mRNA expression and analysis of small amounts of RNA (e.g. small amounts of tissue or even single cells[163]). Using conventional PCR, it is difficult to get quantitative results since the most frequently used dye, ethidium bromide, is rather insensitive. This lack of sensitivity makes the detection of a band to occur when the logarithmic phase of amplification is over and the reaction has reached the plateau phase. In this study Q-PCR with SYBR Green I was employed. SYBR Green I is a dye that fluoresces brightly only when bound to double stranded DNA. This dye is much more fluorescent than ethidium bromide and also has a higher ratio of fluorescence in the presence of double-stranded DNA to the fluorescence in the presence of single-stranded DNA. As a result of these features SYBR Green I Q-PCR analyses the exponential phase of the PCR reaction, where the only rate-limiting factor is the cDNA template. Since SYBR Green I binds to all double stranded DNAs it does not distinguish between one DNA and another. For this reason, important quality control steps, including melting curve analysis and agarose gel electrophoresis, are used to ensure the accurate formation of the expected PCR products. All the results in this study were correlated to the expression of the housekeeping gene GAPDH. The expression of GAPDH is not affected by hypoxia/ischemia[164].

Non-viral transfection [III]

NSPCs were seeded onto polyornithine/laminin coated 96 well plates or 12 mm coverslips in 24 well plates. Six transfection agents were screened: polyethylenimine 25 kD (Sigma-Aldrich), Lipofectamine (Invitrogen, Carlsbad, CA, USA), Superfect (Qiagen, Hilden, Germany), Effectene (Qiagen), ExGen500 (Fermentas, Vilnius, Lithuania) and FuGene6 (Roche Diagnostics). Electrostatic complexes were made by adding transfection agents to plasmid DNA (pDNA, gWiz-β-gal, Gene Therapy Systems, CA, USA). Both transfection agents and DNA were pre-diluted in 150 mM NaCl. These mixtures were incubated at RT (15-30 min) to allow complex formation. Mixtures were then diluted in complete medium and added to cells. For initial comparison, transfection times of 3 h or 48 h were used. An additional time point of 24 h was used to further determine the optimal transfection times for ExGen500 and FuGene6.

The final optimised conditions were: ExGen500 – 10 μl ExGen500 + 1.0 μg pDNA per 10^5 cells, applied for three hours; FuGene6 – 6 μl FuGene6 + 2.0 μg pDNA per 10^5 cells, applied for two days. These optimised conditions were used for all subsequent transfections.

Comments: Methods for viral transduction of NSPCs have been published[165,166]. However, viral vectors can greatly affect the differentiation of NSPCs and block neurogenesis[109,166]. Furthermore, drawbacks associated with viral vectors, such as cytotoxicity, limited transgene capacity, difficulties in producing pure high titre stocks and most importantly patient safety may be problematic if engineered cells are used in a clinical setting[101,102,167,168]. Non-viral transfection is less common[166,169,170], however, it is more compatible with clinical protocols[101,171]. These techniques are also enhanced by the ability to readily change the gene of interest. This would allow screening of multiple transgenes by simply substituting plasmid constructs, which is not possible with viral vectors. Such plasmids may be of virtually any size, may contain bicistronic expression cassettes and may be combined with other plasmids by co-transfection. Although most plasmids confer transient expression, long-term expression can be achieved with non-viral transfection by selecting for random transgene integration, or using specifically designed plasmids which persist episomally[172]. Unfortunately, low levels of transfection with non-viral vectors have limited the analysis of neural progenitor cells by transgene expression[110] and previous experiments in our laboratory achieved transfection in only 0.3% of cells[173].

Assay of β-galactosidase activity [III]

Total activity of the reporter gene product from the gWiz-β-gal plasmid was determined from cell lysates using a chemiluminescent assay for β-galactosidase (Roche Diagnostics). Briefly, following transfection, cells were incubated for two days to allow reporter gene expression. The medium was then removed and cells were frozen at -20°C until the assay was performed. Lysates were prepared by thawing the cells before 100 µl of lysis buffer (20 mM phosphate buffer with 0.1% (v/v) Triton X-100 and 0.02% (w/v) magnesium chloride) was added. Cells were incubated for one hour before 50 µl of the samples was transferred to the wells of a white microtitre plate. To these were added 50 µl of Solution A (50 µl Substrate, 4.95 ml Assay Buffer). Plates were incubated (45 min, RT, rocking) before Solution B (750 µl Enhancer, 3.75 ml Initiator) was added to induce luminescence and the plates were read in a GenIOS plate reader (TECAN, Maennedorf, Switzerland). Solution B was added to wells of the plate in the order and timing that the reader was to measure them to ensure a consistent interval between initiation and reading.

Comments: Traditionally, the enzymatic activity of β-galactosidase is measured using colorimetric substrates. We used a kit employing chemiluminescent substrates (based on 1,2-dioxetanes), which increases the sensitivity of enzymatic detection by several orders of magnitude compared to colorimetric substrates[174]. Briefly, in the first step the substrate becomes deglycosylated by the enzymatic activity in the cell extract. This incubation step is performed at pH 7.8, where bacterial β-galactosidase is highly active. At this neutral pH, the cleaved product is protonated and will not produce a light signal. The stable intermediate accumulates during the reaction, and provides a light signal when the pH is adjusted higher (>12). The light signal peaks immediately and decays with a half-life of approximately 10 min. To ensure comparable results, the light reactions were initiated in the order and timing that the reader was to measure the samples.

X-gal staining and percent transfection [III]

For photomicrographs of X-gal staining, cells were grown and transfected on polyornithine/laminin coated coverslips. Two days after transfection cells were fixed (4% paraformaldehyde in PBS, 4°C, 10 min), washed with PBS, and subsequently incubated

(overnight, 37°C) in X-gal stain (10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, 2 mM magnesium chloride, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, in PBS). After staining, coverslips were washed twice in PBS, and mounted on glass slides. Percentage transfection was determined by counting the fraction of X-gal stained cells (out of at least 500 cells) on eight coverslips per treatment. Coverslips were from at least three independent experiments.

Comments: β-galactosidase can be detected using a variety of substrates. The most common substrate is the indole derivative, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)[175]. When β-galactosidase cleaves the glycosidic linkage in X-gal, a soluble, colourless indoxyl monomer is produced. Subsequently, two of the liberated indoxyl moieties form a dimer which is non-enzymatically oxidised. The resultant halogenated indigo is a very stable and insoluble blue compound[175]. Thus blue coloured cells can easily be distinguished from negative cells using light microscopy.

Lactate dehydrogenase activity [III]

To analyse the effects of transfection on cell viability, release of lactate dehydrogenase (LDH) was assayed (Cytotoxicity Detection Kit, Roche Diagnostics). Briefly, cells were cultured in 24-well plates coated with polyornithine/laminin at 5 x 10^4 cells/cm^2 in N2 medium supplemented with 20 ng/ml bFGF. On the following day the medium was discarded and fresh medium was added to the cultures, after which the cells were transfected with ExGen500 or FuGene6. The medium was collected after 3 h or 6 h. An additional time point of 24 h (which is the longest collection period recommended by the manufacturer) was analysed for FuGene6-transfected cells. Samples were centrifuged for 10 min at 500 g, the supernatants were collected and 4 lots of 100 µl from each sample were transferred to a 96-well plate. To determine the LDH activity in the samples, 100 µl of reaction mixture was added to each well. The plate was incubated for 20 min at RT, protected from light. The absorbance of the samples was measured at 490 nm using a Victor II plate reader (EG&G Wallac, Turku, Finland). Results were derived from four independent experiments.

