Receptor Mechanisms of VEGF-mediated Neuroprotection Following Status Epilepticus

by

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Abstract

VEGF RECEPTOR MECHANISMS IN EPILEPSY

by

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Vascular endothelial growth factor (VEGF) is an angiogenic factor with known neuroprotective effects. Previous research in our laboratory has shown that intrahippocampal VEGF infusions protect neurons from death 24 hours after status epilepticus (Nicoletti et al., 2008). VEGF is unable to cross the blood brain barrier given its large molecular size, which limits its therapeutic utility in human epilepsy. If the VEGF receptor mediating VEGF's neuroprotective effects could be identified, small molecule reagents targeted to specific receptors may be developed for clinical use. *In vitro* studies suggest VEGF's neuroprotective effects are mediated by VEGFR2 (Jin et al., 2000; Matsuzaki et al., 2001). To determine which receptor(s) mediate VEGF's neuroprotective effects, we infused VEGF into the hippocampus simultaneously with 1 of 3 different VEGF receptor inhibitors continuously for 5 days prior to pilocarpine-induced status epilepticus. Tissue was taken 24 hours following status epilepticus and CA1 neuronal damage was evaluated using stereological techniques. VEGFR2 receptor inhibition with the tyrosine kinase inhibitor SU1498/SU5416, which blocks VEGF signaling on intracellular and cell surface VEGFR2, did not alter neuroprotection, while blockade of the VEGFR2 binding site with an anti-VEGFR2 antibody significantly worsened neuronal damage. This may suggest a neuroprotective mechanism of VEGF binding to cell surface VEGFR2. While blockade of the VEGF binding site on cell surface VEGFR1 with an anti-VEGFR1 antibody did not influence neuroprotection, we propose that VEGFR1 activation may

exacerbate neuronal death via increased permeability and inflammation secondary to seizures. These findings suggest a balance between activation of different VEGF receptors may determine optimal therapeutic utility of VEGF in epilepsy.

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Chapter 1: Introduction

Epilepsy

Epilepsy is a neurological disorder characterized by recurrent, unprovoked seizures. Epilepsy is the most common neurological disorder worldwide and affects approximately 3% of the population, or 50 million people (Chang & Lowenstein, 2003; World Health Organization, 2012). Briefly, seizures are triggered by sudden disruption of brain activity leading to over-excitation of cerebral neurons. A diagnosis of epilepsy requires the onset of more than one unprovoked seizure. Onset of epilepsy can occur throughout the lifespan, though typically occurs during childhood or later in life (Annegers et al., 1998). Epilepsy etiology has been classified into three categories based on the probable cause of seizure onset (Engel, 2001). Symptomatic epilepsy has an identifiable cause, such as a traumatic brain injury, stroke, brain aneurysm or tumor. Idiopathic epilepsy describes seizures of unknown cause, most of which are presumed to evolve from genetic abnormalities. Cryptogenic epilepsy also has no known cause although it is believed to be a result of neuropathology undetectable by current diagnostic tools (Lee & Clason, 2008).

Seizure Types

Seizures may be characterized as partial or generalized. Characterization is dependent on epileptic focus within the brain and seizure semiology. Partial seizures occur when epileptiform activity is limited to one cortical area unilaterally, while generalized seizures occur when epileptiform activity is found globally within the brain. Partial seizures can be further categorized into simple partial seizures and complex partial seizures (Lee & Clason, 2008). Simple partial seizures last from a few seconds to a few minutes. Individuals maintain awareness during these events, which include motor or sensory symptoms. Complex partial seizures last

several minutes and are characterized by loss or decreased awareness, which is commonly followed by motor automatisms. Generalized seizures can further be classified as primary generalized seizures and secondarily generalized seizures (Lee & Clason, 2008). Epileptiform activity in primary generalized seizures is found throughout the brain at the ictus (the start of the seizure event). On the other hand, secondarily generalized seizures develop following spreading of epileptiform activity from a complex partial seizure focus to the entire brain. The most common generalized seizure is a generalized tonic-clonic seizure. These seizures begin with increased tone across several muscle groups followed by a clonic phase consisting of whole body muscle contractions lasting up to a few minutes. A post-ictal period of confusion or lethargy is common (Lee & Clason, 2008).

A prolonged generalized seizure is known as status epilepticus. Status epilepticus is defined as continuous or recurring generalized convulsive seizures in which the individual does not return to baseline consciousness between ictal events (Watson, 1991; Dupont & Crespel, 2009). The duration of status epilepticus can vary from 5 minutes to 90 minutes depending on the milieu in which it is studied. Use of electroencephalographic (EEG) technology demonstrates that the epileptiform activity of status epilepticus follows a progressive pattern of changes. Initially, discrete seizures with interictal slowing are seen followed by waxing and waning of interictal discharges, then to ictal discharges interrupted by flat periods (Watson, 1991). The beginning of motor activity evident during status epilepticus correlates with initial phases of EEG epileptiform activity, though motor activity typically diminishes after an hour. Systemic and metabolic changes evolve during status epilepticus as well (Watson, 1991). During the first 30 minutes, blood pressure, serum lactate, and glucose levels increase and pH decreases, leading to acidosis. After about thirty minutes, blood pressure, pH, and lactate levels returns to baseline

and glucose levels decrease or return to baseline. Respiratory failure and hyperthermia may occur after approximately 60 minutes (Watson, 1991). Seizures lasting longer than 60-90 minutes may result in neuronal death (Watson, 1991). The epileptiform activity associated with status epilepticus contributes substantially to the development of chronic epilepsy. One study found that 42% of adults with acute symptomatic status epilepticus developed chronic epilepsy 10 years later. On the other hand, only 13% of adults with acute symptomatic seizures without status epilepticus developed chronic epilepsy in this same time period (Hesdorffer et al., 1998).

Treatments

The most common and comparably innocuous method for the treatment of seizures is the use of antiepileptic drugs (AEDs). There are presently approximately 20 different AEDs available in the United States. The primary function of these medications is to increase seizure threshold, though the mechanism by which each drug operates varies. While drug therapies have proven beneficial for some patients, approximately 30% remain refractory to current pharmacotherapies (Loscher, 2002). This drug-resistant epilepsy is defined as "a failure of adequate trials of 2 tolerated and appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve seizure freedom" (Kwan & Brodie, 2010). At present, there is no available AED to prevent neural damage concomitant with recurrent seizures.

Additional treatments for drug-resistant epilepsy include resective brain surgery and vagal nerve stimulation (VNS). Resection surgery involves removing sections of brain tissue thought to be responsible for seizure generation, thereby potentially eliciting seizure freedom or improved control of seizures following resection. Success rate is best for patients with discrete abnormalities (i.e., tumors, gliosis, hippocampal sclerosis) with no history of generalized seizures (Spencer & Huh, 2008). Approximately 66-70% of patients are seizure-free less than 5

years after surgery (Spencer & Huh, 2008). However, of those who obtain seizure freedom, about 15-20% experience seizure relapse between 5 to 10 years after surgery (Spencer & Huh, 2008). In the VNS procedure, electrodes are used to send pulses of energy to the brain through the vagus nerve. VNS may be employed as adjunctive therapy (to supplement poor seizure control with pharmacotherapy) for patients reluctant to undergo brain surgery or who may not be surgical candidates. While VNS is not a "cure-all", advantages include decreasing AED dosage and seizure frequency and severity (Milby et al., 2009).

Experimental Models of Epilepsy

Various methods have been employed to provoke chronic symptomatic epilepsy in animal models that are useful for investigating the pathophysiology and potential treatment mechanisms of chronic seizures. The two most common methods are the kindling model and the post-status epilepticus model of temporal lobe epilepsy (TLE). The kindling model uses repeated sub-threshold electrical stimulation to limbic regions. Over time, the neuronal responses to these repeated stimulations escalate, progressing into spontaneous generalized seizures (Giblin & Blumenfeld, 2010). The post-status epilepticus model utilizes chemicals to induce status epilepticus, which may then incite chronic epilepsy. Common chemicals include the muscarinic agonist pilocarpine or the glutamate analog kainate.

Physiological and Anatomical Changes

The temporal lobes are the most common focus for localized seizures. Furthermore, mesial temporal lobe epilepsy (MTLE) is the most common form of temporal lobe epilepsy (Manford et al., 1992). In MTLE, epileptiform activity is generated in limbic structures, including the hippocampus, amygdala, and entorhinal cortex, and are propagated outwards to cortical structures (Andre et al., 2000). Animal models of temporal lobe epilepsy demonstrate

that recurrent spontaneous seizures contribute to hippocampal sclerosis with cell loss concentrated in the dentate gyrus and subfields CA1 and CA3 of the hippocampus and mossy fiber sprouting (Mello et al., 1993; Borges et al., 2003). Significant neuronal loss is evident in animal models of continuous seizures lasting more than 60 minutes, with particular damage to layers 3, 5, and 6 of the cerebral cortex, cerebellum, hippocampus, amygdala, and several thalamic nuclei (Watson, 1991). Patients with temporal lobe epilepsy exhibit decreased volumes in limbic system regions, including the hippocampus, parahippocampal region and the amygdale, cerebellum, and frontal regions (Bernasconi et al., 2003; Riederer et al., 2008). Atrophy to these areas undoubtedly contributes to alterations in brain circuitry, which may function to propagate additional seizures.

Induction of epileptic events, or ictogenesis, results from activation of the excitatory glutamate receptors and opening of the voltage-gated sodium channels due to depolarization, thereby causing further depolarization (Sasa, 2006). The long-term biochemical or physical alterations in structures following recurrent ictogenic events is called epileptogenesis (Sasa, 2006). Acquired forms of epilepsy are caused by a provoking event followed by a clinical latent period in which the brain undergoes repair and reorganization into a hyperexcitable state that results in establishment of an epileptic brain (Giblin & Blumenfeld, 2010).

Both physiological and morphological changes contribute to the prolonged effects of acquired epilepsy. Acute physiological changes include alternations in channel signaling, more specifically for GABA and glutamate (Giblin & Blumenfeld, 2010) This phenomenon then modifies the baseline membrane potential to induce an overall hyperexcitable state within the brain (Giblin & Blumenfeld, 2010). Increases in excitotoxic antibodies are also present. Status epilepticus in particular can lead to calcium influx, increased influx through NMDA and voltage-

gated ion channels, and reductions in GABA-ergic interneurons in the hippocampus and entorhinal cortex (Fujikawa et al., 2000; Niqet et al., 2005; Kobayashi & Buckmaster, 2003; Kumar & Buckmaster, 2006). Pilocarpine models of status epilepticus decrease the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels on CA1 pyramidal neurons, further contributing to hyperexcitability (Jung et al., 2007).

Anatomical changes contribute to the remodeling of circuitry following epileptogenesis. Cell loss, increased expression of growth factors, and neurogenesis play a role in the development of abnormal circuits and morphological changes (Giblin et al., 2010). Observed mossy fiber sprouting in the dentate gyrus leads to synaptogenesis of excitatory circuits, which are hypothesized to be mediated by changes in semaphorin expression (Rao et al., 2006; Yang, 2005). Astrocytes undergo morphological changes and are referred to as "reactive astrocytes." These reactive astrocytes develop aberrant overlapping processes with neighboring astroctyes (Oberheim et al., 2008). This reactive gliosis results in glial scar tissue in and adjacent to epileptic brain tissue in a clinical sample. (Oberheim et al., 2008; McKhann et al., 2000). Release of glutamate by astrocytes has been hypothesized to contribute to the hypersynchonous neuronal activity seen in epilepsy (Tian et al., 2005). An increase in inflammatory cytokines, like interleukins, in the brain is identified as a consequence and cause for subsequent seizures (Vezzani et al., 2011). Rodent models of epilepsy illustrate that inflammatory responses contribute to breakdown of the blood brain barrier and enhance neuronal excitability, which may be in part mediated by the astrocytes (Devinsky et al., 2013).

Cognitive and Emotional Consequences

The resulting physiological and anatomical sequelae of chronic seizures disrupt underlying mechanisms mediating cognitive and emotional functioning. Clinical correlates of

epilepsy include intellectual decline, impairments in memory, executive dysfunction, attentional deficits and reduced processing speed (Moore & Baker, 2002). Patients are also at greater risk for developing depression, anxiety and suicidal ideation (McCagh, 2009). The temporal lobes house limbic structures important for cognitive and emotional functioning and, as previously mentioned, are also a major focus for epilepsy-related neuronal damage. The hippocampus is important for learning and memory consolidation, while the amygdala is instrumental in orchestrating emotional responses to stimuli (Kandel et al., 2000). Animal models of temporal lobe epilepsy have demonstrated that seizures contribute to depressive and anxious characteristics and impaired memory (Groticke et al., 2007). Furthermore, since current AEDs function to increase seizure threshold, thereby quieting neuronal excitation, side effects of these medications often include depression and cognitive slowing (Cavanna et al., 2010). The inhibitory effects of AEDs on neurons can exacerbate the emotional and cognitive effects resulting from repeated seizures alone. In addition to anatomical justifications, social factors also play a role in emotional dysfunction comorbid with epilepsy (Moore & Baker, 2002). Social stigmas attached to this disorder encourage a diminished sense of self, and fears of embarrassment from having a seizure in public. These cognitive and emotional correlates likely contribute to the overall poor educational and employment outcomes for these patients (Gauffin et al., 2011). Taken together, these cognitive, emotional, and behavioral impairments in patients with epilepsy contribute to a lower quality of life. Modifying underlying anatomical factors supporting these functional impairments may serve to alleviate these complaints.

Growth Factors and Epilepsy

Growth factors are protein factors that protect or functionally enhance neuronal cells. Several families of growth factors have been identified and some have been implicated in seizure

disorders. The first growth factor family studied in the context of seizures was the neurotrophins. Neurotrophins mediate neuronal plasticity throughout development. More specifically these growth factors are important for neuronal proliferation and survival, axonal growth, neurogenesis and synaptogenesis (Henriques et al., 2010). Neurotrophin family members include nerve growth factor (NGF), brain-derived neurotropic factors (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4/5 (NT-4/5) (Skaper, 2012). The neurotrophins bind to related receptors, including the trk family of receptors (trkA, trkB, and trkC) and the p75 neurotrophin receptor (Skaper, 2012). BDNF and NGF expression are both upregulated following seizures (Isackson et al., 1991) and BDNF is upregulated in areas vulnerable to TLE (hippocampal pyramidal cells, amygdala, entorhinal cortex) (Scharfman et al., 2005). The trkB receptor is the only BDNF receptor known to be upregulated following seizures (Merlio et al., 1993), which suggested a neuroprotective potential for BDNF. While BDNF has demonstrated neuroprotective effects in other neurodegenerative disorders (Chao et al., 2006), it has been known to increase neuronal damage in the hippocampus following a model of severe seizures (Rudge et al., 1998). Further, BDNF has also been shown to increase excitability within the brain (for review see Croll, 2009). These paradoxical findings render BDNF a poor therapeutic candidate in the epilepsy model.

VEGF

The therapeutic potential of vascular endothelial growth factor (VEGF) in epilepsy is currently under investigation given preliminary evidence of its neuroprotective effects (Nicoletti et al., 2008; Storkebaum & Carmeliet, 2004) and ability to "quiet" neural activity following insult (McCloskey et al., 2005). VEGF is an endothelial cell mitogen best known for its trophic effects on endothelial cells, leading to angiogenesis (Carmeliet, 2003). VEGF was first identified for its role in permeabilizing vascular endothelium, thereby inducing vascular leakage (Senger et

al., 1986). It was later discovered to promote the sprouting of new blood vessels from existing blood vessels and facilitate the growth of new blood vessels during development (for review see Yancopoulous et al., 2000). VEGF is essential to normal developmental processes. VEGF knockout mice die by age E8-E9, and mice missing a single allele for VEGF-A die at age E11-E12 (Ferrara et al., 2003; Tammela et al., 2005). Exogenous application of VEGF to brain parenchemya contributes to the breakdown of the blood brain barrier leading to leaky vasculature poorly invested with astroglial endfeet (Croll et al., 2004b). Leukocyte extravasation and vascular permeability precedes angiogenesis, suggesting that inflammation and leakage are necessary for angiogenic processes (Croll et al., 2004a). Indeed, VEGF-mediated angiogenesis can be abrogated by anti-inflammatory therapies (Pipp et al., 2003). VEGF infusions also produce a local inflammatory response mediated by extravasation of monocytes (Croll et al., 2004b). This inflammation is accompanied by upregulation of adhesion molecules like ICAM-1, which initiate leukocyte rolling and sticking (Fabene et al., 2008).

VEGF Isoforms

VEGF, also called VEGF-A, is one of several VEGF family members that share similar biochemical and physical properties. Each VEGF family member mediates different processes and maintains a specific receptor binding profile. In addition to VEGF-A, other VEGF family members include placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E (Roy et al., 2006; Takahashi & Shibuya, 2005; Figure 1). Different isoforms may also exist for VEGF family members. The VEGF-A gene is located on chromosome 6p21.3 and contains 8 encoding exons and 7 introns (Ferrara et al., 2003). Alternative splicing can result in several VEGF-A isoforms including $VEGF_{165}$, $VEGF_{121}$, $VEGF_{189}$, and $VEGF_{206}$, which vary in length of number of amino acids following signal sequence cleavage (with lengths of 165, 121, 189, and

206 amino acids, respectively). Splice variants of $VEGF_{145}$ and $VEGF_{183}$ have been reported, though they are less commonly found (Ferrara et al., 2003). Each isoform is unique in its receptor binding profiles and concomitant effects.

