The role of the intermediate filament (nanofilament) protein nestin in neural progenitor cell and astrocyte differentiation

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If we knew what it was, we were doing, it would not be called research, would it?

Albert Einstein

ABSTRACT

Nestin, a class VI intermediate filament (nanofilament) protein, is commonly used as a marker for neural stem/progenitor cells (NSPCs), but its role in neurogenesis remains elusive. Nestin is also expressed in immature and reactive astrocytes. The up-regulation of intermediate filament proteins glial fibrillary acidic protein (GFAP), vimentin and nestin is a characteristic feature of reactive astrocytes, accompanied by alterations in the expression of many other genes. We found that transgenic mice deficient for nestin have increased number of newly born hippocampal neurons 6 weeks after BrdU *in vivo* labeling, suggesting increased generation and/or survival of newly born neurons. We also showed that *in vitro* nestin deficient astrocytes provide a more pro-neurogenic environment that results in a 2-fold increase in neuronal differentiation of NSPCs.

Astrocytes are highly heterogeneous cells and fulfill a variety of important functions in healthy as well as diseased brain. In addition, astrocytes can exhibit features characteristic of NSPCs and modulate neurogenesis by inhibiting neuronal differentiation of NSPCs through Jagged1-mediated Notch signaling. Given the vast array of astrocyte functions, classification of astrocyte subpopulations on a molecular level is highly desirable. We used single cell quantitative real-time PCR to investigate the heterogeneity of astrocytes with respect to their Notch signaling competence. Our results show that the Notch signal sending but not Notch signal receiving competence of astrocytes depends on GFAP and vimentin. Further, we showed that nestin and heparin binding EGF-like growth factor (HB-EGF) may serve as classifiers of astrocyte subpopulations *in vitro*.

Utilizing our newly developed Bioactive3D and standard 2D cell culture systems, we showed that HB-EGF alters astrocyte morphology towards a more radial glia-like phenotype. HB-EGF affects proliferation, differentiation and expression of Notch signal pathway related genes and leads to the up-regulation of nestin expression. HB-EGF in cell culture media results in partial de-differentiation of astrocytes and therefore should be used with caution.

Keywords: intermediate filament (nanofilament) proteins, nestin, neural stem/progenitor cells, astrocytes, GFAP, vimentin, HB-EGF

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their roman numerals.

I. Lebkuechner I, Andersson D, Möllerström E, Wilhelmsson U, Pekna M, Pekny M. (2014) Heterogeneity of Notch signaling in astrocytes and the effects of GFAP and vimentin deficiency. *Manuscript*

II. Wilhelmsson U, Lebkuechner I, Yang X, Nagy A, Pekny M. (2014) Increased hippocampal neurogenesis in mice deficient for intermediate filament (nanofilament) protein nestin. *Manuscript*

III. Puschmann TB, Zanden C*, Lebkuechner I*, Philippot C, de Pablo Y, Liu J, Pekny M. (2014) HB-EGF affects astrocyte morphology, proliferation, differentiation, and the expression of intermediate filament proteins. *Journal of Neurochemistry, 128(6):878-89*

* Authors contributed equally to this work

CONTENT

ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
ADAM	A disintegrin and metalloprotease
Aldh1L1	Aldehyde dehydrogenase 1 family, member L1
Bioactive3D	3-dimensional cell culture system
BrdU	5-Bromo-2´-Deoxyuridine
CNS	Central nervous system
Dcx	Doublecortin
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EdU	5-Ethynyl-2´-Deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor (also known as ErbB1)
ErbB1-4	v-erb-a erythroblastic leukemia viral oncogene homolog 1-4 (avian) (EGFR signaling pathway receptors)
FCS	Fetal calf serum
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HB-EGF	Heparin binding epidermal growth factor-like growth factor
mRNA	Messenger RNA (ribonucleic acid)
NSC	Neural stem cell
NSPC	Neural stem/progenitor cell
NPC	Neural progenitor cell
RT-qPCR	Quantitative real-time polymerase chain reaction
SGZ	Subgranular zone of the dentate gyrus
SVZ	Subventricular zone of the lateral ventricles

1 INTRODUCTION

1.1 Development of the mammalian central nervous system (CNS)

1.1.1 The major cellular players of the CNS

The mammalian central nervous system (CNS) consists of three parts: the brain, the retina and the spinal cord. Functions of the spinal cord are e.g. to conduct sensory information from the peripheral nervous system to the brain and to control simple muscle reflexes. The brain is responsible for the integration and evaluation of the sensory information and coordination of conscious or unconscious responses and, in addition, for complex functions such as thinking, memory and emotions.

The major cellular players within the mammalian CNS are neurons and glial cells. The CNS consists of roughly the same number of neurons and glial cells, however the neuron-glia ratio depends on the CNS region [1]. In addition, the ratio is increased in organisms of higher taxonomy as is the morphological complexity of astrocytes [2]. Neurons are defined as electrically excitable cells, which convey information through electrical action potentials or through chemical-induced signal transduction via neurotransmitters. Neurons are a highly heterogeneous cell class based on their functions and morphology, with various subpopulations, such as sensory neurons, interneurons or motoneurons. Glial cells are defined as electrically non-excitable cells and comprise macroglial cells, such as astrocytes, oligodendrocytes, NG-2 glia, and microglia in the CNS. Oligodendrocytes are equivalent

to the Schwann cells of the peripheral nervous system and have myelinating function isolating neuronal axons. NG-2 glia are oligodendrocyte precursor cells and the most recent identified subpopulation of glia. Microglia are the immune cells within the CNS. Astrocytes are highly multifunctional cells and play important roles in extracellular ion homeostasis, recycling of neurotransmitters, neurogenesis and also in CNS regeneration.

1.1.2 Neural stem/progenitor cells in CNS development

The development of the CNS starts during early embryogenesis within the one-layered neuroepithelium of the neural plate, which later forms the neural tube. Neural stem cells (NSCs) of the neural tube (neuroephitelial cells), characterized by unlimited self-renewing capability and multi-potency, generate the two major cell classes of the CNS, neurons and macroglia (astrocytes, oligodendrocytes and NG-2 glia), while microglia are generated by mesodermal stem cells. NSCs divide symmetrically, generating two daughter NSCs until the onset of neurogenesis (embryonic day 9-10 in mice). After the onset of neurogenesis, NSCs start to divide asymmetrically to generate one NSC and a more differentiated neural progenitor cell (NPC), which is restricted in its possible lineage development to neurons. With the onset of neurogenesis, the neuroepithelium becomes multilayered and most neuroephitelial cells change their molecular properties (e.g. down-regulation of tight junctions) and start to show some astrocyte features, such as the presence of glutamate transporters, and are named radial glia cells, that give either rise directly to neurons or differentiate

into neural intermediate progenitor cells (capable to generate neurons or macroglial cells) through asymmetric division. In most regions of the mouse CNS, the molecular shift from neuroepithelial cells to radial glia cells occurs between embryonic day 10 – 12. Most radial glia cells are lineage restricted to neurons, astrocytes or oligodendrocytes and they serve as a migration scaffold for neurons. The onset of generation of the macroglial cells (gliogenesis), astrocytes (astrogenesis) and oligodendrocytes (oligodenrocytogenesis), occurs after the onset of neurogenesis in CNS development, with the peak of astrogenesis at perinatal time points and the peak of oligodendrocytogenesis at postnatal time points (Fig. 1; for review see [3]).