Comments: Cell death is classically evaluated by the quantification of plasma membrane damage. The assay we used in this study is based on measuring the activity of LDH,

which is released by damaged cells. LDH is a stable cytoplasmic enzyme and is rapidly released into the cell culture supernatant upon plasma membrane damage. To ensure a cell-free supernatant all samples were centrifuged for 10 min at 500 g before being analysed.

Hippocampal slice cultures [III, IV]

Rat organotypic hippocampal slice cultures (OHSC) were prepared from P9 Sprague-Dawley rats, using the interface method of Stoppini and co-workers [176]. Briefly, rats were decapitated and the hippocampi were rapidly dissected out. Each hippocampus was cut in 400 μm-thick slices using a McIlwain tissue chopper. Slices were transferred to Millicell membranes (Millipore CM, Bedford, USA) and cultured at 36°C in 5% CO_2. OHSCs were cultured in slice medium (50% Basal medium Eagle, 25% Earl's basal salt solution, 23% horse serum, 7.5 mg/ml D-glucose, 1 mM L-glutamine and 25 U/ml penicillin/streptomycin) for 12-14 days before experiments commenced.

Comments: Slice cultures represent an *ex vivo* model suitable for studies of e.g. neurotoxicity and neuroprotection. Using the interface method, hippocampal slices display intact cytoarchitecture for several weeks in culture[177,178].

Ex vivo grafting and differentiation [III]

Rat OHSCs were used for grafting experiments after 7 days *in vitro* (DIV). NSPCs were seeded into 24-well plates, coated with polyornithine, at 5 x 10^4 cells/cm^2 in N2 medium supplemented with 20 ng/ml bFGF. After 24 h, cells were transfected with the eGFP-N1 plasmid, using either ExGen500 or FuGene6. On the following day, the transfected cells were trypsinised, resuspended in N2 medium and seeded onto a defined area over the hippocampal slices, using cloning rings. Four days after grafting, the slices were washed and fixed (4% paraformaldehyde in PBS, 4°C, overnight). Hippocampal slices were pre-incubated for 2 h in PTS buffer (0.1M sodium phosphate buffer, 0.3% Triton X-100 and 1% donkey serum), followed by incubation (4°C, overnight) with primary antibodies. All antibodies in this experiment were diluted in PTS buffer. After five washes in PTS buffer,

hippocampal slices were incubated (4°C, overnight) with corresponding secondary antibodies. Finally, slices were washed five times in PTS buffer and mounted with ProLong Gold (Molecular Probes). Differentiation of grafted NSPCs in OHSCs was analysed using laser confocal microscopy (Leica TCS SP2, Leica Microsystems AG, Wetzlar, Germany). Co-localisation of immunofluorescence and eGFP expression was confirmed using Z-series analysis.

Comments: Acutely after preparation of hippocampal slices a phase of microglial activation commences. The presence of activated microglial in OHSCs has been reported to markedly decrease between 7 and 10 DIV[179]. Meanwhile, astrocytes become activated and GFAP immunoreactivity gradually increases throughout the slice. Coltman *et al* showed that after 10 days in culture, reactive astrocytes were prominent throughout the entire hippocampal slice culture and that astrocytes had formed a dense glial scar around the slice. We chose to perform the *ex vivo* grafting experiment at 7 DIV to limit the effects of activated microglial cells and also to give the grafted cells reasonable conditions for integration due to the relatively thin glial scar observed at this time point.

NMDA-induced excitotoxicity and neuroprotection [IV]

In paper IV, we characterised the cell death in OHSCs exposed to NMDA. Hippocampal slices were cultured in N2 medium with different concentrations of NMDA (in the presence of 2 μM propidium iodide (PI)). After 24 h, OHSCs were washed in PBS and fixed in 4% paraformaldehyde (overnight, 4°C). Subsequently, hippocampal slices were blocked and permeabilised by incubation for two hours in PTS buffer at RT, then incubated overnight (rocking, 4°C) with mouse anti-NeuN antibody, rabbit anti-caspase3A antibody, and goat anti-doublecortin (DCX) antibody. After thorough washing (3 x 30 min in PTS buffer, on a rocker), hippocampal slices were incubated overnight (rocking, 4°C) with donkey anti-mouse Alexa 647-conjugated antibody, donkey anti-rabbit Alexa 488-conjugated antibody and donkey anti-goat Alexa 488-conjugated antibody. In the last step of the staining, the slices were washed thoroughly and mounted using Prolong Gold mounting medium. Co-localisation of PI and/or caspase3A staining with NeuN and DCX immunofluorescence was determined by confocal microscopy (Leica TCS SP2).

To determine the neuroprotective effects of factors secreted from NSPCs, OHSCs were transferred to test media (CM or pentinin) 1 hour before being exposed to 10 μM NMDA for 24 h. The degree of NMDA-induced excitotoxicity was determined by comparing initial PI uptake prior to NMDA exposure (24 h) with that following exposure. Pictures were captured using a digital camera (Olympus DP50, Olympus Inc., Center Valley, PA, USA) coupled to an inverted fluorescence microscope (Olympus IX70), equipped with a red long-pass WG fluorescence filter. Uptake of PI was quantified as the mean pixel intensity of epifluorescence, over the whole slice, or in defined sub-regions (ImageJ v1.29x).

Comments: Propidium iodide is a polar compound that only enters cells with compromised membrane integrity. Inside the cells, PI binds to nucleic acids causing a red fluorescence. The cellular uptake of PI has been widely used as a marker of dead or dying cells in hippocampal slice cultures[180-183]. Propidium iodide is not toxic to neurons and is suitable for long-term exposure[183]. The intensity of PI staining correlates with other methods of determining cell death, including efflux of lactate dehydrogenase, staining for degenerating neurons with Fluoro-Jade and decreases in MAP2 expression[184].

Mass spectrometry [IV]

Samples of medium conditioned by NSPCs (50 μl) were desalted and concentrated using ZipTip™ C18 (Millipore, Bedford, MA, USA) according to the supplier's instructions. Subsequently, the samples were eluted with 3 μl of matrix solution (50 mg/ml 2,5-dihydroxybenzonic acid (Sigma-Aldrich) in acetone:0.1% trifluoric acid in water (4:1 v/v)) directly onto the highly polished, stainless steel, sample probe and left to dry in ambient conditions. The matrix-assisted laser desorption/ionisation (MALDI) analyses were performed using an upgraded Bruker Reflex II instrument (Bruker-Franzen Analytik, Bremen, Germany) equipped with a two-stage electrostatic reflectron, a delayed extraction ion source, a high-resolution detector and a 2 GHz digitizer. The spectra were acquired in reflectron mode. Calibration was performed externally by using a mixture of peptides with known masses. Calibrant peptides were Met-enkephalin, angiotensin II, gamma-MSH, ACTH 18-39, mellitin and insulin (Sigma-Aldrich).

Comments: Mass spectrometry (MS) has emerged as the most accurate and sensitive method in the analysis of peptides and proteins. It provides accurate molecular mass measurements of low quantities of peptides and proteins as well as amino acid sequence information essential for protein identification using databases. The MS analysis of the conditioned medium was performed using a MALDI TOF (time-of-flight) instrument. Advantages of this method include high sensitivity for peptides and proteins and the relative insensitivity to salts, surfactants, and other contaminants. This allows direct analysis of the CM without extensive purification. The MALDI mass spectra are simple to interpret due to the propensity of the method to generate predominantly singly charged ions. However, one drawback is that no sequence information of the peptide/protein required for protein identification can be obtained. In the present study insulin of different origins and subsequently different amino acid sequences was therefore used to identify the insulin cleavage products. An alternative MS approach to identify peptides and proteins in complex mixtures is to use fragmentation. Tandem mass spectrometers have the ability to fragment peptide/protein ions and to record the resulting fragment ion spectra. The peptide/protein is isolated and fragmented and a spectrum containing a pattern more or less unique for the individual peptide/protein is obtained. The fragments are then used to search theoretical fragmentation patterns of peptides/proteins from an *in silico* database.

