THE ROLE OF ASTROCYTES IN STROKE, BRAIN PLASTICITY AND NEUROGENESIS

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ABSTRACT

Astrocytes, one of the most abundant and heterogeneous cell types in the central nervous system, fulfill many important roles in the healthy and injured brain. This thesis investigates the role of astrocytes in the neurogenic niche and the astrocyte response in stroke and neurotrauma. Using gene expression profiling on a global level as well as on a single-cell level and applying it to disease and transgenic models in vivo and in vitro, we have addressed molecular bases of these responses and molecular signatures of the subpopulations of astrocytes. Following injury, stroke or neurodegenerative diseases, astrocytes upregulate intermediate filament (nanofilament) proteins glial fibrillary acidic protein and vimentin along with many other genes, in a process referred to as reactive gliosis. Results presented in this thesis show that mice with attenuated reactive gliosis developed larger infarct volumes following experimental brain ischemia, compared to controls, implying that reactive gliosis is neuroprotective. Using astrocyte and neurosphere co-cultures, we show that astrocytes inhibit neuronal differentiation through cell-cell contact via the Notch signaling pathway and that intermediate filaments are involved in this process. We found that even a very limited focal trauma triggers a distinct brain plasticity response both in the injured and contralesional hemisphere and that this response at least partly depends on activation of astrocytes. Finally, using single-cell gene expression profiling in vitro and in vivo, we show that the astrocyte population is highly heterogeneous, we attempt to define astrocyte subpopulations in molecular terms, and we demonstrate that astrocyte subpopulations respond differentially to a subtle neurotrauma both in the injured and contralesional hemisphere.

Keywords: astrocytes, reactive gliosis, stroke, neurotrauma, brain plasticity, intermediate filaments, nanofilaments, GFAP, vimentin, neurogenesis, neural stem/progenitor cell, single-cell gene expression profiling

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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. Li L, Lundkvist A, Andersson D, Wilhelmsson U, Nagai N, Pardo AC, Nodin C, Ståhlberg A, Aprico K, Larsson K, Yabe T, Moons L, Fotheringham A, Davies I, Carmeliet P, Schwartz JP, Pekna M, Kubista M, Blomstrand F, Maragakis N, Nilsson M, Pekny M.
Protective role of reactive astrocytes in brain ischemia.
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Astrocytes negatively regulate neurogenesis through the Jagged1-mediated Notch pathway.
Stem Cells. 2012 Oct;30(10):2320-9.

III. Ståhlberg A, Andersson D, Aurelius J, Faiz M, Pekna M, Kubista M, Pekny M.
Defining cell populations with single-cell gene expression profiling: correlations and identification of astrocyte subpopulations.
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IV. Andersson D, Wilhelmsson U, Nilsson M, Kubista M, Ståhlberg A, Pekna M, Pekny M.
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Molecular definition of astrocytes in unchallenged and injured hippocampus, a single-cell gene expression study.
Manuscript

CONTENT

ABBREVIATIONS

Aldh1L1	Aldehyde dehydrogenase 1 family, member L1
BBB	Blood brain barrier
CNS	Central nervous system
ECL	Entorhinal cortex lesion
ET_BR	Endothelin B receptor
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
IF	Intermediate filament
MCA	Middle cerebral artery
PCA	Principal component analysis
RT-qPCR	Reverse transcription quantitative real-time PCR
SGZ	Subgranular zone
SOM	Self-organizing map
SVZ	Subventricular zone

INTRODUCTION

Astrocytes, one of the most abundant cell type in the central nervous system (CNS)(Markiewicz & Lukomska, 2006), were for long believed to mainly provide architectural structure, nutrition and homeostasis in the healthy brain. This has changed and astrocytes are today attributed with many essential and controlling functions in the healthy as well as in the injured brain. They are known to control neuronal activity (Araque et al., 1999; Anderson & Swanson, 2000), induce neurogenesis from neural stem cells in the adult brain (Song et al., 2002), or act as a source of neural stem cells themselves (Buffo et al., 2008; Sirko et al., 2013).

