From mice to men - astrocytes and neural progenitor cells in neural plasticity and regeneration

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To my family

ABSTRACT

Astrocytes, a key homeostatic cell type in the mammalian central nervous system (CNS), have various functions in health and diseases. In neurotrauma, stroke, epilepsy or neurodegenerative diseases, astrocytes become reactive, which is known as reactive gliosis. Two characteristic hallmarks of reactive gliosis are hypertrophy of astrocyte processes and up-regulation of the intermediate filament (also known as nanofilament) proteins GFAP, vimentin, nestin and synemin. Reactive astrocytes have a neuroprotective role in the acute stage of CNS pathologies, by handling the acute stress, limiting the tissue damage and restoring the homeostasis of the CNS, but persisting reactive gliosis in some situations can become maladaptive and lead to the inhibition of neural plasticity and other regenerative responses. Animal studies suggested that some reactive astrocytes can act as neural stem cells after injury.

We hypothesized that some reactive astrocytes within the epileptic foci of patients with epilepsy have neural progenitor cell properties. We studied material from surgical resections from patients with pharmacologically intractable epilepsy after we first established protocols for dissociating such tissue into single live cells. We showed that cells with neural progenitor cell properties exist in the epileptic cortex outside the well-established adult neurogenic regions (i.e. outside the subventricular zone and the hippocampal dentate gyrus), and demonstrated that these neural progenitor cells are not glutamate aspartate transporter (GLAST) expressing astrocytes.

Modulation of the astrocyte nanofilament system was proposed as a potential therapeutic strategy in some CNS pathologies. To assess the role of vimentin phosphorylation in neurogenesis, we used *VIM*$^{SA/SA}$ mice, in which the serine sites in the vimentin molecule that are phosphorylated during mitosis had been mutated, which show cytokinetic failure in fibroblasts and lens epithelial cells. We found that mutation of the serine sites phosphorylated in vimentin during mitosis leads to increased neuronal differentiation of neural progenitor cells, and suggest that this is a neural progenitor cell intrinsic phenotype.

We studied three drug candidates that could potentially decrease the expression of GFAP and the other astrocyte nanofilament proteins, and assessed their effect on neurosupportive properties and resilience of astrocytes to oxygen and glucose deprivation (OGD), an in vitro model for brain ischemia. We found that two of them increased survival of neurons co-cultured with astrocytes, which makes them potential candidates for attenuation of reactive gliosis in vivo.

Key words: astrocytes, intermediate filaments, reactive gliosis, GFAP, GLAST, vimentin, neural progenitor cells, neurogenesis, Bioactive3D culture system

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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. **Chen M**, Puschmann TB, Wilhelmsson U, Örndal C, Pekna M, Malmgren K, et al.: Neural progenitor cells in cerebral cortex of epilepsy patients do not originate from astrocytes expressing GLAST.
Cereb Cortex. 2016 Dec 14.

II. Möllerström E, Rydenhag B, Andersson D, Lebkuechner I, Puschmann TB, **Chen M**, et al.: Classification of subpopulations of cells within human primary brain tumors by single cell gene expression profiling.
Neurochem Res. 2015 Feb;40(2):336-52.

III. **Chen M**, Puschmann T, Marasek P, Inagaki M, Pekna M, Wilhelmsson U, et al: Increased neuronal differentiation of neurosphere cells derived from phosphovimentin-deficient mice.
Manuscript.

IV. de Pablo Y, **Chen M**, Möllerström E, Pekna M, Pekny M: Drugs targeting intermediate filaments can improve neurosupportive properties of astrocytes.
Submitted manuscript.

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ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
bFGF	Basic fibroblast growth factor
BrdU	5-bromo-2′-deoxyuridine
CNS	Central nervous system
DCX	Doublecortin
EGF	Epidermal growth factor
FCS	Fetal calf serum
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
MAP2	Microtubule-associated protein-2
OGD	Oxygen and glucose deprivation
SGZ	Subgranular zone
SVZ	Subventricular zone
VIM	Vimentin

1. INTRODUCTION

1.1 Astrocytes and reactive gliosis

1.1.1 The function of astrocytes in healthy brain

The major cellular players within the mammalian central nervous system (CNS) are neurons and glial cells. Neurons are defined as electrically excitable cells, which fire action potentials that propagate to axonal terminals and initiate synaptic transmission. Glial cells are defined as electrically non-excitable cells, which are optimized for housekeeping, control and neural tissue protection (Pekny et al., 2016). Glial cells are composed of macroglia, e.g. astrocytes, oligodendrocytes, NG2 glia, and microglia. Astrocytes, also known as astroglial cells, are the most abundant cell type in the mammalian CNS. In the past, astrocytes were only believed to provide structural and nutritional support for the neurons, and this neuron-centered view has changed during the past 30 years. Astrocytes are now receiving increasing attention from neuroscientists and neurologists.