Electrophysiology [IV]

Electrophysiological experiments were performed on acutely prepared hippocampal slices from 8-16 day-old Wistar rats. The rats were anaesthetised with isoflurane (Abbott) prior to decapitation, in accordance with the guidelines of the local ethical committee for animal research. The brain was removed and placed in an ice–cold solution containing: 124 mM NaCl, 3 mM KCl, 0.5 mM $CaCl_2$, 6 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4 and 10 mM D–glucose. Transverse hippocampal slices (300 μm thick) were cut with a vibratome (HM 650 V, Microm, Germany) in the same ice–cold solution and they were subsequently stored in artificial cerebrospinal fluid (ACSF) containing: 124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 0.5 mM ascorbic acid, 3 mM myo–inositol, 4 mM D,L–lactic acid, and 10 mM D–glucose. A

surgical cut was made between the CA3 and CA1 of the hippocampus. After at least 1 h of storage at 25°C, a single slice was transferred to a recording chamber where it was kept submerged in a constant flow (~2 ml min^{-1}) at 30–32°C. The perfusion solution contained: 124 mM NaCl, 3 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 10 mM D–glucose. All solutions were continuously infused with 95% O_2 and 5% CO_2 (pH ~7.4). Picrotoxin (100 μM, Sigma-Aldrich) was always present in the perfusion solution to block $GABA_A$ receptor-mediated activity. D-2-amino-5-phosphonopentanoate (D-AP5, 50 μM, Ascent Scientific, Weston-Super-Mare, UK) was used as a positive control of NMDA receptor antagonism.

Excitatory postsynaptic currents (EPSCs) were recorded from visually identified CA1 pyramidal neurons using whole-cell patch-clamp recordings, holding the cell at +40 mV. The pipette solution contained: 130 mM Cs–methanesulfonate, 2 mM NaCl, 10 mM HEPES, 0.6 mM EGTA, 5 mM QX–314, 4 mM Mg–ATP and 0.4 mM GTP (pH ~7.3 and osmolality 270–300 mOsm). Patch pipette resistances were 2.5-6 MΩ. EPSCs were recorded at a sampling frequency of 10 kHz and filtered at 1 kHz, using an EPC–9 amplifier (HEKA Elektronik, Lambrecht, Germany). Series resistance was monitored using a 5 ms 10 mV hyperpolarising pulse and it was not allowed to change more than 10%, otherwise the recording was not included in the analysis. Schaffer collateral afferents were activated in stratum radiatum at 0.2 Hz using biphasic constant currant pulses (200 μs + 200 μs, 30-50 μA) delivered through tungsten wires (resistance ~0.5 MΩ, STG 1004, Multi Channel Systems MCS Gmbh, Reutlingen, Germany).

NMDA EPSCs were measured as the mean amplitude at 30-50 ms after the stimulation artefact. The EPSCs were analysed off-line using custom made IGOR Pro (WaveMetrics, Lake Oswego, OR, USA) software.

Comments: Electrophysiological recordings of evoked NMDA receptor-mediated synaptic responses in CA1 pyramidal cells in acute hippocampal slices were performed to test whether pentinin had any antagonistic effects on NMDA receptors. Several precautions were applied to ascertain that uncontaminated NMDA receptor-mediated responses were measured. $GABA_A$ receptors were blocked using the open channel blocker picrotoxin. To maximize the NMDA receptor response by relieving the voltage-dependent magnesium the CA1 pyramidal cells were voltage-clamped at +40 mV. To avoid contamination of the AMPA receptor-mediated response the NMDA receptor-

mediated response was measured between 30 and 50 ms after the synaptic activation, a time when the AMPA receptor-mediated response has vanished. Finally, using the well-known competitive NMDA receptor antagonist D-AP5 we could establish that our measurements were indeed NMDA receptor-mediated.

The CA3-CA1 synapse is probably the most studied synapse in the brain and it expresses NMDA receptors containing both NR2A and NR2B subunits. The type of synaptic activation used presently will activate not only strictly synaptic NMDA receptors, both also to a certain degree (about 30%), extrasynaptic NMDA receptors, which are mainly NR2B-containing[185]. It should be noted that we followed the NMDA responses for 15 minutes after the application of pentinin. This time is certainly sufficient to detect direct antagonistic effects of the pentinin peptide. However, our analysis cannot exclude that pentinin may cause a reduction of the NMDA response on a longer time span, e.g. by causing internalisation of existing receptors or by inhibiting the delivery or production of new NMDA receptors.

RESULTS

Reactive astrocytes induce astrocytic differentiation of adult NSPCs *in vitro* [I]

The aim of this study was to investigate whether reactive astrocytes could influence the lineage selection of NSPCs *in vitro*, using a mechanical lesion model of reactive astrogliosis. We found that secreted factors from lesioned astrocytes induced astrocytic differentiation of adult NSPCs, as assessed by immunoreactivity for the astroglial markers glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS). The induction of astrocytic differentiation was paralleled by a decrease in immunoreactivity for the immature marker nestin. No significant difference in the number of cells immunoreactive for Map2ab or RIP was observed between the different CM, demonstrating that neither neuronal nor oligodendrocytic differentiation was affected by factors secreted by lesioned astrocytes.

The effect of CM from lesioned astrocytes on astroglial differentiation of NSPCs could be due to increased survival, increased proliferation of restricted progenitors and/or instruction of fate commitment by the NSPCs to an astrocytic lineage. In a series of experiments we were able to demonstrate that factors from lesioned astrocytes did not affect cell survival or proliferation, suggesting that the effects of secreted factors from lesioned astrocytes were primarily mediated by an increased rate of conversion from stem/progenitor cells to astrocytes.

Astrocytes derived from NSPCs participate in glial scar formation *in vitro* [I]

To determine the effects of cell-cell interactions between NSPCs and lesioned astrocytes, a co-culture paradigm was employed. Co-culture of GFP^+ NSPCs with lesioned hippocampal or cortical astrocytes significantly increased the number of $GFP^+/GFAP^+$ astrocytes, when compared with non-lesioned cultures. Interestingly, some of the $GFP^+/GFAP^+$ cells were found in direct contact with the polarised astrocytes bordering

the lesion. These NSPC-derived astrocytes were morphologically similar to the cells along the lesion, suggesting that these cells participated in glial scar formation. No effect on neuronal or oligodendrocytic differentiation was found in NSPCs co-cultured with lesioned astrocytes when compared with non-lesioned astrocytes. Moreover, no difference in the number of mitotic NSPCs was observed between the different co-culture paradigms.

LIF and CNTF are released by reactive astrocytes *in vitro* [I]

In order to investigate the molecular mechanisms behind the observed effects on astroglial lineage selection of NSPCs, Western blot analysis was performed on CM from lesioned and non-lesioned astrocytes. The expression levels of CNTF and LIF, proteins that previously have been associated with reactive astrocytes[30,186,187] and astrocytic differentiation of NSPCs[173,188-190], were determined. In these experiments, we found that both CNTF and LIF were up-regulated in CM from lesioned astrocytes.

