PROTECTIVE EFFECT OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN A RAT MODEL OF STATUS EPILEPTICUS

by

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Abstract

VEGF IN A RAT MODEL OF EPILPESY

By

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Epilepsy is the most common neurological disease worldwide. Current treatments involve the administration of anti-epileptic drugs (AEDs) which address seizure frequency but not etiology or resultant pathology. Neurotrophic factors have recently been explored in the treatment of epilepsy. One such neurotrophic factor, vascular endothelial growth factor (VEGF), may have potential as a therapeutic in the treatment of epilepsy. VEGF mediates several biological processes including vascular permeability, angiogenesis, and neuroprotection. Given its various functions, we sought to characterize the role of VEGF in a rat model of status epilepticus. We first investigated whether VEGF was endogenously upregulated after status epilepticus. We found that VEGF was significantly increased in neurons and glia of the hippocampus and temporal cortex 24 hours after status epilepticus. Given that VEGF is primarily known for its angiogenic properties, we next investigated if there was an increase in vascular density after status epilepticus. We found a significant increase in vascular density in the CA1 region of the hippocampus three days after status epilepticus. When we infused Flt-Fc, an immunoadhesin designed to sequester endogenous VEGF, into brain prior to status epilepticus, we found no significant difference in increased vascular density relative to controls. Since VEGF was not responsible for an increase in vascular density in our model, we hypothesized that perhaps it played a neuroprotective role. We again infused

Flt-Fc or a control into brain to characterize VEGF's neuroprotective role. We found a statistically significant decrease in neuronal density in animals treated with Flt-Fc relative to controls. Thus, we concluded that endogenous VEGF did indeed play a neuroprotective role. We also investigated whether exogenous VEGF would provide additional protection to neurons. We found that animals infused with 30ng/d and 60ng/d VEGF had significantly more neurons after status epilepticus than controls. Since VEGF significantly preserved neurons after status epilepticus, we then sought to determine if it also preserved cognitive functioning. We found that VEGF preserved normal anxiety functioning but did not preserve learning and memory. Our findings that exogenous VEGF protein is neuroprotective in this paradigm suggest that it could elucidate a novel approach to cell protection in epilepsy.

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INTRODUCTION

Epilepsy is a common, progressive, neurological disorder for which there is currently no cure. Individuals diagnosed with epilepsy typically experience neuroanatomical and neurophysiological changes at the cellular and molecular level as well as cognitive, emotional, and social changes at the behavioral level. Typical treatments for epilepsy include the administration of anti-epileptic drugs (AEDs) and in severe cases, surgical interventions. Because AEDs address seizure frequency, not etiology or resultant pathology, other avenues of treatment, such as neurotrophic factors, have been explored. This dissertation will focus on one such neurotrophic factor, namely vascular endothelial growth factor (VEGF), and the potential role of this growth factor in new treatments for epilepsy.

Epilepsy

Epilepsy is the most common neurological disease worldwide, affecting 1% to 3% of the population. It is estimated that 45 to 100 million people are afflicted with active epilepsy, which is defined as having a history of the disorder plus a seizure or use of antiepileptic medication in the past five years (Fong & Fong, 2001). Epilepsy is characterized by the periodic and unpredictable occurrence of seizures, which are classified as partial or generalized based on their characteristics. Generalized seizures are characterized by the complete loss of consciousness because the entire cortex is involved whereas partial seizures begin in a limited, focal brain region and do not result in the complete loss of consciousness. Because individuals may experience an alteration in consciousness, there is an additional distinction between simple-partial seizures, in which

there is no alteration of consciousness, or complex-partial seizures, wherein there is an alteration of consciousness.

Partial and generalized classes can be further differentiated based on etiology. Idiopathic epilepsies are typically inherited and result from abnormal neurotransmission (Shneker & Fountain, 2003). However, there are no structural abnormalities associated with idiopathic epilepsies. By contrast, symptomatic epilepsy results from structural abnormalities or a known cause, hence the epilepsy is "symptomatic" of the disease or disorder (Shneker & Fountain, 2003). Cryptogenic defines syndromes for which etiology is unknown but presumed to be symptomatic (Fong & Fong, 2001; Shneker & Fountain, 2003).

Regardless of the syndromic classification or the underlying etiology, episodes of status epilepticus can occur in each. Status epilepticus has been defined as a state of continuing generalized, convulsive seizures or intermittent convulsions in which the individual does not regain consciousness between attacks (Watson, 1991; Shneker & Fountain, 2003). The defined time frame of status epilepticus varies anywhere from five minutes up to ninety minutes depending on the milieu in which it is being treated or investigated (Watson, 1991; Scott et al., 1998; Bassin et al., 2002). In humans, it has been found that if status epilepticus lasts for five minutes, it will likely continue for at least twenty minutes but may spontaneously remit (Bassin et al., 2002). Animal models of epilepsy have demonstrated significant structural brain damage after thirty minutes of seizure activity (Scott et al., 1998). Demonstration of such damage in humans has been complicated by limitations in technology because of the insensitivity of structural magnetic resonance imaging (MRI) to scattered neuronal loss and injury (Duncan, 2002).

Various neuroimaging techniques have been employed to address this issue, however findings have been inconsistent, perhaps as a function of the variability in technique and design. Thus, the extent of neuronal injury in humans due to seizure activity is currently unclear.

Common causes of status epilepticus include, but are not limited to, brain injury, cerebrovascular disease, hypoxia/anoxia, tumor, infectious disease, drug and alcohol withdrawal, systemic and metabolic illnesses, and noncompliance with antiepileptic drugs in patients with epilepsy (Bassin et al., 2001). Based on a prospective population-based study conducted at the Medical College of Virginia, it is estimated that 100,000 cases of status epilepticus occur in the United Status per year (DeLorenzo et al., 1996). Of these cases, approximately 22,000 to 42,000 result in death.

Systemic and metabolic changes result from status epilepticus and typically occur in two stages. The initial phase lasts approximately thirty minutes and is characterized by cardiovascular changes including tachycardia or bradycardia and arrhythmias. Respiratory failure may occur as a result of the underlying etiology causing the status event, such as those previously described, or pulmonary edema (Watson, 1991). Metabolic changes during this phase include respiratory and metabolic acidosis, hypoxia, hyperkalemia, hypoglycemia, and significant increases in serum levels of prolactin, glucagon, insulin, norepinephrine, epinephrine, growth hormone, and cortisol (Watson, 1991). Autonomic nervous system complications include hyperpyrexia, diaphoresis, increased tracheobronchial secretions, and pupillary dilation or constriction. During the second phase, blood pressure tends to return to baseline, although the patient may become hypotensive, and remains at baseline with subsequent seizures. Serum levels of

altered chemicals also return to normal. In most circumstances, status epilepticus is treated pharmacologically, terminating the seizures. However, if status epilepticus cannot be controlled and continues to progress beyond a total of sixty minutes, respiratory functions become compromised once again and hyperthermia may occur (Watson, 1991).

Experimental Models of Epilepsy

The mechanisms underlying the pathophysiology of epilepsy are poorly understood. To begin to understand these mechanisms and their resultant outcomes, it is important to distinguish between ictogenesis and epileptogenesis. Ictogenesis (i.e., the induction of a seizure) is due to excessive discharges from groups of neurons that are initiated by depolarization of voltage-dependent sodium channels and activation of ionic glutamate receptors (Sasa, 2006). Epileptogenesis is the end result of prolonged anatomical and biochemical alterations and reorganizations of neuronal networks that stem from repeated episodes of ictogenesis.

To elucidate these mechanisms, two types of animal models have commonly been employed, which mimic the clinical course of epilepsy. The first approach utilizes chemical convulsants (e.g., pilocarpine or kainic acid) to induce an acute excitotoxic insult, which results in an episode of status epilepticus. The acute phase lasts approximately 24 hours and is followed by a latent period ranging from 4 to 44 days that is characterized by a return to normal electroencephalogram and behavior. This latent period is followed by a chronic period of recurrent spontaneous seizures (Leite et al., 1990; Cavalheiro, 1995). Thus, the animal experiences an acute episode of status epilepticus that can lead to chronic epilepsy. The acute (status epilepticus) model mimics

the development of epilepsy in humans where there is an initial neurological insult, such as an episode of status epilepticus, traumatic brain injury, or brain tumor, which is followed by a latent period that may span several weeks, although more commonly several years. Following the latent period, the individual develops recurrent spontaneous seizures. The second approach takes advantage of a phenomenon known as "kindling" in which frequent, small insults lead to brief repeated seizures (Sutula et al., 1988; Golarai et al., 1992). These small insults are induced either with chemical convulsants or electrical stimulation. Therefore, in this kindling model, animals ultimately develop chronic epilepsy in the absence of an acute status epilepticus event. The chronic (kindling) model mimics forms of human epilepsy in which the individual continuously experiences repeated, brief partial-onset seizures in the absence of an acute neurological insult.

Both types of model produce significant neuropathological and neurophysiological abnormalities similar to those observed in patients with epilepsy although the abnormalities evoked in the kindling model are considerably less extensive. These abnormalities include neuronal loss, subsequent sprouting, and synaptic reorganization in the hippocampus, which are believed to underlie the mechanisms of epileptogenesis (Wuarin & Dudek, 2001). In the acute model, these changes occur during the latent period while in the kindling model they occur concomitant with repeated, brief seizures.

Central Nervous System Pathophysiology

Status epilepticus results in damage to five main brain regions: layers 3, 5, and 6 of the cerebral cortex, cerebellum, hippocampus, subsets of thalamic nuclei, and the

amygdaloid body (Watson, 1991). Of these regions, the hippocampus appears to be the most vulnerable area to neuronal death. Regional vulnerability is also evident within the hippocampus, with CA1 and CA3 being most susceptible to damage. Neuronal death within the hippocampus is thought to occur as a result of excitotoxicity resulting from the increase in extracellular glutamate levels during a seizure (Chapman, 2000). Glutamate binds to N-methyl-D-aspartate (NMDA) receptors, which are ionotropic, causing an influx of calcium. High intracellular calcium levels in turn initiate calcium-dependent processes such as activation of (1) protein kinase C, which results in the destruction of the cell wall, (2) nitric oxide formation, which inhibits mitochondrial respiration through free radicals which are cytotoxic, (3) activation of phospholipase A, which breaks down membrane lipids and causes the release of arachidonic acid possibly leading to cell death, and (4) activation of protease calpain I (Scott et al., 1998; Fujikawa, 2005). Activation of metabotropic glutamate receptors also results in excitotoxicity through potentiation of NMDA and other excitatory membrane currents, potentiation of intracellular calcium release, a decrease in inhibitory membrane currents, and a decrease in GABAergic inhibition (Scott et al., 1998). Glutamate receptor stimulation also initiates the formation of immediate early genes such as c-fos, c-jun, fos-B, and jun-B which may induce apoptosis through activation of p53, cell-death promoting Bcl-2 family members, and endonuclease-induced DNA laddering (Fujikawa, 2005).

A consequence of neuronal loss and neuronal excitability is reorganization of neuronal networks within the hippocampus. Animal models, as well as studies of resected tissue from cases of human mesial temporal lobe epilepsy, have demonstrated that mossy fibers reorganize and sprout into the inner molecular layer of the dentate gyrus

to form new synapses with dendrites of inhibitory interneurons (Cavazos et al., 1991, 1992; Nadler et al., 1980; Sutula et al., 1992). One hypothesis regarding mossy fiber sprouting is that it occurs as a compensatory mechanism to reinstate inhibitory control through feedback connections on interneurons (Ribak et al., 1991; Cavazos et al., 2003). However, as the inner molecular layer continues to degenerate as a result of hilar polymorphic neuronal loss, the synaptic connections form on the spines and dendrites of granule cells, creating a recurrent excitatory collateral circuit (Boyett & Buckmaster, 2001; Cavazos et al., 2003). In addition to these aberrant recurrent collaterals into the molecular layer of the dentate gyrus, other alterations have been observed including increased branching within the hilus, abnormal connectivity between the blades of the dentate gyrus, the formation of aberrant recurrent collaterals into the stratum oriens of CA3, and remodeling of the surface for synaptic contacts of dendritic trees of granule cells, which may alter temporal and spatial summation of postsynaptic potentials (Sutula et al., 1998; Buckmaster & Dudek, 1999; Sutula 2002). In fact, in models of ischemia, which share many similarities with epilepsy regarding mechanisms of brain damage and synaptic reorganization, investigation of membrane potentials of post-ischemic CA3 pyramidal neurons reveals a positive shift in the membrane resting potential which likely functions to facilitate the generation of synchronized burst discharges following an ischemic event (Crepel et al., 2003). These changes in synaptic reorganization may explain the mechanisms which underlie hippocampal hyperexcitability in epilepsy (for review, see Cavazos & Cross, 2006).

Synaptic reorganization is not limited to the mossy fiber pathway. Experimental models have demonstrated that CA1 pyramidal neurons exhibit cellular hyperexcitability

and subsequent death similar to that observed in the dentate gyrus. Lehmann and colleagues (2000) studied sprouting in area CA1 in slices from hippocampi of patients operated on for temporal lobe epilepsy and from pilocarpine-treated rats. They found an increase in axon collaterals of CA1 pyramidal cells and projections via aberrant collaterals to the stratum pyramidale and the stratum radiatum of area CA1 suggesting that the network reorganization contributes to hyperexcitability via increased backward excitation. Evidence in support of this notion comes from the work of Smith and Dudek (2001, 2002) in which they isolated CA1 pyramidal neurons in slice and applied glutamate demonstrating an increased excitatory postsynaptic current in kainate-treated rats. Cavazos and Cross (2006) investigated the possible reorganization of distal axonal projections of CA1 to the subiculum outside of their normal laminar boundaries. They demonstrated significant plasticity of axonal branches of CA1 to subiculum along the septotemporal axis of the hippocampus, allowing for increased connectivity among lamellae above and below the normal circuitry. It has also been demonstrated that there is a significant increase in the number of bursting neurons in the subiculum following status epilepticus, possibly due to the fact that non-bursting neurons are more vulnerable (Wellmer et al., 2002). Thus, the increased synaptic plasticity coupled with the reorganization of the subiculum may play a critical role in icto- and epileptogenesis (Cavazos & Cross, 2006).

Functional Consequences of Epilepsy

Aside from the underlying neuropathology, individuals with epilepsy experience significant neuropsychological and psychosocial problems. Neuropsychological sequelae include learning and memory deficits, language impairments, reduced speed of

processing, compromised levels of attention and concentration, and executive functioning deficits (Goldstein, 1991; Moore & Baker, 2002). These impairments can significantly compromise quality of life. In fact, individuals with epilepsy are more likely to be unemployed resulting in lower socioeconomic status, lower rates of marriage, and greater social isolation (Moore & Baker, 2002).

Although social consequences may be a factor in some cases, comorbid psychiatric disorders commonly occur in many cases of epilepsy, particularly anxiety and depression. When an individual is initially diagnosed with epilepsy, he or she often experiences an adjustment disorder predominantly characterized by a heightened state of anxiety (Jackson & Turkington, 2005). If seizures are not adequately controlled with medications, the individual may also experience a social phobia such as agoraphobia. Depression may result as a consequence of social factors, or may occur independent of social factors. The lifetime prevalence of depression in epilepsy has been estimated to be as high as 55% (Jackson & Turkington, 2005). Suicide is four to five times more common in people with epilepsy than in the general population. In addition to these disorders, some major psychiatric disorders have been found to coexist with epilepsy including chronic interictal psychosis, which closely resembles schizophrenia, and episodic psychotic states (Toone, 2000). These disorders have commonly been referred to as the psychoses of epilepsy.

Current Treatments

Treatments for epilepsy typically include anti-epileptic drugs (AEDs), though vagal nerve stimulation and/or surgery are used in severe cases when AED therapy fails. AEDs function to suppress the occurrence of seizures through decreasing neuronal

excitability, enhancing inhibition through the alteration of ion conductance, or by affecting neural transmission of γ-aminobutyric acid (GABA) or glutamate (Fong & Fong, 2001; Sasa, 2006). Hence, the drugs are anti-ictogenic, not anti-epileptogenic. In addition to the fact that AEDs only address seizure frequency and not etiology or resultant pathogenesis, AEDs have been found to compromise cognitive functioning in patients (Diaz-Arrastia et al., 2002). Although novel AEDS have been developed that carry a lower risk of side effects, fewer drug interactions, and less enzyme induction, they do not eradicate functional deficits.

Although AEDs have remained the treatment of choice, they have been largely unsuccessful in their actions. That is, approximately 50% of patients treated with AEDs continue to experience seizures and a proportion of these individuals experience disease progression with increased seizure frequency and continued cognitive decline (Simonato et al., 2006). In addition, these drugs do little to prevent neuronal damage. The development of reagents that intervene in the basic mechanisms underlying the epileptic process or that function to repair these processes is crucial. The recent molecular studies that have begun to elucidate the pathophysiological mechanisms underlying epileptogenesis and seizure sequelae have pointed treatments in a new direction, namely the role of neuroprotective agents in epileptogenesis.

Role of Neurotrophic Factors in Epilepsy

Neurotrophic factors (NTFs) are endogenously occurring proteins involved in the survival and differentiation of neurons during development or providing tropic and trophic support throughout the lifespan (Twiss et al., 2006). Research suggests that they may be involved in the cellular alterations associated with epileptogenesis. Specifically,

failure of their trophic effects likely plays a role in cell death, while enhancement of their effects may contribute to neurogenesis and axonal sprouting. In addition, recent evidence suggests that they have an acute functional role in modulating excitatory and inhibitory synapses (Schinder & Poo, 2000). Given their functions, NTFs have become a target of interest in the development of new therapeutic agents for epilepsy. Nevertheless, research has demonstrated that NTFs may exert both favorable and unfavorable effects in epileptogenesis (Simonato et al., 2006).

The neurotrophin family is a family of neurotrophic factors comprised of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (for review, see Scharfman, 2005). Neurotrophins were the first NTFs studied in the context of epilepsy. Studies have illustrated that NGF mRNA and protein levels are upregulated in the dentate gyrus and neocortex after seizures, suggesting NGF plays either a pro-epileptogenic role or is expressed by the brain in an attempt at protection (Gall & Isackson, 1989; Bengzon et al., 1992). Similarly, BDNF synthesis is increased and its receptor, trkB, is activated following an epileptic event (Ernfors et al., 1991; Isackson et al., 1991; Gall, 1993; Nibuya et al., 1995; Mudo et al., 1996). Scharfman and colleagues (1999) demonstrated that application of BDNF to slices from pilocarpine-treated rats that experienced status epilepticus enhanced excitatory transmission of mossy fibers to granule cells and induced hyperexcitability of granule cells. Further, a tyrosine kinase antagonist, K252a, blocked these effects, implicating the necessity for activation of trkB receptors in these processes. Similar actions of BDNF and it receptor, trkB, were found in normal hippocampal slices (Kang & Schuman, 1995a, 1995b). Studies of synapsin-Cre conditional $trkB^{-/-}$ mice,

where trkB has been ablated in hippocampal granule cells and CA3 pyramidal neurons, have demonstrated that epileptogenesis is completely abolished (He et al., 2004). Thus, trkB activation is essential for this process to occur. Further evidence in support of a pro-epileptogenic role of BDNF is observations of increased seizure severity after exposure to kainic acid and hyperexcitability in CA3 in transgenic mice that overexpress BDNF (Croll et al., 1999). Other studies, however, have demonstrated that BDNF plays a role in the survival and/or regeneration of hippocampal neurons damaged as a result of status epilepticus (Simonato et al., 2006). It therefore remains controversial whether the net effect of neurotrophins in epilepsy would be advantageous or disadvantageous.

Studies investigating the role of fibroblast growth factors (FGF) in epilepsy have also provided inconsistent results. The best known member of this family, FGF-2, is found to be expressed in hippocampal CA2 pyramidal neurons constitutively (Woodward et al., 1992). It has been found that seizures increase FGF-2 mRNA and protein levels and upregulate its receptor, again suggesting a role in epilepsy (Riva et al., 1992, 1994; Van Der Wal, et al., 1994; Bugra et al., 1994; Follesa et al., 1994). Chronic intracerebroventricular infusion of FGF-2 reduces seizure-induced hippocampal damage (Liu et al., 1993). Further, genetically-altered mice that overexpress FGF-2 sustain less seizure-induced cell damage (Zucchini et al., 2005).

Another protein factor, vascular endothelial growth factor (VEGF), has only recently begun to be examined in the context of epilepsy. A recent study using in situ hybridization demonstrated that neurons increase the synthesis of VEGF mRNA after seizures induced via electroconvulsive shock (Newton et al., 2003). In cell culture, VEGF has been found to protect neurons from a variety of insults, including hypoxia,

ischemia and, most pertinent to epilepsy, glutamate excitotoxicity (Jin et al., 2000, 2001; Matsuzaki et al., 2001). This effect has been shown to be mediated through one of VEGF's receptors, VEGFR2. Specifically, blockade of VEGFR2 synthesis resulted in the blocked induction of the Akt survival pathway in these neurons (Jin et al., 2000; Matsuzaki et al., 2001), which has been shown to be activated after VEGF treatment (Mazure et al., 1997; Gerber et al., 1998). Based on these findings, it is possible that VEGF directly protects cells from excitotoxic damage by increasing signaling pathways important to neuronal survival after seizures. Evidence in support of this notion comes from the finding that cortical cells that are spared after status epilepticus upregulate Akt while cell populations that are damaged after seizures have low levels of Akt (Henshall et al., 2002). Although VEGF may serve a protective role in seizures, it has been found to play diverse roles in the central and peripheral nervous systems, which must be taken into account when exploring this growth factor as a novel therapeutic agent in the context of epilepsy.

Vascular Endothelial Growth Factor

VEGF is a secreted protein mitogen for micro- and macrovascular endothelial cells derived from arteries, veins, and lymphatics (for review, see Ferrara & Davis-Smyth, 1997). VEGF was initially studied for its potent effects as a vascular permeability agent because it was found to induce vascular leak in tumor ascites of guinea pigs (Senger et al., 1983). Since then, VEGF has gained increasing attention as an angiogenic factor, stimulating the development of new blood vessels from pre-existing blood vessels. More recently, however, VEGF has been studied as a mediator of inflammation (Proescholdt et al., 1999; Heil et al., 2000; Croll et al., 2004a) and as a

possible neuroprotective factor (for review, see Carmeliet & Storkebaum, 2002). Given VEGF's variously described roles, its role in epilepsy could be protective or destructive.

VEGF Gene

In order to elucidate VEGF's role in epilepsy, it is important to understand how VEGF is produced endogenously. The human VEGF gene is located on chromosome 6p21.3 and is composed of 8 exons separated by 7 introns with a coding region of approximately 14kb (Vincenti et al., 1996). The promoter region is located near a cluster of potential binding sites for Sp1, AP-1, and AP-2 transcriptional factors. Through alternate exon splicing, the VEGF gene codes for several isoforms of VEGF, such as 121, 145, 165, 183, 189, and 206, each composed of a specific number of amino acids (Tischer et al., 1991). Each VEGF isoform differs in its expression pattern and biological properties. The murine VEGF gene is similar to the human VEGF gene with 8 exons divided by 7 introns encompassing 14kb, however, this gene only codes for 3 isoforms, 120, 164, and 188, which are shorter than human VEGF by one amino acid (for review, see Ferrara & Davis-Smyth, 1997). Because our experiments are conducted in rat, it is important to understand the biophysical characteristics of human and murine isoforms.

$VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ are the most commonly expressed forms of VEGF and have been found in a wide range of tissues while $VEGF_{145}$ and $VEGF_{206}$ are relatively rare and limited to cells of placental origin (Robinson & Stringer, 2001). $VEGF_{183}$ has recently been identified and is a less frequent splice variant. All isoforms bind heparin except $VEGF_{121,}$ which is released as a freely soluble protein. $VEGF_{189}$ and $VEGF_{206}$ bind to heparin with high affinity and are almost completely sequestered in the extracellular matrix after secretion (Zachary & Gliki, 2001). $VEGF_{165,}$ which is the major

isoform found in most mammalian tissue, is secreted as a 46,000 dalton heparin-binding homodimeric glycoprotein. Its intermediate affinity for heparin results in a significant portion remaining bound to the extracellular matrix and cell surface (Robinson & Stringer, 2001). It has been demonstrated that VEGF protein becomes available to endothelial cells as freely diffusible proteins, as is the case with $VEGF_{121}$ and $VEGF_{165}$, or it must be cleaved by plasmin at the COOH terminus, as is the case with the longer forms bound in the extracellular matrix (Keyt et al., 1996). While there is an inverse relation between heparin affinity and diffusibility, there is a direct relation between the affinity of each isoform for heparin-binding and its mitogenic activity. Typically, those with a higher affinity for heparin exhibit greater mitogenic activity for vascular endothelial cells although this is not necessarily the case for all isoforms particularly $VEGF_{165}$ and $VEGF_{189}$ (Ferrara, 2001).

	$VEGF_{121}$	$VEGF_{165}$	$VEGF_{189}$
Heparin Affinity	+	++	++++
Diffusibility	++++	+++	+
Mitogenic Activity	++	++++	+

Table 1. Major VEGF isoforms and their properties (adapted from Ferrara, 2001). It appears that $VEGF_{165}$ has the greatest bioavailability combined with biological potency.

Several factors appear to play a role in the expression of the VEGF gene. A major contributor to its expression is hypoxia, which results in the binding of hypoxia-

inducible factors (HIFs) to the hypoxia response element (HRE) located in the promoter region of the VEGF gene (Carmeliet et al., 1998; Marti & Risau, 1998). The ability of HIFs to upregulate VEGF appears adaptive under hypoxic conditions as VEGF serves to increase vasculature in the hypoxic region that increases the amount of oxygen and nutrients delivered to metabolically-compromised neurons. During status epilepticus, cells become more metabolically active, as their need for glucose and oxygen increases, and they may therefore experience a "relative" state of hypoxia. These hypoxic conditions may lead to VEGF upregulation in an effort to preserve cells during seizures. Although hypoxia is a major contributor to VEGF upregulation, cytokines and growth factors such as platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, transforming growth factors, and interleukins have also been found to upregulate VEGF mRNA or activate its release (Neufeld et al., 1999).

VEGF Family and Receptors

In addition to VEGF, also called VEGFA, hereafter referred to as "VEGF", the VEGF family of growth factors is comprised of placental growth factor (PlGF), VEGFB, VEGFC, VEGFD, and VEGFE (for review, see Tammela et al., 2004). Receptors that are available for the VEGF family include VEGFR1 (also known as Flt-1 {feline sarcoma virus-like tyrosine kinase/fms-like tyrosine kinase}), VEGFR2 (also known as Flk-1 {fetal liver kinase-1} in the mouse or KDR {kinase insert domain receptor} in humans), and VEGFR3 (also known as Flt-4). These receptors are tyrosine kinase receptors that mediate downstream signaling of the mitogen-activated protein kinase kinase (MEK)/extracellular-signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase(PI3-K)/Akt signaling pathways (Gerber et al., 1998; Pedram et al., 1998). In

addition to their tyrosine kinase receptors, VEGF members may also bind to the neuropilins, a family of truncated receptors lacking a catalytic domain (Kawakami et al., 1996; Chedotal et al., 1998). These receptors were initially identified as receptors for class 3 semaphorins, secreted molecules that mediate axonal guidance and retraction during neural development (Chedotal et al., 1998). Because VEGF binds to various receptors, it may exert different effects depending on the receptor to which it binds.

Each of VEGF's family members performs different functions and bind preferentially to specific receptors. PlGF is expressed in the placenta, heart, and lungs and preferentially binds to neuropilin-1 and VEGFR1 (Robinson & Stringer, 2001). VEGF, the first identified member of the VEGF family, is a key regulator of blood vessel growth and thus has a wide-range tissue distribution, though its constitutive expression in normoxic states is low in all adult tissues except kidney. VEGF binds to VEGFR1, VEGFR2, neuropilin-1 (np-1), and neuropilin-2 (np-2). VEGFB, also commonly referred to as VEGF-related factor (VRF), has a wide tissue distribution but is predominantly expressed in striated muscle and brown fat in the myocardium and skeletal muscle. Thus, VEGFB is believed to play a role in cellular energy metabolism (Olofsson et al., 1996). VEGFB binds to VEGFR1. VEGFC and VEGFD have been implicated in the induction of the formation of new lymphatic vessels during development and adulthood (Kukk et al., 1996). In addition to this role, VEGFD is important for lung and skin development. Both VEGFC and VEGFD bind to VEGFR2 and VEGFR3. Much less is known about VEGFE, which collectively categorizes proteins encoded by strains of the open reading frame (orf) of the parapox virus family found in sheep and goats and binds VEGFR2 (Robinson & Stringer, 2001). Of the VEGF family members, VEGF has the widest-

ranging tissue distribution and binds four different receptors and will therefore be utilized in our experiments.

Because VEGF may exert different effects by binding different receptors, it is important to understand receptor distribution in brain. As previously mentioned, VEGF binds VEGFR1, VEGFR2, np-1, and np-2. In brain, VEGFR1 and VEGFR2 are localized predominantly to cerebral vascular endothelium although other localizations have been reported. Specifically, recent studies indicate that VEGFR1 is also expressed on monocytic leukocytes, circulating inflammatory cells, VEGF-treated astroglia, and reactive astrocytes following ischemia (Sawano et al., 2001; Krum et al., 2002). VEGFR2 has been identified on neurons in cultured hippocampal or dorsal root ganglion cells as well as neurons in peri-infarct areas after a focal cerebral ischemic event (Jin et al., 2000; Sondell et al., 1999, 2000). VEGFR2 has also been described on glial cells after cerebral ischemia (Lennmyr et al., 1998; Issa et al., 1999; Plate et al., 1999). VEGFR3 is absent in brain as it is expressed almost exclusively on lymphatic endothelium (Kaipainen et al., 1995), which brain lacks. The neuropilin receptors are heavily localized in brain and can be found on vascular endothelium; however, they are most densely expressed on neurons (Kawakami et al., 1996; Chedotal et al., 1998). Given this distribution pattern, during normoxic states, VEGF preferentially binds VEGFR1 and VEGFR2 on vascular endothelium, neuropilin-1 on neurons, and VEGFR1 on astrocytes. However, when the system becomes compromised, VEGF may also bind to VEGFR1 on leukocytes or cells of leukocytic origin (i.e., microglia) and VEGFR2 on neurons or glial cells.

Evidence suggests that VEGFR1 and VEGFR2 play distinctly different roles at various points throughout the lifespan. It appears that VEGFR1's primary role is as a ligand-binding molecule rather than a signal transduction receptor as evidenced by studies in which VEGFR1 receptors lacking the tyrosine kinase domain were still able to bind VEGF and did not result in lethality or defect in development and angiogenesis (Hiratsuka et al., 1998). In contrast, complete deletion of this receptor resulted in early embryonic lethality (Fong et al., 1995). Additionally, research has shown that VEGFR1 is necessary for endothelial cell morphogenesis during embryonic development since a mutation in VEGFR1 results in the failure of endothelial cells to organize into normal vascular channels (Fong et al., 1995; Matsuzaki et al., 2001). Thus, VEGFR1 activation leads to chemotaxis and tissue factor production.

VEGFR2 also plays a key role in vasculogenesis during development as embryos lacking the VEGFR2 gene die before birth because differentiation of endothelial cells does not take place resulting in the failure of blood vessel formation (Shalaby et al., 1995). In addition to this function, VEGFR2 has been identified as the primary receptor involved in the signal transduction cascade that results in VEGF-mediated angiogenesis in adult animals. Most pertinent to VEGF's potential neuroprotective role in epilepsy, VEGFR2 has been implicated as the primary receptor involved in the signal transduction cascade which activates Erk/MEK and Akt signaling pathways that result in neuroprotection (Rosenstein et al., 2003; Mazure et al., 1997; Gerber et al., 1998). VEGFR2 activation leads to vasculogenesis, angiogenesis, and neuroprotection, and could therefore exert a neuroprotective response after status epilepticus.

Although VEGF exhibits a higher affinity for VEGFR1 and VEGFR2, VEGF also binds neuropilins-1 and neuropilins-2. The precise role of VEGF binding the neuropilins is currently unclear. Based on gene disruption studies in which mice lacking the gene that encodes neuropilin-1 die as a result of the failure to develop a functional cardiovascular system, it has been postulated that the neuropilins play a role in blood vessel development (Neufeld et al., 1999). However, given the fact that neuropilins have a truncated intracellular domain, it is likely that they do not function independently. Indeed, cells expressing neuropilin-1 but no other VEGF receptors failed to exhibit a response in the presence of VEGF. Thus it is likely that the neuropilins serve as co-receptors to the VEGF family (Neufeld et al., 1999). On the other hand, it is also possible that the semaphorins, the other family of neuropilin ligands, play a role in blood vessel development and angiogenesis through their binding with neuropilins expressed on endothelial cells. Indeed, binding of VEGF to neuropilins may mediate effects secondary to competition with semaphorins. Regardless of their precise role of neuropilins in blood vessel development, further evidence in support of the co-receptor theory is provided by the fact that VEGF binds VEGFR2 more effectively in the presence of neuropilin-1, resulting in enhanced migration (Neufeld et al., 1999). The neuropilins also have the ability to form complexes with other VEGF receptors. Therefore they may serve to modulate signaling through other pathways (Fuh et al., 2000; Gluzman-Poltorak et al., 2001). As previously stated, neuropilins are most densely expressed in neurons. Accordingly, another possibility is that VEGF can activate neurons by binding neuropilins localized to neuronal cells.

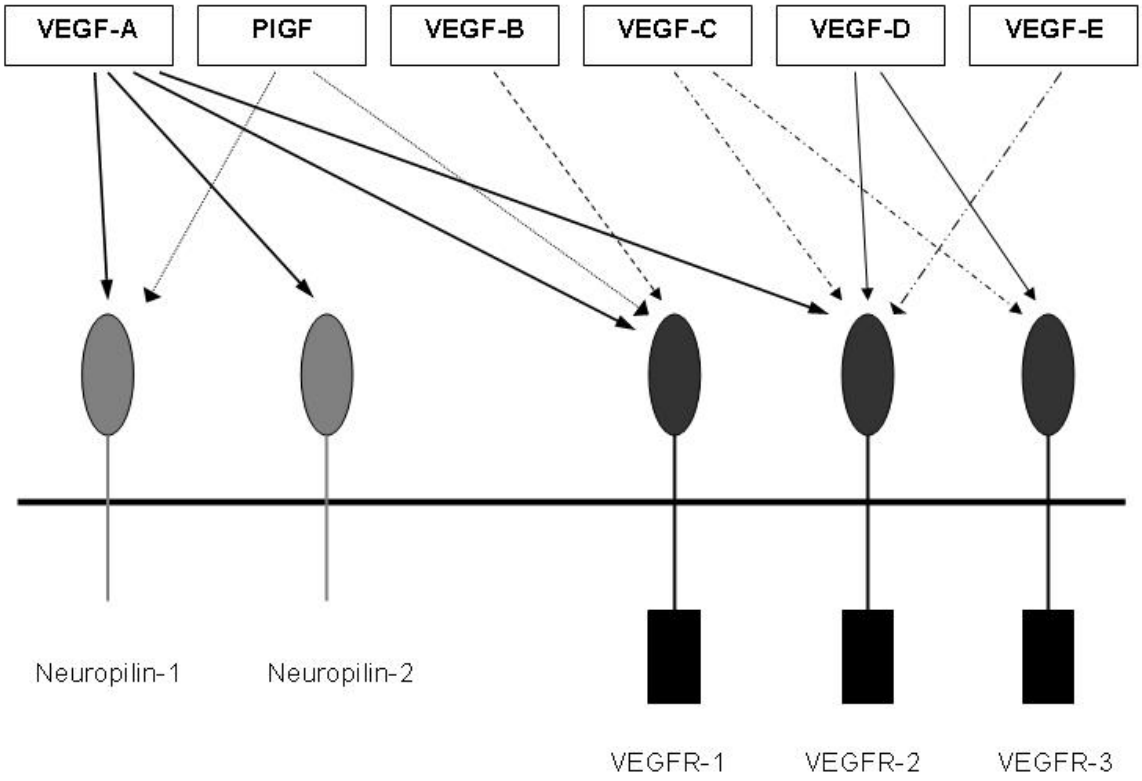

Figure 1. The VEGF family members, their receptors, the receptor tyrosine kinases VEGR-1, VEGFR-2, and VEGFR-3 and co-receptors Neuropilin-1 and Neuropilin-2 (adapted from Croll et al., 2006).

VEGF's Biological Activities

As previously described, VEGF mediates various functions within the central nervous system including vasculogenesis, angiogenesis, inflammation, and neuroprotection, each of which could play a role in seizure sequelae.

Vascular effects of VEGF

The VEGF family of protein factors has been found to have potent effects on vasculature by modulating its structure and function during development, as well as in

adult organisms. Gene deletion studies demonstrate that the lack of even a single VEGF allele during embryonic development results in a deficiency in the formation of secondary vasculature and ultimately death (Ferrara et al., 1996; Carmeliet et al., 1996). VEGF has recently been implicated as an angiogenic factor, responsible for the development of new blood vessels (for review, see Carmeliet & Storkebaum, 2002). Application of VEGF to adult tissue induces the formation of new vasculature from pre-existing vessels. This new vasculature, however, is typically poorly differentiated, disorganized, and grossly abnormal. Since exogenous administration of VEGF results in leaky blood vessels, research efforts are now also focused on VEGF's originally described function as a vascular permeabilizing agent (Senger et al., 1983).

Application of VEGF to adult brain tissue induces permeability of the cerebrovasculature resulting in edema and vascular leak (Croll et al., 2004a). This process occurs rapidly, often within 30 minutes of exposure (Dobrogowska et al., 1998). VEGF's effects on vascular permeability have therefore been implicated in a number of pathological processes including cerebral ischemia, tumor ascites, and post-stroke vasogenic edema (Kovacs et al., 1996; Hayashi et al., 1997; Cobbs et al., 1998; Lennmyr et al., 1998; Issa et al., 1999; Kraft et al., 1999; Lee et al., 1999; Pichiule et al., 1999; Plate et al., 1999; Zebrowski et al, 1999; Slevin et al., 2000). In animal models of stroke, upregulation of VEGF mRNA has been temporally correlated with vasogenic edema (for review, see Croll & Wiegand, 2001). Immunostaining and in situ hybridization illustrate an increased expression of VEGF in both glia and neurons in the ischemic brain (Lennmyr et al., 1998; Issa et al., 1999; Lee et al., 1999; Croll & Wiegand, 2001). It is therefore proposed that VEGF is secreted by the neurons and glia, binds to its receptors

on local endothelium, and mediates the increase in vascular permeability. The reason for the increase in vascular permeability is unclear, although a role for vascular leak in the angiogenic process has been proposed (Dvorak et al., 1999).

Inflammation

Another function of VEGF is the induction of inflammation. VEGF permeabilizes brain vasculature, resulting in the breakdown of the blood brain barrier and the resultant leakage of both proteins and particulates (Dvorak et al., 1995, 1999; Dobrogowska et al., 1998). In brain, this leakage is correlated with the extravasion of leukocytes, resulting in a marked localized inflammatory response (Proescholdt et al., 1999; Croll et al., 2004a). While the mechanism by which this process occurs is currently unknown, it is possible that this effect could be caused by a direct chemoattractant effect on monocytes, which express VEGFR1 (Sawano et al., 2001). However, it has also been postulated that this effect results from the complex pattern of upregulation of multiple inflammatory mediators such as ICAM-1 and Mip-1α, which have both been observed after VEGF administration to brain (Croll et al., 2004a). Thus, VEGF may be part of a pro-inflammatory cytokine cascade and has been shown to be upregulated by the pro-inflammatory cytokines IL-1 and TNF-α (Li et al., 1995; Ryuto et al., 1996; Jung et al., 2001).

Vascular permeability and inflammatory extravasation occur before the appearance of angiogenesis (Proescholdt et al., 1999; Croll et al., 2004a). Based on these findings, it has been proposed that these events precede or facilitate adult angiogenesis (Dvorak et al., 1999). Evidence in support of this notion comes from the finding that monocyte depletion inhibits the development of pathological angiogenesis by VEGF

family members (Ishida et al., 2003; Pipp et al., 2003), and that the anti-inflammatory steroid dexamethasone prevents VEGF-induced pathological angiogenesis (Kasselman et al., 2007). Other ways in which monocytes may contribute to the angiogenic process include the release of cytokines and regulatory factors conducive to endothelial cell proliferation and migration, the enzymatic degradation of vasculature, or transdifferentiation of monocytes into vascular endothelial cells (Shi et al., 1998; Moldovan et al., 2000; Fernandez Pujol et al., 2000; Pakala et al., 2002; Croll et al., 2004a).

Neurotrophic and Neuroprotective Effects of VEGF

In contrast to its potentially deleterious effects in pathological conditions, recent findings illustrate that VEGF has direct neurotrophic effects under numerous conditions (for reviews, see Carmeliet & