# Vascular Remodeling of the Blood Brain Barrier

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Ariel Raina Larson Johnson

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Dr. Grant W. Anderson

January 2011

**Acknowledgements**

I would like to thank Kevin Viken, Dr. Dan Westholm, Gregg Baldeshweiller, Thomas Bastian, Mary Sneve, Arinzechukwu Nkemdirim Okere, and Chelsey Poquette for their technical assistance, and support in lab.

Thanks also to the staff at the Animal Care facility who made all my animal research protocols run seamlessly.

Thanks also to Dr.'s Tongrong He, Zvonimir Katusic, Amy Greene, Ed Perkins, Joseph Prohaska, Janet Fitzakerley, and Greg Rutkowski for their continuing support and guidance throughout my graduate school career. With a special thanks to Dr.'s Lester Drewes and Pat Scott for being members of my Thesis Committee.

I would also like to thank my mom, Cheryl Larson, and my sisters, Kelsey Johnson and Jody Johanessen for all their emotional support as I completed my graduate degree.

Lastly, I would like to thank Dr. Grant W. Anderson for his excellent mentorship, thoughtful guidance, and extraordinary expertise with my graduate school career and life in general!

UMD Department of Chemistry and Biochemistry, Graduate Program for their continued administrative and financial support.

Research supplies and reagents were funded by NIH National Eye Institute Grant.

## Dedication

This thesis is dedicated to my family who has supported me and made it possible for me to further my career in science.

**Abstract**

The human brain contains a vast network of blood vessels, capillaries, and microvessels. The blood brain barrier (BBB) is made up of three main components: resident endothelial cells (ECs), tight junctions, and a basement membrane. This barrier is impermeable to most solutes, bacteria, antibodies, chemicals, and drugs. It does, however, allow for the transport and diffusion of substances that are metabolically necessary in the brain such as glucose and oxygen. The transport of glucose is facilitated by Glut-1, a brain endothelial cell specific glucose transporter. The loss or deficiency of Glut-1 in the BBB has been clinically diagnosed in humans. Glut-1 deficiency syndrome is characterized by a haploinsufficiency of the wild type Glut-1; the dominant non-functional mutation causes the clinical manifestations related to the syndrome. The manifestations begin in infancy and, if undiagnosed, may cause serious developmental delay, acquired microcephaly, seizures, ataxia, and spasticity. The only known treatment is a ketogenic diet which eliminates the brain's need for glucose in metabolism. Incorporation of genetically engineered ECs or endothelial progenitor cells (EPCs) that contain the gene for the wild type Glut-1 into the brain vasculature would correct this syndrome in addition to opening the door for treating other CNS diseases. The proposed mechanism for incorporating new cells into the BBB is via postnatal neovasculogenesis. Postnatal neovasculogenesis occurs in ischemia, hypoxia, and tumor growth. There are two modes of postnatal neovasculogenesis: angiogenesis and vasculogenesis. Angiogenesis is the process by which the resident ECs proliferate when the signal for growth of new vessels is received. Theoretically, during the process of vasculogenesis EPCs are recruited from the bone marrow, differentiate, proliferate,

and migrate to the signaling tissue and incorporate into the new vessel. My research project focused on method development to incorporate cells into the brain neovasculature. I focused this development further to incorporation of cultured and bone marrow-derived ECs and EPCs into the neovasculature through hypoxia-mediated outgrowth or BBB disruption.

We have now developed a method for investigating the effects of hypoxia on vascular remodeling and EPC and EC recruitment into the neovasculature. In this method we utilized two models; direct injection of cultured ECs and EPCs into the brain followed by hypoxia, or osmotic disruption of the BBB followed by injection of ECs and EPCs. Cultured ECs were isolated from brain microvessels. Cultured human EPCs were isolated from peripheral blood. ECs and EPCs display different antigens that allow for immunohistochemical detection. The different combinations of antigens elucidate the different cell types. ECs display antigens for Glut-1, CD31, and von Willebrand Factor. Bone marrow-derived EPCs display antigens for CD31 and Tie2, but do not display antigens for von Willebrand Factor. Immunohistochemistry was used to characterize the cells as EPCs or ECs prior to injection and determine location of the cells in the brain after animals are exposed to hypoxia using the specific antigens for ECs and EPCs.

Keywords: Blood Brain Barrier, Endothelial Progenitor Cells, Postnatal Neovasculogenesis, Vasculogenesis

## Table of Contents

## List of Figures

## CHAPTER 1: Introduction

During embryonic development there is a divergence in the characteristics of vasculature of the brain and the rest of the body. The term blood brain barrier (BBB) refers to the endothelial cells (ECs) and the basement membrane that restrict the movement of solutes into the brain parenchyma. BBB development occurs during the beginning stages of embryogenesis. In a developing embryo the growth of blood vessels occurs via angiogenesis and vasculogenesis. Briefly, angiogenesis is the sprouting of new blood vessels from ECs already present in the vessel, and vasculogenesis is the expansion of the vasculature by incorporation of circulating ECs and EPCs (Asahara, 1997; Folkman, 1995; Asahara, 1999; Dimmeler, 2007).

At the earliest stages of development the neural tube is the rudimentary form of the central nervous system. Ultimately it forms the different regions of the brain and spinal cord. As the neuroepithelium or neural crest cells proliferate and migrate to the "head" of the embryo, blood flow becomes necessary to carry oxygen and nutrients to the expanding regions. This growth of blood vessels into what will become the brain and spinal cord is tightly regulated through the actions of Wnt signaling. BBB formation begins by the migration of the angioblast out of the mesoderm. Once the angioblast has lodged into the area surrounding the neural tube the angioblast then proliferates into the perineural vascular plexus which begins to surround the neural tube. ECs sprout from this plexus and begin to vascularize the inside of the neural tube where the Wnt signals are highest (Stenman, 2008; Lammert, 2008). The two predominant forms of Wnt signaling in BBB development have been identified as signaling through Wnt7a and Wnt7b (Daneman, 2009). Wnt7a and 7b act via

stabilization of β-catenin. Stabilized β-catenin translocates into the nucleus and acts on the Wnt target genes of the angioblast (Liebner, 2008; Polakis, 2008). These target genes are related to the development of the BBB, and the properties that are necessary for maintenance of the BBB. Wnt signaling is believed to be specific for the BBB formation. *In vitro* and *in vivo* studies have shown that Wnt signaling is necessary to maintain BBB phenotype of ECs (Liebner, 2008). In the presence of Wnt signals the BBB ECs continue to express tight junction proteins and glucose transporter 1 (GLUT1) (Liebner, 2008). In the beginning stages of development GLUT1 is expressed in the neuroepithelium surrounding the neural tube. Previous studies have shown that Wnt signaling is also necessary for the expression of GLUT1 to be conferred from the neuroepithelium to the ECs. In the presence of Wnt signals the neuroepithelium no longer expresses GLUT1 while the ECs begin to express GLUT1 in addition to forming tight junctions, adherens, and gap junctions (Daneman, 2009). It has been hypothesized that the change in the expression of GLUT1 at the stage of development forms the BBB. The demand for glucose and movement of glucose across the newly formed BBB is reduced at this point in development because the occlusive nature of the BBB is more important than free diffusion of glucose to the neurons (Daneman, 2009). Maintaining the correct environment for the proper function of the BBB then becomes the job of the neurovascular unit.

The neurovascular unit is composed of ECs that form the basement membrane, astrocytes, pericytes, and neurons (Persidsky, 2006). ECs are capable of forming tight junctions which prevent the uninhibited movement of solutes into the brain parenchyma. ECs are also responsible for junctional adhesion and adherens and gap

junctions. These cells are polar and therefore each face, luminal and abluminal, is different and helps maintain the semi-permeability of the BBB. The next restriction in movement of solutes into the brain parenchyma is provided by the astrocytes. Astrocytes envelop the BBB endothelium. In the absence of astrocytes the ECs that make up the BBB may change (Dropulic, 1987) to be more like ECs in the rest of the body. Pericytes are also important to the proper function of the neurovascular unit. Pericytes have generally been believed to support ECs in the BBB by promoting growth and survival of the vasculature. However, studies have shown that in the presence of hypoxia pericytes migrate away from the endothelium. At the same time there is an increase in the permeability of the BBB (Gonul, 2002), which presents an argument for pericytes to help in the formation of tight junctions between the ECs. Neurons are the outermost part of the neurovascular unit. Proper neuronal function directly relates to blood flow. Neurons would be unable to survive or function without the presence of capillaries forming conduits of oxygen and nutrients to each neuron in the brain. Nor would neurons function and survive without the restrictions of BBB preventing infection and directing solute movement.

The ability of the vasculature in the brain to support each neuron comes from the close proximity of the neurons to the blood vessels which carry nutrients throughout the brain. Each astrocyte in the brain is no more than 20 nanometers away from the extensive vascular network of blood vessels, capillaries, and microvessels (Padridge, 1999). In the human brain this vascular network stretches approximately 400 miles in length if capillaries are placed end-to-end (Lorke, 2008). The vascular network in the brain is different than the vasculature in the rest of the body. The brain vasculature

exhibits the special occlusive properties of the BBB. The BBB restricts the movement of solutes, drugs, and chemicals across the blood vessel wall and into the parenchyma of the brain. The BBB also protects the parenchyma of the brain from bacteria and viruses. Protection from invaders like bacteria and viruses is necessary to maintain the health of the brain because diffusion of antibodies is also restricted and therefore mounting an effective immune response is difficult in the brain (Reese, 1969; Reese, 1967).

However, the BBB is semi-permeable. There is movement of $O_2$, $CO_2$, hormones, and other low molecular weight hydrophobic, lipid soluble molecules that freely diffuse across the barrier to fuel function of the other cells in the brain (Lorek 2008). Although diffusion of small molecules is necessary for the proper function of the brain, cells also require other solutes, like glucose to properly function. Movement of larger molecules is achieved via facilitated or active transport down the concentration gradient through an open ion channel or an ATP-dependent transporter. In facilitated transport the transport protein is present on the cell membrane and binds to the solute causing a conformational change that moves the solute into or out of the cell (Wolburg, 1990). An ATP-dependent transporter contains binding sites for both ATP and a specific solute(s). Initially, the solute binds followed closely by ATP hydrolysis which changes the conformation of the transport protein and moves the solutes into or out of the cell, often against a concentration gradient.

Glucose transporter 1 (GLUT1) is a facilitated transporter that is present in normal ECs of the BBB. Mutations in the GLUT1 gene, *SLC2A1* (in humans), have been identified. These mutations can lead to loss of function, reduction in protein

number and may be dominant or recessive (De Vivo, 2001; Harik, 1988). One mutation that has been characterized is an autosomal dominant mutation that results in a haploinsufficiency of the GLUT1 transport protein in the cell membrane. The mutated *SLC2A1* gene is dominant over the wild type copy, this type of mutation results in elimination or reduction of functional GLUT1 in the BBB (De Vivo, 2000). Such decreased expression of GLUT1 leads to lower amounts of glucose transported across the BBB. The decrease in glucose transport results in less glucose for the brain to utilize in ATP production in glycolysis and the citric acid cycle (Harik, 1988). When the brain is developing it utilizes large amounts of glucose. It follows that the clinical manifestations of a syndrome associated with decreased glucose transport into the brain appear in early childhood. These manifestations can include microcephaly, infantile seizures, and developmental delays. The condition is diagnosed by comparing the cerebrospinal fluid and blood glucose levels. A depressed glucose level in cerebrospinal fluid when compared to blood glucose is diagnostic for GLUT1 deficiency syndrome, as there is less glucose transported into the brain and spinal cord (De Vivo, 1991). GLUT1 deficiency syndrome manifestations can be partially ameliorated by a ketogenic diet, the current treatment for the syndrome. The brain is capable of efficiently transporting ketone bodies with the monocarboxylic acid transporter, MCT1. Ketone bodies can be utilized, albeit less effectively, than glucose for the generation of energy in the brain (Withrow, 1980). A ketogenic diet is based on the intake of high amounts of fat and low amounts of carbohydrates. However, a long term ketogenic diet is not suitable to sustain overall health. Other treatment options have not been extensively examined. Our research was aimed at treating the condition

through gene therapy. The ultimate goal would be to use gene therapy to replace the missing or mutated allele for Glut1 (in mice). This can be done through transfection of a plasmid or artificial chromosome containing the wild type Glut1 gene into resident ECs, or use of genetically engineered cells that contain copies of wild-type *Slc2a1*. The next step is to incorporate the genetically engineered cells into the developing neovasculature. This incorporation would require the growth of new vessels or displacement of resident ECs with the genetically modified ECs.

Incorporation of cells into growing vessels, postnatal neovasculogenesis, occurs via two primary mechanisms, angiogenesis and vasculogenesis. The two processes can be differentiated by the cells that are utilized to expand the vasculature. Angiogenesis is defined as the sprouting of new vessels via the proliferation of resident ECs, while vasculogenesis is characterized by the growth of new vessels via incorporation of circulating bone marrow-derived endothelial progenitor cells (EPCs) at the site of new growth (Asahara, 1997; Folkman, 1995). However, it has been discovered that up to 74% of cells that are incorporated into the neovasculature in a reverse bone marrow transplant with hind limb ischemia are non-bone marrow derived, circulating cells (Dimmeler, 2007). Resident ECs can be characterized by certain markers. These markers have been identified to include Tie2, CD31, VE-Cadherin, Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2), von Willebrand factor (vWF), and CD34 (Yoder, 2008; Yoder, 2009; Ingram, 2010). Tie2 is a cell surface receptor associated with angiopoietins which promote angiogenesis. CD31, or PECAM-1, and CD34 are adhesion molecules present on the surface of ECs specifically for platelets. VEGFR2 or Flk-1, are receptors for VEGF, a potent pro-angiogenic factor that has been

shown to regulate many angiogenic and vasculogenic processes. VE-Cadherin is involved in calcium dependent cell-cell adhesion, and von Willebrand factor (vWF) is a serum glycoprotein that is secreted by ECs into the blood. EPCs have been defined through similar methods, however, the markers that define these cells remain controversial. Most studies agree that EPCs contain makers for CD31, AC133, VEGFR-2, VE-cadherin, c-kit, Tie2, CD34, and CD45, and are positive for acLDL uptake and lectin staining (Yoder 2010, Katusic 2004). CD31, VEGFR-2, VE-cadherin, Tie2, c-kit, and CD34 are also EC markers, but CD45 and CD133are indicative of EPCs. However there is still some debate over the expression levels of CD45 (Estes, 2010). C-kit is a gene that encodes a stem cell factor receptor, and CD45 is a transmembrane protein in the protein-tryosine-phosphatase family also called leukocyte common antigen (Katusic, 2004; Yoon, 2005; Yoder, 2008; Yoder, 2009; Hur, 2004; Asahara, 1997; Friedrich, 2005; Rafii, 2000; Dimmeler, 2005; Karsan, 2007; Finkel, 2005; Miller-Kasprzak, 2007). Most studies agree that EPCs have low expression of CD45. AC133 is a pro-angiogenic marker that when found on circulating cells is indicative of a hematopoietic progenitor cell (Estes, 2010). In order to differentiate between circulating ECs and circulating EPCs and other circulating cells a few different methods can be used (Sbarbati, 1991).

