The effect of astrocytes and reactive gliosis on neurogenesis and astrogenesis in mice

Åsa Widestrand

Center for Brain Repair and Rehabilitation
Department of Clinical Neuroscience and Rehabilitation
Institute of Neuroscience and Physiology
Sahlgrenska Academy
University of Gothenburg
Göteborg 2008

Cover illustration: A neurosphere (green; EGF-receptor, blue; cell nuclei) in the light of an astrocyte (yellow).

Åsa Widestrand
Tryck: Geson, Göteborg 2008
ISBN: 978-91-628-7449-0

Till min familj

THE EFFECT OF ASTROCYTES AND REACTIVE GLIOSIS ON NEUROGENESIS AND ASTROGENESIS IN MICE

Abstract

Astrocytes are the most common cell type in mammalian central nervous system (CNS). Glial fibrillary acidic protein (GFAP) and vimentin constitute intermediate filaments (known also as nanofilaments), a part of the cytoskeleton, in astrocytes. In damaged CNS, astrocytes become reactive and increase the expression of GFAP and vimentin and alter the expression of the host of other genes, a process referred to as reactive gliosis. Reactive astrocytes have a neuroprotective effect in neurotrauma or brain ischemia, but they have also been shown to inhibit CNS regeneration. *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice are deficient in astrocyte intermediate filaments and after neurotrauma or in brain ischemia show less prominent reactive gliosis than wildtype mice.

Mild reactive gliosis occurs in the hippocampus of healthy aging individuals, both in rodents and humans. Neurogenesis in the dentate gyrus of the hippocampus is known to decline during life. We assessed the effect of age-related reactive gliosis on hippocampal neurogenesis in *GFAP*$^{-/-}$*Vim*$^{-/-}$ and wildtype mice (Paper I). We found that attenuated reactive gliosis in aged *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice was linked to higher hippocampal cell proliferation and neurogenesis. Our data suggest that age-related reactive gliosis may be a cause for declining neurogenesis in aging brain.

Our research group previously showed that the attenuation of reactive gliosis in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice improved integration of neural grafts. Here, we addressed whether *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes affect neural progenitor cell differentiation in vitro and survival and differentiation of grafted neural progenitor cells in a neurogenic niche in the brain (Paper II). Using cocultures of neural progenitor cells and astrocytes, we found that *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes increased the number of neural progenitor-derived neurons and astrocytes. When adult hippocampal progenitor cells were grafted in the hippocampal region, *GFAP*$^{-/-}$*Vim*$^{-/-}$ recipients showed more graft-derived astrogenesis and neurogenesis. These findings suggest that attenuation of reactive gliosis may be a suitable strategy for enhancing adult neurogenesis and for increasing the efficiency of neural progenitor cell transplantation.

In the next study (Paper III), we found that the baseline and posttraumatic hippocampal neurogenesis and some aspects of learning and memory were improved in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice compared to wildtype controls. Moreover, we provide evidence that astrocytes constitute an important niche for production of new neural cells in the adult hippocampus.

Key words: astrocytes, intermediate filaments, neural stem cells, neurogenesis, astrogenesis, hippocampus

ASTROCYTER PÅVERKAR NERVCELLSBILDNING I HJÄRNAN

Astrocyter, den vanligaste celltypen i hjärnan, får allt mer uppmärksamhet inom forskningen. Astrocyterna har många olika funktioner såsom kontroll av blodflödet, upptag och återvinning av signalsubstanser och induktion och stabilisering av synapser. Tillsammans med kapillärerna i hjärnan bildar astrocyterna blod-hjärnbarriären, vilken förhindrar att många potentiellt farliga ämnen tar sig in i hjärnan. På senare år har man även funnit att astrocyterna har egenskaper av stamceller och kan dela sig och ge upphov till nya nervceller i vissa delar av hjärnan. Vid skador på hjärnvävnaden men även i samband med det naturliga åldrandet aktiveras astrocyterna och blir "reaktiva": bland annat får de tjockare utskott och aktiverar sitt intermediärfilament-system (också kallat nanofilament-system, en del av cellskelettet). Att astrocyter blir reaktiva är viktigt för att begränsa en skada men det kan också innebära att nervvävnaden i det skadade området på lång sikt får sämre möjligheter att återhämta sig.

I hippocampus, en del av hjärnan som är viktig för inlärning och minne, bildas nya nervceller livet ut, även om graden av nybildning är betydligt lägre i den åldrade hjärnan. Hur mycket nya nervceller som bildas, har visat sig vara mycket beroende av miljön som omger stamcellerna. I denna avhandling har vi undersökt betydelsen av astrocyter för neuronala stamceller i hippocampus. Vi har studerat detta i möss vars astrocyter saknade GFAP och vimentin och därmed också intermediärfilament-system. Dessa möss uppvisar mindre grad av astrocytaktivering.

Det är möjligt att åldersrelaterad astrocytreaktivitet kan ge upphov till försämrad hjärnfunktion hos gamla människor. Vi fann att gamla möss som har mindre grad av astrocytaktivering hade mer nybildning av nervceller i hippocampus än vanliga möss. Detta tyder på att åldersrelaterad astrocytreaktivitet har en negativ påverkan på stamceller.

I framtiden kan aktivering av stamceller bli en ny möjlighet att behandla sjukdomar och skador i hjärnan. Vi fann att transplanterade neuronala stamceller i större grad överlevde och differentierade till nervceller och astrocyter i möss som saknade GFAP och vimentin jämfört med vanliga möss. Detta visar att det kan vara fördelaktigt att kontrollera reaktiva astrocyter i samband med stamcellsaktivering och stamcellstransplanatation.

I frånvaro av transplantation eller åldrande har astrocyter stor betydelse för hur mycket nya nervceller som bildas från stamcellerna. Vi fann att nybildningen av nervceller i hippocampus hos möss som saknade GFAP och vimentin jämfört med kontrollmöss var ökad hos unga friska möss och hos möss som experimentellt tillfogats en specifik skada i hippocampus. Vi fann att vissa aspekter av inlärning och minne var bättre i möss som saknade GFAP och vimentin. Våra data tyder på att astrocyter i möss som saknar GFAP och vimentin bidrar till att skapa en miljö som är extra gynnsam för nybildning av nervceller.

Denna avhandling visar att astrocyter spelar en viktig roll för stamceller i hjärnan. Den visar också att reaktiva astrocyter har betydelse för hur transplanterade stamceller integreras och för den åldrande hjärnan. Vi föreslår att astrocyter är en ny måltavla för framtida behandlingsstrategier vid hjärnskada.

This thesis is based on the following papers:

I Increased cell proliferation and neurogenesis in the hippocampal dentate gyrus of old *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice
Åsa Larsson, Ulrika Wilhelmsson, Marcela Pekna and Milos Pekny
Neurochemical Research, 2004 Nov; 29(11): 2069-73.

II Increased neurogenesis and astrogenesis from neural progenitor cells grafted in the hippocampus of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice
Åsa Widestrand, Jonas Faijerson, Ulrika Wilhelmsson, Peter L. P. Smith, Lizhen Li, Carina Sihlbom, Peter S. Eriksson and Milos Pekny
Stem Cells, 2007 Oct; 25(10): 2619-27

III GFAP and vimentin are negative regulators of the hippocampal neurogenic niche
Maryam Faiz*, Åsa Widestrand*, Ulrika Wilhelmsson, Sofia Linde, Peter L. P. Smith, Daniel Andersson, Helena Christianson, Barbora Slaba, Tulen Pekny, Marcela Pekna and Milos Pekny
Manuscript
*this work represented a joint effort of the first two authors

ABBREVIATIONS

BrdU	5-Bromo-2´-deoxyuridine
CNS	Central nervous system
GCL	Granular cell layer
b-FGF	basic-Fibroblast growth factor (FGF-2)
EGF	Epidermal growth factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GS	Glutamine synthase
IF	Intermediate filament
IGF	Insulin-like growth factor
IGFPB	Insulin-like growth factor binding protein
IL	Interleukin
ML	Molecular layer
PBS	Phosphate buffered saline
SGZ	Subgranular zone
Shh	Sonic hedgehog
TNF-α	Tumor necrosis factor-alpha
Vim	Vimentin
Wnt3	Wingless helix protein-3

TABLE OF CONTENTS

ABSTRACT 5
ASTROCYTER PÅVERKAR NERVCELLSBILDNING I HJÄRNAN 6
ABBREVIATIONS 8
TABLE OF CONTENTS 9
INTRODUCTION 11
BACKGROUND 12
Astrocytes 12
Intermediate filaments (nanofilaments) 13
Reactive gliosis 15
Mammalian neural stem cells 17
The hippocampus – a neurogenic niche 20
Possible functional relevance of adult neurogenesis 21
THE AIM OF THE STUDIES 24
MATERIALS AND METHODS 25
The mice (Paper I, II and III) 25
Cell culture and in vitro analyses (Paper II and III) 25
Neural progenitor cell transplantation (Paper II) 30
SDS-PAGE and Western blot analysis of Wnt3 protein (Paper II) 31
Enthorinal cortex lesion (ECL) as a neurotrauma model (Paper III) 31
BrdU labeling and immunohistochemistry (Paper I, II and III) 31
Assessment of cell proliferation/survival in the hippocampus (Paper I, II and III) 34
Behavior studies (Paper III) 35
RESULTS 38
Increased cell proliferation and neurogenesis in the hippocampal dentate gyrus of old *GFAP$^{-/-}$Vim$^{-/-}$* mice (Paper I) 38
Increased neurogenesis and astrogenesis from neural progenitor cells grafted in the hippocampus of *GFAP$^{-/-}$Vim$^{-/-}$* mice (Paper II) 39
GFAP and vimentin are negative regulators of the hippocampal neurogenic niche (Paper III) 41
DISCUSSION 43
Age-related reactive gliosis might reduce adult hippocampal neurogenesis 43
The astrocyte environment is important for the integration of grafted neural progenitor cells 43
GFAP and vimentin as regulators of the neurogenic niche 44
Enhanced neural plasticity in *GFAP$^{-/-}$Vim$^{-/-}$* mice 45
CONCLUSIONS 47
ACKNOWLEDGEMENTS 48
REFERENCES 51

INTRODUCTION

Progress in neuroscience research has greatly challenged the old picture of glial cells. Astrocytes, the most common cell type in mammalian central nervous system (CNS), were previously considered as providers of matrix and metabolic support for the neuronal network. During the last decades, astrocytes have been attributed with new functions. The role of astrocytes in the generation of new neurons has recently been proposed. It was speculated that astrocytes induce neurogenesis from neural stem cells (Song et al., 2002a). Also, astrocytes or cells with astrocyte properties can themselves act as neural stem cells (Laywell et al., 2000; Seri et al., 2001). Astrocytes also modulate formation of synapses as well as take part in neurotransmission.

In damaged CNS, astrocytes become reactive and increase the expression of the intermediate filament (nanofilament) proteins glial fibrillary acidic protein (GFAP), vimentin and nestin, and alter the expression of the host of other genes, a process referred to as reactive gliosis (Eddleston and Mucke, 1993). The formation of a glial scar may be a necessary step to quickly restore CNS homeostasis and to isolate the comparably intact area from the lesioned region (Eddleston and Mucke, 1993; Ridet et al., 1997). But, reactive gliosis may also constitute a physical and biochemical barrier to neuroregeneration in the injured CNS (Ridet et al., 1997; McGraw et al., 2001; Pekny and Pekna, 2004; Silver and Miller, 2004; Pekny and Nilsson, 2005; Pekny and Wilhelmsson, 2006b; Pekny et al., 2007). We previously showed that the presence of intermediate filaments in astrocytes has a major impact on the ability of astrocytes to become reactive and produce glial scars (Pekny et al., 1999). This thesis addresses the role of astrocyte intermediate filaments and reactive gliosis in neurogenesis and astrogenesis from neural progenitor cells in vitro and in vivo in the hippocampus.

BACKGROUND

Astrocytes

Astrocytes are the most common cell type in the CNS. Astrocytes are interconnected via gap junctions into network known as astrocyte syncytium. Embryonically, astrocytes develop from radial glia cells, which function as a scaffold for the migrating neurons and are important for the correct development of the CNS architecture. In the adult CNS, the radial glia can serve as progenitors of astrocytes (Hof, 1999; Merkle et al., 2004) and neurons (Kornblum and Geschwind, 2001; Merkle et al., 2004).

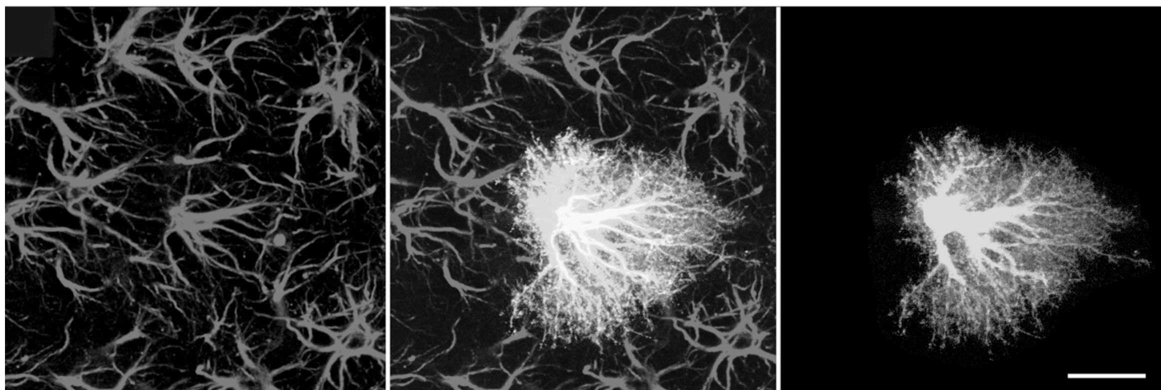

Figure 1. An astrocyte bears a large number of cellular processes and has the appearance of a bush, as demonstrated on this 3D reconstruction picture. Scalebar 20 µm. Picture from (Wilhelmsson et al., 2004).

When visualized with antibodies against GFAP, astrocytes appear as "stars" (Fig. 1, left). However, when filled with dye their "bushy" morphology with a large number of very fine processes is revealed (Fig. 1, right (Bushong et al., 2002; Wilhelmsson et al., 2004)). A human astrocyte can contact over a million neuronal synapses (Oberheim et al., 2006). Astrocytes surround synaptic regions and take up "spilled" neurotransmitters, such as glutamate, and recycle them (Hof, 1999). Astrocytes are important for the maintenance of the CNS homeostasis and are also responsible for induction, maintenance and repair of the blood-brain barrier. The end-feet of the astrocytes cover endothelial cells and induce tight junctions in the endothelial cells that make up the blood-brain barrier. These tight junctions prevent the entry of cells and certain molecules into the CNS. Astrocytes are also important for controlling local blood flow in the CNS by regulating dilation of arteriols (Zonta et al., 2003). Astrocytes have been shown to produce a large number of growth factors and other molecules, which can affect neuronal

subpopulations and their morphology, proliferation and differentiation (Hof, 1999; Barkho et al., 2006). Astrocytes also control neurons more directly by inducing and stabilizing neuronal synapses (Ullian, 2001; Christopherson et al., 2005). Similarly to neurons, astrocytes can release neurotransmitters; for example they release glutamate to increase synaptic strength (Jourdain et al., 2007).

Intermediate filaments (nanofilaments)

The cytoskeleton is composed of three major components: actin filaments, microtubules and intermediate filaments (IFs), known also as nanofilaments. Over fifty IF proteins have been identified, and based on sequence comparison and expression pattern they are divided into six subclasses (Hyder et al., 2008). IFs are built up of alpha-helical subunits that form filaments about 10 nm in diameter. Cells and tissues with missing or abnormal intermediate filaments exhibit structural or physical defects, in particular following stress (reviewed in (Pekny and Lane, 2007)). Such cells and tissues sustain force less well, are unable to adopt and maintain complex shapes, respond less well to swelling, etc. This can lead to dysregulation of other processes, and in many cases can form the basis for more complex patophysiological phenomena (Pekny and Lane, 2007). For example, in the skin, keratin IFs were shown to provide mechanical support and structural integrity to the epidermis and mutations may cause the skin blistering disorder epidermolysis bullosa simplex (Matoltsy, 1975; Bonifas et al., 1991; Pekny and Lane, 2007). Another example are lamin mutations, which are associated with the largest and most diverse number of diseases of any group of human intermediate filament genes, and the diversity ranging from lipodystrophies through cardiomyopathies to premature aging (Pekny and Lane, 2007).

Intermediate filaments in astrocytes

Four different IF proteins are expressed in astrocytes: GFAP, vimentin, nestin and synemin. The expression pattern of these IF proteins depends on the developmental stage and the degree of astrocyte activation (Eliasson et al., 1999; Sultana et al., 2000). Nestin, vimentin and synemin form IFs in astrocyte precursors and in immature astrocytes. In maturing astrocytes, the expression of GFAP increases while that of vimentin decreases and nestin disappears (Schneitzer, 1981; Bignami, 1982; Lendahl et al., 1990). In mature non-reactive astrocytes, GFAP and vimentin are the building blocks of the IFs. In neurotrauma, stroke or neurodegenerative disease, astrocytes become reactive and up-regulate GFAP and vimentin and re-express nestin and some also synemin (Eddleston and Mucke, 1993; Eng, 1994; Lin, 1995; Jing et al., 2007).

