Regulation Of Neuroinflammation After Ischemic Stroke By Astroglial Endothelin Receptor Type-B Signaling

REGULATION OF NEUROINFLAMMATION AFTER ISCHEMIC STROKE BY ASTROGLIAL ENDOTHELIN RECEPTOR TYPE-B SIGNALING

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ABSTRACT

A large body of neuroscientific research has focused on reactive gliosis and glial scar formation because these are among the most prominent features of the cellular response to central nervous system (CNS) injury. Despite much progress in our understanding, controversy remains regarding the relative balance between the protective nature of the astroglial scar and its anti-regenerative features. Recent work suggests that astrocytes are heterogeneous in their resting state and in their reactivity. In traumatic injuries such as stroke and spinal cord injury, proliferative reactive astrocytes protect CNS tissue. By contrast, under neuroinflammatory and/or neurodegenerative conditions, neurotoxic astrocyte phenotypes may contribute to disease progression.

Our previously published work showed that endothelin receptor type-B (ETB_R) signaling was required for reactive astrocyte proliferation in a distal middle cerebral artery occlusion (dMCAO) model of stroke. In the studies for this dissertation, we utilized stem cells derived from the peri-infarct area after stroke to model astroglial ETB_R signaling and identified the array of paracrine factors released in response to endothelin signaling. The secreted factor with gene expression most decreased by astroglial ETB_R-knockout was Angiopoietin-2 (Ang-2), a pro-angiogenic paracrine factor that affects stroke recovery. We found that astrocytes expressed significantly more Ang-2 after stroke. Ang-2 expression was specifically regulated by ETB_R and downstream NFκB and MEK/ERK signaling. After stroke, we discovered that adult microglia, the sentinel immune cells of the brain, increased their expression of Tie-2, the receptor for Ang-2. To evaluate whether paracrine activity controlled by ETB_R functioned in neuroinflammation, we performed astrocyte-specific conditional knockout of ETB_R using GFAP-CreER^{TM}-*EDRNB*-$^{fl/fl}$ mice. Notably, peri-infarct $CD11b^+$ cells, corresponding to microglia/macrophages/monocytes, proliferated significantly less after dMCAO in astrocyte-specific ETB_R-knockout mice as compared with controls. Taken together, our results indicated that astroglial ETB_R signaling may promote Ang-2 release to regulate the proliferation, survival, and/or the inflammatory status of microglia after stroke.

In addition to Ang-2, we demonstrated that astroglial ETB_R regulates the expression of CD109, a TGFβ co-receptor and C3 alpha-2-macroglubulin family member. CD109 is the target of multiple proteases such as furin, and retains many of its functions as a released, soluble protein. In reactive astrocytes, increased expression of CD109 mRNA was reported to indicate a "neuroprotective" phenotype. We show that ETB_R signaling increased astroglial expression of several forms of CD109. Astroglial CD109 expression increased following stroke, and ETB_R-knockout prevented stroke-induced increases in CD109 expression. Consistent with a function for CD109 in altering astroglial neuroinflammatory status, in the presence of TNFα and an ETB_R specific agonist, CD109-knockdown blocked ETB_R-dependent effects on C3d expression. To test the disease modifying potential of soluble (free) CD109, we injected mice with recombinant human CD109 after stroke. At 7 days after dMCAO, CD109 treatment tended to increase the size of cortical infarcts and areas of secondary reactive astrocyte thalamic reactivity. Further research will be required to determine potential differences between the membrane-bound and protease- cleaved forms of CD109 and their effects on neuroinflammation, injury progression and resolution. Improved understanding of paracrine, juxtacrine, and/or autocrine cell signals regulated by astroglial ETB_R may provide valuable targets to treat CNS injury and disease.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1 Glial cell types of the central nervous system

Astrocytes

Astrocytes are macroglial cells of the central nervous system, originally named neuro-glia (nerve-cement), then named more specifically for their star-like appearance when stained for glial fibrillary acidic protein (GFAP) (Golgi, 1873; Virchow, 1858; von Lenhossek, 1893). During development, radial glial cells first give rise to neurons and then take on a glial restricted fate, producing fibrous astrocytes, protoplasmic astrocytes, and oligodendrocytes (Clavreul et al., 2019; Wang and Bordey, 2008). Numerous cell-autonomous factors and cell-cell signaling events balance the proliferation of radial glial cells, the relative numbers of neurons and glia they produce, and the relative proportion of astrocytes and oligodendrocytes they generate (Beattie et al., 2017; Hippenmeyer and Luo, 2010; Hirabayashi et al., 2009). For example, Achaete-scute homolog-1 (Ascl1) is thought of as a canonically pro-neuronal transcription factor, but it also regulates the balance between astrocytes and oligodendrocytes generated in the gray and white matter of the spinal cord (Vue et al., 2014). The transcription factor lethal giant larvae homolog-1 (Lgl1) regulates the production of both radial glia and astroglial cells, but its effects on astroglial specification are highly dependent on interactions with epidermal growth factor receptor (EGFR) signaling (Beattie et al., 2017).

Once generated by radial glial cells, astrocytes undergo clonal expansion to tile the brain. Each cell forms its own territory, overlapping the processes of neighboring astrocytes minimally (Clavreul et al., 2019; Beattie et al., 2017; Oberheim et al., 2009; Wilhelmsson et al., 2006). The sequential development of radial glia and corresponding

cascade of signaling events is necessary to establish the correct balance of neuronal and glial cell numbers. Differentiated neurons require astrocytes to support them trophically, metabolically, and to maintain ionic balance. Additionally, astrocyte endfeet form one part of the tripartite synapse, maintaining the extracellular environment at axonal myelin internodes (Araque et al., 1999; Arizono et al., 2020; Ventura and Harris, 1999). Defined neuronal communication is altered without astroglial transport to remove glutamate from the synapse (Arnth-Jensen et al., 2002; Marcaggi et al., 2003; Takatsuru et al., 2013).

There are two primary categories of astrocytes that reside in the healthy brain. Astrocytes responsible for maintaining the axonal environment at myelin internodes are fibrous and reside within the white matter of the brain, supporting axons and the oligodendrocytes that myelinate them (Butt et al., 1994; Gadea et al., 2009; Raff et al., 1988; Simons and Trajkovic, 2006). Protoplasmic astrocytes exist in the gray matter and have more extensive ramifications which interact with neurons to regulate their extracellular environment as well as synaptic transmission. Both fibrous and protoplasmic astrocytes extend processes that cover the vasculature and contribute to blood-brain barrier (BBB) regulation and function (Boulay et al., 2017; Simard et al., 2003). There is further heterogeneity beyond these two astrocyte subpopulations. Astrocytes are often characterized by expression of the intermediate filament protein glial fibrillary acidic protein (GFAP), but even GFAP is differentially expressed by astrocytes depending on brain region and astroglial subtype (Emsley and Macklis 2006; Meng et al., 2015; Nolte et al., 2001). Additional astroglial diversity has been characterized based on anterior-posterior, dorsal-ventral, and cortical layer-specific astroglial gene expression profiles (Bayraktar et al., 2020; Lanjakornsipan et al., 2018; Miller et al., 2019).

In response to central nervous system pathologies astrocytes become reactive, altering morphology, paracrine signaling, and cellular transporters to limit neuronal injury (Wilhelmsson et al., 2004; Zamanian et al., 2012). Reactive astroglia are not a single homogenous population but have a variety of functions within and between disease states. In diseases such as Alzheimer's and Parkinson's, astrocytes alter gene expression and paracrine signaling in ways that can be toxic to neurons (Liddelow et al., 2017; Yun et al., 2018). In brain injuries such as stroke and stab wound models, astrocytes increase proliferation and trophic factor expression to protect neurons (Anderson et al., 2016 and 2018; Shimada et al., 2012). These two distinct types of reactive astrocytes have been characterized as "neurotoxic" (A1) and "neuroprotective" (A2). It is unlikely that reactive astrocytes fall exclusively into two simple states, but these categories may represent general roles for astrocytes depending on the type of injury, just as pro-proliferative reactive gliosis may have distinct features from non-proliferative (Sirko et al., 2013). It remains to be determined if this reactivity is a spectrum, or if there are numerous discrete reactive states that are generated, depending on the mixture of paracrine signals and the injury type present.

Proliferative reactive astrocytes likely represent a type of neuroprotective astrocyte state. Proliferation of reactive astrocytes has been tied to astroglial vascular contacts and blood-cell infiltration (Bardehle et al., 2013). Specific removal of proliferating reactive astrocytes leads to increased lesion sizes after brain-stab, spinal cord injury, and stroke (Buffo et al., 2008; Bush et al., 1999; LeComte et al., 2015; Shimada et al., 2012; Wanner et al., 2013). Work from our lab and others indicates that Notch1, signal transducer and activator of transcription-3 (STAT3), and endothelin

receptor type-B (ETB_R) regulate astroglial proliferation after stroke (Anderson et al., 2016; LeComte et al., 2015; Shimada et al., 2012). Notch1 is a membrane-bound receptor that interacts with membrane-bound ligands (e.g. Jagged, Delta-Like) in a juxtacrine fashion. This interaction leads to intracellular cleavage of the Notch intracellular domain, which translocates to the nucleus and acts as a transcription factor (Bray, 2016). The transcription factor STAT3 is part of the JAK-STAT pathway downstream from cytokines and paracrine factors such as EGF, leukemia inhibitory factor (LIF), and interleukin-6 (IL-6). Phosphorylation of STAT3 promotes its translocation to the nucleus and STAT3 activation is a characteristic of astrocyte reactivity. Endothelin receptor type-B is a potent regulator of vascular tone in response to endothelin ligands (Lucas et al., 1996; Verhaar et al., 1998). The removal of any one of these signals (Notch1, STAT3, ETB_R) decreases astroglial proliferation in response to traumatic central nervous system injury, and therefore removes the tissue protection derived from proliferative reactive astrocytes. Interestingly, genetic removal of astroglial STAT3 improves aspects of the cellular response in a mouse model of Alzheimer's disease (Reichenbach et al., 2019). Astrocytes are generally not proliferative in Alzheimer's disease. If astroglial STAT3 signaling is detrimental in Alzheimer's but beneficial in traumatic injuries, it indicates that regulators of reactive gliosis are performing disease-specific functions. These functions are detrimental or beneficial depending on the inflammatory context, possibly independent of their promotion of glial proliferation. Importantly, the paracrine activity of proliferating reactive astrocytes and how that activity is regulated by pro-proliferative signaling pathways has not been determined. Aspects of neuroprotection are mediated by the release of paracrine factors such as glial derived neurotrophic factor (GDNF) and

brain derived neurotrophic factor (BDNF), but the full neuroprotective paracrine profile of proliferative reactive astrocytes, and how this profile is regulated, has not been characterized (Koyama et al, 2003a, 2003b, 2005; Rocha et al., 2012; Toyomoto et al., 2005). Determining the paracrine profile downstream of each astroglial pro-proliferative pathway, and the disease-specific impacts of each, may lead to new treatments for CNS injury.

Microglia

Microglia are derived from hypoblast/yolk sac erythromyeloid precursors, an important cellular source of blood cells during development (Ginhoux et al., 2010; Kierdorf et al., 2013). Primitive macrophages enter the central nervous system through the vasculature and leptomeninges prior to neurogenesis (Monier et al., 2007). The transition from erythromyeloid progenitor (c-Kit$^+$ cells) to mature microglial cell is dependent on signaling through the colony stimulating factor-1 receptor (CSF1R). Microglial maturation is characterized by subsequent upregulation of the transcription factors Pu.1 and Irf8 (Ginhoux et al. 2010; Kierdorf et al. 2013). The CSF1R ligands are CSF1 and interleukin-34 (IL-34), cytokines which are thought to have interchangeable trophic functions. There is some evidence that the CSF1 and IL-34 have slightly different mechanisms of action, in part because IL-34 is able to interact with receptor type protein-tyrosine phosphatase zeta (PTPζ) in addition to CSF1R (Nandi et al., 2013). Furthermore, IL-34 is expressed at high levels during microglial cell development as compared with CSF1 expression, indicating that development of the mature microglial phenotype is likely IL-34 dependent and less dependent on CSF1 (Ginhoux et al. 2010). IL-34 and CSF1 are differentially expressed between adult white matter and gray matter, which

drives a corresponding differential dependence of microglia on IL-34 and CSF1 between white and gray matter (Easley-Neal et al., 2019). Microglial progenitors induced by these signals upregulate MMPs, possibly as a mechanism of entry into the brain. Progenitors also express increased TGFβ1, an essential factor that regulates microglial activity, survival, and identity (Kierdorf et al. 2013).

Once in the brain, microglia are maintained locally, renewed by other microglial cells, with blood-cell contribution limited or non-existent in the uninjured brain due the presence of the blood-brain barrier (Ajami et al., 2007). This sets microglial cells apart from many other neural cells within the brain, in that they are not ectodermally-derived like macroglial cells and neurons. In the adult brain, long after CSF1R signaling has induced microglial specification, microglia remain dependent on CSF1R signaling to survive and maintain their identity (Elmore et al., 2014). CSF1R inhibition eliminates microglial cells from the adult brain. Transforming growth factor-β (TGFβ) signaling plays a similar role in maintaining microglial identity and limiting de-differentiation and reactivity (Bohlen et al., 2017; Butovsky et al. 2014; Qin et al. 2018; Zoller et al. 2018). Removal of TGFβ paracrine signals can cause microglia to lose aspects of their unique signature that set them apart from other macrophages and monocytes. They de-differentiate, taking on a pro-neurodegenerative phenotype, leading to lethal demyelination. It is unclear if TGFβ microglial maintenance occurs through microglial expressed TGFβ receptors or TGFβ signaling in other cell types (Hickman et al., 2013). Of interest, TGFβ signaling also induces infiltrating blood-cells to take on microglial-like mRNA signatures in a time-dependent manner after a BBB compromising injury (Qin et

al., 2018). Promotion of a microglial-like phenotype by TGFβ signaling reigns in some of the neurotoxic effects of blood-cell entry into the brain (Cekanaviciute et al., 2014).

Certain aspects of microglial identity are highly specified to neuronal functions. For example, in developing neural pathways of the visual system, there is a surplus of synapses that undergo significant pruning to refine the pathways to support coherent conveyance of visual information. Microglial cells are involved in pruning of these excess synapses. TGFβ3 signaling induces superfluous synapses to express complement C1 components that promote synaptic engulfment by microglia (Bialas and Stevens, 2013). Neuronal synapse pruning during development lends further evidence to the tight regulatory role of TGFβ signaling in microglial functions. TGFβ, along with other paracrine regulators, often have parallel roles in regulating cellular dynamics in both neural development and adult injury/diseases states.

While microglial engulfment of complement-tagged synapses occurs during development and CSF1 regulates the microglial development, similar mechanisms also play a significant role in adult CNS injury and disease. In Alzheimer's Disease, synapses near amyloid beta plaques are tagged by complement for microglial engulfment (Hong et al., 2016). Complement is also a microglial-derived signal which leads to the formation of neurotoxic astrocytes in diseases such as Alzheimer's and Parkinson's (Liddelow et al., 2017; Shi et al., 2017). In models of peripheral nerve injury, sensory neurons release CSF1 which activates microglia and leads to chronic neuropathic pain (Guan et al., 2016). This is in contrast with evidence that IL-34 promotes microglial neuroprotection and improved BBB integrity (Jin et al., 2014; Mizuno et al., 2011). CSF1-receptor inhibitors have been used to reduce microglial inflammatory activity and prevent memory

deficits in Alzheimer's disease mouse models (Dagher et al., 2015); these same inhibitors eliminate microglia from the brain altogether (Elmore et al., 2014). Therefore, as seen with reactive astroctyes, many aspects of developmental signaling (CSF1, TGFβ, and complement) are recapitulated or repurposed in microglial responses to injury and/or disease in adults.

During development, embryonic day 7.5-8.5 (E7.5-8.5) microglial cells briefly express the Tie-2 receptor before expression disappears by E10.5 (Gomez Perdiguero et al., 2015). The function of Tie-2 receptor signaling in regulating microglial development and/or inflammation has not been determined. Macrophages express Tie-2 in certain injury situations, particularly in angiogenic contexts and tumor angiogenesis (Chen et al., 2016; Patel et al., 2013). The timeline of Tie-2 expression in microglia closely corresponds to their initial entry into the brain around E9.0 (Kierdorf et al., 2013). This entry is prior to neurogenesis and angiogenesis, so it is possible that Tie-2 is expressed to induce a pro-angiogenic microglial state in development, similar to pro-angiogenic Tie-2 expressing macrophages (TEMs) (Arnold and Betsholtz, 2013; Dudiki et al., 2020). It is also possible that angiopoietins in the CNS promote entry of microglial precursors, just as angiopoietins are pro-migratory to TEMs. Many injuries and diseases in the brain have vascular involvement and traumatic injuries such as stroke are particularly dependent on angiogenesis for optimal recovery. Given that TEMs appear in a variety of situations requiring angiogenesis, such as dermal wound healing and as pro-angiogenic cells in tumor angiogenesis, it is reasonable to predict that Tie-2 expressing microglia could play similar pro-angiogenic roles (Chen et al., 2016; Patel et al., 2013). If microglia behave similarly to their macrophage counterparts, it is likely that pro-angiogenic microglia

increase Tie-2 expression in conjunction with post-stroke angiogenesis. After stroke, Tie-2 activity in both microglia and the vasculature may function to synchronize pro-angiogenic signaling and inflammatory state in the stroke penumbra.

Oligodendrocytes

Oligodendrocytes are macroglial cells of the central nervous system that generate dense protein and lipid-rich structures called myelin. Oligodendrocytes have multiple processes with myelin at the distal end. The processes wrap and insulate neuronal axons of the CNS to facilitate fast conduction and prevent action potentials from spreading between axons. The myelin covered portion of the axon is referred to as the internode, and each internode is separated by small unmyelinated axon segments called nodes of Ranvier (Bergles and Richardson, 2015). Oligodendrocytes and myelin also support axons through trophic and cell-cell signaling mechanisms that are distinct from the effects of myelin on axonal electrophysiology (Byravan et al., 1994; Du and Dreyfus, 2002; Lappe-Siefke et al., 2003; Wilkins et al., 2003).

In multiple sclerosis (MS) relapses and secondary progressive MS, demyelination and oligodendrocyte cell-death lead to symptoms such as motor dysfunction and visual deficits. Oligodendrocyte progenitors (a.k.a. OPC, $NG2^+$ glial cell, polydendrocyte) remyelinate axons and may improve symptoms after therapy or during disease remission (Baxi et al., 2017). OPCs exhibit plasticity in the healthy adult brain, generating new myelin internodes and contributing to certain types of learning (Hughes et al., 2018; McKenzie et al., 2014). Similar to other glial cells, OPCs proliferate after many CNS injuries including stroke (Bonfanti et al., 2017; McTigue et al., 2001). OPCs are some of

the first cells to respond to LPS injection, and change morphology prior to microglial cells (Rhodes et al., 2006).

Schwann Cells

Schwann cells are the glia of the peripheral nervous system (PNS) that myelinate single axons, but also ensheath single or multiple axons to support neurons and/or facilitate fast saltatory conduction. In contrast with CNS oligodendrocytes which myelinate multiple axons, Schwann cells generate compacted myelin to wrap a single axon and only the largest caliber axons are myelinated. Schwann cell myelin is similar in nature to CNS oligodendrocyte myelin in its facilitation of faster axonal conduction speeds (Salzer, 2015). Smaller caliber axons are ensheathed by a subset of Schwann cells called Remak cells. Remak cells can have a single or multiple troughs that contain the small axon(s) (Jessen and Mirsky, 2019). Similar to CNS astrocytes, Remak cells also secrete trophic factors to promote growth and maintenance of peripheral axons, but do not facilitate faster axonal conduction.

After peripheral nerve injury, Schwann cells withdraw their myelin, phagocytose debris, and secrete factors that promote axon growth (Jessen and Mirsky, 2019). If the connective tissue of the nerve is still aligned after the injury, axons regrow down the nerve and re-innervate their destination sites. Axonal regeneration after an injury is dependent on Schwann cell c-Jun signaling and expression of the trophic factors artemin (*Artn*), brain derived neurotrophic factor-1 (*BDNF1*), glial derived neurotrophic factor (*GDNF*), leukemia inhibitory factor (*LIF*), and nerve growth factor (*NGF*) (Fontana et al., 2012). Notably, this repair process contrasts with that of CNS injuries in which axons do not significantly regenerate.

Muller Cells

Muller cells are glia that provide specialized support to the diverse neural cell types of the retina. Both Muller cells and neurons of the retina arise from a common progenitor in development (Turner and Cepko, 1987). Astrocytes and Muller cells are the neuroglia of the retina that, along with microglial cells, maintain retinal homeostasis. Astrocytes are more prevalent in the nerve fiber and inner nuclear layers of the retina. Muller cells are radially oriented such that they interact with all retinal cell layers. Many functions such as BBB maintenance and regulation of the neural cell environment are shared between Muller glia and astrocytes (Vecino et al., 2016).

Injury to the retina causes glial reactivity in a manner similar to glial responses in the rest of the CNS. Microglial cells respond to retinal high-intensity light-injuries by upregulating trophic factors such as GDNF, NGF, neurotrophin-3 (NT-3), and ciliary neurotrophic factor (CNTF). Additionally, microglial cells induce Muller glia to express increased levels of bFGF, GDNF, and BDNF, that serve to protect retinal neurons after injury (Harada et al., 2002). Muller glial GDNF and BDNF also confer neuronal protection in diabetic retinopathy (Fu et al., 2015). Similar to astrocytes, Muller glia regulate innate immune complement cascades, which affect neurotoxicity and retinal degeneration (Guttenplan et al., 2020; Pauly et al., 2019). Due to the therapeutic accessibility of the retina, many studies manipulate retinal neuroinflammatory responses to model nervous system injury. However, it will be important to understand the distinctions between glial cells of the retina and the CNS in order to translate these therapeutic approaches to CNS injuries and disease.

1.2 Background on stroke

Basics

Stroke is a cerebro-vascular event in which blood flow is blocked to regions of the central nervous system. In most clinical cases this is induced by a thrombus or embolus that occludes an artery. If the blockage leads to rupture of the blood vessel, a hemorrhagic stroke develops. The most common type of stroke is an ischemic stroke, in which the occluded artery does not rupture (Campbell et al., 2019). In ischemic stroke, the flow of nutrients and oxygen to parts of the brain is impaired, but there is not an acute influx of blood into the brain parenchyma. Due to the lack of blood flow and oxygen, oxidative phosphorylation in the affected area drops. Without adenosine tri-phosphate (ATP), neurons cannot pump ions against their concentration gradients and neuronal activity ceases (Lipton and Wittingham, 1979; Santos et al., 1996). Additionally, ATP and adenosine levels increase in the extracellular environment, triggering microglial adenosine receptor signaling (Lu et al., 2012; Monif et al., 2009). The lack of oxygen also triggers hypoxia inducible factor (HIF) activation. Endothelins and HIF are known to induce each other's expression and activity, possibly indicating a positive feedback loop. Under hypoxic and injurious conditions, endothelins are released by endothelial cells, microglial cells, and astrocytes (Hammond et al., 2014; Khamaisi et al., 2015; Tsang et al., 2001; Wang et al., 2018; Wu et al., 2006). Endothelins induce blood vessel vasoconstriction, which could exacerbate stroke, but may also function to protect the area from blood flow that might infiltrate through the leaky vasculature. The longer that cerebral tissue is deprived of nutrients and oxygen, the greater the level of neuronal death and tissue necrosis.

Types of stroke

Ischemic stroke resulting from thrombus involves a blood clot that occludes a cerebral artery. The blockage leads to impaired blood flow and a decrease of nutrient and oxygen delivery within the downstream vascular distribution of the impacted artery. Some redistribution of blood flow can mitigate the damage, such as rerouting of blood at the Circle of Willis, or through anastomosis of anterior, middle, and posterior cerebral arteries.

Hemorrhagic stroke, or stroke with hemorrhagic transformation, is a significant clinical complication because of the limited supportive interventions available. This type of stroke includes intracerebral hemorrhage in which the bleeding occurs within the brain parenchyma, and subarachnoid hemorrhage in which the bleeding occurs outside the parenchyma but underneath the dural and arachnoid meningeal layers (outside of the pial layer). In ischemic stroke, there can also be hemorrhagic transformation, with bleeding developing within the infarcted area. A common clinical consideration in treating ischemic stroke is ensuring that the patient is not at risk of hemorrhaging in response to tPA administration (Yaghi et al., 2017).

A frequent precursor to a major stroke is a transient ischemic attack (TIA), in which blood flow is only temporarily impeded to an area of the brain. Since the transient ischemia often occurs for less than an hour, the tissue is rarely permanently damaged, and an infarct is generally not formed. Clinically diagnosing the source of the clot is essential to determining if the TIA patient is at high risk of a subsequent cerebrovascular event that will result in significant tissue with infarction (Amarenco et al., 2018; Perry et al., 2011).

Similar to TIA, silent cerebral infarcts (SCIs) can be precursors to a major stroke. In contrast to TIAs, SCIs are detected only by MRI. As the name indicates, these small infarcts do not impact enough brain tissue or behaviorally-essential brain area to cause noticeable symptoms and are therefore termed "silent" (DeBaun et al., 2012).

Border zone infarcts (watershed infarcts) are strokes that occur at the edges of the vascular distribution of the major arterial blood flow to the brain. A decrease in cerebral blood flow leads to inadequate perfusion of small blood vessels that anastomose between vascular territories of the three major cerebral arteries: the middle, anterior, and posterior cerebral arteries; this leads to unique stroke patterns following the edges of each major vessels' vascular territories. Infarcts of this type result from haemodynamic compromise, reduced blood flow due to other clinical conditions, or from emboli (Mangla et al., 2011).

Ischemic Stroke Treatment

To date, the only FDA-approved treatment for ischemic stroke is recombinant tissue plasminogen activator (rtPA, also known as Alteplase) and supportive care. Tissue plasminogen factor, expressed by endothelial cells, is a serine protease that induces fibrinolysis (breakdown of blood clots) by converting plasminogen into plasmin (Suzuki et al., 2011). The recombinant form of tPA was developed to break down an arterial thrombus that causes a stroke. Unfortunately, there is a strict time window during which rtPA is therapeutically effective (~4.5 hours) (Barber et al., 2001; O'Rourke et al., 2009). Outside of this time window rtPA can actually increase the risk of hemorrhaging and worsen stroke outcomes (Yaghi et al., 2017). The inability of many people to recognize the symptoms of a stroke and quickly get to a hospital means that the majority of ischemic stroke patients do not receive rtPA treatment (only ~7% treated). Attempts to

expand beyond the limits of the ~4.5 hour effective time window of rtPA have led to only minor improvements in patient outcomes. Minimal added effectiveness, and increased risk of intracerebral hemorrhage mean that expanded time windows are generally not indicated for most patients.

A second treatment option is endovascular and mechanical, in which a catheter is used to access the vasculature and remove the thrombus from major arteries. This approach includes a more flexible treatment timeline than does rtPA, but because of the intervention mechanism, thrombectomy is limited to large arteries. Estimates of the percent of ischemic stroke patients treatable by thrombectomy vary widely from 3% to 22% (Mokin et al., 2019). Notably, with the majority of ischemic stroke patients not receiving a reperfusion treatment, more therapies are needed to treat stroke patients that are not reperfused.

1.3 Cell signaling background

Endothelins

Endothelins are evolutionarily conserved signaling peptides involved in the maintenance of vascular tone. There are three endothelin ligands: ET-1, ET-2, and ET-3. All endothelins start out as precursor proteins termed “big-endothelin” and are converted to their active form by endothelin converting enzyme (ECE) (Davenport et al., 2016; Khamaisi et al., 2015). The peptide ligands are then secreted into the extracellular space by secretory vesicles or are processed to their active form to reside within Weibel-Palade Bodies (WPBs) (Ozaka et al.,1997; Russell et al., 1998). The receptors for endothelin ligands are endothelin receptor type-A (ETB_R) and endothelin receptor type-B (ETB_R). Both receptors are expressed in vascular cells, where endothelin signaling regulates

vascular tone (Bacon and Davenport, 1996; Brain et al., 1998). High densities of endothelin receptor type-A and type-B expressed on smooth muscle cells trigger intracellular $Ca2^{+}$ release, leading to vasoconstriction (Bacon and Davenport, 1996; Merlen et al., 2013; Roubert et al., 1991). By contrast, endothelin receptor type-B expressed on endothelial cells triggers Nitric Oxide release and vasodilation (Dupuis, 2001; Schneider et al., 2007; Verhaar et al., 1998). ETB_R signaling simulates downstream RAC-alpha Serine/Threonine-protein kinase (AKT) and extracellular signal-regulated kinase (ERK) activity, which may account for some of the indirect vasodilatory effects of endothelins. WPBs contain von Willebrand factor (VWF), P-selectin, and endothelins. WPB exocytosis is one of the first responses to vascular injury, triggering blood clotting, inflammatory cell recruitment, and vasoconstriction. Hypoxic/ischemic tissue injuries such as stroke induce the exocytosis of WPBs include hypoxia injuries (Pinsky et al., 1996; Rondaij et al., 2006).

