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Reactive astrocytes and therapeutic potential in focal ischemic stroke

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Abstract

Astrocytes are specialized and the most abundant cell type in the central nervous system (CNS). They play important roles in the physiology of the brain. Astrocytes are also critically involved in many CNS disorders including focal ischemic stroke, the leading cause of brain injury and death in patients. One of the prominent pathological features of a focal ischemic stroke is reactive astrogliosis and glial scar formation. Reactive astrogliosis is accompanied with changes in morphology, proliferation and gene expression in the reactive astrocytes. This study provides an overview of the most recent advances in astrocytic Ca^{2+} signaling, spatial and temporal dynamics of the morphology and proliferation of reactive astrocytes as well as signaling pathways involved in the reactive astrogliosis after ischemic stroke based on results from experimental studies performed in various animal models. This review also discusses the therapeutic potential of reactive astrocytes in a focal ischemic stroke. As reactive astrocytes exhibit high plasticity, we suggest that modulation of local reactive astrocytes is a promising strategy for cell-based stroke therapy.

Keywords

Ischemic stroke; reactive astrocytes; glial scar; Ca^{2+} signaling; morphology; cell proliferation; stroke therapy

Introduction

Astrocytes are the most numerous glial cell type in the central nervous system (CNS). In a normal brain, there are two major types of astrocytes: Fibrous and protoplasmic astrocytes. They can be found in white matter such as the corpus callosum and in grey matter such as the cortex. Glial fibrillary acidic protein (GFAP) is primarily expressed in the thick main processes and has been considered as a ‘pan-astrocyte’ marker (Brenner, 2014), but its expression levels are higher in the fibrous astrocytes than in the protoplasmic astrocytes.

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Transcriptome analysis found that the Aldh1L1gene is most widely and homogenously expressed in the astrocytes, while immunostaining with an anti-Aldh1L1 antibody revealed that the Aldh1L1 protein is highly expressed in the astrocyte cell body and its extensive processes (Cahoy et al., 2008). Thus Aldh1L1 is now considered as a new 'pan-astrocyte' marker.

It has been long recognized that astrocytes play a critical role in the physiology and are very important for overall brain architecture as well as function. Astrocytes can maintain ionic homeostasis by acting as a potassium (K^+) sink to (Djukic et al., 2007). Astrocytes can remove synaptically released glutamate by their glutamate transporters to avoid glutamate excitotoxicity (Huang et al., 2004; Bergles et al., 1999). Astrocytes also mediate Ca^{2+} signaling and intercellular waves through the stimulation of different G-protein coupled receptors (GPCRs) via phospholipase-C/inositol 1,4,5-triphosphate (PLC/IP_3) pathway *in vivo*. These GPCRs include metabotropic glutamate receptors (mGluRs) (Ding et al., 2007; Fellin et al., 2004; Sun et al., 2013), P2Y receptors (Ding et al., 2009; Thrane et al., 2012; Sun et al., 2013; Wang et al., 2006; Nizar et al., 2013), $GABA_B$ receptors ($GABA_BRs$) (Ding et al., 2009; Meier et al., 2008), noradrenergic receptors (Bekar et al., 2008; Ding et al., 2013; Paukert et al., 2014). Due to the intimate physical contact with synapses, astrocytes are considered as a part of the 'tripartite' synapse where they can listen and talk to the synapse by regulating Ca^{2+} increase in response to neuronally released transmitters and by gliotransmitter release (Haydon, 2001). We have also known that astrocytes and the blood vessels have intimate anatomic relationship. This was further confirmed by studies using fluorescence imaging and electron microscopy showing that astrocyte endfeet almost completely cover the cerebral vascular surface (Simard et al., 2003; Petzold et al., 2008; Mathiisen et al., 2010; Kacem et al., 1998). Astrocytic Ca^{2+} signaling is involved in functional hyperemia from *in vitro* studies of brain slice preparations (Zonta et al., 2003; Mulligan and MacVicar, 2004; Gordon et al., 2008); however, its role in the regulation of cerebral blood flow (CBF) *in vivo* is controversial as suggested by results from studies using type 2 IP_3 receptor knockout mice (Jego et al., 2014; Takata et al., 2013; Nizar et al., 2013; Bonder and McCarthy, 2014).

Growing evidence indicates that astrocytes are heterogeneous in morphology, molecular expression and physiological function under normal conditions (Zhang and Barres, 2010; Matyash and Kettenmann, 2010). Morphologically, protoplasmic astrocytes and fibrous astrocytes are different. Protoplasmic astrocytes are complex (sponge like) and highly branched with numerous fine processes and their endfeet wrap around blood vessels, while fibrous astrocytes are less complex and have thicker and less branched processes (Wilhelmsson et al., 2006; Bushong et al., 2002). Numerous studies have found that different genes are expressed among different subsets of astrocytes *in vivo* (Zhang and Barres, 2010). GFAP expression is higher in the astrocytes in corpus callosum, but it is expressed in astrocytes in the cortex at lower levels (Xie et al., 2010). Electrophysiologically, astrocytes exhibit a different current-voltage relationship with one type of astrocytes, known as outward rectifying astrocytes, when compared to the other known as variably rectifying astrocytes (Zhou and Kimelberg, 2000). Astrocytes also exhibit different properties of Ca^{2+} signaling *in vivo.* Two-photon (2-P) in *vivo* Ca^{2+} imaging has

shown that astrocytes in the cortical layer 1 (L1) nearly doubled the Ca^{2+} activity compared to the astrocytes in L2/3 in anaesthetized rats; moreover, Ca^{2+} signals in the processes in the same astrocyte were asynchronous in L1 while those in L2/3 were more synchronous (Takata and Hirase, 2008). The morphological, molecular and functional heterogeneity of astrocytes indicates a diversity among astrocytes and the complex physiological and pathological roles that astrocytes play in the CNS.

Astrocytes respond to different neurological diseases through a common phenomenon of GFAP upregulation, a process termed as reactive astrogliosis. Severe CNS injuries such as stroke, traumatic brain injury (TBI), and spinal cord injury (SCI), as well as neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS) all cause a massive up-regulation of GFAP. Therefore, GFAP is considered a reliable marker to characterize reactive astrocytes. However, given the different causes and onsets of diseases, the temporal and spatial changes of the reactive astrocytes are different. For example, in the AD brain, due to slow disease progression, the reactive astrocytes are more evenly distributed and do not form glial scars. While after ischemic stroke or SCI, reactive astrocytes in the peri-infarct region express higher GFAP and eventually form glial scar, which establishes both a physical and biochemical barrier that separates dead and vital tissues. Thus, the properties of reactive astrocytes in chronic neurodegenerative diseases are different from those seen in acute conditions like focal ischemia and SCI. Although similar phenomena, such as glial scar formation is observed in both focal ischemia and SCI, in experimental animal models of SCI, the injury occurs in the large area of white matter rather than in grey matter as seen in ischemic strokes. The functions and role of reactive astrocytes have been much more extensively studied in SCI than in the focal ischemic stroke (for reviews see Burda and Sofroniew (2014); Sofroniew (2009); Sofroniew and Vinters (2010); Silver and Miller (2004); Rolls et al. (2009)). Thus, this article will review the dynamic changes in astrocytic Ca^{2+} signaling, morphology and proliferation of reactive astrocytes. The article also examines the distribution of reactive astrocytes surrounding the ischemic core, i.e., in the penumbra, in experimental animal models of focal ischemic stroke. Discussion then focuses on the signaling pathways involved in reactive astrogliosis after focal ischemia followed by the therapeutic potential of reactive astrocytes in ischemic stroke. For extensive reviews of reactive astrocytes in various aspects in different neurological diseases, readers are advised to consult a few detailed reviews (Burda and Sofroniew, 2014; Sofroniew and Vinters, 2010; Escartin and Bonvento, 2008; Anderson et al., 2014).

Dynamics of reactive astrocytes in the penumbra after focal ischemia

Focal ischemic stroke, resulting from the blockage of cerebral blood vessels, leads to cell death and brain damage and causes human disability and death (Stapf and Mohr, 2002). After the onset of ischemia, astrocytes undergo numerous pathological alterations over time including rapid swelling (Nedergaard and Dirnagl, 2005; Barber and Demchuk, 2003; Swanson et al., 2004; Li et al., 2014) and enhanced Ca^{2+} signaling (Ding et al., 2009). Astrocytes can also become reactive following ischemia. The hallmark of reactive astrogliosis is the morphological changes and the increased expression levels of GFAP (Nedergaard and Dirnagl, 2005; Panickar and Norenberg, 2005). Reactive astrocytes

eventual form glial scar in the penumbra that demarcates the ischemic core (infarction) from healthy tissue (Hayakawa et al., 2010; Barreto et al., 2011b; Shimada et al., 2011b; Bao et al., 2012; Li et al., 2014). In addition to reactive astrogliosis, focal ischemic stroke is also accompanied by other temporal and spatial dependent changes. As the clinical aim of stroke therapy is to salvage the penumbral cells, understanding the spatial and temporal changes of astrocytes at molecular and cellular levels will provide therapeutic strategy insights.

Ca^{2+} signaling in astrocytes after ischemia—A few *in vitro* and *in vivo* studies have demonstrated how ischemia induces enhanced Ca^{2+} signaling in different stages after ischemia onset (for extensive review see Ding (2013; 2014)). Using acute brain slice preparations and an oxygen-glucose deprivation (OGD) model, Duffy and MacVicar (1996) found that a short episode (5 min) of simultaneous hypoxia and hypoglycemia can induce an intracellular Ca^{2+} increase within an average of 7.5 min and it takes 2.5 min to reach a peak. After reoxygenation, astrocytic Ca^{2+} will remain elevated for a highly variable period of time ranging from several minutes to one hour. In the absence of extracellular Ca^{2+}, astrocytic Ca^{2+} increase can still be observed with a relatively consistent duration. Electrophysiological recordings have shown that hypoxia and hypoglycemia also depolarize astrocytes (Duffy and MacVicar, 1996). The data from brain slice experiments suggest that astrocytes can mediate Ca^{2+} increase from the internal store release and influx of voltage-dependent Ca^{2+} influx. Alternatively, internal Na^{+} accumulation can lead to Ca^{2+} influx through reverse operation of the sodium-calcium exchanger (NCX). In another study using brain slices, OGD induced slow inward currents (SICs), which were mediated by extrasynaptic N-methyl-D-aspartate (NMDA) receptors in rat hippocampal CA1 pyramidal neurons (Dong et al., 2013). Moreover, dialysis of 1,2-**b**is(o-**a**mino**p**henoxy)ethane-N,N,N',N'-**t**etra**a**cetic acid (BAPTA), an Ca^{2+} chelator, into the astrocytes reduced SIC frequency after OGD, implying that astrocytic Ca^{2+} activity stimulate extrasynaptic NMDA receptors. Using Ca^{2+} imaging, it was found that astrocytes are quiescent in spontaneous Ca^{2+} activity in brain slices under normal conditions, but astrocytes exhibits frequent Ca^{2+} elevations during OGD. Surprisingly, within the 10 min of OGD more than 60% of astrocytes have exhibited Ca^{2+} elevations. Moreover, a majority of astrocytes displayed more than two Ca^{2+} transients. To further demonstrate the importance of astrocytic Ca^{2+} activity in ischemia, slice preparations from type 2 IP_3R knockout mice were used. Astrocytes exhibited rare Ca^{2+} transients during OGD and low frequency SICs in CA1 neurons in IP_3R2 knockout mice as compared with wild type (WT) mice.

Using a photothrombosis (PT)-induced ischemia model and *in vivo* two-photon (2-P) imaging, Ding et al. (2009) found that astrocytes exhibit enhanced Ca^{2+} signaling characterized as intercellular Ca^{2+} waves starting ~20 min after PT. Both amplitude and frequency of astrocytic Ca^{2+} signal were significantly increased compared to relative quiescent Ca^{2+} signaling prior to ischemia. In addition, the magnitude of a Ca^{2+} signal was smaller in the penumbral region than the magnitude of a Ca^{2+} signal in the ischemic core. An important feature of the Ca^{2+} signals was that most of them started and returned to the basal level at the same time among the astrocytes in the imaging field, i.e., they exhibited a high degree of synchrony. Pharmacological study has suggested that glutamate and GABA released into the extracellular space following PT stimulated intercellular waves among

astrocytes. The results represent the first direct evidence of an astrocytic Ca^{2+} response after ischemia in an *in vivo* setting. Acute increase in astrocytic Ca^{2+} signals have also been observed after a single vessel occlusion by PT (Zheng et al., 2013). Acute response of astrocytes with enhanced Ca^{2+} signaling thus may reflect the initial step in reactivation of astrocytes after ischemia.

In addition to the acute changes in astrocytic Ca^{2+} signaling ~20 min after PT-induced ischemia, astrocytic Ca^{2+} signaling also changed in the chronic phase following ischemia. Winship et al. studied the neuronal and astrocytic Ca^{2+} signaling and functional rewiring in somatosensory neurons in the ipsilateral hemisphere after a stroke (Winship and Murphy, 2008). Astrocytic Ca^{2+} responses were examined in anesthetized mice to study Ca^{2+} responses in penumbra after two weeks, one month, and two months following PT. Their study observed a significant increase in the prevalence of responses to preferred limb stimulation in the somatosensory cortex. The selectivity of astrocyte responses was also significantly different between regions of overlap between contralateral hindlimb- and contralateral forelimb-evoked intrinsic optical signals and regions without intrinsic optical signals overlap. These results indicate that neuron–astrocyte communication is preserved, or even enhanced in the penumbra. The altered response of the astrocytic Ca^{2+} signaling to limbic stimulation in contralateral hemisphere in chronic phase after ischemia suggests the change of astrocytic Ca^{2+} plasticity after ischemia, which may contribute to brain remodeling during the recovery process. Further elucidation of the mechanisms of astrocytic Ca^{2+} signaling in acute and chronic phases can provide insights for potential therapeutic strategies for improved brain recovery after stroke.