	Immature astrocytes	Non-reactive astrocytes	Reactive astrocytes
GFAP	-	+	++
Vimentin	+	+	++
Nestin	+	-	+
Synemin	+	-	+

Table 1. IF proteins expressed in astrocytes.

IFs are dynamic structures and there is an equilibrium between IF protein as soluble subunits and in form of filaments (reviewed in (Pekny and Wilhelmsson, 2006a; Hyder et al., 2008)). Commonly, phosphorylation by various protein kinases on serine and threonine residues at the N-terminal of the subunits induces filament disassembly and prevents filament assembly. This increases the pool of free phosphorylated subunits that can be incorporated in the filaments after dephosohorylation by phosphatases. Intermediate filament reorganization is important for cell motility and cell division (Pallari and Eriksson, 2006). Mutations in the gene encoding GFAP may cause protein accumulation in the CNS leading to Alexander's disease (Brenner et al., 2001).

The mice deficient in astrocyte intermediate filaments: understanding intermediate filament function and reactive gliosis

In order to better understand the function of the astrocyte IFs and of reactive astrocytes, mice deficient for GFAP and/or vimentin have been generated and characterized (Colucci-Guyon et al., 1994; Pekny et al., 1995; Eliasson et al., 1999; Pekny et al., 1999). Eliasson and colleagues examined non-reactive as well as reactive astrocytes in $GFAP^{-/-}$, $Vim^{-/-}$ and $GFAP^{-/-}Vim^{-/-}$ mice (Eliasson et al., 1999). Non-reactive $GFAP^{-/-}$ astrocytes are devoid of IFs since vimentin cannot self-polymerize into IFs. In $GFAP^{-/-}$ reactive astrocytes, vimentin, nestin and synemin together polymerize into IFs in the absence of GFAP. In $Vim^{-/-}$ astrocytes, the IFs are made of self-polymerized GFAP only. GFAP and nestin in $Vim^{-/-}$ reactive astrocytes cannot co-polymerize. In $GFAP^{-/-}Vim^{-/-}$ astrocytes, non-reactive or reactive, polymerized IFs are absent.

	Composition of IFs:		Reactive astrocytes:
Genotype	Non-reactive astrocytes	Reactive astrocytes	IF Amount/Bundling
Wildtype	GFAP, vimentin	GFAP, vimentin, nestin, synemin	Normal/normal
GFAP-/-	No IFs (non-filamentous vimentin)	Vimentin, nestin, synemin	Decreased/normal
Vim-/-	GFAP	GFAP (non-filamentous nestin, traces of synemin)	Decreased/tight
GFAP-/-Vim-/-	No IFs	No IFs (non-filamentous nestin, traces of synemin)	

Table 2. Composition of IFs in astrocytes.

Reactive gliosis

What is reactive gliosis?

Reactive gliosis occurs in CNS trauma, neurodegenerative disorders, viral infections, stroke or tumors. Reactive astrocytes differentially express hundreds of genes compared to non-reactive astrocytes. These include inflammatory cytokines, growth factors and extracellular matrix molecules (Ridet et al., 1997; Fawcett and Asher, 1999). TNF-α, IL-6 and endothelin-1 are examples of genes implicated in astrocyte activation (Eddleston and Mucke, 1993; Hof, 1999; Raivich et al., 1999; Rogers et al., 2003). In reactive gliosis, astrocytes and other glial cells accumulate in the area of injury and this ultimately gives rise to a glial scar.

Normal physiological aging is associated with mild progressive reactive gliosis and increased GFAP production (Goss et al., 1991; Kohama et al., 1995; David et al., 1997). In humans, a strong upregulation in GFAP production was reported after the age of 65 years, in particular in the hippocampus (David et al., 1997).

The most prominent hallmark of reactive astrocytes is the upregulation of GFAP and vimentin. Nestin and synemin are re-expressed by reactive astrocytes (Clarke et al., 1994; Eliasson et al., 1999; Jing et al., 2007). Visualized by antibodies against GFAP, the processes of reactive astrocytes appear to be thicker and more numerous compared to processes of non-reactive astrocytes (Fig. 2, left and middle panels). By dye-filling astrocytes (Fig. 2, right panel), we have shown that reactive astrocytes increase the thickness of their main cellular processes but they do not extend to occupy a greater volume of tissue than nonreactive astrocytes. Thus, reactive astrocytes stay within their tiled domains (Wilhelmsson et al., 2006).

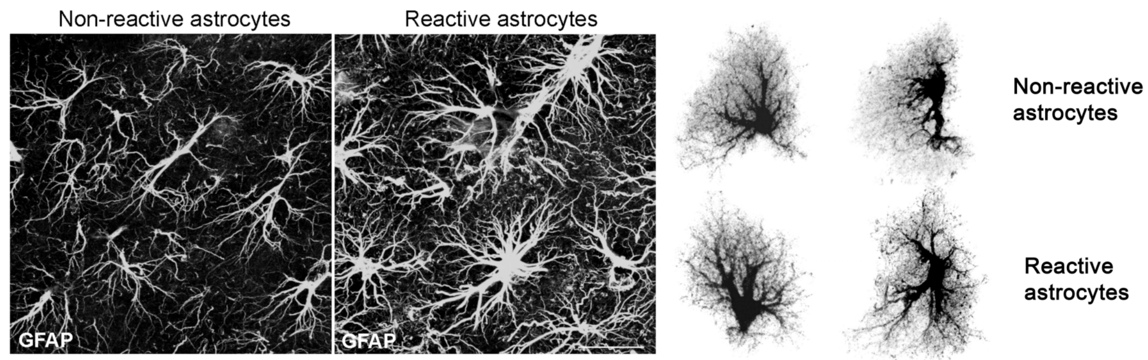

Figure 2. Non-reactive and reactive astrocytes visualized with antibodies against GFAP (left) and dye-filled (right), from Wilhelmsson et al., 2006.

Subpopulations of reactive astrocytes divide and it has been suggested that these dividing astrocytes may arise from endogenous progenitors (Miyake et al., 1988; Alonso, 2005; Buffo et al., 2005; Sofroniew, 2005). However, fate mapping of quiescent astrocytes in the adult brain indicated that these dividing reactive astrocytes derive from mature astrocytes (Buffo et al., 2008).

Reactive gliosis – both friend and foe?

Studies in transgenic mice where reactive astrocytes were ablated show that these cells are essential for spatial and temporal regulation of inflammation after CNS injury (Sofroniew, 2005). Without reactive astrocytes, the lesioned CNS area is separated from the surrounding tissue and inflammation spreads over a larger area, causing secondary damage and increased duration (Faulkner et al., 2004). Reactive gliosis is beneficial during the acute stage after a CNS insult but can severely restrict the functional regeneration (reviewed in (Silver and Miller, 2004; Pekny and Wilhelmsson, 2006b; Pekny and Wilhelmsson, 2006a). The type of injury and localization were shown to have a major influence on the nature of the gliotic scar and on the regenerative response (Ridet et al., 1997; Pekny and Wilhelmsson, 2006a).

Attenuated reactive gliosis in $GFAP^{-/-}Vim^{-/-}$ mice

The Pekny group and collaborators have shown that the IFs are essential for normal formation of glial scars in response to CNS injury (Pekny et al., 1999). Compared to wild-type mice, the *$GFAP^{-/-}Vim^{-/-}$* mice showed prolonged healing after brain or spinal cord injury. The glial scars in these mice were less compact at a time when a dense glial scar formed in the wildtype controls. These findings showed that IF upregulation is an important step in reactive gliosis and that reactive astrocytes are required for proper wound healing.

It was shown that synaptic regeneration after brain injury is improved in *GFAP$^{-/-}$Vim$^{-/-}$* mice (Wilhelmsson et al., 2004). Moreover, the retina of *GFAP$^{-/-}$Vim$^{-/-}$* mice was shown to provide a more permissive environment for the integration of grafted cells, as demonstrated by experiments in which the fate of genetically labeled retinal transplants was followed in the retines of *GFAP$^{-/-}$Vim$^{-/-}$* mice. The transplanted cells migrated, extended processes and sent neurites into the optic nerve to a higher extent in *GFAP$^{-/-}$Vim$^{-/-}$* than in wildtype recipients (Kinouchi et al., 2003).

Mammalian neural stem cells

The concept of neural stem cells

For a long time, the brain was considered a network of neurons where no new neurons were added after birth. This picture of the brain was challenged when J. Altman forty years ago suggested that new neurons were formed also in the adult human brain (Altman, 1962). But how could a new neuron arise? This problem was overcome by the discovery of adult neural stem cells that can give rise to neurons. In the mid 90´s the availability of cell-specific markers used for immunohistochemical identification of newly generated cells established that new neurons in the adult mammalian brain were actually born in at least two distinct areas of the brain: in the dentate gyrus of the hippocampal formation (Cameron et al., 1993; Gage et al., 1995) and in the subventricular zone/olfactory bulb (Lois and Alvarez-Buylla, 1993; Doetsch et al., 1997). Since then, several research groups have demonstrated adult neurogenesis in these areas.

Neural stem cells (Fig. 3) can be defined as cells that are capable of self-replication and can give rise to the three main cell types found in the CNS: neurons, astrocytes and oligodendrocytes (Kornblum and Geschwind, 2001). Each stem cell can divide symmetrically to give rise to two equal stem cells, but it can also divide asymmetrically to form another stem cell and one more differentiated cell. During development, the stem cell pool is expanded through symmetric divisions. After development, stem cells mostly divide asymmetrically, but they retain the ability to divide symmetrically, especially after injury or disease or in order to replace stem cells that have been lost (Morrison and Kimble, 2006). Neural stem cells were first defined as a population of nestin-expressing cells (an IF protein expressed by immature cells of neuroectodermal origin but also by reactive astrocytes and endothelial cells) isolated from adult and embryonic CNS, which can be induced to divide when stimulated by epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Reynolds and Weiss, 1992; Reynolds

and Weiss, 1996; Tropepe et al., 1999). The neural stem cells in the adult brain have characteristics of glia. Doetsch and colleagues have shown that the subventricular zone lining the lateral ventricles in the brain contains GFAP expressing astrocyte-like cells that are the source of new neurons (Doetsch et al., 1997; Doetsch et al., 1999). This applies also to the subgranular zone in the adult hippocampus (Seri et al., 2001; Seri et al., 2004; Steiner et al., 2004). The idea of an astrocyte-like GFAP expressing stem cell in the adult brain is by now fully accepted and has been supported by a number of studies (Laywell et al., 2000; Gotz et al., 2002; Imura et al., 2003; Garcia et al., 2004; Merkle et al., 2004; Imura et al., 2006).

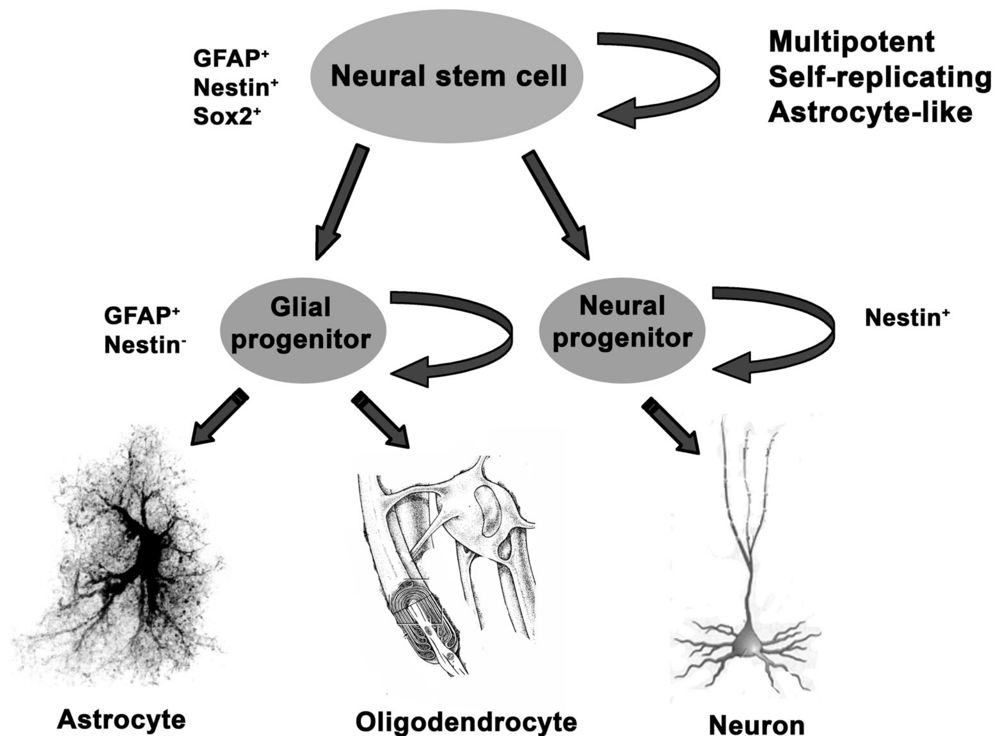

Figure 3. Neural stem cells divide to give rise to new neurons, astrocytes and oligodendrocytes. A possible scenario.

Neurogenesis, astrogenesis and oligodendrogenesis in the adult hippocampus

New neurons continue to be born in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus in mammals, including humans (Kaplan and Bell, 1984; Cameron et al., 1993; Gould et al., 1997; Eriksson et al., 1998). The neural stem cells in the SGZ, often referred to as "radial astrocytes" or "vertical astrocytes" are quiescent, slowly dividing cells (Seri et al., 2004; Steiner et al., 2004). The radial astrocytes divide asymmetrically to generate mostly neuronal doublecortin immunoreactive progenitors that may undergo further division meanwhile migrating the short distance into the adjacent granular cell layer (GCL) (Fig. 4).

The large majority of the immature neurons and astrocytes die at some point between their first and fourth week before they fully differentiate (Kempermann et al., 2003; Steiner et al., 2004). Comparably few neurons are stably integrated and acquire morphological, biochemical and functional characteristics of mature granular neurons (Stanfield and Trice, 1988; Cameron et al., 1993; Seri et al., 2001; van Praag et al., 2002). Astrocytes are also generated and to date there is evidence of few or no newly-born oligodendrocytes in the dentate gyrus of the hippocampus (Steiner et al., 2004).

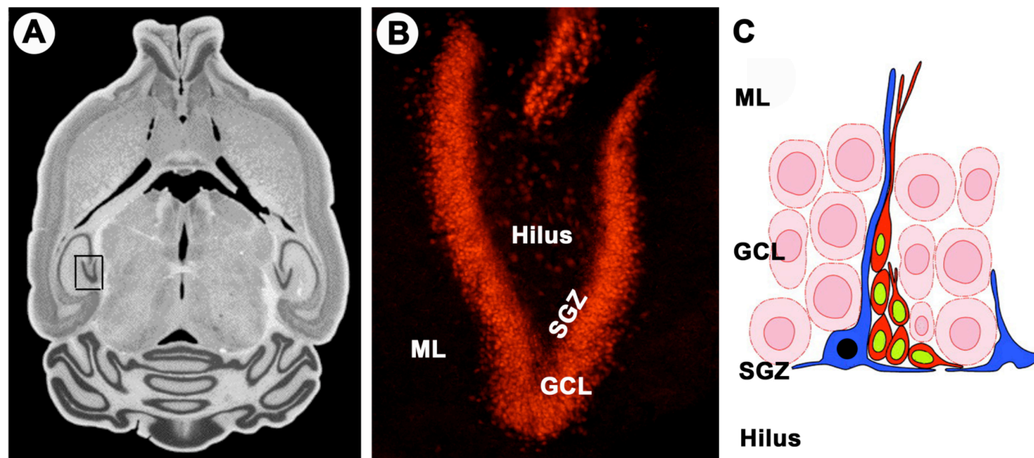

Figure 4. Adult neurogenesis in the hippocampus. A, Horizontal view of a mouse brain. The dentate gyrus of the hippocampal formation is boxed in black. B, The dentate gyrus and its subregions of the hippocampal formation visualized by NeuN immunohistochemistry. C, Radial astrocytes (blue) divide to generate neural progenitors (red) that migrate and become mature granular neurons (pink). ML; molecular layer, GCL; granular cell layer, SGZ; subgranular zone.