In addition to endothelial cells, microglial cells also express endothelin. Like endothelial cells, microglia express ET-1 and ETB_R in response to ischemia (Li et al., 2010; Rogers et al., 1997). There is also evidence that microglia increase ET-1 expression in response to inflammatory stimuli such as lipopolysaccharide (Wu et al., 2006). This indicates that endothelin ligands may not be exclusively coupled to endothelial cell function and vascular tone but may also act as important regulators of inflammatory responses.

Astrocytes do not express endothelins under normal conditions but upregulate endothelin secretion under pathological conditions such as stroke, accumulation of amyloid beta peptide, and in proximity to multiple sclerosis plaques (Ehrenreich et al.,

1993; Hama et al., 1997; Ho et al., 2001; Jiang et al., 1993; Zhang et al., 1994). ET-1 blocks remyelination in multiple sclerosis lesions by promoting increased Jagged-1 expression in astrocytes, which is associated with a putative protective/proliferative reactive astrocyte phenotype. Astrocyte Jagged-1 signals to oligodendrocyte Notch receptors to inhibit remyelination (Hammond et al., 2014). $Jagged^{Hi}$ astrocytes are associated with neuroprotection in stroke, but inhibit myelination in multiple sclerosis, again illustrating the context-dependence of reactive astrocyte phenotypes (LeComte et al., 2015). Furthermore, endothelin signaling can stimulate the migration of subventricular zone progenitors which have similarities to reactive astrocytes (Gadea et al., 2009).

In multiple CNS diseases and disorders, astroglial endothelin signaling serves as a pro-growth trophic factor, inflammatory regulator, and mediator of vascular tone (Jo et al., 2014). In the brain, astrocytes express some of the highest levels of ETB_R relative to other cells, while expressing low levels of ETA_R (Guo et al., 2014; Hammond et al., 2015; John Lin et al., 2017; Zhang et al., 2014). Strong ETB_R expression localizes to reactive astrocytes within the peri-infarct area after ischemic stroke (Lecomte et al., 2015). After stroke, it is likely that astrocytes need an array of receptors to detect BBB compromise and respond to the injury. ETB_R expression may provide a multifaceted response mechanism to detect injury signals and induce both growth and inflammatory responses in reactive astrocytes.

Angiopoietins

Angiopoietins are a family of secreted paracrine factors made up of angiopoietins 1-4 (Ang1-4) and Angiopoietin-like proteins 1-8 (Angptl1-8). Angiopoietin family members

regulate the sprouting of new blood vessels from existing vessels (angiogenesis) and vascular stability. This is notably a separate process from *de novo* development of vasculature from precursor cells (vasculogenesis) which can also be regulated by Tie-2, vascular endothelial growth factor (VEGF), and Angptl2 (Dumont et al., 1994; Richardson et al., 2014; Shalaby et al., 1995). Ang-1 is the primary agonist of the Tie-2 receptor. Ang-1/Tie-2 signaling promotes endothelial cell survival, tight junction integrity, and vascular stability. Ang-2 was originally described as an antagonist of the Tie-2 receptor that promoted destabilization of the vasculature (Maisonpierre et al., 1997). Increasing evidence suggests that Ang-2 acts as an agonist or antagonist depending on context; this includes the presence or absence of inflammation (Akwii et al., 2019; Hegen et al., 2004; Kim et al., 2016). One aspect of this context dependence is expression levels of the Tie-1 receptor, which can be altered by infection and tumor necrosis factor (TNF) signaling. Tie-1 and Tie-2 are differentially expressed within the endothelial cells of growing vasculature to promote tip-cell proliferation, as compared with stable, non-growing blood vessels (Kim et al., 2016; Savant et al., 2015; Yuan et al., 2007). Tie-2 can form homodimers or heterodimerize with the Tie-1 receptor. There is evidence that heterodimerization blocks Ang-2 signaling, but Ang-1 treatment dissociates heterodimer and Tie-2 homodimer formation and signaling (Seegar et al., 2010). An additional important factor in the function of Ang-2 is the presence or absence of VEGF. In the absence of VEGF, Ang-2 promotes vascular collapse and endothelial cell death. In the presence of VEGF, Ang-2 promotes angiogenesis (Zhu et al., 2005).

These different signaling environments might explain why the effects of Ang-2 in the context of stroke are controversial. In the acute phase of stroke, when delivered prior

to ischemic injury, Ang-2 is reported to be protective (Marteau et al., 2013). Alternatively, the canonical role of Ang-2 in downregulating vascular tight junctions and increasing vascular permeability is thought to exacerbate stroke injury (Gurnik et al., 2016). These differences in Ang-2 function could be important to effectively use it in stroke therapies. Translational studies designed to modify post-stroke recovery and tissue regeneration using VEGF hydrogels have injected them 5 days post-infarct (Nih et al., 2018). Both timing of the administration and the contents of the hydrogels is critical because they will interact with endogenous signals. Therefore, it is important to understand related signaling pathways in necrosing tissue as ischemia progresses. Defining how VEGF interacts with endogenous Ang-2 may be critical for mechanistic understanding of the observed treatment effects of the VEGF hydrogels. Other inflammatory signals such as tumor necrosis factor (TNF), which is thought of as pro-inflammatory and cell-death promoting, are similarly context dependent. TNF is an essential factor in developmental and post-stroke angiogenesis (Huang et al., 2016). It promotes angiogenesis, in part, by inducing the expression of Ang-2. The contrasting pro-angiogenic and pro-apoptotic functions of TNF signaling illustrates how a variety of post-stroke signal transducers are highly context-specific and timing-dependent. This also highlights the manner in which the inflammatory and angiogenic paracrine environments after stroke are intricately linked. To better design and properly time the administration of disease-modifying therapies, it will be critical to better understand the full array of cell signals and their spatial and temporal specificity.

TGFβ signaling

The transforming growth factor-β (TGFβ) superfamily consists of cytokine signaling molecules that includes TGFβs (1-3), bone morphogenic proteins (BMPs 1-20), activins (A and B), inhibins (A and B), myostatin, nodal, leftys (1 and 2), Mullerian inhibiting substance (MIS), and glial derived neurotrophic factor (GDNF) family members (Gordon and Blobe, 2008; Eigenbrot and Gerber, 1997). The superfamily is named for the originally discovered cytokine TGFβ1.

TGFβs are characterized by expression in a dimerized form with a large prodomain. The prodomain maintains the factors in a latent (non-signaling form, although latent forms may have some activity). Latent TGFβs associate with the extracellular matrix through latent TGFβ binding proteins (LTBPs). Importantly latent TGFβs are in a "ready-to-activate state", and able to respond to changes in the cellular environment or to injury (Schachtrup et al., 2010). Integrins, thrombospondins, fibrinogen, and environmental changes such as temperature and pH, all promote transformation of latent TGFβ into its active form.

There are three major TGFβ ligands: TGFβ1, TGFβ2, and TGFβ3 (Derynck and Budi, 2019; Massague, 2012). TGFβs bind to and induce heterodimeric complexes of Type I and Type II TGFβ receptors. There are seven type I receptors, five type II receptors, and a small number of type III co-receptors that modulate the signaling of ligands through the type I and II receptors. TGFβ signals through type I receptors, phosphorylating and activating downstream SMAD2 (SMA, small worm phenotype; MAD, Mothers against Decapentaplegic) and SMAD3 signaling. BMPs activate their

type I receptors to signal through downstream SMAD1, SMAD5, and SMAD8 (Akhurst and Hata, 2012). These SMADs cooperate with SMAD4 to translocate to the nucleus, interact with transcription factors, and alter gene expression. The mechanism that inhibits SMAD transcriptional activity is the expression of inhibitory SMADs (6 and 7), which are essential to CNS development (Casellas and Brivanlou, 1998; Imamura et al., 1997; Nakao et al. 1997). The list of signaling pathways that can be activated by TGFβ family members is extensive, and includes: tumor necrosis factor receptor-associated factors (TRAFs), P38/MAPK, RHO, PI3K, AKT, ERK, JNK, and NFκB (Zhang, 2009).

TGFβ has essential roles in the regulation of development and inflammatory responses. The induction of ectoderm and the primitive nervous system requires the inhibition of TGFβ/BMP signaling, and this effect has been shown to cause embryonic stem cells and induced pluripotent stem cells to take on a neural cell fate (Chambers et al., 2009). Increased TGFβ signaling suppresses some aspects of stroke injury (Doyle et al., 2010; Gross et al., 1993; McNeill et al., 1994; Pang et al., 2001). TGFβ promotes a mature microglial state and reigns in certain aspects of microglial activity such as microglial-driven demyelination (Hollander et al., 2017; Qin et al., 2018; Stoll et al., 2004). In other settings, TGFβ promotes astroglial and immune cell reactivity and inflammation (Diniz et al., 2017; Schachtrup et al., 2010; Veldhoen et al., 2008). These effects contrast with the role of TGFβ in limiting reactivity and dampening down inflammation in astrocytes and microglia.

CD109

CD109 is an alpha-2 macroglobulin C3, C4, C5 family member and a TGFβ co-receptor (Finnson et al., 2006; Lin et al., 2002). As a membrane-bound thioester-containing protein, CD109 can be released from the cell membrane through furin cleavage (Janssen et al., 2005). The solubility of CD109, and its role in controlling cellular growth has made it a marker of more aggressive cancers and a target for early cancer diagnosis (Dong et al., 2015; Emori et al., 2013). Studies on the function of CD109 have been limited to determining how it regulates fibroblast proliferation and extracellular matrix production in injuries and diseases of the skin. Two primary models for the actions of CD109 have arisen from these studies. The first is that CD109 binds to TGFβ receptors, localizes the receptors to caveolae, and induces receptor degradation and desensitization of cells to TGFβ signaling (Bizet et al., 2011). The other model proposes that CD109 binds TGFβ and TGFβ receptors, shunting TGFβ signaling away from ALK5 class receptors toward ALK1 receptors (Vorstenbosch et al., 2017). ALK1 signals through SMAD1/5, decreasing extracellular matrix production and increasing cellular proliferation. Notably, many functions of membrane bound CD109 are retained by furin cleaved, soluble CD109 (Li et al., 2016; Litvinov et al., 2011). Thus, soluble CD109 may have widespread or local effects on the injury environment.

In mouse models of rheumatoid arthritis, neutralization of CD109 decreased inflammation, improved symptoms, and led to better outcomes (Song et al., 2019). This illustrates that CD109 is likely to alter the inflammatory activities regulated by TGFβ. Adding complexity to this picture, CD109 was shown to affect EGF signaling (Zhang et al., 2015). Notably, it is closely related to complement C3, a key element in complement

activation, inflammation, and a marker of neurotoxic astrocytes (Janssen et al., 2005; Liddelow et al., 2017).

The role of CD109 in other systems is just being elucidated. In the brain, studies have found expression of CD109 mRNA in neuroprotective astrocytes. To our knowledge no mechanistic studies of the function of CD109 in astrocytes have been reported (Liddelow et al., 2017). CD109 expression causes increased glioma-cell proliferation and decreased survival times in glioma mouse models (Shiraki et al., 2017). Increased CD109 expression in glioblastoma was also associated with radioresistance and poor prognosis (Minata et al., 2019). Gene expression studies indicate that CD109 is primarily highly expressed by endothelial cells in the healthy brain. During brain injury and neuroinflammation, CD109 is one of the most upregulated genes in reactive astrocytes (Zamanian et al., 2012). Additionally, an analysis of the astrocyte glio-vascular endfoot found CD109 mRNA undergoing active translation (Boulay et al., 2017). As vascular-contacting astrocytes are uniquely proliferative and scar forming, and this process is partially regulated by TGFβ, the location of CD109 at the astrocyte vascular enfoot may be significant. Given the location of CD109 in astrocytes, and changes to CD109 after injury, the function of astroglial CD109 in the brain during homeostasis and after injury warrants further investigation.

1.4 Proliferative reactive astrocyte response to injury

Opinions pertaining to reactive astrogliosis and proliferative reactive astrocytes, are, in many ways, tied to the history of the glial scar. This is exemplified by studies that eliminated proliferative reactive astrocytes and prevented the formation of the glial scar (Bush et al. 1999, Wanner et al. 2013). Glial scars usually form in response to traumatic

injuries such as stroke and spinal cord injury and are often the most striking feature of CNS injury. It's commonly thought that the glial scar is inhibitory to mammalian central nervous system regeneration after an injury (Hu et al., 2010; Wang et al., 2018; Yiu and He, 2006). This hypothesis derives from an observed lack of regeneration in the central nervous system after injury, in contrast to notable nerve regeneration seen after peripheral nerve injuries. Historically, mammalian glial scarring was shown to prevent axonal regeneration, while there is clearly axon regeneration through glial scars in multiple other species, including amphibians. In the rat central nervous system axons grow into grafts or "bridges" of peripheral nervous system tissue after spinal cord transection (David et al. 1981; Richardson et al. 1980). Also, the glial scar contains astrocytes that express chondroitin sulfate proteoglycans, which dramatically inhibit axon growth (Fry et al. 2010; Monnier et al. 2003; Snow et al. 2002). Myelin within the central nervous system is generated by oligodendrocytes, whereas peripheral nervous system axons are myelinated by Schwann cells. Oligodendrocytes express multiple factors inhibitory to axonal growth, including the aptly named protein "nogo" (GrandPre et al. 2002; Stephany et al., 2014). These studies highlight differences between the mammalian CNS and PNS tissue, and the CNS tissue of other species.

Recent work, particularly from the Sofroniew lab, is helping to change the negative view of the glial scar. Prior attempts at a therapy for spinal cord injury attempted to eliminate the scar or scar components, in order to promote regeneration (Janzadeh et al., 2017; Rosenzweig et al., 2019). Advances in mouse genetics have allowed for the selective removal of proliferative reactive astrocytes and therefore inhibition of scar formation in response to spinal cord injury (Faulkner et al., 2004; Herrmann et al., 2008).

As expected, wild-type axons grow into the scar but are unable to pass through the scar after spinal cord injury. Surprisingly, genetic removal of proliferative reactive astrocytes and glial scar actually led to decreased axon regeneration as compared to that observed in wild-type mice (Anderson et al., 2016). Multiple strategies were applied to significantly regenerate axons through the scar in wild-type mice, including hydrogel-based delivery of trophic factors and viral delivery of pro-regenerative signals to neurons. While these strategies promoted axon regeneration through the scar and lesion after SCI, they universally failed in mice with inhibited scar formation (Anderson et al., 2018). Importantly, there is some question as to whether delivering such therapies 2 days post-spinal cord injury leads to regeneration prior to full maturation of the glial scar. Some aspects of the scar are not fully mature at this time point, but many of the inhibitory molecules are present within the scar at the time of the reported treatments. Regardless, these studies demonstrate the importance of proliferative reactive astrocytes in spinal cord injury recovery and add to our understanding of the role of the glial scar in regeneration.

Reactive astrocytes proliferate in response to catastrophic injuries of the central nervous system such as stroke, spinal cord injury, and penetrating traumatic brain injury (Frik et al., 2018; Lecomte et al., 2015; Shimada et al., 2012; Wanner et al., 2013). This is in contrast with inflammatory neural disfunction such as Alzheimer's disease, multiple sclerosis, and Parkinson's disease, in which astrocytes become reactive but proliferate minimally (Liddelow and Barres, 2017). This difference has been mirrored in recent work that shows that astrocytes become "neuroprotective" in response to stroke but become largely "neurotoxic" during inflammatory diseases of the brain (Liddelow et al., 2017).

When proliferative reactive astrocytes are eliminated and scar formation is inhibited, injuries to the CNS expand, axon regeneration decreases, and blood-cell infiltration increases. Interestingly, blood-cell infiltration is a primary stimulus of astroglial proliferation in the first place (Frik et al., 2018). After injury, vascular contacting astrocytes proliferate disproportionately as compared to non-vascular contacting astrocytes (Bardehle et al., 2013). Despite potentially neuroprotective features of proliferative reactive astrocytes and changes in the cellular environment upon their removal, research has largely focused on scar formation and ignored paracrine factors released by these cells. With the recent discovery of multiple regulators of astroglial proliferation in stroke, tools are now available to determine how proliferative astrocyte regulators alter astroglial derived paracrine signals. Endothelins regulate vascular tone and our laboratory recently showed that endothelin signaling controls astroglial proliferation through a Notch1-STAT3-ETB_R signaling axis. With the ties between the vasculature and reactive astrogliosis, it is of great interest to determine how endothelins alter astroglial paracrine signaling.

1.5 Progression of blood brain barrier permeability and inflammation after stroke

In the healthy adult brain, the BBB keeps blood components out and participates in the selective trafficking of fluid and molecules into and out of the CNS (Wimmer et al., 2019). The maintenance of this barrier is dependent on tight junctions between endothelial cells and the active transport of selected molecules and cells (i.e. diapedesis) into the brain by endothelial cells. Astrocyte endfeet completely encase the vascular compartment, which includes endothelial cells, extracellular matrix, and pericytes. These barrier functions also act to limit the entry of immune-cells, such as leukocytes, into the

brain. The limited exposure of the brain to the immune system contributes to the perception of the brain's status as an immune-privileged organ (Forrester et al., 2018). When the BBB of the CNS is intact, most immune functions are maintained and performed by microglial cells (Ajami et al., 2007).

After ischemic stroke without hemorrhage, there is still dysregulation of endothelial cell tight junctions; this leads to increased blood cell infiltration and contributes to neuroinflammation after stroke (Gelderblom et al., 2009; Reijerkerk et al., 2011). The compromise of the BBB is a significant event that must be detected, and responded to, by a variety of CNS cell types. Microglial cells in particular have evolved a sensitive system wherein blood components act as damage associated molecular patterns (DAMPs) that they detect and that control their biology (Hickman et al., 2013). The array of receptors that microglia express to detect DAMPs has been referred to as "pattern recognition receptors" (PRRs). The array of PRRs prime microglia to detect the earliest signs of damage such as metabolic dysfunction and BBB permeability early in ischemia. One of the most studied groups of PRRs include the Toll-like receptors (TLRs). TLRs detect pathogen-associated lipopolysaccharide (LPS), specific blood components such as fibrinogen and fibronectin, and necrotic cells (Gulke et al., 2018; Kigerl et al., 2014). Toll-like receptor signaling triggers a variety of downstream paracrine and inflammatory effectors, including those that mediate neuroprotection (Kamigaki et al., 2016). Another notable wing of the PRR system is the P2Y receptors. These receptors detect nucleotides such as ADP and ATP in the extracellular space, as these are often signatures of tissue damage or disease. Microglia express P2Y receptors, and one of these (P2Y12) has been characterized as a microglial-specific marker within the CNS. Another characteristic

receptor of microglia is CD11b, which acts as a receptor for complement proteins and also triggers microglial activation after binding fibrinogen (Adams et al., 2007).

BBB breakdown contributes to the reactive astroglial response to injury and disease. Several studies have shown that blood-cell infiltration directly stimulates reactive astrocyte proliferation after CNS injury (Frik et al., 2018). Along with blood-cells, blood-specific components such as thrombin and fibrinogen enter the brain after stroke. Fibrinogen and other blood-borne factors activate latent TGFβ in the brain, and the activation of TGFβ by fibrinogen stimulates astrocyte scar formation in response to injury (Schachtrup et al., 2010; Zhang et al., 2018).

Due to the immune-privileged nature of the brain and the interplay of blood cell infiltration with neuroinflammation, it is important to understand how BBB dysfunction and neuroinflammation progress after ischemic stroke. An early step in barrier dysfunction after stroke is the shutdown of ionic transporters and the loss of ionic gradients (Yang et al., 1992). Changes in ionic balance lead to increased entry of fluid from the blood into the perivascular space (Chen et al., 2015). Additionally, oxidative stress and metabolic dysfunction promote downregulation of endothelial cell tight junction components, causing further edema (Lochhead et al., 2010; McCaffrey et al., 2009; Witt et al., 2003). Cell signaling changes such as fibrinogen activation of TGFβ, and the presence of other DAMPs (e.g. HSPs, HMGB1, nucleotides), induce microglial inflammatory reactions. Microglial cell numbers peak from 1-3 days after stroke, generally preceding peak blood cell infiltration (Gelderblom et al., 2009). Many of the inflammatory signals released by microglia in response to CNS damage also act to recruit blood-borne immune cells into the brain parenchyma. Endothelins, which are released by

microglia and numerous other CNS cells, including astrocytes, control the diapedesis of monocytes into the brain (Reijerkerk et al., 2012). The infiltration of blood cells into the brain is greatest around 3-days post-stroke (Gelderblom et al., 2009). Blood-cell infiltration into the CNS is toxic to neurons by direct cell-cell contact and release of proteases (Dimitrijevic et al., 2007; Dinkel et al., 2003; Soriano et al., 1999).

An important consideration in developing drugs to protect and regenerate neural tissue after stroke is the propensity of pharmacological agents to increase vascular leak. The most obvious example of this problem is rtPA treatment, which can cause increased blood infiltration, particularly when delivered outside of its specified time window or in patients with other complications such as hypertension, diabetes, or metabolic disease (Dong et al., 2016; Suzuki et al., 2015). Problematically, pro-angiogenic therapies such as VEGF treatment can lead to vascular leakage. Thus, there is great interest in developing drugs that can promote rescue and/or repair without compromising the vasculature or BBB (Rust et al., 2019).

1.6 Adult neural stem cells

Since their discovery, adult neural stem cells (NSCs) have been the focus of intense research. This is in part because of their perceived regenerative potential and the possibility that these cells or their downstream functions could contribute to adult CNS regeneration. These cells have many similarities to adult reactive astrocytes and may offer clues to inducing adult reactive astrocytes to promote regeneration after injury. During development, most NSCs exhaust their self-renewal potential and differentiate. However, a select number of NSCs persist into the adult brain (Reynolds and Weiss, 1992; Spalding et al., 2013). In the adult brain, NSC/NPCs reside in two main niches.

One niche is the walls of the lateral ventricles (the subventricular zone, SVZ), and the other niche is in the subgranular zone (SGZ) of the dentate gyrus (Bond et al., 2015; Fuentealba et al., 2012). SVZ neural stem cells self-renew and give rise to neuroblasts which migrate to the olfactory bulb by way of the rostral migratory stream. Within the olfactory bulb, these neural progenitors differentiate into interneurons that control olfactory processing (Merkle et al., 2014). SGZ stem cells self-renew and generate neuroblasts, but their progeny differentiate into neurons that incorporate into the granule cell layer of the hippocampus, later participating in hippocampal mechanisms associated with learning and memory (Kirschen et al., 2017; Zhuo et al., 2016).

A major question that persisted in the field after the discovery of adult neurogenesis was: where do adult neural stem cells come from? SVZ neural stem cells were recently shown to derive from a select number of cortical radial glial cells during development. Through signals such as vascular cell adhesion molecule-1 (VCAM1), neurotropin-3 (NT-3), Notch2, and β-catenin, selected radial glial cells are triggered during developmental neurogenesis to downregulate proliferation and become slow-proliferating (quiescent) adult neural stem cells. In contrast to other radial glial cells, which lose self-renewal potential and differentiate, these $VCAM1^{+}$ cells lower their division rate to avoid prematurely reaching their hayflick limit, thus allowing them to persist as stem cells into adulthood (Hu et al., 2017). Radial glial cells become the adult NSC, also known as the B-cell or the quiescent NSC of the adult SVZ stem cell niche (Codega et al., 2014). One current model indicates that there are two stem-cell types that self-renew at different rates and maintain the neurogenic potential of the SVZ. One is the quiescent NSC, characterized by a protein profile including expression of CD133, GFAP,

VCAM1, and FGFR, and a lack of EGFR expression (Codega et al., 2014). When the quiescent cell becomes an activated NSC, it loses VCAM1 expression and gains expression of EGFR (Codega et al., 2014). Along with these changes, there is a corresponding increase in cellular proliferation and expression of proliferative markers such as PCNA. The NSCs maintain EGFR expression and become transit amplifying progenitor cells (NPCs), which contribute to the pool of migratory neuroblasts, in part characterized at these later stages by doublecortin (DCX) expression.

In addition to VCAM1, which contributes to the specification of NSCs during development, there are a variety of other factors that maintain the unique state of adult NSCs. These signals are important to balance quiescence, proliferation, and differentiation. If this balance is not maintained, the stem cell pool could be exhausted or proliferate until reaching their Hayflick limit, thereby becoming senescent. Notch signaling is required for NSC self-renewal (Aguirre et al., 2010; Hatakeyama et al., 2014; Shen et al., 2002). Notch2 signaling maintains quiescent neural stem cells, while Notch1 functions to promote a more proliferative, activated stem cell phenotype (Basak et al., 2012; Engler et al., 2018; Wilhelmsson et al., 2012). Notch and VCAM1 illustrate the necessity of cell-cell juxtacrine signaling for stem-cell self-renewal, but a variety of other secreted signals are also essential to stem-cell maintenance. VCAM1 expression is promoted by IL-1β and promotes the maintenance of adult quiescent NSCs (Hu et al., 2017; Kokovay et al., 2012). Endothelial and cerebral spinal fluid-derived neurotrophin-3 (NT-3) also contribute to stem cell quiescence (Delgado et al., 2014). Leucine rich repeats and immunoglobulin like domain proteins (Lrigs), Tenascins, FGF-receptors, and EGF-receptors, each function in pathways known to regulate stem cell dynamics and are

differentially-expressed across the lineages of quiescent NSCs, activated NSCs, and NPCs. Multiple FGF-receptors (e.g. *Fgfr*1, 2, 3) show greater than 5-fold enrichment in qNSCs relative to aNSCs, whereas *EGFR* is more highly-expressed in the aNSCs. *EDNRB* (the gene for ETB_R) is ~3-fold more expressed in qNSCs than in aNSCs, whereas *EDNRA* is ~15-fold more expressed in qNSCs than aNSCs (Codega et al., 2014). Recent data show that endothelin signaling helps to maintain neural stem cells in the SVZ of early postnatal animals (Adams et al., 2020). This evidence suggests that endothelin receptors expressed by neural stem cells may allow these stem cells to persist into adulthood. It remains to be determined if ETB_R and/or ETA_R regulate neural stem-cell proliferation dynamics in the adult SVZ.

Context and framework of Dissertation

Heterogeneity of reactive astrocytes is increasingly a focus of research to improve disease outcomes. Dissecting the molecular mechanisms regulating particular astroglial phenotypes could lead to therapies that alter astroglial reactivity and therefore change the inflammatory environment in CNS diseases and injuries. To accurately model most reactive astrocyte contexts which occur in adulthood/aging, we utilized Reactive astrocyte-derived Neural Stem Cells (Rad-NSCs) to generate adult reactive astrocytes *in vitro*, then model reactive astroglial signaling. This contrasts with a large portion of the reactive astrocyte research using immature *in vitro* cellular models, which are known to exhibit different reactive phenotypes.

In chapter 2 of this dissertation we begin characterizing the paracrine profile of proliferative reactive astrocytes promoted by ETB_R-signaling. Our microarray analysis of ETB_R-knockout astrocytes reveals an extensive profile of downstream paracrine factors

and suggests that ETB_R may promote certain features of neuroprotection and/or represses astroglial neurotoxicity. We particularly focus on Ang-2 secretion by astroctyes, as this factor could be released in response to stroke to promote vascular protection and to regulate the neuroinflammatory environment. We found that microglia respond to Ang-2/Tie-2 signaling with altered cellular survival and inflammatory state (Figure 1.1). These findings indicate that astroglial neuroprotective states may not depend solely on paracrine trophic factors released to neurons, but also on paracrine regulation of microglial activation and paracrine protection of the vasculature.

In chapter 3 we more closely focus on how ETB_R-signaling, and downstream CD109 expression, alter astroglial neuroprotection/neurotoxicity. We show that the expression of multiple forms of CD109 is downstream of ETB_R signaling, including enzymatically released forms. CD109 mRNA is reported to be a marker of neuroprotective astrocytes, so we began characterizing how astroglial CD109 influences astroglial phenotypes (Figure 1.2). CD109 regulated both astroglial Ang-2 and complement C3, consistent with altering astroglial phenotypes. Injection of a soluble form of CD109 tended to increase stroke sizes, though it is unclear if this is through direct interactions with astrocytes or other CNS cell types. Further work is needed to determine how CD109 alters astrocytes directly and the cellular environment through astroglial released CD109. It will also be important to establish if ETB_R and/or CD109 are necessary for neuroprotective astrocytes to perform their functions in traumatic CNS injuries, and, whether signaling through either protein is sufficient to convert neurotoxic astrocytes to neuroprotective.