Molecular characterisation of the perifocal neocortex early and late after stroke [II]

The aim of this study was to screen for differentially expressed genes in the peri-infarct environment early and late after stroke. Special interest was devoted to gene products that potentially render the post-ischemic brain detrimental or beneficial for regeneration, especially secreted peptide factors with autocrine/paracrine effects. At 3 days post-stroke, using Atlas cDNA macroarray analysis, 61 genes were found to be differentially regulated between the stroke lesioned animals and the non-lesioned controls. Thirty-six of these genes were up-regulated, whereas 25 genes were down-regulated. The categories of genes that were mainly up-regulated after stroke were extracellular cell signalling and communication genes, immune system proteins as well as genes involved in protein turnover. The genes up-regulated in the extracellular cell signalling and communication group were fibroblast growth factor 10, pleiotrophin, transforming growth factor β1 (TGF-β1), TRH and insulin-like growth factor binding proteins 2 and 6. Down-

regulations after stroke were mainly seen in gene products coding for ion channels and transport proteins, receptors as well as modulators, effectors and intracellular transducers. However, two genes in the extracellular cell signalling and communication group, VGF nerve growth factor inducible and early growth response 1, were found to be down-regulated.

At the 14 day time-point, fewer genes were found to be differentially regulated compared to the 3 day time-point. Thirty-eight genes were up-regulated after stroke compared to sham operated animals and 34 of these genes were not found to be differentially regulated after the lesion at the 3 day time-point. Notably, no genes were down-regulated 14 days after stroke. In the extracellular signalling and communication group, six genes were up-regulated at the 14 day time-point. These genes were neuropeptide Y, insulin like growth factor II, pleiotrophin, phosphofructokinase, insulin-like growth factor binding protein 2 and TGF-β1.

Q-PCR analysis of individual samples was used to confirm the induction of selected genes from the cDNA array analysis. TRH gene expression was potently induced at 3 days post-stroke. At the 14 day time-point, mRNA levels of TRH were back to baseline levels in stroke-lesioned animals. Analysis of TGFβ1 gene expression showed a marked induction in stroke-lesioned animals at both 3 and 14 days post-stroke.

Immunohistochemistry was employed to characterise the expression of proTRH, the precursor peptide for TRH, in the peri-infarct cerebral cortex and the corresponding non-lesioned neocortex. Neurons displaying proTRH immunoreactivity were found throughout these regions in both sham and stroke-lesioned rats. However, prominent proTRH immunoreactivity was also observed in microglial cells surrounding the infarct.

TRH induces NSPCs to proliferate [II]

In order to assess whether NSPCs had the ability to react to TRH stimulation, we studied the expression of TRH receptors in these cells. Both isoforms of the TRH receptor, TRH-R1 and TRH-R2, were expressed on NSPCs as evidenced by immunocytochemistry. Subsequently, the effects of TRH on NSPC proliferation were investigated using an *in vitro* proliferation assay. Proliferation experiments demonstrated that TRH increased the rate of cell proliferation in a dose-dependent manner. The greatest effect was found at a

TRH concentration of 1 pM. At this dose, TRH had a comparable proliferative effect as the mitogen bFGF (1 nM).

The effects of TRH on proliferation were further confirmed by determining the fraction of NSPCs expressing the mitotic marker pHH3. These experiments showed that 1 pM TRH markedly increased the fraction of pHH3-expressing cells, when compared with control cultures.

Development of an efficient method of non-viral transfection in adult NSPCs without affecting survival, proliferation or differentiation [III]

The aim of this study was to develop an efficient method of gene transfer in NSPCs that did not affect cellular viability, proliferation or differentiation. Initially, six non-viral agents were tested for their ability to transfect NSPCs. Two of these agents, ExGen500 and FuGene6, achieved promising transfection efficiencies and were chosen for further optimisation. After optimisation, ExGen500 transfected 16% and FuGene6 transfected 11% of cultured NSPCs. The toxicity of the two agents was then assessed using the LDH assay. This analysis demonstrated that FuGene6-treated cells did not differ from untransfected cells in their viability, whereas LDH release was significantly increased in ExGen500-treated cells. A similar pattern was observed when cellular proliferation was analysed, where FuGene6-transfected cells did not show a significant reduction when compared with untransfected cells, whereas [^{3}H]thymidine incorporation was significantly reduced in ExGen500-transfected cultures.

A key feature of NSPCs is their ability to differentiate into the three lineages of the CNS: neurons, astrocytes or oligodendrocytes. For this reason, it was important to determine whether non-viral transfection would affect cellular differentiation. Cell-count data showed that the number of differentiated cells (of the three neural lineages) was not significantly different between untransfected, ExGen500- and FuGene6-transfected cells under any of the assayed conditions.

Factors secreted by NSPCs protect neurons against excitotoxicity [IV]

The aim of this study was to analyse whether NSPCs secrete factors with neuroprotective properties. A model of NMDA-induced excitotoxicity in hippocampal slice cultures was employed to investigate neuroprotection. Hippocampal slices were cultured in medium conditioned by NSPCs in the presence of different concentrations of NMDA, together with the cell death marker PI. Exposure to 10 μM NMDA caused a greater than three-fold increase in PI staining. Incubation in medium conditioned by NSPCs led to a marked reduction in NMDA-induced PI staining. This protection against excitotoxicity was demonstrated in all hippocampal regions. However, regional differences were observed and the most effective neuroprotection was found in the dentate gyrus.

NSPCs release pentinin, a novel neuroprotective peptide [IV]

Analysis of CM by mass spectrometry indicated the presence of a pentameric peptide fragment (YTPKT), which corresponded to residues 26-30 of the insulin B chain, and which we termed pentinin. The peptide is a putative breakdown product of insulin, which was contained in the culture medium. The degradation of insulin and the subsequent formation of pentinin can be catalysed by insulin degrading enzyme and this enzyme was expressed in NSPCs.

We tested the neuroprotective properties of pentinin by adding the synthetic peptide to unconditioned medium in the excitotoxicity model. A dose response assay showed that 100 pM gave the highest level of neuroprotection. This dose revealed a potent protective effect of neurons exposed to 10 μM NMDA. In order to determine the cell types that were protected by pentinin, we fixed the hippocampal slices and performed immunofluorescence for markers of neuronally committed progenitors (DCX) and neurons (NeuN). Cells in the dentate gyrus were counted, and the percentage of cells double-labelled for PI and each marker was determined. We found that the percentages of PI-incorporating mature and immature neurons after NMDA-induced toxicity were markedly reduced in the presence of pentinin.

DISCUSSION

Specific aspects of presented findings

Interactions between reactive astrocytes and NSPCs

Reactive gliosis is a significant process in virtually all CNS pathologies, including ischemia. In paper I we examined whether the lineage selection of NSPCs was altered when these cells were exposed to reactive astrocytes, since NSPCs migrate to sites where reactive gliosis is prominent.

The results demonstrate that reactive astrocytes induced astrocytic differentiation of NSPCs *in vitro*, without affecting neuronal or oligodendrocytic lineage selection. This induction was primarily mediated by secreted factors from the reactive astrocytes and was seemingly a result of increased maturation of undifferentiated NSPCs into astrocytes.

Two proteins, CNTF and LIF, were up-regulated in CM from lesioned astrocytes. It is likely that these two proteins contribute to the astrocytic lineage selection of NSPCs observed in this study, since both CNTF and LIF previously have been demonstrated to potently induce astroglial differentiation of NSPCs[173,188-190].