Regulation of adult hippocampal neurogenesis and astrogenesis

The number of granule cells generated in early adulthood is relatively large, in the order of a few thousand per day (West et al., 1991; Cameron and McKay, 2001). The rate at which neurogenesis occurs in aging, is only a fraction of neurogenesis in young individuals (Kuhn et al., 1996; Kempermann et al., 1998; Bondolfi et al., 2004). The rate of neurogenesis (neural progenitor cell proliferation and survival) is also affected by activities such as learning, running, exposure to environmental enrichment, stress and alcohol (Kempermann et al., 1997; Gould and Tanapat, 1999; Gould et al., 1999; van Praag et al., 1999; Crews et al., 2004). Decreased neurogenesis during aging and acute stress has been linked to elevated levels of circulating glucocorticoids (Cameron and McKay, 1999; Gould and Tanapat, 1999). VEGF and IGF-1 increase neurogenesis and it is suggested that the effect on neurogenesis exerted by physical activity is mediated through these factors (Trejo

et al., 2001; Fabel et al., 2003). The dentate gyrus is innervated by glutamatergic, GABAergic, cholinergic and serotonergic neurons and neurons that synthesize nitric oxide. These transmitters can induce changes in neural progenitor proliferation, fate choice and integration (Jang, 2008). Neurogenesis has been the main focus of most studies on neural stem cells in the adult brain. However, many new glial cells are generated from the hippocampal neural stem cells and this process seems to be regulated independently from neurogenesis (Steiner et al., 2004).

The hippocampus – a neurogenic niche

Neural stem cells can be isolated from the hippocampus (Palmer et al., 1995) and the lateral ventricles (Reynolds and Weiss, 1992) but cells with neural stem cell potential can also be isolated from other "non-neurogenic" regions of the adult CNS such as the spinal cord (Shihabuddin et al., 2000), striatum (Palmer et al., 1995), cortex (Palmer et al., 1999) and cerebellum (Lee et al., 2005). In vitro, when cultured with growth factors such as FGF-2, they seem loose their in vivo developmental restriction and show self-renewal and multipotentiality (Shihabuddin et al., 1997; Palmer et al., 1999). Grafting studies support the principle on neurogenic and non-neurogenic CNS region; when progenitors isolated from neurogenic (lateral ventricles and hippocampus) or non-neurogenic (cerebellum and spinal cord) regions were grafted into various regions of the CNS, the progenitors differentiated into neurons only in the neurogenic hippocampus and the olfactory bulb (Suhonen et al., 1996; Shihabuddin et al., 2000; Goh et al., 2003; Emsley et al., 2005). Taken together, these studies demonstrate the high degree of plasticity of the neural stem and progenitor cells and the major impact of the niche on their fate.

Astrocytes are a highly heterogenous population. Aside from a role as neural stem cells, astrocytes might be involved in the generation of neurons by modulating the environment around the neural stem cells, making it more or less permissive for neurogenesis. It has been shown that neural stem cells differentiate into neurons when cocultured with mature hippocampal astrocytes, whereas astrocytes from the spinal cord do not promote neurogenesis (Song et al., 2002a). Also, grafting of hippocampal SGZ astrocytes to the cortex of adult mice induce neurogenesis from endogenous non-neurogenic cortical neural precursors (Jiao and Chen, 2008). These results suggest that different subtypes of astrocytes exist in different parts of the CNS and that they might be essential for the control of neurogenesis.

The molecular mechanisms underlying astrocyte-induced neurogenesis have been investigated (reviewed in (Ma, 2008). A signaling molecule that increases adult hippocampal neurogenesis is Sonic hedgehog (Shh) (Lai et al., 2003). It was recently shown that astrocytes from the adult SGZ express high levels of Shh whereas this factor was almost absent in control astrocytes from the cortex (Jiao and Chen, 2008). Addition of Shh alone was sufficient to stimulate neural progenitor cell proliferation and neurosphere formation from cortical neural progenitors, a response that could be quenched by Shh antagonist (Jiao and Chen, 2008). In vivo, implantation of hippocampal astrocytes to the adult cortex could stimulate endogenous cell proliferation and neurogenesis from endogenous progenitors. Lie and co-workers have shown that winged helix protein-3 (Wnt3) is expressed in adult hippocampal astrocytes both in vitro and in vivo and that overexpression of Wnt3 was sufficient to increase neurogenesis whereas blockade of Wnt signalling dramatically decreased neurogenesis (Lie et al., 2005). In vivo, there is a tight correlation between IGF and neurogenesis (Anderson et al., 2002). IGF activities are modulated by six high-affinity IGF binding proteins (IGFBP-1 to -6), extracellular proteins that regulate the distribution and bioavailability of IGF (Clemmons, 1998; Rechler and Clemmons, 1998). A study in which the effect on neuronal differentiation in co-cultures of neural progenitors and neurogenic or non-neurogenic astrocytes was compared, suggest that neurogenic astrocytes may enhance neuronal differentiation by decreased expression of IGFBP-6 leading to increased IGF signaling (Barkho et al., 2006). Hippocampal astrocytes may also promote synaptic integration and functional maturation (Song et al., 2002b); astrocyte-secreted thrombospondins are interesting candidates for controlling neuronal maturation (Christopherson et al., 2005).

Possible functional relevance of adult neurogenesis

To be able to discuss a possible functional role for adult hippocampal neurogenesis it is important to know the context to which the new neurons are added. The hippocampal formation consists of three layers of neurons, the *dentate gyrus*, *Ammon's horn* (or *Cornu Ammonis*) and *subiculum* (Fig. 5). The dentate gyrus, the site for adult neurogenesis, receives input from the *entorhinal cortex* via a bundle of axons called the *perforant path*. Neurons in the dentate gyrus, the granule cells, send mossy fibres that synapse on neurons in the *CA3* area of the second layer *(Cornu Ammonis)* (McEwen et al., 2002; Monje and Palmer, 2003). The neurons of the CA3 area send axons, *Schaffer collaterals*, to *CA1* that in turn project to the *subiculum*. The subiculum makes connections back to entorhinal cortex and to neocortex. Though here very simplified, these neuronal connections make up

basis of the *the hippocampal trisynaptic circuit* where maturing granular neurons may connect to other neurons and eventually become a part of this neuronal network (Andersen, 1975; Benarroch, 2006). There are also pathways from the cortex that project directly to CA3 and CA1 neurons (Witter, 1993).

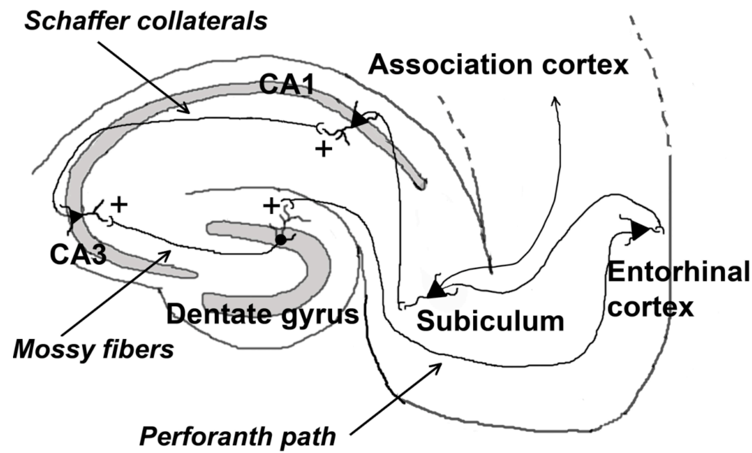

Figure 5. Adult-generated neurons are integrated into the hippocampal neuronal network.

Retroviral labeling of dividing cells in the hippocampus has made it possible to examine morphological and electrophysiological properties of newborn neurons throughout their lifespan (van Praag et al., 2002). The newborn granule cells undergo a period of structural and functional maturation when they differ from the mature granule cells in terms of ion channels properties and response to neurotransmitters, such as GABA (Wang et al., 2000; Schmidt-Hieber et al., 2004; Zhao et al., 2006). Interestingly, it was shown that immature granule cells show a lower threshold for induction of long-term potentiation (Wang et al., 2000; Snyder et al., 2001; Schmidt-Hieber et al., 2004). The fact that a neuron that has recently been activated expresses so called "immediate early genes" has been used to demonstrate that spatial exploration/water maze learning activates newborn hippocampal neurons (Jessberger and Kempermann, 2003; Ramirez-Amaya et al., 2006). It has been shown that by the time the adult-generated neurons are between 4-8 weeks of age, they are more likely to be recruited into the hippocampal network supporting spatial navigation in the Morris water maze task (Kee et al., 2007).

The involvement of the hippocampal formation in learning and memory has been recognized for decades but despite intense research the precise biological substrate for this function is not known. Adult neurogenesis could serve as a special form of plasticity that may help process information about the time and place of new events. A potential role for adult hippocampal neurogenesis in the

encoding of time in new memories has been suggested (Aimone et al., 2006). In animal models, two hippocampus-dependent tests, Morris water maze and different protocols of fear conditioning, have mainly been used to investigate the role of adult hippocampal neurogenesis in learning and memory (reviewed in (Gould et al., 1999; Kempermann et al., 2004)). Many studies show a correlate between the level of hippocampal neurogenesis and learning in different variants of Morris water maze task (Gould et al., 1997; Kempermann et al., 1997; van Praag et al., 2005; Dupret et al., 2008) and fear conditioning (Shors et al., 2001; Saxe et al., 2006; Wojtowicz et al., 2008). However, there are many reports where reduction or ablation of neurogenesis did not correlate with learning or only affected one out of multiple learning and memory tasks (Shors et al., 2002; Merrill et al., 2003; Meshi et al., 2006; Wojtowicz et al., 2008; Zhang et al., 2008). It is important to acknowledge that the addition of new functional neurons is only one dimension of plasticity. Immature cells are an important source of trophic factors that may support many aspects of plasticity (Visnyei et al., 2006; Yasuhara et al., 2006). In addition, for example collateral sprouting may provide substantial contribution to plasticity and function restoration after spinal cord injury (Liu et al., 2008).

THE AIM OF THE STUDIES

This thesis utilizes a mouse transgenic model in which the astrocyte IF proteins GFAP and vimentin were deleted (*$GFAP^{-/-}Vim^{-/-}$* mice) leading to attenuated reactive gliosis. The specific aims of this thesis were:

(I) To study the role of age-related reactive gliosis on the endogenous hippocampal neural stem cell proliferation and neurogenesis (Paper I).

(II) To address the effect of reactive gliosis on differentiation of hippocampal neural progenitor cells in culture and after transplantation into the hippocampus (Paper II).

(III) To study the effect of absence of astrocyte IFs on neural stem cell proliferation, survival, and differentiation, and on learning and memory (Paper III).

MATERIALS AND METHODS

The mice (Paper I, II and III)

Mice carrying null mutations for GFAP and vimentin have previously been described (Eliasson et al., 1999; Pekny et al., 1999). The mice were on a mixed genetic background (C57Bl/6, 129Sv, 129Ola) and housed with their littermates in standard cages in a barrier animal facility and had access to food and water ad libitum. In the study addressing neurogenesis in aged mice (Paper I) we used 18 months old wildtype and *GFAP$^{-/-}$Vim$^{-/-}$* female mice. In the study addressing neurogenesis and astrogenesis from transplanted neural progenitor cells (Paper II) we used 4.5-5 months old wildtype and *GFAP$^{-/-}$Vim$^{-/-}$* female mice and 0-1 day old pups to prepare astrocyte cultures. All mice receiving grafts were null mutants also for the gene encoding recombinase Rag-1 in order to avoid host-versus-graft rejection since the transplanted cells were xenografts (Mombaerts et al., 1992). For in vivo neurogenesis and astrogenesis studies (Paper III), we used 3 months old wildtype and *GFAP$^{-/-}$Vim$^{-/-}$* male mice. A subset of these mice had access to a running wheel. Behavior studies were carried out in additional 3 months old wildtype and *GFAP$^{-/-}$Vim$^{-/-}$* male and female mice. To prepare neurosphere cultures and astrocyte cultures (Paper III) we used wildtype, *GFAP$^{-/-}$*, *Vim$^{-/-}$* and *GFAP$^{-/-}$Vim$^{-/-}$* pups, 4 days of age for neurospheres and 0-1 days of age for astrocyte cultures.

The presence of null mutations in the genes encoding GFAP, vimentin and Rag-1 was determined by PCR and by the Southern blot analysis.

Cell culture and in vitro analyses (Paper II and III)

Neural progenitor cells were cultured as adherent monolayers (Paper II) or as free-floating neurospheres (Paper III). In both these studies, the neural progenitors were cocultured with astrocytes.

Neural progenitor cell cultures (Paper II)

For the transplantation study (Paper II), we used neural progenitor cells derived from adult rat hippocampus, retrovirally transfected to express green fluorescent protein (GFP). The cells were cultured on laminin/poly-L-ornithine coated Petri dishes in Ham's F12 high glucose medium, with 1% N2 supplement, 2 mM L-glutamine and 20 ng/ml recombinant human basic fibroblast growth factor. Penicillin/streptomycin (100 U and 0.1 mg/ml, respectively) and fungizone (0.25 µg/ml) were added to the medium to prevent microbial growth. The cells were cultured at 37°C and 5% CO_2. Prior to grafting, the cells were grown to confluency

in the presence of 5 µM BrdU for 48 hours. The cells were trypsinized, washed and resuspended to a density of 60.000 cells/µl in PBS with 0.15% glucose and 30 ng/ml of basic fibroblast growth factor and kept on ice.

Comments

The neural progenitor cells from the rat hippocampus were obtained from the lab of Dr. F.H. Gage at Salk Institute, La Jolla, CA, USA, (clone HCN-A94/GFPH, passage 12+2). These cell cultures were established from the hippocampus of female adult Fischer 344 rats. Presence of b-FGF promotes proliferation and survival of b-FGF-responsive neural stem cells and their progeny. The cultures consist of an immature population of progenitors that co-expresses a variety of markers of neuronal and glial lineages (Palmer et al., 1995; Palmer et al., 1997).

While all neural progenitor cells expressed GFP in culture prior to transplantation, at 35 days after transplantation about 3% of the transplanted cells showed continued GFP expression. We therefore labeled the neural progenitor cells with BrdU prior to their transplantation.

Assessment of neural progenitor cell differentiation in cocultures (Paper II)

Primary cultures of astrocytes were prepared from 1 day old *$GFAP^{-/-}Vim^{-/-}$* and wild-type mice as described previously (Pekny et al., 1998). At confluency, primary cultures were passaged (1:2) onto coverslips and cultured in normal serum-containing medium. Confluent cultures were washed twice with serum free medium (Dulbecco's modified Eagle's medium/Nut Mix F12 (1:1), 2mM L-glutamine, 1% N2 supplement, 1% penicillin/streptomycin (100 U and 0.1 mg/ml, respectively) and 0.25 µg/ml fungizone and cultured in serum free medium or medium supplemented with 1% fetal calf serum.

Neural progenitor cells, expressing GFP, were added in the cultures at a density of approximately 2.0 x 10^3 cells/cm^2. These GFP^{pos} neural progenitor cells have previously been used in coculture paradigms with astroglial cultures (Song et al., 2002a). For assessment of neural progenitor cell differentiation, the GFP^{pos} cells were cocultured with the astrocytes for six days before lineage selection was assayed. Briefly, cells were fixed (4% paraformaldehyde in PBS, 4°C, 10 minutes), non-specific binding was blocked and cells were immunocytochemically stained (1 hour, room temperature) with antibodies in PBS containing 3% donkey serum and 0.05% saponin (for antibodies used for in vitro detection see Tab. 3). Following three washes in PBS, cells were incubated for 1 hour at room temperature with secondary antibodies.

For quantification of differentiation, GFP^{pos}, $GFP^{pos}Map2ab^{pos}$, $GFP^{pos}RIP^{pos}$ and $GFP^{pos}GFAP^{pos}$ cells from at least twelve, randomly selected, non-overlapping

fields were counted. All differentiation experiments were done in triplicate (n=4). Cell counts were performed using a Nikon eclipse 80i epifluorescence microscope.

For the analysis of Wnt3 expression, we used confluent astrocyte cultures and cocultures with neural progenitor cells in 1% or 10% of fetal calf serum. The percentage of astrocytes displaying the filamentous pattern of Wnt3 immunoreactivity was assessed investigating 200 wildtype and 200 $GFAP^{-/-}Vim^{-/-}$ astrocytes.

Data (presented as mean ± SEM) were evaluated by two-tailed t-test, the differences were considered significant at $p < 0.05$. All images were processed in Photoshop (v. 8.0, Adobe Systems).

Neurosphere culture (Paper III)

Age-matched postnatal day 4 mice were decapitated. The brains were dissected out in Leibovitz medium (Gibco), cut into pieces and enzymatically digested with trypsin. The digested tissue was then mechanically dissociated into a single cell suspension. After centrifuging, the pellets were washed twice in Neurobasal (Gibco) and resuspended in neurosphere media (Neurobasal with L-glutamine (2 mM), penicillin/streptavidin (100 U and 0.1 mg/ml, respectively), B27 (1:50), b-FGF (20 ng/ml), EGF (20 ng/ml), heparin (1 U/ml) and fungizone (0.25 µg/ml)). 10^5 cells were plated per well and cultured in neurosphere media for 7 d at 37 °C and 5% CO_2 for primary neurosphere formation.

For passaging, neurospheres were collected, centrifuged and mechanically dissociated into the single cell suspension from which 10^5 cells/well were plated in neurosphere media and incubated for 7 d at 37°C and 5% CO_2. To assess the number of passages possible, samples were passaged every 7 d and 10^5 cells were replated under the same conditions as primary cultures. Alternatively, secondary neurosphere were formed from dissociated primary neurospheres that were sorted at a concentration of 1 cell/well in 96 well plates using FACS.

