modeling pure vasogenic edema in the rat brain

charles nottingham
Virginia Commonwealth University
MODELING PURE VASOGENIC EDEMA IN THE RAT BRAIN

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

CHARLES UPSHUR NOTTINGHAM
B.S., Biology, Wake Forest University, 2006
Certificate Anatomy and Neurobiology, Virginia Commonwealth University 2007

Director: William C. Broaddus, M.D., Ph.D
Professor, Departments of Neurosurgery, Anatomy & Neurobiology

Virginia Commonwealth University
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1. ADC – apparent diffusion coefficient
2. AQP – aquaporin
3. BBB – blood-brain barrier
4. CED – convection-enhanced delivery
5. CNS – central nervous system
6. CSF – cerebrospinal fluid
7. DAB – diamino benzene
8. DCA – deoxycholic acid
9. DWI – diffusion-weighted imaging
10. EB – Evan’s blue dye
11. ECM – extracellular matrix
12. ECS – extracellular space
13. EM – electron microscopy
14. ERK – extracellular regulated kinase
15. FPI – fluid percussion injury
16. FXR – farnesoid X receptor
17. Gd-DTPA – gadolinium diethylenetriamine pentaacetic acid
18. GFAP – glial fibrillary acidic protein
19. H&E – hematoxylin and eosin
20. IgG – immunoglobin G
21. LM – light microscopy
22. MCAO – middle carotid artery occlusion
23. MMP – matrix metalloproteinase
24. MRI – magnetic resonance imaging
25. MW – molecular weight
26. NGS – normal goat serum
27. PBS – phosphate buffer solution
28. PKC – protein kinase C
29. SER – smooth endoplasmic reticulum
30. TBI – traumatic brain injury
31. TEM – transmission electron microscopy
32. TGF-β – transforming growth factor β
33. VEGF – vascular endothelial growth factor
34. VPF – vascular permeability factor
Abstract

Modeling Pure Vasogenic Edema in the Rat Brain

By Charles Upshur Nottingham, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Major Director: William C. Broaddus, M.D., Ph.D., Hord Associate Professor
Department of Neurosurgery, Department of Anatomy & Neurobiology

Targeted drug delivery to the brain is difficult to achieve using conventional techniques, largely due to the blood-brain barrier’s (BBB) impediment to drug diffusion into the brain parenchyma. In response, development of convection-enhanced delivery (CED) offers the ability to circumvent the BBB and target specific areas of the brain. Predictability of infusate movement in pathological brain states during CED will maximize the effectiveness of this treatment, and therefore modeling of infusate movement must be characterized. Previous work from our lab effectively modeled CED in rats using the middle carotid artery occlusion model of cytotoxic edema. However, previous models examined for vasogenic edema study did not show pure vasogenic edema. The purpose of this study was to develop a model of pure vasogenic edema in the rat brain. In this study, we show that stereotactic 9 µL infusion of 1.0 mM DCA over 45
minutes into the rat corpus callosum reproducibly creates pure vasogenic edema, as observed in the peritumoral white matter surrounding gliomas.
Chapter 1

Introduction

The Blood-Brain Barrier

Neuroscience literature describes the blood-brain barrier (BBB) as both a structural and functional impediment to the movement of molecules from blood vessels into the brain parenchyma. Paul Ehrlich showed the first evidence of the BBB when he demonstrated that intravenously-administered dyes stained many tissues except brain in the late 1800s. Further experiments by his student, Edwin Goldman, showed that the dyes themselves had the ability to stain brain parenchyma when exposed to the brain through non-intravenous methods (Wells and Bonetta 2005). Modern experimental techniques have since elucidated the complex cellular and molecular structures that comprise this barrier between blood and the brain (Huber et al. 2001), which is particularly limiting for high molecular weight (MW) and polar molecules (Bobo et al. 1994).

Histologically, the BBB consists of vascular endothelial cells connected by tight junctions and adherens junctions. Pericytes overlay the endothelial cells and are considered part of the BBB because they participate in its function and share a common basement membrane with the endothelial cells (Ueno 2007). Figure 1-1 provides an
Figure 1-1. Illustration of the BBB and perivascular astrocytic foot processes. Figure modified from Uneo (2007).
illustration of the BBB. Astrocytic foot processes ensheath this structure and participate in its maintenance (Neuwelt 2004). In fact, the CNS microenvironment is necessary for formation and maintenance of tight junctions by endothelial cells (Stewart and Wiley 1981; Argaw et al. 2006). Still, endothelial cells contribute to the metabolic requirement necessary for BBB function, and mitochondria comprise a large intracellular presence in the endothelial cell ultrastructure (Ueno 2007).

Tight junctions between the endothelial cells limit paracellular permeability of molecules with a MW larger than 200-400 Da between the blood vessel lumen and brain parenchyma, while other mechanisms of transport across the endothelium play minor roles (Neuwelt 2004). The network of tight junctions between endothelial cells is also referred to as the zonula occludens (Wang and Margolis 2007). The structure of tight junctions consists of integral, transmembrane proteins that attach cytoplasmic accessory proteins on the intracellular face. Claudins, occludins and junction adhesion molecules all comprise the integral membrane component of tight junctions (Huber et al. 2001). Adherens junctions also consist of transmembrane proteins, namely members of the cadherin family. Cytoplasmic accessory proteins attach tight junctions and adherens junctions to the actin cytoskeleton of the endothelial cell. ZO-1, ZO-2, ZO-3, cingulin, and additional proteins attach tight junctions, while p120 and catenins attach adherens junctions (Doolittle et al. 2005; Ueno 2007).

As suggested above, the responsibility of BBB maintenance lies with astrocytes, endothelial cells and pericytes (Neuwelt 2004; Ueno 2007). Astrocytic interaction with the endothelium is necessary for proper development and maintenance of the BBB.
(Laterra et al. 1990), and astrocytes also maintain a suitable CNS microenvironment for the cells and structures involved. In pathological brain states such as neuro-inflammation, factors secreted by microglia and macrophages (such as IL-1β and histamine) lead to reactive astrogliosis and BBB disruption by interactions with the astrocytes and endothelium (Argaw et al. 2006).

Under normal conditions, protein kinase C (PKC) in the endothelial cell asserts several important functions in tight junction regulation (Huber et al. 2001). One such function is the maintenance of appropriate intracellular calcium levels, which is necessary for proper cadherin function and ZO-1 migration within the cytoplasm. In addition, phosphorylation activity by PKC permits ZO-1 migration and proper sorting of tight junction proteins. PKC also regulates intracellular adhesion molecule 1 (ICAM-1) transcription induced by tumor necrosis factor α (TNFα).

Although proper BBB function is necessary for a normal brain environment (Neuwelt 2004), it provides a major obstacle in conventional drug delivery to CNS pathologies such as tumors and neurodegenerative disorders (Groothuis 2000). As stated above, high MW and polar molecules do not readily pass the BBB, and therapeutic agents often have these characteristics (Bobo et al. 1994). However, novel treatments to circumvent the BBB are being developed such as implantable polymers and convection-enhanced delivery (CED) of drugs (Groothuis 2000; Haroun and Brem 2000). The overlying goal of our lab is to optimize CED in pathological brain states, which will permit appropriate doses of therapeutic agents to reach the desired area.
Convection-Enhanced Delivery

Delivery of medication into the brain has proven a major challenge to clinicians, and scientists continue to search for optimal ways of appropriately delivering drugs to specific targets. Largely due to the BBB, medication from the bloodstream diffuses poorly into the brain parenchyma, especially with increasing molecular weight (Broaddus et al. 2001). In 1977, Vick et al. (Vick et al. 1977; Groothuis et al. 2000) stated that the BBB did not provide a major obstacle to chemotherapeutic delivery and predicted that research on factors other than the BBB, such as dosage and route of delivery, would improve drug delivery. Although supported by some, the large majority of literature supports the idea that the BBB provides a major obstacle to drug delivery (Groothuis 2000).

Common methods of drug delivery rely on simple diffusion of the drug to the target tissue. The ability of a substance to diffuse relies on the properties of both the drug and the target tissue environment. Several properties in particular hold great importance for brain such as MW, polarity, and the BBB (Bobo et al. 1994). For example, diffusivity into normal brain rapidly decreases with increasing MW (Groothuis et al. 1999). Similarly, diffusion of high MW compounds into brain tumor tissue occurs slowly. Novel therapeutics (e.g. polypeptides, proteins or nucleic acids) tend to have high MW, and therefore simple diffusion to pathologic brain states from the vasculature is inadequate. Given orally or intravascularly, the diffusion of compounds into the normal brain is much lower than into other target tissues, and the body acts as a large sink for most of the drug. This sink effect also reduces the ability of the physician to deliver high
doses of a drug to a localized target. Even for compounds with the capability to cross the BBB, any associated toxicity may affect other regions of the body and not localize to the brain (Haroun and Brem 2000).

Infusion of drugs into the brain’s ventricular system does allow the surgeon to bypass the blood-cerebrospinal fluid (CSF) barrier, but has also proven unsuccessful due to the requirement that the compound diffuse from CSF into the brain parenchyma. Even with compounds that would be expected to diffuse readily, diffusion alone cannot adequately deliver medication to the brain (Zovickian et al. 1987). The amount of drug found in brain following intraventricular delivery is high at the ventricular border and sharply decreases with increasing distance from the ventricular wall (Blasberg et al. 1975; Patlak and Fenstermacher 1975; Groothuis and Levy 1997). Intraventricular drug delivery is thus suitable for pathologies in the subarachnoid space such as carcinomatous meningitis (Brown et al. 1996) but not for intraparenchymal delivery (Groothuis 2000).

Localization of drugs to the target area allows for higher dosing and the potential to leave healthy tissue unharmed. One approach uses degradable wafers containing the drug for direct intraparenchymal implantation. MGI Pharma markets 3.85% BCNU-impregnated polymer wafers that are implanted on the margins of the resection cavity after removing primary brain tumors. This therapeutic strategy has undergone phase III clinical trials and has been demonstrated to improve mean survival time to 31 weeks compared to 23 weeks for placebo wafers (Bota et al. 2007). Although this improvement is modest, it still shows the value and promise of local drug delivery as opposed to systemic delivery.
Utilizing the immune system provides another avenue for localization of drugs to the pathological area. Even when given systemically, immunotherapeutics are intended to specifically target a tumor due to their ability to enhance the natural immune response a body creates in response to the tumor. Cancer cells often show resistance to the immune response, also called immune evasion (Melero et al. 2007) and in vivo use of certain cytokines such as IL-2, 4, and 12 is currently under study to enhance the efficacy of the natural immune response. Preliminary results, though, have shown significant systemic toxicity for IL-2 in early clinical trials (Haroun and Brem 2000).

Although the above techniques address the problem of localization, diffusion is still the final common pathway for drugs to distribute throughout the target tissue and no technique has yielded impressive results. As mentioned above, diffusion is only effective within very short (millimeter) distances, the concentration of the drug declines dramatically with increasing distance from its source, and tissue clearance can rapidly deplete the drug before it can diffuse over a long distance. In addition, the large MWs characteristic of many therapeutics exacerbates the shortcomings of diffusion-based techniques. In response to the issue of diffusion, researchers at the NIH began to optimize convection-enhanced delivery (CED), which creates a positive pressure gradient driving the bulk flow of infusate fluid (Bobo et al. 1994).

Bobo et al. (1994) infused increasing volumes of $^{111}$In–labeled transferrin ($^{111}$In-Tf) and $[^{14}$C]sucrose into the corona radiata of cats over time as representative high and low MW compounds. In their paper the authors describe the convection pressure in relation to the flow rate for infusion and the positive linear relationship between volume
of infusate ($V_i$) and volume of distribution ($V_d$) of the infusate attributable to convection. Optimization of CED has since been a major focus to ensure predictable and effective drug delivery with minimal side effects for the patient. Infusion covering an area of even a few centimeters takes numerous hours to complete, which places tremendous strains on the patient (Morrison et al. 1994). Therefore, development of proper technology and determination of adequate dosing, volume, and flow rate have undergone much investigation (Groothuis 2000).

The initial low flow rate is critical to prevent fluid backflow up the needle tract, and small incremental increases in the flow rate can be made during longer infusions (Bobo et al. 1994; Chen 1999). Flow rates and cannula diameter have been the focus of technical optimization due to the greater risk of infection and patient discomfort of long infusions (Morrison et al. 1994; Chen et al. 1999; Krauze et al. 2005a; Linninger et al. 2008). Generally infusion occurs from narrow-diameter cannulas (26-32 gage) (Krauze et al. 2005a) at approximately 0.5 µL/min or lower, and then flow rate is increased incrementally by 0.5 or 1 µL/min to minimize reflux of infusate along the cannula track (Bobo et al. 1994). Morrison first reported high-flow rate infusion without reflux at 3 µL/min over a 12-hour period using large-MW compounds that produced an infusion radius of 1.5 cm (Morrison et al. 1994). Recently, however, Krauze et al. (2005b) developed a “reflux-free” cannula that allows 10-fold higher flow rates without tissue damage or backflow as tested first in agarose gel, and then confirmed in rat and primates. Predictability of $V_d$ relies on the surgeon knowing exactly how much infusate will reach the tissue under a properly-maintained convection pressure.
Another method of increasing the $V_d$ is to increase the infusate concentration, as demonstrated by Kroll et al. (1996). When flow rate and $V_i$ are kept constant but the infusate concentration is increased fivefold, $V_d$ will experience a remarkable 79% increase. The authors of the study suggested that higher doses of the drug would thus enhance therapeutic benefit over a larger target area, which would ideally reduce the time required for infusion (Kroll et al. 1996). Although short infusion times are ideal, other studies have investigated the effect of a long duration of infusion on the brain parenchyma. Laske et al. (1997) performed 87-hour infusions into the primate brainstem at a moderate infusion rate of 1.7 µL/min and showed consistent and reproducible $V_d$. Despite this long infusion, only a mild astrocytic response was observed and blood perfusion to the infused area decreased by less than 5%. These findings indicate that the principal concern of long infusions is the duration of anesthesia administration as opposed to the procedure itself.

Predictability of infusate movement in the tissue is integral to proper clinical use of CED. Fluid movement in gray matter versus white matter differs due to properties of the extracellular matrix and the cellular structures (Morrison et al. 1994, Haar 2005), and fluid movement through pathological brain tissue also differs from normal brain tissue (Haar 2005). Gray matter parenchyma is considered homogenous and primarily consists of cell bodies, and has a tortuous ECM. White matter, however, is considered heterogeneous and consists primarily of tracts of axonal processes surrounded by myelin sheaths, and has a less tortuous ECM than that of gray matter (Morrison et al. 1994, Haar 2005). Fluid movement through the ECS of gray matter is generally isotropic, whereas
fluid movement through white matter ECS occurs preferentially in parallel with the axon tracts and is thus anisotropic (Haar 2005). The ratio of $V_i:V_d$ following CED should presumably follow different patterns depending on the location and type of pathology present (Raghavan et al. 2006).

A number of studies have set out to model infusate movement with respect to predicted distributions and actual distributions observed. Published work from our lab has utilized gel phantoms (Chen et al. 2002) in addition to animal experimentation (Broaddus et al. 1998; Prabhu et al. 1998) to quantifiably model infusate distribution using CED. Several quantifiable parameters affecting distribution have been cited, including tissue hydraulic conductivity, resting pore fraction, irreversible drug loss from tissue, tissue dilation, etc. (Raghavan et al. 2006). Although formulas have been developed based on these variables to predict infusate movement for a given region of tissue, results have shown that our understanding is incomplete (Prabhu et al. 1998; Raghavan et al. 2006; Linninger et al. 2008). Further understanding both of tissue properties and fluid movement through the tissue is thus necessary before effective CED can be achieved.

Previous work in our lab investigated infusate distribution in pathological brain states, namely pure cytotoxic edema and pure vasogenic edema (Haar 2005). Using the middle carotid artery occlusion (MCAO) model of cytotoxic edema, Haar showed that gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA), a mock therapeutic infusate distributes to a larger tissue volume in cytotoxic edema relative to normal brain in rats. Further, clearance of the infusate from brain with cytotoxic edema took longer than
clearance from normal brain. Unfortunately, inconclusive results were observed when Haar infused Gd-DTPA into a model of vasogenic edema, which were in part attributed to intrinsic problems with the model that did not produce pure vasogenic edema. Later, Shukla (2006) optimized a different model but was also unable to create edema that was purely vasogenic. In response to these findings by Haar (2005) and Shukla (2006), purpose of this study was to optimize a different model of pure vasogenic edema.
Cerebral Edema

A feature common to many brain pathologies is edema. Such conditions include traumatic brain injury (TBI), intracerebral hemorrhage, brain tumors and stroke (Hutchinson et al. 2007). Raslan and Bhardwaj (2007) define edema as an appreciable increase in brain water content. Several subtypes have been described, although the three major forms are cytotoxic, vasogenic, and interstitial. Cytotoxic edema results from water increase that is intracellular, whereas fluid confined to the extracellular space (ECS), typically as a result of serum water and proteins extravasated across a breached BBB, defines vasogenic edema (Klatzo 1967). Interstitial edema is also referred to as hydrocephalic edema, and it results from CSF buildup that leads to hydrocephalus (Raslan and Bhardwaj 2007).

A thorough understanding of edema has permitted the development of clinical treatments (Heiss et al. 1996), although further improvements are necessary (Raslan and Bhardwaj 2007). Our lab is particularly interested in drug delivery to edematous brain using CED. The goal of this study in particular is to find a reproducible model of pure vasogenic edema that would be appropriate for our CED investigation. Several models of vasogenic edema exist, but unfortunately the edema consists of a heterogeneous mixture of vasogenic and cytotoxic types, which is not appropriate for our purposes.
Figure 1-2. Illustrations of normal brain (A), vasogenic edema with characteristics ECS expansion (B), and cytotoxic edema with characteristics ECS contraction and intracellular swelling (C). Figure taken from Shukla (2006).
Normal Vasogenic Edema Cytotoxic Edema
**Vasogenic Edema**

The late Igor Klatzo, a leader in the field of edema research, asserted that in normal brain, serum proteins cannot readily cross the BBB like water and electrolytes. Compromise of the BBB, however, will permit serum proteins and fluid to move from the vasculature to the brain parenchyma, hence the term vasogenic edema. The two major extravasated proteins found in the ECS in this circumstance are immunoglobin G (IgG) and albumin (Klatzo 1987). Proteins will remain in the ECS following resealing of the BBB or upon diffusion to an area with the BBB intact, maintaining an edematous state until they have been cleared (Kuroiwa et al. 1985; Klatzo 1987).

Three major mechanisms of extracellular protein clearance include uptake by astrocytes, bulk flow to ventricles, and digestion or transport within luminal vesicles of endothelial cells (Klatzo et al. 1980; Klatzo 1987), although diffusion has been shown to play a minor role (Marmarou et al. 1990). It was previously thought that the astrocytic pathway was the predominant mechanism of edema fluid clearance (Klatzo et al. 1980; Klatzo 1987). However, later studies by other authors showed evidence of albumin clearance along perivascular spaces into the ventricles and dorsally to the subarachnoid space, and the authors posited that these routes provided the major clearance pathway for edema fluid (Ohata et al. 1990; Ohata and Marmarou 1992; Abbott 2004; Marmarou 2007). Astrocytic assistance in edema resolution does occur (Klatzo 1987), particularly via the uptake of albumin through the transforming growth factor (TGF)-β receptor (Ivens et al. 2007); water presumably flows into the astrocytes concurrently with the albumin (Klatzo 1987). Additionally, ECS expansion is thought to put a strain on
metabolic needs of cells other than astrocytes (Klatzo 1987), and mechanical stress to their membranes, particularly in neurons at the vulnerable nodes of Ranvier, has been suggested to induce cellular swelling (Ohata et al. 1990). Therefore, intracellular fluid uptake can latently be observed during vasogenic edema and the resultant pathology will be a heterogeneous mixture of vasogenic and cytotoxic forms (Klatzo 1994).

**Cytotoxic Edema**

Although cellular uptake of fluid in brain occurs predominantly in astrocytes, cytotoxic edema affects neurons and other cells as well. Klatzo attributed the initial stages of cytotoxic edema to tissue deprivation of glucose and oxygen, causing cells to lose control of the Na\(^+/\)K\(^+\) ATPase pump. Due to this event, the cell cannot properly regulate osmolarity and will begin retaining water (Klatzo 1987). Typically ischemia, as seen in stroke, leads to cytotoxic edema, and the time course of cellular swelling (R Katzman et al. 1977) followed later by BBB disruption is well-documented (Gotoh et al. 1985). Experimentally, middle cerebral artery occlusion is a reproducible model of cytotoxic edema. Studies of this model have revealed that the brain begins retaining Na\(^+\) and water concurrent with K\(^+\) loss within 12 hours, and BBB permeability to albumin is observed between 12 to 24 hours (Gotoh et al. 1985; Hatashita and Hoff 1990). Similar to vasogenic edema, cytotoxic edema temporally evolves into a heterogeneous mixture of these two types (Katzman et al. 1977; Gotoh et al. 1985; Hatashita and Hoff 1990; Klatzo 1994).
Aquaporins

Aquaporins (AQPs) are a recently-discovered family of transmembrane protein channels integral to water transport (Papadopoulos and Verkman 2007). A diversity of species including bacteria and mammals express aquaporins, and they exist in many tissues in the human body. The brain relies on AQPs for CSF production and for astrocytic regulation of water and BBB maintenance. These channels have the capacity for bidirectional water transport, a feature that forms the cornerstone of an ongoing debate among edema researchers (Griesdale et al. 2004). Specifically, the evaluation of AQP-4 in edematous brain, expressed on the astrocytic endfeet enveloping capillaries (Nielsen et al. 1997), as protective or harmful to the parenchyma has yet to be fully understood (Marmarou 2007; Papadopoulos and Verkman 2007). Stroke models with overexpression (Yang et al. 2008) and knockout (Manley et al. 2000) of AQP-4 found that AQP-4 overexpression enhances the edema, as evidenced by respective greater and less edema observed in these parameters when compared to normal brain. This channel has thus been implicated as a contributor to the pathogenesis of cytotoxic edema. Indeed, inability of a cell to control its water is characteristic of cytotoxic edema, and AQP-4 would not be expected to provide reprieve for the cell as a passive water channel (Marmarou 2007; Papadopoulos and Verkman 2007).

In contrast to its harmful role in cytotoxic edema, Papadopoulos et al. (2004) found a reduction in edema and ICP in wild-type mice compared to AQP-4 -/- mice in three models of vasogenic edema (intraparenchymal fluid injection, freeze lesion and brain tumor), suggesting a protective role in this pathology. Freeze lesion and brain
tumor models induce edema through BBB breakdown and serum protein extravasation, whereas intraparenchymal fluid injection does not create edema through a compromised BBB. This aspect highlights the role of AQP-4 as an endogenous tool that the brain uses to protect itself in this particular pathology. In fact, VEGF has recently been shown to upregulate AQP-4 mRNA and protein expression in rats following infusion into the substantia nigra, which coincided with return of BBB function (Rite et al. 2008). Although the VEGF-induced BBB breakdown initially harms the dopaminergic neurons of this region (Rite et al. 2007), the authors posited that the subsequent AQP-4 upregulation allowed the region to remove excess water as quickly as possible and thereby reduce the total injury volume (Rite et al. 2008). In humans, Saadoun et al. (2002) reported a large upregulation of AQP-4 in reactive astrocytes of the parenchyma surrounding all but one of the examined high-grade astrocytomas, whereas expression in similar tissue surrounding low-grade astrocytomas equaled that of normal brain. This finding supports the finding by Rite et al. that the brain responds in a self-protective fashion to vasogenic edema through upregulation of AQP-4 (Rite et al. 2008).

**Traumatic Brain Injury**

TBI has been attributed to long-term disabilities in an astounding 5.3 million Americans. The pathophysiology itself is complex, and its specifics can vary depending on a number of factors, including the age, gender and severity of the traumatic event. Nevertheless, TBI pathologies have several common characteristics including excitotoxicity, metabolic deregulation, inflammation and edema (Keck et al. 2007). Edema subsequent to TBI has been shown to be heterogeneous, but with a predominance
of cytotoxic edema (Marmarou et al. 2006). Barzó et al. (1997) describes the edema subsequent to TBI as biphasic, with vasogenic edema appearing in the acute stages followed by cellular swelling. Following treatment of Marmarou’s impact acceleration model in rats, edema was observed on MRI T1 water maps for 2 weeks. ADC values transiently increased up to 60 minutes post-injury before declining to below-baseline values, where they remained for over 2 weeks. Ultrastructural evaluation of this model in the acute phase shows perivascular edema as well as astrocytic swelling (Foda and Marmarou 1994; Vaz et al. 1998) that persists for 24 hours but is not visible thereafter (Foda and Marmarou 1994).

A notable feature of the impact acceleration model is the mild increase in blood pressure from 102 to 123 mmHg that decreases to hypotensive levels from 15 seconds to 30 minutes post-injury (Marmarou et al. 1994). Similarly, experimental fluid percussion injury (FPI) creates an even greater rise in blood pressure that persists over a longer time course (van den Brink et al. 1990; Yuan et al. 1991). Oddly enough, edema following FPI persists for only 5 days post-injury (McIntosh et al. 1990). Van den Brink et al. (1994) posited that the “key event” in edema formation from both FPI and impact acceleration, though, was damage to the vasculature including BBB breakdown and loss of blood pressure autoregulation.

**Intracerebral Hemorrhage**

Edema secondary to a cerebral hemorrhage can compose up to 75% of the perihematomal volume. In the first 48 hours following the event, breakdown of the BBB due to the action of thrombin allows vasogenic edema to accompany the existing lesion.
Adding insult to injury, the breakdown products of the red blood cells, particularly those from hemoglobin, initiate an inflammatory immune response, keeping the region edematous (Thiex and Tsirka 2007). It is widely believed that the edema exacerbates the hemorrhagic injury, largely due to the action of thrombin and its downstream effectors in the event cascade (Xi et al. 1999). Yang et al. (1994) described the progression of cerebral hemorrhagic edema following injection of autologous blood into the rat caudate nucleus as similar to the edema following ischemia. The water content increased progressively for 24 hours and remained constant for 5 days, after which edema began to decrease. Also, the full spread of edema extended beyond the area of BBB disruption observed locally around the clot.

The perihematomal area which experiences inflammation and edema has been the target of studies to reduce the total lesion size. Elevated cytokines, particularly IL-6, IL-10 and IL-1β are observed (Thiex and Tsirka 2007), with some thought to be protective while others are thought to be harmful. IL-6 is thought to have a protective role through inhibition of MMP-9 (Cucullo et al. 2003; Vajtr et al. 2008), whereas Masada et al. (2003) managed to attenuate edema following both autologous blood and thrombin infusion with administration of IL-1 receptor antagonists (Thiex and Tsirka 2007). Collectively, these findings demonstrate the complexity of hemorrhagic edema, particularly with respect to the immune response and subsequent inflammation present in this brain state.
**Peritumoral Edema**

In addition to treatment of brain tumors, treatment of peritumoral edema has also received a great deal of attention. Clinical management of this complex edema correlates to better patient outcome following neurosurgical procedures and limits the volume of brain affected indirectly by the tumor (Stummer 2007). Over time, peritumoral edema causes peritumoral cyst formation, as well (Baggenstos et al. 2007). Alterations of the vasculature and ultimately breakdown of the BBB permit the edema formation, and inhibition of tumor-specific cytokines known to cause BBB breakdown, especially VEGF (Carlson et al. 2007; Stummer 2007) and COX-2, has been shown to attenuate peritumoral edema (Stummer 2007). The vasculature displays ultrastructural evidence of BBB breakdown such as abnormal tight junctions, irregular basal lamina and fenestrated capillaries (Stewart et al. 1985; Shibata 1989; Stummer 2007).