The importance of astrocytes in the maintenance of the CNS homeostasis, nutrition of neuronal cells and neurotransmitter recycling has long been known, as reviewed in (Khakh and Sofroniew, 2015; Parpura et al., 2012; Pekny et al., 2016; Verkhratsky et al., 2014). More recently, astrocytes were shown to control many other functional aspects of the CNS, both in health and disease. These range from the control of blood flow (Mulligan and MacVicar, 2004; Zonta et al., 2003), induction, elimination and functional control of neuronal synapses (Carmona et al., 2009; Christopherson et al., 2005; Chung et al., 2013; Sultan et al., 2015; Ullian et al., 2001; Yang et al., 2016), to the role in neural plasticity responses and regeneration processes [(Cho et al., 2005; Pekny et al., 2016; Widestrand et al., 2007);Fig 1].

1.1.2 Reactive astrocytes in diseased brain

Upon CNS injury, such as neurotrauma, stroke, epilepsy or neurodegenerative diseases, astrocytes become reactive, which is known as reactive gliosis (Pekny and Pekna, 2014; Pekny et al., 2016). Reactive gliosis is accompanied by various morphological and functional changes of astrocytes (Eddleston and Mucke, 1993; Sofroniew, 2009; Sofroniew and Vinters, 2010). The two cellular hallmarks of reactive gliosis are hypertrophy of astrocyte processes and up-regulation of the intermediate filament proteins GFAP, vimentin, nestin and synemin. In the acute stage of the injury, reactive gliosis has a protective role by handling the acute stress, limiting the tissue damage and restoring the CNS homeostasis. However, persisting reactive gliosis in some situations can become maladaptive and lead to inhibition of neural plasticity and other regenerative responses [for the review, see (Pekny and Pekna, 2014; Pekny and Pekna, 2016; Pekny et al., 2016); Fig 1]

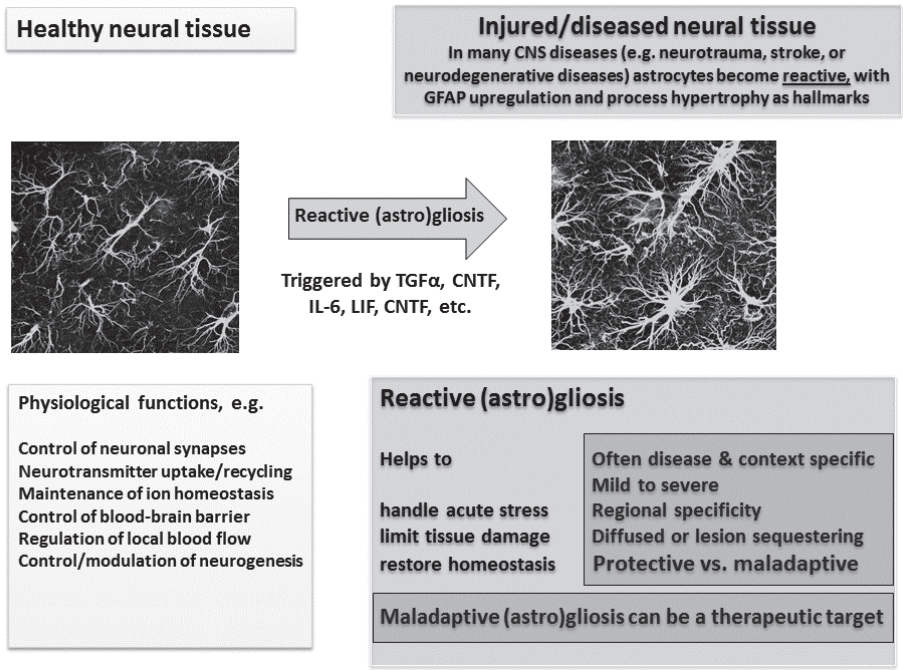

Fig 1. Astrocyte functions in health and disease, adapted from (Pekny and Pekna, 2016)

1.2 The intermediate filament system

1.2.1 The intermediate filaments

The cytoskeleton is composed of actin filaments, intermediate filaments (also known as nanofilaments), and microtubules. Actin filaments are the thinnest cytoskeletal elements of a diameter at around 7 nm, and are important e.g. for cell motility. Microtubules are the thickest cytoskeletal filaments of a diameter at around 25 nm with a tubular structure, and are important e.g. for intracellular transport and cell division. The diameter of intermediate filaments is around 10 nm, hence the name "intermediate". Unlike actin filaments and microtubules that are expressed in almost all eukaryote cells, the expression of individual intermediate filament proteins is highly cell-type specific (Herrmann et al., 2007; Wickstead and Gull, 2011).

In humans, intermediate filament proteins are encoded by at least 65 genes, giving rise to a large protein family (Herrmann et al., 2007). All intermediate filament protein molecules consist of a head, rod and tail domain. The α-helical rod domain is highly conserved, while the N-terminal head and C-terminal tail domains show large variations between intermediate filament proteins. Depending on the amino acid sequence and protein structure, the intermediate filament proteins are divided into six subclasses [(Eriksson et al., 2009; Guerette et al., 2007; Herrmann et al., 2007; Hyder et al., 2008); Table 1].