Comments

The technique to culture neural stem and progenitor cells a free-floating aggregate was developed in the early 90'ies (Reynolds and Weiss, 1992; Ray et al., 1993). This method to culture neural stem cells quickly became popular since it is easy, gives better neuronal survival and doesn't require laminin/poly-L-ornithine coated plates. Similar sphere assays are now being used to culture for example cardiac cells or pancreatic cells. The neurosphere assay is based on the idea that a neural stem cell divide in response to growth factors to generate progeny that form a sphere (= clone). It has been shown that a neurosphere is a heterogeneous structure and includes both the slowly dividing bona fide stem cells and quickly dividing restricted progenitors (Kukekov et al., 1997) and a clone does not

necessarily represent the presence of one multipotent stem cell since neurospheres could also be derived from restricted glial or neural progenitors. It is a very attractive idea that one neurosphere formed in a dish would correspond to one neural stem cell in vivo and that the neurosphere assay could be used to estimate the number of stem cells in vivo (Morshead et al., 1998). However, it was recently suggested that the majority of neurospheres formed were indeed chimeric and not clonal and time-lapse microscopy revealed that free-floating neurospheres are highly movable, can merge and exchange cells with each other (Singec et al., 2006). It is recommended that caution is taken when drawing conclusions from the classical neurosphere assay with plating of 10^2 to 10^5 cells per well and that these data at some point should be correlated with the degree of sphere formation where one cell per well is plated.

Neurosphere counts and size measurements (Paper III)

After 7 d in culture, total numbers of primary and secondary (for *Vim*$^{-/-}$ samples also tertiary) neurospheres per well were counted using an inverted Nikon microscope. To assess size 7-d-old neurospheres were photographed with a Nikon inverted microscope attached to a Leica 290 camera and measured using Leica software.

Neurosphere differentiation (Paper III)

For assessment of differentiation, fifteen 7-d-old neurospheres were pipetted into 24 well plates with glass coverslips coated with laminin/poly-L-ornithine and gently flooded with differentiation media (neurosphere media without b-FGF, EGF and heparin). 1 d after plating, 1% fetal calf serum was added. Cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min at room temperature after 5 d of differentiation for immunocytochemistry or directly lysed with lysis buffer (Qiagen microKit) and collected for quantitative PCR analysis.

Neurosphere and astrocyte cocultures (Paper III)

Primary astrocytes were cultured in Dulbecco's modified Eagle's medium/Nut Mix F12 with 10% fetal calf serum, as previously described (Pekny et al., 1998). Upon confluency, primary astrocytes were trypsinized, stained with a membrane specific live cell dye (Sigma) and replated at ½ confluency on glass coverslips coated with poly-L-ornithine and laminin. One day later, media was changed to differentiation media. After another day, 15 neurospheres were plated on the astrocyte monolayer. Cells were fixed after 5 d of differentiation as previously described.

Immunocytochemistry and quantification (Paper III)

For BrdU detection, fixed cells were washed in PBS and blocked in 3% normal goat serum and 0.01% Triton X-100 in PBS for 30 min at room temperature. For differentiated neurospheres, fixed cultures were washed in PBS and blocked with 10% fetal calf serum and 0.5% triton X-100 in PBS for 1 hr at room temperature. BrdU and cell phenotype markers were detected with antibodies as previously described (Table. 3). DAPI was used to counterstain nuclei (1:10000, Sigma-Aldrich). Beta-III-tubulin quantification was done using a stereology microscope (Leica). All images were processed in Photoshop (v. 8.0, Adobe Systems).

Data were analyzed in SPSS (v. 16.0, SPSS Inc.) or GraphPad (v. 5.0, Prism). One-factor or two-factor ANOVA was used followed by post hoc analysis (Bonferroni or Tukey HSD). Two-tailed t-tests were used for comparisons between two groups. Differences were considered significant at $p < 0.05$ and values are presented as mean ± SEM.

Primary Antibodies	**Source**	**Affinity**	**Dilution**	**Company**
β-actin	Goat	Actin, cyoskeletal protein used as reference gene	1:500 (Western blot)	Abcam
Beta-III-tubulin	Mouse	Neurons	1:100	Covance
BrdU	Mouse	Marker for cells in DNA synthesis phase	1:200	Dako
GFAP	Rabbit	Astrocytes and adult neural stem cells.	1:500	Dako
Map2ab (microtubule-associated protein)	Mouse	Neurons	1:100	Sigma-Aldrich
Rip	Mouse	Oligodendrocytes	1:20	Developmental Studies Hybridoma Bank
O4	Mouse	Oligodendrocyte progenitors	1:100	Chemicon
S100	Rabbit	Astrocytes	1:200	Dako
Wnt3	Goat	Wnt3 expressing cells	1:50 (Immuno) 1:200 (Western blot)	Santa Cruz Biotechnologies
Secondary Antibodies	**Source**	**Affinity**	**Dilution**	**Company**
Donkey-∝-mouse-Alexa Fluor 555 / Alexa Fluor 568/ Alexa Fluor 488/	Donkey	Mouse IgG	1:1000-2000	Molecular Probes
Donkey-∝-rabbit-Alexa Fluor 555	Donkey	Mouse IgG	1:2000	Molecular Probes

Donkey-∝-goat-Alexa Fluor 555	Donkey	Goat IgG	1:2000	Molecular Probes
Donkey-∝-goat (iodine-125 conj.)	Donkey	Goat IgG	1:1000 (Western blot)	Sigma-Aldrich
Goat-∝-mouse-Alexa Fluor 594	Goat	Mouse IgG	1:500	Molecular Probes

Table 3. Antibodies for in vitro immunodetection.

Neural progenitor cell transplantation (Paper II)

The mice were anesthetized and placed in a stereotactic frame. Using a 5 µl Hamilton syringe, 1.0 µl of cell suspension was slowly injected (2 minutes) unilaterally in the hippocampus (at the coordinates mediolateral; -3.0 mm, anteriorposterior; -3.5 mm, dorsoventral; -2.7 mm, using the bregma point on top of the skull as a reference). The syringe was left in place for additional 2 minutes to prevent reflux of the cell suspension. Then, the syringe was slowly raised 0.5 mm and another 0.2 µl was injected and the syringe was left in place for one more minute. After 7 hours on ice, the remaining cell suspension showed 90% viability as assessed by trypan blue exclusion.

Comments

The coordinates used to inject the cells resulted in deposition of the cells adjacent to the lateral molecular layer of the dentate gyrus of the hippocampus (see Fig. 3C in Paper II).

Assessment of the morphology of transplanted cells

While all neural progenitor cells in culture prior to transplantation expressed GFP, 8-40 days after transplantation only about 3% of transplanted cells retain their GFP positivity. We took advantage of the GFP positivity of a subpopulation of these cells to assess their morphology.

GFP^{pos} cells in the molecular layer with processes exceeding 3 lengths of the cell body were counted on a series of seven 35 µm thick horizontal hippocampal sections spaced 140 µm and the length of the longest process per cell was measured and the primary branches extending from such processes were counted. We used laser-scanning confocal microscopy on stacks of confocal images (Leica TCS) taken at 1 µm intervals within the thickness of the section.

Data (presented as a mean ± SEM) were evaluated by two-tailed t-test, the differences were considered significant at $p < 0.05$. All images were processed in Photoshop (v. 8.0, Adobe Systems).

Comments

We previously showed improved growth of processes from transplanted cells in the retina of *GFAP$^{-/-}$Vim$^{-/-}$* mice (Kinouchi et al., 2003). In the present study we used a similar approach to analyse neurite-like processes on GFP^{pos} cells.

SDS-PAGE and Western blot analysis of Wnt3 protein (Paper II)

Western blot was used to analyze Wnt3 protein expression in vivo and in vitro. Hippocampi from wildtype and *GFAP$^{-/-}$Vim$^{-/-}$* mice, 18 months of age, were homogenized in 500 µl lysis buffer (50 mM dithiothreitol, 25 mM Tris HCl, 35 mM Tris base, 0.5 % LDS (detergent), 2.5% glycerol, 12.5 mM EDTA and proteinase inhibitor). The samples were sonicated for 1 minute, agitated, heated and centrifuged to produce a crude protein exctract. Primary astrocyte cultures from wildtype and *GFAP$^{-/-}$Vim$^{-/-}$* mice, 1-day-old, and cocultures from these cells and neural progenitors were lysed in lysis buffer and sonicated for 1 minute. Total protein concentration was determined with the Bradford method using the Coomassie method with absorbance measured at 595 nm. The samples were loaded on a 10% acrylamide gel and the volume was adjusted to gain an equal loading of 30 µg total protein. 1D gel electrophoresis was performed using MOPS SDS running buffer at 200 V constant voltage. For quantitative Western blot analysis, protein samples were transferred from gels to polyvinyldifluoride membranes. The membranes were blocked, washed and incubated with goat anti-Wnt3 antibodies or goat anti-β-actin antibodies followed by several washes and incubation with iodine-125-conjugated donkey anti-goat antibodies (Tab. 3). After exposure to photographic film, the relative intensities of Wnt3 and β-actin protein bands were analyzed using ImageJ (NIH).

Enthorinal cortex lesion (ECL) as a neurotrauma model (Paper III)

Male and female mice, 18 months old, were anesthetized and a stereotaxic retractable wire knife (Scouten wire knife; Kopf) was used to transect the perforant path (Fig. 5) of the entorhinal cortex leading to partial deafferentation of the hippocampus (Stone et al., 1998).

BrdU labeling and immunohistochemistry (Paper I, II and III)

BrdU labeling (Paper I and III)

For in vivo labelling of proliferating cell in the hippocampus of aged mice (Paper I), in young adult running and non-running mice (Paper III) or in mice with ECL (Paper III), we used 5-Bromo-2´-deoxyuridine (BrdU) injected intraperitoneally twice daily for 7 days at a dose of 300 mg/kg body weight.

Comments

BrdU is a nucleoside analogue that can be used as a marker for DNA synthesis and hence proliferating cells. If dividing cells are exposed to BrdU during the S-phase of the cell cycle, they will incorporate BrdU instead of deoxythymidine in their replicating DNA (Nowakowski et al., 1989). If a cell is BrdU immunopositive, it indicates that the cell has passed through the S-phase. The intra-peritoneal doses of BrdU used for detection of proliferating cells and their fate in the dentate gyrus ranged from 25-600 mg/kg body weight (Cameron and McKay, 2001). In rats, 25 mg/kg has shown to be the lowest dose at which the cells are visibly labeled by BrdU. There seems to be a plateau in the number of detectable cells at doses about 100-300 mg/kg and therefore it is suggested that a dose of 300 mg/kg is sufficient to label all dividing cells in the adult dentate gyrus. Since BrdU is injected twice daily during seven days, our labeling paradigm gives an accumulated number of cells having undergone cell division and still alive at the time point when the mice are killed (Cameron and McKay, 2001).

BrdU is incorporated also at sites of DNA repair (Selden et al., 1993). Thus, there was a concern that the use of BrdU as a marker for cell proliferation could give false data. However, the amount of BrdU incorporated due to DNA repair compared to the amount of BrdU incorporated during the complete replication of the genome is very small and should not lead to any significant error (Cameron and McKay, 2001; Cooper-Kuhn and Kuhn, 2002). Given during embryonic development BrdU may cause malformations and altered behavior (Kolb et al., 1999) but in adult rats, even multiple administrations of BrdU at 600 mg/kg did not have any deleterious effects on neurogenesis in the hippocampal dentate gyrus (Cameron and McKay, 2001).

An alternative method study cell proliferation is to assess expression of Ki67. Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0) (Seigneurin and Guillaud, 1991). We used detection of Ki67 to assess proliferation in mice with hippocampal lesions in paper III. However since Ki67 expression is only transient it cannot be used to assess long-term survival and differentiation of cells as in the case with BrdU.

Tissue processing and in vivo immunodetection

Two weeks (Paper I and III) and six weeks (Paper III) after the first BrdU injection, or 35 days after transplantation (Paper II), the mice were deeply anesthetized and perfused transcardially with phosphate buffer followed by 4% paraformaldehyde. The brains were dissected and postfixed in 4% paraformaldehyde, horizontal 50 µm thick sections were prepared by using a vibratome (Paper I and III), or 35 µm (Paper II) or 40 µm (Paper III) thick cryostat sections were obtained. Prior to

antibody incubation, all sections were pre-treated in 0.05% glycine in PBS and permeabilized in 1% BSA and 0.01% Tween 20 in PBS over night. To detect BrdU-labeled cells that had differentiated into neurons or astrocytes, antibodies (Tab. 4) against the respective markers were diluted in 1% BSA and 0.01% Tween 20 in PBS and incubated with the sections over night at 4°C. From lesioned mice (Paper III) we also prepared 8 µm thick paraffin-embedded sections. Paraffin sections were rehydrated and microwave antigen retrieval was done with 0.01 M citrate buffer (pH 6.0) before incubation with antibodies.

Primary Antibodies	**Source**	**Affinity**	**Dilution**	**Company**
BrdU	Mouse	Marker for cells in DNA synthesis phase	1:100	Dako
BrdU (FITC-conjugated and un-conjugated.	Rat	Marker for cells in DNA synthesis phase	1:100	Nordic Biosite
Doublecortin (DCX)	Goat	Migrating neuroblasts (immature)	1:50	Santa Cruz Biotechnologies
ET_BR (Endothelin B receptor)	Rabbit	Most prominent in reactive astrocytes.	1:100	Alomone Labs
GFAP	Mouse	Astrocytes and adult neural stem cells.	1:100	Sigma-Aldrich
Glutamine synthase (GS)	Mouse	Mature astrocytes	1:100	Chemicon
Isolectin (biotinylated)	*Bandeiraea simplicifolia*	Microglia	1:10	Sigma-Aldrich
Ki67 (Clone TEC-3)	Rat	Proliferating cells	1:25	Dako
NeuN (biotinylated and non-biotinylated)	Mouse	Postmitotic neurons	1:100	Chemicon
Olig2	Goat	Oligodendrocyte and astrocyte progenitors	1:200	R&D Systems Inc.
Rip	Mouse	Mature oligodendrocytes	1:20	Developmental Studies Hybridoma Bank
S100	Rabbit, polyclonal	Immature and mature astrocytes	1:200-300	Dako
Wnt3	Goat	Wnt3 expressing cells	1:100	Santa Cruz Biotechnologies
Secondary Antibodies	**Source**	**Affinity**	**Dilution**	**Company**
Streptavidin-Cy3	*Streptomyces avidinii*	Biotin	1:100	Sigma-Aldrich
Streptavidin-Alexa Fluor 594/Alexa Fluor 633	*Streptomyces avidinii*	Biotin	1:1000/ 1:500	Sigma-Aldrich

Donkey-∝-rabbit-biotin	Donkey	Rabbit IgG	1:200	Jackson Immunoresearch Laboratories Inc.
Rabbit-∝-mouse-biotin	Rabbit	Mouse IgG	1:400	Dako
Goat-∝-mouse-Alexa Fluor 568 /Alexa Fluor 488	Goat	Mouse IgG	1:500	Molecular Probes
Goat-∝-rabbit-Alexa Fluor 568 /Alexa Fluor 488	Goat	Rabbit IgG	1:500	Molecular Probes
Goat-∝-rat - Alexa Fluor 488	Goat	Rat IgG	1:500	Molecular Probes
Goat-∝-rat - Cy3	Goat	Rat IgG	1:500	Jackson Immunoresearch Laboratories Inc.

Table 4. The antibodies used for in vivo immunodetection.

Assessment of cell proliferation/survival in the hippocampus (Paper I, II and III)

Central in all studies (Paper I, II and III) is the methodology to assess the number and differentiation of BrdU labeled (BrdU^{pos}) cells, endogenous or grafted, in different regions of the hippocampus. Before any quantification took place, all microscopy slides were blinded for the experimenter.

Assessment of cell proliferation and the number of newly formed neurons in the hippocampal dentate gyrus of aged mice (Paper I)

BrdU^{+} cells were counted in the subgranular zone (SGZ)/hilus, the granular cell layer (GCL) and in the molecular layer (ML) on 50 µm horizontal sections using epifluorescence microscopy (Nikon) and laser-scanning confocal microscopy (Leica). In order to assess neurogenesis, the BrdU^{+} cells in the SGZ and in the GCL were examined for NeuN immunoreactivity, using laser-scanning confocal microscopy. With both methods, the average number of BrdU^{+} or BrdU^{+}NeuN^{+} cells/section in the different regions were calculated and compared between wildtype and $GFAP^{-/-}Vim^{-/-}$ mice.

Differences between groups were evaluated by two-tailed t-test and considered significant at $p < 0.05$. All images were processed in Photoshop (v. 7.0, Adobe Systems).

