The Role of EphB3 in Cortical Vascular Integrity Following Traumatic Brain Injury

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THE ROLE OF EPHB3 IN CORTICAL VASCULAR INTEGRITY FOLLOWING
TRAUMATIC BRAIN INJURY

By
Poincyane Assis-Nascimento

A DISSERTATION

Submitted to the Faculty
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the requirements for the degree of
Doctor of Philosophy

THE ROLE OF EPHB3 IN VASCULAR STABILITY AFTER TRAUMATIC BRAIN INJURY

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Traumatic brain injury (TBI) continues to be a major health concern worldwide. One of the earliest and most profound deficits comes from vascular damage and breakdown of the blood-brain barrier (BBB). Despite the consequences of blood vessel destruction, little is known of the cellular responses that occur in vessel integrity following TBI. B-class ephrins and Eph receptors are membrane bound proteins that initiate bidirectional signals during cell-cell interactions, and are indispensable for developmental and pathological neovascularization. The role of EphB3 and its ligand ephrinB3 was investigated in vascular integrity in a murine controlled cortical impact (CCI) injury model of TBI. We identified a novel pro-apoptotic role for EphB3 in cerebral vascular endothelial cells (cvECs) that lead to increased BBB permeability and immune cell infiltration after CCI injury. In addition, EphB3 also regulated the degree of cvEC-astrocytic end-feet membrane association in the gliovascular unit, as well as the expression of the endothelial intercellular junction proteins ZO-1 and VE-cadherin. In short, our findings demonstrate that blocking EphB3 receptor signaling in the cerebral vasculature increases cvEC survival and reduces BBB permeability representing a potentially important therapeutic strategy for vessel repair and/or stability following TBI.
Dedication

This final project is dedicated to my husband, Uilson Nascimento Jr., to my two children, Matheus and Isabella Nascimento, and to my mother, Mellyzye Aoas, with gratitude for their unending love, faith-filled prayers, moral and emotional support, courage-infusing tolerance and uplifting inspiration throughout my study.
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**Publication Note**

The majority of the work included in Chapters 2 and 5 was modified from a previously published peer-reviewed original basic science manuscript:


The work in Chapters 3 and 4 was modified from a manuscript in progress. Working title: EphB3 has a detrimental role in cerebral vascular endothelial cell survival and blood-brain barrier integrity after CCI injury in mice.

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Chapter 1

Introduction

1.1 The Vascular System

1.1.1 Blood Vessel Development

Vessel development is an early event in embryogenesis starting during the third week of gestation in humans and approximately embryonic (E) day 7 in mice (Plate, 1999). The formation of the vascular network is a highly elaborate process and is an obligatory requirement during all vertebrate development. During embryogenesis, a layer of the paraxial mesoderm attaches to the nervous tissue-deriving ectoderm layer, forming the splanchnopleuric mesoderm. Brain endothelial cells from hematopoietic and angiogenic lineages are derived from this layer, following cues from factors released by the neural tube, and giving rise to the initial blood islands (Ema and Rossant, 2003). The first organs to be formed during mammalian development are the blood vessels and heart, and even as the organs function, the vasculature continues to be dynamically remodeled (Chung and Ferrara, 2011). In the central nervous system (CNS), vessel development occurs in two stages: first, the initial vascularization of the embryonic CNS relies on the process of vasculogenesis, when angioblasts (endothelial cell precursors) unite and undergo a complex remodeling process to form a functional primitive vasculature network. This initial process takes place in the so-called peri-neural vascular plexus (PNVP) and gives rise to extra-cerebral vessels. Then, via inward sprouting, new vessel branches originating from the primary capillary plexus, a process termed angiogenesis, invade the neural tube to establish the intra-cerebral vascular network until the entire brain is fully vascularized (Quaegebeur et al., 2011).
1.1.2 VEGF is a Key Molecule in Vascular Development.

Vascular endothelial growth factor (VEGF) and its receptors are essential regulators of vessel sprouting and permeability. To date the VEGF family comprises six different ligands in mammals, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF)-1, which bind to three different cell surface receptor tyrosine kinases, VEGFR-1, VEGFR-2 and VEGFR-3 (Lange et al., 2016). Of the different ligands, VEGF-A or simply VEGF is the most potent inducer of blood vessel growth for developmental and adult organ remodeling and vascular pathologies. As a consequence of alternative splicing, VEGF is synthesized as three major isoforms named according to the number of consisting amino acids. In humans, these include VEGF-121, VEGF-165 and VEGF-189, whereas the corresponding mouse isoforms are termed VEGF-120, VEGF-164 and VEGF-188, as each isoform consist of one less amino acid (Mackenzie and Ruhrberg, 2012). VEGF-165, a 46 kDa homodimer, is the predominant isoform containing one heparin-binding domain, which makes it partly matrix-bound and partly diffusible. In addition to VEGFR-1 and VEGFR-2, VEGF-165 also binds the co-receptors NRP-1 (neuropilin-1) and NRP-2 (neuropilin-2), which enhance VEGF receptor signaling (Takahashi and Shibuya, 2005). VEGF has indispensible roles in stimulating endothelial cell proliferation, migration and increasing vascular permeability. Additionally, VEGF family members have also been implicated in the formation of lymphatic vessels, monocyte recruitment, hematopoiesis, as well as in the proliferation and survival of non-vascular cells, including neuronal progenitors that express the corresponding VEGF receptors (Lange et al., 2016). During development VEGF signaling is critical for the expansion of angioblasts that combine to form the initial
vessels via vasculogenesis, such as the dorsal aorta. Genetic deletion of even one copy of VEGF is embryonic-lethal because of lack of vessel development. The blood vessels in these mutants appear disorganized; the endothelial cells undergo delayed differentiation and disrupted hematopoietic cell formation (Carmeliet et al., 1996, Ferrara et al., 1996). In addition to its role in vasculogenesis, VEGF is also required for subsequent endothelial cell sprouting and migration that leads to the expansion and patterning of the vessel network via angiogenesis (Mackenzie and Ruhrberg, 2012).

1.1.3 Vessel Branching and Maturation

The process of angiogenesis or vascular collateral growth begins at around gestational week 4 in humans and continues to occur normally in the human body at specific times of development and growth. In mice, angiogenic vessel sprouting starts around E9.5 - E10 and continues after birth until approximately postnatal (P) day 20 (Plate, 1999). Proliferation of new blood vessels also takes place in adults, although it is a relatively infrequent event. In women, angiogenesis is active for a few days each month as new blood vessels form in the lining of the uterus during the menstrual cycle. More importantly, angiogenesis is necessary for the repair or regeneration of tissue during wound healing and after injury (DiPietro, 2016). A larger body of evidence for pathological angiogenesis comes from the cancer field. Tumor angiogenesis begins with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue. This results in the proliferation of a network of blood vessels that penetrate into cancerous growths, activating specific cancer related genes in the host and stimulating protein synthesis, which result in growth and sprouting of new blood vessels (Carmeliet and Jain, 2011). In addition, angiogenesis also occurs in the adult brain under
pathological conditions such as stroke, ischemia and traumatic brain injury (TBI) (Welser et al., 2010). Angiogenesis involves an intricate process of vessel branching and maturation, which relies on a coordinated collective migration of cvECs. One cell, referred to as the endothelial tip cell, takes the lead to guide the proliferating stalk cells that elongate the growing sprout. Radial glia secrete factors such as VEGF and Wnt to facilitate guidance and growth of the angiogenic sprout. The tip cell is exposed to the highest levels of VEGF allowing for signaling by the VEGF receptor VEGFR-2 (KDR/Flk-1), a tyrosine kinase receptor that regulates physiological as well as pathological angiogenesis (Shibuya, 2006). VEGFR-2 signaling instructs the tip cell to extend numerous filopodia to explore the environment and guide the branch toward the source of proangiogenic factors (Quaegebeur et al., 2011). To become mature, fully functional quiescent cells, and stabilize endothelial cell channels, angiogenic cvECs release platelet derived growth factor (PDGF)-B to chemoattract PDGF receptor-β (PDGFR-β)+ pericytes. Pericytes in turn secrete angiopoietin-1 (Ang-1) that binds to the endothelial specific Tie-2 receptors to promote cvEC survival, cell-cell adhesion, and pericyte coverage (Augustin et al., 2009, Quaegebeur et al., 2011).

1.2 The Blood-Brain Barrier (BBB)

1.2.1 The Neurovascular and Gliovascular Units

The BBB is a selectively permeable barrier in the CNS composed of cvECs that form tight junctions and interact with other vascular cells, including pericytes and astrocytes (Abbott et al., 2010). Although supported by other cells, endothelial cells making up the inner lumen of all vessels in the brain are the major component of the BBB. These highly
specialized cvECs possess unique features that differ from endothelial cells in the periphery. Since the characteristics of brain endothelium are induced by organ-specific local signals, the fundamental differences of cvECs are primarily due to their ability to regulate exchanges between the blood and brain parenchyma and to establish the BBB. Due to their high metabolic activity, cvECs have more mitochondria, while lacking pores or fenestrations and fewer caveolae (specialized lipid rafts) that restrict pinocytosis. Importantly, cvECs have specialized tight junction proteins that are found in the basement membrane and control the transfer of substances between cells, a process known as paracellular transport (Prakash and Carmichael, 2015, Wilhelm et al., 2016).

The primary extra-cerebral vessels have layers of smooth muscle to regulate blood flow whereas most of the intra-cerebral microvessels and capillaries are made up solely of cvECs wrapped by pericytes and astrocytic end-feet. Endothelial cells and pericytes are very closely related and share a common basement membrane, a 30 to 40 nm thick lamina composed of collagen type IV, heparin sulfate proteoglycans (HSP), laminin, fibronectin, and other extracellular matrix (ECM) proteins (Daneman and Prat, 2015). Pericytes play essential roles in the survival and quiescence of cvECs and have also been shown to regulate blood flow in these cerebral microvessels (Hamilton et al., 2010). The plasma membranes of astrocytic end-feet are also closely adjacent to the basement membrane and together with neurons, they form a structure referred to as the neurovascular unit (NVU), which are involved in the regulation of cerebral blood flow (McCarty, 2009). Within the NVU organization, further segmental structure can be identified involving single astrocytic glial cells and the neurons they surround, interacting with associated segments of microvessels. This structure is referred to as the gliovascular
unit (GVU) and is capable of regulating blood flow at the arteriolar level as well as BBB functions down to the level of cerebral capillaries (Abbott et al., 2006, Wolburg et al., 2009a, Wolburg et al., 2009b). Both pericytes and astrocytes have been shown to directly interact with cerebral blood vessels (Correale and Villa, 2009, Wolburg et al., 2009a, Armulik et al., 2010). This close perivascular association is important for the modulation of BBB differentiation and maintenance of functions. Some of these features include tight junction adherence (Dehouck et al., 1990, Rubin et al., 1991), expression of Glut-1 glucose transporters (McAllister et al., 2001), differentiated cell transport mechanisms (Abbott et al., 2006), regulation of specialized cerebrovascular enzyme systems (Hayashi et al., 1997, Sobue et al., 1999, Haseloff et al., 2005) and constituting a true gliovascular unit (Wolburg et al., 2009a), with glial and cvECs functionally interacting in a paracrine manner.

1.2.2 Microvascular Association with Pericytes and Astrocytic End-Feet

From the time of development and all throughout adulthood, perivascular pericytes and the end-feets of astrocytes are closely associated with microvessel walls. The distance between a pericyte or an astrocytic end-foot to the capillary endothelium is only about 20 nm (Pardridge, 2005), which allows for regulatory interactions and exchange of factors either directly or through the basal membrane. The ratio of pericytes to cvECs is 3:1 covering approximately 30% of the abluminal surface of cerebral microvascular endothelium (Armulik et al., 2011), while astrocytic end-feet cover about 90%, overlapping areas already ensheathed by pericytes (Correale and Villa, 2009). Together with neuronal input, these two cells regulate cerebral blood flow and wrap nearly the entire surface of capillaries ensuring a tight seal for proper BBB permeability. Both
pericytes and astrocytes have specialized features that are characteristic of their location and functions. Perivascular astrocytic end-feet have a high density of orthogonal arrays of particles (OAPs) containing the water channel aquaporin 4 (AQP4) and the Kir4.1 K\(^+\) channels (Abbott et al., 2006). These specialized channels are involved in ion and volume regulation and their localized expression correlates with the expression of the protein agrin, which serves as an anchor in the basal lamina (Verkman, 2002, Wolburg and Lippoldt, 2002). Agrin has been shown to accumulate in brain microvessels at the time of BBB tightening (Barber and Lieth, 1997, Wolburg et al., 2009a, Wolburg-Buchholz et al., 2009), making the association between cvECs and astrocytic end-feet processes through this anchoring protein important for the integrity of the BBB. Astrocytes are also able to secrete a number of agents including transforming growth factor-β (TGFβ), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF) and angiopoietin 1 (ANG-1) (Abbott et al., 2006, Abbott et al., 2012). Studies in vitro have shown that ANG-1 binding to the endothelial cell-specific receptor tyrosine kinase (TIE)-2 can induce BBB-like phenotype in cultured cvECs (Lee et al., 2003). In vivo, endothelial TIE-2 receptor activation is also mediated by either astrocytic- or pericytic-derived ANG-1 and participates in the regulation of BBB tight junction formation and angiogenesis (Quaegebeur et al., 2011). Pericytes play essential roles in this regulation by organizing the perivascular complex through the stabilization of the vessel wall, regulation of EC survival and growth, and BBB permeability (Hellstrom et al., 2001, Armulik et al., 2005). These specialized cells extend long, finger-like projections that ensheath the endothelium capillary wall. Pericytes have their cell bodies and cytoplasmic processes enveloped by the same basal lamina as cvECs, except for where they make
direct contacts with each other (Diaz-Flores et al., 1991). In these areas, where the basement membrane is lacking, pericytes make direct peg-and-socket contacts with cvEC membranes containing cell-cell junction proteins including N-Cadherin and VE-Cadherin (adherens junction defined in section 1.2.4). Peg-and-socket connections also consist of connexin-43 (CX43) hemi-channels that form gap junctions as well as integrins. Integrins mediate the attachment of both pericytes and cvECs to the ECM of the basement membrane (Stratman et al., 2009), whereas gap junctions allow for the transfer of nutrients and ions between the two cell types (Bobbie et al., 2010). In addition to pericytes and cvECs, astrocytic processes also express integrins and connexins that help guide and stabilize the membrane attachments and support the communications in the astrocytic network (Tanigami et al., 2012). Both gliovascular and neuroglial gap junctions are contributed by CX43 and CX30 (Chew et al., 2010). Pericytic-endothelial membrane associations are also accomplished by adhesion plaques, which are mainly composed of fibronectin. These adhesion plaques facilitate the link between the basement and plasma membranes as well as the underlying actin cytoskeletal networks of pericytes and cvECs, which contribute to BBB permeability (Diaz-Flores et al., 2009).

1.2.3 Transport Across the Blood-Brain Barrier

Brain capillaries are only approximately 40 microns apart and yet they make up the largest endothelial surface area for blood-brain exchange (Pardridge, 2005, 2016). Unlike larger artioles and venules, capillary endothelium is tightly sealed by junctions as well as both pericytic and astrocytic end-feet interactions, creating a strict barrier between blood and brain (Wilhelm et al., 2016). Aside from the free diffusion of small gases such as
oxygen and carbon dioxide, there are two main ways in which molecules can cross this barrier, namely paracellular or transcellular transport. In paracellular transport substances such as water-soluble agents travel across the endothelium through the intercellular junctions found between adjacent cvECs. The other mechanism, transcellular transport, is the preferred route and involves the movement of substances through the lipid membranes across the BBB (Abbott et al., 2006, Wolburg et al., 2009a). Transcytosis is accomplished via specific transport systems located on both the luminal (towards the lumen) and abluminal (away from the lumen) membranes of cvECs, separated by approximately 300 nm of endothelial cytoplasm (Pardridge, 2005, 2016) that vary depending on the size and chemical composition of the substance being transported. The transcellular lipophilic pathway provides an effective diffusive route for lipid-soluble agents, whereas transport proteins, some of which as energy-dependent, allow for substances such as glucose, amino acids, purine bases, nucleosides, and choline to cross the barrier. Certain proteins such as insulin and transferrin are taken up by specific receptor-mediated endocytosis and transcytosis. Albumin, an endogenous plasma protein, encounters great difficulty in crossing the BBB; its only way across is via cationization, which can increase albumin uptake by adsorptive-mediated endocytosis and transcytosis. Overall, all large hydrophilic molecules are generally excluded, unless they can be transported by either specific receptor-mediated transcytosis, or by the less specific adsorptive-mediated transcytosis (Abbott et al., 2006, Wolburg et al., 2009a, Almutairi et al., 2016).
1.2.4 Endothelial Intercellular Junctions

Proper functioning of the BBB crucially depends on a network of both tight (TJ) and adherens junctions (AJ) that contribute to this barrier by regulating the passive diffusion of electrolytes and water-soluble proteins, while ensuring that all of the brain’s metabolic requirements are met (Segura et al., 2009). Tight and adherens junction proteins play distinct roles but are both equally essential for the proper development and repair of the BBB (Tietz and Engelhardt, 2015). Studies have shown that AJs are required for the formation of TJs and are indispensable to the organization and continuous crosstalk between components of these different intercellular junctions. AJs also participate in the regulation of cell-cell contact initiation, maturation, maintenance, plasticity, as well as regulation of tensile forces (Vorbrodt and Dobrogowska, 2003). TJs, on the other hand, function as endothelial gates regulating the flow of solutes and ions through the paracellular route while limiting the free movements of lipids and proteins (Dejana et al., 2009). The intercellular cell junction proteins that are most important for cvECs survival and BBB regulation include claudin-5, zonula occludens (ZO)-1, vascular endothelial (VE)-cadherin, occludin, platelet endothelial cell adhesion molecule (PECAM)-1, and junctional adhesion molecule (JAM)-A. Claudin-5 is the most abundant tetraspanning TJ protein in ECs and its stability and paracellular sealing functions are achieved through proper linking to the actin cytoskeleton via the scaffolding protein ZO-1 (Haseloff et al., 2015). ZO-1 is a large cytoplasmic protein (1745 aa) consisting of 3 PDZ domains on its N-terminus, in addition to other binding sites (Itoh et al., 1999). The last 2 amino acids at the C-terminus of claudin-5 is a PDZ-binding motif that has been shown to bind directly to the PDZ-1 domain of ZO-1 (Itoh et al., 1999, Morita et al., 1999). Claudin-5 is
essential for BBB development as claudin-5 deficient mice die due to BBB leakiness (Nitta et al., 2003) and focal loss of claudin-5 correlates with BBB dysfunction (Zhou et al., 2014). Similarly, occludin is an integral membrane protein localized exclusively to TJs and also links to the actin cytoskeleton by recruiting ZO-1 (Furuse et al., 1993). JAM-A, also known as JAM-1, regulates cell polarity and leukocyte infiltration across the BBB as well as other endothelial barriers (Martin-Padura et al., 1998, Williams et al., 2013). Studies in humans show that loss of vascular JAM-A immunostaining in multiple sclerosis brain tissue correlates with BBB leakiness (Padden et al., 2007). In addition to TJs, ZO-1 is also a central regulator of VE-cadherin dependent AJs (Tornavaca et al., 2015). VE-cadherin, also known as cadherin (Cdh)-5 type 2 or cluster of differentiation (CD) 144, is a type of cadherin encoded by the human gene CDH5 (Suzuki et al., 1991). VE-cadherin functions as a classic cadherin giving cvECs the ability to adhere to one another in a homophilic manner. Cdh-5 also plays an important role in endothelial biology as it regulates both the organization and cohesion of the intercellular junctions (Entrez Gene: CDH5). The cytoplasmic domain of VE-cadherin is also linked to the actin cytoskeleton and intermediate filaments via the catenin complex (Dejana and Vestweber, 2013). Finally, another player normally found on cvECs that contributes to steady-state BBB function is PECAM-1, also known as cluster of differentiation (CD)-31. PECAM-1 is highly expressed in endothelial cell junctions outside of the organized AJs and TJs of the BBB (Graesser et al., 2002, Lyck et al., 2009). CD31 functions as a mechanosensor and accelerates restoration of barrier integrity following injury and other cellular perturbations (Graesser et al., 2002, Privratsky and Newman, 2014). In addition to its expression by cvECs, PECAM-1 is also found on the surface of platelets, monocytes,
neutrophils, and some types of T-cells (Newman et al., 1990); all of which are known to infiltrate the injured brain.