Following any injury to the brain, astrocytes become reactive and increase the expression of the intermediate filament (IF) proteins glial fibrillary acidic protein (GFAP), vimentin and nestin and alter the expression of many other genes, in a process referred to as reactive gliosis. This is thought to function as a way of quickly restoring the homeostasis of the brain, which is crucial for proper neuronal transmission to take place. In severe cases, reactive gliosis can create a glial scar which isolates the injured tissue, but later functions as a major inhibitor of regeneration. Depending on what triggered astrocytes to become reactive, reactive gliosis differs. Previous studies have shown that mice with astrocytes deficient in the two IF proteins GFAP and vimentin (*GFAP*$^{-/-}$*Vim*$^{-/-}$ mice)(Pekny et al., 1999a) show attenuated reactive gliosis, improved integration of neural grafts and neural progenitor cells (Kinouchi et al., 2003; Widestrand et al., 2007) and synaptic regeneration (Wilhelmsson et al., 2004).

This thesis investigates the role of astrocytes in the neurogenic niche, their response to stroke and neurotrauma and addresses the astrocyte heterogeneity on a single-cell level.

BACKGROUND

Astrocytes

Astrocytes, one of the most abundant cell type in the central nervous system (Markiewicz & Lukomska, 2006), were for long believed to mainly provide architectural structure, nutrition and homeostasis in the healthy brain. The last decades have shown that they fulfill many other important roles (Nilsson & Pekny, 2007; Oberheim et al., 2012).

Classically, astrocytes were divided into protoplasmic or fibrous subtypes based on their anatomical location and cellular morphology. Using silver impregnation techniques protoplasmic astrocytes, spread throughout all grey matter, appear as cells with several main branches which in turn give rise to smaller processes. Fibrous astrocytes, on the other hand, located in all white matter, exhibit many fiber-like processes (Sofroniew & Vinters, 2010). They were for long treated as a homogenous group of cells, but are now acknowledged to be highly heterogenous (Matyash & Kettenmann, 2010; Zhang & Barres, 2010). Specialized subtypes of astrocytes have been characterized, including the Bergmann glia of the cerebellum and the Müller glia of the retina, based on morphology, as well as the expression of various proteins, physiological properties, function and response to injury or disease, (Emsley & Macklis, 2006; Zhang & Barres, 2010). Knowing the functional heterogeneity of astrocytes is essential as astrocytes are involved in almost all diseases of central the nervous system (Zhang & Barres, 2010).

Due to the heterogeneity of the astrocytes, no perfect astrocyte-specific marker has been found. The expression of the intermediate filament (IF) protein glial fibrillary acidic protein (GFAP) has for long been the most useful marker to immunohistochemically identify astrocytes, but not all astrocytes in the healthy brain express GFAP. Other astrocyte markers, such as S100β and glutamine synthetase have similar shortcomings (Sofroniew & Vinters, 2010). Recently, the aldehyde dehydrogenase 1 family, member L1 (Aldh1L1), also known as 10-formyltetrahydrofolate dehydrogenase (FDH), was suggested as a pan-astrocyte marker based on transcriptome gene profiling and *in situ* hybridization (Cahoy et al., 2008).