Morphological change of reactive astrocytes and formation of glial scar after ischemia—It has been known that there is little expression of GFAP in the cortex of normal mice (also see previous studies (Zhang et al., 2010; Li et al., 2013; Li et al., 2014) (Figure 1A:A1). Astrocytes undergo dramatic changes in morphology associated with GFAP upregulation over time after an ischemic insult. Thus, GFAP expression has been used to inspect the morphological changes during astrogliosis following ischemia. Using endotelin-1 collagenase type IV-S injections, Mestriner et al. (2015) conducted a detailed study on morphology of reactive astrocytes in the penumbra 30 days after ischemic and hemorrhagic stroke in rats. The following parameters were analyzed based on GFAP staining in the regions of the sensorimotor cortex and dorsolateral striatum: 1) the GFAP immunohistochemistry intensity by measuring the optical density; 2) astrocyte morphology and polarization by measuring the the number (i.e., ramification) and length of primary processes of reactive astrocytes; 3) the density of GFAP+ astrocytes. As expected, optical density and GFAP+ astrocyte density significantly increased after stroke; but more importantly, the ramification and length of primary processes also increased compared with the astrocytes in a sham control group. No differences were found in these measurements based on ischemic and hemorrhagic stroke animals, but a difference was observed among different regions in the brain, indicating a regional heterogeneity in the morphology of reactive astrocytes. However, in this study, the morphological changes during early stages were not studied which could be very important in disease progression. Wagner et al. studied the morphology of reactive astrocytes in rats at day 4 after middle cerebral artery

occlusion (MCAo) (Wagner et al., 2012). Based on the results from GFAP immunostaining, the process volume, process diameter, process length and branching levels in reactive astrocytes in the penumbral region were increased compared with the astrocytes in contralateral hemisphere and in the remote regions away from the ischemic core. In addition, the process volume and diameter in the peri-infarct region were larger than those in the remote regions. However, the mean process length of astrocytes in the contralateral hemisphere was longer than reactive astrocytes in the penumbra and the remote region, and the mean process length of reactive astrocytes in the remote region was slightly but significantly longer than these in the penumbra. On the other hand, the mean process branching was similar in the penumbra and in the remote region. The data suggest that reactive astrocytes become hypertrophic rather than increasing the length of their process at relatively acute time points of the injury.

Since reactive astrogliosis after ischemia is highly dependent on time and location, it is necessary to quantitatively examine the changes with a high temporal resolution. Li et al. (2014) presented a high temporal resolution study on the dynamic changes of reactive astrocytes in the cortex using a PT-induced mouse ischemia model. GFAP expression was examined in mouse brains at days 2, 4, 6, 8, 10, 12, and 14 post PT using confocal microscopy. A significant increase of GFAP expression levels was observed at day 2 post PT (Figure 1A:A2). Astrocytes exhibited a stellate morphology and hypertrophy with highly upregulated GFAP expression up to day 4 after PT (Figure 1A:A3). From day 6 after PT, reactive astrocytes became densely packed and changes to a stream-like structure with their elongated processes directing towards the ischemic core, i.e., a feature of polarization of reactive astrocyte and astroglial scar formation (Figure 1A:A4). In addition, reactive astrocytes became less hypertrophic after day 6 post. After day 10, reactive astrocytes in the penumbra remained similar but with longer processes as compared to days 6-8, indicating the maturation of glial scar (Figure 1A:A5-A8). An significant increase in GFAP expression was also observed in the regions further away from the scar border but with similar morphology to the astrocytes under normal conditions. Thus, the morphology of GFAP+ astrocytes in the penumbra progressed through dynamic modifications over time with dramatic changes occurring between days 2-4 after PT.

In healthy mouse brains, astrocytes exhibit non-overlapping domains (Bushong et al., 2002). However, in an epileptic brain, it has been shown that reactive astrocytes interdigitate, i.e., they exhibit overlapping domains between the neighboring astrocytes (Oberheim et al., 2008). However, in an electrically induced mouse lesion model, reactive astrocytes in the cortex exhibit minimal interdigitation and are similar to those in the cortex of control animals (Wilhelmsson et al., 2006). Thus, it is likely that the severity of brain injury determines the degree of overlapping between reactive astrocytes. Although there is no report to indicate that reactive astrocytes interdigitate or remain non-overlapping domains after stroke, it is likely that they tend to interdigitate as stroke causes severe reactive gliosis and cell proliferation. Whether reactive astrocytes interdigitate after focal ischemia, and if they do, whether the degree of interdigitation is temporal and spatial dependent, are worth further investigation.

Reactive astrocyte proliferation after ischemia—Cell proliferation including reactive astrocyte proliferation in response to ischemic stroke has been well documented using PT-induced focal ischemia and MCAo models (Nowicka et al., 2008; Barreto et al., 2013; Haupt et al., 2007; Li et al., 2013; Shen et al., 2012; Stoll et al., 1998; Schroeter et al., 2002). Bromodeoxyuridine (Brdu) labeling and immunostaining can be used to assess the spatial and temporal changes in cellular proliferation (Barreto et al., 2011; Zamanian et al., 2012; Haupt et al., 2007; Panickar and Norenberg, 2005; Nowicka et al., 2008; Li et al., 2013). To precisely study the rate of proliferation of astrocytes and microglia at different times after ischemia, Li et al. (2014) developed a 'time-block' protocol to label proliferating cells in the penumbra at different times after ischemia. Mice were administered with Brdu at days 1, 3, 4, 5, 9, 11, and 13 post PT for two consecutive days and transcardially perfused after one day from the last injection. In normal mice, there were almost no Brdu+ cells in the cortex. The density of Brdu+ cells exhibited a temporal and spatial dependent manner (Li et al., 2014). Brdu+ cells significantly increased from day 1 to day 2 post PT and reached their peak during days 3 and 4 post PT; then, they declined over time and persisted until day 14 (Figure 1A-B). The region closest to the ischemic core had a higher density of Brdu+ cells than those regions further away from the ischemic core. These data demonstrated that the rate of proliferating cells generated in the penumbra is highly dependent on the location and time. Nevertheless, Brdu+ cell estimated from this labeling protocol may underestimate the total number of proliferating cells since a single daily injection of Brdu may not label all the proliferating cells (Wanner et al., 2013).