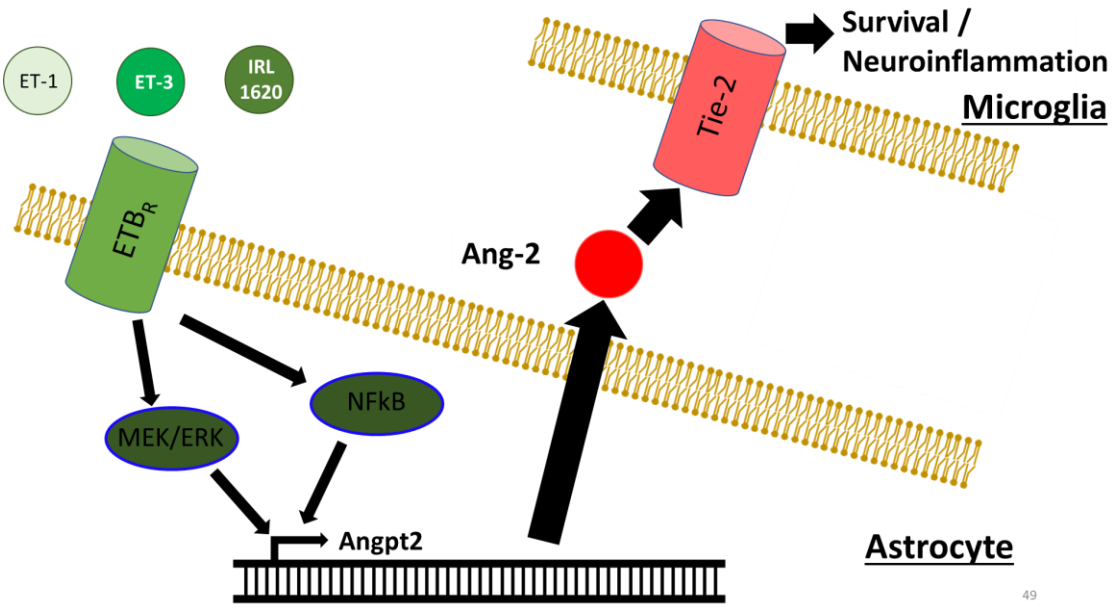
ET-1
ET-3
IRL 1620
ETB$_R$
Tie-2
Survival / Neuroinflammation
Microglia
Ang-2
NFkB
MEK/ERK
Angpt2
Astrocyte
49

Figure 1. 1.

A model illustrating the main findings of chapter 2 of this dissertation. Endothelin ligands released in the extracellular mileau, or ETB_R-specific drugs such as IRL-1620, interact with astroglial ETB_R receptors. ETB_R signaling activates downstream NFκB and MEK/ERK signaling. This increases transcription and secretion of Ang-2, which then signals through microglial Tie-2 receptors. Tie-2 signaling promotes the survival/proliferation of microglial cells under hypoxic conditions and also alters microglial inflammatory state.

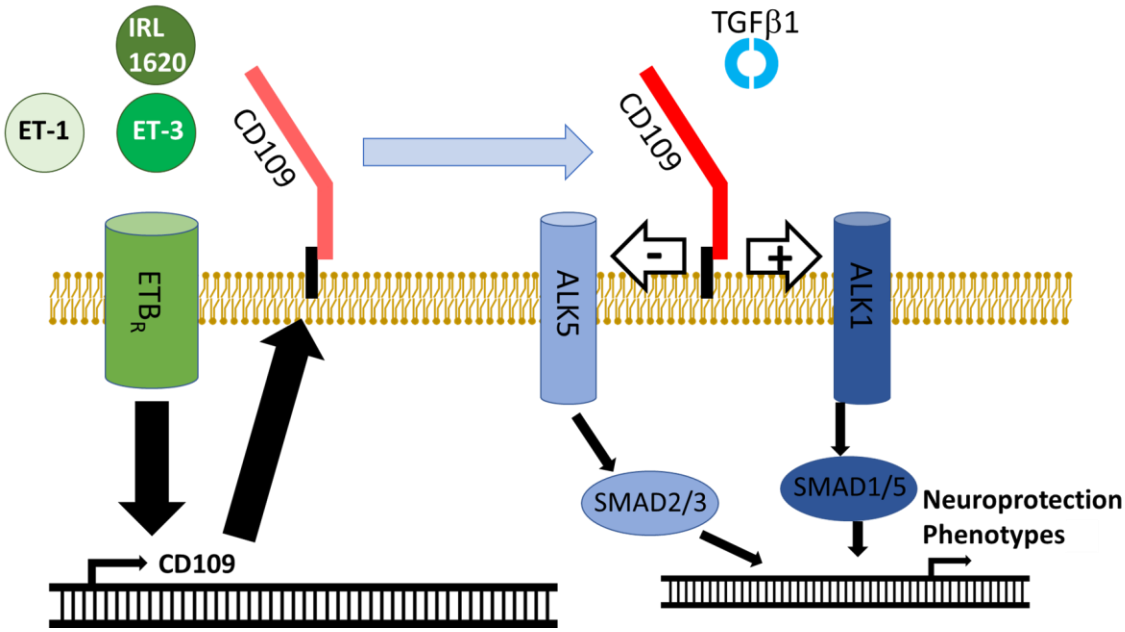
IRL
1620
ET-1
ET-3
CD109
ETBR
CD109
TGFβ1
CD109
ALK5
ALK1
SMAD2/3
SMAD1/5
Neuroprotection
Phenotypes
CD109

Figure 1. 2.

A model illustrating the main findings of chapter 3 of this dissertation. Endothelin ligands released in response to stroke, or ETB_R-specific drugs such as IRL-1620, stimulate astroglial ETB_R receptors. ETB_R signaling increases astroglial CD109 expression. CD109 acts to alter TGFβ signaling, thereby regulating astroglial neurotoxic/neuroprotective phenotypes in response to injuries and/or inflammatory factors.

Chapter 1 References

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CHAPTER 2: ETB_R signaling promotes angiopoetin-2 production by reactive astrocytes and regulates neuroinflammation after ischemic stroke.

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ABSTRACT

Reciprocal communication between reactive astrocytes and microglial cells provides local, coordinated control over critical processes such as neuroinflammation, neuroprotection, and scar formation after CNS injury, but is poorly understood. The vasoactive peptide hormone endothelin (ET) is released and/or secreted by endothelial cells, microglial cells and astrocytes early after ischemic stroke and other forms of brain injury. Using a genetic loss-of-function screen to identify paracrine effectors regulated by astroglial endothelin receptor B (ETB_R) signaling, we identified angiopoetin-2 (Ang-2) as a factor produced by reactive astrocytes in response to ET. In experiments with primary adult astrocytes stimulated by IRL1620, a specific ETB_R agonist, we found ERK1/2 and NFκB mediated the effects of ET on Ang-2 production. To determine astroglial Ang-2 levels *in vivo*, reactive astrocytes expressing the high affinity glutamate transporter

(GLAST, EEAT1) were isolated by magnetic-activated cell sorting 3 days after stroke. Astrocytes obtained from the ipsilateral hemisphere expressed significantly more Ang-2 compared with astrocytes isolated from the contralateral hemisphere, or from cortices of sham-operated (control) mice. Notably, analysis of microglia sorted from CX3CR1-eGFP mice demonstrated increased cell surface expression of Tie-2, the Ang-2 receptor, on microglial cells from ipsilateral tissue. Targeted loss of ETB_R in reactive astrocytes from GFAP-CreER^{TM}-*EDNRB*-$^{fl/fl}$ mice significantly decreased the number of proliferating microglial cells in the peri-infarct area. By contrast, Notch1-cKO in reactive astrocytes did not decrease microglial proliferation after stroke. Our data indicate paracrine effectors, such as Ang-2, acting downstream of astroglial ETB_R signaling, regulate microglial cell dynamics and neuroinflammation after stroke and perhaps other forms of CNS injury.

INTRODUCTION

To regulate blood brain barrier (BBB) integrity, neuroinflammation, and neuroprotection during CNS injury, microglial cells and reactive astrocytes engage in a complex array of autocrine, juxtacrine, and paracrine signaling (reviewed by Sofroniew, 2015). Resting microglia are highly dynamic cells, constantly sampling the extracellular space with their processes and responding within minutes to BBB disruption (Nimmerjahn et al., 2005). In ischemic stroke, sentinel microglial cells sense damage signals produced by occluded blood vessels (e.g. reactive oxygen species, thrombin, complement, ECM components) (Chamak and Mallat, 1991; Milner and Campbell, 2003; Moller et al., 2000; Roy et al., 2008; Schafer et al., 2012; Suo et al., 2002; Wang et al., 2006; Webster et al., 2000) and released from necrotic neurons (e.g. ATP, HMGB1, peroxiredoxins) (Davalos et al.,

2005; Gulke et al., 2018; Kim et al., 2006; Kim et al., 2007; Lu et al., 2012; Lu et al., 2018; Monif et al., 2009; Muhammad et al., 2008). These Damage Associated Molecular Patterns (DAMPS) alter microglial cell phenotype and initiate reciprocal signaling between nearby microglia and astrocytes. Delineating glial signaling mechanisms during different phases of injury progression and resolution has exciting potential to help identify new treatment targets for CNS injuries such as stroke. Notably, however, glial cell communication and its functional effects in most forms of CNS injury remain poorly understood.

During cerebral ischemia, vascular endothelial cells as well as astrocytes and microglia release and secrete endothelins (ET1-3), which are potent vasoactive peptides that constrict blood vessels by signaling through two G-protein-coupled endothelin receptors: type A and B (ETA_R and ETB_R) (reviewed by Davenport et al, 2016). ET signaling in the developing CNS affects the survival, proliferation and migration of a variety of cell types that include neural crest derivatives, neuroblasts, and melanocyte precursors (Lee et al., 2003; Nataf et al., 1996; Shin et al., 1999). In adults, in addition to controlling vascular tone, ETs regulate critical processes during tissue remodeling and repair such as inflammation, fibrosis, and angiogenesis (Bagnato and Spinella, 2003; Clozel and Salloukh, 2005; Leask, 2010; Li et al., 2010; MacCumber et al., 1990; Siren et al., 2000; Salani et al., 2000). Astroglial proliferation is significantly greater after stroke, penetrating traumatic brain injury, and spinal cord injury than in neuroinflammatory/neurodegenerative diseases such as Multiple Sclerosis, Alzheimer's Disease, or Parkinson's Disease (Gadea et al, 2009; Herrmann et al., 2008; Kamphuis et al., 2012; LeComte et al., 2015; Lepore et al., 2008; Shimada et al., 2011 Simon et al.,

2011; Sirko et al., 2013; Wanner et al., 2013). Results from our laboratory and others have shown that astroglial ETB_R signaling is an important regulator of reactive astrocyte proliferation (Gadea et al., 2008; Lecomte et al., 2015).

Of interest, glial cell phenotype(s) and function(s) differ between types of injury and appear to correlate with changes in astroglial proliferation. For example, in a lipopolysaccharide (LPS)-based model of neuroinflammation where little astrocyte proliferation is observed, microglia secrete TNFα, IL1α, and C1q that induce an astroglial state that is toxic to neurons (Liddelow et al., 2017). Although the production of many inflammatory factors such as lipocalin-2 is shared between several injury systems, it appears that reactive glia assume a more protective phenotype after stroke. The neuroprotective phenotype is associated with secretion of factors such as BDNF, GDNF, and IGF-1, that increase the survival of jeopardized neurons (Chen et al., 2019; Cunningham and Su, 2002; Koyama et al., 2003; Madathil et al., 2013; Rocha et al., 2012; Saha et al., 2006). Given that proliferating reactive astrocytes require ETB_R, we sought to better understand ETB_R function(s) in terms of regulation of paracrine activity after brain injury.

To identify astroglial paracrine activity controlled by ETB_R signaling, we performed microarray assays to determine changes in gene expression for adult astrocyte paracrine factors following *EDNRB*-cKO. Our screen identified multiple genes for secreted factors involved in neuroinflammation, neuroprotection, blood brain barrier regulation, and angiogenesis, including angiopoietin-2 (Ang-2). We show that ischemic stroke causes astrocytes to release Ang-2 and microglia to increase expression of the

Ang-2 receptor Tie-2. Importantly, we show that Tie-2 signaling alters the microglial inflammatory state and may serve as a marker of microglial activation.

RESULTS

Loss of ETB_R signaling in adult astrocytes impacts gene expression linked to cell-cell communication and paracrine response(s) to injury.

To identify paracrine activity downstream of ETB_R signaling in reactive astrocytes, we performed a loss-of-function gene expression screen using clonal lines of Reactive astrocyte-derived Neural Stem Cells (Rad-NSCs). To obtain cell lines capable of inducible, conditional *EDNRB* KO (LeComte et al., 2015; Rattner et al., 2013), Rad-NSCs were isolated from hGFAP-CreER^{TM}:*EDNRB*$^{flox/flox}$ mice at 3 d after distal Middle Cerebral Artery Occlusion (dMCAO) surgery to induce stroke. For Affymetrix cDNA microarray assays, two Rad-NSC clones from different mice were expanded in culture and differentiated into astrocytes by incubation in serum-containing medium for 7 days. For *EDNRB*-cKO, cultured astrocytes were exposed to 4-hydroxytamoxifen (4-OH-TM) for 10 days. Successful gene knockout in hGFAP-CreER^{TM}-*EDNRB*-cKO astrocytes was confirmed by a 103.18-fold decrease in *EDNRB* expression in cells treated with 4-OH-TM compared with vehicle-treated (control) cells (Figure 2.1). Furthermore, by western blot assays, we demonstrated reduced ETB_R levels in both clones after 4-OH-TM treatment relative to vehicle-treated controls (Data not shown). In agreement with our prior work on astroglial ETB_R signaling, Go-enrichment analysis revealed significant changes in genes related to cellular proliferation after *EDNRB* knockout ($P = 1.92x10^{-5}$) (Figure 2.1).

The top 15 cell-cell signaling related genes that increased or decreased after astroglial *EDNRB*-cKO are listed in the heatmap in Figure 2.1D. Notably, many genes we identified were reported to regulate immune cells (*Glycam1, Lipocalin-2, Sucnr1),* inflammation (*Ptger3, Tgfbr3, CD109, C1s1, Pla2g4a, Metrnl*), angiogenesis and BBB integrity (*Anpt2, Renbp, Alcam*). In addition, several cell signaling-related genes with expression changes outside the top 15 have functions in inflammatory and/or angiogenic cell signaling pathways (*C1ra, CD59a, Tgfb3, Vegfa*) (CD59a: Inafuku et al., 2018; Harhausen et al., 2017; Meri et al., 1990; C1ra: Bialas and Stevens, 2013; Gaboriaud et al., 2014; TGFβ3: Bialas and Stevens, 2013; Vegfa: Nih et al., 2018; Hiratsuka et al., 2005; Kaya et al., 2006). For example, *Angpt2* is an important regulator of vascular permeability and angiogenesis (Benest et al., 2013; Gurnik et al., 2016; Parikh et al., 2006), but was also shown to promote the migration of peripheral macrophages (i.e. outside the CNS) (Chen et al., 2016; Forget et al., 2014). Among paracrine effectors, angiopoietin-2 mRNA levels decreased the most in adult astrocytes after *EDNRB* loss (-11.53-fold, $P = 0.01$) (Figure 2.1), prompting us to investigate the expression and function(s) of astroglial-derived Ang-2 *in vivo* after cerebral ischemia.

Astrocyte Ang-2 expression increases *in vivo* after stroke.

To determine astrocyte-specific Ang-2 levels *in vivo*, we isolated GLAST$^+$ cells from the cerebral cortex by Magnetic Activated Cell Sorting (MACS). Astrocytes obtained by GLAST-MACS were counted by hemocytometer and cell lysate Ang-2 levels were determined by ELISA. Adult astrocytes sorted from the cortices of uninjured animals had low to undetectable levels of Ang-2 (Figure 2.2C). In contrast, in mice with stroke we observed a significant increase in astroglial Ang-2 expression 3 days after dMCAO

surgery (Uninjured mice, 1.89 ± 0.68 pg/ml, $n = 3$; Stroke mice, 10.36 ± 1.91 pg/ml, $n = 5$; $P < 0.05$; Figure 2.2C). In addition, reactive astrocytes sorted from the ipsilateral hemisphere after stroke had significantly more Ang-2 relative to astrocytes isolated from the contralateral hemisphere (Contra, 5.61 ± 1.88 pg/ml, $n = 5$; Ipsilateral, 15.10 ± 2.98 pg/ml, $n = 5$; $P \leq 0.05$; Figure 2.2D).

Endothelin signaling through ETB_R promotes secretion of Ang-2 by astrocytes.

Adult cortical astrocytes predominantly express ETB_R compared to ETA_R but are capable of ETA_R-mediated signaling (Guo et al., 2014; Hammond et al., 2015; John Lin et al., 2017; Zhang et al., 2014). To determine the relative contribution(s) of ETA_R and ETB_R over control of astroglial Ang-2 secretion, cultures of Rad-NSC derived astrocytes were treated with endothelin (ET-1, 100 nM) in the presence or absence of selective inhibitors for ETA_R (BQ123) and ETB_R (BQ788). Under serum-free conditions, ET-1 treatment alone significantly increased Ang-2 secretion (Vehicle, 59.14 ± 6.74 pg/ml; ET-1 78.51 ± 5.73 pg/ml; $n = 3$; $P \leq 0.05$; Figure 2.3A; ANOVA). Selective inhibition of ETA_R during ET-1 stimulation had no significant impact on Ang-2 secretion relative to ET-1 treatment ($P = 0.43$), but inhibition of ETB_R reduced Ang-2 levels to baseline (Vehicle vs ET-1 + ETB_R inhibition, $P = 0.81$; ET-1 vs ET-1 + ETB_R inhibition, $P < 0.05$; Fig. 2.3A). This result was consistent with reported low levels of ETA_R in astrocytes and the differing roles of ETA_R and ETB_R signaling (Hammond et al., 2015; Lange et al., 2007; Zhang et al., 2014; Zhang et al., 2016; John Lin et al., 2017). ETB_R is a G-protein coupled receptor known to signal through G_q coupling, but also is promiscuous in its G-coupling and downstream signaling (Takagi et al., 1995; Urtatiz et al., 2016). In various cell types, ETB_R has been reported to signal through various downstream transcriptional effectors

including HIF, ERK, and NFκB (Cramer et al., 2001; Hung et al., 2014; Spinella et al., 2014; Heimlich et al., 2015; Grimshaw et al., 2002). We therefore pretreated astrocytes with inhibitors to these pathways for 1 hour prior to ET-1 treatment. Pretreatment with inhibitors to NFκB and MEK/ERK each significantly decreased angiopoietin-2 secretion relative to ET-1 treatment alone (ET-1 + NFκB inhibitor at 0.33-fold of ET-1, $P < 0.05$; ET-1 + MEK/ERK inhibition at 0.18-fold of ET-1, $P < 0.01$; $n = 3$; Fig. 2.3C). TGFβ1 signaling is known to block aspects of astrocyte reactivity and reset astrocytes to a "resting" state (Liddelow et al., 2017; Diniz et al., 2017). As expected, treatment with TGFβ1 (1 ng/ml) also significantly decreased Ang-2 secretion (Vehicle 57.84 ± 2.73 pg/ml, TGFβ1 19.78 ± 7.22 pg/ml; $P < 0.01$, Fig. 2.3D).

Astrocyte-specific ETB_R knockout does not alter microglial CD68 expression.

Changes in microglial proliferation suggested that other aspects of microglial cell reactivity might be altered by astroglial loss of *EDNRB*. CD68 expression positively correlates with phagocytic and pro-inflammatory microglial functions (Nicoll et al., 2003 and 2005; Song et al., 2011; Barois et al., 2002; Yeo et al., 2019). Therefore, we performed immunohistochemistry for GFAP, Iba1, and CD68. We assessed CD68 staining outside and inside the infarct area as demarcated by the presence of $GFAP^+$ astrocytes. $Iba1^+$ cell expression of CD68 increased markedly inside of the infarct area, whereas CD68 expression was lower in tissue outside the infarct zone; this tissue contained $GFAP^+$ astrocytes. We found that CD68 expression decreased with increasing distance from the infarct, however, microglial CD68 expression did not differ between control and *EDNRB*-cKO mice (Supplemental Fig. 2.3).

Adult microglia upregulate the Tie-2 receptor after stroke.

Ang-1 and Ang-2 both bind the Tie-2 receptor, and often in an antagonistic fashion (Kim et al., 2016; Yuan et al., 2009). Whereas Tie-2 expression by microglial cells was documented early during their development from the fetal yolk sac (Ginhoux et al., 2010; Gomez et al., 2015; Kierdorf et al., 2013), expression by microglia in the adult brain was unreported. To determine whether adult cortical microglia express Tie-2, and whether or not its expression changed in the context of stroke injury, we employed a combination of MACS and FACS. We first enriched for microglia/macrophages by CD11b MACS from Cx3cr1-GFP mice 3 days post-stroke. We then used FACS to determine the Tie-2 expression profile of microglia after stroke. Also, cells were incubated with an antibody to TMEM-119 to specifically mark microglial cells and then Tie-2 antibody to detect the level of cell surface Tie-2. Of interest, we detected two separate cell populations, which expressed differing levels of GFP (i.e. Cx3cr1) (Fig. 2.4A). Notably, the $Cx3cr1^{Hi}$ population corresponded to the TMEM-119^{+} microglial cells (Fig. 2.4B). After stroke, cell surface Tie-2 expression was increased on TMEM-119^{+} cells ipsilateral to the infarct zone relative to TMEM-119^{+} cells from the contralateral hemisphere (Figures 2.4C and 2.4D); this indicated that Tie-2 might be a marker of microglial activation after CNS injury. In agreement with this concept, at 3 days after dMCAO surgery, double immunohistochemical stains for Tie-2 and TMEM-119 demonstrated the strongest staining for cells residing in close proximity to the stroke infarct (Fig. 2.4E).

Ang-2 increases alters microglial inflammatory activity.

After stroke and other forms of brain injury, secretion of cytokines, chemokines, and inflammatory modulators represent a key function for microglial cells (Zhao et al., 2017). Accordingly, we next determined whether or not Ang-2 treatment affected the paracrine activity of microglia. By ELISA, treatment of a mouse microglial cell-line (SIM-A9) with Ang-2 did not alter IL-6 and TNFα, both major inflammatory mediators (Figures 2.5A and 2.5B). To determine if the effects of Ang-2 were dependent on signaling through the Tie-2 receptor, we used Bay-826, an inhibitor specific to Tie-2. In the presence of Ang-2 (100 ng/ml), Bay-826 treatment significantly inhibited microglial secretion of IL-6 (Figure 2.6B; $P < 0.05$); these data indicated that the pro-inflammatory microglial cell phenotypes may be mediated by Tie-2 signaling. We therefore measured Tie-2 expression in microglia treated with astrocyte conditioned medium (ACM) and/or CSF1 (100 ng/ml), a factor released in response to CNS injuries. CSF1 increased microglial Tie-2 expression and ACM limited CSF1-induced Tie-2 expression (Figures 2.5C and 2.5D).

Astrocyte-specific loss of ETB_R reduces microglial cell proliferation after stroke.

Previously we reported astrocyte-specific knockout of *EDNRB* significantly decreased reactive astrocyte proliferation 3 d after stroke (Lecomte et al., 2015). In subsequent studies of the peri-infarct area, we identified a subset of non-astrocyte (GFAP-negative) cells with reduced proliferation (Ki67) after astroglial-specific EDNRB knockout (Figure 2.6C). Signaling cross-talk between reactive astrocytes and microglial cells is an important determinant of CNS injury outcomes (Bohlen et al., 2017; Guan et al., 2016; Liddelow et al., 2017; Welser-Alves et al., 2013; Yu et al., 2020). To test whether

decreased reactive astrogliosis following GFAP-*EDNRB*-cKO affected also microglial cell dynamics, we performed immunohistochemistry for proliferative $Ki67^+/CD11b^+$ cells 3 days after dMCAO. Of interest, we found that astroglial-specific knockout of *EDNRB* significantly decreased the proportion of $Ki67^+$ cells that were $CD11b^+$ (Oil vehicle controls 0.39 ± 0.12, $n = 9$; Tamoxifen Knockouts 0.24 ± 0.12, $n = 10$; $P < 0.05$; Fig. 2.6D). $CD11b^+$ cells include circulating monocytes, neutrophils, NK cells, granulocytes, and macrophages as well as microglia (Duan et al., 2016; Fu et al., 2011; Hardersen et al., 2018). Notably, however, previous studies have shown that peripheral blood-derived cells do not proliferate upon entering the central nervous system after injury; this suggests that the $Ki67^+/CD11b^+$ cells we counted were primarily microglial cells (Li et al., 2018).

To examine whether other stroke injury-related signals to reactive astrocytes also affected microglial cell dynamics we studied cells in GFAP-*Notch1*-cKO mice 3 days after stroke. In contrast to the pattern observed in *EDNRB*-cKO mice, the number of $Ki67^+/CD11b^+$ cells in GFAP-Notch1-cKO mice did not change after *Notch1*-cKO ($P = 0.24$; supplemental Figure 2.6A). Furthermore, we examined the number of proliferating $CD45^+$ blood lineage cells as well as $NG2^+$ oligodendrocyte progenitor cells in GFAP-*Notch1*-cKO mice 3 d after stroke. Similar to the $CD11b^+$ cells, the number of $Ki67^+/CD45^+$ or the Ki67+/NG2+ cells did not change after *Notch1*-cKO ($CD45^+/Ki67^+$ $P = 0.57$; $NG2^+/Ki67^+$ $P = 0.80$; Supplemental Figures 2.6B and 2.6C); this indicated that microglial cell proliferation was affected by astroglial ETB_R signaling, but not by Notch1 signaling in reactive astrocytes.

Direct cortical injection of recombinant murine Ang-2 did not alter microglial cell proliferation in a consistent, predictable manner.

To investigate whether or not Ang-2 could directly impact microglial cell proliferation *in vivo*, we injected one cortical hemisphere of Cx3cr1-GFP mice with vehicle (n = 5) and the other with recombinant murine Ang-2 (100 ng/ml in vehicle, n = 5). The hemisphere that received Ang-2 injection was randomized in a blinded manner to control for any possible lateralization effects. Injection of Ang-2 increased microglial proliferation in 3 out of 5 mice injected, whereas proliferation was unchanged in the one mouse, and it actually decreased in the other (Supplemental Figure 2.2). Thus, direct Ang-2 injection into the cerebral cortex of otherwise healthy mice did not significantly alter the proliferation of GFP^+ cells. Although the brain stab/injection model we employed clearly differs from a model of cerebral ischemia (i.e. dMCAO), our results do suggest that other factors may be required for Ang-2 to change the proliferation of microglia after brain injury.

METHODS

Distal middle cerebral artery occlusion (dMCAO) surgery. Mice were induced with 4% isoflurane and maintained with 1.5-2% isoflurane in oxygen. Body temperature was maintained with a heated pad. Under sterile conditions, an incision was made vertically, between the ear and eye. The temporalis muscle was cut from its attachment on the lateral portion of the skull. The muscle was retracted and a rectangular portion of the skull was removed. The dMCA was visualized and a 10-0 suture (03171, Fine Science Tools) was used to permanently tie off and occlude the artery. The artery was cut to confirm occlusion (i.e. lack of distal blood-flow). The muscle was then returned to its resting

position, and the skin was closed with Vetbond (3M). GFAP-CreER^{TM}-*EDNRB*-$^{fl/fl}$ or C57BL/6J background animals were injected with Buprenorphine and allowed to recover for the indicated time periods before either cell isolations or 4% paraformaldehyde (AAJ19943K2, Affymetrix) transcardiac perfusion and preparation of the brain for immunohistochemistry.

Generation of Reactive astrocyte-derived Neural Stem Cells (Rad-NSC). Mice underwent dMCAO surgery and recovered for 3 d before brains were removed for dissection. Brains were cut coronally, cortical tissue ipisilateral to the stroke injury was separated from the rest of the brain, and meninges were excluded. Brains were digested in a solution of Papain (20 units/mL, LS003126; Worthington), L-Cysteine (0.2 mg/mL, C7352; Sigma), DNase (20 μg/mL, Worthington), $CaCl_2$ (1.5 mM), and EDTA (1.5 mM) in DMEM-F12 (Hyclone) for 20 minutes in a 37°C incubator at 5% CO_2. The digestion solution was neutralized by trypsin inhibitor (1.5 mg/mL, 17075-029; Gibco) and BSA (1.5 mg/mL, BP1605-100; Fisher) in DMEM-F12. Cells were resuspended in NSC medium: Neurobasal-A base medium (10888022, ThermoFisher) supplemented with recombinant human basic FGF (20 ng/mL, produced in our lab), EGF (10 ng/mL, Gibco), B27 supplement (Gibco), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. To obtain cell populations with uniform and predicable Cre-mediated knockout, individual cells were maintained until initial neural sphere formation. Subsequently, single spheres were pipetted into separate wells for clonal expansion.