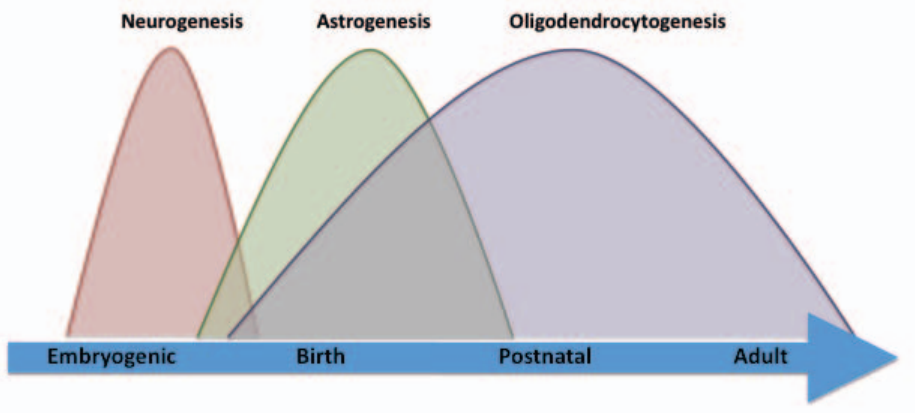

Fig.1 Development of the major cell types within the CNS. *During embryogenesis neurogenesis starts first, followed by astrogenesis that lasts into perinatal and postnatal time points. Oligodendrocytogenesis starts perinatal and lasts into adulthood. Modified after Verkhratsky & Butt, Glial Physiology and Pathophysiology, 2013.*

1.2 Adult neurogenesis

1.2.1 Neurogenesis in the adult CNS occurs in two distinct regions

The traditional view of neurogenesis in the CNS was that generation of neurons from neuroepithelial cells and/or radial glia cells occurs only during embryogenesis and perinatal stages. Studies by J. Altman 50 years ago provided the first evidence of postnatal neurogenesis in rat hippocampi [4]. Functional integration of newly born neurons in adults was first shown in songbirds in 1984 [5] and in 1992 NSCs were first isolated from adult mammalian brain [6, 7].

The capacity of the healthy adult mammalian CNS to generate and functionally integrate new neurons into existing neuronal networks is extremely limited under physiological conditions in comparison to the developing CNS. In addition, within the healthy CNS adult neurogenesis occurs in two distinct neurogenic niches only, namely the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Fig. 2), while gliogenesis in the adult brain is not restricted to specific niches. Newly born neurons in the SVZ migrate along the rostral migratory stream into the olfactory bulb and replace interneurons throughout the lifespan. Newly born neurons in the SGZ of the dentate gyrus are capable to integrate into existing hippocampal neuronal networks but remain within the hippocampus (for review see [8]). The survival and functional integration of newly born neurons is affected by intracellular factors, such as cell-cycle regulators and transcription factors, extracellular factors, such as cell-cell signaling mechanisms, released growth factors or hormones (for review see [9]) but also by

external stimuli of the environment, such as learning and memory, enriched environment, physical activity, stress or diet [10-15].

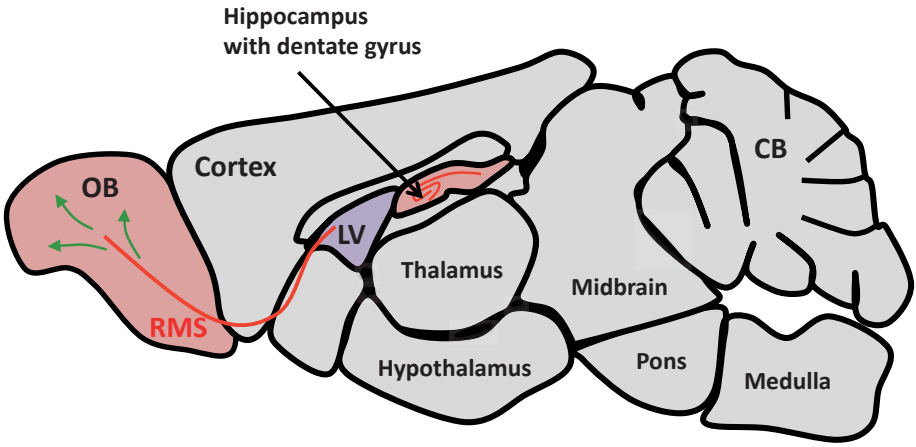

Fig. 2 The neurogenic niches within the adult rodent brain. *New-born neurons are either generated in the subventricular zone of the lateral ventricles (LV) and migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB), where they replace interneurons, or in the subgranular zone of the dentate gyrus within the hippocampus, where they are integrated into functional neuronal networks. CB, cerebellum.*

1.2.2 Neural stem/progenitor cells in the adult brain

Adult hippocampal neurogenesis has been implicated in mechanisms for learning and memory and disturbed adult neurogenesis within the SGZ of the dentate gyrus has been associated with depression, neuroinflammation and epilepsy. Adult neurogenesis within the SVZ of the lateral ventricles has been implicated in olfaction. Identification of the neural stem/progenitor cells (NSPCs), that give rise to new neurons in the adult CNS, was one of the main research subjects in the past years, however the matter of the origin of adult-born neurons is still under debate.

Recently two non-mutually exclusive models of the origin of adult-born neurons were proposed, suggesting that newly born neurons in the hippocampus arise either from radial glia-like cells (also known as type I cells or type I progenitors) or from non-radial precursors (also known as type II cells) that are defined by their expression of the progenitor cell marker Sox2 (Fig. 3A). Both cell types reside within the SGZ and are considered to be quiescent, slowly dividing NSCs. Radial glia-like cells express the astrocyte marker glial fibrillary acidic protein (GFAP) and also the stem/progenitor cell marker nestin. However, radial glia-like cells or non-radial precursors do not directly generate newly born neurons but divide asymmetrically and produce intermediate progenitor cells (also known as type D cells). Intermediate progenitor cells generate neuroblasts that are integrated into the granular cell layer and mature into granule cells. Radial glia-like astrocytes within the SGZ also maintain the functional properties of normal astrocytes. The different origin of newly born neurons in the adult may be an indicator for the existence of several subpopulations of adult neural stem cells within the CNS.

In the SVZ of the lateral ventricles NSCs (also named radial glia-like cells, type B cells or SVZ astrocytes) give rise to newly born neurons (Fig. 3B). Radial glia-like cells express stem cell specific markers, such as nestin and Sox2, but also astrocyte specific markers, such as GFAP and the glutamate aspartate transporter (GLAST). Radial glia-like cells are suggested to be quiescent, slowly dividing NSCs that give rise to type C cells (or transient-amplifying progenitor cells) that have a high proliferative capability, function as intermediate

progenitor cells in the adult SVZ and generate immature neuroblasts (type A cells), which subsequently migrate along the rostral migratory stream into to olfactory bulb to replace interneurons (for review see [16, 17]).

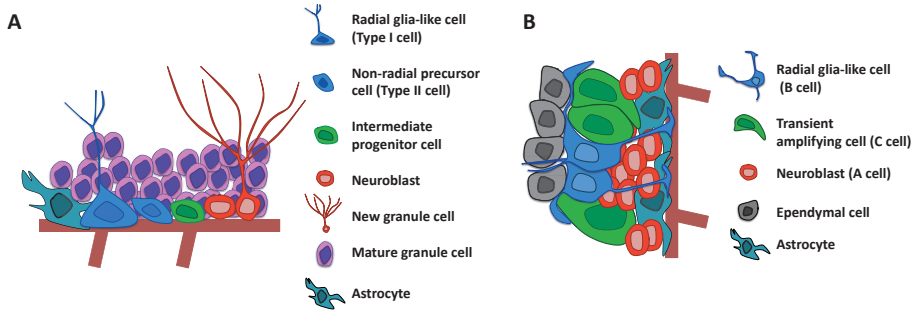

Fig. 3 Schematic illustration of the neurogenic niche in the SGZ of the dentate gyrus (A) and the SVZ of the lateral ventricles (B). *A) Radial glia-like cells (Type I cells) and non-radial precursors (Type II cells), located in the granular cell layer of the dentate gyrus, generate intermediate progenitor cells. Proliferation of intermediate progenitor cells leads to generation of neuroblasts that develop into new granule cells and are finally integrated as mature granule cells in the neuronal hippocampal network. B) Radial glia-like cells (B cells) in the SVZ generate transient amplifying cells (C cells), which generate immature neuroblasts (A cells) that migrate along the rostral migratory stream into the olfactory bulb to replace interneurons.*

1.2.3 The cellular environment of the adult neurogenic niches

Transplantation studies of NSPCs or neural grafts into various regions of the adult CNS showed, that neuronal differentiation of the transplanted NSPCs or grafts only occurs in the two adult neurogenic niches [18-21]. These findings point to the cellular environment as a key modulator for neurogenesis in adult CNS.

