THE ROLE OF REACTIVE ASTROCYTES IN BRAIN ISCHEMIA AND NEUROTRAUMA

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ABSTRACT

Astrocytes are the most abundant cell type in the central nervous system (CNS) and increasing evidence now suggests that they play an active role in various brain functions. Astrocytes are involved in the induction and maintenance of the blood brain barrier, as well as the induction and stabilization of neuronal synapses. Moreover, astrocytes control the extracellular ionic homeostasis, recycle neurotransmitters and are interconnected through gap junctions into a network. Astrocytes become reactive, a process known as reactive gliosis, in CNS pathologies, such as ischemia, neurotrauma or neurodegeneration. Major features of reactive gliosis include hypertrophy of astrocyte processes, upregulation of glial fibrillary acidic protein (GFAP) and vimentin and re-expression of nestin. GFAP, vimentin and nestin are constituents of intermediate filaments (IFs), which are part of the cytoskeleton. It remains largely unclear whether reactive astrocytes are beneficial or detrimental in CNS pathologies. In this thesis, the role of reactive astrocytes was studied in brain ischemia and neurotrauma by using a mouse model in which the *GFAP* and *vimentin* genes were ablated. These *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice are devoid of astrocyte IFs and show attenuated reactive gliosis following CNS injury. We found that, after neurotrauma, reactive astrocytes produce synemin, another IF protein, and that synemin needs vimentin to form IFs. We propose that synemin expression is part of the response of astrocytes to neurotrauma and thus, synemin might be a useful marker of reactive astrocytes. When subjected to brain ischemia, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice have larger infarct volume than wildtype controls, which suggests that reactive astrocytes are protective in brain ischemia and limit the extent of the infarct. The absence of IFs affects vesicle trafficking in astrocytes. *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes have a decreased number of vesicles displaying directional mobility and fewer vesicles that travel for a long distance compared to wildtype astrocytes. This suggests that IFs may act as a structure supporting highly mobile vesicles in astrocytes. At an early stage after neurotrauma, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice show a greater loss of synapses compared to wildtype. At a later stage, however, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice show highly improved synaptic regeneration compared to wildtype controls. Thus, reactive astrocytes seem to be protective at an early stage after neurotrauma but inhibit regeneration later on.

Keywords: astrocytes, intermediate filaments, GFAP, vimentin, reactive gliosis, brain ischemia, neurotrauma

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Astrocyter, den mest förekommande celltypen i nervsystemet, får allt mer uppmärksamhet inom forskningen. Astrocyter kan tillskrivas en rad olika funktioner såsom kontroll av blodflödet, induktion och stabilisering av synapser (nervcellskontakter) och upptag av kemiska ämnen som har frisatts i synapser för att förhindra överaktivering av nervceller. Tillsammans med kapillärerna i hjärnan bildar astrocyter blod-hjärnbarriären, vilken hindrar många blodburna substanser från att ta sig in i hjärnvävnaden. Det har även föreslagits att astrocyter kan inducera nybildning av nervceller från stamceller och att astrocyter själva kan vara stamceller. Vid skador på hjärnvävnad aktiveras astrocyter. De blir reaktiva; får tjockare utskott (förgreningar) och ett ökat uttryck av GFAP och vimentin, två proteiner som bygger upp intermediärfilament som i sin tur bildar nätverk och utgör en del av cellskelettet. Huruvida reaktiva astrocyter är av godo eller ondo vid olika sjukdomstillstånd är till mestadels oklart.

I denna avhandling har vi undersökt betydelsen av reaktiva astrocyter i två skademodeller: hjärninfarkt, där blodtillförseln till delar av hjärnan stoppas, och hjärntrauma där vissa nervbanor i hjärnan klipps av. Vi har studerat detta i möss vars astrocyter saknade GFAP och vimentin och därmed också intermediärfilament. Dessa möss uppvisar dessutom mindre grad av astrocytaktivering vid skada.

Vi fann att astrocyter uttrycker intermediärfilament-proteinet synemin efter hjärntrauma, och att synemin påverkar sammansättningen av intermediärfilament-nätverket. Vi föreslår därför att synemin kan vara en ny markör för reaktiva astrocyter. Vid hjärninfarkt hade mössen som saknade GFAP och vimentin större infarktvolym än kontrollmössen, vilket tyder på att reaktiva astrocyter skyddar hjärnvävnaden och begränsar infarkten. Astrocyter lagrar och frisätter kemiska ämnen som påverkar hjärnfunktionen. En del av dessa ämnen frisätts genom diffusion genom cellmembranet medan andra transporteras och frisätts av vesiklar (blåsor i celler) Vi fann att avsaknaden av GFAP och vimentin påverkade transporten av vesiklar inuti astrocyter. Detta kan påverka astrocyternas sätt att kommunicera med andra celltyper och kan leda till ändrad hjärnfunktion. Vid ett

tidigt stadium efter hjärntrauma hade mössen som saknade GFAP och vimentin färre synapser än kontrollmössen. Efter en längre tids återhämtning visade mössen som saknade GFAP och vimentin en högre grad av återbildning av synapser. Detta tyder på att reaktiva astrocyter är skyddande i ett tidigt skede efter hjärntrauma men hämmar återbildningen av synapser vid ett senare stadium.

Reaktiva astrocyter kan alltså vara både av godo och av ondo för återhämtning efter skada. Mer forskning behövs för att klargöra när astrocytaktivering bör förstärkas respektive dämpas.

LIST OF ORIGINAL PAPERS

This thesis is based on the following published papers or manuscripts, which are referred to throughout the thesis by their roman numerals:

I. Runfeng Jing, Ulrika Wilhelmsson, William Goodwill, Lizhen Li, Yihang Pan, Milos Pekny and Omar Skalli.
Synemin is Expressed in Reactive Astrocytes in Neurotrauma and Interacts Differentially with Vimentin and GFAP Intermediate Filament Networks.
Submitted

II. Lizhen Li, Andrea Lundkvist, Daniel Andersson, Ulrika Wilhelmsson, Nobuo Nagai, Andrea Pardo, Christina Nodin, Anders Ståhlberg, Karina Aprico, Kerstin Larsson, Takeshi Yabe, Lieve Moons, Andrew Fotheringham, Ioan Davies, Peter Carmeliet, Joan P. Schwartz, Marcela Pekna, Mikael Kubista, Fredrik Blomstrand, Nicholas Maragakis, Michael Nilsson and Milos Pekny.
Protective Role of Reactive Astrocytes in Brain Ischemia.
Manuscript

III. Maja Potokar, Marko Kreft, Lizhen Li, Daniel Andersson, Tina Pangršič, Helena H. Chowdhury, Milos Pekny and Robert Zorec.
Cytoskeleton and Vesicle Mobility in Astrocytes.
Traffic. In press

IV. Ulrika Wilhelmsson, Lizhen Li, Marcela Pekna, Claes-Henric Berthold, Sofia Blom, Camilla Eliasson, Oliver Renner, Eric Bushong, Mark Ellisman, Todd E. Morgan and Milos Pekny.
Absence of Glial Fibrillary Acidic Protein and Vimentin Prevents Hypertrophy of Astrocytic Processes and Improves Post-Traumatic Regeneration.
Journal of Neuroscience 24:5016-5021, 2004

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ABBREVIATIONS

ANP	atrial natriuretic peptide
CNS	central nervous system
ET_BR	endothelin B receptor
GFAP	glial fibrillary acidic protein
GLT-1	glutamate transporter 1
IF	intermediate filament
MAP-2	microtubule-associated protein-2
MCA	middle cerebral artery
PAI-1	plasminogen activator inhibitor-1
tPA	tissue plasminogen activator
Vim	vimentin

INTRODUCTION

Astrocytes

Astrocytes are the most abundant cell type in the central nervous system (CNS), in the human brain outnumbering the neurons by several fold (Bignami, 1991). Astrocytes are receiving more and more attention in research. Increasing amount of evidence now suggests that astrocytes play an active role in the brain. Astrocytes communicate with other cell types in the CNS, such as endothelial cells, neurons, oligodendrocytes, microglia and ependymal cells. Astrocytes are involved in a wide range of activities both in the developing and adult CNS. They induce and maintain blood-brain barrier properties in endothelial cells, which is important for keeping the homeostasis of the CNS (Janzer and Raff, 1987; Ballabh et al., 2004). Astrocytes control the extracellular homeostasis of K^+ and other ions. Astrocytes induce and stabilize neuronal synapses (Ullian et al., 2001; Christopherson et al., 2005) and control blood flow (Zonta et al., 2003; Takano et al., 2006) as well as recycle of neurotransmitters, such as glutamate, GABA or glycine. Astrocytes are interconnected through gap junctions into a syncytium that communicates via the spreading of Ca^{2+} waves (Verkhratsky and Kettenmann, 1996). Moreover, it has been speculated that astrocytes induce neurogenesis from neural stem cells (Song et al., 2002) and act as neural stem cells themselves (Laywell et al., 2000; Seri et al., 2001).

Intermediate filaments

The cytoskeleton is a web of fibers that is important for cell shape and movement and a host of other functions. The individual fiber systems of the cytoskeleton are microtubules, intermediate filaments (IFs) and actin filaments. The function of IFs is still largely unknown. More than 50 IF proteins have been identified and are divided into six classes based on the sequence homology.

Table 1. The major IF proteins and examples of where they are expressed.

IF protein class	IF protein	Cell type/tissue
I	acidic keratins	epithelial cells
II	basic keratins	epithelial cells
III	GFAP	astrocytes
	vimentin	astrocytes, mesenchyme
	desmin	muscle cells
	peripherin	neurons
IV	neurofilament (NF)-L, -M, -H	neurons
V	lamin A, B and C	nuclear envelope
VI	nestin	astrocytes, neural stem cells
	synemin	astrocytes, muscle

IFs are composed of different IF proteins depending on the cell type as well as developmental and activation state of the cell (Fuchs and Cleveland, 1998). Monomers of IF proteins contain a well conserved α-helical rod domain flanked by globular N- and C-terminal domains. Formation of IFs starts by pairing of two monomers into a dimer. Two dimers form a tetramer that associates with other tetramers into ropelike IFs. The assembly and disassembly of IFs through phosphorylation and dephosphorylation affects the equilibrium between the pool of unpolymerized IF proteins and the assembled IF network. One well-described function of IFs is the mechanical stability and resilience they provide to muscles and skin. It was first shown in mice that the expression of mutant keratin caused abnormalities resembling the human skin disease epidermolysis bullosa (Vassar et al., 1991). Point mutations in *keratin* and *desmin* genes were then identified in patients with epidermolysis bullosa and myopathies, respectively (Coulombe et al., 1991; Lane et al., 1992; Goldfarb et al., 1998).

Astrocyte intermediate filaments

The composition of astrocyte IFs depends on developmental stage and degree of activation of astrocytes. Immature astrocytes have IFs composed of vimentin, nestin and synemin (Bignami et al., 1982; Pixley and de Vellis, 1984; Dahlstrand et al., 1995; Sultana et al., 2000). During maturation, the expression of nestin is replaced by glial fibrillary acidic protein (GFAP) and in mature non-reactive astrocytes, IFs are formed by GFAP and vimentin. Upon astrocyte activation (see below), astrocytes upregulate GFAP and vimentin and re-express nestin.

The traditional view of astrocytes as star-shaped cells was largely based on immunostaining with antibodies against GFAP. This reveals bundles of IFs in the cell body and the major processes but does not visualize the fine cellular processes (Bushong et al., 2002; Ogata and Kosaka, 2002). Bushong et al. (2002) showed that instead of being star-shaped, astrocytes have a "bushy" appearance as sometimes proposed many decades ago based on the visualization of astrocytes by impregnation techniques. Visualization of astrocytes with antibodies against GFAP reveals only approximately 15% of the total volume that astrocytes access.

Reactive gliosis

Astrocytes become activated (this process is known as reactive gliosis) in CNS pathologies, such as neurotrauma, ischemia, tumors or neurodegeneration. This process is triggered by cell death, the inflammatory response and plasma proteins. Reactive astrocytes undergo morphological changes and the expression of various molecules is altered. (Eddleston and Mucke, 1993; Ridet et al., 1997; Eng et al., 2000). Two well known features of reactive gliosis are hypertrophy of astrocyte processes and upregulation of GFAP and vimentin and re-expression of nestin (Eng and Ghirnikar, 1994; Eng et al., 2000), three proteins that form astrocyte IFs. A number of molecules have been implicated to induce reactive gliosis, for example transforming growth factor-β (TGF-β), interleukin 1 (IL-1), interferon-γ (IFN-γ), basic fibroblast growth factor 2 (FGF2) and endothelin-1 (Giulian et al., 1988; Yong et al., 1991; DiProspero et al., 1997; Lagord et al., 2002; Rogers et al., 2003). Reactive astrocytes migrate towards the injury and participate in the formation of a

glial scar, which consists predominately of reactive astrocytes and proteoglycans. The glial scar might provide beneficial functions for stabilizing fragile CNS tissue after injury, such as secluding the injury site from healthy tissue, however, axons cannot regenerate beyond the glial scar (Silver and Miller, 2004).

Transgenic mouse models to study reactive gliosis

Several mouse models in which astrocytes can be eliminated have been generated to study the role of astrocytes in CNS pathologies. In transgenic mice expressing the herpes simplex virus thymidine kinase under the control of the GFAP promoter, treatment with ganciclovir led to the ablation of dividing astrocytes and severe developmental abnormalities (Delaney et al., 1996). Using the same approach to eliminate reactive astrocytes adjacent to a brain stab injury, another study showed vastly increased infiltration of leukocytes, impaired blood brain barrier repair and increased neuronal degeneration (Bush et al., 1999).

Another approach to study the function of astrocytes in health and diseases is the ablation of the *GFAP* and *vimentin* genes in mice (Colucci-Guyon et a., 1994; Pekny et a., 1995; Eliasson et al., 1999). Mice lacking GFAP and/or vimentin develop and reproduce normally. Non-reactive astrocytes in mice deficient for GFAP ($GFAP^{-/-}$) are devoid of IFs since vimentin cannot self-polymerize into IFs. Reactive astrocytes in $GFAP^{-/-}$ mice have decreased amount of IFs, which are composed of vimentin and nestin. Astrocytes in mice lacking vimentin ($Vim^{-/-}$) also have decreased amount of IFs, which are formed only by GFAP since GFAP and nestin cannot co-polymerize. Astrocytes of mice lacking both GFAP and vimentin ($GFAP^{-/-}Vim^{-/-}$) are completely devoid of IFs (Eliasson et al., 1999; Table 2). $GFAP^{-/-}$ and $Vim^{-/-}$ mice show normal wound healing in the CNS, while $GFAP^{-/-}Vim^{-/-}$ mice show attenuated reactive gliosis following CNS injury (Pekny et al., 1999). These findings suggest that IF upregulation is an important step in reactive gliosis and that reactive astrocytes are needed for proper wound healing in the brain and in the spinal cord.

Table 2. IFs in reactive and non-reactive astrocytes in mice of different genotypes (from Eliasson et al., 1999).

Mice	Composition of IFs		IF amount and appearance in reactive astrocytes
	Non-reactive astrocytes	Reactive astrocytes	
wildtype	GFAP, vimentin	GFAP, vimentin, nestin	Normal amount and appearance
GFAP$^{-/-}$	No IFs	vimentin, nestin	Decreased amount, normal appearance
Vim$^{-/-}$	GFAP	GFAP	Decreased amount, tighter bundling
GFAP$^{-/-}$*Vim*$^{-/-}$	no IFs	no IFs	-

Stroke

Stroke can be divided into two major categories: hemorrhagic and ischemic. Ischemic stroke, which account for approximately 80% of all stroke cases, is caused by occlusion of a blood vessel resulting in total or partial blockage of blood supply to a part of the brain. Ischemic stroke can arise from thrombosis, embolism or systemic hypoperfusion. Thrombosis is the formation of a blood clot or thrombus. This will gradually narrow the lumen of the blood vessel and impede the blood flow. Embolism occurs when an embolus (a particle such as a blood clot, fat or a plaque broken off from an atherosclerotic blood vessel) migrates from one part of the body and causes blockage of blood flow in another part of the body. Systemic hypoperfusion is the reduction of blood flow to all parts of the body, caused by e.g. cardiac arrest or myocardial infarction.

Cerebral ischemia and astrocytes

Cerebral ischemia is an ischemic condition where the brain or parts of the brain do not receive enough blood flow to maintain a normal delivery of oxygen and nutrients, leading to cell dysfunction and death. The neurological outcome is determined by the extent of neural cell death, which depends on the severity and duration of the ischemia and the brain areas affected (Lipton, 1999). The affected

brain area will develop an infarct core, which is generally considered to be beyond rescue, and a surrounding ischemic penumbra, defined as the ischemia border zone that remains metabolically active but electrically mostly silent (Astrup et al., 1981). The ischemic penumbra has some residual blood supply via collaterals, making it a potentially rescuable area upon reperfusion (Astrup et al., 1981).

Astrocyte changes are among the earliest and most dramatic responses to ischemic injury (Petito and Babiak, 1982). Astrocytes in the infarct core are dead, while astrocytes in the ischemic penumbra are viable and undergo reactive gliosis in the periphery of the penumbra. The lack of blood flow causes malfunction of ion pumps on astrocytes and subsequent disturbed ion homeostasis with increased Ca^{2+} levels intracellularly and extracellularly elevated K^+ levels. As discussed by Nedergaard and Dirnagl (2005), astrocyte malfunction may act as a determining factor of neuronal death in ischemia and cause a stepwise expansion of the infarct volume. For example, reduced glutamate uptake by astrocytes leads to increased extracellular glutamate level and triggers excitotoxic neuronal death (Anderson and Swanson, 2000). Reduced ability of astrocytes to take up K^+, which accumulates during neuronal depolarization, may initiate waves of peri-infarct depolarizations, or spreading depression, that further increase the infarct (Nedergaard and Hansen, 1993). The disturbed ion homeostasis also leads to passive influx of water into cells, causing astrocyte swelling and cytotoxic edema. Moreover, disruption of the blood brain barrier causes vasogenic edema, which further decreases the blood flow and may increase the intracranial pressure and the infarct. Glycogen is the main energy reserve in the brain and is stored predominantly in astrocytes (Ignacio et al., 1990; Dringen et al., 1993). During energy crisis, glycogen is metabolized to lactate, which is released from astrocytes and taken up by neurons as an energy substrate (Hamprecht et al., 1993; Hamprecht and Dringen, 1995). Thus, astrocytes are important as energy source supplier for neurons in situations of energy failure such as ischemia.

The most common cause of ischemic stroke in human is occlusion of the middle cerebral artery (MCA) (Chambers et al., 1987; Saito et al., 1987; Virley,

2005). The MCA is one of the three branches of the internal carotid artery and supplies the cerebral cortex, basal ganglia and internal capsule.

Despite intense efforts, both experimentally and clinically, few therapeutic strategies exist to fight the consequences of cerebral ischemia. Today, only a small fraction of stroke patients are offered specific treatment, which includes thrombolysis with recombinant tissue plasminogen activator (tPA). A number of clinical trials based on neuroprotective strategies, including administration of glutamate receptor antagonists and blockers of voltage-gated Ca^{2+}- and Na^{+}-channels have so far been unsuccessful. Exploration of non-neuronal mechanisms in ischemic injuries seems highly appropriate and glial cells constitute a promising target for new treatments (Nedergaard and Dirnagl, 2005; Rothstein et al., 2005).

Exocytosis and vesicle transport in astrocytes

Exocytosis is the release of molecules, stored in vesicles, through the cell membrane. For example, in neuronal synapses, exocytosis is Ca^{2+} triggered and serves interneuronal signaling. Constitutive exocytosis serves the release of extracellular matrix components or plasma membrane proteins that are to be incorporated into the plasma membrane.

Astrocytes release many neuroactive substances such as neurotransmitters, neurotrophins, eicosanoids and neuropeptides by which they signal and respond to the environment (Volterra and Bezzi, 2002). Astrocytes can initiate intercellular communication by elevation of intracellular Ca^{2+} concentration, caused by intrinsic Ca^{2+} oscillations or by stimulation of receptors, for example by neurotransmitters released during synaptic activity (Pasti et al., 1997; Kang et al., 1998; Zhang et al., 2003). One consequence of such increase in intracellular Ca^{2+} is the release of glutamate (Parpura et al., 1994; Bezzi et al., 1998) by which astrocytes modulate neuronal excitability and synaptic functions (Newman, 2003). It has been shown that astrocytes express several SNARE proteins, key elements in exocytosis (Parpura et al., 1995; Zhang et al., 2004). Moreover, astrocytes have been shown to contain a vesicular compartment that is involved in the uptake and regulated exocytosis of glutamate (Bezzi et al., 2004).

The mechanisms of vesicle transport through the cytoplasm to exocytotic sites on the plasma membrane remain largely undefined. In neurons and excitable secretory cells, this transport involves an interaction with the cytoskeleton, in particular microtubules and actin filaments. It is not known whether vesicle mobility in astrocytes also involves cytoskeleton components. Vesicle mobility can be studied by using fluorescently tagged atrial natriuretic peptide, proANP-Emd (Han et al., 1999), to label single exocytotic vesicles containing native ANP (Krzan et al., 2003; Kreft et al., 2004; Potokar et al., 2005). ANP is a member of the natriuretic peptide family and plays an important role in the regulation of blood pressure homeostasis and salt and water balance. Besides its location in myocytes, ANP has also been identified in neurons (McKenzie et al., 1990) and astrocytes (McKenzie, 1992). In myocytes, ANP is stored in vesicles and released by exocytosis (Klein et al., 1993) triggered by muscarinic and vasopressin receptor activation (Sonnenberg and Veress, 1984). In the brain, ANP regulates the Na^+ balance and blood pressure (Buckley et al., 1994) and is upregulated in astrocytes in brain infarction (Nogami et al., 2001). In astrocytes, ANP is released by Ca^{2+}-dependent exocytosis (Krzan et al., 2003).

By labeling exocytotic vesicles with proANP-Emd (Han et al., 1999), it was shown that exocytotic vesicles in astrocytes display two types of mobility: nondirectional mobility, which probably involves free diffusion, and directional mobility, which may involve the cytoskeleton, as in secretory cells (Tvarusko et al., 1999; Potokar et al., 2005).

MATERIALS AND METHODS

The following materials and methods were used in the papers included in this thesis. Some of the methods are described more in detail below.

Induction of focal brain ischemia, measurement of infarct volume, comparison of cerebrovascular architecture and monitoring of blood pressure and heart rate

Entorhinal cortex lesion

Electrically induced brain injury

Electron microscopy

Astrocyte-enriched primary cultures from wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice

RNA preparation from cultures, brains and lesioned brain parts

DNA array analysis

Reverse transcription and quantitative real-time PCR

Depolarization of actin filaments, microtubules and IFs, transfection of astrocytes with proANP-EMD, vesicle tracking

Transfection of SW13-c12 cells with GFAP, vimentin and/or synemin, binding and cosedimentation assays

Dye filling of astrocytes in brain slices

Immunohistochemical analysis of the distribution of synemin, endothelin B receptor and S100

Immunocytochemical analysis of the distribution of endothelin B receptor, vinculin, vimentin, and GFAP

Preparation of total, cytoskeletal and cytosolic protein samples, SDS-PAGE, Western blot analysis and densitometry

Scrape loading/dye transfer

Glutamate uptake assay

ELISA

Mice

The *GFAP* and *vimentin* genes in mice were disrupted via targeted mutation in embryonic stem cells (Colucci-Guyon et al., 1994; Pekny et al., 1995). Mice deficient for GFAP and vimentin (*GFAP*$^{-/-}$*Vim*$^{-/-}$) were obtained by cross-breeding of mice lacking GFAP and mice lacking vimentin (Eliasson et al., 1999; Pekny et al., 1999b).

Primary astrocyte-enriched cultures

Primary astrocyte-enriched cultures were prepared from 1-2 days old mice (Pekny et al., 1998). Whole mouse brains were dissected out, freed of meninges under a dissection microscope and mechanically disintegrated when forced through an 80 μm nylon mesh into medium consisted of DMEM (D5671; Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal calf serum (Invitrogen, Paisly, UK), 2 mM L-glutamine and penicillin-streptomycin (Invitrogen). The cultures were maintained at 37^{o}C and 5% CO_2 with the first medium change on day four after plating and thereafter every three days.

Induction of focal brain ischemia and measurement of infarct volume

Proximal MCA transection. Focal cerebral ischemia was induced by proximal MCA transection as described (Chiamulera et al., 1993; Fotheringham et al., 2000) with slight modifications. Mice were anesthetized with isofluorane in oxygen, and body temperature was maintained at 37°C with a heating pad. Under the operating microscope, the left MCA was exposed, occluded at two points by bipolar coagulation, and transected to ensure permanent disruption. The proximal end of the MCA was coagulated approximately 2.5 mm from the MCA/anterior cerebral artery branch (as determined after brain dissection). After surgery, mice were housed in single cages, which were placed on a heating pad for 1 hr before being returned to their normal environment. For measurement of infarct volume, 0.5 mm thick fresh frontal brain slices were incubated for 30 min in 0.125% triphenyltetrazolium chloride solution (T4375, Sigma) in buffer containing 1.35% dimethylsulfoxide, 2

mM $MgCl_2$, 0.1 M Na_2HPO_4, and 0.1 M NaH_2PO_4 (pH 7.4) at 37°C, and fixed in 4% buffered formaldehyde (pH 7.4) (Fotheringham et al., 2000). In each brain slice, the infarcted area in white matter was measured with a Nikon SMZ-U stereomicroscope and image-analysis software (Easy Image, Bergström Instrument, Göteborg, Sweden). The infarct volume was calculated by integrating infarct areas on all adjacent brain slices with detectable infarction.

Distal MCA transection. Distal MCA transection was performed as described (Welsh et al., 1987; Nagai et al., 1999) in two independent series of experiments. One set of experiments was performed in wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. The second set of experiments was performed in wildtype, *GFAP*$^{-/-}$, *Vim*$^{-/-}$, and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. Briefly, mice were anesthetized by intraperitoneal injection of ketamine (75 mg/ml, Apharmo, Arnhem, Netherlands) and xylazine (5 mg/ml, Bayer, Leverkusen, Germany), and body temperature was maintained at 37°C with a heating pad. Under the operating microscope, the MCA was ligated at point with 10-0 Ethylon nylon thread (Neuilly, France) and transected distally to the ligation point. After surgery, the mice were returned to their cages, which were placed on a heating pad (37°C) for 1 hr. 7 days after MCA transaction, deeply anesthetized mice were perfused through the left ventricle with 4% phosphate-buffered formaldehyde (pH 7.4). The brains were postfixed overnight and embedded in paraffin. 8 μm thick frontal sections were stained with hematoxylin and erythrosine. Infarcted areas were assessed by delineating the ischemic region, which could be clearly discriminated by its lighter appearance and high proportion of cells with pycnotic nuclei. The infarcted area was measured on all sections (320 μm apart) on which it was detectable (4-10 sections per mouse) as described above, and the total infarct volume was calculated.

Entorhinal cortex lesion

Unilateral entorhinal cortex lesion was performed on anesthetized mice by insertion of a retractable wire knife (Scoutenwire knife; Kopf, Tujunga, CA, USA) (Stone et al., 1998) into the entorhinal cortex (0.2 mm anterior, 3.6 mm lateral of

lambda, and 1 mm ventral from dura). The extended knife was lowered 2 mm ventrally twice at angles 30° and -135° to avoid the hippocampus. The wound was closed and the mice were kept in heated cages until they recovered from anesthesia.

The unilateral entorhinal cortex lesion (Matthews et al., 1976; Caceres and Steward, 1983; Steward and Vinsant, 1983) interrupts the axonal connections (the perforant path) between the entorhinal cortex and the projection area in the outer molecular layer of the dentate gyrus of the hippocampus, where degenerating neurons trigger reactive gliosis. The distance between these two regions allows assessment of both the response of astrocytes and regeneration in the hippocampus, which is not directly affected by the lesion.

Electrically induced brain injury

Electrically induced brain injury of the cerebral cortex was performed as described before (Enge et al., 2003). Deeply anesthetized mice were placed in a stereotactic frame (Kopf), a hole was drilled through the skull and a fine-needle electrode was inserted through the skull 2.25 mm laterally of bregma and lowered 1.0 mm (measured from the meningeal level) into the cortex of the right hemisphere. A second electrode was attached to the root of the tail. Using Lesion maker (Ugo Basile, Comerio, Italy), a direct current of 5 mA was applied for 10 s. The mice were kept in heated cages until they recovered from anesthesia.

RNA preparation from astrocyte cultures, brains and cortical lesions

When confluent, the astrocyte cultures were harvested by scraping in RNase-free PBS and centrifuged at 500 x g for 5 min at 4°C. The supernatant was discarded and the cell pellet stored at -70°C. TRIZOL Reagent (Invitrogen) was added to each sample - frozen cell pellets, whole brains or lesioned frontotemporal cortex. The cell pellets were homogenized by vortexing, the tissues were homogenized by Polytron PT 2100 (Kinematica AG, Switzerland) at 4°C and extracted four times by phenol/chloroform. Glycogen (co-precipitant) and isopropanol were added to precipitate the RNA. The pellet was washed with 80% ethanol, dried, resuspended in

RNase-free water and stored at -70°C. The purity of the RNA was assessed by gel electrophoresis on 1% agarose gel containing MOPS buffer (CPG Inc. NJ, USA) and 1 M formaldehyde.

DNA array analysis

Confluent primary astrocyte cultures were harvested by scraping in RNase-free PBS and centrifuged at 500 x g for 5 min at 4°C. The supernatant was discarded and the cell pellet stored at -70°C. TRIZOL Reagent (Gibco BRL, Gaithersburg, MD, USA) was added to the frozen cell pellet, which was homogenized by vortexing and extracted four times by phenol/chloroform. Glycogen (co-precipitant) and isopropanol were added to precipitate the RNA. The pellet was washed with 80% ethanol, dried, resuspended in RNase-free water and treated with DNase I at 37°C for 30 min. The yield was measured by a spectrophotometer at 260 nm and the purity was assessed by gel electrophoresis on 1% agarose gel containing 1 M formaldehyde. The poly A^+ RNA enrichment was performed using streptavidin magnetic beads and biotinylated oligo(dT). cDNA probes were generated using a mixture of gene-specific primers, MMLV (Moloney Murine Leukemia Virus) reverse transcriptase and ^{33}P-labeled dATP (10mCi/ml, BF1001, Amersham). The arrays (Atlas cDNA Expression Arrays, Clontech) were hybridized with the cDNA probe over night at 68°C and exposed to phosphor screens (Molecular Dynamics, Buckinghamshire, UK) overnight. The screens were scanned in a Storm instrument (Storm 820, Molecular Dynamics) and the image was analyzed using AtlasImage 1.5 (Clontech).

Reverse transcription and quantitative real-time PCR

cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) with a mixture of random hexamers and oligo(dT) primers, according to the manufacturer's instructions, the incubation time at 42°C was increased from 30 to 60 min. The reverse transcription was run in

duplicates in 10 µl reactions (59) using 1.5 µg of total RNA extracted from primary astrocyte cultures, brains or tissue from cortical lesions.

Gene specific SYBR-Green based PCR assays were designed for microtubule-associated protein-2 (MAP-2), endothelin B receptor (ET_BR), connexin 43 (Cx43) and plasminogen activator inhibitor-1 (PAI-1). Formation of expected PCR products was confirmed with agarose gel electrophoresis and melting curve analysis. Real-time PCR experiments were run on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) and analyzed as described elsewhere (Pfaffl, 2001; Stahlberg et al., 2004). All gene expression data were normalized against total RNA concentration (Bustin, 2000). Statistical significance between $GFAP^{-/-}Vim^{-/-}$ mice and wild-type mice was tested with Student's *t*-test.

Quantitative real-time PCR for synemin was performed with a TaqMan assay utilizing a fluorigenic probe conjugated to TAMRA and 6FAM. GAPDH served as endogenous control, using the primers and probe provided by ABI. Quantitative PCR was performed with an ABI Prism 7700 and data were analyzed with the ABI 7700 Sequence Detection System using the comparative CT method. Values are expressed as means ± SEM of three to four separate experiments; differences were analyzed by *t*-test or one-way ANOVA.

Transfection with pro-ANP-Emd and tracking of vesicles

Cells were transfected with DNA proANP-Emd using FuGene transfecting reagent (Roche, Mannheim, Germany) as recommended by the manufacturer.

Vesicle tracking was analyzed with custom software (ParticleTR, Celica, Slovenia). We estimated current time (time from the beginning of tracking for a single vesicle), step length (displacement of a vesicle in the time interval 300 ms), track length (the total length of the analyzed vesicle pathway), velocity, maximal displacement and the directionality index (maximal displacement/total track length) of vesicles as described previously (Wacker et al., 1997; Potokar et al., 2005). The analysis of vesicle mobility was performed for epochs of 15 s. Statistical significance was determined with the two-tailed *t*-test for equal variances.

Dye filling of astrocytes

75 μm horizontal brain slices were stored in PBS at 4°C and examined with an Olympus BX50WI microscope using infrared-differential interference contrast optics [Olympus, Melville, NY; 60X water objective numerical aperture (NA) 1.4]. Astrocytes in the outer molecular layer of the dentate gyrus of the hippocampus were identified by the shape and size of their somata and filled with 5% aqueous lucifer yellow (Sigma-Aldrich) using 1 s pulses of negative current (0.5 Hz) for 1–2 min. For immunolabeling of GFAP in the dye-filled astrocytes, the slices were washed in PBS and permeabilized for 1 hr at room temperature in PBS containing 1% BSA, 0.25% Triton X-100, and 3% normal donkey serum followed by incubation with guinea pig antibodies against GFAP (Sigma-Aldrich; 1:100) for 48 hr at 4°C in PBS containing 1% BSA, 0.1% Triton X-100, and 0.3% normal donkey serum. After several washes in PBS, donkey anti-guinea pig antibodies conjugated with Rhodamine Red-X (Jackson ImmunoResearch, West Grove, PA; 1:300) was added to the slices, incubated overnight at 4°C, and then mounted in gelvatol (Harlow and Lane, 1988). The slices were examined using a Radiance2000 laser scanning confocal system (Bio-Rad, Hercules, CA) attached to a Nikon E600FN microscope (Kanagawa, Tokyo, Japan) with a 60X oil immersion objective (NA 1.4). Image visualization and analysis were performed using Imaris 3.3 (Bitplane, Zurich, Switzerland) and ImageJ (National Institutes of Health, Bethesda, MD) software. Quantification of neuropil volume reached by a dye-filled astrocyte was performed on three-dimensional reconstructed cells using Imaris 3.3 software. The number and character of cell processes reaching outside a 40 μm wide circle centered around the soma was assessed by using ImageJ software on superimposed serial images.

RESULTS AND DISCUSSION

Paper I – Synemin is Expressed in Reactive Astrocytes in Neurotrauma and Interacts Differentially with Vimentin and GFAP Intermediate Filament Networks

Synemin is upregulated in wildtype reactive astrocytes in neurotrauma

To assess whether synemin is expressed in reactive astrocytes, we used the entorhinal cortex lesion model and examined reactive astrocytes in partially deafferented dentate gyrus of the hippocampus. In wildtype mice, synemin immunoreactivity was detected in reactive astrocytes at 4 and 14 days after entorhinal cortex lesion, while it was not detected in non-reactive astrocytes.

We propose that synemin expression is part of the response of astrocytes to neurotrauma and possibly also to other brain and spinal cord pathologies. Thus, along with nestin (Dahlstrand et al., 1992) and endothelin B receptor (Rogers et al., 1997), synemin might be a useful marker of reactive astrocytes.

Synemin immunoreactivity is undetectable in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes, synemin protein expression is very low in *GFAP*$^{-/-}$*Vim*$^{-/-}$ and *Vim*$^{-/-}$ neonatal brains

In *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, synemin immunoreactivity was undetectable in astrocytes in partially deafferented hippocampus 4 and 14 days after entorhinal cortex lesion.

Western blot analysis of total protein extracts from neonatal brains of wildtype mice revealed bands of 210 and 170 kDa, corresponding to α- and β-synemin, respectively. Brains of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice had about 95% lower levels of α-synemin and no detectable amount of β-synemin. Quantitative real-time PCR analysis showed comparable cerebral levels of synemin mRNA between wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice and between cultured reactive astrocytes from wildtype, *GFAP*$^{-/-}$*Vim*$^{-/-}$, *GFAP*$^{-/-}$ and *Vim*$^{-/-}$ mice. In contrast, Western blot analysis showed

that synemin was present at levels comparable to wildtype in astrocytes expressing vimentin (wildtype and *GFAP*$^{-/-}$ cultures of reactive astrocytes) but undetectable in the absence of vimentin (*Vim*$^{-/-}$ and *GFAP*$^{-/-}$*Vim*$^{-/-}$ cultures of reactive astrocytes).

These findings indicate that the levels of synemin protein in *Vim*$^{-/-}$ astrocytes are regulated post-transcriptionally. This may be a general mechanism by which IF protein levels are regulated in the absence of polymerization partners, as post-transcriptional regulation is also responsible for decreased nestin, NF-L and keratin protein levels after the genetic ablation of vimentin, NF-M and of specific keratin pair members, respectively (Reichelt et al., 1997; Elder et al., 1998; Magin et al., 1998; Eliasson et al., 1999; Jacomy et al., 1999; Tao et al., 2003).

More dispersed and less bundled IF network in synemin-positive cells

Synemin was present in some GFAP-positive and in some GFAP-negative cells. In synemin-containing cells, whether GFAP-positive or negative, the IF network appeared more dispersed and less bundled than in synemin-negative cells. In both GFAP-positive and negative cells and in *GFAP*$^{-/-}$ cultures, synemin was distributed throughout the IF network.

This suggests that synemin may regulate bundling of IF networks. The degree of bundling of the IF network might affect some properties of astrocytes, such as cell motility. Indeed, we have previously demonstrated reduced motility of *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes compared to wildtype (Lepekhin et al., 2001).

Synemin binds unpolymerized GFAP and vimentin equally well but interacts differently with GFAP and vimentin IFs

Our finding that synemin protein is stable in *GFAP*$^{-/-}$, but not in *GFAP*$^{-/-}$*Vim*$^{-/-}$ or *Vim*$^{-/-}$, astrocytes raised the question of whether synemin can interact with GFAP. Overlay and dot-blot assays showed that synemin bound to unpolymerized GFAP and vimentin equally well.

When synemin was mixed with GFAP or vimentin in conditions that allowed assembly of IFs, synemin was mostly recovered in the pellet after ultracentrifugation. Subjecting synemin alone to this treatment resulted in recovery of synemin in the supernatant.

These results did not rule out the possibility that synemin cosediments with GFAP because it binds to GFAP IFs rather than copolymerizes with GFAP. This possibility was examined by mixing synemin with GFAP or vimentin IFs assembled in the absence of synemin ("pre-formed" IFs). After incubation with pre-formed vimentin IFs, most of the synemin was recovered in the supernatant. However, after incubation with pre-formed GFAP IFs, most of the synemin was recovered in the pellet. This suggests that, *in vitro*, synemin interacts with GFAP IF like an associated protein rather than like a polymerization partner.

Synemin needs vimentin in order to incorporate into GFAP-containing IF networks

To further investigate how synemin associates with GFAP networks, we performed transfection experiments in SW13-cl2 adrenocortical carcinoma cells, which are devoid of cytoplasmic IFs. When SW13-cl2 cells were transiently transfected with GFAP and synemin cDNA, synemin did not incorporate into the filamentous portion of a GFAP network, in contrast to its known capacity to form filamentous network with vimentin (Bellin et al., 1999; Titeux et al., 2001).

To investigate whether vimentin affects the incorporation of synemin into GFAP networks, SW13-cl2 cells were stably transfected with GFAP (SW13-cl2/G) or vimentin (SW13-cl2/V) cDNA. In SW13-cl2/G cells transiently transfected with vimentin cDNA or in SW13-cl2/V cells transiently transfected with GFAP cDNA, immunofluorescence staining with anti-GFAP and anti-vimentin yielded identical patterns, consistent with the ability of GFAP and vimentin to coassemble into heteropolymeric networks (Quinlan and Franke, 1983; Wang et al., 1984).

After transient transfection of SW13-cl2/G cells with synemin cDNA, synemin failed to incorporate into the GFAP network but instead formed numerous

punctate aggregates throughout the cytoplasm. However, after cotransfection of these cells with vimentin and synemin cDNA, synemin was incorporated into the vimentin/GFAP IF network in >90% of the cotransfected cells. In SW13-cl2/V cells cotransfected with GFAP and synemin, synemin and GFAP integrated within the same IF network.

These results suggest that one function of vimentin in astrocytes is to permit the incorporation of synemin into GFAP-containing IFs.

Taken together, these findings demonstrate that neurotrauma induces synemin expression in reactive astrocytes and that synemin needs vimentin in order to integrate into IF networks. Integration of synemin into IFs seems to lead to more dispersed and less bundled IFs and these might affect functional properties of reactive astrocytes, such as cell motility.

Paper II – Protective Role of Reactive Astrocytes in Brain Ischemia

Larger infarct volume in *GFAP*$^{-/-}$*Vim*$^{-/-}$ than in wildtype mice after MCA transection

Seven days after permanent focal brain ischemia induced by middle cerebral artery (MCA) transection, the infarct volume was 2-3.5-fold larger in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice than in wildtype controls. Apart from astrocytes, vimentin is also expressed in endothelial cells. In *Vim*$^{-/-}$ mice, endothelial cells are devoid of IFs and astrocytes have decreased amount of IFs, as have reactive astrocytes in *GFAP*$^{-/-}$ mice. To assess whether partial deficiency of IFs in astrocytes or a deficiency of IFs in endothelial cells also affects the infarct volume, we examined single *GFAP*$^{-/-}$ and *Vim*$^{-/-}$ mice and found that they had comparable infarct volume as wildtype mice. Medium blood pressure and heart rate at MCA transection and during 60 minutes thereafter did not differ between wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. The lines of anastomoses between branches of MCA and anterior cerebral artery were also

comparable in wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. This implies that the increased infarct size after MCA transection in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice is a consequence of the absence of IFs in astrocytes.

These results are consistent with the findings that healing after brain or spinal cord trauma was prolonged and regeneration improved in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, which lack IFs in reactive astrocytes, but not in *GFAP*$^{-/-}$ or *Vim*$^{-/-}$ mice, which have only a partial IF deficiency (Pekny et al., 1999a; Menet et al., 2003; Wilhelmsson et al., 2004).

Absence of ET_BR-immunoreactivity in astrocytes in the ischemic penumbra of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice

The expression of endothelin B receptor (ET_BR) by astrocytes in the injured CNS was proposed as one of the steps leading to astrocyte activation and reactive gliosis (Ishikawa et al., 1997; Baba, 1998; Koyama et al., 1999; Peters et al., 2003). In the ischemic penumbra and in the corpus callosum, 7 days after MCA transection, ET_BR were highly expressed by reactive astrocytes in wildtype mice but were essentially undetectable in astrocytes of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. Similarly, ET_BR were readily detectable in cultured wildtype astrocytes where, most interestingly, the ET_BR-immunoreactivity co-localized with bundles of IFs. In contrast, ET_BR-immunoreactivity was undetectable in the cytoplasm of *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes. These findings suggest that IFs might be required for production, stability or distribution of ET_BR in reactive astrocytes.

IFs determine ET_BR distribution in reactive astrocytes

To determine whether IFs are required for production, stability or distribution of ET_BR in reactive astrocytes, we performed quantitative real-time PCR and Western blot analyses on cultured astrocytes from *GFAP*$^{-/-}$*Vim*$^{-/-}$ and wildtype mice. In the *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes, the amounts of ET_BR mRNA was increased by 43% compared to wildtype, whereas the amount of ET_BR protein was comparable. Thus,

in the absence of IFs, astrocytes contain normal amount of ET_BR, which do not associate with IFs but seem to be distributed throughout the cell.

Endothelin-3-induced blockage of gap junctions is attenuated in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes

Endothelins are blockers of astrocyte gap junctional communication in culture (Giaume et al., 1992; Blomstrand et al., 1999) or in acute brain slices (Blomstrand et al., 2004). To determine if the altered cytoplasmic distribution of ET_BR affects astrocyte gap junctional communication and thus the function of the astrocyte network, we stimulated ET_BR with its selective ligand, endothelin-3. We found no difference in basal astrocyte gap junctional communication between wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes. However, endothelin-3-mediated inhibition of astrocyte gap junctional communication was less prominent in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes compared to wildtype. Gap junctions are made of connexins, with connexin 43 being the predominant gap junction protein in astrocytes (Saez et al., 2003). We used quantitative real-time PCR to compare connexin 43 mRNA levels in primary cultures of astrocytes. We found that connexin 43 was upregulated by 44% in *GFAP*$^{-/-}$*Vim*$^{-/-}$ compared to wildtype astrocytes, which further supports the link between astrocyte IFs and gap junctional communication.

Astrocyte gap junctional communication was proposed to promote secondary expansion of focal ischemic injury since open astrocyte gap junctions can mediate the propagation of cell death signals or undesirable backflow of ATP from living to dying cells (Rawanduzy et al., 1997; Budd and Lipton, 1998; Cotrina et al., 1998; Lin et al., 1998). Thus, less efficient ET_BR-mediated inhibition of astrocyte gap junctional communication in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice may contribute to larger infarct volume in these mice after focal brain ischemia.

Reduced glutamate transport in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice

Astrocytes are responsible for the clearance of glutamate released from neurons. The ability of astrocytes to remove glutamate was proposed to reduce the infarct size by limiting the excitotoxic cell death (Nedergaard and Dirnagl, 2005). In order to determine if glutamate transport is altered in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, we examined total glutamate uptake and glutamate uptake mediated by glutamate transporter 1 (GLT-1), the primary glutamate transporter in astrocytes, in freshly dissected hemicortices of wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. We found that both total and GLT-1-mediated glutamate uptake was reduced in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice compared to wildtype controls.

Glutamate that is taken up in astrocytes is converted by glutamine synthase to glutamine. Despite similar levels of glutamine synthase (Wilhelmsson et al., 2004) in wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes, the latter have increased levels of glutamine (Pekny et al., 1999a). Thus, we can speculate that the reduced ability of the *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice to remove glutamate from the ischemic brain tissue is a consequence of intracellular glutamine accumulation and leads to increased ischemic infarcts.

tPA inhibitor PAI-1 is downregulated in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes

To further address on a molecular level the effect of attenuated reactive gliosis, we performed the DNA array analysis and compared the expression of 1200 genes between primary astrocytes derived from wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. Only a single gene, *plasminogen activator inhibitor* (*PAI-1*), an inhibitor of tissue plasminogen activator (tPA), fulfilled the criteria of a threefold or higher downregulation in *GFAP*$^{-/-}$*Vim*$^{-/-}$ compared to wildtype astrocytes.

Quantitative real-time PCR analysis of PAI-1 mRNA levels in primary *GFAP*$^{-/-}$*Vim*$^{-/-}$ and wildtype astrocytes maintained in the presence of either 1% or 10% of serum, the latter mimicking some aspects of reactive gliosis, revealed a 69% and 86% reduction in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes in the presence of 1% and 10% serum, respectively.

PAI-1 inhibits tPA, which has a neurotoxic effect in the ischemic penumbra, probably mediated through activation of microglia and activation of NMDA receptors (Sheehan and Tsirka, 2005). Mice deficient in tPA develop smaller infarcts than wildtype controls in a transient focal ischemia model and administration of tPA to both tPA-deficient and wildtype mice leads to increased infarct volume (Tsirka et al., 1995). Similarly, mice overexpressing PAI-1 show reduced infarcts compared to wildtype controls after focal brain ischemia (Nagai et al., 2005). Thus, reduced levels of PAI-1 mRNA could enhance the neurotoxic effects of tPA in the ischemic penumbra of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice.

Taken together, these results indicate that reactive astrocytes are protective in brain ischemia and limit the extent of the infarct. The absence of IFs in reactive astrocytes seems to compromise several functional aspects of astrocytes, such as astrocyte gap junctional communication, uptake of glutamate and protection against tPA-mediated neurotoxicity.