ELISA. Clonal Rad-NSC populations were expanded in NSC medium. Rad-NSCs were plated onto dishes previously coated with 5 μg/ml Laminin (Corning) and 5 μg/ml Poly-d-lysine (Corning) at a density of 1 million cells per 10 cm^2 dish (Nunc, Thermo Fisher

Scientific). NSCs were differentiated into astrocytes in DMEM-F12 (Hyclone) medium containing 10% fetal bovine serum (Atlanta Biologics) for 17 days. Astrocytes were then treated with ET-1 (100 nM, Sigma), IRL1620 (10 nM, ToCris), BQ-788 (8 μM, Sigma or Caymen Chemicals), and/or BQ-123 (1 μM, Sigma or Abcam). Additionally, astrocytes were maintained for 21 days in 10% serum for 1 hr pretreatment with DMSO (vehicle control) or TCS-7009 (1 μM, HIF2a inhibitor; Tocris Bioscience) or U0126 (10 μM, MEK/ERK inhibitor; Cell Signaling Technologies) or Repsox (1 uM, TGFβRI/ALK5 inhibitor; Stemcell Technologies) or K02288 (80 nM, BMPR/ALK1/2/6 inhibitor; Caymen Chemicals) or NFκB inhibitor (10 nM, Calbiochem) or PX-478 (25 μM, HIF1α inhibitor; Caymen Chemicals), followed by treatment with ET-1 (100 nM) or PBS (vehicle control). Before application of treatments, astrocytes were washed PBS and then for 5 min at 37°C with PBS. Treatments were added in serum-free DMEM-F12 base medium for 48 hrs to generate conditioned medium; this medium was filtered (0.22μm) and analyzed by ELISA.

Magnetic-Activated Cell Sorting (MACS). Following dMCAO surgery, mice recovered for 3 d before brains were removed for dissection. Olfactory bulb, hindbrain, and meninges were excluded and the hemispheres contralateral and ipsilateral to the stroke were separated for cell isolations. We adapted our cell isolation protocol from Foo et al. (2011). Cortical hemispheres were digested separately in a 37°C incubator at 5% CO_2 for 1 hr. The enzymatic dissociation solution contained Hanks Buffered Salt Solution (HBSS) supplemented with 30% D-Glucose, 1 M $NaHCO_3$, 50 mM EDTA, L-Cysteine 1 mg/mL, Sigma; DNase (20 μg/mL, Worthington), and Papain (20 U/mL, Worthington). The enzymatic tissue digest was neutralized by washing 3x with “low” ovomucoid

inhibitory solution that was diluted from a concentrated stock to a 1x final concentration in HBSS containing 30% D-Glucose and 1 M $NaHCO_3$. Low ovomucoid stock solution (10x): 0.2 mg/mL ovomucoid trypsin inhibitor, Worthington; 1.5% BSA, Miltenyi Biotech; all dissolved into 1 x Dulbucco's PBS, pH 7.4. Cells were triturated gently in the low ovomucoid solution and passed through a filter to generated a single cell suspension (70 μm, Falcon). The low ovomucoid solution was underlaid with a "high" ovomucoid solution; this 1x high ovomucoid solution was diluted from a concentrated stock solution in HBSS supplemented with 30% D-Glucose and 1 M $NaHCO_3$. High ovomucoid stock solution (10x): 3% BSA solution, Miltenyi; 0.0333 g/ml ovomucoid trypsin inhibitor, Worthington; all dissolved into 1x DPBS (pH 7.4). After high ovomucoid solution was underlaid, cell suspensions were centrifuged at 300 x g for 7-minutes. Cell pellets were resuspended in 140 ul MACS buffer (1 x Miltenyi BSA solution in DPBS) containing 20 μl FcR blocking solution (Miltenyi Biotech). FcR block was incubated for 10 min at 4℃. After blocking, 40 μl of biotinylated anti-GLAST antibody (Miltenyi Biotech) was added and incubated for 20 min at 4℃. Cells were washed with 5 ml of MACS buffer, then centrifuged at 300 x g for 7 min. Cell pellets were re-suspended in 160 ul of MACS buffer. After resuspension, 40 ul of paramagnetic-bead conjugated streptavidin (Miltenyi Biotec) was added and incubated for 20 min at 4℃. Labeled cells were washed with 5 ml of MACS buffer, centrifuged (300 x g, 7 min), and re-suspended in 3 ml of MACS buffer. A MACS column (L/S column, Miltenyi Biotech) was primed with 3 ml of MACS buffer prior to loading on the labeled cell suspension. The MACS columns were washed 3x with MACS buffer (3 ml, each). $GLAST^+$ cells were eluted in 5 ml of MACS buffer, centrifuged at 300 x g for 7 min, re-suspended in 400 μl of DPBS, and counted by

hemocytometer. After quantification, the cells were centrifuged 1,000 x g for 10 minutes. The final $GLAST^+$ cell pellet was lysed in hypotonic lysis buffer compatible with ELISA: 20 mM HEPES pH 7.5, 5% Glycerol, 1.5 mM $MgCl_2$, 10 mM KCL, 10 mM NaCl, supplemented with 1 x protease (Pierce) and phosphatase (Sigma) inhibitors.

Microarray analysis. For microarray assays, Rad-NSCs were plated and differentiated into astrocytes for 7 d (as above). On day 7, cells were fed with either 4 hydroxy tamoxifen (4-OH-TM, 1 μM; Tocris Bioscience) or ethanol (vehicle control) in DMEM-F12 with 10% FBS. This treatment was renewed 3 times, every 3 d. After 10 d of treatment (17 d total differentiation), cells were washed and switched to DMEM-F12 base medium with no serum. After 48 hrs of incubation in serum-free medium, astrocyte-conditioned medium (ACdM) was harvested for analysis, and cells were lysed using RNA lysis-buffer (Zymo Research).

Probe set statistics and identification of differential expression was performed by the Molecular Bioinformatics Shared Resource of the University of Vermont College of Medicine Vermont Integrative Genomics Resource, using Partek Genomics Suite ® Version 6.6 (Partek Inc., St. Louis, MO). Probe-level intensities were calculated using the Robust Multichip Average (RMA) algorithm, including background-correction, normalization (quantile), and summarization (median polish), for each probe set and sample. Sample quality was assessed based on 3': 5' ratio, relative log expression (RLE), and normalized unscaled standard error (NUSE). Principal Component Analysis (PCA) was also used to identify outlier samples that would potentially introduce latent variation into the analysis of differential expression across sample groups. Multivariate Principal Component Analysis was performed on the normalized data set using the covariance

matrix. Univariate linear modeling of sample groups was performed by ANOVA. The magnitude of the response (fold-change calculated using the least square mean) and the p-value associated with each probe set and binary comparison were calculated, as well as a "step-up" adjusted p-value for the purpose of controlling false discovery rate (Benjamini, Y, and Y Hochberg, 1995). Data were analyzed by Gene Set Enrichment (Partek), which uses a right-tailed Fisher's Exact test with a null hypothesis that data are changing together strictly by chance. The alternative is that the data change in concert because they are part of a biological gene set of pathway. Functional clusters and pathways were identified using the DAVID bioinformatics resource (Huang et al. 2009).

Stereotaxic injections. Cx3cr1-GFP mice (Jackson Labs) were induced with 4% isoflurane and maintained with 1.5-2% isoflurane in oxygen. A heated pad was used to maintain body temperature. For studies of Ang-2 in brain stab injury, vehicle was injected into one hemisphere, whereas Ang-2 was injected into the other hemisphere. Under sterile conditions, a midline incision was made over the dorsal surface of the skull and the skull was drilled bilaterally. A 26 Gauge needle (SGE010RNS, World Precision Instruments) was inserted bilaterally: AP = +0.62, Depth = -2.00, Lat = +/- 2.5 mm relative to Bregma (KOPF stereotaxic device). The needle was allowed to sit for 2 min before beginning of injection. Injection of 1 μl rmAng-2 in sterile PBS (100 μg/ml; 7186-AN-025/CF, R&D Systems) was performed at 0.1 μl/min (UMP-2 and Micro4 Pump Controller) for 10 minutes, for a total of 100 ng Ang-2. After injection, the needle was left in place for 5 minutes before being slowly retracted. Animals were closed and allowed to recover for 48 hours before harvesting brains for immunohistochemistry. Horizontal (perpendicular to needle stab) serial sectioning was performed for 30 um

sections, with each serial section separated by 60 μm. Tissue was stained for Ki67. Four representative 40x images were taken within 400 μm of the needle track per section, with 5 sections represented per mouse. Counts were performed for GFP^+, $Ki67^+$, and $GFP^+/Ki67^+$ cells. All injections and counts were performed by a blinded investigator.

Stroke tissue processing and cell-counts: Fixed brain tissue was cryosectioned at 30 μm per section, with 270 μm between each section. Tissue was stained for CD11b and Ki67. For counts, 3 representative 40x images were taken per section within the dorsal portion of the peri-infarct area; this region typically has the highest level of cellular proliferation after stroke. For 3 sections from each brain, blinded counts were performed for $Ki67^+$ and $CD11b^+/Ki67^+$ cells. Counts of $CD11^+/Ki67^+$ cells were normalized to the total number of $Ki67^+$ cells.

Microglial Cell Culture: Human Microglia (Celprogen 37089-01), and immortalized microglia (SIM-A9, END001) were cultured in DMEM-F12 medium supplemented with 10% FBS and 5% Horse Serum. Prior to plating ELISA experiments, the serum content was reduced to 1% FBS (v/v) and 0.5% Horse Serum (v/v) for at least 24 hrs. Microglia were lifted by trypsinization (GIBCO), passed through a single cell filter (40 μm, Falcon), and plated at 200,000 cells per well in a 6-well dishes (Nunc) in reduced serum (1% FBS and 0.5% Horse Serum). Cell culture dishes were coated with 5 μg/ml Laminin and 5 μg/ml Poly-D-lysine (both from Corning). After 24 hrs, the serum-containing medium was removed, cells were washed with PBS and switched to serum-free defined medium (AdM), 50:50 Neurobasal-A/DMEM with heparin sulfate (SIGMA) B25 (made in house) or B27 (Invitrogen) and L-Glutamine (GIBCO) and Pen/Strep (GIBCO). After 24 hrs in AdM (to remove serum), cells were treated in AdM and put into normoxia or

hypoxia (1% oxygen). After 48 hour medium was removed and cells were centrifuged at 1000 x g for 10 min. Cell pellets and adherent cells were kept for standard cyquant assay (Invitrogen) and conditioned medium was analyzed by ELISA.

Statistical analysis

Statistical analyses were performed with GraphPad Prism Software (Version 7.01). Groups were compared by Student's T-test and multiple comparisons were made by ANOVA. *P*-values of less than 0.05 were considered significant.

DISCUSSION

In our prior studies of the peri-infarct area after stroke, we reported a subpopulation of proliferating reactive astrocytes that expressed GLAST, the RC2 antigen, and high levels of Jagged-1 and ETB_R. Similar to conditional knockout of the Notch1 receptor, astroglial-specific loss of ETB_R demonstrated its requirement for survival of proliferating reactive astrocytes. As the population of proliferating reactive astrocytes is characteristically anti-inflammatory and neuroprotective in the context of stroke (Shimada et al., 2011), traumatic brain injury (Frik et al., 2018), and spinal cord injury (Anderson et al., 2016), we hypothesized that astroglial ETB_R signaling promotes paracrine activity associated with a neuroprotective astrocyte phenotype.

Our microarray analysis of adult astrocytes after *EDNRB*-cKO revealed significant changes in gene expression related to cell-cell signaling. Notably, we observed significantly reduced expression for genes associated with neuroprotection (e.g. *CD109*) (Liddelow et al., 2017), angiogenesis (e.g. *Angpt2*) (Zhu et al., 2005), BBB homeostasis (e.g. *Alcam*) (Lecuyer et al., 2017), and axon guidance (e.g. *Sema3c*)

(Hernandez-Montiel et al., 2008). Due to the potential for increased vascular permeability, administration of factors such as VEGF early after stroke can be problematic (Marteau et al., 2012; Zhang et al., 2000). Notably, however, treatment with Ang-2 prior to stroke was shown to protect the blood brain barrier and reduce stroke injury (Marteau et al., 2013).

Genes with expression upregulated by *EDNRB-cKO* were largely pro-inflammatory in nature. Multiple genes encoded proteins that regulate inflammatory responses of microglia/monocyte/macrophages (e.g. *Lcn2, Glycam1, Sucnr1, Metrnl*) (Bridgewood et al., 2019; Jin et al., 2014; Keiran et al., 2019; Williams et al., 2017) and prostaglandin signaling (e.g. *Ptger3*) (Aronoff et al., 2004), and some were anti-angiogenic (e.g. *Serpinf1*) (Wietecha et al., 2015). Particularly notable were increases in gene expression related to complement and TGFβ signaling (e.g. *Tgfb3*). In the developing brain, TGFβ-3 was demonstrated to upregulate synaptic C1q expression during the process of synaptic pruning (Bialas and Stevens, 2013). Additionally, astroglial *EDNRB-cKO* increased expression of C1r and C1s; these proteins complex with C1q to form complement C1, a key initiator of the complement cascade (Dunkelberger and Song, 2010). In regard to neurotoxicity, the results suggested *EDNRB* loss in reactive astrocytes may promote TGFβ/complement signaling that tags neuronal synapses and/or neurons for elimination.

Among paracrine factors, angiopoietin-2 (Ang-2) mRNA levels decreased the most in response to ETB_R knockout. Perhaps best known as a positive regulator of angiogenesis, Ang-2 was also shown to affect peripheral monocyte/macrophage migration and angiogenic activity (Chen et al., 2016; Forget et al., 2014; Patel et al.,

2013). By ELISA, we found that reactive astrocytes isolated by GLAST-MACS significantly increased Ang-2 levels in cortical tissue ipsilateral to the stroke injury. In contrast, Ang-2 was barely detectable in astrocytes sorted from the contralateral (uninjured) hemisphere or from healthy, uninjured mice.

ETB_R is highly expressed in reactive astrocytes, whereas ETA_R is expressed at comparably lower levels (Guo et al., 2014; Hammond et al., 2015; John Lin et al., 2017; Zhang et al., 2014). Our experiments with pharmacological inhibitors specific to ETA_R and ETB_R confirmed that ETB_R promotes Ang-2 expression and secretion, whereas ETA_R inhibition had no effect on Ang-2 secretion. ET-1 interacts with both ETA_R and ETB_R, whereas ET-3 selectively interacts with ETB_R. Thus, ET-1 and ET-3 may have different effects after stroke and/or other brain injuries.

In experiments to determine downstream effectors of ETB_R signaling, we found that ET-1 induced Ang-2 secretion by primary astrocytes in a NFκB and MEK/ERK-dependent manner. ELISA of TNF and IL-6 in CdM from SIMA9 cells that were exposed to Ang-2 suggested that high (>100 ng/ml) levels of Ang-2 could have a modest pro-inflammatory effect on microglia. In the presence of 100 ng/ml Ang-2, treatment of microglia with a specific Tie-2 inhibitor (Bay826) decreased TNF and IL-6 secretion. As Ang-1 is the major agonist ligand for Tie-2, some of this effect, may be due to decreased autocrine Ang-1 signaling. In peripheral monocytes and macrophages, Ang-1 was reported to support differentiation of cells toward a pro-inflammatory (i.e. M1) phenotype and increase secretion of TNF and to a lesser extent IL-6 (Seok et al., 2013). In endothelial cells, Ang-2 may function as a Tie-2 antagonist at low doses and a Tie-2 agonist at high doses (e.g. >50 ng/ml) (Fiedler and Augustin, 2006). As a receptor ligand,

there is thus potential for Ang-2 to act as an antagonist (supporting M2, anti-inflammatory phenotypes in microglia/monocytes), or as an agonist (promoting M1, pro-inflammatory phenotypes), depending on its local concentration and the signaling milieu in a particular injury/disease environment.

In normal, healthy brain recent findings have demonstrated regional differences in subpopulations of microglia (Stratoulias et al., 2019), and astrocytes in the spinal cord (Hochstim et al., 2008) and the brain (Lin et al., 2017). We and others have demonstrated that several subpopulations of reactive astrocytes can be identified based on their proximity to tissue damage (i.e. stroke infarct core), proliferation status, and environmental milieu (Lecomte et al., 2015; Liddelow et al., 2017). In regard to Ang-2 and its function(s) *in vivo*, it will be important to determine which astrocytes secrete it and where. Based on the regulation of astroglial Ang-2 secretion by ETB_R and that the proliferating reactive astrocytes nearest to the infarct core express the highest levels of ETB_R, it is likely that reactive astrocytes expressing the most Ang-2 may actually be deleted from the peri-infarct area in our GFAP-CreER^{TM}-ETB_R-cKO mouse model after stroke. Accordingly, experiments in GFAP-CreER^{TM}-Angpt2-cKO mice may provide further insight into the effects of astrocyte-secreted Ang-2 on microglia, the BBB, angiogenesis and recovery post-stroke. In addition, single cell analyses of reactive astrocytes and microglia sorted from injured CNS tissues using cell surface markers such as ETB_R and Tie-2 may provide insight into the specific functions of diverse, interacting glial subpopulations.

In a rat model of stroke, Leonard and Gulati (2013) demonstrated that systemic treatment with IRL-1620 reduced stroke size and improved stroke outcomes. They

posited that agonizing ETB_R had direct beneficial effects on neuron survival in the acute phase of injury and potentially benefit also angiogenesis and/or neurogenesis to enhance stroke outcome (Leonard and Gulati, 2013). Our data indicate that direct effects on the paracrine activity of neuroprotective reactive astrocytes and indirect effects on microglial dynamics may be responsible for some of the benefits observed after IRL-1620 treatment of stroke.

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CHAPTER 2 FIGURES AND TABLES

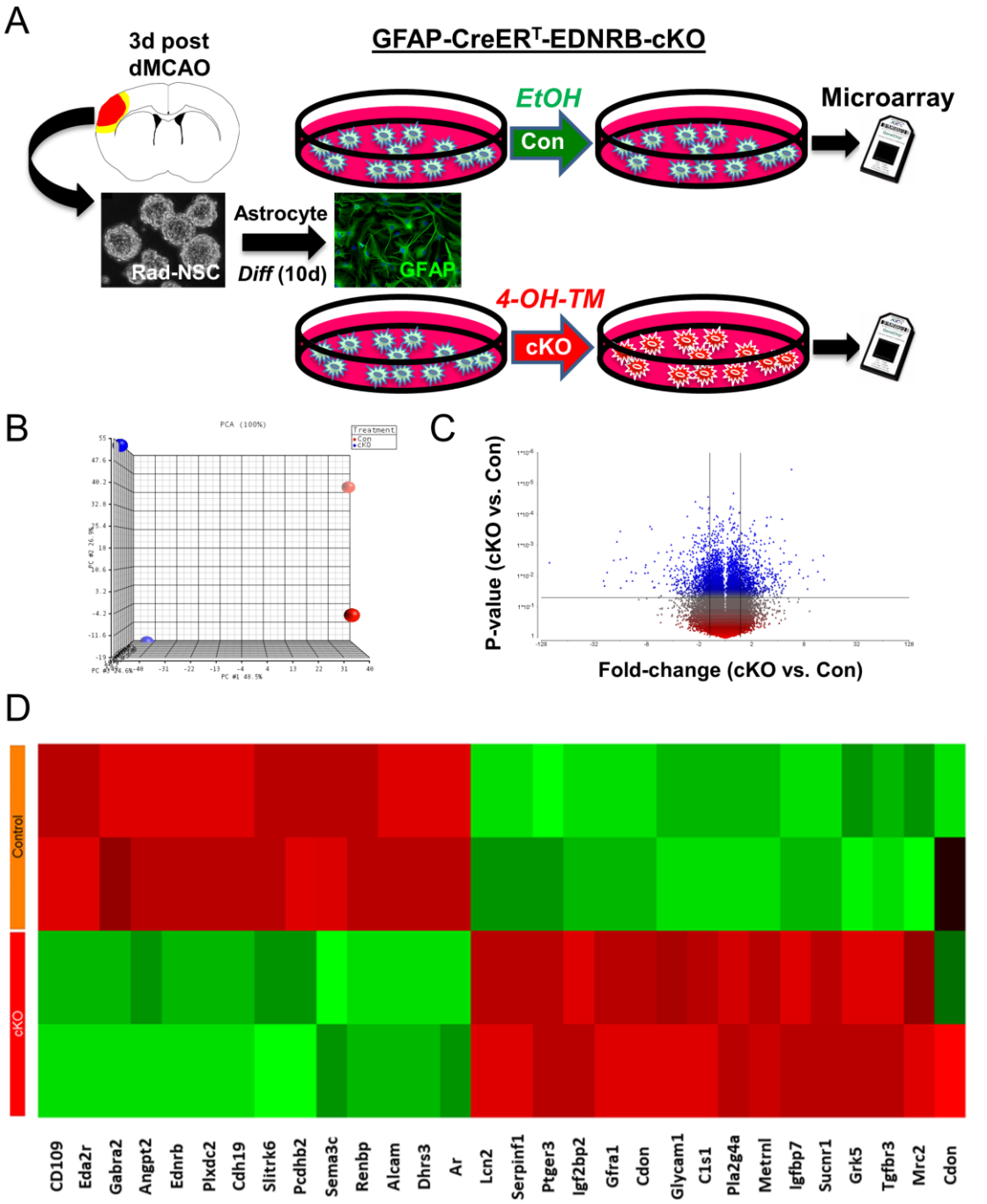


Figure 2. 1.

Microarray analysis of cultured *EDNRB*-knockout reactive astrocytes revealed cell-cell signaling related changes in gene expression. **A)** Schematic illustrating workflow for generating reactive astrocyte derived neural stem cells (Rad-NSCs) from the peri-infarct area. Reactive astrocytes were generated by differentiation of Rad-NSCs from GFAP-CreER^{TM}-*EDRNB*-$^{fl/fl}$ mice. Differentiated astrocytes were treated with ethanol-vehicle or 4-OH-tamoxifen (1 μM) to knockout *EDNRB.* After 2wks astrocytes were lysed, and RNA was purified for analysis. **B)** Principal Component Analysis (PCA) of control and knockout astrocytes derived from two separate Rad-NSC clones. **C)** Volcano plot illustrating significance and magnitude of mRNA expression changes after astoglial *EDNRB*-knockout. **D)** Heat-map of the top astroglial cell-cell signaling-related genes altered by *EDNRB*-knockout.

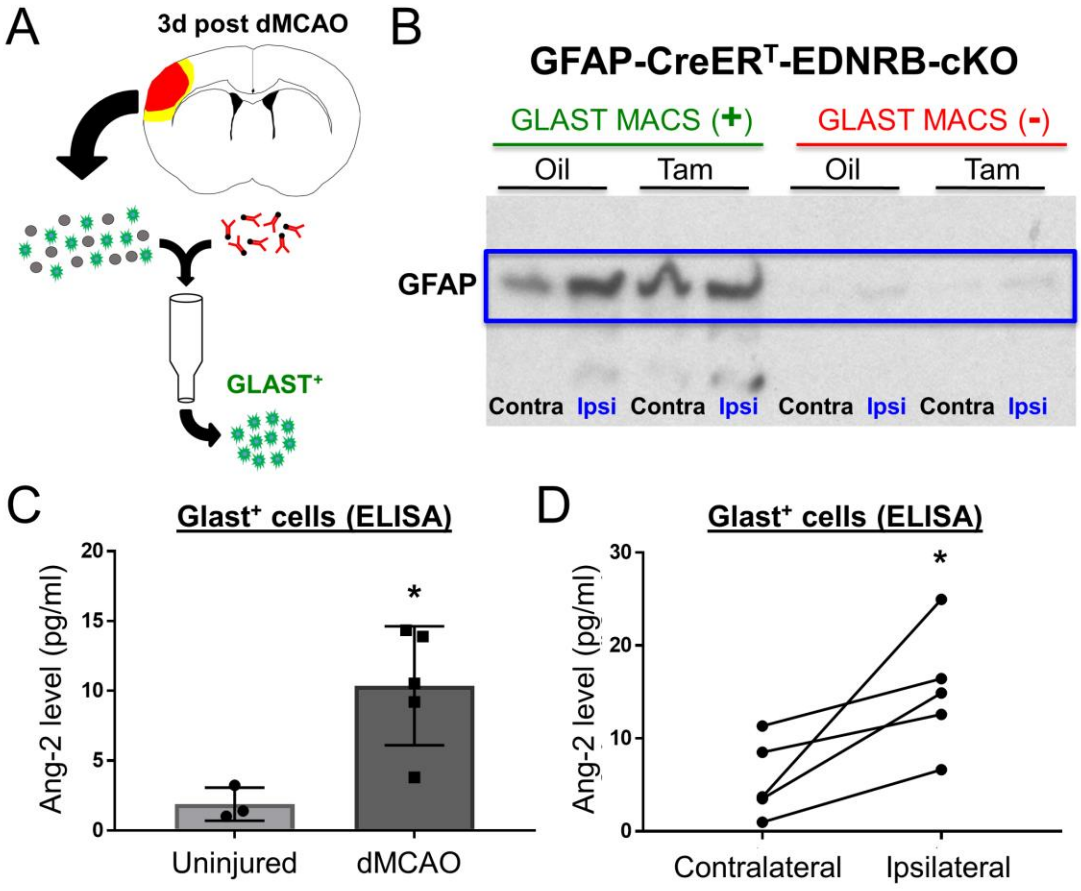

A
3d post dMCAO
GLAST+
B
GFAP-CreERT-EDNRB-cKO
GLAST MACS (+)
GLAST MACS (-)
Oil
Tam
Oil
Tam
GFAP
Contra
Ipsi
Contra
Ipsi
Contra
Ipsi
Contra
Ipsi
C
Glast+ cells (ELISA)
Ang-2 level (pg/ml)
20
15
10
5
0
*
Uninjured
dMCAO
D
Glast+ cells (ELISA)
Ang-2 level (pg/ml)
30
20
10
0
*
Contralateral
Ipsilateral

Figure 2. 2.

Astrocytes isolated by Magnetic Activated Cell Sort (MACS) against GLAST express increased Ang-2 expression 3 d after stroke. **A)** Schematic of GLAST-MACS workflow, cell lysis, and Ang-2 ELISA. **B)** Immunoblot for GFAP showing the enrichment of astrocytes in the $GLAST^+$ cell fraction after GLAST-MACS. **C)** Comparison of uninjured control animals ($n = 3$) and dMCAO animals ($n = 5$). Astroglial Ang-2 expression significantly increased following stroke. **D)** Astroglial Ang-2 expression significantly increased in the hemisphere ipsilateral to the stroke ($n = 5$) compared with that of the contralateral hemisphere ($n = 5$). $^*P < 0.05$.

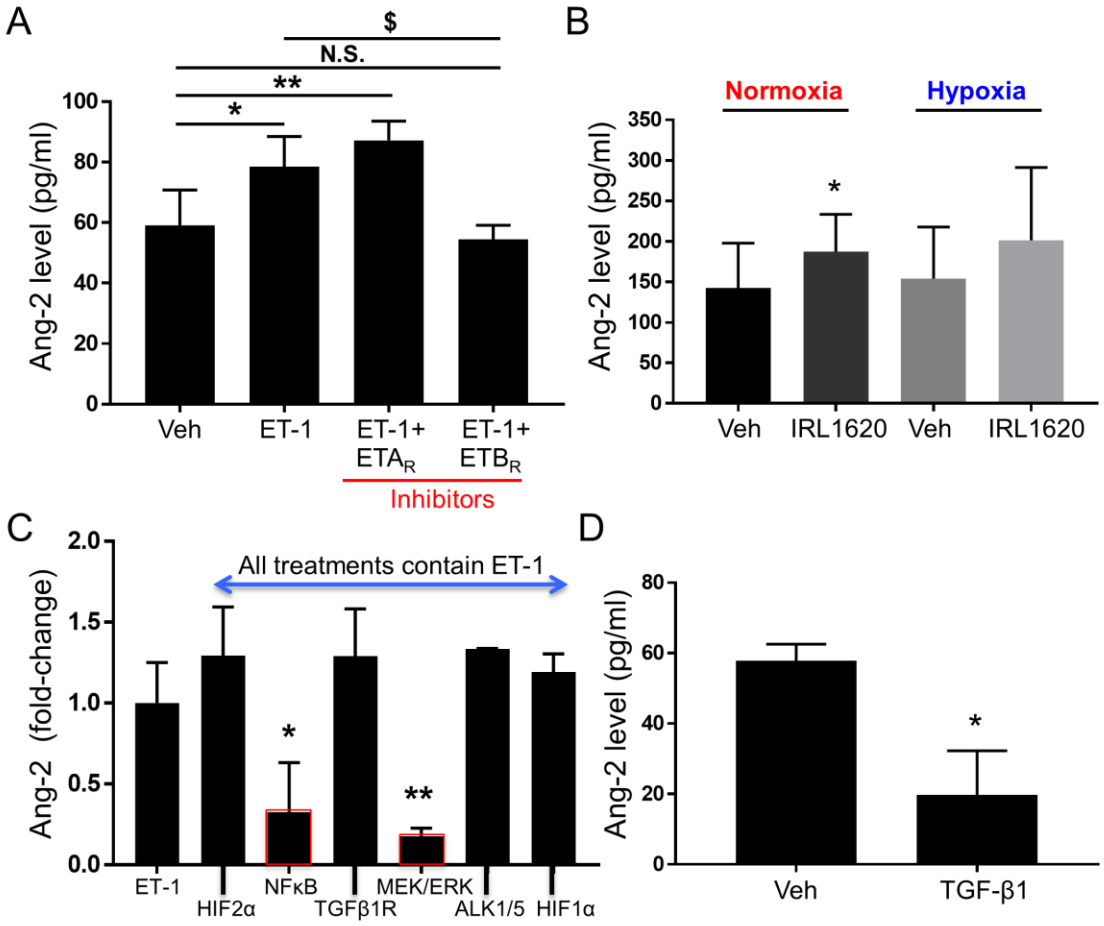

A
$
N.S.
**
*
Ang-2 level (pg/ml)
100
80
60
40
20
0
Veh
ET-1
ET-1+ ETA$_R$
ET-1+ ETB$_R$
Inhibitors
B
Normoxia
Hypoxia
Ang-2 level (pg/ml)
350
300
250
200
150
100
50
0
*
Veh
IRL1620
Veh
IRL1620
C
All treatments contain ET-1
Ang-2 (fold-change)
2.0
1.5
1.0
0.5
0.0
*
**
ET-1
HIF2α
NFκB
TGFβ1R
MEK/ERK
ALK1/5
HIF1α
D
Ang-2 level (pg/ml)
80
60
40
20
0
*
Veh
TGF-β1

Figure 2. 3.