The major cellular players that influence the micro-environment of the adult neurogenic niches are astrocytes, endothelial

and ependymal cells, microglia, mature neurons and the progeny of adult NSCs and/or NPCs. NPCs with dividing capability were shown to be located closely to the capillaries of the vasculature, implicating that vascular derived signals and molecules regulate proliferation of NPCs [22, 23]. Astrocytes that are in cell-cell contact with cells of the vascular system and connected through gap junctions, can modify the effects of blood-derived molecules and can regulate the disposal of endothelial-derived cytokines or growth factors. Moreover, astrocytes can control proliferation and differentiation of adult NPCs and affect migration, maturation and synapse formation through diffusible or membrane-attached factors [24]. Besides astrocytes, microglia are also known to actively regulate neurogenesis in the adult CNS through phagocytosis of apoptotic newly generated neurons in the adult SGZ [25].

1.3 Astrocytes

1.3.1 The functions of astrocytes in healthy brain

Astrocytes are the most abundant glial cell type within the CNS [26]. They constitute 50% of all glial cells, besides oligodendrocytes, NG-2 glia and microglia. Under physiological conditions astrocytes are often (and wrongly) referred to as “quiescent” astrocytes, since they were believed to provide mainly structural support for the CNS. However, over the 30 last years, astrocytes were shown to be highly complex and multifunctional cells involved in various cellular

processes, mechanisms and cell-cell interactions necessary for a normally functioning brain [27, 28].

Astrocytes are in direct cell-cell contact with neurons, oligodendrocytes, microglia, endothelial cells, pericytes and other astrocytes, thereby influencing and modulating the environment in the brain. Named “astro-cyte” because of their star-shaped morphology, it was later shown that astrocytes exhibit a more “bushy” morphology with many fine processes [29-31]. This morphology allows one human astrocyte to be in contact with up to two million synaptic terminals [2], thereby regulating neuronal activity via for example recycling of neurotransmitters, such as glutamate [32]. In addition, astrocytes are directly involved in synaptogenesis by affecting synaptic plasticity and pruning of new synapses [33-36] and were recently shown to actively engulf synapses in the developing as well as adult CNS depending on neuronal activity [37]. Homeostatic functions of astrocytes include regulation of extracellular H^+ (for review see [38, 39]) and K^+ concentration (for review see [40]) as well as regulation of the H_20 transport (for review see [41]). Astrocytes locally regulate cerebral blood flow [42, 43] and their end-feet are in contact with endothelial cells, thereby regulating the formation and maintenance of the blood-brain-barrier [44].

Astrocytes cannot communicate with each other electrically through action potentials like neurons, but they are connected by gap junctions into so-called astrocyte syncytia and communicate through Ca^{2+} waves and other molecules [45]. Release of molecules by astrocytes (gliotransmission) affects neuronal differentiation,

proliferation and morphology (see also 1.2.3 The cellular environment of the adult neurogenic niches) and also synaptic strength [46-48]. Recently it was shown, that astrocytes affect neurogenesis by actively inhibiting neuronal differentiation of NSPCs through Jag-1 mediated Notch signaling [49].

1.3.2 Astrocytes in diseased brain – reactive astrocytes

Upon CNS injury, neuroinflammation, neurotrauma, such as stroke, or neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease, astrocytes become reactive [50]. This phenomenon is called reactive gliosis and is accompanied by various morphological and functional changes of astrocytes [27, 51, 52]. Hallmarks of reactive gliosis are hypertrophy of astrocyte processes, up-regulation of the cytoskeletal intermediate filament proteins GFAP and vimentin and re-expression of nestin. However, the extent of astrocyte activation depends on the type and severity of CNS damage and changes in morphology, proliferation and alteration of gene expression during reactive gliosis is context dependent and differs under various pathophysiological conditions [53-55]. Astrocyte activation ranges from reversible and time-limited changes in gene expression, such as up-regulation of GFAP and re-expression of the intermediate filament proteins synemin and nestin [56, 57], to the irreversible generation of a glial scar due to proliferation of reactive astrocytes near the injury site [52, 58]. A glial scar protects the surrounding, healthy tissue from spreading CNS damage [52, 58], but also forms a physical barrier for axonal regeneration after the acute

stage of injury [59]. However, the (patho-) physiological response of the CNS is not restricted to astrocytes becoming reactive, but involves a multicellular reaction of all cell types within the CNS [60].

1.3.3 Astrocytes are heterogeneous

Astrocytes exhibit a high degree of heterogeneity [61, 62] and given the highly complex functions of astrocytes in healthy as well as diseased CNS, classification of astrocyte subpopulations on a molecular level is highly desirable. So far, astrocytes have been classified as protoplasmic or fibrous astrocytes, based on their morphological structure and their occurrence in gray and white matter, respectively [51]. In addition, Bergmann Glia in the cerebellum and Müller Glia in the retina have been classified as subpopulations of astrocytes based on their morphology, physiological properties, expression of specific markers and their response to injury [62, 63]. However, it is still a matter of debate, if and how many other astrocyte subpopulations exist, which of these exist already *in vivo* and if some of these can be found exclusively *in vitro*.

The most common used astrocyte-specific markers are GFAP, S100β and glutamine synthetase. The aldehyde dehydrogenase 1 family, member L1 (Aldh1L1; also known as 10-formyltetrahydrofolate dehydrogenase) was suggested as an additional astrocyte marker, that in healthy brain is present in more astrocytes than GFAP [64].

1.4 The intermediate filament (nanofilament) system

1.4.1 Intermediate filaments

The cytoskeleton of most eukaryotic cells consists of microfilaments (actin filaments), microtubules and the intermediate filament system. The actin filaments are the thinnest of all cytoskeletal components and important for determination of the cell shape as well as cell motility, while microtubules are the thickest cytoskeletal components and are important for e.g. intracellular transport and cell division. In contrast to actin filaments and microtubuli, the expression of intermediate filament proteins is highly tissue-specific (Fig. 4) and fulfills functions beyond mechanical stability (for review see [65, 66]).

Intracellular localization and function of mitochondria, the Golgi and the nucleus as well as intracellular protein targeting depend on intact intermediate filaments. In addition, the intermediate filament cytoskeleton was shown to have cytoprotective functions in stress and injury situations and act as signaling platform controlling various cell responses to stress. Over 70 cytoplasmic and nuclear intermediate filament proteins are known and their expression is highly dynamic and depends on the developmental stage, the cell- and tissue-type as well as on the physiological condition (e.g. healthy versus diseased cell/tissue). All intermediate filament protein molecules consist of a rod, head and tail domain. The α-helical rod domain is highly conserved, while the flanking N-terminal head and C-terminal tail domains differ both in amino acid sequence and length. Intermediate filament proteins usually form polymers by either homo-polymerization or hetero-polymerization with each other, a process,

which is regulated by phosphorylation/dephosphorylation of threonine and/or serine residues of the N-terminal head domain (for review see [65]).

Intermediate filament proteins can be divided into 6 different classes depending on their amino acid sequence and protein structure. Type I intermediate filament proteins comprise acidic keratins, while type II comprise basic keratins. The astrocyte intermediate filament proteins GFAP and vimentin belong to class III, while the neuronal intermediate filament proteins neurofilament-L, -M, -H and α-internexin belong to class IV. Intermediate filament proteins comprising the nuclear envelope (lamin A, B, C) belong to class V. Nestin, a stem cell marker and astrocyte intermediate filament protein belongs to type VI intermediate filament proteins (Fig. 4).