Separation of cells can be achieved using flow cytometry and cell sorting or density gradient centrifugation. Flow cytometry separates cells based on antigens present on the cell surface labeled with fluorescent markers. Given that there are a limited number of markers that can be used to differentiate between circulating EPCs and ECs, separation with this method is difficult. Another approach is to separate cells

with density gradient centrifugation. Blood is collected and poured over a density gradient usually generated with Ficoll Paque Plus (Hebbel, 2000; Caplice, 2002). This slurry is then centrifuged to separate the different components of the blood based on their densities. The gradient separates into specific layers; plasma, circulating monocytes, granulocytes, and RBCs with platelets. After separation, the monocyte layer, which is said to contain circulating EPCs and ECs, can be identified based on density and the cells in the layer can be further characterized with surface antigens (Katusic, 2004; Hebbel, 2000; Caplice, 2002). ECs, which have also been proposed to circulate in the blood to some extent, can be harvested by homogenizing tissue and isolating the microvessels. Once isolated, the microvessels and circulating monocytes can be cultured *ex vivo* and characterized for EC specific markers.

There are several different mechanisms currently used to study neovascularization namely, ischemia (stroke, hind-limb ischemia, myocardial infarction, hypoxia), tumor formation, and vascular homeostasis in the mouse model (Asahara, 1999; Finkel, 2005). Most commonly, hind limb ischemia is used to encourage new blood vessel growth in mice. Hind limb ischemia is achieved by unilateral ligation of the femoral artery. In this method the mouse serves as its own control, because the contralateral side maintains unligated, normal vasculature. The ligated side provides a large amount of tissue that has very limited access to the blood supply and therefore oxygen and nutrients. In this model the tissue is thought to signal to the limited, functional vasculature that there is a need for expansion of the vascular network in order to supply the tissue with oxygen and nutrients (Limbourg, 2009; Scholz, 2002). Stroke has also been shown to induce neovascularization (Chopp, 2002).

New blood vessel growth also occurs in the presence of tumors. Tumor vascularization has been shown to occur via vasculogeneic and angiogeneic mechanisms. Bone marrow-derived cells as well as resident ECs participate in the expanding tumor vasculature (Asahara, 1999). Another method for inducing neovascularization in the brain and elsewhere in the body is via exposure to chronic mild hypoxia (LaManna, 1994). In this method the animal is housed in the presence of 10% oxygen for days to weeks at a time. Such treatment results in hypoxia-dependent signals for new blood vessel growth in the brain (LaManna, 1995). This new blood vessel growth may occur via vasculogenesis or angiogenesis (LaManna, 1995).

In order to maintain tissue oxygen homeostasis the body must have a systemic response to hypoxia. Intracellularly, hypoxia inducible factor -1 (HIF-1) regulates the downstream cellular response of production and secretion of growth factors. Extracellularly, the growth factors are secreted and act on cells in the blood stream, surrounding cells in the tissue, or on resident ECs. HIF-1 is made up of two subunits, HIF-1 α and HIF-1β. HIF-1β is constitutively expressed (Semenza, 1997). HIF-1α expression is regulated by intracellular oxygen concentration. Under normoxic conditions hydroxylases cleave hydroxyl groups from HIF-1α. This cleavage opens up HIF-1α for ubiquitination and degradation (Maxwell, 1999). But, as intracellular oxygen concentration decreases, as is the case in hypoxia, HIF-1α remains hydroxylated (or activated) and is translocated into the nucleus. When hydroxylated HIF-1α enters the nucleus it dimerizes with HIF-1β. Once HIF-1α and HIF-1β dimerize the subsequent complex upregulates expression of genes carrying a HIF-1 response element (*HRE*) in the promoter region. These genes include *VEGF*, platelet derived growth factor B

(*PDGF-B*), placental growth factor (*PLGF*), stroma derived factor 1 (*SDF-1*), and angiopoietin 2 (*Ang2*) (Semenza, 2010). These growth factors act intracellularly and are secreted to act on other cells. There are known receptors for all of the HIF-1 regulated growth factors on resident vascular ECs. Some of these receptors are also present on circulating or bone marrow-derived EPCs and ECs. A down-stream effect of hypoxia is an increase in vascular density. This increase occurs through either proliferation of resident vascular ECs as well as recruitment of circulating EPCs and ECs.

Hypoxia has effects other than increasing the vascular density. It is also known to increase the oxygen carrying capacity of the blood, and is therefore used by professional athletes to improve their ability to perform. The functional extent of the hypoxia can be tested by the change in the oxygen carrying capacity of the blood via the levels of hematocrit, hemoglobin, and ceruloplasmin (LaManna, 1996). Hematocrit is the amount of packed red blood cells, measured via centrifugation of blood samples. Hemoglobin and ceruloplasmin are metalloproteins contained by red blood cells, measured by colorimetric reactions. An increase in hematocrit, hemoglobin, and ceruloplasmin confirms the body's response to a need for more oxygen. In hypoxia, hematocrit, hemoglobin, and ceruloplasmin increase allowing the blood to carry more oxygen and aids in maintaining tissue oxygen homeostasis (LaManna, 1996). When the levels of hematocrit, hemoglobin, and ceruloplasmin in a hypoxic animal are compared to those of a normoxic animal the extent of the hypoxic effect can be seen. Therefore, the hypoxic effect can be measured through hematocit, hemoglobin, and ceruloplasmin levels as well as vascular density; but the hypoxic effect is not permanent. Following

hypoxia and approximately 3 weeks after return to normoxia in a rat model the vasculature density and blood levels of hematocrit and hemoglobin return to pre-treatment levels (LaManna, 1996).

Another method for inducing neovascularization or vascular repair is disrupting vascular homeostasis. Osmotic disruption of the blood vessels requires an immediate response from resident ECs and circulating ECs to repair the damage. Using this form of disruption we hypothesized that it would be possible to promote binding and incorporation of circulating (injected/labeled) EPCs or ECs for repair of the injured vasculature. This method of disruption has been shown to increase incorporation of marrow stromal cells in the brain (Chopp, 2008). Osmotic disruption of the BBB is a clinically approved method for targeting vasculature of brain tumors to facilitate the ability of chemotherapeutic drugs to cross the BBB and reach the parenchyma of the tumor (Rapoport, 2000; Rapoport, 1970; Rapoport, 1973). Reversible osmotic disruption occurs by injecting a highly concentrated mannitol solution into the vasculature which causes a rush of water out of the ECs that line the blood vessels, capillaries, and microvessels. The osmotic flow of water out of the ECs into the lumen of the blood vessel causes shrinking of the cells and ultimately, breakage of the tight junctions that maintain the impermeable nature of the BBB (Huttner, 1984; Christophidis, 1988; Sokoloff, 1979). Previous studies have shown that reversible osmotic disruption of the BBB allows for leakage of Evan's Blue dye-bound albumin into the surrounding brain parenchyma (Huttner, 1984; Christophidis, 1988; Pokorny, 2003; Fortin, 2008).

Chronic, acute hypoxia has been shown to have an effect on almost all tissue types in the body. Also, chronic, acute hypoxia has been shown to increase the vascular density of the brain parenchyma as compared to normoxic littermates (LaManna, 1994; LaManna, 1995; LaManna, 1996). It follows that both angiogenesis and vasculogeesis may be involved in the expanding vasculature of the brain after exposure to hypoxia. How vasculogenesis occurs in the brain is still unknown, but it is possible that the hypoxic cells in the brain signal to the current vasculature to expand. An acute lack of oxygen in the tissue may encourage incorporation of circulating cells instead of proliferation of resident cells. Another possibility is that the hypoxia causes the disruption of the BBB, and that this disruption signals for the incorporation of circulating cells at the sites of disruption. Hypoxic cells in the brain parenchyma signal for new blood vessel growth and expansion may occur through incorporation of circulating EPCs or circulating ECs. In order to test this, labeled cells can be injected into the circulation during and after exposure to chronic, acute hypoxia. The cells will theoretically circulate in the blood much like progenitor cells recruited from the bone marrow. Circulating cells may have the ability to respond to signals from the hypoxic tissue and incorporate into the expanding vasculature, in addition to or instead of proliferation of resident ECs.

The purpose of my research was to determine whether it is possible to incorporate cultured cells into the expanding or damaged vasculature of the brain. With the ability to incorporate cultured cells into the vasculature comes the possibility for engineering the BBB by altering gene expression in the cultured cells prior to injection and incorporation into the brain vasculature. This alteration may prove effective in the

gene therapy treatment of diseases like GLUT1 deficiency syndrome if treated at an early age and the injected cells are encouraged to proliferate in development of the vasculature in the brain. We hypothesized that ex vivo expanded human EPCs or ECs isolated from strain-matched, mouse brain microvessels will incorporate into the expanding mouse brain neovasculature. To facilitate incorporation cells were injected via retrograde cannulation and ligation of the external carotid artery, along with exposure to chronic, acute hypoxia or osmotic disruption of the BBB via mannitol.

## CHAPTER 2: Materials and methods

### Brain microvessel EC isolation

All instruments were sterilized before use and all pipette tips were coated in 1% PBSA before contacting microvessels. Briefly, brains were harvested from four eight week old male 129-E mice (Charles River Laboratories, Wilmington, MA, USA) and placed in chilled sterile 1X Advanced DMEM-F12 (Invitrogen, Carlsbad, CA, USA). All subsequent steps were performed in a tissue culture hood to maintain sterility. The brains were minced with a sterile razor blade in a sterile glass petri dish rinsed with 1% PBSA (Invitrogen, Fischer, Pittsburgh, PA, USA, Sigma, St. Louis, MO, USA). After mincing, the brain matter was transferred with 3-4 mL of sterile 1X Advanced DMEM-F12 to a sterile Potter-Elvehjem homogenizer with a sterile Teflon pestle. The brains were homogenized with 5-8 slow strokes using a homogenizer at 300 rpm. The homogenate was then transferred to a sterile 1% PBSA soaked 50mL centrifuge tube and centrifuged (Allergra X-12 or Allergra X-15R, Model No., Beckman-Coulter, Brea, CA, USA) at 500 xg for 5 min; the supernatant was aspirated off the top leaving the microvessels and the fatty tissue of the brain on the bottom. The pellet was carefully resuspended in sterile 18% dextran (Sigma, St. Louis, MO) and Advanced DMEM-F12 and re-centrifuged at 5400 xg for 15 min. After centrifuging, the microvessels remained at the bottom of the tube with the fatty layer on top. The fatty layer and supernatant were carefully aspirated and the remaining pellet was resuspended in 3-4 mL sterile 1X HBSS (Gibco/Invitrogen). The suspension was then passed over a 29 μm sterile nylon filter cut to fit a CellEctor (Bellco Glass, Vineland, NJ, USA). The filter and deposited microvessels were washed several times with sterile 1X HBSS. The

microvessels along with other contaminants remain on the top of the filter. The filter was removed and carefully rinsed with 5mL sterile 1X HBSS into sterile 1%PBSA pre-soaked plastic petri dish. 20U/mL DNAse I (Sigma), 1mg/mL collagenase/dispase (Roche), and 0.147 µg/mL Tosyl-L-lysyl-chloromethane hydrochloride(TLCK) (Sigma) were added to the microvessel suspension. The microvessels were then incubated at 37 C for 90-120 min with occasional agitation. After the digestion was complete the microvessels were passed over a sterile 1% PBSA soaked 110 µm nylon filter fitted to a CellEctor. The digested microvessels passed through the filter, and the filter was rinsed with up to 5 mL of sterile 1X HBSS. The filtrate was transferred to a sterile, 1% PBSA soaked 15mL centrifuge tube and the filter rinsed again with 5 mL of sterile 1X HBSS, again the filtrate was transferred to a different sterile, 1% PBSA soaked 15mL centrifuge tube. This process was repeated until there were 4 tubes containing filtrate. The tubes containing the filtrate were centrifuged at 200 xg for 5 minutes. The pellet at the bottom contained the microvessels. The supernatant was carefully aspirated off so as not to disturb the microvessel pellet. Subsequently the pellet was resuspended in 0.5 mL of sterile 1X HBSS and carefully layered over the pre-formed Percoll gradient (for a total of 3mL per centrifuge tube). A sterile Percoll (GE Healthcare Life Sciences, Piscataway, NJ) gradient was used to separate the microvessels from other debris in the sample. Density gradient beads (Amersham BioSciences, Piscataway, NJ) were used for comparison to the sample to determine which layer contained microvessels in the samples tubes. The gradient was formed by adding 1.5 mL of sterile Percoll to 0.5mL of sterile 1X HBSS in a sterile 3.5 mL 1% PBSA soaked polycarbonate thick walled centrifuge tube (13 X 51 mm, Beckman). This was centrifuged at 26000 xg in an

Optima TLX Personal Benchtop ultracentrifuge (Beckman-Coulter) with a TLA-100.3 rotor (Beckman-Coulter) for 30 min to make a pre-formed gradient. The tubes were then centrifuged at 1000 xg for 10 min to separate red blood cells, granulocytes, nonvital cells, and cell debris from the microvessels at density 1.035-1.051 g/mL. The microvessels were removed and transferred to sterile, 1% PBSA soaked 1.5 mL eppendorf centrifuge tubes and then washed with sterile 1X HBSS two times at 100 xg and finally with fresh, sterile brain microvessel EC (BMEC) culture media (Advanced DMEM-F12 with 10% heat inactivated horse serum (Invitrogen), 10% FBS (Gibco-Invitrogen), 1% antibiotic-antimycotic solution (Invitrogen), 0.1 U/mL heparin sulfate (Fischer), and 100 µg/mL EC growth supplement (BD Biosciences, Bedford, MA, USA)). After the final wash the pellet was resuspended in 4 mL of 37 C BMEC media and plated on a freshly coated rat tail collagen (Gibco) 12-well plate with 1mL of cell suspension per well. Media was changed initially after 72 hours then every 48 hours. Only enough media was made initially to last through 10 days of culture.