ET-1 signals through ETB_R, and downstream NFκB and MEK/ERK, to stimulate Ang-2 production by cultured astrocytes. **A)** Cultured astrocytes were treated with ET-1 (100 nM), and vehicle, ETA_R inhibitor (BQ-123, 1 μM), or ETB_R inhibitor (BQ-788, 8 μM). Inhibition of ETB_R blocked the ET-1 dependent increase in astroglial Ang-2 secretion (Veh vs. ET-1 + ETB inhibitor: $P = 0.81$). **B)** Treatment with IRL1620 significantly increased astroglial Ang-2 expression in normoxia but not hypoxia ($n = 3$ for all groups). **C)** Prior to treatment with ET-1 (100 nM), astrocytes were pretreated for 1 hr with inhibitors to multiple signaling pathways: HIF2α (TCS-7009, 1 μM), NFκB (10 nM), TGFβRI/ALK5 (Repsox, 1 μM), MEK/ERK (U0126, 10 μM), ALK1/2/6 (K02288, 80 nM), HIF1α (PX-478, 25 μM). NFκB or MEK/ERK inhibition significantly reduced astroglial Ang-2 expression. **D)** TGFβ1 treatment significantly decreased Ang-2 secretion. $n = 3$ for both groups. *$P < 0.05$; **$P < 0.01$.

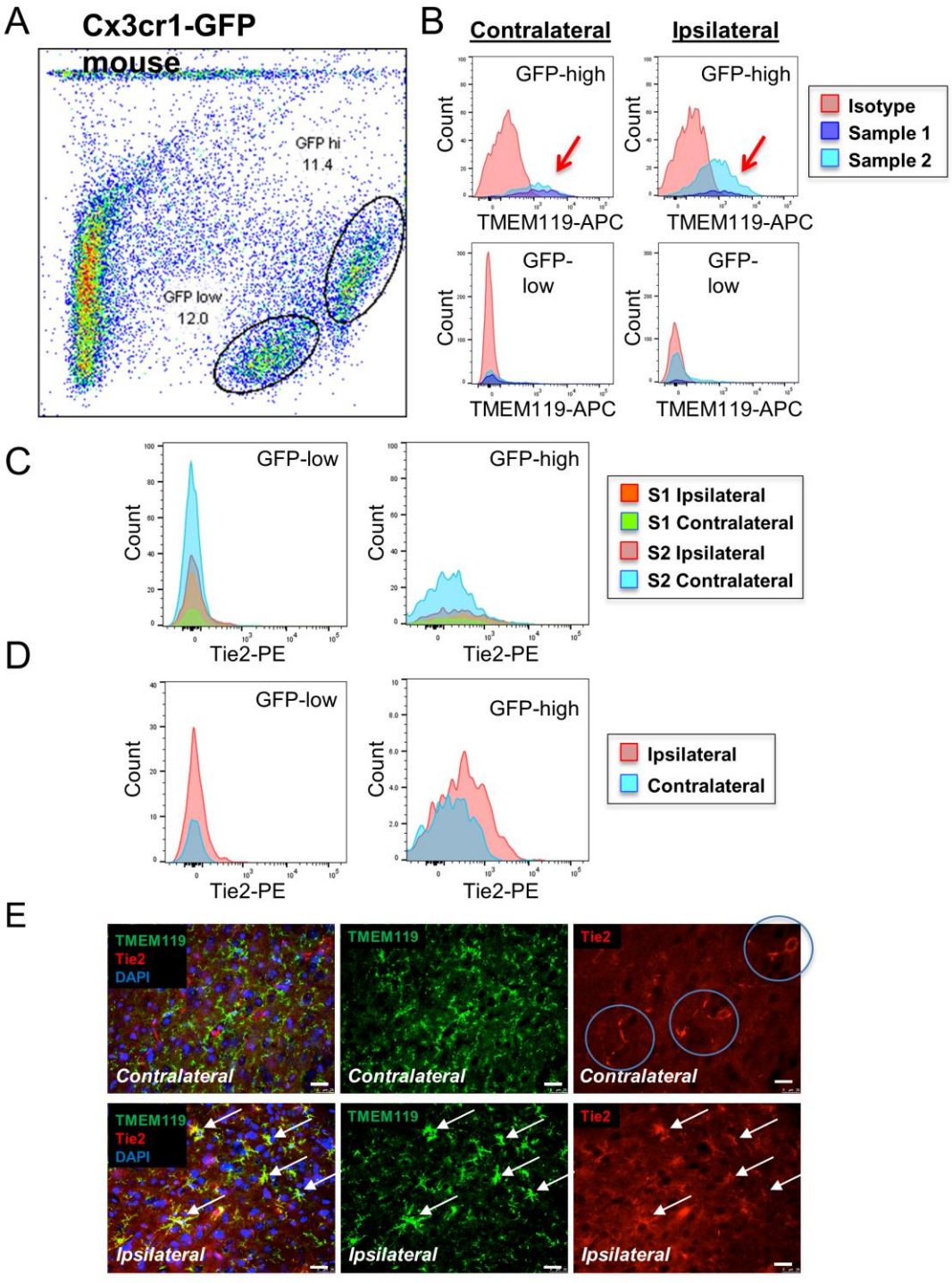

A
Cx3cr1-GFP mouse
GFP hi
11.4
GFP low
12.0
B
Contralateral
Ipsilateral
GFP-high
GFP-high
Count
Count
TMEM119-APC
TMEM119-APC
Isotype
Sample 1
Sample 2
GFP-low
GFP-low
Count
Count
TMEM119-APC
TMEM119-APC
C
GFP-low
GFP-high
Count
Count
Tie2-PE
Tie2-PE
S1 Ipsilateral
S1 Contralateral
S2 Ipsilateral
S2 Contralateral
D
GFP-low
GFP-high
Count
Count
Tie2-PE
Tie2-PE
Ipsilateral
Contralateral
E
TMEM119
Tie2
DAPI
Contralateral
TMEM119
Contralateral
Tie2
Contralateral
TMEM119
Tie2
DAPI
Ipsilateral
TMEM119
Ipsilateral
Tie2
Ipsilateral

Figure 2. 4.

Cx3cr1-GFP^{High} microglia express Tie-2 in both cerebral hemispheres 3 d after dMCAO surgery. **A)** Cell population was enriched for microglia by MACS against CD11b. By FACS, we observed two populations of Cx3cr1-GFP^{+} cells; Cx3cr1-GFP^{High} and Cx3cr1-GFP^{low}. **B)** The Cx3cr1-GFP^{high} cell population expressed the microglial marker TMEM119, both ipsilateral and contralateral to the ischemic infarct. Cx3cr1^{low} cells expressed less TMEM119. **C, D)** The Cx3cr1-GFP^{high} population (TMEM119^{+} microglia) expressed the Tie-2 receptor in both hemispheres, ipsilateral and contralateral to the stroke. **E)** Immunohistochemistry for Tie-2 and TMEM119, demonstrated prominent Tie-2 expression in the vasculature (blue circles) contralateral to the stroke injury. TMEM119^{+} microglia (green cells) co-expressed Tie-2 (red, white arrows) ipsilateral to the stroke injury.

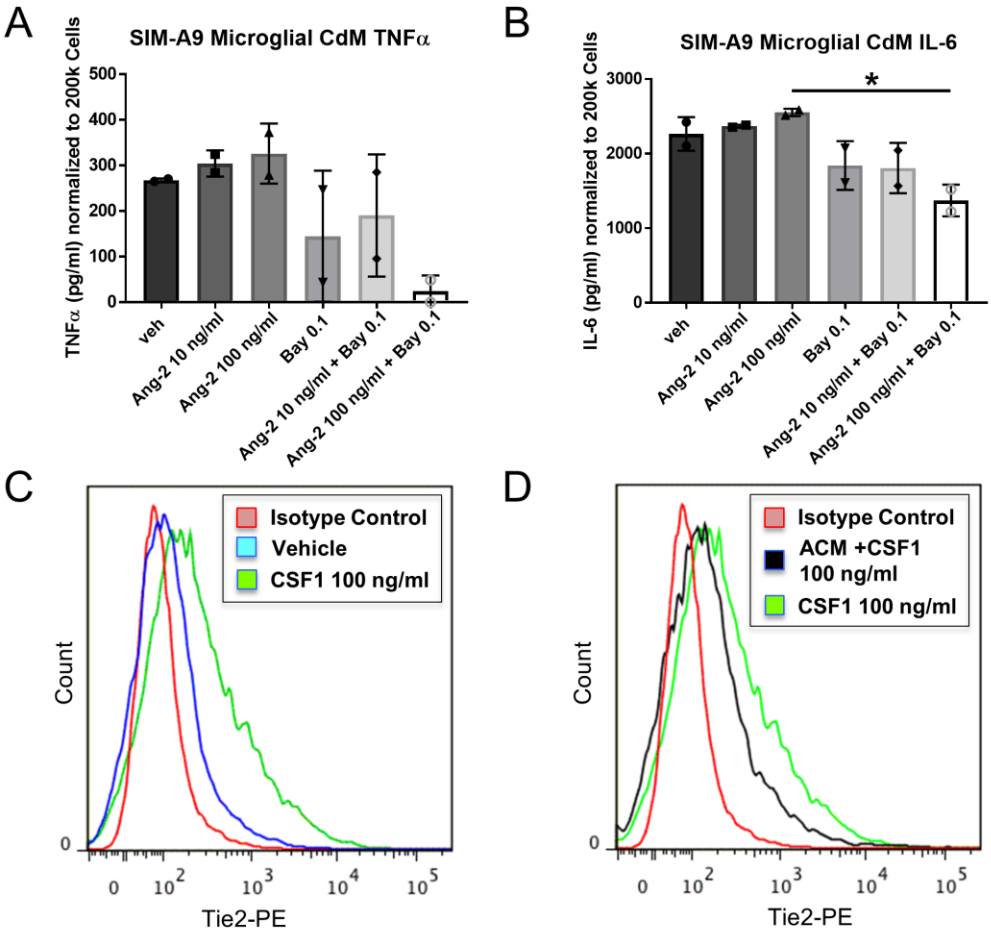

A
SIM-A9 Microglial CdM TNFα
TNFα (pg/ml) normalized to 200k Cells
500
400
300
200
100
0
veh
Ang-2 10 ng/ml
Ang-2 100 ng/ml
Bay 0.1
Ang-2 10 ng/ml + Bay 0.1
Ang-2 100 ng/ml + Bay 0.1
B
SIM-A9 Microglial CdM IL-6
IL-6 (pg/ml) normalized to 200k Cells
3000
2000
1000
0
*
veh
Ang-2 10 ng/ml
Ang-2 100 ng/ml
Bay 0.1
Ang-2 10 ng/ml + Bay 0.1
Ang-2 100 ng/ml + Bay 0.1
C
Isotype Control
Vehicle
CSF1 100 ng/ml
Count
0
0
10^2
10^3
10^4
10^5
Tie2-PE
D
Isotype Control
ACM +CSF1
100 ng/ml
CSF1 100 ng/ml
Count
0
0
10^2
10^3
10^4
10^5
Tie2-PE

Figure 2. 5.

Microglia alter cytokine secretion in response to Ang-2/Tie-2 signaling and Tie-2 expression in response to CSF1. **A)** Treatment with Ang-2 or Tie-2 inhibitor did not significantly change microglial TNFα secretion. **B)** In the presence of Ang-2 (100 ng/ml), Tie-2 inhibition (BAY-826 0.1 μM) significantly lowered microglial IL-6 secretion (Ang-2 100 ng/ml treatment: 2,550 ± 34.96 pg/ml IL-6 normalized to 200,000 cells, Ang-2 100 ng/ml + BAY-826 0.1 μM: 1,371.15 ± 151.01 pg/ml IL-6 normalized to 200,000 cells, $n = 2$, Ang-2 100 ng/ml vs. Ang-2 100 ng/ml + BAY-826 0.1 μM; $*P < 0.01$ by 1-way ANOVA). **C)** Treatment with CSF1 (100 ng/ml, green-line) increased the expression of Tie-2 relative to vehicle treatment (blue line). Isotype control for PE (red line). **D)** Astrocyte conditioned medium limits the effects of CSF1 on induction of cell surface Tie-2 (black line). Isotype control (red line) and CSF1 treatment (green line) are as in **C**.

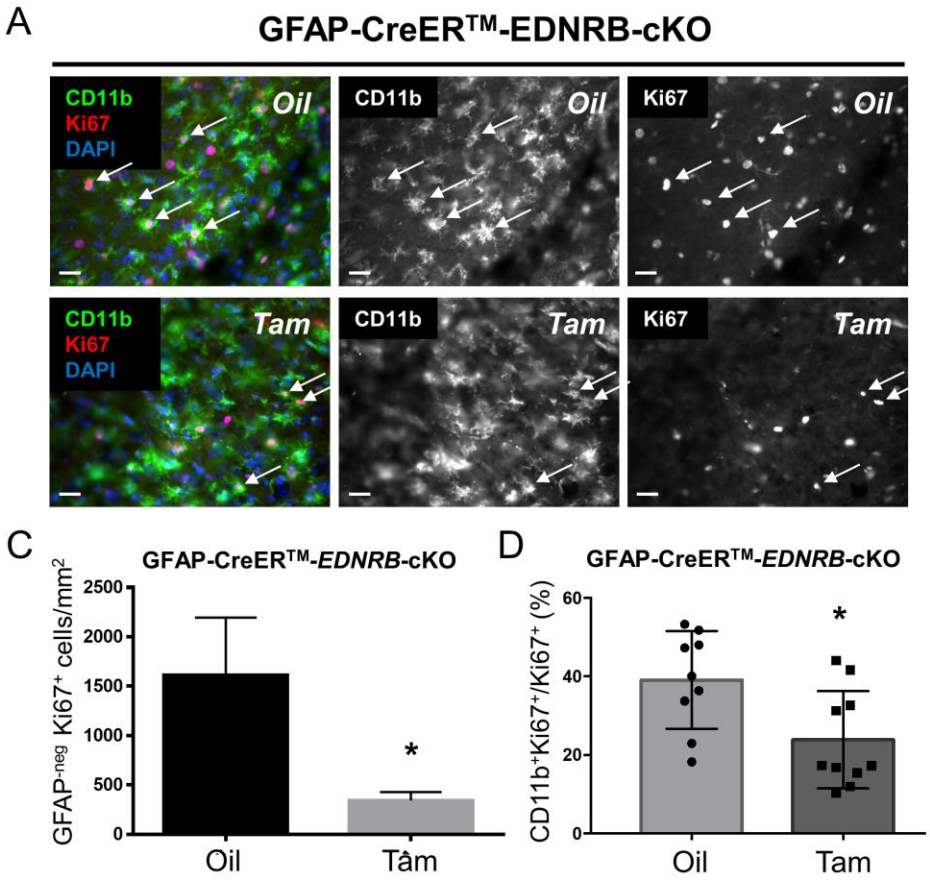
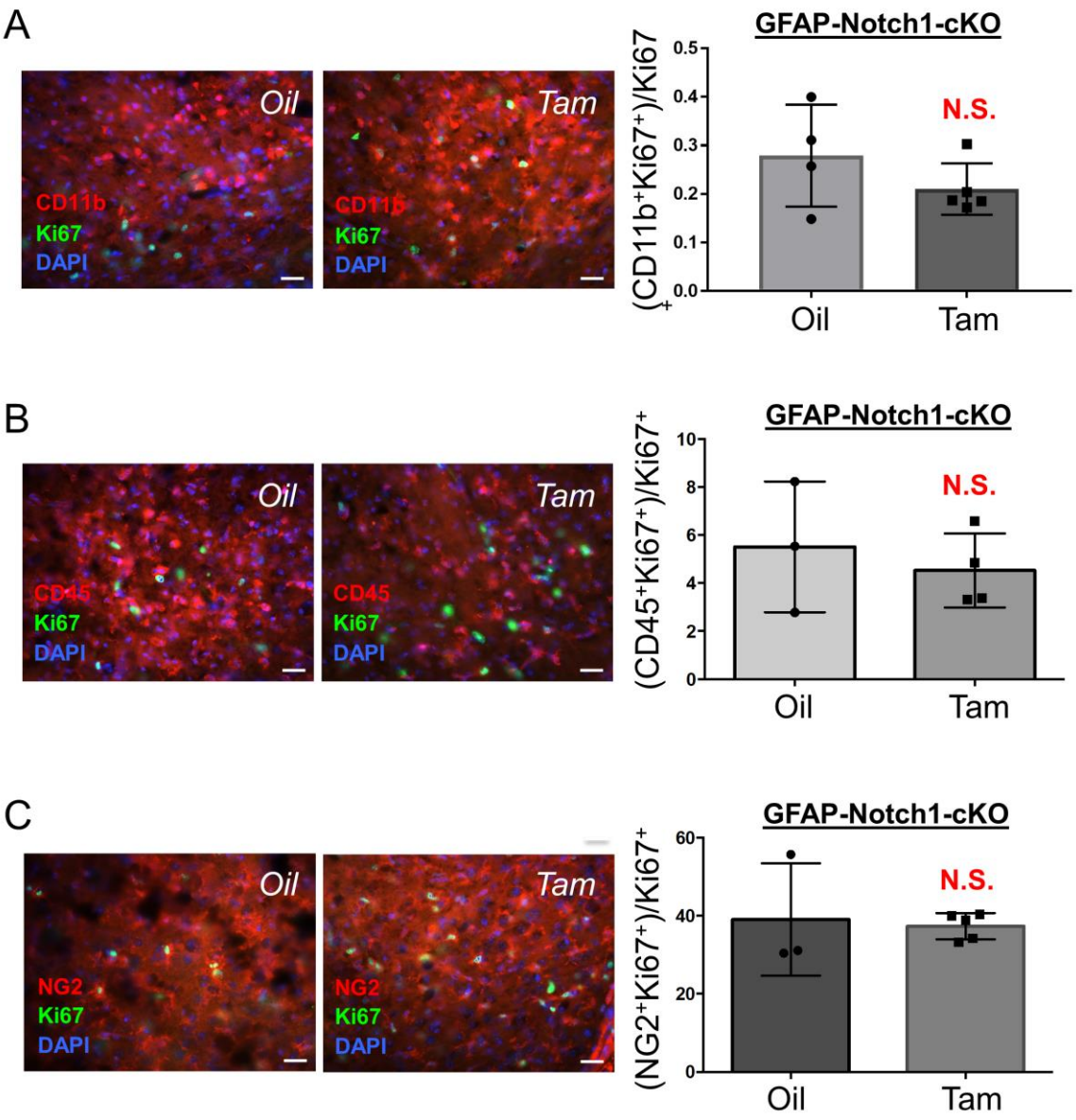

A
GFAP-CreER™-EDNRB-cKO
CD11b
Ki67
DAPI
Oil
CD11b
Oil
Ki67
Oil
CD11b
Ki67
DAPI
Tam
CD11b
Tam
Ki67
Tam
C
GFAP-CreER™-EDNRB-cKO
GFAP-neg Ki67+ cells/mm2
2500
2000
1500
1000
500
0
*
Oil
Tâm
D
GFAP-CreER™-EDNRB-cKO
CD11b+Ki67+/Ki67+ (%)
60
40
20
0
*
Oil
Tam

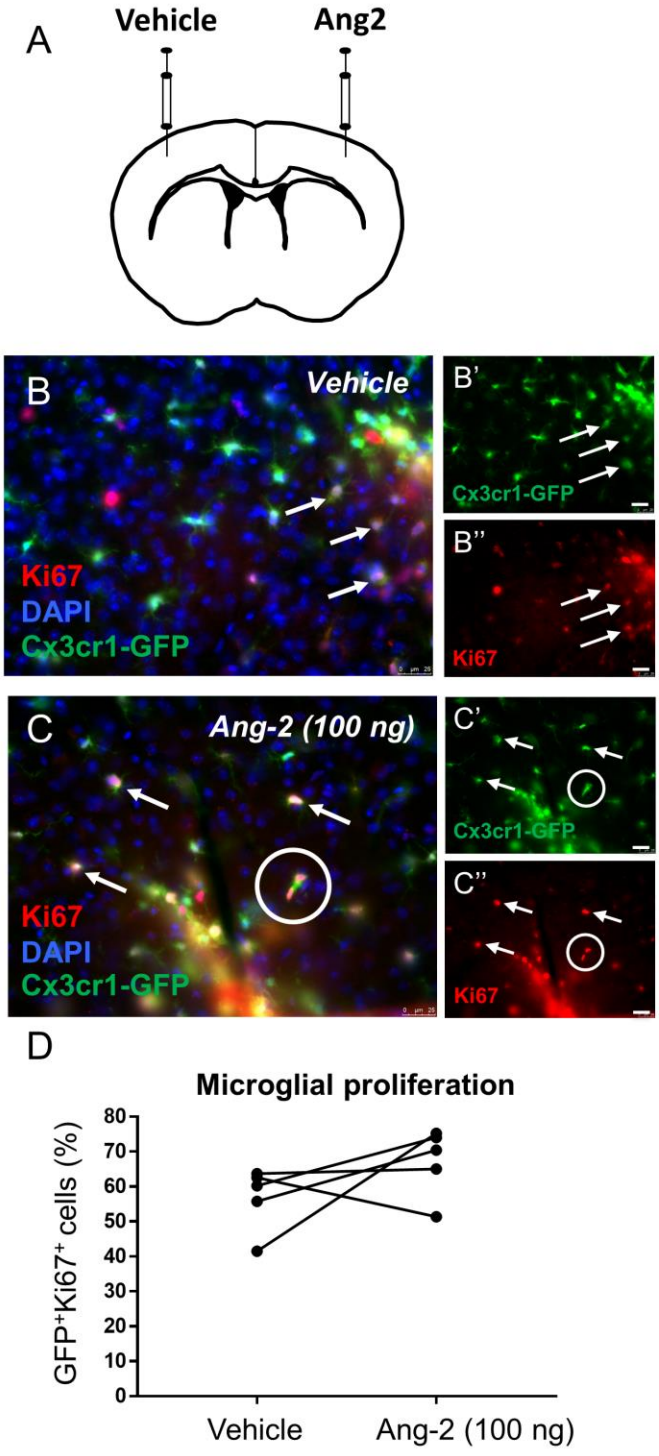
Figure 2. 6.

Astroglial-specific *EDNRB*-knockout reduced the number of proliferating microglial-like cells. **A)** Immunohistochemistry for microglia/macrophages/monocytes (CD11b, green) and proliferating cells (Ki67, red) 3 d post-stroke at the infarct edge, in vehicle-injected (Corn oil, control) and tamoxifen(tam)-injected GFAP-CreER^{TM}-*EDNRB*-$^{fl/fl}$ mice. Arrows indicate proliferative CD11b^{+} cells. **B)** *EDNRB* loss significantly decreased the number of proliferating non-astroglial cells (GFAP^{-neg}/Ki67^{+}) (Control: n = 3; Knockout n = 3) . **C)** *EDNRB* loss significantly decreased the proportion of double-positive (Ki67^{+}/CD11b^{+}) (Controls: n = 9; Knockouts: n = 10). Scale bars = 25 μm. *$P < 0.05$.


A
Oil
Tam
CD11b
Ki67
DAPI
GFAP-Notch1-cKO
(CD11b+Ki67+)/Ki67+
N.S.
Oil
Tam
B
Oil
Tam
CD45
Ki67
DAPI
GFAP-Notch1-cKO
(CD45+Ki67+)/Ki67+
N.S.
Oil
Tam
C
Oil
Tam
NG2
Ki67
DAPI
GFAP-Notch1-cKO
(NG2+Ki67+)/Ki67+
N.S.
Oil
Tam

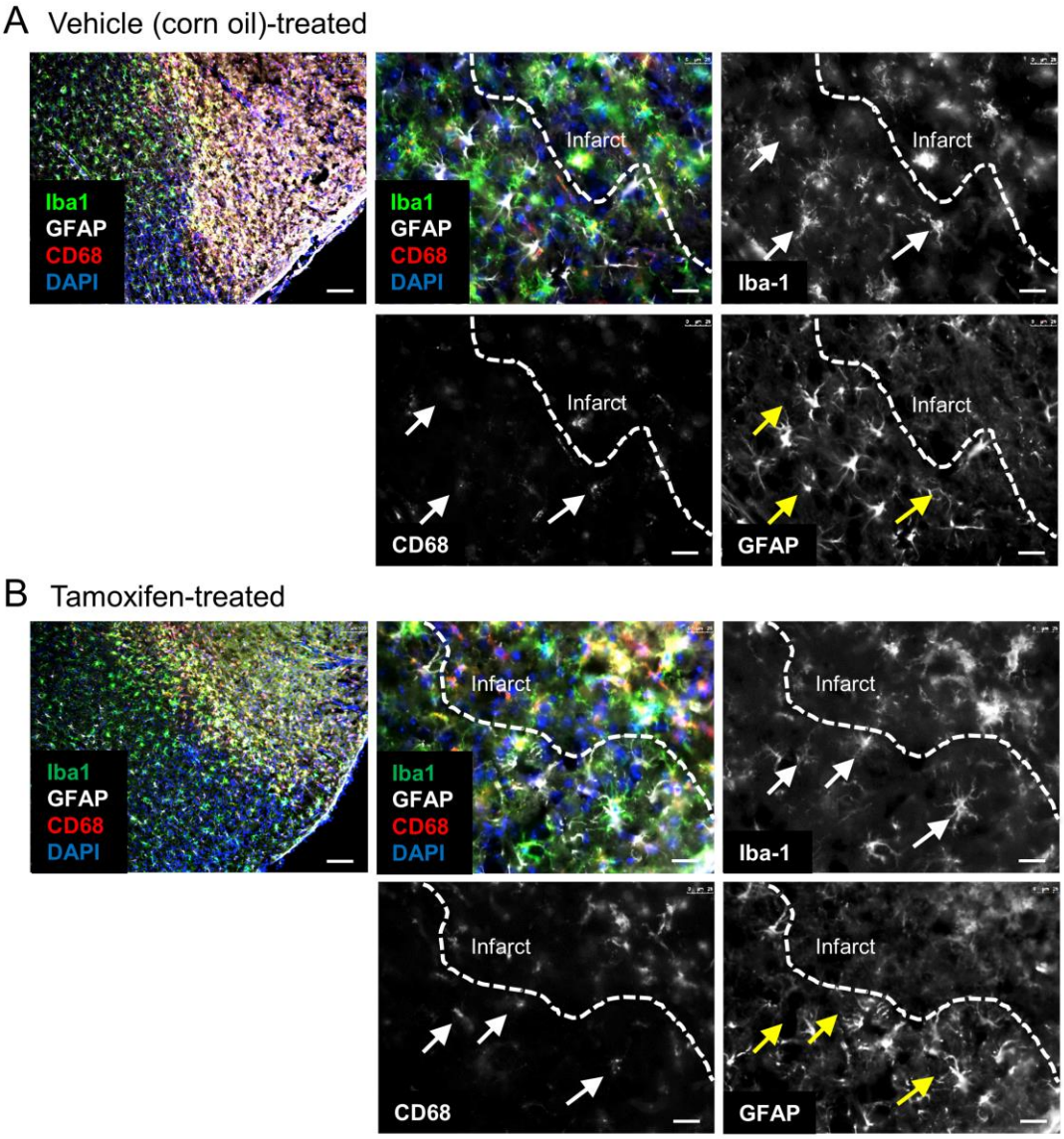
Supplemental Figure 2. 1.