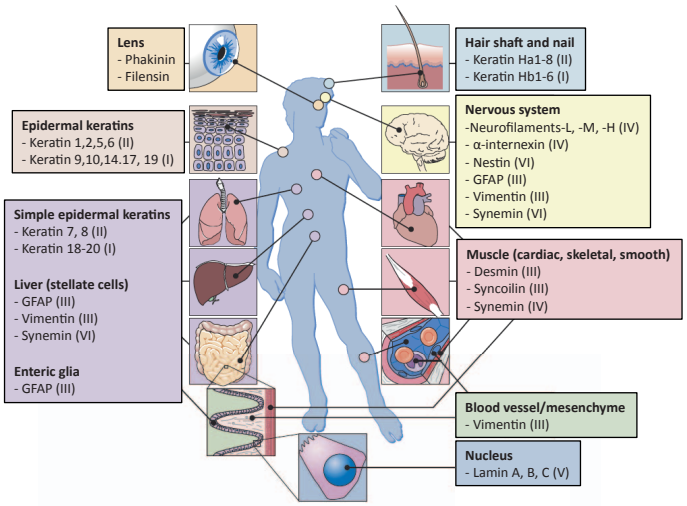

Fig. 4 Expression and classification of intermediate filament proteins*. The expression of intermediate filament proteins is tissue-specific and depends on the developmental stage. All intermediate filament proteins can be classified into 6 different subclasses. Modified after Toivola et al. 2005.*

1.4.2 Nestin

Expression of nestin was first described in neuroephitelial cells [67, 68] but ranges from NSPCs, immature astrocytes in the developing CNS [68, 69] to developing heart, muscle, kidney and testis tissue [70-72]. In the adult brain, nestin expression is maintained in NSPCs and radial glia [73] and is induced in reactive astrocytes after injury, stroke and in neurodegenerative diseases [56, 74]. Nestin expression is often used as a marker specific for NSPCs [68, 75, 76], however its functional role in neurogenesis remains elusive.

Nestin was shown to regulate apoptosis and myoblast differentiation via interaction with Cdk5 [77-79]. Further, assembly and disassembly of nestin with its polymerization partners (nestin cannot self-polymerize [56]) is regulated by the Cdc2 kinase. Together with cyclin B, Cdc2 comprises the maturation/M-phase promoting factor complex, which regulates the transition within the cell-cycle, implicating that nestin is important for dividing and migrating cells [80-82].

1.4.3 Glial fibrillary acidic protein (GFAP)

Expression of GFAP within the CNS occurs in astrocytes [83] and radial glia [84-86] and in the peripheral nervous system in non-myelin forming Schwann cells and enteric glia cells, but is also expressed in ependymal cells, kidney, testis, epidermis, osteocytes, chondrocytes, pancreatic and liver tissue [87-93]. After injury GFAP expression is up-regulated in reactive astrocytes.

GFAP, alone or together with vimentin, is implicated to play a role in many fundamental cellular processes, such as astrocyte motility and migration [94-96], proliferation [97-101], vesicle trafficking [102-106], modulation of neuronal activity [107, 108], synaptic plasticity [109], glutamate transport and synthesis [110-113], neurite outgrowth [114-117] and cytoprotective functions after injury [30, 118-121].

So far 10 different splice variants of GFAP are known and literature about GFAP gene expression usually relates to the most abundant splice form GFAPα (for review see [122]).

GFAP usually polymerizes with vimentin in astrocytes, but cannot polymerize with nestin in the absence of vimentin. GFAP replaces nestin as a polymerization partner for vimentin after maturation of astrocytes [56]. However, in vimentin deficient astrocytes it has been shown that GFAP can polymerize into abnormally bundled intermediate filaments [56].

1.4.4 Vimentin

Expression of vimentin occurs in astrocytes, in cells of mesenchymal origin, leukocytes, epithelial cells, fibroblasts and developing muscle. Functions of vimentin comprise maintaining cell shape, compartmentalization and anchoring of organelles such as the nucleus and the endoplasmatic reticulum (for reviews see [65, 66, 123, 124]). Similar to GFAP, vimentin plays important roles in many fundamental cellular processes, such as during wound healing [125, 126], in mechanotransduction of shear stress [127], cyto-protection after CNS injury [30], migration and serves as a signaling platform (for

review see [123]). In addition, vimentin is implicated to play a role in intracellular vesicle trafficking [102-106] and in astrocyte motility [95].

In astrocytes, Vimentin cannot polymerize into homodimers in the absence of GFAP and forms heterodimers with nestin in immature astrocytes and with GFAP in mature astrocytes [56].

1.4.5 The role of intermediate filament proteins in diseases

The pivotal role of intermediate filament proteins in cellular functions and tissue integrity becomes obvious in the disease context. So far, 98 clinical disorders are associated with (a) mutation(s) of intermediate filament protein genes (Human intermediate filament database; www.interfil.org; [128]). To name only a few, mutations in keratin 14 or keratin 5 (type I & II intermediate filament proteins) cause several forms of epidermis bullosa simplex, an inheritable skin blistering disorder. Mutations in GFAP (type III intermediate filament protein) can cause Alexander's disease, a progressive neurological disorder, which is fatal within about 10 years after disease onset. Characteristic features are accumulation of Rosenthal fibers in astrocytes and chronic gliosis. Mutations in peripherin (type III intermediate filament protein) and neurofilament-L, -M, -H (type IV intermediate filament proteins) are associated with the neurodegenerative disease amyotrophic lateral sclerosis (also known as Lou Gehrig's disease), a disorder characterized by loss of motoneurons in the spinal cord and the CNS.

1.5 Signaling pathways

1.5.1 Notch signaling pathway

The Notch signaling pathway is throughout species a highly conserved and interconnected mechanism. The Notch signaling pathway has a fundamental role during tissue and organ development by regulating proliferation, differentiation and apoptosis. It was recently shown, that Notch signaling promotes the differentiation of NPCs into glial cells [129], decreases neuronal differentiation and proliferation [130] and that Notch signaling is necessary to maintain the pool of existing NSPCs [131-135]. Dysregulation of Notch signaling has been linked to several diseases, developmental defects and cancers, such as leukemia (for review see [136-139]).

The Notch signaling machinery of mammals consists of 4 known receptors (Notch1-4) and two Jagged (Jag) ligands (Jag1, Jag2), 3 Delta-like (Dll) ligands (Dll1, Dll3, Dll4) and 2 Delta-like homologue (Dlk) ligands (Dlk1, Dlk2). The Notch receptor consists of an extracellular domain and an intracellular domain. Shortly, activation of the Notch signaling pathway occurs via binding of one of the ligands to the Notch extracellular domain of one of the receptors (Fig. 5). The membrane-tethered receptor is enzymatically cleaved by an ADAM/TACE metalloprotease releasing the ligand-bound Notch extracellular domain, which is subsequently transendocytosed into the Notch ligand expressing cell. This transendocytotic step is essential for eliciting Notch signaling. A second enzymatic cleavage by the γ-secretase complex releases the membrane-tethered Notch intracellular domain from the plasma membrane, which is subsequently

translocated to the nucleus and affects gene expression of Notch regulated down-stream targets, such as the transcriptional regulators Hairy and Enhancer of split (HES; HES1 and HES5). The transendocytosed Notch extracellular domain undergoes lysosomal degradation, while the ligand is either recycled and represented at the membrane or also degraded (for review see [136-139]).