The findings in paper I provide a mechanism for astroglial differentiation of NSPCs following brain injury[91,191,192]. In a previous study, Gage *et al* (1995) demonstrated that hippocampal NSPCs that were grafted into the adult rat dentate gyrus showed a clear localisation in the area damaged by the grafting procedure and that many of these cells displayed a glial phenotype[87]. Neuronal differentiation was only observed in undamaged regions of the dentate gyrus and no glial differentiation was observed in these areas. One explanation to these findings could be that reactive astrocytes induced astroglial differentiation of NSPCs in the damaged areas. This hypothesis is supported by a recent study where NSPCs were transplanted into the adult brain following a cortical impact injury. Cells grafted to the ipsilateral side differentiated into both neuronal and astroglial cells, whereas only neuronal differentiation was observed on the contralateral side[193].

The presence of NSPC-derived astrocytes in direct contact with morphologically similar polarised astrocytes along the lesion suggested that these cells participated in glial scar formation *in vitro*. It has been hypothesised that NSPCs from the SVZ could participate in

formation of the astrocytic scar following a lesion of the fimbria fornix[194]. Furthermore, ependymal cells can generate migratory cells that differentiate into astrocytes and participate in glial scar formation following spinal cord injury, however these cells do not display multipotency *in vivo*[195]. Recently, astrocytes derived from NSPCs were found in the glial scar after transplantation into the injured spinal cord. These cells were suggested to modulate the scar, making it more permissive for regeneration[196]. Furthermore, grafted neural stem cells have also been found to attenuate reactive gliosis in the brain (Evan Snyder, personal communication).

The majority of $GFAP^+/GFP^+$ cells were found in contact with non-lesioned astrocytes, suggesting that NSPC-derived astrocytes may have functions other than participating in glial scar formation. *In vivo*, newborn astrocytes (derived from migrating NSPCs or from glial progenitors present in the lesioned area) could contribute to an injured area in several ways. For example, following an ischemic lesion they could: 1, replace lost or injured cells; 2, provide trophic support to increase cell survival in the peri-infarct region; 3, maintain ion homeostasis; and, 4, induce and/or stabilise synapses. Activation of astrocytes has also been suggested to underlie the ectopic migration of neuroblasts from the SVZ towards damaged brain regions[72]. Furthermore, rats housed in an enriched environment following focal ischemia, previously shown to improve function after an ischemic lesion, displayed increased numbers of reactive astroglia in the peri-infarct region suggesting beneficial effects of these newborn reactive astrocytes[197]. Whether lesion-induced astrocytic differentiation of NSPCs should be seen as aberrant lineage selection or as a means to increase survival and regeneration is still to be determined.

The findings in paper I also highlight the potential problems of unwanted differentiation if NSPCs are grafted into sites of reactive gliosis. An example of this is cell-based therapies in which the aim is to produce new neurons from grafted stem/progenitor cells. In this scenario, it might be preferable to use progenitor cells with a high degree of lineage commitment in order to avoid non-neuronal lineage selection.

Molecular characterisation of the peri-infarct neocortex

In paper I we investigated the interactions between NSPCs and reactive astrocytes, an important factor in the post-ischemic environment. These experiments were followed by

an unbiased molecular screening paradigm in paper II, aiming to characterise the peri-infarct neocortex following MCAO. Special interest was devoted to identifying factors that may render the region around an ischemic lesion permissive to plasticity and regeneration. Factors with these properties would represent interesting pharmacological targets for the treatment of stroke.

Most gene expression studies of the post-ischemic environment have focused on the initial 24 h period where gene changes are likely to reflect the extensive tissue damage that takes place around this time point. We were interested in later time-points, 3 and 14 days, which coincide with structural plasticity and the activation/recruitment of NSPCs[37,198,199].

Using cDNA array analysis, considerably more genes were found to be differentially regulated at 3 days than at 14 days post-stroke. Furthermore, the pattern of expression changes was different. Interestingly, 11 genes in the extracellular cell signalling and communication group were differentially regulated after stroke. Several of these factors could be involved in modulating the endogenous response of plasticity and regeneration after an ischemic lesion in the brain.

Q-PCR analysis of individual samples was performed to confirm the results of selected genes in the cDNA array analysis. Genes were chosen on the basis of their potential involvement in lesion-induced proliferation and neurogenesis. The Q-PCR analyses demonstrated that the expression of both TGF-β1 (3 and 14 days post-stroke) and TRH (3 days post-stroke) was markedly up-regulated after MCAO.

Previous studies have shown that TGF-β1 is induced after an ischemic lesion in the brain[200-203] and administration of the related factor TGF-α reduces stroke damage[204]. Interestingly, TGF-β1 has also been shown to modulate neural progenitor cell proliferation and neurogenesis[205-207].

Apart from direct effects on the NSPC population, TGF-β1 may exert indirect effects through influencing the environment of newly-born recruited immature cells. Indeed, TGF-β1 has been shown to have pleiotrophic functions in the CNS, including anti-inflammatory and neovascularisation-promoting effects[208,209], cell survival-promoting actions[210], stimulation of neuronal process outgrowth[211] and extracellular matrix molecules synthesis[212].

Stroke-induced TRH expression has not previously been demonstrated using a robust quantitative method. However, a previous DNA array study lists TRH among the genes

up-regulated 24 h after ischemia[213], suggesting that TRH up-regulation lasts at least for several days. Interestingly, our results indicate that microglial cells are involved in the up-regulation of TRH expression after ischemia.

Exogenous administration of TRH, TRH analogues or precursor peptide ameliorates the reduced local cerebral blood flow and glucose utilisation in peri-infarct areas, reduces infarct volume and improves functional recovery after stroke[214-216].

The expression of TRH followed the general pattern of lesion-induced proliferation with up-regulation in the sub-acute phase and attenuation in the chronic phase post-stroke[217-219], suggesting that this factor could be involved in mediating this process. However, effects of TRH on structural plasticity and regeneration have not been reported.

TRH stimulates proliferation in NSPCs

Induction of NSPC proliferation is a novel role for TRH in the brain. Interestingly, a recent study has shown that the TRH peptide is present in adult hippocampal stem/progenitor cells[122]. This suggests that TRH may act as an autocrine or paracrine modulator of NSPC proliferation.

Three main intracellular pathways are activated when TRH binds to its receptors, mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and activation of cAMP-responsive element binding protein (CREB). All of these pathways have previously been shown to be important for proliferation in NSPCs[220-222].

Several studies have shown that TRH promotes neuroprotection after various lesions, including ischemia[215,223]. It is possible that some of the beneficial effects of TRH are mediated by changing the dynamics of NSPCs. Yonemori *et al* administered a TRH-analogue, JTP-2942, one week after occlusion of the MCA and demonstrated long-lasting improvement of the motor and neurologic deficits[223]. Interestingly, the beneficial effects of JTP-2942 were observed four weeks after cessation of drug administration. Furthermore, no difference was observed in ipsilateral cerebral shrinkage between the MCA-occluded groups. These findings are supported by another observation of delayed functional recovery after cessation of TRH administration[224]. This delayed effect on functional recovery could be related to the activation of NSPCs, leading to neuronal regeneration and/or modulation of the post-ischemic environment.

Several factors which stimulate NSPC proliferation are induced after a lesion in the brain. These factors include IGF-1, VEGF and bFGF[199,225]. It has been suggested that diffusible factors produced in the ischemic area of the brain can induce proliferation of NSPCs in both the SVZ and the hippocampal dentate gyrus[199]. It is possible that TRH exerts similar effects, potentially together with other factors since lesion-induced proliferation could be controlled by the synergistic action of several mitogens.

Given that TRH displays neuroprotective properties[227], this peptide may have beneficial effects on the survival of recruited immature cells. Indeed, we have found that TRH protects NSPCs against hypoxia *in vitro*. This suggests that TRH can promote the survival of both mature and immature cells in the peri-infarct region.