Astrocytes are essential for cell-cell communication in the neural tissue, being directly in contact with neurons, oligodendrocytes, microglia, as well as with endothelial cells and pericytes of blood vessels. Astrocytes, unlike neurons, cannot signal via action potentials. Instead, they are connected via gap junctions into syncytia and communicate through propagated waves of Ca^{2+} and other active substances (Parpura & Verkhratsky, 2012). In the human brain a single astrocyte can have up to two million synapses within its domain (Oberheim et al., 2009). Astrocyte cellular processes enwrap synapse terminals (Araque et al., 1999) and modulate neuronal activity by recycling molecules involved in neurotransmission (Anderson & Swanson, 2000), releasing gliotransmitters that regulate the activity of neighbouring cells, including neurons (Parpura et al., 1994; Schell et al., 1995; Beattie et al., 2002). This concept of the 'tripartite synapse' was recently called into question as it appears only to occur in the immature brain (Sun et al., 2013). Astrocytes affect synapse plasticity by having an active part in the formation, maintenance and pruning of synapses (Ullian et al., 2001; Christopherson et al., 2005; Stevens et al., 2007; Kucukdereli et al., 2011). Astrocytes control cerebral blood flow (Zonta et al., 2003; Takano et al., 2006) and are thought to induce and maintain the blood brain barrier (BBB) properties in endothelial cells, which is essential for the regulation of the microenvironment to allow for reliable neuronal signaling (Abbott et al., 2006). Astrocytes have also been shown to regulate neurogenesis by instructing neural stem cells to adopt neuronal fate (Song et al., 2002) and by acting as neural stem cells themselves (Doetsch et al., 1999; Buffo et al., 2008; Sirko et al., 2013).

Intermediate filaments (nanofilaments)

The cytoskeleton provides the cell with structure and shape. Eukatyotic cells contain three kinds of cytoskeletal filaments: the microfilaments, the intermediate filaments (IFs) and the microtubules. Of these, the IFs are the least understood, partly due to having more than 70 different genes coding for IF proteins (Goldman et al., 2012) and are composed of different IF proteins depending on cell type, developmental and activity state of the cell (Fuchs & Cleveland, 1998). IFs have been shown to give the cell the means to withstand mechanical and non-mechanical stress, thus preserving cellular functions (Parry et al., 2007). IF dysfunction can result in various diseases,

such as epidermolysis bullosa simplex (EBS), caused by mutations in keratin IF proteins (Omary et al., 2004; Pekny & Lane, 2007). IFs also regulate cell-adhesion, migration and function as signaling platforms (Jones et al., 1998; Lepekhin et al., 2001; Ivaska et al., 2007).

Four different IF proteins are expressed in astrocytes: GFAP, vimentin, nestin and synemin. Their expression is dependent on developmental stage as well as astrocyte activity (Eliasson et al., 1999; Sultana et al., 2000; Jing et al., 2007). Astrocyte precursors express vimentin, nestin and synemin. In maturing astrocytes vimentin expression is decreased while nestin and synemin are progressively replaced by GFAP (Pixley & de Vellis, 1984; Lendahl et al., 1990; Sultana et al., 2000). Following neurotrauma, stroke or neurodegenerative diseases, vimentin and nestin are re-expressed, as is synemin in some cells (Pekny & Nilsson, 2005; Jing et al., 2007; Luna et al., 2010).

Reactive gliosis

A part of the response of the CNS to neurotrauma, stroke or neurodegenerative diseases is activation of astrocytes, a process referred to also as reactive gliosis or astrogliosis (Eddleston & Mucke, 1993; Nilsson & Pekny, 2007; Sofroniew & Vinters, 2010). It is thought to be an attempt of the CNS to quickly restore homeostasis. The classical hallmark of reactive gliosis is the upregulation of GFAP and vimentin in astrocytes (Pekny et al., 1999b). Depending on the severity of the injury, the effects of reactive gliosis on the morphological level can range from slight, to moderate, to very prominent. In the first case, more cells show expression of GFAP (Sofroniew & Vinters, 2010). In more severe cases of reactive gliosis, GFAP and vimentin are upregulated and there is a typical hypertrophy of the cellular processes of astrocytes and re-expression of the IF proteins nestin and synemin (Eliasson et al., 1999; Jing et al., 2007); the IF network becomes very prominent, especially in the soma and main cellular processes (Pekny & Nilsson, 2005). In its most extreme form, reactive gliosis results in proliferation of astrocytes and demarcation of the injury via glial scar formation in an attempt to isolate it (Eddleston & Mucke, 1993; Sofroniew, 2009), and constitutes a major impediment to axonal regeneration in the CNS (Ridet et al., 1997). Reactive gliosis is also accompanied by the alteration in the expression of many genes (Eddleston & Mucke, 1993; Zamanian et al.,

2012) and this expression depends on the nature of CNS injury, suggesting that reactive gliosis is disease specific (Zamanian et al., 2012; Sirko et al., 2013).