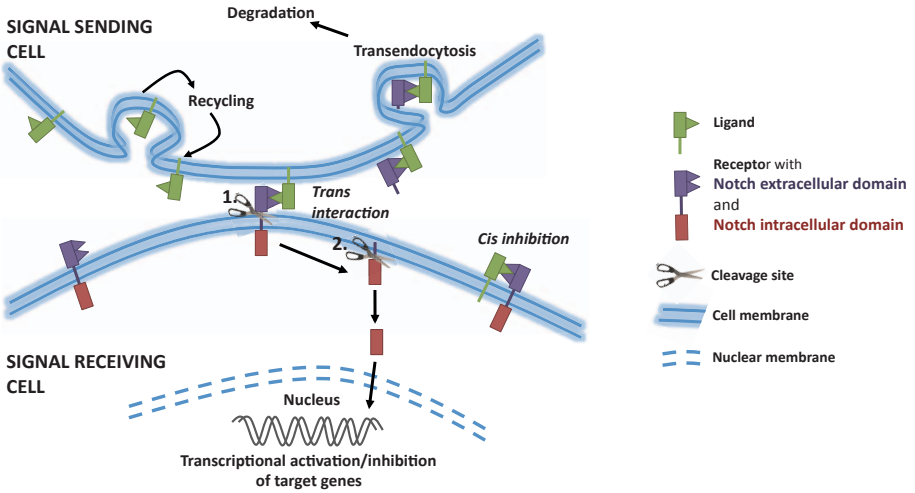

Fig. 5 Simplified illustration of receptor/ligand binding of the Notch signaling cascade. *Ligands undergo constitutive cycles of recycling and representation at the membrane of the signal sending cell. The Notch extracellular domain of the receptor of the signal receiving cell binds to the ligand of the signal sending cell (trans interaction) and is subsequently cleaved by ADAM/TACE ("the 1. scissor") which releases the Notch extracellular domain for transendocytosis together with the ligand into the signal sending cell. A second cleavage step by the γ-sectretase complex ("the 2. scissor") releases the Notch intracellular domain from the membrane of the signal receiving cell. The Notch intracellular domain is translocated to the nucleus were it regulates transcription of downstream target genes of the Notch signaling pathway. Binding of receptor and ligand, presented on the membrane of the same cell is indicated as Cis inhibition.*

Regulation of the Notch signaling activity occurs on several levels, but lacks an enzymatic amplification step. This might be one reason for the gene dosage sensitivity, due to haploinsufficiency (a diploid organism has one functional copy of a gene, while the other is inactive) of Notch signal pathway related genes. Positive and/or negative transcriptional feedback loops may amplify only small differences in ligand expression that in turn affect the signaling between two neighboring cells. In addition, Notch signal activity is regulated via *cis* or *trans* interactions of the ligands and receptors.

Trans interactions occur between two neighboring cells, one being the signal sending cell (ligand expressing cell) while the other receives the signal (receptor expressing cell), *cis* interactions have been proposed to be inhibitory and occur when ligand and receptor are presented at the same cell. The ratio of *cis* and *trans* interactions can modulate signaling activity between neighboring cells. Other crucial steps of Notch signal activity regulation are ubiquitination, glycosylation and phosphorylation of the receptors and ligands, which mark ligands or receptors for degradation or endocytosis with subsequent intracellular trafficking. In addition, Notch signal activity is always interconnected with multiple signaling pathways, such as the EGFR signaling pathway, and the integration of multiple signaling mechanisms result in cell fate determinations, such as proliferation, differentiation or apoptosis [136-138, 140]. Cross-talk between Notch signaling pathway and the EGFR signaling pathway is necessary to keep the balance between NSC pool and NPC pool [141], since Notch signaling controls self-renewal and identity of NSCs [132, 142, 143],

while EGFR signaling promotes proliferation and migration of NPCs [144].

1.5.2 HB-EGF- a ligand for the EGFR signaling pathway

Heparin binding epidermal growth factor-like growth factor (HB-EGF) is one of several known ligands of the EGFR signaling pathway. Other ligands comprise the epidermal growth factor (EGF), transforming growth factor α (TGF-α), amphiregulin, heregulin, epiregulin, betacellulin, epigen and neuregulin 1-4. Further, four receptors are known: ErbB1 (also known as EGFR) and ErbB2-4. Activation of the EGFR signaling pathway leads to a highly complex cascade of down-stream signaling and affects proliferation, migration, differentiation or apoptosis of neighboring cells (for review see [145]).

The receptors of the EGFR signaling pathway belong to the tyrosine kinase receptor family and are transmembrane receptors. ErbB1 and ErbB4 are able to build functional homo- or heterodimers, while ErbB2 has no ligand binding capability and is functional only as a heterodimer. Similar, ErbB3 is only functional as a heterodimer, since the tyrosine kinase activity of ErbB3 is defect. All known ligands of the ErbB receptors contain a EGF domain and are extracellularly presented as membrane-bound ligands (for review see [145]). In case of HB-EGF, the membrane bound proHB-EGF is cleaved (so called ectodomain shedding) by matrix metalloproteinases or a disintegrin and metalloproteinases (ADAMs) and the soluble HB-EGF is released into the extracellular space. Subsequently, soluble HB-EGF binds to the ErbB receptors at neighboring cells and activates the EGFR

signaling cascade [146]. However, membrane bound proHB-EGF can also bind ErbBs and activate EGFR signaling in a juxtacrine fashion [147]. HB-EGF is a known ligand for the receptors ErbB1 and ErbB4. Activation of the EGFR signaling cascade via HB-EGF binding to ErbB1 results also in activation of the Ras-MAPK pathway affecting proliferation. Activation of EGFR signaling via HB-EGF binding to ErbB4 is associated with regulation of differentiation (for review see [145]).

Sixty percent of HB-EGF deficient mice die shortly after birth [148] and the expression pattern of HB-EGF during CNS development suggests a role in maturation of neurons and glial cells [149]. In addition, HB-EGF *in vivo* has a neuroprotective function after ischemia [150, 151] and was shown to improve functional recovery after stroke [152] and wound healing [153, 154]. HB-EGF *in vitro* increases migration of astrocytes in a scratch-wound model when used with the co-factor insulin-like growth factor 1 [155] and functions as a potent mitogen for fibroblasts, smooth muscle cells and keratinocytes [156]. Recently it was reported that HB-EGF induces de-differentiation of Müller glia into multipotent progenitor cells in zebra fish retina [157]. In addition, HB-EGF was suggested as a replacement for serum in primary astrocyte cultures [158]. Taken together, the functional complexity of EGFR signaling, the expression of HB-EGF during development as well as its possible role in neuroprotection after injury make HB-EGF a highly interesting molecule and a possible target for treatment strategies in CNS regeneration.

2 METHODOLOGICAL CONSIDERATIONS

In this chapter methodological considerations of some state-of-the-art methods used in the Papers I-III are given. For detailed experimental procedures of all used techniques, please refer to the Material and Methods parts of the respective paper.

2.1 Mouse models – genetic ablation of intermediate filament proteins in mice (Paper I-III)

To study the role of intermediate filament proteins GFAP, vimentin and nestin we used mice deficient for GFAP and vimentin (*GFAP*$^{-/-}$*Vim*$^{-/-}$, Paper I) or nestin (*Nes*$^{-/-}$, Paper II).

We used *Nes*$^{-/-}$ mice and their respective wild-type controls on a mixed genetic background (C57BL/6(B6)-129/Sv). The *Nes*$^{-/-}$ mice are viable and reproduce normally [159]. CNS organization in *Nes*$^{-/-}$ mice shows no major defects, but nestin seems to be essential for the distribution of acetylcholine receptors at neuromuscular junctions [159]. In contrast to the *Nes*$^{-/-}$ mice used here, Park et al. [160] reported that *Nes*$^{-/-}$ mice generated in their lab showed embryonic lethality and *in vitro* experiments decreased self-renewal capability of *Nes*$^{-/-}$ NSCs derived from embryonic CNS. However, for the generation of our mice, several ES clones were used to generate several mouse lines deficient for nestin in parallel, while the *Nes*$^{-/-}$ mice of Park et al. were generated from only a single ES clone. All *Nes*$^{-/-}$ mice lines generated in the lab of Prof. Andras Nagy showed the same phenotype and in

immunocytochemical and Western blot analyses of *Nes*$^{-/-}$ mice nestin protein was not detectable.

GFAP$^{-/-}$*Vim*$^{-/-}$ mice and their respective wild-type controls are on a mixed genetic background (C57Bl/6-129Sv-129Ola) and they are viable and reproduce normally [161-164]. Since nestin cannot polymerize with itself into intermediate filaments [56], *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes are completely devoid of intermediate filaments; the mice show attenuated reactive gliosis and glial scar formation [162]. However, synaptic and axonal regeneration [30, 165, 166], transplantation of neural grafts and neural stem cells are improved [167, 168], and in addition, basal and posttraumatic hippocampal neurogenesis is increased [49, 169].

2.2 *In vitro* - the cell culture systems

2.2.1 Bioactive3D – a novel in vivo-like 3-dimensional cell culture system – compared to conventional 2-dimensional culture systems (Paper III)

To study the function of astrocytes *in vitro,* primary astrocytes are commonly cultured in 2-dimensional (2D) systems on coated or un-coated plastic or glass surfaces in the presence of a high percentage (10%) of fetal calf serum (FCS). Those 2D-cell culture systems constitute a highly artificial and stressful environment, where astrocytes are forced to grow without the 3-dimensional (3D) support. Astrocytes in 2D-cell cultures resemble reactive astrocytes, since they exhibit increased proliferation, up-regulate the expression of GFAP and vimentin, re-express nestin and alter gene expression, cell

morphology and function. Over the last years, several attempts have been made, to design 3D cell culture systems that provide a less stressful culture environment by giving structural 3D-support to preserve the cell morphology and function that allow *in vitro* studies in a more *in vivo*-like environment. Up to date, available 3D-cell culture systems are based on alginate scaffolds, collagen gels or hydrogels [170-172] or are comprised of polymer, ceramics or macroporus scaffolds [173, 174], all of which exhibit several disadvantages. Gels prevent extensions of cell processes and prolong diffusion of released molecules. Further, cell type specific coating of gels is impossible. Rigid polymer, ceramics or macroporus scaffolds can lead to unwanted clustering and compartmentalization of cells [173, 174]. To circumvent these problems and to create a more *in vivo*-like environment for cultured astrocytes, we utilized bioactively-coated 3D-nanofiber scaffolds and established a novel 3D-cell culture system (Bioactive3D). Nanofibers were prepared by electrospinning using a biocompatible polyether-based polyurethane resin in a 60:40 mixture of tetrahydrofuran and *N,N*-dimethylformamide and the nanofibres were subsequently coated with poly-L-ornithine and laminin [175].

Astrocytes grown in Bioactive3D maintain their complex *in vivo*-like morphology (Fig. 6) and motility of cell filopodia is supported. Further, we demonstrated that astrocytes grown in Bioactive3D are less exposed to cell culture stress compared to astrocytes, grown in conventional 2D-cell culture systems, which results in decreased expression of GFAP, vimentin, synemin and nestin, reduced proliferation and altered expression of genes involved

in regulation of proliferation, cell shape determination and cell motility [176]. The Bioactive3D cell culture system allows us to study the function of primary astrocytes *in vitro* since these astrocytes are less reactive and keep some *in vivo*-like properties.

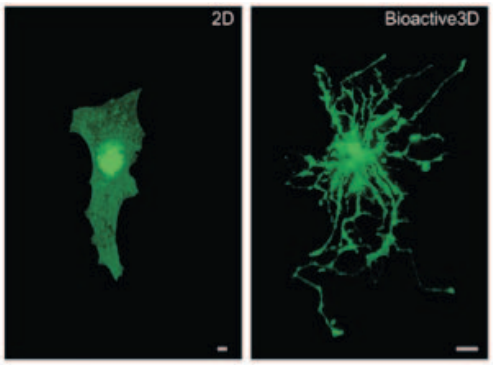

Fig. 6 Morphological differences of astrocytes cultured in 2D and Bioactive3D. *Primary astrocytes cultured in Bioactive3D show in vivo-like morphology (right) compared to flat and polygonal shaped astrocytes cultured in conventional 2D cell culture systems (left). Puschmann et al. 2013.*

2.2.2 Neurosphere cultures (Paper II)

The possibilities to investigate intrinsic molecular properties of NSCs *in vivo* are limited and often impossible. One *in vitro* system, to study the cell intrinsic mechanisms of NSCs is the neurosphere assay that has been commonly used to address cell proliferation, self-renewal and differentiation of NSCs into neurons, astrocytes and oligodendrocytes. The neurosphere assay is based on the theory that NSCs, cultured in presence of growth factors but without FCS, divide symmetrically and build non-adherent, free-floating clusters of multipotent daughter stem cells that are able to differentiate after growth factor removal. However, it was reported, that neurospheres are heterogeneous cell clusters consisting of symmetrically, slowly dividing NSCs, but also of more restricted progenitor cells, which divide rapidly. Further it was shown, that the free-floating

neurospheres are able to merge and even exchange cells with each other, questioning the pure “stemness” and ”clonality” of the cells within one neurosphere (for review see [177]). Therefore caution is advised, when using neurosphere forming capacity (the number of neurospheres formed) or the size of neurospheres as an indicator of how many NSCs may exist *in vivo* and what their proliferative capability is. Further it needs to be considered, that cells that are not NSCs can acquire stem cell properties when cultured in the presence of growth factors and they can also form neurospheres.

2.2.3 The establishment of NSC lines from adult mice (Paper II)

To investigate the functional role of nestin in cell intrinsic properties of adult NSCs, we established neural stem cell lines of adult *Nes*$^{-/-}$ and the corresponding wild-type control mice in our lab. The NSC lines were established from brains (without olfactory bulb and cerebellum) of 4-5 week old mice of both genders. At 4-5 weeks of age mice have reached maturity and are considered adult animals. The established cell lines were cultured for at least 25 passages to verify their stemness, proliferative capability and their capability to generate neurons and astrocytes. Adult NSC lines generated from male or female animals did not show differences in their stemness, proliferative capability or their capability to generate neurons and astrocytes. However, the NSC lines used in paper II were derived from male animals and all experiments were performed with NSCs of passage 5-7. Proliferation was assessed using a hemocytometer and differentiation capability was assessed by immunocytochemical

detection of cell type specific markers (βIII-tubulin for neurons and S100β for astrocytes). Directional migration towards a SDF-1α gradient was assessed as previously described [178] using a chemotaxis chamber.

2.2.4 Astrocyte co-cultures with rat NSCs derived from adult hippocampus (Paper II)

To investigate the effect of the astrocyte (wild-type and *Nes*$^{-/-}$) environment on neuronal differentiation of NSCs, primary mouse astrocytes were co-cultured with NSCs. The NSCs were derived from adult rat hippocampus and retrovirally transfected to express green fluorescent protein (GFP). The NSC cell line (clone HCN-A94/GFPH) was kindly provided by F.H. Gage (Salk Institute, La Jolla, CA, USA). For all co-culture experiments NSCs of passage 19-20 were used, a passage in which GFP expression was detected in only a fraction of all NSCs. Therefore, proliferating NSCs were labeled with 5-bromo-2-deoxyuridine (BrdU) for 48h before plating them on top of primary astrocytes. Total cell number was evaluated by counting BrdU positive cells, while differentiation properties of NSCs were addressed with antibodies against GFAP for astrocytes and βIII-tubulin for neurons. It needs to be considered that BrdU has a low genotoxicity and changes the methylation pattern of the DNA, which may influence the differentiation of NSCs (see also paragraph 2.4). The reason, why rat NSCs were used instead of mouse NSCs was primarily because the rat NSC line was available in our laboratory and has been used in similar experiments with *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes before [168].

2.3 Quantitative real-time PCR (RT-qPCR) of individual astrocytes (Paper I)

Heterogeneity of cells on the levels of mRNA, proteins, metabolites or lipids within an apparently homogenous cell type is acknowledged since many years. However, technical limitations in the past prevented transcriptomical, proteomical, metabolomical or lipidomical analyses on the level of individual cells. Up to date, single cell analyses were mainly based on immunocytochemical, immunohistochemical or flow-cytometric methods restricting the number of analyzed genes, proteins or metabolites per cell to only a few, which makes it almost impossible to decipher the complexity of biological processes, such as signaling pathways, in detail. The rapid pace of technological development within the last few years makes it now possible to address heterogeneity on single cell level revealing unique molecular signatures of individual cells (for review see [179]).