VEGF Receptors

The different known signal-transducing receptors for the VEGF family members include VEGFR1 (Flt-1), VEGFR2 (Flk-1 or KDR), and VEGFR3 (Figure 1). VEGFR1, VEGFR2, and VEGFR3 are tyrosine kinase receptors and are capable of mediating downstream signaling cascades (Ferrara et al., 2003; Roy et al., 2006; Takahashi & Shibuya, 2005). In particular, these VEGF tyrosine kinase receptors activate cascades traditionally mediated by growth factors, such as the ERK/MAPK pathway and the Akt pathway (Gerber et al., 1998b). While VEGFR1 is largely found on vascular endothelium, its expression has also been seen on monocytic leukocytes, astroglia (Krum et al., 2002), and microglia (Forstreuter et al., 2002). VEGFR1 is also expressed by reactive astrocytes following ischemia (Lenmyr et al., 1998), and grafting or stab injury (Krum & Rosenstein, 1999) in the adult brain. VEGFR2 is found on vascular endothelium, though VEGFR2 may also be expressed on neurons and astrocytes in the brain following insults such as ischemic stroke (Issa et al., 1999), as well as on microglia in cerebral ischemia (Lenmyr et al., 1998; Krum et al., 2002; Croll & Wiegand, 2001). In contrast, VEGFR3 is not present in the brain and is predominantly expressed on lymphatic endothelium (Kaipainen et al., 1995), highlighting its regulatory role in lymphangiogenesis.

In addition to binding to the signal transducing tyrosine kinase receptors, VEGF can bind to the non-catalytic receptors neuropilin-1 (NP-1) and neuropilin-2 (NP-2). The neuropilin receptor family members also mediate effects of the semaphorins, which are critical to neuronal development (Chedotal et al., 1998). The neuropilins can bind to and form complexes with other

VEGF receptors (Fuh et al., 2000). It has been suggested that the neuropilin receptors function to bring local VEGF in close proximity to the high-affinity tyrosine kinase receptors, thereby enhancing activation of these VEGF receptors (Croll et al., 2005).

As previously stated, the VEGF isoforms exhibit different receptor binding profiles. $VEGFA_{165}$ can bind to most known VEGF receptors (NP-1, NP-2, VEGFR1, and VEGFR2), while $VEGF_{121}$ fails to bind to VEGFR2. PlGF is known to bind to only VEGFR1 and NP-1, and VEGFB to VEGFR1. VEGF-C, D, and E expression in the brain has not been shown (Figure 1).

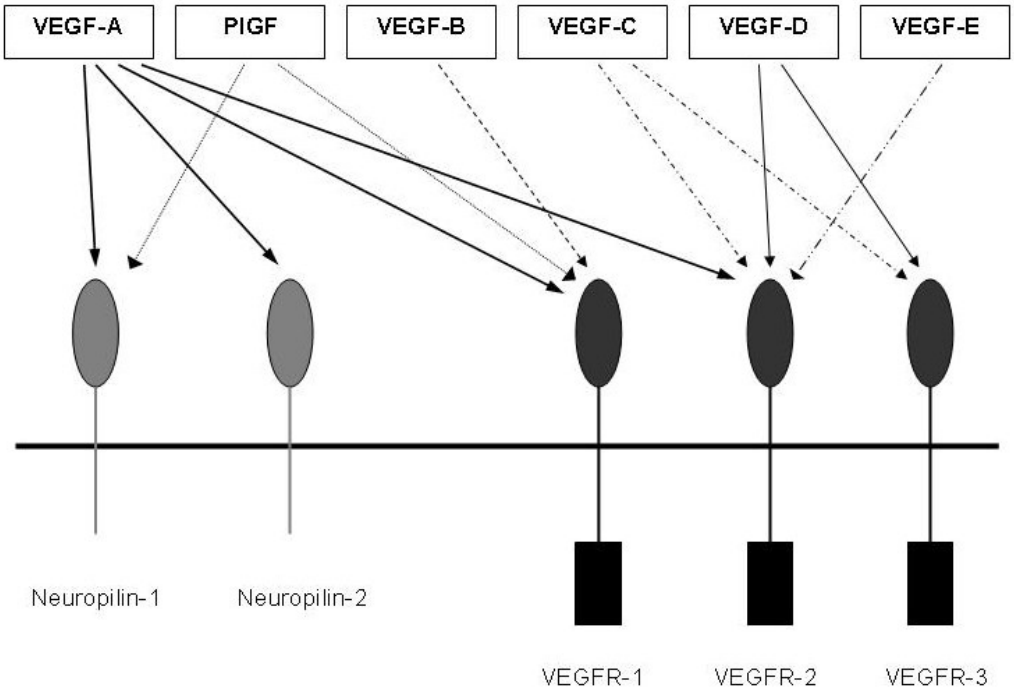

Figure 1: VEGF family members and corresponding VEGF receptors (adapted from Croll et al., 2006).

Neuroprotective Effects of VEGF

More recently, neurotrophic effects of VEGF have been identified. A neurodevelopmental role of VEGF was initially proposed after finding VEGFR2 upregulation in

progenitor cells in retinal development (Yang & Cepko, 1996). Application of VEGF to retinal cultures enhances the number of amacrine cells and photoreceptors with no apparent increase to non-neuronal cells (Yourey et al., 2000). VEGF induces neuritic outgrowth in ventral mesencephalon explants and upregulates MAP-2 (a structural protein important for development), which together suggests a role for VEGF in neuronal development (Silverman et al., 1999). *In vitro* application of VEGF to neuronal cell cultures promotes cell survival (Oosthuyse et al., 2001).VEGF's neurotrophic effects have also been demonstrated in the peripheral nervous system. VEGF application to adult mouse superior cervical ganglion and dorsal root ganglion *in vitro* enhances axonal outgrowth (Sondell et al., 1999).

A neuroprotective role for VEGF has also been described (for reviews see Storkenbaum et al., 2004; Rosenstein & Krum, 2004). Depletion of oxygen to cells following neuronal insult contributes to hypoxia and subsequent ischemia. The hypoxia-inducible factor-1α (HIF-1α) has been located on the promotor region of the VEGF gene. While mRNA levels for VEGF are low in the naïve adult brain (Monacci et al., 1993), they are upregulated following ischemia (Lee et al., 1999) indicating a possible role in post-insult support. Neuronal damage is reduced by application of VEGF to an ischemic adult rat brain (Hayashi et al., 1998). Deletion of HIF-1α promotes motor neuron degeneration (Oosthusye et al., 2001). VEGF levels in neurons, astrocytes and endothelial cells are elevated in the penumbra of brains collected from patients following an acute ischemic stroke (Margaritescu et al., 2011). Survival of serum-deprived hippocampal cells is augmented following the addition of VEGF (Jin et al., 2000a) and protective effects of VEGF are as good as a glutamate receptor antagonist on a rat hippocampal cell line undergoing hypoxia (Svensson et al., 2002). Further, an *in vitro* model of cerebral ischemia noted increased expression of VEGFR2 and NP-1 and not VEGFR1 on a hippocampal

cell line (Jin et al., 2000b).VEGF slows degeneration in animal models of motor neuron disease (Storkebaum et al., 2005) and motor neuron loss concomitant with spinal cord ischemia was reduced with VEGF treatment (Lambrechts et al., 2003).

Given VEGF's role in vascular leakage, a deleterious role of VEGF in enhancing ischemic lesions may be suggested. Intravenous VEGF injections result in blood brain barrier leakage and hemorrhaging in ischemic tissue (Zhang et al., 2000). However, neuronal damage is reduced with local application of VEGF following middle cerebral artery (MCA) occlusion in an adult brain (Hayashi et al., 1998). This preservation of neuronal tissue was accompanied by a decrease in edema formation and infarct volume. These findings highlight the delicate balance between VEGF's detrimental vascular and inflammation effects and its valuable neuroprotective effects.

VEGF application enhances proliferation of reactive astrocytes and microglia and induces hypertrophy in astrocytes even in the absence of inflammation (Krum et al., 2002; Croll et al., 2004b; Ackerman et al., 2003) Continuous VEGF infusions (3 days) *in vivo* stimulate increases in VEGFR1 and not VEGFR2 mRNA and expression on astrocytes (Mani et al., 2005). Further, antisense against VEGFR1 and not VEGFR2 decreases the astrocytic marker GFAP (Mani et al., 2005). VEGF-induced activation of VEGFR1 on astrocytes is thought to be mediated by the Erk protein kinase, since pharmacological inhibitors of this pathway eliminated this GFAP expression (Mani et al., 2005).

VEGF's role in neuronal protection can also be appreciated at the clinical and behavioral level. Individuals homozygous for particular haplotypes in the VEGF promoter region were at a 1.8 fold greater risk of amyotrophic lateral sclerosis (ALS) and have lower VEGF plasma levels than controls (Lambrechts et al., 2003). Transgenic mice expressing low VEGF levels were more

vulnerable to persistent paralysis following spinal cord ischemia as compared to wild-type controls (Oosthuyse et al., 2001) and secondary behavioral symptoms were reduced in rats treated with VEGF 6 weeks after sustaining a spinal cord contusion injury (Widenfalk et al., 2003).

VEGF and Epilepsy

Severe seizures may result in deterioration of brain tissue. Such an anatomical effect can best be seen in the hippocampus, a structure vulnerable to traumatic brain damage (Mello et al., 1993; Borges et al., 2003). Breakdown of the blood brain barrier and concomitant inflammation are characteristics of severe seizures, like status epilepticus (Janigro, 2012). Heightened levels of VEGF mRNA are noted following seizure induction by electroconvulsant shock therapy (Newton et al., 2003) indicating that seizure generation may contribute to VEGF expression. This idea is supported by findings of significant VEGF expression in resected brain tissue of epileptic patients with intractable epilepsy (Boer et al., 2008) and prominent VEGF upregulation in the hippocampus of temporal lobe epilepsy patients (Rigau et al., 2007). Indeed, VEGF protein expression is increased on neurons and glia following pilocarpine-induced seizures in an animal model, with neuronal expression resolving in days and glia expression resolving in one week (Nicoletti et al., 2008). VEGF protein expression is also increased in the hippocampus, temporal cortex, amygdala, and thalamus after pilocarpine-induced status epilepticus (Nicoletti et al., 2008). Increased metabolic activity and subsequent elevated need for glucose and oxygen following seizures contributes to the ictal-related hypoxia. The resultant hypoxia may play a role in activating VEGF production given the presence of the HIF -1α hypoxia-inducible transcription factor on the promoter region of the VEGF gene (Semenza, 2001). HIF-1α upregulation has

been shown in CA regions and the dentate gyrus of the hippocampus following an animal model of electroconvulsive seizures (Girgenti, 2009).

VEGF can rescue neurons from death following glutamate-induced toxicity *in vitro*, a physiological state often associated with seizures (Matsuzaki et al., 2001). Administration of exogenous VEGF protein to the hippocampus has been shown to rescue neurons from death in the 24 hours following a pilocarpine model of severe seizures (Nicoletti et al., 2008). While this rescue is accompanied by preservation of behavioral functioning one month following seizures, neurons themselves were not preserved during this same time period (Nicoletti et al., 2010), suggesting other effects of VEGF on hippocampal functioning after seizures.

VEGF may play a role in "quieting" neuronal processes following severe seizures. Application of VEGF to rat hippocampal cell slices decreases amplitude of excitatory pathways in CA1 and CA3 (McCloskey et al., 2005). VEGF application to hippocampal slices of epileptic rats decreases spontaneous discharges while similar findings were not found in disinhibited slices of naïve brains (McCloskey et al., 2005), demonstrating a preferential effect of VEGF in mediating neuroprotection in the epileptic brain. Hence, the upregulation of endogenous VEGF that accompanies seizures may function to reduce excitability in the epileptic brain.

VEGF's ability to decrease excitability may confound the mechanistic interpretation of VEGF-mediated neuroprotection. Neuroprotection is traditionally thought to be due to direct effects on neurons, but other processes can indirectly lead to neuronal preservation. For example, inflammatory responses may contribute to decreases in seizure threshold and may play a role in mediating protection following seizure (Vezzani & Granata, 2005). Inflammatory responses activate microglia, and microglia-derived interleukins can stimulate production of neurotrophic factors from astrocytes, thereby facilitating CNS repair.

It is unlikely that VEGF's neuroprotective effects in epilepsy are mediated by increased vascular density since VEGF did not increase vascular density in the *in vivo* status epilepticus model (Nicoletti et al., 2008). It is more likely that VEGF receptor activation on neurons or glia in the brain may play a role in protection, since inactivated VEGF had no protective effect (Nicoletti et al., 2008). VEGF's neuroprotective effects in CA1 of the hippocampus are present 24 hours following pilocarpine-induced status epilepticus. However, neuronal preservation was not noted 1 month after seizure induction (Nicoletti et al., 2010), which may suggest a limited time course of protection. The transient nature of protection may indicate that VEGF plays a role in delaying the onset of cell death.

Certain cell death pathways are thought to mediate seizure-related neuronal death. Such pathways may be dependent on seizure duration and length of time following seizures. Necrotic cell death is a passive process by which cells die from lack of energy sources, which often leads to cell lysis and inflammation (Fujikawa, 2005). Apoptotic cell death, or programmed cell death, is an energy-dependent process which leads to mitochondrial death and DNA fragmentation (Pollard et al., 1994). Unlike necrosis, apoptosis is a controlled process and does not contribute to additional damage to neighboring cells or to the organism (Taylor et al., 2008). However, given the activation of multiple pathways following seizure-induced excitotoxicity, other cellular organelles, like lysosomes, may also be activated. Lysosomes can activate a process called autophagy (Shacka et al., 2007). Autophagy is the decomposition of cellular parts due to lysosomal mechanisms, which form autophagic vacuoles capable of carrying injured cell parts to the lysosomes for degradation (Shacka et al., 2007). However, over-accumulation of vacuoles can lead to autophagic cell death (Shacka et al., 2007).

Necrotic cell death is seen 4-6 hours after seizure onset, while apoptotic cell death is found 24 and 72 hours after seizure onset (Henshall, 2000a). It is hypothesized that short duration seizures contribute to apoptosis because such mechanisms have not yet depleted energy resources. Evidence for apoptotic cell death includes enhanced activation of the bcl-2 and caspase apoptotic proteins following temporal lobe seizures (Henshall et al., 2002) and increases in BAX and caspase 3 in drug-refractory epileptic brains (Henshall, 2000b). However, biochemical markers for autophagy are present 24 hours after pilocarpine-induced status epilepticus (Cao et al., 2009), though autophagic morphology resembles both necrosis and apoptosis (Shaka et al., 2007).

The VEGFR1 and VEGFR2 activation-induced downstream Akt pathway may act as a caspase-9 inhibitor, thereby suggesting a potential role of VEGF in anti-apoptotic mechanisms (Romashkova & Makarov, 1999). Application of VEGF *in vitro* promotes survival of serum-starved endothelial cells by provoking expression of the anti-apoptotic proteins Bcl-2 and A1 (Gerber et al., 1998a). The phosphatidylinositol (PI)-3-kinase-AKT pathway has been implicated as the mediating signal of VEGF's anti-apoptotic effects (Gerber et al., 1998b).

Mechanisms of VEGF-Mediated Neuroprotection

It is likely that VEGF mediates neuroprotection via VEGFR1, VEGFR2, or both given their localization on neuronal tissue and their capacity to mediate downstream signaling cascades. VEGFR1 is also present in adult brain, though localization is thought to be limited to vasculature, astroglia, monocytes, and microglia (Krum & Rosenstein, 2002; Sawano et al., 2001; Forstreuter et al., 2002). VEGFR1 expression is increased by a HIF-dependent mechanism during hypoxia, similar to that which increases VEGF expression (Ferrara et al., 2003). More recently, a neuroprotective role of VEGFR1 has been described in a model of motor neuron

disease (Poesen et al., 2008). However, VEGFR1 is only weakly autophosphorylated in response to VEGF binding (Waltenberger et al., 1994). As such, it has been suggested that VEGFR1 acts as a "decoy" receptor sequestering VEGF and preventing its binding to VEGFR2 and its concomitant effects on vascular endothelium (Park, 1994).

Preliminary *in vitro* research supports a neurotrophic role of VEGFR2. Antisense treatment *in vitro* against VEGFR2 hinders protection, while antisense against VEGFR1 shows no effect (Matsuzaki et al., 2001). VEGF more readily activates ERK pathways via VEGFR2 than via VEGFR1 in models testing VEGF's effects receptor activation pathways (Grummer et al., 2009; Chen et al., 2006). Activation of both the VEGFR2-mediated Akt and Erk downstream pathways have also been suggested following *in vitro* models of neuronal insult (Jin et al., 2000; Matsuzaki et al., 2001; Gomes et al., 2007). At rest, adult hippocampal neurons do not express VEGFR2, yet VEGFR2 is upregulated following status epilepticus (Nicoletti et al., 2005). This upregulation of hippocampal immunoreactivity to VEGFR2 after status epilepticus may permit VEGFR2-mediated signaling on hippocampal neurons. VEGFR2 upregulation in neurons has been previously reported during various insults, most notably ischemia (Croll & Wiegand, 2001). Blockade of VEGFR2 with SU5416 increases the number of apoptotic neurons in the dentate hilus in a model of traumatic brain injury (Lee & Agoston, 2009). Furthermore, VEGFR2 may mediate seizure suppression given findings that transgenic mice overexpressing VEGFR2 exhibited a higher seizure threshold (Nikitidou et al., 2012).

Membrane-bound growth factor receptors are capable of internalizing and maintaining their signaling following ligand binding (Berger & Ballmer-Hofer, 2011). Like other tyrosine kinase receptors, membrane-bound VEGFR2 can translocate to the nucleus following VEGF-induced phosphorylation while this has not been demonstrated with VEGFR1 (Santos et al.,

2007). These findings have been replicated in models of *in vitro* wounding of endothelial cells (Santos et al., 2007), and following proliferation of tumor and leukemia cells (Santos & Dias, 2004; Blazquez et al., 2006). This VEGFR2 translocation can function to regulate its own transcription (Dominques et al., 2011) and is hypothesized to control cell survival (Vincent et al., 2005). It is possible that VEGFR2 internalization contributes to its biological effects in some way, although this potential mechanism of action is not well-understood.

An alternative possibility is that a glially-mediated mechanism of protection is involved. Glia have been identified as important mediators of synaptic transmission in the hippocampus (Hulsmann et al., 2003). Immunoreactivity for VEGF surrounds glial cells after pilocarpine-induced spontaneous seizures (Croll et al., 2004), and VEGF receptor expression is increased on glia following neuronal insult. It is possible that glial hypertrophy in the hippocampus may allow glia to invaginate the synaptic cleft and sequester local neurotransmitters, like glutamate, thereby diminishing excitotoxicity.

Therapeutic Utility of VEGF

VEGF itself has little therapeutic value to humans during central nervous system disease because of its large molecular size, rendering it unable to cross the blood brain barrier. Identifying the receptor(s) mediating VEGF-induced neuroprotection would permit development of small molecule drugs designed to upregulate and/or downregulate the responsible receptor(s). Possible receptors include NP-1, VEGFR1, and VEGFR2. NP-1 is the only VEGF receptor constitutively expressed on neurons, although VEGFR1 and VEGFR2 can be found in brain vasculature and VEGFR1 has been reported on glia (Krum et al., 2002). It is important to note that an optimal method for studying VEGF receptor activation is to identify phosphorylation of the target receptor (VEGFR1 or VEGFR2) given that receptor phosphorylation must occur to

incite receptor activation. Receptor expression may change after insult, as VEGFR2 has been noted on neurons after stroke and seizures (Croll & Wiegand, 2001: Nicoletti et al., 2005). It is likely that VEGF-induced protection is mediated by one or both of the high affinity tyrosine kinase receptors VEGFR1 or VEGFR2 given its potential for downstream signaling. However, a pro-survival role of the neuropilins may also exist. Co-expression of neuropilin-1 and VEGFR2 augments the effects of $VEGF_{165}$ binding to VEGFR2 (Soker et al., 1998). In addition, binding of VEGF to the neuropilins prevents their interaction with their semaphorin ligands (Carmeliet & Storkebaum, 2002). This competitive inhibition of semaphorin activity could serve to protect neurons.

Prior models of VEGF-mediated neuroprotection in epilepsy have utilized continuous infusions of VEGF for several days prior to induction of status epilepticus (Nicoletti et al., 2008). However, neuroprotective treatment interventions would be most useful in clinical settings if they could be initiated after the onset of acute severe seizures. If neuroprotection could also be demonstrated with VEGF treatment immediately following status epilepticus, a more practical use of VEGF in critical clinical settings could be explored.

Specific Aims

VEGF has been shown to protect neurons from cell death after pilocarpine-induced status epilepticus (Nicoletti et al., 2008). Because VEGF is a large protein, it does not cross the blood brain barrier, which limits its therapeutic utility in human epilepsy. If the receptor mechanism mediating VEGF's neuroprotective effects could be identified, it would be possible to develop small molecule reagents specific for that mechanism to serve as neuroprotective agents. To elucidate the receptor(s) mediating neuroprotection, we pursued the following specific aims:

Specific Aim 1: *Establish effects of* in vivo *blockade of VEGFR2 signaling on VEGF-induced neuroprotection following status epilepticus.* VEGF's neuroprotective effects *in vitro* have previously been shown to be mediated by activation of VEGFR2 (Matsuzaki et al., 2001; Gomes et al., 2007). In two separate sets of experiments, animals will be treated with intrahippocampal infusions of either the VEGFR2 specific tyrosine kinase inhibitor SU1498/SU5416 or a neutralizing antibody against VEGFR2 (both with and without simultaneous VEGF administration) during pilocarpine induced status epilepticus. Hippocampal cell loss will be analyzed by stereological procedures to illustrate potential neuroprotection following VEGFR2 inhibition.

Specific Aim 2: *Determine a functional role of VEGFR1 in VEGF- mediated neuroprotection during status epilepticus.* Research from the Carmeliet lab has suggested a protective role of VEGFR1 in an *in vivo* model of motor neuron disease (Poesen et al., 2008). To support a role for VEGFR1 activation in neuroprotection following status epilepticus, antibodies against VEGFR1 will be infused into the hippocampus both with and without simultaneous administration of VEGF during pilocarpine-induced status epilepticus. Hippocampal neuronal loss will be assessed using stereological techniques to determine whether VEGFR1 blockade prevents VEGF's neuroprotective effects.

Specific Aim 3: *Determine potential neuroprotective effects of VEGF treatment immediately following status epilepticus.* Pre-application of VEGF has shown to be protective in a pilocarpine model of status epilepticus. It is possible that pre-treatment with VEGF results in changes in VEGF receptor density, expression, or distribution. Therefore, the effects of VEGF on neuroprotection when administered after seizures could be different. To investigate the potential for neuroprotective effects of VEGF treatment following status epilepticus, bolus VEGF or

inactivated VEGF control will be injected into the hippocampus immediately following status epilepticus. Preservation of hippocampal neurons following VEGF post-treatment will be assessed using stereological techniques.

Chapter 2: Methods

General Methods

Subjects

All subjects were adult male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY, USA) weighing about 300-400 grams. Animals were housed three per cage within a temperature-stabilized animal facility with food and water available *ad libitum*. Animals were maintained on a 12-h light/dark cycle (lights on 07:00). Animals were acclimated to the living environment at least one week prior to manipulations. All experiments were approved by the Queens College Institutional Animal Care and Use Committee which operates under federal and state animal care guidelines. All experiments conformed to international guidelines on the ethical use of animals, and every effort was made to minimize both the number of animals used and animal suffering.

Proteins

VEGF: The VEGF used for protein infusions was human recombinant $VEGFA_{165}$ (a generous gift from Regeneron Pharmaceuticals, Tarrytown, NY, USA). VEGF was stored frozen until use then diluted in sterile phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) to attain a dosage of 45ng/day delivered at 0.5µl/h via an osmotic minipump (Alzet Minipump Model 2002, Durect Corporation, Cupertino, CA). This dose was determined from previous data (Nicoletti et al., 2008) demonstrating significant neuroprotection and minimal angiogenic effects at this dosage. Inactivated VEGF was used as a control in some experiments. VEGF was inactivated by approximately 10 repeated freeze-thaw cycles of the VEGF protein.

SU1498/SU5416: SU1498 (Sigma-Aldrich) is a small tyrosine kinase inhibitor that selectively and potently blocks VEGFR2 (IC_{50}= 750nm) as compared to other tyrosine kinase receptors (e.g., PDGF, IC_{50} = >50µm; Strawn et al., 1996). SU1498 was stored frozen until use then

diluted in 75% dimethyl sulfoxide (DMSO) in 25% PBS and a dosage of 20µg/day was delivered intrahippocampally at 0.5µl/h via an osmotic minipump. SU1498 requires DMSO as a diluent, which few labs have applied directly to the brain. We therefore conducted pilot studies to evaluate the morphological effects of DMSO alone in the hippocampus. Animals infused with 75% DMSO intrahippocampally for six days did not show gross anatomical abnormalities as compared with animals receiving PBS, suggesting that DMSO could safely be used as a diluent for intrahippocampal infusions at this concentration. Unfortunately, at the time of the last VEGFR2 kinase inhibitor experiment, SU1498 was unobtainable from manufacturers due to discontinuation of production. SU5416 was used instead in this one experiment. SU5416 is a compound similar to SU1498 made by the same manufacturer. It is another small molecule VEGFR2 tyrosine kinase inhibitor, albeit somewhat less potent than SU1498 (e.g., IC_{50} for SU5416 = 1.04µm; Fong et al., 1999). Like SU1498, SU5416 is preferential for VEGFR2 but can also inhibit the activity of some other receptors, albeit it with lower efficacy (i.e., IC_{50} for FGF = 50µm; Fong et al., 1999).

VEGFR1 and VEGFR2 Neutralizing Antibodies: VEGFR1 and VEGFR2 neutralizing antibodies (R&D Systems, Minneapolis, MN) were stored frozen until use then diluted to 20µg/ml and delivered intrahippocampally at 0.5µl/h via an osmotic minipump. This dose was determined from previously published data demonstrating effectiveness of this antibody in an *in vivo* model of traumatic brain injury (Krum et al., 2008).

IgG1 Isotype Control Antibody: IgG1 (R&D Systems) was stored frozen until use then diluted to 20µg/ml and used as an isotype control antibody for the VEGFR1 and VEGFR2 antibodies. This was delivered intrahippocampally at 0.5µl/h via an osmotic minipump

Phosphate Buffered Saline (PBS): PBS was purchased in powder form and diluted in distilled water. PBS was autoclaved prior to use. PBS was also used as a control solution for VEGF when inactivated VEGF was unavailable.

Cannula and Pump Implantation for Protein Infusion

Animals were implanted unilaterally with cannulae or bicannulae (two fused cannulae, 1mm apart) into the hippocampus (Plastics One, Roanoke, VA, USA).Bi-cannulae were used for experiments requiring simultaneous infusions of two reagents and in which one of the reagents necessitated dilution into DMSO. DMSO reagents were not placed in the same pump as VEGF to avoid any potential destabilizing effects of DMSO on the VEGF protein. Animals were anesthetized by intraperitoneal injections of 75mg/kg sodium pentobarbital (Sigma-Aldrich). The scalp was shaved, cleaned with alcohol, and treated with iodine. Animals were placed into a stereotaxic apparatus. A longitudinal incision was made along the scalp. Three burr holes were drilled and two anchor screws (Plastics One) were attached to the skull. Cannulae were 4mm in length and sterilized prior to use. Each cannula was attached to a polyvinyl catheter (Plastics One) containing sterile PBS and heat-sealed to prevent leakage. Cannulae were implanted unilaterally into the dorsal hippocampus (3.8 mm posterior and 2.7 mm lateral relative to bregma), so that each tip would be positioned in the lateral portion of the dentate hilus. This location was chosen based on data demonstrating that VEGF diffuses over a 1.5 mm radius (Croll et al., 2004), and hence good coverage of the dorsal hippocampus could be accomplished from this cannula location. The cannulae were secured into place on the skull with dental acrylic and surrounding screws. Wounds were closed with polyvinyl sutures and a topical antibacterial ointment was applied. Animals then recovered under heat lamps.

Pumps were added one week after cannula implants to allow for surgical trauma to settle. Animals were briefly anesthetized with 2% isoflurane in 100% oxygen to allow for pump implantation. An incision was made in the nape of the neck. The heat-sealed end of the catheter was exposed and snipped off with scissors, and an Alzet osmotic minipump (Durect Corporation, Palo Alto, CA, USA) infusing the proper solution at a rate of.5µl/h was attached to each catheter (for a total of 1 pump per animal with single cannula and two pumps per animal with bicannulae). Pumps were re-inserted into the sub-cutaneous space in the nape of the neck, and the incision was closed with polyvinyl sutures. We have previously determined, using green dye in the pump, that it takes 21-22 hours for the solution in the pump to reach the brain (Croll et al., unpublished observations).

Acute Seizure Induction

Acute seizures were induced five days after pump implantations using 350mg/kg pilocarpine hydrochloride (Sigma-Aldrich) intraperitoneally administered 30 minutes after a subcutaneous injection of 1mg/kg atropine methylbromide (Sigma-Aldrich) to prevent peripheral side effects. After injection with pilocarpine, animals were monitored continuously for tracking of progression through various seizure stages. Seizures were scored from stages 1-8 based on a previously published modification of Racine's (1972) scale (Rudge et al, 1998). Briefly, stages 1-4 were identical to Racine's stages such that stage 1 was behavioral arrest, stage 2 was tremors and gnawing; stage 3 was unilateral forelimb clonus; and stage 4 was bilateral forelimb clonus. The scale was expanded to define stage 5 as transient whole-body tonus. Stages 6 and 7 were consistent with status epilepticus defined as more than 5 minutes of continuous seizures without intervening return to normal behavior. Stage 6 was characterized by continuous rearing and falling while stage 7 involved episodes of stage 5 seizures. Stage 8 was defined as status

epilepticus-induced death (Rudge et al., 1998). Latency to seizure onset, as measured by length of time to achieve the first stage 4-7 seizure, was also recorded. Seizures were truncated with 10mg/kg diazepam (Henry Schein, Melville, NY, USA) intraperitoneally after either 1 hour of status epilepticus or when prolonged tonic seizures were noted. Any animal that did not achieve status epilepticus within 90 minutes of pilocarpine injection was treated with diazepam. Animals were carefully monitored and hydrated with dextrose-saline or saline two hours following diazepam injections. In addition, animals were given fresh fruit for at least 24 hours after status to encourage rehydration and resumption of feeding. The experimental timeline is diagrammed below (Figure 2):

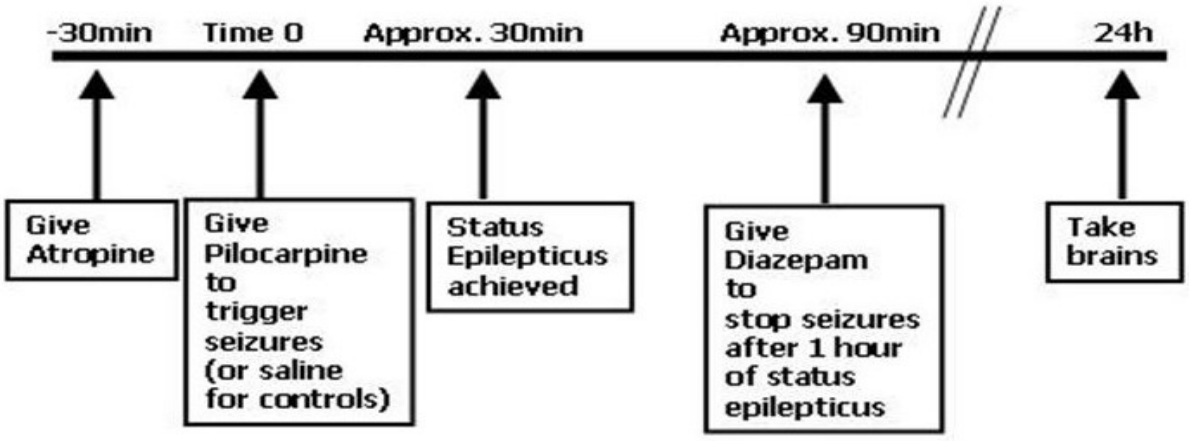

Figure 2: Experimental timeline for induction of seizures.

Bolus VEGF Injections

Surgical procedures for bolus VEGF injections were similar to that of the cannula implants described above with several exceptions. 4mm guide cannulae (33GA, Plastics One) with dummy tops were unilaterally implanted into the dorsal hippocampus, and secured with dental acrylic and two anchor screws. The wound was partially closed with polyvinyl sutures permitting the dummy top of the guide cannula to be exposed. One to two weeks later, animals were injected with pilocarpine to induce status epilepticus. Bolus VEGF 1 µl injections of

60ng/ml VEGF) or inactivated VEGF control was injected over a 10 minute period through the guide cannulae using a microliter syringe 30 to 60 minutes after status epilepticus was truncated with diazepam The syringe was withdrawn following a 2 minute rest period.

Tissue Collection

24 hours after seizures, animals were deeply anesthetized with a pentobarbital-based euthanasia solution (Euthasol, Henry Schein). The chest cavity was opened and a needle was inserted into the left ventricle of the heart. Heparinized isotonic (0.9%) saline was transcardially perfused to exsanguinate the animals. The animals were then perfusion fixed with 4% paraformaldehyde first in acetate then borate buffer (approximately 200mL of each) as previously described (Croll et al., 1999). The brains were removed and placed in 30% sucrose in borate buffer for 3-7 days at 4^{o} C until they were sectioned. The brains were sectioned coronally using a sliding microtome (Microm International, Walldorf, Germany) at 50µm and stored in 24-well plates in ethylene glycol-based cryoprotectant (Watson et al., 1986) at -20^{o} C until they were stained.

Stereological Quantifications of Neuronal Density

Stereological techniques were used to quantify density of CA1 pyramidal cells. Sections were mounted in a one in six series, hydrated through graded ethanols, and stained with 1.6%/1% Methylene Blue/Azure II solution following immersion in 1% periodic acid.

Only animals that achieved status epilepticus were included in CA1 damage scores. Estimates of CA1 density were determined by the optical fractionator method (West et al., 1991). The region of interest within the hippocampus was in the diffusion range of 1.5mm radius from the cannula tip. The area of interest was defined as the CA1 pyramidal cell layer between area CA2 and the subiculum in the medial-lateral axis, and from immediately prior to the initial

appearance of the CA1 pyramidal cell layer to the portion of the hippocampus where the dorsal and ventral portions of area CA3 unite in the rostral-caudal axis. Within the rostral-caudal axis the area of interest was defined by 2.3mm rostral from bregma and 4.3mm caudal from bregma. Stained sections were viewed with an Olympus BX-51 microscope and Optronic video camera. Using StereoInvestigator stereology software (MicroBrightfield, Inc., Williston, VT) a 25μm^2 counting frame was moved along a randomly placed grid (100μm^2) systematically. Cell nucleoli within CA1 of the hippocampus were counted if they were focused within a portion of the section, excluding 3μm thick upper and lower guard zones. Cells with a darkly stained nucleolus surrounded by a lightly stained nucleus and cytoplasm qualified for counting. Pyknotic neurons with a dense profile were not deemed viable and were therefore not counted. The total number of viable neurons in the previously defined region of interest were estimated with the formula N=sum Q-x1/tsfx1/asf/1/ssf where the number of neurons counted (sum Q-) was multiplied by the reciprocal value of the sampling probabilities based on the proportion of section thickness (tsf), cell layer area (asf), and total number of sections (ssf). Stereological analyses were conducted by an examiner blind to the treatment groups.

Immunofluorescent Staining

Rat hippocampal tissue sections were isolated and fixed with 4% paraformaldehyde. Tissue sections were heated in 10mM Sodium Citrate for 10 minutes for antigen retrieval, then permeabilized and blocked in PBST with 1% BSA, 0.25% Triton X-100 at room temperature for 1 hour, followed by double staining with antibodies for anti-phospho-VEGFR2 and anti-NeuN (neuronal marker). Images were captured at 63X magnification by using a Leica confocal microscope TCS SP2.

Western Blot

Western blots were also used to determine protein densities following application of Sugen1498. A similar experiment to that described above was conducted, however fresh hippocampal tissue was collected instead of tissue for histology at 24 hours post-status epilepticus. Fresh tissue was immediately frozen in dry ice. Protein was extracted with use of a BioMasher disposable micro homogenizer in a lysis buffer as previously described (Rockwell, et al., 2004). Protein concentrations were determined with a bicinchoninic acid test (BCA) according to manufacturer's instructions (Pierce, Rockford, IL). Equal amounts of protein from each lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were blocked and incubated overnight at 4^0C with the indicated primary antibodies. Immunoreactive bands were detected with anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase and visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Endogen, Rockford, IL). All Western blot analyses on the tissue from the experiment were conducted by Tianfeng Hao from Patricia Rockwell's laboratory at Hunter College of CUNY in a collaborative effort.

Statistical Analysis

To determine significant differences between groups, either an independent groups t-test (comparing two treatment groups) or a 2 (VEGF) x 2 (inhibitor) independent groups factorial analysis of variance (ANOVA) were used depending on the design of the experiment. All analyses were conducted using SPSS software (Version 15) at $\alpha = .05$.

Specific Aim Methods

Specific Aim 1: Explore the Neuroprotective Role of VEGFR2

Specific Aim 1a: Investigate effects of VEGFR2 blockade with the VEGFR2 specific tyrosine kinase inhibitor SU1498 or SU5416 during status epilepticus. A VEGFR2-specifc tyrosine kinase inhibitor (either SU1498 or SU5416) was administered alone or concomitant with VEGF intrahippocampally during status epilepticus to determine potential effects of inhibition of VEGFR2 signaling on VEGF-mediated neuroprotection. Animals were surgically implanted with bicannulae directed into the dorsal hippocampus. Each bicannulae was attached to 2 pumps. One pump carried either a Sugen compound in 75% DMSO or PBS in 75% DMSO. The other pump carried either active VEGF protein or freeze-thaw inactivated VEGF (45ng/day) dissolved in PBS (see General Methods). The DMSO and inactivated VEGF groups served as the control groups while the VEGF and SU1498/SU5416 groups served as the experimental groups in a 2 x 2 factorial design as illustrated below:

	Vehicle Control (75% DMSO)	SU1498/SU5416
Inactivated $VEGFA_{165}$ (Control)	n=8	n=8
$VEGFA_{165}$	n=8	n=8

Protein infusions continued for 5 days at which time pilocarpine was administered to induce status epilepticus using the timeline diagrammed below. Animals were sacrificed and tissue was collected 24-hours following severe seizures. Tissue sections were stained by Tianfeng Hao in Patricia Rockwell's lab with an immunofluorescent antibody to phosphoVEGFR2 to validate functional inhibition of VEGFR2 with SU1498 following status epilepticus. A second series of sections were mounted and stained with methylene blue. In a second experiment, protein analysis

by Western blot was conducted to analyze markers of oxidative stress and inflammation (see General Methods for more detailed procedures). Data were statistically analyzed using 2x2 factorial ANOVAs. Post-hoc treatment simple main effects were analyzed using Fisher's LSD. This study was completed with 2 cohorts of subjects. We ensured the absence cohort effects and the data was therefore merged.

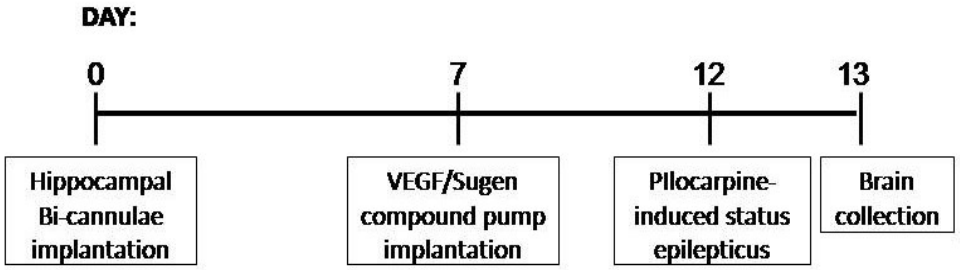

Figure 3: Experimental timeline of VEGF/Sugen compound administration, seizure induction, and brain collection.

Specific Aim 1b: Investigate effects of VEGFR2 blockade with a neutralizing antibody against VEGFR2 during status epilepticus. To further explore effects of VEGFR2 blockade during VEGF-mediated neuroprotection, a neutralizing antibody against VEGFR2 was administered either alone or concomitant with VEGF during pilocarpine induced status epilepticus. An alternative mechanism of VEGFR2 inhibition is warranted given that the SU1498/SU5416 agent can block VEGFR2 signaling by both the surface-bound VEGFR2 receptor and also on intracellularly localized VEGFR2 whereas anti-VEGFR2 only blocks VEGF binding to VEGFR2 at the cell membrane. Animals were implanted with a cannula directed into the dorsal hippocampus. Each cannula was attached to a catheter and pump. Each pump carried either active VEGF protein (45ng/day) or freeze-thawed inactivated VEGF control and either anti-VEGFR2 antibody (20μg/ml) or antibody control matched for IgG isotype. Some animals

received PBS as the control for the VEGF protein, but no statistical difference was detected between results from animals receiving the PBS or inactivated VEGF controls. Because VEGF and the VEGFR2 neutralizing antibody both requires PBS as their vehicle solution, both reagents were administered through the same pump and cannula. The inactivated VEGF and IgG isotype groups served as the control groups while the VEGF and the anti-VEGFR2 antibody groups served as the experimental groups in a 2 x 2 factorial design as illustrated below. This study was completed with 2 cohorts of subjects. We ensured the absence cohort effects and the data was therefore merged.

	IgG Isotype Control	Anti-VEGFR2 Antibody
Inactivated VEGF Control (or PBS)	n=8	n=8
$VEGFA_{165}$	n=8	n=8

Protein infusions continued for 5 days at which time pilocarpine was administered to induce status epilepticus as illustrated in the timeline diagrammed below. Animals were sacrificed and tissue was collected 24-hours following severe seizures. Brain tissue was sectioned, mounted, and stained with methylene blue. Pyramidal neurons was counted using StereoInvestigator stereology software to determine whether effects of VEGFR2 inhibition with a VEGFR2 neutralizing antibody is similar to that of inhibition with SU1498 and if this antibody alters VEGF's neuroprotective profile. To further test this hypothesis, Tianfeng Hao from Patricia Rockwell's laboratory stained tissue immunocytochemically in both conditions for VEGFR2 and VEGFR2 phosphorylation to determine localization and activation of VEGFR2, thereby verifying inhibition loci (see General Methods for more detailed procedures). Data were statistically analyzed using 2x2 factorial ANOVAs. Post-hoc treatment effects were analyzed

using Fisher's LSD. This study was completed with 3 cohorts of subjects. We ensured the absence of cohort effects and the data was therefore merged.

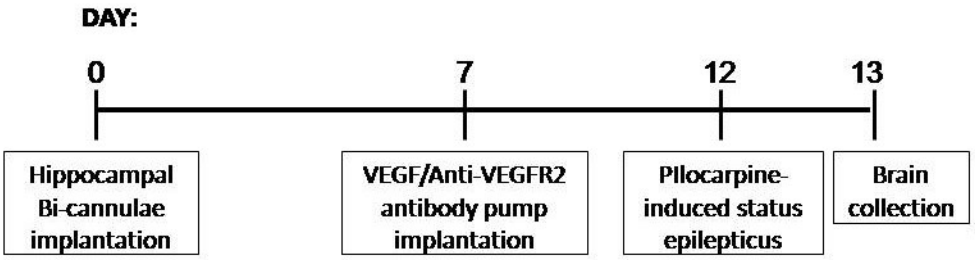

Figure 4: Experimental timeline of VEGF/Anti-VEGFR2 administration, seizure induction, and brain collection.

Specific Aim 2: Explore the Neuroprotective Role of VEGFR1

Specific Aim 2: Investigate effects of VEGFR1 blockade with a large molecule neutralizing antibody against VEGFR1 during status epilepticus. To establish effects of VEGFR1 inhibition during VEGF-mediated neuroprotection, a neutralizing antibody against VEGFR1 was administered either alone or simultaneously with VEGF during pilocarpine induced status epilepticus. Animals were implanted with a cannula directed into the dorsal hippocampus. Each pump carried active VEGF protein (45ng/day) and either anti-VEGFR1 antibody (20μg/ml) or antibody control matched for IgG isotype. The IgG isotype + VEGF group served as the control group while the anti-VEGFR1 antibody + VEGF group served as the experimental group in a 2 group independent groups design as illustrated below.

	IgG Isotype Control	Anti-VEGFR1 Antibody
$VEGFA_{165}$	n=8	n=8

Protein infusions continued for 5 days at which time pilocarpine was administered to induce status epilepticus as illustrated in the timeline diagrammed below. Animals were sacrificed and tissue was collected 24-hours following severe seizures. Brain tissue was sectioned, mounted,

and stained with methylene blue. Pyramidal neurons were counted using StereoInvestigator stereology software to determine the effects of VEGFR1 inhibition with a VEGFR1 neutralizing antibody during VEGF-mediated neuroprotection following status epilepticus. (See General Methods for more detailed procedures). Data was statistically analyzed using an independent groups t-test.

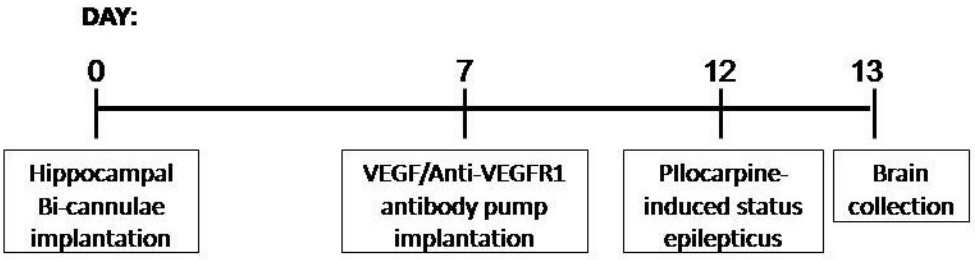

Figure 5: Experimental timeline of VEGF/Anti-VEGFR1 administration, seizure induction, and brain collection.

Specific Aim 3: Explore Effects of VEGF Post-Treatment

Specific Aim 3: Determine potential neuroprotective effects of acute VEGF administration immediately following status epilepticus. Neuroprotective effects of pre-application of VEGF have been established in a pilocarpine model of status epilepticus; however, effects of VEGF post-treatment have not yet been explored. To determine effects of VEGF post-treatment, guide cannulae were implanted into the dorsal hippocampus. One week later animals were injected with pilocarpine to induce status epilepticus. Bolus VEGF or inactivated VEGF control were injected through the guide cannulae using a microliter syringe 30 to 60 minutes after status epilepticus is truncated with diazepam. The design is illustrated below.

	Pilocarpine
Inactivated VEGF	n=8
$VEGFA_{165}$	n=8

Brains were collected 24 hours later as illustrated in the experimental timeline below. Brain tissue were sectioned, mounted, and stained with methylene blue. Hippocampal neuronal density estimates were made usingStereoInvestigator Software to determine neuroprotective potential of exogenous VEGF administration post-ictally. (See General Methods for more detailed procedures). Data were statistically analyzed using an independent groups t-test. This study was conducted with only 1 cohort and therefore findings will be deemed preliminary.

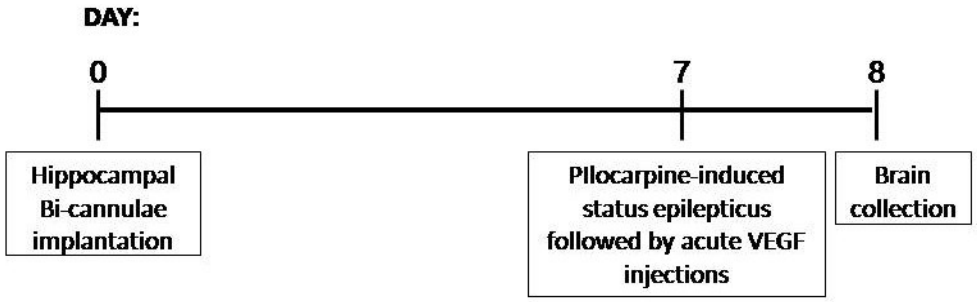

Figure 6: Experimental timeline of seizure induction, VEGF administration, and brain collection.

Chapter 3: Results

Specific Aim 1: Determine if VEGF-mediated neuroprotection is mediated by VEGFR2

Specific Aim 1a: Investigate effects of VEGFR2 blockade on VEGF-mediated neuroprotection using the VEGFR2 specific tyrosine kinase inhibitor SU1498 (or SU5416) during status epilepticus.

Previous research in our laboratory has demonstrated that VEGFR2 is upregulated 24 hours following status epilepticus (Nicoletti et al., 2005). We chose to verify that VEGFR2 is indeed upregulated following *both* status epilepticus and VEGF administration, and to determine whether it is also activated. We found that VEGFR2 was both upregulated and activated after status, and that the presence of VEGF enhanced phosphorylation of the upregulated VEGFR2 following status epilepticus (Figure 7).

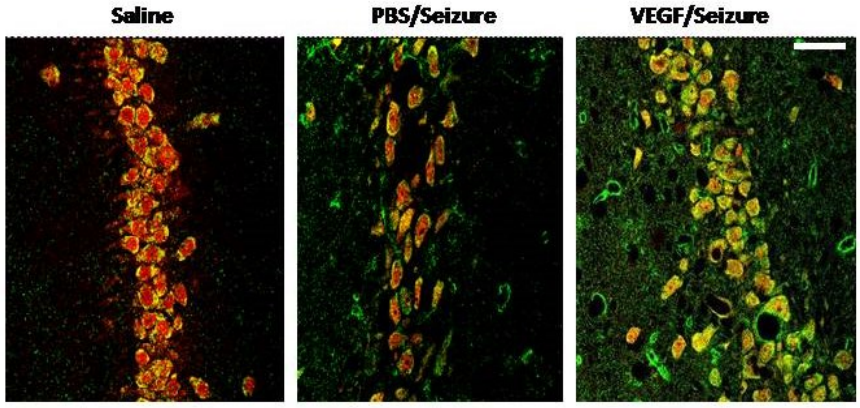

Figure 7: VEGF increased VEGFR2 activation in hippocampal neurons 24 hours post-status epilepticus. Green is phospho-VEGFR2; red is NeuN, a neuronal marker; yellow is colocalization. Scale bar = 40 μm.

Experiment 1: Confirming tolerability of DMSO: SU1498/SU5416 requires DMSO as a diluent, which few labs have applied directly to the brain. We therefore conducted pilot studies to evaluate the morphological effects of DMSO alone in the hippocampus. Animals infused with 75% DMSO intrahippocampally for six days did not show gross anatomical abnormalities as

compared with animals receiving PBS (Figure 8). While control animals in these experiments will still need to receive DMSO so that comparisons can be made against the appropriate vehicle, this pilot study suggested that DMSO could safely be used as a diluent for intrahippocampal infusions at this concentration.

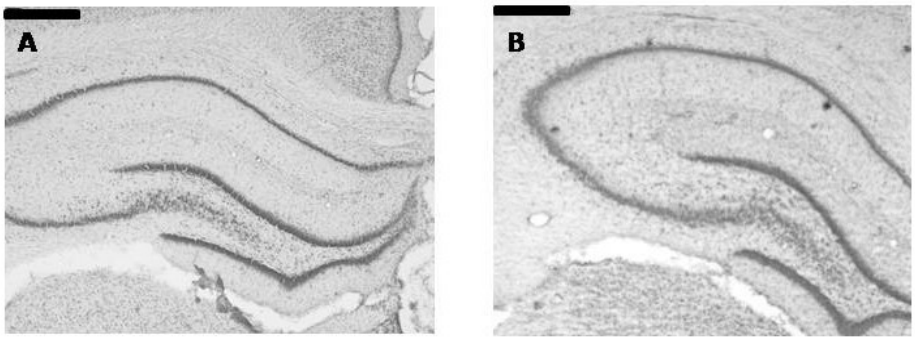

Figure 8: Six days of 75% DMSO infusion at a rate of 0.5µl per hour resulted in no gross pathology in the hippocampus as evidenced by apparently normal anatomy in the DMSO-infused rat. Photomicrographs of Nissl-stained sections adjacent to the cannula track are shown for a A) PBS-infused and B) DMSO-infused hippocampus. Scale bar = 1mm.

Experiment 2: Validation of SU1498's inhibition of VEGFR2 activation: To ensure that the SU1498 functioned as an inhibitor of the VEGFR2 receptor in our paradigm, SU1498-infused tissue was immunostained with an antibody against phosphorylated VEGFR2 (Figure 9). Fluorescent images revealed that the SU1498 compound markedly diminished phosphorylation of VEGFR2 in CA1 of the hippocampus relative to DMSO alone. While SU1498 has the potential to block VEGFR2 signaling at the cell surface and intracellularly, SU1498 demonstrated preferential blockade of phosphorylation of VEGFR2 intracellularly in this model. This intracellular signaling appeared to be nuclear or perinuclear, suggesting internalization of VEGFR2.

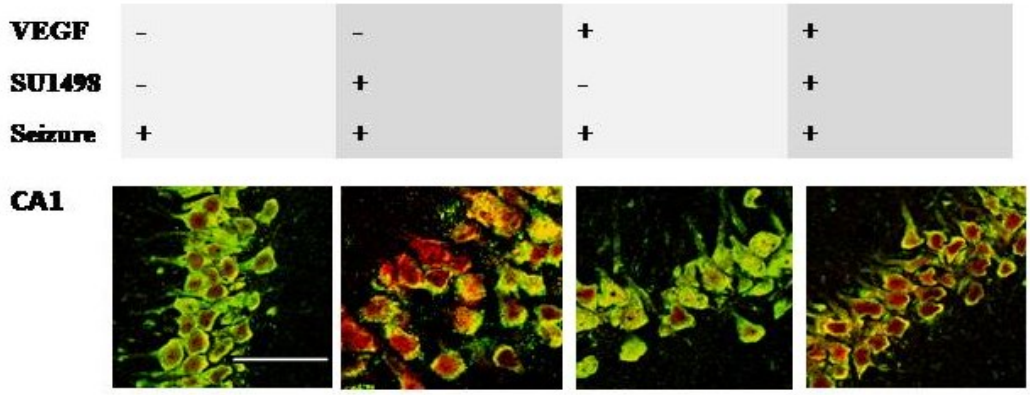

Figure 9: The VEGFR2 kinase inhibitor SU1498 decreases VEGFR2 phosphorylation following status epilepticus. Immuno-fluorescent images of hippocampal CA1 neurons reveal almost complete inhibition of intracellular VEGFR2 signaling and attenuated VEGFR2 phosphorylation on the cell membrane. Green is phospho-VEGFR2; red is NeuN, a neuronal marker. Scale bar = 30 µm.

Experiment 3: Neuroprotection: Treatment with SU1498/SU5416 failed to prevent VEGF's neuroprotective effects following status epilepticus, as assessed by stereological counts to evaluate CA1 pyramidal cell density (Figure 10A, effect of SU1498/SU5416: $F(1,10) = 0.605$, $p = .455$; effect of VEGF: $F(1,10) = 3.725$, $p = .082$; interaction effect $F(1, 10) = 0.946$, $p = .354$).

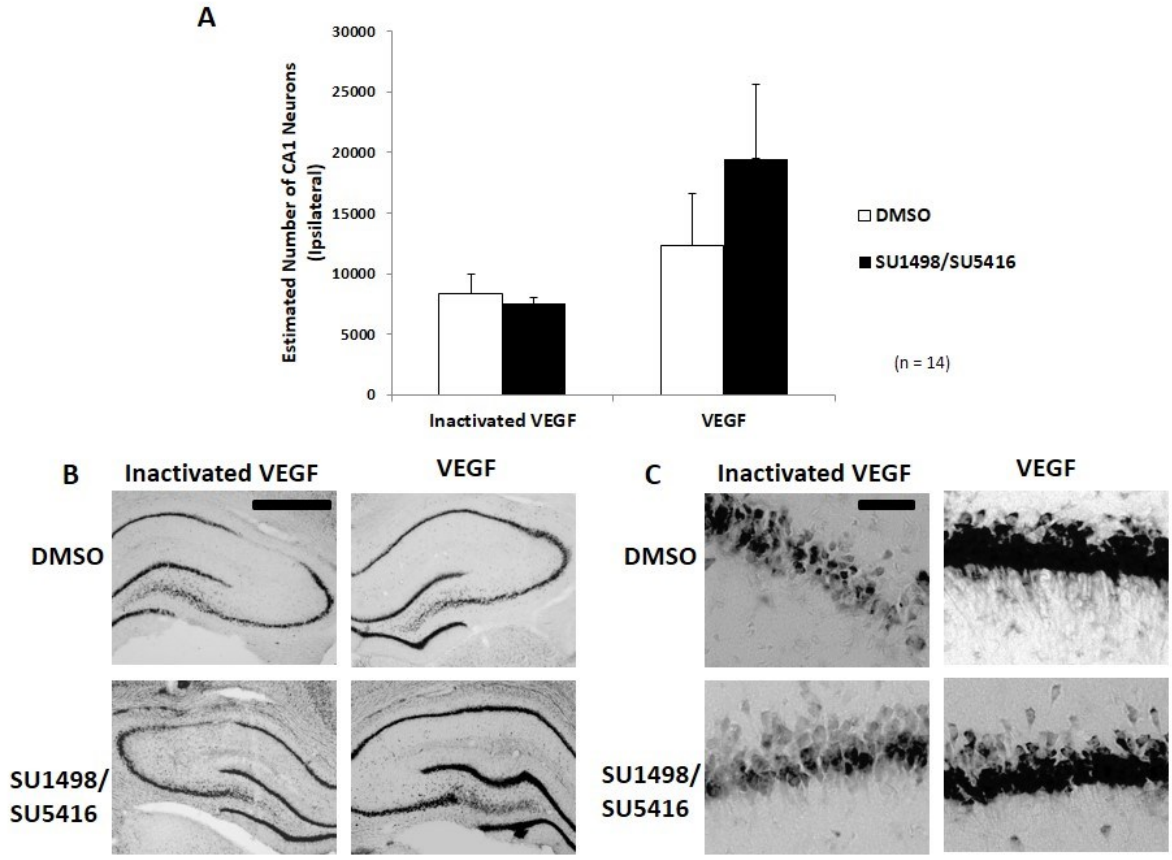

Figure 10: The VEGFR2 kinase inhibitor SU1498/SU5416 did not prevent VEGF-mediated neuroprotection following status epilepticus. **A**) Graph of stereological estimates of pyramidal cell density in CA1 of the hippocampus demonstrates that inhibition of VEGFR2 with SU1498 did not prevent VEGF-mediated neuroprotection (effect of SU1498/SU5416: $F(1,10) = 0.605$, $p = 0.455$; effect of VEGF: $F(1,10) = 3.725$, $p = 0.082$;. interaction effect $F(1, 10) = 0.946$, $p = 0.354$). B & C) Photomicrographs of Methylene Blue stained sections at 4x (**B**, scale bar = 1mm) and 60x (**C**, scale bar = 50 µm) of DMSO-infused and SU1498/SU5416-infused hippocampi with or without simultaneous administration of VEGF 24h post-status epilepticus.

E*xperiment 4: Markers of inflammation and oxidative stress:* Oxidative stress and inflammation were analyzed using immunofluorescent analysis and Western Blot techniques. Evidence of oxidative stress during pilocarpine-induced status epilepticus was detected with the biochemical marker heme-oxygenase-1 (HO-1). Inflammation during pilocarpine-induced status epilepticus was found with the biochemical marker cyclooxygenase-2 (COX-2), suggesting an increased

immune response. Results further show that oxidative stress and inflammation were most pronounced in the hippocampi of seized animals receiving the DMSO and PBS controls alone (Figure 11). In contrast, there was a pronounced decrease in both HO-1 and COX-2 in hippocampal tissue from seized animals receiving SU1498 alone or in combination with VEGF. VEGF alone decreased HO-1 but not COX-2 in this seized tissue, suggesting a functional role of VEGF in decreasing oxidative stress but not inflammation.

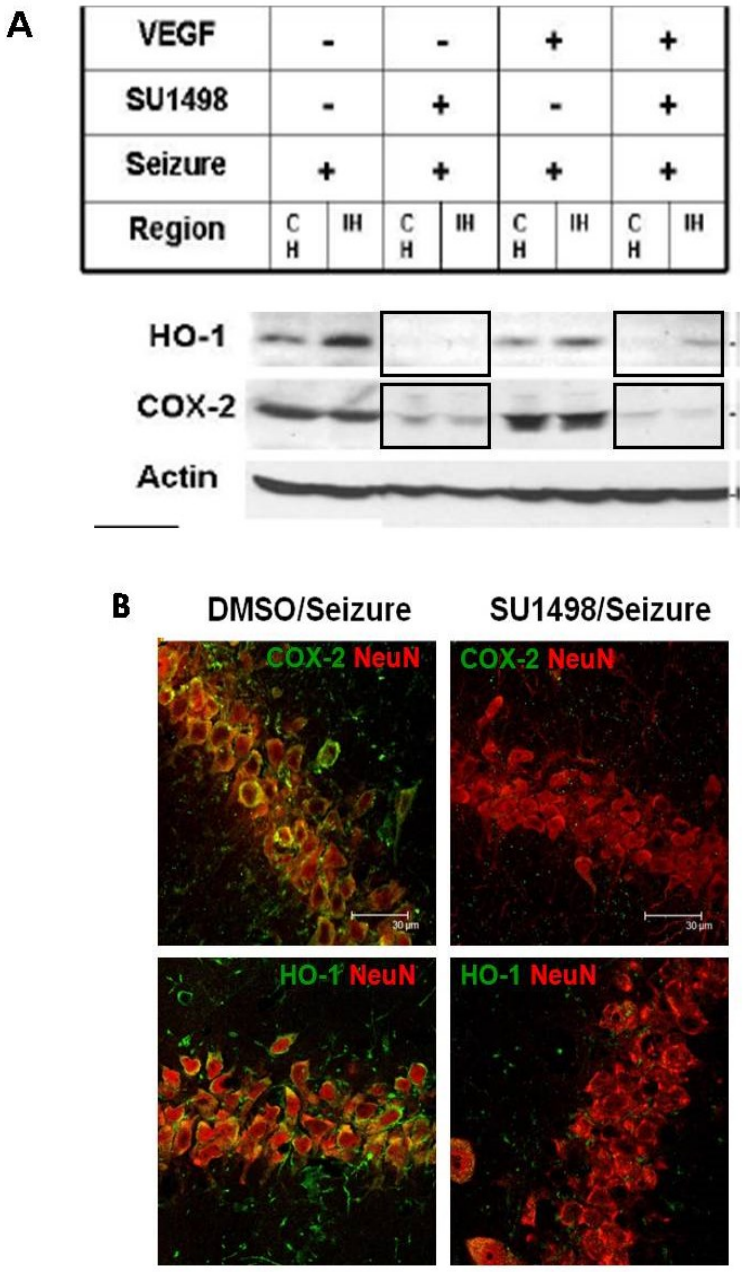

Figure 11: Hippocampal tissue from seized animals was probed for oxidative stress and inflammation using the biochemical markers HO-1 (heme-oxygenase-1) and COX-2 (cycloxygenase-2), respectively. Evidence of oxidative stress and inflammation was seen in DMSO-infused tissue and was reduced in the SU1498-infused tissue. A) Western Blot analysis and B) Immunofluorescent staining, Scale bar = 30µm. Note: IH=ipsilateral hippocampus; CH=contralateral hippocampus

Seizure severity and latency to seize: Neither VEGF nor SU1498/SU5416 had any effect on the severity of seizures achieved. However there was a tendency for VEGF to inhibit the severity of seizures in the presence of the DMSO control as compared to SU1498/SU5416 (Figure 12A: $F(1, 33) = 3.154$, $p=.085$). Furthermore, there was a tendency for both VEGF and SU1498/SU5416, independently, to delay the onset of tonic-clonic seizure behavior (stage 4 or higher). While our findings did not demonstrate any significant effect of VEGF or SU1498/SU5416 on seizure severity, there was a tendency for VEGF in combination with SU1498/SU5416 to decrease seizure severity as well as a tendency for both VEGF and SU1498/SU5416 independently, to delay the onset of seizures. Decreased seizure severity could be reasonably expected to impact neuronal loss. Seizure latency may also present as a possible confounding variable for VEGF's protective effects. In fact, there was a significant positive correlation between latency to seize and neuronal density, suggesting that latency to seize was predictive of neuronal loss in this experiment ($r = .814$, $n =14$, $p<.01$).

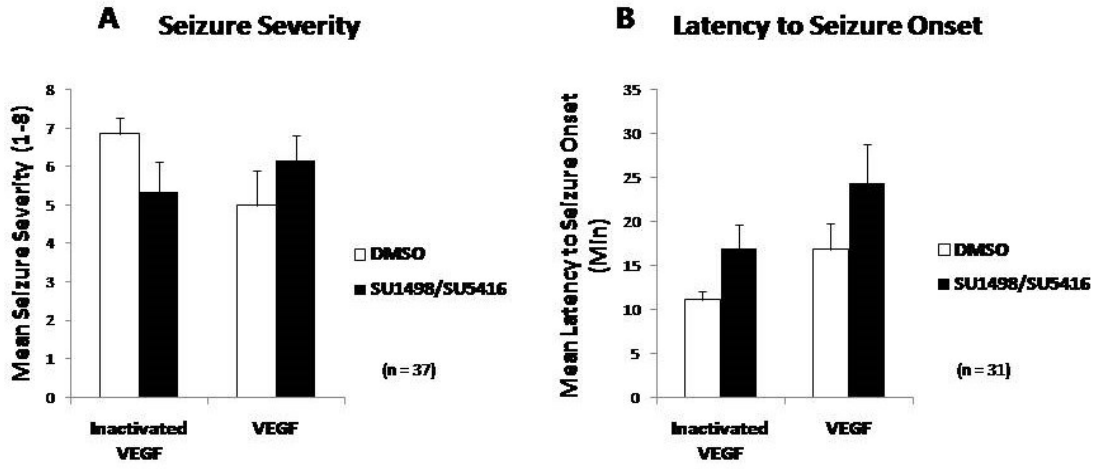

Figure 12: **A**) Neither VEGF nor SU1498/SU5416 independently significantly affected seizure severity (effect of VEGF: $F(1, 33) = 0.473$, $p = 0.497$; effect of SU1498: $F(1,33) = 0.60$, $p = 0.808$). There was a tendency for VEGF in combination with DMSO to decrease seizure severity (interaction effect: $F(1, 33) = 3.154$, $p = 0.085$) **B**) There was a tendency for both VEGF ($F(1,27) = 3.16$, $p = 0.087$) and SU1498/SU5416 ($F(1, 27) = 3.183$, $p = 0.086$) to delay onset of tonic-clonic seizures (interaction effect: $F(1,27)= 0.076$, $p = 0.785$).

Specific Aim 1a Interim Discussion:

Our results demonstrate that the VEGFR2 inhibitor SU1498 (or SU5416) did not prevent VEGF-mediated neuroprotection following pilocarpine-induced status epilepticus. This finding could suggest that VEGF-mediated neuroprotection may not act via a VEGFR2 signaling system. However, like most kinase inhibitors, the Sugen compounds, while highly preferential for VEGFR2, have been demonstrated to weakly inhibit other signaling systems. In addition, given that the Sugen compounds inhibit the signaling of both extracellularly and intracellularly localized VEGFR2 signaling, a potential neuroprotective role of cell membrane-bound VEGFR2 warrants investigation.

Specific Aim 1b: Investigate effects on neuronal loss of VEGFR2 blockade with a large molecule neutralizing antibody against VEGFR2 during status epilepticus.

Experiment 1: Validation of anti-VEGFR2's inhibition of VEGFR2 activation: Tissue was immunostained against both the phosphorylated form of VEGFR2 and against all VEGFR2 protein to ensure that the VEGFR2 antibody should only function to inhibit activation of membrane-bound VEGFR2.Antibody reagents have limited access to intracellular compartments, and preliminary work in the Rockwell lab suggested that VEGFR2 was also localized to intracellular compartments. Fluorescent images demonstrate that the anti-VEGFR2 neutralizing antibody successfully blocked VEGFR2 phosphorylation near the cell surface without altering receptor density (Figure 13).

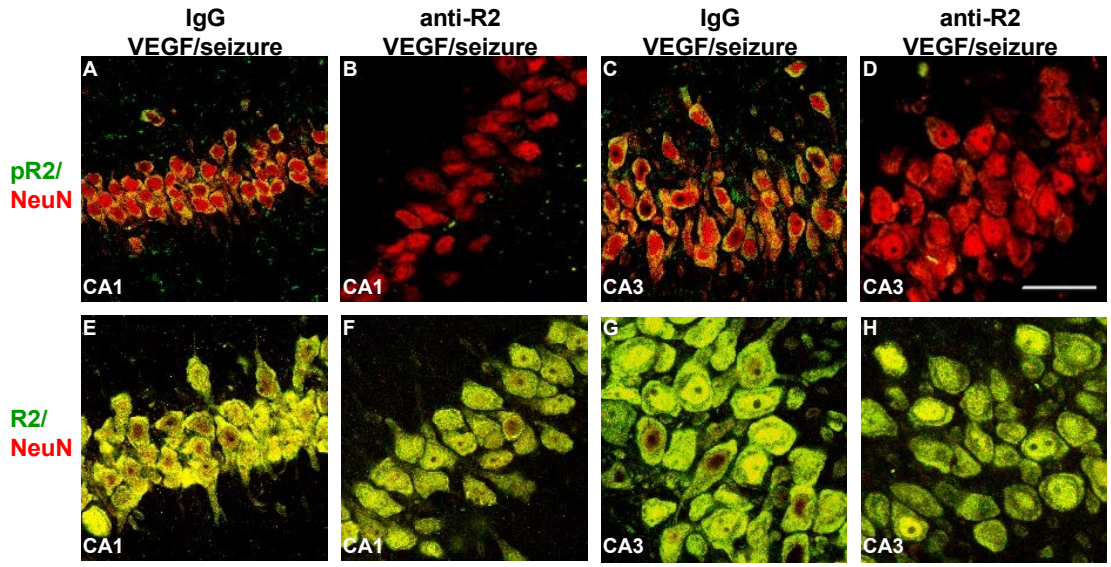

Figure 13: Anti-VEGFR2 blocks VEGFR2 phosphorylation following status epilepticus. Immuno-fluorescent images of hippocampal CA1 neurons demonstrate attenuation of VEGFR2 phosphorylation at the cell membrane. Green phospho-VEGFR2 (A-D) and VEGFR2 (E-G); red is NeuN. Scale bar = 30 μm.

Experiment 2: Neuroprotection: Treatment with the anti-VEGFR2 antibody during status epilepticus significantly increased neuronal damage following stereological measurements of CA1 neuronal density (Figure 14A, effect of AntiR2 antibody: $F(1, 10) = 6.419$, $p = 0.03$). However, VEGF alone did not protect neurons from death in this seizure model (Figure 14A, effect of VEGF: $F(1, 10) = 0.079$, $p = 0.784$). There was no interaction effect between VEGF and anti-VEGFR2 antibody treatments (Figure 14A, interaction effect: $F(1, 10) = 0.012$, $p = 0.916$).

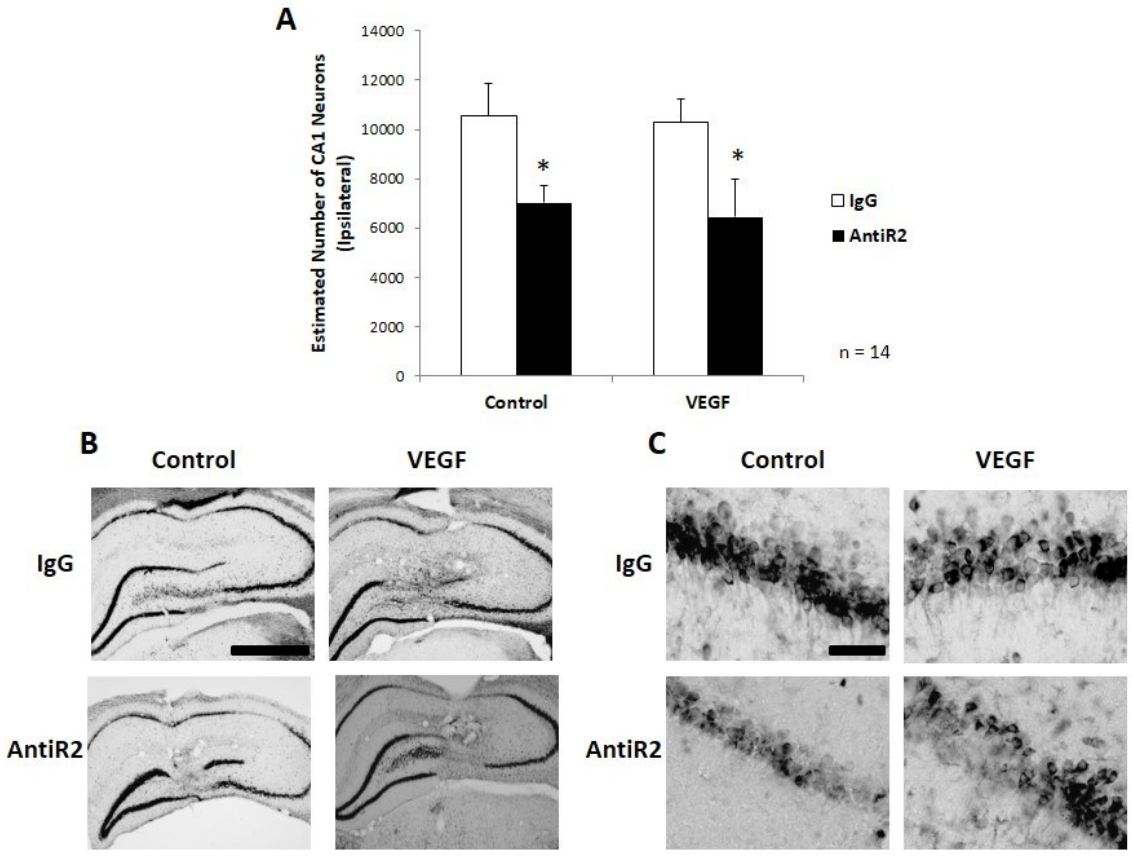

Figure 14: Treatment with the anti-VEGFR2 antibody (antiR2) contributed to greater neuronal damage following status epilepticus as compared to treatment with the IgG control antibody. **A**) Graph of stereological estimates of pyramidal cell density in CA1 of the hippocampus demonstrates that inhibition of VEGFR2 with anti-VEGFR2 caused greater neuronal loss (effect of anti-VEGFR2: $F(1,10) = 6.419$, $p = 0.03$; effect of VEGF: $F(1,10) = 0.079$, $p = 0.784$;. interaction effect $F(1, 10) = 0.012$, $p = 0.916$). **B&C**) Photomicrographs of methylene blue-stained sections at 4x (**B**, scale bar = 1mm) and 60x (**C**, scale bar = 50 μm) from IgG control-infused and anti-VEGFR2 antibody-infused hippocampi with or without simultaneous administration of VEGF 24h post-status epilepticus. Scale bar = 100μm. * anti-VEGFR2 is significantly different than IgG, $p < 0.05$.

Seizure severity and latency to seize: Neither VEGF nor the VEGFR2 neutralizing antibody had an effect on the severity of seizures achieved (Figure 15A, effect of VEGF: $F(1, 51) = 1.163$, $p = 0.286$; effect of AntiR2: $F(1,55) = 0.845$, $p= 0.362$; interaction effect: $F(1, 51) = 2.477$, $p = 0.122$). Neither VEGF nor the VEGFR2 antibody had an effect on the latency to onset of tonic-

clonic seizure behavior (stage 4 or higher), (Figure 15B, VEGF: F(1, 25) = 0.14, p = 0.711; AntiR2: F(1, 25) = 0.618, p = 0.439; Interaction effect: F(1,25) = 0.121, p = 0.731). Furthermore, latency to seizure onset was not predictive of neuronal loss in this experiment (r = 0.04, n = 14, p = 0.892

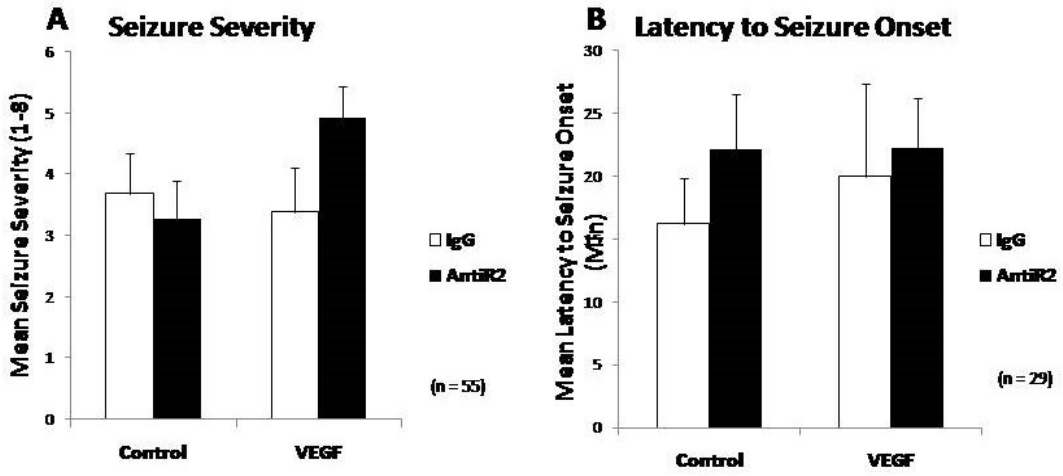

Figure 15: A) Neither VEGF nor the VEGFR2 antibody independently significantly affected seizure severity (effect of VEGF: F(1, 51) = 1.163, p = 0.286; effect of AntiR2: F(1,55) =0.845, p= 0.362). There was no interaction effect (F (1, 51) = 2.477, p =0.122). **B)** Neither VEGF nor the VEGFR2 antibody significantly effects seizure latency. Latency VEGF: F (1,25) = 0.14 ,p= 0.711) and AntiR2: F(1, 25) = 0.618, p = 0.439); interaction effect: F(1,25) = 0.121, p = 0.731).

Contribution of the VEGFR2 antibody to seizure severity: Although the VEGFR2 antibody did not influence seizure severity score or latency to seize, a non-parametric analysis revealed that animals receiving VEGF plus the VEGFR2 antibody were significantly more likely to reach stage 4 or greater seizures (Figure 16, $G^2 = 10.47$, $p < .05$).

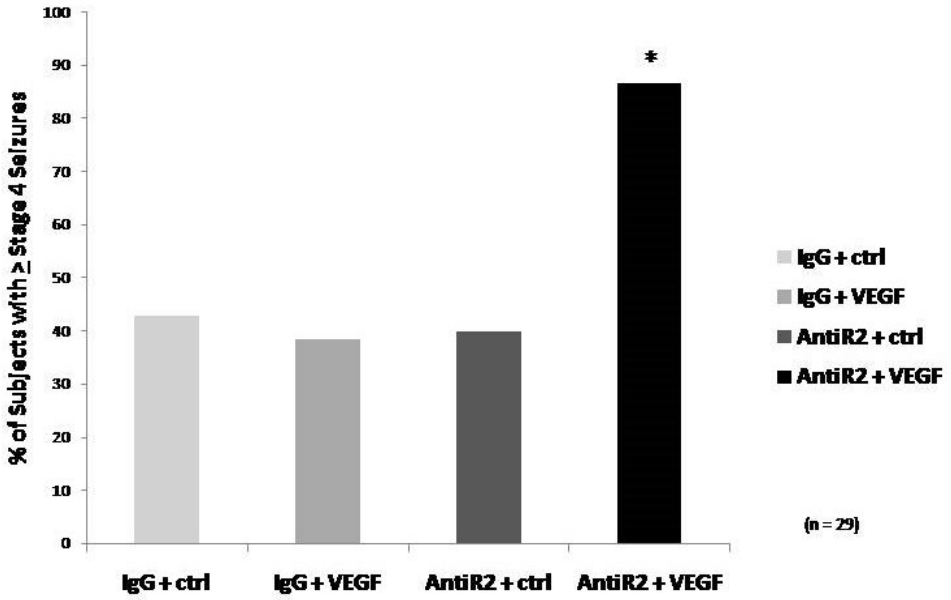

Figure 16: Animals treated with anti-VEGFR2 in combination with VEGF were more likely to advance to Stage 4 seizures or greater as (G^2 = 10.47, p <.05). (*Anti-VEGFR2 +VEGF significantly different than IgG + control, IgG + VEGF, and anti-VEGFR2 + control, p <0.05.

Experiment 3: VEGFR2 distribution: Tissue was immunostained against VEGFR2 to elucidate VEGFR2 distribution. Application of the anti-VEGFR2 antibody blocked VEGFR2 localization into the nucleus, which was not demonstrated with SU1498 application (Figure 17).

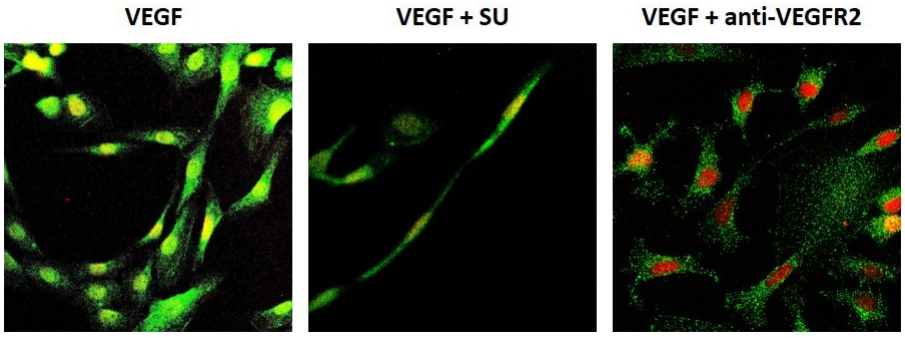

Figure 17: The anti-VEGFR2 antibody (but not SU1498) blocks VEGFR2 localization into the nucleus. Green fluorescence (FITC) is VEGFR2 immunoreactivity; red fluorescence (7-ADD) is a nuclear marker.

Specific Aim 1b Interim Discussion:

Our findings indicate that application of the anti-VEGFR2 antibody, a large molecule VEGFR2 inhibitor, leads to greater neuronal damage in the CA1 region of the hippocampus following pilocarpine-induced status epilepticus. The VEGFR2 antibody blocks VEGFR2 only on the cell surface by preventing VEGF binding to VEGFR2. The Sugen compound, which blocks VEGFR2 signaling directly, and does it both intracellularly and extracellularly, did not prevent VEGF-mediated neuroprotection. Therefore it is possible that VEGF mediates protection via a VEGFR2 mechanism specifically localized to cell surface VEGFR2 that involves either VEGF binding to VEGFR2 or specific activation of membrane-bound VEGFR2. Additionally, neither VEGF nor the VEGFR2 antibody affected seizure severity or latency to seize. However, animals were more likely to reach status epilepticus when given simultaneous infusions of VEGF and the VEGFR2 antibody. This finding may suggest that membrane-bound VEGFR2 may function to decrease the severity of seizures and not necessarily mediate a direct neuroprotective mechanism. Regression analyses between neuronal death and seizure severity could not be analyzed in this model, however, since neuronal death only occurred in animals achieving status epilepticus. That is, all animals demonstrating any neuronal loss would have the same seizure severity, "status".

One interesting possibility for the detrimental effects of anti-VEGFR2 but not SU1498 in this model is the removal of VEGFR2 as a binding site for VEGF. This effect of anti-VEGFR2 would result in higher extracellular levels of VEGF that would then be available to bind VEGFR1 or neuropilin-1. SU1498 would not have this same effect, as VEGF would still be free to bind VEGFR2, but would not transduce a signal. Because of this possibility, we next probed

the effects of anti-VEGFR1 to determine the effects of removing VEGFR1 as a VEGF binding site in this model.

Our findings also show that VEGFR2 was localized to the nucleus with SU1498 treatment, but not with anti-VEGFR2 antibody treatment. It is hypothesized that removal of the VEGFR2 binding site for VEGF via interference by the VEGFR2 antibody prevents VEGF from binding to VEGFR2 and prevents receptor internalization. This lack of VEGFR2 internalization may play a role in increased neuronal damage with the antibody against VEGFR2, although the mechanism by which this might happen is currently unclear.

Interestingly, VEGF did not demonstrate neuroprotective effects in this particular model. However, all animals in this model received IgG istotype as a control or as a diluent for the experimental treatment. It is possible that the IgG antibody may inhibit VEGF's protective effects by unknown mechanisms, perhaps by modulating immune system contributions to the hippocampal damage observed after status epilepticus.

Specific Aim 2: The role of VEGFR1 in VEGF-mediated neuroprotection in a rat model of status epilepticus

Specific Aim 2a: Investigate effects of VEGFR1 blockade with a large molecule neutralizing antibody against VEGFR1 with simultaneous VEGF treatment during status epilepticus.

Experiment 1: Neuroprotection: Pretreatment with the VEGFR1 antibody in combination with VEGF pretreatment during status epilepticus did not prevent VEGF-mediated neuroprotection as determined by stereological measurements of CA1 neuronal density (Figure18A: $t(9) = 1.502$, $p = 0.167$).

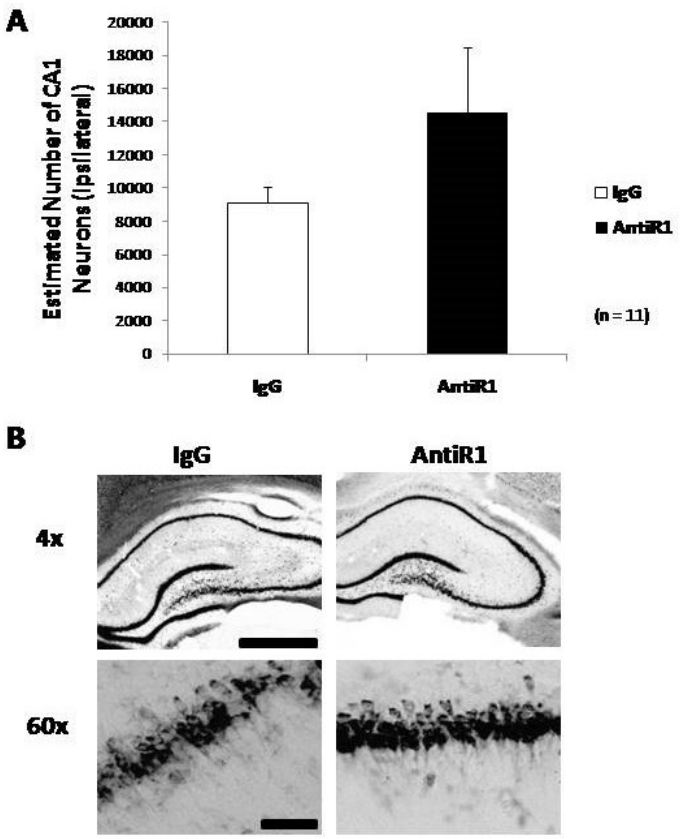

Figure 18: A) There was no significant difference in neuronal damage 24 hours after status epilepticus between animals that received treatment with the VEGFR1 antibody as compared to animals that received the IgG isotype control (both groups received simultaneous VEGF infusions) ($t(9) = 1.502$, $p = 0.167$;. **B**) Photomicrographs of methylene blue stained 4x and 60x sections of IgG control-infused and anti-VEGFR1 antibody-infused hippocampi 24h post-status epilepticus. Scale bar = 100μm.

Seizure severity and latency to seize: There was no significant difference in seizure severity between animals that received pretreatment with the VEGFR1 antibody as compared to those receiving the IgG control antibody (Figure 19A, $t(29) = 0.412$ $p = 0.683$). Furthermore, there was no difference in the latency to reach onset of tonic-clonic seizure behavior (stage 4 or higher) between animals receiving the VEGFR1 antibody as compared to the IgG control (figure 19B, $t(16) = 0.814$). Latency to seizure onset was also not predictive of neuronal loss in this experiment ($r= 0.11$, $n = 11$, $p = 0.747$)

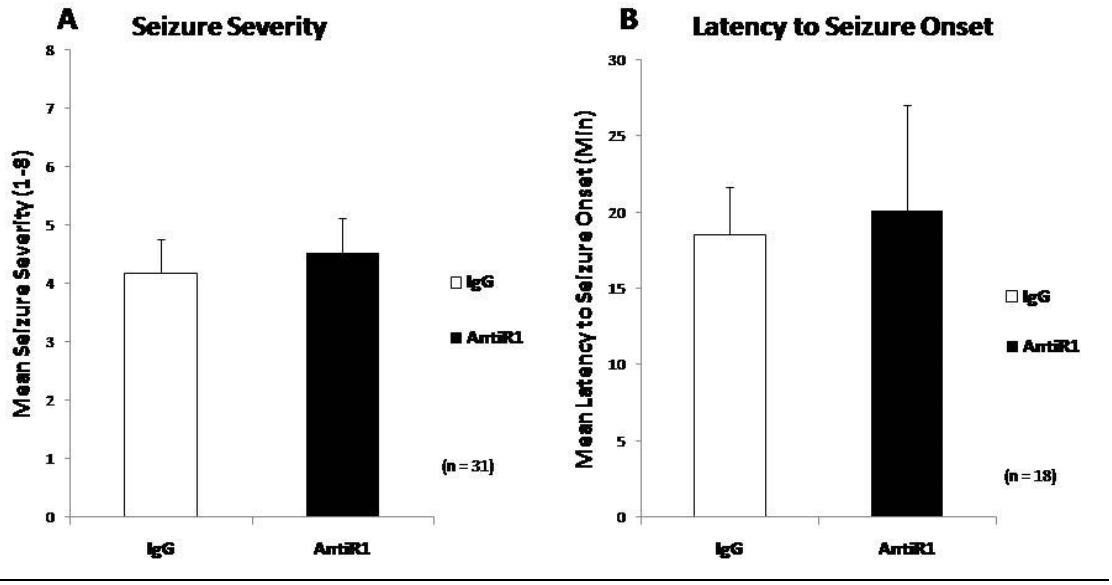

Figure 19: There was no significant difference in either **A)** seizure severity ($t(29) = 0.412$, $p = 0.683$) or **B)** latency to stage 4 seizures or higher ($t(16) = 0.204$, $p = 0.841$) between animals receiving pretreatment of the VEGFR1 antibody as compared to the IgG isotype control.

Contribution of the VEGFR1 antibody to seizure severity: Unlike findings with the VEGFR2 antibody, the VEGFR1 antibody did not influence the likelihood of achieving stage 4 or greater. Seizures (Figure 20, $\chi^2 = 0.01$, $p > 0.05$).

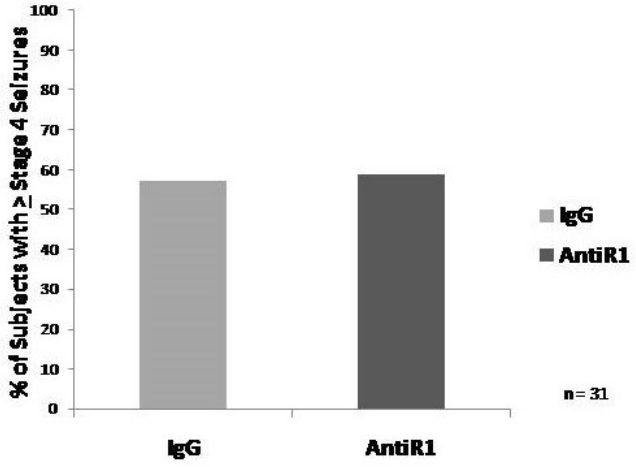

Figure 20: There was no difference in the likelihood that animals treated with anti-VEGFR1 or with IgG to advance to Stage 4 seizures or greater as (χ^2 (1, n = 31) = 0.01, p >.05)

Specific Aim 2a interim discussion:

These results demonstrate that inhibition of ligand binding to extracellular VEGFR1 during simultaneous VEGF infusions has no significant effect on VEGF's neuroprotective effects. This could suggest that VEGF's neuroprotective effects may be acting via a mechanism independent of VEGFR1 and may also not be influenced by an increase in VEGF available to other receptors with the high affinity VEGFR1 receptor not available to attract VEGF. Indeed, if anything, our findings did not demonstrate any effect of anti-VEGFR1 on seizure severity or latency to seize relative to the IgG isotype control-treated group. One interesting note is that the VEGFR1 receptor may not be as important an influence after VEGF pre-treatment as it would normally be, because VEGF infusion upregulates VEGFR2. This possibility may contribute to the lack of effect of VEGFR1 inhibition in this model.

Specific Aim 3: Determine if treatment with VEGF after status epilepticus can protect neurons, or if pre-treatment is necessary.

Specific Aim 3a: Determine potential neuroprotective effects of acute VEGF administration immediately following status epilepticus.

Experiment 1: Neuroprotection: In this ingle trial preliminary study, there was no significant difference in CA1 neuronal densities between animals that received bolus injections of inactivated VEGF control or active VEGF immediately following status epilepticus (Figure 21A, $t(6) = 0\ .054$, $p = 0.959$).

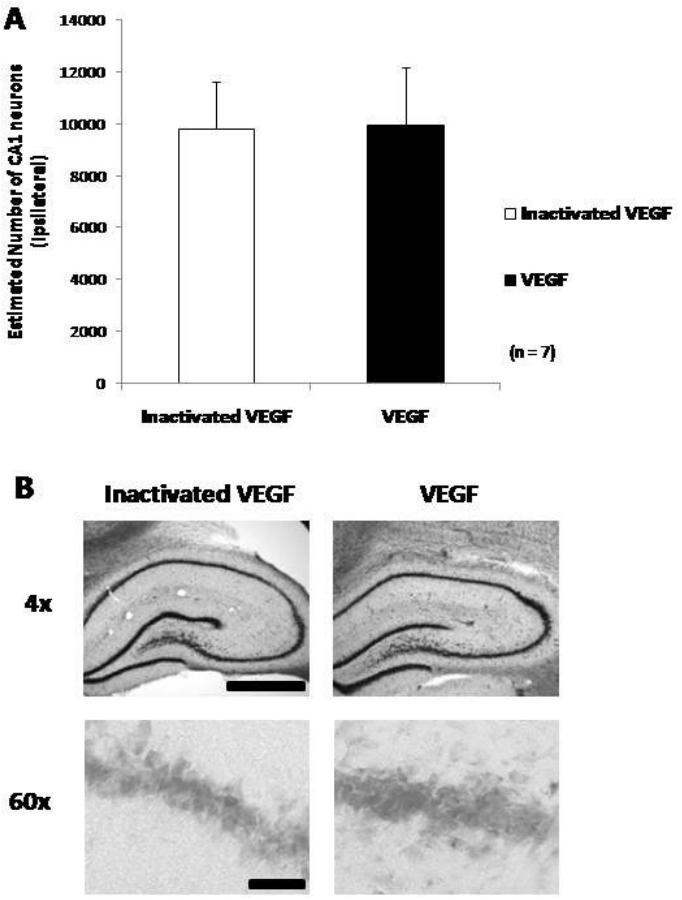

Figure 21: A&B) There was no significant difference in neuronal density in CA1 between animals receiving intrahippocampal injections of inactivated VEGF control and active VEGF immediately following status epilepticus ($t(5) = 0.054$, $P = 0.959$). A) Graph of stereological estimates of CA1 neuronal density. B) Photomicrographs of Methylene Blue stained sections at 4x and 60x 24 hours post-status epilepticus.

Seizure severity and latency to seize: As would be expected from a post-treatment paradigm, there was no significant difference in seizure severity (Figure 22A, $t(19) = 0.612$ $p = 0.548$) or seizure latency (Figure 22B, $t(14) = 0.998$, $p = 0.335$) between animals that received post-treatment of VEGF as compared to post-treatment of inactivated VEGF immediately following status epilepticus.

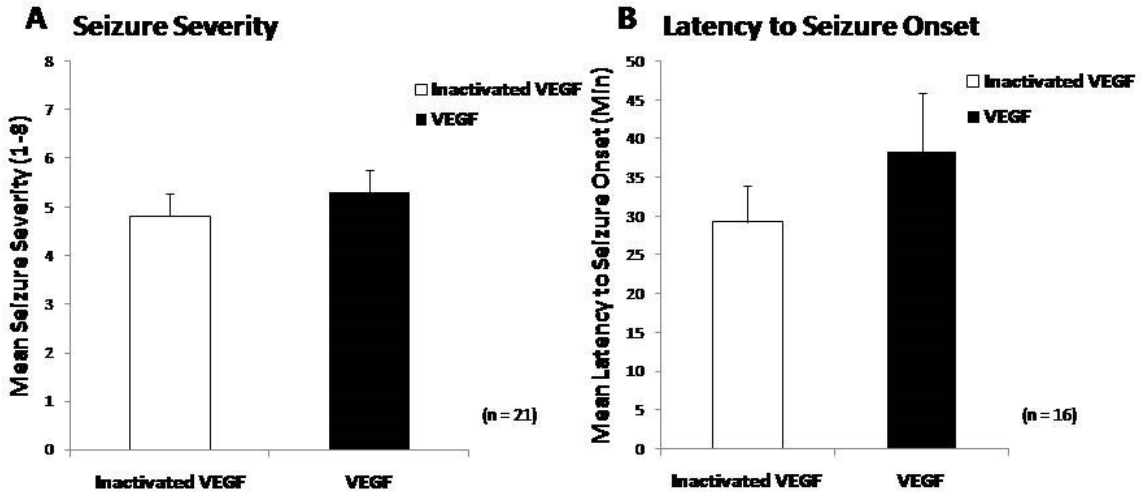

Figure 22: There was no significant difference in either **A)** seizure severity ($t(19) = .612$ $p = 0.548$) or **B)** latency to stage 4 seizures or higher ($t(14) = 0.998$, $p = 0.335$) between animals receiving post-treatment of the inactivated VEGF control or active VEGF, suggesting that groups were well-balanced for VEGF treatment assignment.

Specific Aim 3a interim discussion:

While pre-treatment with exogenous VEGF protects neurons from death 24 hours after status epilepticus, post-treatment with VEGF immediately following status epilepticus does not protect neurons from death 24 hour later. Given the finding that VEGF pre-treatment protects while VEGF post-treatment does not in our preliminary study, it is possible that pretreatment with VEGF serves to "dampen" seizure activity. This is further supported by previous findings demonstrating a trend for VEGF pre-treatment to lower the severity of seizures. Furthermore, preliminary findings have suggested that VEGFR1 may downregulate, and VEGFR2 may upregulate, following VEGF administration (unpublished). Therefore it is also possible that VEGF receptor alterations due to VEGF pre-treatment may be the reason for protection, and not necessarily the VEGF itself.

Chapter 4: Discussion

Epilepsy is a neurological disorder characterized by repeated unprovoked seizures. Acute severe seizures and/or repeated seizures will contribute to neuronal loss in limbic regions of the brain, especially the hippocampus. The protein VEGF has been shown to have neuroprotective effects in several models of neurological disorders and continues to be under exploration as a novel therapeutic mechanism. More specifically, VEGF has demonstrated neuroprotection in both *in vitro* and *in vivo* models of epilepsy. With use of an *in vivo* rat model, our lab has demonstrated that infusions of VEGF into CA1 during pilocarpine-induced status epilepticus decreases seizure-related neuronal death in this region 24 hours after seizures (Nicoletti et al., 2008). The current dissertation describes studies designed to test which VEGF receptor mediates VEGF's protective effects.

Abbreviated Summary of Current Results

In our experiments, we asked which VEGF receptor mediated VEGF's protective effects by administration of selective VEGF receptor inhibitors with VEGF during status epilepticus. We inhibited VEGFR2 both with 1) SU1498/SU5416, a tyrosine kinase inhibitor that can inhibit VEGFR2 signaling regardless of whether the receptor is localized to the cell surface or intracellularly (with preferential intracellular blockade) and with 2) an anti-VEGFR2 antibody. VEGFR1 activation was inhibited with an anti-VEGFR1 antibody. Both antibody reagents served to block VEGF binding to its receptors on the cell surface. In our studies, the Sugen compounds failed to prevent VEGF-mediated neuroprotection. While SU1498/SU5416 did not block VEGF-mediated protection, there is evidence that it decreased oxidative stress and inflammation. This observation was made even in animals with minimal neuronal damage. In contrast, application of the anti-VEGFR2 antibody, which blocks VEGF binding to extracellular

VEGFR2 only, contributed to more neuronal loss when simultaneously infused with either VEGF or inactivated VEGF control. Animals treated with anti-VEGFR2 in combination with VEGF were more likely to advance to more severe seizures. Simultaneous infusions of VEGF with the anti-VEGFR1 antibody, which blocked binding of VEGF to extracellular VEGFR1, did not contribute to any changes in neuronal density 24 hours following status epilepticus. Of note, VEGF administered with IgG alone, the vehicle control for the neutralizing antibodies, did not appear to be neuroprotective. Furthermore, neuronal perseveration was not seen when VEGF treatment was delayed until immediately following status epilepticus.

Speculated Neuroprotective Mechanisms

VEGF may protect neurons directly or act via an indirect mechanism. VEGF may directly prevent the activation of cellular death pathways in neurons given qualitative observations in our lab that animals with VEGF infusions within CA1 have fewer pyknotic cells (cells undergoing degeneration). Short duration seizures likely contribute to apoptotic cell death, and not necrotic cell death, given that energy resources are unlikely to be depleted within this timeframe. Activation of Akt pathways (e.g., VEGFR1 and VEGFR2) can inhibit caspase-9 (Romashkova & Makarov, 1999), thereby inhibiting caspase-9-driven apoptosis. In addition, VEGF promotes expression of the anti-apoptotic proteins Bcl-2 and A *in vitro* (Gerber et al., 1998). Although these effects suggest anti-apoptosis, the alternative process of autophagy cannot be ruled out given the presence of autophagy-related markers 24 hours following pilocarpine-induced status epilepticus (Cao et al., 2009).

VEGF could also potentially protect neurons via an indirect mechanism. For example, VEGF may serve to "quiet" circuit excitation secondary to seizures. Alterations in astroglia parameters described in our laboratory (Lenzer et al., 2011) following VEGF application may

initiate the sequestering of local neurotransmitters, such as glutamate, thereby decreasing excitotoxicity. Additionally, the profile of receptors expressed on cells could be modified following VEGF infusions into the hippocampus. Our laboratory has observed marked increases in VEGFR2 expression on astrocytes after seizures and induction of low levels of VEGFR2 in neurons in the same animals (Nicoletti et al., 2005). These shifts in receptor expression on specific cells may act as a compensatory mechanism that could serve to rescue neurons from death. Finally, it is possible that VEGF functions to change interactions between different cells (i.e., neurons, glia, astrocytes, etc.) via changes in the ratio of signaling in various receptor pathways in different cells.

The possibility that a combination of the preceding possibilities function together to mediate neuroprotection cannot be ruled out. A systems level approach achieved by *in vivo* experimentation must be implemented in order to elucidate this possibility. For my dissertation, we chose to explore the possible receptor mechanism(s) by which VEGF protects. This goal was accomplished by studying hippocampal neuronal density following status epilepticsin animals receiving pre-treatment of VEGF in combination with various VEGF receptor blockers.

Interpretation of Results

VEGF Receptor Distribution

The VEGF receptors identified within the naïve adult rat brain include VEGFR1, VEGFR2, NP-1, and NP-2. VEGFR1 is largely found on the vascular endothelium, though expression has been seen on inflammatory monocytic leukocytes, microglia and astroglia (Krum et al., 2002; Forstreuter et al., 2002). VEGFR1 has also been identified on reactive astrocytes following ischemia (Lennmyr et al., 1998) and monocytes following neuronal insult (Lennmyr et al., 1998). VEGFR2 is found on the vascular endothelium in the naïve adult brain, though

upregulation on neurons and astrocytes following seizures and ischemia have been noted (Croll & Wiegand, 2001; Nicoletti et al., 2005). Both NP-1 and NP-2 are localized to neurons and have the capacity to bind to and form complexes with VEGFR2 (Fuh et al., 2000). The function of these complexes is unproven, but they may function to bring local VEGF into close proximity to, and enhance activation of, VEGFR2. Receptor profiling may be modified following VEGF application. Exogenous VEGF may increase VEGFR2 expression on glia in the naïve adult brain (Krum et al., 2002) and augment VEGFR2 activation in hippocampal neurons one day following seizure induction (Nicoletti et al., 2005). In contrast, preliminary studies suggest that VEGF treatment may lead to a decline in VEGFR1 expression (unpublished observations). Together, the findings suggest that a compensatory mechanism may be at play to alter receptor expression and distribution in response to a neurological insult, such as seizures, and/or following introduction of exogenous VEGF in the system.

Role of VEGFR1 and VEGFR2 in Neuroprotection

Past research has suggested a direct role of VEGFR2 in mediating VEGF's neuroprotective effects on neurons. For example, VEGFR2 inhibition has been shown to increase death of hilar neurons in the dentate gyrus in an animal model of traumatic brain injury (Lee & Aroston, 2009). In the current model, inhibiting VEGFR2 signaling on receptors located both within the cell and on the cell membrane (with preferential blockade of intracellular VEGFR2), while not blocking the ligand binding site, does not alter VEGF-mediated neuroprotection. However, a direct block of the ligand binding site on extracellular VEGFR2 prevents VEGF's neuroprotective effects. It is therefore possible that VEGF mediates neuroprotection either specifically via activation of membrane-bound cell surface VEGFR2, or by preventing the sequester of VEGF by VEGFR2. Additionally, prevention of VEGF binding and signaling

through membrane-bound VEGFR1 during VEGF infusions did not alter neuronal death. Therefore it is unlikely that VEGFR1 is directly mediating neuroprotection.

Given the significant neuronal loss that occurs when VEGF is not able to bind to VEGFR2, it is possible that exogenous and endogenous VEGF becomes available to bind to other receptors such as VEGFR1. Based on our data, we could speculate that VEGFR1 localized to monocytes or glia may play a role in increased circuit vulnerability to seizures or their sequelae. Indeed, while treatment with a neutralizing antibody against VEGFR1 had no statistically significant effect in our assay, the mean neuronal count for the anti-VEGFR1 antibody was slightly higher than that of the IgG isotype control.

Internalization of VEGFR2

Previous findings have illustrated internalization of VEGF receptors following VEGF binding to VEGFR2. VEGFR2 may be internalized following ligand binding on the cell surface (Berger & Ballmer-Hofer, 2011). Our findings showed that VEGFR2 was localized to the nucleus during co-treatment with SU1498, but not with the anti-VEGFR2 antibody. It is hypothesized that removal of the VEGFR2 binding site for VEGF with the VEGFR2 antibody (which should then prevent extracellular VEGF from binding to VEGFR2) contributed to the inability of this receptor to internalize. This lack of internalized VEGFR2 may play a role in the increased neuronal damage observed after treatment with the antibody against VEGFR2. This possibility requires further evaluation in future experiments.

VEGFR2 Modulation of Seizure Severity

VEGF may serve to indirectly “quiet” neurons, thereby decreasing the excitotoxicity that contributes to neuronal death. We speculate that this "quieting" effect may occur via VEGFR2 activation on astroglia. Previous results from our laboratory have demonstrated that VEGFR2 is

upregulated on astrocytes 24 hours after status epilepticus (Nicoletti et al., 2005). Astrocyte proliferation and glial hypertrophy have also been shown following exogenous VEGF application (Krum et al., 2002; Croll et al., 2004; Lenzer et al., 2011). It is possible that glial hypertrophy may allow glia to invaginate the synaptic cleft and sequester local neurotransmitters, such as glutamate, thereby decreasing excitotoxicity and diminishing glutamateric tone. This "quieting" effect, whether glial or not, is supported by findings that application of VEGF to rat hippocampal slices diminishes the amplitude of excitatory pathways (McCloskey et al., 2005). While administration of VEGF to hippocampal cells from epileptic rats decreases spontaneous discharges, similar results are not seen with naïve rats (McCloskey et al., 2005). The reason for this apparent discrepancy is currently unknown, but given that the distribution of VEGF receptors in the hippocampus changes after seizures (Nicoletti et al., 2005), it is perhaps not unexpected. Animals pre-treated with VEGF in our laboratory have shown a tendency to have less severe seizures and decreased seizure latency relative to controls, although this finding has not been consistent or statistically significant.

Our current experiments support the hypothesis that VEGF must be present prior to seizures in order for VEGF to have neuroprotective effects. The presence of VEGF during, and not after, seizures may prepare the system to dampen hyperexcitabilty in the presence of imminent seizure activity. Exogenous VEGF independently increases expression of VEGFR2 on astroglia (Krum et al., 2002) and, as discussed previously, VEGF could then better activate VEGFR2 to alter glial hypertrophy. The increase in VEGFR2 may also shift activation of VEGF receptors in the hippocampus from a predominantly VEGFR1-mediated event to a predominantly VEGFR2-mediated event. That is, large quantities of upregulated VEGFR2 on glia and neurons could then compete with the higher affinity VEGFR1 on blood vessels for VEGF binding. Pre-

application of VEGF may allow enough time for glial hypertrophy or VEGFR2 overexpression to occur prior to the induction of seizures. Furthermore, if VEGF binding to the increased VEGFR2 on astrocytes following severe seizures contributes to neuroprotection, then blockade of VEGF binding to VEGFR2 would prevent neuroprotection, consistent with the findings of this dissertation.

Changes in receptor expression on neurons may also function to quiet seizures. Preliminary observations from Pat Rockwell's laboratory (personal communication) suggest that VEGF decreases VEGFR1 on CA3 neurons, which are highly vulnerable to hyperexcitability. This finding suggests a potential quieting effect of VEGF via downregulation of CA3 receptors.

The hypersynchrony of neuronal firing and spreading of seizure activity observed in epilepsy is thought to be secondary to the release of glutamate by astrocytes (Tian et al., 2005). We have proposed that activation of VEGFR2 on astrocytes may produce a quieting effect, thereby increasing seizure threshold and indirectly promoting neuroprotection. This hypothesis is supported by a recent study demonstrating that exogenous VEGF reduced ictal-like events and interictal discharges in an *in vitro* mouse model of epileptiform activity in CA1 (Cammalleri, et al., 2011). The authors speculated that this reduction in discharges by VEGF occurred via reduced glutamate release after VEGFR2 activation. A quieting role of VEGFR2 is also corroborated by findings that transgenic mice overexpressing VEGFR2 demonstrated a higher threshold for focal seizure induction (Nikitidou et al., 2012). However, these researchers postulated that modulation in synaptic transmission and plasticity in hippocampal circuitry was responsible for this quieting effect, but not via glia given the absence of GFAP co-immunolabeling. The effect of their manipulation on VEGFR1 levels was not studied.

VEGFR1 Activation and Vascular Permeability

Vascular permeability is identified as both a trigger and a consequence of severe seizures. In general, application of VEGF results in blood brain barrier breakdown and the formation of tortuous dilated leaky blood vessels. This vascular permeability may be seen at sub-angiogenic doses, which are similar to those administered in the current model (Croll et al., 2004). Therefore, exogenous VEGF in our model may play a role in enhancing vascular permeability and indirectly influencing neuronal survival following status epilepticus. Since VEGF is secreted by neurons and glia following status epilepticus, local VEGF receptors may mediate this vascular permeability. The VEGF family member PlGF has been shown to induce this process, and since PlGF binds to VEGFR1 and NP-1, but not to VEGFR2, it is likely that activation of VEGFR1 may play an important role in mediating vascular permeability (Pipp et al., 2003), although a role for VEGFR2 in permeability has also been proposed. Vascular permeability has been hypothesized to promote seizure activity (Rigau et al., 2007) and may therefore be detrimental to neuronal survival.

As mentioned above, we hypothesize that there may be more unbound VEGF available in the central nervous system if VEGF is prevented from binding to cell surface VEGFR2 by an antibody. This free VEGF may potentially bind to available VEGFR1 in the system to enhance vascular permeability. This hypothesis represents one potential explanation for our finding that simultaneous infusions of VEGF and the anti-VEGFR2 antibody, but not the Sugen compounds, contributed to more severe seizures.

One caveat in the interpretation of our data is that we infused an IgG isotype control antibody as a control for both the anti-VEGFR1 and anti-VEGFR2 antibodies. To our surprise, VEGF did not demonstrate the expected neuroprotective properties when co-infused with this

control. Past research has shown that IgG leakage in the brain parenchyma secondary to the blood brain barrier breakdown is common in both clinical and animal models of seizures and is thought to contribute to neuronal impairment (Rigau et al., 2007). In addition, the extravasation of IgG has been associated with astrocyte activation and subsequent neuronal hypersynchrony, which could be a mechanism of hyperexcitability (Seiffert et al., 2004). These findings further support our suspicions that the IgG used as a "control" in several of our models may have actually had detrimental effects, thereby masking the potential neuroprotection secondary to VEGF in our models. However, in prior studies it was not proven whether it was extravasated IgG or other blood components that contributed to neuronal impairment. Given our data, it is possible the IgG itself interferes with neuroprotective mechanisms in the context of severe seizures.

VEGFR1 Activation and Inflammation

Inflammatory cytokines are upregulated by seizures and have been shown to exacerbate subsequent seizures (Vezzani et al., 2005). Local application of sub-angiogenic doses of VEGF (similar to that used in the current experiments) independently contributes to inflammatory responses (Croll et al., 2004) by upregulating a number of pro-inflammatory proteins such as the adhesion molecule ICAM-1 and the chemokine MIP-1α. In addition, previous research has shown that inflammatory cytokines such as IL-1 and TNF-α can upregulate VEGF, further driving inflammation. The resulting upregulation of pro-inflammatory mediators contributes to extravasation of leukocytes that infiltrate the brain parenchyma and incite inflammatory processes (Croll et al., 2004).

Inflammatory events at the blood brain barrier (i.e., increased production of ICAM adhesion molecules) are important for promoting the entrance of leukocytes into the blood

stream and consequently into the central nervous system parenchyma (Vezzani et al., 2005). VEGFR1 expression on microglia has been demonstrated in other neurodegenerative disorders. For example, VEGFR1 inhibition with an anti-VEGFR1 antibody reduced microglia mobility and was neuroprotective in a rat model of Alzheimer's disease (Ryu et al., 2009).

Given these findings, VEGF may serve to induce effects of these inflammatory cytokines, thereby aggravating seizures and indirectly causing detrimental effects like neuronal death. The inflammatory cells located at the region of VEGF infusion have been characterized as monocytic and macrophagic (Croll et al., 2004). Given that VEGFR1 expression has been reported on monocytic leukocytes, VEGF may be trophic to this cell type (Sawano et al., 2001).Therefore, it is speculated that VEGF-induced inflammatory responses are mediated primarily by activation of VEGFR1.

Because we have hypothesized that blockade of VEGF binding to VEGFR2 with the anti-VEGFR2 antibody may lead to increased binding of VEGF to VEGFR1, it follows that elevations in local monocyte infiltration and microglia activation may result. These increases in local monocyte/macrophage activation could potentially contribute to increased excitation and neuronal death.

Our revised hypothesis based on the data collected in the current experiments is that VEGFR1 activation does not mediate VEGF's neuroprotective effects and instead may contribute to seizure-related damage. This is additionally supported by research showing that PlGF expression is augmented in temporal lobe patients as compared to control and greater in temporal lobe epilepsy patients, especially following an initial injury. PlGF levels were also increased several hours after pilocarpine-induced status epilepticus in a rat model (Xu et al., 2012). However, other laboratories have demonstrated a neuroprotective role of VEGF-B, a

VEGF analog capable of binding to VEGFR1 only. Poesen, et al. found that intracerebroventricular administration of VEGF-B prolonged survival in an animal model of motor neuron disease (2008). A role for VEGF-B in inhibiting apoptosis has also been proposed (Li et al., 2008). However, a fundamental difference exists between their models and the current model. While they utilized a VEGF analog to study VEGFR1 neuroprotection, we blocked VEGFR1 binding and signaling. In addition, we were working in an acute and severe brain insult model that included inflammation and vascular permeability as inherent elements of the seizure event, whereas the studies using VEGFR1 agonists used different types of models.

Summary of Proposed VEGF Receptor Mechanisms in Neuroprotection

VEGF produces greater tyrosine kinase activity at VEGFR2 as compared to VEGFR1 (Park et al., 1994), but has a greater affinity for VEGFR1 as compared to VEGFR2 (Shibuya, 2001). In normal brain tissue, VEGF tyrosine kinase receptors are most densely localized to cerebral vasculature. However, VEGF administration increases the expression of VEGFR2 on glia, and status epilepticus robustly upregulates VEGFR2 on glia and neurons. This upregulation of VEGFR2 may function to shift some VEGF activity from the vasculature to the neural cells, as well as shift the VEGF binding profile from predominantly VEGFR1 to predominantly VEGFR2. We suggest a role for VEGF binding to extracellular VEGFR2 in neuroprotection because blockade of VEGF binding to cell surface VEGFR2 inhibited neuronal survival. One hypothesis is that the removal of this binding site may prevent VEGFR2 from translocating to the nucleus, thereby inhibiting a possible cell survival pathway. We also propose, however, that the protection resulting from blockade of VEGFR2 binding may be indirect since blockade of VEGFR2 activation (but not binding) did not result in neuroprotection. That is, it might be the shift back to a predominantly VEGFR1 binding profile that ultimately favors cell death.

VEGFR1 is thought to underlie increases in vascular permeability and inflammation, and freeing VEGF from VEGFR2 so that it will bind to VEGFR1 could enhance the inflammation and vascular leak occurring after seizures. Inflammation and permeability are both implicated in the maintenance of hyperexcitability within the epileptic brain. Hyperexcitabilty contributes to neuronal death in areas susceptible to damage following seizures, such as CA1 of the hippocampus. Our current experiments showed that blockade of the ligand binding site on extracellular VEGFR2 during VEGF infusions contributed to the development of significantly more severe seizures. It could therefore be speculated that either membrane-bound VEGFR2 functions to increase seizure threshold *or* that increased VEGFR1 activation decreases the threshold for severe seizures.

Inhibition of VEGF binding to VEGFR1 during simultaneous infusions of exogenous VEGF did not significantly change neuronal density estimates. This may be because VEGF is still available to bind to both VEGFR2 and NP-1. The potential direct neuroprotective effects of VEGFR2 activation on astrocytes or neurons may be enough to compensate for the potentially damaging effects of VEGFR1 in mediating vascular permeability and inflammation. The possibility of protection via VEGF binding to NP-1, which may be colocalized with VEGFR2 to enhance VEGFR2 activation, should not be ruled out.

NP-1 is the only VEGF receptor constitutively expressed on neurons in the normal adult brain. NP-1 also functions as a receptor for the semaphorins (specifically semaphorin3a), which are known to mediate a cell death pathway (Gu et al., 2002). VEGF binding to NP-1 in our status epilepticus model may prevent NP-1 activation by the semaphorins and therefore impede cell death via this mechanism. Future experiments will be necessary to elucidate any potential role of NP-1 in VEGF-mediated neuroprotection.

Study Limitations

While VEGF mediated neuroprotection decreased following infusions of the large molecule anti-VEGFR2 antibody during status epilepticus, the same was not seen following infusions of the small tyrosine kinase inhibitors, SU1498 & SU5416. Although these reagents are known to be preferential for inhibition of VEGFR2 tyrosine kinases, they are not 100% specific to this receptor. For example, SU5416 can also inhibit RET, FLT-3, (Mologni et al., 2006), and Kit (Smolich et al., 2001) and SU1498 can inhibit PDGF, EGFR, and HER-2 (Charnock-Jones, 2005). The possibility that small effects at other receptors contributed to their effects in these experiments cannot be ruled out.

In the current experiments, we did not evaluate the diffusion rate nor radius of SU1498/SU5416 diffusion from intrahippocampal infusions. It is possible that the kinetics and distribution of these tyrosine kinase inhibitors may differ from the established 1.5mm diffusion radius of VEGF infusions. Additionally, the area of CA1 immediately adjacent to the cannula tip may be receiving SU1498/SU5416 at higher (or lower) concentrations than the more distal portions of CA1, and this difference may vary across the 5 days of continuous administration. Therefore, future experiments may include evaluation of the rate and radius of SU1498/SU5416 diffusion as well as establishment of a dose response curve to determine the effectiveness of the SU1498/SU5416 at different concentrations. In fact, previous research has shown that SU5416 can reduce apoptotic neurons in an *in vivo* model of traumatic brain injury at a much smaller dosage than that administered in the current experiment (Lee and Agoston, 2009),

VEGF did not protect neurons 24 hours after status epilepticus when infused with IgG, the proper isotype control antibody for the neutralizing VEGFR antibodies used in our studies. As previously discussed, we suspect that IgG may have had unexpected deleterious effects on

neuronal survival. In the future, we plan to determine any detrimental effects of IgG alone or in combination with pilocarpine-induced status epilepticus in order to determine the effects of this isotype control in our model.

In the anti-VEGFR1 study, we were unable to include inactivated VEGF control groups due to limited resources at the time. To determine the effects of neuronal density secondary to VEGF administration and secondary to the VEGFR1 neutralizing antibody, it would be ideal to repeat this study with the inclusion of these groups.

Given the interpretation of our findings, we suspect that VEGFR1 mediates detrimental effects via increased vascular permeability and/or inflammation. In order to further investigate this hypothesis, we hope to stain our tissue for markers of permeability and inflammation. Should these processes be decreased following administration of the anti-VEGFR1 antibody, we may conclude that VEGFR1 does at least partially mediate these processes.

While pre-treatment with exogenous VEGF protects neurons from death 24 hours after status epilepticus, post-treatment with VEGF immediately following status epilepticus does not protect neurons from death 24h later in the current experiment. It is, however, possible that the dosage of acute injections was not protective in the current experiment. We plan to test a range of dosages to determine potential optimal dose of VEGF post-treatment for neuroprotection.

In the preceding studies, neuronal density was measured only for animals that reached status epilepticus (stage 6). In the future, we hope to measure CA1 densities from animals that achieved both status epilepticus and subacute seizures after a period longer than 24 hours (i.e., 1 week or 1 month) in order to determine any long term effects of the various treatment modalities described above. Although neuron loss is subtle or non-existent after more mild seizures at this time point, longer-term time points might reveal compromise in the cell layers.

Conclusion

While VEGF has the potential to be of therapeutic value to humans in the context of central nervous system disease, its utility is limited by its large molecular size, which prevents it from crossing the blood brain barrier. If the particular VEGF receptor mediating VEGF's neuroprotective effects could be identified, specific small molecule drugs could be developed to function at the target receptor. In the current studies, inhibition of VEGF binding to the ligand binding site on cell surface VEGFR2 with use of a large antibody exacerbated neuronal damage following pilocarpine-induced status epilepticus. Blockade of the VEGFR2 binding site in the presence of VEGF also contributed to more severe seizures. These findings suggest a neuroprotective mechanism of VEGF binding to extracellular VEGFR2. This finding may also suggest further investigation for a therapeutic role of agonism of this receptor-ligand pair. In contrast, if VEGFR1 activation aggravates neuronal death secondary to enhanced permeability and inflammation as we have proposed, then novel drugs should function to inhibit this receptor on the vascular endothelium, monocytes, and microglia. Therefore, both receptors remain as potential therapeutic targets, with one targeted for agonism and one for antagonism. It may be the balance of activation between the two VEGF receptors, rather than the signaling of either receptor alone, that ultimately determines VEGF's therapeutic potential in epilepsy.

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