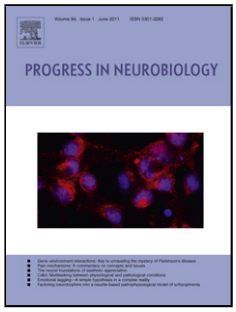

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Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke

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Abstract

Astrocytes are the most abundant cell type within the central nervous system. They play essential roles in maintaining normal brain function, as they are a critical structural and functional part of the tripartite synapses and the neurovascular unit, and communicate with neurons, oligodendrocytes and endothelial cells. After an ischemic stroke, astrocytes perform multiple functions both detrimental and beneficial, for neuronal survival during the acute phase. Aspects of the astrocytic inflammatory response to stroke may aggravate the ischemic lesion, but astrocytes also provide benefit for neuroprotection, by limiting lesion extension via anti-excitotoxicity effects and releasing neurotrophins. Similarly, during the late recovery phase after stroke, the glial scar may obstruct axonal regeneration and subsequently reduce the functional outcome; however, astrocytes also contribute to angiogenesis, neurogenesis, synaptogenesis, and axonal remodeling, and thereby promote neurological recovery. Thus, the pivotal involvement of astrocytes in normal brain function and responses to an ischemic lesion designates them as excellent therapeutic targets to improve functional outcome following stroke. In this review, we will focus on functions of astrocytes and astrocyte-mediated events during stroke and recovery. We will provide an overview of approaches on how to reduce the detrimental effects and amplify the beneficial effects of astrocytes on neuroprotection and on neurorestoration post stroke, which may lead to novel and clinically relevant therapies for stroke.

Abbreviation List

AQP: aquaporin
BBB: blood brain barrier
BDNF: brain-derived neurotrophic factor
bFGF: basic fibroblast growth factor
CNTF: ciliary neurotrophic factor

CSPG: chondroitin sulfate proteoglycan
CST: corticospinal tract
DLX: distal-less homeobox
EPO: erythropoietin
GDNF: glia-derived neurotrophic factor
GFAP: glial fibrillary acidic protein
GLAST: glutamate aspartate transporter
GLT: glutamate transporter-1
HMGB: high-mobility group box
IL: interleukin
iNOS: inducible nitric oxide synthase
KLK: Kallikrein-related peptidase
LTD: long-term depression
LTP: long-term potentiation
Mash: mammalian achaeteschute homolog
MCAo: middle cerebral artery occlusion
MMP: matrix metalloproteinases
MSC: marrow stromal or mesenchymal cells
mTOR: mammalian target of rapamycin
NGF: nerve growth factor
NMDAR: N-methyl-D-Aspartate receptor
NO: nitric oxide
OGD: oxygen-glucose deprivation
SDF: stromal cell-derived factor-1
SGZ: subgranular zone
Shh: sonic hedgehog
SOD: superoxide dismutase
SVZ: subventricular zone
TGF: transforming growth factor
TIMP: tissue inhibitors of metalloproteinases
TNF: tumor necrosis factor
tPA: tissue plasminogen activator
TSP: thrombospondins EGF: vascular endothelial growth factor

1. Introduction

Stroke is the third leading cause of death in the United States and the leading cause of serious, long-term disability. Each year, Approximately 795,000 Americans suffer strokes, and more than 4,000,000 people have survived a stroke and live with some form of neurological impairment or disability (Pearson-Fuhrhop and Cramer, 2010). One of the most common impairments after stroke is hemiplegia of the contralateral side to the affected cerebral hemisphere. Of stroke

survivors, 50% have some hemiparesis, 30% are unable to walk without assistance, 26% are dependent in activities of daily living at 6 months after stroke, and approximately 15% to 30% are left permanently disabled (Duncan *et al.*, 2005). Long-term disability from stroke not only affects functional status, but also has profound emotional and social effects on stroke survivors and their families, and has major economic consequences (Zorowitz *et al.*, 2009).

Currently, intravenous administration of recombinant tissue plasminogen activator (tPA) is the only FDA approved therapy for acute ischemic stroke; however, due to the narrow therapeutic time window of 4.5 hours after stroke onset and the risk of subsequent hemorrhage , only approximately 5% of patients benefit from this treatment (Fang *et al.*, 2010). For decades, the primary approach and goal of therapy for stroke have focused on neuroprotection, to salvage ischemic neurons in the brain from irreversible injury, however, despite showing efficacy in experimental stroke models, all these efforts have failed to provide significant benefit in clinical trials of stroke (Han *et al.*, 2013; Rother, 2008). The lack of translational success of neuroprotective agents is often attributed to differences between pre-clinical studies and clinical trials, such as population type (young animals in homogeneous population with no comorbidities, vs. elderly patients in heterogeneous population with numerous comorbidities); ischemic territory (restricted territory of MCA in animals vs. various vascular territories in humans); scope for optimization (optimized therapeutic time window, dose, and route of administration for animal studies, while not optimized for clinical studies); occlusion duration (controlled duration of occlusion in animal studies vs. variable occlusion duration in humans); primary endpoint (animal studies use infarct volume, while human studies use functional testing) (Minnerup *et al.*, 2012; Stroke Therapy Academic Industry Roundtable, 2001). The consideration of using older animals and animals with comorbidities such as diabetes and hypertension, optimized dosage and

time window of administration, as well as multiple physiological and neurological measurements, will hopefully improve the chances of successful translation for neuroprotection (Turner *et al.*, 2013). More importantly, despite the fact that stroke affects all cellular elements of the brain, i.e., vascular cells, neurons, astrocytes, oligodendrocytes, microglia and ependymocytes, and induces signaling responses that occur within and between different cell types, most clinical trials were often performed using a single agent against single purported mechanism of action specifically targeting the neurons. Protecting neurons alone may be insufficient to improve neurological outcome after stroke. To accomplish this and to broaden treatment targets, we must consider therapeutic approaches that benefit multiple cell types, and in our view, particularly, astrocytes (Li *et al.*, 2014). Astrocytes are likely to be essential targets for manipulation, because they are the most abundant subtypes of glial cells, by several fold outnumber neurons in the CNS, and are in contact with and interact and affect all parenchymal cells. Therefore, an increasing number of studies focus on the roles of astrocytes in stroke in recent years. Brain astrocytes are classically divided into several major types according to morphology and spatial organization: radial astrocytes surrounding ventricles, protoplasmic astrocytes in gray matter, and fibrous astrocytes located in white matter (Privat *et al.*, 1995), as well as Bergmann glia in the cerebellum, velate astrocytes in the granule layer of the cerebellum, interlaminar astrocytes in the supragranular layers of the cerebral cortex, among others (Reichenbach and Wolburg, 2013). These cellular subtypes may differ not only phenotypically but also functionally, however, in this review, we will only use an umbrella term, astrocyte, neglecting the complexity, variety and distribution of assorted astrocytes.

2. Astrocytes in normal brain

As an integral part of the neuron-glia system, astrocytes provide many housekeeping functions, including structural support, formation of blood brain barrier (BBB), neuronal metabolism, maintenance of the extracellular environment, regulation of cerebral blood flow, stabilization of cell-cell communications, neurotransmitter synthesis, and defense against oxidative stress (Ransom and Ransom, 2012). Astrocytic finely branched processes envelop all cellular components throughout the CNS, and contact all parts of neurons, e.g., soma, dendrites, axons and synaptic terminals. Astrocytes thus, function as a syncytium of interconnected cells in the CNS (Nagy and Rash, 2000; Scemes *et al.*, 2000), and discrete microdomains within the astrocytic syncytium may interact autonomously with one another and with neurons (Giaume and Liu, 2012; Verkhratsky, 2010).

2.1. Astrocytes and synapses

The astrocytic processes envelop the pre- and post-synaptic terminals, forming a physical barrier that limits diffusion of the neurotransmitter away from the synapse, termed as "tripartite synapse" (Araque *et al.*, 1999). To maintain the extracellular concentration of neurotransmitters, astrocytes rapidly remove the K^+ accumulated as a result of neuronal activity, take up the glutamate released during neurotransmission, and convert the glutamate to glutamine and release it back into presynaptic terminals (Verkhratsky and Kirchhoff, 2007). Glutamate plays a key role in the regulation of synaptic activity and causes a response in astrocytes (Cornell-Bell *et al.*, 1990). Activation of astrocytes by neurotransmitters released from the presynaptic terminal triggers additional release of transmitters from the astrocytic compartment, which can directly participate in the synaptic event (Newman, 2003). Astrocytes play direct and interactive roles with neurons in synaptic transmission through the regulated release of synaptically active molecules including glutamate, purines (ATP and adenosine), GABA, and D-serine (Halassa *et*

al., 2007; Nedergaard *et al.*, 2003). Glutamate released from astrocytes activates presynaptic NMDA receptors and promotes increased excitatory communication between neurons (Jourdain *et al.*, 2007; Shigetomi *et al.*, 2008). Astrocytes also release several other neuroactive molecules, such as D-serine, ATP, adenosine, GABA, tumor necrosis factor α (TNF-α), prostaglandins, proteins and peptides (Perea *et al.*, 2009), that can regulate the activity and expression of receptors on the postsynaptic neuron through gliotransmitter activity, and play a role in dampening activity and promoting the removal of nonadvantageous connections (Pascual *et al.*, 2005). In addition, because a single astrocyte may be in contact with thousands of synapses, astrocyte calcium elevations and subsequent glutamate release lead to the synchronous excitation of clusters of neurons, indicating that gliotransmission may contribute to neuronal synchronization (Fellin *et al.*, 2004). Astrocytes also express numerous neurotransmitter receptors, ion channels, and second messenger systems and can thereby respond to and integrate input from neurons (Nilsson *et al.*, 1993; Porter and McCarthy, 1997; Verkhratsky and Kettenmann, 1996). Therefore, astrocytes are not only involved in passive homeostatic control of adequate conditions for synaptic function, but also actively modulate neuronal excitability and synaptic transmission in synaptic function, contributing to the brain functional output through the coordinated activity of complex neuronal networks comprising both neurons and glia (Haydon, 2001; Perea *et al.*, 2009).