Experimental tumor models have provided a wealth of knowledge about the edema present in this pathology. Within the tight junctions of edematous peritumoral vasculature, altered protein expression of occludins (Papadopoulos et al. 2001) and claudins (Liebner et al. 2000) has been observed, creating the paracellular portal through which the serum ultrafiltrate extravasates (Papadopoulos et al. 2001). Edema generated locally around the tumor then spreads radially away from the tumor, mostly parallel to white matter tracks (Stummer 2007), as the intratumoral pressure is greater than that of surrounding parenchyma (Li et al. 2006). This edema is said to be predominantly vasogenic because extravasated fluid resides mostly in the ECS (Papadopoulos et al. 2004).
Models of Vasogenic Edema

The literature documents many experimental models of vasogenic edema. Many involve a breach of the BBB to permit extravasation of serum fluid and proteins, a characteristic that is observed in clinical practice as seen on MRI with gadolinium (Gd)-enhanced T1 imaging. However, direct intraparenchymal infusion places a proteinaceous fluid infusate directly into the ECS without BBB disruption (Haar 2005). Clinical edema is complex and variable depending on the broader pathology, and development of various edema models permits accurate study of a diverse range of injuries (Haar 2005).

Cold Lesion

Cold lesion is a highly reproducible model of vasogenic brain edema that has been used since the 1950s (Bartkowski et al. 1985). The injury is produced by placement of a cold metal probe onto the exposed scalp or underlying dura mater following craniotomy. The severity of the lesion can vary depending on the temperature of the probe, typically between -68°C to -80°C (Houkin et al. 1996; Stoffel et al. 2004), and the duration of probe placement, which is usually 30 seconds (Papadopoulos et al. 2004; Stoffel et al. 2004; Nag 1996). Following placement of the probe over the exposed skull, a necrotic lesion immediately forms in the brain region directly underneath the probe. The total lesion expands to a maximal volume at 24 hours post-operatively that is maintained for several days (Stoffel et al. 2004). Apoptosis is observed for 24 hours within the primarily necrotic core of the lesion, but at 72 hours only the margin of the necrotic lesion contains apoptotic cells (Murakami et al. 1997).
Lesion expansion is attributed to the development of vasogenic edema surrounding the necrotic lesion as a result of BBB breakdown and serum extravasation. BBB breakdown occurs immediately post-injury and persists up to 24 hours as seen both from Evan’s Blue extravasation and Gd-enhanced T1 MR imaging (Houkin et al. 1996; Murakami et al. 1997). Specifically, altered tight junction protein expression occurs in a temporal fashion. Interestingly, caveolin-1 expression is increased up to 4 days post-injury before returning to control level, whereas occludin and claudin-5 show varying levels of decrease before returning to normal (Nag et al. 2007). Decreased expression of junction adhesion molecule (JAM)-A has also been observed. Further, an important regulator of tight junction molecules similarly displays an altered expression profile following cold injury. Both endothelial (e) and inducible (i) nitrous oxide synthase (iNOS) (Nag et al. 2001) are overexpressed in astrocytes (Gotoh et al. 1998) for 4 days after cold injury.

**Intraparenchymal Albumin Infusion**

Unlike many other models of vasogenic edema, direct intraparenchymal infusion creates vasogenic edema while the BBB remains intact. Albumin, the major component of serum ultrafiltrate in vasogenic edema, dissolved in mock cerebrospinal fluid (CSF) is distributed directly to the extracellular space where it remains for 8 days as visualized by MRI (Fatouros et al. 1990) and TEM (Ohata et al. 1990). Without albumin, the same volume of mock CSF remains only for 72 hours (Groeger and Marmarou 1989). Especially in comparison with other models, the edema produced is thought to be purely vasogenic; cellular pathology is not observed and edema clearance occurs primarily along
perivascular routes instead of through cellular uptake (Ohata et al. 1990). This model has been used to examine pathways of protein and fluid clearance in vasogenic edema (Naruse et al. 1990; Ohata et al. 1990; Ohata and Marmarou 1992), and more recently by our lab to model infusate movement in an edematous state (Haar 2005). Unfortunately, intrinsic characteristics of the model did not permit adequate results for that particular study.

Mannitol

Over the last half century, a number of studies have documented reversible BBB opening using hyperosmolar agents. Due to the BBB, many therapeutic agents cannot reach the brain parenchyma and conventional methods of delivery have thus proven inadequate. Because of their ability to reversibly open the BBB, manipulation of hyperosmolar agents to improve drug delivery has received a great deal of attention (Kroll and Neuwelt 1998; Rapoport 2000). In addition, clinicians have used this same manipulation in attempts to lower acute intracranial pressure. This technique remains controversial and no conclusion of its clinical effectiveness has been solidified (Kaufmann and Cardoso 1992; Kroll and Neuwelt 1998; Rapoport 2000).

Clinically, a single bolus of 1.4 mM (25%) mannitol is administered to the internal carotid artery to induce BBB disruption that reduces edema and elevated ICP. Each month this can be done once a day for 2 days but for a maximum of 12 months. This BBB opening is mild, short-lived, and thought to attenuate an existing pathology where vasogenic edema is present. From this method, the BBB remains open widely for 40 minutes and returns to normal between 6-8 hours post-infusion (Siegal et al. 2000).
Experimentally, a single dose of 1.4 mM mannitol via the intracarotid infusion to healthy rat brain will also open the BBB. This opening occurs maximally for 5 minutes before returning towards normal (Cosolo et al. 1989). Studies from Shukla (2006) in our lab have shown that 3 intracarotid infusions of 1.4 mM mannitol given 30 minutes apart can produce extensive BBB disruption and create large edema in the rat brain. EB extravasation is observed within 2 hours following the last infusion, and extensive edema is observed by hyperintense T2 signal on MRI throughout the ipsilateral cortex, caudate, and corpus callosum. Significant mass effect is also observed. The extensive edema does lead to cellular pathology within 24 hours, and many rats do not survive the procedure even in the acute stages (Shukla 2006). This model does not meet the criteria of pure vasogenic edema, and consequently our lab sought other models.

**Vascular Endothelial Growth Factor (VEGF)**

Vascular endothelial growth factor (VEGF) is a powerful cytokine that induces neovascularization. The protein was originally discovered for its ability to increase vascular permeability around tumors, and was therefore named vascular permeability factor (VPF) (Murohara et al. 1998). VEGF upregulation occurs in most brain tumors, particularly in aggressive ones (Jain et al. 2007). Blockage of VEGF by steroids (Heiss et al. 1996), antibodies and exogenously-applied VEGF receptors (Gorski et al. 1999) can effectively attenuate peritumoral edema and reduce mass effect of brain tumors (Jain et al. 2007). Vascular permeability is observed within 1 minute following injection as the result of a leaky BBB, and neovascularization occurs much later (Dvorak 2002). *In vitro* studies have demonstrated a biphasic nature of BBB opening due to VEGF exposure to
cultured vascular endothelial cells. An initial “rapid and transient phase” occurs over the
first 2 hours, followed by a “delayed and sustained phase” from 6-24 hours (Behzadian et
al. 2003).

The edema response to VEGF is largely due to the inflammatory response that it
creates. Croll et al demonstrated vascular leak and leukocyte after one day of continuous
intracortical VEGF infusion via mini-osmotic pumps, and levels of both increased
throughout the 7-day period. The inflammatory response included increased IgG
extravasation, leukocyte extravasation, and ICAM-1 (an important adhesion molecule in
diapedesis). All of these inflammatory elements contributed to the leaky vasculature that
preceded neovascularization by several days (Croll et al. 2004). Intraventricular delivery
of VEGF has also been shown to induce edema and neovascularization in the
subventricular zone. Similar to Croll, Harrigan et al. (2003) used mini-osmotic pumps
for continuous intraventricular VEGF infusion, and showed increase brain water content
as well as neovascularization.

Bile Acids

Bile acids are cholesterol derivatives synthesized in the gut that circulate through
the enterohepatic circulation. Although their primary function is the emulsification of
lipids, they assert essential roles in the biochemical regulation of glucose and lipid
metabolism. Bile acids exert these actions through a number of common intracellular
signaling pathways, including protein kinase C (PKC) and FAS-receptor. In addition,
bile acids bind the nuclear farnesoid X receptor (FXR) through which they can affect
other intracellular pathways (Scotti et al. 2007). Depending on concentration and type,
bile acids can paradoxically promote cell (Dent et al. 2005) survival or induce apoptosis (Redlak et al. 2003). Conjugated bile acids (bile acids covalently attached to taurine or glycine) have been shown to upregulate the cell survival proteins Akt and extracellular regulated kinase (ERK)1/2 in human hepatocytes; this interaction is observed in the normal gut and thought to be essential for proper gastrointestinal function (Dent et al. 2005). Alternatively, ovarian cancer cells in vitro demonstrate a dose-dependent apoptotic response to deoxycholic acid (DCA) (Redlak et al. 2003).

DCA is a secondary bile acid, meaning that it is synthesized by flora in the gut (Scotti et al. 2007), and several studies have utilized DCA for its ability to produce brain edema in experimental animals (Seiffert et al. 2004). Its relative hydrophobicity permits DCA to integrate into lipid membranes, from which point it is thought to alter tight junctions between opposing ends of vascular endothelial cells and thus permit serum protein and fluid extravasation (Greenwood et al. 1991; Todd et al. 1997; Seiffert et al. 2004). As mentioned above, bile acids (including DCA) are known to alter PKC signaling in many cell types of the visceral organs and also alter intracellular calcium fluxes in these cells (Scotti et al. 2007). Tight junction maintenance depends on modulation by PKC and proper calcium regulation (Huber et al. 2001), and DCA may alter the tight junction integrity through those mechanisms.

Greenwood et al. (1991) showed a dose-response of DCA on RBC lysis and on BBB function. RBCs exposed to increasing concentrations of DCA demonstrated that DCA exerted “lytic action” at 1.5 mM and above. In a separate set of experiments, increasing concentrations of DCA were perfused intracarotidly with [14C]mannitol in
exsanguinated animals. The group showed that a minimum concentration of 1.0 mM DCA was necessary to induce BBB disruption as evidence by mannitol extravasation (mannitol concentration used was not high enough to contribute to BBB disfunction). However, concentrations of 2.0 mM DCA and higher caused pathologic morphology in the vascular endothelial cells. The authors concluded 1.0 mM DCA was the optimal concentration for BBB disruption while maintaining the integrity of vascular endothelial cells.

Perfusion of DCA over the surface of the cortex has also been shown to induce BBB disruption resulting in vasogenic edema (Seiffert et al. 2004; Tomkins et al. 2007). A concentration of 1.0 mM DCA caused immediate albumin extravasation as evaluated by EB staining (Seiffert et al. 2004). Cortical perfusion with 2.0 mM DCA caused albumin extravasation, as well as increased T1 and T2 signals on MRI (Tomkins et al. 2007). The authors also demonstrated that albumin uptake by astrocytes did not occur until 6-8 hours post-perfusion, suggesting that extravasated albumin remained in the extracellular space along with the extravasated fluid for that duration.

The aforementioned VEGF and DCA models have not demonstrated that the edema produced is purely one type or a heterogeneous mixture of vasogenic and cytotoxic types. We designed experiments using these two substances with the intent of creating pure vasogenic edema, and defined criteria to evaluate whether or not this state was created. Establishment of pure vasogenic edema will permit the study of CED in this specific brain state by our lab, and the model can certainly be applied to other studies of vasogenic edema as well.
Chapter 2

Hypothesis and Specific Aims

**Rationale:** Thorough examination of many models of edema has not shown that an existing model can reproducibly create pure vasogenic edema. Several new approaches were attempted based on existing models, such that issues with old ones were ideally eradicated. We attempted to create pure vasogenic edema in the rat brain by modification of existing models of VEGF infusion and DCA infusion. Further, we compared these models to a real case of peritumoral edema.

**Hypothesis I:** Direct intraparenchymal infusion of VEGF will demonstrate a dose-dependence and time-dependence on BBB disruption and edema formation in the rat striatum.

**Specific Aims:**

1. Establish a dose at which BBB disruption will occur.
   a) Demonstrate the time course over which BBB disruption occurs.
2. Establish whether significant edema is produced by BBB disruption.
   a) Characterize the edema as purely vasogenic, purely cytotoxic, or heterogeneous
**Hypothesis II:** Direct intraparenchymal infusion of DCA will demonstrate a dose-dependence and time-dependence on BBB disruption and edema formation in the rat corpus callosum.

**Specific Aims:**

1. Establish a dose at which BBB disruption will occur.
   
a) Demonstrate the time course over which BBB disruption occurs.

2. Establish whether significant edema is produced by BBB disruption.
   
a) Characterize the edema as purely vasogenic, purely cytotoxic, or heterogeneous.

**Hypothesis III:** Peritumoral edema in the rat corpus callosum is purely vasogenic.

**Specific Aims:**

1. Establish whether significant peritumoral edema exists in the corpus callosum of rats bearing T9 gliomas
   
a) Characterize the edema as purely vasogenic, purely cytotoxic, or heterogeneous.
Chapter 3

Methods

All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Virginia Commonwealth University. Male Sprague-Dawley rats (Harlan, Indianapolis, Indiana) with an average weight range of 300 – 350 grams were used for all infusions of saline, VEGF, and DCA. Methods used were similar or equalent to those used by Haar (2005), Shukla (2006), and Parry (2007). In addition to these groups, brain tissue from female Fisher 344 rats (Harlan, Indianapolis, Indiana) weighing approximately 140 grams and bearing T9 glioma tumors was donated by Dr. Zhi-jian Chen from his experiments; methods for surgery, tumor implantation, sacrifice, and tissue processing in these animals are summarized under the heading “Donation of Tumor-Bearing Rats” at the end of this chapter.

Stereotactic Infusion

Male Sprague-Dawley rats were anesthetized by intraperitoneal administration of 90 mg/kg ketamine : 10 mg/kg xylazine (10 : 1 mixture of ketamine [100 mg/mL] :
xylazine [50 mg/mL]). Lubricating veterinary ointment was applied to the eyes and the animal was placed on a heating pad in a stereotactic frame. A 10 mm midline incision was made at the scalp, centered approximately at bregma. An infusion burr hole was stereotactically created 3mm lateral, 1mm posterior to bregma using a roto-tool and fine drill bit for all infusions. A 28-gauge needle attached to a 100 µL Hamilton syringe driven by a syringe pump (Bioanalytical Systems, West Lafayette, Indiana) was lowered 6mm below the surface of the brain for striatum infusions and 2.5 mm below the surface of the brain for corpus callosum infusions and remained in the brain parenchyma for 5 minutes before infusion. Five minutes after infusion, the infusion needle was slowly raised 1 mm every minute until out of the brain. The incision was then closed with absorbable chromic gut, averaging 6 interrupted sutures along the length of the incision and the rat was placed under a heat source and monitored until alert and mobile. Animals sacrificed less than 24 hours post-operatively were not given analgesic. For animals sacrificed 24 hours or longer post-operatively, buprenorphine (0.03 mL, 0.3 mg/mL) was administered subcutaneously every 12 hours up to 72 hours.

20 µL of 0.1 µg/mL, 1 µg/mL, and 5 µg/mL VEGF in PBS was infused into the right striatum at a flow rate of 0.25 µL/min for 80 minutes and given intravenous Evan’s Blue (EB) dye before being sacrificed at 1 hour post-infusion (n=2 for each concentration). Brains were then harvested and then grossly section in the coronal plane at the needle track with a razor blade to look for EB staining. Brain tissue at needle tract was then stained with hematoxylin and eosin by the Department of Anatomic Pathology at Virginia Commonwealth University, Richmond, Virginia. A parallel set of animals
(n=2) received 20 µL of 0.1 µg/mL VEGF in PBS, and was given EB before sacrifice at 20 hours post-infusion. These brains received gross sectioning as previously described, but were not stained with hematoxylin and eosin.

6 and 12 µL of DCA was infused at 2.0 mM in 0.9% saline into the right striatum at a flow rate of 0.2 µL/min for 30 and 60 minutes, respectively. Animals (n=2 per volume group) were given EB and sacrificed at 1 hour, after which brains received gross sectioning, as described above for stereotactic VEGF infusion. For a second group, 6, 9, 12, and 18 µL of DCA was infused at 2.0 mM in 0.9% saline into the right corpus callosum at a flow rate of 0.2 µL/min for 30, 45, 60, and 90 minutes, respectively. Animals (n=2 per volume group) were given EB and sacrificed at 1 hour, after which brains received gross sectioning as described above for stereotactic VEGF infusion. A third set of animals (n=2 per time group) received 18 µL of 2.0 mM DCA and were given EB and sacrificed 5 hours, 1 day, 1 week, and 2 weeks post-infusion. Brains were examined by gross sectioning. A fourth set of animals (n=2) received 9 µL of 2.0 mM DCA were sacrificed 1 hour post-infusion. Brains were sectioned by vibratome and parallel samples were processed for GFAP immunohistochemistry and electron microscopy preparation, as described below. 2 more animals received 9 µL of 2.0 mM DCA and were saved for MRI at 1 day post-infusion, after which animals were sacrificed. Brains were sectioned by vibratome and parallel samples were processed for GFAP immunohistochemistry and electron microscopy preparation.

6 and 12 µL of 1.0 mM DCA in saline was infused into the right striatum at a flow rate of 0.2 µL/min for 30 and 60 minutes, respectively. Animals (n=2 per volume
group) receiving 6 and 12 µL were given EB and sacrificed at 1 hour, after which brains were examined by gross sectioning. In a separate experiment, 6, 9, and 12 µL of 1.0 mM DCA in saline was infused into the right corpus callosum at a flow rate of 0.2 µL/min for 30, 45, and 60 minutes, respectively. Animals (n=2 per volume group) receiving 6 and 12 µL of 1.0 mM DCA were given EB and sacrificed at 1 hour, after which brains were examined by gross sectioning. Animals (n=2 per time group) receiving 9 µL of 1.0 mM DCA were given EB and sacrificed at 1 hour and 1 day, after which brains were sectioned by vibratome and parallel samples were processed for GFAP immunohistochemistry and electron microscopy preparation.

9 µL of 0.5 mM DCA was infused at a flow rate of 0.2 µL/min for 45 minutes. One group of animals (n=3) receiving 0.5 mM DCA was saved for MRI 1 hour and then sacrificed. A second group of animals (n=3) receiving 0.5 mM DCA was saved for MRI at 1 day and then sacrificed. A third group of animals (n=2) receiving 0.5 mM DCA was given EB and sacrificed at 1 hour. A fourth group of animals (n=2) receiving 0.5 mM DCA was given EB and sacrificed at 1 day. For the third and fourth groups, brains were sectioned by vibratome and parallel samples were processed for GFAP immunohistochemistry and electron microscopy preparation.

18 µL of 0.9% saline (vehicle) was infused at a flow rate of 0.2 µL/min for 90 minutes into the right corpus callosum. Animals (n=2) were sacrificed at 1 hour post-infusion, and brains were sectioned by vibratome and parallel samples were processed for GFAP immunohisto-chemistry and electron microscopy preparation.
**Osmotic Pump Implantation**

Alzet mini-osmotic pumps (DURECT Corporation, Cupertino, California) were filled with 200 μL of 1 μg/mL VEGF in PBS, and Alzet micro-osmotic pumps (DURECT Corporation, Cupertino, California) were filled with 90 μL of 1 μg/mL VEGF in PBS. Pumps were attached to Alzet brain infusion kit II (DURECT Corporation, Cupertino, California) and primed by incubation in sterile 0.9% saline at 37°C for 24 hours before implantation.

Each rat was anesthetized by intraperitoneal administration of 90 mg/kg ketamine : 10 mg/kg xylazine (10 : 1 mixture of ketamine [100 mg/mL] : xylazine [50 mg/mL]). Lubricating veterinary ointment was applied to the eyes and the animal was placed on a heating pad in a stereotactic frame. A 10 mm midline incision was made at the scalp, centered approximately at bregma. An infusion burr hole was stereotactically marked 3mm lateral, 1mm posterior to bregma. From the posterior of the incision site, subcutaneous pocket was made and the pump was placed inside. Then, the first burr hole was created 3 mm posterior to the infusion mark using a roto-tool and fine drill bit. The burr hole was manually threaded with a metal screw, the rise of which was calculated and compared to the thickness of the skull to prevent injury to the brain. The cannula of the brain infusion kit was placed onto the infusion burr hole and temporarily fixed with superglue. After 5 minutes, the stereotactic arm was raised, and dental cement (Hygenic, Akron, OH) was applied around the base of the guide cannula and nylon screw. Allowing enough time to set, the incision was then closed with absorbable chromic gut,
averaging 6 interrupted sutures along the length of the incision. Buprenorphine (0.03 mL, 0.3 mg/mL) was administered subcutaneously every 12 hours up to 72 hours.

Animals (n=2) receiving micro-osmotic pump implantation were given EB and sacrificed at 3 days post-implantation, and animals (n=2) receiving mini-osmotic pump implantation were given EB and sacrificed at 3 days post-implantation. All brains were harvested and examined by gross sectioning, as described above.

_Evan’s Blue Dye Injection_

EB dye was utilized to mark BBB disruption on all rats and brains were examined when sectioned. EB dye (2% in saline) was delivered at 2ml/kg via the penile vein within 1 hour prior to sacrifice.

_Sacrifice and Perfusion_

Euthasol (150 mg/kg plus 19 mg/kg phenytoin) was administered by intraperitoneal injection following IACUC guidelines. The rat was transcardially perfused through a blunt, large-bore needle placed into the left ventricle and positioned within the ascending aorta. Approximately 400 mL of normal saline (0.9%) was used to flush the vasculature, followed by approximately 400 mL of fresh 4% paraformaldehyde. Brains were harvested and placed in fresh 4% paraformaldehyde for 48 hours, after which they were removed, washed with Millonig’s solution, and stored in the latter solution until processing for LM or EM studies.
**Light Microscopy Preparation**

Selected samples for LM study were cut via vibratome into consecutive 50 µm thick slices. From each block, approximately 3 slides of 4 sections each were sampled at -0.4, 0, and +0.4 mm relative to the infusion site (0).

**Tissue Preparation for Immunohistochemistry Analysis**

Semi-serial coronal sections were used for single label immunohistochemistry analysis. These sections were rinsed 3 X 10 minutes in PBS and endogenous peroxidase activity was blocked with 0.3% H$_2$O$_2$ in PBS for 30 min. Sections were rinsed 3 X 10 min in PBS, and pre-incubated for 60 min with 0.2% Triton X (Sigma Chemical Co., St. Louis, MO) in 10% normal goat serum (NGS)/PBS. The tissue was then incubated overnight in mouse anti-glial fibrillary protein (GFAP) antibody at a dilution of 1:1,500 (Zymed Laboratories) in 1% NGS/PBS at a dilution of 1:600. After 3 X 10 min washes in PBS containing 1% NGS, the sections were incubated in a 1:200 dilution of or biotinylated anti-mouse rat adsorbed immunoglobulin (diluted 1:200 in 1% NGS/PBS (Vector, Burlingame, CA) for 60 min followed by 3 X 10 min rinses in PBS. After incubation in an avidin–biotin peroxidase complex (ABC standard Elite kit, Vector, Burlingame, CA) at a dilution of 1:200 for 60 min and rinsing in PBS and 0.1 M phosphate buffer 3 X 10 min, the sections were processed for visualization of the immunohistochemical complex using 0.05% diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO), 0.01% hydrogen peroxide, 0.3% imidazole in 0.1 M PBS. Next the sections were mounted, serially, on gelatin-coated glass slides, dehydrated and coverslipped.
Qualitative Representation of GFAP Immunoreactivity

Tissue stained for GFAP was visualized using the Nikon E800 M Eclipse Microscope with SpotRT camera (Diagnostic Instruments), which was used for image acquisition. Microscopy was performed at the VCU – Dept. of Anatomy & Neurobiology Microscopy Facility, supported, in part, with funding from NIH-NINDS Center core grant (5P30NS047463). Slides stained for GFAP were qualitatively assessed according to morphological characteristics. At 4x and 20x magnification, the corpus callosum was inspected for evidence of dark, interconnecting astrocytic processes, the presence of which was defined as a GFAP increase.

Transmission Electron Microscopy Preparation

Selected samples for electron microscopy (EM) study were taken from parallel sampling sites as those used in LM preparation. Selected slices were washed with 0.1M Sorensen's phosphate buffer at 4°C then postfixed with 1% osmium tetroxide in 0.1M phosphate buffer at 4°C for 1 hour. Samples were then washed with buffer and dehydrated with a cold ethanol series: 50%, 70%, 80%, and 95%, for 5 minutes each. Next, slices were dehydrated with 100% ethanol at 20°C, followed by 3 changes of 100% propylene oxide for 10 minutes each. Slices were placed in a 1:1 propylene oxide:PolyBed solution for 4 hours, and then transferred to pure PolyBed resin overnight. Each section was embedded on vinyl slides and placed in a 60°C oven overnight. After LM examination, sections were chosen and excised from the cortical and deep gray region for remounting on blocks and further sectioning. Using the LKB 2120 Ultramicrotome, 900Å sections were cut with a diamond knife. Each section was then
placed on Formvar-coated slotted grids and stained with uranyl acetate and lead citrate for TEM study. A JEOL JEM-1230 (JEOL USA, Inc.) transmission electron microscope was used to capture images via the Ultrascan 4000 camera (Gatan, Inc.).
Magnetic Resonance Imaging

Implantation of Guide Cannula

Rats were anesthetized by intraperitoneal administration of 90 mg/kg ketamine : 10 mg/kg xylazine (10 : 1 mixture of ketamine [100 mg/mL] : xylazine [50 mg/mL]). Lubricating veterinary ointment was applied to the eyes and the animal was placed on a heating pad in a stereotactic frame. A 10 mm midline incision was made at the scalp, centered approximately at bregma. An infusion burr hole was stereotactically marked 3 mm lateral, 1 mm posterior to bregma for all in-bore infusions. The first burr hole was created 3 mm posterior to the infusion mark using a roto-tool and fine drill bit. The burr hole was manually threaded with a metal screw, the rise of which was calculated and compared to the thickness of the skull to prevent injury to the brain. A nylon screw (PlasticsOne, Roanoke, VA) was placed into this hole and the infusion burr hole was then created. An outer guide cannula (PlasticsOne, Roanoke, VA) containing a stylette was placed onto the infusion burr hole and temporarily fixed with superglue. Several minutes following, the stereotactic arm was raised, and dental cement (Hygenic, Akron, OH) was applied around the base of the guide cannula and nylon screw. Allowing enough time to set, the incision was then closed with absorbable chromic gut, averaging 6 interrupted sutures along the length of the incision. The stylette was removed from the guide cannula and a dummy cap – previously cut to match the length of the guide – was placed into the guide. Buprenorphine (0.03 mL, 0.3 mg/mL) was then administered subcutaneously every 12 hours up to 72 hours, with the rat placed under a heat source and monitored until alert and mobile.
In-bore Infusion

Each rat to be scanned was either kept under isoflurane anesthesia if imaging was conducted on the same day as DCA or saline infusion, or anesthetized by intraperitoneal administration of 90 mg/kg ketamine : 10 mg/kg xylazine (10 : 1 mixture of ketamine [100 mg/mL] : xylazine [50 mg/mL]) for all subsequent follow-ups. After baseline scanning, a 6 mm infusion needle (PlasticsOne, Roanoke, VA) was lowered into the guide cannula such that the tip of the needle was 3 mm below the surface of the brain. A volume of 9 µL (n=3) of 2.0 mM DCA in saline was infused into the right corpus callosum at a flow rate of 0.2 µL/min for 45 minutes with one group of rats (n=3). Animals were scanned for up to 3 hours post-infusion, such that T2-weighted, diffusion-weighted, T1-weighted images were taken sequentially every 30 minutes. The infusion needle remained in the animal for post-infusion scanning and the guide cannula was recapped after the final scan. The rat was then placed under a heat source and monitored until alert and mobile. One of these animals was received follow-up scanning at 1 day, 5 days, and 7 days post-infusion. A second animal received follow-up scanning at 1 day. The third died between 2-3 hours post-infusion. After scanning, animals were placed under a heat lamp and monitored until normal respiration had returned before extubation. Animals were then monitored until awake and alert before being placed by in the vivarium.

Imaging Following Stereotactic Infusion

All animals that were examined by MRI following stereotactic infusion were anesthetized by intraperitoneal administration of 90 mg/kg ketamine : 10 mg/kg xylazine
(10 : 1 mixture of ketamine [100 mg/mL] : xylazine [50 mg/mL]). Each animal received was subjected to a single set of T2-weighted, diffusion-weighted, T1-weighted images. Although described above in the stereotactic infusion section, a brief summary is provided. From the 9 µL of 2.0 mM DCA infusion group, 2 animals received scanning at 1 day post-infusion. From the 9 µL of 1.0 mM DCA infusion group, 3 animals received scanning at 1 hour post-infusion and 3 additional animals received scanning at 1 day. Similarly, 3 animals received scanning at 1 hour post-infusion and 3 additional animals received scanning at 1 day following infusion of 9 µL of 0.5 mM DCA.

**Imaging Placement**

Each rat to be scanned was either kept under isoflurane anesthesia for in-bore infusion, or anesthetized by intraperitoneal administration of 90 mg/kg ketamine : 10 mg/kg xylazine (10 : 1 mixture of ketamine [100 mg/mL] : xylazine [50 mg/mL]) when scanning occurred following stereotactic infusion and for all subsequent follow-ups. Each rat was placed in an acrylic imaging tube, secured via plastic ear bars, and fitted with a surface coil. Images were acquired with a 2.4 T, 40-cm bore magnet (Bruker Medical, Inc., Billerica, MA) equipped with a 12 cm inner-diameter, actively shielded gradient insert (maximum gradient strength: 25 G/cm). An actively decoupled RF coil set was used for RF excitation/reception and was comprised of a 7 cm inner-diameter “birdcage” design resonator and a 2 cm diameter circular surface coil.
**T2 images**

T2-weighted images with echo time (TE) of 100 msec, and repetition time (TR) of 3000 msec, were obtained on 5 contiguous coronal slices 2-mm thick with a resolution of 96 by 96.

**EPI DWI**

DWI (TR: 1500 msec/ TE: 41 msec) images were taken which employed an echo planar based spin echo sequence appropriately modified to include diffusion-sensitizing gradients along the readout (horizontal) direction with a duration of 9 msec and a gradient separation of 20 msec. Each data set consisted of 5 coronal slices of the forebrain and midbrain region, each slice being 2-mm thick with a resolution of 128 by 128 and a 30-mm$^2$ field of view. Diffusion weighting factors, or b values of 0 and 1042 s/mm$^2$ were used (maximum gradient strength of 23 G/cm). ADC maps were processed from DWI images using the MRVision software package (MRVision Co., Winchester, MA).

**SE DWI**

DWI (TR: 1500 msec/ TE: 39 msec) images were taken which employed a spin echo sequence appropriately modified to include diffusion-sensitizing gradients along the readout (horizontal) direction with a duration of 4 msec and a gradient separation of 25 msec. Each data set consisted of 5 coronal slices of the forebrain and midbrain region, each slice being 2-mm thick with a resolution of 128 by 128 and a 30-mm$^2$ field of view. Diffusion weighting factors, or b values of 4.2, 160.5, 557.6, and 1195.6 s/mm$^2$ were used (maximum gradient strength of 23 G/cm). ADC maps were processed from DWI images using the MRVision software package (MRVision Co., Winchester, MA).
T1 images

A two-dimensional T1 imaging series was generated with a spin echo, echo-planar imaging sequence preceded by an inversion recovery preparation period using a hyperbolic secant inversion pulse. Data was obtained on 3 coronal sections at a thickness of 2.5mm and slice separation of 3.0mm. Inversion recovery times were: 30, 60, 150, 300, 700, 1300, and 2500 msec.

Data Analysis

Average T1 and ADC values were obtained from regions-of-interest (ROI) outlined in the corpus callosum from the slice containing the cannula track. T1 Maps were calculated from the inversion recovery images. These T1 maps were converted to water maps by means of the following equation (Fatouros and Marmarou 1999) using the MRVision software:

\[ f_w = \frac{T1}{(0.407 + 0.907 \times T1)} \times 100 \]

Here \( f_w \) is the percent water content. This formula is applicable at a field strength of 2.4 T. Mean \( f_w \) and ADC values from edematous regions for each infusate concentration and time period were compared to contralateral brain using t-tests and graphical analysis.

Donation of Tumor-Bearing Rats

Brain tissue from T9 glioma-bearing female Fisher 344 (Harlan, Indianapolis, IN) rats (weighing approximately 120 – 140 grams) was donated by Dr. Zhi-jian Chen. Two of the animals had been tumor-bearing for 22 days, and one of the animals had been tumor-bearing for 15 days. Tissue was sectioned on a vibratome and parallel sections were processed for GFAP and EM as described above for each animal.
T9 glioma cells were given to Dr. Chen by Dr. Martin Graf (Department of Neurosurgery, Virginia Commonwealth University, Richmond, Virginia). Cells were cultured in DMEM supplemented with 10% FBS and non-essential amino acids, as adherent monolayers at 37°C, and passed biweekly with trypsin in the absence of antibiotics. Cell lines were routinely screened for mycoplasma contamination (MycoTect, Gibco BRL). All tissue culture reagents and supplements were obtained from Gibco BRL (Grand Island, NY) unless noted otherwise. Monolayers of tumor cells were trypsinized, counted on a hemacytometer and viability was assessed by trypan blue exclusion. Cells were washed twice in phosphate buffered saline (PBS) and the concentration was adjusted appropriately (Graf et al. 2005).

Before tissue donation to this project, each rat received implantation of a guide cannula as described above, and after 24 hours was infused with $5 \times 10^4$ T9 glioma cells into the right striatum (Graf et al. 2005). Cells were permitted to grow for 1 week before MRI scanning to confirm tumor formation. Two animals received follow-up scans up to 21 days post-infusion of T9 cells, and the third received follow-up scans up to 14 days post-infusion. For follow-up scans, animals were anesthetized as previously described, but were also administered Gd-DTPA intravenously through the lingual vein before scanning for enhancement on T1-weighted images. For the purposes of this study, T2-weighted images and Gd-enhanced T1-weighted images and were analyzed. All animals were sacrificed by transcardial perfusion and tissue was fixed with 4% paraformaldehyde as described above at 1 day after the final follow-up MRI scans. After brains were harvested, they were donated to this project for aforementioned tissue processing. For
analysis in this study, qualitative examination was performed on T2-weighted images, Gd-enhanced T1-weighted images, tissue stained for GFAP, and tissue processed for EM.
Chapter 4

Results

VEGF

Blood-Brain Barrier Integrity

VEGF was infused stereotactically into the striatum, and infusions with micro-and mini-osmotic pumps were also performed into the cortex. BBB integrity was then evaluated by gross sectioning of the brain to visualize EB staining following intravenous administration. No EB extravasation, as demonstrated in Figure 4-1, A, was observed ipsilaterally or contralaterally following stereotactic infusion of 0.1 µg/mL VEGF at 1 and 20 hours, 1 µg/mL VEGF at 1 hour, and 5 µg/mL VEGF at 1 hour. Similarly, no ipsilateral or contralateral EB stain was observed following 1 µg/mL VEGF infusion both through micro-osmotic pumps after 3 days, and mini-osmotic pumps after 5 days. It was also noted that needle tracts and cannula tracts (ipsilateral) from all infusions showed no evidence of EB extravasation.

Hematoxylin and Eosin Staining

Hematoxylin and Eosin (H&E) stains were also performed on representative tissue from all stereotactic infusion parameters by the department of anatomical pathology. No Virchow-Robins spaces or other evidence of fluid extravasation was
Figure 4-1. Representative images showing a brain with no Evan’s Blue Dye extravasation (A) following VEGF infusion compared to a brain infused with 2.0mM DCA where Evan’s Blue Dye extravasation was present (B). Blue color in (B) shows extravasated Evan’s Blue bound to serum proteins in the corpus callosum on the left side of the image.
Figure 4-2. Photomicrograph of H&E-stained ipsilateral striatum following stereotactic infusion of 0.1 µg/mL VEGF. Red blood cells in needle tract noted with arrow.
observed ipsilaterally or contralaterally in any tissue sample. Needle tract containing red blood cells was observed ipsilaterally in representative tissue for 1 hour post-infusion of 0.1 µg/mL VEGF (Figure 4-2).

**Deoxycholic Acid**

**Blood-Brain Barrier Integrity**

EB extravasation was observed by gross brain sectioning in the ipsilateral corpus callosum following 30-, 45- and 60-minute infusion of 2.0 mM DCA (Figure 4-1, B). For this same concentration, 30- and 60- minute infusion into the striatum also demonstrated EB extravasation. Gross brain sectioning of 1.0 mM DCA infusion did not show EB extravasation in ipsilateral corpus callosum (30-minute infusion), but 30-minute infusion of this same concentration into the striatum did show EB extravasation ipsilaterally. However, EB extravasation was observed at 1 hour post-infusion of 1.0 mM DCA in the ipsilateral corpus callosum in sections containing needle tract when sectioning tissue on the vibratome; it was thereby determined that 45-minute infusion of 1.0 mM DCA caused EB extravasation. 1 day post-infusion of 1.0 mM DCA, no EB extravasation was observed. No EB extravasation in the ipsilateral or contralateral corpus callosum was observed following 45-minute infusion of 0.5 mM DCA upon gross sectioning or vibratome sectioning at 1 hour and 1 day post-infusion.

Stereotactic saline infusion into the corpus callosum over 90 minutes did not show EB extravasation upon gross sectioning or vibratome sectioning when animals were sacrificed at 1 hour post-infusion.
Table 4-1. Table demonstrating observed EB extravasation in the corpus callosum for groups of rats receiving 9 µL of 0.5 mM, 1.0 mM, or 2.0 mM DCA infusion when examined at 1 hour and 1 day. No observed EB staining is indicated by (-). Observed EB staining is indicated by (+).
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<thead>
<tr>
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Magnetic Resonance Imaging

T2 Imaging

T2 images from representative animals are presented in Figure 4-3, showing 1 hour and 1 day post-infusion of 0.5 mM, 1.0 mM, and 2.0 mM DCA. Infusion of 0.5 mM DCA produced hyperintensity in the ipsilateral corpus callosum at 1 hour post infusion. Lateral extension of the hyperintensity from the site of infusion was observed as seen in the 2.0 mM DCA group described above; no medial extension across the midline was observed. By 1 day, no ipsilateral hyperintensity was apparent in any animals. Contralaterally, no hyperintensity for the same area of the corpus callosum was apparent in any animal for each time point.

Infusion of 1.0 mM DCA produced hyperintensity in the ipsilateral corpus callosum at 1 hour post infusion. Lateral extension of the hyperintensity from the site of infusion was observed as seen in the 2.0 mM DCA group described above; no medial extension across the midline was observed. By day 1, similar ipsilateral hyperintensity was observed in 1 animal but not in the other two. In addition, hyperintensity along the needle tract was observed in this animal and another animal. Contralaterally, no hyperintensity for the same area of the corpus callosum was apparent in any animal for each time point.

Following 45-minute infusion of 2.0 mM DCA, T2 hyperintensity was observed in the ipsilateral corpus callosum in all animals at 1 hour. The observed hyperintensity was localized to the corpus callosum such that the hyperintensity extended primarily in a
Figure 4-3. T2-weighted MR images following infusion of 0.5 (A, B), 1.0 (C, D), and 2.0 (E, F) mM DCA at 1 hour (A, C, E) and 1 day (B, D, F). At 1 hour post-infusion, all concentration groups display T2 hyperintensity in the ipsilateral (right side of image) corpus callosum. Only the 2.0 mM DCA group displays ipsilateral T2 hyperintensity at 1 day post-infusion.
1 Hour Post-Infusion  1 Day Post-Infusion

0.5mM DCA

A

B

1.0mM DCA

C

D

2.0mM DCA

E

F
lateral fashion from the infusion site. However, in one animal there was medial extension slightly beyond the midline axis of the brain in addition to the lateral extension. By 1 day, the ipsilateral corpus callosum also exhibited hyperintensity, and was confined to a lateral extension from the site of infusion without cross of the midline. Contralaterally, no hyperintensity for the same area of the corpus callosum was apparent in any animal for each time point.

**Water Content**

The location of each region selected for water content measurement was dictated by the area of T2 hyperintensity ipsilaterally and by the symmetrical region on the contralateral side. Graphs of water content for each DCA dose are seen at the tops of **Figure 4-4** for 1 hour post-infusion and **Figure 4-5** for 1 day post-infusion.

Infusion of 0.5 mM DCA over 45 minutes produced higher average water content in the ipsilateral corpus callosum (76.0%) relative to contralateral 71.7%) but the difference in these values did not attain significance (p=0.088). For this same treatment, the ipsilateral and contralateral corpus callosum did not show a significant difference in water content 1 day post-infusion. Average values for ipsilateral and contralateral were 72.5% and 72.5%, respectively.

Infusion of 1.0 mM DCA over 45 minutes produced significantly higher (p=0.014) ipsilateral water content of 77.1% compared to 71.8% of the contralateral corpus callosum at one hour post-infusion. 1 day following this same treatment, ipsilateral and contralateral water contents were 76.4% and 73.4%, respectively, but the difference in these values did not attain significance.
Figure 4-4. Graphs displaying percent water (top) and ADC values (bottom) for the contralateral and ipsilateral corpus callosum at 1 hour post-infusion of DCA. The ipsilateral corpus callosum for 1.0 and 2.0mM DCA has significantly higher percent water, indicating the presence of edema. Furthermore, all ipsilateral ADC values are significantly higher than contralateral, suggesting a predominance of vasogenic edema following 1.0 and 2.0mM DCA infusion. The ipsilateral corpus callosum following 0.5mM DCA infusion does not demonstrate significant increase in percent water content, but does show significant increase in ADC.
Corpus Callosum % Water at 1 Hour Post-Infusion (n=3)

Corpus Callosum ADC at 1 Hour Post-Infusion (n=3)
Figure 4-5. Graphs displaying percent water (top) and ADC values (bottom) for the contralateral and ipsilateral corpus callosum at 1 day post-infusion of DCA. The ipsilateral corpus callosum for 2.0mM DCA has significantly higher percent water, indicating the presence of edema. Furthermore, significantly higher ADC values for this group suggest the predominance of vasogenic edema. The 0.5 and 1.0 mM DCA groups show no significant increase in percent water or ADC.
Corpus Callosum % Water at 1 Day Post-Infusion (n=3)

Corpus Callosum ADC at 1 Day Post-Infusion (n=3)
Following in-bore (infusion inside the MRI on cannulated animals, as previously described in the methods section) 45-minute infusion of 2.0 mM DCA, water content of the ipsilateral corpus callosum was increased significantly (p=0.035, n=3) relative to the contralateral corpus callosum at 1 hour post-infusion. Average ipsilateral water content was 76.1% compared to 70.9% for contralateral. One of these animals was also used in conjunction with two additional animals that received 45-minute stereotactic infusions on the benchtop (as used in 0.5 mM and 1.0 mM DCA infusions) of 2.0 mM DCA to assess water content and ADC values 1 day post-infusion. At this time point, water content of the ipsilateral corpus callosum was 77.3%, which was significantly higher (p=0.0041) than contralateral at 72.0%.

**Apparent Diffusion Coefficient**

The location of each region selected for ADC measurement was the same as those used to measure water content, as dictated by T2 imaging. Graphs of ADC for each DCA dose are seen at the tops of Figure 4-4 for 1 hour post-infusion and Figure 4-5 for 1 day post-infusion.

Following 0.5 mM DCA infusion, ADC of the ipsilateral corpus callosum was significantly higher (902 cm$^2$/sec x 10^{-6} and 647 cm$^2$/sec x 10^{-6}, respectively; p=0.030) than contralateral. Ipsilateral and contralateral ADC measurements were not significantly different at 1 day. Average ipsilateral and contralateral ADCs were 723 cm$^2$/sec x 10^{-6} and 702 cm$^2$/sec x 10^{-6}, respectively.

For the 1.0 infusion group, the changes in ADC occurred in a similar fashion to water content changes. Average ADC of the ipsilateral corpus callosum (834 cm$^2$/sec x
was significantly higher (p=0.036) at 1 hour post-infusion than contralateral (663 cm$^2$/sec x 10$^{-6}$). Measurement at 1 day showed average ADC of the ipsilateral corpus callosum to be higher than contralateral, but the difference in these values did not attain significance. Average ipsilateral and contralateral ADCs were 811 cm$^2$/sec x 10$^{-6}$ and 704 cm$^2$/sec x 10$^{-6}$, respectively.

ADC for the 2.0 mM DCA infusion group were significantly higher in the ipsilateral corpus callosum relative to contralateral at both 1 hour (p=0.0049) and 1 day (p=0.0013). Average ipsilateral and contralateral ADCs at 1 hour were 945 cm$^2$/sec x 10$^{-6}$ and 728 cm$^2$/sec x 10$^{-6}$, respectively, and at 1 day were 955 cm$^2$/sec x 10$^{-6}$ and 710 cm$^2$/sec x 10$^{-6}$, respectively.

**GFAP Staining**

In the corpus callosum contralateral to infusion, no difference in GFAP staining was observed between 1 hour and 1 day post-infusion for all doses of DCA. Further, no morphological characteristics of GFAP upregulation were visible. Cell bodies and astrocytic processes were occasionally visible, and no interconnecting processes were observed. The rats receiving saline infusion showed similar GFAP staining in the contralateral and ipsilateral corpus callosum, both of which were similar to contralateral corpus callosum GFAP staining in DCA-infused animals. **Figure 4-6** shows GFAP staining in representative photomicrographs of each dose and time parameter at 4x magnification. **Figure 4-7** shows 20x magnification of the same images seen in **Figure 4-6**.
The ipsilateral corpus callosum following 0.5 mM DCA displayed no evidence of GFAP upregulation at both 1 hour and 1 day post-infusion. The region of interest did not appear darker at 4x magnification, and at 20x magnification no morphological difference relative to control or ipsilateral corpus callosum outside of the region was observed.

GFAP staining within the ipsilateral region of interest following 1.0 mM DCA was no different from contralateral at 1 hour post-infusion, both at 4x and 20x magnification. At 1 day, dark GFAP staining was observed when viewed at 4x magnification, and visualization at 20x magnification showed dark staining but no interconnecting astrocytic foot processes.

Ipsilateral to infusion of 2.0 mM DCA, astrocytes of the corpus callosum displayed morphological characteristics of GFAP upregulation at both 1 hour and 1 day post-infusion within the same region of interest as that of MRI studies. At 4x magnification, dark GFAP staining was observed in a similar region to that of T2 hyperintensity. Long foot processes were visible and highly interconnecting at 20x magnification. Outside of this region on the ipsilateral side, the callosum was no different from control.

**Electron Microscopy**

Parallel tissue samples taken from each brain used for GFAP immunohistochemistry were prepared for electron microscopy. Briefly, these were ipsilateral and contralateral corpus callosum samples taken from animals sacrificed 1 hour and 1 day post-infusion of 0.5 mM, 1.0 mM and 2.0 mM DCA.
**Figure 4-6.** Representative tissue stained for GFAP showing dose-dependence of the corpus callosum to 0.5 (C,D), 1.0 (E,F), and 2.0 (G,H) mM DCA infusion compared to contralateral (A,B) at 1 hour (A, C, E, G) and 1 day (B, D, F, H) post-infusion. Increased GFAP staining is present in (F), (G), and (H). Images taken at 4x magnification.
Figure 4-7. Representative tissue stained for GFAP showing dose-dependence of the corpus callosum to 0.5 (C,D), 1.0 (E,F), and 2.0 (G,H) mM DCA infusion compared to contralateral (A,B) at 1 hour (A, C, E, G) and 1 day (B, D, F, H) post-infusion. Large presence of cell bodies with few interconnections visible in (D). Dark background staining with few astrocytic processes are seen in (F). Prominent, interconnecting astrocytic processes in (G) and (H) produce a spider-web appearance. Images taken at 20x magnification.
Contralateral corpus callosum tissue samples taken from both 1 hour (Figure 4-8) and 1 day post-infusion (Figure 4-9) of all three DCA concentrations showed evidence of normal, healthy tissue ultrastructure. Myelin closely surrounded neurons, and close apposition of perivascular astrocytic endfeet to blood vessels was observed. No perivascular spaces were observed, nor was there other evidence of ECS expansion. Further, blood vessel morphology showed no abnormalities. Intracellular organelles such as smooth endoplasmic reticulum (SER) were observed within the perivascular astrocytic endfeet without large electron-lucent areas indicative of swelling. Glial somata did not show evidence of swelling, either. Neural filaments were apparent and not compacted both at the node of Ranvier and within the intermodal segment. Intra-axonally, mitochondria displayed normal cristae and no evidence of impaired axonal transport was observed. Around the node, myelin paranodal loops were closely apposed to each other and to the paranodal axon segment. Additionally, myelin ensheathment of the internodal segments of the axons was observed.

Only two ultrastructural differences were observed in the ipsilateral corpus callosum following 0.5 mM DCA at 1 hour post-infusion (Figure 4-10) relative to contralateral. Astrocytic endfeet surrounding blood vessels appeared swollen; intracellular areas with little presence of organelles comprised the swollen appearance. However, astrocytic endfeet tightly apposed blood vessels as seen contralaterally. In addition, blood vessel morphology was normal relative to contralateral, except luminal pinocytic vesicles were observed inside blood vessels. Myelin and neurons displayed no differences from
Figure 4-8. Electron micrograph images of contralateral corpus callosum at 1 hour post-infusion of DCA. **A** demonstrates a blood vessel with the visible lumen (“L”). Thick arrow indicates a neuron in cross section with normal myelin ensheathment and axoplasm. **B** demonstrates a node of Ranvier ensheathed by tight, apposing paranodal loops (thick arrow). Small arrow in A and B indicate artifactual space.
Figure 4-9. Electron micrograph images of contralateral corpus callosum at 1 day post-infusion of DCA. A demonstrates a blood vessel. Thin. arrow indicates a neuron in cross section with normal myelin ensheathment and axoplasm. Thick arrow demonstrates normal intracellular components of an astrocytic endfoot. B demonstrates a node of Ranvier ensheathed by tight, apposing paranodal loops (thin arrow).
contralateral at the node, paranode, or internode. At 1 day post-infusion of 0.5 mM DCA (Figure 4-11), the ipsilateral corpus callosum displayed the same ultrastructural characteristics as that of 0.5 mM DCA infusion at 1 hour. Perivascular astrocytic endfeet were swollen but tightly apposed to blood vessels, which were similar to contralateral. Myelin and neurons displayed no differences from contralateral, as well. No pinocytic vesicles were observed.

Ipsilaterally at 1 hour post-infusion of 1.0 mM DCA (Figure 4-12), ECS expansion was evident and perivascular spaces were observed, as well as swollen perivascular astrocytic endfeet. Pinocytic vesicles appeared on the lumen of some blood vessels. Proximal to the needle tract, intra-axonal electron-dense areas were observed inside of some neurons indicating impaired axonal transport, although others displayed features similar to contralateral. These electron densities were observed in the internode and at the node, but no blebbing at the node was observed. In addition, the myelin-neuron interface was no different from contralateral. Regions lateral to the needle tract but still within the area identified as hyperintense by MRI T2-weighted imaging displayed the ECS expansion and swollen perivascular astrocytic endfeet, but neurons were similar to those observed contralaterally. Normal intracellular organelles and axoplasm, no blebbing at the nodes, and normal myelin and axon structural features were apparent throughout the axons.

1 day following 1.0 mM DCA infusion (Figure 4-13), ipsilateral ultrastructural differences relative to contralateral were observed. Evidence of ECS expansion was observed in some samples but not others, although swollen perivascular astrocytic
Figure 4-10. Electron micrograph images of ipsilateral corpus callosum at 1 hour post-infusion of 0.5 mM DCA. A demonstrates a blood vessel. Arrow indicates a pinocytic vesicle. Asterisk (*) demonstrates swelling with a perivascular astrocytic end foot. B demonstrates a normal node of Ranvier.
Figure 4-11. Electron micrograph images of contralateral corpus callosum at 1 day post-infusion of 0.5 mM DCA. **A** demonstrates a blood vessel. Asterisk (*) demonstrates swelling with a perivascular astrocytic end foot. **B** shows a normal node of Ranvier.
endfeet apposed to blood vessels of normal morphology were observed. Pinocytic vesicles were visible, as well. Nodes displayed normal axoplasm and morphology. Internodes and paranodes displayed normal ensheathment by myelin as well as normal organelles, and no intra-axonal electron-dense areas were observed.

When a concentration of 2.0 mM DCA was infused, multiple ultrastructural differences existed ipsilaterally relative to contralateral at 1 hour post-infusion (Figure 4-14). Tissue proximal to the needle tract was in poor condition after processing, but tissue in areas displaying heavy GFAP upregulation were able to be observed. ECS expansion, perivascular spaces, and swelling of perivascular astrocytic endfeet were all observed. Pinocytic vesicles were observed on the luminal face of some blood vessels. Examples of nodal blebbing were observed, although normal myelin ensheathment to the axon was observed. No intra-axonal electron-dense bodies or were observed but some abnormal mitochondrial cristae were observed.

Ipsilateral to infusion at 1 day following 2.0 mM DCA infusion (Figure 4-15), ECS expansion and perivascular spaces were observed. Swollen perivascular astrocytic endfeet were also present, as were interendothelial vacuolizations within swollen endothelial cells. Intra-axonal clustering of organelles and disruption of the cellular membrane was observed in some axons, although others displayed no difference from contralateral. Further, some myelin degeneration was observed although myelin tightly apposed other axons.

Following infusion of each concentration of DCA, no ultrastructural abnormalities were observed in glial somata at 1 hour or 1 day post-infusion, similar to control.
**Figure 4-12.** Electron micrograph images of ipsilateral corpus callosum at 1 hour post-infusion of 1.0 mM DCA. **A** demonstrates a blood vessel. Arrow indicates a normal neuron in cross section. Perivascular space noted by “P.” **B** demonstrates a normal node of Ranvier, but ECS expansion is evident (“E”).
Figure 4-13. Electron micrograph images of ipsilateral corpus callosum at 1 day post-infusion of 1.0 mM DCA. **A** demonstrates a blood vessel. Asterisk (*) demonstrates swelling with a perivascular astrocytic end foot. **B** demonstrates a normal node of Ranvier.
**Figure 4-14.** Electron micrograph images of ipsilateral corpus callosum at 1 hour post-infusion of 2.0 mM DCA. A demonstrates a blood vessel surrounded by ECS expansion (“E”). Thick arrow indicates healthy neuron, whereas think arrow indicates a demyelinating axon. B demonstrates a node of Ranvier with blebbing (arrow).
**Figure 4-15.** Electron micrograph images of ipsilateral corpus callosum at 1 day post-infusion of 2.0 mM DCA.  **A** demonstrates a swollen pericyte (arrow) surrounded by ECS expansion (“E”). Thick arrow indicates a necrotic neuron with electron-dense axoplasm. **B** demonstrates a blood vessel and swollen astrocytic end foot (*).
**T9 Glioma-Bearing Rats**

Consistent results were observed among all tumor-bearing rats, and as with stereotactic DCA infusions, the region of interest was the corpus callosum ipsilateral to tumor.

On MRI (Figure 4-16), Gd-enhanced T1-weighted imaging (Figure 4-16, A) qualitatively showed tumor boundaries localized to the right striatum, and T2-weighted imaging (Figure 4-16 B) showed peritumoral hyperintensity surrounding the tumor mass and within the ipsilateral corpus callosum. No T2 hyperintensity was observed in the contralateral corpus callosum. Mass effect was observed on in both T1-weighted and T2-weighted images.

GFAP immunohistochemistry qualitatively demonstrated GFAP upregulation ipsilateral to the tumor in the corpus callosum at 4x and 20x magnification, as seen by dark color coloring and interconnecting astrocytic cell processes. Contralateral hemispheres showed no evidence of GFAP upregulation. Images of tissue stained for GFAP can be seen in Figure 4-17.

EM analysis qualitatively demonstrated no ultrastructural abnormalities in the corpus callosum contralateral to the tumor (Figure 4-18, A). Ipsilaterally (Figure 4-18, B, C, D), however, ultrastructural differences in the corpus callosum relative to contralateral were observed. ECS expansion was evident and some perivascular spaces were observed. However, axons and myelin displayed normal ultrastructural morphology.
Figure 4-16. Parallel MRI Gd-enhanced T1-weighted (A) and T2-weighted (B) images of coronal slices from a rat brain at 20 days post-implantation of T9 glioma. Tumor boundary is visible in hyperintensities visible in both both A and B, particularly in the bottom middle slice of each image set. Top left and top middle slices in B demonstrate hyperintensities localized to the corpus callosum.
**Figure 4-17.** Photomicrographs of coronally-sectioned tissue stained for GFAP from animal implanted with T9 glioma. Ipsilateral is seen in A (4x) and B (20x), and contralateral is seen in C (4x) and D (4x). Contralateral corpus callosum displays similar qualitative staining profile as observed in the contralateral corpus callosum following DCA infusions. Ipsilateral shows dark staining (A) and evident spider-web appearance in the cellular morphology (B).
Ipsi

Contra

4x Magnification

20x magnification

A

B

C

D
**Figure 4-18.** Electron micrograph images of contralateral (A) and ipsilateral (B, C, D) corpus callosum in animals implanted with T9 gliomas. Thick arrows in A, C, and D demonstrate normal neuronal and myelin ultrastructure. B and D demonstrate blood vessels (“L” indicates lumen). Thin arrow in B demonstrates healthy endothelial cell and “E” indicates ECS expansion. Large perivascular space (“P”) exists in D.
Chapter 5

Discussion

**VEGF**

The literature documents VEGF as a potent cytokine capable of inducing vascular leakage followed by angiogenesis (Folkman et al. 1995). Despite these findings, VEGF infusion did not produce noticeable extravasation of serum proteins in this study. Serum protein extravasation was evaluated by visualization of EB in the infusion site following intravenous EB administration and transcardial perfusion. EB binds irreversibly to serum proteins, and one would expect that protein extravasation across a compromised BBB would be visible when EB is bound. The lack of visible blue staining of the parenchyma in any of the parameters tested suggests that stereotactic infusion of VEGF does not induce significant BBB breakdown.

EB extravasation is a common method of evaluating BBB breakdown and albumin extravasation (Shukla 2006), but others have evaluated BBB breakdown following VEGF infusion using different techniques. In one study (Dobrogowska et al. 1998), albumin extravasation and BBB integrity were evaluated 10 minutes, 30 minutes and 24 hours following stereotactic infusion of 10 µL of 4µg/mL VEGF into the mouse cortex; these same evaluations were performed on untreated animals and at 10 and 30
minutes following stereotactic saline infusion. Ultrastructural analysis by transmission electron microscopy (TEM) was performed and immunogold staining for albumin permitted its quantification using TEM. The authors found small amounts of albumin in the subendothelial space and perivascular neuropil of all saline-infused animals, which was quantitatively similar to albumin present in untreated animals. VEGF infusion caused an increase of albumin extravasation to the subendothelial space but not to the perivascular neuropil. In addition, ultrastructural analysis of the endothelial cells showed small differences in their structure compared to saline and normal brain but no tight junction variability.

The experiment performed by Dobrogowska et al. (1998) is similar to stereotactic infusions performed for this thesis of 20 µL of 1 and 5 µg/mL VEGF into the striatum, after which animals received EB and were sacrificed 1 hour post-infusion. Due to the closeness of concentrations and time parameters, the larger volume of infusion, and the fact that EB binds albumin irreversibly (Shukla 2006), one would expect that albumin would extravasate and be visible by EB. Because we did not observe EB extravasation in the area of infusion upon sectioning, this finding suggests that stereotactic infusion of VEGF did not produce significant BBB disruption and albumin extravasation, which contradicts the findings of Dobrogowska et al. However, there may be a difference in sensitivity between gross visualization of EB and immunogold visualization of albumin at the ultrastructural level. Dobrogowska et al. observed significant albumin extravasation in the subendothelial space but not in the perivascular neuropil (Dobrogowska et al. 1998). If albumin localizes only to this specific area and does not
extend to the surrounding neuropil following VEGF infusion then EB staining localized to this small region might not be visible grossly. This would suggest that immunogold labeling of albumin is a more sensitive technique for visualizing extravasated albumin than grossly viewing EB.

Another interesting aspect of the paper is that the authors neither stated nor implied that saline and VEGF infusions induced edema nor did they show perivascular spaces at the ultrastructural level (Dobrogowska et al. 1998), which are seen in the albumin infusion model of edema (Ohata et al. 1990). The authors showed some alterations of the endothelial cell morphology but no alteration of tight junctions (Dobrogowska et al 1998). Although VEGF infusion by these authors caused albumin extravasation and altered endothelial cell morphology, the lack of morphological characteristics of edema could mean that the amount of serum protein extravasation was insufficient to cause edema. Determination of water content or another evaluation of fluid extravasation for the infused tissue could show whether the BBB alteration and albumin extravasation was sufficient to produce edema.

Some of our other VEGF experiments utilized micro-and mini-osmotic pumps to allow continuous infusion of 1 μg/mL VEGF into the rat cortex for 3 and 5 days, respectively. Again, no EB extravasation was observed grossly in the infused region upon sectioning. In a similar experiment, Croll et al. (2004) showed increased inflammatory response to VEGF infusion by micro-osmotic pump that increased over 7 days. Dose-dependent IgG and leukocyte extravasation, as well as altered vascular morphology were observed in the rat cortex infused for 1-7 days with 2.5, 5, 10 and 20
µg/mL VEGF relative to PBS using the same model micro-osmotic pumps as in our experiment. Immunohistochemical stains for leukocytes, IgG, and endothelial cells were used to visualize them by light microscopy. Additionally, Western blotting quantitatively revealed IgG and ICAM-1, an adhesion molecule necessary for diapedesis by leukocytes, upregulation.

The lower two concentrations used by Croll et al. were within the same order of magnitude but larger nonetheless than those used in our experiment. A dose-dependence was observed (Croll et al. 2004) and therefore the 1 µg/mL VEGF infusion used in our experiment would not be expected to induce the magnitude of effects seen by Croll et al. The authors noted modest leukocyte and IgG extravasation 1 day and robust extravasation on days 3-7 for all concentrations but with a dose-dependence on the intensity of staining at each time point. Performance of this experiment with 1 µg/mL VEGF would tell either that this concentration was sufficient to induce leukocyte and/or IgG extravasation, or that a minimum dose for extravasation exists between 1 and 2.5 µg/mL VEGF. If 1 µg/mL VEGF is sufficient to induce these effects as seen by the methods of Croll et al., then we could conclude that gross EB visualization is a less-sensitive tool for evaluation of vascular leakage than immunostaining for IgG and leukocytes. Further, if 1 µg/mL VEGF infusion is not sufficient to induce vascular leakage, then gross EB evaluation should be performed on the higher concentration parameters and compared to determine if this technique is as sensitive as immunohistochemistry for IgG and leukocytes.
The issue of specificity may also be a concern when comparing our methods to those of Croll et al. (2004). EB binds with high affinity to albumin (Radius and Anderson 1980), and because albumin is the predominant protein in plasma (Vert and Domurado 2000), we measured predominantly albumin extravasation. Other authors have measured both albumin extravasation by EB visualization with fluorescence microscopy or other techniques, and IgG extravasation by immunohistochemistry for a given experiment to evaluate vascular permeability (Shimamura et al. 2006; Chen et al. 2008).

VEGF is known for its role as a BBB permeability factor in the inflammatory response (Proescholdt et al. 1999), so VEGF-induced vascular leakage could show preference for components of the immune system. Dobrogowska et al. (1998) showed that VEGF infusion permitted albumin extravasation but did not show evidence of edema, suggesting that the amount of albumin leakage subsequent to stereotactic VEGF infusion was insufficient to cause edema. Repetition of this experiment to determine whether or not an immune response occurred would be useful, as Croll et al. similarly did not observe edema within 24 hours of exogenous VEGF introduction. In these experiments (Croll et al. 2004), the authors showed IgG and leukocyte extravasation with as little as 1 day of continuous infusion but morphological evidence of edema was evident only after 7 days of continuous infusion. As mentioned above, repetition of the Croll et al. experiments could point to the presence or absence of a sensitivity difference between gross EB visualization and immunohistochemistry techniques, but they may also indicate a specificity preference for immune system components during VEGF-induced
vascular leakage. Croll et al. even showed that ICAM-1 was upregulated, which specifically permits leukocytes to extravasate during diapedesis (Croll et al. 2004). For repetition of these experiments, one would need to use techniques with similar sensitivity to measure IgG and albumin extravasation. In addition, repetition of the Dobrogowska et al. (2004) experiments with staining for IgG compared to that of albumin would provide further information on the question of preferential BBB permeability in VEGF-induced vascular leakage.

The purpose of this study was to determine if VEGF could be used as a model of pure vasogenic edema in the rat brain. We first set out to decide if VEGF could induce BBB breakdown and serum protein extravasation before investigation of water content. No evidence of serum protein extravasation using gross EB visualization was observed after multiple parameters of VEGF infusion followed by intravenous EB administration, and therefore no analysis of water content was performed. Because similar experiments did show evidence of serum protein extravasation using different evaluation techniques, further experiments as described above should be performed to compare the sensitivities of these techniques, and to investigate the specificity of vascular leakage before attempting experiments to evaluate edema.

**Deoxycholic Acid**

Contralateral Control

All brain infusions were performed in the right cerebral hemisphere of the rat brains. The left cerebral hemisphere served as a control because no effect was expected or observed contralateral to the infusion for the same brain region. For visualization of
EB staining, no EB was present in the contralateral symmetrical region so internal controls were used for comparison.

On MRI, observed brain water content of the contralateral corpus callosum was consistent for each experimental parameter, and was similar to human brain as seen by Fatouros and Marmarou (1999); human corpus callosum is approximately 68% water. These authors attributed the presence of myelin, a lipid, in white matter to the smaller T1 signals relative to greater T1 signals observed in gray matter, which had water content of 77.1% and 80.3% in representative regions (lentiform and caudate nuclei, respectively). This suggests that the contralateral corpus callosum is an adequate control for comparison with the ipsilateral corpus callosum for MRI experiments.

Two animals received stereotactic saline infusions into the corpus callosum, and were given EB and sacrificed 1 hour post-infusion. No EB extravasation occurred ipsilateral or contralateral to infusion. In addition, GFAP staining ipsilateral to infusion for these animals showed no difference from contralateral following saline infusion, and both sides displayed no difference from each contralateral corpus callosum from DCA-infused animals. This suggests that the contralateral corpus callosum is an appropriate control for comparison with the ipsilateral corpus callosum in EB and GFAP experiments.

On the EM, consistency between all contralateral corpus callosum samples from treated animals at both 1 hour and 1 day was observed. Morphologic observations of the contralateral samples were consistent with normal ultrastructural characteristics such as tight ensheathment of axons by myelin, tightly-apposed paranodal loops (Dupree et al.
2005), apposition of perivascular astrocytic end feet to pericytes and endothelial cells (Ohata et al. 1990), lack of glial and axonal swelling (Singleton et al. 2002), and lack of NF compaction (Gallyas et al. 2006). Further, glial somata all displayed normal and consistent ultrastructure for both time points. These ultrastructural observations permitted the use of internal controls for EM experiments.

**Evaluation of Edema**

As described in the introduction, exogenous DCA application to the brain has been shown to disrupt the BBB (Greenwood et al. 1991), allowing serum albumin extravasation and the formation of vasogenic edema (Seiffert et al. 2004). However, these studies did not thoroughly examine the purity of vasogenic edema. In experiments by Greenwood et al., no examination of cellular swelling was performed following intracarotid DCA perfusion (Greenwood et al. 1991). Seiffert et al. showed EB extravasation, increased T1 and T2 signal intensities on MRI, and GFAP increase in response to cortical perfusion with DCA (Seiffert et al. 2004). ADC measurements would tell whether the increased tissue water content occurred predominantly in the intracellular or extracellular compartments of the tissue. In fact, the GFAP increase, as a marker for astrocytic reactivity, suggests the presence of cellular edema, although this increase did not occur immediately, and therefore investigation into the exact location of the excess fluid would permit definition of the edema as purely vasogenic or cytotoxic, or a heterogeneous mixture of each.

Our studies utilized multiple techniques to provide specific information regarding tissue fluid components, tissue water content, location of excess tissue fluid, and
 ultrastructural analysis following infusion of 0.5 mM, 1.0 mM and 2.0 mM DCA after 1 hour and 1 day. From the results of these individual techniques, an assessment of edema formation and edema type was made for each of these 6 parameters. EB staining suggested that extravasated serum proteins and fluid contributed to any observed edema. Edema was defined as a significant increase in water content on MRI in the region of hyperintensity; if this was observed, a significant ADC increase was then required to show the predominance of vasogenic edema. ADC increase indicates a predominant increase in extracellular fluid, but this signal may represent average water diffusivity throughout the tissue. Therefore further examination of the tissue is necessary to determine if there is intracellular swelling in addition to interstitial fluid accumulation when water content and ADC are significantly increased. GFAP upregulation indicated astrocytic cell reaction, along with indications of both interstitial fluid accumulation and cellular swelling that were observed by EM.

0.5 mM DCA

It has previously been shown that DCA concentrations below 1.0 mM did not disrupt BBB function when perfused through the carotid artery in exsanguinated rats, as evidenced by no observed extravasation of intravenous \[^{14}\text{C}]\text{mannitol}\ (\text{Greenwood et al. 1991}). \text{Greenwood et al. also demonstrated in this study that the concentration of} \[^{14}\text{C}]\text{mannitol did not contribute to BBB disruption. The study further showed that DCA concentrations below 1.5 mM did not cause "lytic action" on RBCs (Greenwood et al. 1991), which was consistent with our ultrastructural observations on the effects of a similar range of concentrations of DCA infused intraparenchymally. In our study, no EB}
staining was observed following 0.5 mM DCA infusion both at 1 hour and 1 day. This result suggested that no functional disruption of the BBB occurred, which is consistent with the aforementioned study (Greenwood et al. 1991). As a whole, our results taken in the context of the other studies cited suggest that any potential increase in tissue water content following 0.5 mM DCA consists solely of the infusate fluid.

At 1 hour, ipsilateral T2 hyperintensity was observed in the corpus callosum, but average water content increased without attaining significance, which demonstrated that significant edema was not present. Interestingly, the significant increase in ADC showed an increase in extracellular fluid, although no perivascular spaces or other evidence of ECS expansion was observed ultrastructurally. In fact, the observation of swollen astrocytic end feet without evidence of ECS expansion suggested the contrary - that cellular fluid accumulation occurred. However, GFAP upregulation was not observed, suggesting that the presence of infusate fluid did not cause the astrocytes to become reactive. Therefore this ultrastructural observation of cellular fluid accumulation indicates that the astrocytes made an insignificant contribution to fluid clearance, as further supported by the observation that glial somata did not appear swollen. This would be expected based on documented observations that astrocytes uptake extravasated proteins in vasogenic edema, thereby lowering the extracellular oncotic pressure (Stummer 2007). The insignificance of the observed water content increase does correlate with both the lack of GFAP upregulation and the lack of evidence for ultrastructural ECS expansion. In addition, no ipsilateral GFAP upregulation was observed at 1 day despite swelling of perivascular astrocytic end feet. No difference
between contralateral and ipsilateral was observed by MRI, and no ultrastructural observations of ECS expansion, perivascular spaces, glial somata, or neuronal pathology were observed, as well.

Taken together, these results suggest that the observed swellings due to 0.5 mM DCA infusion made an insignificant contribution to overall distribution of infusate. Further, the results of this experiment suggest that the infusion of 0.5 mM DCA does not produce edema at 1 hour and 1 day, and is therefore inappropriate for studies of vasogenic edema. However, replication of this experiment may permit water content increase to attain significance if additional trials produce similar T1 values on MRI to those already documented in this study, and suggest the presence of vasogenic edema due solely to infusate fluid at 1 hour post-infusion.

1.0 mM DCA

Consistent with the findings of Greenwood et al. (1991) and Seiffert et al. (2004), we observed EB staining in the ipsilateral corpus callosum at 1 hour post-infusion of 1.0 mM DCA. In addition, MRI revealed T2 hyperintensity and significant increase in water content consistent with the results of Seiffert et al. (2004). We therefore determined that this infusion parameter induced edema by disruption of the BBB and extravasation of serum albumin, and the significant ADC increase suggested a predominance of vasogenic edema. As mentioned above, the infusate fluid likely contributes to the total edema, which would suggest that the total edema volume in this parameter is a combination of infusate fluid and extravasated serum fluid.
At 1 hour, no GFAP upregulation was observed, but we did see swelling of perivascular astrocytic end feet at the ultrastructural level. However, no glial somata swelling was observed. The lack of GFAP upregulation suggests that the end foot swelling did not contribute significantly to the observed edema. Further, perivascular spaces and ECS expansion were apparent, and no other ultrastructural evidence of cellular swelling was observed. These GFAP and ultrastructural findings are consistent with the significant ADC increase, and the results as a whole suggest the existence of pure vasogenic edema for this parameter. Our finding is consistent with the findings of Greenwood et al. (1991), who endorsed 1.0 mM DCA as an optimal concentration for BBB disruption that permits albumin extravasation without necrotic tissue damage.

Our findings show that this edematous state is dynamic in a time-dependent manner. 1 day post-infusion of 1.0 mM DCA we observed no functional BBB disruption, and no significant difference in both water content and ADC between ipsilateral and contralateral, although both averages were greater than contralateral. Some tissue samples showed evidence of ECS expansion but could be attributed to artifact of tissue process, as no perivascular spaces were observed. We therefore concluded that the DCA-induced BBB disruption is temporary, and that the brain had cleared the edema fluid seen at 1 hour post-infusion, making the 1-day time point inappropriate for study of vasogenic edema.

Ultrastructurally, no abnormalities were observed in myelin, axons, or glial somata, suggesting that the edema produced by this treatment did not produce permanent tissue pathology. However, GFAP upregulation and swollen perivascular astrocytic end
feet were observed, indicating cellular swelling. Astrocytic function of extravasated proteins may be the cause of this observed astrocyte reaction. Because MRI in no way indicated cellular swelling and other ultrastructural characteristics were normal, the observed astrocytic reaction is likely due to a small amount of protein clearance still persisting at this time, which is consistent with previous findings that several days are required for removal of albumin from the ECS (Ohata et al. 1990; Fatouros et al. 1990).

These other studies have shown that clearance of proteinaceous fluid from the brain takes 8 days (Ohata et al. 1990; Fatouros et al. 1990). The major clearance pathway is currently thought to be through perivascular spaces (Ohata et al. 1990), but astrocytes were previously thought to clear the majority of fluid (Klatzo 1987). The combination of multiple mechanisms in edema clearance is well-supported nonetheless (Klatzo 1987) (Ohata et al. 1990). At 1 day post-infusion of 1.0 mM DCA, our results showed neither significant edema nor perivascular spaces, suggesting that a large amount of fluid had cleared through the perivascular spaces as observed by Ohata et al. (1990). Swollen astrocytes indicated that the astrocytic mechanism of clearance was also apparent, although these did not show prominence until 1 day. Seiffert et al. (2004) also observed GFAP increase following their 1.0 mM DCA perfusion over the cortex. In a later study by this same group, they showed that astrocytes take up extravasated serum albumin through the TGF-β receptor within 6-8 hours following 2.0 mM DCA perfusion over the cortex (Ivens et al. 2007), and interstitial water would presumably follow the albumin (Klatzo 1987). This time course of albumin uptake by astrocytes explains why significant astrocytic swelling was not observed at 1 hour. Due to our observations in the
scope of work, we suggest that the fluid clearance following 1.0 mM DCA is attributable to perivascular pathways and to astrocytic uptake, with the former predominating and removing the majority of edematous fluid before the latter.

2.0 mM DCA, 1 Hour Post-infusion

Infusion of 2.0 mM DCA produced an ipsilateral necrotic lesion in the corpus callosum proximal to the needle tip, which was surrounded by a region of mixed vasogenic and cytotoxic edema, with a predominance of the former. All MRI measurements (T2 signal, water content, and ADC) suggested a predominance of vasogenic edema throughout the region of interest at both time points. However, astrocytic swelling was observed ultrastructurally, suggesting that the edema was not purely vasogenic. Observed astrocyte morphology was consistent with documented morphology of reactive astrocytes (Gomide et al. 2005), particularly through our observation of interconnecting astrocytic processes. Interestingly we did not observe ultrastructural abnormalities in glial somata at both time points, but these somata were sparse relative to axons and blood vessels and perhaps further investigation could show evidence of pathology.

Tissue necrosis due to 2.0 mM DCA is consistent with findings of the aforementioned Greenwood et al. (1999) paper, in which the authors showed cell membrane lysis and pathological cerebral blood vasculature following intracarotid perfusion of DCA at concentrations of 1.5 mM and higher in exsanguinated rats. Additionally, Ivens et al. (2007) showed BBB disruption by EB extravasation with 2.0
mM DCA perfusion over the rat cortex, which we similarly observed with intraparenchymal infusion.

These findings are consistent with our previous results showing BBB disruption and vasogenic edema formation following a DCA infusion with a lower concentration (1.0 mM). Together, they suggest a dose-dependence of DCA on BBB disruption, edema formation and cytotoxicity following direct intraparenchymal delivery. In addition, we suggest that infusion of 2.0 mM DCA does not produce pure vasogenic edema and is therefore not optimal for study of this brain state.

**Tumor-Bearing Rats**

The qualitatively-assessed characteristics of the T9 glioma-bearing rats was consistent with known features of peritumoral edema in the corpus callosum. Peritumoral edema typically resides in the white matter (Raslan and Bhardwaj 2007), which is where we observed our findings. Mass effect, ipsilateral T2-weighted hyperintensity, and ipsilateral GFAP upregulation in combination with ultrastructural observations (e.g. perivascular spaces and expanded ECS ipsilaterally) suggested the presence of ipsilateral edema, predominantly of the vasogenic type. To further support this claim, no evidence of necrosis was observed in throughout the tissue.

The upregulated GFAP suggests astrocytic reaction to the proteinaceous fluid extravasation. As mentioned before, however, astrocytic reaction to proteinaceous fluid extravasation specifically involves uptake of the extravasated albumin without the fluid in an effort to lower extracellular oncotic pressure (Stummer 2007). Together, our observed results in combination with the documented astrocytic response to peritumoral
edema suggest a purely vasogenic edema state in the ipsilateral corpus callosum due to the brain tumor. Still, measurement of water content would have quantitatively given evidence for significant edema, and ADC measurement would have similarly provided quantitative evidence suggesting a predominance of vasogenic edema.

Further, the effect on the ipsilateral corpus callosum observed at 1 hour following 1.0 mM DCA infusion parallel this peritumoral vasogenic edema state, with the exception that GFAP was not upregulated significantly following DCA infusion at this time. However, GFAP increased at the 1 day time point from this same treatment. As suggested above, the astrocytic response to extravasated albumin following this treatment takes longer than 1 hour but less than 24 hours to occur. In the tumor, several days of gadolinium enhancement around the tumor margin demonstrate that the BBB was compromised for this time and presumably serum albumin would have extravasated. This would have permitted enough time for the astrocytic response (upregulated GFAP) to occur in the tumor, which is consistent the time-dependent GFAP increase observed following 1.0 mM DCA infusion. It is important to remember that the observed BBB disruption following a single DCA infusion was transient, whereas tumors continuously maintain a disrupted BBB (Groothuis 2000). This is consistent with our observation of no edema at 1 day post-infusion of 1.0 mM DCA, despite the observed edema at 1 hour post-infusion.

Conclusion
The purpose of these studies was to develop a model of pure vasogenic edema. Preliminary experiments with several parameters of VEGF infusion did not indicate that this technique would produce the desired result. Alternatively, DCA infusion experiments displayed a dose-dependence and time-dependence on the formation of edema. Taken as a whole, we observed that infusion into the corpus callosum of 9 µL of 1.0 mM DCA over 45 minutes produces pure vasogenic edema 1 hour post-infusion. Further, we suggest that the tumor-bearing rats exhibited peritumoral edema that was purely vasogenic. Therefore, we suggest that this specific infusion parameter provides an optimal model for the study of vasogenic edema, particularly as it pertains to the vasogenic edema in the white matter surrounding brain tumors.
Literature Cited


Vita

Charles Upshur Nottingham was born on September 10, 1984, in Roanoke, Virginia, and is an American citizen. He graduated from Patrick Henry High School, Roanoke, Virginia, in 2002. In 2006 he received his Bachelor of Science in Biology with a minor in Chemistry from Wake Forest University, Winston-Salem, North Carolina. He participated in research at the Wake Forest University Baptist Medical Center, Winston-Salem, North Carolina, in the lab of Dr. Christopher P. Turner, Ph.D., from which he co-authored three publications.

He will have completed the degree requirements for Master of Science in Anatomy and Neurobiology at Virginia Commonwealth University on the Medical College of Virginia campus, Richmond, Virginia, in August 2008. Following completion of this program, he will matriculate into the Virginia Commonwealth University School of Medicine Class of 2012.