1.3 Traumatic Brain Injury

1.3.1 Definition

Traumatic brain injury (TBI) is a devastating disorder that occurs when an external mechanical force causes insult to the brain potentially leading to dysfunction that results in tearing of tissue, hemorrhage and other physical brain damage which can cause long-term complications or even death (Maas et al., 2008). TBI commonly yields either temporary or permanent impairment of cognitive, physical, and psychosocial functions, which is correlated with reduced or altered state of consciousness. The injury ranges from mild to severe and consists of a broad spectrum of symptoms and disabilities depending on the severity of the trauma. A minor concussion can result in a brief loss of consciousness or mental status whereas more severe injuries can cause extended periods of unconsciousness or permanent brain damage (Malec et al., 2007). Mild to moderate TBI is most common in injuries where no skull fracture occurs although closed head injuries can also result in severe deficits and possibly permanent neurological damage. Open head or penetrating TBI occurs when an object penetrates the skull causing a direct injury from the head impact, and usually pushing of skull fragments into the brain. These are usually more severe and often accompanied by additional complications, such as infections, depending on the nature of the penetrating object (Collaborators et al., 2008, Niedzwecki et al., 2008, Steyerberg et al., 2008, Pop and Badaut, 2011).
1.3.2 Epidemiology

TBI is a major source of death and disability worldwide. Every year, nearly 2 million individuals are victims of TBI, contributing to over 30% of all injury-related deaths in the United States alone (Centers for Disease and Prevention, 2013). As of 2010, TBI epidemiology studies indicate that each year approximately 235,000 Americans are hospitalized for non-fatal TBI, 1.1 million are treated in emergency departments, and about 50,000 are fatal incidents (Corrigan et al., 2010). The leading causes of TBI result from falls, motor vehicle accidents, assault or self-inflicted violence, and sports. These causes vary depending on the country, socioeconomic status, region, gender and age group. In the United States the individuals at greatest risk for TBI include males, young children and elderly, as well as individuals of low economic status (Centers for Disease and Prevention, 2013, Coronado et al., 2015). For the age category, children younger than 10 years of age and elderly over the age of 74 are at highest risk of TBI that result mostly from falls. The propensity for men to incur TBI is nearly twice that of women, which can be attributed to risk-taking behavior and high-risk activities commonly engaged in by men including sports and traffic related accidents. For TBI incidents that result in death the rates among males are even more pronounced, estimated approximately 3.4 times higher than those for females. According to previous statistics, uninsured individuals with low socioeconomic status present nearly twice the risk of acquiring TBI compared to persons with private insurance (Corrigan et al., 2010). Additionally, TBI presents a huge economic burden with total estimated costs of approximately $76.5 billion per year (Coronado et al., 2012).
1.3.3 Pathophysiology

The pathophysiology of TBI is divided into 2 subcategories: (1) primary injury, which occurs immediately at the time of trauma, and (2) secondary injury, which occurs after the trauma and produces effects that may continue from hours to months or even years after the initial incident. The primary injury is a result of the direct or indirect mechanical force on brain tissues that disrupts cellular integrity leading to tissue distortion and destruction. The acceleration, deceleration, or rotational forces that result when the impact injury is delivered to the head at rest also contribute the initial injury. The secondary injury is attributable to the biomolecular and physiological changes that follow the primary insults (Greve and Zink, 2009, Pop and Badaut, 2011, Pearn et al., 2016). These include but are not limited to the release of excitatory amino acids, oxygen radical reactions, disruption of calcium homeostasis and nitric oxide production; all of which result in a massive depolarization of neurons, glial cells, and cvECs (Greve and Zink, 2009). This massive depolarization is followed by cell death, thought to result from both apoptotic and necrotic signals, and treatment strategies have been developed to block these processes with marginal success. Additionally, the integrity of blood vessels and the BBB is compromised at the injury epicenter and the sustained forces that result from the accumulation of fluid in the brain (edema) and the localized collection of blood outside the blood vessels (hematomas) can continually disrupt brain function by distorting brain tissue, elevating intracranial pressure (ICP), and reducing cerebral blood flow (CBF) which is associated with poor outcome and death (Chen et al., 2003, Faul et al., 2016). Despite the consequences of blood vessel collapse and destruction, which are
often accompanied with functional deficits, little is known of the cellular signs and responses that occur in vessel integrity or remodeling following TBI.

1.3.4 Vascular and BBB Changes Following TBI

The brain, due to its size, requires large amounts of oxygen and glucose in order to function properly. Maintenance of cortical tissue health and stability including adequate supply of blood and nutrients is accomplished via the brain’s vasculature, and is thus an important consideration for repairing tissue damage resulting from TBI. The BBB is an integral part of the NVU and the resulting dysfunction from the primary damage to the vasculature dramatically alters cerebral homeostasis, causing a deregulation in the uptake of molecules, severely decreased cerebral blood flow due to vasoconstriction in areas surrounding the impact site (injury penumbra), as well as edema and hypoxia. These TBI induced alterations contribute greatly to secondary injury pathophysiology, including overall cell death and tissue loss. The BBB is a very dynamic structure and post trauma disruptions vary dramatically depending on injury severity. There are clinical and experimental findings indicating that BBB breakdown is both a common and prominent pathological feature in TBI patients. In fact, there is a consensus in the literature that BBB damage following TBI leads to increased vessel leakiness and permeability, which allows for the infiltration of immune cells from the periphery that results in neuroinflammation and cytotoxicity (Das et al., 2012, Alves, 2014, Pearn et al., 2016, Price et al., 2016). There are several underlying events involved in TBI-induced cerebral vascular and BBB alterations, including disruption of TJ seals, widening of intercellular spaces, changes in endothelial transport properties, ECM degradation, altered associations of gliovascular cells, and peripheral cell infiltration (Alves, 2014, Price et
The impact-induced shearing stress that occurs after TBI leads to destabilization of the actin cytoskeleton resulting in disruptions in the organization of cvEC intercellular junctions as well as their interactions with pericytes and astrocytic end-feet. This in turn causes widening of paracellular spaces, which results in changes in endothelial transport properties, peripheral leukocyte infiltration and increased extravasation of blood-borne factors into the brain tissue. One of the ways in which BBB breakdown can be assessed after injury is through IgG or Evans blue (EB) extravasation assays, where the increased amount of these large molecules in the brain correlates with vessel disruption and BBB damage. EB dye has a high affinity to serum albumin, a macromolecule that does not normally cross the BBB. Because this inert tracer is not taken up by cells, it can be injected either intravenously (i.v.) or intraperitoneally (i.p.) and used to assess vascular permeability in animal models by quantifying the amount of dye absorbed by the tissue of interest (Manaenko et al., 2011). TBI studies in rats have shown a biphasic BBB permeability to albumin and other high molecular weight molecules peaking at 4 to 6 hours and then again at 2 to 3 days post-injury (dpi) (Baskaya et al., 1997, Tado et al., 2014). Another study, also in rats, showed high levels of IgG extravasation near the injury site at 1 and 3 dpi, which decreased by 7 dpi (Badaut et al., 2015). While it is not currently known what exactly causes these two temporally distinct surges in BBB permeability, the first peak in macromolecule extravasation contributes to vasogenic edema formation whereas the second peak coincides with observed increases in peripheral cell infiltration and immune response (Pop and Badaut, 2011). An influx of neutrophils is observed during the initial 24 hours post injury, and significant increases in macrophage infiltration is observed at 1 and 3 dpi in mice, which is subsequently reduced...
by 7 dpi (Jin et al., 2012). In addition to the paracellular interactions by TJ and AJs, vessel stability and adequate BBB function also requires proper endothelial wrapping by pericytes and astrocytic end-feet, as described in previous sections. Astrocytes and pericytes support cvEC function at the NVU, which directly regulates vascular permeability. Also, with the help of neurons, these cells support the basement membrane surrounding the cvECs (Logsdon et al., 2015). Astrocytic and pericytic associations with the vasculature rely on direct interactions with the ECM and its components. Normal astrocytic end-feet interactions with the endothelial basement membrane and the ECM have been shown to regulate leukocyte infiltration into the brain parenchyma (Owens et al., 2008), whereas pericytes contribute directly to the synthesis of essential ECM proteins including laminin, nidogen and fibronectin (Stratman et al., 2009). TBI results in disruptions in the associations of gliovascular cells related to injury-induced ECM degradation (Badaut and Bix, 2014). Loss of brain pericytes contributes to vessel leakiness and breakdown of the BBB, accompanied by extravasations of circulating plasma proteins, as well as hypoxia (Bell et al., 2010). Perivascular astrocytes undergo swelling after trauma as a result of an acute upregulation in AQP4 expression. This results in increased water uptake and also contributes to brain edema triggered by BBB breakdown. Likely as a result of this swelling, agrin is lost from the cvEC abluminal surface adjacent to astrocytic end-feet, contributing further to BBB damage (Abbott et al., 2006). In essence, TBI induced alterations in BBB function and permeability are very complex as it involves the different NVU cellular components and their individual as well as combined response to the injury. This complexity is directly reflected by the lack
of thorough understanding in injury-induced changes and reparative mechanisms in cerebral vasculature.

1.3.5 Endothelial Progenitor Cells (EPCs) and TBI

In the past decade there is increasing evidence that pathological neovascularization in the adult involves both angiogenesis and vasculogenesis (Guo et al., 2009, Timmermans et al., 2009, Xue et al., 2010). Postnatal vasculogenesis is mediated by endothelial progenitor cells (EPCs) that exist in adult life normally residing in the vascular niche of the bone marrow (BM), with a very low percentage (few thousand cells/mL blood) found circulating in healthy subjects. Following injury, EPCs are mobilized from the BM into the circulation and migrate to tissue repair sites contributing to neovascularization and healing (Balaji et al., 2013). In TBI, bone-marrow derived EPCs are also released into circulation stimulated by cytokines and chemokines as well as growth factors such as VEGF that are increased after injury. These endothelial progenitors have been shown to infiltrate the injured brain, concentrating at the injury site, and are believed to participate in vascular stabilizing and/or repairing events of neovascularization. Additionally, EPCs can differentiate into mature cvECs that have the potential to integrate into the brain’s vasculature also possibly contributing to vessel repair (Guo et al., 2009, Timmermans et al., 2009, Xue et al., 2010). Although both mature and progenitor endothelial populations have been shown to play essential roles in vascular integrity and BBB stability, their individual contributions and mechanisms of repair after TBI are very poorly understood. It is believed that once at the site of injury EPCs secrete stimulating factors that aid in the angiogenic response ultimately inducing cvEC proliferation and vessel branching. Studies have tried to enhance this angiogenic response after injury by infusing VEGF at different
time points following CNS injury. VEGF treatments have been shown to enhance both neurogenesis and angiogenesis in the injured brain (Thau-Zuchman et al., 2012); however, its role on bone marrow-derived EPC infiltration and proliferation has not yet been described. Previous studies have also demonstrated that local administration of adult EPCs derived from either rat adipose tissue and/or BM following traumatic injury participates in adult brain neovascularization and promotes tissue reconstruction (Guo et al., 2009, Xue et al., 2010). Transplantation of EPCs after TBI has also been shown to improve behavioral performance and reduce the volume of the injury cavity (Xue et al., 2010, Zhang et al., 2013). Additionally, clinical trials utilizing EPCs for ischemic and wound treatment have also been favorable (Zhang et al., 2002, Balaji et al., 2013). Not surprisingly, EPCs have been proposed as a potential regenerative tool for treating human vascular disease and vascular repair following trauma, as well as a possible target to restrict vessel growth in tumor pathology. Since clinical trials are primarily therapeutic innovations, they do not provide mechanistic information of how EPCs promote wound healing and revascularization. Conflicting results have been observed in the field pertaining to proper identification and characterization of EPCs in vascular biology (Timmermans et al., 2009), as well as from the therapeutic use of cultured EPCs from different sources and in different models of CNS injury. Unfortunately, these controversies amongst researchers have halted further advancement of the field. Studies are still needed to properly identify the EPC population that infiltrates the traumatically injured brain, which will provide a better consensus on the population best suited for curative transplantation studies. Better understanding of the brain’s endogenous response to EPCs and their temporal peaks in homing and proliferation in different TBI models
will also further our knowledge of their contribution to vascular integrity. This information can then be useful for designing new therapeutic strategies to enhance the survival and/or proliferation of this innate EPC population.

1.3.6 Injury-induced Cell Death and Proliferation

Of all TBI-induced cellular changes that result from both primary and secondary injury, cell death either through necrosis or apoptosis and proliferation accounts for the majority of them. Necrotic cell death is known to occur primarily as an immediate consequence of the mechanical forces from the physical impact whereas apoptosis continually takes place in regions surrounding the injury site via different cellular mechanisms and over prolonged time periods (Raghupathi et al., 2000). Neurons are especially susceptible to both types of cell death and countless efforts have been made to repair post-traumatic neuronal loss. Glutamate excitotoxicity, which occurs almost immediately after TBI, and is highly associated with gradual pathogenesis, is one of the major mechanisms of secondary injury leading to the post-traumatic loss of neural tissue (Chodobski et al., 2011). This excessive glutamate activation of excitatory receptors correlates with increased production of nitric oxide (NO) and oxidative stress (Lau and Tymianski, 2010) also resulting in the death of other cell types in addition to neurons. Injury-induced apoptosis of cvECs and oligodendrocytes has also been linked to increases in glutamate after trauma (Parfenova et al., 2006, Johnstone et al., 2013). As described previously, secondary injury can last for several weeks or months and the accompanying effects continually contribute to additional neuronal cell death (Pearn et al., 2016). On the opposite end of the scale, cell proliferation is also a hallmark of TBI, with different cell types expanding at varying time points and injury paradigms. Injury-
induced proliferation of neural stem cells (NSCs) originating from the sub-ventricular zone (SVZ) and the hippocampal dentate gyrus (DG), two areas where adult neurogenesis is known to occur, has been widely documented in the literature (Kernie and Parent, 2010, Gao and Chen, 2013). NSC proliferation correlates with injury severity and is significantly increased after both moderate and severe controlled cortical impact (CCI) injury in mice (Theus et al., 2010, Wang et al., 2016). Additionally, proliferation of other cell types such as neuron-glial (NG)-2+ cells is dramatically increased at 1 day after moderate mouse CCI injury (Susarla et al., 2014), whereas microglia and astrocyte expansion peaks at 3 days after TBI in both humans and mice, a time where overall bulk proliferation takes place (Engel et al., 2000, Myer et al., 2006, Susarla et al., 2014). TBI-induced proliferation of glial fibrillary acidic protein (GFAP)+ astrocytes increases significantly starting as early as 24 hours post-injury (hpi) and remains high until nearly a month later (Susarla et al., 2014). Although not widely accessed in TBI models, proliferation of cvECs and potential EPCs has been demonstrated as early as 24 to 48 hpi in peri-ischemic cortical regions using an acceleration impact model of TBI in rats (Morgan et al., 2007). A significant accumulation of BrdU+ cvECs was also detected around the injury area 2 weeks after CCI injury in rats (Wu et al., 2011) and as far as 3 months after closed-head TBI in mice (Thau-Zuchman et al., 2012), which were further enhanced with exogenous stimulating factors. In summary, TBI studies demonstrate that cell proliferation seems to follow the initial stage of necrotic cell death but overlaps with apoptosis in peri-contusional areas and surrounding the site of injury. Both proliferation and cell death continues well after the first week following TBI and understanding these
temporal patterns in different injury models can contribute to the design of repair strategies.

1.3.7 Controlled Cortical Impact (CCI) Injury as a Model for TBI in Mice

TBI continues to compromise the health and quality of life of the individuals affected as well as their family members, despite the numerous efforts devoted to addressing this problem. To better study the widely variable effects of TBI as well as to develop potential therapeutic treatments, researchers have developed numerous different types of both in vitro and in vivo modeling systems. Some of the most widely accepted mouse models of TBI include the fluid percussion injury (FPI), penetrating ballistic-like brain injury (PBBI), weight drop, and CCI models (Xiong et al., 2013). Our studies utilize the CCI model of TBI in mice. Experimental CCI employs a pneumatically or electromagnetically driven device that allows for precisely controlled and reproducible impacts delivered directly onto the cortical brain surface. This model was first characterized in mice by Smith and colleagues (Smith et al., 1995) after being previously described in the ferret (Lighthall, 1988) and in the rat (Dixon et al., 1991). Most CCI devices used consist of a metal tip piston attached to a shaft that is held on a crossbar allowing for different mounting positions (beveled and vertical aligned with the surface of the brain). To ensure uniform injuries, velocity, depth, and impact duration can be pre-programmed and then verified by the sensor contained in most devices. Additionally, the experimental CCI model provides important clinical relevance as it demonstrates many aspects of TBI that are observed physiologically including cognitive and neurologic motor dysfunction, in addition to cortical contusion, inflammation, oxidative stress, axonal injury, apoptosis, and BBB disruption (Osier et al., 2015).
1.4 Ephrins and Eph receptors

1.4.1 Description and Family Classification

Erythropoietin-producing human hepatocellular (Eph) receptors make up the largest subfamily of tyrosine kinases receptors. Both ephrin ligands and their receptors are membrane bound proteins that interact to initiate bidirectional signaling cascades in both the ligand- and the receptor-containing cells (Pasquale, 2004, Klein, 2012). There are two classes of ephrins and Eph receptors, namely A and B class, separated by their ligand structural differences and binding preferences. The EphA and EphB receptors have conserved domain structures consisting of a an extracellular ligand-binding domain, followed by a cysteine-rich domain, EGF-like motifs and two fibronectin type III repeats. The cytoplasmic domain of Eph receptors contain SRC homology 2 (SH2) binding sites, a juxtamembrane region that regulates kinase activity, a typical tyrosine kinase domain, a sterile alpha motif (SAM) protein-protein interaction domain and a PDZ [post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (ZO-1)] binding motif (Coulthard et al., 2012, Boyd et al., 2014). The A class contains 5 ephrins (A1–A5) that are tethered to the plasma membrane via a glycosyl phosphatidyl inositol (GPI) moiety and 10 EphA (A1-A10) receptors, 8 of which are well-characterized. The B class consists of 3 ephrins (B1–B3) that span the plasma membrane and have a short cytoplasmic tail containing a PDZ-binding motif, in addition to 6 EphB receptors (B1-B6) (Pasquale, 2004, Coulthard et al., 2012). Eph-ephrin signaling is indispensable for a vast of physiological and pathological regulatory processes both during development and adult postnatal life. Some of their CNS functions include but are not limited to angiogenesis, cell migration, neurite outgrowth, cell
survival and proliferation, cell adhesion and motility, and synaptic plasticity (Goldshmit et al., 2006).

1.4.2 Roles in CNS Cell Survival and Proliferation

Eph receptors and their ephrin ligands are expressed in nearly all tissues of the mammalian embryo, and participate in a wide spectrum of developmental and injury-induced processes (Palmer and Klein, 2003). Different members of the Eph-ephrin family have been shown by our lab and others to play important roles in cell survival and proliferation in the vertebrate CNS. Of particular interest to our studies is the ligand-receptor binding partners, ephrinB3 and EphB3, which employ anti-proliferative and cell death functions at varying stages of CNS development, adult homeostasis, as well as in post-injury pathophysiology. During development, the proliferation of neural progenitors is inhibited by EphB3 in the SVZ demonstrated by significant increases in both BrdU+ and Ki67+ cells along the ventricles of P1 EphB3−/− mice (del Valle et al., 2011). In the adult mouse brain, EphB3 limits the expansion of neural stem progenitor cells (NSPCs), also in the SVZ, by regulating p53 both during homeostasis and after CCI injury (Theus et al., 2010). The ligand, ephrinB3, participates in the regulation of adult neurogenesis by negatively contributing to the control of cell cycle progression and apoptosis (Ricard et al., 2006). EphB3−/− mice show reduced counts of TUNEL+ NSPCs in the adult mouse SVZ (Theus et al., 2010) as well as in mature NeuN+ cortical neurons during the first week after CCI injury (Theus et al., 2014). EphB3 also plays a post-trauma cell death role in the spinal cord (Tsenkina et al., 2015). PLP-GFP+ oligodendrocyte survival is improved in the spinal cord in the absence of EphB3 at 7 days following SCI in mice (Tsenkina et al., 2015). In all mentioned cases, EphB3-mediated cell death can be rescued
upon infusion of ephrinB3 starting at the time of injury; thus confirming its dependence receptor function. Dependence receptors are transmembrane proteins that have dual opposing roles depending on the availability of their corresponding ligand. By definition, dependence receptors induce apoptotic cell death under stress conditions in the absence of their ligand. When the ligand is present, these receptors promote normal development and tissue homeostasis by inducting positive signals such as cell survival, migration and differentiation (Mehlen and Bredesen, 2004, Goldschneider and Mehlen, 2010). The intracellular downstream mechanisms activated by the different dependence receptors upon ligand binding vary; nonetheless, despite the different sequences and structures these receptors share general functional similarities when ligand deprived. They function as caspase substrates making caspases or caspase-like molecules a requirement for the induction of cell death. Caspase binding leads to proteolytic cleavage of the receptor, a change in its conformation, and the release/exposure of an addiction/dependence domain (ADD), which is required for dependence receptor induced apoptosis and can amplify caspase activity (Mehlen and Bredesen, 2004, Furne et al., 2009). There are several currently identified members of the dependence receptors family known to play important roles in both physiological and pathological conditions. Our work has previously led to the identification of two Eph receptors, EphA4 and EphB3, as new members of the dependence receptor family (Furne et al., 2009, Nelersa et al., 2012, Theus et al., 2014, Tsenkina et al., 2015). In the absence of the ligand, which in both of these cases is ephrinB3, the receptors are cleaved; an apoptotic death signal is induced and amplified in neurons (Theus et al., 2014) and oligodendrocytes (Tsenkina et al.,
The role of ephrinB3 or EphB3 in either cvEC proliferation or survival, however, has not been investigated to date.