Assessment of survival, migration and differentiation of transplanted BrdU^{pos} neural progenitor cells (Paper II)

In this study we used BrdU to label the neural progenitor cells prior to transplantation. This means that all BrdU^{pos} cells originate from the graft. In order

to study survival and migration of the transplanted BrdU^{pos} neural progenitor cells (Paper II), the BrdU^{pos} neural progenitor cells were quantified in the SGZ, in the GCL and in the Ammon's horn of the hippocampus. On the same sections, the migration of cells was assessed from the transplantation site by quantifying BrdU^{pos} neural progenitor cells 250 µm and 500 µm away from the needle track. A series of 7 horizontal sections through the hippocampus, spaced 140 µm was examined for each mouse using epifluorescence microscopy.

To assess the number of the BrdU^{pos} transplanted neural progenitor cells that had differentiated into astrocytes and neurons and were alive by day 35 after transplantation, we quantified BrdU^{pos} cells positive for S100, GS and NeuN. We examined 25 BrdU^{pos} cells in the SGZ and 50 BrdU^{pos} cells in the GCL using laser-scanning confocal microscopy. Data (presented as mean ± SEM) were evaluated by two-tailed t-test, the differences were considered significant at $p < 0.05$. All images were processed in Photoshop (v. 8.0, Adobe Systems).

Assessment of endogenous neural progenitor cell proliferation/survival, neurogenesis and astrogenesis in the hippocampal dentate gyrus (Paper III)

To assess cell proliferation/survival in standard-housed and running mice at 2 weeks or 6 weeks, BrdU^{pos} cells were counted in the SGZ and the GCL using epifluorescence microscopy. For each mouse we examined a series of 6 horizontal sections through the hippocampus, spaced 160 µm. Doublecortin positive (DCX^{pos}) cells were assessed in the same way. Using laser-scanning confocal microscopy, we examined 25 BrdU^{pos} cells in the SGZ and 50 BrdU^{pos} cells in the GCL for expression of NeuN and S100. The number of neurons and astrocytes for each mouse was calculated by multiplying the number of BrdU^{pos} cells by the percentage of cells expressing NeuN or S100.

In ECL mice, Ki67^{pos} cells in the whole lesioned dentate gyrus were examined for S100 and isolectin (microglia and endothelial cells) expression on 8 paraffin sections per mouse. BrdU^{pos} cells in the SGZ and GCL were examined for S100 and NeuN expression on two vibratome sections per mouse. Laser-scanning confocal microscopy was used for quantifications in ECL mice.

Data (presented as mean ± SEM) were evaluated by two-tailed t-test or Mann-Whitney test, the differences were considered significant at $p < 0.05$. All images were processed in Photoshop (v. 8.0, Adobe Systems).

Behavior studies (Paper III)

We used two behavioral tests, Morris water maze and trace fear conditioning, since these are both reported to be dependent on hippocampal function and

possibly on hippocampal neurogenesis (Shors et al., 2001; Schimanski and Nguyen, 2004; Dupret et al., 2008).

Morris water maze task

Mice were trained to locate a submerged platform 1 cm below the surface in one of the quadrants in a water maze with diameter of 100 cm. The water was made opaque with white non-toxic paint and the temperature was constant at 18°C. The mice were trained for eight consecutive days with 5 daily trials (with 20 s rest between trials) starting from different positions, until they were able to find the platform within approximately 10-20 s. On the probe trial days (probe 1 and probe 2 performed on day 9 and 15, respectively), the platform was removed and all mice performed a single trial of 60 s. The latency to the first platform crossing, the number of crossings and the time spent in the platform quadrant were recorded. Video recordings of training day 1-8 and on the probe trials 1 and 2 were done using video-tracking system (2020 Plus tracking system, HVS Image).

Trace fear conditioning

Three-month-old male and female mice were conditioned (Automatic Reflex Conditioner, Ugo Basile). On day 1, mice were exposed to eight 30 s presentations of the tone (670 Hz, 80 dB) followed by a trace period of 20 s prior to a brief foot-shock (0.3 mA, 2 s). Between each tone-shock pairing there was an inter-trial period of 2 min. On day 2 we introduced a novel context by changing spatial and olfactory cues. Subjects were allowed to explore the novel environment for 3 min without tone presentation, followed by 3 min of continuous tone presentation. Freezing was scored at 10 s intervals throughout the entire session. 2-4 months later, the mice were once more scored for freezing in the same novel context as on day 2.

Comments to behavior studies

Morris water maze and trace fear conditioning are both methods that assess hippocampal function, yet these two tests are in their nature very different: Morris water maze relies on spatial cues whereas trace fear conditioning utilizes spatial, olfactory and auditory cues and also involves the perception of time. Interpreting behavior data is not always straightforward and the performance of the experimental animals is sensitive to factors such as handling prior to and during testing, physical status and factors attributed to the environment where they are tested (Crabbe et al., 1999; Crawley, 1999). In the case of Morris water maze, repeated swimming in 18°C water may constitute a considerable physical challenge except for the challenge of learning to find the platform. Impaired

physical strength, poor swimming technique or on the contrary, mice enjoying floating in water, are factors that potentially can influence the way a mouse or a rat learns the task (Brandeis et al., 1989; Crawley, 1999). Whenever a foot-shock is used to condition animals in different protocols of fear conditioning (with or without a trace period), increased pain sensitivity may enhance the acquisition and expression of conditioned fear (Good and Westbrook, 1995; Sandkuhler, 2002). It is therefore desirable to assess possible differences in pain sensitivity before drawing conclusions from fear conditioning data. For this thesis, we used both the baseline testing and the hotplate test to assess this potential confounding factor. We concluded that pain sensitivity was not increased in $GFAP^{-/-}Vim^{-/-}$ mice (data not shown). The use of a trace period between the tone and the foot-shock as in trace fear conditioning seems to add a special dimension of hippocampus dependence and we found it therefore suitable to use this set-up (Paper III) (Misane et al., 2005; Bangasser et al., 2006).

RESULTS

Increased cell proliferation and neurogenesis in the hippocampal dentate gyrus of old *GFAP$^{-/-}$Vim$^{-/-}$* mice (Paper I)

Mild reactive gliosis occurs in the hippocampus of healthy, aging individuals, both in rodents and humans (Goss et al., 1991; David et al., 1997; Morgan et al., 1997; Unger, 1998). Neurogenesis in the dentate gyrus of the hippocampus is known to decline gradually during adulthood and at old age the rate of neurogenesis is only a fraction of that in the young mice (Kuhn et al., 1996; Bondolfi et al., 2004). Based on the results presented in this paper we hypothesize that age-related reactive gliosis could be one cause for declining neurogenesis in the aging brain.

To evaluate the impact of age-related reactive gliosis on hippocampal neurogenesis, we injected twice daily for 7 days 18-month-old wildtype and *GFAP$^{-/-}$Vim$^{-/-}$* mice with the cell proliferation marker BrdU. Labeled cells were counted in the subgranular zone, hilus, granular cell layer and the molecular layer of the hippocampal dentate gyrus. Neuronal differentiation of BrdU labeled cells was assessed in the subgranular zone and the granular cell layer of the hippocampus.

We found that the number of proliferating/surviving cells in the granular cell layer of the dentate gyrus was increased by 34% in *GFAP$^{-/-}$Vim$^{-/-}$* mice compared to wildtype mice and that the absolute number of newly formed neurons was increased by 36% in *GFAP$^{-/-}$Vim$^{-/-}$* mice compared to wildtype mice. In the other regions of the dentate gyrus we could not detect any difference in cell proliferation/survival or neurogenesis (subgranular zone and granuar cell layer only).

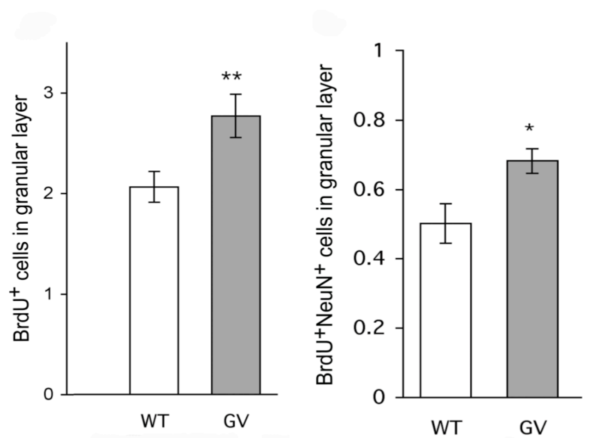

Figure 5. Increased hippocampal cell proliferation/survival and neurogenesis in aged GFAP$^{-/-}$ Vim$^{-/-}$ mice.

We conclude that attenuation of age-related reactive gliosis has a positive effect on hippocampal neurogenesis.

Increased neurogenesis and astrogenesis from neural progenitor cells grafted in the hippocampus of *GFAP$^{-/-}$Vim$^{-/-}$* mice (Paper II)

We have previously shown that attenuation of reactive gliosis such as in *GFAP$^{-/-}$Vim$^{-/-}$* mice improves the integration of transplanted retinal cells (Kinouchi et al., 2003). These results raised the question whether *GFAP$^{-/-}$Vim$^{-/-}$* astrocytes affect neural progenitor cell differentiation in vitro and whether this attenuation of reactive gliosis in recipient mice is beneficial for the survival and differentiation of grafted neural progenitor cells (a defined cell population versus a multitude of cells types from disintegrated retinas) also in the brain. We also assess the expression of Wnt3, an important regulator of adult hippocampal neurogenesis (Lie et al., 2005), in wildtype and *GFAP$^{-/-}$Vim$^{-/-}$* astrocytes. Here, we report that attenuation of reactive gliosis increases neurogenesis and astrogenesis from hippocampal neural progenitor cells — both in vitro and in vivo.

To evaluate the effect of attenuated reactive gliosis on neural progenitor cells, we assessed differentiation of hippocampal neural progenitor cells in cocultures with *GFAP$^{-/-}$Vim$^{-/-}$* or wildtype astrocytes in a situation resembling reactive gliosis (the presence of serum). To evaluate the effect of attenuated reactive gliosis on graft integration, we injected hippocampal neural progenitor cells into the hippocampus of adult *GFAP$^{-/-}$Vim$^{-/-}$* and wildtype mice. 35 days post-implantation we assessed survival, integration and differentiation of the grafted cells into neurons and glia in different regions of the hippocampus. We also assessed the length of neurite-like cellular processes and their extent of branching of GFP^{pos} grafted cells in the molecular layer of the hippocampus. Wnt3 protein expression was analyzed in vitro and in vivo using Western blotting and immunocytochemistry.

We found that *GFAP$^{-/-}$Vim$^{-/-}$* astrocytes, compared to wildtype, improved neurogenesis by 65% and astrogenesis by 124% in cocultures of neural progenitor cells and astrocytes. In vivo, attenuation of reactive gliosis in the *GFAP$^{-/-}$Vim$^{-/-}$* recipients increased the number of graft-derived neurons, astrocytes and glia progenitors in the granular cell layer of the hippocampus by 45%, 91% and 78%, respectively (Fig. 6). We also found that the incidence of GFP^{pos} cells with a neurite-like process exceeding 3 cell body lengths and bearing at least one branch was twice as high in *GFAP$^{-/-}$Vim$^{-/-}$* compared to wildtype recipients. A distinct Wnt3 immunoreactivity was detected in astrocytes in wildtype mice but was much weaker in astrocytes in *GFAP$^{-/-}$Vim$^{-/-}$* mice (Fig. 7). The Wnt3 protein levels were not different in the hippocampus of *GFAP$^{-/-}$Vim$^{-/-}$* and wildtype mice or in cultures with *GFAP$^{-/-}$Vim$^{-/-}$* and wildtype astrocytes.

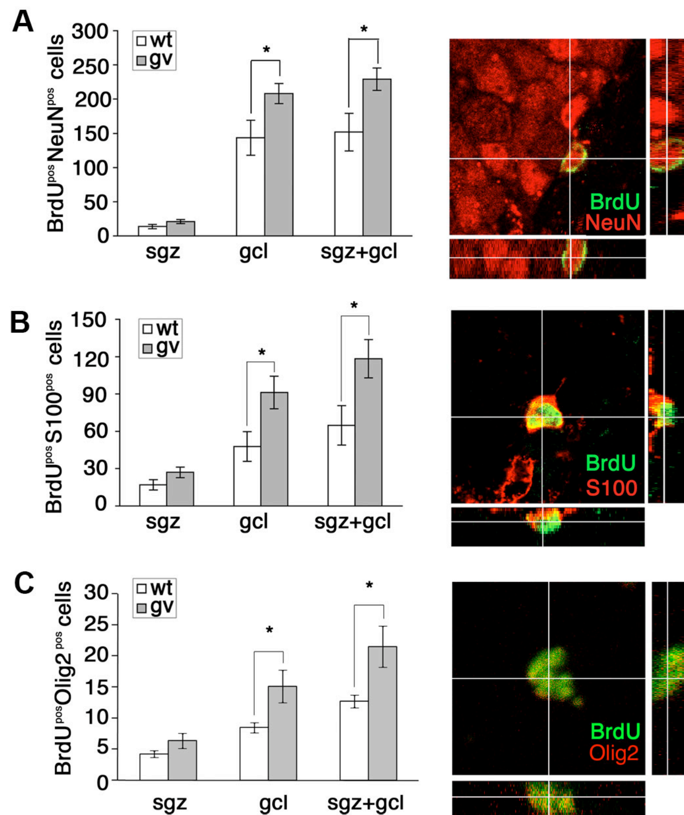

Figure 6. The number of graft-derived cells that had differentiated into neurons (A), astrocytes (B) and glia progenitors (C) and that stayed alive at 35 days after grafting was increased in $GFAP^{-/-}Vim^{-/-}$ recipients.

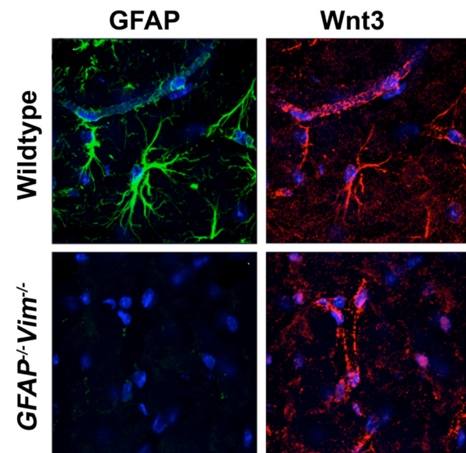

Figure 7. Wnt3 immunoreactivity in astrocytes was detected in wildtype mice (expressing GFAP) but was virtually absent in $GFAP^{-/-}Vim^{-/-}$ mice. In addition to this, the Wnt3 immunoreactivity was associated with blood vessels in both wildtype and $GFAP^{-/-}Vim^{-/-}$ mice.

We conclude that reactive gliosis negatively influences survival and differentiation of neural progenitor cells, both in culture and after grafting. Thus, the data from the retina were now confirmed in the brain using neural progenitor cells (rather than disintegrated retinas) as the source of cells. The present study highlights modulation of the host's astrocyte environment as an important tool to improve graft survival and differentiation.

GFAP and vimentin are negative regulators of the hippocampal neurogenic niche (Paper III)

Genetic ablation of intermediate filaments in astrocytes can theoretically affect the hippocampal neural stem cell niche by two mechanisms. First, such modulation of the hippocampal astrocytes can make the astrocytes surrounding the neural stem cells more supportive for neurogenesis. For example, it has been shown that neural stem cells may exist in many CNS regions but whether neurogenesis can occur or not is a matter of the type of astrocytes present in a particular region (Song et al., 2002a). This suggests that astrocytes are a heterogeneous population of cells, different astrocyte subtypes support neurogenesis to different extent and that absence of astrocyte intermediate filaments might create more neurogenesis-supporting astrocytes. Second, absence of GFAP and vimentin in the neural stem cells could also change their stem cell features. Here we attempted to distinguish between the two.

To evaluate the effect of absence of GFAP and vimentin on neurogenesis and astrogenesis in adult mice, we used the same BrdU-labeling paradigm as in paper I. Apart from assessing basal neurogenesis and astrogenesis in standard-housed mice, we also assessed how the hippocampal neurogenic niche responds to wheel running and to partial deafferentation of the hippocampus (entorhinal cortex lesion). We assessed learning and memory in two different hippocampal-dependent cognitive tasks, Morris water maze and trace fear conditioning. In vitro, we used the neurosphere assay to assess presence of neurosphere-capable progenitor cells in the brains of wildtype, *GFAP$^{-/-}$*, *Vim$^{-/-}$* and *GFAP$^{-/-}$Vim$^{-/-}$* 4-day old pups and evaluated the extent of neuronal differentiation within such neurospheres. To assess the effect of the astrocyte environment on neuronal differentiation we cocultured wildtype, *GFAP$^{-/-}$*, *Vim$^{-/-}$* or *GFAP$^{-/-}$Vim$^{-/-}$* neurospheres with wildtype, *GFAP$^{-/-}$*, *Vim$^{-/-}$* or *GFAP$^{-/-}$Vim$^{-/-}$* astrocytes.