Paper III – Cytoskeleton and Vesicle Mobility in Astrocytes

Microtubule disassembly predominantly affects directional mobility

To address the role of microtubules in vesicle transport in astrocytes, we depolymerized microtubules with nocodazole (Vasquez et al., 1997) in rat astrocytes transfected with the proANP-Emd DNA in order to label single vesicles (Han et al., 1999; Krzan et al., 2003; Potokar et al., 2005). Vesicles were previously characterized, based on their translocation, as directional (translocation for more than 1 μm) or nondirectional (translocation for less than 1 μm) (Potokar et al., 2005). In untreated cells, vesicles displayed both directional and nondirectional mobility. In cells with depolymerized microtubules, however, vesicles displayed only nondirectional mobility. Microtubule depolymerization significantly reduced the mobility of vesicles, including their average velocity, track length (the length of

the pathway that vesicle traveled in 15 s), and maximal displacement (a measure for the net translocation of vesicles in 15 s). These results show that microtubules play a role in directional mobility of vesicles in astrocytes and are compatible with this function of microtubules documented in other cell types.

Actin filaments contribute to vesicle mobility

To examine the role of actin filaments, we depolymerized actin filaments with the *Clostridium spiroforme* toxin in astrocytes transfected with proANP-Emd. Vesicles in untreated cells displayed both directional and nondirectional mobility. After depolymerization of actin filaments, vesicles displayed only nondirectional mobility. Average velocity, track length, and maximal displacement of the vesicles were decreased in cells with depolymerized actin filaments. These findings indicate that actin filaments participate in directional mobility of vesicles in astrocytes.

IFs affect the directional mobility of vesicles

Although they are not thought to participate in vesicle transport, IFs interact with microtubules (Chang and Goldman, 2004) and play an important role in astrocyte motility (Lepekhin et al., 2001). To determine if they contribute to vesicle mobility, we depolymerized IFs in rat astrocytes with the phosphatase inhibitor calyculin A. This treatment reduced vesicle mobility. The average velocity, track length, and maximal displacement of vesicles were all significantly lower in cells with depolymerized IFs.

Since the reduced mobility could reflect a nonspecific action of calyculin A that affected the phosphorylation of other substrates (Chang and Goldman, 2004), we analyzed proANP-Emd-labeled vesicles in astrocytes from wildtype and $GFAP^{-/-}Vim^{-/-}$ mice, which are devoid of IFs (Eliasson et al., 1999). In wildtype and $GFAP^{-/-}Vim^{-/-}$ astrocytes, the mean track lengths and the mean maximal displacements of vesicles, displaying both directional and nondirectional mobility, were similar. However, the fraction of vesicles displaying directional mobility was

lower in *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes compared to wildtype controls (21% vs. 34%), suggesting a role for IFs in vesicle mobility. In *GFAP*$^{-/-}$*Vim*$^{-/-}$ cells, we found five times less vesicles with track length higher than 9 µm compared to wildtype cells. Thus, it is possible that IFs act as a scaffold representing a conduit for highly mobile vesicles.

Lack of astrocyte IFs affects the microtubular system

Next, we addressed whether genetic ablation of astrocyte IFs triggered a compensatory response from the microtubular system. We used quantitative real-time PCR to assess the expression of microtubule-associated protein-2 (MAP-2), which was previously implicated in reactive gliosis (Geisert et al., 1990). MAP-2 expression was 52% higher at postnatal day 1 (P1) *GFAP*$^{-/-}$*Vim*$^{-/-}$ brains and 45% higher in primary astrocyte-enriched cultures prepared from P1 *GFAP*$^{-/-}$*Vim*$^{-/-}$ brains compared to wildtype brains and astrocyte cultures, respectively. To establish whether the elevated MAP-2 expression in *GFAP*$^{-/-}$*Vim*$^{-/-}$ persists in adult animals in a pathological context, we used the electrically induced cortical lesion as a neurotrauma model. Four days after injury, in the tissue surrounding the lesion, MAP-2 expression was 64% higher in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice compared to wildtype. Thus, genetic ablation of astrocyte IFs seems to trigger a partial compensatory response of the microtubular system.

MAP-2 was proposed to have a direct effect on stabilization of microtubules and their crosslinking as well as crosslinking of microtubules with IFs (Itoh et al., 1997). Thus, the increased MAP-2 expression in IF-free astrocytes may be a compensatory response to the IF absence and it might explain the relatively minor difference between the mobility of vesicles in *GFAP*$^{-/-}$*Vim*$^{-/-}$ and wildtype astrocytes.

Paper IV – Absence of Glial Fibrillary Acidic Protein and Vimentin Prevents Hypertrophy of Astrocytic Processes and Improves Post-Traumatic Regeneration

Absence of IFs attenuates hypertrophy of astrocyte processes

We used antibodies against glutamine synthase to visualize astrocytes in the molecular layer of the dentate gyrus of the hippocampus. To confirm the astrocyte specificity of the antibodies, we co-stained astrocytes of wildtype mice with GFAP antibodies and found that glutamine synthase was expressed only in astrocytes. ELISA showed comparable levels of glutamine synthase in primary cultures of wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes, and glutamine synthase was distributed homogenously in both types of cells. Thus, antibodies against glutamine synthase could be used to evaluate the morphological appearance of astrocytes.

The degree of hypertrophy of astrocyte processes was assessed by measuring the longest process in astrocytes in the molecular layer of the dentate gyrus of the hippocampus at 4 and 14 days after entorhinal cortex lesion. On the unlesioned side, the length of astrocyte processes was similar in wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. On the lesioned side, however, reactive astrocytes in wildtype mice showed more prominent signs of hypertrophy with glutamine synthase-positive processes longer than in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice at both 4 and 14 days after lesion.

By using antibodies against another astrocyte marker, S100, we found again significantly longer processes in wildtype compared with *GFAP*$^{-/-}$*Vim*$^{-/-}$ reactive astrocytes at 4 days after entorhinal cortex lesion, and this trend was present also at 14 days. Thus, the degree of hypertrophy of astrocyte processes was significantly altered in the absence of IFs.

Absence of IFs alters the morphology but not the volume of brain tissue accessed by individual astrocytes

To further evaluate the effect of the absence of IFs on hypertrophy of cellular processes of reactive astrocytes and to determine whether this has an impact on the

volume of tissue they access, we performed dye-filling followed by three-dimensional reconstruction of astrocytes *in situ* (Bushong et al., 2002). Dye filling reveals the terminal processes that failed to be visualized by immunostaining and shows the true action radius of individual astrocytes, with a bushy appearance. We found no difference in the volume of tissue accessed by wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ reactive astrocytes in the molecular layer of the dentate gyrus of the hippocampus on the lesioned side. However, the appearance of *GFAP*$^{-/-}$*Vim*$^{-/-}$ reactive astrocytes was clearly different from wildtype. *GFAP*$^{-/-}$*Vim*$^{-/-}$ reactive astrocytes had 37% fewer long processes that could be followed over a 20 μm radius as well as 83% fewer processes that remained straight for most of their length. Thus, the absence of IFs does not affect the volume of tissue accessed by reactive astrocytes but affects the morphology of their cellular processes.

Increased regeneration in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice after entorhinal cortex lesion

To assess the extent of synaptic loss and regeneration after entorhinal cortex lesion, we quantified synaptic complexes in the outer molecular layer of the dentate gyrus of the hippocampus by electron microscopy. On the injured side, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice had only one-fourth as many synaptic complexes as wildtype mice by day 4. These findings indicate that the absence of astrocyte IFs exacerbates the loss of synaptic complexes early after entorhinal cortex lesion. Between day 4 and 14, the number of synaptic complexes on the lesioned side showed a mild tendency towards recovery in wildtype mice, consistent with findings in rats (Matthews et al., 1976; Steward and Vinsant, 1983), while the number of synaptic complexes in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice increased by 77%, reaching the levels measured on the contralateral side.

Upregulation of ET_BR in wildtype, but not *GFAP*$^{-/-}$*Vim*$^{-/-}$, mice after entorhinal cortex lesion

The upregulation of ET_BR by astrocytes in the injured CNS was proposed to be one of the steps leading to astrocyte activation and hypertrophy (Ishikawa et al., 1997; Baba, 1998; Koyama et al., 1999; Peters et al., 2003). By using antibodies against ET_BR, we found weak and almost exclusively endothelial immunostaining in the molecular layer of the dentate gyrus of the hippocampus on the side contralateral to entorhinal cortex lesion, fully comparable between wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. As expected, on the lesioned side at both 4 and 14 d after entorhinal cortex lesion, ET_BR were highly upregulated in reactive astrocytes in wildtype mice. However, no signs of ET_BR upregulation were found in astrocytes of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. Thus, the upregulation of ET_BR by reactive astrocytes is IF dependent and associated with post-traumatic regeneration.

Taken together, these findings suggest that reactive astrocytes play a beneficial role in the acute stage after neurotrauma but inhibit synaptic regeneration later on.

CONCLUSIONS

Paper I

Synemin is expressed in reactive astrocytes in neurotrauma and we propose synemin as a new marker of reactive astrocytes.

Synemin needs vimentin in order form IFs in astrocytes.

Paper II

Reactive astrocytes are protective in focal brain ischemia and limit the extent of the infarct.

Our data suggest that the absence of astrocyte IFs in brain ischemia influences glutamate transport, ET_BR-mediated control of gap junctions and expression of PAI-1, an inhibitor of tPA.

Paper III

Astrocyte IFs play a role in long-range directional vesicle mobility, probably by acting as a scaffold for the moving vesicles.

Paper IV

IFs are of major importance for hypertrophy of cellular processes in reactive astrocytes.

Reactive astrocytes are protective at an early stage after neurotrauma but inhibit synaptic regeneration later on.

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REFERENCES

Anderson CM, Swanson RA (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32:1-14.

Astrup J, Siesjo BK, Symon L (1981) Thresholds in cerebral ischemia - the ischemic penumbra. Stroke 12:723-725.

Baba A (1998) Role of endothelin B receptor signals in reactive astrocytes. Life Sci 62:1711–1715.

Ballabh P, Braun A, Nedergaard M (2004) The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis 16:1-13.

Bellin RM, Sernett SW, Becker B, Ip W, Huiatt TW, Robson RM (1999) Molecular characteristics and interactions of the intermediate filament protein synemin. Interactions with alpha-actinin may anchor synemin-containing heterofilaments. J Biol Chem 274:29493-29499.

Bezzi P, Gundersen V, Galbete JL, Seifert G, Steinhauser C, Pilati E, Volterra A (2004) Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate. Nat Neurosci 7:613-620.

Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391:281-285.

Bignami A (1991) Glial cells in the central nervous system. In: Discussions in neuroscience (Magistretti P, ed), pp 1-45. Amsterdam: Elsevier.

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

Blomstrand F, Aberg ND, Eriksson PS, Hansson E, Ronnback L (1999) Extent of intercellular calcium wave propagation is related to gap junction permeability and level of connexin-43 expression in astrocytes in primary cultures from four brain regions. Neuroscience 92:255-265.

Blomstrand F, Venance L, Siren AL, Ezan P, Hanse E, Glowinski J, Ehrenreich H, Giaume C (2004) Endothelins regulate astrocyte gap junctions in rat hippocampal slices. Eur J Neurosci 19:1005-1015.

Buckley MG, Markandu ND, Sagnella GA, MacGregor GA (1994) Brain and atrial natriuretic peptides: a dual peptide system of potential importance in sodium balance and blood pressure regulation in patients with essential hypertension. J Hypertens 12:809-813.

Budd SL, Lipton SA (1998) Calcium tsunamis: do astrocytes transmit cell death messages via gap junctions during ischemia? Nat Neurosci 1:431-432.

Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, Mucke L, Johnson MH, Sofroniew MV (1999) Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. Neuron 2:297–308.

Bushong EA, Martone ME, Jones YZ, Ellisman MH (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci 22:183-192.

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169-193.

Caceres A, Steward O (1983) Dendritic reorganization in the denervated dentate gyrus of the rat following entorhinal cortical lesions: A Golgi and electron microscopic analysis. J Comp Neurol 214:387-403.

Chambers BR, Norris JW, Shurvell BL, Hachinski VC (1987) Prognosis of acute stroke. Neurology 37:221-225.

Chang L, Goldman RD (2004) Intermediate filaments mediate cytoskeletal crosstalk. Nat Rev Mol Cell Biol 5:601-613.

Chiamulera C, Terron A, Reggiani A, Cristofori P (1993) Qualitative and quantitative analysis of the progressive cerebral damage after middle cerebral artery occlusion in mice. Brain Res 606:251-258.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120:421-433.

Colucci-Guyon E, Portier MM, Dunia I, Paulin D, Pournin S, Babinet C (1994) Mice lacking vimentin develop and reproduce without an obvious phenotype. Cell 79:679-694.

Cotrina ML, Kang J, Lin JH, Bueno E, Hansen TW, He L, Liu Y, Nedergaard M (1998) Astrocytic gap junctions remain open during ischemic conditions. J Neurosci 18:2520-2537.

Coulombe PA, Hutton ME, Letai A, Hebert A, Paller AS, Fuchs E (1991) Point mutations in human keratin 14 genes of epidermolysis bullosa simplex patients: genetic and functional analyses. Cell 66:1301-1311.

Dahlstrand J, Collins VP, Lendahl U (1992) Expression of the class VI intermediate filament nestin in human central nervous system tumors. Cancer Res 52:5334-5341.

Dahlstrand J, Lardelli M, Lendahl U (1995) Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. Brain Res Dev Brain Res 84:109-129.

Delaney CL, Brenner M, Messing A (1996) Conditional ablation of cerebellar astrocytes in postnatal transgenic mice. J Neurosci 16:6908-6918.

DiProspero NA, Meiners S, Geller HM (1997) Inflammatory cytokines interact to modulate extracellular matrix and astrocytic support of neurite outgrowth. Exp Neurol 148:628-639.

Dringen R, Gebhardt R, Hamprecht B (1993) Glycogen in astrocytes: possible function as lactate supply for neighboring cells. Brain Res 623:208-214.

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

Eliasson C, Sahlgren C, Berthold CH, Stakeberg J, Celis JE, Betsholtz C, Eriksson JE, Pekny M (1999) Intermediate filament protein partnership in astrocytes. J Biol Chem 274:23996-24006.

Eng LF, Ghirnikar RS (1994) GFAP and astrogliosis. Brain Pathol 4:229-237.

Eng LF, Ghirnikar RS, Lee YL (2000) Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem Res 25:1439-1451.

Enge M, Wilhelmsson U, Abramsson A, Stakeberg J, Kuhn R, Betsholtz C, Pekny M (2003) Neuron-specific ablation of PDGF-B is compatible with normal central nervous system development and astroglial response to injury. Neurochem Res 28:271-279.

Fotheringham AP, Davies CA, Davies I (2000) Oedema and glial cell involvement in the aged mouse brain after permanent focal ischaemia. Neuropathol Appl Neurobiol 26:412-423.

Fuchs E, Cleveland DW (1998) A structural scaffolding of intermediate filaments in health and disease. Science 279:514-519.

Geisert EE, Jr., Johnson HG, Binder LI (1990) Expression of microtubule-associated protein 2 by reactive astrocytes. Proc Natl Acad Sci U S A 87:3967-3971.

Giaume C, Cordier J, Glowinski J (1992) Endothelins Inhibit Junctional Permeability in Cultured Mouse Astrocytes. Eur J Neurosci 4:877-881.

Giulian D, Woodward J, Young DG, Krebs JF, Lachman LB (1988) Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. J Neurosci 8:2485-2490.

Goldfarb LG, Park KY, Cervenakova L, Gorokhova S, Lee HS, Vasconcelos O, Nagle JW, Semino-Mora C, Sivakumar K, Dalakas MC (1998) Missense mutations in desmin associated with familial cardiac and skeletal myopathy. Nat Genet 19:402-403.

Hamprecht B, Dringen R (1995) Energy metabolism. In: Neuroglia (Kettenmann H, Ransom BR, eds), pp 473-487. New York: Oxford University Press.

Hamprecht B, Dringen R, Pfeiffer B, Kurz G (1993) The possible roles of astrocytes in energy metabolism of the brain. In: Biology and pathology of astrocyte-neuron interactions (Fedoroff S, Juurlink BH, Doucette R, eds), pp 83-91. New York: Plenum.

Han W, Ng YK, Axelrod D, Levitan ES (1999) Neuropeptide release by efficient recruitment of diffusing cytoplasmic secretory vesicles. Proc Natl Acad Sci U S A 96:14577-14582.

Ignacio PC, Baldwin BA, Vijayan VK, Tait RC, Gorin FA (1990) Brain isozyme of glycogen phosphorylase: immunohistological localization within the central nervous system. Brain Res 529:42-49.

Ishikawa N, Takemura M, Koyama Y, Shigenaga Y, Okada T, Baba A (1997) Endothelins promote the activation of astrocytes in rat neostriatum through ET(B) receptors. Eur J Neurosci 9:895–901.

Itoh TJ, Hisanaga S, Hosoi T, Kishimoto T, Hotani H (1997) Phosphorylation states of microtubule-associated protein 2 (MAP2) determine the regulatory role of MAP2 in microtubule dynamics. Biochemistry 36:12574-12582.

Janzer RC, Raff MC (1987) Astrocytes induce blood-brain barrier properties in endothelial cells. Nature 325:253-257.

Kang J, Jiang L, Goldman SA, Nedergaard M (1998) Astrocyte-mediated potentiation of inhibitory synaptic transmission. Nat Neurosci 1:683-692.

Klein RM, Kelley KB, Merisko-Liversidge EM (1993) A clathrin-coated vesicle-mediated pathway in atrial natriuretic peptide (ANP) secretion. J Mol Cell Cardiol 25:437-452.

Koyama Y, Takemura M, Fujiki K, Ishikawa N, Shigenaga Y, Baba A (1999) BQ788, an endothelin ET(B) receptor antagonist, attenuates stab wound injury-induced reactive astrocytes in rat brain. Glia 26:268–271.

Kreft M, Stenovec M, Rupnik M, Grilc S, Krzan M, Potokar M, Pangrsic T, Haydon PG, Zorec R (2004) Properties of Ca(2+)-dependent exocytosis in cultured astrocytes. Glia 46:437-445.

Krzan M, Stenovec M, Kreft M, Pangrsic T, Grilc S, Haydon PG, Zorec R (2003) Calcium-dependent exocytosis of atrial natriuretic peptide from astrocytes. J Neurosci 23:1580-1583.

Lagord C, Berry M, Logan A (2002) Expression of TGFbeta2 but not TGFbeta1 correlates with the deposition of scar tissue in the lesioned spinal cord. Mol Cell Neurosci 20:69-92.

Lane EB, Rugg EL, Navsaria H, Leigh IM, Heagerty AH, Ishida-Yamamoto A, Eady RA (1992) A mutation in the conserved helix termination peptide of keratin 5 in hereditary skin blistering. Nature 356:244-246.

Laywell ED, Rakic P, Kukekov VG, Holland EC, Steindler DA (2000) Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. Proc Natl Acad Sci U S A 97:13883-13888.

Lepekhin EA, Eliasson C, Berthold CH, Berezin V, Bock E, Pekny M (2001) Intermediate filaments regulate astrocyte motility. J Neurochem 79:617-625.

Lin JH, Weigel H, Cotrina ML, Liu S, Bueno E, Hansen AJ, Hansen TW, Goldman S, Nedergaard M (1998) Gap-junction-mediated propagation and amplification of cell injury. Nat Neurosci 1:494-500.

Lipton P (1999) Ischemic cell death in brain neurons. Physiol Rev 79:1431-1568.

Matthews DA, Cotman C, Lynch G (1976) An electron microscopic study of lesion-induced synaptogenesis in the dentate gyrus of the adult rat. I. Magnitude and time course of degeneration. Brain Res 115:1-21.

McKenzie JC (1992) Atrial natriuretic peptide-like immunoreactivity in astrocytes of parenchyma and glia limitans of the canine brain. J Histochem Cytochem 40:1211-1222.

McKenzie JC, Cowie RJ, Inagami T (1990) ANP-like immunoreactivity in neuronal perikarya and processes associated with vessels of the pia and cerebral parenchyma in dog. Neurosci Lett 117:253-258.

Nagai N, De Mol M, Lijnen HR, Carmeliet P, Collen D (1999) Role of plasminogen system components in focal cerebral ischemic infarction: a gene targeting and gene transfer study in mice. Circulation 99:2440-2444.

Nagai N, Suzuki Y, Van Hoef B, Lijnen HR, Collen D (2005) Effects of plasminogen activator inhibitor-1 on ischemic brain injury in permanent and thrombotic middle cerebral artery occlusion models in mice. J Thromb Haemost 3:1379-1384.

Nedergaard M, Hansen AJ (1993) Characterization of cortical depolarizations evoked in focal cerebral ischemia. J Cereb Blood Flow Metab 13:568-574.

Nedergaard M, Dirnagl U (2005) Role of glial cells in cerebral ischemia. Glia 50:281-286.

Newman EA (2003) New roles for astrocytes: regulation of synaptic transmission. Trends Neurosci 26:536-542.

Nogami M, Shiga J, Takatsu A, Endo N, Ishiyama I (2001) Immunohistochemistry of atrial natriuretic peptide in brain infarction. Histochem J 33:87-90.

Ogata K, Kosaka T (2002) Structural and quantitative analysis of astrocytes in the mouse hippocampus. Neuroscience 113:221-233.

Parpura V, Fang Y, Basarsky T, Jahn R, Haydon PG (1995) Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP-25 in cultured astrocytes. FEBS Lett 377:489-492.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte-neuron signalling. Nature 369:744-747.

Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. J Neurosci 17:7817-7830.

Pekny M, Leveen P, Pekna M, Eliasson C, Berthold CH, Westermark B, Betsholtz C (1995) Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. EMBO J 14:1590-1598.

Pekny M, Eliasson C, Chien CL, Kindblom LG, Liem R, Hamberger A, Betsholtz C (1998) GFAP-deficient astrocytes are capable of stellation in vitro when cocultured with neurons and exhibit a reduced amount of intermediate filaments and an increased cell saturation density. Exp Cell Res 239:332-343.

Pekny M, Eliasson C, Siushansian R, Ding M, Dixon SJ, Pekna M, Wilson JX, Hamberger A (1999a) The impact of genetic removal of GFAP and/or vimentin on glutamine levels and transport of glucose and ascorbate in astrocytes. Neurochem Res 24:1357-1362.

Pekny M, Johansson CB, Eliasson C, Stakeberg J, Wallen A, Perlmann T, Lendahl U, Betsholtz C, Berthold CH, Frisen J (1999b) Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin. J Cell Biol 145:503-514.

Peters CM, Rogers SD, Pomonis JD, Egnazyck GF, Keyser CP, Schmidt JA, Ghilardi JR, Maggio JE, Mantyh PW (2003) Endothelin receptor expression in the normal and injured spinal cord: potential involvement in injury-induced ischemia and gliosis. Exp Neurol 180:1-13.

Petito CK, Babiak T (1982) Early proliferative changes in astrocytes in postischemic noninfarcted rat brain. Ann Neurol 11:510-518.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Pixley SK, de Vellis J (1984) Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. Brain Res 317:201-209.

Potokar M, Kreft M, Pangrsic T, Zorec R (2005) Vesicle mobility studied in cultured astrocytes. Biochem Biophys Res Commun 329:678-683.

Quinlan RA, Franke WW (1983) Molecular interactions in intermediate-sized filaments revealed by chemical cross-linking. Heteropolymers of vimentin and glial filament protein in cultured human glioma cells. Eur J Biochem 132:477-484.

Rawanduzy A, Hansen A, Hansen TW, Nedergaard M (1997) Effective reduction of infarct volume by gap junction blockade in a rodent model of stroke. J Neurosurg 87:916-920.

Ridet JL, Malhotra SK, Privat A, Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 20:570-577.

Rogers SD, Peters CM, Pomonis JD, Hagiwara H, Ghilardi JR, Mantyh PW (2003) Endothelin B receptors are expressed by astrocytes and regulate astrocyte hypertrophy in the normal and injured CNS. Glia 41:180-190.

Rogers SD, Demaster E, Catton M, Ghilardi JR, Levin LA, Maggio JE, Mantyh PW (1997) Expression of endothelin-B receptors by glia in vivo is increased after CNS injury in rats, rabbits, and humans. Exp Neurol 145:180-195.

Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433:73-77.

Saez JC, Contreras JE, Bukauskas FF, Retamal MA, Bennett MV (2003) Gap junction hemichannels in astrocytes of the CNS. Acta Physiol Scand 179:9-22.

Saito I, Segawa H, Shiokawa Y, Taniguchi M, Tsutsumi K (1987) Middle cerebral artery occlusion: correlation of computed tomography and angiography with clinical outcome. Stroke 18:863-868.

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153-7160.

Sheehan JJ, Tsirka SE (2005) Fibrin-modifying serine proteases thrombin, tPA, and plasmin in ischemic stroke: a review. Glia 50:340-350.

Silver J, Miller JH (2004) Regeneration beyond the glial scar. Nat Rev Neurosci 5:146-156.

Song H, Stevens CF, Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39-44.

Sonnenberg H, Veress AT (1984) Cellular mechanism of release of atrial natriuretic factor. Biochem Biophys Res Commun 124:443-449.

Stahlberg A, Hakansson J, Xian X, Semb H, Kubista M (2004) Properties of the reverse transcription reaction in mRNA quantification. Clin Chem 50:509-515.

Steward O, Vinsant SL (1983) The process of reinnervation in the dentate gyrus of the adult rat: A quantitative electron microscope analysis of terminal proliferation and reactive synaptogenesis. J Comp Neurol 214:370-386.

Stone DJ, Rozovsky I, Morgan TE, Anderson CP, Finch CE (1998) Increased synaptic sprouting in response to estrogen via an apolipoprotein E-dependent mechanism: implications for Alzheimer's disease. J Neurosci 18:3180-3185.

Sultana S, Sernett SW, Bellin RM, Robson RM, Skalli O (2000) Intermediate filament protein synemin is transiently expressed in a subset of astrocytes during development. Glia 30:143-153.

Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M (2006) Astrocyte-mediated control of cerebral blood flow. Nat Neurosci 9:260-267.

Titeux M, Brocheriou V, Xue Z, Gao J, Pellissier JF, Guicheney P, Paulin D, Li Z (2001) Human synemin gene generates splice variants encoding two distinct intermediate filament proteins. Eur J Biochem 268:6435-6449.

Tsirka SE, Gualandris A, Amaral DG, Strickland S (1995) Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. Nature 377:340-344.

Tvarusko W, Bentele M, Misteli T, Rudolf R, Kaether C, Spector DL, Gerdes HH, Eils R (1999) Time-resolved analysis and visualization of dynamic processes in living cells. Proc Natl Acad Sci U S A 96:7950-7955.

Ullian EM, Sapperstein SK, Christopherson KS, Barres BA (2001) Control of synapse number by glia. Science 291:657-661.

Vasquez RJ, Howell B, Yvon AM, Wadsworth P, Cassimeris L (1997) Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. Mol Biol Cell 8:973-985.

Vassar R, Coulombe PA, Degenstein L, Albers K, Fuchs E (1991) Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. Cell 64:365-380.

Verkhratsky A, Kettenmann H (1996) Calcium signalling in glial cells. Trends Neurosci 19:346-352.

Virley D (2005) Choice, methodology, and characterization of focal ischemic stroke models: the search for clinical relevance. Methods Mol Med 104:19-48.

Volterra A, Bezzi P (2002) Release of transmitters from glial cells. In: The tripartite synapse: Glia in synaptic transmission (Volterra A, Magistretti PJ, Haydon PG, eds), pp 164-182. Oxford: Oxford University Press.

Wacker I, Kaether C, Kromer A, Migala A, Almers W, Gerdes HH (1997) Microtubule-dependent transport of secretory vesicles visualized in real time with a GFP-tagged secretory protein. J Cell Sci 110 (Pt 13):1453-1463.

Wang E, Cairncross JG, Liem RK (1984) Identification of glial filament protein and vimentin in the same intermediate filament system in human glioma cells. Proc Natl Acad Sci U S A 81:2102-2106.

Welsh FA, Sakamoto T, McKee AE, Sims RE (1987) Effect of lactacidosis on pyridine nucleotide stability during ischemia in mouse brain. J Neurochem 49:846-851.

Wilhelmsson U, Li L, Pekna M, Berthold CH, Blom S, Eliasson C, Renner O, Bushong E, Ellisman M, Morgan TE, Pekny M (2004) Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration. J Neurosci 24:5016-5021.

Yong VW, Moumdjian R, Yong FP, Ruijs TC, Freedman MS, Cashman N, Antel JP (1991) Gamma-interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo. Proc Natl Acad Sci U S A 88:7016-7020.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40:971-982.

Zhang Q, Pangrsic T, Kreft M, Krzan M, Li N, Sul JY, Halassa M, Van Bockstaele E, Zorec R, Haydon PG (2004) Fusion-related release of glutamate from astrocytes. J Biol Chem 279:12724-12733.

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