Since we have found that TRH can promote both proliferation and cell survival in NSPCs *in vitro*, we hypothesise that this peptide is involved in both the activation and protection of NSPCs following a lesion in the brain.

Efficient non-viral gene transfer in NSPCs

Factors that have both neuroprotective and regenerative properties represent interesting targets for the treatment of stroke. We demonstrate that TRH displays both these properties and treatment after stroke with TRH or a TRH-analogue may hold promise for the future. However, TRH has many central functions and side effects can be prominent following systemic administration of TRH[228,229]. Local administration of TRH may therefore be preferable in a clinical setting. An interesting delivery protocol for factors with these properties would be to use genetically engineered cells. A paradigm of this sort could combine local delivery of TRH, for example, with potential effects on functional improvement mediated by the grafted cells[91,230].

We therefore wanted to develop efficient methods for non-viral transfection of NSPCs. Such methods could be used to analyse molecular mechanisms in these cells and to create a foundation for the use of NSPCs in neuroprotective gene therapy. Genetically engineered NSPCs are suitable for cell-based gene therapy strategies, since these cells possess many of the characteristics desired in such graft cells, including ability to survive, migrate and integrate in the injured brain, without causing tumour formation[231]. Furthermore, NSPCs have immunological characteristics (low levels of MHC class I and

undetectable MHC class II expression) that make them suitable for allogenic transplantation[232].

Low levels of transfection with non-viral vectors have limited the analysis of neural progenitor cells by transgene expression[110]. The protocols developed in paper III represent substantial improvements in the efficiency of non-viral transfection of NSPCs.

In many applications it is essential that the method of gene transfer in itself does not alter the characteristics of the transfected cells. Therefore, it was important that these higher transfection rates were not associated with perturbed differentiation, toxicity or reduced proliferation. The findings in paper III show that both ExGen500 and FuGene6 efficiently transfected NSPCs without affecting the pattern of lineage selection. This is in contrast to some viral vectors, which can have profound effects on neural progenitor cell fate[109,166].

Cells treated with FuGene6 showed no reduction in viability or rate of proliferation. Thus, the choice of ExGen 500 over FuGene 6 would only be appropriate if achieving a higher transfection efficiency outweighed the disruption of these growth characteristics.

These methods can be used, and represent useful tools, to study the effects of transgene expression on lineage selection and injury responses in cultured NSPCs as well as to yield mechanistic data on these effects. In addition, the findings in paper III provide a foundation for future gene therapy strategies using NSPCs. Although the transfection efficiencies achieved in this thesis would not be suitable in all scenarios, transient expression from a sub-population of grafted cells is well suited to strategies in which engineered cells secrete factors that provide cellular protection to endogenous and grafted cell populations. However, if higher percentages of transfected cells are needed, enrichment of transgene-expressing cells can be achieved by FACS sorting.

We intend to use these methods of non-viral transfection to induce the expression of secreted TRH in NSPCs. Non-viral transfection, using ExGen500 or FuGene6, would allow the production of genetically-engineered graft cells, capable of generating the three neural lineages, which may be applied in a clinically-compatible model of neural repair using TRH.

NSPCs reduce excitotoxicity via the novel neuroprotective peptide pentinin

Studies have suggested that NSPCs may influence the outcomes of pathological events by other means than cell replacement, e.g. through the secretion of growth factors[233,234]. This could be an important feature of recruited endogenous NSPCs, as well as for grafted NSPCs, potentially modulating the environment around a lesion. However, in areas where NSPCs reside in close proximity to neurons, such as the dentate gyrus, these endogenous factors could contribute to local neuroprotection.

In paper IV, we examined the influence of factors produced by adult NSPCs on NMDA-induced excitotoxicity, one of the most important mediators of cell death after ischemia[13], in hippocampal slice cultures. We found that medium conditioned by NSPCs provided a significant degree of neuroprotection, and indeed completely abolished NMDA-mediated cell death in the dentate gyrus.

Mass spectrometric analysis of CM demonstrated that the NSPCs cleave insulin, resulting in a truncated form of the protein and the C-terminal pentapeptide, which we termed pentinin.

We hypothesised that pentinin may have neuroprotective properties through analogy with the tripeptide GPE, an N-terminal peptide of IGF-1, which protects neurons in different disease paradigms[235-237]. In addition, a C-terminal peptide of mechano-growth factor, a splice variant of IGF-1, also has neuroprotective properties in a NMDA/OHSC model, as well as *in vivo*[238].

Indeed, pentinin displayed neuroprotective properties and treatment with 100 pM of the peptide resulted in a potent reduction of NMDA-induced excitotoxicity in both mature and immature neurons.

Pentinin may be produced *in vitro* by the cleavage of insulin and we demonstrated that NSPCs express insulin degrading enzyme (IDE). This enzyme is known to produce this pentapeptide as a breakdown product[239]. IDE has been identified in several subcellular locations, but is primarily cytosolic. However, membrane-bound and secreted forms of IDE have been reported in studies performed *in vitro* [240,241].

Insulin processing usually occurs in endosomes, as part of insulin receptor recycling. Although a proportion is fully degraded by lysosomes, both intact insulin and fragments are secreted by diacytosis[242]. Interestingly, it has also been reported that insulin B chain

lacking these five residues is fully active, *in vitro*[243], suggesting that both fragments may have a role, although it should be noted that IDE further cleaves the B chain to make smaller fragments.

The expression of IDE is not unique to NSPCs. IDE is in fact expressed throughout the body, with its highest expression in testes, tongue and brain[244]. However, it remains unknown whether the processing of insulin fragments (e.g. lysosomal degradation versus secretion), especially pentinin, is differentially regulated. Interestingly, the expression of IDE, in muscle and liver, is high during development and gradually declines with age[245].

The molecular mechanism of the observed neuroprotection was investigated using electrophysiological recordings of excitatory neurons in the hippocampus. These recordings showed that pentinin does not have a direct antagonistic effect on NMDA-signalling, indicating that the actions of pentinin are downstream of the receptor.

Recent studies have demonstrated that NMDA-receptors containing the NR2A subunit promote cell survival upon activation, whereas activation of NMDA-receptors with the NR2B subunit results in excitotoxicity[226]. This difference could relate to coupling of the two subunits to different intracellular proteins[246,247]. In this way, a similar influx of Ca^{2+} through the receptor could activate different systems and give diverse outcomes. It is possible that pentinin interferes with the intracellular activation of proteins after NMDA-receptor stimulation, either by potentiating NR2A-mediated cell survival or by inhibiting NR2B-mediated cell death. Other proteins that pentinin potentially could interact with to reduce cell death after an excitotoxic lesion include nNOS, PARP-1, AIF and CREB.

Effects on these systems could be mediated by a membrane-bound receptor. It is possible that pentinin can bind to insulin- or IGF-receptors and in this way promote cell survival. However, if the effects of pentinin are mediated by insulin receptors, this interaction should be different from the actions of full-length insulin since this protein is present in unconditioned medium.

Interestingly, we recently found that pentinin markedly reduced LDH release in NSPCs following hypoxia *in vitro* (J. Faijerson et al, unpublished results). Robust neuroprotection in different model systems is important to validate pentinin as a target for future drug development.

The results presented in paper IV suggest a new role for NSPCs as modulators of lesions in the brain and imply the presence of a novel neuroprotective factor in the CNS.

Cellular interactions in the post-ischemic environment

The interactions between NSPCs and different components in the post-ischemic brain are likely to be very complex and probably vary depending on several factors, including the extent of damage, the anatomical localisation and the time that has passed after the lesion. This thesis has characterised some of these important interactions independently to understand their relative contributions. The results demonstrate that NSPCs are affected by factors present in the post-ischemic environment, but also that these cells can modulate this environment in a beneficial way. In Figure 4, an integrated view of the findings in this thesis is presented.