We found that the absence of GFAP and vimentin in *GFAP$^{-/-}$Vim$^{-/-}$* mice resulted in 26% lower dentate gyrus cell proliferation and in a similar number of newly generated neurons and astrocytes at 2 weeks. At 6 weeks, *GFAP$^{-/-}$Vim$^{-/-}$* mice had 74% more surviving newly generated neurons in the GCL of the hippocampus. In running *GFAP$^{-/-}$Vim$^{-/-}$* and wildtype mice, cell proliferation increased to comparable levels despite the lower proliferation levels in standard-housed *GFAP$^{-/-}$Vim$^{-/-}$* mice. (Data summarized in Tab. 5). After entorhinal cortex lesion, *GFAP$^{-/-}$Vim$^{-/-}$* mice showed increased progenitor cell proliferation and increased neurogenic fate choice. The number of newly generated astrocytes was not different between *GFAP$^{-/-}$Vim$^{-/-}$* and wildtype mice during standard housing, after running or after entorhinal cortex lesion.

GFAP$^{-/-}$Vim$^{-/-}$ and wildtype mice showed comparable learning of Morris water maze task but *GFAP$^{-/-}$Vim$^{-/-}$* mice performed better in trace fear conditioning.

Our astrocyte-neurosphere coculture experiments showed that the IF absence in astrocytes, but not in neural progenitor cells, leads to increased neurogenesis (Fig. 8).

Condition	Cell proliferation/survival		Neurogenesis	
	Wildtype	*GFAP$^{-/-}$Vim$^{-/-}$*	Wildtype	*GFAP$^{-/-}$Vim$^{-/-}$*
Standard 2 weeks	—	⇓ - 26%	—	⇒
Standard 6 weeks	—	⇑ + 40%	—	⇑ + 74%
Running vs standard	⇑ + 29%	⇑ + 65%	⇑ + 77%	⇑ + 67%

Table 5. Hippocampal cell proliferation/survival and neurogenesis in GFAP$^{-/-}$Vim$^{-/-}$ and wildtype mice.

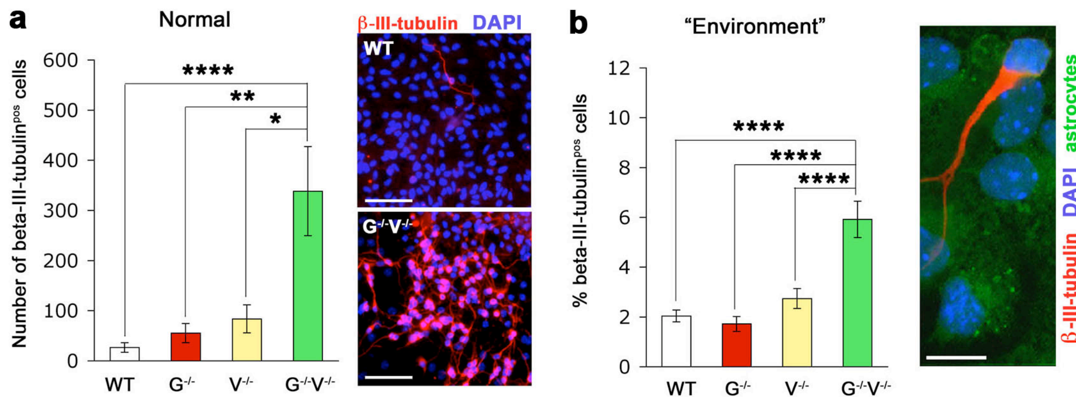

Figure 8. Differentiating neurospheres from GFAP and vimentin deficient pups (G$^{-/-}$V$^{-/-}$) give rise manyfold more neurons than do neurospheres from the other genotypes (a). In coculture with astrocytes, the environment provided by G$^{-/-}$V$^{-/-}$ astrocytes results in more neuronal differentiation (b).

We conclude that the *GFAP$^{-/-}$Vim$^{-/-}$* hippocampal niche is highly supportive for neurogenesis. Upon stimulation, such as injury or running, the *GFAP$^{-/-}$Vim$^{-/-}$* hippocampal niche shows enhanced plasticity. GFAP and vimentin may on their own regulate intrinsic neural stem cells features but the effect of these intrinsic factors on neurogenesis is minimal in comparison with the effect of the astrocyte environment and absence of both GFAP and vimentin (*GFAP$^{-/-}$Vim$^{-/-}$*) is required to create this neurogenic environment.

DISCUSSION

Age-related reactive gliosis might reduce adult hippocampal neurogenesis

Reactive gliosis is usually associated with disease or trauma in the CNS. However, normal physiological aging is also associated with a mild progressive reactive gliosis that is in particular prominent in the hippocampus (David et al., 1997). It is possible that age-related reactive gliosis, when superimposed upon genetic and environmental factors, determines whether the brain aging leads to disease (Mattson et al., 2002). Paper I shows that progenitor cell proliferation is increased by 34% and the number of newborn neurons by 36% in the hippocampus of old *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. Thus, there is a correlation between attenuation of age-related reactive gliosis and higher levels of hippocampal cell proliferation and neurogenesis.

It has been shown that astrocytes derived from old brains compared with astrocytes from young brains are considerably less supportive for neurite outgrowth (Rozovsky et al., 2005). Interestingly, Rozovsky and co-workers could link this change to the levels of GFAP: diminishing the GFAP levels of old astrocytes restored neurite outgrowth from embryonic neural progenitors, whereas overexpression of GFAP in young astrocytes modeled these effects of aging by reducing neurite outgrowth in these progenitors. Vimentin is also up-regulated in the aging brain but to a lesser extent than GFAP (Porchet et al., 2003). Also, since vimentin is not exclusive to astrocytes but is also expressed by endothelial cells and microglia, it is more difficult to establish the contribution of vimentin for age-related reactive gliosis.

The astrocyte environment is important for the integration of grafted neural progenitor cells

Why is grafting of neural cells only sometimes successful? The source of the cells and their degree of differentiation is a part of the answer (Sheen et al., 1999; Darsalia et al., 2007). Also, the environment in which the cells are grafted is essential (Goh et al., 2003; Emsley et al., 2005). It is necessary to understand the factors that modulate graft survival and integration if grafting of neural cells is to be successfully used as a treatment for disese or trauma in the CNS. We have previously shown the impact of astrocyte environment on integration of neural grafts in the retina (Kinouchi et al., 2003). Paper II shows that attenuated reactive gliosis increases neurogenesis and gliogenesis from adult hippocampal progenitor

cells grafted in the hippocampus. Using coculture experiments we demonstrate that *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes increase neural progenitor cell differentiation. The role of the increased astrogenesis from grafted neuronal progenitor cells in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice and the instructive or trophic functions these might have is currently incompletely understood. Is has been shown that grafting of immature glial cells or glial restricted precursors may be beneficial for neuroregeneration (Davies et al., 2008; White and Jakeman, 2008). Based on the findings presented in Paper II, we suggest that it is important to control reactive gliosis in order to increase graft survival and integration.

GFAP and vimentin as regulators of the neurogenic niche

We and others have previously identified the astrocyte environment as an important factor determining the degree of CNS regeneration (Song et al., 2002a; Kinouchi et al., 2003; Wilhelmsson et al., 2004; Cho et al., 2005; Jiao and Chen, 2008). Since GFAP and vimentin are expressed not only by astrocytes but also by neural stem cells and some of their progeny (Bignami, 1982; Steindler and Laywell, 2003), genetic ablation of astrocyte intermediate filaments can affect either the neural stem cells, the astrocyte niche, or both.

Intermediate filament proteins are known to interact with a large number of signaling proteins (Pallari and Eriksson, 2006) and it is possible that absence of GFAP and vimentin could change cell intrinsic properties. The first cells building up a neurosphere are probably generated from a GFAP and vimentin expressing stem cell and thus, absence of GFAP and/or vimentin may have an impact on whether a neurosphere will form or not. In Paper III, we show that absence of either GFAP or vimentin is enough to create cells (bona fide stem cells or restricted neural or glial progenitors) that are more prone to give rise to neurospheres. The presence of a neurosphere is not merely a product of intrinsic features of the cell from which the neurosphere arose: as a neurosphere grows it might eventually create its own astrocyte environment that affects the proliferation and differentiation of progenitors within that neurosphere. Two examples may serve to illustrate how IF proteins may act as regulators of neural stem cell division, differentiation and survival. It has been shown that nestin can act as an organizer of survival-determining signaling molecules and that a high level of nestin protects a cell from apoptosis (Sahlgren et al., 2003; Sahlgren et al., 2006). In the avian nervous system, transitin, a nestin-like IF protein in neural stem cells has been shown to play an important role for distribution of Numb between the two daughter cells after cell division, making the Numb-containing daughter cell a

stem cell whereas the other daughter cell differentiate into the neuronal lineage (Wakamatsu et al., 2007). On a correlative level, low expression of GFAP has been linked to higher levels of cell proliferation and neurogenesis: the rate of neurogenesis during embryonic development is high and the neural stem cells are GFAP negative, whereas in the adult brain the rate of neurogenesis is comparably low and the majority of the neural stem cells are at least transiently GFAP positive (Imura et al., 2003; Garcia et al., 2004). Also, a negative regulation of GFAP expression by TLX has shown to promote proliferation in neural stem cells (Shi et al., 2004).

It has been shown that neural stem cells may exist in many CNS regions but whether neurogenesis can occur or not is a matter of the type of astrocytes present in a particular region (Song et al., 2002a; Jiao and Chen, 2008). Paper II showed that $GFAP^{-/-}Vim^{-/-}$ astrocytes compared to wildtype astrocytes are more supportive for neuronal differentiation in vitro, an observation that is further supported by coculture data in Paper III, suggesting a synergistic effect of the absence of GFAP and vimentin. Interestingly, Paper III suggests that the neurogenic effect of $GFAP^{-/-}Vim^{-/-}$ astrocytes is strong enough to over-run any possible intrinsic differences between wildtype, $GFAP^{-/-}$, $Vim^{-/-}$ and $GFAP^{-/-}Vim^{-/-}$ neurosphere samples.

Enhanced neural plasticity in $GFAP^{-/-}Vim^{-/-}$ mice

Young $GFAP^{-/-}Vim^{-/-}$ mice that are not exposed to wheel running or challenged by injury seem to have a more "economic" way of generating their new hippocampal neurons: less progenitor cell proliferation in combination with better neuronal survival (Paper III). Upon stimulation, such as wheel running, the hippocampal neural progenitors in $GFAP^{-/-}Vim^{-/-}$ mice compared to wildtype mice show a more powerful proliferation response. Such a scenario is supported by the fact that entorhinal cortex lesioned $GFAP^{-/-}Vim^{-/-}$ mice (Paper III) show increased proliferation of a population of cells highly enriched in neural progenitor cells. Since $GFAP^{-/-}Vim^{-/-}$ mice have more neurosphere forming cells in their brains, at least at an early age, this taken together with in vivo data suggests that $GFAP^{-/-}Vim^{-/-}$ mice may have more post-mitotic neural stem cells but that these remain quiescent in absence of any challenge. A recent paper shows how two morphologically distinct populations of Sox2^{pos} stem cells might regulate the generation of newly born neurons (Suh et al., 2007). If this two population model is applied to neurogenesis in wildtype and $GFAP^{-/-}Vim^{-/-}$ mice, it may explain how a sparse cell proliferation can yield a high number of neurons and astrocytes and how neurogenesis can be quickly up-regulated to accommodate new demands.

Neurogenesis is only one aspect of plasticity. The ability to grow axons to the right targets and form synapses finally determines if there is a role to play for the newborn neurons. The exact quality and the function of the 74% extra granular cell layer neurons in $GFAP^{-/-}Vim^{-/-}$ mice is not known but it is possible that the improved performance of $GFAP^{-/-}Vim^{-/-}$ mice in the hippocampus-dependent task trace fear conditioning may be explained by this increased neurogenesis and/or other accompanying elements of increased plasticity.

CONCLUSIONS

(I) Adult *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice show increased basal and neurotrauma-triggered hippocampal neurogenesis.

(II) Attenuation of age-related reactive gliosis has a positive effect on hippocampal cell proliferation and neurogenesis.

(III) *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes increase neurogenesis and astrogenesis from neural progenitor cells in cocultures of neural progenitor cells and astrocytes.

(IV) Attenuation of reactive gliosis increases neurogenesis and astrogenesis from grafted hippocampal progenitor cells.

(V) The absence of both GFAP and vimentin is required to create an astrocyte environment which allows increased neurogenesis from neural progenitor cells.

ACKNOWLEDGEMENTS

Just as neurogenesis is dependent on the astrocyte environment, the writing of this thesis was also very much a product of environment:

I would like to express my gratitude to the **Center for Brain Repair and Rehabilitation (CBR)**, **Department of Clinical Neuroscience and Rehabilitation**, **Institute of Neuroscience and Physiology** and the former **Institute of Medical Biochemistry** at University of Gothenburg for all of its support.

Special thanks:

Milos Pekny, my supervisor, for your never-ending enthusiasm and devotion to science, for inspiration and for making me a scientist.
Marcela Pekna, for support and valuable input to the manuscripts of this thesis.
Michael Nilsson, för bihandledarskap och för ditt fantastiska arbete för CBR.

Ulrika Wilhelmsson, för att du delat med dig av din erfarenhet, för kul pratstunder under de många år som vi delat "cell" och för ditt jordnära sätt att hantera vardagens och forskningens klurigheter. **Daniel Andersson**, för trogen vänskap, skön humor och fin dialekt. **Maryam Faiz**, for great friendship, for your open-minded attitude, scientific commitment and fun times in the cell culture room. **Pete Smith,** for great friendship and for educating me in British culture of all kinds. **Anders Ståhlberg**, för stort kunnande, för vänskap och för att du piffar upp labbet med "dagens vits". **Noriko Shinjyo**, for nice company in the lab - also during weekends. The newcomers **Yolanda de Pablo, Hana Sourkova, Anna Stokowska** and **Alison Atkins** – good luck and enjoy your stay in the lab and in Sweden!

Lizhen Li och **Yalda Rahpeymai Bogestål** för allt roligt vi haft (och har!) tillsammans, för stöd och för stor vänskap under de år vi varit "labsystrar". **Carina Sihlbom,** för hjälp med Western blot och roliga diskussioner om föräldraskap och forskning. **Sofia Linde,** för din tid i labbet och för ditt bidrag till manuskript III i denna avhandling. Tack även **Linda Persson**, **Andrea Lundkvist, Albert Hietala** och alla ni andra **tidigare Pekny/Pekna-gruppmedlemmar** som på olika sätt förgyllt min tid som doktorand!

Jonas Faijerson för medförfattarskap och för givande diskussioner om de kluriga astrocyterna. ***Peter Erikson****, för medförfattarskap och din förmåga att inspirera, för ditt generösa sätt och för allt ditt arbete du lade ner på att sätta CBR på kartan. Din tid här blev alldeles för kort.*

All the PI:s at CBR and their respective groups members and collaborators at the 4th floor: **Klas Blomgren, Michael Nilsson** and **Georg** and **Christi Kuhn** for competence and for creating a nice atmosphere. Särskilt tack **Marie Kalm, Christina Nodin, Helene Andersson, Cecilia Bull, Nina Hellström, Åsa Persson,**

Matilda Zetterström-Axell och **Jenny Nyberg** för roliga pratstunder och för stöd och uppmuntran.

Ann-Marie Alborn och **Birgit Linder** för många fina geler med och utan band och för skratt och gott sällskap i labbet.

All people at the **Institute of Biomedicine** for many nice dissertation parties and long coffee breaks. Special thanks to the **Sven Enerbäck** group: **Gunilla Petersson, Zahra Arani, Rickard Westergren, Mikael Heglind, Daniel Nilsson, Martin Lidell** and **Valentina Sukonina** for fun lunch and coffee breaks through all these years. Tack även hela grupp **Anne Uv** med både nuvarande och tidigare medlemmar för stort support: särskilt tack **Anna Nilton**, för en härlig tid som toast-mamas och **Erika Tång Hallbäck** och **Tina Chavoshi** för skratt och givande pratstunder. Grupp **Jan-Olof Karlsson** och grupp **Michael Nilsson** (tyroidea-gruppen) för att ni livar upp souterrängvåningen. Tack även **alla ni andra i Medkem-huset** för trevligt sällskap i korridoren och under luncher och fikaraster.

Anki Nyberg, **Ulla Ohlson** med flera, för all hjälp viktiga paper och för ert kunnande om hur allt funkar här på institutionen.

Alla ni **djuskötare** för omsorg om möss och för sällskap under långa dagar på "barriären".

Grupp Jörgen Engel, särskilt **Caroline Wass** och **Kenn Johannessen** för lån av utrustning och för värdefulla tips.

Center for Cellular Imaging for supply and support of confocal microscopy.

Bandet **Moonshine** för avstressande onsdagkvällar med skönt tryck, fantastiska spelningar och för en stabilt växande vänskap som klarar både riggning och soundcheck med lågt blodsocker och tillskott av små barn.

"flickornasmat" för djupaste vänskap. Samtidigt som ni vet hur man på bästa sätt tar vara på livets glädjeämnen är ni ett utmärkt stöd när saker och ting inte blir riktigt som man tänkt sig. Under de 10 år som vi känt varandra har vi tillsammans alltmer förstått oss på oss själva, våra sambos/äkta män och vår omvärld.