Astroglial-specific *Notch1*-knockout did not affect proliferation of non-astroglial cell types. **A)** Representative immunohistochemistry for microglia/macrophages/monocytes (CD11b, red) and a nuclear proliferation marker (Ki67, green) in vehicle-treated (Oil, control) and tamoxifen-treated (TAM, knockout) GFAP-creER^{TM}-*Notch1*-$^{fl/fl}$ mice. The number of proliferating microglia-like cells did not differ between control (n = 4) and knockout mice (n = 5) (P = 0.24). **B)** Representative immunohistochemistry for hematopoietic-lineage cells (CD45, red) and a nuclear proliferation marker (Ki67, green) in control (Oil-treated) and knockout (TAM-treated) GFAP-creER^{TM}-*Notch1*-$^{fl/fl}$ mice. Quantification of proliferative hematopoietic-lineage cells shows no significant difference (P = 0.57) between control (n = 3) and knockout mice (n = 4). **C)** Representative immunohistochemistry for Oligodendrocyte Progenitor Cells (OPCs, i.e. polydendrocytes) and pericytes (NG2, red) and a nuclear proliferation marker (Ki67, green) in control (Oil-treated) and knockout (TAM-treated) GFAP-creER^{TM}-*Notch1*-$^{fl/fl}$ mice. The number of proliferative OPCs and/or pericytes did not differ between control (n = 3) and knockout mice (n = 5) (P = 0.80). N.S. = not significant. Scale bars = 25 μm.


A
Vehicle
Ang2
B
Vehicle
Ki67
DAPI
Cx3cr1-GFP
B'
Cx3cr1-GFP
B''
Ki67
C
Ang-2 (100 ng)
Ki67
DAPI
Cx3cr1-GFP
C'
Cx3cr1-GFP
C''
Ki67
D
Microglial proliferation
GFP+Ki67+ cells (%)
80
70
60
50
40
30
20
10
0
Vehicle
Ang-2 (100 ng)

Supplemental Figure 2. 2.

Stereotaxic injection of Ang-2 did not affect microglial proliferation. **A)** Schematic for stereotaxic injection paradigm. The hemisphere that received Ang-2 injection was randomized in a blinded manner to control for any possible lateralization effects. **B)** Representative images of immunohistochemistry of vehicle-treated Cx3cr1-GFP mice stained for a proliferative marker (Ki67, red) and nuclei (DAPI, blue). Arrows indicated proliferative GFP^+ cells. **C)** Representative images of immunohistochemistry of Ang-2 treated (100 ng injection) Cx3cr1-GFP mice stained for a proliferative marker (Ki67, red) and nuclei (DAPI, blue). Arrows indicated proliferative GFP^+ cells. Circle indicates GFP^+ cells that just divided. **D)** The number of proliferating Cx3cr1-GFP^+ cells did not differ (n = 5 both groups) (P = 0.12). N.S. = not significant. Scale bars = 25 μm.


A Vehicle (corn oil)-treated
Iba1
GFAP
CD68
DAPI
Infarct
Iba-1
CD68
GFAP
B Tamoxifen-treated
C
GFAP-ETB$_R$-cKO
(inside infarct)
N.S.
Iba1$^+$CD68$^+$/Iba1$^+$ (%)
Oil
TAM
D
GFAP-ETB$_R$-cKO
(outside infarct)
N.S.

Supplemental Figure 2. 3.

Astroglial-specific *EDNRB*-knockout did not alter expression of CD68 in $Iba1^{+}$ cells at the peri-infarct edge. **A)** Representative images of the infarct edge 3 d post-stroke in vehicle treated (corn oil, control) GFAP-CreER-*EDNRB*-$^{fl/fl}$ (left panel, 10x; scale bar = 100 μm) (right panels, 40x; scale bars = 25 μm) **B)** Representative images of the infarct edge 3 d post-stroke in tamoxifen-treated (TAM, knockout) GFAP-CreER-*EDNRB*-$^{fl/fl}$ (left panel, 10x; scale bar = 100 μm) (right panels, 40x; scale bars = 25 μm) **A, B)** Immunohistochemical assays for astrocytes (GFAP, gray), microglia (Iba1, green), and a marker of inflammatory/phagocytic cells (CD68, red). **C, D)** Quantification of CD68 reactivity in Iba1-expressing cells showed no difference in the number of cells expressing high levels of CD68 either inside the infarct (n = 3 Oil-controls, n = 4 TAM-knockout mice; P = 0.38) or outside the infarct (n = 3 Oil-controls, n = TAM-knockout mice; P = 0.95), as demarcated by the edge of GFAP reactivity.

CHAPTER 3: CD109 expression in astrocytes is controlled by endothelin receptor type-B signaling and alters neuroinflammation

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Disclosures: J.L.S. holds patent interests in regenerative medicine and is founder of Samba BioLogics, Inc.

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ABSTRACT

Following ischemic stroke, reactive astrocytes proliferate and form a glial scar that limits progressive damage to the central nervous system (CNS). Recently, a paradigm was proposed in which discrete subpopulations of reactive astrocytes found in different inflammatory/neurodegenerative diseases were characterized as neurotoxic, whereas those observed after stroke or spinal cord injury were deemed neuroprotective (stroke). Accordingly, the new model has inspired research directed at identifying markers that effectively parse the subpopulations and also to determine their paracrine activity. Of special interest, the neuroprotective subpopulation was shown to express high levels of CD109 mRNA. In several different forms of CNS injury, our group and others have demonstrated that proliferating reactive astrocytes possess neuroprotective function(s). After ischemic stroke, reactive astrocyte proliferation and survival are regulated, in part, by Endothelin receptor type-B (ETB_R) signaling. Here we show that astroglial ETB_R signaling promotes the expression of CD109 after stroke. For reactive glia, CD109 was primarily expressed on aquaporin-4^+ endfeet of astrocytes that were associated with the microvasculature. In studies of primary adult astrocytes with shRNA-mediated knockdown of CD109, reduction of CD109 significantly impacted astroglial expression of the neurotoxic marker complement C3 (C3) in response to TNFα and ETB_R signaling.

Treatment with recombinant CD109 after stroke tended to increase infarct sizes as well as neuroinflammatory activity in areas of the brain distal to the infarct. Our results suggest that soluble (free) CD109 may serve as a modulator of neuroinflammation after stroke. Further research is needed to better understand the cell-autonomous effects of membrane-bound CD109 on reactive astrocyte phenotypes and the non-cell-autonomous effects of cleaved/released CD109 during neuroinflammation and injury progression after stroke.

INTRODUCTION

Reactive astrocytes display heterogeneous phenotypes that vary between CNS injuries and diseases and also across an individual injury environment, such as ischemic stroke. Recently, complement $C3^+$ astrocytes were described as "neurotoxic astrocytes" based on their secretion of a paracrine protein factor(s) that trigger neuronal cell death in neuroinflammatory diseases (Alzheimer's, multiple sclerosis, Parkinson's) (Liddelow et al. 2017; Yun et al., 2018). $C3^+$ neurotoxic astrocytes are induced by inflammatory factors such as IL-1α, TNFα, and C1q that are released by microglial cells in response to bacterial antigens (e.g. lipopolysaccharide, LPS) or central nervous system (CNS) degeneration. To treat neuroinflammatory/neurodegenerative diseases, therapies are being pursued to block the neurotoxic inducing factors or neutralize complement factors associated with neurotoxicity (Alawieh et al., 2020; Guttenplan et al., 2020; Qian et al., 2019; Yun et al., 2018).

Neurotoxic astrocytes have been contrasted with astrocytes bearing a "neuroprotective" phenotype. Primarily observed after stroke and spinal cord injury (Anderson et al., 2016; LeComte et al., 2015; Tyzack et al., 2017), the neuroprotective phenotype is characterized by a relatively high rate of proliferation as compared with

reactive astrocytes described in neuroinflammatory disease (Kamphuis et al., 2012; LeComte et al., 2015; Lepore et al., 2008; Liddelow and Barres, 2017; Liddelow et al., 2017; Shimada et al., 2011; Simon et al., 2011; Sirko et al., 2013). Although further definition is clearly needed, putative markers for neuroprotective astrocytes include the RC2 antigen (phosphorylated form of Nestin, an intermediate filament protein), high expression of Jagged-1 (Notch-ligand), phosphorylated Signal Transducer and Activator of Transcription-3 (STAT3), high levels of endothelin receptor type B (ETB_R), and proteins associated with cell proliferation, such as Ki67 (Herrmann et al., 2008; Shimada et al., 2010; Shimada et al., 2011; Wanner et al., 2013; LeComte et al., 2015). After CNS injury, proliferative/neuroprotective astrocytes are linked to tissue preservation, reduction of inflammation and injury progression, glial scar formation, and injury resolution (Anderson et al., 2016; Bush et al., 1999; Shimada et al., 2011). Numerous signals found within the injury environment have been shown to induce reactive astrogliosis (Sofroniew, 2009; Pekny and Pekna, 2016) and promote reactive astrocyte proliferation (e.g. EGF, HB-EGF, Jagged1, Shh, and endothelin) and scar formation (e.g. TGFβ) (Schachtrup et al., 2010; Shimada et al., 2011, 2012; Sirko et al., 2013; LeComte et al., 2015; Zhang et al., 2018). Importantly, a wide variety of signals are similarly found under injury conditions of sterile inflammation (e.g. stroke and spinal cord injury), trauma (e.g. brain stab injury), and neuroinflammatory/neurodegenerative diseases such as Alzheimer's and Parkinson's. The specific signals that drive reactive astrocytes to become neuroprotective or neurotoxic remain poorly understood.

Previously, CD109 gene expression in reactive astrocytes was reported to correlate with the neuroprotective phenotype (Liddelow et al., 2017; Zamanian et al.,

2012). CD109 is a TGFβ co-receptor and a member of the alpha (2) macroglobulin/complement C3, C4, C5 family (Li et al., 2016; Lin et al., 2002). CD109 interacts with both TGFβ ligands and receptors and was demonstrated to shunt signaling from SMAD2/3 to alternative SMAD1/5 signaling (Vorstenboschet al., 2017). CD109 is cleaved by membrane-bound proteases such as furin and retains many of its biological functions as a free, soluble protein (Li et al., 2017). Research suggests that CD109 modulates TGFβ1 and EGFR signaling in its membrane tethered form and that released CD109 retains TGFβ1 modifying abilities but loses EGFR modulation (Zhang et al., 2015). Consistent with its function altering EGFR signaling, CD109 can also alter STAT3 activity and possibly interacts with Gp130 signaling (Chuang et al., 2017). Notably, whereas CD109 expression levels may identify astrocytes with different molecular phenotypes, the function(s) of CD109 in the brain and during reactive astrogliosis have not been determined.

Here we demonstrate that ETB_R signaling in reactive astrocytes promotes CD109 expression. In loss-of-function experiments with reactive astrocytes from GFAP-$CreER^T$-*EDNRB*-$^{fl/fl}$ mice, tamoxifen treatment decreased multiple forms of CD109. By contrast, IRL1620, a specific ETB_R agonist, increased the membrane-localized form of CD109 as well as its cleaved, soluble form. We found that astrocyte CD109 knockdown by shRNA decreased the secretion of Angiopoietin-2, a mitogen/survival factor for microglia. Of special interest, we found that CD109 and IRL1620 mediated TNFα-induced expression of complement C3 and C3d. Delivery of soluble CD109 to mice 24-hours post-stroke tended to exacerbate primary cortical stroke-injury as well as secondary thalamic inflammation.

RESULTS

We first identified CD109 as a putative downstream effector of ETB_R signaling in reactive astrocytes using a loss-of-function screen for *EDNRB* (the gene encoding ETB_R). Astroglial *EDNRB*-knockout in cultured cells isolated from GFAP-CreER^{TM}-*EDNRB*-$^{fl/fl}$ mice decreased CD109 gene expression by ~4.0-fold (Chapter 2, Figure 2.1). To confirm that *EDNRB*-knockout also affected CD109 protein, we immunoblotted cell lysates from two separate, clonal transgenic reactive astrocyte populations following treatment with 4-hydroxy tamoxifen (4-OH-TM). Although baseline CD109 expression levels varied between clones, *EDNRB*-knockout similarly decreased both cleaved and un-cleaved forms of CD109 (Figure 3.1A). To further investigate the potential relationship between ETB_R and CD109, we treated adult astrocytes with IRL1620, a truncated form of endothelin that binds ETB_R, but not ETA_R. Treatment of cultured astrocytes with 100 nM IRL1620 increased expression of cell-associated CD109 as well as the released form of CD109, detected in serum-free astrocyte-conditioned medium (Figure 3.1B).

To investigate the effects of CD109 loss-of-function in reactive astrocytes, we re-differentiated astrocytes from Reactive astrocyte-derived NSC that were isolated from peri-infarct cortical tissue 3 d after stroke (as reported previously, Shimada et al., 2012). To generate CD109 knockdown cell lines, Rad-NSC from the same clone were split into separate culture wells and then transduced with control lentiviral shRNA vector (shScrambled) or shRNA specific to CD109 (shCD109). By immunoblot assays, astrocytes with CD109 knockdown had decreased expression for multiple CD109 isoforms (i.e. cleavage products). Notably, several CD109 isoforms of similar size decreased after *EDNRB*-knockout in astrocytes from GFAP-CreER^{TM}-*EDNRB*-$^{fl/fl}$ mice

(Figure 3.1A, 3.1B); these results confirmed that the antibody we used detected multiple full-length, cleaved, and released forms of CD109 (Figure 3.1B).

To detect CD109 during reactive astrogliosis at 3 d after stroke, we performed double immunohistochemical stains for CD109 and Glial Fibrillary Acidic Protein (GFAP, an intermediate filament protein,) or the aquaporin-4 water channel (AQP4). At the blood brain barrier (BBB), astrocytes and endothelial cells form a gliovascular unit that is critical to maintain the appropriate balance of fluid and ions in the brain. Accordingly, AQP4 is typically highly-expressed at astrocyte endfeet, which wrap the cerebral microvasculature (Simard et al., 2003). After stroke, we observed co-localization of CD109 with AQP4 at blood vessels both ipsilateral and contralateral to the ischemic injury (Figure 3.2A). Notably, our observations were consistent with a prior ribotagging study that reported CD109 mRNA was actively translated at gliovascular endfeet. To determine if CD109 was limited only to glial endfeet and/or expressed elsewhere in the cell body, we examined reactive astrocytes at the infarct edge, within the peri-infarct area, and within the outlying brain parenchyma. At 3 d after stroke, we observed that the majority of $GFAP^{+}$ astrocytes that stained for CD109 were located proximal to the infarct and in close contact with the vasculature (Figure 3.2B). Of interest, we also observed rare $GFAP^{+}CD109^{+}$astrocytes that expressed CD109 throughout the entire cell body, but did not appear to contact blood vessels (Figure 3.2C).

To further characterize astroglial expression of CD109 and its cleavage products, we performed dMCAO surgeries on control (corn oil-injected) and tamoxifen-treated GFAP-$CreER^{TM}$-*EDNRB*-$^{fl/fl}$ mice (*EDNRB*-cKO). We isolated astrocytes from the stroke and non-stroke hemispheres of control and knockout mice by magnetic-activated cell

sorting with antibodies directed against the Glutamate Aspartate Transporter (GLAST MACS) (Figure 3.3A). By immunoblot for vehicle controls, CD109 (~60 kDa cleavage) was significantly higher in astrocytes from the stroke vs. the non-stroke hemisphere (Vehicle-treated uninjured-hemisphere: 16,488.52 ± 2,950.03; Vehicle-treated stroke-hemisphere: 28,551.66 ± 4,703.08, $n = 4$ per group; $P < 0.05$) (Figures 3.3E, 3.3F). Although CD109 is reported as a marker of reactive astrocytes, it was also detected in astrocytes isolated from cortical tissue contralateral to the stroke (Figure 3.3E). In *EDNRB*-knockout animals, astroglial CD109 levels appeared to be elevated in cells from the uninjured hemisphere relative to those of vehicle controls (Figure 3.3E). Notably, in *EDNRB*-knockout animals, CD109 expression levels were not significantly different between cells isolated from stroke and non-stroke hemispheres (TM-treated uninjured-hemisphere: 24,837.52 ± 7,554.83; TM-treated stroke-hemisphere: 18,312.55 ± 5,343.51; $n = 4$ per group; $P = 0.54$) (Figure 3.3E, 3.3G). We detected multiple bands corresponding to different cleavage products of CD109, consistent with our previous immunoblots of lysates from cultured *EDNRB*-knockout and shCD109 astrocytes. The pattern of CD109 expression paralleled that of GFAP expression, which also tended to increase in response to stroke and presented higher baseline levels in *EDNRB*-knockout mice relative to levels observed in vehicle-treated control mice (Figure 3.3B).

CD109 mRNA is a reported marker of a neuroprotective astrocyte phenotype; we therefore utilized shCD109 Rad-NSC derived astrocytes to assess the expression of inflammatory factors. Western blots for the neurotoxic marker complement C3 (C3) and its cleavage products containing the C3d domain (C3d), showed that clones expressed variable baseline levels of C3d (Figure 3.4A). This is consistent with the differences we

observed in the expression of CD109 between astrocytes derived from different Rad-NSC clones (Figure 3.1A). Astroglial stimulation with tumor necrosis factor-α (TNFα), an inducer of neurotoxicity, increased several forms of C3 (shCT TNFα-treatment 185 kDa C3: Up 5.38-fold ± 3.67; shCD109 TNFα-treatment 185kDa C3: Up 6.95-fold ± 2.57) (Figure 3.4). Treatment with IRL1620 simultaneous with TNFα consistently decreased C3 expression relative to TNFα treatment alone, particularly cleavage products at 26-28 kDa (shCT TNFα-treatment: 0.81-fold ± 0.05 of vehicle-treatment; shCT TNFα + IRL1620-treatment: 0.60-fold ± 0.05 of vehicle treatment; $n = 2$; $P < 0.05$) (Figures 3.4A; Figure 3.4D). Importantly, in astroglial CD109 knockdown cells stimulated with TNFα, IRL1620 treatment was unable to decrease expression of C3 (shCD109 TNFα-treatment: 0.71-fold ± 0.08 of vehicle-treatment; shCD109 TNFα + IRL1620-treatment: 0.77-fold ± 0.06 of vehicle treatment; $n = 2$; $P = 0.95$) (Figure 3.4C, $P = 0.989$; Figure 3.4E, $P = 0.952$).

In order to assess the impact of CD109 signaling on astroglial paracrine and neuroinflammatory responses, we utilized shRNA CD109 knockdown astrocytes to measure angiopoietin-2 (Ang-2) secretion. We previously characterized a novel function of Ang-2 in regulating microglial inflammatory activity. ELISA of conditioned medium demonstrated that CD109 knockdown may decrease astroglial Ang-2 secretion (shCT IRL1620-treated: 1.20-fold ± 0.16 of shCT-vehicle; shCD109 IRL1620-treated: 0.43-fold ± 0.23 of shCT-vehicle; $n = 2$; $P = 0.09$) (Figure 3.5A). TGFβ1 interacts with CD109 and we previously showed that TGFβ1 treatment significantly decreases astroglial Ang-2 secretion. We found that CD109 knockdown altered the ability of TGFβ1 treatment to

repress Ang-2 secretion (shCT TGFβ1-treated: 0.15-fold ± 0.07 of shCT Vehicle-treated; shCD109 TGFβ1-treated: 1.57-fold ± 1.05 of shCD109 Vehicle-treated; $n = 2$; $P = 0.31$) (Figure 3.5A).

Astrocytes express soluble CD109 *in vitro* in response to ETB_R signaling, suggesting that soluble CD109 is being released in response to stroke and could impact stroke progression. To study the impact of secreted CD109 on stroke, we injected soluble recombinant human CD109 (rhCD109) 24-hours post-stroke and analyzed the effect stroke outcomes after 7 d of stroke recovery. CD109 treatment tended to increase stroke sizes at 7 d post-stroke as measured by the necrotic GFAP-negative portion of the cortex (Vehicle-treated Stroke size: 4.94 ± 1.45 mm^2; CD109-treated stroke size: 12.23 ± 5.21 mm^2; $n = 6$ vehicle-treated, $n = 7$ CD109-treated; $P = 0.24$) or by Cresyl violet staining for viable neural tissue (Vehicle-treated Stroke size: 4.81 ± 1.87 mm^2; CD109-treated stroke size: 11.91 ± 5.65 mm^2; $n = 5$ both groups; $P = 0.27$) (Figures 3.6A, 3.6B, and 3.6C). After stroke, neuroinflammation is often observed in secondary areas such as the thalamus; this is due to reciprocal connections between the thalamus and cortical tissue with infarction. We noted GFAP, IBA1, and CD68 reactivity in the thalamus ipsilateral to stroke and minimal reactivity in the contralateral thalamus (Figure 3.7). When the reactive GFAP area in the thalamus ipsilateral to the infarct was measured, there was a trend towards increased GFAP reactive area in CD109 treated animals as compared to controls (Vehicle-treated: 1,212,520 ± 359,227 μm^2; CD109-treated: 2,276,554 ± 917,740 μm^2; $n = 5$ both groups; $P = 0.31$) (Figure 3.7A, 3.7B, and 3.7D).

METHODS

Mice

All mice were bred, maintained, and treated in accordance with NIH and AAALAC guidelines and a protocol approved by an Institutional Animal Care and Use Committee (IACUC, University of Vermont, Burlington, VT). GFAP-CreER^{TM}:*EDNRB*$^{fl/fl}$ mice were bred and genotyped using a commercial kit (RED-Extract-N-Amp kit, Sigma), as described previously (LeComte et al., 2015). GFAP-CreER^{TM} mice express Cre recombinase under control of the human GFAP promoter; these mice undergo conditional knockout of *EDNRB* (gene sequence encoding ETB_R) upon intraperitoneal injection with tamoxifen (0.1 mg/g animal weight, dissolved in ethanol and corn-oil).

Distal middle cerebral artery occlusion (dMCAO) surgery.

Mice were anesthetized with an isoflurane/oxygen mixture (1-4%, to effect) and body temperature was maintained at 37°C using a water-circulated, heated pad (T/pump and pads, Gaymar). Incisions were made to uncover the lateral portion of the skull between the eye and ear. Using a drill, a rectangular portion of skull was then removed to reveal the distal middle cerebral artery. Nylon Suture (10-0, 12051-10, Fine Science Tools) was used to ligate and occlude the artery. To confirm a lack of blood flow, the artery was cut distal to the ligature. The wound was closed using Vetbond (3M) and pain was managed after surgery by Buprenorphine injections. Mice were monitored daily for any signs of pair or distress and normal patterns of feeding, drinking, nesting, and ambulation until euthanization.

CD109 treatment

At 8-12 weeks of age, dMCAO surgeries were performed as above on male C57BL/6J mice (Cat# 000664, Jackson Labs). At 24 hrs after stroke, mice were re-anesthetized with isoflurane. The chest of the mouse was shaved and sterilized to visualize the apex of the heart. For injection into the arterial circulation, 100 μl of vehicle (normal saline) or saline containing CD109 (10 μg) was slowly infused over 1 min into the heart (left ventricle lumen). Needle entry into the heart was confirmed by flash of blood before injection. After treatment, all mice were returned to the vivarium in individual cages and recovered for 6 d. At 7 days after stroke, mice were euthanized and transcardiac-perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS), and brains dissected for immunohistochemistry. Stroke size was determined by cresyl violet staining as described previously (Bakondi et al., 2009). The area of GFAP-reactivity in the thalamus was traced by a blinded observer and measured with software (ImageJ).

Generation of Reactive astrocyte-derived neural stem cells (Rad-NSC).

Whole brains were dissected 3 d after dMCAO and meninges were removed. Brains were cut coronally to visualize the stroke area and cortical material with infarction was separated from the rest of the brain. Cells were dissociated with a solution of Papain (20 U/mL, LS003126; Worthington), L-Cysteine (0.2 mg/mL, C7352; Sigma), DNase (20 μg/mL, Worthington), $CaCl_2$ (1.5 mM), and EDTA (1.5 mM) in DMEM-F12 base medium (Hyclone) for 20 minutes in a 37°C incubator at 5% CO_2. The papain digest was neutralized with soybean trypsin inhibitor (1.5 mg/mL, 17075-029; Gibco), and bovine serum albumin (BSA, 1.5 mg/mL, BP1605-100; Fisher) in DMEM-F12. The final cell solution was resuspended in neural stem cell medium (NSC medium): Neurobasal-A

(Gibco); human basic FGF (20 μg/mL, produced in-house); EGF (Gibco); 1 x B27 supplement (Gibco); 2 mM L-glutamine (Gibco); and 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). The cells were grown in NSC medium until initial neural sphere formation. After sphere formation, single spheres were pipetted into separate wells for clonal expansion and identification of clones with predictable Cre-mediated gene knockout. Clonal populations were maintained in NSC medium prior to differentiation and other assays.

Rad-NSC differentiation into reactive astrocytes

For studies in GFAP-CreER^{TM}-*EDNRB*-cKO astrocytes, cultured Rad-NSC were plated onto dishes previously coated with Laminin/Poly-d-Lysine (5 μg/ml each, Corning 354232 and 354210). Rad-NSC were differentiated astrocytes by 7 d incubation in DMEM-F12 base medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). For gene knockout, differentiated astrocytes were maintained in differentiation medium containing vehicle (EtOH) or 4-OH-Tamoxifen (1 μM, Tocris) for an additional 10 d. To generate conditioned medium, astrocytes were washed twice with PBS and then incubated in serum-free DMEM-F12 for 2 d.

Lentiviral shRNA knockdown

Lentiviral shRNA knockdown experiments were performed with a scrambled-sequence shRNA (control) or shRNA specific to CD109 (Mission Lentiviral particles, Sigma). Cultured Rad-NSC were infected over 3 d. Transduced cells were selected in NSC medium containing puromycin (2 μg/ml, MP Biomedicals). To generate knockdown

stem-cell lines, selection was performed for a minimum of 1 wk. Puromycin was withdrawn from the medium for at least 3 d prior to astrocyte differentiation.

Magnetic-Activated Cell Sorting (MACS)

At 3 d after dMCAO surgery, brains were dissected. Olfactory bulb, hindbrain, and meninges were removed and the hemispheres contralateral and ipsilateral to the stroke injury were reserved for separate cell isolations. Based on a protocol adapted from Foo et al. (2011), ipsilateral and contralateral cortices were digested separately in a 37℃ incubator at 5% CO_2 for 1 hr. The digestion solution contained Hanks Buffered Salt Solution (HBSS) supplemented with 30% D-Glucose, 1 M $NaHCO_3$, 50 mM EDTA, L-Cysteine 1 mg/mL, Sigma; DNase (20 μg/mL, Worthington), and Papain (20 U/mL, Worthington). Enzymatic activity of the resulting tissue digest was neutralized by 3 washes with an inhibitory "low" ovomucoid solution that was diluted to a 1:10 final concentration in HBSS containing 30% D-Glucose and 1 M $NaHCO_3$.

Low ovomucoid solution (10x stock): 0.2 mg/mL ovomucoid trypsin inhibitor, Worthington; 1.5% BSA, Miltenyi Biotech; all dissolved into 1 x Dulbucco's PBS, pH 7.4. Cells were triturated gently in the low ovomucoid solution and filtered to a single cell suspension (70 μm, Falcon). The low ovomucoid solution was underlaid with a "high" ovomucoid solution; this solution was similarly diluted at 1:10 in HBSS supplemented with 30% D-Glucose and 1 M $NaHCO_3$. High ovomucoid solution (10x stock): 3% BSA solution, Miltenyi; 0.0333 g/ml ovomucoid trypsin inhibitor, Worthington; all dissolved into 1x DPBS (pH 7.4). Once underlaid, cells were centrifuged at 300 x g for 7-minutes. Cell pellets were resuspended in 140 ul MACS buffer (1 x Miltenyi BSA solution in DPBS) containing 20 μl FcR blocking solution

(Miltenyi Biotech). Cells were incubated in FcR block for 10 min at 4°C. After the blocking step, 40 ul of biotinylated anti-GLAST antibody (Miltenyi Biotech) was added, and the solution was incubated for an additional 20 min at 4°C. Cells were washed with 5 ml of MACS buffer and then centrifuged at 300 x g for 7 min. The resulting cell pellet was re-suspended in 160 ul of MACS buffer. After the addition of 40 ul of paramagnetic-bead conjugated streptavidin (Miltenyi Biotec), the cells were incubated for 20 min at 4°C. Labeled cells were washed with 5 ml of MACS buffer, centrifuged (300 x g, 7 min), and re-suspended in 3 ml of MACS buffer. A MACS column (L/S column, Miltenyi Biotech) was primed with 3 ml of MACS buffer prior to loading on the labeled cell suspension. After applying the cell solution, the MACS column was thrice washed with MACS buffer (3 ml, each). $GLAST^+$ cells were eluted in 5 ml of MACS buffer, centrifuged at 300 x g for 7 min, re-suspended in 400 ul of DPBS and counted by hemocytometer. After quantification, the cells were collected by centrifugation (1,000 x g, 10 min). The final $GLAST^+$ cell pellet was lysed in RIPA buffer: 25 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 (w/vol), 0.5% Sodium Deoxycholate (w/vol), 0.1% SDS (w/vol), pH 7.4, supplemented with 1 x protease (Pierce) and phosphatase (Sigma) inhibitors.