Although it is well known that the common feature of reactive astrocytes is increased GFAP expression levels, whether reactive astrogliosis is merely through the upregulation of GFAP or through cell proliferation is still not clear. The proliferation of reactive astrocytes can be studied using GFAP and Brdu double staining. In the study from Li et al. (2014), they showed that Brdu+ cells and GFAP+ astrocytes dramatically increased from day 2 post PT and reached peak at day 4; however, overall, the GFAP+Brdu+ proliferating astrocytes only accounted for a small percentage of total Brdu+ cells, which reached a peak value of about 6% from days 3 to 4 post PT and then declined sharply (Figure 1D). Based on the labeling protocol, the GFAP+Brdu+/Brdu+ ratio at each time point represents the relative rate of the generation of reactive astrocytes. On the other hand, the GFAP+Brdu+/GFAP+ ratio also reached the highest level within days 3 to 4 post PT. These data demonstrated that focal ischemic stroke increase the population of proliferating reactive astrocytes in a highly temporal dependent manner. The results indicate that glial scar is formed largely from the existing astrocytes through the upregulation of GFAP, rather than from newly generated astrocytes through proliferation. Although proliferation rate reduces dramatically after 8 days following PT, the morphology of reactive astrocytes maintains straight processes pointing to ischemic core for a prolonged time based on the GFAP expression, suggesting an irreversible change of reactive astrocytes in the penumbra (Haupt et al., 2007; Li et al., 2014). This phenomenon also suggests that the expression of certain genes cannot be reversed during the recovery from focal ischemic injury and glial scar formation causes substantial tissue remodeling and permanent and persistent structural changes in the penumbra following ischemia.

Different responses of astrocytes to ischemia—In addition to the heterogeneity in astrocyte morphology, even in the same anatomical location of the brain such as within the cortex (Benesova et al., 2009; Matyash and Kettenmann, 2010), the astrocytes respond to ischemic stroke in different manners. As mentioned before, it is well know that protoplasmic astrocytes are different from fibrous astrocytes in morphology and GFAP expression. Their sensitivity to ischemia is also different. Lukaszevicz et al. (2002) studied the reactivity and fate of protoplasmic and fibrous astrocytes after permanent MCAo in mice. Loss of GFAP was observed within minutes in the ischemic core. The fibrous astrocytes exhibited a transient and limited hypertrophy without discernible cell death as compared to protoplasmic astrocytes. Their results demonstrated that protoplasmic astrocytes have a higher sensitivity to ischemia than the fibrous cortical astrocytes, with a rapid demise of the protoplasmic astrocytes. In another study using imaging of GFP+ optic nerve astrocytes and hippocampal astrocytes in neonatal whole-mount preparations, no reactive astrogliosis was observed in white matter nor grey matter GFP+ astrocytes during a 180-min reperfusion following modelled ischemia (Shannon et al., 2007). Furthermore, it was observed that the white matter astrocytes are much more sensitive to ischemia-reperfusion injury than the grey matter astrocytes. Even though the results from two groups were contradictory, the discrepancy could be attributed to the differences in the experimental models used and in the characteristics of the fibrous astrocytes of the optic nerve vs corpus colossal. In summary, astrocytes respond diversely to ischemia. Another study from Benesova et al., (Benesova et al., 2009) using the GFAP-EGFP mice revealed that there are two populations of cortical astrocytes in terms of their volume changes in response to OGD in brain slice preparations, the first exhibiting a large volume but the second a small volume increase. Furthermore, patch-clamp measurement of resting membrane potential (V_m) showed that one group significantly depolarized during OGD, while the second group only exhibited small changes in V_m toward more negative values. Their study provided evidence that there are two astrocytic populations in the cortex with different responses to ischemia. Given the complex of heterogeneity of astrocytes, further studies to identify the different responses such as morphological change and proliferation of astrocytes in response to ischemia should be performed using lineage analysis in the transgenic mice that express fluorescent markers in different types of astrocytes.

Reactive astrogliosis and behavioral recovery

After ischemia, the brain undergoes spontaneous recovery with improvement in behavioral deficits over time (Badan et al., 2003; Li et al., 2004; Clarkson et al., 2013). Focal ischemia-induced reactive astrocytes exhibit heterogeneity in morphology, GFAP expression and proliferation capability in a spatiotemporal dependent manner. This can be seen in Figure 2 where GFAP expression levels and proliferation capability eventually decrease after the first 4-day acute phase in a spatiotemporal dependent manner. One outstanding question is whether behavioral deficits are correlated to reactive astrogliosis and glial scar formation, and if so, are reactive astrocytes beneficial to functional recovery? To explore this, behavioral tests were conducted (Li et al., 2014). The time courses of forelimb shift asymmetricity, strength, and sensory motor impairments were evaluated. The functional deficits have a time window similar to that of infarct expansion, brain swelling, the fast cell proliferation and reactive astrocyte generation. Functional deficits improved from day 6 post

PT when glial scar began to form, implying that glial scarring might have a beneficial effect by preventing the expansion of the ischemic core. Thus, the behavioral study results suggest that reactive astrocyte proliferation and astrogliosis correlate with neuronal remodeling and functional recovery. This can be supported by a study from Hayakwa et al. (2010). In their study, the effects of fluorocitrate, a metabolic inhibitor of astrocytes, on reactive gliosis and behavioral recovery were assessed. They found that fluorocitrate-treated mice have a significant decline in reactive astrocytes and neurovascular remodeling as well as a corresponding enhanced behavioral deficit compared with stroke-only controls, suggesting that reactive astrocytes in the penumbra may improve neurovascular remodeling, and thus promote stroke outcomes (Hayakawa et al., 2010). In another study using WT and glial fibrillary acidic protein and vimentin in double knockout ($GFAP^{-/-}Vimentin^{-/-}$) mice, Liu et al. (2014) compared the behavioral outcomes, axonal remodeling of the corticospinal tract (CST), and the spatio-temporal change of chondroitin sulfate proteoglycan (CSPG) expression after PT (Liu et al., 2014). The results showed that motor functional recovery and biotinylated dextran amine-positive CST axonal length in the denervated side of the cervical gray matter were significantly reduced in $GFAP^{-/-}Vimentin^{-/-}$ mice as compared with WT mice. Immunostaining showed that the $GFAP^{-/-}Vimentin^{-/-}$ mice exhibited attenuated astrocytic reactivity and a significant increase in CSPG in both hemispheres. However, these $GFAP^{-/-}Vimentin^{-/-}$ mice showed a decrease in the ischemic lesion boundary zone when compared to the WT mice. This study suggests that attenuation of reactive astrogliosis reduces CST axonal remodeling in the denervated spinal cord and thus impairs or delays neurological recovery. These studies indicate that targeting reactive astrocytes might, therefore, lead to an important therapeutic strategy that can improve stroke outcomes.

Signaling pathways of reactive astrogliosis after ischemia

Over the last two decades, advancements in genetics and molecular biology have equipped researchers with unique tools, which have resulted in an extensive characterization of reactive astrocytes following CNS injury. Studies have identified a plethora of genes and molecular markers for reactive astrocytes and the list still continues to grow (Ridet et al., 1997; Zamanian et al., 2012; Colangelo et al., 2014). Moreover, many studies have provided clues that have shed some light on key signaling pathways that may regulate changes to genes and molecular markers during reactive astrogliosis in the brain and spinal cord following injury (Sofroniew and Vinters, 2010; Kang and Hebert, 2011; Karimi-Abdolrezaee and Billakanti, 2012). Despite such extensive characterization, an across-the-board understanding of the key signaling pathways regulating distinct aspects of the reactive astrogliosis still remains inadequate. Further complicating things is the highly context dependent nature of astrogliosis in the injured CNS (e.g., acute phase vs chronic phase, and white matter vs gray matter), which limits the one-size-fits-all approach for its manipulation in various CNS disorders (Zhang and Barres, 2010; Kang and Hebert, 2011; Anderson et al., 2014). To keep things in perspective, the following section is a brief summary of the major signaling mechanisms that have been identified to regulate reactive astrogliosis in stroke.