Alla andra härliga **vänner** för trevliga middagar, fikor, semestrar…

Familjen Jonsson och **Figge** för stort stöd, omtanke och för livliga familjemiddagar med inspiration av Sydeuropa.

Morfar Knut, för din omtanke och visdom och för att du tror på mig och på vetenskapen.

Min bror Petter med familj, för stort stöd, för er smittande livsglädje och för att ni ger mig perspesktiv på tillvaron.

Mamma och pappa, för er ovillkorliga kärlek och för en varm och förnuftig (?) uppfostran. Tack också för fantastisk omsorg om min lilla familj, barnvakt under spelningar med bandet och för hjälp med allt möjligt mellan himmel och jord.

Pär Fredrik, min livskamrat ♥, för lycka och stor kärlek, för att du vet vad som är viktigt och för den bästa tiden i mitt liv. Med dig vid min sida törs jag ta mig an livets utmaningar. Tack för förståelse och stort tålamod under denna intensiva period.

Victoria, min underbara lilla flicka och mitt enda riktigt lyckade experiment. Jag kan inte sätta ord på min kärlek till dig.

The studies in this thesis were supported by grants from the Swedish Medical Research Council, LUA/ALF Göteborg, the Region of Västra Götaland (RUN), Hjärnfonden, Frimurare Foundation, the Swedish Society for Medicine, the Swedish Society for Medical Research, the King Gustaf V Foundation, Torsten and Ragnar Söderberg's Foundations, Trygghansa, Rune and Ulla Amlöv's Foundation, Volvo Assar Gabrielsson Fond, the Swedish Stroke Foundation, Wilhelm and Martina Lundgren's Foundations, the Tornspiran Foundation, the Emil and Maria Palm Foundation and the Mannheimer Foundations.

REFERENCES

Aimone, J. B., Wiles, J. and Gage, F. H. (2006). Potential role for adult neurogenesis in the encoding of time in new memories. *Nat Neurosci* **9**, 723-7.

Alonso, G. (2005). NG2 proteoglycan-expressing cells of the adult rat brain: possible involvement in the formation of glial scar astrocytes following stab wound. *Glia* **49**, 318-38.

Andersen, P. (1975). Organization of hippocampal neurons and their interconnections. . In *The Hippocampus* vol. 1 (ed. R. L. I. K. H. Pribram), pp. 155-175. New York: Plenum Press.

Anderson, M. F., Aberg, M. A., Nilsson, M. and Eriksson, P. S. (2002). Insulin-like growth factor-I and neurogenesis in the adult mammalian brain. *Brain Res Dev Brain Res* **134**, 115-22.

Bangasser, D. A., Waxler, D. E., Santollo, J. and Shors, T. J. (2006). Trace conditioning and the hippocampus: the importance of contiguity. *J Neurosci* **26**, 8702-6.

Barkho, B. Z., Song, H., Aimone, J. B., Smrt, R. D., Kuwabara, T., Nakashima, K., Gage, F. H. and Zhao, X. (2006). Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. *Stem Cells Dev* **15**, 407-21.

Benarroch, E. E. (2006). Limbic System: Emotion and Memory. In *Basic Neurosciences with Clinical Applications*. Philadelphia: Butterworth Heinemann Elsevier.

Bignami, A., Raju, T. and Dahl, D. (1982). Localization of vimentin, the non-specific intermediate filament protein, in embryonal glia and in early differentiating neurons. In vivo and in vitro immunofluorescence study of rat embryo with vimentin and neurofilament antisera. *Developmental Biology* **91**, 286-295.

Bondolfi, L., Ermini, F., Long, J. M., Ingram, D. K. and Jucker, M. (2004). Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice. *Neurobiol Aging* **25**, 333-40.

Bonifas, J. M., Rothman, A. L. and Epstein, E. H., Jr. (1991). Epidermolysis bullosa simplex: evidence in two families for keratin gene abnormalities. *Science* **254**, 1202-5.

Brandeis, R., Brandys, Y. and Yehuda, S. (1989). The use of the Morris Water Maze in the study of memory and learning. *Int J Neurosci* **48**, 29-69.

Brenner, M., Johnson, A. B., Boespflug-Tanguy, O., Rodriguez, D., Goldman, J. E. and Messing, A. (2001). Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease. *Nat Genet* **27**, 117-20.

Buffo, A., Rite, I., Tripathi, P., Lepier, A., Colak, D., Horn, A. P., Mori, T. and Gotz, M. (2008). Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. *Proc Natl Acad Sci U S A* **105**, 3581-6.

Buffo, A., Vosko, M. R., Erturk, D., Hamann, G. F., Jucker, M., Rowitch, D. and Gotz, M. (2005). Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. *Proc Natl Acad Sci U S A* **102**, 18183-8.

Bushong, E. A., Martone, M. E., Jones, Y. Z. and Ellisman, M. H. (2002). Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J Neurosci* **22**, 183-92.

Cameron, H. A. and McKay, R. D. (1999). Restoring production of hippocampal neurons in old age. *Nat Neurosci* **2**, 894-7.

Cameron, H. A. and McKay, R. D. (2001). Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J Comp Neurol* **435**, 406-17.

Cameron, H. A., Woolley, C. S., McEwen, B. S. and Gould, E. (1993). Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* **56**, 337-44.

Cho, K. S., Yang, L., Lu, B., Feng Ma, H., Huang, X., Pekny, M. and Chen, D. F. (2005). Re-establishing the regenerative potential of central nervous system axons in postnatal mice. *J Cell Sci* **118**, 863-72.

Christopherson, K. S., Ullian, E. M., Stokes, C. C., Mullowney, C. E., Hell, J. W., Agah, A., Lawler, J., Mosher, D. F., Bornstein, P. and Barres, B. A. (2005). Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* **120**, 421-33.

Clarke, S. R., Shetty, A. K., Bradley, J. L. and Turner, D. A. (1994). Reactive astrocytes express the embryonic intermediate neurofilament nestin. *Neuroreport* **5**, 1885-8.

Clemmons, D. R. (1998). Role of insulin-like growth factor binding proteins in controlling IGF actions. *Mol Cell Endocrinol* **140**, 19-24.

Colucci-Guyon, E., Portier, M. M., Dunia, I., Paulin, D., Pournin, S. and Babinet, C. (1994). Mice lacking vimentin develop and reproduce without an obvious phenotype. *Cell* **79**, 679-94.

Cooper-Kuhn, C. M. and Kuhn, H. G. (2002). Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. *Brain Res Dev Brain Res* **134**, 13-21.

Crabbe, J. C., Wahlsten, D. and Dudek, B. C. (1999). Genetics of mouse behavior: interactions with laboratory environment. *Science* **284**, 1670-2.

Crawley, J. N. (1999). Behavioral Neuroscience. In *Current Protocols in Neuroscience*, (ed. J. N. Crawley, Grefen, C.R., McKay, R.D., Rogawski, A.M., Sibley, D.R. and Skolnick, P.). Hoboken, NJ, USA: John Wiley & Sons, In.

Crews, F. T., Nixon, K. and Wilkie, M. E. (2004). Exercise reverses ethanol inhibition of neural stem cell proliferation. *Alcohol* **33**, 63-71.

Darsalia, V., Kallur, T. and Kokaia, Z. (2007). Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged rat striatum. *Eur J Neurosci* **26**, 605-14.

David, J. P., Ghozali, F., Fallet-Bianco, C., Wattez, A., Delaine, S., Boniface, B., Di Menza, C. and Delacourte, A. (1997). Glial reaction in the hippocampal formation is highly correlated with aging in human brain. *Neurosci Lett* **235**, 53-6.

Davies, J. E., Proschel, C., Zhang, N., Noble, M., Mayer-Proschel, M. and Davies, S. J. (2008). Transplanted astrocytes derived from BMP- or CNTF-treated glial-restricted precursors have opposite effects on recovery and allodynia after spinal cord injury. *J Biol* **7**, 24.

Doetsch, F., Caille, I., Lim, D. A., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703-16.

Doetsch, F., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* **17**, 5046-61.

Dupret, D., Revest, J. M., Koehl, M., Ichas, F., De Giorgi, F., Costet, P., Abrous, D. N. and Piazza, P. V. (2008). Spatial relational memory requires hippocampal adult neurogenesis. *PLoS ONE* **3**, e1959.

Eddleston, M. and Mucke, L. (1993). Molecular profile of reactive astrocytes--implications for their role in neurologic disease. *Neuroscience* **54**, 15-36.

Eliasson, C., Sahlgren, C., Berthold, C. H., Stakeberg, J., Celis, J. E., Betsholtz, C., Eriksson, J. E. and Pekny, M. (1999). Intermediate filament protein partnership in astrocytes. *J Biol Chem* **274**, 23996-4006.

Emsley, J. G., Mitchell, B. D., Kempermann, G. and Macklis, J. D. (2005). Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Prog Neurobiol* **75**, 321-41.

Eng, L. F. a. G., R.S. (1994). GFAP and astrogliosis. *Brain Pathology* **4**, 229-237.

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. and Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nat Med* **4**, 1313-7.

Fabel, K., Tam, B., Kaufer, D., Baiker, A., Simmons, N., Kuo, C. J. and Palmer, T. D. (2003). VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci* **18**, 2803-12.

Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, N. B. and Sofroniew, M. V. (2004). Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci* **24**, 2143-55.

Fawcett, J. W. and Asher, R. A. (1999). The glial scar and central nervous system repair. *Brain Res Bull* **49**, 377-91.

Garcia, A. D., Doan, N. B., Imura, T., Bush, T. G. and Sofroniew, M. V. (2004). GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat Neurosci* **7**, 1233-41.

Goh, E. L., Ma, D., Ming, G. L. and Song, H. (2003). Adult neural stem cells and repair of the adult central nervous system. *J Hematother Stem Cell Res* **12**, 671-9.

Good, A. J. and Westbrook, R. F. (1995). Effects of a microinjection of morphine into the amygdala on the acquisition and expression of conditioned fear and hypoalgesia in rats. *Behav Neurosci* **109**, 631-41.

Goss, J. R., Finch, C. E. and Morgan, D. G. (1991). Age-related changes in glial fibrillary acidic protein mRNA in the mouse brain. *Neurobiol Aging* **12**, 165-70.

Gotz, M., Hartfuss, E. and Malatesta, P. (2002). Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. *Brain Res Bull* **57**, 777-88.

Gould, E., McEwen, B. S., Tanapat, P., Galea, L. A. and Fuchs, E. (1997). Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J Neurosci* **17**, 2492-8.

Gould, E. and Tanapat, P. (1999). Stress and hippocampal neurogenesis. *Biol Psychiatry* **46**, 1472-9.

Gould, E., Tanapat, P., Hastings, N. B. and Shors, T. J. (1999). Neurogenesis in adulthood: a possible role in learning. *Trends Cogn Sci* **3**, 186-192.

Hof, P. R., Trapp, B.D., de Velles J., Claudio, L. & Colman, D.R. (1999). Cellular and molecular neuroscience. In *Fundamental Neuroscience*, (ed. L. R. Squire). San Diego: Academic press.

Hyder, C. L., Pallari, H. M., Kochin, V. and Eriksson, J. E. (2008). Providing cellular signposts--post-translational modifications of intermediate filaments. *FEBS Lett* **582**, 2140-8.

Imura, T., Kornblum, H. I. and Sofroniew, M. V. (2003). The Predominant Neural Stem Cell Isolated from Postnatal and Adult Forebrain But Not Early Embryonic Forebrain Expresses GFAP. *J Neurosci* **23**, 2824-32.

Imura, T., Nakano, I., Kornblum, H. I. and Sofroniew, M. V. (2006). Phenotypic and functional heterogeneity of GFAP-expressing cells in vitro: differential expression of LeX/CD15 by GFAP-expressing multipotent neural stem cells and non-neurogenic astrocytes. *Glia* **53**, 277-93.

Jang, M.-H., Song, H. and Ming, G.-L. (2008). Regulation of adult neurogenesis by neurotransmitters. In *Adult neurogenesis*, (ed. G. K. Fred H. Gage, Hongjun Song). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Jessberger, S. and Kempermann, G. (2003). Adult-born hippocampal neurons mature into activity-dependent responsiveness. *Eur J Neurosci* **18**, 2707-12.

Jiao, J. and Chen, D. F. (2008). Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. *Stem Cells* **26**, 1221-30.

Jing, R., Wilhelmsson, U., Goodwill, W., Li, L., Pan, Y., Pekny, M. and Skalli, O. (2007). Synemin is expressed in reactive astrocytes in neurotrauma and interacts differentially with vimentin and GFAP intermediate filament networks. *J Cell Sci* **120**, 1267-77.

Jourdain, P., Bergersen, L. H., Bhaukaurally, K., Bezzi, P., Santello, M., Domercq, M., Matute, C., Tonello, F., Gundersen, V. and Volterra, A. (2007). Glutamate exocytosis from astrocytes controls synaptic strength. *Nat Neurosci* **10**, 331-9.

Julien, J. P. and Mushynski, W. E. (1998). Neurofilaments in health and disease. *Prog Nucleic Acid Res Mol Biol* **61**, 1-23.

Kaplan, M. S. and Bell, D. H. (1984). Mitotic neuroblasts in the 9-day-old and 11-month-old rodent hippocampus. *J Neurosci* **4**, 1429-41.

Kee, N., Teixeira, C. M., Wang, A. H. and Frankland, P. W. (2007). Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. *Nat Neurosci* **10**, 355-62.

Kempermann, G., Gast, D., Kronenberg, G., Yamaguchi, M. and Gage, F. H. (2003). Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development* **130**, 391-9.

Kempermann, G., Kuhn, H. G. and Gage, F. H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature* **386**, 493-5.

Kempermann, G., Kuhn, H. G. and Gage, F. H. (1998). Experience-induced neurogenesis in the senescent dentate gyrus. *J Neurosci* **18**, 3206-12.

Kempermann, G., Wiskott, L. and Gage, F. H. (2004). Functional significance of adult neurogenesis. *Curr Opin Neurobiol* **14**, 186-91.

Kinouchi, R., Takeda, M., Yang, L., Wilhelmsson, U., Lundkvist, A., Pekny, M. and Chen, D. F. (2003). Robust neural integration from retinal transplants in mice deficient in GFAP and vimentin. *Nat Neurosci* **6**, 863-8.

Kohama, S. G., Goss, J. R., Finch, C. E. and McNeill, T. H. (1995). Increases of glial fibrillary acidic protein in the aging female mouse brain. *Neurobiol Aging* **16**, 59-67.

Kolb, B., Pedersen, B., Ballermann, M., Gibb, R. and Whishaw, I. Q. (1999). Embryonic and postnatal injections of bromodeoxyuridine produce age-dependent morphological and behavioral abnormalities. *J Neurosci* **19**, 2337-46.

Kornblum, H. I. and Geschwind, D. H. (2001). Molecular markers in CNS stem cell research: hitting a moving target. *Nat Rev Neurosci* **2**, 843-6.

Kuhn, H. G., Dickinson-Anson, H. and Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* **16**, 2027-33.

Kukekov, V. G., Laywell, E. D., Thomas, L. B. and Steindler, D. A. (1997). A nestin-negative precursor cell from the adult mouse brain gives rise to neurons and glia. *Glia* **21**, 399-407.

Lai, K., Kaspar, B. K., Gage, F. H. and Schaffer, D. V. (2003). Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. *Nat Neurosci* **6**, 21-7.

Laywell, E. D., Rakic, P., Kukekov, V. G., Holland, E. C. and Steindler, D. A. (2000). Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc Natl Acad Sci U S A* **97**, 13883-8.

Lee, A., Kessler, J. D., Read, T. A., Kaiser, C., Corbeil, D., Huttner, W. B., Johnson, J. E. and Wechsler-Reya, R. J. (2005). Isolation of neural stem cells from the postnatal cerebellum. *Nat Neurosci* **8**, 723-9.

Lendahl, U., Zimmerman, L. B. and McKay, R. D. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 585-95.

Lie, D. C., Colamarino, S. A., Song, H. J., Desire, L., Mira, H., Consiglio, A., Lein, E. S., Jessberger, S., Lansford, H., Dearie, A. R. et al. (2005). Wnt signalling regulates adult hippocampal neurogenesis. *Nature* **437**, 1370-5.

Lin, R. C. S., Matesic, D.F., Marvin, M., McKay, R.D. and Brustle, O. (1995). Re-expression of the intermediate filament nestin in reactive astrocytes. *Neurobiological Disease* **2**, 79-85.

Liu, Z., Li, Y., Zhang, X., Savant-Bhonsale, S. and Chopp, M. (2008). Contralesional axonal remodeling of the corticospinal system in adult rats after stroke and bone marrow stromal cell treatment. *Stroke* **39**, 2571-7.

Lois, C. and Alvarez-Buylla, A. (1993). Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci U S A* **90**, 2074-7.