Genetic ablation of IFs in astrocytes

One approach to study the role of astrocytes in health and disease is to genetically ablate *GFAP* and *vimentin* (Colucci-Guyon et al., 1994; Pekny et al., 1995; Eliasson et al., 1999). Mice lacking *GFAP* and/or *vimentin* develop and reproduce normally. Non-reactive astrocytes in *GFAP*$^{-/-}$ mice are deficient in IFs as vimentin cannot self-polymerize, whereas reactive astrocytes in *GFAP*$^{-/-}$ mice contain reduced amounts of IFs composed of vimentin and nestin (Eliasson et al., 1999; Pekny et al., 1999a). Reactive astrocytes in *Vim*$^{-/-}$ contains reduced amounts of IFs, composed solely of GFAP into abnormally compacted IFs since GFAP and nestin cannot co-polymerize and nestin does not self-polymerize into IFs (Eliasson et al., 1999). Mice deficient of both *GFAP* and *vimentin*, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, are devoid of astrocytic IFs (Pekny et al., 1999b) and show attenuated reactive gliosis and scar formation after neurotrauma (Pekny et al., 1999b). Compared to wildtype, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice show improved posttraumatic regeneration of neuronal synapses and axons (Menet et al., 2003; Wilhelmsson et al., 2004), and integration of neural grafts and neural progenitor cells (Kinouchi et al., 2003; Widestrand et al., 2007), despite a more severe synaptic loss at the initial stage after neurotrauma (Wilhelmsson et al., 2004).

RESULTS AND DISCUSSION

Paper I – Protective role of reactive astrocytes in brain ischemia

Astrocytes are believed to play a major role in the brain and spinal cord pathologies. Although it has never been directly proven, astrocytes are thought to exert a neuroprotective effect in stroke by shielding neurons from oxidative stress (Kraig et al., 1995). In the absence of a suitable experimental model, a direct proof has been lacking. To address the role of reactive astrocytes in stroke, we subjected *GFAP*$^{-/-}$, *Vim*$^{-/-}$, and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, to experimental brain ischemia induced by middle cerebral artery (MCA) transection. After 7 days of ischemia, infarct volume was 2- to 3.5-fold larger in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice than in wildtype, *GFAP*$^{-/-}$, or *Vim*$^{-/-}$ mice, implying that the increased infarct size seen in the *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice was a consequence of the absence of IFs in astrocytes. Endothelin B receptor (ET_BR) expression by astrocytes in the injured CNS was proposed as one of the steps leading to astrocyte activation and reactive gliosis (Koyama et al., 1999). Whereas ET_BR immunoreactivity was strong in cultured astrocytes and reactive astrocytes around the ischemic penumbra in wildtype mice and colocalized extensively with bundles of IFs, it was undetectable in the cytoplasm of *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes. Compared to wildtype, *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes also showed reduced ET_BR-mediated inhibition of astrocyte gap-junctional communication which has been proposed to promote secondary expansion of focal injury via propagation of cell death signals or undesirable backflow of ATP from living to dying cells (Lin et al., 1998). In addition, in comparison with wildtype, *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes showed lower glutamate transport, as well as reduced expression of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of the tissue plasminogen activator (tPA) which has neurotoxic effect in the ischemic penumbra (Sheehan & Tsirka, 2005).

In summary, we have shown a neuroprotective effect of reactive gliosis in brain ischemia, which limits the extent of the infarct following MCA transection. The absence of IFs in reactive astrocytes seems to result in an altered gap junctional communication, and reduced glutamate transport.