1.4.3 Roles in The Vasculature

Blood vessels express several Eph and ephrin family members and control a variety of critical vascular processes including developmental and postnatal angiogenic remodeling, pathological vasculo-/angio-genesis and neovascularization. Eph-ephrin signaling also has essential roles in cvECs as well as in supporting pericytes, perivascular astrocytes and smooth muscle cells (Kuijper et al., 2007, Salvucci and Tosato, 2012). EphB4 and EphrinB2 are the most vastly studied Eph-ephrin molecules in the vascular system as gene-targeted deletion of ephrinB2 is embryonic lethal resulting from underdeveloped vessels and poor vascular organization (Adams et al., 1999). EphB4 receptor along with its ligand ephrinB2, expressed in venous and arterial ECs, respectively, are essential for arterial-venous specification and vascular remodeling in both health and disease (Herbert et al., 2009, Abengozar et al., 2012, Salvucci and Tosato, 2012). Moreover, ephrinB2 also participates in assembly of the vessel wall and in establishing proper cvEC-pericyte interactions (Foo et al., 2006), whereas activation of EphB4 enhances the pro-angiogenic potential of EPCs (Foubert et al., 2007). Direct paracellular endothelial contact, endothelial-mural cell, as well as blood vessel-tissue interactions are indispensable for vascular morphogenesis and are also in part controlled by the Eph-ephrin signaling system (Kuijper et al., 2007). Ephrins have been shown to regulate the recruitment of pericytes both during developmental and pathological conditions, which is a critical step in the process of vessel maturation of newly formed branches. The targeted deletion of ephrinB2 in pericytes and smooth muscle cells results
in perinatal lethality, which is associated with developmental defects in small-diameter blood vessels that are not properly wrapped by pericytes. Ineffective pericytic ensheathing results in leaky microvessels, which in turn causes diffuse tissue edema, hemorrhaging and BBB dysfunction (Foo et al., 2006). Different studies by Goldshmit Y. and colleagues (2006) demonstrated that another member of the Eph receptor family, EphA4, is upregulated in astrocytes after CNS injury and plays a role in the interaction between cvECs and astrocytic end-feet. Astrocytes of EphA4−/− mice are not as tightly associated with blood vessels as the wild-type astrocytes and this reduced association results in prolonged leakage of the blood-spinal cord barrier following SCI (Goldshmit et al., 2006). Given that Eph receptors and their ephrins regulate several types of cell-cell interactions generally by modulating cytoskeletal dynamics (Park and Lee, 2015), the actin cytoskeleton and its binding proteins are a potential target for Eph-ephrin modulation of TJs and/or AJs that connect cvECs to properly seal the BBB. Additionally, ephrins and Eph receptors have been shown to interact with other key vascular proteins such as CXCR4/SDF-1 to regulate endothelial movement and morphogenesis of blood vessels (Salvucci et al., 2006) and VEGFR-2 to mediate its internalization, which is necessary for activation and downstream signaling of the VEGFR-2 receptor and required for tip cell filopodial extension (Sawamiphak et al., 2010). EphrinB ligands can also induce capillary sprouting in vitro with a similar efficiency as VEGF, demonstrating a stimulatory role of ephrins in the remodeling of the developing vascular system (Adams et al., 1999). Developmental and pathological blood vessel integrity reveals yet another layer of vascular regulation by different members of the Eph-ephrin family (Salvucci and Tosato, 2012). In the developing vascular system EphB2 and EphB3, in addition to
ephrinB2, regulate cerebral vascular angiogenesis, which involves cvEC proliferation, chemotactic migration, and functional maturation. Adams and colleagues showed EphB2/EphB3 double homozygotes have fewer, small diameter cerebral vessels where many are underdeveloped and arrested at the primary capillary plexus stage (Adams et al., 1999). A role for EphB3 was also suggested in ocular angiogenic disease in humans where differences in receptor expression correlates with increased ephrinB2 levels and changes in vascular density (Umeda et al., 2004). Although a role for EphB3 in post-traumatic vascular integrity has not yet been defined, the current literature suggests that this receptor may participate in the maturation process of angiogenic vessel branching and that its role after injury might differ from initial vascular development.

1.4.4 As Therapeutic Targets

Pathological angiogenesis in the adult CNS is mostly associated with ocular neovascular diseases and tumor angiogenesis contributing to the growth of brain cancers (Salvucci and Tosato, 2012, Prakash and Carmichael, 2015). It is now clear that Eph-ephrin interactions participate in several aspects of neovascular regulation including cvEC sprouting, proliferation, vascular remodeling and vessel stabilization, making Eph receptors and their ligands desirable therapeutic targets for post trauma vascular remodeling such as stroke and TBI. The multi-domain structure of Eph receptors have led to the development of different modulatory strategies to control Eph-ephrin signaling in either the ligand- and/or the receptor-containing cells. Some of these approaches include the use of small molecule kinase inhibitors targeting the ATP binding site in the Eph receptor kinase domain, receptor/ligand downregulation with siRNAs, miRNAs or biological factors such as ligands and antibody agonists (Barquilla and Pasquale, 2015,
Tognolini and Lodola, 2015). Additionally, chemical compounds or peptides that can directly bind to the ephrin-binding domain of Eph receptors and abrogate signal have recently received individual attention from researchers in the field (Riedl and Pasquale, 2015). Although a work in progress, the combined interest and efforts in this later approach resulted in the development of several agents, including peptides that can bind with high affinity and selectivity to the extracellular ligand-binding domain of Eph receptors and some seem promising for medical applications (Riedl and Pasquale, 2015). As it pertains to the vasculature, most of the Eph-ephrin therapeutic studies currently available are aimed at anti-angiogenic signaling mechanisms to restrict tumor vessel growth (Boyd et al., 2014). Understanding cell-specific roles for different ephrins and their preferred receptors in a CNS injury paradigm, such as TBI, will provide much needed insight for the development of temporally appropriate pro-angiogenic therapy via modulation of Eph-ephrins signaling.
Chapter 2

CCI injury leads to cerebral vascular damage and increased proliferation of both residential cerebral vascular endothelial cells (cvECs) and bone-marrow derived endothelial progenitor cells (EPCs)

2.1 Introductory Remarks

Following traumatic brain injury (TBI) the integrity of blood vessels is compromised at the injury epicenter and the resulting pathophysiological changes are associated with poor patient outcome and possibly even death (Faul and Coronado, 2015, Logsdon et al., 2015). Despite the consequences of blood vessel collapse and destruction, which are often accompanied with functional deficits, little is known of the cellular responses that occur in vessel integrity or remodeling following TBI. Most of the knowledge in the field comes from developmental vessel formation where neovascularization in the CNS is known to occur via two processes, namely vasculogenesis and angiogenesis. Although the cellular mechanisms are not completely understood, it has been long established that angiogenesis of residential cvECs can occur in the adult brain under pathological conditions such as stroke, ischemia, tumor formation, and TBI (Welser et al., 2010). Within the last decade an increasing body of evidence suggests that the process of neovascularization after stroke and TBI also includes vasculogenesis, where circulating endothelial progenitor cells (EPCs) from bone marrow and peripheral blood contribute to vessel repair and possibly new vessel growth (Zhang et al., 2002, Guo et al., 2009, Timmermans et al., 2009, Xue et al., 2010). However, the cellular responses of residential cvECs and peripheral EPCs after TBI remain poorly defined. In TBI the disruption of the blood brain barrier (BBB) leads to the invasion of numerous peripheral cells into CNS tissues, including monocytes, macrophages, T-cells, B-cells, and other inflammatory cell
types (Das et al., 2012, Abdul-Muneer et al., 2013). TBI can also enhance the production and release of several cytokines and chemokines that participate in both pro- and anti-inflammatory responses (Chodobski et al., 2011). Furthermore, the CNS responds to trauma by enhancing growth factor production that can directly affect neovascularization. For example, increased levels of vascular endothelial growth factor (VEGF) are known to stimulate cell proliferation and survival as well as vessel growth, maintenance and branching; in addition to being a major regulator of neurogenesis (Quaegebeur et al., 2011, Thau-Zuchman et al., 2012). In the adult TBI mouse, the acute effect of VEGF infusion on residential cell ECs and infiltrating EPCs remains poorly defined. Our studies examine acute vascular changes that occur as a result of TBI. We take advantage of cutting edge scientific tools, such as 3-D light-sheet microscopy and endothelial cell-specific inducible transgenic mice. Additionally, we demonstrate how VEGF alters the TBI microenvironment as it relates to proliferation and peripheral infiltration of EPCs and cell ECs proliferation.

2.2 Results

2.2.1 Graded CCI injury leads to progressive tissue damage and vascular loss in the CCI injured cortex.

To begin examining vascular damage and remodeling in the murine cortex after TBI, we first needed to establish the extent of pathological tissue and vascular damage induced by our CCI injury device. For this reason, we subjected adult male mice to graded piston velocities of 2, 4, and 6 m/s, while maintaining a standard depth of 0.5 mm and impact duration. The amount of gross tissue damage was qualitatively evaluated in hematoxylin
and eosin (H&E) stained coronal brain sections at 3 and 7 days post-injury (dpi). We observed minor gross tissue loss at 2 m/s at either time point, while the higher velocities, 4 and 6 m/s, presented graded and progressive cortical tissue loss accompanied by visible damage in some subcortical regions (Fig. 2.1 a–f). Although H&E histological assessment revealed little to no gross tissue damage at 3 dpi, we observed vascular damage even at the lowest velocity (2 m/s) tested (Fig. 2.1 g–j). Analysis of cerebral vascular endothelial cells (cvECs) in the 2 m/s CCI-injured Cdh5-zG mice using 3-D ultramicroscopy revealed an absence of cells in the injury epicenter at 3 dpi (Fig. 2.1 g,h). CvECs (green) were preserved in the injury penumbra (Fig. 2.1 g,i; arrow heads) and were found to retain their vessel formation as demonstrated by the presence of lectin-594 (red) following peripheral infusion (Fig. 2.1 h,j). At the higher impact velocities of 4 and 6 m/s vessel loss was also observed in the cortex with a graded increase in the injury epicenter and penumbra that matched velocity intensities. The moderate injury paradigm resulted in some tissue damage while preserving cvECs at the surrounding penumbra regions; thus for all experiments described in this study, we used a moderate (4 m/s) injury paradigm.

2.2.2 CCI injury leads to increased proliferation of both cvECs and infiltrating EPCs.

TBI-induced tissue damage suggests that vessel repair and regeneration would require expansion of the cvEC an/or EPC pools through active proliferation. We first examined overall cortical proliferation by both immunohistochemistry (IHC) and flow cytometry on
Figure 2-1. Graded CCI injury leads to graded cortical damage and vascular loss at the injury epicenter. H&E stained coronal brain sections at impact velocities of 2 m/s (a, d), 4 m/s (b, e), and 6 m/s (c, f) at 3 (a-c) and 7 (d-f) dpi. Higher magnification H&E stained images of injured cortex (a’-c’). Light-sheet 3D images of the CCI injured cortex in Cdh5-zG mice infused with Lectin-594 (g-j). Arrowheads depict injury penumbra (g). Sagittal view of the injury penumbra reveals the presence of ECs (i) but not of infusible vessels (j).

Abbreviations: Hipp, hippocampus, Th, thalamus.
our EC-specific inducible Cdh5-zGreen transgenic mice. Confocal images revealed a drastic increase in EdU+ cells (red) around blood vessels (green) at the injury penumbra during the first 3 days after injury as compared to sham controls (Fig. 2.2 a–b). Flow cytometry analysis at the same time point showed a consistent and significant increase in total cell proliferation at 3 dpi with approximately 20% more total viable EdU+ cells after injury compared with sham controls (Fig. 2-2 c-d). Next, we assessed proliferation of mature cvECs and infiltrating EPCs in the cortex at 3 and 7 dpi also by flow cytometry. EPCs, once committed to the endothelial fate turn off the progenitor marker CD133 (Prominin-1) and turn on the mature EC marker CD144 (VE-Cadherin)(Asahara and Kawamoto, 2004). Thus, mature cvECs were identified as CD45-/CD144+ cells whereas infiltrating EPCs were identified as CD309+/CD133+ cells out of the CD45-/CD144- population (refer to chapter 5 for details in marker selection). A homogenous viable cell population was selected using the forward and side scatter and proliferation of both populations was assessed as a percentage of total cells for both 3 and 7 day time points (Fig. 2.2 e-f). We quantified the TruCounts (refer to chapter 5) for each individual cell type as well as the number of EdU+ cells within each gate so that the percent of EdU+ cells could be calculated. The CD45+ population, used as an internal control, showed a significant increase in proliferation at both time points; while YFP+ neurons, our negative control, showed no change in proliferation after CCI injury (Fig. 2.2 e). Proliferation assessment of both the cvECs and EPCs endothelial populations showed a significant increase in the ratio of EdU+ cells out of the total cell populations at 3 and 7 dpi as compared with sham control, with the greatest proliferation (over 20%) occurring at 3 dpi for EPCs (Fig. 2.2 f). Furthermore, EPCs represent a greater population of proliferating
Figure 2-2. Proliferating EdU+ infiltrating cells (CD45+), cvECs and EPCs are increased at 3 and 7 dpi. Immunostained coronal brain section of sham (a) and CCI-injured (b) Cgh5-zG (green) cortex shows DAPI staining (blue) and proliferating cells (red). Inset shows high-magnification images of subcortical layers and injury penumbra (a-b). Flow cytometry analysis revealed a nearly 20% increase in total viable EdU+ cells at 3 dpi (d) compared with sham (c) mice. CD45+ infiltrating cells were significantly proliferating at 3 and 7 dpi, while YFP+ neurons were not proliferating (e). Mature cvECs and infiltrating EPCs were significantly proliferating at 3 and 7 dpi (f). Statistical analysis: unpaired two-tailed Student's t-test with 95% confidence interval comparing CCI group to corresponding sham for each time point. N-values: (a, b) n=3; (c-f) n=9-12. * p<0.05, ** p<0.01, *** p<0.001 as compared with sham mice. Abbreviations: Ctx, cortex; cc, corpus callosum; Hipp, hippocampus.
cells than the cvEC population. These studies suggest that the brain retains an intrinsic proliferative response of both mature and progenitor endothelial populations to repair the damaged vasculature in the traumatically injured brain. Enhancement of this natural response and further expansion of these populations may improve recovery and help to better stabilize the injury penumbra.

2.2.3 VEGF infusion enhances both mature and progenitor endothelial populations during the first week after CCI injury.

Vascular endothelial growth factor (VEGF) is an important mediator of endothelial cell proliferation and survival. In these studies, we assessed the potential of VEGF-A administration to enhance EPC infiltration and/or proliferation of both EPC and cvEC populations. WT mice received moderate CCI (4m/s) and 0.03 mg/Kg VEGF-A or PBS (vehicle) for 7 days followed by analysis of the ipsilateral cortex using flow cytometry at 7 dpi. The gating strategy for data analysis and quantification of EdU+ cells was described above; mature cvECs were identified as CD45-/CD144+ cells and infiltrating EPCs as CD309+/CD133+ cells out of the CD45-/CD144- population. Infusion of VEGF-A significantly increased the numbers of cvECs and EPCs compared to vehicle-infused controls (Figure 2.3 a-b). In addition to cell numbers, the proliferation of residential cvECs and infiltrating EPCs was also examined at 7 dpi following cortical infusion with VEGF to evaluate its potential on vasculogenic and/or angiogenic responses. Exogenous VEGF stimulation resulted in a significant increase in proliferation of the residential cvEC population but not of the infiltrating EPCs at 7 dpi (Fig. 2-3 c-d).
2.3 Discussion

These studies examined a complex aspect of traumatic brain injury (TBI) where endothelial cell proliferation and infiltration participate in defining the progressive pathology associated with vascular damage. Our results demonstrated a significant loss in blood vessels at the injury epicenter and variable damage in the penumbra where vessel regeneration could occur. Cerebral vascular endothelial cell (cvEC) proliferation and

Figure 2-3. VEGF infusion acutely after CCI injury results in increased cvEC and EPC numbers and enhanced proliferation of the mature population. Flow cytometry analysis of WT CCI injured animals infused for 7 days with vehicle (PBS) or VEGF-A165 (a-d). TruCount quantification of CD45+/CD144+ cvECs number (a) and proliferation (c) were both significantly increased compared to vehicle at 7dpi. CD309+/CD133+ EPC numbers were significantly increased upon VEGF infusion (b) but not the EdU+ population compared to CCI injured vehicle-infused controls (d). * p<0.05, ** p<0.01 as compared with vehicle-treated mice. Statistical analysis: unpaired two-tailed Student's t-test with 95% confidence interval. N-values: Vehicle-treated n=8, VEGF-treated n=6 for (c-d). For EdU counts n=8 for vehicle and n=3 for VEGF-treated groups (e-f).
endothelial progenitor cell (EPC) invasion are both thought to contribute to vessel repair and/or regeneration after TBI (Melero-Martin et al., 2007, Guo et al., 2009, Chen et al., 2013). We employed a flow cytometric method to quantitatively assess these two cell populations in the murine controlled cortical impact (CCI) injured brain. We found that in the first week post-CCI injury proliferating EPCs represent a large number of the expanding vascular cell population. Furthermore, though only a small percentage of cvECs proliferate after CCI compared to EPCs, administration of VEGF could further enhance the proliferative response of this residential cvEC population.

These findings suggest that the contribution of EPCs to vessel repair may be an early event as injury induced EPC infiltration has been reported to take place primarily within the first 3 days after injury (Guo et al., 2009). These cells are believed to provide a better angiogenic niche by secreting growth factors and are also thought to integrate into damaged vessels and differentiate into mature cvECs thus ‘patching’ broken vessels (Kirton and Xu, 2010). Our results revealed a significant increase in EPC numbers at 7 dpi but not in proliferation upon VEGF infusion. Though we cannot rule out the possibility that the bulk proliferation may have taken place earlier after injury and reduced by 7 dpi, it is likely that VEGF may also increase EPC infiltration into the injured brain. If so, localized cortical administration of VEGF after TBI may serve as a chemo-attractant to increase the mobilization and infiltration of circulating and/or bone marrow-derived EPCs to the injury site. Either way, VEGF induced expansion of this progenitor endothelial population acutely after TBI may further enhance their vasculogenic properties to improve vessel repair and/or reduce progressive vascular loss.
Interestingly, VEGF infusion significantly enhanced the proliferative potential of the mature residential cvEC population up to 7 dpi, which is consistent with previous observations in rat TBI models (Nag et al., 1997, Morgan et al., 2007) and mouse ischemic injury (Hayashi et al., 2003). These findings suggest that augmentation of this natural proliferative response may be increased at least in the first week following injury, which may provide a potential therapeutic window for enhancing neovascularization after TBI. Our results would benefit from physiological and behavioral assessment at later time points to determine whether our findings would translate into functional improvements. Nonetheless, we demonstrate that VEGF stimulation differentially affects these two endothelial cell populations after TBI, where EPCs may participate in the earlier phase of vessel stabilization and residential cvECs may be involved in the later repair mechanisms.
Chapter 3

The role of EphB3 and ephrinB3 in the proliferation and survival of cerebral vascular endothelial cells in the CCI injured brain

3.1 Introductory Remarks

During developmental and early postnatal stages ephrins and Eph receptors are widely expressed in a variety of different tissues including the vasculature (Liebl et al., 2003, Kuijper et al., 2007); and their cell guidance roles have been well defined (Cramer and Miko, 2016). These membrane-bound molecules are known for their regulatory roles in cell-cell interactions, such as cell adhesion, cell mobility, and others, which are indispensable functions in the pathophysiology and recovery of the traumatically injured brain. In addition to their cell guidance roles our lab and others have shown that both members of the B-class ephrins and Eph receptors have anti-proliferative and cell death functions in the CNS. EphB3 inhibits the proliferation of neural progenitor cells in the mouse subventricular zone during development (del Valle et al., 2011) and in the adult brain via p53 regulation during both homeostasis and following TBI (Theus et al., 2010). Moreover, EphB3 has also been shown to mediate cell death through its dependence receptor functions in adult neurogenesis after TBI (Theus et al., 2014) as well as in oligodendrocyte survival after spinal cord injury (Tsenkina et al., 2015). EphrinB3, one of the ligands for the EphB3 receptor, also negatively regulates cell proliferation and survival of neuronal progenitors during adult neurogenesis (Ricard et al., 2006). The role of ephrinB3 and EphB3 in the proliferation and/or survival of cvECs and EPCs; however, have not been adequately investigated. Another feature of ephrins and Eph receptors is their ability to interact with other vascular molecules such as vascular endothelial growth
factor receptor-2 (VEGFR-2). This interaction regulates VEGFR-2 internalization, which is necessary for activation and downstream signaling upon binding of the VEGF ligand (Sawamiphak et al., 2010). However, it is not yet known whether VEGF induced expansion of endothelial cells is modulated by Eph-ephrin, potentially via modulation of VEGFR-2 receptors. These studies examine cell specific expression and function of ephrinB ligands and Eph receptors in the cerebral vasculature as well as their potential role in the proliferation and/or the survival of the cvEC and EPC populations after CCI injury. Here, we demonstrate a novel cell death role for EphB3 in cvECs after TBI where the absence of the receptor results in improved endothelial cell survival at the injury penumbra.

3.2 Results

3.2.1 EphB3 and ephrinB3 are expressed on cerebral vascular endothelial cells (cvECs) of both sham and CCI injured adult mouse brain.

We evaluated the expression of ephrinBs and EphB receptors in cvECs using quantitative reverse transcriptase (qRT) polymerase chain reaction (PCR). RNA was extracted from purified primary cultured and FACS sorted cvECs from sham and CCI injured cortices at 1 dpi. In sorted cells RNA expression for all three ephrinB ligands (B1, B2, and B3) and the two Eph receptors, EphA4 and EphB3, was detected in both sham and injured conditions, although significant differences between sham and CCI injury cortices were only observed for ephrinB3 expression. We found that both EphB3 and ephrinB3 mRNA expression is present in sorted cvECs from sham and CCI injured mice, and all ephrins and Eph receptors tested are down-regulated in cultured brain ECs (Fig. 3-
1). To ensure that no DNA contamination and/or primer dimers were present in our samples, no template controls were run for each primer set. Relative expression values were nearly zero when GAPDH Ct (no template control) was subtracted from each sample confirming a positive expression for all conditions.

3.2.2 Cortical vessel density is significantly reduced following CCI injury in mice but is unaffected by the absence of either ephrinB3 or EphB3.

