Vascular and Metabolic Changes in Epilepsy and the Effects of Vascular Endothelial Growth Factor

Kerri A. Scorpio M.A.

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2012

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Susan D. Croll, Ph.D.

Date Chair of Examining Committee

Maureen O'Connor

Date Executive Officer

Carolyn Pytte Ph.D

Brenda Anderson Ph.D

Jeffrey Goodman Ph.D

Daniel McCloskey Ph.D.

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

EFFECT OF VEGF ON VASCULATURE IN A RAT MODEL OF EPILPESY

By

KERRI A. SCORPIO

Advisor: Professor Susan D. Croll

Preliminary research has shown possible vascular morphological changes in the hippocampus of adult rats and humans after seizure activity. Research has shown high blood vessel density associated with high seizure frequency (Rigau et al., 2007) and vessel diameter enlargement has been observed with SE. We expected long term vascular morphological changes present in the hippocampus in rats after status epilepticus to be similar to the vascular morphological changes present in humans with temporal lobe epilepsy. We found a greater mean density of blood vessels in the rat hippocampus after SE. In human temporal lobe epilepsy tissue we found a greater mean density of blood vessels in the CA3 region of the hippocampus.

Research has shown that one day after SE, VEGF protein protects neurons from damage (Croll et al., 2004a; Nicoletti et al., 2008). We were interested in whether VEGF might also drive changes in post-seizure vascular parameters. We predicted that VEGF treatment would lead to less dense blood vessels after SE, closer to that of controls. As predicted, we found less dense blood vessels after SE with VEGF. This could be a mechanism of VEGF's neuroprotection.

During SE there may be an increased need by neurons for oxygen and glucose and this can result in increased metabolic load and increased vasculature. One potential component of VEGF's neuroprotective actions could be its role in decreasing electrical or metabolic activity during or after seizures. When applied to hippocampal slices, VEGF significantly decreases

circuit excitability (McCloskey et al., 2005). VEGF may decrease metabolic activity via glucose transporters. We measured Glut-1 and Glut-4 expression after SE and found less Glut-1 and Glut-4 expression with VEGF. This change could be a mechanism of VEGF's neuroprotection by decreasing metabolic load and vascular need.

To explore if the transporter expression was functional the amount of glucose uptake in the hippocampus was measured. A study by Sheth et al., (2009) showed glucose uptake is significantly increased in the brain of an animal with seizures compared to controls. Our results were consistent in showing greater glucose uptake in the hippocampus after SE and VEGF may reduce glucose uptake.

Foreword

Tuesday September 11, 2012

For this I would like to thank my advisor and mentor Dr. Susan D. Croll for her support in getting me to where I am. I would also like to thank my dissertation committee: Dr. Carolyn Pytte, Dr. Brenda Anderson, Dr. Jeffrey Goodman, and Dr. Daniel McCloskey for their support. I would like to thank Dr. Joan C. Borod whom I have considered a mentor throughout this process, for her support. Finally, I would like to thank the CUNY Graduate Center for their support of my research through the Doctoral Student Research Grant for the 2011-2012 year.

The product of my hard work and dedication will now have a permanent place on Fifth Avenue in the center of the greatest city. No matter where I end up in life I will always call this city home. May it always continue to shine.

Table of Contents

List of Figures

Chapter 1

Epilepsy

According to the American Epilepsy Society, epilepsy is one of the most common neurological diseases worldwide and affects 1% to 3% of the population. It is characterized by recurring seizures due to abnormal electrical activity in the brain. The symptoms of epilepsy are caused by episodic disturbances that are the result of hyperexcitable neurons firing in the brain (Lezak, 1995).

There are two types of seizures, generalized seizures and partial seizures. Generalized seizures mainly involve disordered muscular contraction and neuronal discharge in widespread areas of in the brain, while partial seizures reflect a neuronal discharge from a more focal area of the brain. Simple partial seizures arise from an even more localized area of the brain and may have motor symptoms but may not include impairment or loss of consciousness. Complex partial seizures usually include loss or impairment of consciousness along with motor symptoms and may have evolved from simple partial seizures. Most complex partial seizures arise in the temporal lobe and this condition is referred to as temporal lobe epilepsy. However, for about 20% of individuals with epilepsy the seizures originate in other areas of the brain, typically the frontal lobe (Lishman, 1987). The seizures can involve the whole cortex and may be non-convulsive or convulsive, in which case there are various motor manifestations (Lezak, 1995; Walsh & Darby, 1999; Williamson et al., 1985).

The abnormal firing of the neurons can be caused by a host of different conditions such as genetic defects, birth trauma, head trauma, tumors, infections, and cerebrovascular accidents. For people prone to seizures, certain stimuli can invoke seizure activity. High amounts of

alcohol consumption, illness, lack of sleep, and physical exhaustion can trigger seizure activity (Lezak, 1995).

The hippocampus, a major structure within the temporal lobe, is part of the limbic system and has been widely known to be involved in epileptic activity. The limbic system structures appear to work together in controlling memory and emotional behavior and could explain some of the memory and emotional deficits observed in those with temporal lobe epilepsy (Lezak, 1995).

Functional Consequences of Epilepsy

Cognitive dysfunction present in epilepsy does not manifest the same characteristics for every patient with epilepsy. Cognitive dysfunction depends on the location where seizures originate and what areas are affected by the seizure episode, which can vary for each case. There is usually, however, some form of cognitive dysfunction that is associated with seizure activity. Studies have shown that as the rate of seizures and the presence of epileptiform activity. increase, scores on cognitive tests decline (Bornstein et al., 1988; Dodrill, 1980). The cognitive deficits seem to be correlated with decreased blood flow to the areas affected by the seizure (Homan et al., 1989). The most common cognitive deficits that seem to be associated with epilepsy are attention, memory, and learning problems. Individuals with temporal lobe epilepsy that includes involvement of the left temporal lobe tend to perform poorly on verbal material of learning and recall (Fowler et al., 1987). Visuospatial learning tends to be poor in those with seizure involvement of the right temporal lobe. Short-term memory also tends to be impaired for those with temporal lobe epilepsy. Impairments in both verbal and visuospatial memory can be found in those with temporal lobe epilepsy (Lezak, 1995; Walsh & Darby, 1999).

The occurrence of personality and behavior disorders increases in those with epilepsy compared to those without a history of seizure disorder. They are more likely to suffer from affective disorders and have a higher rate of suicide attempts (Mittan, 1986; Pincus & Tucker, 1985). For those who suffer from seizures as a result of brain damage, there are hosts of factors that can contribute to higher rates of depression and suicide attempts, including reduced social desirability and increased rates of affective disorders (Lezak, 1995).

Some epidemiological studies have suggested that patients with temporal lobe epilepsy have a higher frequency of psychopathology (Walsh & Darby, 1999). They can have a variety of unpleasant personality characteristics, such as irritability, outbursts of anger, obsessional thinking, humorlessness, and religiosity, to name a few (Bear et al., 1982; Waxman & Geschwind, 1975). They may also exhibit excessive attention to details and it may be difficult for them to shift sets or topics in conversations (Blumer & Benson, 1975; Heilman et al., 1993). There may also be a higher prevalence of affective disorders such as anxiety and depression, even compared to other seizure disorders (Koch-Weser et al., 1988). Those with temporal lobe epilepsy may also be prone to schizophrenic-like psychotic episodes with paranoid delusions and hallucinations and dissociative episodes sometimes encompassing multiple personalities (Neppe & Tucker, 1992). For those with a temporal lobe seizure focus on the left side, behavior seems to include more ideational thinking and self-criticism. Those with left sided focus are also more prone to schizophrenic-like psychotic episodes than those with right-sided focus (Walsh & Darby, 1999).

Seizure Pathophysiology

The hippocampus has been one of the most studied structures for epileptic activity. In temporal lobe epilepsy, the hippocampus has been widely associated with neuronal loss,

particularly in the CA1 area. Metabolic abnormalities of the hippocampus have been found in temporal lobe epilepsy. A metabolite, N-acetyl aspartate, is thought to be a neuronal marker, and reductions in N-acetyl aspartate/choline and N-acetyl aspartate/creatine are usually interpreted as neuronal loss or increased gliosis. Measured by magnetic resonance spectroscopy, a reduction in N-acetyl aspartate/choline and N-acetyl aspartate/creatine levels has been shown in the hippocampus of those with temporal lobe epilepsy compared to the levels in those without epilepsy (Connelly et al., 1994; Matthews et al., 1990).

Many individuals with temporal lobe epilepsy also have Ammon's horn sclerosis, which is one of the most common types of neuropathological damage represented by neuronal loss and gliosis in the hippocampal areas of CA1, CA3, CA4, and the dentate gyrus. Since Ammon's horn sclerosis is common among those with temporal lobe epilepsy, it has been theorized that it is caused by arterial spasm and anoxia that can occur during convulsive seizure activity. Development of epilepsy can have a genetic component, yet many individuals with Ammon's horn sclerosis with temporal lobe epilepsy have no genetic history of epilepsy (Duncan, 1993). It seems that for these individuals the sclerosis preceded the seizure activity. Repeated seizure activity occurring after the sclerosis may or may not increase atrophy in the hippocampal areas (Cendes et al., 1993). It seems that Ammon's horn sclerosis is the result of possible central nervous system infection, encephalitis/meningitis, or other causes of cerebral anoxia that could later progress into temporal lobe epilepsy (Uesugi et al., 2002).

Other areas beyond the hippocampus, however, such as the parahippocampal region along with the perirhinal cortex and entorhinal cortex, have also been studied to understand their possible role in seizure origin in the temporal lobe (Bartolomei et al., 2005). A review by Scharfman (2002) has shown that the parahippocampal area is highly predisposed to seizure

activity. Seizures can be easily induced in the perirhinal cortex and the entorhinal cortex is often involved in hippocampal seizure activity. The entorhinal cortex is located in the medial area of the temporal lobe (Bragin et al., 1999; De Guzman et al., 2004). The entorhinal cortex has reciprocal connections with the hippocampus and plays a role in processing sensory information. The neurons from superficial layers II and III send glutamatergic afferents to the hippocampus and the CA1 region (Scharfman, 2000; Du et al., 1995; Du et al., 1993). With its many glutamatergic synapses, it is possible that spontaneous excitatory activity could lead to seizure-like activity after a prolonged time period, thus making this area highly prone to seizures (Bartolomei et al., 2005; Dawodu et al., 2005).

After status epilepticus in animal models there seems to be a vulnerability of neurons in layer III of the cortex. Loss of layer III neurons in the entorhinal cortex seems to play a role in propagating seizures in the temporal lobe. Abnormal discharges have been demonstrated in those regions of the entorhinal cortex suffering neuron loss (Dawodu et al., 2005).

In animal studies, abnormalities found in the hippocampus can be found in the entorhinal cortex as well. Electrolytic lesions in the hilus of the dentate gyrus lead to seizures (White et al., 1987) and electrolytic lesions of the entorhinal cortex have been shown to lead to seizures as well (Dasheiff & McNamara, 1982). Not only does an injection of kainic acid directly in the hippocampus induce seizure activity but an injection of kainic acid directly into the entorhinal cortex also induces seizure activity (Miettinen et al., 1998). In electrophysiological studies, in addition to the hippocampus, entorhinal cortical cells have been shown to be hyperexcitable in animal models of temporal lobe epilepsy (Scharfman, 2002). There can be considerable neuronal cell loss in the entorhinal cortex accompanying seizure activity that sometimes is even more striking than hippocampal damage. It seems possible that damage to the entorhinal cortex

leading to such pathology could be a trigger for widespread cell loss found in temporal lobe epilepsy (Scharfman, 2002).

There have been several studies that have demonstrated that there is a reduction in the volume of the entorhinal cortex in those with temporal lobe epilepsy. The amount of atrophy of the entorhinal cortex could correlate with its apparent sensitivity to seizure activity. A study by Bartolomei et al. (2005) used magnetic resonance imaging to study the amount of atrophy in individuals with temporal lobe epilepsy and how it might relate to seizure activity. The results showed a relationship between entorhinal cortex volume and its involvement in initiating seizure activity. The degree of atrophy of the entorhinal cortex was found to be correlated with the amount of involvement the entorhinal cortex has in generating seizures. The results suggest that the more atrophic the entorhinal cortex, the more likely it is to interact with the hippocampus in generating seizures (Bartolomei et al., 2005).

It has been widely known that the hippocampus is vulnerable to seizure activity and studies have begun to show that the hippocampus and entorhinal cortex have reciprocal signals and there are intercortical networks between deep and superficial cortical layers. Any loss of layer III neurons in the entorhinal cortex could result in disruptions in the circuitry that could facilitate seizure spread (Scharfman, 2002; Dawodu et al., 2005).

Metabolic dysfunction has also been implicated in the pathogenesis of temporal lobe epilepsy. A study by Kann et al. (2005) explored the cellular metabolic functions in the *ex vivo* hippocampus of individuals with temporal lobe epilepsy during neuronal activation. Neuronal activation was elicited by electrical stimulation or glutamate application and NAD(P)+ fluorescence was used to monitor oxidative and glycolytic energy metabolism (Lipton, 1973; Schuchmann et al. 2001; Kann et al. 2003a, b; Shuttleworth et al. 2003). Pathological variances

in NAD(P)+ oxidation were observed in individuals with temporal lobe epilepsy mainly in the dentate gyrus, CA3, and CA1 regions. Pilocarpine-treated rats have also demonstrated these changes, suggesting that these metabolic alterations are consistent across species (Du et al., 1995; Mello et al., 1993). These disruptions may be the result of dysfunction in cellular energy metabolism rather than because of neuronal cell loss (Henry et al., 1994; O'Brien et al., 1997). It could be that in chronic epileptic tissue there are defects on the mitochondrial enzyme level in neurons that could lead to deficits in glucose utilization. There could also be dysfunction on the level of astrocytic glycolysis and/or neuronal-astrocytic metabolic coupling. Metabolic dysfunction in neurons and glial cells might significantly affect ATP homeostasis and excitability as well as intrinsic anti-oxidative mechanisms. Under certain conditions, the disturbances may favor neuronal vulnerability and the manifestation of seizures and status epilepticus (Kann et al., 2005).

Models of Epilepsy

Ictogenesis, or the induction of seizures, is due to excessive discharges from groups of neurons that can be initiated by depolarization of voltage-dependent sodium channels and activation of ionic glutamate receptors (Sasa, 2006). Repeated episodes of ictogenesis can lead to epileptogenesis, the result of prolonged anatomical and biochemical alterations and reorganizations of neuronal networks. In rat models of temporal lobe epilepsy, after the initiation of a prolonged seizure event, known as status epilepticus there is a period of latency that can last from 4-44 days and this is followed by a chronic period of recurrent spontaneous seizures (Leite et al., 1990; Cavalheiro, 1995). Status epilepticus can trigger an epileptogenic process that results in spontaneous recurrent seizures later in life. These events can lead to the development of chronic epilepsy.

During the chronic period, three to four weeks after status epilepticus, neuronal loss is most evident in pyramidal layers of CA1, CA3, and CA4 and has been associated with severe dilatation of lateral ventricles surrounding the shrunken dorsal hippocampus (Rigau et al., 2007). The acute status epilepticus model in rats mimics the development of epilepsy in humans.

In the rat model, metabolic and mitochondrial functions in the hippocampus have been characterized and compared to that of pharmaco-resistant human temporal lobe epilepsy individuals (Kann et al., 2005). Hippocampal tissue from human temporal lobe epilepsy individuals is generally characterized by neuronal loss and astrogliosis, network reorganization, and alterations of receptor and ion channel functions (Babb et al., 1984; Sutula et al., 1989; Steinhauser & Seifert, 2002). In the pilocarpine-treated rat model, neuronal loss is predominant in the entorhinal cortex and in the hippocampal formation, with the hilus, CA3, and CA1 areas generally most affected. This pattern corresponds to histopathological alterations seen in tissue from human temporal lobe epilepsy individuals (Kann et al, 2005). Neuroimaging studies have also shown a decrease in glucose utilization (suggesting hypometabolism) in seizure foci (interictally) and could explain dysfunction of mitochondrial oxidative and/or glycolytic energy metabolism (Kuhl et al., 1980; O'Brien et al., 1997).

Metabolic changes and pathology

During seizures the brain becomes more active and as a result requires more energy. Glucose levels may play a role during this process as glucose has an important role in energy supply for brain function. Glucose must cross several membranes before it can be available as an energy source for neurons. Glucose transporters on cerebral blood vessels, astrocytes and neurons facilitate the process by which glucose is able to pass through or between endothelial cells that form cerebral blood vessels, leave the blood, and enter the brain parenchyma (Pellerin,

2010). Astrocytes and neurons display different glutamate receptors-transporters and different Glut isoforms. Two Glut isoforms, Glut1 and Glut3, are known to be expressed in the brain on cells other than vasculature. Glut1 is predominantly localized in astrocytes. Astrocytes have several projections in contact with neurons, especially in the peri-synaptic area where they ensheath the synapse. Astrocytes may play a role in the regulation of energy supply to neurons as a result of glucose uptake by astrocytic end-feet (Pellerin, 2010). Glut 4 is expressed in muscles, fat cells, brain, and kidney. Glut 4 seems to work between intracellular membrane sites and cell surface membranes. It is less specifically localized and more broadly localized within systems (Ploug & Ralston, 2002).

Glut-1 is a specific glucose transporter on the membrane of astrocytic end-feet, which contact blood vessels. The astrocytic end-feet are sites for glucose uptake as it leaves the blood to enter the brain parenchyma. Glut1 is a member of the Na+ independent glucose transporter gene family (Mueckler, 1994). The Glut1 gene is most commonly expressed in the brain at the microvascular endothelium under normal physiologic conditions. The microvascular endothelium comprises the blood brain barrier in vivo (Gerhart et al., 1989; Brightman, 1977). Studies have shown glucose uptake by astrocytes in vivo following neuronal activation (Pellerin et al., 2007). Astrocytic glucose transport has been shown to be stimulated by glutamate, perhaps because of increased demand for energy in the face of this excitatory transmitter (Porras et al., 2004). Cultured astrocytes respond to glutamate by activating the glucose transporter Glut1 and this response may be mediated by sodium influx (Loaiza et al., 2003; Pellerin & Magistretti, 1997). The effects of glutamate on glucose transport by astrocytes and neurons help to explain why glucose uptake in vivo correlates better with the expression of Glut1 than Glut3 (Duelli et

al., 2001). Glutamate has been shown to strongly inhibit glucose transport in neurons (Porras et al., 2004).

During neuronal activation, glutamate can prevent glucose from entering neurons, perhaps via AMPA activation and the resultant influx of sodium (Porra et al. 2004, Pellerin & Magistretti, 1997; Voutsinos-Porche et al. 2003). In epithelial cells, Glut1 modulation by metabolic stress is associated with the activation of the AMP-activated protein kinase which is an enzyme that may be sensitive to the increased ATP expenditure caused by glutamate-induced sodium entry in neurons (Porras et al., 2004;). This pattern suggests that glutamate redistributes glucose toward astrocytes and away from neurons (Porras et al., 2004; Abbud et al., 2000).

Astrocytes appear to metabolize glucose into lactate, and neuronal firing may be supported preferentially by lactate. Lactate production is stimulated by glutamate and glutamate activation of glycolysis and, along with stimulated glucose transport, can release lactate and make lactate available to neurons as an energy supply (Pellerin, 2010; Pellerin & Magistretti, 1994; Loaiza et al., 2003). Lactate can sustain firing in cultured neurons more effectively than glucose (Bouzier-Sore et al., 2003).

Astrocytic lactate production by glutamate (Pellerin & Magistretti, 1994) may explain the large increase in local extracellular lactate that follows cortical activation (Hu & Wilson, 1997). Support for the astrocyte-to-neuron lactate shuttle has come from in vivo experiments in knock-out mice (Voutsinos-Porche et al., 2003). It has been shown that lactate does not interfere with the effect of glutamate on glucose transport. In vitro studies have shown a preferential use of lactate over glucose by neurons (Bouzier-Sore et al., 2003). In vivo studies in the hippocampus have shown that stimulation of glutamatergic afferents cause a decrease in extracellular lactate, presumably attributable to neuronal uptake (Hu & Wilson, 1997).

One argument against the astrocyte-to-neuron lactate shuttle has suggested that glucose metabolism, by competing for NAD+, should obstruct lactate usage by neurons (Chih et al., 2001). Early during neuronal activation, a specific mechanism triggered by glutamate and dependent on the influx of sodium via AMPA receptors actively prevents glucose from entering the neurons. Lactate may be used by neurons if the glycolytic flux is small but it seems unlikely as there are a large number of glucose transporters on these cells (Vannucci et al., 1997). A surge of NAD+ as a result of neuronal activation and the rapid inhibition of glucose uptake in neurons should create a thermodynamic sink for lactate in favor of glucose. When the lactate from astrocytes reaches the neuron, NAD+ depletion may further inhibit glycolytic flux (Porras et al., 2004; Cruz et al., 2001).

Changes in astrocytic/vascular Glut1 expression have been observed in varying pathophysiological conditions such as cerebral ischemia and possibly epilepsy (for review see Boado, 1998). Upregulation of Glut1 has been observed following glucose deprivation in brain endothelial cultured cells. Neuropathological conditions may be related to changes in glucose metabolism or glucose transport to the brain because neurons may require more glucose and oxygen as well as more blood supply when metabolic activity is increased (Boado, 2001).

During seizure activity there is increased metabolic activity that could result in an increase in oxidative production and an increased need for glucose. A study by Sheth et al. (2009) showed that glucose uptake is significantly increased in the brains of animals with seizures compared to controls. There may be an increase in the amount of glucose uptake by astrocytic end-feet thus resulting in greater amounts of lactate to be released to neurons. If there is an increase in metabolic load then there may be an increased need for more blood supply, which could lead to alterations in vasculature to try to meet that need.

Vascular changes in epilepsy

The possible need for a greater vascular supply during epileptic activity could result in an angiogenic process in order to attract more blood vessels. Preliminary research has shown possible vascular morphological changes in the hippocampal fissure, CA1, and CA3 regions of the hippocampus after status epilepticus. Specifically, high blood vessel density has been associated with high seizure frequency (Rigau et al., 2007). One week after status epilepticus induction in the rat model of temporal lobe epilepsy there was a 30% increase in microvessel density in the hippocampus as compared to controls. An average 35% increase in microvessel density in rats that developed spontaneous seizures was identified compared to controls (Marcon et al., 2009). High blood vessel density could contribute to the development of epilepsy if there is damage to existing vessels or leakage of newly developed ones. Vessel diameter enlargement has also been observed, primarily in the hippocampal fissure region, during status epilepticus compared to controls. This result was found in animals 2 days, 4 days, and 14 days after status epilepticus induction. The length of vessels increased by 29% at 14 days after status epilepticus compared to 2 days after status epilepticus (Ndode-ekane et al., 2010). It has been proposed that angiogenesis, inflammation, and blood brain barrier damage contribute to epileptogenesis and/or ictogenesis in experimental and human epilepsy (Ates et al., 1999; Roch et al., 2002), so that these changes may not just be compensatory, but also pathogenic.

Angiogenesis is the development of new blood vessels from pre-existing vasculature and although it is essential for organ growth and repair, an imbalance in this process may contribute to pathology. Vascular endothelial cells can proliferate in the brain resulting in the growth of new vessels as demonstrated under various disease conditions such as epilepsy and ischemia (Greenberg & Jin, 2005; Rigau et al., 2007). After brain injury, angiogenesis occurs in the most

damaged hippocampal areas with the highest need of tissue repair. Recent data has shown that endothelial cell proliferation and angiogenesis occur after epileptogenic brain injuries. The formation of new vessels may also affect neuronal function (Marcon et al., 2009).

In both adult epileptic rats and in human temporal lobe epilepsy tissue, increased vascular density was found to be associated with areas of blood brain barrier damage consistent with an increase in the levels of angiogenic factors causing an increase in vascular permeability (Rigau et al., 2007; Nagy et al., 2008). In human temporal lobe epilepsy, blood vessel density in hippocampal tissue has been found to be higher compared to that of non-epileptic hippocampal tissue (Rigau et al., 2007). Vessels in epileptic tissue appear longer, more tortuous and more numerous, especially in layers containing neuronal cell bodies, compared to non-epileptic tissue. Vessel density has been found to be positively correlated with seizure frequency (Rigau et al., 2007). The less frequent the seizure activity the lower the density of vessels. In the rat model of temporal lobe epilepsy triggered by pilocarpine-induced status epilepticus, the vascular network has been shown to increase with more numerous microvessels accompanied by the immunohistochemical expression of angiogenic markers (Rigau et al., 2007).

Increased vessel density may compensate for elevated metabolic needs of neurons during seizures leading to increased cerebral blood flow and delivery of oxygen and nutrients to the tissue. However, increased metabolic activity may not explain all of the need for increased vascular investment in epileptic tissue. It seems plausible that vascular damage and angiogenesis induced by epileptogenic brain insult are most prominent in regions with the most severe neurodegeneration because of the need for more oxygen, glucose, and immune cells to facilitate the clean-up and repair of damaged areas (Fabene et al., 2003; Rigau et al., 2007).

Leaky vessels in the regions of angiogenesis suggest newly formed vessels do not express a mature blood brain barrier (Alon et al., 1995). Maintenance of the blood brain barrier is dependent on normal functioning of pericytes, perivascular microglia, astrocytes, and the basal lamina. Under normal conditions the blood brain barrier provides protection of the central nervous system by regulating the entry of plasma-borne substances and immune cells into the nervous system. Central nervous system injuries including seizures and ischemic events can cause transient changes in the physiologic and structural features of the blood brain barrier. In adult rats, angiogenesis and blood brain barrier opening has been induced during epileptogenesis and these vascular changes could be facilitory for inflammation.

Inflammation is often triggered by the presence of an array of pro-inflammatory molecules produced by cells in response to infection or pathological threat. Experimental studies show that inflammatory reactions in the brain can enhance neuronal excitability (Vezzani et al., 1999), impair cell survival (Viviani et al., 2003; Allan et al., 2005), and increase the permeability of the blood brain barrier (Allan et al., 2005; Naldini & Carraro, 2005). Inflammatory processes have been shown to be initiated in the brain by events such as status epilepticus and can persist during epileptogenesis in the absence of the initial seizure activity. Inflammatory reactions can occur in epilepsy disorders that do not feature an inflammatory pathophysiology (Sheng et al., 1994; Maldonado et al., 2003) such as temporal lobe epilepsy and this raises the possibility that activation of inflammatory pathways is a common factor contributing to the etiopathogenesis of seizures. Seizures provoke a pattern of inflammatory mediators in the brain and increase cytokines. Experimental findings have found an induction of cytokines in glia and neurons after acute seizures in areas involved in the seizure activity (Eriksson et al., 1999; Vezzani et al.,

1999; De Simoni et al., 2000; Plata-Salaman et al., 2000). These events could contribute to the microvascular changes observed during epileptogenesis.

In the rodent model, seizure activity increases pro-inflammatory cytokines such as IL-1beta and TNF-alpha. IL-1beta and TNF-alpha messenger RNA and protein levels are rapidly ($\leq$ 30min) increased after seizure induction. Expression of IL-1beta and TNF-alpha during seizures may mediate neuronal excitability and the resultant generation of seizures, and may also contribute to neuronal cell loss, astrogliosis, and blood brain barrier damage (Vezzani et al., 1999; De Simoni et al., 2000; Vezzani et al., 2000). Intracerebral application of IL-1beta in adult rats induces new blood vessel growth in the cerebral cortex indicating that this cytokine elicits angiogenic effects (Giulian et al., 1988).

IL-1beta and its receptor IL-1 receptor type 1 are rapidly upregulated in the brain following seizure activity (Jankowsky & Patterson, 2001; Vezzani & Granata, 2005). They are also responsible for activation of downstream cascades of inflammatory events (Dinarello, 1996; Nguyen et al., 2002). Their activation has relevant functional consequences on neuronal excitability and cell survival. IL-1beta decreases the threshold of seizure induction (Dube et al., 2005), prolongs epileptic activity (Vezzani et al., 1999), and promotes excitotoxic neuronal cell death (Allan et al., 2005). IL-1beta can affect the permeability properties of the blood brain barrier (Abraham et al., 1996; de Vries et al., 1996) via disruption of the tight junction organization (Del Maschio et al., 1996) or production of nitric oxide and matrix metalloproteases in endothelial cells (Allan et al., 2005). During acute phases of status epilepticus, upregulation of IL-1beta occurs in both microglial cells and astrocytes. Previous studies have shown IL-1beta has pro-icotogenic and pro-neurotoxic properties that are mediated by IL-1 receptor type 1 and the effects seem to occur via either direct effects of IL-1beta on neurons or indirect effects on IL-

1beta mediated by astrocytes (Ravizza et al., 2007). Chronic exposure of brain tissue to elevated IL-1beta levels increases glutamate mediated neuronal cell death (Allan et al., 2005; Viviani et al., 2003). IL-1beta inhibits glial reuptake of glutamate (Ye & Sontheimer, 1996) and may increase its glial release via TNF-alpha production thus resulting in elevated extracellular glutamate levels (Bezzi et al., 2001; Marcon et al., 2009).

IL-1beta and IL-1 receptor type-1 immunoreactivity in perivascular astrocytic endfeet impinging on blood vessels and in endothelial cells of microvasculature in areas of blood brain barrier damage have been reported in both hippocampal tissue of temporal lobe epilepsy individuals and rat chronic epileptic tissue (Zucker et al., 1983; van Vliet et al., 2006; Ravizza et al., 2007). Clinical evidence in brain tissue of individuals with epilepsy that show both IL-1beta and IL-1 receptor type 1 is overexpressed (Ravizza et al., 2006).

IL-1 therefore represents one potential factor mediating vascular and inflammatory changes after seizures, but there are likely to be others. Because vascular changes may play an important role in epilepsy, elucidating the potential role of other pro-inflammatory and pro-angiogenic factors in epilepsy could lead to a better understanding of epilepsy pathogenesis. Growth factors and cytokines that are known to act on vascular cells, such as vascular endothelial growth factor, are especially good candidates for other factors that may play an important role in epilepsy and vascular changes.

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a vascular growth factor that has also been shown to protect neurons from cell death. It is a secreted protein and mitogen for micro and macrovascular endothelial cells (Ferrara & Davis-Smyth, 1997). VEGF acts as a potent angiogenic factor, stimulating the development of new blood vessels from pre-existing blood

vessels (Carmeliet & Storkebaum, 2002). Angiogenesis is crucial for normal development and homeostasis and is involved in a number of pathological conditions. It is essential for repair, remodeling, and regeneration of tissues (Veikkola et al., 1999), both by providing increased metabolic support, and by delivering immune cells to damaged regions. VEGF is a regulator of both physiological and pathological neovascularization (Ferrara & Davis-Smyth, 1997). VEGF has also been studied as a mediator of inflammation, vascular permeability and as a possible neuroprotective factor (Proescholdt et al., 1999; Heil et al., 2000; Croll et al., 2004a). Receptors for VEGF in the brain have been localized to vascular endothelium, neurons, and glia (Croll et al., 2004a).

The VEGF family includes: VEGF, PIGF, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGF mRNA is expressed in the brain and its transcription is induced by a variety of growth factors and cytokines. These include: platelet-derived growth factor-BB, epidermal growth factor, tumor necrosis factor alpha, TGF-beta1 and IL-1beta (Veikkola et al., 1999). Receptors for members of the VEGF family of proteins include VEGFR1 (Flt-1), VEGFR2 (Flk-1 or KDR), VEGFR3 (Flt-4), and the neuropilins. Except for neuropilins, all of the VEGF receptors are tyrosine kinase receptors that mediate downstream signaling of the mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathways (Gerber et al., 1998; Pedram et al., 1998). VEGF (also known as VEGF-A), the original VEGF family member, binds to VEGFR1, VEGFR2, and the neuropilins, but not VEGFR3.

VEGFR1 and VEGFR2 are densely localized to vascular endothelium including to cerebral endothelium. Neuronal localization of VEGFR2 has also been reported in cultured hippocampal or dorsal root ganglion cells, and on neurons after various brain insults, including

after seizures (Nicoletti et al., 2008). VEGFR2 has been described on glial cells, most notably after cerebral ischemia. VEGFR2 is important in vasculogenesis during development (Shalaby et al., 1995) and has been identified as the primary receptor involved in the signal transduction cascade that results in VEGF-mediated angiogenesis in adult animals (Shalaby et al., 1995). VEGFR1 has been shown to be localized to vascular endothelium but has also been described on circulating inflammatory cells and VEGF-treated astroglia. Gene deletion studies have shown VEGFR1 and VEGFR2 null mutants are early embryonic lethal and show a lack of vasculature (Croll et al., 2004a). VEGFR1 and VEGFR2 bind to VEGFA (Kawakami et al., 1996; Chedotal et al., 1998). There are five known isoforms of VEGFA in humans: VEGF 121, 145, 165, 189, and 206 (Ferrara & Davis-Smyth, 1997). All isoforms of VEGFA bind with high affinity to both VEGFR1 and VEGFR2, though binding to the neuropilins is isoform-specific, with the shortest isoform, VEGF-121, failing to bind to neuropilin. Glial cells do not appear to express neuropilins, but do express the other VEGF receptors (Gluzman-Poltorak et al., 2000).

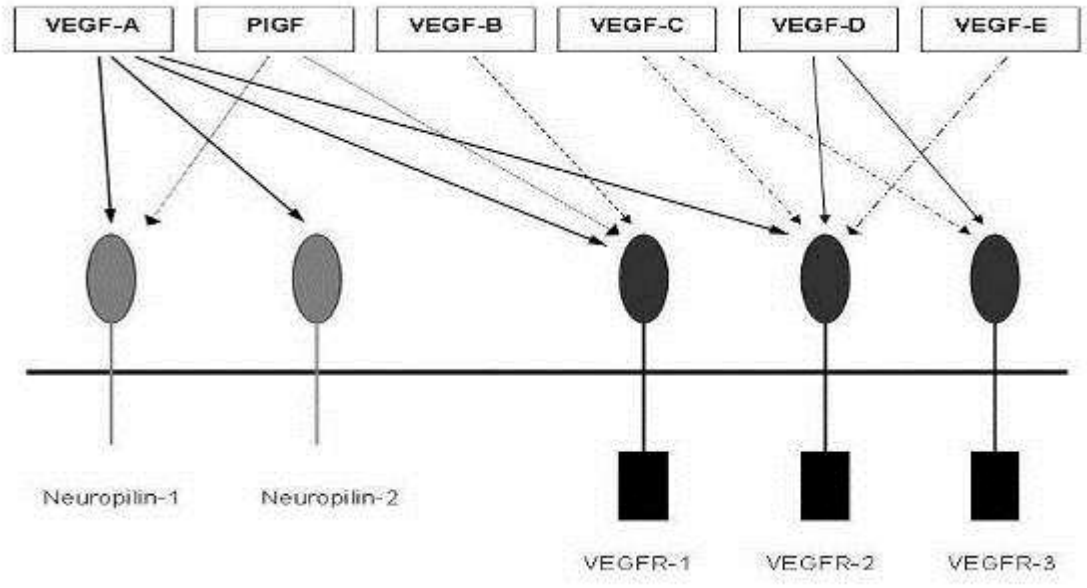

Figure 1. The VEGF family members, their receptors, the receptor tyrosine kinases VEGR-1, VEGFR-2, and VEGFR-3 and co-receptors Neuropilin-1 and Neuropilin-2 (adapted from Croll et al., 2006).

Under various disease conditions, such as epilepsy, endothelial cells can proliferate and lead to the growth of new vessels, and VEGF may contribute to these processes. In addition, blood-brain barrier disruption is common in neurological disease, and VEGF induces a dramatic leak of the blood brain barrier (Croll et al., 2004b). Indeed, vascular permeability precedes, and may be involved in, the initiation of pathological angiogenesis (Croll et al., 2004b; Krum et al., 2002; Bauters et al., 1994; Takeshita et al., 1994). VEGF-treated vasculature has been shown to be grossly abnormal with profound permeability and disorganized and dilated morphology (Krum et al., 2002; Rosenstein et al., 1998; Springer et al., 1998; Croll et al., 2004b).

The increased vascular density observed in human temporal lobe epilepsy may be based on up-regulation of VEGF in neurons after acute, short or long lasting seizures and result in the increase of vascular density (Rigau et al., 2007). High expression of VEGF and tyrosine kinase receptors suggests angiogenesis may be recurrently activated by spontaneous seizures. Experimentally, VEGF is upregulated after seizures in rats (Nicoletti et al., 2008), in concordance with results obtained in human tissue.

VEGFR2 becomes expressed in response to brain insult and VEGF signaling (Neufeld et al., 1999; Ferrara & Gerber, 2001). Quantification of VEGFR2-immunopositive blood vessels in the hippocampal regions of CA1, CA2, CA3, dentate gyrus, and hilus has been conducted in tissue after brain insult both short term (14-33 days) and long term (90-180 days) in a study by Dombrowski et al. (2006). Results of this study showed that in tissue after brain insult more VEGFR2-immunoreactive cells and blood vessels were observed compared to normal controls. A hypoxic response, as a result of brain insult, activates VEGF and VEGFR-2 mechanisms in neurons, glial, and endothelial cells involved in adaptive processes such as angiogenesis. Blood

vessel density after seizures has been found to be approximately twice that of normal controls for all hippocampal areas for both short and long term groups. This is in line with studies showing blood vessel proliferation can be attributed to decreased cerebral blood flow (Dombrowski et al., 2006) and oxygen delivery several weeks after the initial insult (Fukuhara et al., 2001; Dombrowski et al., 2006).

Studies have reported that after an initial insult VEGFR2 expression increases as early as 6 hours after seizures and peak expression is observed after 5-7 days, returning to normal after approximately 3 weeks (Dombrowski et al., 2008). However, similar expression has been observed as early as 2 weeks and as late at 12+ weeks after initial insult. This suggests that an adaptive process of VEGFR-2 induced angiogenesis may be active as early as two weeks after insult and may continue for up to 12 weeks (Marti & Risau, 1998; Issa et al., 1999; Marti & Risau, 1999). Newly formed vessels may have fewer and more immature endothelial cells and may have different structural properties such as a permeable blood brain barrier which could contribute to VEGF's involvement in pathological angiogenesis (Rosenstein et al., 1998; Fischer et al., 2002). However increased expression may also indicate certain adaptive mechanisms for recovery as studies have reported less cell loss in the hippocampus (Dombrowski et al, 2008; Cobbs et al., 1998; Jin et al., 2000a).

Evidence suggests that angiogenesis occurs as an adaptive response to hypoxia (Fukuhara et al., 2001; Luciano et al., 2001). During hypoxic conditions VEGF expression is induced, driven by a response element on the VEGF promoter for the hypoxia inducible transcription factor HIF-1alpha (Semenza, 2000). Indeed, upregulation of VEGF mRNA occurs both in vivo and in vitro under hypoxic conditions (Marti et al., 2000; Chiarugi et al., 1999; El Awad et al., 2000). This pathway provides a mechanism by which tissues can increase oxygen levels by

inducing new blood vessel growth (Veikkola et al., 1999). VEGF can also be induced in response to intracellular signals of metabolic stress or to extracellular stimuli such as certain cytokines (Pages & Pouyssegur, 2005) that are known to occur during seizure activity in both the human and experimental model (Kann et al., 2005; Vezzani & Granata, 2005). Therefore, there are multiple mechanisms available for the induction of VEGF after seizures.

While VEGF has been shown to be involved in pathological angiogenesis, (Vezzani et al., 1999), there has been growing evidence suggesting VEGF has a neuroprotective role in various pathological conditions such as epilepsy. VEGF's effects may be in protecting against neuronal cell damage or death and mediating pathways for cell survival (Croll et al., 2004b). Endogenous VEGF may have a mild effect in protecting against cell loss during seizures as suggested by Nicoletti et al., 2008. Greater neuronal cell loss was shown during seizures when endogenous VEGF was blocked. This then leads to the notion that infusion of exogenous VEGF would provide additional protection to neurons. The dual role of VEGF may also be the result of actions at two different receptors. VEGFR1 may induce inflammation and vascular leak (Luttun et al., 2002). VEGFR2, however, has been shown to be the receptor that mediates the protective effects of VEGF in vitro (Matsuzaki et al., 2001).

VEGF has been shown to protect cultured hippocampal neurons against glutamate excitotoxicity (Matsuzaki et al., 2001). It is possible that VEGF directly protects cells from excitotoxic damage via the PI3-K/Akt pathway and MEK/ERK pathway. Inhibition of PI3-K inhibits VEGF-induced activation of Akt without affecting ERK. Conversely, MEK inhibitors block activation of ERK without affecting Akt. VEGF increases activity of one of the pathways, which is sufficient to prevent neuronal death (Matsuzaki et al., 2001; Mazure et al., 1997; Gerber et al., 1998). This protective effect has been shown to be mediated through VEGFR2. VEGFR2

been implicated as the primary receptor involved in the signal transduction cascade that activates Erk/MEK and Akt signaling pathways and results in neuroprotection (Rosenstein et al., 2003; Mazure et al., 1997; Gerber et al., 1998).

Inhibition of VEGFR2 signaling blocks axonal outgrowth in response to VEGF (Sondell et al., 2000). Signaling through VEGFR2, PI3-kinase, and Akt protects cells against death from deprivation of oxygen and glucose (Jin et al., 2000a). It also protects cortical neuron cultures that are subjected to hypoxia. In hypoxic neurons, caspase-3 levels increase when VEGF is blocked adding to the evidence of a direct effect of VEGF on pathways mediating cell survival (Jin et al., 2001; Croll et al., 2004a). VEGF also has mitogenic effects on astrocytes. The proliferative effect of VEGF on astrocytes seems to be mediated by VEGFR1 (Storkebaum et al., 2004).

One further bit of evidence of VEGF protecting adult neurons has been shown by the deletion of the hypoxia response element from the VEGF promoter, which results in motor neuron disease in adult mice (Oosthuyse et al., 2001). Hypoxia is a major contributor to VEGF gene expression and the insufficient levels of VEGF seem to result in a lack of neuroprotection. Reduced VEGF levels may promote motor neuron degeneration by limiting neural tissue perfusion and VEGF-dependent neuroprotection (Storkebaum et al., 2004).

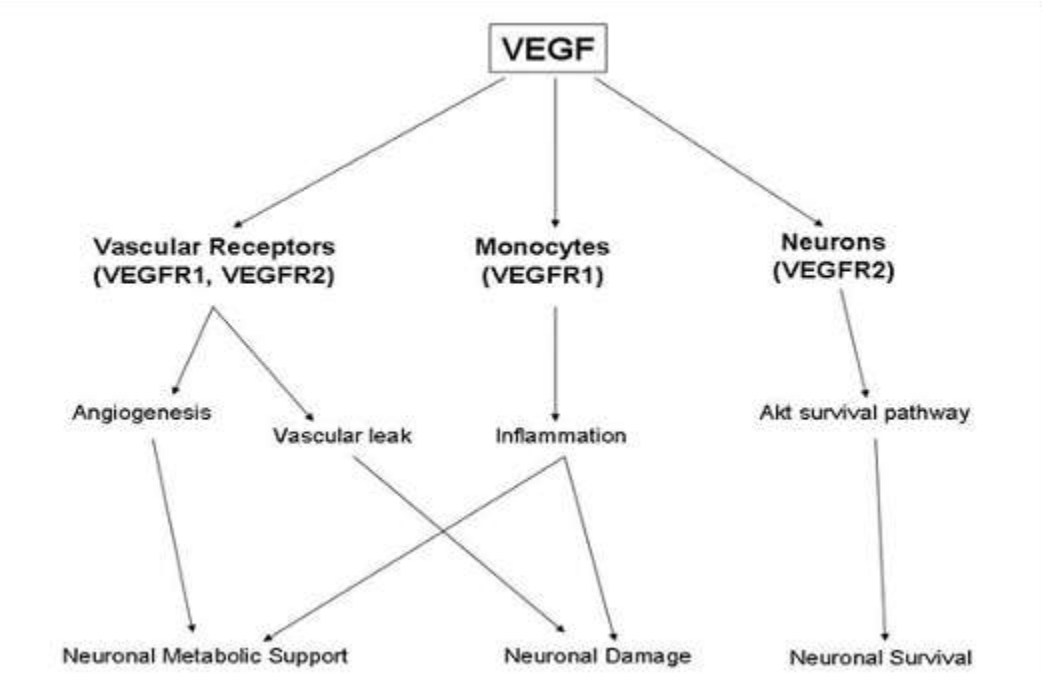

Figure 2. VEGF mediates various effects based on its receptor localization. During adverse conditions in which the system has become compromised, binding to specific receptors leads to beneficial effects while binding to other receptors leads to detrimental consequences (adapted from Croll et al., 2006)

During status epilepticus cells become more metabolically active and increase the need for glucose and oxygen and may therefore experience a state of relative hypoxia. Prolonged seizures can lead to hypoxic states due to breathing compromise during tonus and hypoxia can trigger VEGF upregulation. This hypoxic state could lead to upregulation of VEGF through HIF-1alpha, which would then induce increased vascular flow metabolically supporting their increased activity. The upregulation of VEGF during the hypoxic state could lead to VEGF's neuroprotective effects in part by eliminating the potentially damaging hypoxic state.

Studies of glioblastoma tumor cells have shown anti-VEGF treatment leads to major vessel remodeling, resulting in reduced perfusion and an increase in hypoxia in tumor microenvironments and activation of the PI3K-Wnt signaling pathways according to a study by

Keunen et al. (2011). In this well-defined model, it becomes possible to carefully study the impact of VEGF inhibition or enhancement on metabolism in local tissues. Hypoxia in these cells leads to a metabolic shift in tumors toward glycolysis that is reflected in an increase in lactate production and a stabilization of hypoxia-inducible factor 1alpha (HIF1alpha), accompanied by an increase in cell invasion into the normal brain. Upregulated gene transcripts in this model include those in the PI3K and Wnt signaling pathways and increases in tumor hypoxia leading to the activation of alternative angiogenic pathways. The PI3K/Akt pathway is known to be involved in several steps of both anaerobic and aerobic glycolysis regulation such as the localization of glucose transporters at the cell surface and maintenance of hexokinase function (Beckner et al., 2005; Bjerkvig et al., 2009). The activation of Akt in hypoxia may increase intracellular glucose and stimulate anaerobic glycolysis and lactate production and promote cell invasion (Keunen et al., 2011).

In epilepsy, little is known about the effects of VEGF upregulation on metabolism or vascular density of the brain. VEGF treatment has, in fact, been shown not to induce an increase in vascular density 24 hours after status epilepticus (Nicoletti et al., 2008). However, this finding does not rule out the possibility that VEGF alters other vascular parameters with the potential to impact brain function after seizures, particularly given that VEGF does appear to impact seizures and/or their sequelae. For instance, when applied to hippocampal slices, VEGF significantly decreases circuit excitability (McCloskey et al., 2005), suggesting that one potential component of VEGF's neuroprotective actions could be its role in decreasing electrical or metabolic activity during or after seizures. While this could be a direct effect of VEGF on neurons, VEGF could also reduce the amount of glucose uptake by astrocytic end-feet at the blood-brain barrier, thus resulting in less production of lactate being released to neurons and reducing the neuronal

metabolic load. This hypothesis of VEGF limiting glucose transport could serve as one potential explanation about how glucose restriction may reduce seizure susceptibility by reducing brain glycolytic energy (Greene et al., 2001). This effect forms the basis for one well-established mechanism of limiting seizures, the ketogenic diet. This diet induces changes in excitability that lead to reduced seizure occurrence in chronic epilepsy by decreasing the consumption of glucose so that the brain needs to rely on ketones, rather than glucose, for fuel. By understanding the acute impact of VEGF after seizures, it might be possible to better understand its longer-term therapeutic potential in epilepsy.

The vascular and metabolic changes observed in epilepsy, as well as the changes seen with the administration of VEGF lead to important questions that can be addressed. It is unclear whether acute treatment of VEGF around the time of the initiating seizure has long-lasting effects on vasculature and/or glucose uptake in the hippocampus. To investigate this further in a rodent model, one could take advantage of the fact that a single initiating status epilepticus event leads to chronic epilepsy starting approximately 1 month after status.

As previously mentioned, research has shown a relationship between angiogenesis and seizure activity. High blood vessel density has been associated with high seizure frequency in the hippocampal fissure, CA1, and CA3 regions during both the latent and chronic period after status epilepticus induction (Rigau et al, 2007). Vessel diameter enlargement has also been observed during status epilepticus (Ndode-Ekane et al., 2010). It therefore may be of interest to examine and provide further evidence for the effect of status epilepticus on vascular morphology (density and diameter) in the hippocampus during the chronic epileptogenic phase one month after initiating a seizure event, and how VEGF alters that effect. It may also be of interest to examine vascular parameters in human temporal lobe epilepsy, specifically the hippocampus, as

a comparator of the rat model of epilepsy in the temporal lobe. Histopathological alterations in the pilocarpine treated rat model have been seen to correspond with alterations seen in tissue from temporal lobe epilepsy individuals.

In addition, because components of the blood-brain barrier modulate metabolic load, one potential component of VEGF's neuroprotective actions could be a role in decreasing metabolic activity during or after seizures. By studying the long-term glucose transport effects of acutely administered VEGF, we could determine whether VEGF induces changes in the tissue that lead to alterations in metabolic load.

Specific Aims

Previous research has demonstrated vascular changes associated with seizure activity. Persistent angiogenic processes, activated by seizures, can be deleterious for brain tissue, probably due to increased blood brain barrier permeability. Chronic blood brain barrier permeability can lead to neovascular uncoupling, inflammation, and excitability. VEGF, while involved in the induction of vascular permeability, inflammation and vascular leak, has been shown to protect neurons after seizures. The role of VEGF and its possible effects on long-term vascular morphology is, however, unclear.

Functional effects of VEGF on vasculature after seizures are also unclear. VEGF's neuroprotective mechanism may work by reducing neuronal excitability as VEGF has been shown to reduce neuronal excitability when applied to hippocampal slices. Reduction in neuronal excitability by VEGF during seizure activity may reduce the need by neurons for increased oxygen and glucose. The following aims were employed to address these questions:

Specific Aim 1: To examine the effect of status epilepticus on vascular morphology (density and diameter) in the hippocampus one month after the initiating seizure event.

Specific Aim 2: To examine vascular parameters in human temporal lobe epilepsy, specifically the hippocampus, as a comparator to the rat model of epilepsy in the temporal lobe.

Specific Aim 3: To examine the effect of early VEGF treatment on vascular morphology (density and diameter) in the hippocampus one month after status epilepticus.

Specfic Aim 4: To examine the effect of early VEGF treatment on glucose transport in the hippocampus one month after status epilepticus, including glucose uptake via transporters.

Specific Aims Rationale

Specific Aim 1: To examine the effect of status epilepticus on vascular morphology (density and diameter) in the hippocampus one month after initiating seizure event.

During the latent period after acute seizure activity (from 4 days to 2-4 weeks after seizure induction) the number, size, and complexity of microvessels has been shown to be increased. Vascular density and diameter measurements were shown to be increased during this time period (Ndode-Ekane, et al., 2010). Angiogenic processes increase during this period. We focused on the chronic period (beginning 21-28 days after seizure induction) during which time spontaneous seizures develop. We investigated the changes in vascular morphology in this time period to determine if an increase in density and diameter is present. It has been suggested that angiogenesis continues during the chronic period and is either chronic or recurrently activated by the spontaneous seizures.

In determining vascular changes, our primary focus was on the hippocampus. The hippocampus is highly susceptible to seizure activity and plays a critical role in learning and memory. We specifically focused on the hippocampal fissure, CA1, and CA3 regions of the hippocampus as high blood vessel density has been associated with high seizure frequency in these areas (Rigau et al, 2007).

<u>Specific Aim 2:</u> To examine vascular parameters in human temporal lobe epilepsy, specifically the hippocampus, as a comparator to the rat model of epilepsy in the temporal lobe.

In human temporal lobe epilepsy vessel density has shown to be increased, and is often characterized by many complex tortuous microvessels. Blood brain barrier permeability and leak occurs as well as increased inflammation in temporal lobe epilepsy. We expected that in the rat model of temporal lobe epilepsy, vascular morphology in the chronic period with spontaneous seizures would be similar to vascular morphology in human temporal lobe epilepsy. To determine that this would be the case in our hands, we quantified vasculature in the human hippocampus using the same stereological methodology as that used to quantify rat vasculature in Specific Aim 1.

We focused on the hippocampus region, more specifically CA1, CA3, and fissure, to be consistent with measurements in the rat hippocampus. The focus was also on the hippocampal region due to its susceptibility to seizures and the functional consequences of seizure damage to the hippocampus on learning and memory.

<u>Specific Aim 3:</u> To examine the effect of VEGF on vascular morphology (density and diameter) in the hippocampus one month after status epilepticus.

VEGF has been shown to mediate both angiogenesis and neuroprotection in the brain. Continuous intra-hippocampal infusion of VEGF has been shown to protect hippocampal neurons from cell death 24 hours after status epilepticus without significantly increasing vascular density. One month after status epilepticus, however, neuronal preservation was not sustained (Nicoletti et al., 2010). Therefore, any long-term changes in vascular morphology one month

after status epilepticus as a result of VEGF treatment would likely be the result of changes occurring during the initial seizure event or due to prolonged changes in excitability.

In determining the effects of early VEGF treatment on long-term vascular changes we specifically focused on the hippocampal fissure, CA1 and CA3 regions of the hippocampus. High blood vessel density has been associated with high seizure frequency in these areas (Rigau et al., 2007) and any significant changes as a result of VEGF treatment would mostly likely have been observed in these areas.

Specific Aim 4: To examine the effect of VEGF on glucose transport, including glucose uptake via transporters, in the hippocampus after status epilepticus.

During status epilepticus, there may be an increased need by neurons for oxygen and glucose, which could result in a relative hypoxic state. This hypoxic state may lead to an increase in the amount of glucose uptake by astrocytic end-feet, which may play a role in regulation of energy supply and can result in increased metabolic load and increased vasculature.

VEGF is upregulated in neurons during status epilepticus and can be induced in response to metabolic stress. VEGF has been shown to reduce neuronal excitability (McCloskey et al., 2005) and may reduce the need by neurons for increased oxygen and glucose. While this response is likely to occur during the initial seizure event, there may be a carryover effect that can be observed long-term if VEGF was able to eliminate the initial metabolic increase via glucose transporters. If VEGF reduces metabolic load during status epilepticus then there may be a reduction in the need for increased oxygen and glucose and a reduction in the amount of glucose uptake by glucose transporters. A change in glucose transporter expression and the reduction in glucose uptake as a result of VEGF's effect may carryover long-term if the metabolic increase was eliminated during the initial seizure event.

We studied the potential long-term effects of VEGF on glucose transport by staining for Glut-1 and Glut-4, as a confirmatory to any changes observed in Glut-1, in the hippocampus one month after status epilepticus. We also explored glucose uptake at this same time point to verify the functionality of the transporters.

Chapter 2

General Methods

1. Subjects, Protein, and Surgeries

Subjects

All subjects were adult male Sprague Dawley rats 10 and 13 weeks old and weighing 250-350g. Animals were housed 2 to 3 per cage within a temperature-stabilized animal facility with food (Rat LabDiet 5001, Purina Mills, LLC, St. Louis, MO) and water was available ad libitum. Animals were maintained on a 12:12 light:dark cycle (lights on 07:00) and were acclimated to their colony environment at least one week prior to any manipulations.

Proteins

The active VEGF used for protein infusions was human recombinant VEGF-A165 (generously provided by Regeneron Pharmaceuticals, Inc.). VEGF was stored frozen until used and then diluted in sterile phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) to attain a dose of 45ng/day in a 12µl volume delivered .5µl/hour via Alzet (Durect Corporation, Palo Alta, CA) osmotic minipump. PBS was autoclaved before being used as a diluent for protein reagents. The dose was chosen based on pilot data (not shown) demonstrating no significant effect of VEGF in our model when infused at a dose of 15ng/d or lower. Inactivated VEGF was used as a control. VEGF was inactivated by repeated freeze-thaw cycles, which has previously been shown to eliminate VEGF's bioactivity, rather than by heat, which results in a precipitate.

Pump implantation and protein infusion

Animals were anesthetized by an injection of 65mg/kg sodium pentobarbital. The scalp was shaved and treated with iodine. Animals were placed in a stereotaxic apparatus and a

longitudinal incision was made along the scalp. For unilateral infusions, two burr holes were drilled and anchor screws (Plastics One, Roanoke, VA) were inserted. A sterile 4mm cannula (Plastics One), with an attached heat-sealed polyvinyl catheter (Plastics One) containing sterile PBS, was implanted unilaterally into the dorsal hippocampus (3.8mm posterior and 2.7mm lateral as measured from bregma, so that the tip was positioned in the lateral portion of the dentate hilus) of each animal. This location was chosen based on data demonstrating that VEGF diffuses over a 1.5mm radius (Croll et al., 2004a). Dental acrylic was then applied to secure the cannula and anchor screws in place. Polyamid nylon suture thread (CP Medical) was used to close the incision, topical antimicrobial ointment was applied, and animals were placed under a heat lamp to recover.

After one week animals were re-anesthetized with 2.5% isoflurane in oxygen and an incision was made at the nape of the neck. The heat-sealed tip of the catheter was snipped and an Alzet osmotic minipump (Durect Corporation, Palo Alta, CA) containing active VEGF or inactive VEGF, infusing 0.5 µl per hour, was attached to the catheter with cyanoacrylate glue. The pump was inserted into the subcutaneous space at the nape of the neck and the incision was closed with nylon sutures. Animals were placed under a heat lamp to recover.

Acute seizure induction

Five days following pump implantations for protein infusions, animals were pre-treated with 1mg/kg atropine methylbromide (Sigma-Aldrich) injected subcutaneously 30 minutes prior to receiving either 350mg/kg pilocarpine hydrochloride (Sigma-Aldrich) or an equivalent volume of saline intraperitoneally. Seizures were scored from stages 1-4 based on Racine's scale (1972) and modified to include stage 5, defined as sudden but transient, whole-body tonus, stage 6, defined as status epilepticus, stage 7, defined as status with a period of tonus, and stage 8, death

occurring during status epilepticus (Rudge et al., 1998). Status epilepticus was defined as seizures with no intervening return to normal behavior for greater than five minutes. Status epilepticus was truncated with 10mg/kg diazepam after 60 minutes. Animals not achieving status epilepticus received diazepam 90 minutes after pilocarpine. Status epilepticus is typically induced 30 minutes after pilocarpine administration, therefore, animals that achieved status epilepticus and those that did not, received diazepam injections in approximately the same time frame. Animals were hydrated immediately following diazepam injections with 3cc of a glucose and saline solution and received apple slices for further hydration. Animals received hydration injections daily for one week.

2. Tissue Collection and Processing

2-NBDG injection: The 2-Deoxy-D-Glucose (NBDG) was light protected and was mixed with PBS at a 1:1 dilution (5mg of 2-NBDG to 5ml of PBS). Each animal received a 1cc injection of the 2-NBDG solution 90 minutes prior to fixed tissue collection to measure glucose uptake. Animals were injected intraperitoneally. Animals were placed in 3 different enriched environments for 30 minutes each prior to fixed tissue collection. This was to stimulate glucose uptake in each animal.

Fixed Tissue Collection for Histology: One month after induction of status epilepticus, animals were euthanized using Euthasol, a pentobarbital-based euthanasia solution. The chest cavity was opened, a needle was inserted into the left ventricle of the heart, and an incision was made in the right auricle for release of fluids. The animals were exsanguinated with 200ml heparinized isotonic (0.9%) saline perfused through the heart. Following exsanguination, animals were perfusion-fixed first with 200ml of 4% paraformaldehyde in acetate and then 200ml of 4%

paraformaldehyde in borate buffer, as previously described (Croll et al., 1999). The brains were removed and placed in 30% sucrose borate buffer at 4°C until sectioned.

Sectioning

After 3-7 days in the buffered sucrose solution, brains were sectioned coronally at 50µm using a sliding microtome (American Optical Company, Buffalo, New York). Sections were placed in a 24-well plate and stored in an ethylene glycol-based cryoprotectant solution (Watson et al., 1986) at -20 degrees Celsius until stained.

Histology

Immunocytochemistry: Sections were immunostained for anti-rat endothelial cell antigen-1 (RECA) for vasculature (mouse monoclonal, 1:500, Serotec, Raleigh, NC; secondary antibody horse-anti-mouse, 1:1500, Vector Laboratories) using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Additional sections were immunostained with anti-glucose transporter-1 (rabbit polyclonal, 1:1000, Millipore, Billerica, MA; secondary antibody goat-anti-rabbit, 1:1500, Vector Laboratories) and anti-glucose transporter-4 (rabbit polyclonal, 1:500, Millepore, Billerica, MA; secondary antibody goat-anti-rabbit, 1:1500, Vector Laboratories). All tissues were exposed using a nickel sulfate-intensified diaminobenzidene (DAB) chromagen reaction.

3. Anatomical Quantification

Vasculature: Vascular density and vascular diameters in hippocampal fissure, CA1, and CA3 regions of the hippocampus were measured in RECA-immunostained tissue sections. Images were viewed under a Nikon Eclipse E400 microscope (Morrell Instruments, Melville, NY) captured with a digital video camera into Pictureframe software and imported into the public access image analysis program, NIH Image J (National Institutes of Health, Bethesda,

MD). Vascular density was measured as the proportion of area occupied by RECA-positive lumens at 40x magnification using area fraction as an estimate of volume fraction, which has been mathematically demonstrated, by point-count stereology using a randomly-oriented acetate-grid overlay. Vascular diameters were measured by taking the smallest diameter across cross-sectional vascular profiles, and the perpendicular distance across longitudinally-oriented vessels. Both measures were taken using the NIH Image J length function on those vessels randomly selected by the grid used in point-count stereology.

Vasculature in human tissue: Vascular density in the CA1, CA3, and dentate gyrus regions of the hippocampus was measured in previously Nissl stained tissue sections. Images were viewed under a Nikon Eclipse E400 microscope (Morrell Instruments, Melville, NY) captured with a digital video camera into Pictureframe software and imported into the public access image analysis program, NIH Image J (National Institutes of Health, Bethesda, MD). Vascular density was measured as the proportion of area occupied by blood vessels at 40x magnification using area fraction as an estimate of volume fraction, which has been mathematically demonstrated, by point-count stereology using a randomly-oriented acetate-grid overlay.

Glucose Transporters: Glucose transporter 1 and glucose transporter 4 on vasculature in the CA1 and dentate gyrus regions of the hippocampus were measured in GLUT-1 and GLUT-4-immunostained tissue sections. Images were viewed under a Nikon Eclipse E400 microscope (Morrell Instruments, Melville, NY) captured with a digital video camera into Pictureframe software and imported into the public access image analysis program, NIH Image J (National Institutes of Health, Bethesda, MD). GLUT-1 on vasculature was measured as the proportion of a randomly selected area occupied by transporters at 10x magnification by threshold technique in

Image J using binary settings. GLUT-4 on vasculature was measured as the proportion of a randomly selected area occupied by transporters at 40x magnification by threshold technique in Image J using binary settings.

2-NBDG glucose uptake: Glucose uptake in the CA1, CA3, and dentate gyrus areas of the hippocampus was measured in tissue sections of animals that have been injected with fluorescently-tagged 2-NBDG prior to sacrifice. Images were viewed under a fluorescent microscope and captured with a digital video camera into SPOT software and imported into the public access image analysis program, NIH Image J (National Institutes of Health, Bethesda, MD). Glucose uptake was measured as the proportion of a randomly selected area occupied by fluorescence at 40x magnification by threshold technique in Image J using binary settings.

4. Data Analysis

To determine if there were any statistical differences between groups, quantitative data was analyzed with an independent groups t-test, one-way analysis of variance (ANOVA) or a factorial analysis of variance (ANOVA), depending on the design of the experiment. All statistical analyses were conducted using SPSS software (version 11.5) using an alpha value of 0.05.

Specific Methods

Specific Aim 1: Effect of status epilepticus on vascular morphology (density and diameter) in the hippocampus one month after initiating seizure event.

Experiment 1:

In order to determine if there were changes in vascular morphology (density and diameter), animals initially received injections of pilocarpine to induce status epilepticus or

saline as a control as previously described (See General Methods). The experiment consisted of 2 groups as shown in the table below.

Group 1	N=10	Pilocarpine
Group 2	N=14	Saline

Animals were sacrificed 28 days after initial injections and tissue was perfusion fixed and sectioned for RECA immunostaining for vascular analysis in the hippocampus as previously described (See General Methods).

Specific Aim 2: Vascular parameters in human temporal lobe epilepsy, specifically the hippocampus, as a comparator to the rat model of epilepsy in the temporal lobe.

In order to determine vascular parameters in human temporal lobe epilepsy as a comparator to the rat model of epilepsy, human tissue previously collected, sectioned, stained and mounted on gelatin coated slides was used (See General Methods). 2 groups were used as shown in the table below.

Group 1	N=4	TLE
Group 2	N=3	Control

Specific Aim 3: Effect of VEGF on vascular morphology (density and diameter) in the hippocampus one month after status epilepticus.

Experiment 1:

To determine the effect of VEGF on vascular morphology (density and diameter) a cannula attached to a catheter and pump was inserted in the hippocampus containing either active

VEGF or inactive VEGF (See General Methods). 5 days later animals received injections of pilocarpine to induce status epilepticus or saline as a control as previously described (See General Methods). The experiment consisted of 4 groups as shown in the table below.

	VEGF	Inactive VEGF
Pilocarpine	N=14	N=10
Saline	N=10	N=14

Animals were sacrificed 28 days after initial injections and tissue was perfusion fixed and sectioned for RECA immunostaining for vascular analysis in the hippocampus as previously described (See General Methods).

Specific Aim 4: To examine the effect of VEGF on glucose transport, including glucose uptake via transporters in the hippocampus after status epilepticus.

Experiment 1:

We chose to examine glucose transporter 1 as Glut-1 is predominantly localized in astrocytes and astrocytes may play a role in the regulation of energy supply to neurons. To determine the effect of VEGF on glucose transporter-1 expression, a cannula attached to a catheter and pump was inserted in the hippocampus containing either active VEGF or inactive VEGF (See General Methods). 5 days later animals received injections of pilocarpine to induce status epilepticus or saline as a control as previously described (See General Methods). The experiment consisted of 4 groups as shown in the table below.

	VEGF	Inactive VEGF
Pilocarpine	N=9	N=9
Saline	N=6	N=6

Animals were sacrificed 28 days after initial injections and tissue was perfusion fixed and sectioned for GLUT-1 immunostaining for glucose transporter analysis in the hippocampus as previously described (See General Methods).

Experiment 2:

We chose to examine glucose transporter-4 to confirm if any changes observed in glucose transporter-1 expression would also be observed in other glucose transporters in the brain. Any changes in Glut-4 expression would also help determine that any effects observed in transporter expression would not be attributed solely to the blood brain barrier. To determine the effect of VEGF on glucose transporter-4 expression, a cannula attached to a catheter and pump was inserted in the hippocampus containing either active VEGF or inactive VEGF (See General Methods). 5 days later animals received injections of pilocarpine to induce status epilepticus or saline as a control as previously described (See General Methods). The experiment consisted of 4 groups as shown in the table below.

	VEGF	Inactive VEGF
Pilocarpine	N=5	N=4
Saline	N=5	N=6

Animals were sacrificed 28 days after initial injections and tissue was perfusion fixed and sectioned for GLUT-4 immunostaining for glucose transporter analysis in the hippocampus as previously described (See General Methods).

Experiment 3:

We chose to explore glucose uptake to verify the functionality of the glucose transporters. To explore the effect of VEGF on glucose uptake a cannula attached to a catheter and pump was inserted in the hippocampus containing either active VEGF or inactive VEGF (See General Methods). 5 days later animals received injections of pilocarpine to induce status epilepticus or saline as a control as previously described (See General Methods). The experiment consisted of 4 groups as shown in the table below.

	VEGF	Inactive VEGF
Pilocarpine	N=3	N=3
Saline	N=3	N=2

Animals were sacrificed 28 days after initial injections. Animals were injected with 2-NBDG 90 minutes prior to perfusion fixed tissue collection. Tissue was sectioned and mounted within one week of perfusion fixed tissue collection for fluorescence analysis as previously described (See General Methods).

Chapter 3

Specific Aims Results

Specific Aim One

Effect of status epilepticus on vascular morphology (density and diameter) in the hippocampus one month after initiating seizure event.

During the latent period after acute seizure activity (from 4 days to 2-4 weeks after seizure induction) the number, size, and complexity of microvessels has been shown to be increased. Vascular density and diameter measurements were shown to be increased during this time period (Ndode-Ekane, et al., 2010). Angiogenic processes increase during this period. We focused on the chronic period (beginning 21-28 days after seizure induction) during which time spontaneous seizures develop.

In order to determine any differences in vascular morphology in the hippocampus one month after initiating seizure event, the experiment consisted of two groups as shown in the table below.

Group 1	N=10	Pilocarpine
Group 2	N=14	Saline

Tissue was stained for anti-RECA-1, a vascular marker in rats, to evaluate vascular density and diameter analysis in the CA1, CA3, and hippocampal fissure regions of the hippocampus. Measurements were taken at random points across layers in CA1, CA3, and hippocampal fissure regions.

Vascular Density

Data revealed a significantly greater mean vascular density in animals one month after pilocarpine-induced status epilepticus (group effect $F=7.370$, $p<0.05$, see Figure 3). Therefore, there were a significantly greater numbers of vessels in the hippocampus one month after status epilepticus.

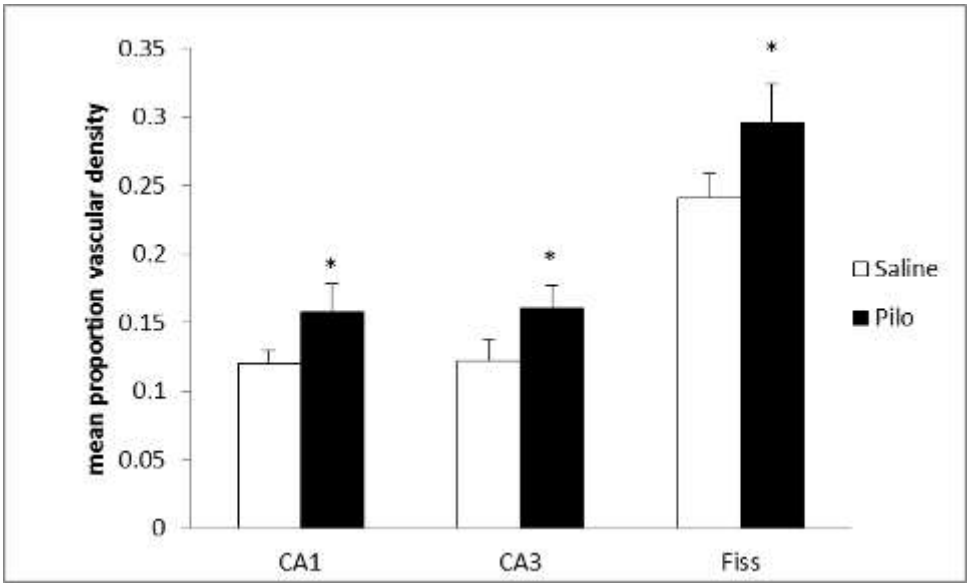

Figure 3: Vascular density one month after status epilepticus.
Graph illustrates a significantly greater amount of vascular density in the pilocarpine-induced status epilepticus group. * Statistically significant difference from saline group ($p<0.05$), error bars are SEM

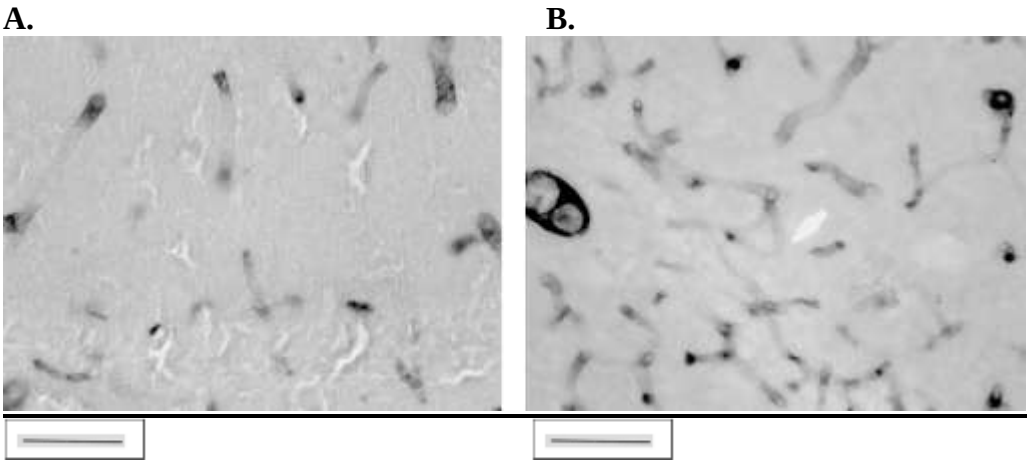

Figure 4: CA1 of the hippocampus one month after saline or pilocarpine-induced status epilepticus
Sections from a saline-treated (**A**) rat and a pilocarpine-treated (**B**) rat one month after status epilepticus. Sections were immunostained with anti-RECA-1. An increase in vascular density is seen after pilocarpine-induced status epilepticus. Scale bar = 50μm.

Vascular Diameter

Data revealed no statistically significant differences in vascular diameter in the hippocampus one month after animals received pilocarpine-induced status epilepticus or saline (group effect $F=0.816$, $p=0.371$, see Figure 5). Therefore, while there were a significantly greater number of vessels in the hippocampus one month after status epilepticus, there was no statistically significant change in vessel diameter, suggesting that there were more, rather than larger, vessels contributing to the increased vascular density.

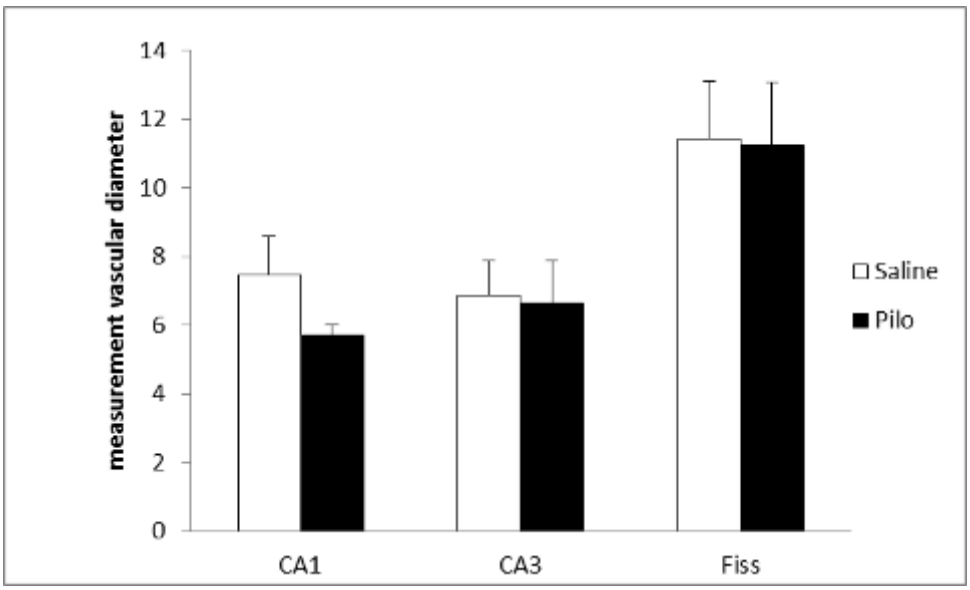

Figure 5: Vascular diameter one month after status epilepticus
Graph illustrates no significant difference in vessel diameter in the pilocarpine-induced status epilepticus group and the saline group ($p>0.05$), error bars are SEM

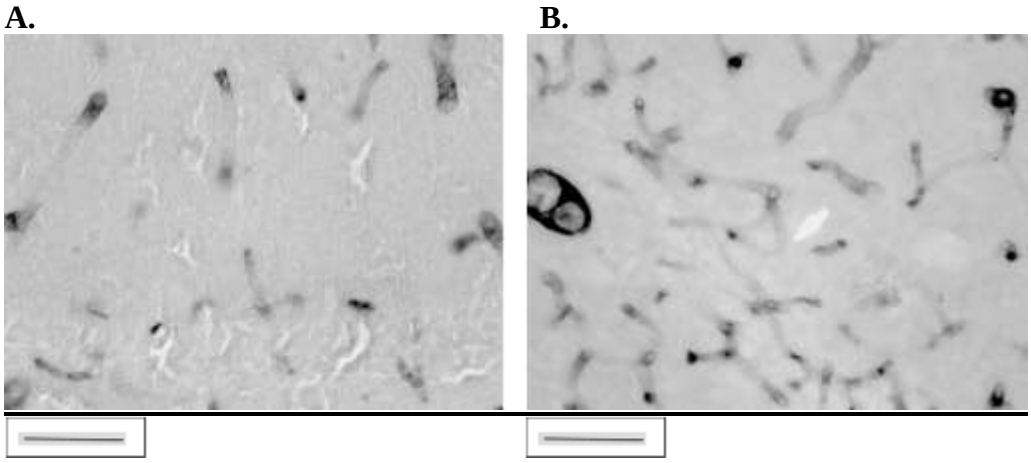

Figure 6: CA1 of the hippocampus one month after saline or pilocarpine-induced status epilepticus
Sections from a saline-treated (**A**) rat and a pilocarpine-treated (**B**) rat one month after status epilepticus. Sections were immunostained with anti-RECA-1. No significant increase in vascular diameter is seen after pilocarpine-induced status epilepticus. Scale bar = 50µm.

Specific Aim 2

Vascular parameters in human temporal lobe epilepsy, specifically the hippocampus, as a comparator to the rat model of epilepsy in the temporal lobe.

In human temporal lobe epilepsy vessel density has shown to be increased, and is often characterized by many complex tortuous microvessels. Blood brain barrier permeability and leak occurs as well as increased inflammation in temporal lobe epilepsy. In order to determine vascular parameters in human temporal lobe epilepsy as a comparator to the rat model of epilepsy, 2 groups were used as shown in the table below.

Group 1	N=4	TLE
Group 2	N=3	Control

We expected that in the rat model of temporal lobe epilepsy, vascular morphology in the chronic period with spontaneous seizures would be similar to vascular morphology in human temporal lobe epilepsy.

Vascular Density

Data revealed a significantly greater mean vascular density in humans with temporal lobe epilepsy compared to controls, although this effect was limited to the CA3 region of the hippocampus (group effects: Hilus $F(1,5)=0.304$, $p=0.605$; CA1 $F(1,5)=0.773$, $p=0.420$; CA3 $F(1,5)=84.396$, $p<0.000$, overall MANOVA for group shows significantly more vessels in TLE than controls, $p<0.02$, see Figure 7). Therefore, there were significantly more vessels in the hippocampus of TLE patients compared to controls.

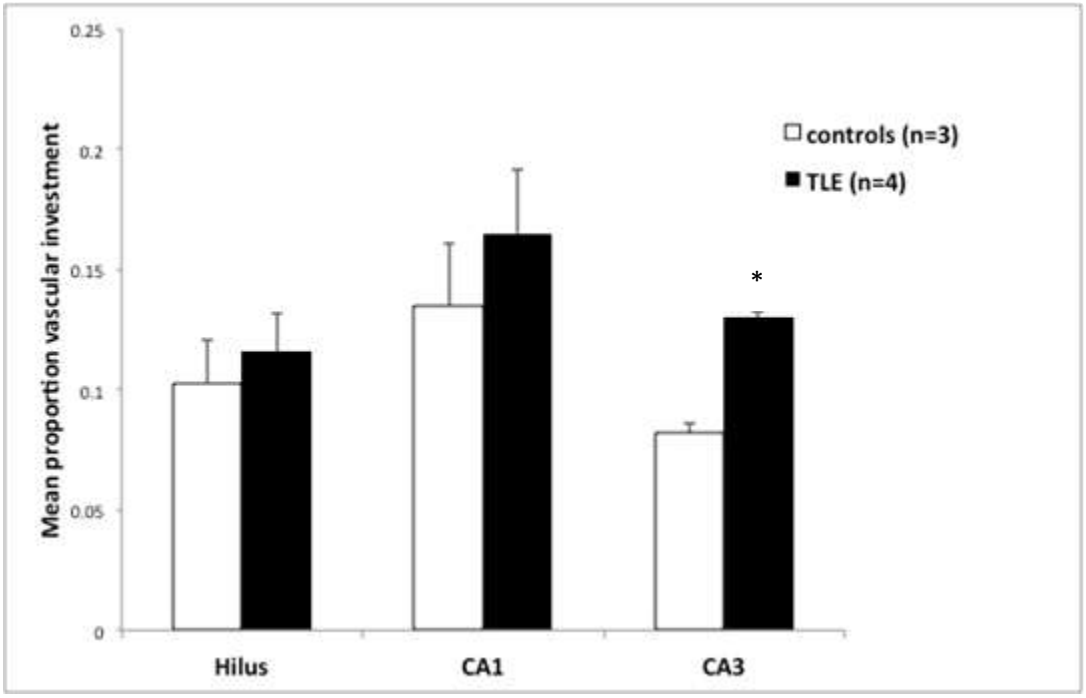

Figure 7: Vascular density in human TLE patients
TLE patients show significantly greater vascular investment in the CA3 region of the hippocampus than control patients ($p<0.05$), error bars are SEM

Specific Aim 3:

Effect of VEGF on vascular morphology (density and diameter) in the hippocampus one month after status epilepticus.

VEGF has been shown to mediate both angiogenesis and neuroprotection in the brain. Continuous intra-hippocampal infusion of VEGF has been shown to protect hippocampal neurons from cell death 24 hours after status epilepticus without significantly increasing vascular density. One month after status epilepticus, however, neuronal preservation was not sustained (Nicoletti et al., 2010). Therefore, any long-term changes in vascular morphology one month after status epilepticus as a result of VEGF treatment would likely be the result of changes occurring during the initial seizure event or due to prolonged changes in excitability. To determine the effect of VEGF on vascular morphology (density and diameter), the experiment consisted of 4 groups as shown in the table below.

	VEGF	Inactive VEGF
Pilocarpine	N=14	N=10
Saline	N=10	N=14

Tissue was stained for anti-RECA-1 for vascular density and diameter analysis in the CA1, CA3, and hippocampal fissure regions of the hippocampus. Measurements were taken at random points across layers in CA1, CA3, and hippocampal fissure regions.

Vascular Density

As to whether there were long-term changes in vascular density as a results of VEGF treatment, data revealed a significantly greater mean vascular density in the hippocampus in animals one month after pilocarpine-induced status epilepticus receiving inactive VEGF treatment than VEGF treatment (treatment effect, $F=5.665$, $p< 0.05$, see Figure 8). Therefore, there were a significantly greater number of vessels in the hippocampus one month after status epilepticus in the inactive VEGF treatment group than the VEGF treatment group.

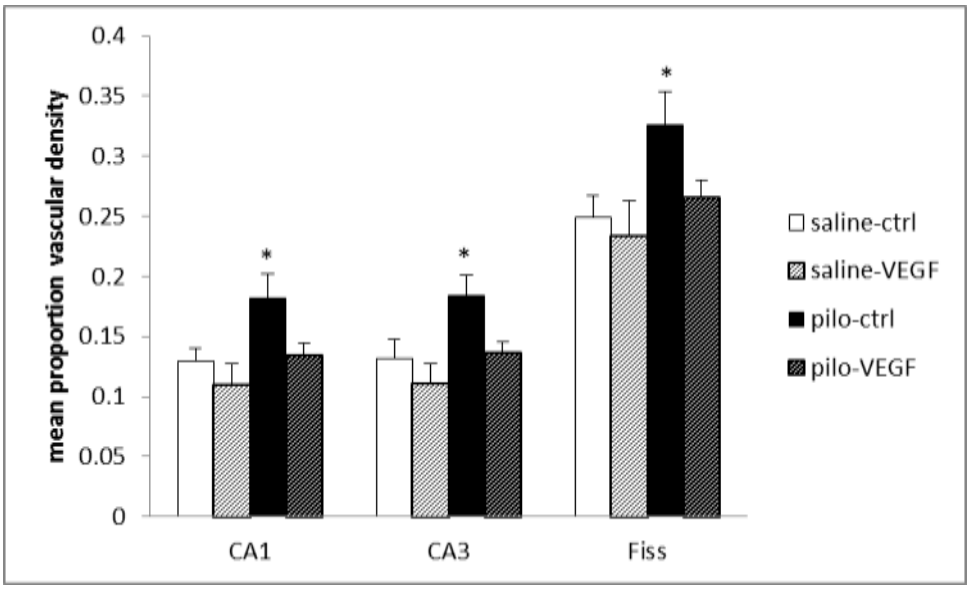

Figure 8: VEGF effect on vascular density one month after status epilepticus
Graph illustrates a statistically significant greater vascular density one month after status epilepticus in the inactive VEGF group than the VEGF group. * statistical difference from VEGF group ($p<0.05$), error bars are SEM

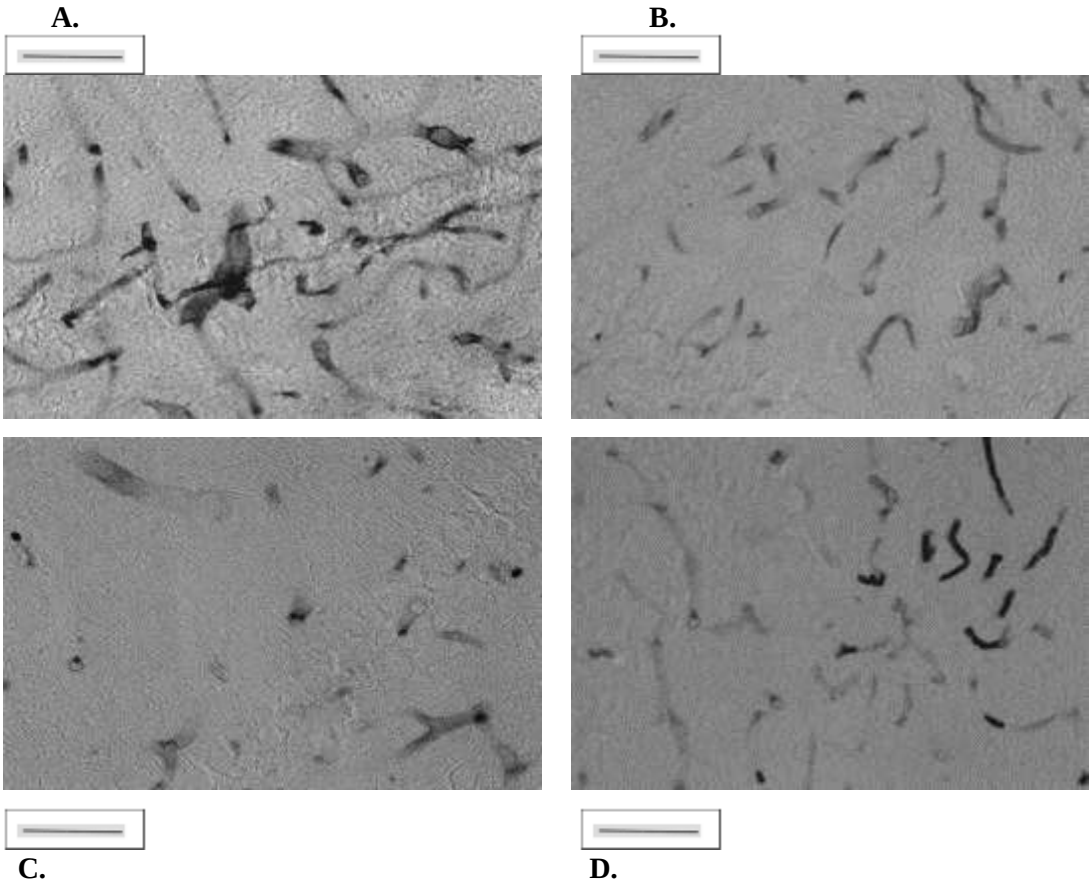

Figure 9: CA1 of the hippocampus one month after saline or pilocarpine-induced status epilepticus with VEGF or inactive VEGF treatment

Sections from an inactive VEGF-treated **(A)** rat one month after status epilepticus and a VEGF-treated (**B**) rat one month after status epilepticus. Controls were inactive VEGF-treated (**C**) with saline and VEGF-treated (**D**) with saline. All were immunostained with anti-RECA-1. Significantly greater vascular density is seen in the inactive VEGF-treatment after pilocarpine-induced status epilepticus. Scale bar = 50μm.

Vascular Diameter

As to whether there were long-term changes in vascular diameter as a results of VEGF treatment, data revealed no statistically significant difference in vessel diameter one month after status epilepticus in animals receiving either VEGF treatment or saline treatment (treatment group, F=0.227, p=0.636, see Figure 10). Therefore, while there were a significantly greater number of vessels in the hippocampus one month after status epilepticus in the inactive VEGF

group than VEGF group, there was no statistically significant change in vessel diameter with inactive VEGF or VEGF treatment. In addition, there was no significant group (saline vs. pilocarpine) by treatment (VEGF vs. inactive VEGF) interaction.

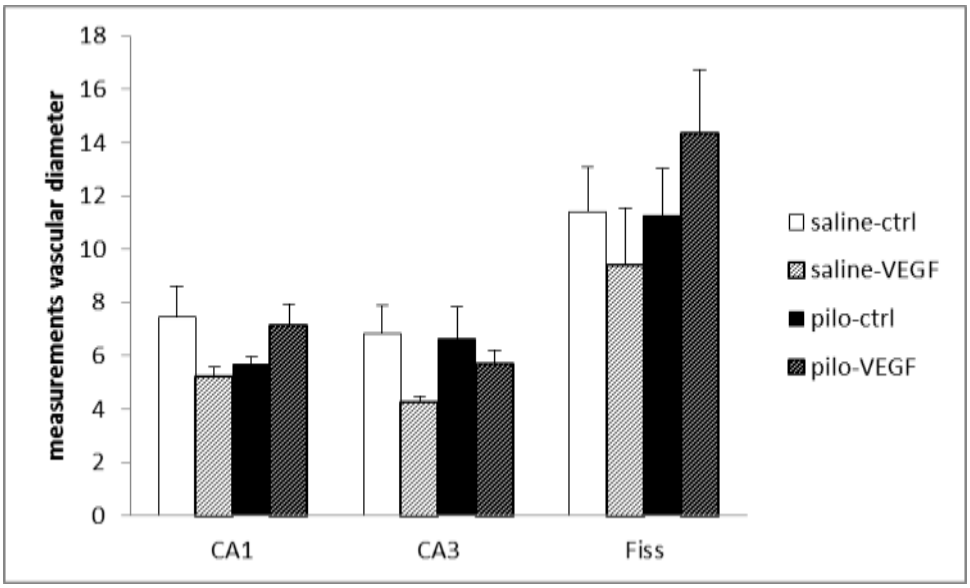

Figure 10: Effect of VEGF on vascular diameter one month after status epilepticus
Graph illustrates no statistically significant difference in vascular diameter in the VEGF treatment and inactive VEGF treatment groups ($p>0.05$), error bars are SEM

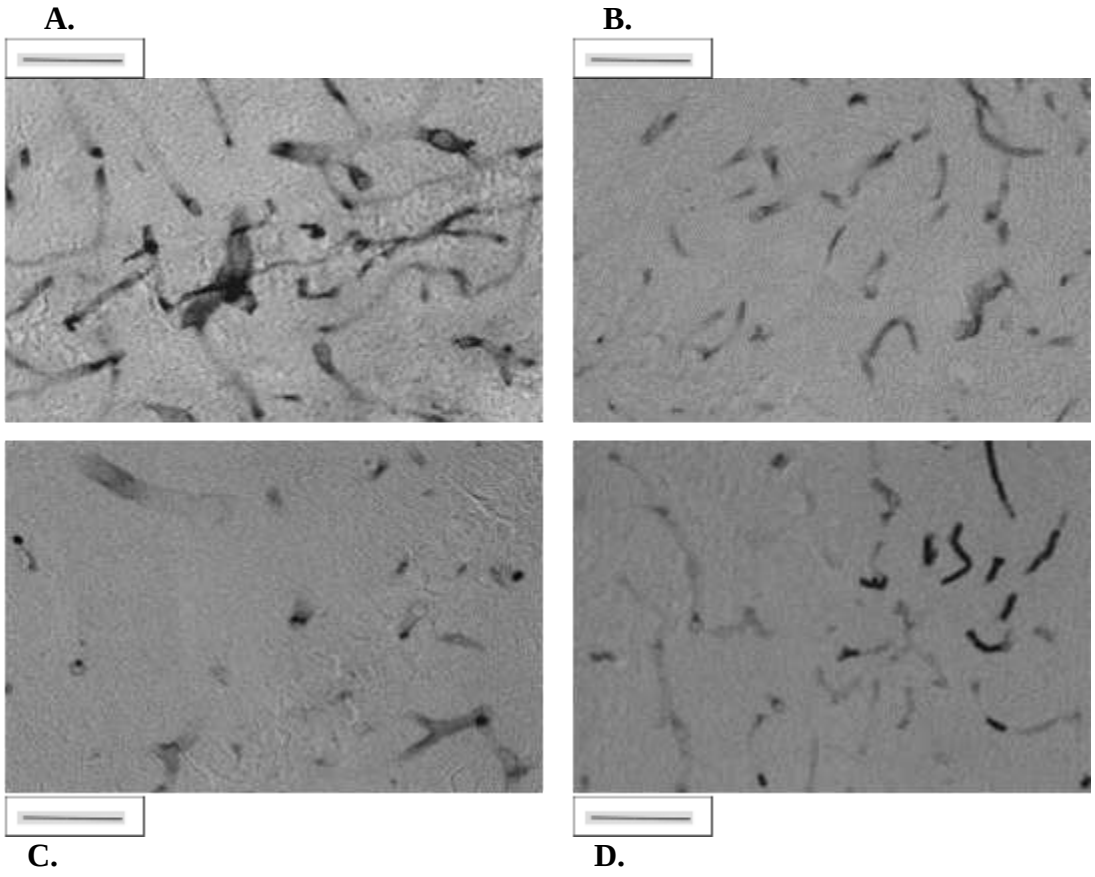

Figure 11: CA1 of the hippocampus one month after saline or pilocarpine-induced status epilepticus with VEGF or inactive VEGF treatment

Sections from an inactive VEGF-treated **(A)** rat one month after status epilepticus and a VEGF-treated (**B**) rat one month after status epilepticus. Controls were inactive VEGF-treated (**C**) with saline and VEGF-treated (**D**) with saline. All were immunostained with anti-RECA-1. No change in vascular diameter is seen in the inactive VEGF-treatment after pilocarpine-induced status epilepticus. Scale bar = 50μm.

We also looked at seizure score to determine if there any significant differences between treatment groups regarding scores on the Racine seizure scale. We found no significant difference between treatment groups ($F=0.037$, $p=0.849$, See Figure 12).

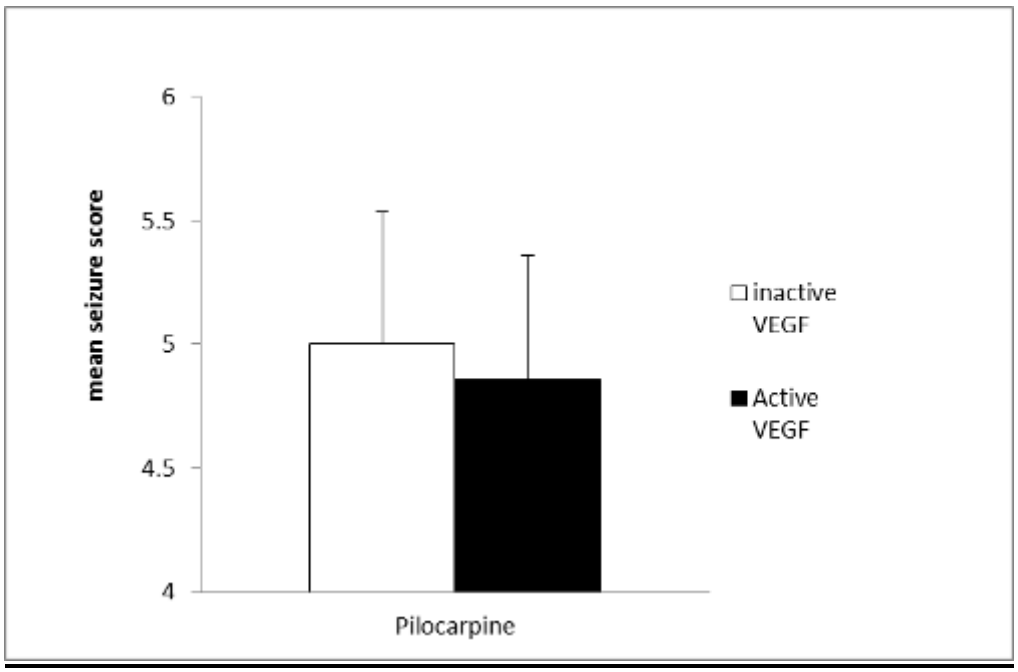

Figure 12: Mean seizure score
Graph illustrates no statistically significant difference between treatment groups on mean seizure score, $p>0.05$, error bars are SEM

Specific Aim 4

To examine the effect of VEGF on glucose transport, including glucose uptake via transporters in the hippocampus after status epilepticus

During status epilepticus, there may be an increased need by neurons for oxygen and glucose, which could result in a relative hypoxic state. This hypoxic state may lead to an increase in the amount of glucose uptake by astrocytic end-feet, which may play a role in regulation of energy supply and can result in increased metabolic load and increased vasculature.

VEGF is upregulated in neurons during status epilepticus and can be induced in response to metabolic stress. VEGF has been shown to reduce neuronal excitability (McCloskey et al., 2005) and may reduce the need by neurons for increased oxygen and glucose. While this response is likely to occur during the initial seizure event, there may be a carryover effect that can be observed long-term if VEGF was able to eliminate the initial metabolic increase via glucose transporters. If VEGF reduces metabolic load during status epilepticus then there may be a reduction in the need for increased oxygen and glucose and a reduction in the amount of glucose uptake by glucose transporters. A change in glucose transporter expression and the reduction in glucose uptake as a result of VEGF's effect may carryover long-term if the metabolic increase was eliminated during the initial seizure event.

Experiment 1

Glucose Transporter-1

We chose to examine glucose transporter 1 as Glut-1 is predominantly localized in astrocytes and astrocytes may play a role in the regulation of energy supply to neurons. To determine the effect of VEGF on glucose transporter-1 expression the experiment consisted of 4 groups as shown in the table below.

	VEGF	Inactive VEGF
Pilocarpine	N=9	N=9
Saline	N=6	N=6

Tissue was stained for anti-GLUT-1 for glucose transporter analysis in the CA1 and DG regions of the hippocampus. Measurements were taken at random points across layers in CA1 and DG regions.

As to whether there is an increase in glucose transporter-1 expression after status epilepticus, data revealed a statistically significant difference in glucose transporter expression in the hippocampus of animals receiving pilocarpine-induced status epilepticus than animals receiving saline one month after initial induction (group effect, $F=16.332$, $p<0.05$, see Figure 13). Therefore, there was a significantly greater amount of glucose transporter-1 expression in the hippocampus one month after status epilepticus.

As to the effect of VEGF on glucose transporter-1 expression, data revealed a statistically significant difference in glucose transporter expression one month after status epilepticus in animals receiving inactive VEGF treatment than VEGF treatment (treatment effect, $F=13.196$, $p<0.05$, see Figure 14). Therefore, there was a significantly greater amount of glucose transporter-1 expression in the hippocampus one month after status epilepticus in the inactive VEGF treatment animals than VEGF treatment animals.

There was also a statistically significant group (pilocarpine vs. saline) by treatment (VEGF vs. inactive VEGF) interaction with a statistically significant greater amount of glucose

transporter expression in the inactive VEGF treatment pilocarpine group than the VEGF treatment saline group (group x treatment effect, $F=20.139$, $p<0.05$, see Figure 14).

Data also revealed significantly greater glucose transporter-1 expression the CA1 region of the hippocampus than the DG region (region, $F=4.870$, $p<0.05$).

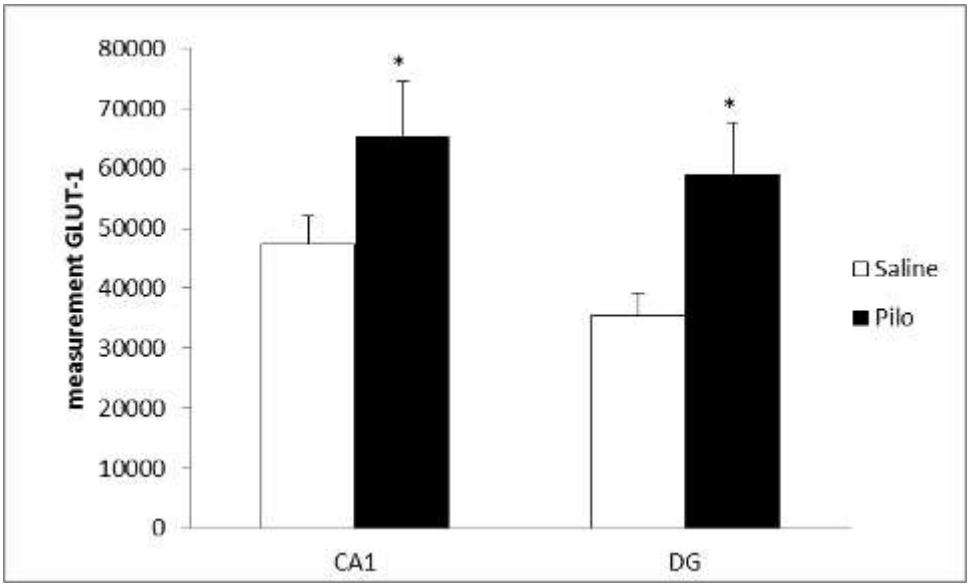

Figure 13: Glucose transporter-1 expression one month after status epilepticus
Graph illustrates a significantly greater amount of glucose transporter expression in the pilocarpine-induced status epilepticus group. * Statistically significant difference from saline group ($p<0.05$), error bars are SEM

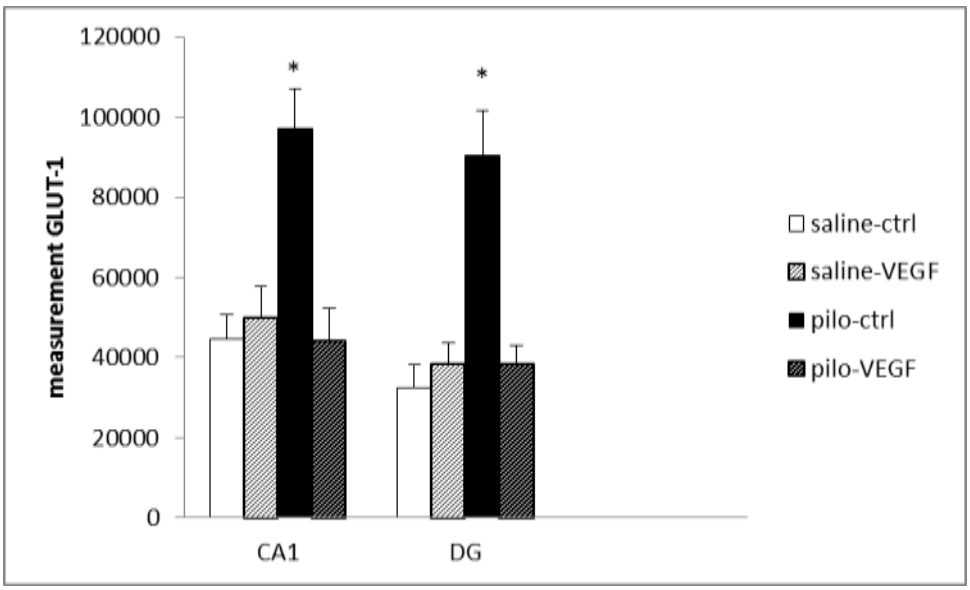

Figure 14: Effect of VEGF on glucose transporter-1 expression one month after status epilepticus
Graph illustrates significantly greater amount of glucose transporter expression in the inactive VEGF treatment group than the VEGF treatment group, * statistically significant from VEGF group, $p<0.05$ There was also a statistically significant group (pilocarpine vs. saline) by treatment (VEGF vs. inactive VEGF) interaction with a statistically significant greater amount of glucose transporter expression in the inactive VEGF treatment pilocarpine group than the VEGF treatment saline group, * statistically significant from VEGF saline group ($p<0.05$), error bars are SEM

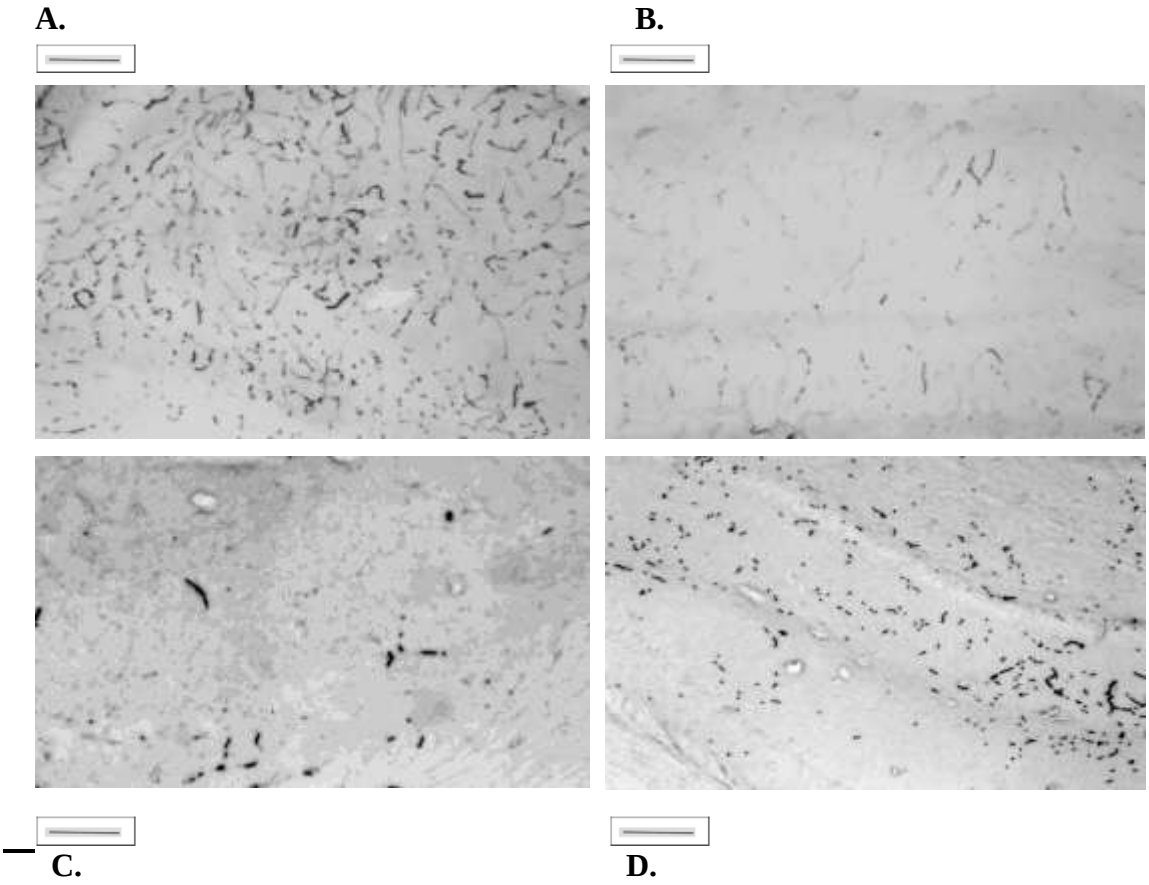

Figure 15: GLUT-1 transporter expression in the CA1 region one month after saline or pilocarpine-induced status epilepticus with VEGF or inactive VEGF treatment
Sections from an inactive VEGF-treated **(A)** rat one month after status epilepticus and a VEGF-treated (**B**) rat one month after status epilepticus. Controls were inactive VEGF-treated (**C**) with saline and VEGF-treated (**D**) with saline. All were immunostained with anti-GLUT-1. Significantly greater glucose transporter expression is seen in the inactive VEGF treatment group than the VEGF treatment group. Scale bar = 100μm

We normalized for blood vessel density using Glut-1. Data revealed a statistically significant group (pilocarpine vs. saline) by treatment (VEGF vs. inactive VEGF) interaction with the pilocarpine inactive VEGF group having a greater mean density than the pilocarpine VEGF group. (group x treatment effect, $F=7.890$, $p<0.05$, See Figure 16).

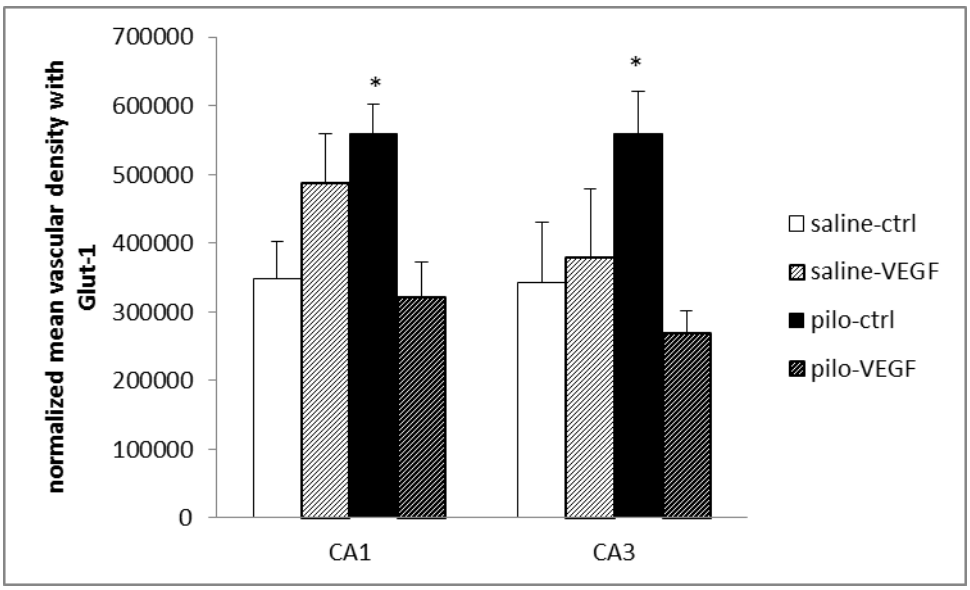

Figure 16: Normalized density with Glut-1
Graph illustrates significantly greater amount of density in the inactive VEGF treatment pilocarpine group than the active VEGF treatment pilocarpine group, * statistically significant from VEGF group, $p<0.05$, error bars are SEM

Experiment 3

Glucose Transporter-4

We chose to examine glucose transporter-4 to confirm if any changes observed in glucose transporter-1 expression would also be observed in other glucose transporters in the brain. Any changes in Glut-4 expression would also help determine that any effects observed in transporter expression would not be attributed solely to the blood brain barrier. To examine the effect of VEGF on glucose transporter-4 expression the experiment consisted of 4 groups as shown in the table below.

	VEGF	Inactive VEGF
Pilocarpine	N=5	N=4
Saline	N=5	N=6

Tissue was stained for anti-GLUT-4 for glucose transporter analysis in the CA1 and DG regions of the hippocampus. Measurements were taken at random points across layers in CA1 and DG regions.

As to whether there is an increase in glucose transporter-4 expression after status epilepticus, data revealed a trend toward significance in glucose transporter expression in the hippocampus. This suggests a slightly greater amount of glucose transporter expression in animals receiving pilocarpine-induced status epilepticus than animals receiving saline one month after initial induction (group effect, $F=3.35$, $p=0.086$).

As to the effect of VEGF on glucose transporter-4 expression, data revealed a statistically significant difference in glucose transporter expression one month after status epilepticus in animals receiving inactive VEGF treatment than VEGF treatment (treatment effect, $F=13.46$, $p<0.05$, see Figure 17). Therefore, there was a significantly greater amount of glucose transporter-4 expression in the hippocampus one month after status epilepticus in the inactive VEGF treatment animals than VEGF treatment animals.

There was also a trend toward significance in the group (pilocarpine vs. saline) by treatment (VEGF vs. inactive VEGF) interaction. This suggests a slightly greater amount of glucose transporter expression in the inactive VEGF treatment pilocarpine group than the VEGF treatment saline group (group x treatment effect, $F=4.46$, $p=0.051$).

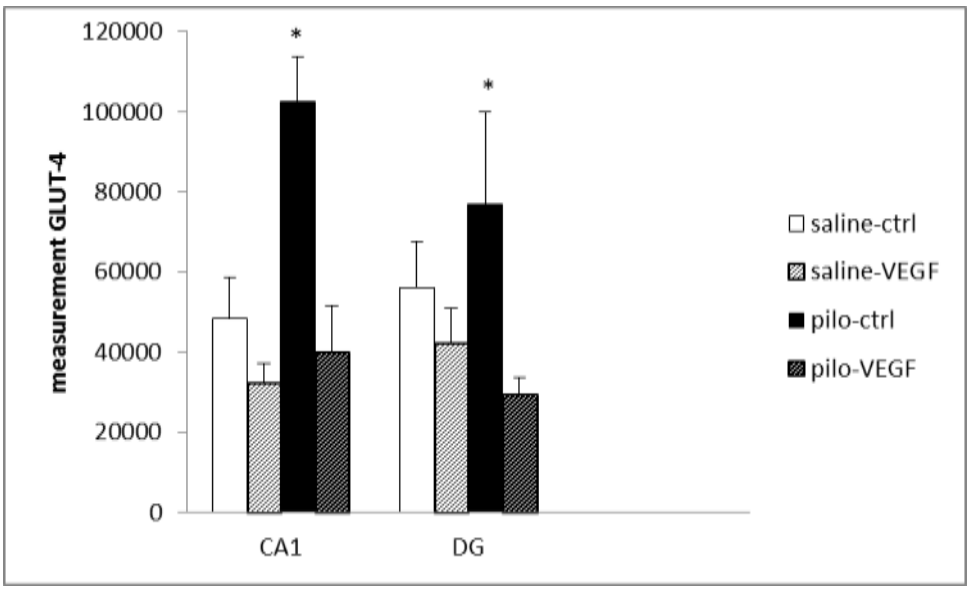

Figure 17: Effect of VEGF on glucose transporter-4 expression one month after status epilepticus
Graph illustrates significantly greater amount of glucose transporter expression in the inactive VEGF treatment group than the VEGF treatment group, * statistically significant from VEGF group ($p<0.05$), error bars are SEM

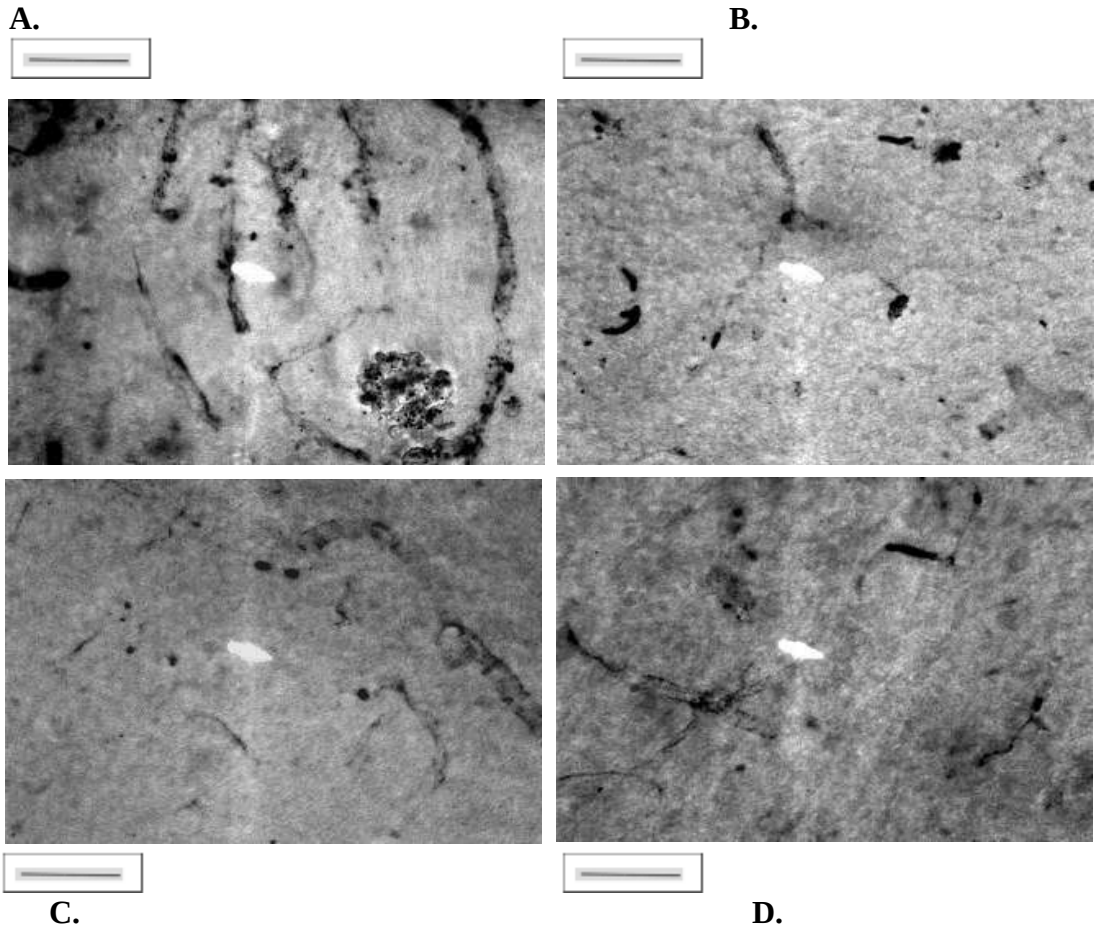

Figure 18: GLUT-4 transporter expression in the CA1 region one month after saline or pilocarpine-induced status epilepticus with VEGF or inactive VEGF treatment
Sections from an inactive VEGF-treated **(A)** rat one month after status epilepticus and a VEGF-treated (**B**) rat one month after status epilepticus. Controls were inactive VEGF-treated (**C**) with saline and VEGF-treated (**D**) with saline. All were immunostained with anti-GLUT-4. Significantly greater glucose transporter expression is seen in the inactive VEGF treatment group than the VEGF treatment group. Scale bar = 50μm

We normalized for blood vessel density using Glut-4. Data revealed a no statistically significant results for group (pilocarpine vs. saline) or treatment (VEGF vs. inactive VEGF). There were no significant interaction effects. ($p>0.05$, See Figure 19).

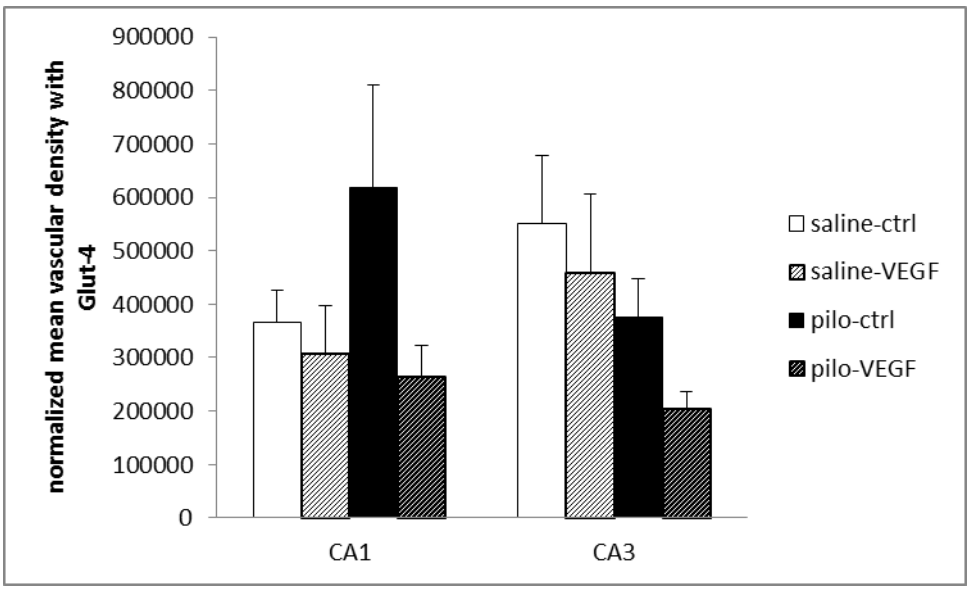

Figure 19: Normalized density with Glut-4
Graph illustrates no significant difference in density in the pilocarpine x saline groups or active VEGF x inactive VEGF groups, $p>0.05$, error bars are SEM

Experiment 3: Glucose Uptake

We chose to explore glucose uptake to verify the functionality of the glucose transporters. To explore the effect of VEGF on glucose uptake, the experiment consisted of 4 groups as shown in the table below.

	VEGF	Inactive VEGF
Pilocarpine	N=3	N=3
Saline	N=3	N=2

Animals were injected with 2-NBDG 90 minutes prior to perfusion fixed tissue collection. Tissue was sectioned and mounted within one week of perfusion fixed tissue collection for fluorescence analysis as previously described (See General Methods).

Measurements were taken at random points across layers in CA1, CA3, and DG regions of the hippocampus.

As to whether there is an increase in glucose uptake after status epilepticus, data revealed a statistically significant difference in glucose uptake in the hippocampus of animals receiving pilocarpine-induced status epilepticus than animals receiving saline one month after initial induction (group effect, $F=16.635$, $p<0.05$, see Figure 20). Therefore, there was a greater amount of glucose uptake in the hippocampus one month after status epilepticus.

As to the effect of VEGF on glucose uptake, data revealed no statistically significant difference in glucose uptake one month after status epilepticus in animals receiving either VEGF treatment or inactive VEGF treatment (treatment group, $F=0.355$, $p=0.57$, see Figure 21). Therefore, while there was a greater amount of glucose uptake in the hippocampus one month after status epilepticus there was no significant change with VEGF added.

There was a significant treatment (VEGF vs. inactive VEGF) by group (saline vs. pilocarpine) interaction with a statistically significant greater amount of glucose uptake in the inactive VEGF treatment pilocarpine group than the VEGF treatment saline group (group x treatment interaction, $F=6.668$, $p<0.05$, see Figure 21).

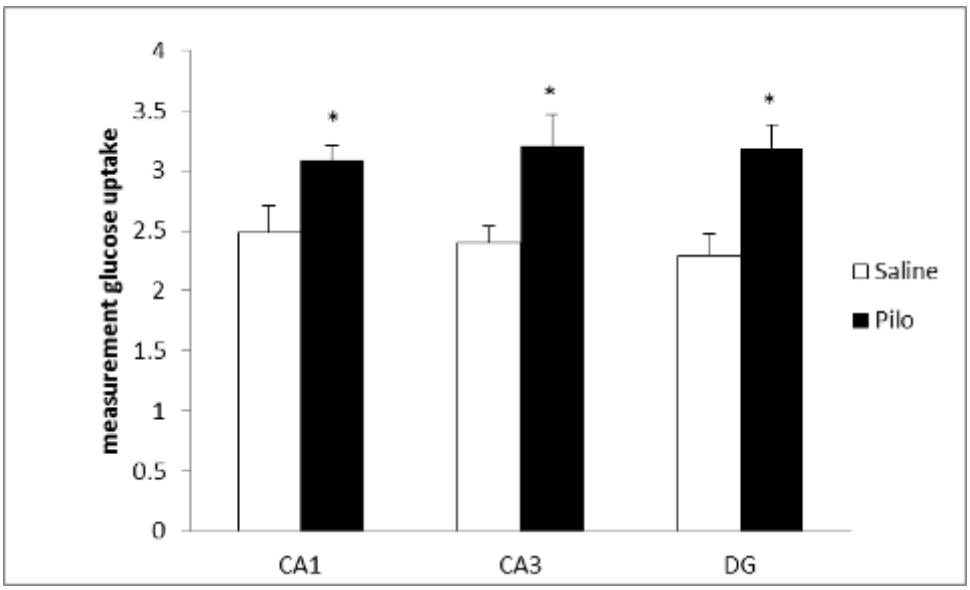

Figure 20: Glucose uptake one month after status epilepticus
Graph illustrates a significantly greater amount of glucose uptake in the pilocarpine-induced status epilepticus group. * Statistically significant difference from saline group ($p<0.05$), error bars are SEM

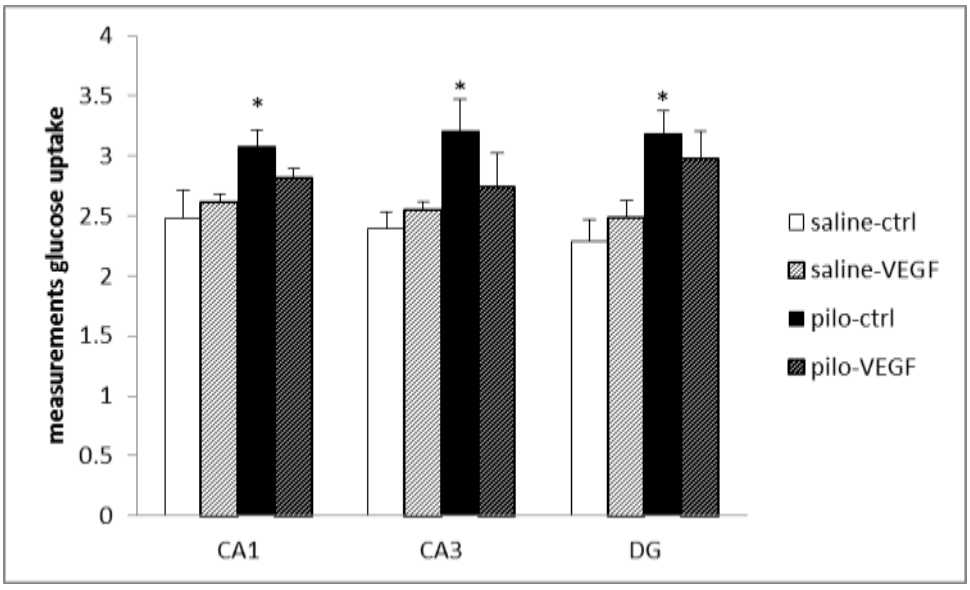

Figure 21: Effect of VEGF on glucose uptake one month after status epilepticus
Graph illustrates significantly greater amount of glucose uptake in the inactive VEGF treatment pilocarpine group than the VEGF treatment saline group ($p<0.05$), error bars are SEM

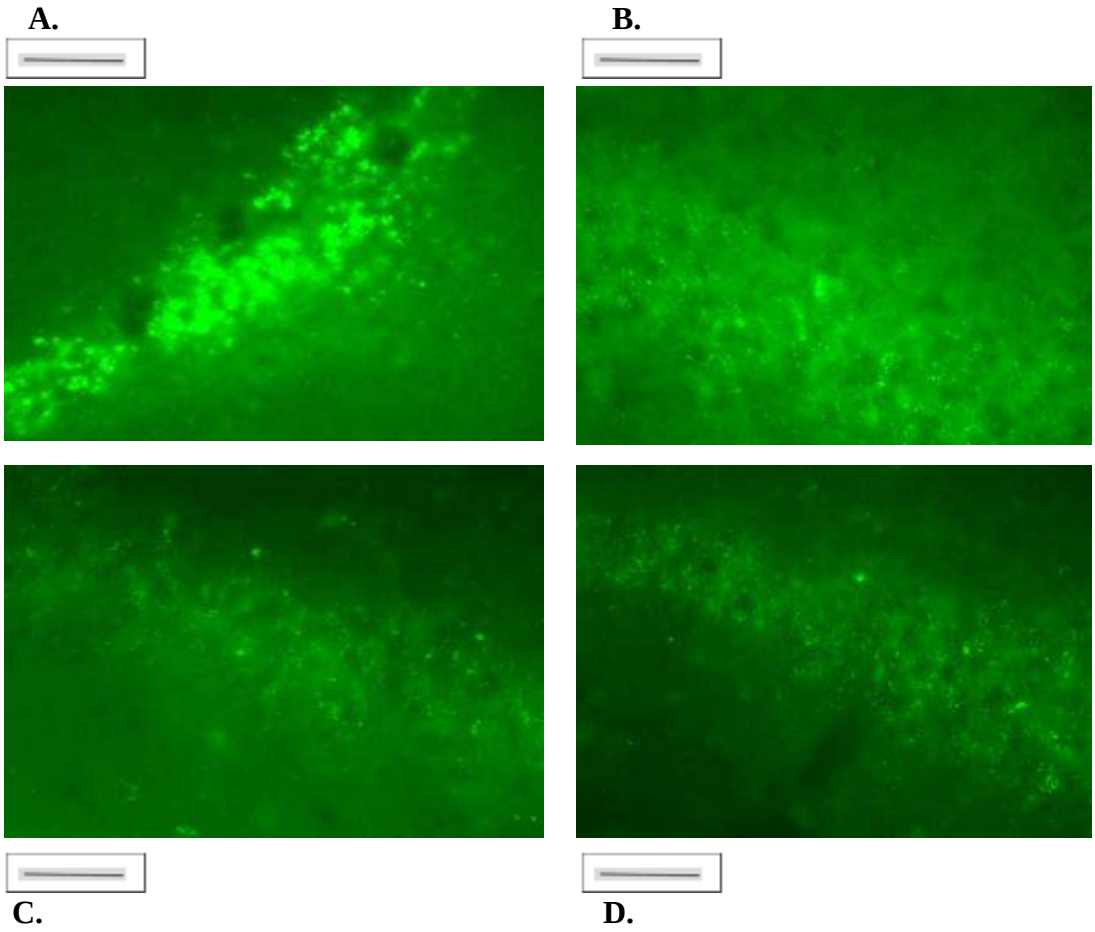

Figure 22: Glucose uptake in CA1 region one month after status epilepticus
Sections from an inactive VEGF-treated **(A)** rat one month after status epilepticus and a VEGF-treated (**B**) rat one month after status epilepticus. Controls were inactive VEGF-treated (**C**) with saline and VEGF-treated (**D**) with saline. All injected with 2-NBDG fluorescent glucose marker. Significantly greater glucose uptake is seen in the inactive VEGF-treated pilocarpine group. Scale bar = 50µm

Chapter 4

Discussion

Overview

Vascular morphological changes, angiogenesis, inflammation, and blood brain barrier damage may contribute to epileptogenesis in both experimental and human epilepsy and these changes may be pathogenic. Vessels may increase in density and size, vessels may become more tortuous, and there may be an increase in vascular permeability. Our results have shown significant changes in vascular density in both human epilepsy and in animals assessed one month after status epilepticus. Growth factors and cytokines that are known to act on vascular cells, such as vascular endothelial growth factor, may play a role in these vascular changes in epilepsy.

In epilepsy, vascular endothelial growth factor (VEGF) may alter vascular parameters with the potential to impact brain function. VEGF has also been shown to protect neurons from cell death. In epilepsy, our results have shown a lower mean vascular density in experimental groups receiving active VEGF than experimental groups receiving inactive VEGF. This might seem to be counterintuitive given VEGF's well-known role as a pro-angiogenic factor, but given that VEGF receptors are dramatically upregulated on neurons and glial cells after epilepsy (Nicoletti et al., 2008), VEGF's effects could be more potent on these cell types than on vasculature in this context. That is, the neural cells could become a "sink" for VEGF binding in the context of epilepsy, so that parameters of the vasculature could be more driven by neural activity than direct effects of VEGF on vascular VEGF receptors. One component of VEGF's neuroprotective action on neural cells could be in decreasing electrical or metabolic activity during or after seizures, as suggested by ex vivo work (McCloskey et al., 2005).

During epileptic events the brain becomes more active and as a result requires more energy. Changes in glucose metabolism or glucose transport may occur because neurons require more glucose and oxygen as well as more blood supply when metabolic activity is increased. Our results have shown a significantly greater mean glucose uptake and glucose transporter-1 expression in pilocarpine-treated experimental groups (seized groups) than in saline-treated controls. Results have also shown greater mean glucose transporter-1 and glucose transporter-4 expression in experimental groups receiving inactive VEGF than in experimental groups receiving active VEGF.

Vasculature after status epilepticus

We found that there was a significantly greater mean density of blood vessels in the hippocampus one month after status epilepticus relative to in normal animals, and also in CA3 of human TLE patients relative to human controls. These results are consistent with literature that has shown high blood vessel density associated with high seizure frequency and an increase in microvessel density in rats that developed spontaneous seizures (Rigau et al., 2007; Marcon et al., 2009). Measurements of vascular diameter were used to elucidate whether changes in vascular density were caused by increased vascular size or increased number of blood vessels in our seized rats. We found no significant change in vascular diameter, suggesting that there were more blood vessels present in the hippocampus rather than larger blood vessels. Although we found no significant change in vascular diameter, previous research (Ndode-Ekane et al., 2010) has found significant vessel length increase 2 weeks post status epilepticus. This suggests there are longer term morphological changes including density and diameter occurring.

A greater number of blood vessels present after status epilepticus may be due to an angiogenic process designed to attract more blood vessels, or possibly make blood vessels more

tortuous. This process might begin at the initial insult of seizure activity and then be sustained throughout the one month period after the initial insult, particularly given that earlier work from our laboratory showed increased vascular density in the first week after status epilepticus (Nicoletti et al., 2008). Therefore, the greater vessel density we observed one month after status could be the result of chronic angiogenesis; however, there is no direct evidence to support this idea. One alternative explanation is that the vessels formed in the first week after status matured and stabilized, becoming relatively permanent. Another alternate explanation is de novo angiogenesis. Spontaneous seizure activity has been reported to begin to occur in the rat model of epilepsy one month after initial insult. Therefore, a resulting increase in vessel density may be due to an angiogenic process that is activated by new spontaneous seizures, and the vessels developed during this process would therefore be new. If spontaneous epileptic activity is leading to an increase in metabolic need and hyperexcitability in the neurons and other cells of the brain, then a greater vascular supply would be needed and the angiogenic process would occur in order to attract more blood vessels to deliver additional glucose and oxygen to these overactive brain regions.

The greater amount of vascular density observed could be attributable to hippocampal shrinkage. A loss of hippocampal volume could lead to an observation of a greater amount of vessels present in a given area. However, it seems unlikely vessel density is the result of hippocampal shrinkage. Nicoletti et al., 2008 showed a reduction in neuronal cells 24 hours after seizure induction compared to controls with hippocampal volume between both groups remaining equal. Nicoletti et al., 2010 also showed no significant difference in hippocampal volume between status epilepticus control and status epilepticus VEGF groups at one month.

The one month time point is the same time point we measured, therefore, it has already been shown there is no change in hippocampal volume at the one month time point.

While vascular changes have been reported in epileptic rats, literature has also shown vascular changes in human temporal lobe epilepsy tissue. Literature has shown greater blood vessel density in human temporal lobe epilepsy hippocampal tissue compared to that of non-epileptic hippocampal tissue (Rigau et al., 2007). In our examination of human temporal lobe epilepsy tissue, we were able to confirm this increase in blood vessel density, albeit only at a statistically significant level in the CA3 region. Unlike the rat tissue used which was in the early stages of epilepsy, the only human tissue available was from individuals with severe refractive chronic temporal lobe epilepsy, making direct comparisons between our rat and human tissue difficult.

VEGF's effect on vasculature after status epilepticus

VEGF has been shown to protect neurons 24 hours after initial seizure insult (Nicoletti et al., 2008), however, this neuroprotection was not sustained one month after the initial insult (Nicoletti et al., 2010). Behavioral function has been shown to be preserved long term by VEGF after seizures (Nicoletti et al., 2010). As for vascular changes, 24 hours after initial seizure insult VEGF treatment has not been shown to induce any changes in vascular density as reported by Nicoletti et al. (2008). This does not rule out the possibility that VEGF does have an effect on vascular parameters long term.

The reduced mean vascular density for the VEGF group that we observed one month after status could be the result of VEGF's neuroprotective effect, as VEGF has been shown to protect hippocampal neurons against glutamate excitotoxicity (Matsuzaki et al., 2001). VEGF has also been shown to significantly decrease circuit excitability when applied to hippocampal

slices suggesting that VEGF could be decreasing electrical activity during or after seizures (McCloskey et al., 2005). Decreased electrical activity is likely to translate to decreased metabolic activity, thereby leading to a decreased need for oxygen and glucose and therefore less need for more blood vessels.

VEGF is a potent angiogenic factor (Carmeliet & Storkebaum, 2002), therefore, its infusion would be expected to induce an increase in vascular density or diameter in the hippocampus. However, in the current study exogenous VEGF was no longer present one month after status epilepticus. Rather, it had only been present for five days before and a week and a half after status. Therefore, acute effects normally associated with VEGF would not have been expected in our experiment. Of course, the lack of effect of VEGF on vascular density reported 24 hours after status epilepticus when exogenous VEGF was still present suggests that increased vascular density is not necessarily a given after administration of exogenous VEGF, particularly at the sub-angiogenic doses defined in earlier work (Croll et al., 2004) and then used in our experiments (see Nicoletti et al., 2008). Although we know that VEGF receptors were increased on neurons and glia in the first few days after seizures, it's possible that this "sink" for VEGF could have been present throughout our entire infusion period. Additional experiments would be needed to further assess this possibility.

Although the acute neuroprotective effects of VEGF do not last over time, the report of sustained functional improvement by VEGF is intriguing (Nicoletti et al., 2010). It is unclear if the early protective changes as a result of VEGF treatment are only on neurons or are also on vasculature or vascular changes. It seems more likely that the entire hippocampal system is involved and that interaction between systems (i.e. between neurons, vasculature, glia, etc.) could be involved in the effects of VEGF over time. In terms of vasculature, one could propose

that the effect of early VEGF treatment in reducing the number of vessels long-term may result if VEGF initially quiets the system, thereby preventing post-status plasticity and the resultant development of recurrent excitation. That is, VEGF may have the effect of quieting neuronal excitability and preventing recurrent excitation over time, perhaps preventing the development of the chronic epileptic state. There is, however, no evidence that VEGF alters the severity of the initial status epilepticus insult. It would be of great value if treatment with VEGF during the latent period following an acute, initiating seizure event could prevent the establishment of all changes leading to or associated with the development of chronic epilepsy. More work will need to be done to determine if this possibility is indeed a major contributor to our results.

Glucose transport

If VEGF is suppressing metabolic activity during or after status epilepticus, and therefore preventing seizure-induced increases in vascular density, one might expect a corresponding dampening effect on glucose uptake and glucose transport. Increased vasculature as a result of seizures may be supporting an increase in glucose utilization that VEGF can then prevent.

We examined glucose transporter expression and glucose uptake one month after status epilepticus. We found a significantly greater amount of glucose transporter-1 (Glut-1) expression in the hippocampus one month after pilocarpine-induced status epilepticus relative to one month after control injections of saline. We also found a statistical trend toward a greater amount of glucose transporter-4 (Glut-4) expression in the hippocampus one month after status epilepticus. These findings support the idea that increased metabolic activity as a result of status epilepticus results in an increase in glucose transporter expression. This interpretation is also supported by our finding of a significantly greater amount of glucose uptake into the hippocampus one month after status epilepticus compared to one month after saline injection.

This finding suggests that the increase in glucose transporter expression is present to mediate an increase of glucose to the more metabolically active seized hippocampus.

It is possible the increase in hippocampal glucose uptake could be attributed to increases in blood perfusion through vasculature, perhaps due to changes in vascular tortuosity. We examined tissue histologically for evidence of poor tissue perfusion (eg. trapped red blood cells, etc.), and found when visually observed, that there were no differences between the treatment groups in poor perfusion-related findings such as pooled red blood cells

When looking at VEGF treatment, we also found a significantly lower amount of glucose transporter-1 and glucose transporter-4 expression in the VEGF group compared to the animals treated with inactivated VEGF as a control. This supports the idea that increased VEGF during and shortly after status epilepticus led to a quieting of metabolic activity resulting in less transporter expression long-term.

It is possible, of course, that VEGF suppressed actual baseline metabolic activity necessary for normal neuronal function. However, there would likely be hypoxic damage as a result of VEGF causing decreases in glucose levels below that necessary for normal activity to occur. If VEGF directly suppresses the metabolic activity of the epileptic hippocampus it could explain why the ketogenic diet works in quieting seizure activity. We would have expected to see a decrease in the active VEGF saline group if exogenous VEGF alone suppressed baseline metabolic activity, but our data did not show a decrease in this group. However, we do not have data to demonstrate whether or not VEGF acutely decreases the metabolic activity of the epileptic hippocampus, because our animals no longer had VEGF treatment at the time points we studied. Future work studying acute effects of VEGF on chronic epilepsy, rather than the effects of early VEGF on later epilepsy, would be necessary to answer this question.

Conclusions

Our finding of lower vascular density in the hippocampus of post-status animals who had received early VEGF treatment versus those who hadn't suggests that VEGF may alter vascular parameters, and these changes can be observed even when exogenous VEGF is no longer present. VEGF could have played a role in reducing the severity of the initial status epilepticus, however, this was not observed behaviorally. There are, perhaps, differences in electrical activity between animals receiving active VEGF and animals receiving inactive VEGF during initial status epilepticus, which in future research could be measured using EEG technology. McCloskey et al. (2005) demonstrated in vitro, VEGF administration to hippocampal slices decreases neuronal excitability of neurons. Nicoletti et al. (2004) observed in vivo; animals treated with VEGF exhibited behaviorally less severe seizures. In our current work it was observed behaviorally that animals treated with VEGF seemed to exhibit less severe seizures long term. EEG could determine if VEGF reduced the severity of the initial electrographic status epileticus event.

Although the exogenous VEGF did not stop or diminish the initial status epilepticus event, as observed behaviorally, it is possible that VEGF prevented long term vascular remodeling, and that this prevention of abnormal vasculature could have contributed to the preservation of function seen long term. While Nicoletti et al. (2010) did not find long term neuronal preservation with VEGF, they did find long term preservation of behavioral function. If VEGF is preventing long term vascular remodeling and preventing eventual epileptogenesis from occurring, then this effect could contribute to why behavioral function is preserved.

We do not know if VEGF is preventing vascular remodeling during the latent phase between the initial status epilepticus event and the period one month later, although our results

support that it may. Future research would be necessary to see if there are any vascular changes with VEGF observed during the period between initial status epilepticus and one month. These results could lend further support to the idea that VEGF is preventing initial vascular remodeling.

Along with possibly preventing vascular remodeling, VEGF may also be decreasing metabolic or electrical activity during or after status epilepticus that could also contribute to long-term preservation of function. Our findings may support this as glucose transport was shown to be greater without VEGF treatment present. Increased glucose transporter expression suggests glucose transporters may be accommodating for an increased need for oxygen and glucose during status epilepticus and an increase in vasculature, which we also observed. There is also evidence to support that increased glucose levels can contribute to an increase in damage during a cerebral insult, specifically in cerebral ischemia. Severe hyperglycemia (>400mg/dl) has been shown to be associated with increased volume of blocked and damaged tissue (Duverger & MacKenzie, 1988). It is possible that increased glucose levels during status epilepticus could be associated with increased damage in hippocampal tissue and that VEGF may prevent eventual damage by preventing the increase in glucose levels. A possible mechanism of VEGF's neuroprotection could be decreasing glucose transporter expression which could in turn decrease metabolic capacity of the tissue, making the region non-permissive for increased metabolic and excitatory activity.

Overall reduction in metabolic or electrical activity by VEGF could contribute in preventing vascular remodeling by reducing the need for more blood vessels. While our current research suggests overall reduction of metabolic activity by VEGF, as supported by our results of glucose transporter expression and glucose uptake levels at one month, it is unclear how soon after the initial status epilepticus event these changes occur. It would be necessary to measure

glucose transporter expression and glucose uptake at varying time points between the initial status epilepticus event and one month in order to lend greater support that VEGF is reducing metabolic activity during the time frame that initial vascular remodeling may be occurring thus possibly preventing epileptogenesis from occurring.

Even if VEGF is not preventing vascular remodeling or preventing epileptogenesis, the current research has shown that early VEGF is still having a long-term effect on vasculature and metabolic activity. The effects of VEGF long term shown here on vasculature and metabolic activity coupled with results from Nicoletti et al. (2010) of long term preservation of behavior lends strong support to the idea that VEGF does have some long term protective effect after status epilepticus. If the vasculature or metabolic system is not the mechanism by which VEGF is generating the protective effects, VEGF is still having an impact long term on these systems. VEGF may be producing its effects by decreasing activity in the overall system of the brain. VEGF may also be directly generating its effects through astrocytes, which through a cascade effect lead to the changes seen in metabolic activity and vasculature. That is, VEGF's effects on metabolism may be indirect.

While it is necessary to determine how long after the initial event these effects start to occur it is also necessary to determine if these effects persist beyond one month, to three months or six months. Not only would it be necessary to determine if the effects persist beyond one month, but it would also be necessary to determine whether VEGF given acutely after an initial insult, unlike what is shown in the current experiments, would produce the same long term effects. This would be crucial to help determine VEGF’s long term efficacy as a potential treatment for chronic epilepsy. If VEGF could prevent long term changes after seizures, it could potentially be used as an anti-epileptogenic, or as an aid to prevent the progression of epilepsy

from a more mild to a more severe form. Future research will be necessary to determine whether VEGF carries promise as an anti-epileptogenic therapeutic.

Chapter 5

REFERENCES

Abraham, C.S., Deli, M.A., Joo, F., Megyeri, P., Torpier, G. (1996). Intracarotid tumor necrosis factor-alpha administration increases the blood–brain barrier permeability in cerebral cortex of the newborn pig:quantitative aspects of double-labelling studies and confocal laser scanning analysis. *Neuroscience Letters, 208*, 85–88.

Abbud W, Habinowski S, Zhang JZ, Kendrew J, Elkairi FS, Kemp BE, Witters LA, Ismail-Beigi F. (2000). Stimulation of AMP-activated protein kinase (AMPK) is associated with enhancement of Glut1-mediated glucose transport. *Arch Biochem Biophys, 380*, 347–35.

Alon, T., et al. (1995). Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat. Med., 1*, 1024–1028.

Allan, S.M., et al. (2005). Interleukin-1 and neuronal injury. *Nat. Rev., Immunol. 5*, 629–640.

Arras, M., Ito, W.D., Scholz, D., Winkler, B., Schaper, J., Schaper, W. (1998). Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J. Clinical Invest., 101*(1), 40-50.

Ates, N., Esen, N., IIbay G. (1999). Absence epilepsy and regional blood-brain-barrier permeability: the effects of pentylenetetrazole-induced convulsions. *Pharmacol Res, 39*(4), 305-310.

Attwell D, Laughlin SB. (2001). An energy budget for signaling in the grey matter of the brain. *J Cereb Blood Flow Metab, 21*, 1133–114.

Babb TL, Brown WJ, Pretorius J, Davenport C, Lieb JP, Crandall PH. (1984). Temporal lobe volumetric cell densities in temporal lobe epilepsy. *Epilepsia, 25*, 729–40.

Baik EJ, Kim EJ, Lee SH, et al. (1999). Cyclooxygenase-2 selective inhibitors aggravate kainic acid induced seizure and neuronal cell death in the hippocampus. *Brain Res, 843*, 118–129.

Bartolomei, F., Khalil, M., Wendling, F., Sontheimer, A., Regis, J., Ranjeva, J. P., Guye, M., & Chauvel, P. (2005). Enthorinal cortex involvement in human mesial temporal lobe epilespy: An electrophysiologic and volumeric study. *Epilepsia, 46*(5), 677-687.

Bauters C, Asahara T, Zheng LP, et al. (1994). Physiological assessment of augmented vascularity induced by VEGF in ischemic rabbit hindlimb. *Am J Physiol , 267*, 1263–1271.

Bear, D., Levin, K., Blumer, D., et al. (1982). Interictal behavior in hospitalized temporal lobe epileptics: Relationship to idiopathic psychiatric syndromes. *Journal of Neurology, Neurosurgery, and Psychiatry, 45*, 481-488.

Beckner ME, et al. (2005) Glycolytic glioma cells with active glycogen synthase are sensitive to PTEN and inhibitors of PI3K and gluconeogenesis. *Lab Invest, 85*, 1457–1470

Becker KJ. (1998). Inflammation and acute stroke. *Curr Opin Neurol , 11*, 45–49.

Bezzi, P., Domercq, M., Brambilla, L., Galli, R., Schols, D., De Clercq, E., Vescovi, A., Bagetta, G., Kollias, G., Meldolesi, J., Volterra, A., 2001.CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. *Nat. Neurosci., 4*, 702–710.

Bjerkvig R, Johansson M, Miletic H, Niclou SP (2009) Cancer stem cells and angiogenesis.

Semin Cancer Biol, 19, 279–284.
Blumer, D. & Benson, D.F. (1975). Personality changes in frontal and temporal lobe lesions. In D.F. Benson & D. Blumer (Eds.), Psychiatric aspects of neurologic disease, New York: Grune & Stratton.
Boado, R.J. (1998a). Molecular biology of brain capillaries. In: Pardridge, W.M. (Ed.), An Introduction to the Blood–Brain Barrier: Methodology and Biology. Cambridge University Press, Cambridge, pp. 151–162.
Boado, J. R. (2001). Amplification of blood–brain barrier GLUT1 glucose transporter gene expression by brain-derived peptide. *Neuroscience Research*, *40*, 337–342.
Bornstein, R.A., & Suga, L.J. (1988). Educational level and neuropsychological performance in healthy elderly subjects. *Developmental Neuropsychology*, *4*, 17-22.
Bouzier-Sore AK, Voisin P, Canioni P, Magistretti PJ, Pellerin L. (2003). Lactate is a preferential oxidative energy substrate over glucose for neurons in culture. *J Cereb Blood Flow Metab, 23*, 1298–1306.
Bragin, A., Engel, J. Jr., Wilson, C.L., Fried, I., Mathern, G.W. (1999). Hippocampal and entorhinal cortex high-frequency oscillations (100—500Hz) in human epileptic brain and in kainic acid treated rats with chronic seizures. *Epilepsia*, *40*(2), 127-137.
Brightman, M.W. (1977). Morphology of blood–brain barrier interfaces. *Exp. Eye Res. (Suppl.), 25*, 1–2.
Carmeliet P, Storkebaum E. (2002). Vascular and neuronal effects of VEGF in the nervous system: Implications for neurological disorders. *Semin Cell Dev Biol, 13*, 39–53.
Cavalheiro, E.A. (1995). The pilocarpine model of epilepsy. *Italian Journal of Neuroscience*, *16*(1-2), 33-37.
Cendes, F., Andermann, F., Gloor, P., et al. (1993). Atrophy of mesial structures in patients with temporal lobe epilepsy: Cause or consequence of repeated seizures? *Annals of Neurology*, *34*, 795-801.
Chabardes S, Kahane P, Minotti L, Tassi L, Grand S, Hoffmann D, et al. (2005). The temporopolar cortex plays a pivotal role in temporal lobe seizures. *Brain, 128*, 1818–31.
Chedotal, A., Del Rio, J.A., Ruiz, M., et al. (1998). Semaphorins III and IV repel hippocampal axons via two distinct receptors. *Development*, *125*, 4313-4323.
Chiarugi V, Magnelli L, Chiarugi A, et al. (1999). Hypoxia induced pivotal tumor angiogenesis control factors including p53, vascular endothelial growth factor and the NFKB-dependent inducible nitric oxide synthase and cyclo-oxygenase-2. *J Cancer Res Clin Oncol, 125*, 525–528.
Chih CP, Lipton P, Roberts Jr EL. (2001). Do active cerebral neurons really use lactate rather than glucose? *Trends Neurosci*, *24*, 573–578.
Chopp M, Li Y, Jiang N, et al. (1996). Antibodies against adhesion molecules reduce apoptosis after transient middle cerebral artery occlusion in rat brain. *J Cereb Blood Flow Metab, 16*, 578–584.
Cobbs, C. S., Chen, J., Greenberg, D.A., Graham, S.H. (1998). Vascular endothelial growth factor expression in transient focal cerebral ischemia in the rat. *Neuroscience Letters*, *249*, 79-82.
Connelly, A., Jackson, G. D., Duncan, J. S., King, D., Gadian, D. G. (1994). Magnetic resonance spectroscopy in temporal lobe epilepsy. *Neurology*, *44*, 1411-1417.
Croll, S. D., Ransohoff, R. M., Cai, N., Zhang, Q., Martin, F. J., Wei, T., Kasselman, L. J.,

Kintner, J., Murphy, A. J., Yancopoulos, G. D., & Wiegand, S. J. (2004a). VEGF-mediated inflammation precedes angiogenesis in adult brain. *Experimental Neurology, 187*, 388-402.

Croll, S. D., Goodman, J. H., & Scharfman, H. E. (2004b). Vascular endothelial growth factor (VEGF) in seizures: A double-edged sword. *Advances in Experimental Medicine and Biology, 548*, 57-68.

Croll S. D., Wiegand SJ. (2001). Vascular growth factors and cerebral ischemia. *Mol Neurobiol, 23*, 121–35.

Croll, S. D., Suri, C., Compton, D. L., Simmons, M.V., Yancopoulos, G. D., Lindsay, R. M., Wiegand, S. J., Rudge, J. S., & Scharfman, H. E. (1999). Brain-derived neurotrophic factor transgenic mice exhibit passive avoidance deficits, increased seizure severity and in vitro hyperexcitability in the hippocampus and entorhinal cortex. *Neuroscience, 93*, 1491-1506.

Cruz F, Villalba M, Garcia-Espinosa MA, Ballesteros P, Bogonez E, Satrustegui J, Cerdan S. (2001). Intracellular compartmentation of pyruvate in primary cultures of cortical neurons as detected by (13)C NMR spectroscopy with multiple (13)C labels. *J Neurosci Res, 66*, 771–781.

Dasheiff, R. M. & McNamara, J. O. (1982). Intradentate colchicine retards the development of amygdala kindling. *Annal of Neurology, 11*(4), 347-352.

Dautry, C., Vaufrey, F., Brouillet, E., Bizat, N., Henry, P.G., Conde, F., Bloch, G., Hantraye, P. (2000). Early N-acetylaspartate depletion is a marker of neuronal dysfunction in rats and primates chronically treated with mitochondrial toxin 3-nitropropionic acid. *Journal of Cerebral Blood Flow Metabolism, 20*, 789-799.

Dawodu, S., & Thorn, M. (2005). Quantitative neuropathology of the entorhinal cortex region in patients with hippocampal sclerosis and temporal lobe epilepsy. *Epilepsia, 46*(1), 23-30.

DeGraba TJ. (1998). The role of inflammation after acute stroke: Utility of pursuing anti-adhesion molecule therapy. *Neurology, 51*, 62–68.

De Guzman, P., D'Antuono, M., Avoli, M. (2004). Initiation of electrographic seizures by neuronal networks in entorhinal and perirhinal cortices in vitro. *Neuroscience, 123*(4), 875-886.

De Simoni MG, Perego C, Ravizza T, et al. (2000). Inflammatory cytokines and related genes are induced in the rat hippocampus by limbic status epilepticus. *Eur J Neurosci, 12*, 2623–2633.

De Stefano, N., Matthews, P.M., Arnold, D.L. (1995). Reversible decrease in N-acetylaspartate after acute brain injury. *Magnetic Resonance Medicine, 34*, 721-727.

De Vries, H.E., Blom-Roosemalen, M.C., van Oosten, M., de Boer, A.G., van Berkel, T.J., Breimer, D.D., Kuiper, J. (1996). The influence of cytokines on the integrity of the blood–brain barrier in vitro. *J. Neuroimmunol, 64*, 37–43.

Del Maschio, A., Zanetti, A., Corada, M., Rival, Y., Ruco, L., Lampugnani, M.G., Dejana, E., 1996. Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. *J. Cell Biol, 135*, 451–497.

Del Zoppo GJ, Wagner S, Tagaya M. (1997). Trends and future developments in the pharmacological treatment of acute ischaemic stroke. *Drugs, 54*,9–38.

Dinarello, C.A. (1996). Biologic basis for interleukin-1 in disease. Blood, 87, 2095–214.

Dodrill, C.B. (1980). Neuropsychological evaluation in epilepsy. In J.S. Lockard & A.A. Ward,

Jr. (Eds.), Epilepsy: A window to brain mechanisms. New York: Raven Press.
Dombrowski, S. M., Deshpande, A., Dingwall, C., Leichliter, A., Leibson, Z., & Luciano, M. G. (2008). Chronic hydrocephalus-induced hypoxia: Increased expression of vegfr-2 and blood vessel density in hippocampus. *Neuroscience, 152*, 346-359.
Dombrowski SM, Schenk S, Leichliter A, Leibson Z, Fukamachi K, Luciano MG (2006) Chronic hydrocephalus-induced changes in cerebral blood flow: mediation through cardiac effects. *J Cereb Blood Flow Metab, 26*,1298 –1310.
Draznin B, Sussman K, Kao M, Lewis D, Sherman N. (1987). The existence of an optimal range of cytosolic free calcium for insulin-stimulated glucose transport in rat adipocytes. *J Biol Chem, 262*,14385–1438.
Du F, Eid T, Lothman EW, Kohler C, Schwarcz R. (1995). Preferential neuronal loss in layer III of the medial entorhinal cortex in rat models of temporal lobe epilepsy. *J Neurosci, 15*, 6301–13.
Du, F., Whetsell, W.O. Jr., Abou-Khalil, B., Blumenkopf, B., Lothman, E.W., Schwarcz, R. (1993). Preferential neuronal loss in layer III of the entorhinal cortex in patients with temporal lobe epilepsy. *Epilepsy Research, 16*(3), 223-233.
Dubé, C., Vezzani, A., Behrens, M., Bartfai, T., Baram, T.Z., (2005). Interleukin-1beta contributes to the generation of experimental febrile seizures. *Annals of Neurology, 57*, 152–155.
Duelli R, Maurer MH, Staudt R, Sokoloff L, Kuschinsky W (2001) Correlation between local glucose transporter densities and local 3-Omethylglucose transport in rat brain. *Neuroscience Letters, 310*, 101–104.
Duncan, D.A. (1993). The neuropathology of temporal lobe epilepsy. Journal of *Neuropathological Experimental Neurology, 52*, 433-443.
El Awad B, Kreft B, Wolber EM, et al. (2000). Hypoxia and interleukin-1 stimulate vascular endothelial growth factor production in human proximal tubular cells. *Kidney Intl, 58*, 43–50.
Eriksson, C., Van Dam, A.M., Lucassen, P.J., Bol, J.G., Winblad, B.,Schultzberg, M., 1999. Immunohistochemical localization of interleukin-1beta, interleukin-1 receptor antagonist and interleukin-1beta converting enzyme/caspase-1 in the rat brain after peripheral administration of kainic acid. *Neuroscience, 93*, 9915–9930.
Fabene, P.F., et al. (2003). Magnetic resonance imaging of changes elicited by status epilepticus in the rat brain: diffusion-weighted and T2-weighted images, regional blood volume maps, and direct correlation with tissue and cell damage. *NeuroImage, 18*, 375–389.
Ferrara N, Davis-Smyth T. (1997). The biology of vascular endothelial growth factor. *Endocr Rev, 18*, 4–25.
Ferrara N, Gerber HP. (2001). The role of vascular endothelial growth factor in angiogenesis. *Acta Haematol, 106*, 148 –156.
Ferriero DM. (2004). Neonatal brain injury. *N Engl J Med, 351*, 1985–95.
Ferriero, D. M. (2005). Protecting neurons. *Epilepsia, 46*(7), 45-51.
Feuerstein GZ, Wang X, Barone FC. (1997). Inflammatory gene expression in cerebral ischemia and trauma. Potential new therapeutic targets. *Ann NY Acad Sci, 825*, 179–193.
Fischer S, Marti HH. (2002). Hypoxia-induced vascular endothelial growth factor expression causes vascular leakage in the brain. *Brain, 125*, 2549 –2557.
Fowler, P.C., Richards, H.C., Boll, T.J., et al. (1987). A factor model of an extended Halstead

Battery and its relationship to an EEG lateralization index for epileptic adults. *Archives of Clinical Neuropsychology, 2*, 81-92.

Fukuhara T, Luciano MG, Brant CL, Klauscie J. (2001). Effects of ventriculoperitoneal shunt removal on cerebral oxygenation and brain compliance in chronic obstructive hydrocephalus. *J Neurosurg, 94*, 573–58.

Gerber HP, McMurtrey A, Kowalski J, et al. (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *Biol Chem, 273*, 30336–30343.

Gerhart, D.Z., LeVasseur, R.J., Broderius, M.A., Drewes, L.R. (1989). Glucose transporter localization in brain using light and electron immunocytochemistry. *J. Neurosci. Res, 22*, 464–472.

Giulian, D., et al. (1988). Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. *J. Neurosci., 8*, 2485–2490.

Gluzman-Poltorak, Z., Cohen, T., Herzog, Y. et al. (2000). Neuropilin-2 and neuropilin-1 are receptors for the 165-amino acid form of vascular endothelial growth factor (VEGF) and of placenta growth factor-2, but only neuropilin-2 functions as a receptor for the 145-amino acid form of VEGF. *Biological Chemistry, 275*, 18040-18045.

Greenberg, D.A., Jin, K., (2005). From angiogenesis to neuropathology. Nature, 438, 954–959.

Greene, A.E., Todorova, M.T., McGowan, R., Seyfried, T.N. (2001). Calorie restriction inhibits seizure susceptibility in epileptic EL mice by reducing blood glucose. *Epilepsia, 42*(11), 1371-1378.

Hayashi T, Abe K, Itoyama Y. (1998). Reduction of ischemic damage by application of vascular endothelial growth factor in rat brain after transient ischemia. *J Cereb Blood Flow Metab*.

Heil, M., Clauss, M., Suzuki, K., Buschmann, I.R., Willuweit, A., Fischer, S., Schaper, W. (2000). Vascular endothelial growth factor (VEGF) stimulates monocyte migration through endothelial monolayers via increased integrin expression. *European Journal of Cell Biology, 79(*11), 850-857.