One approach to target heterogeneity among astrocytes is by means of mRNA expression levels using quantitative real-time PCR (RT-qPCR) in individual astrocytes. The amount of mRNA molecules per gene can vary between only a few copies to up to 50.000 molecules per individual astrocyte [180]. This difference is masked in RT-qPCR analyses of astrocyte populations, but on the level of individual cells gives us an accurate representation of variations as well as correlations of gene expression within astrocytes to classify subpopulations and/or reveal a detailed picture of signaling mechanisms (Fig. 7).

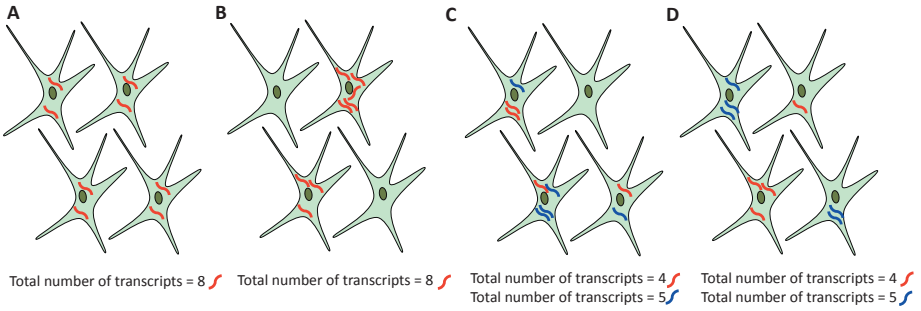

Fig. 7 Heterogeneity of mRNA expression and correlation. *RT-qPCR analyses of cell populations cannot distinguish between A) and B) or C) and D).*

The mRNA content of one individual mammalian cell is usually about 1pg of mRNA, which corresponds to approximately 300.000 mRNA molecules of about 10.000 existing genes [181, 182]. RT-qPCR is highly sensitive and detection of only one mRNA molecule, corresponding to only femtograms of mRNA, within one cell is possible; further advantages are the wide dynamic range and high reproducibility of RT-qPCR [183-185]. Technical variability of RT-qPCR analyses due to minimal mRNA material was shown to be minute in comparison to the biological variability occurring in gene expression of individual cells [186].

Genes of interest, also splice variants of the same gene, can be specifically selected and pre-amplification of mRNA makes it possible to analyze up to 100 genes per individual cell [187].

The classification of possible subpopulations of heterogeneous cell types by RT-qPCR is based on mRNA levels. Differences in mRNA levels do not necessarily lead to alterations in protein expression, morphological or functional differences. Posttranslational

mRNA modifications affect translation of mRNA into proteins; further, transcription is a highly dynamic process and half-life for specific mRNA molecules is gene-dependent. In addition, protein modifications such as phosphorylation, dephosphorylation as well as glycosylation alter the functional properties of proteins.

2.4 BrdU labeling of proliferating cells and cell fate determination (Paper II and III)

To detect the small population of dividing neural stem cells in the adult mammalian brain, BrdU *in vivo* labeling is commonly used. BrdU is incorporated into the replicating DNA of dividing cells as well as during DNA repair mechanisms as a thymidine analogue, followed by immunohistochemical detection. Incorporation of BrdU into dividing cells *in vivo* allows to address the existing number of NSPCs in the CNS and to follow the survival and cell fate determination of neural stem cells in CNS over time. The amount of incorporated BrdU during DNA repair mechanisms is much lower compared to the amount incorporated during DNA replication and is therefore negligible. BrdU is known to be genotoxic, which may result in malformations when administered during embryogenic development [188], however the toxicity is considered to be very low and it was shown that hippocampal neurogenesis was not affected in adult rats by multiple BrdU injections [189, 190]. The ideal dosage of BrdU, administered intraperitonally and shown to be non-toxic, for the detection of all dividing cells lays between 100-300mg/kg [189].

We administered BrdU intraperitonally to label and follow the cell fate of newly born cells in the adult brain of *Nes*$^{-/-}$ mice *in vivo* at a dose of 200mg/kg. Total number of BrdU positive cells was calculated to address the existing pool of proliferating NSPCs within different regions of the adult brain. For cell fate determination, double labeling of BrdU with S100β was used to determine the number of newly formed astrocytes, double labeling of BrdU with NeuN was used to address newly generated neurons, double labeling of BrdU with GFAP was used to address the number of NSCs (corresponding to type 1 cells also known as radial glia-like cells) and double labeling of BrdU with doublecortin (Dcx) was used to address the number of neuronal precursor and immature neurons (corresponding to type 2-3 cells also known as non-radial precursors or IPCs) [191].

BrdU labeling can also be used for detection of proliferating cells *in vitro* (see paragraph 2.2.3). An alternative possibility to label proliferating cells *in vitro* is by using 5-ethynyl-2`-deoxyuridine (EdU) instead of BrdU (Paper III). The mechanism of incorporation into the DNA of replicating cells as a thymidine analogue remains the same.

3 AIMS OF THE THESIS

I) To study (i) the heterogeneity of astrocytes on a single cell level with a particular focus on the Notch signaling pathway, (ii) the effect of the intermediate filament (nanofilament) proteins GFAP and vimentin on the Notch signaling competence of individual astrocytes, and (iii) to identify possible astrocyte subpopulations based on mRNA expression of individual astrocytes (Paper I).

II) To investigate the role of the intermediate filament protein nestin (i) in neural stem/progenitor cell proliferation, differentiation and directional migration *in vitro*, and (ii) in the neurogenesis in the two adult neurogenic niches, the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles (Paper II).

III) To address the effect of heparin binding EGF-like growth factor (HB-EGF) on (i) astrocyte morphology, (ii) proliferation, (iii) differentiation, (iv) expression of intermediate filament proteins and (iv) mRNA expression of selected genes in our newly developed 3 dimensional cell culture system (Bioactive3D) and in conventional 2 dimensional cell culture systems (Paper III).

4 RESULTS AND DISCUSSION

Paper I

Heterogeneity of Notch signaling in astrocytes and the effects of GFAP and vimentin deficiency

Astrocytes are a multifunctional and highly heterogeneous cell type and fulfill many roles in healthy and injured CNS. Recently, we showed, that astrocytes actively participate in the regulation of neurogenesis by inhibiting neuronal differentiation of NSPCs through Jag1-mediated Notch signaling and that this regulation is dependent on the cytoplasmic intermediate filament proteins GFAP and vimentin [49]. Here we show the heterogeneity of astrocytes with regard to their Notch signal sending competence on a single cell level.

We found that most astrocytes are competent to receive Notch signals (defined as astrocytes that express the Notch1 receptor), while only a minority of astrocytes is competent to send Notch signals (defined as astrocytes that express one of the Notch ligands, Jag1 or Dlk2) and even fewer astrocytes can send as well as receive Notch signals (defined as astrocytes that express one of the Notch ligands, Jag1 and Dlk2, and the Notch1 receptor). Only Notch signal sending competence seems to depend on GFAP and vimentin since fewer *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes have Notch signal sending competence, whereas the size of the Notch signal receiving competent population of *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes is comparable to wild-type astrocytes.

Gene expression of Notch signal pathway genes *Notch1, Dlk2* and *Sox2* is differentially regulated in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes, with up

to a 1.5-fold increase in *Dlk2* mRNA levels in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes compared to wild-type, while fewer *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes express *Dlk2* in general. Further, *Sox2* and *Dlk2* mRNA expression is only correlated in wild-type astrocytes but is absent in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes. Since *Dlk2* was reported as an inhibitory Notch ligand that modulates the activity of other Notch ligands, *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes may send more inhibitory Notch signals compared to wild-type astrocytes, which affect gene expression and correlation of the remaining Notch signal pathway related genes. The Notch signal pathway lacks an enzymatic amplification step but is sensitive to the gene dosage, therefore already small changes in the amount of mRNA molecules of Notch pathway genes may affect the signaling outcome in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes.