Table 1. The major intermediate filament proteins and examples of their tissue specificity

Type	Protein	Site of expression
I	Acidic keratins	Epithelial cells
II	Neutral or basic keratins	Epithelial cells
III	Vimentin	Fibroblasts, white blood cells, glial cells
	Glial fibrillary acidic protein (GFAP)	Astrocytes
	Desmin	Muscle
	Peripherin	Peripheral neurons
IV	Neurofilament proteins	Neurons
V	Nuclear lamins	Nuclear lamina of all cell types
VI	Nestin	Neural stem cells, astrocytes
	Synemin	Muscle, astrocytes

1.2.2 Intermediate filaments and reactive astrocytes

Four intermediate filament proteins are expressed in astrocytes: GFAP, vimentin, nestin and synemin. The expression of these four intermediate filament proteins in astrocytes depends on the type of astrocytes, developmental stage and the degree of astrocyte activation. Nestin and synemin are expressed in astrocyte precursors and in immature astrocytes (Lendahl et al., 1990; Pekny et al., 2014; Sultana et al., 2000). As astrocytes mature, GFAP expression increases while vimentin expression decreases, and nestin expression disappears (Bignami et al., 1982; Lendahl et al., 1990). In mature non-reactive astrocytes, GFAP and vimentin are the building blocks of the intermediate filament bundles. In neurotrauma, stroke, epilepsy or neurodegenerative diseases, astrocytes become reactive and up-regulate all the four intermediate filament proteins (Eddleston and Mucke, 1993; Eng and Ghirnikar, 1994; Jing et al., 2007; Lin et al., 1995).

We and others previously demonstrated that mice carrying null mutations in genes encoding GFAP and vimentin (*GFAP*$^{-/-}$*Vim*$^{-/-}$ mice), have astrocytes devoid of astrocyte intermediate filaments (Eliasson et al., 1999; Pekny et al., 1999), and exhibit better posttraumatic regeneration of neuronal synapses and axons (Cho et al., 2005;

Wilhelmsson et al., 2004), improved functional recovery after spinal cord injury (Menet et al., 2003), reduced photoreceptor degeneration in the retinal detachment model (Verardo et al., 2008), and reduced pathological neovascularization in oxygen-induced retinopathy (Lundkvist et al., 2004). We and others also demonstrated that in *GFAP*$^{-/-}$ *Vim*$^{-/-}$ mice, grafts can better integrate into the host retina (Kinouchi et al., 2003), differentiation of transplanted neural stem cells into neurons and astrocytes is enhanced (Widestrand et al., 2007). In addition, we found that in *GFAP*$^{-/-}$*Vim*$^{-/-}$mice, hippocampal neurogenesis is increased in naïve mice (Wilhelmsson et al., 2012), after neonatal hypoxic-ischemic injury (Jarlestedt et al., 2010), or after neurotrauma (Wilhelmsson et al., 2012). Thus, in a variety of injury models, the benefits of reactive gliosis in the acute stage of CNS injury is balanced against restricted regenerative potential at the later stage, and hence modulation of reactive gliosis targeting the intermediate filament system might lead to enhanced recovery after CNS injury.

1.2.3 Phosphorylation of intermediate filaments

The structure of intermediate filaments is dynamic, and the highly-controlled network reorganization by assembly and disassembly of intermediate filament proteins is essential for the cell motility, cell division and other cellular functions. (Herrmann and Aebi, 1998; Herrmann et al., 2007; Inagaki et al., 1996; Ivaska et al., 2007; Omary et al., 2006). The intermediate filament disassembly, which is regulated by phosphorylation of serine/threonine residues in the amino-terminal head domain of intermediate filament proteins (Inagaki et al., 1996; Sihag et al., 2007), was reported to be essential for the efficient separation of the two daughter cells during mitosis (Goto et al., 2003; Kawajiri et al., 2003; Yamaguchi et al., 2005; Yasui et al., 1998; Yasui et al., 2001). Some of the key phosphorylation sites and the protein kinases involved have already been identified, as described before (Chou et al., 1990; Goto et al., 1998; Goto et al., 2003; Goto and Inagaki, 2007; Inada et al., 1999; Kawajiri et al., 2003; Kosako et al., 1997; Nishizawa et al., 1991; Tsujimura et al., 1994; Yamaguchi et al., 2005).

Phosphovimentin-deficient mice (*VIM*$^{SA/SA}$ mice), i.e. mice expressing vimentin in which all the serine sites that are phosporylated during mitosis were substituted by alanine residues, show cytokinetic failures in fibroblasts and lens epithelial cells resulting in aneuploidy, chromosomal instability, and increased expression of cell senescence markers (Matsuyama et al., 2013). *VIM*$^{SA/SA}$ mice exhibit some signs of pre-mature aging, including cataract development in lens, delayed skin wound healing, and subcutaneous fat loss in old age (Matsuyama et al., 2013; Tanaka et al., 2015).

1.3 Mammalian adult neurogenesis

1.3.1 The neurogenic niches in the adult mammalian brain

Decades ago it was firmly believed that the adult mammalian brain was an organ without any neurogenesis, and this belief had not been challenged until 1960's (Altman, 1962). After decades of studies, it is now well established that adult mammalian neurogenesis exists throughout life, and is restricted to two brain niches: the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (Cameron et al., 1993; Cameron and McKay, 2001; Conover and Notti, 2008; Doetsch et al., 1999; Eriksson et al., 1998; Ernst and Frisen, 2015; Kirschenbaum et al., 1999; Spalding et al., 2013; Wang et al., 2011). In the adult human brain, neural progenitor cells were also reported in the striatum (Ernst et al., 2014).