An acute interruption of blood flow results in rapid energy depletion and cell death. The infarct core is surrounded by the penumbra and the peri-infarct region. Many different cell types, including astrocytes and inflammatory cells, are activated and recruited to the lesioned area. Furthermore, endogenous or exogenous NSPCs are activated and migrate towards the lesion. This migration is guided by stromal cell-derived factor 1α (SDF-1α), a chemoattractant produced by reactive astrocytes and inflammatory cells in the lesioned environment[73,248]. The recruitment of NSPCs into lesioned areas represents a novel cytoprotective mechanism, providing bystander support to cells and tissues after an insult. These recruited NSPCs give trophic support to injured cells by releasing various protective factors, including pentinin. The presence of such neuroprotective factors may also be important for secondary prevention if a new ischemic lesion occurs.

Reactive astrocytes form a glial scar to separate the lesion from healthy tissue. The formation of this barrier is beneficial in the acute phase[30,249]. However, the glial scar represents a major obstacle for regeneration at later time points[30,249,250]. Therefore, lesion-induced modulation of astrocytes may be an interesting target for future therapeutic interventions. In addition to forming the glial scar, reactive astrocytes release a wide range of molecules. These factors, including CNTF and LIF, mediate glial scar formation and can direct recruited NSPCs towards the astrocytic lineage. Astrocytes derived from recruited cells (NSPCs or glial progenitors) participate in the formation of the glial scar, potentially altering its properties[196]. However, whether incorporation of NSPC-derived astrocytes in the glial scar is beneficial or detrimental for regeneration remains to be established.

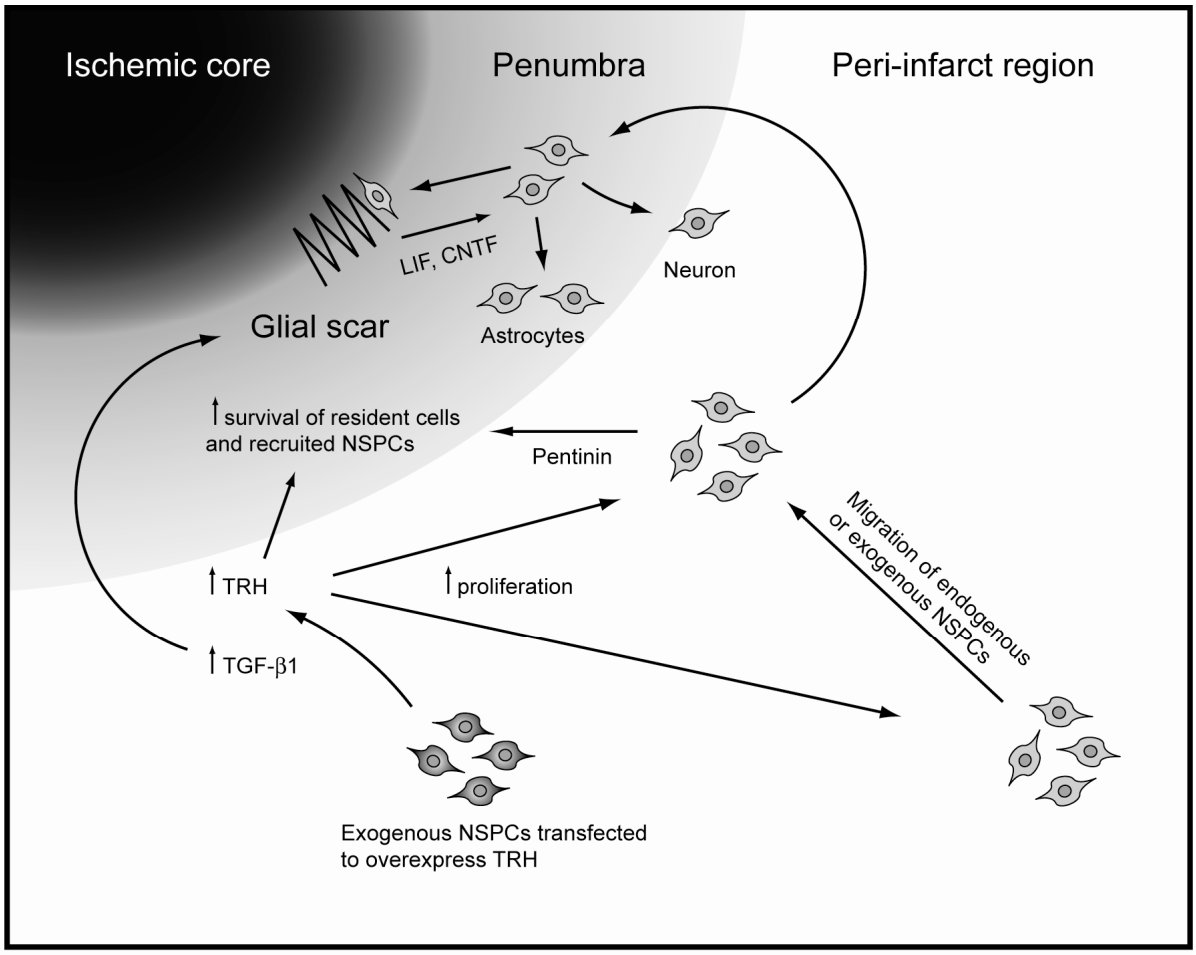

Figure 4. An integrated view of the main findings in the thesis studies. Briefly, neural stem/progenitor cells (NSPCs) are activated after a stroke and migrate towards the lesion. Secreted factors in the post-ischemic environment induce proliferation and astrocytic differentiation of the cells. Furthermore, recruited cells secrete neuroprotective factors, including pentinin, which promote the survival of both resident cells and recruited NSPCs. Cellular survival and regeneration may be further enhanced by grafting NSPCs genetically engineered to secrete TRH.

Besides involvement in the glial scar, NSPC-derived astrocytes could also promote cell survival, synapse formation, cellular migration and homeostasis (e.g. by regulating extracellular K^+ and glutamate concentrations).

The expression of TRH is dramatically increased around the lesion and promotes the survival of both resident and recruited cells. In addition, TRH promotes regeneration by stimulating the proliferation of migrating NSPCs and resident stem/progenitor cells in the SVZ. Furthermore, TRH could potentially also be involved in mediating lesion-induced proliferation in the hippocampal dentate gyrus.

Cellular survival and regeneration may be further enhanced by grafting NSPCs genetically engineered to secrete TRH. In addition to promoting the survival of injured cells around

the lesion, TRH can also increase the survival and proliferation of the grafted cells in an autocrine manner. Moreover, grafted cells could improve plasticity and participate in cell replacement in addition to secreting TRH.

Microglial cells are involved in the lesion-induced up-regulation of TRH. Furthermore, microglia have been reported to secrete IDE *in vitro*, suggesting that the neuroprotective peptide pentinin could be produced from insulin in areas populated by these cells. However, activated microglial cells also secrete pro-inflammatory cytokines and have been shown to impair neurogenesis[251,252]. This indicates that microglial activation is a complex phenomenon and could result in either promotion or inhibition of regeneration, potentially depending on the severity of the insult.

TGF-β1 expression is also increased and can be secreted by several different cell types after ischemia, including reactive astrocytes, neurons and inflammatory cells[202,253]. The expression of this cytokine stimulates glial scar formation and modulates the inflammatory response[254,255]. Furthermore, secreted TGF-β1 could be involved in promoting cell survival and regulating regeneration in the post-ischemic environment.