To our knowledge, this is the first study to address heterogeneity of signaling competence in individual astrocytes and it heralds the importance of single cell analyses for a better understanding of cell-cell interactions.

Nestin and HB-EGF are possible classifier for astrocyte subpopulations

Astrocytes have complex functions in healthy and diseased CNS and are also able to de-differentiate and regain stem cell characteristics after injury or neurotrauma. This heterogeneity of astrocytes makes classifications of astrocyte subpopulations based on expression of specific markers highly desirable since up to date no

specific markers are known to classify possible astrocyte subpopulations *in vivo* or *in vitro*.

Nestin (Nes) and *HB-EGF* mRNA expression can be used to classify both wild-type and *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocyte subpopulations. *Nes* and *HB-EGF* mRNAs are both expressed in immature astrocytes [56, 158] and are re-expressed in CNS after injury [74, 150, 151, 192]. In addition, nestin serves as a marker for NSCs [193], reactive astrocytes and astrocytes in a de-differentiated state [194, 195]. Here we showed that *in vitro Nes* serves as a classifier in wild-type and *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes in the same manner, while the function of HB-EGF as a classifier seems to depend on the expression of GFAP and vimentin. *Nes*^{POS} and *HB-EGF*^{POS} subpopulations of both wild-type and *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes may resemble either a more immature astrocyte subpopulation or they represent reactive astrocytes. Since nestin usually polymerizes with vimentin, both theories go in line with the finding, that *vimentin* mRNA levels were decreased in *Nes*^{NEG} and *HB-EGF*^{NEG} subpopulations in wild-type astrocytes.

We propose *Nes* and *HB-EGF* mRNA as a possible classifier for astrocyte subpopulations *in vitro*.

Paper II

The absence of nestin does not affect proliferation of adult NSPCs in vivo and in vitro

The intermediate filament protein nestin is commonly used as a marker of neural stem/progenitor cells (NSPCs) and is expressed in NSPCs in the adult subgranular zone (SGZ) of the dentate gyrus in the

hippocampus and in the subventricular zone (SVZ) of the lateral ventricles [3, 16]. Nestin is also re-expressed when astrocytes become reactive [56, 74]. However, the role of nestin in embryonic and adult neurogenesis remains elusive. Here we investigated the role of nestin in neurogenesis in *Nes*$^{-/-}$ mice.

We found that nestin does not affect proliferation of adult NSPCs or neuroblasts from the two adult germinal zones in *Nes*$^{-/-}$ and wild-type mice *in vivo.* Proliferation, differentiation and directional migration of NSPCs derived from adult *Nes*$^{-/-}$ and wild-type brains was comparable. Previous reports suggested, that nestin is essential for proliferation of neural progenitor cells [196] and required for normal zebra fish brain and eye development through control of neural progenitor cell apoptosis [197]. However, those findings were based on knock down techniques in embryonic stem cells and the same mechanisms might not apply to adult NSPCs. Recently, nestin was shown to not be essential for the development of the CNS [159], suggesting, that the role of nestin during CNS development can be compensated by other proteins.

Increased adult neurogenesis in the hippocampal dentate gyrus of mice deficient for nestin

The hippocampus of *Nes*$^{-/-}$ mice had 48%-58% more neurons compared to wild-type mice 6 weeks after BrdU administration, whereas we did not see a difference 2 weeks after BrdU administration. These data imply that the process of survival of newly born neurons is altered at a later stage of neuronal maturation and

integration in *Nes*$^{-/-}$ mice. Survival of newly born neurons is dependent on the activity and functional integration into the cellular environment. Increasing evidence supports the theory of astrocytes as key modulators of the neurogenic niches, since astrocytes can share characteristic properties with neural stem cells [198-200] and can also create a neurogenesis-supportive milieu [201]. The molecular mechanisms by which astrocytes control neurogenesis, neural plasticity and regeneration are affected by the expression of the intermediate filament proteins GFAP and vimentin. In concordance with our recent finding, that *GFAP*$^{-/-}$*Vim*$^{-/-}$ deficient astrocytes provide a more pro-neurogenic milieu compared to wild-type astrocytes [49], we show here that adult rat NSCs gave rise to a higher percentage of neurons in co-cultures with *Nes*$^{-/-}$ compared to wild-type astrocytes. In addition, under differentiation conditions, neuronal differentiation of P4.5 *Nes*$^{-/-}$ neurosphere-derived cells was increased compared to wild-type. These data suggest that *Nes*$^{-/-}$ astrocytes provide a more pro-neurogenic environment than wild-type astrocytes. Moreover, the results from co-culture experiments suggest that this pro-neurogenic effect depends on cell-cell contact.

Astrocytes in the unchallenged hippocampus do express no or very low levels of nestin, while neural stem cells in the hippocampus are nestin positive. Therefore, the pro-survival environment in *Nes*$^{-/-}$ mice may rather be provided by other cells, such as neural stem cells than astrocytes. The survival and integration of adult-born neurons may depend on several factors, such as excitation through NMDA receptors [202], activation of TrkB receptors by brain-derived

neurotrophic factor [203, 204], Notch signaling [132] or intracellular activation of Cdk5 [205, 206]. The exact role of nestin in this process remains to be elucidated.

As astrocytes control the microenvironment of neurons and in particular synaptic function, not least by an active uptake of neurotransmitters, it is not surprising that they regulate also the survival of newly born neurons and their integration into the existing networks. Our results provide support for such a scenario, although the mechanisms by which nestin and perhaps other intermediate filament proteins and their networks control this process remain elusive. Further analysis of the role of nestin in this process may provide important clues on how intermediate filament proteins and the intermediate filament network affect the ability of astrocytes to control neurogenesis and perhaps also other aspects of neuronal function.

Paper III

HB-EGF affects astrocyte proliferation and alters astrocyte morphology towards a radial glia-like phenotype

Astrocytes are usually cultured in 2D-cell culture systems in the presence of a high percentage of FCS. FCS is an essential, but highly undefined, component to maintain and expand astrocyte cultures and it is known to alter astrocyte properties, such as gene expression, proliferation and morphology. Recently, HB-EGF was suggested as a replacement for serum in astrocyte cultures [158]. Additionally, it was shown, that HB-EGF induces de-differentiation of Müller glia into multipotent progenitors in zebra fish retinas [157].

Here we utilized the Bioactive3D system [176] to address the effect of HB-EGF on astrocyte morphology, proliferation, differentiation, expression of intermediate filament proteins and mRNA expression of selected genes.

In the presence of HB-EGF we observed a stronger increase in proliferation in Bioactive3D compared to 2D cultured astrocytes, which may be due to the lower baseline reactivity of astrocytes in the Bioactive3D culture system [176]. Alterations of astrocyte morphology induced by HB-EGF lead to a more radial glia-like phenotype. A similar de-differentiating phenotype was observed after conditioned media to the cell cultures from astrocytes in an *in vitro* injury model [194] and it was also reported that HB-EGF is released after injury *in vivo* [207].

HB-EGF affects astrocyte intermediate filament expression and leads to partial astrocyte de-differentiation

In line with the observed morphological changes towards a radial glia-like phenotype, we found that HB-EGF leads to an increase in the expression of the intermediate filament protein nestin, a marker for reactive astrocytes [192], neural progenitor cells [193] and also de-differentiating astrocytes [194, 195] in both cell culture systems. Notch signaling enhances nestin expression in human gliomas [208] and here we show that expression of Notch signal pathway related genes was differentially affected in both culture systems. mRNA expression of the Notch signal pathway ligand *Dll1* was up-regulated in both culture systems, while its suppressor *Hes1* was down-regulated only in

Bioactive3D. This may be an indication of a HB-EGF-induced amplified Notch signaling cascade in Bioactive3D compared to 2D cultures.

Taken together, the changes in astrocyte morphology towards a radial glia-like phenotype, increased nestin protein expression and the differential expression of Notch signal pathway related genes, indicate that the addition of HB-EGF leads to a partial de-differentiation of astrocytes *in vitro*. Thus, HB-EGF should be used as a component of astrocyte culture media only with caution.

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