In rodents, it was demonstrated that newly born neurons in the SVZ migrate along the rostral migratory stream into the olfactory bulb where they replace the interneurons throughout life (Angot et al., 2008; Kirschenbaum et al., 1999). Newly born neurons in the SGZ of the hippocampal dentate gyrus are capable of integrating into existing hippocampal neuronal networks but remain within the hippocampus (Gage, 2000; Kempermann et al., 2004). The rate of neurogenesis is affected by activities such as learning, exercise, exposure to environmental enrichment, stress and alcohol (Crews et al., 2004; Gould and Tanapat, 1999; Gould et al., 1999; Kempermann et al., 1997; van Praag et al., 1999). Neurogenesis in the dentate gyrus of the hippocampus regulates

formation of newly acquired memories as well as forgetting through hippocampal circuit remodeling (Akers et al., 2014; Chung et al., 2015).

1.3.2 The neurogenic niche provides neurogenesis-permissive environment

Transplantation studies of neural progenitor cells into various regions of the adult CNS showed that neuronal differentiation of the transplanted neural progenitor cells only occurs in the two adult neurogenic niches (Emsley et al., 2005; Goh et al., 2003; Shihabuddin et al., 2000; Suhonen et al., 1996). Only astrocytes isolated from neurogenic niche had the neuronegesis-permissive properties (Jiao and Chen, 2008; Song et al., 2002). These findings point to the cellular environment as a key modulator for neurogenesis in the adult CNS.

Extensive studies have been done to investigate the molecular mechanisms underlying astrocyte-induced neurogenesis, and several signaling pathways have been identified. For example, astrocytes enhance proliferation and differentiation of adult neural progenitor cells through increased insulin-like growth factor (IGF) signaling by decreased expression of insulin-like growth factor binding protein-6 (IGFBP-6) (Barkho et al., 2006). Wnt3 was shown to be over-expressed in adult hippocampal astrocytes and was proposed to lead to increased neurogenesis (Lie et al., 2005). Sonic hedgehog (SHH) was reported to be expressed by SGZ astrocytes and stimulate neural progenitor cell proliferation (Jiao and Chen, 2008). Our laboratory previously demonstrated that the astrocyte intermediate filament system is important for Notch signaling from astrocytes to neural progenitor cells, and astrocytes devoid of intermediate filaments support increased differentiation of neural progenitors into neurons, astrocytes or oligodendrocytes in the hippocampus of adult mouse brain (Lebkuechner et al., 2015; Widestrand et al., 2007; Wilhelmsson et al., 2012).

1.3.3 Neurogenic regions outside the well-established neurogenic niches

Whether there are neurogenic regions other than the well-established neurogenic niches is still under debate. During the recent decades, in vitro neurosphere studies indicated that it was possible to isolate multipotent neural progenitor cells from live human brain tissue obtained from surgical resections (Arsenijevic et al., 2001; Nunes et al., 2003). However, studies on adult human brains, by means of 5-bromo-2′-deoxyuridine (BrdU) labeling of proliferating cells, determination of the ^{14}C isotope incorporated into DNA during cell proliferation, as well as magnetic resonance spectroscopy, did not reveal any evidence of neurogenesis in human cortical regions (Eriksson et al., 1998; Manganas et al., 2007; Spalding et al., 2005). Interestingly, in rodents some reactive/de-differentiated astrocytes which are found in areas with reactive gliosis after e.g. traumatic brain injury or stroke, seem to exhibit properties of neural progenitor cells (Buffo et al., 2008; Shimada et al., 2012). These findings widened our view of adult neurogenesis.

2 AIMS OF THE STUDIES

I. To study whether the activation of astrocytes in human epilepsy leads to de-differentiation of these cells, and whether some reactive astrocytes would acquire properties of neural progenitor cells (Paper I). To establish a protocol for obtaining viable single cells from freshly isolated human brain tissue (Paper II).

II. To study whether the vimentin phosphorylation deficit in *VIM*$^{SA/SA}$ mice alters astrocyte morphology, proliferative capacity and cell motility, and whether the phosphovimentin-deficient astrocyte niche affects neuronal differentiation of neural progenitor cells in vitro or neurogenesis in vivo (Paper III).

III. To study the drug candidates that could potentially decrease the expression of GFAP and other astrocyte intermediate filament proteins, and to assess their effect on neurosupportive properties and resilience of astrocytes to oxygen and glucose deprivation (OGD; Paper IV).

3 MATERIAL AND METHODS

3.1 Human research specimens and tissue dissociation (Paper I and II)

Human cerebral cortex tissue was obtained from 19 patients operated for pharmacologically intractable epilepsy (2.5-45 years old, 10 females, 9 males; Paper I). Tumor tissue was collected from a grade IV astrocytoma (49 years old male patient); tumor and tumor penumbra tissue was collected from a grade III oligodendroglioma (63 years old male patient; paper II). Brain surgery took place at Sahlgrenska University Hospital, Gothenburg, Sweden. During the surgery, the resected tissue was divided into a research specimen and a specimen that was used for histopathological diagnosis. Research specimens were collected and immediately transferred into cold Hybernate A medium (Invitrogen) for transportation to the cell culture facility. Research specimens were collected in accordance with the ethical guidelines of the Sahlgrenska University Hospital and the approval was obtained from the Regional ethical review board in Gothenburg (Dnr 179-08).