Intermediate filaments—The intermediate filament GFAP is predominantly expressed in astrocytes and is essential for astroglial cyto-architecture and cellular functions. Following CNS injury, including ischemic stroke, the peri-lesional astrocytes become reactivated and

dramatically upregulate their GFAP in a temporally and spatially dependent manner. As a result, the role of intermediate filaments in the reactive astrogliosis has generated a widespread interest among neuroscientists trying to understand glial biology in the injured CNS. As a result, a number of genetic deletion studies of astrocyte intermediate filaments (especially GFAP and vimentin) have been undertaken to elucidate the importance of the intermediate filaments in ischemic injury, reactive astrogliosis and glial scar formation using mice with either a single deletion of GFAP or vimentin and a double deletion of GFAP and vimentin. Hanbury et al. (2003) found that deletion of GFAP is neuroprotective against metabolic insult and excitotoxicity which was attributed to increased production of the glial derived neurotrophic factor (GDNF) by GFAP knockout astrocytes (Hanbury et al., 2003). Pekny et al. showed that single deletion of either $GFAP^{-/-}$ or $vimentin^{-/-}$ from astrocytes produced little to no effect on reactive astrogliosis and resulted in scars similar to those found in WT animals after brain injury (Pekny et al., 1998; Pekny et al., 1999). In ischemia, studies demonstrated that $GFAP^{-/-}$ mice exhibited a more significant decrease in cortical cerebral blood flow, and relatively larger infarct volume than the WT mice after MCAo. Their results indicated that GFAP-null mice have a high susceptibility to cerebral ischemia and suggested that astrocytes and GFAP play an important role in the progression of brain damage after ischemia (Nawashiro et al., 2000). Their higher susceptibility to cerebral ischemia in GFAP-null mice could have resulted from the reduction in both astrocytic (EAAT1) and neuronal (EAAT3) glutamate transporter subtypes (Hughes et al., 2004). Thus, these results from single GFAP deletion were highly ambiguous. However, studies using $GFAP^{-/-}$/$Vimentin^{-/-}$ double knockout mice have indicated that GFAP and vimentin are essential for retaining beneficial effects of reactive astrocytes after ischemia. These studies show that in acute ischemia, double deletion of GFAP and vimentin result in decreased glutamate uptake, increased susceptibility to oxidative stress and significantly increased infarct volumes (2-3 times compared to WT mice). Hence, the deletion of both intermediate filament proteins (GFAP and vimentin) in the acute stage disrupts glial scar formation and promotes neuronal regeneration after traumatic brain injury and spinal cord injury (Li et al., 2007; de Pablo et al., 2013). Whereas in chronic stages of stroke, attenuation of astroglial reactivity by GFAP and vimentin deletion impaired axonal remodeling and functional recovery (Liu et al., 2014). Together these studies suggest that GFAP and vimentin are essential to retain beneficial function of reactive astrocytes in ischemic as well as recovering nervous tissue.

p38 MAPK pathway—Mitogen activated protein kinases (MAPK) are an important family of enzymes transducing a wide range of extracellular signals like inflammation, growth factors, and toxic stimuli as well as integrating corresponding cellular responses (Kaminska, 2005). Among MAPKs, p38 is of particular importance since it is known to transduce cellular inflammation (Cuadrado and Nebreda, 2010). In an ischemic stroke, p38 MAPK can become activated in neurons, microglia and astrocytes (Nozaki et al., 2001; Ferrer et al., 2003; Krupinski et al., 2003). Furthermore, p38 inhibitors have proven effective in reducing infarct volume and improving functional recovery after experimental ischemia (Barone et al., 2001b; Barone et al., 2001a). Compared to neurons and microglia, the activation of p38 MAPK in astrocytes is delayed, reaching its peak during the chronic stages of injury following primary ischemic insult (Piao et al., 2002; Irving et al., 2000)., A similar

pattern has been seen in GFAP upregulation during ischemia-reperfusion (Yamashita et al., 1996). Further, activation of p38 MAPK in astrocytes has been reported to induce matrix metalloprotease-9 (MMP-9) expression, reactive oxygen species (ROS) and cytokine production features that are manifested during astrocyte reactivation (Saha et al., 2007; Ralay Ranaivo et al., 2012; Nahirnyj et al., 2013). Recently, a study performed using an astrocyte-specific p38 MAPK knockout mouse model demonstrated a key role of p38 MAPK signaling in reactive astrogliosis induced by ischemic stroke (Roy Choudhury et al., 2014). This study demonstrated that delayed activation of p38 MAPK following ischemic injury corresponds with upregulation of GFAP in the penumbra region signifying an important role of p38 MAPK in signaling reactive astrogliosis following ischemic stroke. To further confirm this, the authors developed a novel conditional astrocyte specific p38 MAPK knockout mice line using a human GFAP promoter driven Cre-recombinase system and floxed p38 MAPK. Their results demonstrated that conditional deletion of p38 MAPK from astrocytes attenuated injury (both OGD and scratch injury) and induced GFAP over expression, MMP-9 secretion and astrocyte migration *in vitro*. *In vivo*, conditional GFAP-Cre/LoxP p38 mice had significantly fewer and smaller astrocytes in the penumbra following ischemia compared to WT animals. The conditional hGFAP Cre/LoxP p38 mice also had a significantly reduced expression of GFAP in the ischemic hemisphere compared to WT mice indicating attenuated reactive astrogliosis. However, attenuation of reactive astrogliosis by conditional deletion of p38 MAPK had no significant effect on motor functional recovery in the mice. Nito et al. (2012) found that p38 MAPK may be involved in the regulation of aquporin-4 expression in astrocytes and could potentially cause the development of lethal cerebral edema following ischemic injury. Taken together, these studies indicate that p38 MAPK signaling may be a critical transducing pathway in signaling reactive astrogliosis in ischemic stroke, and targeting p38 MAPK may provide an excellent opportunity to modulate reactive astrogliosis. However, extensive studies are still necessary to completely elucidate the role of p38 MAPK signaling in astrogliosis.

On the other hand the role of another important member of MAPK in terms of cerebral ischemia, the Jun N-terminal kinase pathway (JNK), still remains unclear. Early activation of JNK in neurons and microglia during ischemia has been reported (Lennmyr et al., 2002), and its inhibition in the acute phase has proven protective (Borsello et al., 2003; Gao et al., 2005). Gadea et al. (2008) found that the JNK pathway plays a part in endothelin-1 induced astrocyte proliferation and reactive astrogliosis (Gadea et al., 2008). However, Murata et al. (2012) found that delayed inhibition of the JNK pathway following ischemic injury resulted in increased infarct volume and worsened the outcome after stroke. This seemingly contradictory information suggests the different roles of JNK in acute brain injury vs chronic stroke outcomes. Due to lack of effective pharmacological inhibitors and genetic models, many questions remain unanswered as to how and when JNK functions. Research is needed to determine if JNK is sequestrated or integrated with p38 MAPK pathway during activation. We know that activation of JNK induces changes in astrocytes akin to p38 MAPK activation that leads to the production of MMPs and cytokines as well as astrocyte migration. Future research should focus on how JNK impacts long-term stroke outcomes.