To begin examining how ephrinB3 and EphB3 may influence the cortical vasculature, we first assessed vessel density in sham and CCI-injured animals in the presence and absence of ephrinB3 or EphB3. EC-specific inducible transgenic Cdh5-zG mice crossed with WT, ephrinB3⁻/⁻ and EphB3⁻/⁻ mice were used for vessel identification (green vessels

**Figure 3-1. Expression of ephrins and Eph receptors in cvECs.** Quantitative (q)-RT-PCR analysis of relative RNA expression of ephrins and Eph receptors in either CD45⁻/CD144⁺ cerebral vascular endothelial cells (cvECs) sorted from WT sham and CCI-injured cortices at 1 dpi by FACS or from pure primary brain endothelial cultures from naïve animals. Data expressed as ΔCt expression (2⁻ΔCt) normalized to GAPDH. EphrinB3 expression is significantly reduced after CCI injury and further downregulated in cultured cells compared to sham. Statistical Analyses: One-way ANOVA with Newman-Keuls multiple comparison post-hoc test where P < 0.05 equals significance. N-values: Sham n=4, CCI n=3 (both run in triplicates); cultured cells n=3 (from 6 grouped animals). * p<0.05, ***p<0.001 as compared with cvECs sorted from WT sham cortices.
on Fig. a and d). The surface-tracing feature of Imaris 3D image analysis software was used to compute vessel area and volume fraction on six non-overlapping fields at the injury penumbra of CCI-injured animals (Fig. 3-2 d-e and f) and corresponding regions

Figure 3-2. Cortical vessel density is reduced acutely after CCI injury. Confocal images of Cdh5-zG-WT sham (a-b) and CCI-injured cortex (d-e) at 3 dpi. Dashed border depicts quantification region. Cdh5 vessels (green) in examples of six non-overlapping fields (60X) for both sham (b) and CCI-injured (e) brains. Imaris 3-D imaging software surfacing feature showing computational overlay surfacing (white) of cortical penumbra vessels (c, f). Vessel density fraction quantification (area/(100 μm)²) reveals a significant injury-induced decrease in vessel density of WT mice at 3 dpi, which is independent of ephrinB3 or EphB3 (g). No significant changes in vessel volume (volume/(100 μm)³) were observed at 3 dpi (h).

Statistical Analyses: One-way ANOVA with Bonferroni’s multiple comparison post-hoc test where P < 0.05 equals significance. N-values: WT sham n=10 and CCI n=12; EphB3⁻/⁻ sham n=8 and CCI n=10; ephrinB3⁻/⁻ sham n=5 and CCI n=7. * p<0.05, **p<0.01 as compared with corresponding sham for each genotype.
on sham controls (Fig. 3-2 a-b and c). Quantification of vessel area fraction (per 100 squared micron) revealed a significant decrease in vessel density compared to sham controls; however, no differences were observed in EphB3−/− and ephrinB3−/− mice as compared to WT animals (Fig. 3-2 g). Vessel volume assessment (per 100 cubed micron) did not reveal significant changes either after CCI injury compared to sham controls, or across genotypes, at least for the 3-day time point analyzed (Fig. 3-2 h).

3.2.3 The absence of EphB3 results in increased cvEC numbers after CCI injury but has no effect on cvEC or EPC injury-induced proliferation.

To provide additional confirmation for our vessel density assessment we used a more sensitive flow cytometric approach to quantify cvECs numbers in the injured cortex of WT, EphB3−/− and ephrinB3−/− mice. Using BD TruCounts as a single platform technique for cell quantification (see chapter 5), we observed a significant decrease in the number of cvECs in both WT and ephrinB3−/− CCI-injured cortices as compared with their respective sham controls at 3 dpi (Fig. 3-3 a), which is consistent with the previously observed reductions in vessel density. Interestingly, we did not observe a significant reduction in EphB3−/− mice, which was a significant increase as compared with WT CCI injured mice (Fig. 3-2 a). This suggests that EphB3 may participate in the regulation of cvEC numbers after CCI injury by affecting cvEC proliferation and/or survival. Both members of the B-class of ephrins and Eph receptors have been shown to play anti-proliferative roles in the CNS (Ricard et al., 2006, Theus et al., 2010, del Valle et al., 2011). To examine changes in cvEC and EPC proliferation after CCI injury, WT, ephrinB3−/− and EphB3−/− mice were pulsed with 50 mg/Kg EdU for 3 days following injury onset and examined by flow cytometry at 3 dpi also using TruCount methods.
Assessment of the residential cvEC population revealed a significant increase in the percent of EdU+ cells after CCI injury across all 3 genotypes compared to their corresponding sham controls; however, no differences were observed in the absence of either EphB3 or ephrinB3 (Fig. 3-3 b). We also assessed whether proliferation of the EPC

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Figure 3-3. EphB3 affects cvEC numbers but not proliferation acutely after CCI injury. TruCount flow cytometry quantification of cortical cvECs (as CD45-/CD144+) and infiltrating EPCs (CD309+/CD133+) at 3 dpi in WT, EphB3−/− and ephrinB3−/− mice (a and c). Quantification of cvECs reveals a significant decrease in cell number in CCI-injured compared to sham controls, but a significant increase in the absence of EphB3 (a). EPC numbers are significantly increased at 3dpi but no difference is observed across genotypes (c). Flow cytometry quantification of EdU+ cortical cvECs at 3 days after CCI injury in WT, EphB3−/− and ephrinB3−/− mice (b and d). %EdU+ cells were calculated as a ratio of the total CD45+/CD144+ population for cvECs (b) or CD309+/CD133+ for EPCs (d). No significant differences in %EdU+ cells were observed across genotypes although CCI injury resulted in dramatic increases in proliferation in both cvECs (b) and EPCs (d) compared to sham controls. Statistical Analyses: One-way ANOVA with Newman-Keuls multiple comparison post-hoc test. P < 0.05 was considered significant. N-values: WT sham n=12 and CCI n=15; EphB3−/− sham n=5 and CCI n=6; ephrinB3−/− sham n=14 and CCI n=15. * p<0.05, ** p<0.01, *** p<0.001 as compared with corresponding genotypic sham controls. # p<0.05 as compared with WT CCI 3 dpi.
population was affected in the presence or absence of ephrinB3 or EphB3. Our results demonstrate a significant increase in both total numbers (Fig. 3-3 c) and proliferation (Fig. 3-3 d) of infiltrating endothelial progenitors into the injured cortex at 3 dpi across all three genotypes. Once again, we observed no difference between genotypes in either total EPCs (Fig. 3-3 c) or in the percent of EdU+ progenitors (Fig. 3-3 d) compared to the WT controls. These results confirm that both mature and progenitor endothelial populations are expanding in the first 3 days after injury, but neither ephrinB3 nor EphB3 regulate this process.

3.2.4 The absence of EphB3 results in increased cvEC survival after CCI injury.

We next examined whether EphB3 could regulate cvEC survival. Our lab has previously shown that EphB3 receptors can induce cell death of several cell types after CNS injury via dependence receptor functions (Theus et al., 2010, Theus et al., 2014, Tsenkina et al., 2015). We performed stereological quantification of TUNEL+ cvECs at 1 dpi on WT and EphB3−/+ brain sections immunostained with the endothelial marker Glucose transporter (Glut)-1. Immunohistochemical confocal images reveal a visible decrease in total overall TUNEL+ cells (red) in the absence of EphB3 (Fig. 3-4 a-b) as well as less TUNEL+ cvECs (green) (Fig. 3-4 c-d). Quantification of TUNEL+ cells on Glut-1+ (green) vessels consistently shows a significant increase in cell death in the injured brains compared to shams for both genotypes. Comparison between WT and EphB3−/+ injured brains reveal a significant reduction in Glut-1+/TUNEL+ cells at the injury penumbra in the absence of EphB3 (Fig. 3-4 e), suggesting a novel cell death role for this Eph receptor in cvECs after CCI injury.
3.2.5 VEGF-induced increase in cvECs is independent of either EphB3 or ephrinB3.

Eph and ephrins have been shown to modulate VEGFR-2 internalization and downstream signaling in the presence of VEGF (Sawamiphak et al., 2010). In these studies we wanted to assess whether the VEGF induced increase in endothelial cell numbers and proliferation observed in chapter 2 is modulated by ephrinB3 and/or EphB3. VEGF-A (0.03 mg/Kg) filled osmotic pumps were implanted on the ipsilateral cortex of WT, ephrinB3<sup>-/-</sup> and EphB3<sup>-/-</sup> CCI-injured mice at the time of injury, and vehicle (PBS)-infused WT animals were used as controls. The injured cortex was processed for flow cytometry analysis at 7 dpi using the same gating strategy as described previously with

Figure 3-4. EphB3 affects cvEC survival acutely after CCI injury. Immunohistochemistry of TUNEL<sup>+</sup> (red) and Glut-1<sup>+</sup> cvECs (green) at 1dpi of WT (a, c) and EphB3<sup>-/-</sup> brains (b, d) reveal overall reduced TUNEL staining in the absence of EphB3 (a-b) as well as less double labeled TUNEL<sup>/</sup>/Glut-1<sup>/</sup> cvECs compared to WT (c-d). Stereological quantification of TUNEL<sup>+</sup>/Glut-1<sup>+</sup> cvECs at 1dpi shows a significant decrease a in the number of TUNEL<sup>+</sup> cvECs in EphB3<sup>-/-</sup> mice compared to WT CCI injured animals (e) as seen in higher magnification panels (c-d, f). Statistical analysis: One-way ANOVA with Bonferroni’s post-hoc test. P < 0.05 was considered significant. N-values: shams n=3 and CCI groups n=6. ** p<0.01, *** p<0.001 as compared with corresponding genotypic sham controls. # p<0.05 as compared with WT CCI 1 dpi. Abbreviations: Ctx, cortex; Hipp, hippocampus.
TruCount beads. Our results show that infusion of VEGF-A significantly increased the number of CD45+/CD144+ mature cvECs in all three genotypes compared to vehicle-infused WT controls. When comparing the knockout VEGF-infused brains with WT; however, we did not observe any significant differences in cvEC numbers. This suggests that neither the ephrinB3 ligand nor the EphB3 receptor modulate VEGF-induced expansion of the endothelial population after CCI injury.

Figure 3-5. VEGF-induced increase in cvECs is independent of either EphB3 or ephrinB3. Flow cytometry quantification of CD45+/CD144+ cvECs in WT, EphB3+/− and ephrinB3+/− mice infused with VEGF165 for the first 7 days after CCI injury. VEGF infusion results in a significant increase compared to vehicle but no differences were observed across genotypes. Statistical Analyses: One-way ANOVA with Bonferroni's post-hoc test with 95% confidence interval. N-values: WT vehicle n=8, WT VEGF n=6, n=3 for both VEGF-infused knockout groups. * p<0.05, ** p<0.01, as compared with CCI-injured Vehicle-infused controls.

3.3 Discussion

These studies examined the role of ephrinB3 and EphB3 in the mouse cerebral vasculature following a moderate CCI injury paradigm. Our studies reveal that both ephrinB3 and EphB3 mRNA are expressed in sham and CCI injured cvECs. CCI injury
resulted in a significant decrease in vessel density at the injury penumbra in WT, EphB3\(^{-/-}\) and ephrinB3\(^{-/-}\) mice; however, greater numbers of cvECs were preserved in the EphB3\(^{-/-}\) injury penumbra as compared with WT mice. Increased cvEC numbers resulted from reduced cell death and not increased proliferation, suggesting that CCI injury may lead to EphB3-mediated cell death in mature cvECs. EphB3 has been shown to function as a pro-apoptotic “dependence” receptor in other CNS cell types after CCI injury (Theus et al., 2014, Tsenkina et al., 2015). We also found that the previously observed VEGF-induced expansion of both EPCs and cvECs is not ephrinB3- or EphB3-dependent. In summary, our studies suggest that EphB3 may interact with ephrins to regulate vessel stability and potentially BBB integrity after TBI.

Cerebral vascular injury is a very common feature after TBI, prevalent in nearly all patients, and contributes to functional deficits that result in chronic disability (Kenney et al., 2016). In addition to humans, CCI studies in rats have also shown dramatic vascular deficits and a significant reduction in vessel density of the injured cortex (Chen et al., 2003). Our results are consistent with these findings where cortical vessels and cvECs are decreased at the injury penumbra as compared with sham controls. Though damage to the vessels alone is not responsible for all of the pathophysiology following TBI, it does influence and contribute greatly to the characteristic secondary injury component resulting from the trauma (Villapol et al., 2014). Secondary injury occurs in the first hours after the initial shock and can persist for months, resulting in neurochemical, metabolic, and cellular changes (Kumar and Loane, 2012). Brain swelling, intracranial hemorrhage, increased pressure, reduced oxygen circulation and release of cytokines that promote inflammation are only some examples of secondary injury that lead to cell death.
as consequences of leaky and damaged blood vessels after TBI (Pearn et al., 2016). Cerebral vascular disruption is a characteristic feature of several other models of CNS injury, especially stroke. In this model, vascular pathology develops in the neurovascular unit just before and/or during a stroke and progresses throughout ischemia and reperfusion ultimately culminating in tissue damage and cell death (Zhang et al., 2012). One of the B-class members of ephrins and Eph receptors, ephrinB3 has been shown to play important post-ischemic roles in a stroke model where its absence resulted in enhanced brain injury. Though the vasculature was only grossly analyzed in these animals, ephrinB3−/− mice showed significantly increased ischemic injury with enhanced infarct volumes. This phenotype was accompanied by reduced functional recovery and motor coordination deficits compared to WT controls, that persisted up to 4 weeks after cerebral ischemia (Doeppner et al., 2011). In our TBI model, we did not observed significant changes in vessel density or cvEC numbers at acute time points in the absence of ephrinB3 compared to WT. Although, we cannot rule out the possibility that ephrinB3 may have a more chronic role in the CCI-injured vasculature. Furthermore, functional deficits in motor coordination were not assessed in ephrinB3−/− mice due to the well-known hopping phenotype (simultaneous movement of both hind legs) as a result of developmental defects in spinal reflex and corticospinal tracts (Kullander et al., 2001, Kullander et al., 2003). In a different study, deletion of the EphA2 receptor showed an opposite role in a murine ischemic stroke-induced brain inflammation where the presence of the receptor directly contributed to BBB damage and neuronal death (Thundyil et al., 2013). This suggests that members of the ephrin/Eph receptor family may have diverse functions in regulating brain vasculature and additional studies are warranted. In our CCI
injury model, we observed a detrimental role for EphB3 at acute time points where its absence resulted in increased cvECs as a result of reduced cell death. Our studies are the first to report a cell death role for EphB3 in the brain vasculature following TBI providing new direction for potential mechanistic intervention to help prevent vascular loss after CNS injury.

EphB3 has also been shown to play a cell death role in other cells of the CNS including cortical neurons and spinal oligodendrocytes. Neurons in the adult mouse cortex showed reduced TUNEL staining after TBI in EphB3−/− mice, which correlated with functional improvements in motor deficits after CCI injury (Theus et al., 2014). Similarly, fewer oligodendrocytes were shown to undergo cell death following spinal cord injury in mice in the absence of EphB3 receptors, also resulting in improved locomotor behavior in the EphB3−/− as compared with wild type mice (Tsenkina et al., 2015). Our findings that EphB3 functions as a pro-apoptotic receptor is consistent with previous studies, suggesting that cvECs may undergo dependence receptor-mediated cell death after CCI injury. Dependence receptors are activated in conditions of reduced ligand interactions, where they function as caspase substrates. Caspase binding leads to proteolytic cleavage of the receptor, and subsequent apoptotic cell death (Mehlen and Bredesen, 2004, Furne et al., 2009). Reduced ligand availability can result either from injury-induced changes in ligand expression levels and/or compromised ligand-receptor cell-cell contact. In the case of Eph-ephrin interactions, where both ligands and receptors are membrane bound, cell-cell contact is crucial for adequate ephrin ligand-mediated activation of Eph receptors. After TBI, mechanical forces resulting from the initial impact are known to disrupt paracellular communication (Greve and Zink, 2009), thus
interfering with Eph-ephrin signaling and creating a suitable environment for Eph
dependence receptor-mediated cell death to occur (Furne et al., 2009). In addition to
tissues disruption, Theus and colleagues also showed that CCI injury also resulted in
reduced cortical expression levels of the ephrinB3 ligand, another prerequisite for EphB3
to function as a dependence receptor (Theus et al., 2014). Our qPCR analyses support
these reductions in cortical ephrinB3 expression after CCI injury, specifically in cvECs at
1 dpi compared with sham cvECs. Thus, alterations in the ligand-to-receptor ratios and
reduced cell–cell interactions that result from CCI injury support an environment for
EphB3-mediated cell death in the brain vasculature. Additional studies are needed to
confirm a dependence receptor role for EphB3 in cvECs after TBI. In particular,
ephrinB3 infusion may reverse the number of apoptotic cvECs after CCI injury
potentially leading to both improved vessel stability and BBB integrity. This is supported
by previous in vivo ephrinB3 infusion studies in both brain and spinal cord, where
addition of the ligand back into the system rescued the EphB3-induced cell death
phenotype. Osmotic infusion of ephrinB3 into the injured mouse brain resulted in
significant reductions in overall cortical TUNEL+ cells and TUNEL+ neurons as well as
reduced infarct volume, which translated into improved motor functions after CCI injury
(Theus et al., 2014). Similarly, EphB3-mediated oligodendrocyte cell death was also
blocked following administration of ephrinB3 to the injured spinal cord (Tsenkina et al.,
2015). These studies confirm a dependence receptor cell death role for EphB3 in both
neurons and oligodendrocytes after CNS injury, making this receptor a suitable
therapeutic target. Therefore, verifying a dependence receptor function for EphB3 in
cvECs will provide therapeutic implications for vascular repair in the injured brain as well as recovery potential for surrounding tissues.

In addition to cell death, both ephrinB3 and EphB3 function as anti-proliferative factors in the developing and injured CNS. In particular, ephrinB3 regulates neural stem/progenitor cell (NSPC) proliferation and survival in the adult subventricular zone (SVZ) through interactions with EphB3 (Ricard et al., 2006, Theus et al., 2010). EphB3 was also shown to limit the expansion of SVZ-derived NSPCs during development (del Valle et al., 2011) and in the TBI brain (Theus et al., 2010). In cvECs and EPCs, we found that ephrinB3 nor EphB3 regulate CCI-injury induced proliferation. Other mediators of vessel integrity (i.e. VEGF) that are known to increase EC survival and proliferation (Thau-Zuchman et al., 2012) have been shown to interact with other ephrin/Eph receptor family members. In particular, ephrinB2 was shown to be required for clathrin-dependent internalization and full signaling activity of VEGFR-2, upon binding of VEGF (Pitulescu and Adams, 2014). Similarly ephrinB2 was also shown to control VEGF-induced angiogenesis and lymphangiogenesis. Conversely, the absence of ephrinB3 or EphB3 has no effect on VEGF induced changes in cvEC proliferation in the CCI injured brain. This reinforces the diversity in signaling mechanisms regulated by different Eph-ephrin family members, especially in injured conditions. EphB4-ephrinB2 binding partners, for example, have been shown to be essential for vessel development in arterial-venal delineation (Adams et al., 1999) as well as in branching and repair under pathological conditions (Zhu et al., 2015, Lv et al., 2016). Our studies demonstrate additional roles in the vasculature for a different pair of Eph-ephrins, EphB3-ephrinB3, in EC survival and vessel integrity after CCI injury.
Chapter 4

EphB3 plays a detrimental role in blood-brain barrier (BBB) integrity after CCI injury

4.1 Introductory Remarks

The central nervous system (CNS) is innervated by an intricate network of microvessels, which are composed of tubular endothelial structures wrapped around by pericytes. Together with astrocytic end-feet and neuronal processes, these cvECs and pericytes form highly functional neurovascular units (NVUs), which establish the blood-brain barrier (BBB) (Quaegebeur et al., 2011). After TBI, one of the earliest and most profound deficits result from damage to the vascular network and breakdown of the BBB, which ultimately underlies much of the progressive pathophysiology associated with TBI (Greve and Zink, 2009). Unfortunately, little is known of the cellular signs and responses that regulate vessel integrity and/or remodeling in the injured brain. Our previous findings reveal a cell death role for EphB3 in cvECs after TBI suggesting that this receptor plays a critical role in vessel integrity in the CCI injured cerebral cortex; however, its role in BBB permeability has not yet been defined. BBB integrity is heavily dependent on an intact NVU where proper ensheathing of blood vessels by pericytes and astrocyte end-feet help to form a barrier and regulates blood flow (Dalkara and Alarcon-Martinez, 2015). Additionally, endothelial intercellular junctions form a tight vascular seal that prevents the passive diffusion of electrolytes and large proteins ensuring that all of the brain’s metabolic requirements are met via a constant blood and nutrient supply (Segura et al., 2009). These include both tight (TJ) and adherens junction (AJ) proteins such as VE-cadherin, PECAM-1, zona occludens (ZO)-1 and others. EphB receptor
tyrosine kinases and ephrinB ligands regulate several types of cell-cell interactions in the developing and adult brain, in both normal and pathological conditions, generally by modulating cytoskeletal dynamics (Park and Lee, 2015). Thus, the actin cytoskeleton and its binding proteins are a potential target for ephrin-Eph regulation of tight (TJ) and/or adherens junctions (AJ) that connect cvECs to properly seal the BBB. Ephrins and Eph receptors are membrane bound proteins that elicit bi-directional signaling upon cell-cell interaction, which make them good candidates for regulating membrane association between NVU cells and/or endothelial intercellular junctions. These studies examine the role of EphB3 and ephrinB3 in BBB integrity and permeability to peripheral cells after CCI injury. We demonstrate a detrimental role for both EphB3 receptor and its ligand ephrinB3 in post-trauma BBB integrity and permeability, which contributes to the loss in cortical vessel stability observed after TBI.