The brain tissue was dissociated using MACS brain tumor dissociation T kit (Miltenyi Biotech) according to manufacturer's protocol. The resulting single cell suspension was purified with MACS myelin removal microbeads (Miltenyi Biotech) to remove the myelin debris. To obtain GLAST-positive astrocytes for cell culture, the isolated cells were sorted using MACS anti-GLAST (ACSA-1) microbeads (Miltenyi Biotech; Paper I). To obtain single cells for mRNA expression profiling, the isolated cells were sorted by a FACS Aria II (Becton-Dickinson) with single cell sorting function into separate wells of a 96-well plate (Paper II).

3.2 Mice (Paper III and IV)

To study the impact of vimentin phosphorylation to astrocyte cell morphology and functions (Paper III), we used phosphovimentin-deficient mice (*VIM*$^{SA/SA}$ mice), i.e. mice

expressing vimentin in which all the serine sites that are phosporylated during mitosis were substituted by alanine residues (Matsuyama et al., 2013; Tanaka et al., 2015), and their respective wild-type controls on a C57BL/6 genetic background. *VIM*$^{SA/SA}$ mice are viable, and show phenotypes as described before (Matsuyama et al., 2013; Tanaka et al., 2015).

To study the effect of drug candidates on neurosupportive properties and resilience of astrocytes to oxygen and glucose deprivation (OGD) in vitro (Paper IV), we used wild-type mice and mice carrying a null mutation in the GFAP and vimentin genes [(*GFAP*$^{-/-}$ *Vim*$^{-/-}$); (Colucci-Guyon et al., 1994; Eliasson et al., 1999; Pekny et al., 1995; Pekny et al., 1999)] on a mixed C57BL/6-129Sv-129Ola genetic background. *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice are viable, and show phenotypes as described before (Pekny and Pekna, 2014).

All mice were housed in standard cages in a barrier animal facility and had free access to food and water. All the experiments were conducted according to protocols approved by the Ethics Committee of the University of Gothenburg (Dnr. 247-2014).

3.3 Cell culture and in vitro analysis

3.3.1 Neurosphere culture (Paper I and III)

The neurosphere in vitro assay was developed in 1990's and it soon became a commonly used method to evaluate the two major neural stem cell properties: self-renewal and multipotency, i.e. ability to differentiate into neurons, astrocytes and oligodendrocytes (Brewer and Torricelli, 2007; Reynolds and Weiss, 1992). The neurosphere assay is based on the concept that neural stem cells could divide symmetrically and form free floating spherical clusters (neurospheres) in a serum-free culture medium, in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

To assess the multipotency of the neurosphere cells, neurospheres or dissociated neurosphere cells were plated on poly-L-ornithine and laminin coated coverslips or

culture dishes, in presence of 0.1%-1% fetal calf serum (FCS) without growth factors. The differentiation was assessed by immunocytochemical detection of cell type specific markers (βIII tubulin and MAP2 for neurons, GFAP for astrocytes, and O4 for oligodendrocytes).

3.3.2 Neurosphere cells and neurons co-cultured with astrocytes (Paper III and IV)

In rodents, cortical neurogenesis completes at embryonic day 15, the onset of gliogenesis occurs after the onset of neurogenesis, with the peak of astrogenesis perinatally and the peak of oligodendrogenesis postnatally (Gotz and Huttner, 2005; Semple et al., 2013). Therefore, in our in vitro studies, mouse cortical neurons were prepared from E17.5 mouse cortex to reduce astrocyte contamination, mouse cortical astrocytes were prepared from P0.5-2.5 mouse cortex, and multipotent neurosphere cells were prepared from P2.5-3.5 mouse forebrain.

To investigate the effect of astrocyte environment on neuronal differentiation (Paper III), neurosphere cells were plated on top of primary astrocytes. To distinguish the neurosphere cells and co-cultured astrocytes, 0.5 μM BrdU was added into the neurosphere culture medium 48 h prior to dissociation. The neuronal differentiation was assessed by quantification of βIII-tubulin labeled cells as a fraction of BrdU-positive cells.

To investigate the astrocyte neurosupportive properties (Paper IV), neurons were plated on top of primary astrocytes. Astrocyte neurosupportive properties were assessed by counting the surviving neurons and measuring the neurite length.

3.4 Bioactive3D – a novel three-dimensional cell culture system that allows to maintain some of the in vivo properties of astrocytes (Paper III)

The traditional 2-dimensional (2D) cell cultures constitute a highly artificial and stressful environment, where astrocytes are forced to grow on a flat culture dish without the 3-

dimensional (3D) support. Astrocytes in 2D cell cultures resemble reactive astrocytes, by exhibiting increased proliferation, upregulated expression of GFAP and vimentin, and re-expressing nestin (Andreasson et al., 2016; Puschmann et al., 2013).

We previously developed a novel 3D cell culture system (Bioactive3D) and demonstrated that Bioactive3D culture system allows the cultured astrocytes to maintain some in vivo-like properties (Andreasson et al., 2016; Puschmann et al., 2013; Puschmann et al., 2014a; Puschmann et al., 2014b). Astrocytes cultured in Bioactive3D system retain aspects of their complex in vivo-like morphology (Fig 2), have minimal baseline reactivity and show reduced cell proliferation (Puschmann et al., 2013). Neurons cultured in Bioactive3D system extended their neurites to all dimensions and formed complex structures, and astrocyte contamination in the neuron cultures in the Bioactive3D system was minimized due to their reduced proliferation (Puschmann et al., 2014a).

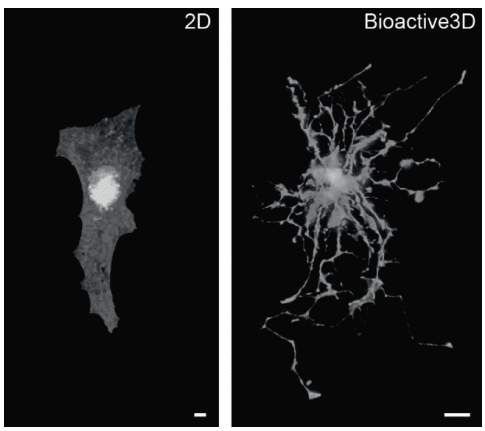

Fig 2. Astrocytes cultured in 2D and Bioactive3D show distinct morphology, adapted from (Andreasson et al., 2016)

Primary astrocytes cultured in the Bioactive3D system, which is composed of polyurethane nanofibers coated with poly-L-ornithin and laminin, preserve some of the complex morphological and biochemical features of in vivo astrocytes that are normally lost upon 2D culture (compare the left and right panel). Space bar, 10 µm. The primary astrocytes were derived from mice expressing enhanced green fluorescein protein under the control of GFAP promoter (Andreasson et al., 2016; Pekny and Pekna, 2014; Puschmann et al., 2013).

3.5 Assessment of cell proliferation and survival in the mouse hippocampus (Paper III)

To detect a small population of dividing neural progenitor cells in the adult mouse brain, BrdU in vivo labeling is used. BrdU, a thymidine analogue, is incorporated into the replicating DNA of dividing cells. Immunohistochemical detection of BrdU-positive

cells in brain sections allows to visualize the neural progenitor cells and follow their survival and differentiation over time. For detection of all dividing cells in vivo, the dose of intraperitoneally administered BrdU ranges between 100-300 mg/kg, which is non-toxic to rodents (Cameron and McKay, 2001; Wojtowicz and Kee, 2006).

We administered BrdU intraperitoneally (300mg/kg) to label and follow the cell fate of newly born cells in the hippocampal dentate gyrus of adult $Vim^{SA/SA}$ mice and their respective controls. Double labeling using anti-BrdU and anti-doublecortin (DCX) antibodies was applied to visualize and count the neuronal precursors and immature neurons. Double labeling using anti-BrdU and anti-NeuN was applied to visualize and count the surviving newly generated neurons.

4 RESULTS AND DISCUSSION

4.1 Paper I and II

In the adult human brain, neural progenitor cells are present in the subventricular zone (Curtis et al., 2007; Wang et al., 2011), the hippocampus (Eriksson et al., 1998; Spalding et al., 2013) and the striatum (Ernst et al., 2014). In rodents, reactive astrocytes were proposed to exhibit properties of neural progenitor cells (Buffo et al., 2008; Shimada et al., 2012). However, the origin of multipotent neural progenitor cells obtained from human brain cortical surgical resections (Arsenijevic et al., 2001; Kirschenbaum et al., 1994; Nunes et al., 2003) is still under debate. In Paper I, we addressed the following questions: Can activation of astrocytes in epilepsy lead to de-differentiation of some of these cells? Do some astrocytes, when activated, de-differentiate into cells with neural progenitor cell properties capable of giving rise to all three cell types of the neurogenic lineage – i.e. neurons, astrocytes and oligodendrocytes? We hypothesized that some reactive astrocytes within the epileptic foci of patients with epilepsy have properties of neural progenitor cells. Within Paper II, we developed protocols for dissociating human brain tissue into single live cells.

In Paper I, we assessed the capacity of cells dissociated from fresh cortical tissue from patients who underwent surgical treatment for pharmacologically intractable epilepsy to form neurospheres and the differentiation potential of the neurosphere cells. Neurospheres were generated from 57% of cases (8/14). Upon differentiation, the neurosphere cells gave rise to neurons, oligodendrocytes and astrocytes. Sorting of dissociated cells showed that only cells negative for astrocyte marker glutamate aspartate transporter (GLAST) formed neurospheres. The origin of these cells in the human epileptic cortex warrants further investigation. This finding is an important step in our understanding of the processes ongoing in the epileptic tissue and of the role of astrocytes in epilepsy.