Immunohistochemistry

Paraformaldehyde (PFA)-perfused brains were post-fixed (4% PFA in PBS) for 24 hrs and then incubated in PBS with 30% sucrose at 4°C until equilibration (sinking). The fixed brains were embedded in O.C.T. compound and frozen using an ethanol slurry on dry-ice. Sections were cut at 30 μm thickness, with 270 μm between each section. Tissue sections were stored at -80°C until staining procedures. For immunohistochemistry,

individual sections were thawed on a slide warmer for 20 min, washed with PBS, and blocked/permeabilized for 1 hr (PBS with 5% Goat Serum [vol/vol] and 0.5% Trition-X [vol/vol]). Fresh blocking buffer was used for primary antibody incubations (either overnight at 4℃ or for 2 hr at room temperature) and secondary antibody incubations (1:1000 dilutions of cross-adsorbed goat polyclonal antibodies, 1 hr at room temperature). The following primary antibodies were used: GFAP (Rabbit anti-GFAP, Z0334, DAKO; Mouse anti-GFAP, G3893, Sigma; Rat anti-GFAP, 13-0300, ThermoFisher), AQP4 (PA5-85767, ThermoFisher), CD109 (sc-271085, Santa Cruz), Iba1 (019-19741, WAKO), and CD68 (MCA1957GA, BioRad). Tissue sections were washed 3-times with PBS after primary antibody incubations. Secondary Alexa-Fluor polyclonal goat antibodies against mouse (A-11032 and A-11029, Thermofisher), rat (A-11007 and A-11006, ThermoFisher), and Rabbit (A-11037 and A-11034, ThermoFisher) were incubated at room temperature for 1-hour. Sections were then washed again 3-times with PBS, then mounted with DAPI-mount (Southern Biotech 010020). Mouse CD109 staining was performed using Vector Labs Mouse on Mouse kit (BMK-2202), according to manufacturer instructions.

Immunoblotting

The protein concentration of cell lysates in RIPA buffer was determined by BioRad-DC protein assay according to the manufacturer's instructions. Novex Bis-Tris gels (Invitrogen) were loaded for equal protein, or equal cell number in the case of GLAST MACS samples. Following electrophoresis, proteins were transferred to PVDF membranes (Millipore) and blocked for 1 hr in TBS-T buffer (20 mM Tris,150 mM NaCl, and 0.1%TWEEN 20) containing 5% BSA (w/vol). Primary antibodies were

diluted in the same blocking buffer and incubated overnight at 4℃. Washes were done with TBS-T and secondary antibodies conjugated to horse radish peroxidase were incubated in blocking buffer for 1 hr at room temperature. Primary antibodies were used against: CD109 (Rabbit Polyclonal BS-6067r, Bioss; Mouse Monoclonal, SC-281085, Santa Cruz), GFAP (Rabbit Polyclonal anti-GFAP Z0334, DAKO), β-actin (A5441, Sigma), and Complement C3d (Goat anti-C3d AF2655, R&D Systems).

Statistical analysis

Statistical analyses were performed with GraphPad Prism Software (Version 7.01). Groups were compared by Student's T-test and multiple comparisons were made by ANOVA. *P*-values of less than 0.05 were considered significant.

DISCUSSION

Among other markers, neurotoxic astrocytes (termed A1) were reported to express complement C3 and neuroprotective astrocytes (termed A2) were reported to express mRNA for CD109 (Liddelow et al., 2017; Clarke et al., 2018; Smith et al., 2020). Whereas several inflammatory factors and LPS were shown to induce a neurotoxic astroglial phenotype, the signaling environment or factors necessary to induce a neuroprotective astroglial state are less understood. Here we demonstrated that primary adult astrocytes express several forms of CD109 protein, both cleaved and uncleaved, and reactive astrocytes significantly increased CD109 in response to stroke.

Previously, we found that ETB_R signaling regulates a population of proliferative, $RC2^+$ reactive astrocytes in the peri-infarct area after stroke (LeComte et al., 2015). The $RC2^+$ astrocytes reduced immune cell invasion into the peri-infarct area, consistent with a neuroprotective phenotype (Shimada et al., 2011). Importantly, the results shown here

demonstrate astroglial ETB_R signaling regulates CD109 *in vivo* and, potentially, reactive astrocyte phenotype(s) after CNS injury. In the absence of stroke injury, astrocytes isolated from tamoxifen-treated GFAP-CreER^{TM}-*EDNRB*-cKO mice exhibited a "pre-reactive" or inflammatory state with increased GFAP and CD109 compared with cells from vehicle-treated mice. Notably, in contrast with cells from vehicle-treated control mice, $GLAST^+$ reactive astrocytes isolated from the cortices of GFAP-CreER^{TM}-*EDNRB*-cKO mice did not increase CD109 levels after stroke.

In agreement Liddelow et al. (2017), we observed that treatment of astrocytes with TNFα, an inducer of neurotoxic astrocytes, increased the level of full length complement C3. Notably, in the presence of TNFα, ETB_R-specific agonist (IRL1620) reduced complement C3 expression. Also, IRL1620 treatment significantly reduced the cleaved C3d form of complement; this form is critical in the progression of complement cascades and microglial cell activation. However, in TNFα-treated astrocytes with shRNA knockdown of CD109, IRL1620 did not affect C3d levels. These data indicate that CD109 may influence protease activity associated with the complement cascade during neuroinflammation, or complement itself. It is worth noting that CD109 and complement C3 share homology in several domains and are both cleaved by furin and related proteases (Hagiwara et al., 2010; Misumi et al., 1991).

In light of possible neuroprotective functions for CD109, we experimented with the soluble (free) form of CD109 as a potential therapeutic for ischemic stroke. Although not significant, at 7 d after stroke onset, our data trended toward an increase in stroke volume after treatment, rather than a decrease. This observation agrees with a recent paper from Song et al. (2019) demonstrating pro-inflammatory effects of CD109 in

rheumatoid arthritis. In their model, treatment with neutralizing antisera to CD109 was shown to improve both histological and behavioral measures of injury. It is important to note that astroglial membrane-bound forms of CD109, acting as co-receptors, may function differently compared with various cleaved forms that are released into the injury environment by protease activity. In this regard, the membrane-bound form of CD109 could promote neuroprotective outcomes, whereas the soluble (free) form could promote neuroinflammation with negative consequences.

Further characterization of the astroglial CD109 co-receptor, its interaction partners, and the function(s) of its membrane-bound and cleaved forms will help better define its role(s) in glial cell phenotype and neuroinflammation in the context of different CNS injuries and diseases. Direct sorting of $CD109^{high}$- and $CD109^{low}$-expressing astrocytes from different injury/disease systems could reveal if the cellular mRNA signature for CD109 truly correlates with neuroprotective function(s). Further studies of CD109 cleavage by furin, thrombin, mesotrypsin and other proteases will be essential to determining if CD109 cleavages occur in parallel to those for complement and/or alter the generation of neuroprotective and neurotoxic astrocytes.

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CHAPTER 3 FIGURES

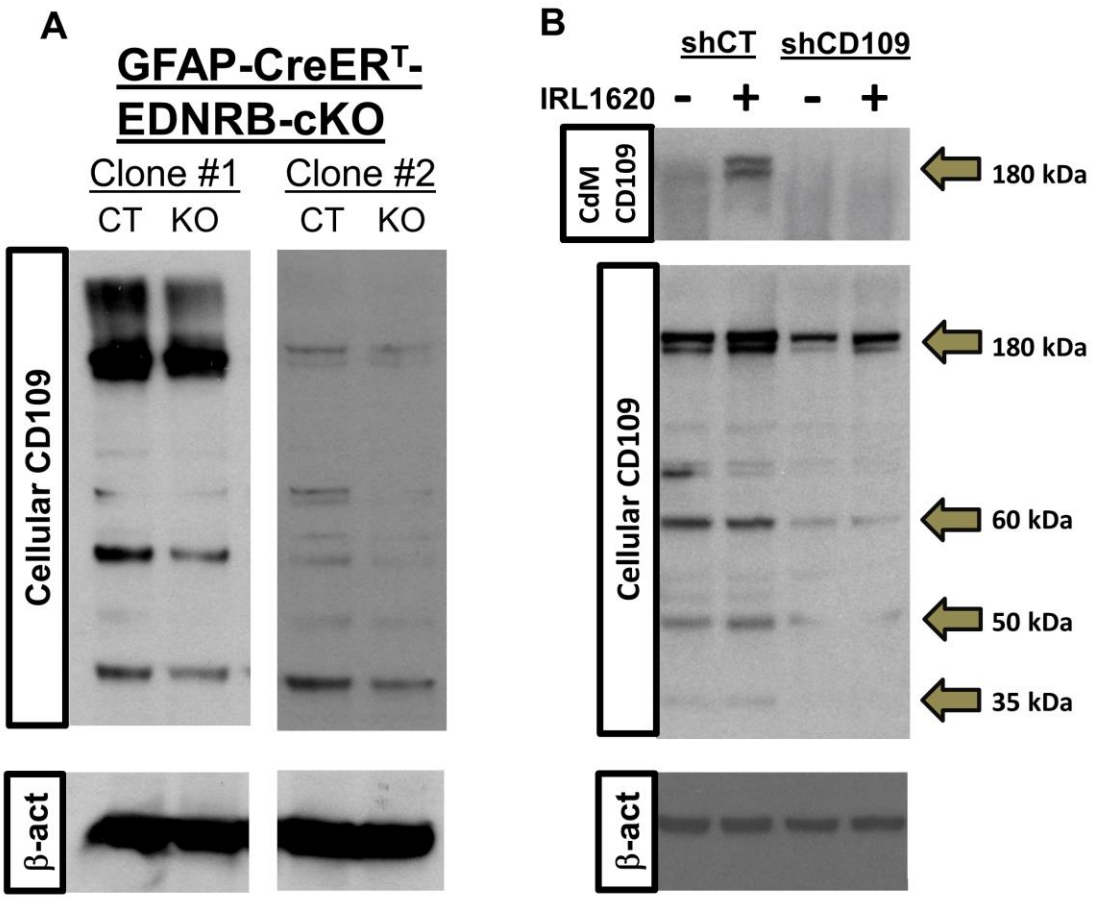
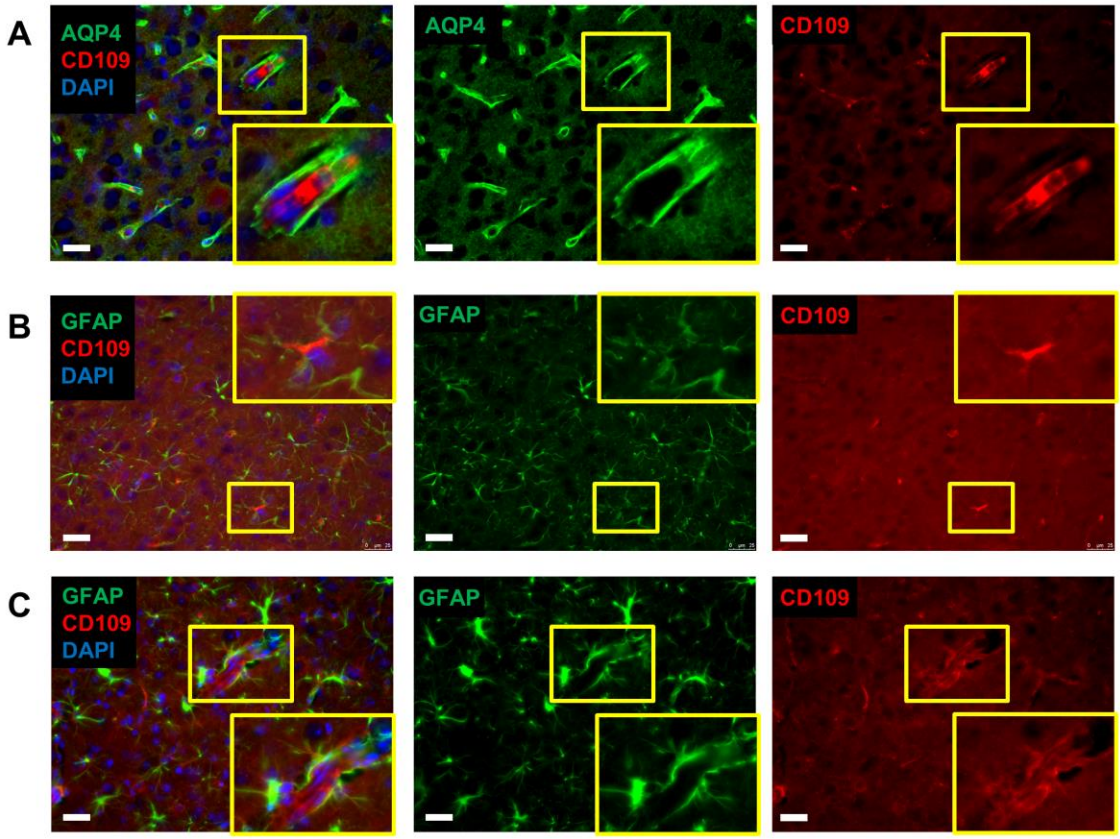


Figure 3. 2.

Immunoblotting of *EDNRB*-knockout and IRL1620 treated astrocytes demonstrates that ETB_R signaling controls multiple isoforms of CD109. **A)** Immunoblots of two Rad-NSC clones derived from GFAP-creER^{TM}-*EDNRB*-$^{fl/fl}$ mice, differentiated into astrocytes, and treated with ethanol-vehicle (CT) or 4-OH-tamoxifen (KO) to induce *EDNRB*-knockout. *EDNRB*-knockout reduced multiple cellular isoforms of CD109. **B)** Clonal Rad-NSC populations were split and transduced with lentiviral particles containing non-targeting, scrambled shRNA (shCT) and particles containing shRNA specific to CD109 (shCD109). Control and knockdown Rad-NSCs were differentiated into astrocytes, treated with an ETB_R-specific agonist (IRL1620), and astrocyte-conditioned medium (ACdM) and cell lysates were immunoblotted for CD109. IRL1620 treatment increased cellular and released CD109. shCD109 knockdown confirmed antisera specificity for multiple CD109 isoforms detected by immunoblot.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CD109
DAPI
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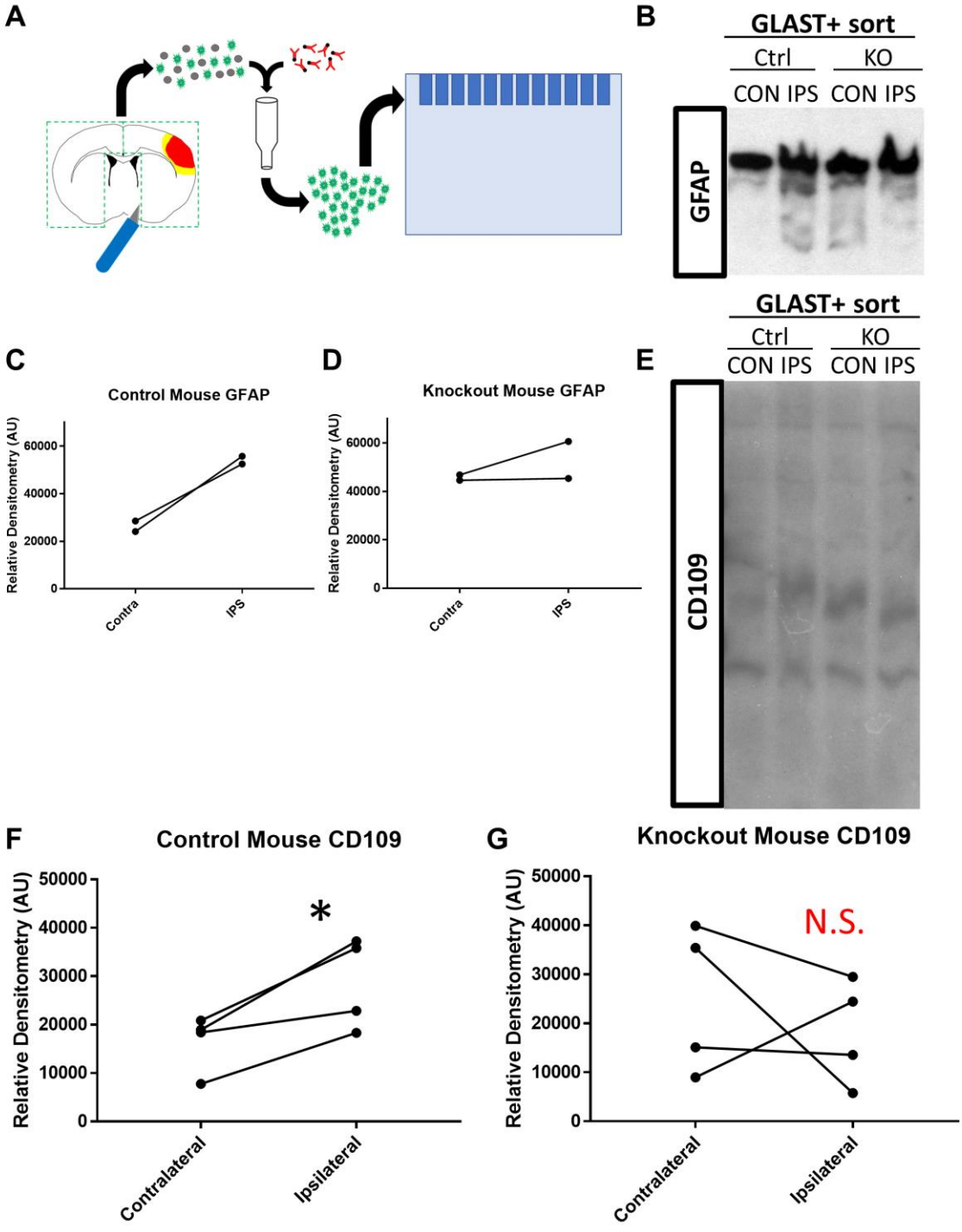
GFAP
CD109
DAPI
GFAP
CD109
C
GFAP
CD109
DAPI
GFAP
CD109

Figure 3. 3.

CD109 is predominantly expressed by astrocytes that contact the vasculature. **A)** Images of immunohistochemistry for astrocyte vascular contacts and endfeet by aquaporin-4 (AQP4) and CD109 in proximity to the ischemic infarct 3 d after dMCAO (Inset: 1.8x). **B)** Immunohistochemistry for GFAP and CD109, showing rare instance of a $GFAP^{+}/CD109^{+}$ astrocyte cell body with no visible vascular contacts (Inset 2.3x). **C)** Immunohistochemistry for GFAP and CD109 showing astrocytes contacting the vasculature in proximity to the infarct 3-days post-stroke (Inset: 1.55x). Scale bars = 25 μm.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Knockout Mouse CD109
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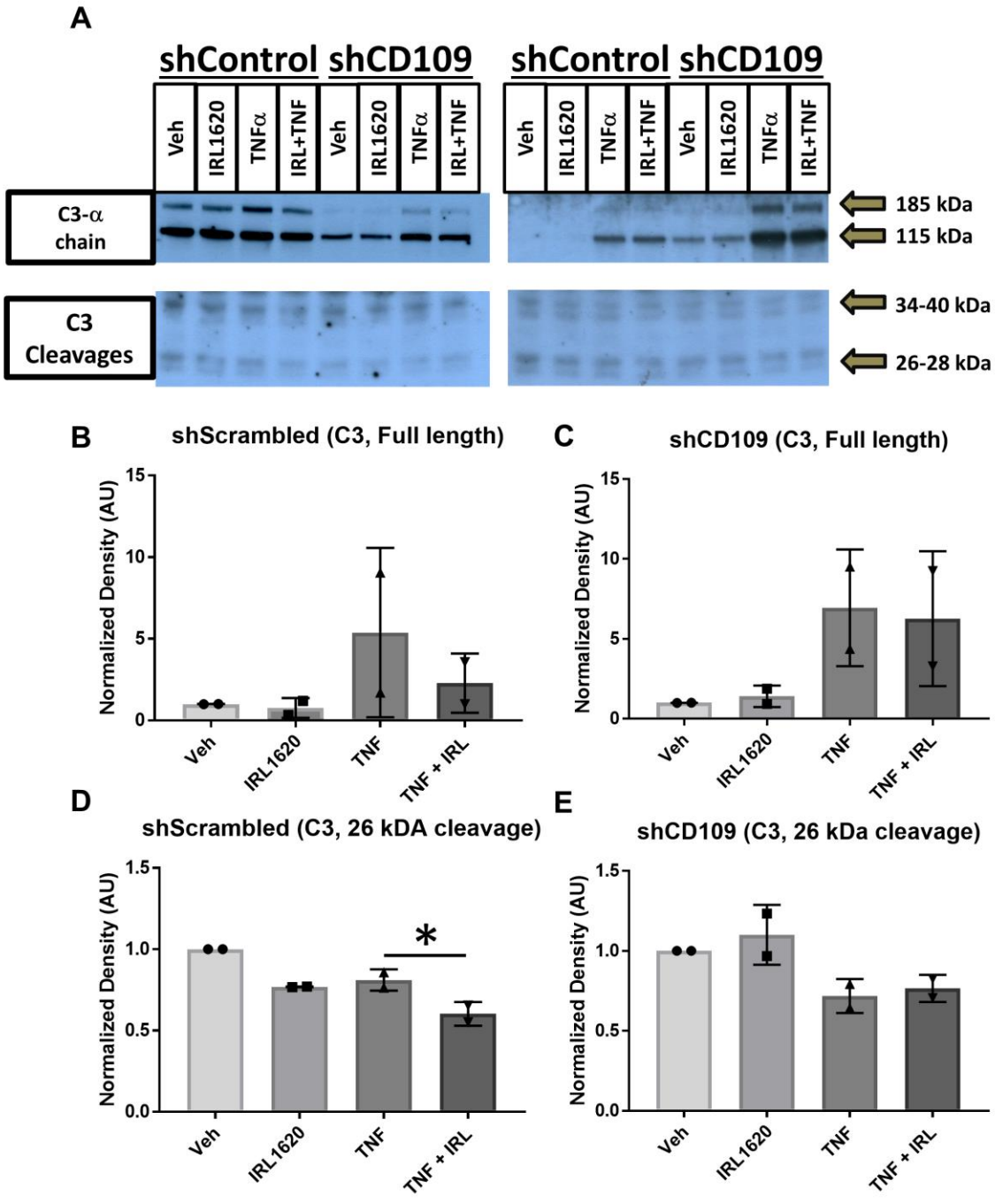
N.S.
Contralateral
Ipsilateral

Figure 3. 4.

Astrocytes increase CD109 expression during stroke and markers of inflammation in *EDNRB*-knockout mice. **A)** Schematic illustrating the GLAST-MACS protocol to isolate and immunoblot astrocytes from stroke (ipsilateral, IPS) and non-stroke (contralateral, CON) hemispheres of oil-control (Ctrl) and Tam-knockout (cKO) mice. **B)** Immunoblot showing GFAP expression by astrocytes isolated by GLAST-MACS. Cells were counted by hemocytometer **C)** Quantification of GFAP immunoblots from control mouse hemispheres contralateral and ipsilateral to stroke (n =2 for both groups). **D)** Quantification of GFAP immunoblots from astrocyte-specific *EDNRB*-knockout mouse hemispheres contralateral and ipsilateral to stroke (n =2 for both groups). **E)** Representative immunoblot of CD109 from GLAST-MACS astrocytes. **F)** Quantification of astrocyte CD109 (60 kDa band) in hemispheres contralateral and ipsilateral to stroke in control mice (Control contralateral mean: 16,488.52 ± 2,950.03; Control ipsilateral mean: 28,551.66 ± 4,703.08; Contralateral vs. Ipsilateral P = 0.03, n = 4 for both groups). **G)** Quantification of astrocyte CD109 (60 kDa band) in hemispheres contralateral and ipsilateral to stroke in astrocyte-specific *EDNRB*-knockout mice (Knockout contralateral mean: 24,837.52 ± 7,554.84; Knockout ipsilateral mean: 18,312.55 ± 5,343.51; Contralateral vs. Ipsilateral P = 0.54; n = 4 for both groups).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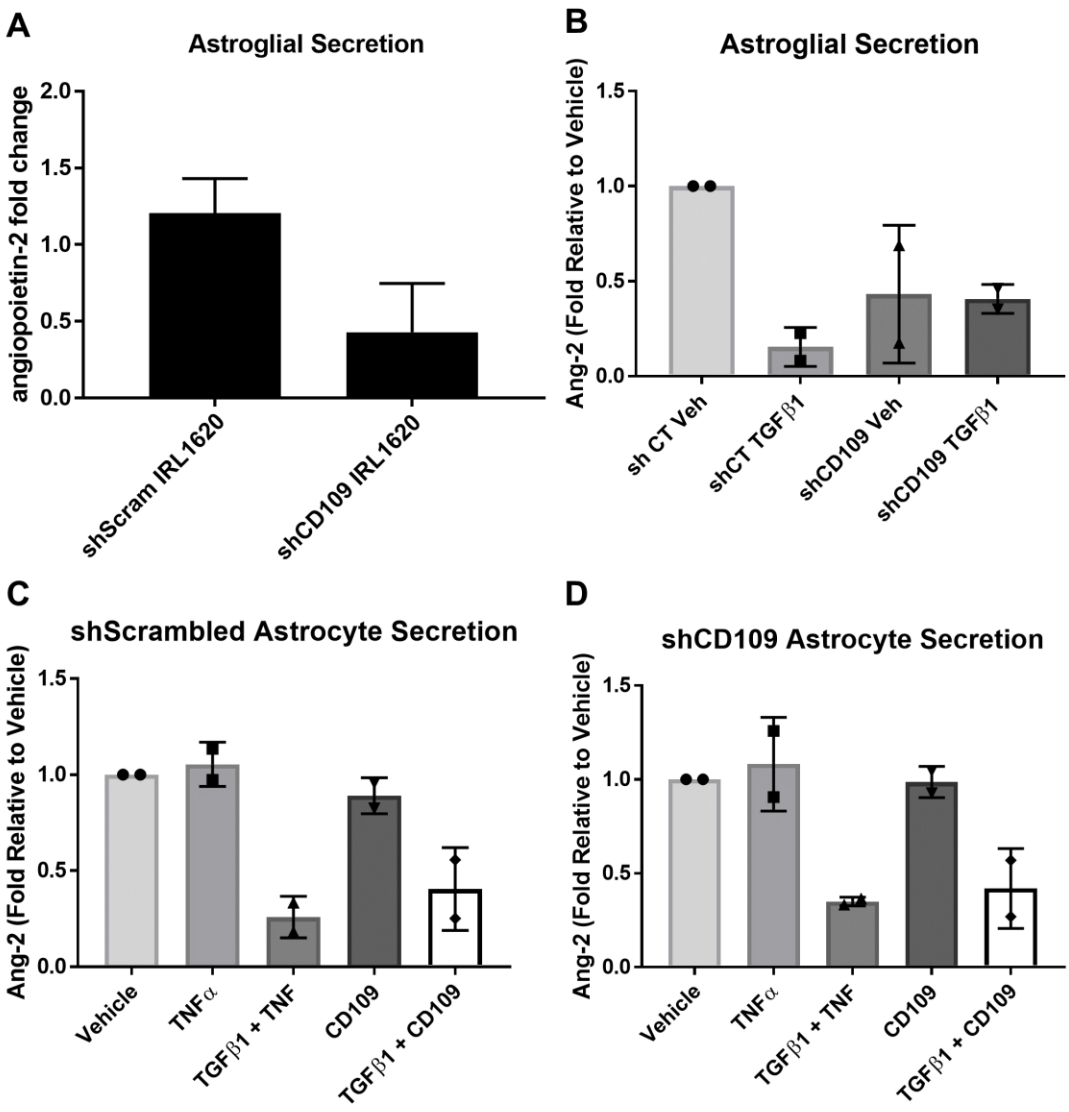
26-28 kDa
B
shScrambled (C3, Full length)
C
shCD109 (C3, Full length)
Normalized Density (AU)
Veh
IRL1620
TNF
TNF + IRL
D
shScrambled (C3, 26 kDA cleavage)
E
shCD109 (C3, 26 kDa cleavage)
*

Figure 3. 5.