Notch signaling pathway—Notch signaling is a highly conserved pathway critical for the maintenance and self-renewal of progenitor cells and to inhibit precocious neurogenesis (Yoon and Gaiano, 2005). In developing CNS, Notch 1 is responsible for astrogliogenesis by forming a transcriptional activation complex with a GFAP gene thereby directing neural stem cells to differentiate into astrocytes (Ge et al., 2002) . Mature astrocytes are quiescent and do not divide in a healthy brain; however, they acquire a proliferative phenotype following cerebral injury (Barreto et al., 2011; Li et al., 2014). In an ischemic stroke, It was reported that Notch 1 signaling was activated in astrocytes in the peri-infarct region by 24 h (Marumo et al., 2013) and tamoxifen induced deletion of Notch 1 from GFAP positive cells resulted in a decreased number of proliferating astrocytes following injury. The study also suggested that Notch 1 signaling in reactive astrocytes may aid in suppression of immune response into the peri-infarct area as Notch 1 knockout animals had significantly higher invading immune cells (Shimada et al., 2011a). Some speculation existed among researchers concerning the source of Notch 1 in the peri-infarct area, as some scientists believed that NG2+ cells also expressed a Notch 1 positive stain along with reactive astrocytes, but genetic fate mapping analysis revealed that a subpopulation of reactive astrocytes—not NG2+ cells—activated Notch1 signaling in response to injury and became proliferative (Komitova et al., 2011; Marumo et al., 2013). In addition, it was suggested that activation of Notch 1 signaling in reactive astrocyte after stroke may redirect the cells to dedifferentiate into astrocytes derived neural stem cells (Shimada et al., 2012a). However, an important gap in knowledge still exists regarding Notch 1 signaling in regulating reactive astrocytes. Many questions remain to be explored such as: Why does only a subset of reactive astrocytes express Notch 1 signaling and become proliferative? What is the temporal and spatial pattern of Notch 1 signaling in the brain following ischemia? What is the fate of astrocyte-derived neural stem cells? Does inhibiting Notch 1 induced astrocyte proliferation have any effect on functional recovery? Until these gaps are filled, the definitive role of Notch 1 in reactive astrogliosis cannot be completely understood.

STAT3 signaling—Signal transducer and activator of transcription 3 (STAT3), a member of the Jak-STAT family, is a major downstream signaling pathway activated by peptides like hormones, growth factors or cytokines (Nicolas et al., 2013). Astrocyte marker GFAP is a STAT3 regulatory target and the role of STAT3 in reactive astrogliosis has been extensively studied in SCI models (Herrmann et al., 2008; Sofroniew, 2009; Karimi-Abdolrezaee and Billakanti, 2012; Wanner et al., 2013). Studies have showed that GFAP-cre-promoted deletion of STAT3 attenuates astrocyte hypertrophy, and astrocyte proliferation eventually causes failure of formation of a compact glial scar following spinal cord injury. In addition, studies indicate that STAT3 might be critical in migration of the astrocytes to the damaged site and may prevent its further spread (Okada et al., 2006; Herrmann et al., 2008; Wanner et al., 2013). In ischemic stroke, reperfusion induced increase in ROS and inflammatory cytokines are known to be powerful stimulants of STAT3 signaling (Lei et al., 2011; Zhu et al., 2013). But cell type specific activation of STAT3 in the post ischemic brain has been a matter of extensive debate. While some reports suggest neurons are the predominant source of activated STAT3 (Suzuki et al., 2001; Wen et al., 2001), others have shown that STAT3 is also activated in astrocytes along with neurons (Justicia et al., 2000; Choi et al., 2003). These conflicts in reports have been attributed to differences in insult models chosen and

brain regions observed. More recent information, however, suggests that STAT3 can regulate reactive astrogliosis induced by diverse forms of neurotoxic insults where activation of STAT3 in astrocytes and subsequent increase in GFAP expression can be induced by direct neuronal death even in the absence of astrocyte damage (Sriram et al., 2004; O'Callaghan et al., 2014). Despite reports establishing STAT3 signaling as an important regulator of reactive astrogliosis in spinal cord injury, its role in driving astrogliosis in ischemic stroke remains open to exploration. This further reiterates the idea that reactive astrogliosis is highly dependent on its relationship to the context and its anatomical location in the CNS.

Transforming growth factor Beta (TGF-β) signaling—An injury related peptide, TGF-β has been shown to increase in immunoreactivity in both the infarct and penumbra in stroke patients (Krupinski et al., 1996) as well as animal models of focal ischemic stroke (Wang et al., 1995; Ruocco et al., 1999; Ali et al., 2001). Despite opposing reports, TGF-β has been mostly regarded as neuroprotective particularly against the N-methyl-d-aspartate receptor (NMDA), which can induce neuronal cell death (Prehn et al., 1993; Ruocco et al., 1999). It is suggested that the protective action of TGF-β is mostly mediated by astrocytes (Buisson et al., 1998; Docagne et al., 1999). This was further confirmed when deletion of TGF-β in the CNS resulted in loss of astrocyte glutamate transporters, like GLT-1 (EAAT2) and GLAST (EAAT1) causing decreased glutamate uptake and enhanced neuronal death. Treatment of astrocytes with TGF-β reversed the phenotype suggesting a beneficial role of TGF-β signaling in astrocytes (Koeglsperger et al., 2013). Further, most recent data suggests that the early activation of TGF-β in astrocytes after injury promotes anti-inflammatory response and aids in neuroprotection (Cekanaviciute et al., 2014). Other studies have shown that TGF-β signaling in astrocytes induces the production of GFAP, CSPGs and promotes scar formation (Moon and Fawcett, 2001; Wang et al., 2008; Schachtrup et al., 2010) with aging being an important determinant of the outcome (Doyle et al., 2010; Yu et al., 2014).

In addition to the above mentioned signaling pathways, CD36, a class B scavenger receptor, has recently been reported to be involved in astrocyte activation and glial scar formation in ischemic stroke patients. Bao et al. (2012) demonstrated that functional absence of CD36 receptor reduced astrocyte proliferation, delayed wound healing and attenuated injury induced GFAP overexpression and glial scar formation in the brain.