Astrocytes are active participants in the structural plasticity during both initial establishment and ongoing remodeling of synaptic connections within the nervous system (Haber *et al.*, 2006). Astrocytes also release several distinct soluble factors to control maturation of synapses. Astrocyte-derived glypicans 4 and 6 are necessary and sufficient to promote glutamate receptor clustering and receptivity and to induce the formation of postsynaptically functioning CNS

synapses (Allen *et al.*, 2012). Astrocyte-derived TNF-α regulates the insertion of glutamate receptors into postsynaptic membranes, and activity-dependent neurotrophic factors increase the density of NMDA receptors in the membrane of postsynaptic neurons, indicating that astrocytes have a powerful ability to regulate synaptic strength in response to alterations in neuronal activity (Stellwagen and Malenka, 2006).

Dendritic spines are quite dynamic, responding to many events with rapid changes as long-term potentiation (LTP) is associated with increases in spine size, and long-term depression (LTD) is associated with decreases in spine size. Using time-lapse confocal imaging, it has been demonstrated that astrocytes can rapidly extend and retract fine processes to engage and disengage from motile postsynaptic dendritic spines coordinating with changes in spines (Haber *et al.*, 2006), indicating the active role of astrocytes in modulating synaptic plasticity. Neurotrophic factors play critical roles in LTP as well as in learning and memory. For example, activity-dependent secretion of BDNF enhances synaptic plasticity (Akaneya *et al.*, 1997). BDNF is secreted in its precursor form (pro-BDNF) and is then cleared from the extracellular space through rapid uptake by nearby astrocytes (Bergami *et al.*, 2008), suggesting that astrocytes exert an important function in the neuronal clearance of pro-BDNF secreted upon neuronal activity and subsequent recycling of the endocytic neurotrophin, thus regulating both its spatial and temporal availability. Recycling of BDNF by astrocytes may thus contribute to the regulation of synaptic plasticity by glia.

2.2. Astrocytes and gap junctions

In addition to modulating the local environment, astrocytes also have the structural and morphological elements for integrating and modulating homeostasis in larger brain regions. Astrocytes are extensively coupled into a cellular network (homocellular and heterocellular

junctions) via gap junction intercellular communication (Rouach *et al.*, 2000). Gap junction channels are built of connexin (CX) proteins, of which the CX-43 and -30 subtypes are the major forms in astrocytes (Giaume and McCarthy, 1996; Rouach *et al.*, 2002). Gap junctions are relatively freely permeable to substances up to approximately 1.2 kDa, ions, and metabolites such as glutamate and glutamine can diffuse from regions of high concentration to regions of low concentration. Gap junctions interconnect astrocytes, allow direct intracellular movement of metabolic substrates and support astrocytic spatial K^+ buffering to modulate and synchronize large scale activity, which are essential for astrocyte function (Gardner-Medwin, 1983). Gap junction channels enriched in the endfeet of astrocytes, that enwrap the walls of blood vessels, provide a perivascular route that facilitates intercellular trafficking between neighboring endfeet (Simard *et al.*, 2003). Interestingly, Cx43/Cx30 double knockout leads to astrocyte endfeet edema and weakens the BBB, which opens upon increased hydrostatic vascular pressure and shear stress (Ezan *et al.*, 2012), indicating that the astrocytic gap junction is necessary to maintain BBB integrity. Both gap channel function and Cx43 are important for normal neuronal proliferation (Sutor and Hagerty, 2005) and migration during CNS development (Cina *et al.*, 2009; Liu *et al.*, 2012). Since astrocytic gap junction channels play important roles in extracellular glutamate and potassium removal during synaptic activity, astrocytic gap junctions significantly contribute to control synaptic strength and synaptic plasticity (Rouach *et al.*, 2008). The communication between glia and neurons also can be through a newly discovered cell-to-cell communication system involving small, membrane-enveloped nanovesicles, exosomes, which are capable of carrying signaling molecules in the forms of protein, mRNA and miRNA to serve as the platforms for complex intercellular communication and subsequently regulate functions of targeted cells (Fruhbeis *et al.*, 2013; Xin *et al.*, 2014).

2.3. Astrocytes and blood brain barrier

Astrocytic endfeet of the basal process cover almost the entire surface of intraparenchymal capillaries in the adult brain (Abbott *et al.*, 2006; Mathiisen *et al.*, 2010). Together with cerebral capillary endothelial cells that form tight junctions and are surrounded by a basal lamina and perivascular pericytes, astrocyte endfeet are involved in the formation and integrity of BBB, a diffusion barrier that impedes the influx into brain parenchyma of certain molecules on the basis of polarity and size, that allows oxygen and hormones to permeate into the brain while preventing the passage of other molecules due to possible harmful effects (Abbott *et al.*, 2006; Ballabh *et al.*, 2004; Daneman and Prat, 2015). The perivascular astrocytic covering may control the exchange of water and solutes between blood and brain, and free diffusion is limited to narrow clefts between overlapping endfeet. Astrocytes abundantly express aquaporin 4 (AQP4), the water channel protein in the pericapillary processes of endfeet, which play a major role in the exchange of water and solutes between blood and brain (Fukuda and Badaut, 2012). In addition, astrocytes make extensive contacts with and have multiple bidirectional interactions with blood vessels. In the brain, astrocytes produce and release various molecular mediators, such prostaglandins, nitric oxide (NO), and arachidonic acid, that can regulate blood vessel diameter and blood flow in a coordinated manner (Gordon *et al.*, 2011; Gordon *et al.*, 2007; Iadecola and Nedergaard, 2007). Moreover, astrocytes provide a cellular link between the neuronal circuitry and blood vessels. Astrocytic processes are in contact with both blood vessels and synapses. This neurovascular coupling enables astrocytes to work as primary mediators to relay signals that regulate local CNS blood flow in response to changes of neuronal activity in order to coordinate oxygen and glucose delivery (Attwell *et al.*, 2010; Koehler *et al.*, 2009; Schummers *et al.*, 2008). During neural activity, neurons release glutamate acting on astrocytic metabotropic glutamate

receptors (mGluRs) to raise astrocytic Ca^{2+}, resulting in downstream production of arachidonic acid and the formation and release of vasoactive substances, thereby increasing the blood flow (Mulligan and MacVicar, 2004; Zonta *et al.*, 2003). On the other hand, astrocytic glutamate transporters also contribute to functional hyperemia through mechanisms independent of calcium rises and cyclooxygenase activation (Petzold *et al.*, 2008). Thus, both direct astrocyte interactions and astrocyte-derived factors are essential for modulating and maintaining the BBB function in the brain.

3. Astrocytes in ischemic brain

3.1. Astrocytic activation and glial scar formation after stroke

In the ischemic brain, astrocytes undergo important morphological modifications, such as hyperplasia and hypertrophy. Within minutes after injury, cytokines produced by injured neurons in the core and penumbra of the lesion and glial cells in the core, such as transforming growth factor (TGF)-α (Junier *et al.*, 1994; Rabchevsky *et al.*, 1998; Scarisbrick *et al.*, 2012), ciliary neurotrophic factor (CNTF) (Winter *et al.*, 1995), interleukin (IL)-1 (Herx and Yong, 2001), IL-6 (Brunello *et al.*, 2000; Cardenas and Bolin, 2003; Chiang *et al.*, 1994; Odemis *et al.*, 2002), and Kallikrein-related peptidase 6 (KLK6) (Scarisbrick *et al.*, 2012), trigger astrocyte activation. These astrocytes, also referred to as, reactive astrogliosis (For detail, see (Sofroniew, 2009), exhibit cellular hypertrophy, proliferation, increased expression of the intermediate filament proteins including glial fibrillary acidic protein (GFAP), vimentin and nestin, and have altered expression of many other molecules involved in cell structure, gene transcription, energy metabolism, intracellular signaling and membrane transporters (Clarke *et al.*, 1994; Fuchs and Cleveland, 1998; Holmin *et al.*, 1997; Li and Chopp, 1999; Ridet *et al.*, 1997; Yasuda *et al.*, 2004). Interestingly, vimentin and nestin are expressed during development and are usually

down-regulated in the mature CNS (Sancho-Tello *et al.*, 1995; Valles *et al.*, 1996). Activated astrocytes exhibit elongated processes in the area surrounding the ischemic infarction (Kajihara *et al.*, 2001). Within a few days after the insult, around ischemic lesions in the brain, a glial scar, a physical and functional wall is forming around the necrotic brain tissue of the infarct (Bidmon *et al.*, 1998; Silver and Miller, 2004). This scar is mainly generated by reactive astrocytes. Depending on the severity of lesion, minor forms of reactive astrogliosis can resolve over time, while in more severe cases, the scar formation can be permanent (Sofroniew, 2009).

The functional role of glial scar after stroke is controversial. In the scar, reactive astrocytes express a broad range of inhibitory molecules against axonal regeneration, such as chondroitin sulfate proteoglycans (CSPGs) (Gris *et al.*, 2007; McKeon *et al.*, 1991; Smith and Strunz, 2005), which have been recognized as major barriers to CNS axon extension and thus regeneration failure in the CNS, at least in mammals. However, the glial scar may also seclude the injury site from viable tissue, preventing a cascading wave of uncontrolled tissue damage (Faulkner *et al.*, 2004), and may also restrict diffusible factors secreted from the damaged region into remote area (Bush *et al.*, 1999). After injury, CSPG expression is rapidly upregulated by reactive astrocytes, forming an inhibitory gradient that is highest at the center of the lesion and diminishes gradually into the penumbra (McKeon *et al.*, 1991). Thus, while there is no doubt that the glial scar can limit regeneration in the adult mammal by inhibiting axonal sprouting, the glial scar may on the other hand be important for isolating the injury site and preventing its extension, thus protecting cells against harmful substances released from the infarct core. In injured brains of mice lacking both GFAP and vimentin ($GFAP^{-/-}Vim^{-/-}$), astrocytes show similar abundance and access comparable volumes of brain tissue as astrocytes of wild-type (WT) mice (Wilhelmsson *et al.*, 2004), but do not exhibit the reactive phenotype with characteristic hypertrophic processes as

astrocytes in WT mice (Li *et al.*, 2008; Lu *et al.*, 2011; Wilhelmsson *et al.*, 2004). In $GFAP^{-/-}$ $Vim^{-/-}$ mice subjected to unilateral Rose Bengal induced cerebral cortical photothrombotic stroke, we found that CSPG expression was decreased in the ischemic lesion boundary zone, but significantly increased in the areas remote from the lesion including the contralesional cerebral hemisphere and the cortical area outer lesion boundary zone in the ipsilesional hemisphere, compared with WT mice; attenuation of glial scar formation surrounding the ischemic core region may lead to an extension of the cellular responses to areas remote from the lesion (Liu *et al.*, 2014).

3.2. Roles of astrocytic gap junction in stroke

The role of astrocyte gap junctions in stroke also remains controversial, with evidence that both beneficial and harmful substances may pass through them and influence stroke in opposite ways (Nakase and Naus, 2004). In the acute phase of the ischemic setting, although gap junction connections in astrocytes are significantly reduced during ischemic conditions, astrocytic gap junctional channels remain open following stroke (Cotrina *et al.*, 1998; Martinez and Saez, 2000), allowing substances such as pro-apoptotic factors to diffuse from dying cells through the channels, leading to secondary propagation of brain injury to the neighboring cells in cerebral ischemia (Lin *et al.*, 1998), which may thereby expand the size of ischemic lesions. For example, cellular Ca^{2+} overload, or perturbation of intracellular Ca^{2+} compartmentalization, can cause cytotoxicity, stimulating several important apoptotic pathways both directly and indirectly through activation of caspases (Orrenius *et al.*, 2003). Such apoptotic signal spreading from ischemic astrocytes in the ischemic core may contribute to further damage of neurons and glial cells in the penumbral regions, where apoptosis is prominent (Li *et al.*, 1995a; Li *et al.*, 1995b; Li *et al.*, 1995c; Li *et al.*, 1995e). Blocking gap junction using carbenoxolone and reducing the

synthesis of specific connexins using antisense oligodeoxynucleotides significantly decrease the spread of hypoxic injury induced cell death in cultured organotypic hippocampal slices (Frantseva *et al.*, 2002). Consistently, treatment with the gap junction blockers octanol or halothane reduced neuronal death and infarct volume in rats after permanent focal ischemia (Rawanduzy *et al.*, 1997; Saito *et al.*, 1997) and transient global ischemia (Rami *et al.*, 2001). In contrast, a protective role of gap junctions has been shown in other studies. In mixed cultures of neurons and astrocytes, inter-astrocytic gap junctional communication decreases neuronal vulnerability to oxidative injury by a mechanism involving stabilization of cellular calcium homeostasis and dissipation of oxidative stress (Blanc *et al.*, 1998). Blocking gap junctions during a glutamate insult to co-cultures of astrocytes and neurons results in increased neuronal injury (Naus *et al.*, 2001; Ozog *et al.*, 2002). Astrocytic neuroprotection is also demonstrated with impaired gap junction coupling, attenuated intercellular calcium signaling, enhanced neuronal apoptosis, and increased infarct volumes after experimental ischemic stroke in mice lacking one allele of connexin-43, the major constituent of astrocytic gap junctions (Nakase *et al.*, 2003; Naus *et al.*, 2001), and mice in which the connexin-43 gene was specifically deleted in astrocytes (Nakase *et al.*, 2004), respectively, indicating that reactive astrocytes may reduce neuronal apoptosis in the penumbra following ischemic insult by regulating extracellular conditions through their gap junctions.

3.3. Astrocytes in maintaining BBB after stroke

Brain edema is a major contributor to the early symptoms of stroke, as well as to the mortality of this disease. Early astrocytic response to ischemia includes astrocyte swelling, which occurs initially as an osmotic consequence of increased uptake of glutamate, K^+ ions, and lactate in the endfeet around the microvessels (Kimelberg, 2005; Landis, 1994). As a result, astrocytic

swelling leads to an increase in intracerebral pressure, reduction of vascular perfusion, and exacerbation of the ischemic event (Sykova, 2001). After ischemia, the BBB disruption may occur by several mechanisms, including active solute transport, vesicular transport, opening of paracellular channels, and physical disruption of astrocyte-endothelial junctions (del Zoppo and Hallenbeck, 2000; Petito, 1979). Digestion of BBB matrix proteins by astrocyte matrix metalloprotease 2 and other matrix metalloproteases contributes to the physical disruption of the BBB (Mun-Bryce and Rosenberg, 1998; Rosenberg *et al.*, 1998). AQP4 is the primary route by which water moves in and out of astrocytes in response to these osmotic changes, and therefore plays an important role in astrocyte swelling and cerebral edema formation (Fukuda and Badaut, 2012). Absence of AQP4 in astrocytic endfeet leads to decreased hypertrophy of astrocytes due to decreased water entry as well as migration toward the site of the injury (Auguste *et al.*, 2007; Saadoun *et al.*, 2005). AQP4 deletion in mice reduces astrocyte swelling and brain edema, and enhances neurologic outcome after focal ischemia (Manley *et al.*, 2000). There is a biphasic leakage of the BBB, with an early opening within hours following hypoxia/ischemia, followed by a refractory phase and then a second opening the next day (Huang *et al.*, 1999; Kuroiwa *et al.*, 1985). Interestingly, AQP4 expression in perivascular astrocyte endfeet is increased during the secondary edema resolution phase (Fukuda *et al.*, 2012; Tourdias *et al.*, 2011). Thus, functional integrity of astrocytes is essential for maintaining the BBB during ischemia, and reestablishment of astrocytic water channels is critical for BBB repair during stroke recovery.

4. Astrocytes in neuroprotection

Ischemic damage results from a cascade of cellular and molecular events triggered by sudden lack of blood flow and subsequent reperfusion of the ischemic territory. The onset of ischemia produces an irreversible core region, in where the blood flow is severely reduced, by almost

90% , leading to generalized cell death within minutes due to the energy failure, as insufficient adenosine triphosphate (ATP) supply is available to maintain homeostatic ionic gradients and metabolic (Lipton, 1999). In the ischemic penumbra, where the blood flow is partially reduced, typically in the range of 35% of baseline, neurons remain viable for a prolonged period of time after the insult, but the neurons are stressed and critically vulnerable to pathogenic events that may tip their fragile metabolic balance (Moskowitz *et al.*, 2010). Although the general consensus remains that the infarct core is not salvageable, it is believed that penumbral tissue can be salvaged through flow restoration and/or restoration of cellular homeostasis through manipulation with pharmacological compounds. Thus, the existence of a penumbra implies that therapeutic salvage of neuroprotection is theoretically possible after stroke (Lo, 2008).

4.1. Protecting astrocytes during ischemia

Because neurons are not viable without astrocytes, neuroprotective therapeutics should consider being beneficial for protecting both neurons and astrocytes (Chen and Swanson, 2003). Astrocytes are generally more resistant than neurons to oxygen-glucose deprivation (OGD) in the culture media, an in vitro model of ischemia. Most neurons in astrocyte-neuronal co-cultures will die after 60-90 min of OGD, while astrocytes are irreversibly injured after 4-6 hours (Almeida *et al.*, 2002; Goldberg and Choi, 1993). It has been demonstrated in animal models of stroke that astrocyte viability is maintained longer than neurons during ischemia (Gurer *et al.*, 2009), thus astrocytes are better preserved than neurons in the boundary zone to the infarct (Li *et al.*, 1995d), even within the ischemic core, a proportion of astrocytes remain viable and metabolically active at early phases after reperfusion post temporary focal cerebral ischemia (Thoren *et al.*, 2005). The surviving astrocytes are thus providing a possibility to reestablish neuronal integrity leading to a return of neuronal function in the ischemic penumbra. Neuroprotective effects of melatonin

against free radical damage have been extensively studied. Melatonin treatment may provide neuroprotection against toluene neurotoxicity by increasing the survival of glial cells (Baydas *et al.*, 2003). It has been also demonstrated that enhancing astrocyte survival by adrenomedullin gene delivery provides neuroprotection against cerebral ischemia injury (Xia *et al.*, 2004). Therefore, therapeutic approaches aimed to protect astrocytes in combination with neuroprotective strategies may provide a synergistic and efficient protection to the ischemic brain.

4.2. Neuroprotection by reactive astrogliosis after stroke

Reactive astrogliosis is a hallmark of various pathologies, including stroke. Reactive astrocytes are traditionally thought to be detrimental to neurological outcome after stroke. Within minutes after injury, reactive astrocytes produce and release inflammatory mediators such as cytokines and chemokines, cytokines including IL-6, TNF-α, IL-1α and β and interferon γ (Basic Kes *et al.*, 2008; Nayak *et al.*, 2012; Orzylowska *et al.*, 1999; Tuttolomondo *et al.*, 2008). These cytokines may induce neuronal death (Venters *et al.*, 2000) and contribute to infarct progression in the post-ischemic period, either directly or via induction of neurotoxic mediators such as nitric oxide (Stoll *et al.*, 1998), and increased BBB permeability (Yang *et al.*, 1999). Indeed, activation of nuclear factor kappa-B (NF-κB) in astrocytes may contribute to neuronal degeneration by inducing the production and release of inflammatory cytokines, reactive oxygen molecules and excitotoxins (Mattson, 2005), and inactivation of astroglial NF-κB promotes neuronal survival ischemic injury (Dvoriantchikova *et al.*, 2009). Additionally, in response to ischemia, reactive astrocytes may also produce free radicals, including NO (Buskila *et al.*, 2005; Catania *et al.*, 2003; Endoh *et al.*, 1994; Kader *et al.*, 1993), superoxide and peroxynitrite (Gursoy-Ozdemir *et al.*, 2004; Love, 1999), which may induce neuronal apoptosis or necrotic death (Sugawara and

Chan, 2003). Following ischemia, reactive astrocytes also increase expression of S-100β throughout the penumbral region, which enhances the expression of inducible nitric oxide synthase (iNOS), leading to NO-mediated neuronal death (Matsui *et al.*, 2002).

However, in addition to the detrimental role during the early stage of ischemic onset, reactive astrocytes may also play a beneficial role in the brain. As noted above, astrocytic glial scar formation isolates the injury site from healthy tissue, preventing a cascading wave of uncontrolled further tissue damage (Bush *et al.*, 1999; Faulkner *et al.*, 2004). Reactive astrocytes restrict the lesion and minimize the area of inflammation in the acute stage after CNS injury (Sofroniew, 2005), and may also restrict diffusible factors secreted from the lesion region into remote area. The astrocytic nanofilament system is a structural component of the cytoskeleton and serves as an important signaling platform in situations linked to cellular stress (Pallari and Eriksson, 2006; Pekny and Lane, 2007; Wilhelmsson *et al.*, 2006). $GFAP^{-/-}Vim^{-/-}$ astrocytes exposed to OGD and reperfusion exhibit reduced ability to eliminate reactive oxygen species and increased cell death than WT astrocytes, indicating that astrocyte intermediate filament system is important for the cell response to oxidative stress (de Pablo *et al.*, 2013). GFAP knockout mice exhibit more an extensive and a profound decrease in cortical cerebral blood flow, and larger lesions than their wild-type littermates following focal ischemia (Nawashiro *et al.*, 2000). Reactive gliosis and glial scar formation are attenuated in $GFAP^{-/-}Vim^{-/-}$ mice, and healing after trauma takes longer and post-traumatic synaptic loss is more prominent (Pekny *et al.*, 1999; Wilhelmsson *et al.*, 2004). The astrocyte intermediate filament system influences viscoelastic properties of astrocytes (Potokar *et al.*, 2007), intracellular vesicle trafficking (Cho *et al.*, 2005a; Potokar *et al.*, 2010; Vardjan *et al.*, 2012), and is important for astrocyte response to hypo-osmotic stress (Ding *et al.*, 1998), spontaneous astrocyte motility (Lepekhin *et al.*, 2001), and for

the interaction of astroglial cells with microglia and blood borne monocytes (Hyder *et al.*, 2011; Kraft *et al.*, 2013; Nakazawa *et al.*, 2007). Induction of middle cerebral artery occlusion (MCAo) in $GFAP^{-/-}Vim^{-/-}$ mice is associated with loss of barrier functions of astroglial scar formation along the margins of infarct , and leads to increased spread of inflammation and increased lesion volume compared to that in WT mice (Li *et al.*, 2008). Astrocytic transforming growth factor-beta (TGF-β) is anti-inflammatory and a neuroprotective cytokine that is upregulated after stroke. In mice with TGF-β signaling specifically inhibited in astrocytes, photothrombotic motor cortex stroke leads to excessive inflammation, infarct expansion and worse motor outcome (Cekanaviciute *et al.*, 2014). These data suggest that reactive astrocytes play an essential role in neuroprotection by limiting inflammation in the peri-infarct cortex and preserve brain function during the subacute period after stroke.

4.3. Neuroprotection by astrocytic anti-excitotoxicity after stroke

In addition to their role in glial scar formation, astrocytes also respond to ischemia by promoting neuroprotection. Reactive astrocytes provide important metabolic support to neurons during cerebral ischemia, and disruption of astrocyte function may contribute to neuronal death (Rossi *et al.*, 2007). A recent in vitro study demonstrated that $GFAP^{-/-}$ $Vim^{-/-}$ astrocytes exposed to oxygen-glucose deprivation and reperfusion exhibit increased cell death and confer a lower degree of protection to co-cultured neurons than WT astrocytes (de Pablo *et al.*, 2013), suggesting that reactive astrocytes are protective to neurons during brain ischemia. Astrocytes protect neurons from oxidative stress via a glutathione dependent mechanism (Chen *et al.*, 2001c). Glutathione is a central component in the antioxidant defense of cells, acting both to directly detoxify reactive oxygen species and as a substrate for various peroxidases in the protection against reactive oxygen species (Dringen, 2000). Astrocytes contain the highest

concentrations of antioxidants of glutathione, and provide substrate of glutathione precursors to neighboring neurons for glutathione synthesis (Dringen *et al.*, 1999). Hence, when neurons are co-cultured with astrocytes, neuronal glutathione increased (Rathinam *et al.*, 2012). In rats subjected to MCAo, glutathione levels in the ischemic side of the cortex decreased with time after ischemia. Depletion of cellular glutathione with buthionine sulfoximine, a selective inhibitor for gamma-glutamylcysteine synthetase, exacerbated cortical infarction and edema after ischemia (Mizui *et al.*, 1992). In vitro studies demonstrated that glutathione depletion increases reactive oxygen intermediates and neuronal cell death (Colell *et al.*, 1998; Wullner *et al.*, 1999). Astrocytes from $Gclm^{-/-}$ mice, which lack the modifier subunit of glutamate cysteine ligase and, as a consequence, have very low glutathione levels, are much less effective at protecting neurons from oxidative stress and apoptotic cell death (Giordano *et al.*, 2009).

Glutamate toxicity is an important mechanism of neuronal death in ischemic stroke (Choi, 1988). Increased extracellular glutamate concentrations in the ischemic brain result in the local hyper-activation of ionotropic glutamate receptors, thereby triggering neuronal cell death via excessive Na^+ and Ca^{2+} influx into neurons (Sattler and Tymianski, 2001). Astrocytes are primarily responsible for glutamate uptake from the extracellular space using the astrocyte specific Na^+ dependent glutamate transporters, glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) (Anderson and Swanson, 2000). In rat cerebral cortical cultures, concentrations of glutamate one hundred-fold higher were required to produce neurotoxicity in the presence of abundant astrocytes, indicating that astrocytes protect neurons from glutamate neurotoxicity by glutamate uptake (Rosenberg and Aizenman, 1989). Thus, in $GFAP^{-/-}Vim^{-/-}$ mice, impaired astrocyte activation leads to decreased glutamate uptake abilities, and increased infarct volume after MCAo (Li *et al.*, 2008). Loss of glutamate transport activity and

immunoreactivity for the astrocyte-specific GLT-1 in astrocytes occurred at early reperfusion times, hours to days before the death of neurons, and upregulation of GLT-1 expression in astrocytes with ceftriaxone protected CA1 neurons from forebrain ischemia (Ouyang *et al.*, 2007).

4.4. Neuroprotection by astrocyte-derived neurotrophins after stroke

In response to ischemia, reactive astrocytes produce multiple neurotrophic factors to protect neurons (Hansson and Ronnback, 2003; Markiewicz and Lukomska, 2006; Swanson *et al.*, 2004), including nerve growth factor (NGF) (Lee *et al.*, 1998; Lindholm *et al.*, 1992; Lu *et al.*, 1991), CNTF (Asada *et al.*, 1995), basic fibroblast growth factor (bFGF) (Frautschy *et al.*, 1991; Ho and Blum, 1997), brain-derived neurotrophic factor (BDNF) (Tokumine *et al.*, 2003), glia-derived neurotrophic factor (GDNF) (Nicole *et al.*, 2001; Wang *et al.*, 1997; Yamagata *et al.*, 2002), vascular endothelial growth factor (VEGF) (Wick *et al.*, 2002), and erythropoietin (EPO) (Bernaudin *et al.*, 2000). In vitro studies have demonstrated that astrocytes synthesize and release neurotrophic factors into the culture media (Furukawa *et al.*, 1987; Yamakuni *et al.*, 1987). Therefore, application of astrocyte-conditioned media results in decreased infarct volume after MCAo (Kinoshita *et al.*, 1990). Up-regulated erythropoietin secretion from preconditioning of astrocytes promotes neuronal survival in ischemic injury (Wu *et al.*, 2013), suggesting that neurotrophic factors released by astrocytes following ischemia are important for neuroprotection after stroke.

5. Astrocytes in neurorestoration

In the early stage after stroke, recovery may be attributable to the resolution of brain edema, absorption of damaged tissue or reperfusion of the ischemic penumbra, and attenuation of inflammation and excitotoxicity, while the recovery after the initial 2 weeks is likely due to

neurovascular plasticity. Although the adult brain of mammals is a highly inhibitory environment for neuronal regeneration, the ischemic brain responds dynamically to stroke. Profound neurorestorative processes including angiogenesis, neurogenesis, neurite plasticity and synaptogenesis, are induced in brain tissue in response to focal cerebral ischemia (Cramer and Chopp, 2000; Zhang and Chopp, 2009). Specific cellular and molecular events open a crucial time window to create a unique regeneration-permissive microenvironment in the post-acute ischemic phase (Carmichael, 2006); they induce substantial structural and functional remodeling of the remaining intact brain tissue and lead to neurological recovery. As an active participant of these events, astrocytes play important roles in mediating endogenous repair after stroke, leading to functional improvement.

5.1. The functional roles of reactive astrogliosis and glial scar for stroke recovery

Because the glial scar provides a physical barrier for axonal growth, and contains factors inhibitory to axon growth cones (Fitch and Silver, 2008), the glial scar is thought to be detrimental for neurological recovery after stroke. Indeed, in $GFAP^{-/-}Vim^{-/-}$ mice, axonal regeneration and functional recovery after spinal cord trauma is improved (Menet *et al.*, 2003), and these mice also show increased hippocampal neurogenesis in adulthood (Larsson *et al.*, 2004), and in older mice (Kinouchi *et al.*, 2003). Genetic ablation of GFAP and vimentin when combined with neuronal overexpression of Bcl-2 improves regeneration of the severed optic nerve in the postnatal period (Wilhelmsson *et al.*, 2012). $GFAP^{-/-}Vim^{-/-}$ mice also support better integration of neural grafts (Widestrand *et al.*, 2007) and increased neuronal and astrocytic differentiation from neural stem cells transplanted in the hippocampus (Sutherland *et al.*, 2012). Thus, at least in some disease contexts, the benefits of reactive gliosis at the acute injury phase seem to be counteracted by restricted regenerative potential during the later repair stages (Pekny

et al., 2014). In adult mice after ablation of astrocyte reactivation and scar formation in astrocytes adjacent to a forebrain stab injury, injured tissue that was depleted of GFAP-positive astrocytes exhibits a pronounced increase in local neurite outgrowth. However, also present were a prolonged 25-fold increase in infiltration of CD45-positive leukocytes, failure of BBB repair and substantial neuronal degeneration (Bush *et al.*, 1999), indicating that reactive astrogliosis may also positively contribute to endogenous repair mechanisms following a stroke.

Considering the multifaceted effects of reactive astrocytes as inhibiting axonal growth and supporting neuronal survival, it is important to address the question of how reactive astrocytes affect neurological recovery post stroke? We therefore examined whether the absence of the two major astrocytic intermediate filament proteins, GFAP and Vimentin, would impact functional recovery and axonal remodeling after stroke in $GFAP^{-/-}Vim^{-/-}$ mice (Liu *et al.*, 2014). In this study, we performed a unilateral photothrombosis to the forelimb motor area to generate a consistent focal cortical ischemia of equivalent size in both WT and $GFAP^{-/-}Vim^{-/-}$ mice. We found that motor functional recovery and corticospinal tract (CST) axonal length in the denervated side of the cervical gray matter were significantly reduced, while CSPG expression was significantly increased in the lesion remote areas in both hemispheres, but decreased in the ischemic lesion boundary zone in $GFAP^{-/-}Vim^{-/-}$ mice, compared to WT mice.

Consistent with previous studies (Menet *et al.*, 2003; Pekny *et al.*, 1999; Wilhelmsson *et al.*, 2004), our results indicated that mice deficient in both GFAP and vimentin genes exhibit attenuated astrocytic reactivity after cortical stroke. Unexpectedly, our behavioral functional data showed that the attenuated glial scar did not lead to improved functional recovery after stroke, as in the increased functional restoration observed in $GFAP^{-/-}Vim^{-/-}$ mice subjected to spinal cord injury (Menet *et al.*, 2003). This indicated that unlike spinal cord injury, the glial scar formation

in the infarct proximal boundary region is not a major barrier factor for neurological recovery after cerebral stroke. After spinal cord injury, extension of axons to bridge damaged tissue, may enhance functional recovery. Thus, reduced scar formation and reduced CSPG at the site of the damage may foster neurite growth and functional recovery. In contrast, after induction of stroke, reduction of the adjacent glial scar and reduction of CSPG, likely has no beneficial effect, since there is no neural cell survival in the ischemic infarct core area, thus, no necessity or benefit of promoting neurite extension and growth crossing the glial scar into the lesion boundary zone. Thus, our data suggest that the glial scar may have restorative effects, and restricting extension of the ischemic lesion, and thereby promoting neurological recovery post stroke. Thus, the reduced CST axonal remodeling and neurological recovery in $GFAP^{-/-}Vim^{-/-}$ mice after stroke may be attributed to upregulated CSPG expression in the remote areas surrounding a subpopulation of astrocytes and neurons, although the specific types of these astrocytes and neurons remain to be further characterized.

5.2. Role of astrocytes in neurogenesis and neuronal plasticity after stroke

New neurons could be helpful in the ischemic brain to restore the disrupted neuronal network and impaired neurological function. In the adult mammalian brain, neurogenesis is mainly restricted to two neurogenic niches: the subventricular zone (SVZ) of the lateral ventricles (Doetsch *et al.*, 1999) and the subgranular zone (SGZ) of the hippocampus (Kaplan and Hinds, 1977). In response to stroke, neurogenesis in the SVZ is significantly upregulated (Jin *et al.*, 2001; Lin *et al.*, 2015; Zhang *et al.*, 2001). Neuroblasts migrate toward the area of ischemic damage in the striatum (Arvidsson *et al.*, 2002; Parent *et al.*, 2002; Zhang *et al.*, 2007a) and cortex (Jin *et al.*, 2003; Ohab *et al.*, 2006), since reactive astrocytes as well as activated microglia in the ischemic area secrete the neuroblast attracting chemokine stromal cell-derived

factor-1 (SDF-1) after MCAo and hypoxic-ischemic injury (Imitola *et al.*, 2004; Thored *et al.*, 2006). In the ischemic striatum the number of astrocytes is increased after stroke, and the long processes of astrocytes form a network linking the SVZ and the ischemic striatum (Teramoto *et al.*, 2003). These astrocytes may contribute to guiding neuroblasts migrating to the infarcted brain area (Parent *et al.*, 2002; Zhang *et al.*, 2007b).

Astrocytes may also play essential roles in regulating neurogenesis. Astrocytes actively regulate adult neurogenesis both by instructing neuronal fate commitment and by promoting proliferation of adult neural stem cells in culture (Song *et al.*, 2002). A recent in vitro study demonstrated that when neural stem cells treated with astrocyte-conditioned medium obtained from astrocytes are stimulated by lipopolysaccharide, both proliferation and differentiation of these neural stem cells are significantly increased (Wang *et al.*, 2011). SVZ astrocytes are the primary precursors for new neurons generated during regeneration and under normal conditions in the adult rodent brain (Doetsch *et al.*, 1999). Hippocampal neurogenesis under basal conditions as well as after injury is also increased in $GFAP^{-/-}Vim^{-/-}$ mice (Larsson *et al.*, 2004). In addition, both in vitro and in vivo studies have demonstrated that astrocytes can be efficiently reprogrammed into neurons. Astrocytes in the adult mouse brain parenchyma carry a latent neurogenic program, which may be elicited by stroke, or by blocking Notch signaling, that could potentially obviate the need for neuronal replacement strategies (Magnusson *et al.*, 2014). Interestingly, the reprogrammed glia-derived neurons may establish a glutamatergic neuronal network (Blum *et al.*, 2011), and it is possible to drive these cells toward the genesis of fully functional, synapse-forming, glutamatergic, i.e., excitatory, and GABAergic, i.e., inhibitory, neurons, respectively (Heinrich *et al.*, 2012).

Neurite extension may depend on a balance of growth-promoting and growth-inhibiting molecules in the extracellular matrix after injury. The CNS response to stroke is a multicellular process that changes continually over time and is regulated by a multitude of extracellular and intracellular molecular signaling events. Depending on the timing and local environments after stroke, reactive astrocytes may be beneficial or detrimental (Lo, 2008). Astrocytes, together with microglia, also release trophic factors such as bFGF, NGF, CNTF (Hansson and Ronnback, 2003), GDNF (Schaar *et al.*, 1993) and BDNF (Bejot *et al.*, 2011), thus promoting neuronal plasticity, synaptic formation, and rebuilding of the nervous system to improve functional outcome after injury. On the other hand, reactive astrocytes may release proteolytic molecules, such as matrix metalloproteinases, to degrade CSPGs (Zuo *et al.*, 1998), consistent with our data that attenuated astrocytic activation induced increased CSPG expression and CST axonal remodeling in $GFAP^{-/-}$ $Vim^{-/-}$ mice after stroke (Liu *et al.*, 2014).

Astrocyte-released trophic factors may act to protect neuronal survival during the acute phase after stroke, while they may also contribute to neural repair and plasticity later (Ridet *et al.*, 1997). For example, bFGF increases the outgrowth rate of axons and dendrites and may also increase the complexity (branching) of the neurites of primary cultured neurons (Zhang *et al.*, 2011). BDNF gene expression is upregulated in reactive astrocytes one day after MCAo (Zamanian *et al.*, 2012), and BDNF protein levels are increased eight days after infarction by microsphere injection (Bejot *et al.*, 2011). Over-expression of BDNF in ischemic rat brain promotes neurite outgrowth (Zhang *et al.*, 2011). In addition to the neuroprotective effects in acute ischemic stroke, delayed treatment with EPO enhances neurogenesis and angiogenesis and improves neurological function in rats (Wang *et al.*, 2004), and the enhanced neurological recovery is associated with structural remodeling of ischemic brain tissue and contralesional CST

axonal sprouting in mice subjected to MCAo (Reitmeir *et al.*, 2011), demonstrating the EPO also plays important restorative roles during stroke recovery. CNTF is a potent neural cytokine with very low expression in the CNS, predominantly by astrocytes. CNTF is rapidly increased when neuron-astrocyte contacts are lost after neuronal death in stroke, while once new neurons, or processes of surviving neurons make contact with astrocytes, CNTF expression is repressed (Mattson *et al.*, 1989). This regulation may make CNTF a possible target for astrocytic promotion of axonal outgrowth and plasticity in stroke treatment.

5.3. Role of astrocytes in synaptogenesis and synaptic plasticity after stroke

As astrocytic processes envelope synapses and regulate synaptic efficacy, astrocytes secrete numerous factors indispensable for maintaining synapses after injury and promoting synaptogenesis. Cholesterol produced and secreted by astrocytes is required as a building material for new membranes for the assembly of presynaptic components such as synaptic vesicles and release sites (Mauch *et al.*, 2001; Thiele *et al.*, 2000). Hippocampal neurons form more synapses when cultured in the presence of astrocytes in serum-free medium, in which a significant increase in agrin expression is detected in astrocytes (Tournell *et al.*, 2006). Astrocytes also control synaptogenesis via thrombospondins (TSPs)-1 and -2. TSP-1 and -2 act as a permissive switch that control the timing of CNS synaptogenesis by enabling neuronal molecules to assemble into synapses within a specific window of CNS development (Christopherson *et al.*, 2005). Although TSP1 and TSP2 levels are normally low in the adult brain, the expression of these proteins are increased by reactive astrocytes and activated microglia after focal cerebral ischemia/reperfusion in mice (Lin *et al.*, 2003). Additionally, TSPs-1/2 double knockout mice show defects in synaptogenesis and axonal sprouting post-stroke (Liauw *et al.*, 2008), indicating the importance of astrocytes in promoting the formation,

plasticity and repair of synapses that are necessary for stroke recovery.

5.4. Role of astrocytes in angiogenesis and BBB repair after stroke

Astrocytes may also mediate stroke recovery through enhancing angiogenesis and BBB repair. One of the most important facets of early neurovascular damage is BBB leakage. VEGF is a potent mitogen for endothelial cells and is rapidly produced in the brain in response to both hypoxia and cytokines. After focal cerebral ischemia, endogenous VEGF expression is upregulated in both neurons and astrocytes for up to 2 weeks (Bernaudin *et al.*, 2002; Sun *et al.*, 2003). VEGF increases BBB permeability and thereby exacerbates BBB leakage in the acute phase; however, late administration of VEGF enhances angiogenesis in the ischemic brain, improving neurological recovery in stroke (Zhang *et al.*, 2000). Sonic hedgehog (Shh) released from astrocytes promotes BBB formation and integrity by upregulating tight junction proteins in capillary endothelial cells (Tian and Kyriakides, 2009). In a coculture system of brain microvascular endothelial cells with astrocytes, OGD-activated astrocytes increase Shh secretion and promote cerebral angiogenesis following ischemia (Wang *et al.*, 2014b). Down-regulation of Shh expression in astrocytes disrupts the BBB (Alvarez *et al.*, 2011), suggesting that stimulation of astrocytic Shh production could promote restoration of BBB integrity. Shh further induces upregulation of angiopoietin-1 in astrocytes, which is necessary for vessel maturation (He *et al.*, 2013).

Matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), are upregulated in reactive astrocytes following ischemic stroke (Cunningham *et al.*, 2005). In the initial stage of stroke, MMPs damage the BBB by degrading neurovascular matrix and cause edema, hemorrhage and neuronal death, but they have a beneficial role during neurovascular repair phases for promoting angiogenesis of new blood vessels and restoration of

the blood brain barrier. MMP-9 is upregulated in peri-infarct cortex, and is colocalized with markers of neurovascular remodeling at 7-14 days after focal cerebral ischemia in rats; inhibition of MMPs at 7 days after stroke suppresses neurovascular remodeling, increases ischemic brain injury and impairs functional recovery at 14 days (Zhao *et al.*, 2006). MMP-9 deficient mice show prolonged BBB leakage (Tian and Kyriakides, 2009). Experiments in knockout mice also demonstrate that that astrocyte-derived TSP-2 is critical for the maintenance of physiological MMP-2 and MMP-9 levels and contributes to the repair of the BBB (Tian *et al.*, 2011).

High-mobility group box 1 (HMGB1), a DNA binding protein that acts as an inflammatory mediator, is upregulated and released from astrocytes in peri-infarct cortex after MCAo (Kim *et al.*, 2008). Metabolic inhibition of reactive astrocytes with fluorocitrate (Jain, 2003) or siRNA suppression of HMGB1 in astrocytes (Hayakawa *et al.*, 2010) induce a significant decrease in HMGB1-positive reactive astrocytes and neurovascular remodeling as well as a corresponding worsening of behavioral recovery. In addition, HMGB1 signaling may also promote endothelial activation (Hayakawa *et al.*, 2012), and endothelial cell migration and sprouting (Treutiger *et al.*, 2003).

6. Astrocytes for neuroprotective therapeutic strategies

Given the neurological deficits post stroke are in general attributed to the death or dysfunction of neurons, therefore, saving neurons, i.e. neuroprotection, is a reasonable, if not a primary therapeutic goal. Thus, over the past decades, enormous efforts and impressive advances have been made in the laboratory on the development of neuroprotective agents for acute ischemic stroke treatment, focusing on therapeutic mechanisms involving excitotoxicity and ionic imbalance, oxidative stress, neuroinflammation and apoptotic-like pathways in neurons. However, all of these neuroprotective agents have failed to demonstrate efficacy in clinical trials

of stroke (Han *et al.*, 2013). Although many factors have been identified that likely contribute to the failure in clinical translation of these neurocentric stroke therapies, it is possible that saving neurons alone is not sufficient to benefit all cell types in the brain after stroke, especially astrocytes. Given their essential role detrimental or beneficial, in neuroprotection during ischemia, astrocytes may be worthy therapeutic targets.

6.1. Therapies aimed at protecting astrocytes

Glutamate uptake by astrocytes is a critical mechanism for preventing excitotoxic neuronal death after stroke. However, an excess of glutamate can also cause death of astrocytes. It has been demonstrated both in vitro and in vivo that glutamate-induced apoptosis of astrocytes is efficiently inhibited by FK506, an inhibitor of calcineurin, and an immunosuppressive drug, suggesting that FK506-mediated neuroprotection in ischemia may be attributed to modulation of glutamate-induced astrocyte death early after reperfusion (Szydlowska *et al.*, 2006).

Activation of the glial-specific purinergic receptor, P2Y1R, which increases mitochondrial O_2 consumption and ATP production (Wu *et al.*, 2007), reduces both astrocyte swelling neuronal damage and cell death, and thereby reduces size of brain infarcts in a photothrombotic mouse model of stroke (Zheng *et al.*, 2013; Zheng *et al.*, 2010), suggesting that stimulation of astrocyte ATP production is potentially a robust therapeutic strategy to treat brain damage. In addition, administration of TGF-α, a known mitogenic growth factor, which promotes sequential conversion of mature astrocytes into neural progenitors and stem cells (Sharif *et al.*, 2007), leads to reduced infarct size and improved functional recovery in rats following MCAO (Justicia *et al.*, 2001).

Although EPO has not demonstrated a therapeutic effect for acute ischemic stroke in a Phase III double-blinded, placebo-controlled trial (Ehrenreich *et al.*, 2009), it could still be a potential

agent to improve functional outcome after stroke, since the reason of the failure of the clinical may be attributed to the combination treatment of EPO with tPA in a substantial percentage of treated patients, while in subgroup analysis the patients not receiving tPA and treated with EPO had an improvement in their NIHSS scores at 30 and 90 days after stroke. EPO may inhibit astrocyte swelling in the penumbra through an effect on AQP4 water permeability, thereby preserving astrocyte function and increasing the potential of tissue salvation (Gunnarson *et al.*, 2009).

Results from several studies suggest that attenuated astrocyte response often correlates with decreased infarct size. For example, focal cerebral ischemia induced astrocyte proliferation and delayed neuronal death is attenuated by nonspecific cyclin-dependent kinase inhibition (Wang *et al.*, 2008). In focal cerebral ischemic rats, neuron loss in the ischemic core 24 h after reperfusion and astrocyte proliferation in the boundary zone 14 days after ischemia are reduced by treatment of pranlukast, a cysteinyl leukotriene receptor 1 antagonist (Fang *et al.*, 2006), or caffeic acid (Zhou *et al.*, 2006). Discrepancy in these findings may be due to differences in time of neuronal loss and astrogliosis observed. In addition, it is also difficult to determine cause and effect, since the extent of astrogliosis likely reflects the severity of the injury.

6.2. Therapies aimed at astrocytic anti-excitotoxicity and antioxidants

Glutamate is the principal excitatory neurotransmitter in the nervous system. Given that astrocytes protect neurons from glutamate excitotoxicity and oxidative stress, several studies have demonstrated the therapeutic potential of increasing astrocyte glutamate transport after stroke. Increasing glutamate transporter GLT-1 expression in astrocytes reduces neuronal injury in both hippocampal slice culture and ischemic rats treated with ceftriaxone, a GLT-1 transporter activator (Chu *et al.*, 2007; Ouyang *et al.*, 2007). Administration of carnosine, a naturally

occurring dipeptide with multiple neuroprotective properties, significantly improves neurological function and decreases infarct size in mice subjected to MCAo, via preserving the expression of GLT-1 on astrocytes, and decreasing the glutamate levels resulting in attenuated excitotoxicity (Shen *et al.*, 2010b). Tamoxifen, a selective estrogen receptor modulator, enhances the expression and function of GLT-1in rat astrocytes (Lee *et al.*, 2009). Administration of tamoxifen reduces infarct volume and improves neurobehavioral outcome after reversible MCAo in adult male rats (Kimelberg *et al.*, 2000; Zhang *et al.*, 2005) or permanent MCAO in adult female rats (Mehta *et al.*, 2003).

Additionally, genetic overexpression of heat shock protein 72 (HSP72) or mitochondrial superoxide dismutase 2 (SOD2), two well-studied neuroprotective proteins, specifically in astrocytes reduces neuronal vulnerability to forebrain ischemia by preservation of the astrocytic GLT-1 and reduced oxidative stress (Shen *et al.*, 2010b). Similarly, selective over-expression of excitatory amino acid transporter 2 (EAAT2, also known as GLT-1) in astrocytes enhances neuroprotection from moderate hypoxia-ischemia (Chu *et al.*, 2007).

Glutathione is the most important antioxidant molecule found in the brain that protects cells from toxins such as free radicals. Pyruvate, an endogenous metabolite of glycolysis, is an anti-toxicity agent. In the neuron-astrocyte co-culture but not in the pure neuronal cultures, pyruvate protects neurons from glutamate-induced toxicity by up-regulating the synthesis of glutathione in astrocytes (Weller *et al.*, 2008). Treatment with dehydroascorbic acid, a BBB permeable oxidized form of ascorbic acid, reduces infarct volume and neurological deficits in mice after both permanent and transient MCAo (Xu *et al.*, 2010). Propofol increases the ability of astrocytes to accumulate intracellular ascorbate from dehydroascorbic acid (Miao *et al.*, 2011), therefore, propofol reduces infarct volume, decreases neurological deficit scores and attenuates

neuron apoptosis in rats after MCAo (Huang *et al.*, 2001). Thus, as important producers of antioxidants in the brain, treatments targeting astrocytes to enhance their anti-oxidative function may protect neuronal survival and promote neurological outcome after stoke.

6.3. Therapies aimed at astrocyte-derived growth factors

As noted above, ischemic lesion in the brain stimulates increased expression and release of several neurotrophic factors from reactive astrocytes supplying the neurons with appropriate factors necessary for their survival and maintenance. It is reasonable to further enhance the expression of these neurotrophic factors from activated astrocytes to reduce the ischemic lesion by pharmacological treatments. For example, galectin-1, a member of the family of beta-galactoside binding proteins, induces astrocyte differentiation and strongly inhibits astrocyte proliferation, and then the differentiated astrocytes greatly enhance their production of BDNF (Wang *et al.*, 2009). Endogenous galectin-1 was found to be markedly upregulated, paralleled with increased astrocytic BDNF production under ischemic conditions both in vitro and in vivo, while brain infusion of galectin-1 to rats subjected to photochemical cerebral ischemia enhances the expression and secretion of astrocytic BDNF, reduces neuronal apoptosis in ischemic boundary zone and improves functional recovery (Daskalopoulos *et al.*, 2002). Administration of the transglutaminase inhibitor cystamine also increases BDNF levels and phosphorylation of TrkB in brain, and enhances neuronal progenitor cell proliferation, neuronal survival, and axonal plasticity in mice brain following photothrombotic stroke (Qu *et al.*, 2010). Pyruvate is the end product of glycolysis, and it is known to be cytoprotective through antioxidant and anti-inflammatory mechanisms. Studies in both in vitro and in vivo ischemic models indicate that pyruvate may stabilize hypoxia-inducible factor-1α in both neurons and astrocytes, and thereby drives endogenous pyruvate EPO synthesis, and protects the brain against ischemia-reperfusion

injury (Li *et al.*, 2015b).

7. Astrocytes for neurorestorative therapeutic strategies

The entire brain appears primed for recovery. Spontaneous recovery after a stroke is often evident in experimental animals and patients, however, recovery is generally incomplete, indicating that the endogenous remodeling of the CNS is not sufficient to restore neurological function. Therefore, pharmacological or cell-based therapeutic approaches aim to capitalize on these recovery events to further stimulate and amplify endogenous restorative mechanisms. The goal of these restorative therapies is to promote repair and restoration of function within surving neural tissue, and not necessarily to salvage acutely threatened brain tissue before the onset of death. The major advantage of restorative therapies is the extended therapeutic time window in days to weeks or even months after an ischemic event, which allows for the combination of different rehabilitative approaches to maximize the therapeutic benefit in a large number of patients.

7.1. Therapies aimed at glial scar and inhibitory molecules

Despite its initial protective role in limiting lesion site, astrocytes form a glial scar along the ischemic lesion and produce proteoglycans that act as physical and biochemical barriers to inhibit outgrowth of regenerating axons (Bidmon *et al.*, 1998). Therefore, inhibitory treatments of glial scar have been considered useful for neuronal regeneration. Several studies have attempted to degrade or suppress the production of inhibitory components from the scar tissue.β-adrenergic receptors directly regulate astrogliosis and glial scar formation. Stimulation of β-adrenergic receptors with agonists leads to increased astrogliosis, while treatment with antagonists reduces astrocytic activation (Hodges-Savola *et al.*, 1996). Astrocytes express CD36, a class B scavenger receptor which mediates free radical production and tissue injury, in a

temporally and spatially restricted pattern in the peri-infarct area post-ischemic stroke (Cho *et al.*, 2005b). CD36 deficiency attenuates the proliferation of astrocytes and stroke-induced GFAP up-regulation and scar formation, indicating that CD36 is a mediator of injury-induced astrogliosis and scar formation (Bao *et al.*, 2012). The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase, involved in cell proliferation, migration, autophagy and protein synthesis. In cultured astrocytes exposed to OGD, mTOR blockade by its inhibitor rapamycin, attenuates astrocyte migration, proliferation and production of inflammation mediators (Li *et al.*, 2015a), suggesting that targeting the mTOR pathway in astrocyte activation may represent a potential therapeutic strategy against deleterious neurotoxic processes of reactive astrogliosis in ischemic stroke. Since the reactive astrocytes are an important source of neurotrophic factors that support neuronal survival under lesion conditions, contrary strategies have also been proposed to further increase the process of reactive astrogliosis, thereby enhance synthesis and release of neurotrophic factors from the astrocytes and improve neurological outcome after stroke. For example, selegiline, an irreversible monoamine oxidase B inhibitor, potentiates astrocytic reaction to injury and increases the expression of NGF, thereby protects cortical tissue from ischemic insult after MCAo in rodents (Semkova *et al.*, 1996), and facilitates recovery in stroke patients (Sivenius *et al.*, 2001). Thus, regulation of the reactive astrocytes may be utilized to improve functional recovery after stroke. The therapeutic effects of either inhibition or further increase of astrocytic reactivity to reduce the detrimental effects and/or amplify the beneficial effects, probably depend on the timing and severity of the ischemic infarct.

Neuroplasticity plays an essential role in rehabilitation and functional recovery after stroke. Neurocan is a powerful inhibitor of axonal growth cones and neurite extension among CSPGs, the major constituent of the glial scar. Enzymatic disruption of neurocan with chondroitinase

ABC, which cleaves glycosaminoglycan side chains, infused directly into the infarct cavity for 7 days, beginning 7 days after MCAo, improves gross anatomical, histological, and functional outcome in the chronic phase of experimental stroke in rats (Hill *et al.*, 2012). In addition, using an in vitro interface model of activated astrocytes, lentiviral delivery of RNAi targeting two key CSPG synthesis enzymes, chondroitin polymerizing factor and chondroitin synthase-1, reduces CSPG levels and enhance axon growth (Tuinstra *et al.*, 2013). Ephrin-A5 is expressed in reactive astrocytes in the peri-infarct region, and inhibits axonal sprouting; while inhibition of ephrin-A5 in the peri-infarct area 7 days after stroke promotes axonal outgrowth and behavioral recovery in mice (Overman *et al.*, 2012). Such delayed treatments provide a promising opportunity with a long therapeutic window to improve recovery after stroke.

7.2. Therapies aimed at astrocyte-mediated neurogenesis

Although ischemic stroke induces endogenous neurogenesis and some of these newly generated neuroblasts change their route of migration and target the ischemic boundary regions, this endogenous neurogenesis is limited and survival of these neurons is extremely poor, and the vast majority of neuroblasts die (Jin *et al.*, 2003; Zhang *et al.*, 2004). Some studies have specifically targeted astrocytes and astrocyte-derived molecules to amplify endogenous neurogenesis and to improve the ischemic microenvironment to be receptive to integration of these newly arriving cells within the tissue. For example, treatment with cilostazol, a type 3 phosphodiesterase inhibitor, increases BDNF expressing in astrocytes and neural progenitor cell generation both in the ipsilateral SVZ and peri-infarct area (Sasaki *et al.*, 2004).

Astrocytes isolated from rodent postnatal brain can be redirected toward neurons following forced expression of transcription factor Pax6 (Heins *et al.*, 2002), proneural genes neurogenin-2 and mammalian achaeteschute homolog 1 (Mash1) (Berninger *et al.*, 2007), or distal-less

homeobox 2 (DLX2) (Heinrich *et al.*, 2010). Interestingly, neurons reprogrammed from astrocytes can acquire electrical properties compatible with a mature neuronal phenotype of glutamatergic, GABAergic or dopaminergic neurons (Addis *et al.*, 2011; Berninger *et al.*, 2007). An in vivo study also demonstrates that a single transcription factor SOX2 is sufficient to reprogram resident astrocytes into proliferative neuroblasts in the adult mouse brain even in aged brains, while when supplied with BDNF and noggin or when the mice are treated with a histone deacetylase inhibitor, iANBs develop into electrophysiologically mature neurons, which functionally integrate into the local neural network (Niu *et al.*, 2013). Recently, reprogramming of astrocytes in the glial scar into neurons demonstrates the therapeutic potential toward regeneration of nervous tissue after brain injury (Guo *et al.*, 2014). Reactive glial cells, including both astrocytes and NG2 glia, in the glial scar can be reprogrammed into functional neurons in the adult mouse cortex with a retrovirus-encoded single transcription factor, NeuroD1 (Guo *et al.*, 2014). Importantly, conversion of reactive astrocytes into neurons may not only contribute to replace neuronal populations lost, but also reduce the regeneration-inhibitory factors to create a more permissive environment for neuronal growth and synaptic integration.

7.3. Therapies aimed at astrocyte-mediated angiogenesis and neurovascular repair

Post-stroke angiogenesis not only improves blood perfusion in the brain ischemic boundary tissue, but also supports the brain parenchymal cell, including astrocytes, release of neurotrophic factors, to promote neurogenesis, which thereby enhances brain remodeling and subsequent improves long-term neurological function after stroke (Zhang and Chopp, 2009). Therefore, enhancing angiogenesis is a key restorative mechanism and represents an important therapeutic strategy for ischemic stroke. Thus, enhancement of astrocyte mediated angiogenesis, as a restorative therapeutic target has been tested in several studies.

Treatment with ecdysterone improves neurologic function by enhancing astrocyte activation and angiogenesis after focal cerebral ischemia in rats (Luo *et al.*, 2011). Transgenic overproduction of omega-3 polyunsaturated fatty acids in mice improves post-stroke revascularization and enhances endogenous angiogenesis by inducing angiopoietin 2 production in astrocytes, which subsequently promoted endothelial cell proliferation and BBB formation, suggesting that omega-3 polyunsaturated fatty acid supplementation is a potential angiogenic treatment capable of augmenting brain repair and improving long-term functional recovery after cerebral ischemia (Wang *et al.*, 2014a). Treatment with selegiline, an inhibitor of B-type monoamine oxidase, enhances Notch-Jagged signaling in astrocytes of the peri-infarct region, improves the functional integrity of the neurovascular unit, and reduces peri-lesional edema following focal ischemia in rats (Nardai *et al.*, 2015). Therefore, astrocytes as a mediator in angiogenesis may provide a treatment target for the development of new neurorestorative therapies of stroke.

7.4. Astrocytes in mediating cell-based therapies for ischemic stroke

Cell-based therapies have shown promise to improve neurological recovery in stroke treatment. Among cells under intense investigation are bone-marrow stromal or mesenchymal cells (MSCs) (Chen *et al.*, 2001a; Chopp and Li, 2002), human umbilical cord blood cells (Chen *et al.*, 2001b), neural stem cells (Abe, 2000), and embryonic stem cells (Wei *et al.*, 2005). MSCs are among the most exciting emerging therapies for improving functional recovery after stroke (Joyce *et al.*, 2010; Li and Chopp, 2009). Rather than cell replacement, exogenous cell transplantation triggers and amplifies the endogenous neurorestorative processes including angiogenesis (Chen *et al.*, 2004), neurogenesis (Chen *et al.*, 2004), synaptogenesis (Shen *et al.*, 2007) and axonal remodeling (Liu *et al.*, 2007; Liu *et al.*, 2010) within the brain and spinal cord that may contribute to neurological recovery after stroke.

Astrocytes may play an important role in many restorative events associated with cell-based therapies to promote neurological recovery. In MCAo rats treated with MSCs, the processes of astrocytes remodel from hypertrophic star-like to tadpole-like shape and orient parallel to the ischemic regions, while axonal projections emanating from individual parenchymal neurons exhibit an overall orientation parallel to elongated radial processes of reactive astrocytes (Li *et al.*, 2006), suggesting that astrocytes may support and guide axonal growth in the ischemic boundary zone and the subventricular zone after stroke. MSCs increase astrocyte survival via upregulation of phosphoinositide 3-kinase/threonine protein kinase and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathways and stimulate astrocyte trophic factor gene expression including BDNF, VEGF and bFGF in cultures after ischemia (Gao *et al.*, 2005b). MSCs secrete soluble factors that increase Cx43 expression and thereby enhance gap junction channels of astrocytes (Gao *et al.*, 2005a). MSCs induce bone morphogenetic proteins 2/4 in astrocytes after OGD, which promotes an astrocytic phenotype in adult subventricular progenitor cells (Xin *et al.*, 2006). MSC administration increases GDNF expression of astrocytes in the ischemic boundary area in rats following MCAo in vivo and in astrocyte primary culture after OGD in vitro (Shen *et al.*, 2010a), as well as increases VEGF, Ang1 and its receptor Tie2 express levels in astrocytes (Zacharek *et al.*, 2007). Muti-trophic factor expression by astrocytes after MSC treatment in vivo and in vitro, provides a basis for astrocytic mediation of enhaced neuronal survival, angiogenesis and vascular integrity in the ischemic brain.

Therapeutic benefits of MSC treatment may also be mediated by increase of astrocyte-derived tPA activity. MSC transplantation post stroke induces increased activation of tPA and down-regulation of plasminogen activator inhibitor-1 (PAI-1) levels in astrocytes in the ischemic

boundary zone (Xin *et al.*, 2011; Xin *et al.*, 2010). This increased tPA activity may be mediated by expression of Shh-Gli transcription factors in astrocytes stimulated by MSCs (Ding *et al.*, 2013; Stecca and Ruiz i Altaba, 2005). tPA may promote brain plasticity via proteolytic cleavage of the precursor forms of pro-BDNF and pro-NGF to their mature active forms of BDNF and NGF, respectively (Lee *et al.*, 2007), as well as tPA binding to N-methyl-D-Aspartate receptor (NMDAR), which can subsequently enhance neurite remodeling (Gakhar-Koppole *et al.*, 2008). Moreover, MSCs also diminish glial scar formation after stroke (Li *et al.*, 2005; Pavlichenko *et al.*, 2008). Down-regulation of neurocan expression, which is one of the main components of CSPGs expressed in the glial scar (Shen *et al.*, 2008), may also promote axonal regeneration and facilitate the neurorestorative effects of MSCs in the ischemic brain.

8. Summary

As astrocytes are the most abundant subtype of cells in the CNS, they are structurally and functionally involved in normal brain physiology, and ischemic pathological responses. They are a positively active participant of the tripartite synapses and the neurovascular unit, and make contact and communicate with all type of brain cells. Astrocytes, therefore, play important supporting and regulating roles in brain function and physiology. The astrocytic responses to ischemic stroke are extremely complex and incompletely understood. After a stroke, astrocytic inflammatory action may aggravate the ischemic lesion during the acute phase, while the glial scar in the peri-infarct area may obstruct axonal regeneration and subsequently reduce the functional outcome during the post-acute phase. However, on the other hand, astrocytes also play beneficial roles in neuroprotection, by limiting lesion extension, by reducing excitotoxicity and releasing neurotrophins. Astrocytes have potent neurorestorative effects after stroke by contributing to angiogenesis, neurogenesis, synaptogenesis, and axonal remodeling. Thus, the

detrimental and beneficial effects of astrocytes on functional outcome and neurological recovery after stroke designate astrocytes as a promising therapeutic target of pharmacological and cell-based approaches. Whether to reduce or further emphasize the astrocytic reactivity and function, probably depends on the timing of the ischemic lesion, the location of the astrocytes and the specific subtype of the astrocytes. Thus, to develop successful clinically relevant neuroprotective and neurorestorative strategies, additional research efforts on astrocytes are needed, in addition to the current neurocentric strategies.

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- Astrocytes play essential roles in maintaining normal brain function.
- Astrocytes perform multiple functions both detrimental and beneficial on neurological recovery after stroke.
- These detrimental and beneficial effects of astrocytes designate astrocytes as a promising therapeutic target for neuroprotection and neurorestoration.