Ma, D. K., Ming, G.-L., Gage, F.H., Song, H. (2008). Neurogenich Niches in the Adult Mammalian Brain. In *Adult neurogenesis*, (ed. G. K. Fred H. Gage, Hongjun Song), pp. 207-225. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Matoltsy, A. G. (1975). Desmosomes, filaments, and keratohyaline granules: their role in the stabilization and keratinization of the epidermis. *J Invest Dermatol* **65**, 127-42.

Mattson, M. P., Chan, S. L. and Duan, W. (2002). Modification of brain aging and neurodegenerative disorders by genes, diet, and behavior. *Physiol Rev* **82**, 637-72.

McEwen, B. S., Magarinos, A. M. and Reagan, L. P. (2002). Studies of hormone action in the hippocampal formation: possible relevance to depression and diabetes. *J Psychosom Res* **53**, 883-90.

McGraw, J., Hiebert, G. W. and Steeves, J. D. (2001). Modulating astrogliosis after neurotrauma. *J Neurosci Res* **63**, 109-15.

Merkle, F. T., Tramontin, A. D., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (2004). Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc Natl Acad Sci U S A* **101**, 17528-32.

Merrill, D. A., Karim, R., Darraq, M., Chiba, A. A. and Tuszynski, M. H. (2003). Hippocampal cell genesis does not correlate with spatial learning ability in aged rats. *J Comp Neurol* **459**, 201-7.

Meshi, D., Drew, M. R., Saxe, M., Ansorge, M. S., David, D., Santarelli, L., Malapani, C., Moore, H. and Hen, R. (2006). Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. *Nat Neurosci* **9**, 729-31.

Misane, I., Tovote, P., Meyer, M., Spiess, J., Ogren, S. O. and Stiedl, O. (2005). Time-dependent involvement of the dorsal hippocampus in trace fear conditioning in mice. *Hippocampus* **15**, 418-26.

Miyake, T., Hattori, T., Fukuda, M., Kitamura, T. and Fujita, S. (1988). Quantitative studies on proliferative changes of reactive astrocytes in mouse cerebral cortex. *Brain Res* **451**, 133-8.

Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S. and Papaioannou, V. E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869-77.

Monje, M. L. and Palmer, T. (2003). Radiation injury and neurogenesis. *Curr Opin Neurol* **16**, 129-34.

Morgan, T. E., Rozovsky, I., Goldsmith, S. K., Stone, D. J., Yoshida, T. and Finch, C. E. (1997). Increased transcription of the astrocyte gene GFAP during middle-age is attenuated by food restriction: implications for the role of oxidative stress. *Free Radic Biol Med* **23**, 524-8.

Morrison, S. J. and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* **441**, 1068-74.

Morshead, C. M., Craig, C. G. and van der Kooy, D. (1998). In vivo clonal analyses reveal the properties of endogenous neural stem cell proliferation in the adult mammalian forebrain. *Development* **125**, 2251-61.

Nowakowski, R. S., Lewin, S. B. and Miller, M. W. (1989). Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. *J Neurocytol* **18**, 311-8.

Oberheim, N. A., Wang, X., Goldman, S. and Nedergaard, M. (2006). Astrocytic complexity distinguishes the human brain. *Trends Neurosci* **29**, 547-53.

Pallari, H. M. and Eriksson, J. E. (2006). Intermediate filaments as signaling platforms. *Sci STKE* **2006**, pe53.

Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F. and Gage, F. H. (1999). Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* **19**, 8487-97.

Palmer, T. D., Ray, J. and Gage, F. H. (1995). FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol Cell Neurosci* **6**, 474-86.

Palmer, T. D., Takahashi, J. and Gage, F. H. (1997). The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci* **8**, 389-404.

Pekny, M., Eliasson, C., Chien, C. L., Kindblom, L. G., Liem, R., Hamberger, A. and Betsholtz, C. (1998). GFAP-deficient astrocytes are capable of stellation in vitro when cocultured with neurons and exhibit a reduced amount of intermediate filaments and an increased cell saturation density. *Exp Cell Res* **239**, 332-43.

Pekny, M., Johansson, C. B., Eliasson, C., Stakeberg, J., Wallen, A., Perlmann, T., Lendahl, U., Betsholtz, C., Berthold, C. H. and Frisen, J. (1999). Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin. *J Cell Biol* **145**, 503-14.

Pekny, M. and Lane, E. B. (2007). Intermediate filaments and stress. *Exp Cell Res* **313**, 2244-54.

Pekny, M., Leveen, P., Pekna, M., Eliasson, C., Berthold, C. H., Westermark, B. and Betsholtz, C. (1995). Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. *Embo J* **14**, 1590-8.

Pekny, M. and Nilsson, M. (2005). Astrocyte activation and reactive gliosis. *Glia* **50**, 427-34.

Pekny, M. and Pekna, M. (2004). Astrocyte intermediate filaments in CNS pathologies and regeneration. *J Pathol* **204**, 428-37.

Pekny, M. and Wilhelmsson, U. (2006a). GFAP and Astrocyte Intermediate Filaments. In *Handbook of Neurochemistry*, (ed. A. Lajtha), pp. 289-314. New York: Springer Science+Business Media.

Pekny, M. and Wilhelmsson, U. (2006b). Intermediate filaments in astrocytes in health and disease. In *Intermediate filaments*, (ed. J. M. Paramio), pp. 10-26. New York and Georgetown: Springer Science+ Business Media and Landes Bioscience.

Pekny, M., Wilhelmsson, U., Bogestal, Y. R. and Pekna, M. (2007). The role of astrocytes and complement system in neural plasticity. *Int Rev Neurobiol* **82**, 95-111.

Porchet, R., Probst, A., Bouras, C., Draberova, E., Draber, P. and Riederer, B. M. (2003). Analysis of glial acidic fibrillary protein in the human entorhinal cortex during aging and in Alzheimer's disease. *Proteomics* **3**, 1476-85.

Raivich, G., Bohatschek, M., Kloss, C. U., Werner, A., Jones, L. L. and Kreutzberg, G. W. (1999). Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res Brain Res Rev* **30**, 77-105.

Ramirez-Amaya, V., Marrone, D. F., Gage, F. H., Worley, P. F. and Barnes, C. A. (2006). Integration of new neurons into functional neural networks. *J Neurosci* **26**, 12237-41.

Ray, J., Peterson, D. A., Schinstine, M. and Gage, F. H. (1993). Proliferation, differentiation, and long-term culture of primary hippocampal neurons. *Proc Natl Acad Sci U S A* **90**, 3602-6.

Rechler, M. M. and Clemmons, D. R. (1998). Regulatory Actions of Insulin-like Growth Factor-binding Proteins. *Trends Endocrinol Metab* **9**, 176-83.

Reynolds, B. A. and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-10.

Reynolds, B. A. and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* **175**, 1-13.

Ridet, J. L., Malhotra, S. K., Privat, A. and Gage, F. H. (1997). Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci* **20**, 570-7.

Rogers, S. D., Peters, C. M., Pomonis, J. D., Hagiwara, H., Ghilardi, J. R. and Mantyh, P. W. (2003). Endothelin B receptors are expressed by astrocytes and regulate astrocyte hypertrophy in the normal and injured CNS. *Glia* **41**, 180-90.

Rozovsky, I., Wei, M., Morgan, T. E. and Finch, C. E. (2005). Reversible age impairments in neurite outgrowth by manipulations of astrocytic GFAP. *Neurobiol Aging* **26**, 705-15.

Sahlgren, C. M., Mikhailov, A., Vaittinen, S., Pallari, H. M., Kalimo, H., Pant, H. C. and Eriksson, J. E. (2003). Cdk5 regulates the organization of Nestin and its association with p35. *Mol Cell Biol* **23**, 5090-106.

Sahlgren, C. M., Pallari, H. M., He, T., Chou, Y. H., Goldman, R. D. and Eriksson, J. E. (2006). A nestin scaffold links Cdk5/p35 signaling to oxidant-induced cell death. *EMBO J* **25**, 4808-19.

Sandkuhler, J. (2002). Fear the pain. *Lancet* **360**, 426.

Saxe, M. D., Battaglia, F., Wang, J. W., Malleret, G., David, D. J., Monckton, J. E., Garcia, A. D., Sofroniew, M. V., Kandel, E. R., Santarelli, L. et al. (2006). Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci U S A* **103**, 17501-6.

Schimanski, L. A. and Nguyen, P. V. (2004). Multidisciplinary approaches for investigating the mechanisms of hippocampus-dependent memory: a focus on inbred mouse strains. *Neurosci Biobehav Rev* **28**, 463-83.

Schmidt-Hieber, C., Jonas, P. and Bischofberger, J. (2004). Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. *Nature* **429**, 184-7.

Schneitzer, J., Franke, W.W. and Schachner, M. (1981). Immunocytochemical demonstration of vimentin in astrocytes and ependymal cells of developing and adult mouse nervous system. *The Journal of Cell Biology* **90**, 435-447.

Seigneurin, D. and Guillaud, P. (1991). [Ki-67 antigen, a cell cycle and tumor growth marker]. *Pathol Biol (Paris)* **39**, 1020-8.

Selden, J. R., Dolbeare, F., Clair, J. H., Nichols, W. W., Miller, J. E., Kleemeyer, K. M., Hyland, R. J. and DeLuca, J. G. (1993). Statistical confirmation that immunofluorescent detection of DNA repair in human fibroblasts by measurement of bromodeoxyuridine incorporation is stoichiometric and sensitive. *Cytometry* **14**, 154-67.

Seri, B., Garcia-Verdugo, J. M., Collado-Morente, L., McEwen, B. S. and Alvarez-Buylla, A. (2004). Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. *J Comp Neurol* **478**, 359-78.

Seri, B., Garcia-Verdugo, J. M., McEwen, B. S. and Alvarez-Buylla, A. (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci* **21**, 7153-60.

Sheen, V. L., Arnold, M. W., Wang, Y. and Macklis, J. D. (1999). Neural precursor differentiation following transplantation into neocortex is dependent on intrinsic developmental state and receptor competence. *Exp Neurol* **158**, 47-62.

Shi, Y., Chichung Lie, D., Taupin, P., Nakashima, K., Ray, J., Yu, R. T., Gage, F. H. and Evans, R. M. (2004). Expression and function of orphan nuclear receptor TLX in adult neural stem cells. *Nature* **427**, 78-83.

Shihabuddin, L. S., Horner, P. J., Ray, J. and Gage, F. H. (2000). Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci* **20**, 8727-35.

Shihabuddin, L. S., Ray, J. and Gage, F. H. (1997). FGF-2 is sufficient to isolate progenitors found in the adult mammalian spinal cord. *Exp Neurol* **148**, 577-86.

Shors, T. J., Miesegaes, G., Beylin, A., Zhao, M., Rydel, T. and Gould, E. (2001). Neurogenesis in the adult is involved in the formation of trace memories. *Nature* **410**, 372-6.

Shors, T. J., Townsend, D. A., Zhao, M., Kozorovitskiy, Y. and Gould, E. (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus* **12**, 578-84.

Silver, J. and Miller, J. H. (2004). Regeneration beyond the glial scar. *Nat Rev Neurosci* **5**, 146-56.

Singec, I., Knoth, R., Meyer, R. P., Maciaczyk, J., Volk, B., Nikkhah, G., Frotscher, M. and Snyder, E. Y. (2006). Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. *Nat Methods* **3**, 801-6.

Snyder, J. S., Kee, N. and Wojtowicz, J. M. (2001). Effects of adult neurogenesis on synaptic plasticity in the rat dentate gyrus. *J Neurophysiol* **85**, 2423-31.

Sofroniew, M. V. (2005). Reactive astrocytes in neural repair and protection. *Neuroscientist* **11**, 400-7.

Song, H., Stevens, C. F. and Gage, F. H. (2002a). Astroglia induce neurogenesis from adult neural stem cells. *Nature* **417**, 39-44.

Song, H. J., Stevens, C. F. and Gage, F. H. (2002b). Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. *Nat Neurosci* **5**, 438-45.

Stanfield, B. B. and Trice, J. E. (1988). Evidence that granule cells generated in the dentate gyrus of adult rats extend axonal projections. *Exp Brain Res* **72**, 399-406.

Steindler, D. A. and Laywell, E. D. (2003). Astrocytes as stem cells: Nomenclature, phenotype, and translation. *Glia* **43**, 62-9.

Steiner, B., Kronenberg, G., Jessberger, S., Brandt, M. D., Reuter, K. and Kempermann, G. (2004). Differential regulation of gliogenesis in the context of adult hippocampal neurogenesis in mice. *Glia* **46**, 41-52.

Stone, D. J., Rozovsky, I., Morgan, T. E., Anderson, C. P. and Finch, C. E. (1998). Increased synaptic sprouting in response to estrogen via an apolipoprotein E-dependent mechanism: implications for Alzheimer's disease. *J Neurosci* **18**, 3180-5.

Suh, H., Consiglio, A., Ray, J., Sawai, T., D'Amour, K. A. and Gage, F. H. (2007). In Vivo Fate Analysis Reveals the Multipotent and Self-Renewal Capacities of Sox2(+) Neural Stem Cells in the Adult Hippocampus. *Cell Stem Cell* **1**, 515-528.

Suhonen, J. O., Peterson, D. A., Ray, J. and Gage, F. H. (1996). Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature* **383**, 624-7.

Sultana, S., Sernett, S. W., Bellin, R. M., Robson, R. M. and Skalli, O. (2000). Intermediate filament protein synemin is transiently expressed in a subset of astrocytes during development. *Glia* **30**, 143-53.

Trejo, J. L., Carro, E. and Torres-Aleman, I. (2001). Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus. *J Neurosci* **21**, 1628-34.

Tropepe, V., Sibilia, M., Ciruna, B. G., Rossant, J., Wagner, E. F. and van der Kooy, D. (1999). Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev Biol* **208**, 166-88.

Ullian, E. M., Sapperstein, S.K., Christophersen, K.S. and Barres, B.A. (2001). Control of synapse number by glia. *Science* **291**, 657-661.

Unger, J. W. (1998). Glial reaction in aging and Alzheimer's disease. *Microsc Res Tech* **43**, 24-8.

Wakamatsu, Y., Nakamura, N., Lee, J. A., Cole, G. J. and Osumi, N. (2007). Transitin, a nestin-like intermediate filament protein, mediates cortical localization and the lateral transport of Numb in mitotic avian neuroepithelial cells. *Development* **134**, 2425-33.

van Praag, H., Christie, B. R., Sejnowski, T. J. and Gage, F. H. (1999). Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc Natl Acad Sci U S A* **96**, 13427-31.

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D. and Gage, F. H. (2002). Functional neurogenesis in the adult hippocampus. *Nature* **415**, 1030-4.

van Praag, H., Shubert, T., Zhao, C. and Gage, F. H. (2005). Exercise enhances learning and hippocampal neurogenesis in aged mice. *J Neurosci* **25**, 8680-5.

Wang, S., Scott, B. W. and Wojtowicz, J. M. (2000). Heterogenous properties of dentate granule neurons in the adult rat. *J Neurobiol* **42**, 248-57.

White, R. E. and Jakeman, L. B. (2008). Don't fence me in: Harnessing the beneficial roles of astrocytes for spinal cord repair. *Restor Neurol Neurosci* **26**, 197-214.

Wilhelmsson, U., Bushong, E. A., Price, D. L., Smarr, B. L., Phung, V., Terada, M., Ellisman, M. H. and Pekny, M. (2006). Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury. *Proc Natl Acad Sci U S A* **103**, 17513-8.

Wilhelmsson, U., Li, L., Pekna, M., Berthold, C. H., Blom, S., Eliasson, C., Renner, O., Bushong, E., Ellisman, M., Morgan, T. E. et al. (2004). Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration. *J Neurosci* **24**, 5016-21.

Visnyei, K., Tatsukawa, K. J., Erickson, R. I., Simonian, S., Oknaian, N., Carmichael, S. T. and Kornblum, H. I. (2006). Neural progenitor implantation restores metabolic deficits in the brain following striatal quinolinic acid lesion. *Exp Neurol* **197**, 465-74.

Witter, M. P. (1993). Organization of the entorhinal-hippocampal system: a review of current anatomical data. *Hippocampus* **3 Spec No**, 33-44.

Wojtowicz, J. M., Askew, M. L. and Winocur, G. (2008). The effects of running and of inhibiting adult neurogenesis on learning and memory in rats. *Eur J Neurosci* **27**, 1494-502.

Yasuhara, T., Matsukawa, N., Hara, K., Yu, G., Xu, L., Maki, M., Kim, S. U. and Borlongan, C. V. (2006). Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. *J Neurosci* **26**, 12497-511.

Zhang, C. L., Zou, Y., He, W., Gage, F. H. and Evans, R. M. (2008). A role for adult TLX-positive neural stem cells in learning and behaviour. *Nature*.

Zhao, C., Teng, E. M., Summers, R. G., Jr., Ming, G. L. and Gage, F. H. (2006). Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. *J Neurosci* **26**, 3-11.

Zonta, M., Angulo, M. C., Gobbo, S., Rosengarten, B., Hossmann, K. A., Pozzan, T. and Carmignoto, G. (2003). Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat Neurosci* **6**, 43-50.