Therapeutic potential of the reactive astrocytes in stroke

Despite tremendous efforts and advances in translational research, the treatment strategies for stroke are still limited. Tissue plasminogen activator (tPA) is the only FDA approved drug currently available for acute stroke treatment, but it is only effective within a narrow therapeutic window, that is, within a few hours after a stroke. Beyond this window, treatment of a stroke is primarily dependent on supportive care, secondary prevention and rehabilitation. Manipulation of the functional recovery process has emerged as an alternative strategy in stroke research. Since reactive astrocytes have intimate contact with neurons and vasculature, and undergo dynamic changes in Ca^{2+} signaling, morphology, proliferation and gene expression, modulation of reactive astrocytes might be a promising strategy for stroke therapy. Knowledge regarding these dynamic changes and signaling pathways that trigger

reactive gliosis and modulate reactive astrocyte function may provide information for effective therapeutic strategies for ischemic brain disorders. However, most studies on the signaling pathway of reactive gliosis have been based on data obtained only from certain time point specific studies after ischemia. A recent study (Zamanian et al., 2012) on the genomic analysis of reactive astrocytes at different times after MCAo should help to define the properties of reactive astrocyte at different stages of response. On the other hand, the heterogeneity of astrocytes and reactive astrocytes in different regions adds another layer of complexity (Anderson et al., 2014). A recently developed approach, termed translating ribosome affinity purification (TRAP) provides a novel tool to study the translational status of transcripts present in reactive astrocytes after a stroke (Heiman et al., 2008; Doyle et al., 2008; Zhou et al., 2013; Hupe et al., 2014). This technology bypasses the lengthy and possibly disruptive cell purification procedures and purifies mRNAs that are associated with ribosomes in the entire cell body, thus enabling study of a translatome profile using high-throughput RNA-seq. With the development of astrocyte-specific TRAP mice (Heiman et al., 2008; Doyle et al., 2008), it would be feasible to study translatome and identify the critical signaling pathways involved in reactive gliosis at different times and in different brain regions after an ischemic stroke.

Data from signaling pathway and gene profile studies provide the base for modulation of reactive astrocytes through gain-and-loss of function approach. However, stroke outcomes can only be improved when large number of reactive astrocytes can be manipulated. Two *in vivo* genetic molecular approaches can be used to achieve this goal, i.e., the use of inducible knockout mice and viral transduction. Inducible knockout mice can be used to control temporal expression of genes by knocking down astrocyte-specific genes at a specific time point after ischemia using commercially available astrocyte-specific Cre reporter mice (Mori et al., 2006). Recent advances in self-complementary adeno-associated viruses (scAAV) have provided another promising approach to express transgenes in reactive astrocytes. scAAV can be transduced into the whole brain through intravenous administration in neonatal mice (Foust et al., 2009; Zhang et al., 2011). Since blood-brain barrier becomes permeable after ischemic stroke, this approach can be adapted in adult mice to knock down or overexpress certain genes in an entire penumbra. Combined with an astrocyte-specific promoter (Xie et al., 2010), scAAV would be an ideal approach for molecular manipulation in a large amount of reactive astrocytes in the penumbra, overcoming the disadvantages of invasive and low efficiency delivery of single strand AAV. Data of dynamics on reactive astrocyte and proliferation will provide the necessary information for the time frame of genetic manipulation after ischemia.

Many studies have shown that reactive astrocytes exhibit stem cell-like properties (Buffo et al., 2008; Sirko et al., 2013; Robel et al., 2011; Dimou and Gotz, 2014; Shimada et al., 2012b). They can express neural stem cell related proteins such as nestin and Sox2 (Shimada et al., 2012b) and doublecortin (DCX), an immature neural stem cell marker (Ohab et al., 2006; Magnusson et al., 2014). These studies suggest that reactive astrocytes can potentially transform into neurons under specific conditions such as overexpression of neurogenic factors. It has been recently reported that reactive astrocytes indeed can be converted to neuroblasts and neurons by forced expression of a single transcriptional factor such as Sox2

(Su et al., 2014), neurogenin-2 (Berninger et al., 2007; Heinrich et al., 2010), NeuroD1 (Guo et al., 2013), or a combination of multiple transcriptional factors such as ASCL1, LMX1B and NURR1 (Addis et al., 2011). The results of dynamics of reactive astrocytes provide an important implication for optimal timing to convert reactive astrocytes into neurons using genetic manipulation techniques such as astrocyte-specific inducible transgenic mice and viral transduction, which can possibly aid improvement of stroke outcomes in experimental as well as clinical studies on stroke therapy. However, astrocyte-to-neuron conversion has not been done in reactive astrocytes in the context of strokes.

In another aspect, growing evidence suggests that an ischemic stroke dramatically increases neurogenesis in the subventricular zone (SVZ) and subgranular layers in dentate gyrus (Tobin et al., 2014). It is suggested that there is an association between endogenous neurogenesis and improved stroke outcomes (Lagace, 2012). Recent studies indicate that reactive astrocytes play an important role in neurogenesis after a stroke. Young et al. (2013) first showed that an ischemic stroke causes substantial reactive astrogliosis in SVZ. The hypertrophic reactive astrocytes and their tortuous processes disrupt the neuroblast migratory scaffold and cause SVZ reorganization after a stroke; thus, reactive astrocytes in SVZ might play a role in modulating neurogenesis and stroke recovery. Strokes also elicit a latent neurogenic program in striatal astrocytes in a mouse model. It is reported that striatal astrocytes produce neuroblasts and Notch 1 signaling is reduced in astrocytes after a stroke (Magnusson et al., 2014); furthermore, blocking Notch signaling triggers astrocytes in the striatum and the medial cortex to enter a neurogenic program even in the absence of a stroke, indicating that attenuated Notch 1 signaling is necessary for neurogenesis by striatal astrocytes. Future study is needed to explore whether astrocyte-derived neuroblasts can be differentiated into mature neurons and contribute to improvement of stroke outcomes.

Conclusions

Current neuron-centric strategies have not resulted in major breakthroughs in stroke therapy. Reactive astrogliosis is one of the most prominent pathological features in strokes and is an adaptive defense response that is both beneficial and detrimental to the injured CNS. Especially during the early time after ischemic injury, the main function of reactive astrocytes is to preserve integrity of the nervous tissue. However, with time, the process becomes increasingly unregulated and maladaptive, and progresses to form into a compact glial scar. The established glial scar further accentuates inflammation, generating a highly toxic microenvironment that inhibits migrating axons and interferes with long term motor functional recovery. Given the dualistic nature of reactive astrogliosis, a comprehensive understanding of reactive astrogliosis is extremely vital to the designing strategies that can aid in modulating reactive astrogliosis for healing tissue. The primary goal of research will be to enhance and retain the therapeutically beneficial functions of reactive astrocytes while completely eliminating undesired outcomes like glial scar formation. Since reactive astrocytes exhibit high plasticity in morphology, proliferation and gene expression, modulation of function, signaling pathways, neurogenic potential and neurogenesis through local reactive astrocytes is an attractive strategy for stroke therapy. Astrocyte-specific molecular genetic techniques including TRAP, inducible knockout animals, viral transduction and lineage analysis can elucidate the mechanisms of astrocyte-based stroke

therapy, and targeting reactive astrocytes is a promising strategy to improve stroke outcomes.