Paper II – Astrocytes negatively regulate neurogenesis through the Jagged1-mediated Notch pathway

In this study, we investigated the role of astrocyte membrane-associated factors in the regulation of neurogenesis. Adult neurogenesis is restricted to two specific neurogenic niches: the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. Increasing evidence suggests an important role for astrocytes in the neurogenic niche as they share certain properties with neural stem cells (Laywell et al., 2000; Seri et al., 2001; Buffo et al., 2008) and create an environment conducive to neurogenesis (Song et al., 2002). Astrocytes regulate neurogenesis by the secretion of various factors of which several have been characterized (Lie et al., 2005; Barkho et al., 2006; Lu & Kipnis, 2010), while the astrocyte membrane-associated factors have been far less studied (Song et al., 2002). Ablation of IF proteins GFAP and vimentin in mice has been shown to create an environment more permissive to transplantation of neural grafts or neural stem cells (Kinouchi et al., 2003; Widestrand et al., 2007) and increased axonal and synaptic regeneration (Menet et al., 2003; Wilhelmsson et al., 2004; Cho et al., 2005). In addition, neuronal differentiation of neural progenitor cells is increased when cocultured with *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes (Widestrand et al., 2007). Although the altered distribution of Wnt3 in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes could be associated with changed secretion of this pro-neurogenic factor and thus explain this finding, it could also be explained by a direct cell-cell signal from astrocyte to neural stem/progenitor cells.

We show that neurosphere cells plated on top of *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes showed enhanced neuronal differentiation compared to when plated on top of wildtype, *GFAP*$^{-/-}$, or *Vim*$^{-/-}$ astrocytes. This effect was shown to be dependent on direct cell-cell contact and could be abolished by mixing *GFAP*$^{-/-}$*Vim*$^{-/-}$ and wildtype astrocytes which suggests the presence of an inhibitory signaling from wildtype astrocytes to neurosphere cells. Compared to wildtype astrocytes, *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes showed similar levels of membrane bound Jagged1, the principal Notch ligand, but lower total expression levels of Jagged1, as well as decreased Notch signaling capacity, total endocytosis and Notch ligand-mediated internalization of the Notch

extracellular domain. When *GFAP*$^{-/-}$*Vim*$^{-/-}$ neurosphere cells were cultured in the presence of immobilized Jagged1, neuronal differentiation was decreased to levels comparable to wildtype neurosphere cells. This decrease was abolished by adding to the culture a γ-secretase inhibitor which prevents activation of the Notch receptor, implying that the proneurogenic effect of *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes is mediated via the Notch signaling pathway.

No difference in number of proliferating cells in the SGZ and granule cell layer (GCL) was seen in the hippocampus of adult wildtype and *GFAP*$^{-/-}$ *Vim*$^{-/-}$ mice 24 hours after labeling of dividing cells, suggesting that reduced Jagged1-mediated Notch signaling from *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes in the adult hippocampus does not affect neural stem pool maintenance or proliferation. But, at 6 weeks after the first labeling of proliferating cells, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice showed a increase in number of labeled cells and a higher number of newly born neurons compared with wildtype mice, implying an enhanced survival of newly formed cells in the dentate gyrus of the hippocampus in mice deficient of astrocytic IFs. Lastly, two weeks after being subjected to entorhinal cortex lesion (ECL), *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice showed decreased number of newborn cells in the SGZ and GCL on the lesioned side compared to wildtype mice, however, the number of newly born neurons was higher in *GFAP*$^{-/-}$*Vim*$^{-/-}$ compared to wildtype mice. Thus, while the lesion-triggered proliferative response in the hippocampus was lower, the cell fate was more directed towards neuronal lineage in *GFAP*$^{-/-}$*Vim*$^{-/-}$ compared to wildtype mice.

In summary, we conclude that astrocytes inhibit neuronal differentiation of neural stem/progenitor cells through cell-cell contact. Notch signaling from astrocytes to neural stem/progenitor cells plays an essential role in this process and is dependent on IFs.

Paper III – Defining cell populations with single-cell gene expression profiling: correlations and identification of astrocyte subpopulations.

In contrast to neurons, we have limited knowledge about the functional diversity of astrocytes and its underlying molecular basis. Cell diversity has commonly been studied using immunohistochemical analysis and gene expression profiling. The first method is restricted to few markers and cannot be used in a truly quantitative manner, and the second method only reflects global transcript levels, consequently any important heterogeneity among the cells remains undetected. With single-cell gene expression profiling it is possible to study heterogeneity among and within cell types in a precise manner. Reverse-transcription quantitative real-time PCR (RT-qPCR) has the sensitivity to detect a single mRNA molecule.

We applied single-cell gene expression profiling as a novel research tool to identify and characterize distinct subpopulations of cells and demonstrated how gene correlations can be applied to determine gene interactions. We collected single cells derived from primary mouse astrocyte cultures and dissociated mouse neurospheres by flow cytometry, lysed them, and analyzed them by RT-qPCR. We found that the majority of cells in the primary astrocyte cultures and cells from the dissociated neurospheres expressed mRNA encoding for markers characteristic of astrocytes as well as markers characteristic for neural stem/progenitor cells, implying that the activation might be linked to a transition into a more stem cell like state as suggested previously (Buffo et al., 2008). In primary astrocytes, the transcription of genes encoding proteins associated with astrocyte activation seems to be regulated by a common mechanism where *vimentin* and *GFAPδ* have key functions in cell lineage determination.

Paper IV – Plasticity response in the contralesional hemisphere after subtle neurotrauma: gene expression profiling after partial deafferentation of the hippocampus

Neurotrauma or focal brain ischemia are known to trigger molecular and structural response in the uninjured hemisphere. Several studies showed that the gene expression profiles in the contralesional hemisphere are altered both within hours (Hori et al., 2012) and days after injury (Buga et al., 2008). These responses are thought to have implications for tissue repair processes as well as for the recovery of function (Kim et al., 2005; Buga et al., 2008). However, whether subtle indirect injury to the brain elicits any detectable contralesional changes in gene expression, in particular the expression of genes involved in neural plasticity, is unknown.

In this study we sought to determine the gene expression profile of selected genes known to be involved in neural plasticity in the affected and contralesional hippocampus at 4 and 14 days following stereotactically performed unilateral entorhinal cortex lesion (ECL). In this injury model, hippocampus is not directly injured but is indirectly affected via partial deafferentation and Wallerian degeneration (Turner et al., 1998; Deller et al., 2007). To elucidate the role of activated astrocytes in the contralesional response to ECL, we made use of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, which exhibit attenuated reactive gliosis.

We could see that a partial deafferentation of the hippocampus leads to upregulation of *GFAP* and *vimentin* mRNA in the affected as well as contralesional hippocampal tissue. These findings demonstrate that even a very subtle focal injury to the CNS induces astrocyte activation also in the contralateral hemisphere. Further, this glial cell response is less pronounced on the contralesional side but has the same temporal pattern in both hemispheres.

We show that genes involved in synaptic re-organization and plasticity, namely *ezrin*, *thrombospondin 4* and *synaptotagmin* (Arber & Caroni, 1995;

Dunkle et al., 2007; Gardzinski et al., 2007; Lavialle et al., 2011) are upregulated both in the affected and contralesional hippocampal tissue. Of these three genes, only *thrombospondin 4* was significantly affected by the absence of GFAP and vimentin, such as the 4 days post injury upregulation observed in wildtype mice was abrogated in both hemispheres in *GFAP*$^{-/-}$ *Vim*$^{-/-}$ mice. Thus, presence of GFAP and vimentin and normal gliosis are necessary for the upregulation of *thrombospondin 4* in response to injury in both the affected and contralesional brain tissue.