Heilman, K.M., Watson, R.T., & Valenstein, E. (1993). Neglect and related disorders. In K.M. Heilman & E. Valenstein (Eds.), Clinical Neuropsychology (3rd ed.). New York: Oxford University Press, 18, 887–895.

Henry TR, Babb TL, Engel J Jr, Mazziotta JC, Phelps ME, Crandall PH. (1994). Hippocampal neuronal loss and regional hypometabolism in temporal lobe epilepsy. *Ann Neurol, 36*, 925–27.

Homan, R.W., Paulman, R.G., Devous, M.D., et al. (1989). Cognitive function and regional cerebral blood flow in partial seizures. *Archives of Neurology, 46*, 964-970.

Hu Y, Wilson GS. (1997). A temporary local energy pool coupled to neuronal activity: fluctuations of extracellular lactate levels in rat brain monitored with rapid-response enzyme-based sensor. *J Neurochem, 69*, 1484–149.

Issa R, Krupinski J, Bujny T, Kumar S, Kaluza J, Kumar P. (1999). Vascular endothelial growth factor and its receptor, KDR, in human brain tissue after ischemic stroke. *Lab Invest, 79*, 417– 425.

Jankowsky, J.L., Patterson, P.H., 2001. The role of cytokines and growth factors in seizures and their sequelae. *Prog. Neurobiol, 63*, 125–14.

Jin K, Mao XO, Batteur SP, et al. (2001). Caspase-3 and the regulation of hypoxic neuronal

death by vascular endothelial growth factor. *Neuroscience, 108,* 351–358.
Jin KL, Mao XO, Greenberg DA. (2000a). Vascular endothelial growth factor rescues HN33 neural cells from death induced by serum withdrawal. *J Mol Neurosci, 14*, 197–203.
Jin KL, Mao XO, Greenberg DA. (2000). Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. *Proc Natl Acad Sci USA, 97*, 10242–10247.
Kann, O., Kovacs, R., Njunting, M., Behrens, C. J., Otahal, J., Lehmann, T. N., Gabriel, S., & Heinemann, U. (2005). Metabolic dysfunction during neuronal activation in the ex vivo hippocampus from chronic epileptic rats and humans. *Brain, 128*(10), 2396-2407.
Kann, O., Schuchmann S., Buchheim, K., Heinemann U. (2003a). Coupling of neuronal activity and mitochondrial metabolism as revealed by NAD(P)H fluorescence signals in organotypic hippocampal slice cultures of the rat. *Neuroscience, 119*, 87-100.
Kann, O., Kovacs, R., Heinemann U. (2003b). Metabotropic receptor-mediated CA^{2+} signaling elevates mitochondrial stimulates CA^{2+} and stimulates oxidative metabolism in hippocampal slice cultures. *Journal of Neurophysiology, 90*, 613-621.
Kasselman LJ, Ransohoff RM, Cai N, et al. Vascular endothelial growth factor (VEGF)-mediated inflammation precedes angiogenesis in adult rat brain. Soc Nsci Abstr. 2002.
Kawakami, A., Kitsukawa, T., Takagi, S., et al. (1996). Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. *Journal of Neurobiology, 29*, 1-17.
Keunen OJ, Oudin M, Sanzey A, Abdul-Rahim M, Fack SA, et al. (2011) Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. *PNAS, 108,* 3749–3754.
Koch-Weser, M., Garron, D.C., Gilley, D.W. (1988). Prevalence of psychologic disorders and surgical treatment of seizures. *Archives of Neurology, 45*, 1308-1311.
Krum JM, Mani N, Rosenstein JM. (2002). Angiogenic and astroglial responses to vascular endothelial growth factor administration in adult rat brain. *Neuroscience, 110*, 589–604.
Kuhl DE, Engel J Jr, Phelps ME, Selin C. (1980). Epileptic patterns of local cerebral metabolism and perfusion in humans determined by emission computed tomography of 18FDG and 13NH3. *Ann Neurol, 8*, 348–60.
Lee, S.K., Kim, D.W., Kim, K.K., Chung, C.K., Song, I.C., Chang, K.H. (2005). Effect of seizure on hippocampus in mesial temporal lobe epilepsy and neocortical epilepsy: an MRS study. *Neuroradiology, 47*, 916-923.
Leite, J.P., Bortolotto, Z.A., Cavalheiro, E.A. (1990). Spontaneous recurrent seizures in rats: an experimental model of partial epilepsy. *Neuroscience Biobehavioral Review, 14*(4), 511-517.
Lezak, M.D. (1995). Neuropsychological Assessment (3rd ed.). New York: Oxford University Press.
Li J, Perrella MA, Tsai JC, et al. (1995). Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. *J Biol Chem, 270,* 308–312.
Lipton, P. (1973). Effects of membrane depolarization on nicotinamide nucleotide fluorescence in brain slices. *Biochem J, 136*, 999-1009.
Lishman, W.A. (1987). Organic psychiatry (2nd ed). Oxford: Blackwell.
Loaiza A, Porras OH, Barros LF. (2003). Glutamate triggers rapid glucose transport stimulation in astrocytes as evidenced by real-time confocal microscopy. *J Neurosci, 23*, 7337–7342.

Luciano MG, Skarupa DJ, Booth AM, Wood AS, Brant CL, Gdowski MJ. (2001). Cerebrovascular adaptation in chronic hydrocephalus. *J Cereb Blood Flow Metab, 21*, 285–294.

Luttun, A., Tjwa M., Carmeliet, P. (2002). Placental growth factor (PIGF) and its receptor Flt-1 (VEGFR-1): novel therapeutic targets for angiogenic disorders. *Ann NY Academy of Science, 979*, 80-93.

Magistretti PJ, Pellerin L, Rothman DL, Shulman RG. (1999). Energy on demand. *Science, 283,* 496– 497.

Maldonado, M., Baybis, M., Newman, D., Kolson, D.L., Chen, W., McKhann II, G., Gutmann, D.H., Crino, P.B., 2003. Expression of ICAM-1, TNF-alpha, NF kappa B, and MAP kinase in tubers of the tuberous sclerosis complex. *Neurobiol. Dis, 14*, 279–290.

Marcon, J., Gagliardi, B., Balosso, S., Maroso, M., Noe, F., Morin, M., Lerner-Natoli, M., Vezzani, A., & Ravizza, T. (2009). Age-dependent vascular changes induced by status epilepticus in rat forebrain: Implications for epileptogenesis. *Neurobiology of Disease, 34*, 121-132.

Marti HJ, Bernaudin M, Bellail A, et al. (2000). Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am J Pathol, 156,* 965–976.

Marti HH, Risau W. (1998). Systemic hypoxia changes the organspecific distribution of vascular endothelial growth factor and its receptors. *Proc Natl Acad Sci U S A, 95*, 15809 –15814.

Marti HH, Risau W. (1999). Angiogenesis in ischemic disease. *Thromb Haemost* 82(*Suppl 1),* 44–52.

Matsuzaki H, Tamatani M, Yamaguchi A, et al. (2001). Vascular endothelial growth factor rescues hippocampal neurons from glutamate-induced toxicity: Signal transduction cascades. *FASEB J , 15*, 1218–1220.

Matthews, P.M., Andermann, F., Arnold, D.L. (1990). A proton magnetic resonance spectroscopic study of focal epilepsy in humans. *Neurology, 40,* 985-989.

Mazure NM, Chen EY, Laderoute KR, et al. (1997). Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signlaing pathway in Ha-rastransformed cells through a hypoxia-inducible factor-1 transcriptional element. *Blood, 90*, 3322–3331.

McCloskey, D.P., Croll, S.D., & Scharfman, H.E. (2005). Depression of synaptic transmission by vascular endothelial growth factor in adult rat hippocampus and evidence for increased efficacy after chronic seizures. *Journal of Neuroscience, 25*(39), 8889-8897.

Mello LE, Cavalheiro EA, Tan AM, Kupfer WR, Pretorius JK, Babb TL, et al. (1993). Circuit mechanisms of seizures in the pilocarpine model of chronic epilepsy: cell loss and mossy fiber sprouting. *Epilepsia, 34,* 985–95.

Miettinen, R., Kotti, T., Tuunanen, J., Toppinen, A., Riekkinen, P., Halonen, T. (1998). Hippocampal damage after injection kainic acid into the rat entorhinal cortex. *Brain Research, 813(*1), 9-17.

Mittan, R.J. (1986). Fear of seizures. In S. Wilson & B.P. Hermann (Eds.), Psychopathology in epilepsy. Social dimensions. New York: Oxford University Press.

Mueckler, M. (1994). Facilitative glucose transporters. *Eur. J.Biochem. 219,* 713–720.

Naldini, A., Carraro, F., 2005. Role of inflammatory mediators in angiogenesis. *Curr Drug Targets Inflamm. Allergy 4,* 3–8.

Nagy, J.A., et al. (2008). Vascular permeability, vascular hyperpermeability and angiogenesis. *Angiogenesis 11*, 109–119.

Ndode-Ekane, X. E., Hayward, N., Grohn, O., & Pitkanen, A. (2010). Vascular changes in epilepsy: Functional consequences and association with network plasticity in pilocarpine-induced experimental epilepsy. *Neuroscience, 166*, 312-332.

Neppe, V.M. & Tucker, G.J. (1992). Neuropsychiatric aspects of seizure disorders. In S.C. Yudofsky & R.E. Hales (Eds.), American Psychiatric Press textbook of neuropsychiatry (2nd ed.). Washington, D.C.: American Psychiatric Press.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J, 13*, 9 –22.

Nguyen, M.D., Julien, J.P., Rivest, S., 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration, *Nat. Rev. Neurosci, 3*, 216–227.

Nicoletti, J.N., Lenzer, J., Salerni, E.A., Shah, S.K., Elkady, A., Khalid, S., Quinteros, D., Rotella, F., Betancourth, D., Croll, S.D. (2010). Vascular endothelial growth factor attenuates status epilepticus-induced behavioral impairments in rats. *Epilepsy Behavior. Nov; 19*(3), 272-277.

Nicoletti, J.N., Shah, S.K., McCloskey, D.P., Goodman, J.H., Elkady, A., Atassi, H., Hylton, D., Rudge, J.S., Scharfman, H.E., Croll, S.D. (2008). Vascular endothelial growth factor is up-regulated after status epilepticus and protects against seizure-induced neuronal loss in hippocampus. *Neuroscience. Jan 2, 151*(1), 232-241.

O'Brien TJ, Newton MR, Cook MJ, Berlangieri SU, Kilpatrick C, Morris K et al. (1997). Hippocampal atrophy is not a major determinant of regional hypometabolism in temporal lobe epilepsy. *Epilepsia, 38*, 74–80.

Oosthuyse B, Moons L, Storkebaum E, et al. (2001). Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet, 28*,131–138.

Pages G, Pouyssegur J. (2005). Transcriptional regulation of the Vascular Endothelial Growth Factor gene–a concert of activating factors. *Cardiovasc Res, 65*, 564–73.

Pedram, A., Razandi, M., Levin, E.R. (1998). Extracellular signal-regulated protein kinase/Jun kinase cross-talk underlies vascular endothelial cell growth factor-induced endothelial cell proliferation. *Journal of Biological Chemistry, 273*(41), 26722-26728.

Pellerin, L. (2010). Food for thought: the importance of glucose and other energy substrates for sustaining brain function under varying levels of activity. *Diabetes Metabolism. Oct, 36*(3), 59-63.

Pellerin, L., Bouzier-Sore, A.K., Aubert, A., Serres, S., Merle, M., Costalat, R., Magistretti, P.J. (2007). Activity dependent regulation of energy metabolism by astrocytes: an update. *Glia, Sept 55*(12), 1251-1262.

Pellerin L, Magistretti PJ. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci USA, 91*, 10625–10629.

Pellerin L, Magistretti PJ. (1997). Glutamate uptake stimulates Na+ ,K+- ATPase activity in astrocytes via activation of a distinct subunit highly sensitive to ouabain. *J Neurochem, 69*, 2132–213.

Pincus, J.H. & Tucker, G.J. (1985). Behavioral neurology. (3rd ed.). New York: Oxford

University Press.
Pitkänen A, Sutula TP. (2002). Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. *Lancet Neurol, 1*, 173–81.
Plata-Salaman, C.R., Ilyin, S.E., Turrin, N.P., Gayle, D., Flynn, M.C., Romanovitch, A.E., Kelly, M.E., Bureau, Y., Anisman, H., McIntyre, D.C., 2000. Kindling modulates the IL-1beta system, TNF-alpha, TGF-beta1, and neuropeptide mRNAs in specific brain regions. *Brain Res. Mol Brain Res, 75*, 248–258.
Ploug, T. & Ralston, E. (2002). Exploring the whereabouts of Glut-4 in skeletal muscle (Review). *Molecular Membrane Biology, 19*, 39-49.
Porras, O. H., Loaizi, A., & Barros, L. F. (2004). Glutamate mediates acute glucose transport inhibition in hippocampal neuron. The Journal of Neuroscience, 24(43), 9669-9673.
Proescholdt MA, Heiss JD, Walbridge S, et al. (1999). Vascular endothelial growth factor (VEGF) modulates vascular permeability and inflammation in rat brain. *J Neuropathol Exp Neurol, 58*, 613–627.
Racine, R. J. (1972). Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalography and Clinical Neurophysiology, 32*, 281-294.
Ravizza, T., Boer, K., Redeker, S., Spliet, W.G., van Rijen, P.C., Troost, D., Vezzani, A., Aronica, E.(2006). The IL-1beta system in epilepsyassociated malformations of cortical development. *Neurobiol. Dis, 24*, 128–143.
Ravizza, T., Gagliardi, B., Noe, F., Boer, K., Aronica, E., & Vezzani, A. (2007). Innate and adaptive immunity during epileptogenesis and spontaneous seizures: Evidence from experimental models and human temporal lobe epilepsy. *Neurobiology of Disease, 29*(1), 142-160.
Rigau, V., Morin, M., Rousset, M. C., Bock de, F., Lebrun, A., Coubes, P., Picot, M. C., Baldy-Moulinier, M., Bockaert, J., Crespel, A., & Lerner-Natoli Mireille. (2007). Angiogenesis is associated with blood-brain barrier permeability in temporal lobe epilepsy. *Brain, 130,* 1942-1956.
Roch, C., Leroy, C., Nehlig, A., Namer, I.J. (2002). Magnetic resonance imaging in the study of the lithium-pilocarpine model of temporal lobe epilepsy in adult rats. *Epilepsia, 43*(4), 325-335.
Rosenstein, J.M., Mani, N., Khaibullina, A., Krum, J.M. (2003). Neurotrophic effects of vascular endothelial growth factor on organotypic cortical explants and primary cortical neurons. *Journal of Neuroscience, Dec 3; 23*(35), 11036-11044.
Rosenstein JM, Mani N, Silverman WF, et al. (1998). Patterns of brain angiogenesis after vascular endothelial growth factor administration in vivo and in vivo. *Proc Natl Acad Sci USA, 95*, 7086–7091.
Rudge, J. S., Mather, P. E., Pasnikowski, E. M., Cai, N., Corcorn, T., Acheson, A., Anderson, K., Lindsay, R. M., & Wiegand, S. J. (1998). Endogenous BDNF protein is increased in adult rat hippocampus after a kainic acid induced excitotoxic insult but exogenous BDNF is not neuroprotective. *Experimental Neurology, 149,* 398-410.
Ryuto M, Ono M, Izumi H, et al. (1996). Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1. *J Biol Chem, 271*, 28220–28228.
Sasa, M. (2006). A new frontier in epilepsy: novel antiepiletogenic drugs. *Journal of Pharmacological Science, 100*(5), 487-494.

Scharfman, H.E. (2002). The parahippocampal region in temporal lobe epilepsy. In The Parahippocampal Region: Organization and Role in Cognitive Function. Edited by Witter, M. & Wouterlood, F. Oxford; New York: Oxford University Press, 321-336.

Scharfman, H.E. (2000). Epileptogenesis in the parahippocampal region. Parallels with the dentate gyrus. *Annals of the New York Academy of Sciences, 911*, 305-327.

Schaper, W. & Buschmann, I. (1999). Arteriogenesis, the good and bad of it. *Cardiovascular Research, 43*(4), 835-837.

Scholz, D., Ito, W., Fleming, I., Deindl, E., Sauer, A., Wiesnet, M., Busse, R., Schaper, J., Schaper, W. (2000). Ultrastructure and molecular histology of rabbit hind-limb collateral artery growth (arteriogenesis). *Virchows Arch, 436*(3), 257-270.

Schuchmann, S., Kovacs, R., Kann, O., Heinemann U., Buchheim, K. (2001). Monitoring NAD(P)H autofluorescence to assess mitochondrial metabolic functions in rat hippocampal-entorhinal cortex slices. *Brain Res Brain Res Protoc, 7*, 267-276.

Seiffert, E., Dreier, J.P., Ivens, S., Bechmann, I., Tomkins, O., Heinemann,U., Friedman, A., 2004. Lasting blood–brain barrier disruption induces epileptic focus in the rat somatosensory cortex. *J. Neurosci, 24*, 7829–7836.

Semenza, G.L. (2000). HIF-1: using two hands to flip the angiogenic switch. *Cancer Metastasis Review, 19*(1-2), 59-65.

Shalaby F, Rossant J, Yamaguchi TP, et al. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice, *Nature, 376*, 62–66.

Sheng, J.G., Boop, F.A., Mrak, R.E., Griffin, W.S., 1994. Increased neuronal beta-amyloid precursor protein expression in human temporal lobe epilepsy: association with interleukin-1 alpha immunoreactivity. *J. Neurochem, 63*, 1872–1879.

Sheth, R.A., Josephson, L., Mahmood, U. (2009). Evaluation and clinically relevant applications of a fluorescent imaging analog to fluorodeoxyglucose positron emission tomography. *Journal of Biomedical Optics, 14*(6), 064014-1-064014-7.

Shulman RG, Hyder F, Rothman DL. (2001). Cerebral energetics and the glycogen shunt: neurochemical basis of functional imaging. *Proc Natl Acad Sci USA, 98*, 6417– 642.

Shuttleworth, C.W., Brennan A.M., Connor, J.A. (2003). NAD(P)H fluorescence imaging of postsynaptic neuronal activation in murine hippocampal slices. *Journal of Neuroscience, 23*, 3196-3208.

Silverman WF, Krum JM, Mani N, Rosenstein JM. (1999). Vascular, glial and neuronal effects of vascular endothelial growth factor in mesencephalic explant cultures. *Neuroscience, 90*, 1529–1541.

Sondell M, Lundborg G, Kanje M. (1999). Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. *J Neurosci, 19*, 5731–5740.

Sondell M, Sundler F, Kanje M. (2000). Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. *Eur J Neurosci 12*, 4243–4254.

Springer ML, Chen AS, Kraft PE, et al. (1998). VEGF gene delivery to muscle: Potential role for vasculogenesis in adults. *Mol Cell, 2*, 549–558.

Steinhäuser C, Seifert G. (2002). Glial membrane channels and receptors in epilepsy: impact for generation and spread of seizure activity. *Eur J Pharmacol, 447*, 227–37.

Storkebaum, E., Lambrechts, D., & Carmeliet, P. (2004). Vegf: once regarded as a specific angiogenic factor, now implicated in neuroprotection. *BioEssays, 26*, 943-954.

Sutula, T., Cascino, G., Cavazos, J., Parada, I., Ramirez, L. (1989). Mossy fiber synaptic reorganization in the epileptic human temporal lobe. *Ann Neurology, 26*(3): 321-330.

Takeshita S, Zheng LP, Brogi E, et al. (1994). Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *Clin Invest, 93*, 662–670.

Tian, G.F., Azmi, H., Takano, T., Xu, Q., Peng, W., Lin, J., Oberheim, N., Lou, N., Wang, X., Zielke, H.R., Kang, J., Nedergaard, M. (2005). An astrocytic basis of epilepsy. *Nat. Med. 11*, 973–98.

Uesugi, H., Shimizu, H., Arai, N., Maehara, T., Mizutani, T., Kawai, K., & Nakayama, H. (2002). Pathological effect of seizures on the hippocampus in cases with temporal lobe epilepsy caused by brain tumors. *Psychiatry and Clinical Neurosciences, 56*, 557-559.

Van Vliet, E.A., da Costa Araujo, S., Redeker, S., van Schaik, R., Aronica, E., Gorter, J.A., 2006. Blood–brain barrier leakage may lead to progression of temporal lobe epilepsy. Brain.

Vannucci, S.J., Maher, F., Simpson, I.A. (1997). Glucose transporter proteins in brain: delivery of glucose to neurons and glia. *Glia, 21*(1): 2-21.

Veikkola, T., & Alitalo, K. (1999). Vegfs, receptors and angiogenesi. *Cancer Biology, 9*, 211-220.

Vezzani, A., Baram, T.Z. (2007). New roles for interleukin-1beta in the mechanism of epilepsy. *Epilepsy Curr. 7*, 45–50.

Vezzani A, Conti M, De Luigi A, et al. (1999). Interleukin-1beta immunoreactivity and microglia are enhanced in the rat hippocampus by focal kainate application: Functional evidence for enhancement of electrographic seizures. *J Neurosci, 19*, 5054–5065.

Vezzani, A., Granata, T.(2005). Brain inflammation in epilepsy: experimental and clinical evidence. *Epilepsia 46*, 1724–1743.

Vezzani A, Moneta D, Conti M, et al. (2000). Powerful anticonvulsant action of IL-1 receptor antagonist on intracerebral injection and astrocytic overexpression in mice. *Proc Natl Acad Sci USA, 97*, 11534–11539.

Voutsinos-Porche B, Bonvento G, Tanaka K, Steiner P, Welker E, Chatton JY, Magistretti PJ, Pellerin L. (2003). Glial glutamate transporters mediate afunctional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. *Neuron, 37*, 275–286.

Viviani, B., et al. (2003). Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J. Neurosci,. 23*, 8692–8700.

Walsh, K. & Darby, D. (1999). Neuropsychology: A Clinical Approach (4th ed.). Edinburgh: Churchill Livingstone.

Watson, R. E. Jr., Wiegand, S. J., Clough, R. W., & Hoffman, G. E. (1986). Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides, 7*, 155-159.

Waxman, S.G., & Geschwind, N. (1975). The interictal behavior syndrome temporal lobe epilepsy. *Archives of General Psychiatry, 32*. 1580-1586.

White, J.D. & Gall, C.M. (1987). Differential regulation of neuropeptide and proto-octogene

mRNA content in the hippocampus following recurrent seizures. *Molecular Brain Research, 3*(1), 21-29.

Williamson, P.D., Spencer, D.D., Spencer, S.S., et al. (1985). Complex partial seizures of frontal lobe origin. *Annals of Neurology, 18,* 497-504.

Ye, Z.C., Sontheimer, H., 1996. Cytokine modulation of glial glutamate uptake: a possible involvement of nitric oxide. *Neuroreport, 7,* 2181–2185.

Zhang RL, Chopp M, Jiang N, et al. (1995). Anti-intercellular adhesion molecule-1 antibody reduces ischemic cell damage after transient but not permanent middle cerebral artery occlusion in the Wistar rat. *Stroke, 26,*1438–1442.

Zucker, D.K., Wooten, G.F., Lothman, E.W., 1983. Blood–brain barrier changes with kainic acid-induced limbic seizures. *Exp. Neurol., 79,* 422–433.