4.2 Paper III

It was previously reported that mutation of the serine sites that are phosporylated in vimentin during mitosis (*VIM*$^{SA/SA}$) leads to cytokinetic failures in fibroblasts and lens epithelial cells in mice, resulting in chromosomal instability and increased expression of cell senescence markers (Matsuyama et al., 2013; Tanaka et al., 2015). In this study, we investigated morphology, proliferative capacity and cell motility of *VIM*$^{SA/SA}$ astrocytes, and their effect on the differentiation of neural progenitor cells.

We found that *VIM*$^{SA/SA}$ astrocytes expressed lower levels of vimentin protein, but showed a well-developed intermediate filament network, exhibited normal cell morphology, proliferation, and motility in the in vitro wound closing assay. Thus, phosphorylation of vimentin during mitosis does not seem to play a role in the control of morphology, cell division and motility of astrocytes. This is in contrast to *VIM*$^{SA/SA}$ lens epithelial cells or fibroblasts that showed disrupted intermediate filament network and formation of unbreakable intermediate filament-bridges during mitosis. Vimentin is highly expressed in astrocytes, lens epithelial cells and fibroblasts. In astrocytes, vimentin forms intermediate filaments together with GFAP and nestin. Given that GFAP is highly expressed in postnatal astrocytes but not in lens epithelial cells or fibroblasts (Boyer et al., 1990; Matsuyama et al., 2013; Tanaka et al., 2015), it is possible that in *VIM*$^{SA/SA}$ astrocytes GFAP compensates for the relative lack of vimentin and/or affects the intermediate filament dynamics. We have already crossed the *VIM*$^{SA/SA}$ mice with *GFAP*$^{-/-}$ mice, and will assess the role of vimentin phosphorylation in the absence of GFAP in astrocytes.

Interestingly, we found a 2-4-fold increased neuronal differentiation of *VIM*$^{SA/SA}$ neurosphere cells, both in a standard 2D and in Bioactive3D cell culture systems, and determined that this effect was neurosphere cell autonomous, and not dependent on co-cultured astrocytes. Using BrdU in vivo labeling to assess neural progenitor cell proliferation and differentiation in the hippocampus of adult mice, we found a modest increase (by 8 %) in the fraction of newly born and surviving neurons. It would be noted

that the neural sphere cells were isolated from the whole forebrain of P2.5-3.5 mouse pups, including the hippocampus and the subventricular zone, but we only assessed the newly born and surviving neurons in the adult mouse hippocampus. Therefore, it would be interesting to assess the neurosphere cells isolated from the adult mouse hippocampus. The prominent in vitro effect of $VIM^{SA/SA}$ mutation can also possibly be mitigated or masked in vivo by niche-related factors that remain to be identified. Even in Bioactive3D, a cell culture system closer to the in vivo situation, the increased neurogenesis from neurosphere cells was very robust and more prominent compared to the modest increase in hippocampal neurogenesis in $VIM^{SA/SA}$ mice. This result was conceivably due to the absence of other niche constituents such as endothelial cells and microglia in the in vitro system. Notch signaling, which regulates the neural stem cell proliferation and differentiation (Liu et al., 2010; Wilhelmsson et al., 2012), is worth further investigation. Interestingly, in the experiment assessing the number of surviving neurons 6 weeks following BrdU administration, we found that the increase in the fraction of surviving neurons in $VIM^{SA/SA}$ mice was due to a decrease in the size of the subpopulation of non-neuronal BrdU-positive cells. It is essential to further identify that cell population in future studies. It will be also interesting to connect the difference of the in vivo phenotype to a behavioral correlation, such as learning and memory. Our preliminary data showed that $VIM^{SA/SA}$ mice had unimpaired short-term and long-term memory in the novel object recognition test, but other tests can be further applied.

In conclusion, mutation of the serine sites phosphorylated in vimentin during mitosis has no or minimal effect on astrocytes, but leads to an increased neuronal differentiation of neural progenitor cells. The functional significance of this finding and the involvement of phosphovimentin in the functions of other cellular elements which express vimentin in the neurogenic niche, such as the endothelial cells and microglia, warrants further investigation.

4.3 Paper IV

As modulation of reactive gliosis targeting the intermediate filament system might lead to enhanced recovery after CNS injury, we hypothesized that pharmacological modulation of the astrocyte nanofilament system improves survival of neurons when co-cultured with astrocytes under standard conditions or when exposed to oxygen and glucose deprivation, an in vitro model of brain ischemia. Several drugs were reported to decrease GFAP expression in vitro and in vivo (Bargagna-Mohan et al., 2010; Cho et al., 2010; Middeldorp et al., 2009). Clomipramine (8 μM) treatment of astrocyte cultures for 10 days decreased the amount of GFAP by 50% (Cho et al., 2010). Epoxomicin, and other proteasome inhibitors, decreased GFAP mRNA and soluble protein levels in human astrocytoma U343 cells at doses ranging from 5 to 100 nM as well as GFAP protein levels in a rat model for induced astrogliosis (Middeldorp et al., 2009). In astrocyte cultures, withaferin A downregulated soluble vimentin and GFAP at 0.5 μM and systemic delivery of withaferin A down-regulated expression of both vimentin and GFAP in mouse retinas (Bargagna-Mohan et al., 2010).