Common features of insults in the CNS

Although the focus of this thesis has been to investigate the interactions between NSPCs and the post-ischemic environment, the results may also have implications on other insults in the CNS. Practically all lesions and diseases in the brain are accompanied by an activation of astrocytes. This coincides with the activation of endogenous NSPCs, indicating that astrocytic differentiation of immature cells induced by reactive astrocytes might be common after brain insults. Incorporation of NSPC-derived astrocytes in the glial scar may modulate the permissiveness of regeneration after various lesions. Furthermore, reactive astrocytes have been observed in proximity to the neural stem cell niches in the brain[256,257], implying that reactive gliosis could affect both recruited and resident NSPCs. Interestingly, neural progenitor cells in the dentate gyrus have been shown to generate increased numbers of astrocytes following global ischemia and temporal lobe epilepsy[67,258].

Several studies have demonstrated that TRH expression is increased after traumatic lesions to the brain or spinal cord[259-261]. The dual role of this peptide with effects on both cellular survival and regeneration is also interesting in the treatment of these insults. Furthermore, local TRH treatment by genetically engineered NSPCs may be preferable over systemic pharmacological therapy following traumatic lesions in the CNS.

In addition to its important role in ischemia, excitotoxicity is also important in other lesions such as trauma and epilepsy[262,263]. Seizures in the hippocampus are common in epilepsy and local cleavage of insulin yielding pentinin may attenuate seizure-induced excitotoxicity. Furthermore, excitotoxicity has been linked to the degeneration of neurons in Parkinson's, Alzheimer's and Huntington's disease[264-267], suggesting that factors like pentinin could modulate pathology also in these diseases.

Interestingly, IDE seems to have implications for Alzheimer's disease (AD). Progressive accumulation of amyloid β-protein (Aβ) is an early and invariant feature of AD. IDE potently degrades Aβ and reduces the extracellular concentration of $A\beta_{40}$ and $A\beta_{42}$ *in vitro*[240,241]. The expression of IDE in the hippocampus is significantly reduced in AD patients carrying a mutation in the apolipoprotein-E gene[268]. In addition, a marked reduction in IDE expression was found in a transgenic mouse model of the disease[269].

Attenuation of IDE expression in transgenic mice decreases the degradation of Aβ and insulin, in brain and liver respectively, suggesting that IDE hypofunction may underlie or contribute to some forms of Alzheimer's disease and type 2 diabetes mellitus[270]. Together, these results indicate that induction of IDE function, by drug treatment or gene therapy, may be an attractive treatment paradigm for both AD and type 2 diabetes mellitus.

Future clinical perspectives

Despite intensive research, all clinical trials of neuroprotective drugs for the acute treatment of stroke have been unsuccessful and therefore the interest in different aspects of neural plasticity has increased. The concept of brain plasticity include not only synaptogenesis and dendritic branching, but also neurogenesis[38]. The discovery of ongoing neurogenesis and the dynamic regulation of multipotent NSPCs in the adult mammalian brain have opened up new possibilities for therapy in the lesioned brain.

These strategies are generally based on either stimulation of endogenous NSPCs or grafting of exogenous cells. Activation of endogenous NSPCs by the administration of stimulatory factors is very appealing, partly because of its relatively non-invasive nature. Several factors have been demonstrated to promote regeneration following experimental ischemia, resulting in reconstitution and functional recovery. These factors include granulocyte-colony stimulating factor (G-CSF), bFGF, EGF and erythropoietin (EPO) [81,271,272].

G-CSF is a very interesting factor with actions on both haematopoiesis and neurogenesis. Treatment with this factor has resulted in functional improvements in different experimental paradigms of ischemia and clinical trials using G-CSF have recently been initiated (Wolf-Rüdiger Schäbitz, personal communication).

However, prolonged administration of growth factors is associated with risks, such as tumour formation, ectopic neurogenesis and increased cell loss after brain insults. Sustained intracerebroventricular administration of EGF has been shown to induce pronounced hyperplasia in the ventricular wall[273]. Furthermore, signalling through the EGF-receptor can result in the formation of malignant brain tumours[274]. Brain derived neurotrophic factor (BDNF) can increase neuronal excitability and infusion of this factor gives rise to ectopic hippocampal neurogenesis which might contribute to seizure development[275,276]. Moreover, delivery of BDNF and GDNF can exacerbate neuronal cell loss after ischemia[277,278].

Thus, it seems that local administration of growth factors during a limited time period is preferable in a clinical setting. Local and transient administration could be achieved through a cell-based gene therapy paradigm using the non-viral transfection protocols developed in this thesis.

Grafting of NSPCs or other immature cell populations, such as bone marrow stromal cells, into the brain can improve functional deficits after an ischemic lesion[91,230,279]. Cells are recruited to the ischemic area, but relatively few cells differentiate into mature cells. The observed functional improvements have therefore been attributed functions other than cell replacement from the grafted cells. These other functions include trophic effects of the immature cell populations, leading to increased cell survival of endogenous cells and improved plasticity. We hypothesise that the secretion of trophic factors is the most important role for activated NSPCs following a lesion in the brain. In addition, a novel

neuroprotective peptide, pentinin, was discovered to be released by NSPCs in Paper IV. This peptide may be important for the protection of endogenous cells following insults in the brain. Furthermore, pentinin is an interesting candidate for drug development and ongoing studies are aimed at determining the efficacy of treatment with this peptide after experimental focal ischemia.

Grafting of NSPCs, or pharmacological treatment with factors secreted from these cells, may be a viable strategy for future treatment of stroke patients. In addition, grafted cells may be genetically engineered to enhance the secretion of protective factors such as TRH, potentially improving the efficacy of treatment.

CONCLUSIONS

The results of this thesis demonstrate that reactive astrocytes induce astrocytic differentiation of NSPCs, and that these cells can participate in glial scar formation *in vitro*. Moreover, we have identified several interesting genes, including TRH, that are up-regulated in the peri-infarct region after stroke and that TRH is a regulator of NSPC proliferation. In order to further explore the effects of these factors in NSPCs, as well as to create a foundation for gene therapy after ischemia, we developed an efficient method for non-viral transfection. We also characterised the neuroprotective effects of factors secreted from NSPCs and identified the novel protective peptide pentinin.

Responses to given aims:

I. Secreted factors from reactive astrocytes induced astrocytic differentiation of adult NSPCs *in vitro*. This induction was not due to changes in cell survival or proliferation, suggesting that the effect was primarily mediated by an increased rate of conversion from stem/progenitor cells to astrocytes. These findings suggest a new mechanism for astroglial differentiation of NSPCs following an insult in the brain.

II. NSPC-derived astrocytes were demonstrated to participate in glial scar formation in an *in vitro* model of reactive astrogliosis.

III. Several genes coding for cell signalling molecules, including TRH, were identified by cDNA array analysis to be up-regulated in the peri-infarct region after focal ischemia.

IV. TRH was demonstrated to potently induce proliferation in cultured NSPCs. This suggests that TRH may be involved in inducing NSPC proliferation after an ischemic lesion. TRH can also promote cell survival, making it an interesting target for future therapeutic interventions.

V. An efficient method of non-viral transfection of NSPCs was developed. This method did not affect viability, proliferation or differentiation of the cells, and creates a foundation for future gene therapy interventions after experimental stroke.

VI. Factors secreted by NSPCs displayed neuroprotection after NMDA-induced excitotoxicity. We identified a novel neuroprotective peptide, pentinin, which displayed potent protective properties and reduced NMDA-induced cell death in both immature and mature neurons.

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