4.2 Results

4.2.1 Reduced protein permeability across the BBB in EphB3−/− mice after CCI injury.

Eph receptors have previously been shown to play a negative role in BBB integrity after CNS injury in a model of ischemic stroke (Thundyil et al., 2013). To examine whether EphB3 or its ligand ephrinB3 affects BBB integrity in our CCI injury model we employed an Evans blue (EB) brain tissue extravasation assay in WT, EphB3−/− and ephrinB3−/− mice. CCI injured mice were injected with EB at either 1 or 3 dpi and the amount of EB extravasation was measured using a spectrophotometer. CCI injury resulted in a significant increase in EB extravasation into the injured brains of all groups tested compared with sham controls at both 1 and 3 dpi (Fig. 4-1 a-c). Given the dark
intensity of the EB dye, differences in absorption cannot be visually observed in the absence of either ligand or receptor. However, quantitative assessment of EB extravasation from the injured cortex revealed a significant 20% and 30% reduction in EB absorption at 1 and 3 dpi, respectively, in EphB3−/− mice when compared with injured WT mice (Fig. 4-1 b-c). In ephrinB3−/− mice we observed a similar 40% reduction in EB absorption at 3 dpi but not at 1 dpi (Fig. 4-1 b-c). These studies show that EphB3 negatively influences BBB integrity after CCI injury that could involve interactions with ephrinB3.

4.2.2 EphB3 regulates macrophage infiltration in the acutely CCI injured brain.

We also examined peripheral cell infiltration using flow cytometry analysis and TruCount quantification (refer to Chapter 5) of CD45high/CD11b+ macrophages at 3 dpi in WT, EphB3−/− and ephrinB3−/− sham and CCI-injured mice (Fig. 4-2 a-f). We observed a significant increase in the total number of CD45+ infiltrating leukocytes in the cortex of WT animals at 3 dpi compared to sham controls (Fig. 4-2 a,d and g). These findings are consistent with the increases in CD45high population reported in Chapter 2 at the same time point. Increased CD45high/CD11b+ macrophage numbers were observed in all three genotypes when compared with their respective sham controls (Fig. 4-2 a-f, g). In EphB3−/− mice, a significant 38% fewer infiltrating CD45high/CD11b+ cells were observed at 3 dpi as compared with WT injured mice, but not in ephrinB3−/− injured mice (Fig. 4-2 g). This supports a novel role for EphB3 in peripheral cell infiltration following TBI induced BBB breakdown. To evaluate whether changes in macrophage infiltration resulted from proliferative differences in the knockout mice, we assessed the ratio of EdU+...
Figure 4-1. EphB3 plays a detrimental role in BBB integrity acutely after CCI injury. Representative images of sham and CCI (3dpi) injured brains 3 h following Evans blue (EB) injections (a). EB brain tissue extravasation assay reveals a significant increase in EB absorption of WT, EphB3−/− and ephrinB3−/− animals at both 1 and 3 days after CCI injury compared to sham controls (b-c). EB absorption is significantly reduced in CCI injured EphB3−/− mice compared to both WT and ephrinB3−/− animals at 1 dpi (b). At 3 dpi, EB absorption is reduced in both EphB3−/− and ephrinB3−/− mice compared to CCI-injured WT controls (c). Statistical Analysis: One-way ANOVA with Newman-Keuls multiple comparison post-hoc test, P < 0.05 was considered significant. N-values 1dpi (b): Sham groups n=5, CCI groups: WT n=13, EphB3−/− n=11, ephrinB3−/− n=15; 3dpi (c): WT sham n=11 and CCI n=14; EphB3−/− sham n=5 and CCI n=8; ephrinB3−/− sham and CCI n=6 each. * p<0.05, ** p<0.01, *** p<0.001 as compared with corresponding genotypic sham controls. # p<0.05, ## p<0.01 as compared between CCI groups at either 1 or 3 dpi.
CD45^{high}/CD11b^{+} cells at 3 dpi. We observed significant increases in macrophage proliferation after CCI injury across all three groups as compared with sham controls, but not between genotypes (Fig. 4-2 h). These findings suggest that the absence of EphB3 can directly reduce both protein and cellular infiltration across the BBB but not macrophage proliferation after TBI.

4.2.3 EphB3 participates in astrocytic but not pericytic membrane interactions with cvECs in the cerebral cortex.

Ephrins and Eph receptors are well known for their chemo-repulsive functions in the CNS in both naïve and injured states (Niclou et al., 2006). We investigated whether the ephrin/Eph expressing astrocyte and/or pericyte end-feet interact with Eph/ephrin expressing cvECs in the gliovascular unit using high-resolution confocal microscopy and membrane co-localization analysis. Six stereological non-overlapping confocal images were taken at the injury penumbra of sham and CCI injured WT, ephrinB3^{-/} and EphB3^{-/} Cdh5-zG mice co-labeled with anti-GFAP (i.e. astrocyte membranes) or anti-PDGFR\(\beta\) (pericyte membranes) antibodies. We observed a large amount of membrane interaction between astrocytes or pericytes (red) with Cdh5^{+} vessels (green) in both sham and CCI injured cortices (Fig. 4-3 a-h). In the WT CCI injury penumbra we observed significantly greater amount of membrane interactions for both astrocytes and pericytes at 3 dpi as compared with sham controls (Fig. 4-3 a-d, i-j). These results suggest that blood vessel ensheathing by cells of the gliovascular unit is increased acutely after TBI, most likely as a self-repairing mechanism to ‘patch’ the leaky BBB. Deficiencies in ephrinB3 or EphB3 in non-injured conditions did not alter the ability of pericyte membranes to interact with cvEC vessels (Fig. 4-3 j); however, GFAP-labeled membranes in astrocytes were
Figure 4-2. EphB3 negatively regulates immune cell infiltration after CCI injury. Flow cytometry histograms of infiltrating CD45^high/CD11b^+ macrophages in sham (a-c) and CCI-injured (d-f) WT (a,d), EphB3^-/- (b,e) and ephrinB3^-/- (c,f) mice at 3dpi (a-f) gated on viable/singlets. Flow cytometric TruCount quantification reveals a significant CCI-injury induced increase in macrophage infiltration (g) and proliferation (h) in all three genotypes compared to each genotypic sham control at 3 dpi. EphB3^-/- but not ephrinB3^-/- mice display a significant decrease in CD45^high/CD11b^+ cells compared to WT (g). No significant differences were observed between genotypes in percent EdU^+ cells out of total CD45^high/CD11b^+ population (h). Statistical Analysis: One-way ANOVA with Newman-Keuls multiple comparison post-hoc test, P < 0.05 was considered significant. N-values cell counts (g): WT sham, ephrinB3^-/- sham and CCI n=12, WT CCI n=14, EphB3^-/- sham n=8 and CCI n=9; %EdU (h): EphB3^-/- sham, ephrinB3^-/- sham and CCI n=8, WT sham n=10 and CCI n=12, EphB3^-/- CCI n=9. ** p<0.01, *** p<0.001 as compared with corresponding genotypic sham controls. # p<0.05, ## p<0.01 as compared with either EphB3^-/- or WT CCI, respectively.
significantly increased in EphB3<sup>−/−</sup> mice as compared with WT sham controls (Fig. 4-3 i). This suggests a novel role for EphB3 in regulating astrocytic wrapping of cortical blood vessels prior to adulthood. After injury, no further increases in GFAP-Cdh5 interactions were detected in either ephrinB3<sup>−/−</sup> or EphB3<sup>−/−</sup> mice as compared to their corresponding genotypic sham controls (Fig. 4-3 i). PDGFR<sub>β</sub>-Cdh5 interactions, on the other hand, were significantly increased after injury in both WT and ephrinB3<sup>−/−</sup> groups, although differences between genotypes were not significant (Fig. 4-3 j). These results demonstrate that ephrins and Eph receptors participate in the regulation of membrane ensheathing in cells of the gliovascular unit, where TBI increases glial-vascular membrane interactions in the cortical penumbra.

4.2.4 Differential expression of adherens (AJ) and tight junction (TJ) proteins in the presence and absence of EphB3 or ephrinB3 in the adult mouse cortex.

Tight and adherens junction proteins play distinct roles in BBB formation and are essential for the proper repair of the BBB (Tietz and Engelhardt, 2015). To examine how CCI injury impacted the AJ protein VE-Cadherin and TJ protein ZO-1, we examined expression in WT, EphB3<sup>−/−</sup> and ephrinB3<sup>−/−</sup> sham and CCI-injured mice at 1 and 3 dpi. We found that VE-Cadherin was significantly upregulated in non-injured ephrinB3<sup>−/−</sup> and EphB3<sup>−/−</sup> tissues as compared to WT sham mice. After CCI injury, VE-Cadherin expression was significantly increased by 3 dpi in WT mice as compared with sham controls. No differences were observed between genotypes at 1 or 3 dpi; however, VE-cadherin expression was decreased at 1 dpi in ephrinB3<sup>−/−</sup> tissues as compared to both ephrinB3<sup>−/−</sup> sham and CCI-injured 3 dpi groups (Fig. 4-4 a). We observed a similar trend in ZO-1 expression, where sham EphB3<sup>−/−</sup> and ephrinB3<sup>−/−</sup> tissues were increased as
Figure 4-3. Increased astrocytic-cvEC membrane association in the absence of EphB3.
Immunohistochemistry confocal images of sham and CCI-injured Cdh5-zG brains at 3dpi (a-h). High magnification panels show close association of red pericytes (PDGFRβ+) and astrocytes (GFAP+) with green Cdh5+ blood vessels at the injury penumbra or corresponding sham region (e-h). Split panels of Cdh5+ vessels (green) and either pericytes or astrocytes (red) shows increased membrane interaction after CCI-injury compared to sham controls (a-d). Coloc 2 FIJI-Image J quantification of astrocytic (i) and pericytic (j) membrane association with cvEC in sham and CCI injured (3 dpi) WT, EphB3-/- and ephrinB3-/- Cdh5-zG mice (i-j). Data presented as Mender’s colocalization coefficient normalized to WT sham. Astrocytic end-feet ensheathing is significantly increased after injury in WT mice but do not increase further in the absence of either ephrinB3 or EphB3 (i). Increased astrocytic membrane colocalization in the absence of EphB3 in sham animals (i). Pericytic ensheathing is significantly increased after injury in WT and ephrinB3-/- mice but not in EphB3-/- (j). Statistical Analyses: One-way ANOVA with Newman-Keuls multiple comparison post-hoc test, P < 0.05 was considered significant. N-values GFAP (i): WT and EphB3-/- sham n=8, WT CCI n=12, EphB3-/- CCI n=10, ephrinB3-/- sham n=5 and CCI n=6; PDGFRb (j): WT sham n=8 and CCI n=11, EphB3-/- sham n=7 and CCI n=10, ephrinB3-/- sham and CCI n=5 each. * p<0.05, as compared with corresponding genotypic sham controls.
compared with WT sham mice (Fig. 4-4 b). However, we did not observe significant
differences in ZO-1 expression at either 1 or 3 dpi in any genotype as compared to their
respective controls (Fig. 4-4b). These results suggest that while the AJ protein VE-
cadherin and TJ ZO-1 may be altered between WT and ephrinB3\textsuperscript{−/−} or EphB3\textsuperscript{−/−} mice in
sham conditions, neither protein contributes to our observed differences in BBB
permeability between genotypes after TBI.

4.3 Discussion

In these studies we examined whether the absence of EphB3 or ephrinB3 affects the
integrity of the BBB in both sham and CCI injured mice. We found that permeability of
the BBB to serum albumin was significantly reduced at 1 dpi in EphB3\textsuperscript{−/−} mice and at 3
dpi in both ephrinB3\textsuperscript{−/−} and EphB3\textsuperscript{−/−} animals compared to WT controls. Additionally, the
invasion of larger infiltrating peripheral cells across the BBB was also significantly
reduced in the absence of EphB3. Assessment of membrane colocalization revealed
increased pericytic and astrocytic membrane associations with cvECs/vessels at the injury
penumbra. EphB3 was shown to limit membrane interactions of astrocytic end-feet with
cvECs, although CCI injury did not lead to further enhancement of these associations.
Intercellular junction proteins VE-Cadherin and ZO-1 were also upregulated in the
absence of either ephrinB3 or EphB3 as compared with WT in sham mice, but CCI had
little to no effect on ZO-1 levels. Conversely, VE-cadherin was significantly increased by
3 dpi in WT mice but not in ephrinB3\textsuperscript{−/−} or EphB3\textsuperscript{−/−} mice from their sham controls. We
did observe a significant decrease in VE-cadherin at 1 dpi in ephrinB3\textsuperscript{−/−} mice. Altogether,
our studies reveal a detrimental role for EphB3 in BBB integrity and permeability after
Figure 4-4. Differential expression of intercellular junction proteins VE-Cadherin and ZO-1 in the absence of ephrinB3 or EphB3. Western blot analysis of sham and CCI injured cortical extracts from WT, ephrinB3<sup>−/−</sup> and EphB3<sup>−/−</sup> mice at 1 and 3 dpi. Both VE-Cadherin (top band, a) and ZO-1 (b) expression levels are higher in ephrinB3<sup>−/−</sup> and EphB3<sup>−/−</sup> sham animals compared to WT sham controls (a-b). After CCI injury, VE-cadherin increases significantly by 3dpi in WT mice but is downregulated in the absence of ephrinB3 at 1 dpi. (a). ZO-1 remains unchanged after CCI injury across the three genotypes (b). Statistical Analysis: One-way ANOVA with Newman-Keuls multiple comparison post-hoc test, P < 0.05 was considered significant. N-values: WT sham n=8, CCI 1dpi n=5 and 3dpi n=6, EphB3<sup>−/−</sup> sham n=6, CCI 1dpi n=4 and 3dpi n=3, ephrinB3<sup>−/−</sup> sham n=6, CCI 1dpi n=4 and 3dpi n=3. * p<0.05, ** p<0.01, *** p<0.001 as compared with WT sham controls. # p<0.05, ## p<0.01, as compared to ephrinB3<sup>−/−</sup> CCI 1dpi or ephrinB3<sup>−/−</sup> sham control, respectively.
CCI-injury, which is consistent with the loss in cortical vessel stability previously observed after TBI.

Our observation that EphB3 signaling negatively regulates BBB integrity after TBI, suggests that B-class ephrins and Eph receptors may participate in vessel stability and repair. The BBB is regulated by neurovascular and gliovascular units that involve direct associations between cvECs with both pericytes and astrocytes (Abbott et al., 2006, Wolburg et al., 2009a). Armulik and colleagues showed that pericytes induce polarization of astrocytic processes surrounding CNS blood vessels by expressing specific cues that mediate the attachment of astrocytic end-feet to the vasculature (Armulik et al., 2010). Considering these findings, our observed increases in astrocytic membrane association with blood vessels at the injury penumbra may result from increased pericytic membrane interactions. It may be arguable that the increases in either pericytic or astrocytic membrane interactions may be a result of injury-induced increases in PDGFRβ or GFAP protein expression. This possibility would have to be tested experimentally, potentially by using transgenic mice with fluorescently tagged pericytes or astrocytic end-feet combined with labeled cortical vessels. However, our observations of similar levels of GFAP expression in wild type and EphB3−/− CCI injured reduce this possibility. Interestingly, our studies show that EphB3 regulates astrocytic but not pericyte interactions with cvECs suggesting that these processes may be controlled independently. A number of proteins are known to regulate pericyte and astrocyte attachment to cerebral capillaries, although the specific cellular mechanisms are not completely understood. Both astrocytes and pericytes have been shown to influence the formation of new vessels during retinal development by affecting the composition of the extracellular matrix (ECM) (Bonkowski
et al., 2011). For example, the ECM component heparin sulfate proteoglycans (HSPGs) are known to interact with a plethora of ligands, including ephrins (Irie et al., 2008, Holen et al., 2011, Sarrazin et al., 2011). HSPGs contain one or more covalently attached heparin sulfate (HS) chains and are known to play several roles in cell adhesion, as well as in the determination of cell shape by modulating the links between the ECM and the actin cytoskeleton (Sarrazin et al., 2011). Interestingly, ephrinB3 has the ability to bind to heparin with a relatively good affinity as well as to cell-surface HSPGs. This interaction results in a repulsive effect directly affecting cell adhesion and spreading (Holen et al., 2011), which is important in cell migration. However, ephrinB3 binding to HSPGs in the endothelial ECM after CNS injury may interfere with proper re-attachment of pericytes and astrocytic end-feet. Thus, the increased perivascular cell interactions with cortical blood vessels after CCI injury may result from decreased ephrinB3 expression on cvECs. In other words, it is possible that EphB3-ephrinB3 signaling between neighboring cvECs and astrocytic end-feet is mediated by an inhibitory regulation of HSPGs in the ECM, where the absence of either the ligand or the receptor would result in more extensive cvEC-astrocyte membrane association as well as endothelial intercellular junctions.

Ephrins and Eph receptors are both membrane-anchored proteins, which make them attractive candidates for cell-cell interactions in the gliovascular unit. B-class Eph receptors and ephrins have been shown to regulate cell structure through the modulation of cytoskeletal dynamics (Park and Lee, 2015). The increased astrocyte-cvEC membrane interactions in EphB3-/- sham animals suggest a potential developmental role for Eph-ephrins in astrocytic end-feet ensheathing of cortical blood vessels. Eph-ephrin signaling regulates cytoskeletal organization, where Eph receptor signaling can lead to actin de-
polymerization and membrane retraction (Zimmer et al., 2003, Groeger and Nobes, 2007). This may be a way in which EphB3 contributes to BBB permeability in the CNS, where its reduced expression, or absence as in the case of EphB3\(^{-/-}\) animals, could potentially provide a pre-injury protection, and thus reduced vascular damage following TBI. However, we do not observe differences in BBB permeability to macromolecules or cell infiltration between WT and knockout non-injured mice. Furthermore, after CCI injury we observed differential regulation of macromolecules and cell infiltration between genotypes, that is more likely the result of injury-induced temporal changes in ligand-receptor expression.

Another member of the Eph family, EphA2, was shown to exacerbate ischemic brain injury in a mouse model of focal stroke, where EphA2\(^{-/-}\) mice showed reduced post-stroke BBB leakage, decreased brain edema and infiltrating immune cells (Thundyil et al., 2013). Comparably, EphA2 activation of in cultured human brain microvascular endothelial cells led to TJ disassembly, whereas Eph receptor inactivation promoted TJ formation (Zhou et al., 2011). The protection by EphA2 deletion observed by Thundyil and colleagues was also associated with regulation of intercellular junction proteins, where the expression of ZO-1 was significantly higher in both sham and injured brains compared to WT controls (Thundyil et al., 2013). Similarly, our studies also revealed higher expression levels of these endothelial junction proteins in non-injured ephrinB3\(^{-/-}\) and EphB3\(^{-/-}\) mice compared to WT. It is possible that ephrinB3-EphB3 signaling may play a developmental role in the regulation of endothelial paracellular junctions, potentially via HSPGs and the ECM, where their absence would result in the higher expression of these intercellular junctions proteins as observed in our studies. However,
the higher pre-injury levels of VE-cadherin and ZO-1 in both ephrinB3\(^{-/-}\) and EphB3\(^{-/-}\) animals does not seem to affect the injury-induced changes in BBB permeability to infiltrating macrophages, which is reduced in the absence EphB3 but not ephrinB3. Compiling evidence demonstrate the ability of Eph-ephrin family members to associate with integrins, cadherins, and claudins, which are involved in the regulation of intercellular permeability and cell adhesion (Arvanitis and Davy, 2008). Claudins, the most abundant TJ proteins, can directly bind to Eph and ephrins and regulate paracellular transport (Tanaka et al., 2005a, b). Claudin-4 has been shown to bind to both ephrinB1 (Tanaka et al., 2005b) and EphA2 (Tanaka et al., 2005a) and the interaction between these proteins affected intercellular adhesion and led to reduced TJ integration resulting in increased paracellular permeability. Claudin-5 was also shown to be required for ephrinB1 localization in ECs of the human heart (Swager et al., 2015). We’ve shown that the absence of either ephrinB3 or EphB3 affects endothelial intercellular junctions resulting in increased expression in the mouse brain. Although a specific interaction between ephrinB3 or EphB3 with claudins or cadherins in the brain microvasculature has not yet been reported, our findings suggests this as a possible mechanism. In addition to ZO-1, another TJ scaffolding protein partitioning defective phenotype (Par)-6 also associates with B-class ephrins resulting in the loss of TJs (Lee et al., 2008). Thus, investigating claudin-5, Par-6, and ZO-1/ephrinB3 or EphB3 protein complexes to further understand ephrinB3-EphB3 mechanistic regulation of vascular integrity after TBI represent a promising target for new therapeutic strategies.
Chapter 5

A quantitative flow cytometric method for the simultaneous analysis of TBI induced temporal changes of distinct cell populations in the mouse cortex

5.1 Introductory Remarks

TBI is a progressive disorder that leads to dynamic temporal changes in different cell populations. These changes often result in significant neurological deficits that are initiated at the time of injury by the external mechanical forces to the brain. Early cell death is followed by the expansion of certain cell types accompanied by peripheral cell infiltration, which results from damaged blood vessels and BBB breakdown. This complex environment makes quantitative analysis of specific cell populations very challenging as total numbers and cellular composition between sham and CCI injured tissues are completely different. Flow cytometry is a sensitive and reliable technique that has been used for decades primarily in the field of immunology (De Rosa et al., 2001, Perfetto et al., 2004), but has recently been implemented by multiple fields including neuroscience. The use of light scatter properties to measure relative size and internal complexity of particles of interest allows for detailed measurements of diverse cell populations simultaneously within specific tissues. Cell viability, DNA content and protein expression can also be assessed with the use of fluorescently labeled dyes or antibodies (Lugli et al., 2010, Bendall et al., 2012). Flow cytometry can therefore be a powerful tool to analyze the temporal flux of multiple cell types at a given time point in the progressive TBI pathology. Data interpretation however, is heavily dependent on the gating strategy utilized by the experimenter (Mair et al., 2016). Thus, without proper cell identification and accurate quantification of the target population, flow cytometry can
also lead to misleading or erroneous results. To overcome these challenges, appropriate controls and optimized computational strategies are essential to demonstrate the relative contributions of each cell type to CNS injury. In this study, we use TruCount tubes that contain a set number of beads per tube to compute total number of cells/μL as opposed to percentages of capped events. TruCount quantification strengthens our understanding of the environmental contributions that lead to temporal differences in cell numbers. Together, these new and improved parameters provide an adequate and powerful flow cytometric assessment of cellular dynamics in TBI tissues.

5.2 Results

5.2.1 Leukocyte common antigen (CD45) is a good exclusion marker as CCI injury leads to differential changes in the CD45⁻ and CD45⁺ sub-populations of cells in the cortex.

To begin assessing cells in the cortex of CCI-injured versus non-injured animals by flow cytometry we used a live/dead fixable near-IR (L/D) stain to include only viable cells in our analysis. To exclude cellular debris, the dissociated cells for each sample were gated on a forward by side scatter plot (Fig. 5-1 a) and single cells (i.e., singlets) were chosen from a homogenous population based on size and granularity. A clear and reproducible separation was observed between the viable (left gated, L/D stain negative) and non-viable (right peak, L/D stain positive) cells, showing viability of over 80% of the singlet population (Fig. 5-1 b). In addition to the L/D stain, DAPI was also applied following fixation in order to provide confirmation that the viable cells are nucleated. We found that nearly all (97%) of the viable singlets were DAPI⁺ (Fig. 5-1 c). To examine injury-induced changes in a specific cell population, we used a conventional endothelial cell
(EC) marker CD31 (or PECAM-1) to label ECs in both sham and CCI injured animals. FITC-conjugated CD31 antibody labeled a large population of viable cells in CCI injured brains; however, no differences in the percent of viable CD31+ cells were observed at 7 dpi compared to non-injured controls (Fig. 5-1 d). This result did not support our previously observed visible losses in cortical cvECs at the injury epicenter (Fig. 2-1). Although this is a very common EC marker, there is evidence that some cell specific markers may cross react with other cell types after injury (Jin et al., 2012). In fact, CD31 is also known to be expressed by hematopoietic cells, such as monocytes and macrophages that infiltrate the injured brain. To overcome this possibility, we used CD45

Figure 5-1. CCI injury leads to differential changes in the percent of CD45- and CD45+ subpopulations of cells in the cortex. Flow cytometry forward by side scatter plot showing exclusion of cellular debris (a). Viable (b) and nucleated (c) cells were selected for using a live/dead stain followed by DAPI staining. No difference was observed in viable CD31+ (PECAM-1) cells between sham and CCI-injured mice at 7 dpi; however, excluding CD45+ cells from the analysis results in a significant decrease in CD45-/CD31+ cvECs (d). CCI injury increased the percent of CD45high (infiltrating leukocytes) and CD45low (residential microglia), while reducing the population of CD45- cells (e-h). Statistical analysis: unpaired two-tailed Student’s t-test with 95% confidence interval comparing CCI group to corresponding sham for each time point. Different time points after CCI injury were compared using One-way ANOVA with Bonferroni’s post-hoc test. N-values: n=3 biological replicates. * p<0.05, ** p<0.01, *** p<0.001 as compared with sham or non-injured mice.
(leukocyte common antigen) as an exclusion marker to eliminate the infiltrating leukocytes and residential microglia from our analysis. Re-examination of our results revealed a significant decrease (54%) in CD45/CD31+ ECs after injury compared with sham controls (Fig. 5-1 d), highlighting the importance of using appropriate exclusion markers to accurately label particular cell populations in injury conditions. Next, to evaluate the extent of cell infiltration in the injured cortex, we assessed the differences in the CD45+ and CD45- cell populations at 3 and 7 dpi compared to sham animals (Fig. 5-1 e–h). In non-injured conditions, we found that >67% of the viable cells are CD45-; while approximately 31% are CD45low (microglia) and only <2% are CD45high (infiltrating leukocytes) (Fig. 5-1 e). At 3 dpi, there is a 53% decrease in the CD45low population accompanied by a 13% increase in CD45high cells (Fig. 5-1 f). By 7 dpi, the percent of CD45high cells returns to nearly non-injured levels, although the CD45low population remains high (Fig. 5-1 g). Overall, we observed a dramatic decrease in viable CD45- cells contrasted by a significant increase in CD45+ cells compared to non-injured controls at both time points (Fig. 5-1 h).

5.2.2 Testing antibody specificity and cross-reactivity in the CCI-injured mouse cortex using flow cytometry to identify injury-induced changes in the endothelial cell population.

To improve our labeling of cerebral vascular endothelial cells (cvECs) in the CCI injured cortex we compared two EC markers, CD31 or CD144. We used a PE-Cy7-conjugated CD45 antibody to measure the percent of cross reactivity of either EC marker with the CD45+ population in CCI-injured cortex using flow cytometry (Fig. 5-2 a-b). Our results reveal that at 3 dpi, a time when CD45+ leukocytes are abundantly present in
the injured brain, CD31 cross reacts with nearly 33% of the viable CD45^+ population (Fig. 5-2 a), whereas CD144 shows <4% cross-reactivity with CD45^+ cells (Fig. 5-2b). Thus, CD144 is a more specific marker for cvECs in the CCI-injured mouse cortex as compared with CD31. Scatter plot shows a distinct population of CD45^-/CD144^+ cvECs in the CCI injured brain at 7 dpi (Fig. 5-2 c) compared to its isotype control (Fig. 5-2 d). This cell marker combination and gating strategy was used to label the mature residential cvEC population throughout our studies.

Figure 5-2. VE-Cadherin (CD144) is a better marker for cvEC identification than PECAM-1 (CD31) in the CCI-injured mouse cortex. Flow cytometry histograms of CCI injured brains from WT mice gated on viable/CD45^+ cells (a-b). After injury, ~33% CD45^+ cells express CD31 (a), which is significantly reduced (<4%) in the CD144^+ population (b). Scatter plots depict a clear separation of CD45^-/CD144^+ cvECs (c), and the isotype control antibody (d). N-value: n=3 biological replicates.
5.2.3 Identification of endothelial progenitor cells (EPCs) in the injured mouse cortex by flow cytometry.

To improve the accuracy and specificity EPC identification, CD133 (prominin-1) was used as a progenitor marker. Using a phycoerythrin (PE)-conjugated anti-CD133 antibody, we also examined PE intensity levels compared to the corresponding isotype control in the presence and absence of blood. PE is a bright fluorescent dye with a high staining index; however, excessive amounts of red blood cells (RBCs) in injured tissue can potentially affect our PE analysis. This is particularly true when labeling small cell populations with low antigen expression, such as the CD133 in the EPC population, where PE fluorescence (572nm wavelength) has a similar absorbance wavelength to RBCs (600nm wavelength). We observed a clear separation of the CD133 population from its corresponding isotype control (Fig. 5-3 a-b), but no significant differences were seen in the background of the PE channel between the non-perfused (Fig. 5-3 a) and the perfused (Fig. 5-3 b) brains. The lower peak height observed in the perfused sample is a result of fewer CD133+ EPCs present in the sample given that circulating EPCs in blood are removed with perfusion; however, this did not affect the fluorescence intensity confirming that the levels of RBCs in the brains do not significantly dim the PE intensity in our samples. To narrow our population down to vascular progenitors, CD309 (VEGFR-2), was used as a secondary marker. Flow cytometry scatter plots of injured brain tissue reveal the CD309+/CD133+ EPC population (Fig. 5-3 c) compared to the isotype control (Fig 5-3 d). The CD309+/CD133+ cells were gated out of the CD45–/CD144– population to exclude any progenitor cells of the glial lineage as well as endothelial cells that have differentiated into the mature phenotype. Therefore, EPCs
were identified as CD45⁻/CD144⁻/CD309⁺/CD133⁺ cells. We found that although this EPC population represents a very small percentage of the total endothelial cell population in the mouse brain (only approximately 1% of the total viable CD45⁻ population), the percentage of EPCs was nearly doubled after CCI injury (Fig. 5-3 e).

Figure 5-3. Identification of endothelial progenitor cells (EPCs) in the mouse cortex by flow cytometry. Flow cytometry overlay histogram of PE-conjugated CD133⁺ EPCs (gray) in non-perfused brains (gated on viable/CD309⁻ cells) has a MFI of 4.6 with 11.8% overlap with its corresponding isotype control (white, a). The overlay histogram of PE-conjugated CD133⁺ EPCs (gray) in perfused brains (gated on viable/CD309⁻ cells), with a MFI of 5.9 and 9.9% overlap with its corresponding isotype control (white), is not significantly different from non-perfused tissues (b). Flow cytometry scatter plot showing separation of CD309⁺ (VEGFR-2)/CD133⁺ (Prominin-1) EPCs gated on viable CD45⁻/CD144⁻ cells (c) compared to corresponding isotype control (d). Quantification of the percent EPCs gated on CD45⁻ cells at 7 dpi shows a significant increase compared to non-injured controls (e). Statistical analysis: unpaired two-tailed Student's t-test with 95% confidence interval. N-values: n=3 biological replicates. *** p<0.001 as compared with non-injured mice.
5.2.4 TruCount beads provide an accurate method for quantifying cortical cell numbers after CCI injury.

When comparing the percent CD45⁻/CD144⁺ cells in the ipsilateral injured cortex to the non-injured contralateral side, there’s a conflicting trend toward an increase in the cvEC population (Fig. 5-4 a). To ensure that we have reproducible and accurate counts of our cell populations of interest, we quantified the number of cells per volume of sample using standard percent of total population versus quantitative TruCount analysis. TruCount tubes contain an absolute number of fluorescent beads per tube, thus allowing us to calculate the number of cells per volume in any given sample. By simply gating on the beads, we can compute the number of fluorescent beads analyzed per total events in each sample (Fig. 5-4 b). The volume analyzed (Vₐ) can then be computed by dividing the number of beads analyzed by the total number of beads in the tube multiplied by the total sample volume. Once the Vₐ is known, the number of cells per volume of sample (cells/μL) can finally be quantified by dividing the number of events within each desired population (i.e. gate) by the Vₐ. TruCount quantification of the same CD45⁻/CD144⁺ cvEC population is now significantly decreased at 7 dpi compared to the contralateral hemisphere when analyzed as either fold change from control (Fig. 5-4 c) or actual cells/μL (Fig. 5-4 d). These findings are consistent with the observed reduction in cvECs following CCI injury (Fig. 2-1). Minor discrepancies are observed when analyzing cell populations, such as infiltrating CD45⁺ cells, where the percent, fold change or TruCount cell numbers are all dramatically increased as a result of CCI injury (Fig. 5-4 e–g). Cells that represent smaller injury-induced changes compared to sham populations are more vulnerable to the misrepresentations resulting from percentage comparisons. We also
tested this difference in method of analysis in residential non-infiltrating and non-proliferating cortical neurons. Analysis of cortices from Thy-1-YFP transgenic mice showed a distinct population of YFP⁺ cells gated out of viable CD45⁻ singlets (Fig. 5-h).

**Figure 5-4. TruCount beads provide an accurate method for quantifying cortical cell numbers after CCI injury.** Flow cytometry quantification of percent CD144⁺ cvECs in the ipsi cortex was unchanged compared to contralateral (a). Scatter plot of TruCount beads (red) shows a distinct separation from cells (b). Quantification of CD144⁺ cvECs using TruCount beads revealed a significant decrease in both the fold change (c) and actual cell numbers (d) compared with the contralateral cortex. Analysis of the CD45⁺ cell population shows a similar increase in the ipsilateral injured cortex as compared with the contralateral cortex using percent change (e) and fold change based on TruCount beads (f) or cell number (g) analysis; however, the values are still different between methods. Scatter plot of Thy-1-YFP fluorescing neurons (green) show distinct population of cells (h). Opposite results were observed in this smaller control population of cells where percent change (i) was increased, while TruCount analysis showed decreased in fold change (j) or cell numbers (k). Statistical analysis: unpaired two-tailed Student's t-test with 95% confidence interval. N-values: (a, c-g) n=9; (i-k) n=6. * p<0.05, *** p<0.001 as compared with contralateral cortex.
Examining neurons in the ipsilateral cortex as compared to the contralateral cortex showed an actual increase in YFP+ cells at 7 dpi (Fig. 5-4 i). Conversely, using TruCount analysis we observed a significant reduction in the number of neurons after CCI as compared with contralateral controls (Fig. 5-4 j-k). These findings validate that because of the dynamic changes in cell survival, proliferation, differentiation and infiltration that occurs after TBI, TruCount analysis with flow cytometry provides a powerful tool to accurately quantify changes of multiple cell types at a given time after CNS injury.

5.2.5 Evaluation of protein expression levels by mean fluorescence intensity (MFI) using flow cytometry

In addition to TruCount quantification we also assessed whether flow cytometry could provide a measurement of protein expression levels by mean fluorescence intensity (MFI) comparable to western blot (WB). MFI provides a means to determine the average level of protein expression per cell. We examined three different endothelial proteins, PECAM-1, VEGFR-2, and VE-Cadherin at 3 and 7 dpi using flow cytometry and MFI (Fig. 5-5 a,c,e) and WB analysis (Fig. 5-5 b,d,f). We observed a very similar trend between both methods for all three proteins, where a significant increase in PECAM-1, VEGFR-2, and VE-Cadherin was observed at 3 dpi as compared with sham controls. By 7 dpi, protein expression returned to sham levels (Fig. 5-5 a–f) with the only exception being VEGFR-2 that remained significantly high at 7 dpi (Fig. 5-5 c-d). These findings support the use of flow cytometry as a quantitative method for protein analysis in the injured brain. The only possible discrepancy is that our results show differences in fold change, which could be attributed to analysis of MFI per cell versus whole tissue as in the case of WB.
**Figure 5-5. Changes in protein expression after CCI injury measured as MFI by flow cytometry are consistent with WB analyses of the whole injured cortex.** The expression of endothelial proteins PECAM-1 (CD31) (a, b), VEGFR-2 (CD309) (c, d), and VE-Cadherin (CD144) (e, f) was measured as change in MFI (a, c, e) and Western blot analysis (b, d, f) at 3 and 7 dpi as compared with sham controls. A significant increase was observed in the expression of all three proteins analyzed at 3 dpi followed by a significant decrease for both PECAM-1 (a-b) and VE-Cadherin (e-f) at 7 dpi. VEGFR-2 levels remained significantly increased at 3 and 7 dpi (c, d). Statistical analysis: unpaired two-tailed Student's t-test with 95% confidence interval comparing CCI group to corresponding sham for each time point. Different time points after CCI injury were compared using One-way ANOVA with Bonferroni's post-hoc test. N-values: (a, c, e) n=3-9 biological replicates; (b, d, f) n=3 biological replicates. * p<0.05, ** p<0.01, *** p<0.001 as compared with sham controls.
5.3 Discussion

These studies examined a quantitative method using TruCount beads for examining cell numbers using a “conventional” flow cytometer that caps a predetermined number of events (i.e. cells). We found that the bulk infiltration of CD45$^{\text{high}}$ cells occurs up to 3 dpi is reduced by 7 dpi, which supports previous findings (Jin et al., 2012). We identified cvECs as a CD45$^-$/CD144$^+$ mature population and EPCs as CD309$^+$/CD133$^+$ cells out of the CD45$^-$/CD144$^-$ population. Using TruCount methods, mature cvECs were significantly reduced at 3 dpi, whereas EPCs were significantly increased after CCI injury, making up about 2% of the viable non-leukocytic cell population in the injured TBI brain. In addition, these studies demonstrated that protein expression levels could be measured using flow cytometry by examining the mean fluorescent intensity (MFI) per cell. Our results showed a significant upregulation in the average levels of PECAM-1, VEGFR-2, and VE-Cadherin at 3 dpi, which was consistent with WB analyses.

In identifying the cvEC populations with the commonly used PECAM-1 marker (i.e. CD31) in the TBI brain we found that anti-CD31 cross-reacts with a population of infiltrating cells after CCI injury (Newman et al., 1990). VE-cadherin (CD144), on the other hand, is exclusively expressed in vascular ECs (Suzuki et al., 1991) and does not cross react with the infiltrating CD45$^+$ population. In addition to infiltrating leukocytes, CD31 is also expressed by bone marrow derived EPCs, thus preventing the proper discrimination between cvECs and EPC after TBI. We also showed that the exclusion marker VE-cadherin (i.e. CD144) can be used for cvECs since its only turned on once cells are committed to the endothelial lineage (Asahara and Kawamoto, 2004). Prominin-1 (CD133) has been used to label EPCs, in combination with a variety of different
cellular markers and studies in CNS injury models have yielded different results (Timmermans et al., 2009). We used CD133 as an inclusive progenitor marker in combination with CD309, which allowed us to limit our selection to vascular progenitors. CD45 was used as an exclusion marker for hematopoietic cells since these cells are also mobilized from bone marrow into the brain after TBI (Deng et al., 2011) and express CD309 (Ziegler et al., 1999). EPCs were therefore identified as CD45−/CD144+/CD309+/CD133+ cells. Our observed increase in EPCs is consistent with previous TBI studies in both rats and humans where EPCs are increased between 1 and 3 dpi in rats (Guo et al., 2009) and in the first week in TBI patients (Liu et al., 2007). Likewise, the CCI-induced reductions in the mature cvEC population at 3 dpi are also consistent with the overall reductions in blood vessel density observed by Chen and colleagues in the peri-lesional cortex within the first week following CCI injury in rats (Chen et al., 2003).

We also showed that utilizing a single platform volumetric standard, i.e., TruCount beads, provides a precise method for the quantification of cell numbers in CCI injured brain samples. Although flow cytometry is a method of gold standard in the hematopoietic and immunology fields, it remains underappreciated and not a common practice in TBI studies. In fact, a Pubmed search using keywords “flow cytometry” and “immunology” result in over 60,000 hits of which only 10% are related to bone marrow-derived cells. A Pubmed search using “traumatic brain injury” identifies over 22,000 hits, where only 77 of those include the term “flow cytometry”. Furthermore, of those 77 hits, the majority are related to immunological aspects of TBI where only 8 involved cell infiltration and only 2 involved bone marrow-derived cell infiltration into the traumatic injured brain, to date. Of those 8 papers involving cell infiltration, 3 of the 8 examined
their samples using percent change or events (for example, Lee J et al. J Trauma Acute Care Surg. 77(5):709-715 (2014); Almolda B et al. Glia. 62(7):1142-61 (2014); Cadosch D et al. Injury. 41(6):e4-9. (2010)), while others used numbers in an undefined manner (for example, Hua R et al. World J Emerg Med. 3(4):294-8 (2012); Clausen F et al. J Neurotrauma. 24(8):1295-307 (2007)). We believe these approaches can lead to misleading results as demonstrated in our manuscript (Assis-Nascimento et al., 2016), which is mainly based on variances between sham and TBI total cell populations as a result of cell death, proliferation and infiltration. Thus, the percent change in any particular cell cannot be compared between sham and TBI groups or even between post injury time points; however, these comparisons can be made using quantified cell numbers. In summary, our studies demonstrate that combining flow cytometry with the TruCount method and specific cell markers can be a useful tool for analyzing temporal changes in protein levels and multiple cell populations within the complex pathology of the injured brain.
Chapter 6

Conclusions

These studies evaluated the role of the receptor tyrosine kinase, EphB3, and its ligand ephrinB3 in vascular integrity following TBI. Our findings show that traumatic cortical injury leads to cvEC death, as well as reductions in both cortical vascular density and in numbers of cvECs lining the lumen of blood vessels. Despite the cellular losses, proliferative expansion of both mature residential cvECs and bone marrow-derived infiltrating EPCs was also observed, suggesting that both cell types participate in post-traumatic vascular repair. A deleterious role for EphB3 was revealed in regulating cvEC survival as well as in BBB stability and permeability after CCI injury. The absence of the EphB3 receptor resulted in reduced macromolecule extravasation into the injured brain as well as decreased peripheral infiltration of inflammatory leukocytes. These findings correlated with injury-induced cvEC preservation resulting from reduced EC death and increased cvEC numbers. Our findings also suggest a role for EphB3 in astrocytic end-feet wrapping and/or attachment to cortical vessels, which highlights a potential intrinsic mechanism to patch leaky capillaries and can be possibly enhanced as a therapeutic intervention. Overall, these studies demonstrated that ephrinB3-EphB3 signaling in cvECs and astrocytes that makeup the gliovascular unit exacerbates cortical vascular damage including BBB integrity after CCI injury.

As the main functional cells in the CNS, neurons have rightfully been the primary focus in TBI research for several decades. Recently, the vasculature and other gliovascular cells forming the neurovascular unit have received greater attention in the TBI field resulting in a paradigm shift in the importance of the vascular response to
injury. Although this has opened new possibilities for therapeutic intervention and drug treatment strategies, specific links between the vascular response to TBI and the progression of chronic disease, as well as mechanisms of post injury vessel repair have yet to be elucidated. To date, most of the mechanistic information on pathological vascular changes has been extrapolated from cancer and stroke models. ECs in the ischemic boundary zone have been shown to proliferate as early as 12 to 24 h following stroke, leading to angiogenesis in peri-infarcted regions 3 days after the ischemic injury (Hayashi et al., 2003). Similarly, in humans, angiogenesis was also observed 3 to 4 days following the ischemic insult (Beck and Plate, 2009). Our observations are consistent with these findings showing increased endothelial proliferation at 3 days following CCI injury, which persisted for up to 7 days. The formation of new vessels in the adult brain after stroke and TBI is not restricted to angiogenesis, but involves vasculogenesis contributed by circulating EPCs from bone marrow (Zhang et al., 2002, Guo et al., 2009). Several studies have demonstrated beneficial effects of EPC transplantation after TBI including brain neovascularization and tissue reconstruction (Guo et al., 2009), improved behavioral performance and reduced injury cavity volume (Xue et al., 2010), improved neurologic functions (Zhang et al., 2013), as well as restored BBB integrity (Huang et al., 2013). However, the individual contributions of these two different endothelial populations to vascular repair following TBI has yet to be carefully assessed. Our studies suggest that EPCs and cvECs have different roles in stabilizing/repairing the cerebral vasculature after CCI injury. EPCs appear to participate in the acute injury followed by a more sub-acute involvement of the residential cvECs, which have been shown to continually proliferate for months after TBI (Thau-Zuchman et al., 2012). Further
understanding of how vasculogenesis and angiogenesis separately contribute to vessel repair and stability after injury will allow researchers to individually target these different endothelial populations in a more temporally effective manner. This may minimize some of the undesired side effects that often result from global, non-specific therapeutic treatments. For example, VEGF is an important mediator of EC proliferation and survival, also required for subsequent EC angiogenic sprouting (Mackenzie and Ruhrberg, 2012) and has been long considered for therapeutic use to repair vascular injury. Post-injury acute VEGF treatments have yielded positive results showing chronic enhancement in angiogenesis and neurogenesis after TBI (Thau-Zuchman et al., 2012). However, other studies revealed a dichotomous role for VEGF as a negative regulator of pericyte function and vessel maturation, where VEGF ablated pericyte coverage of nascent vascular sprouts, leading to vessel destabilization (Greenberg et al., 2008). In our studies, VEGF-induced effects were exclusive to cvECs and not EPCs at 3 days, although both populations continually expanded during the first week after CCI injury. These findings reveal that progenitor and residential ECs respond differently to pro-angiogenic factors, demonstrating the complexity of TBI pathology and the importance of pinpointing temporal roles of individual cells types and how they contribute to progressive damage or repair.

Both developmental and pathological angiogenesis temporally overlap with neurogenesis and are equally important in mediating functional recovery after experimental stroke and TBI (Xiong et al., 2010). Ephrins and Eph receptors have been shown to play essential roles in both of these processes, where upon binding of their membrane-bound ephrin ligands, Eph receptors activate direct signaling pathways that
affect the cellular cytoskeleton, leading primarily to cell repulsion. During development and tissue maintenance, these repulsive signals are indispensible for a variety of regulatory processes such as establishing proper tissue boundaries, aiding in cell migration and axon guidance, and halting proliferation to allow for cell differentiation, among others (Kania and Klein, 2016). Unfortunately, after injury these same signals can have a negative affect on tissue recovery. For example, ephrinB3 has been shown to contribute to myelin-derived axonal growth inhibition and limited recovery after CNS trauma. In fact, genetic deletion of this ligand in mice significantly increased axonal regeneration of retinal ganglion fibers that extended past the injury site following an optic nerve crush (Duffy et al., 2012). After CCI injury in mice, the receptor EphB3 has been shown to negatively regulate neural stem progenitor cell (NSPC) proliferation in the subventricular zone (SVZ) (Theus et al., 2010), as well as neuronal cell death in the injured cortex (Theus et al., 2014). Similarly, our findings also revealed a negative role for EphB3 in cvEC survival and BBB permeability after CCI injury. As both ephrins and Eph receptors are membrane bound, the ability of EphB3 to interact with ephrinBs is dependent on cell-cell interactions; which in the context of the BBB includes neighboring ECs, astrocytes and pericytes. Several members of the Eph-ephrin family are expressed by different gliovascular cells (Zhuang et al., 2010, Salvucci and Tosato, 2012). However, a further understanding of the specific receptor-ligand interactions as well as mechanistic cell-cell regulation by more promiscuous family members such as EphB3 and ephrinB3 has been a challenge partially due to their high homology within respective subclass (Bergemann et al., 1998). This high sequence similarity also makes expression studies extremely challenging considering the poor quality or specificity of many anti-
ephrin and anti-Eph antibodies currently available. Due to these limitations we were unable to identify whether the EphB3-dependent increases in astrocytic end-feet interaction with cortical blood vessels observed in our studies is initiated by the astrocyte or the endothelial cell itself; further studies are needed to discern between these two possibilities. Astrocytic end-feet wrapping of cvECs is important for effective adherent tight junction formation (Dehouck et al., 1990, Rubin et al., 1991) and different receptor tyrosine kinases have been shown to participate in the regulation of this process (Lee et al., 2003, Thundyil et al., 2013). Similar to EphB3, another member of the Eph family, EphA2, aggravates ischemic brain injury in a mouse model of focal stroke, where its absence results in reduced post-stroke BBB leakage, decreased brain edema and less infiltrating immune cells. This protection by EphA2 deletion associated with higher expression levels of the tight junction protein ZO-1 (Thundyil et al., 2013). Our studies revealed increased protein expression levels of the endothelial junction proteins ZO-1 and VE-Cadherin in the absence of either ephrinB3 or EphB3 in non-injury conditions. Although we cannot rule out the possibility that these animals normally have tighter BBB formations from development, the injury-induced phenotypes demonstrated in our studies are nearly exclusive to the EphB3 receptor and not the ephrinB3 ligand. Given that both junction proteins are linked to the actin cytoskeleton (Fanning et al., 1998, Itoh et al., 1999, Dejana and Orsenigo, 2013) and that the absence of either the ephrinB3 ligand or the EphB3 receptor affects them in a similar manner, it is likely that EphB3-ephrinB3 signaling may regulate endothelial TJs indirectly via modulation of the actin cytoskeleton. Different studies have demonstrated that activation of Eph receptors can lead to actin de-polymerization and membrane retraction by via cytoskeletal re-
organization (Zimmer et al., 2003, Groeger and Nobes, 2007). EphB3 signaling after CCI injury may contribute to the de-stabilization EC AJs and TJs via de-polymerization of the actin cytoskeleton. Since ZO-1 links transmembrane AJ and TJ proteins to the actin cytoskeleton, one possibility is the sequestering of this scaffolding protein by EphB3 through a PDZ interaction. ZO-1 contains 3 PDZ domains on its N-terminus (Itoh et al., 1999) and all EphB receptors, including EphB3, have a C-terminus PDZ-binding motif. Increased ZO-1 binding to EphB3 can potentially result in its reduced ability to bind to endothelial TJ and/or AJ proteins, which may in turn cause cytoskeleton de-stabilization and a leakier BBB. Unlike the astrocytic interactions, we did not observe any genotypic differences in pericytic membrane associations with cvECs, despite the developmental differences previously reported in targeted deletions of ephrinB2 from pericytes, where microvessel association with pericytes was shown to be ephrinB2-dependent (Foo et al., 2006). Pericyte deficiency results in increased BBB permeability to water and a range of both low and high molecular weight tracers, which occurs via endothelial transcytosis (Armulik et al., 2010). Thus, an increased pericyte-cvEC membrane association acutely after TBI may suggest a repair mechanism to re-seal the damaged BBB. Although independent of EphB3-ephrinB3 signaling, our findings revealed a significant CCI-injury induced increase in membrane association between cvECs and pericytes at the penumbra region. This points to a possible mechanism of vascular repair that can be potentially augmented following brain injury to improve recovery.

Given the multifaceted environmental alterations that occur as a result of primary and secondary injury effects, TBI produces a constantly changing environment. Additionally, the multidimensional cellular redistribution that takes place in the injured brain increases
the potential variability between different CNS injury models making development of therapeutic agents highly challenging. Despite the challenges, several efforts have been made to target different members of the Eph-ephrin family in a variety of different disease models. In the last decade, several studies have aimed at developing small molecule antagonists of Eph receptors (Tognolini and Lodola, 2015), including monoclonal antibodies and antibody fragments for inhibiting or activating the ephrin-Eph signaling (Lamminmaki et al., 2015). Both the ligand-binding site in the extracellular domain of Eph receptors as well as the intracellular kinase domain for ATP-binding, represent potential binding sites for therapeutic peptides and small molecules (Noberini et al., 2012). Studies by Pasquale and others also suggest that giving inhibitors of ephrin-Eph bindings can benefit several biological outcomes (Noberini et al., 2012, Riedl and Pasquale, 2015). Our findings highlight EphB3 as an important therapeutic target for blocking cvEC death and possibly progressive vascular damage after TBI. Similarly, other researchers have also considered pharmacological ways to block EphB3 signaling as a remedial intervention in different systems. Studies in the cancer field have recently described an anti-Eph nucleic acid aptamer, called GL43.T, which is able to bind EphB3 at a high-affinity and prevent its activation (Amero et al., 2016). Aptamers are single-stranded DNA or RNA molecules that have the ability to bind to pre-selected targets including proteins. In their study, Amero et al found that the GL43.T aptamer inhibited glioma cell vitality and interfered with ephrinB1 inhibition of chemotactic serum-stimulated cell migration (Amero et al., 2016). In a different study, Qu et al used lentiviral expressing vectors with an active small interfering RNA (siRNA) targeting the EphB3 sequence and found that its inhibition following spinal cord injury resulted in
improved nerve functional recovery and regeneration (Qu et al., 2014). Based on the previously reported deleterious roles for EphB3 in our CCI injury model of TBI, blocking this particular Eph receptor acutely after CCI-injury will most likely provide increased neuronal survival, enhanced proliferation of neural stem cells, improved synaptic stability and plasticity, in addition to improved vascular integrity and reduced BBB leakiness. Furthermore, given the cell death role of EphB3 via dependence receptor mechanisms, in both neurons and oligodendrocytes following CNS trauma (Theus et al., 2014, Tsenkina et al., 2015), targeting this particular function may also provide a promising alternative for therapeutic intervention. An interesting characteristic of Eph dependence receptors is that kinase activity is not required for mediating cell death, but rather the receptor is cleaved in the absence of its ephrin ligand generating an addiction/dependence domain (ADD), also known as a cell death domain, which can be identified as a small molecular weight band by Western blot analysis (Furne et al., 2009, Theus et al., 2014). The release/exposure of this cleaved death domain is required for dependence receptor induced apoptosis and can amplify caspase activity (Mehlen and Bredesen, 2004, Furne et al., 2009). Thus, using small molecules to either prevent EphB3 receptor cleavage by hindering the cleavage-binding site and/or by targeting the destruction of the ADD represents a promising therapeutic possibility with great clinical implications for EphB3-mediated endothelial cell death in the traumatic injured brain.
Materials and Methods

7.1 Transgenic Animals

All procedures related to animal use and care were approved by the University of Miami Animal Use and Care Committee. Animals were housed in a 12 h light/dark cycle and food and water were supplied ad libitum. Mouse lines were all in C57BL/6 genetic background. Adult (ages of 2-4 months) male mice were used for all experiments. Cdh5-zG mice were generated by crossing Cdh5 (pac)-CreERT2 (Tg (Cdh5-cre/ERT2) 1Rha, MGI: 3848982) (Sorensen et al., 2009) with Rosa zGreen reporter mice (007906 B6.Cg-Gt (ROSA) 26Sor < tm6 (CAG-ZsGreen1) Hze>)/J; The Jackson Laboratory, Bar Harbor, ME). Thy-1-YFP mice were purchased from Jackson Laboratory (JAX Mice Database-003782 B6.Cg-Tg (Thy-1-YFP) HJrs/J). The generation of ephrinB3 knockout (ephrinB3^{−/−}) and EphB3 knockout (EphB3^{−/−}) mice and genotyping using PCR analysis has been previously described (Henkemeyer et al., 1996, Orioli et al., 1996, Yokoyama et al., 2001). Cdh5-zG-ephrinB3^{−/−} and Cdh5-zG-EphB3^{−/−} mice were generated by crossing the Cdh5-zG mice with the ephrinB3^{−/−} and EphB3^{−/−} mutant mice.

7.2 Surgeries

In preparation for controlled cortical impact (CCI) injury, mice were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal (i.p.) injections. A 5 mm craniotomy was aseptically made using a portable drill over the right parieto-temporal cortex (−2.5 mm caudal and 3 mm lateral from bregma, epicenter). The injury was generated using a 3 mm beveled stainless steel tip piston attached to an eCCI-6.3
device (Custom Design & Fabrication), at 2, 4 and 6 m/s velocities, depth of 0.5 mm and impact duration of 150 ms. Surgical sham mice received only the opening and re-suturing of the skin. After CCI injury the skin was sutured using 5-0 coated vicryl sutures (Ethicon, J391) and animals were placed on a warm heating pad until fully recovered from anesthesia. The surgeries for all flow cytometry studies were performed at a velocity of 4 m/s.

### 7.3 Tamoxifen Preparation and Treatment

Fifty mg/ml Tamoxifen (Tamoxifen Free Base, MP Biomedicals, 156738) was prepared in a 10% absolute ethanol and 90% sunflower oil (Sigma, S5007) solution and vortexed vigorously. The solution was placed in a 55 °C water bath for 3 h, vortexed intermittently every h. Tamoxifen aliquots were pre-warmed to 37 °C for 30 min prior to animal injections. Adult Cdh5-zG male mice received 6 i.p. injections at a dose of 0.18 mg/g body weight over 8 days; a 2 day interval was given between the first and second treatments and 5 consecutive injections followed from days 3 to 8. Animals were used experimentally 1 week after the last injection.

### 7.4 Hematoxylin and Eosin Histological Staining

Anesthetized sham or CCI injured animals received intracardiac perfusion with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA) at 3 and 7 days post-CCI injury (dpi). The brains were removed and post-fixed in 4% PFA at 4 °C overnight. Brains were then incubated for 24 h in a 30% sucrose solution at 4 °C. Following dehydration, the cerebellum/midbrain was removed and the remaining brain tissue was embedded in clear frozen section compound (VWR, 95057-838) for cryostat
sectioning. Thirty micron cryostat sections were stained using a standard hematoxylin and eosin (H&E) histological staining. Briefly, sections were rinsed in distilled water for 5 min to remove all embedding compound then immersed in hematoxylin for 3 min. Sections were rinsed again in running distilled water for 5 min and then immersed in eosin for 30–45 s followed by 3 min incubations of increasing ethanol (EtOH) concentrations: 70% EtOH (1X), 95% EtOH (2X), and 100% EtOH (2X). The sections were immersed in methyl salicylate for 5 min followed by 3 xylene incubations of 3 min each. Finally, the slides were mounted with a xylene-based mounting medium and samples were imaged using a bright field Olympus BX50 microscope equipped with Olympus SC30 digital color camera and Olympus analysis getIT software for image capturing.

7.5 Tissue Preparation for Ultramicroscope Imaging

Mice were deeply anaesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) cocktail and their blood was flushed with approximately 50 mL ice cold, filtered PBS via intracardiac perfusion. Mice were mildly fixed with 1% PFA and 15 mL of DyLight 594 labeled Lycopersicon Esulentum (Tomato) Lectin (DL 1177; Vector Laboratories, Burlingame, CA), was slowly flushed through the heart to label infusible vessels. Following a 5 min Lectin binding period, 50 mL of ice cold 4% PFA was perfused to completely fix all tissues. Brains were removed and post-fixed in 4% PFA overnight in 4 °C, protected from light. To avoid PFA auto-fluorescence brains were washed in PBS for 24 h. The ipsilateral injured cortex was then dissected and was placed in a 30 mL glass amber packer bottle (VWR; Suwanee, GA) for the remaining steps. Brains were first dehydrated in increasing concentrations of tetrahydrofuran (THF) diluted in distilled
water: (1) 50% THF for 2 h; (2) 80% THF for 2 h; (3) 100% THF overnight (16-18 h); and (4) 100% THF for 24 h. Dehydrated brains were then cleared in a 1/3 benzyl alcohol (Sigma-Aldrich, 402834), 2/3 benzyl benzoate (Sigma-Aldrich, B6630) (BABB) solution for at least 2–4 h prior to imaging. All incubation steps were done at 4 °C on a shaker. Cleared cortices were imaged on a LaVision BioTec Ultramicroscope based around an Olympus MVX10 zoom microscope body including light sheet excitation lines OPSL (50 mW): 488 nm, OPSL (50 mW): 561 nm and diode laser (50 mW): 647 nm. The camera used was an Andor Neo scientific low noise sCMOS camera with GFP/FITC: 525/50, TRITC/Alexa594: 620/60 and Alexa 647/Cy5: 700/80 detection channels. 3D image analysis was performed using Imaris 8.1.2 (Bitplane) software.

7.6 Preparation of Cell Homogenate

At either 3 or 7 days following surgery the mice were euthanized using ketamine/xylazine cocktail overdose as described earlier; their brains were immediately extracted and placed in cold Hank's Balanced Salt Solution, without calcium chloride, magnesium chloride or magnesium sulfate (HBSS−/−/−) (Gibco, 14175). The meninges, cerebellum, olfactory bulbs, and midbrain were removed and the injured cortex (or the corresponding sham cortices) were dissected, minced into small pieces using a sharp sterile blade and centrifuged at 1200 rpm for 5 min. The media was aspirated and the tissue was then incubated in an enzymatic solution (Worthington, Lakewood, NJ), containing 30 U/mL papain (Worthington, LK003188) and 40 μg/mL DNase I (Worthington, LK003170) in Earl's Balanced Salt Solution (EBSS) (Worthington, LK003188) for 70 min at 37 °C. Following incubation the digested brain tissue passed 10 times through an 18-gauge needle (B-D, 305195) and successively 10 times through a 21-
gauge needle (B-D, 305165) until fully homogenized. The brain cells were then mixed with 1.7 volumes of freshly prepared, ice cold 22% bovine serum albumin (BSA in PBS pH 7.4) and centrifuged at 2600 rpm for 10 min at 4 °C. After centrifugation a thick myelin/lipid layer formed on the top of the vial, which was carefully aspirated and discarded. The cell pellet was washed in 2 mL of Hank's Balanced Salt Solution with calcium and magnesium chloride (HBSS+/+) (Gibco, 14025), filtered through the cell-strainer cap of 5 mL polystyrene round-bottom tubes (Falcon, 352235) and centrifuged once more at 1200 rpm for 5 min. Homogenized cortical cells were then re-suspended in 1 mL of the same HBSS+/+ solution for further staining and flow cytometric analysis.

### 7.7 Flow Cytometry

Cortical cell homogenates were re-suspended in 1 mL HBSS+/+ solution with 1 μL live/dead fixable Near-IR dead Cell Stain (Life Technologies, L10119 Eugene, Oregon, USA) per sample, an amine reactive dye that binds proteins and works well for both live and fixed/permeabilized cells. Cells were then incubated on ice for 30 min, protected from light. Cells were blocked in FcR blocking solution (MACS Miltenyi Biotec, 130-092-575 in 0.5% BSA) for 15 min at 4 °C and pre-conjugated antibodies were then subsequently added for surface staining for 20 min at 4 °C, protected from light. The primary antibodies included: PE-Cy7 anti-Mouse CD45 (affymetrix eBioscience, 25-0451) 1:100, FITC anti-Mouse CD31 (PECAM-1) (BD Pharmigen, 553372) 1:50, PE anti-Mouse CD133 (Biolegend, 141203) 1:200, BV421 Rat anti-Mouse CD144 (VE-Cadherin) (BD Horizon, 562795) 1:100, all diluted in FcR blocking solution. For intracellular staining, cells were fixed for 20 min with 0.5 mL Cytofix on ice (BD Cytofix/Cytoperm, 554714) and incubated with anti-mouse vascular endothelial growth
factor receptor-2 (VEGFR-2) antibody (CD309–Cell Signaling, 2479S) for 20 min at room temperature (RT), protected from light. VEGFR-2 antibody was diluted 1:300 in BD perm/wash buffer. Following the primary antibody incubation for intracellular staining, cortical cells were centrifuged at 1200 rpm for 5 min, washed with BD perm/wash buffer and incubated with the secondary antibody donkey anti-rabbit Alexa Fluor 594 IgG (Life Technologies, A21207) diluted 1:500 in BD perm/wash buffer for 30 min at RT, protected from light. Cells were once again centrifuged at 1200 rpm for 5 min, washed in BD perm/wash buffer and resuspended in 0.5 mL flow cytometry staining buffer (eBioscience, 00-4222-26). Approximately 10 to 15 min prior to analysis, the samples were transferred to BD TruCount tubes (BD Biosciences, 340334, San Jose CA) and run on a special order BD LSRII flow cytometer configured with a 405 nm, 488 nm, 532 nm, and 640 nm laserline using BD FACS Diva 8.0.1 software. Data were analyzed in Kaluza 1.3 (Beckman Coulter). Fluorescence minus one (FMO) staining and the corresponding isotype controls were used to determine positive staining from background for all antibodies. Finally, 1 mg/mL 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; ThermoFisher Scientific, 62248) was used for the quantification of nucleated cells at 1:1000 dilution, added to the corresponding samples 15 min prior to analysis.

7.8 Cell Proliferation Analysis

Cell proliferation was assessed using the Click-it EdU labeling and imaging kits (Molecular Probes by Life Technologies) in Alexa Fluor (AF)-647 for flow cytometry (C10634) and AF-594 for immunohistochemistry (IHC, C10339). Mice were pulsed with 3 i.p. injections of 50 mg/kg EdU (Molecular Probes by Life Technologies, E10187) on days 1, 2 and 3 following CCI injury for animals processed at 3 dpi, and on days 2, 4, and
6 for animals processed at 7 dpi. EdU staining for both procedures was performed according to manufacturer's instructions for each kit. For the flow cytometric analyses, EdU staining was done after the CD309 intracellular staining step on fixed and permeabilized cells and then transferred to BD TruCount tubes to be analyzed as described above. For the IHC assay, Cdh5-zG mice were perfused and their brains were processed for cryostat sectioning in the same way as described before. Images were acquired on an Olympus FV1000 confocal microscope with 5 channel detection and spectral unmixing modes equipped with 458, 488, 514, 543, and 635 nm laser lines.

7.9 Cell Infiltration Analysis

Sham and CCI surgeries were performed on WT and knockout mice followed by intracardiac PBS perfusion to flush out blood at 3 dpi. Cortical cell homogenates were acquired and processed for flow cytometry as described previously. After the L/D stain FcR blocking steps, the cells were incubated for 20 min at 4 °C with PE-Cy7 anti-Mouse CD45 (affymetrix eBioscience, 25-0451) 1:100 and BV-650 anti-Mouse CD11b (BioLegend, 101239) 1:200, pre-conjugated antibodies for surface staining; both diluted in FcR blocking solution and protected from light. Approximately 10 to 15 minutes prior to analysis, the samples were transferred to BD TruCount tubes (BD Biosciences, 340334, San Jose CA) and run on a special order BD LSRII flow cytometer as previously described. Data were analyzed in Kaluza 1.3 (Beckman Coulter). Fluorescence minus one (FMO) staining and the corresponding isotype controls were also used to determine positive staining from background for all antibodies.
7.10 VEGF Infusion

Mice were anesthetized with ketamine and xylazine as described above and positioned in a stereotaxic frame. A 5-mm craniotomy and CCI injury were performed in the same stereotaxic location and velocity as previously described. Following injury, Alzet osmotic pumps (Alzet Durect Corp, 1007D, Cupertino, CA, USA) were preloaded with 0.03 mg/Kg mouse VEGF-A165 (Shenandoah Biotechnology, 200-34-1) or PBS vehicle control, placed directly over the injury using a stereotactic holder, and secured to cranium with surgical glue (Locite 454 Prism Surf 3G, 45404). Pumps were placed under the skin of the dorsal neck region for an infusion over a 7-day period (12 μl total volume dispensed per day at 0.5 μl/hr rate; 0.84ug mVEGF-A total dose per animal). At 7 dpi mice were euthanized, their brains were quickly extracted and processed for flow cytometric analysis.

7.11 Fluorescence Activated Cell Sorting (FACS)

Sham and CCI-injured tissues were prepared as for flow cytometry at 1 dpi exactly as described previously. Cortical cells were incubated for surface staining for 20 min at 4°C with PE-Cy7 anti-Mouse CD45 (affymetrix eBioscience, 25-0451) 1:100 and BV421 Rat anti-Mouse CD144 (VE-Cadherin) (BD Horizon, 562795) 1:100, pre-conjugated antibodies, diluted in FcR blocking solution and protected from light. Cells were centrifuged at 1200 rpm for 5 min, washed and resuspended in 0.5 mL flow cytometry staining buffer (eBioscience, 00-4222-26). Samples were run on a Beckman Coulter MoFlo Astrios EQ using a 100um nozzle at 25psi at a sort rate of about 10000 events/second using IsoFlow (Beckman Coulter, 8546859) as sheath on the instrument.
Debri were gated out using a Forward Scatter Area x Side Scatter Area plot. Aggregates were excluded using a Forward Scatter Height x Forward Scatter Width and a Sideward Scatter Height x Sideward Scatter Width plot. CD45+ cells were excluded by gating on CD45− cells in a Forward Scatter Area x CD45 log area plot. EC were sorted based on BV421 expression using CD45 PE-Cy7 log Area by a CD144 BV421 log Area plot. Post sort purities for CD45−/CD144+ cvEC population was >95%. Cells were collected directly into 250μl TRI Reagent (Zymo Research, R2050-1-50) for subsequent RNA extraction.

7.12 RNA Extraction and qRT-PCR Analysis

Sorted cvECs were processed for RNA extraction and purification using Direct-zol RNA Mini-Prep kit (Zymo Research, R2070) according to manufacture’s protocol, including the optional DNA digestion step. RNA concentrations were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific; Wilmington, DE). Reverse transcriptase (RT) reactions were done according to manufacture’s instructions using the Omniscript RT kit (Quiagen, 205113) with random primers (Promega, C118A) and

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Size</th>
<th>5’ Primer Sequence</th>
<th>3’ Primer Sequence</th>
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<td>CGGCTGCGAACAATGCTAC</td>
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</tr>
<tr>
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</tr>
<tr>
<td>EphA4</td>
<td>123 bp</td>
<td>GAATTTCGCGACGTGTCACC</td>
<td>CTCCCACCCTCCTCCAG</td>
</tr>
<tr>
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<td>160 bp</td>
<td>CTCCACTGTAAACCAGCCAG</td>
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<td>GAGGCCGGTGCTAGATGTGCAG</td>
<td>TCGGCAGAAGGGCGGAGATGA</td>
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Table 7.1. Primers used for qRT-PCR expression analysis.
incubating the samples at 37°C for 1 h. No RT samples were used as negative control for each animal. Samples were then prepared for qPCR analysis using the Maxima SYBR Green qPCR kit (Thermo Scientific, KO251) according to manufacture’s instructions on 96-well plates (Bio-Rad, SS9601) and covered with adhesive films (VWR, 89024-752). Samples were run on an Eppendorf Mastercycler EP Realplex (SA Biosciences) and analyzed using Realplex software version 2.2. Delta (Δ) Ct was calculated by subtracting the corresponding GAPDH Ct from each sample Ct and data were presented as 2*-ΔCt expression. The qPCR primers used are listed on Table 3.1. All primers were designed using Primer3 software (Untergrasser et al., 2012) integrated into the Primer-BLAST web service (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Sayers et al., 2012). The web tool performs a genome-wide BLAST search for specificity, as well as self-complementarity and primer dimer analysis. The primers were designed to span over exon-exon junctions in order to avoid amplification of contaminant genomic DNA and pre-mRNA, as follows: ephrinB1 qPCR product spans over the exon 4-5 junction; ephrinB2 qPCR product spans over exon1-2 junction; ephrinB3 qPCR product spans over exon1-2 junction; ephrinA4 qPCR product spans over exon 2-3 junction. In order to ensure generation of a single amplicon per qPCR reaction, the primers were selected based on the melting curve analysis performed using Realplex software version 2.2 (SA Biosciences).

7.13 Vessel Area and Volume Fraction

EC-specific inducible Cdh5-zG-WT, Cdh5-zG-ephrinB3−/− and Cdh5-zG-EphB3−/− transgenic mice were treated with tamoxifen in the same way as described above. Sham or CCI injured animals were anesthetized and received intracardiac perfusion with PBS
and 4% PFA as previously described. At 3dpi the brains were removed, post-fixed in 4% PFA at 4°C overnight, and then incubated for 24 h in a 30% sucrose solution at 4°C. The cerebellum was removed and the remaining brain tissue was embedded in clear frozen section compound (VWR, 95057-838) for cryostat sectioning. Thirty-micron stereological cryostat sections were washed 3X with PBS for 5 min each wash and mounted with Fluorogel with Tris buffer (Electron Microscopy Sciences, 17985-10). Six non-overlapping 60X confocal images of the vessels in the penumbra region were randomly obtained for each CCI injured animal or corresponding region on sham controls. Images were acquired on an Olympus FV1000 confocal microscope with 5 channel detection and spectral unmixing modes equipped with 458, 488, 514, 543, and 635 nm laser lines. Vessel area fraction was computed using the surface-tracing feature of Imaris 8.1.2 (Bitplane) 3D image analysis software. Automatic surface segmentation was conducted on Cdh5-zG positive expression and set with surface area detail of 0.414 μm. Segmentation was done with specific thresholding levels kept constant across all images and was originally determined according to control. Vessel area fraction was determined as the rendered surface area per (100 μm)², whereas vessel volume was computed as the rendered volume per (100 μm)³.

7.14 Primary Endothelial Cultures

The protocol for culturing primary cortical endothelial cells was adapted from previously described methods (Milner et al., 2008, Tigges et al., 2012). Six WT naïve mice were euthanized using ketamine/xylazine cocktail overdose as described in chapter 2. Their brains were immediately extracted and placed in cold Minimum Essential Medium (MEM-HEPES, Sigma M7278). The meninges, cerebellum, olfactory bulbs, and
midbrain were removed and the cortices were dissected, minced into small pieces using a sharp sterile blade and centrifuged at 1200 rpm for 5 min at 4°C. The media was aspirated and the tissue was then incubated in an enzymatic solution (Worthington, Lakewood, NJ), containing 30 U/mL papain (Worthington, LK003188) and 40 μg/mL DNase I (Worthington, LK003170) in Earl's Balanced Salt Solution (EBSS) (Worthington, LK003188) for 70 min at 37°C. Following incubation the digested brain tissue passed 10 times through an 18-gauge needle (B-D, 305195) and successively 10 times through a 21-gauge needle (B-D, 305165) until fully homogenized. The brain cells were then mixed with 1.7 volumes of freshly prepared, ice cold 22% bovine serum albumin (BSA in PBS pH 7.4, Sigma A2153) and centrifuged at 2600 rpm for 10 min at 4°C. After centrifugation a thick myelin/lipid layer formed on the top of the vial, which was carefully aspirated and discarded. The blood vessel pellet was washed in 5 mL of freshly prepared endothelial cell growth medium (ECGM) consisting of 40 μg/ml heparin (Sigma, H3149-10KU), 2.5 μg/ml L-ascorbic acid (Sigma, A4544-25G), 4 mM L-glutamine (Sigma, G6392-10VL), 37.5 μg/ml endothelial cell growth supplement (ECGS) (Millipore, 02-102), 1% Penicillin/Streptomycin (Sigma, P7539), and 10% Fetal bovine serum, FBS defined (HyClone, SH30070.03) all diluted in Ham’s F12 media (Sigma, N4888). Cells were resuspended again in 4 mL ECGM and platted onto 2 wells (2 mL per well) of a 6-well plate coated with rat tail collagen type I (Sigma, C3867) and incubated at 37°C, 5% CO₂. Twenty-four hours post seeding cells were washed once with pre-warmed Ham’s F12 and media was replaced with fresh ECGM containing 4 μg/ml puromycin (Axxora, 53-79-2, LKT-P8168-M010) and incubated for 3 days. Puromycin is an inhibitor of protein synthesis and will kill cells. Brain endothelial cells are protected
because they express high levels of the multi-drug (MDR) resistance proteins to pump out the puromycin, thus selectively killing cells other than cvECs. After 3 days cells were washed once again with pre-warmed Ham’s F12 and media to remove the puromycin and replaced with fresh ECGM. Cells were allowed to reach confluency and passaged twice prior to being used experimentally.

7.15 Deoxynucleotidyl Transferase-dUTP Nick End Labeling (TUNEL) IHC

WT, ephrinB3−/− and EphB3−/− sham or CCI injured animals were anesthetized and received intracardiac perfusion with PBS and 4% PFA (refer to Chapter 2). At 1 dpi the brains were removed, post-fixed in 4% PFA at 4°C overnight, and then incubated for 24 h in a 30% sucrose solution at 4°C. The cerebellum was removed and the remaining brain tissue was embedded in clear frozen section compound (VWR, 95057-838) for cryostat sectioning. Thirty micron stereological cryostat sections were washed with PBS for 10 min at RT and then permeabilized with 1% Triton-X in PBS for 30 min. Brain sections were then blocked with 5% BSA in PBS for 30 min, also at RT and GLUT-1 (Glucose Transporter-1) Rabbit Polyclonal (Millipore, 07-1401) primary antibody was added overnight at 4°C, diluted 1:100 in 5% BSA. Following removal of the primary antibody sections were washed with PBS and fixed with 4% PFA for 15 min at RT. This ensures that the antibody is cross-linked to the tissue and will not be washed off with the additional steps. Sections were washed in PBS twice for 5 min/wash and then permeabilized for 5 min at -20°C with a 2:1 ratio Ethanol: Acetic acid solution. Following two additional PBS washes sections were pre-treated with Proteinase K buffer consisting of 1M Tris pH 8.0 and 0.5 M EDTA pH 8.0, for 10 min at RT and then incubated with 12 mg/ml Proteinase K enzyme diluted in Proteinase K buffer (20 μl/ ml)
for 15 min. Sections were washed with PBS twice for 5 min/each and Equilibrium buffer
(Apoptag Red In Situ Apoptosis detection kit, S7165) was added for 15 min at 37°C in
humidified chamber. Then TdT enzyme diluted in reaction buffer was added for 1 h at
37°C, again in a humidified chamber. Stop/Wash Buffer was added to all sections for 10
min at RT followed by three PBS washes for 1 min each. Working strength A594 anti-
digoxigenin conjugate, combined with Donkey anti-Rabbit A488 (Life Technologies,
A21206) 2° antibody (1:500) was applied to each section for 30 min at RT in a
humidified chamber. Sections were washed three times with PBS and Hoechst nuclear
stain (Sigma, 33258) diluted 1:500 in dH2O for 10 min at RT, protected from light.
Sections were washed again 3x with PBS and Mount sections with mounted with
Fluorogel with Tris buffer (Electron Microscopy Sciences, 17985-10).

7.16 Stereological Assessment of Cell Death

Unbiased stereological analysis of Glut-1+/TUNEL+ cvECs at the injury penumbra
was assessed using Stereoinvestigator software and an Olympus BX51 microscope
equipped with a CCD camera, LUDL motorized stage, mercury burner, DAPI, 488, 594
single and triple filters at 63X objective. Four 30 μm sections, 250 μm apart
encompassing levels -1.6 mm to -2.6 mm from bregma were quantified per animal using
Stereoinvestigator v10.56 (Microbrightfield, VT, USA). The ipsilateral cortical region
stretching from the innermost corner of the dentate gyrus to the outermost boundary of
CA3 was contoured at 4X magnification. At 63X (NA1.42) a sampling grid of 75 μm X
75 μm was placed over the region of interest (ROI) and Glut-1+/TUNEL+ cells were
counted within a 25 μm X 25 μm frame. Data presented as estimated counts per 100
(μm³) and normalized to WT sham controls.
7.17 Macromolecule Extravasation Assay

A 0.5% sterile Evans blue (EB; Sigma, E2129) solution was prepared in PBS and passed through a 0.2 μm filter to remove any particulate matter that has not dissolved. Mice having undergone either sham or CCI surgery (as described in Chapter 2) were injected with 200 μL EB either intravenously (i.v.) or intraperitonealy (i.p.). No differences were observed between using i.v. or i.p. administration of EB. Anesthetized sham or CCI injured animals received intracardiac perfusion with phosphate buffered saline (PBS, pH 7.4) 3 h after the EB injection to remove any excess dye. Brains were removed and the ipsilateral and contralateral hemispheres were incubated separately in 500 μL Formamide (Invitrogen, 15515-026) each for 24 h at 55°C. Samples were centrifuged the to pellet the tissue and the absorbance was measured at 610 nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific; Wilmington, DE). Formamide was used to blank the instrument. EB absorbance was normalized to the contralateral hemisphere for each animal.

7.18 Analysis of Membrane Interactions

WT and knockout EC-specific inducible Cdh5-zGreen transgenic mice were treated with tamoxifen in the same way as described before. Sham or CCI injured animals were anesthetized and received intracardiac perfusion with PBS and 4% PFA (refer to Chapter 2). At 3dpi the brains were removed, post-fixed in 4% PFA at 4 °C overnight, and then incubated for 24 h in a 30% sucrose solution at 4°C. The cerebellum was removed and the remaining brain tissue was embedded in clear frozen section compound (VWR, 95057-838) for cryostat sectioning. Thirty micron stereological cryostat sections were
washed with PBS for 10 minutes at root temperature (RT) and then permeabilized with 1% Triton-X in PBS for 30 min. Brain sections were then blocked with 5% BSA in PBS for 30 min, also at RT and primary antibodies were added overnight at 4°C, diluted in 5% BSA. Rabbit monoclonal anti-PDGFRβ antibody (1:200) clone Y92 (Abcam, ab32570) was used to label pericytes and rabbit polyclonal antibody to GFAP (1:500) was used to label astrocytes (DAKO, Z0344), on separate sections. Following primary antibody incubation sections were washed 3X with PBS for 5 min each wash and goat anti-rabbit A647 F(ab’’) 2 Fragment (Life Technologies, A21246) secondary antibody was applied for 1 h at RT diluted 1:500 in 5% BSA, protected from light. Sections were washed 3X with PBS and Hoechst stain (Sigma, 33258) was applied at 1.2 µg/ml for 10 min at RT, protected from light, diluted 1:500 in dH2O. Sections were washed 3X with PBS for 5 min each wash and mounted with Fluorogel with Tris buffer (Electron Microscopy Sciences, 17985-10). Following IHC staining, six non-overlapping confocal images were obtained of the penumbra region for each CCI injured animal or corresponding region on sham controls. Images were acquired on an Olympus FV1000 confocal microscope with 5 channel detection and spectral unmixing modes equipped with 458, 488, 514, 543, and 635 nm laser lines. Coloc 2 plugin (Daniel J. White, Tom Kazimiers, Johannes Schindelin) of FIJI-ImageJ imaging analysis software (Schindelin et al., 2012) was used to quantify the amount of overlap between the two channels. Coloc2 uses pixel intensity spatial correlation for analysis, automatic thresholding and significance testing. The Mander’s split colocalization coefficients determine the proportion of signal in a channel, which colocalizes with the other channel. Z-stack images were used for analysis. All images were acquired using identical parameters including the spatial sampling rate, laser
intensities, PMT and offset levels to avoid different signal:noise levels. Coloc2 parameters were set as PSF=3 and run iterations set as 10 for robust Costes auto threshold determination. This method determines which threshold pair gives a Pearson’s correlation coefficient of zero for the pixels below the thresholds and is fully reproducible among similar datasets. Results were graphed as Mander’s Colocalization coefficients and normalized to WT sham control for each group.

7.19 Western Blot Analysis

The ipsilateral cortex of sham and CCI injured animals from both 3 and 7 dpi, was homogenized in radio-immunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and benzonase nuclease (Millipore Corporation, Billerica, MA) and mixed by rocking at 4 °C for at least 15 min. The homogenized tissue was centrifuged and the supernatant was collected for each sample. Samples were then diluted, standardized to protein concentrations, separated on 8% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were then blocked with 5% milk in 0.1 M phosphate buffer with 0.1% Tween-20 for 1 h at RT and incubated overnight at 4°C with the following primary antibodies: anti-ZO-1 1:1000 (Invitrogen, 40-2200), anti-claudin-5 1:5000 (Life Technologies, 352500), anti-VE-Cadherin 1:200 (Santa Cruz, SC-28644), anti-VEGFR-2 1:200 (Cell Signaling, 2479L), anti-PECAM-1 1:100 (Santa Cruz, SC-1506), and anti-β-tubulin 1:30,000 (Sigma-Aldrich, T4026), all diluted in 5% milk. The membranes were then incubated for 1 h at RT with HRP-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) and the bands were visualized using SuperSignal substrate (ThermoScientific, Pittsburg, PA). ImageJ was used to perform density analysis. Protein
measurements were standardized to β-tubulin and normalized to average WT sham signals.

### 7.20 Mean Fluorescence Intensity (MFI) by Flow Cytometry

Cortical cell homogenates from sham and CCI injured mice were acquired and processed for flow cytometry as described above. The following pre-conjugated antibodies were added for surface staining: PE-Cy7 anti-Mouse CD45 (affymetrix eBioscience, 25-0451) 1:100, used as an exclusion marker, FITC anti-Mouse CD31 (PECAM-1) (BD Pharmigen, 553372) 1:50, and BV421 Rat anti-Mouse CD144 (VE-Cadherin) (BD Horizon, 562795) 1:100, all diluted in FcR blocking solution. For intracellular staining, cells were fixed for 20 min with 0.5 mL Cytofix on ice (BD Cytofix/Cytoperm, 554714) and incubated with anti-mouse vascular endothelial growth factor receptor-2 (VEGFR-2) antibody (CD309–Cell Signaling, 2479S) for 20 min at room temperature (RT), protected from light. VEGFR-2 antibody was diluted 1:300 in BD perm/wash buffer. Following the primary antibody incubation for intracellular staining, cortical cells were centrifuged at 1200 rpm for 5 min, washed with BD perm/wash buffer and incubated with the secondary antibody donkey anti-rabbit Alexa Fluor 594 IgG (Life Technologies, A21207) diluted 1:500 in BD perm/wash buffer for 30 min at RT, protected from light. Cells were once again centrifuged at 1200 rpm for 5 min, washed in BD perm/wash buffer and resuspended in 0.5 mL flow cytometry staining buffer (eBioscience, 00-4222-26). All samples were run on a special order BD LSRII flow cytometer and the mean fluorescence intensity (MFI) for individual populations of interest was computed for each sample using Kaluza 1.3 analysis software (Beckman Coulter).
7.21 Statistical Analysis

Unpaired two-tailed Student's t-test with 95% confidence interval was used to compare cell populations from sham and CCI injured animals for the studies where only those two groups were being compared. One-way ANOVA with either Bonferroni's or Newman-Keuls multiple comparison post-hoc tests were used for statistical analyses including three or more experimental groups. Significant group differences were reported *p < 0.05, **p < 0.01, and ***p < 0.001 as compared to sham mice and as #p < 0.05, ##p < 0.01, and ###p < 0.001 as compared to other CCI groups. Statistical analyses were performed with GraphPad Prism software, version 5.0 where error bars represent ±1 standard error of the mean (SEM) for all graphs. P < 0.05 were considered significant for all comparisons.