In this paper, we examined the effect of epoxomicin, clomipramine and withaferin A, three candidate drugs for targeting the astrocyte nanofilament system, on astrocyte resilience to ischemic stress in vitro, and astrocyte neurosupportive properties. Clomipramine decreased protein levels of GFAP, vimentin and nestin in astrocytes, and did not affect astrocyte resilience to oxidative stress. Both clomipramine and epoxomicin promoted the attachment and survival of neurons co-cultured with astrocytes under standard culture conditions. Moreover, epoxomicin increased neurosupportive properties of astrocytes after oxygen and glucose deprivation. Our data identify clomipramine and epoxomicin as potential candidates for astrocyte modulation to improve outcome after CNS injury.

In summary, this study suggests that pharmacological modulation of the astrocyte intermediate filament system by epoxomicin and clomipramine can increase the

neurosupportive properties of astrocytes without compromising their ability to cope with ischemic stress.

5 CONCLUSIONS

This thesis invested the role of astrocytes and the intermediate filaments in neural plasticity, neuroregeneration and neuroprotection. Specifically, we have found that

1. Cells with neural progenitor cell properties exist in human epileptic tissue outside the two neurogenic niches, and they are not GLAST-positive astrocytes.

2. Mutation of the serine sites phosphorylated in vimentin during mitosis in mice has no or minimal effect on astrocytes, but leads to an increased neuronal differentiation of neural progenitor cells both in vitro and in vivo.

3. Both clomipramine and epoxomicin promote the survival of neurons co-cultured with astrocytes under standard culture conditions, and epoxomicin increases neurosupportive properties of astrocytes after oxygen and glucose deprivation.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Astrocyter är en viktig celltyp i det centrala nervsystemet (CNS) och kontrollerar många neurologiska funktioner, som upptagning och återvinning av signalsubstanser, induktion och eliminering av kopplingar mellan hjärnans nervceller, samt styr blodförsörjningen till hjärnvävnaden. Vid skador och sjukdom i CNS, som neurotrauma, stroke, epilepsi och neurodegenerativa sjukdomar, aktiveras astrocyter, vilket också kallas reaktiv glios. Två viktiga kännetecken för reaktiv glios är förtjockning av astrocyternas utskott och uppreglering av de proteiner som utgör intermediära filament i astrocyter (också kallade nanofilament som utgör en del av cytoskelettet): glial fibrillary acidic protein (GFAP), vimentin, nestin och synemin. Under den akuta fasen av hjärnskada kan reaktiv glios begränsa skadan och skydda omgivande hjärnvävnad, men långvarig reaktiv glios kan hämma hjärnans plasticitet och utväxten av nervutskott. Att aktivera astrocyter vid den akuta fasen och att dämpa kronisk reaktiv glios därefter kan därför vara en potentiell terapeutisk strategi vid skador och sjukdom i CNS.

Vår forskargrupp har utvecklat transgena möss som saknar GFAP och vimentin, och dessa möss saknar därmed nanofilament i astrocyter. Dessa möss uppvisar en dämpad reaktiv glios, och en ökad nybildning av neuroner i hippocampus, ett av de två områden i den hjärnan där neurala stamceller finns även i vuxen ålder.

Det har föreslagits att reaktiva astrocyter vid hjärnskador kan också fungera som neurala stamceller utanför de delarna av hjärnan där neurala stamceller normalt finns hos vuxna. Det är därför mycket intressant att utröna om fenomenet förekommer hos människor. I delarbete I isolerade vi celler från kirurgiska resektioner av vävnad från epilepsipatienter (protokoll för isolation av celler från vävnaden utvecklades inom delarbete II), och studerade deras potential som neurala stamceller. Vi upptäckte att celler med stamcellspotential fanns i vävnaden även utanför de etablerade neurogena områdena hos patienter som opererades för epilepsi och att dessa celler inte var reaktiva astrocyter.

I delarbete III studerade vi betydelsen av fosforylering av vimentin, en viktig regleringprocess för proteins funktioner som nanofilament, genom att använda transgena möss med mutaterade fosforyleringställen (*Vim*$^{SA/SA}$ möss) som gör att vimentin inte kan fosforyleras. Vi visade en ökad differentiering av stamceller till neuroner hos *Vim*$^{SA/SA}$ neurala stamceller jämfört med normala neurala stamceller. Denna ökning var oberoende av om astrocyterna i stamcellernas omgivning var *Vim*$^{SA/SA}$ eller från kontrollmöss.

I delarbete IV utvärderade vi om farmakologisk reglering av nanofilament i astrocyter kan påverka astrocyters förmåga att stödja neuroners överlevnad och deras tillväxt av nervcellsutskott. Vi använde tre läkemedel som rapporterats att minska mängden GFAP i astrocyter och studerade deras effekt på astrocyters förmåga att stödja neuroners överlevnad och tillväxt under normala förhållanden och i en modell av hjärnischemi med minskade nivåer av syre och glukos. Två av de tre testade läkemedlen stödde överlevnaden av neuroner samodlades med astrocyter. Studien föreslår dessa två läkemedel som potentiella kandidater att använda för att modulera astrocyter och reaktiv glios för att förbättra återhämtning efter CNS-skador.

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