**EPC culture**

EPCs were obtained from Dr.'s Zvonomir Katusic and Tongrong He at the Mayo Clinic, Rochester MN. Their lab had isolated the cells from human peripheral blood and cultured the cells for 6-7 passages before freezing them in liquid nitrogen. The frozen cells were then transported to University of Minnesota, Duluth in a standard cell storage solution (EGM-2, 20% FSC, 8%DMSO) with dry ice where the remainder of the research with these cells took place. Upon arrival the cells were returned to liquid nitrogen until they were removed for culture. The cells were thawed in a 37 C water bath for 2 min then plated on human fibronectin coated 100mm plates (BD Biosciences)

with 8 mL of EPC media, EGM-2MV (Lonza, Conshohocken, PA, USA). The media was changed every 48 hours and the cells were allowed to grow to confluence before they were used for injections of staining and characterization with immunohistochemistry. The cells were not passaged again because they were already passaged 6-7 times before we received them and there was concern for senescence of the EPC phenotype.

After defining the cell types that were injected next it was necessary to determine what would be the best method for injection of the cells to localize to the brain. As the blood circulates through the body it gets filtered in the lungs, liver, kidney, and spleen. Therefore, in order for the greatest number of injected cells to reach the brain the injection site must circumvent all of the body's natural filtration. The carotid artery is an accessible artery that brings blood to the head and face. As the common carotid artery progresses up the neck it splits into the external carotid artery and internal carotid artery. The external carotid artery brings blood to the face and scalp. The internal carotid artery brings blood to the cerebral arteries and the Circle of Willis. We hypothesized that cannulating the internal carotid artery would allow for direct delivery of cells and solutes to the brain. However, maintaining blood flow to both brain hemispheres is also important. Therefore, the external carotid artery was cannulated toward the bifurcation in the common carotid artery. This allows for the external carotid artery to be ligated while maintaining the blood flow to the both brain hemispheres. The continued blood flow will bring signaling factors, blood, and solutes to the brain. In this way it will be possible for cells to have direct access to the brain prior to the rest of the body.

**Direct injection into the brain vasculature**

To start, 9-10 week old 129-E mice for the brain microvessel EC injection, and 12-13 week old NOD-SCID (Jackson Laboratories, Bar Harbor, ME, USA) mice were anesthetized with isoflurane; 3% isoflurane was mixed with 0.8% oxygen and 0.3% NO for induction in a flow through acrylic box until eye touch, toe pinch negative. After induction, isoflurane was turned down to 1.5-1.8% and delivered via a nose cone with proper exhaust. Respiration was monitored throughout the entire procedure via observation of chest movement. The eyes of the mouse were covered with an ophthalmic ointment in order to prevent drying and corneal ulcers. The mouse was placed on its back and limbs were taped down. The level of anesthesia was confirmed with toe pinch. The area below the jaw and above the line between the limbs was shaved and then sterilized by wiping three times with alcohol swabs. A sterile drape was placed over the body ensuring a sterile environment was maintained. Using autoclaved instruments, an incision was made from just below the jaw down the center to the line between the limbs. The skin was pulled apart to expose the tissue below. The smooth muscle group over the esophagus and trachea was not disrupted. The right and left carotid arteries are located on either side of this smooth muscle group. The fascia was separated and the left common carotid artery was isolated via slinging with sutures. The vessel was clamped on the bottom end of the visual field; closest to the heart. After clamping the common carotid at the proximal end with a (Braintree Scientific, Braintree, MA, USA) clip, the bifurcation of the internal and external carotid arteries was isolated and a clip was placed on the internal carotid artery. Three sutures

were placed around the external carotid artery. One was tied at distal end of the external carotid artery, the top of the field of view closest to the brain, permanently ligating the artery. Another was placed at the closest point to the bifurcation, at the proximal end of the external carotid artery, to secure the cannula. Lastly, a much smaller suture was placed in the middle, to also secure the cannula and ultimately permanently ligate the proximal end of the external carotid artery. Subsequently, a small incision was made in the external carotid artery between the distal and medial sutures with microsurgical scissors. A cannula was made up of a combination of SUBL-160, 0.01" internal diameter (Braintree Scientific) tubing fitted on a 29 gauge needle followed by MRE010, 0.005" internal diameter (Braintree Scientific) tubing to fit in the vessel. The cannula was inserted with sterile heparinized (1%) PBS in the syringe via the small incision in the external carotid artery down to the bifurcation. The cannula was secured in the vessel with the medial and proximal sutures. The clamp on the internal carotid artery was released and the pressure from the blood was allowed to push back on the PBS heparin solution. At which time, 0.1 cc of solution was slowly injected from a 1cc syringe (Monoject, Mansfield, MA, USA) in order to prevent clotting around the injection site and ensure the cannula was secured in the artery. The clip on the common carotid was then removed. The syringe containing the PBS heparin solution was switched with another 5cc syringe containing a 1.4 M mannitol (Sigma) solution warmed to 37 C. Mannitol was injected at a rate of 0.45 mL/min for 30 seconds (Christophidis, 1989). Mannitol injection was followed immediately by injection of Evan's Blue Dye (2 g/100 mL) to determine if the cannulation was effective and also to confirm BBB disruption using mannitol. Brains were harvested after 24-72

hours to confirm the presence of Evan's Blue Dye in the brain parenchyma. The next step was to determine what would be the best method for injection of the cells to localize to the brain. As the blood circulates through the body it gets filtered in the lungs, liver, kidney, and spleen. Therefore, in order for the greatest number of injected cells to reach the brain the injection site must circumvent all of the body's natural filtration. The carotid artery is an accessible artery that brings blood to the head and face. As the common carotid artery progresses up the neck it splits into the external carotid artery and internal carotid artery. The external carotid artery brings blood to the face and scalp. The internal carotid artery brings blood to the cerebral arteries and the Circle of Willis. We hypothesized that cannulating the internal carotid artery would allow for direct delivery of cells and solutes to the brain. However, maintaining blood flow to both brain hemispheres is also important. Therefore, the external carotid artery was cannulated toward the bifurcation in the common carotid artery. This allows for the external carotid artery to be ligated while maintaining the blood flow to the both brain hemispheres. The continued blood flow will bring signaling factors, blood, and solutes to the brain. In this way it will be possible for cells to have direct access to the brain prior to the rest of the body.

**Hypoxic hypoxia chamber**

Hypobaric hypoxia of 10% $O_2$ was achieved using a Sterlite Ultra 116 quart plastic box and lid and a hypoxia generator (Hypoxico Altitude training systems, Everest Summit Hypoxic Generator, Hypoxico Inc., New York, NY). The box lid had two holes in the top, one for the hose from the hypoxia generator and one for the oxygen sensor (MAXO2 Model OM-25RME, Maxtec, Inc., Salt Lake City, UT), both

loose fitting to allow for air movement out. The Hypoxico hypoxia generator is generally used by athletes to help increase the oxygen carrying capacity of their blood, usually set up as a tent for acclimatization during sleep. The basic function of the Hypoxico hypoxia generator is to generate air containing various percentages of $O_2$ to mimic the percent of oxygen encountered at different altitudes and the effects lower oxygen levels have on the body. The oxygen sensor was first calibrated at normal, room oxygen levels (approximately 20.9% $O_2$) then the hose from the hypoxia generator and the oxygen sensor were both passed through the lid of the hypoxia chamber. Specialized mouse boxes with small, 0.5", diameter holes drilled in the sides allowed for adequate air perfusion throughout the mouse cage. Cages containing FVB/NJ (Jackson Laboratories, Bar Harbor, ME, USA) mice at 4-6 weeks of age were placed inside the chamber and the lid of the box was secured. The oxygen level was allowed to equalize at around 10% with minute adjustments made using the Hypoxico hypoxia generator controls to achieve an atmosphere of approximately 10% oxygen. Mice were continuously exposed to mild hypoxia for 21 days except to change food, water, and bedding.

**Hematocrit, hemoglobin, and ceruloplasmin determination**

Hematocrit was measured by centrifuging heparinized blood samples in sealed capillary tubes. The height of the packed red blood cells was compared to the total volume to give the percentage of packed red blood cells or hematocrit. Hemoglobin and ceruloplasmin levels were measured by colorimetric analysis with a standard curve generated from known concentrations of hemoglobin and ceruloplasmin added to Drabkin's Reagent (Sigma) or Ceruloplasmin Reagent (Sigma), respectively. For

hemoglobin levels the colorimeter was calibrated against a blank containing the reagent at 540 nm followed by standard curve generation using absorbance of known concentrations of hemoglobin added to 5.0 mL of Drabkin's Reagent. Once calibrated and a standard curve obtained, 5.0 mL of Drabkin's Reagent was added to a test tube followed by adding 20 µL of whole blood. The sample and reagent were incubated for 15 min at room temp followed by colorimetric absorbance measurement at 540 nm. The absorbance for the samples was compared to the standard curve to determine hemoglobin concentrations of the sample.

**CFSE dye labeling of cells in culture and injection via internal carotid artery**

Cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) a fluorescent dye that enters the cytoplasm and binds to intracellular molecules. CFSE was incubated with adherent cells on the day of injection. The cells were labeled when adherent so as not to damage the cell surface markers. The cells were washed with 37 C 1X PBS then incubated with 1X PBS containing 2µL/mL of the stock 5mM CFSE at 37 C for 15min. The dye was replaced with pre-warmed BMEC media or EGM-2MV (Lonza), depending on the cell type, for 30 min at 37 C. The cells were then rinsed again with 1X PBS and either fixed for staining and characterization or trypsinized for injection. The cells were counted with a hemocytometer to assure approximately the same number of cells were injected into each mouse. After labeling, the cells were kept in 1.5 mL eppendorf tubes in a rotating 37 C chamber until the external carotid artery retrograde cannulation was completed (the method used to previously to confirm administration of solutes directly to the brain). There were two different treatments that we tested, mannitol and hypoxia. If mannitol was the treatment

the syringe containing 1% heparinized 1X PBS was exchanged for one containing 1.4 M mannitol which was injected at a rate of 0.45 mL/min for 30 seconds. The syringe was then switched back to one containing 1% heparinized PBS injected to rinse the mannitol out of the tubing and needle. Subsequently, the labeled cells were transferred to a 1cc syringe. This syringe was switched with the syringe containing 1% heparinized PBS. The CFSE labeled cells were slowly injected in a volume not exceeding 250 µL. If hypoxia was the treatment the original syringe containing 1% heparinized PBS was exchanged for a 1cc syringe containing the CFSE labeled cells. Following injection, the cannula was slowly removed and the vessel was permanently ligated. In order to prevent potential for any suture opening the smaller suture and the larger braided nylon suture holding the cannula in place were tightened and left in place. The ends of all sutures left in place were cut very short. All instruments and slings were removed, and the mouse was sutured using 5-0 braided nylon suture. Immediately the mice were treated with buprenorphine (Midwest Veterinary Supply, Burnsville, MN) injected subcutaneously and every 12 hours thereafter. Lastly, the mice were removed from the anesthesia and placed back in their cages to recover on a 37 C heater. Blood was continually delivered to the brain via the right carotid artery and the unligated left internal carotid artery. Both brain hemispheres received adequate blood supply because of arterial redundancies present in the circle of Willis.

Due to the short time-frame of the surgery the body temperature was not monitored. Once balance was recovered, the mice were removed from the heating platform and returned to the animal facility. The water in the cage was treated with 11 mg/kg amoxicillin (Midwest Veterinary Supply) to prevent infection. If water was

changed over the course of the experiment the water was re-treated with same concentration of amoxicillin. Mice are allowed to recover for 1 day and placed in the hypoxic hypoxia chamber (as previously described) for either 24 hours or 72 hours. Or if injected with mannitol, tissues were harvested at 24 hours or 72 hours post surgery.

**Tissue Harvesting and Fixation**

All mice were euthanized via $CO_2$ inhalation and tissues harvested. Animals were perfused with 1X PBS followed by 10% neutral buffered formalin (Sigma) and post-fixed in 10% formalin for 24 hours. The tissue was then washed 2 X 5min with 1X PBS and then into 30% sucrose for 24 hours. After 24 hours in 30% sucrose the tissue was transferred into a room temperature 50/50 (v/v) 30% sucrose/OCT (Ted Pella, Redding, CA, USA) solution and ultimately embedded in OCT for cryotome sectioning. Before embedding, the brain hemispheres were separated sagittally and then embedded with the sagittal face placed down in the block. The tissue was then sectioned with a cryotome (Microm HM 525, Waldorf, Germany) at a 10 µm thickness and placed on silane coated slides. As soon as possible after sectioning, immunohistochemistry was performed on the slides.

**Immunohistochemistry**

Fixed, perfused brain tissue sections were washed 2 x 5min with TBS 0.025% Triton X-100. The slides were then blocked with normal goat block (1.5% Normal Goat Serum and antibody diluent (0.1% BSA, 0.01% Sodium Azide in 1X PBS)) for 2h. The normal goat block was removed but the slides were not rinsed. Primary antibodies, rabbit anti Glut1 (1:400, provided by Dr. Lester Drewes Laboratory University of Minnesota, Duluth, School of Medicine) diluted with antibody diluent was applied, and

slides were incubated in a humidified chamber overnight at 4 C. Slides were rinsed 2 x 5min with TBS 0.025% Triton X-100. Secondary antibody, FITC conjugated goat anti rabbit (1:500), or Texas Red conjugated goat anti rabbit (1:500, Abcam) diluted with 1% BSA in TBS) was added, which secondary depended on the experiment. The secondary antibodies were incubated in humidified chamber at room temperature for 2h. Slides were rinsed 3 x 5min with TBS which was decanted and a drop of Hard Set Mounting Media with DAPI (Vectashield) was placed on the tissue slide followed by a coverslip. All slides were viewed with a fluorescent or confocal microscope.

A similar procedure was followed for immunohistochemistry with cultured cells. Isolated brain microvessel ECs and EPCs in culture were characterized by immunohistochemical analysis. The cultured cells, ranging for 1 day to 10 days were immunofluorescently labeled with antibodies for Glut1 and CD31. Cells were cultured on 4 or 12 well plates coated with human fibronectin (BD Biosciences) for EPCs, or rat tail collagen (Sigma) for BMECs. Brain microvessel ECs have difficulty growing on glass coverslips. Therefore, the cells remained adherent during staining and fluid was removed by aspiration. Briefly, the cells were initially rinsed with 1X TBS to remove residual media, followed by 10% formalin at -20 C for 30 min, and 3 X 5 min washes with TBS 0.025% Triton X-100. Primary antibodies for rat anti-CD31 (1:50, BD Biosciences), rabbit anti-vWF (1:800, Abcam), and rabbit anti-Glut1 (1:400, Gerhart, 1989) were incubated on the cells for 1 hour at 37 C. The cells were rinsed 3x 5 min with 1X TBS. Subsequently the cells were incubated with secondary antibodies in solution at 4 C overnight; they include FITC conjugated goat anti rabbit directed against rabbit anti Glut1 or vWF primary antibody (1:500), FITC conjugated goat anti-rat IgG

directed against rat anti CD31 primary antibody (1:500), and Texas Red conjugated goat anti rabbit directed against rabbit anti-Glut1 or vWF (1:500, Abcam) diluted with 1% BSA in TBS. Secondary antibodies were applied in as little light as possible so as not to photo-bleach the cells. The wells were gently rinsed to remove excess unbound secondary antibody. After rinsing, cells were covered by 1 drop of Hard Set Mounting Media with DAPI (Vectashield, VectorLabs) followed by a round glass coverslip. Excess mounting media was removed via gentle aspiration at the edge of the coverslip. Plates were visualized immediately after staining.