ETB_R signaling interacts with TNFα and CD109 to alter expression of the neurotoxic marker, complement C3. **A)** Immunoblots from two different clones for complement C3 and cleavages containing the complement C3d sequence. Cells were plated at equal density, and blots represent equally loaded volumes of conditioned medium. **B)** Quantification of fold-change relative to vehicle for the 185 kDa isoform of C3 in treated shControl (shScrambled) cells. **C)** Quantification of fold-change relative to vehicle of the 185 kDa isoform of C3 in treated shCD109 cells. **D)** Quantification of fold-change relative to vehicle of the 26 kDa isoform of C3 in treated shControl cells, demonstrates that IRL1620 lowers C3 in the presence of TNFα (shCT cells TNFα-treatment: 0.81-fold ± 0.05 of vehicle treatment; shCT cells TNFα + IRL1620-treatment: 0.60-fold ± 0.05 of vehicle treatment; $n = 2$; $P < 0.05$). **E)** Quantification of fold-change relative to vehicle of the 26 kDa isoform of C3 in treated shCD109 cells, finds that IRL1620 did not lower C3d in the context of TNFα and CD109-knockdown (shCD109 cells TNFα-treatment: 0.71-fold ± 0.08 of vehicle-treatment; shCD109 cells TNFα + IRL1620 treatment: 0.77-fold ± 0.06 of vehicle treatment; $n = 2$; $P = 0.95$).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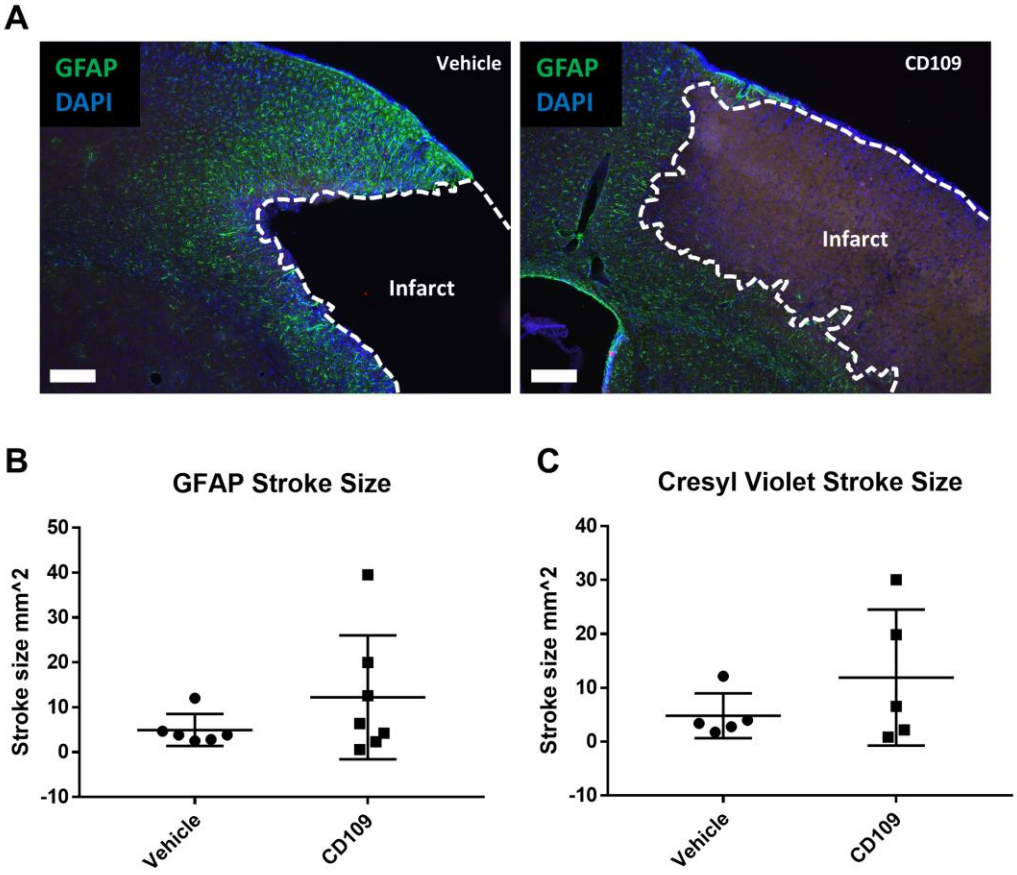
shScram IRL1620
shCD109 IRL1620
B
Astroglial Secretion
Ang-2 (Fold Relative to Vehicle)
1.5
1.0
0.5
0.0
sh CT Veh
shCT TGFβ1
shCD109 Veh
shCD109 TGFβ1
C
shScrambled Astrocyte Secretion
Ang-2 (Fold Relative to Vehicle)
1.5
1.0
0.5
0.0
Vehicle
TNFα
TGFβ1 + TNF
CD109
TGFβ1 + CD109
D
shCD109 Astrocyte Secretion
Ang-2 (Fold Relative to Vehicle)
1.5
1.0
0.5
0.0
Vehicle
TNFα
TGFβ1 + TNF
CD109
TGFβ1 + CD109

Figure 3. 6.

Knockdown of CD109 alters astroglial Ang-2 expression in response to IRL1620 and TGFβ1. **A)** ELISA quantification of Ang-2 in condition medium of control and CD109-knockdown astrocytes in response to IRL1620. **B)** ELISA quantification of Ang-2 in condition medium of control and CD109-knockdown astrocytes in response to TGFβ1. **C and D)** ELISA quantification of Ang-2 in condition medium of control and CD109-knockdown astrocytes in response to TNFα, TGFβ1, and CD109.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GFAP
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Vehicle
Infarct
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-10
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CD109
C
Cresyl Violet Stroke Size
Stroke size mm^2
40
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20
10
0
-10
Vehicle
CD109

Figure 3. 7.

At 7 d after stroke, infarct size increases in response to CD109 treatment as measured by GFAP-negative necrotic area and Cresyl violet staining for viable neural tissue. **A)** Immunohistochemistry for GFAP (green) and nuclear DAPI (blue), in mice treated with transcardiac injections of vehicle and CD109 24-hours after dMCAO surgery. Brains were harvested 7 d after dMCAO (scale bar = 250 μm). **B)** Measures of GFAP-negative necrotic stroke areas 7 d post-stroke in mice treated with vehicle and CD109 (Vehicle mean: 4.94 ± 1.46 mm^2; CD109 mean: 12.23 ± 5.21; n = 6 vehicle, n = 7 CD109; vehicle vs. CD109 P = 0.24). **C)** Measures of unstained Cresyl violet brain necrotic stroke areas 7 d post-stroke in mice treated with vehicle and CD109 (Vehicle mean: 4.81 ± 1.87 mm^2; CD109 mean: 11.91 ± 5.65; n = 6 vehicle, n = 7 CD109; vehicle vs. CD109 P = 0.27).

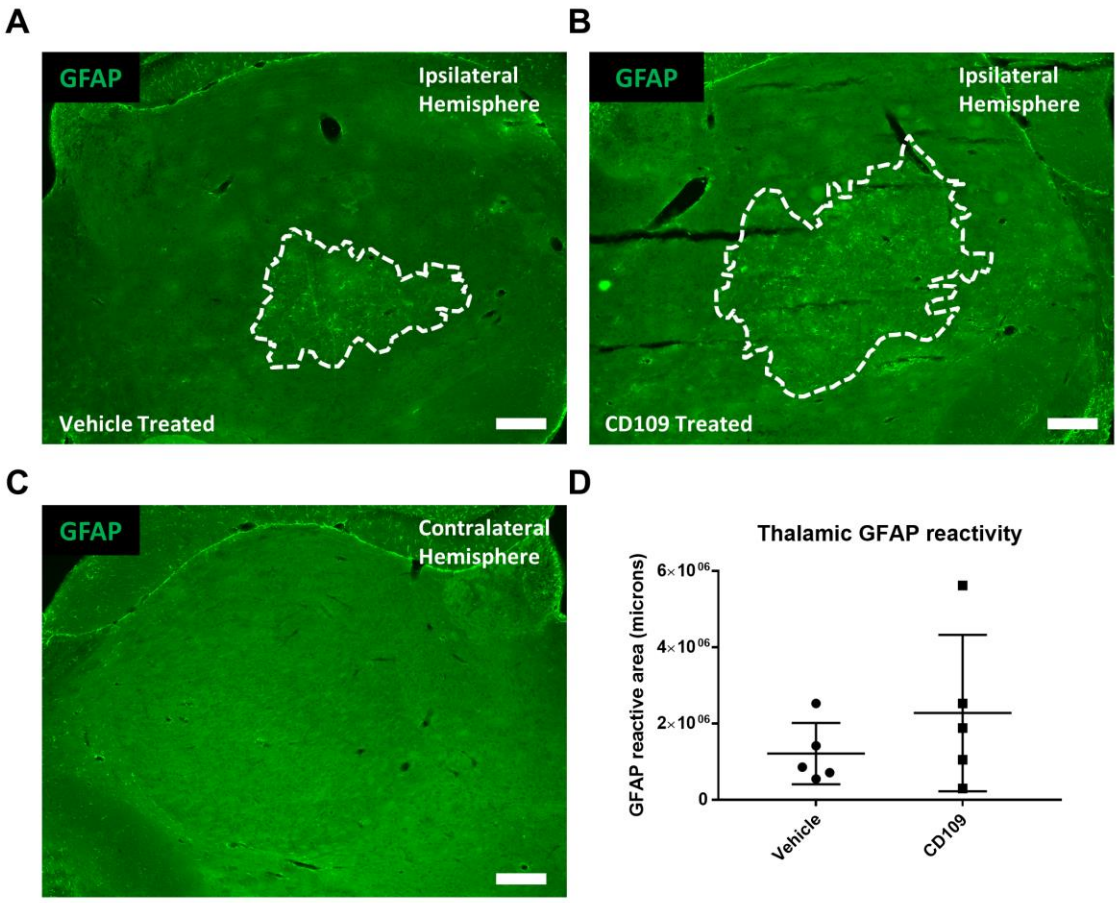
A
GFAP
Ipsilateral Hemisphere
Vehicle Treated
B
GFAP
Ipsilateral Hemisphere
CD109 Treated
C
GFAP
Contralateral Hemisphere
D
Thalamic GFAP reactivity
GFAP reactive area (microns)
6×10^06
4×10^06
2×10^06
0
Vehicle
CD109

Figure 3. 8.

Secondary $GFAP^+$ inflammation in the thalamus ipsilateral to stroke after CD109 treatment. **A)** Immunohistochemistry for GFAP (green) in the thalamus ipsilateral to stroke, in mice treated with transcardiac injections of vehicle and 24-hours after dMCAO surgery (scale bar = 250 μm). **B)** Representative 10x image of immunohistochemistry for GFAP (green) in the thalamus ipsilateral to stroke, in mice treated with transcardiac injections of CD109 (10 μg) and 24-hours after dMCAO surgery (scale bar = 250 μm). **C)** GFAP staining in the thalamus contralateral to the stroke. **D)** Measures of GFAP-reactive area 7-days post-stroke in mice treated with vehicle and CD109 (Vehicle mean: 1,212,520 ± 359,227 μm^2; CD109 mean: 2,276,554 ± 917,740; n = 5 for both groups; vehicle vs. CD109 P = 0.31).

CHAPTER 4: Conclusions and further directions

Rad-NSCs as a cell culture model

In multiple pathologies of the central nervous system, damage to neurons drives functional decline. Importantly, however, there is not significant neuronal regeneration in the adult CNS. Neuronal replacement is viewed as a promising therapeutic avenue and achieving significant survival and distribution of immature neural stem cells in the adult CNS is notoriously difficult. As such, transplantation of immature stem cells has had limited success in addressing neuronal loss in adult diseases and injuries. Furthermore, many diseases involve diffuse neuronal loss and stem cell replacement is better suited to localized transplantation. These hurdles have led the field to pursue adult stem cell-like sources that might improve transplantation or that could be targeted for direct *in vivo* conversion into neurons (Heinrich et al., 2014; Mattugini et al., 2019; Wu et al., 2020). In traumatic injuries to the CNS, multiple labs found that reactive astrocytes take on a stem cell-like phenotype and can be cultured as neural spheres (i.e. Rad-NSCs). Although they are multipotent for neurons, oligodendrocytes, and astrocytes in culture, Rad-NSCs are restricted to a glial fate when cultured and transplanted back into the brain. These cells have been transduced with pro-neural transcription factors *in vivo* and converted into neurons (Brulet et al., 2017; Mattugini et al., 2019). A drawback to this approach is that pro-neural transcription factors may only be able to reprogram in the presence of injury, even if the injury simply results from the needle injecting the pro-neural constructs. This indicates that neural conversion therapies could be restricted to localized traumatic injuries and might be minimally effective in diffuse neuroinflammatory diseases.

Difficulty in accessing injured brain areas for viral transduction will further challenge application of this therapy in humans.

With increased focus on the role(s) of glial cells in CNS injury and disease, the glial-restricted nature of Rad-NSCs may provide a valuable tool for disease modeling and treatment. For the experimental work of this dissertation, we harnessed the glial-restricted nature of Rad-NSCs to generate large numbers of astrocytes *ex vivo* and to model astroglial paracrine signaling post-stroke. This was advantageous over other models that are derived from immature animals, prone to contaminating non-astroglial cell types, are prohibitively expensive, and/or that generate too few viable cells. Recently developed embryonic and iPSC methods for generating differentiated neural cell types produce cells with immature phenotypes that don't recapitulate cellular phenotypes in adult disease (Miller et al., 2013). By contrast, our Rad-NSC system allowed us to generate large numbers of adult astocytes from transgenic animals and investigate critical glial signaling mechanisms in stroke.

It is worth noting some potential drawbacks to our approach. For example, our current differentiation protocol to generate astrocytes from Rad-NSCs involves exposure of the stem cells to fetal bovine serum. Exposure to serum alters the inflammatory profile of reactive astrocytes. In fact, serum exposure represents a significant drawback to many standard astrocyte culture methods. An important future direction for our laboratory and others will be to develop a Rad-NSC astrocyte differentiation protocol that uses defined factors without serum.

Having outlined these drawbacks, our culture model has proven reliable repeatedly. Our *in vitro* results match *in vivo* results out of our lab and multiple other

labs. Rad-NSC derived astrocytes do not proliferate significantly in culture after differentiation, whereas immature astrocytes do proliferate in culture after isolation. Therefore, Rad-NSC astrocytes more closely match the non-proliferative phenotype of resting adult astrocytes *in vivo*. Importantly, we found a significant increase in *Lcn2* gene expression (4.92-fold) in response to *EDNRB* knockout. In amyotrophic lateral sclerosis (ALS) models, LCN2 is an adult specific reactive astrocyte marker that was not expressed by immature reactive astrocytes *in vitro* or *in vivo* (Tripathi et al., 2017). Finding differences in *Lcn2* expression in our astrocytes indicates that our model may recapitulate adult reactive astrogliosis. Furthermore, results presented here showing *in vivo* Ang-2 expression changes and also changes in *Lcn2* gene expression demonstrate the value of the Rad-NSC platform in modeling reactive astrogliosis.

Astroglial gene expression changes in response to *EDNRB*-knockout

Assessing the essential paracrine factors released by adult reactive astrocytes after an injury is difficult due to problems associate with producing adult astrocytes in large numbers. Our Rad-NSC derived astrocytes enabled us to knockout *EDNRB*, a critical regulator of the reactive astroglial response to stroke, and to perform a microarray-based screen of control and *EDNRB*-KO astroctyes. Our goal was to identify reactive astroglial genes regulated by this pro-proliferative signaling pathway. It is worth noting that the reactive glial response likely results from an ensemble of damage-associated molecular signals. Our approach targeted ETB_R, elucidating a paracrine profile downstream of this particular endothelin signaling pathway. Future studies knocking out ETB_R in conjunction with other signaling receptors commonly activated after stroke (TNFR, Notch1, TGFβR,

Ptch), will be important for determining how the array of injury signals generated after stroke interact and promote specific reactive astrocyte phenotypes.

Our microarray data confirmed the efficacy of the GFAPcre^{ERT}-*EDNRB*-cKO Rad-NSC model with ~103-fold decrease in *EDNRB* mRNA in knockout cells (reduced ~103-fold; Figure 2.1). Consistent with our prior research, genes associated with proliferation and growth were also impacted. However, as we expected, there was not a simple relationship between the single signaling pathway we studied and single cellular function. ETB_R signaling regulates a reactive astroglial mRNA profile that includes, but is not limited to, cellular proliferation. Of the significant gene expression changes associated with cell-cell signaling, we found 3 broad gene categories that were disproportionately impacted: 1. Cell-cell adherence and guidance signals (*Cdh19, Sema3c, Spon1, Pcdhb2, Plxdc2, Glycam1, Cdon*); 2. Inflammatory signals, with members of a TGFβ-complement signaling axis particularly represented (*Ptger3, Tgfb3, Tgfbr3, C1s1, C1ra, Pla2g4a, Eda2r, CD109*); 3. Vascular signals that regulate endothelial growth and BBB permeability (*Angpt2*, *Renbp*, *Alcam*, *Serpinf1*). These signals are notable because several gene knockout mouse models that decrease reactive astrocyte proliferation also increase BBB permeability. Many of the genes that changed after *EDNRB*-knockout regulate BBB permeability and/or blood-cell extravasation. It is worth noting that a number of these genes have functions that significantly overlap in all three categories listed above. For example, genes associated with inflammation and injury such as those involved in TGFβ and TNFα signaling (e.g. *Eda2r, Tgfbr3, CD109, Tgfb3*) also regulate angiogenesis and the BBB. Furthermore, genes that regulate axon

guidance signaling such as plexin-semaphorin signaling (*Sema3c*, *Plxdc2*) may also control angiogenesis.

It is notable that proliferative astrocytes with a high level of ETB_R signaling, mirror many aspects of putative "neuroprotective" astrocytes that appear in traumatic injuries to the CNS. Stroke results in significant endothelin release, and our gene array data suggest that ETB_R-regulated genes are driving these some of these "neuroprotective" features. This paradigm is further illustrated by the number of changes relating to a TGFβ-complement-neurotoxicity signaling axis (*Tgfbr3*, *C1s1*, *C1ra*, *CD109*, *Eda2r*, *Tgfb3*) that has been described, in large part, by the labs of Beth Stevens and Ben Barres. The function of this axis is demonstrated in developing retinal ganglion cells that express complement C1 at their excess synapses in response to TGFβ3. Microglia detect the C1 expression and phagocytose the synapses (Bialas and Stevens, 2013). Like many other developmental signals, this paradigm is recapitulated during injuries to the adult nervous system and diseases such as complement-driven microglial synapse removal in Alzheimer's disease. Of interest, we found that ETB_R knockout significantly altered expression for a variety of genes linked to the TGFβ-complement signaling axis and increased the expression of many putative "neurotoxic" complement-related signals (*C1ra*, *C1s1*, *Tgfb3*). One explanation is that ETB_R signaling represses features of this neurotoxic axis and that removal of ETB_R signaling unleashes a neurotoxic astrocyte phenotype. Alternatively, there are multiple instances of ETB_R and ETA_R signaling having antagonistic effects. Removal of ETB_R may increase signaling of endothelins through ETA_R, thereby inducing neurotoxicity. The possibility that ETA_R signaling drives features of neurotoxic phenotypes in reactive astrocytes warrants further investigation.

One hypothesis is that mechanisms driving astroglial proliferation also promote neuroprotective glial phenotype. Our lab previously showed that Notch1 is necessary for astroglial proliferation and is upstream of ETB_R signaling. In many tissues, Notch1 acts a stem-cell regulator by maintaining self-renewal and multipotency. Endothelins direct neural crest cell migration and differentiation in development. While Notch is upstream of ETB_R and possibly neuroprotection, some research indicates instead that Notch is upstream of neurotoxic phenotypes (Qian et al., 2019). It is possible that Notch1 potentiates multiple reactive astrocyte phenotypes, and that ETB_R/ETA_R are amongst the receptors directing the type of astrocyte reactivity. Shh is another signal known to potentiate reactive astrocyte proliferation and a stem-cell like phenotype, but how Shh interacts with the better characterized Notch1-STAT3-ETB_R reactive astrocyte signaling pathway has not been elucidated (Sirko et al., 2013). For treatment of CNS injury, it will be important to characterize mechanisms that promote reactive astroglial proliferation and the specific factors that control neuroprotective or neurotoxic astroglial phenotypes.

Astroglial Ang-2 Expression

Infiltration of serum components and/or blood-cells after stroke are associated with neuronal cell death and increased infarct size. Reducing BBB permeability and inducing angiogenesis are essential to recovery after a stroke. With a portion of vascular distribution to the brain occluded and subsequent death of downstream vasculature, it is essential that collateralization and angiogenesis re-establish vascular supply to the infarct and peri-infarct areas. Ang-2 is critical to promoting post-stroke angiogenesis and restricting vascular permeability. Importantly, Ang-2 can act as an agonist or antagonist for Tie-2 depending on the context, including alterations in inflammatory status and

expression levels of Tie-1. There is some evidence that juvenile brains regenerate better after traumatic CNS injury, in part because of higher levels of Ang/Tie-2 signaling (Brickler et al., 2018). Ang-2 may act as a vaso-protectant in the acute phase after stroke, whereas VEGF promotes vascular permeability (Marteu et al., 2013). Unfortunately, part of the process of angiogenesis promoted by Ang-2 is the downregulation of tight junctions between endothelial cells and increased vascular permeability. These different facets of angiogenesis and Ang-2 signaling after stroke may account for conflicting studies regarding the beneficial nature of Ang-2 signaling after stroke (Gurnik et al., 2016). Also, there may be distinctions between the acute functions of Ang-2 after stroke versus stroke recovery.

Our work adds a layer of complexity and consideration to these observations. We show that astrocytes express Ang-2 acutely in response to endothelins and in response to stroke. Astrocytes, which are intimate participants in BBB regulation, may secrete Ang-2 as a part of acute stroke vascular protection. In stroke, the primary infarct area is devoid of astrocytes, has vascular disfunction, and blood cell infiltration (Shimada et al., 2011). It is possible that acute therapeutic delivery of Ang-2 into the primary infarct area is acting as a replacement for absent astroglial Ang-2 vaso-protection. It is also possible that astrocyte and endothelial cell-derived Ang-2 have different functions post-stroke that will require further research. Our data show microglial expression of the Ang-2 receptor Tie-2. Thus, any therapeutic interventions altering Ang-2 or Tie-2 signaling will need to account for possible changes in microglial activity. As therapies promoting angiogenesis and protection of the vasculature progress, it will be important to account for potentially different effects of Ang-2 signaling through microglial or endothelial Tie-2 receptors.

We have established the control of astroglial Ang-2 expression by ETB_R, but an important next step is to determine the function of Ang-2/Tie-2 signaling in microglia *in vivo* after stroke. Two specific mouse genetic models that would allow us to begin characterizing this mechanism. The first would be a GFAP-cre^{ERT}-*Angpt2*-cKO mouse, where Ang-2 secretion by astrocytes could be specifically removed prior to performing the dMCAO surgery. The second genetic approach would be to use TMEM119-cre^{ERT}-*Tie2*-cKO mice to specifically remove the Tie-2 receptor from microglial cells prior to dMCAO surgery. The necessary mice for these crosses recently became commercially available (TMEMcre^{ERT}) or have been generated by academic labs (*Tie2*-flox). Tamoxifen induction of the gene knockouts can be performed in adult mice prior to stroke, thus avoiding developmental compensatory effects.

Immunohistochemistry and FACS for microglia, using TMEM119 as a microglial-specific marker, could be used to assess microglial numbers, phenotype, and distribution after stroke in the presence or absence of astroglial Ang-2 or microglial Tie-2. These two genetic models may help to determine the mechanistic effects of Ang-2/Tie-2 signaling on microglial survival *in vivo* and confirm some of our *in vitro* data. Any differences in microglial survival and inflammation between the astroglial Ang-2 knockout and microglial Tie-2 knockout could indicate the presence of other sources of Ang-2 that alter microglial activity. These differences could also help to clarify Ang-2 specific effects on microglia, as opposed to the effects of other Tie-2 ligands such as Ang-1. Apart from characterizing post-stroke inflammation, both mouse models could address questions regarding astroglial Ang-2 and microglial Tie-2 signaling in vascular and microglial development.

Astroglial CD109

Increased CD109 mRNA in reactive astrocytes is observed in several CNS disorders and CD109 is reported as a marker of neuroprotective reactive astrocytes. Complement C3, which often used as a marker of neurotoxic astrocytes, is under study in many neurological disorders. The function of CD109 is poorly understood. In fact, to our knowledge there are no mechanistic studies of CD109 in the brain. If CD109 is truly a reactive gliosis marker and a marker of certain cancers, more work is needed to determine its exact function within the brain.

Two functions for CD109 described outside of the nervous system make it essential to study it the CNS. Epidermal CD109 was shown to alter TGFβ signaling through downstream SMADs. Latent TGFβ activation is an important signaling event that triggers astrocytes to form a glial scar (Schachtrup et al., 2010; Zhang et al., 2018). This functions to protect CNS tissue from injury, and modulation of TGFβ signaling may affect the progression of CNS disease. TGFβ-induced astroglial scar formation occurs in the presence of high astroglial CD109 expression, but the function of CD109 in glial scar formation remains unknown. The second potential function of CD109 relates to its complement system activity. CD109 has homology with other complement proteins that include complement C3. Complement proteins tend to interact with one another, promoting or inhibiting the complement cleavage cascade. Complements have essential functions in synapse elimination and neurotoxicity, so understanding if CD109 modulates complement activity will be important for ascertaining its potential neuroprotective functions.

We show that ETB_R signaling promotes increased astroglial CD109 expression. Thus, if CD109 is a marker of neuroprotective astroglia, ETB_R signaling acts as a potent activator of neuroprotection. This is consistent with our microarray data showing that ETB_R knockout lowers *CD109* and that there is a corresponding rise in the highly neurotoxic factor *Lcn2* (Tripathi et al., 2017). Also, if astrocyte-derived Ang-2 is an ETB_R-induced vascular-stability signal after stroke, this adds evidence suggesting ETB_R induces a vasoprotective astrocyte phenotype. ETB_R is part of a STAT3 signaling axis which is protective in spinal cord injury and amyotrophic lateral sclerosis (Anderson et al., 2016; Tyzack et al., 2017). Future studies to determine if CD109 is linked to STAT3 signaling will be important to define functions of CD109 in neuroprotection.

Paracrine signals are essential to neurotoxic/neuroprotective phenotypes, and CD109 knockdown by shRNA significantly decreased astroglial Ang-2 secretion. This is consistent with studies of rheumatoid arthritis, where blocking CD109 alters inflammation and improves histological outcomes. This indicates that CD109 alters inflammatory activity in multiple tissues.

To specifically address questions regarding CD109-mediated neuroprotection, more specific tools and methods will be necessary. Unfortunately, there is currently no mouse model with a floxed CD109 gene. A GFAP-cre^{ERT}-*CD109*-cKO mouse would allow for astroglial-specific knockout of CD109. Astrocyte CD109-cKO in uninjured, stroked, and lipopolysaccharide injected mouse models could help to clarify the function(s) of CD109 in reactive gliosis. The specific CD109 cleavage patterns that we identified are of great interest. Mutagenic studies using CRISPR or similar methods could be used to generate Rad-NSCs or iPSC-derived astrocytes with uncleavable CD109.

Alteration of astroglial phenotype and paracrine secretion in uncleavable CD109 astrocytes may reveal the functional consequences of this cleavage. It's particularly important to assess the neuronal toxicity of secreted factors from astrocytes with CD109 knockdown or uncleavable CD109.

Overview

There has been significant progression in our understanding of glial cells, glial scarring, and CNS injury. The historic view of the glial scar was that it inhibits axonal growth and is therefore inhibitory to CNS generation. However, the glial scar is now widely recognized as a mix of pro and anti-axonal growth factors, with functions essential to protecting CNS tissue from further damage. Astrocytes take on a stem cell-like phenotype in response to CNS injuries as they contribute to the formation of the glial scar. There have been many attempts to harness this stem cell-like nature to regenerate neurons or to transplant neuronally-fated stem cells, but with limited positive outcomes. In our approach, we take lessons from other stem cell transplantation attempts, particularly those utilizing mesenchymal stem cells. The factors secreted from these cells and their derivatives are often the source of their therapeutic benefits, rather than engraftment and cellular replacement. Secreted factors provide a more controllable therapeutic, in part, because they can be produced consistently in recombinant form. Additionally, stem cell therapies suffer from significant variability, host anti-graft reactions, and often high costs.

Reactive astrocytes secrete beneficial (neuroprotective) and/or harmful (neurotoxic) paracrine factors, but the features of the injury environment that influence the choice between neuroprotective and neurotoxic secreting astrocytes have not been

determined. Data from spinal cord injury and neurotoxic inflammatory models indicate that multiple secreted factors act as an ensemble to produce particular astroglial phenotypes. Determining the ensemble of factors and cell-autonomous regulators involved in promoting neuroprotection or neurotoxic astroglial phenotypes has significant implications for treating disease. Therapeutics altering the activity of singular signaling molecules have provided significant benefit to patients with diseases such as arthritis and Crohn's disease, but have shown less benefit in stroke. Inducing a neuroprotective astrocyte phenotype would alter an entire paracrine profile, rather than a singular paracrine factor, and could provide significant benefit to a variety of diseases of the central nervous system.

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