We also report that the expression of genes coding for complement proteins C1q and C3, which are involved in the elimination of synapses from maturing, injured or degenerating neurons (Stevens et al., 2007; Berg et al., 2012) and thus participate in synaptic plasticity, was both upregulated in the deafferented tissue in response to ECL, and that *C1q* mRNA was upregulated also in the contralesional hippocampal tissue.

In conclusion, we show that genes associated with astrocyte activation and neural plasticity show very pronounced response to even a very mild and indirect injury to the brain tissue, and that this response is clearly detectable also in the contralesional hemisphere. In addition, we conclude that the upregulation of some plasticity-related genes is dependent on reactive gliosis.

Paper V – Molecular definition of astrocytes in unchallenged and injured hippocampus, a single-cell gene expression study

Attempts that aim at molecular classification of astrocyte subpopulations are ongoing in a number of laboratories with the emergence of new astrocytes markers, such as Aldh1L1 (Cahoy et al., 2008; Zamanian et al., 2012). Expression profiling of individual astrocytes would advance our understanding of the heterogeneity of these cells and their functions in the healthy and diseased CNS.

Here we have studied the heterogeneity of astrocytes and their response to trauma by applying single-cell gene expression profiling by reverse transcription quantitative real-time PCR (RT-qPCR) on freshly isolated cells as a novel approach to molecular characterization of astrocytes and their

subpopulations. The cells were isolated from the hippocampus of adult healthy mice or from the ipsilateral or contralateral hippocampus of adult mice 4 days after partial deafferentation of the hippocampus by unilateral ECL. The cells were individually analyzed for the mRNA levels of selected genes known to be expressed in non-reactive and reactive astrocytes.

In hippocampus from the unchallenged mice, we observed a substantial overlap between *GFAP*, the classical marker of astrocytes, and *AldhL1*, which persisted after injury. We also the saw correlations between the five astrocyte markers *GFAP*, *GS*, *GLT-1*, *GLAST*, and *Aldh1L1*, in individual cells isolated from unchallenged mice. Combining our current results, showing co-regulation between *GFAP* and *vimentin* only in cells derived from affected and contralesional hippocampus, but not from unchallenged mice, with the data generated in our *in vitro* study (Paper III), suggests that *GFAP* and *vimentin* are co-regulated only in reactive astrocytes.

In a response to partial hippocampal deafferentation, the subpopulations of cells expressing *GFAP*, *GLT-1*, *GLAST*, or *Aldh1L1*, all decreased in both affected and contralesional hippocampus, which could, at least partly, be explained by the expansion of the *C1qc* positive microglial population (Schafer et al., 2000; Lynch et al., 2004; Depboylu et al., 2011). Interestingly, the proportion of *GFAP* positive astrocytes that express the astrocyte markers *GLT-1*, *GLAST*, or *Aldh1L1*, was decreased in the hippocampus on the injured side, and to some degree also in the contralesional hippocampus. While the expression of *GFAP* in *GFAP* positive cells increased after injury, the expression in these cells of *GS*, *GLT-1* and *GLAST*, decreased, while the expression of *Aldh1L1* remained stable. These findings point to the existence of two subpopulations of astrocytes after injury: reactive astrocytes that increase expression of GFAP while decreasing the expression of *GLT-1* and *GLAST*, and *GFAP* expressing astrocytes that show less mature phenotype with undetectable expression of *GLT-1, GLAST* as well as *Aldh1L1*, in line with the concept that some astrocytes show a more immature phenotype following injury (Buffo et al., 2008).

In conclusion, our results show that distinct subpopulations of astrocytes can be identified in the uninjured and injured hippocampus, and that these subpopulations respond differentially to injury. Further, the gene expression profiles of individual astrocytes from the injured and contralesional side are

surprisingly similar and these findings are in line with the notion that astrocytes are important modulators of brain plasticity in the injured and contralesional hemisphere.

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