## CHAPTER 3: Results

### *Isolated brain microvessel-associated cells express endothelial cell specific markers*

Isolated brain microvessels were expanded *in vitro* and characterized for expression of EC specific markers. After 9 days of culture the morphology was no longer that of microvessels but had become a confluent monolayer of endothelial cells (Figure 1). Media was changed every 48-72 h. Immunohistochemistry was used to confirm expression of endothelial cell specific markers, CD31 (Figure 2a) and Glut1 (Figure 2b) at day 0, 3, and 9. The fluorescence and therefore the expression of CD31 and Glut1 was not as intense as the fluorescent staining of the microvessels in brain sections (Figure 1).

### *Endothelial progenitor cells express endothelial progenitor cell specific markers*

EPCs were a gift from Dr.'s Zvonimir Katusic and Tonrong He, collaborators on the project. The EPCs had been isolated from human peripheral blood, expanded and passaged 6-7 times before we received them. Immunohistochemical analysis showed that *ex vivo* expanded EPCs maintain expression for the EPC marker, CD31. But the EPCs did not express GLUT1 or vWF, endothelial cell specific markers (data not shown) (Katusic, 2004).

### *Development of a method for direct delivery of injected cells to the brain vasculature*

Delivery of injected cells to the brain with current methods is difficult. One concern regarding incorporation has been the volume of injected or available circulating cells that reach the target tissue. Cells can be injected via the tail vein, but these cells must travel through the entire circulatory system, through spleen, lungs, and liver before they reach the brain. As the cells pass through the spleen, lungs, and liver they may be

filtered out of the circulation or incorporated into neovascularization at those sites. In order to encourage the incorporation at the expanding vasculature in the brain, the cells can be injected via the carotid artery which will allow for the cells to first go to the brain then continue to heart, lungs, and the rest of the circulation. It was our hypothesis that by forcing the majority of our injected cells to first pass through the brain following or during treatment with hypoxia or mannitol that the cells would home to the locations of disruption or neovascularization in the brain. However, the carotid artery branches into the external and internal carotid arteries at a bifurcation just before it enters the brain. After the bifurcation, in human anatomy, the internal carotid artery continues up into the sinus, the ophthalmic arteries, and the Circle of Willis. As it exits the Circle of Willis it enters the anterior and middle cerebral arteries which supply blood to the cerebrum, corpus collusm, dentate gyrus and cerebellum (Smith, 2003). At the same time, the external carotid artery supplies blood to the neck, face, and base of the skull (Smith, 2003). Therefore, selective ligation and retrograde cannulation of the external carotid artery into the internal carotid artery will allow for more of the injected cells to reach the anterior and medial portions of the brain (Figure 3). Also circulation of blood to the brain is maintained (Toborek, 2009).

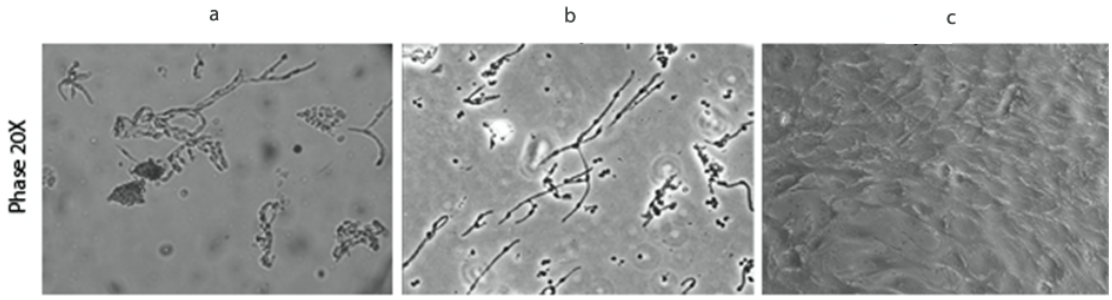


**Figure 1 - Isolated microvessels are capable of expansion ex vivo. Isolated brain microvessels are relatively pure at the (a) time of harvest. (b) Day 1 after harvest the microvessels begin to show signs of adhering to the substrate. (c) Day 10 brain microvessel endothelial cells become confluent after adhering and proliferating.**

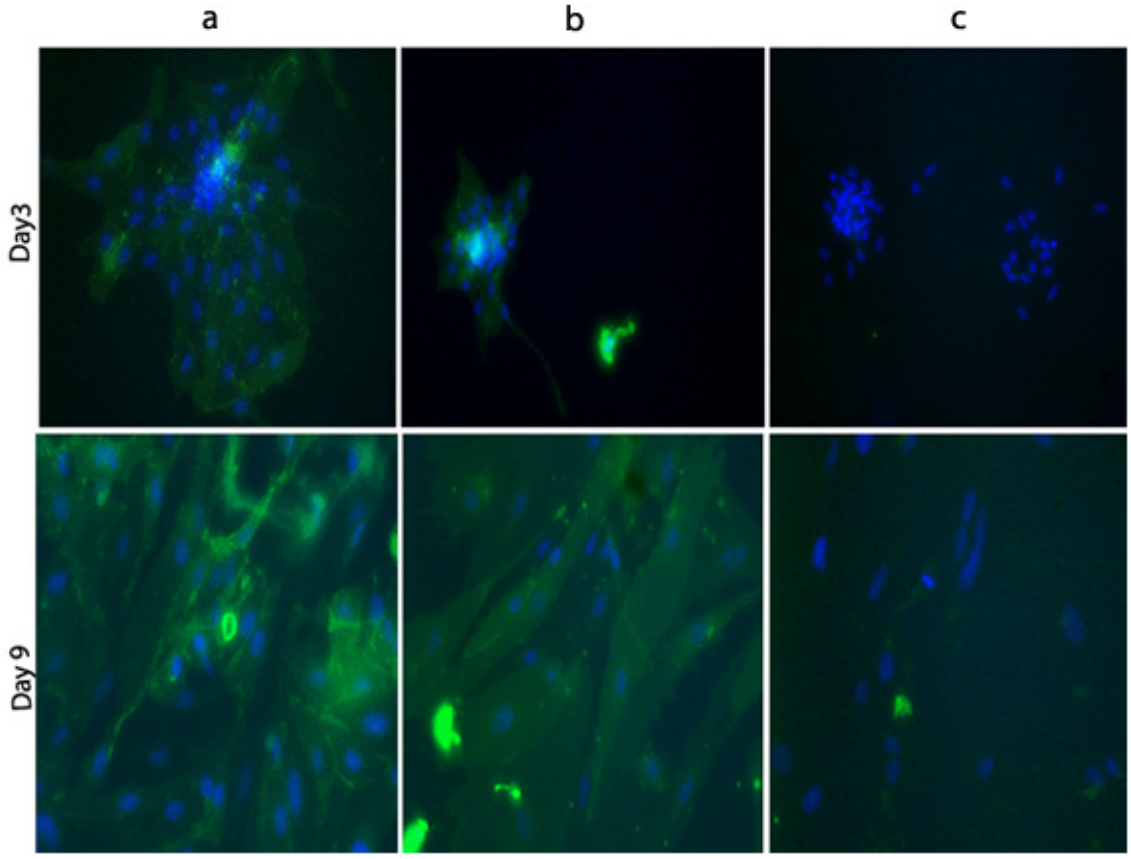


**Figure 2 - Isolated brain microvessel endothelial cells continue to express mature EC markers. Cultures from Days 3 and 9 after isolation and plating of brain microvessel isolations. (a) CD31 and (b) Glut1 (green) staining was used to confirm endothelial cell type. (c) Negative control shows that the endothelial cells do not autofluoresce, however, some debris is present in the Day9 culture negative control and Glut1 images. Nuclei are labeled with DAPI (blue).**

To determine the effectiveness of cannulation and the approximate localization of injections mannitol was injected followed by Evan's Blue Dye. Mannitol was injected via the temporary cannula in the external carotid artery followed immediately by Evan's Blue Dye. Mannitol disrupted the BBB and the Evan's Blue Dye binds to albumin and leaks across the disrupted BBB. We found that the left side of the brain showed blue staining, indicating that mannitol had ipsilaterally disrupted the BBB and Evan's Blue bound by albumin had leaked across the disrupted barrier into the surrounding cortical tissue. Notably, there was no blue staining on the contralateral side indicating that mannitol did not disrupt the BBB on the contralateral side. Localization of blue staining was noted around the left ventricle in the anterior portion of the cortex and around the dentate gyrus in the posterior portion of the cortex (Figure 4). The approximate location (indicated by the black line) of the highest concentration of blue staining in a whole brain was found ipsilateral to the injection in the left internal carotid artery and a few hundred microns lateral of the sagittal plane (Figure 4). We concluded that injection via the internal carotid artery was an effective method to deliver cells and drugs or other solutes to the brain.

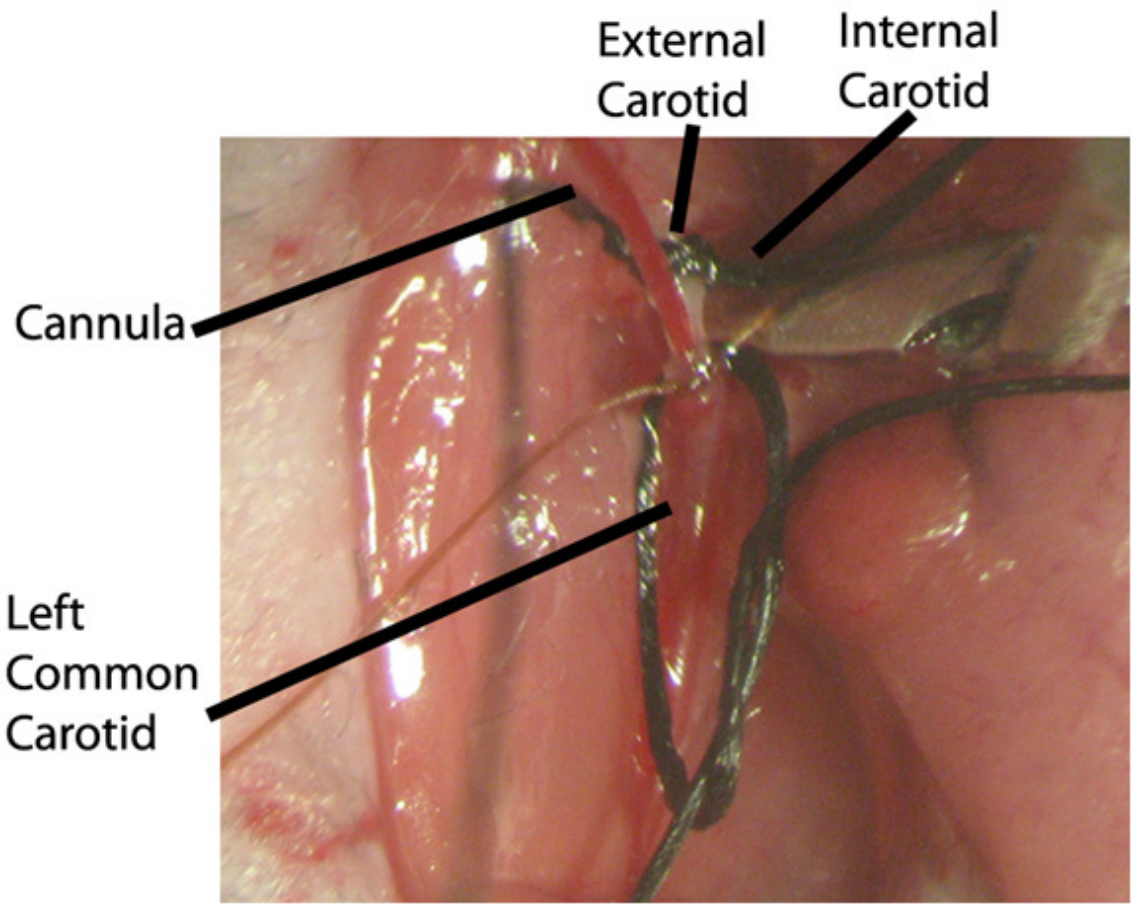


**Figure 3 – Successful carotid artery cannulation. The external carotid artery is ligated by the top left black nylon tie. The internal carotid artery is temporarily ligated by the surgical clip in the top right. The cannula is fed retrograde into the external carotid artery between the ligation and the bifurcation. The cannula is held in place by the two loose ties, one light brown one black nylon (untightened).**

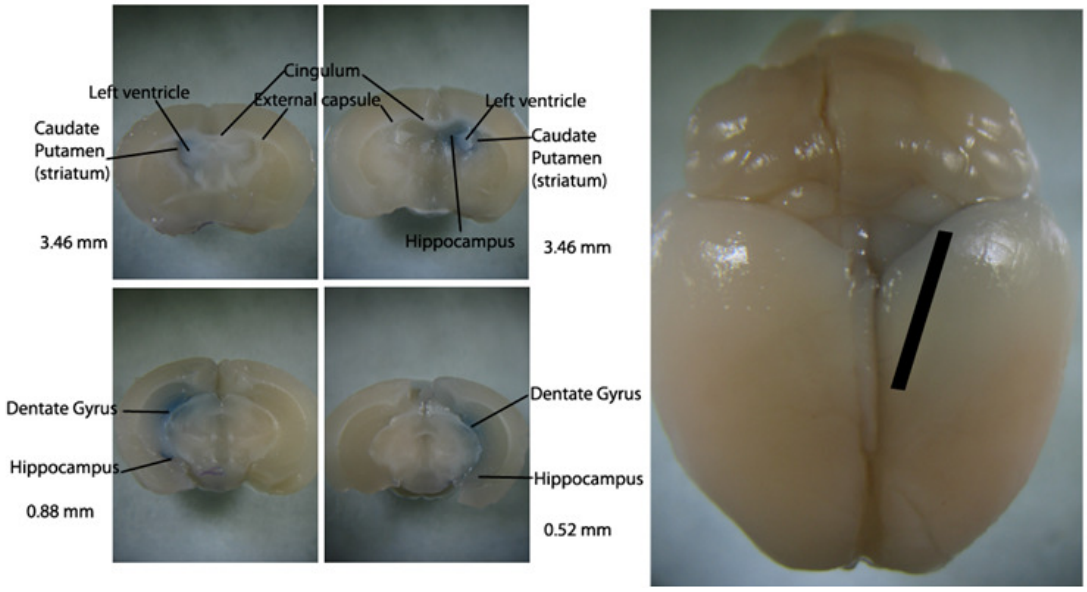


**Figure 4 – Evan's Blue is localized to the ipsilateral side of the brain after injection with mannitol followed by Evan's Blue dye. Injection into the external carotid artery via retrograde cannulation into the internal carotid artery of 1.4M mannitol followed by Evan's Blue dye to monitor osmotic opening of the blood brain barrier. The location of the Evan's Blue dye approximates where the majority of injected cells localize. The black line approximates the localization in an unsectioned brain.**

***EPCs and cultured brain microvessel ECs injected via internal carotid artery retrograde cannulation are localized in microvessels of the ipsilateral side of the brain when concurrently injected with mannitol***

We previously confirmed that mannitol delivered through the carotid artery disrupted the BBB and allowed leakage of Evan's Blue Dye-bound albumin into the parenchyma of the brain. Mannitol is an FDA approved method of BBB disruption that is used in coordination with chemotherapy to allow the drugs access to the tumor parenchyma. Mannitol injection osmotically disrupts the BBB because the high salt concentration induces shrinking of the endothelial cells and pulls the tight junctions apart. We hypothesized that injected cells (ECs and EPCs) would incorporate into the damaged vasculature from pre-treatment with mannitol. In order to prevent filtration of the cells by the spleen or liver, we injected via the internal carotid artery. This type of injection increased the number of cells that were available for incorporation into the injured vasculature. Mannitol was injected at a controlled rate over 30 seconds, followed by a flush of the tubing and needle with less than 100 µL of 1% heparinized PBS. Within 30 seconds CFSE-labeled EPCs and cultured brain microvessel ECs were injected through the cannulated external carotid artery into the internal carotid artery. The animals were then allowed to recover for 24 hours or 72 hours. At 24 or 72 hours mice were anesthetized and the brain was harvested for IHC characterization of injected cells. The brain was cryoembedded and sectioned. The sections were stained for Glut1 with a Texas Red secondary antibody. Glut1 secondary antibody labeled the microvessels with red fluorescence and the CFSE-labeled cells that were injected

fluoresced green, and lastly DAPI was used to label intact nuclei and fluoresced blue. Co-localization of the CFSE-labeled injected EPCs or cultured brain microvessel ECs, Texas Red-labeled Glut1, and DAPI-labeled nuclei was present. In some cases the CFSE + cells appeared more rounded like they were in the lumen of the vessel. In other cases they appeared thinner in morphology like they had become part of the vessel wall. However, there was very limited Glut1 staining on the CFSE + cells, meaning that the cells were present in the vasculature but had not yet begun to express markers for a normal microvessel. Also, there were nuclei co-localized with the CFSE + fluorescence meaning that the cells were viable. We found that the injection of the cells via the internal carotid artery in coordination with mannitol injection resulted in localization and incorporation of EPCs and cultured brain microvessel ECs in the brain microvasculature (Figure 5). A time course of 24h and 72h was also done to determine if the cells that incorporated into the injured vasculature would stay over a longer period of time. We found that with increased recovery time the number of incorporated cells decreased (Figure 6).

***Microvessel density is increased in the cortex and cerebellum of mice exposed to hypoxic hypoxia for 21 days***

To determine if brain microvessel density was increased in response to hypoxic hypoxia, FVB/NJ mice were exposed to chronic hypoxic hypoxia for a period of 21 days while age-matched FVB/NJ were exposed to normal oxygen levels, as a normoxic control. After 21 days, the hematocrit (Figure 7a), hemoglobin (Figure 7b), ceruloplasmin (Figure 7c) of the blood, as well as the microvessel density (Figure 8) in

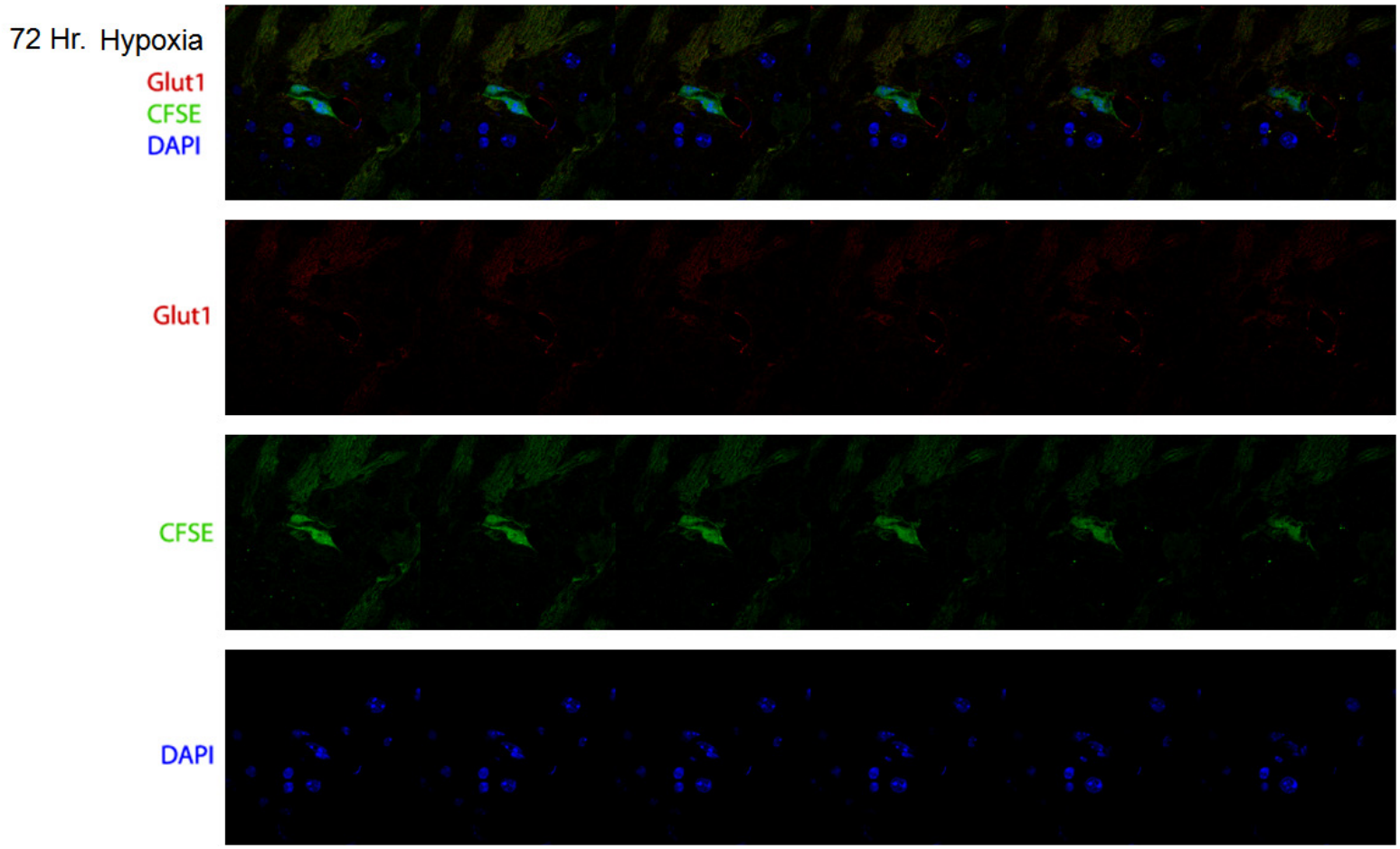


**Figure 5 - Isolated brain microvessels incorporate into brain microvasculature after exposure to 24 hour hypoxia. Colocalization of CFSE (green) labeled cultured brain microvessel endothelial cells with Glut1 (red) labeled brain microvessels from the cortex of a CFSE labeled cell injected mouse treated with 24 h of hypoxia before harvest. A series of images taken with confocal microscopy as the focus progresses through the tissue section. CFSE labeled injected brain microvessel endothelial cells colocalize with the Glut1 stained vessel wall.**

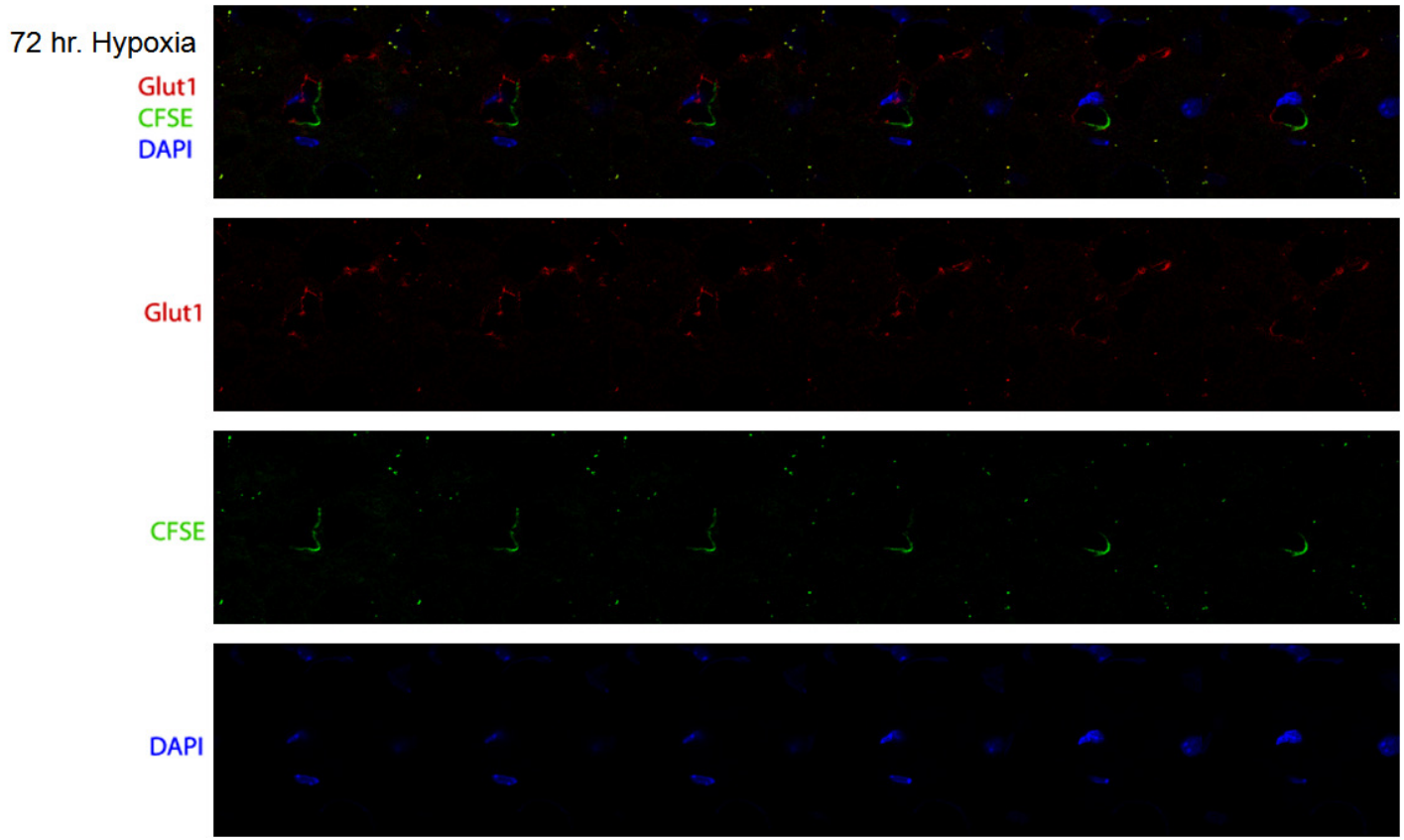


**Figure 6 - Isolated brain microvessels incorporate into brain microvasculature after exposure to 72 hour hypoxia. Colocalization of CFSE (green) labeled cultured brain microvessel endothelial cells with Glut1 (red) labeled brain microvessels from the cortex of a CFSE labeled cell injected mouse treated with 72 h of hypoxia before harvest. A series of images taken with confocal microscopy as the focus progresses through the tissue section. CFSE labeled injected brain microvessel endothelial cells colocalize with the Glut1 stained vessel wall.**

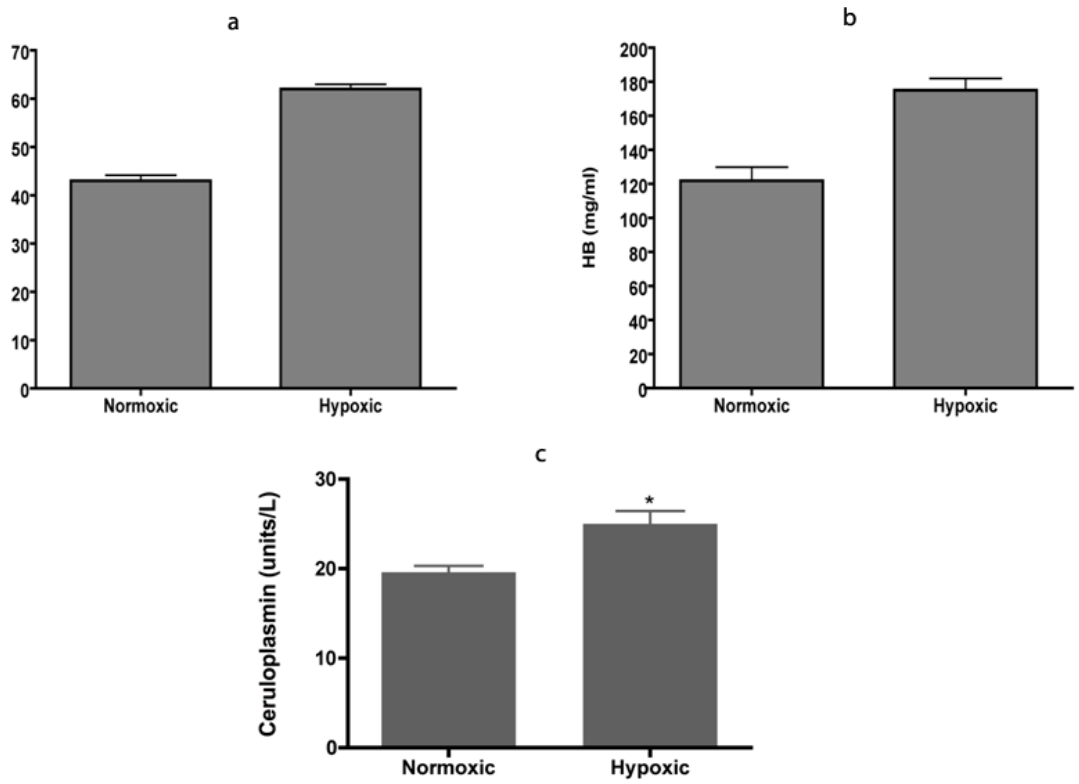


**Figure 7 - Hematocrit, hemoglobin, and ceruloplasmin activity levels increase in hypoxia. Mice treated with chronic acute hypoxic (10% $O_2$) conditions for 21 days had an increase in the levels of (a) hematocrit($t = 11.68$, $p<0.05$), (b) hemoglobin ($t=4.48$, $p<0.05$), and (c) ceruloplasmin ($p<0.027$) as compared with normoxic (20% $O_2$) controls.**

the cerebellum and cortex were measured. Hematocrit significantly increased in hypoxia (62% +/- 1) over normoxia (42.83% +/- 2.25) ($t = 11.68$, $p<0.05$)(Figure 7a).

Hemoglobin levels were also significantly increased by treatment with hypoxia (174.99 mg/mL +/- 12.08) versus normoxia (121.92+/- 16.77 (Figure 7b) ($t=4.48$, $p<0.05$). There was also a significant increase in ceruloplasmin levels with hypoxia treatment (Figure 7c). Lastly, a visual increase in the microvessel density was seen with immunohistochemistry (Figure 9). This visual difference was quantified to show a statistically significant increase in the microvessel density in hypoxic brain sections (0.18 microvessels/$\mu m^2$ +/- 0.034) versus normoxic brain sections (0.13 microvessels/ $\mu m^2$ +/- 0.028) ( $t = 2.83$, $p<0.05$) (Figure 8). We concluded that FVB/NJ mice responded to hypoxic hypoxia by increasing hematocrit, hemoglobin, ceruloplasmin, and microvessel density in the brain.

***EPCs and cultured brain microvessel endothelial cells injected via internal carotid artery retrograde cannulation localize to microvessels in the brain with concurrent hypoxic hypoxia treatment***

Our previous data indicated that animals in a hypoxic hypoxia environment showed an increase in microvessel density. Therefore, we hypothesized that the growth of new vasculature, postnatal neovasculogenesis, under hypoxic conditions occurred at least in part via incorporation of circulating cells. In order to increase the number of cells that were available for incorporation into the brain vasculature, we chose to inject via the internal carotid artery. CFSE-labeled EPCs and brain microvessel ECs were injected via external carotid artery retrograde cannulation into the internal carotid

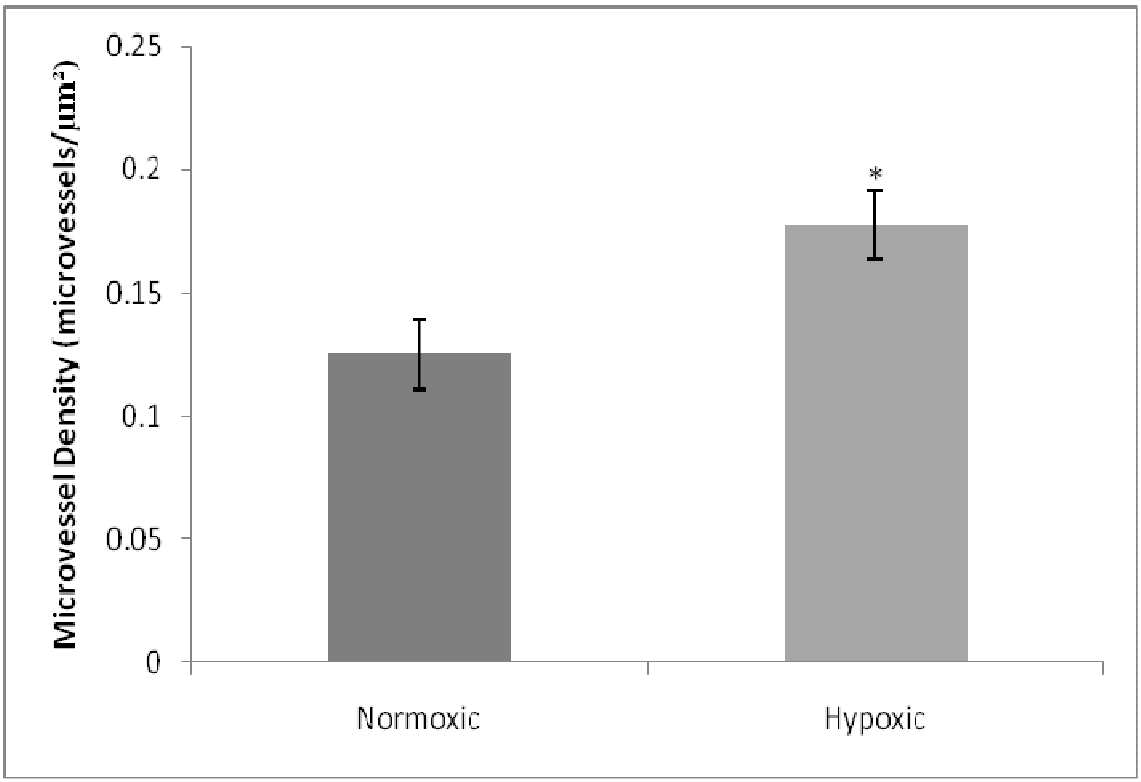


**Figure 8 - Microvessel Density increases significantly between hypoxia and normoxia conditions. Microvessel Density in the cortex of mice treated with chronic acute hypoxia (10% $O_2$) for 21 days (0.18 microvessels/µm$^2$ +/- 0.03) showed a significant increase as compared to control mice at normoxic (20% $O_2$) conditions (0.13 microvessels/ µm$^2$ +/- 0.03) ( t = 2.83, p<0.05).**

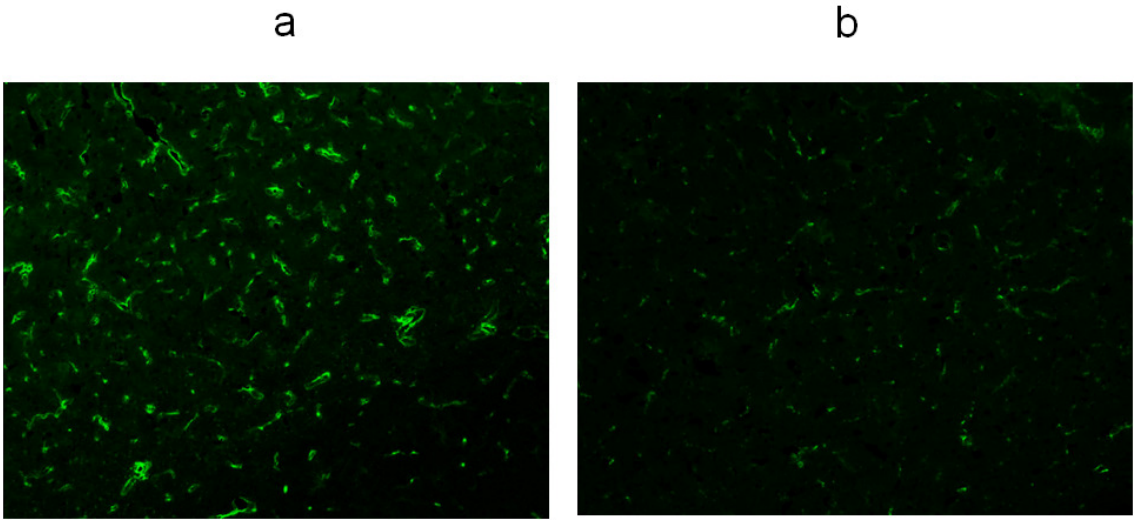


**Figure 9 - Exposure to chronic, acute hypoxia leads to in an increase in the microvasculature of the cortex in FVB mice. Brain cryosections of mice treated with (a) chronic, acute hypoxic conditions (10% $O_2$) for 21 days had an increase in the microvascular density as compared to (b)normoxic (20% $O_2$) controls.**

artery. The total number of cells injected ranged from 46,000-70,000 cells per injection. The CFSE dye was used to track the injected cells. Hypoxia treatment began 24 hours prior to injection. The animals were allowed 24 hours of recovery after the injection before being placed in hypoxia for 24 hours or 72 hours. Animals were anesthetized and their brain harvested for characterization of injected cells. Injected cells were labeled with CFSE prior to injection which fluoresces green, while brain microvessels were labeled using immunofluorescence for Glut1 with a Texas Red secondary antibody. We found that the injection of the EPCs and ECs via the internal carotid artery and with hypoxic hypoxia treatment resulted in localization and incorporation into the microvasculature. However, the incorporation of EPCs was less than that of ECs. We hypothesized that the lesser incorporation of EPCs was because EPCs do not already possess all the markers of brain microvessel ECs. While ex vivo expanded brain microvessel ECs did express all the markers that were present on the current vasculature of the brain and therefore encouraged increased incorporation. However, during *in vitro* characterization of brain micrvoessel ECs we noted a decrease in the fluorescence when the ECs were labeled with Glut1. So although they still expressed some of the markers of brain microvessel ECs *in vivo* they were not phenotypically identical to *in vivo* microvessel ECs. We also tested if a longer recovery time 24 hours (Figure 5) or 72 hours (Figure 6) led to a reduced number of injected cells present or a change in the phenotype of the injected cells. We found that there was an indirect relationship between recovery time and the number of injected cells (Figure 10), i.e. as the recovery time increased the number of injected cells present in the

microvasculature decreased. We also noted that the injected cells did not seem to increase in expression of Glut1 over time. However, 72 hours may not be long enough to induce a phenotypic change matching that of the *in vivo* brain microvessel ECs. Lastly, we compared the number of CFSE + cells in the brain between the two treatment groups. We found that the number of CFSE + cells with mannitol treatment was lower than the number with hypoxia treatment (Figure 10). We hypothesize that the decrease in the number of CFSE + cells is because the circulating cells do not respond to damage of vasculature as readily as they respond to hypoxic signals from the brain tissue.

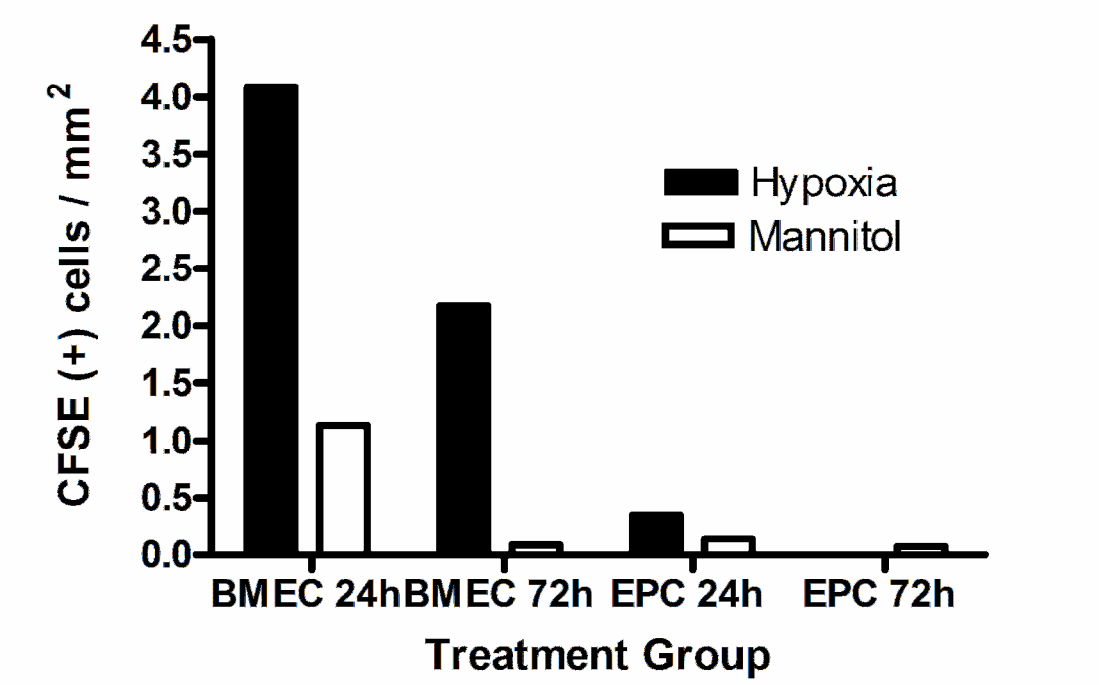


**Figure 10 - Treatment with hypoxia increases incorporation of circulating cells.**
**BMECs incorporate better than EPCs. The average number of CFSE (+) BMECs is higher than outgrowth EPCs. The number of CFSE (+) cells was also higher when treated with hypoxia versus mannitol.**

## CHAPTER 4: Discussion

The structure of vasculature in the brain is strictly maintained to prevent access of molecules, solutes, hormones, gases, antibodies, bacteria, and viruses to the parenchyma of the brain. Prevention of molecule movement is achieved through tight junctions between the ECs lining the vasculature. Tight junctions and ECs form the selectively permeable BBB. Small molecules such as hormones and gases are allowed to freely diffuse across the BBB. Everything else requires a transport mechanism to facilitate movement into or out of the ECs. Ultimately some of the solutes may cross the basement membrane and have an effect on neurons or astrocytes. Facilitated transport, active transport, and free diffusion are some of the transport mechanisms. Active and facilitated transport require transport proteins. Active transport requires energy in the form of ATP to move solutes and large molecules commonly against their concentration gradient. Free diffusion occurs through ion channels which is how solute homeostasis is maintained. Facilitated transport is the movement of molecules into or out of the cell without the use of ATP as an energy source. Facilitated transport occurs via binding of the molecule to be transported by the transport protein. Binding causes a conformational change and moves the molecule to the other side of the membrane.

One of the facilitated transport proteins present in the brain vasculature is Glut1. Glut1 transports glucose out of the blood and across the BBB. Transportation across the BBB makes glucose available for energy generation in neurons and astrocytes (Kalaria, 1988). The gene encoding GLUT1, *SLC2A1*, is present in two copies in each cell in the body. GLUT1 is specifically expressed in brain endothelium while other

glucose transport proteins are present in other tissues. Mutations in the *SLC2A1* gene have been identified that result in non-functioning GLUT1 proteins (Wang, 2000). Non-functioning GLUT1 results in limited transport of glucose across the BBB which reduces available glucose for energy generation in the brain. Glucose is utilized in the citric acid cycle and glycolysis for generation of ATP. ATP is necessary for most biological functions, and is especially important during development. When glucose transport into the brain is significantly reduced during brain development the individual will develop infantile seizures, microcephaly, developmental delays, and other related neurologic problems (De Vivo, 1991). Reduction of ATP production from glycolysis and the citric acid cycle can be ameliorated with a ketogenic diet. A ketogenic diet reduces the need for glucose because ketone bodies can be utilized for energy generation (Withrow, 1980). However, this diet is not healthy for an individual to maintain long-term. Gene therapy is another possibility for treatment of genetic disorders where one or both copies of the wild-type allele is missing or mutated. A gene encoding wild-type Glut1 can be inserted into a plasmid or artificial chromosome engineering (ACE). Therefore, we hypothesized that one way to facilitate gene therapy for the cerebrovasculature is to encourage incorporation of cells transfected with the gene encoding wild-type Glut1. Incorporation of engineered cells requires the ability to specifically home cells containing the gene plasmid to the vasculature in significant amounts. Ischemia (hind limb, myocardial, stroke, and hypoxia), tumors, and vascular homeostasis are several different methods that are currently used to encourage new blood vessel growth for research purposes. Each mode of new blood vessel growth occurs in slightly different ways.

Expansion of the vasculature can occur via incorporation of circulating EPCs or circulating ECs (vasculogenesis) as well as proliferation of resident ECs (angiogenesis). The debate continues over which mechanism predominates. We examined NOD-SCID, FVB/NJ, and 129-E mice for neovascularization. FVB/NJ mice responded to hypoxia by increasing CD31+/Glut1+ vasculature in the brain (hypoxic: 0.18 microvessels/µm2 +/- 0.034, normoxic: 0.13 microvessels/ µm2 +/- 0.028, $t = 2.83$, $p<0.05$) (Figure 8). The hypoxic effect was secondarily confirmed through hematocrit (hypoxic: 62% +/- 1, normoxic 42.83% +/- 2.25, $t = 11.68$, $p<0.05$) (Figure 7a) and hemoglobin concentration (hypoxic: 174.99 mg/mL +/- 12.08, normoxic 121.92 +/- 16.77, $t=4.48$, $p<0.05$) (Figure 7b) in the peripheral blood. Given these data, hypoxia is effective at increasing vascular density and having secondary effect on properties of the blood.

Vascular damage requires angiogenesis and/or vasculogenesis for repair. A known method of inducing vascular damage in the brain is reversible osmotic disruption with hyperosmolar mannitol. Mannitol injections are used in humans to deliver chemotherapeutic drugs directly to the brain. In humans a catheter is fed up and into the brain via the internal carotid artery. We therefore decided to try injection into the internal carotid artery in mice for direct delivery of solutes and cells to the brain. The effectiveness of the injection was determined by injecting mannitol followed by Evan's Blue Dye and looking for Evan's Blue in the brain parenchyma. C57 BL/6 mice were given an injection of mannitol via retrograde cannulation of the external carotid artery into the circulating blood in the internal carotid artery and common carotid artery (Figure 3). The mannitol injection was followed immediately by injection with Evan's Blue Dye (Huttner, 1984; Christophidis, 1988; Pokorny, 2003; Fortin, 2008). Evan's

Blue Dye binds to albumin. After a period of recovery, harvested brain tissue showed a presence of Evan's Blue Dye (Figure 4). The presence of Evan's Blue Dye in the brain parenchyma confirmed that injection into the internal carotid artery directly delivered solutes to the brain. Evan's Blue Dye presence also confirmed that mannitol had induced reversible vascular damage by breaking tight junction in the BBB and allowing leakage of Evan's Blue Dye-bound albumin into the brain parenchyma.

After identification of methods for encouraging the expansion or damaging of current vasculature we needed to decide which cells we would use for injection. Current research identified circulating EPCs and ECs as incorporating into vasculature in addition to proliferation of resident ECs. Therefore we chose to test EPCs and a primary cell culture of brain microvessels ECs. We chose EPCs because they are progenitor cells that are capable of differentiating into several different types of hematopoietic cells, and they have been shown to incorporate into vessels in ischemia or damage repair (Asahara, 1999; Asahara, 2002; Yoder, 2009; Katusic, 2004; Finkel, 2005; Yoon, 2005; Miller-Kasprzak, 2007; Karsan, 2007; Sbarbati, 1991). EPCs were isolated from human peripheral blood. Our collaborators, Dr.'s Zvonimir Katusic and Tongrong He had previously isolated the EPCs and characterized them via their cell surface markers. The EPCs that we used were CD31, CD45, CD133, VEGFR2, VE-cadherin, C-kit, Tie2, and CD34 positive as well as being positive for acLDL uptake and lectin binding (Katusic, 2004).

Previous studies indicated that fully differentiated ECs were also present in physiologic circulation (Estes, 2010). Therefore, we chose to test ex vivo expanded brain microvessel ECs. We hypothesized that brain microvessel ECs would adhere to

and incorporate into the expanding or damaged cerebrovasculature. After ex vivo expansion cells were characterized for cell surface markers of mature microvessel ECs to confirm that their phenotype was maintained for the extent of culture. Our results showed that after 9 days of expansion brain microvessel ECs continued to express Glut1 (Figure 2b), CD31 (Figure 2a), and have an intact nucleus. Brain microvessel ECs *in vitro* had also changed their morphology from a vessel to a monolayer (Figure 1). The expression of CD31 and Glut1 confirms that the isolated cells retain the phenotype of the original brain microvessel. However, the fluorescence associated with the positive staining for both CD31 and Glut1 *in vitro* was less than brain microvessels *in vivo*. Changes in fluorescence levels indicate that the cultured cells may be beginning to undergo a phenotypic change. Prolonged culture may result in a complete phenotypic loss. The intact nucleus of the cells in culture indicates that they are healthy and more clearly shows the flattened nuclei characteristic of a monolayer of cells.

After confirming the phenotypes of the cultured cells it was necessary to determine if incorporation of injected ("circulating") cells was possible with our methods of vascular expansion and damage. We hypothesized that injection of cells into the mouse would result in incorporation of injected cells in response to hypoxia or vascular damage. In order to follow the cultured EPCs and brain microvessel ECs *in vivo*, cells were labeled with a carboxyfluroescin dye (CFSE) that was retained by the cells over several generations (Bronner-Fraser, 1985; Takeichi, 1986; Lyons, 2000; Lyons, 1996; Parish, 1990). Post-labeling, the cells were trypsinized and injected via two methods. The first method of injection was through the tail vein. This method did not show any cells that homed to the brain vasculature despite exposure to hypoxic

hypoxia (data not shown). Likely, the reason for the lack of injected cells present in the brain was filtration of the blood in the kidney, spleen, liver, and lungs. The quantity of injected cells that actually reached the brain was very limited. In order to circumvent the filtration we injected via retrograde cannulation of the external carotid artery into the circulating blood in the internal carotid artery and common carotid artery (Toborek, 2008) (Figure 3). This type of injection would allow the cells immediate and direct access to the brain allowing the largest quantity of labeled, injected cells to reach the brain vasculature. Injection into the internal carotid artery was also the method used to deliver mannitol directly to the brain to disrupt the BBB. Internal carotid artery injection was the most effective method for delivery of cells and solutes to the brain. Therefore, injection of cells via this method was used in coordination with hypoxia and mannitol treatment to facilitate incorporation of injected cells.

Achieving neovascularization in the brain has its own set of challenges and uncertainties regarding exactly what occurs when new blood vessels are formed. Present studies fail to agree on whether the cerebrovasculature expands via angiogenesis or vasculogenesis. Theoretically, both mechanisms may be present in the expansion or repair of the cerebrovasculature. However, it makes more sense for the cerebrovasculature to be expanded via angiogenesis because of the impermeable nature of the BBB. Maintenance of this barrier protects the brain, and proliferation seems to maintain the barrier during expansion. However, if the BBB is disrupted the brain can be exposed to contaminants that are present in the blood but not normally allowed to cross the BBB. Disruption of the tight junctions that maintain the BBB may signal for immediate vascular repair. Repair can occur through angiogenesis, vasculogenesis, or

the immediate reformation of the tight junctions in the BBB. In this situation, incorporation of circulating cells into the vasculature seems more likely because incorporating is a "faster fix" for a hole in the barrier than the proliferation of resident ECs. In order to see if incorporation of circulating cells occurred, signals for neovascularization or vascular repair were used. Two proposed methods for signaling neovascularization and vascular repair in the cerebrovasculature are chronic, acute hypoxia and physically breaking the tight junctions between the ECs using osmotic disruption.

We previously showed that hypoxic hypoxia was able to increase microvessel density in the mouse cerebrovasculature (Figure 9). We also concluded that injection of cells into the internal carotid artery was effective at circumventing the body's natural filtration in the kidney, liver, lungs, and spleen (Figure 3). Therefore, we hypothesized that injection of EPCs and ECs into the internal carotid artery, in combination with hypoxia 24 hours before and 24 or 72 hours after injection will result in incorporation of injected cells into the expanding cerebrovasculature. Using these methods together there were injected cells present in the expanding cerebrovasculture at the time of tissue harvest. The hypoxic brain signaled for an increase in the microvessel density. Injection into the internal carotid artery was effective in trapping and incorporating the injected cells. Treatment with hypoxia began 24 hours prior to injection to ramp up the body's response to the hypoxia in hopes that this would induce signaling from the hypoxic tissue around the body. The animals were allowed 24 hours to recover post-operatively because the injection and hypoxia were taxing on their bodies. After 24 or 72 hours of hypoxic treatment the mice were anesthetized and the brain harvested for

immnofluorescent detection of CFSE labeled injected cells and cerebromicrovasculature (Figures 5 and 6). There was a significant number of CFSE+ cells that were in or had become part of the cerebromicrovasculature ipsilateral to the injection site. However, we cannot definitively say that the cells present will remain part of the permanent cerebromicrovasculature. In fact, as the recovery time increased the number of CFSE+ cells that remained in the cerebromicrovasculature decreased (Figure 10). Also, the CFSE+ cells did not co-localize with Glut1. Glut1 positive staining was usually found on a cell that was in contact with the CFSE+ cells. Using confocal microscopy the CFSE+ cells in contact with the Glut1+ cells had the morphology of a microvessel (Figures 5 and 6). However, since the CFSE+ cells did not display markers for Glut1 we cannot conclude that the cells are fully incorporated into the expanded cerebromicrovasculature.

We previously showed that mannitol disrupts the BBB reversibly, allowing for Evan's Blue Dye-bound albumin to accumulate in the brain parenchyma when injected into the internal carotid artery (Huttner, 1984; Christophidis, 1988; Pokorny, 2003; Fortin, 2008) (Figure 4). Therefore, we hypothesized that reversible osmotic disruption is another method for encouraging incorporation of injected cells recruited for vascular repair. The temporary breakage of the tight junctions between ECs of the brain vasculature caused by injection of the hypertonic salt solution may induce signals related to vascular repair. If the induction of vascular damage is followed by injection with the types of cells that physiologically respond to signals for repair, then the injected cells may help repair the damaged vasculature (Chopp, 2008). Inducing vascular damage with mannitol followed by injection of cultured EPCs and brain

microvessel ECs resulted in a significant accumulation of injected cells in the damaged vasculature (Figure 10). To confirm that presence of CFSE+ cells was due to vascular damage 1X PBS was injected instead of mannitol. PBS injection was immediately followed by injection of CFSE+ cells. When PBS was injected there was no BBB damage and no incorporation of CFSE+ cells into the cerebromicrovasculature (data not shown).

Subsequently, we compared the incorporation of EPCs to brain microvessel ECs. Vasculogenesis is defined as the incorporation of bone marrow-derived circulating EPCs in neovascularization or vascular homeostasis and repair. Angiogenesis is defined as the sprouting of new vessels from resident ECs in the nearby vasculature. Our data indicates that EPCs and brain microvessel ECs are present in the cerebromicrovasculature, however, EPC presence was less than brain microvessel EC (Figure 10). EPCs were derived from human peripheral blood and injected into NOD-SCID mice. Crossing species from human to mice may have impaired the cells ability to respond to signals such as hypoxia or vascular damage. Secondly, the EPCs were passaged 7-8 times and may have lost or have a reduced number of receptors to respond to signals from hypoxia or vascular damage.

Cultured brain microvessel ECs exhibited the same markers as those of cerebrovasculature, but as the time in culture increased the fluorescent intensity associated with these markers decreased (Figure 2a and 2b). The decrease in fluorescence is presumably from a decrease in the expression of cell surface markers. The decrease in the cell surface markers may result in decreased adhesion and incorporation in response to hypoxia or vascular damage signaling from resident ECs.

The presence of brain microvessel ECs in the cerebromicrovasculatre was higher than EPCs, but it is not possible to say whether vasculogenesis or angiogenesis is responsible at this point (Figure 10). Further studies are necessary to determine what role EPCs and ECs play in hypoxic cerebrovascular expansion and vascular repair.

In summary, we used two methods to alter normal brain vascular density. Chronic, acute hypoxia resulted in increased vascular density in the brain. And reversible osmotic disruption with mannitol reversibly damaged the vasculature in the brain. Both methods have the possibility of using angiogenesis or vasculogenesis to expand or repair the current vasculature. Therefore, different cell types circulating in the blood may be recruited to repair damaged vasculature or expand the current vasculature, including EPCs and ECs. We chose to test incorporation of EPCs and ECs. Previous studies showed that EPCs circulate in the blood and home to sites in need of repair in the current vasculature (Katusic, 2004). We used EPCs isolated from human peripheral blood, labeled them, and reintroduced them into the circulation. The cells were reintroduced to test if injected EPCs play a role in expansion or repair of the vasculature in the brain. Brain vasculature has unique characteristics. Therefore, in addition to EPCs we used ex vivo expanded brain microvessel ECs, labeled them, and reintroduced them into the circulation. The reintroduced brain microvessel ECs contained all the same markers as the ECs in the resident vasculature.

The injection site is also important in determining if incorporation is likely to occur and how frequent the incorporation will be. If the cells are injected in the tail vein the majority of the cells are filtered through the liver, spleen, lungs, and kidneys. The volume of cells that actually reach the brain vasculature is very limited and

therefore few cells can incorporate. However, injection of cells into the internal carotid artery allowed direct access to the brain vasculature and encouraged incorporation. The number of injected cells that reached the cerebromicrovasculature was highest with this type of injection. Combining the methods for inducing vascular damage with mannitol or vasculature expansion with hypoxia and the optimal injection site into the internal carotid artery created the most advantageous environment for significant incorporation to occur. The cell types that were injected were chosen based on supporting literature and the target site for incorporation. Incorporation was identified by fluorescently labeling the cells prior to injection and co-localizing the fluorescence with markers found only on cerebrovasculture. In conclusion, brain neovascularization induced by chronic, acute hypoxia or reversible osmotic disruption with mannitol are both capable of recruiting injected EPCs and ECs, however, whether or not the cells are fully incorporated into the cerebromicrovasculature remains unknown.

**REFERENCES:**


Aicher, A., et al. "Nonbone Marrow-Derived Circulating Progenitor Cells Contribute to Postnatal Neovascularization Following Tissue Ischemia." Circ Res 100.4 (2007): 581-9. Print.

Asahara, T., et al. "Bone Marrow Origin of Endothelial Progenitor Cells Responsible for Postnatal Vasculogenesis in Physiological and Pathological Neovascularization." Circ Res 85.3 (1999): 221-8. Print.

Asahara, T., et al. "Isolation of Putative Progenitor Endothelial Cells for Angiogenesis." Science 275.5302 (1997): 964-7. Print.

Bellavance, Marc-André, Marie Blanchette, and David Fortin. "Recent Advances in Blood–Brain Barrier Disruption as a Cns Delivery Strategy." The AAPS Journal 10.1 (2008): 166-77. Print.

Biegel, D., D. D. Spencer, and J. S. Pachter. "Isolation and Culture of Human Brain Microvessel Endothelial Cells for the Study of Blood-Brain Barrier Properties *in vitro*." Brain Res 692.1-2 (1995): 183-9. Print.

Brightman, M. W., et al. "Osmotic Opening of Tight Junctions in Cerebral Endothelium." J Comp Neurol 152.4 (1973): 317-25. Print.

Brightman, M. W., and T. S. Reese. "Junctions between Intimately Apposed Cell Membranes in the Vertebrate Brain." J Cell Biol 40.3 (1969): 648-77. Print.

Brockmann, K., et al. "Autosomal Dominant Glut-1 Deficiency Syndrome and Familial Epilepsy." Ann Neurol 50.4 (2001): 476-85. Print.

Bronner-Fraser, M. "Alterations in Neural Crest Migration by a Monoclonal Antibody That Affects Cell Adhesion." J Cell Biol 101.2 (1985): 610-7. Print.

Campen, M. J., et al. "Heart Rate Variability Responses to Hypoxic and Hypercapnic Exposures in Different Mouse Strains." J Appl Physiol 99.3 (2005): 807-13. Print.

Chen, L., K. R. Swartz, and M. Toborek. "Vessel Microport Technique for Applications in Cerebrovascular Research." J Neurosci Res 87.7 (2009): 1718-27. Print.

Cosolo, W. C., et al. "Blood-Brain Barrier Disruption Using Mannitol: Time Course and Electron Microscopy Studies." Am J Physiol 256.2 Pt 2 (1989): R443-7. Print.

Daneman, R., et al. "Wnt/Beta-Catenin Signaling Is Required for Cns, but Not Non-Cns, Angiogenesis." Proc Natl Acad Sci U S A 106.2 (2009): 641-6. Print.

De Vivo, D. C., et al. "Defective Glucose Transport across the Blood-Brain Barrier as a Cause of Persistent Hypoglycorrhachia, Seizures, and Developmental Delay." N Engl J Med 325.10 (1991): 703-9. Print.

Dehouck, B., et al. "A New Function for the Ldl Receptor: Transcytosis of Ldl across the Blood-Brain Barrier." J Cell Biol 138.4 (1997): 877-89. Print.

Descamps, L., et al. "Receptor-Mediated Transcytosis of Transferrin through Blood-Brain Barrier Endothelial Cells." Am J Physiol 270.4 Pt 2 (1996): H1149-58. Print.

Dropulic, B., and C. L. Masters. "Culture of Mouse Brain Capillary Endothelial Cell Lines That Express Factor Viii, Gamma-Glutamyl Transpeptidase, and Form

Junctional Complexes *in vitro*." *In vitro* Cell Dev Biol 23.11 (1987): 775-81. Print.
Estes, M. L., et al. "Identification of Endothelial Cells and Progenitor Cell Subsets in Human Peripheral Blood." Curr Protoc Cytom Chapter 9: Unit 9 33 1-11. Print.
Folkman, J. "Angiogenesis in Cancer, Vascular, Rheumatoid and Other Disease." Nat Med 1.1 (1995): 27-31. Print.
Gonul, E., et al. "Early Pericyte Response to Brain Hypoxia in Cats: An Ultrastructural Study." Microvasc Res 64.1 (2002): 116-9. Print.
Harik, N., et al. "Time-Course and Reversibility of the Hypoxia-Induced Alterations in Cerebral Vascularity and Cerebral Capillary Glucose Transporter Density." Brain Res 737.1-2 (1996): 335-8. Print.
Harik, S. I., R. A. Behmand, and J. C. LaManna. "Hypoxia Increases Glucose Transport at Blood-Brain Barrier in Rats." J Appl Physiol 77.2 (1994): 896-901. Print.
Harik, S. I., M. A. Hritz, and J. C. LaManna. "Hypoxia-Induced Brain Angiogenesis in the Adult Rat." J Physiol 485 ( Pt 2) (1995): 525-30. Print.
He, T., et al. "Transplantation of Circulating Endothelial Progenitor Cells Restores Endothelial Function of Denuded Rabbit Carotid Arteries." Stroke 35.10 (2004): 2378-84. Print.
Hur, J., et al. "Characterization of Two Types of Endothelial Progenitor Cells and Their Different Contributions to Neovasculogenesis." Arterioscler Thromb Vasc Biol 24.2 (2004): 288-93. Print.
Jiang, B. H., et al. "Transactivation and Inhibitory Domains of Hypoxia-Inducible Factor 1alpha. Modulation of Transcriptional Activity by Oxygen Tension." J Biol Chem 272.31 (1997): 19253-60. Print.
Kalaria, R. N., et al. "The Glucose Transporter of the Human Brain and Blood-Brain Barrier." Ann Neurol 24.6 (1988): 757-64. Print.
Khakoo, A. Y., and T. Finkel. "Endothelial Progenitor Cells." Annu Rev Med 56 (2005): 79-101. Print.
Klepper, J., et al. "Defective Glucose Transport across Brain Tissue Barriers: A Newly Recognized Neurological Syndrome." Neurochem Res 24.4 (1999): 587-94. Print.
Kozler, P., and J. Pokorny. "Evans Blue Distribution in the Rate Brain after Intracarotid Injection with the Blood-Brain Barrier Intact and Open to Osmosis." Sb Lek 104.3 (2003): 255-62. Print.
Lammert, E. "Developmental Biology. Brain Wnts for Blood Vessels." Science 322.5905 (2008): 1195-6. Print.
Larrivee, B., and A. Karsan. "Involvement of Marrow-Derived Endothelial Cells in Vascularization." Handb Exp Pharmacol.180 (2007): 89-114. Print.
Liebner, S., et al. "Wnt/Beta-Catenin Signaling Controls Development of the Blood-Brain Barrier." J Cell Biol 183.3 (2008): 409-17. Print.
Limbourg, A., et al. "Evaluation of Postnatal Arteriogenesis and Angiogenesis in a Mouse Model of Hind-Limb Ischemia." Nat Protoc 4.12 (2009): 1737-46. Print.
Lin, Y., et al. "Origins of Circulating Endothelial Cells and Endothelial Outgrowth from Blood." J Clin Invest 105.1 (2000): 71-7. Print.

Lorke, D. E., et al. "Entry of Oximes into the Brain: A Review." Curr Med Chem 15.8 (2008): 743-53. Print.
Lyons, A. B. "Analysing Cell Division *in vivo* and *in vitro* Using Flow Cytometric Measurement of Cfse Dye Dilution." J Immunol Methods 243.1-2 (2000): 147-54. Print.
Maxwell, P. H., et al. "The Tumour Suppressor Protein Vhl Targets Hypoxia-Inducible Factors for Oxygen-Dependent Proteolysis." Nature 399.6733 (1999): 271-5. Print.
Mead, L. E., et al. "Isolation and Characterization of Endothelial Progenitor Cells from Human Blood." Curr Protoc Stem Cell Biol Chapter 2 (2008): Unit 2C 1. Print.
Miebach, S., et al. "Isolation and Culture of Microvascular Endothelial Cells from Gliomas of Different Who Grades." J Neurooncol 76.1 (2006): 39-48. Print.
Miller-Kasprzak, E., and P. P. Jagodzinski. "Endothelial Progenitor Cells as a New Agent Contributing to Vascular Repair." Arch Immunol Ther Exp (Warsz) 55.4 (2007): 247-59. Print.
Nagy, Z., H. Peters, and I. Huttner. "Fracture Faces of Cell Junctions in Cerebral Endothelium During Normal and Hyperosmotic Conditions." Lab Invest 50.3 (1984): 313-22. Print.
Nose, A., and M. Takeichi. "A Novel Cadherin Cell Adhesion Molecule: Its Expression Patterns Associated with Implantation and Organogenesis of Mouse Embryos." J Cell Biol 103.6 Pt 2 (1986): 2649-58. Print.
Padridge, W. “Blood-brain barrier biology and methodology.” Journal of NeuroVirology (1999) 5, 556 -569
Pappius, H. M., et al. "Osmotic Opening of the Blood-Brain Barrier and Local Cerebral Glucose Utilization." Ann Neurol 5.3 (1979): 211-9. Print.
Peichev, M., et al. "Expression of Vegfr-2 and Ac133 by Circulating Human Cd34(+) Cells Identifies a Population of Functional Endothelial Precursors." Blood 95.3 (2000): 952-8. Print.
Persidsky, Y., et al. "Blood-Brain Barrier: Structural Components and Function under Physiologic and Pathologic Conditions." J Neuroimmune Pharmacol 1.3 (2006): 223-36. Print.
Polakis, P. "Formation of the Blood-Brain Barrier: Wnt Signaling Seals the Deal." J Cell Biol 183.3 (2008): 371-3. Print.
Rapoport, S. I. "Effect of Concentrated Solutions on Blood-Brain Barrier." Am J Physiol 219.1 (1970): 270-4. Print.
Rapoport, S. I. "Osmotic Opening of the Blood-Brain Barrier: Principles, Mechanism, and Therapeutic Applications." Cell Mol Neurobiol 20.2 (2000): 217-30. Print.
Reese, T. S., and M. J. Karnovsky. "Fine Structural Localization of a Blood-Brain Barrier to Exogenous Peroxidase." J Cell Biol 34.1 (1967): 207-17. Print.
Risau, W., and H. Wolburg. "Development of the Blood-Brain Barrier." Trends Neurosci 13.5 (1990): 174-8. Print.
Rupnick, M. A., A. Carey, and S. K. Williams. "Phenotypic Diversity in Cultured Cerebral Microvascular Endothelial Cells." *In vitro* Cell Dev Biol 24.5 (1988): 435-44. Print.

Sbarbati, R., et al. "Immunologic Detection of Endothelial Cells in Human Whole Blood." Blood 77.4 (1991): 764-9. Print.
Scholz, D., et al. "Contribution of Arteriogenesis and Angiogenesis to Postocclusive Hindlimb Perfusion in Mice." J Mol Cell Cardiol 34.7 (2002): 775-87. Print.
Seeger, F. H., et al. "Cxcr4 Expression Determines Functional Activity of Bone Marrow-Derived Mononuclear Cells for Therapeutic Neovascularization in Acute Ischemia." Arterioscler Thromb Vasc Biol 29.11 (2009): 1802-9. Print.
Semenza, G. L. "Vascular Responses to Hypoxia and Ischemia." Arterioscler Thromb Vasc Biol 30.4: 648-52. Print.
Seyfried, D. M., et al. "Mannitol Enhances Delivery of Marrow Stromal Cells to the Brain after Experimental Intracerebral Hemorrhage." Brain Res 1224 (2008): 12-9. Print.
Shaked, Y., et al. "Genetic Heterogeneity of the Vasculogenic Phenotype Parallels Angiogenesis; Implications for Cellular Surrogate Marker Analysis of Antiangiogenesis." Cancer Cell 7.1 (2005): 101-11. Print.
Simper, D., et al. "Smooth Muscle Progenitor Cells in Human Blood." Circulation 106.10 (2002): 1199-204. Print.
Smith, Q. R., and D. D. Allen. "*In situ* Brain Perfusion Technique." Methods Mol Med 89 (2003): 209-18. Print.
Song, L., and J. S. Pachter. "Culture of Murine Brain Microvascular Endothelial Cells That Maintain Expression and Cytoskeletal Association of Tight Junction-Associated Proteins." *In vitro* Cell Dev Biol Anim 39.7 (2003): 313-20. Print.
Stenman, J. M., et al. "Canonical Wnt Signaling Regulates Organ-Specific Assembly and Differentiation of Cns Vasculature." Science 322.5905 (2008): 1247-50. Print.
Timmermans, F., et al. "Endothelial Progenitor Cells: Identity Defined?" J Cell Mol Med 13.1 (2009): 87-102. Print.
Walenta, K., et al. "*In vitro* Differentiation Characteristics of Cultured Human Mononuclear Cells-Implications for Endothelial Progenitor Cell Biology." Biochem Biophys Res Commun 333.2 (2005): 476-82. Print.
Walter, D. H., et al. "Impaired Cxcr4 Signaling Contributes to the Reduced Neovascularization Capacity of Endothelial Progenitor Cells from Patients with Coronary Artery Disease." Circ Res 97.11 (2005): 1142-51. Print.
Wang, D., P. Kranz-Eble, and D. C. De Vivo. "Mutational Analysis of Glut1 (*Slc2a1*) in Glut-1 Deficiency Syndrome." Hum Mutat 16.3 (2000): 224-31. Print.
Ward, N. L., et al. "Cerebral Angiogenic Factors, Angiogenesis, and Physiological Response to Chronic Hypoxia Differ among Four Commonly Used Mouse Strains." J Appl Physiol 102.5 (2007): 1927-35. Print.
Weston, S. A., and C. R. Parish. "New Fluorescent Dyes for Lymphocyte Migration Studies. Analysis by Flow Cytometry and Fluorescence Microscopy." J Immunol Methods 133.1 (1990): 87-97. Print.
Withrow, C. D. "The Ketogenic Diet: Mechanism of Anticonvulsant Action." Adv Neurol 27 (1980): 635-42. Print.

Wu, Z., F. M. Hofman, and B. V. Zlokovic. "A Simple Method for Isolation and Characterization of Mouse Brain Microvascular Endothelial Cells." J Neurosci Methods 130.1 (2003): 53-63. Print.

Yoder, M. C. "Is Endothelium the Origin of Endothelial Progenitor Cells?" Arterioscler Thromb Vasc Biol 30.6: 1094-103. Print.

Yoon, C. H., et al. "Synergistic Neovascularization by Mixed Transplantation of Early Endothelial Progenitor Cells and Late Outgrowth Endothelial Cells: The Role of Angiogenic Cytokines and Matrix Metalloproteinases." Circulation 112.11 (2005): 1618-27. Print.

Zhang, Z. G., et al. "Bone Marrow-Derived Endothelial Progenitor Cells Participate in Cerebral Neovascularization after Focal Cerebral Ischemia in the Adult Mouse." Circ Res 90.3 (2002): 284-8. Print.