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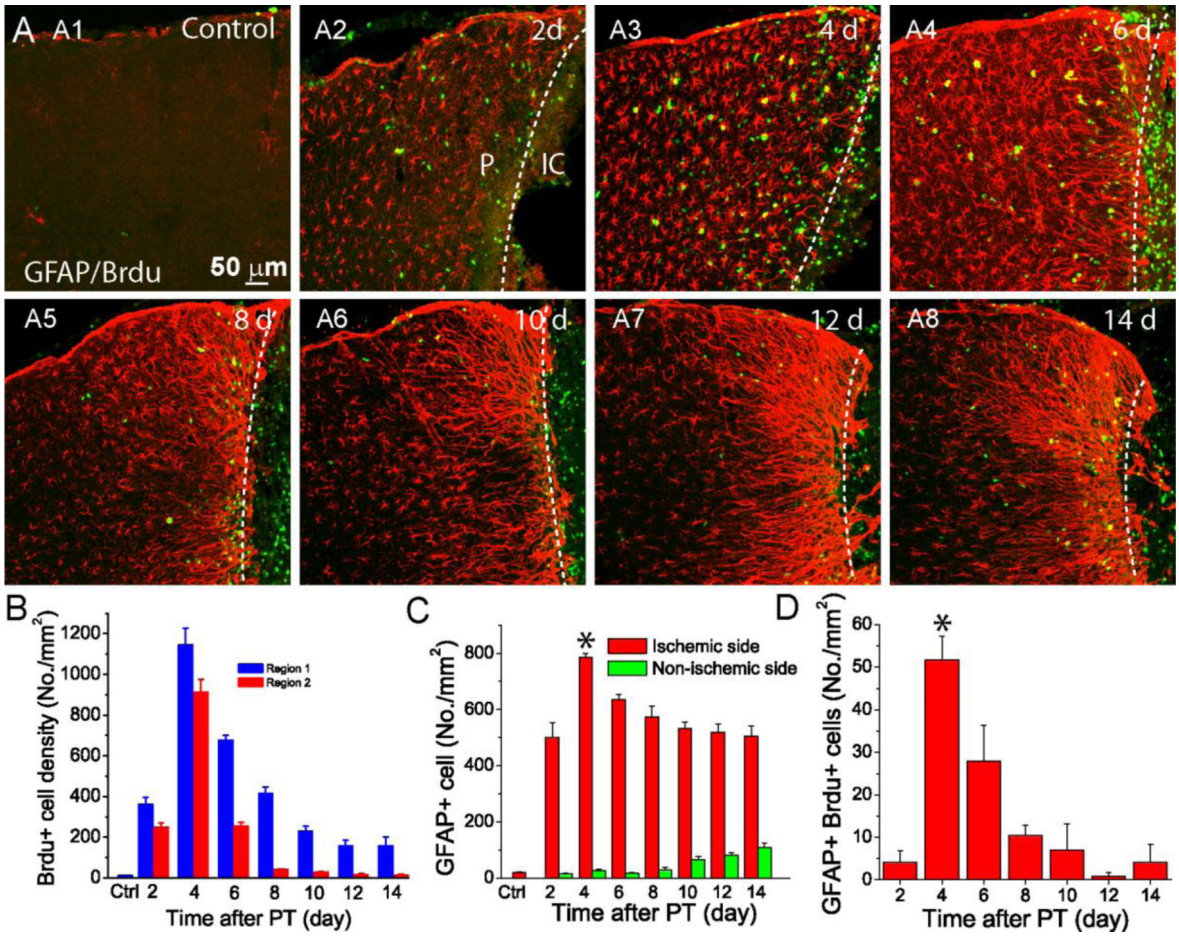

Figure 1. Time course of astrocyte proliferation and morphological changes after stroke

A) Confocal images of GFAP and Brdu expression in the penumbra from mice at indicated times after PT. **B)** Brdu+ cell density presented as cell number per mm^2 in Region 1(R1) and Region (R2) with an area of 200 μm×200 μm in the L2/3 of the cortex. R1 is located 0–200 μm from the edge of ischemic core, and R2 is located 200–400 μm from the edge of ischemic core (see the left panel of A2). **C-D)** The density of GFAP+ (C) and GFAP+Brdu+ (D) stained cells in the penumbra. The cells were counted in the penumbral region of 200 μm×400 μm in L2/3 of the cortex located 0-400 μm from the edge of ischemic core. Dash lines separate ischemic core (IC) and penumbra (P). Data were adapted from Li et al., 2014b.

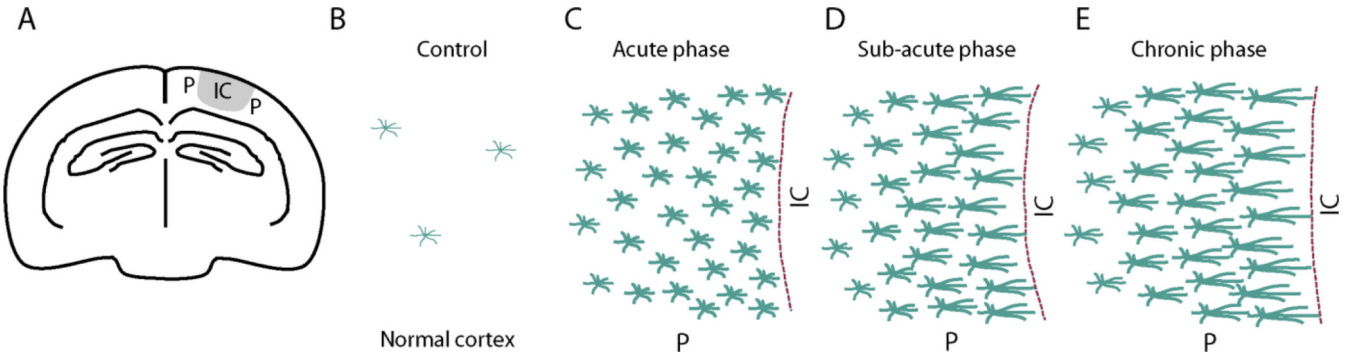

Figure 2. Schematic representations of dynamic reactive astrocytes and glial scar formation in the penumbra at different stages after a focal ischemic stroke

A) Illustration of ischemic core and penumbra after PT in the mouse brain. The grey region indicates the ischemic core (IC) and the surrounding regions are penumbra (P). **B)** In control conditions, very low percentage of astrocytes expresses GFAP. **C)** In the acute phase after focal ischemia (days 1-4 post ischemia), astrocytes exhibit stellate morphology and hypertrophied GFAP positive processes and a high proliferating rate. **D)** During subacute phase after focal ischemia (days 4-8 post ischemia), astrocytes exhibit elongated processes pointing to the ischemic core, and a glial scar forms. The proliferating rate decrease significantly at this stage. **E)** Chronic phase after focal ischemia exceeds the 8-day post ischemia period. Astrocytes further extend processes toward the ischemic core and the glial scar matures during this period. The GFAP expression levels in reactive astrocytes surrounding the glial scar decrease and reactive astrocytes lose the capability of proliferation. Reactive astrocytes are heterogeneous in morphology, molecular expression, and proliferation depends on their location. Figure 2B-E were adapted from Ding (2015).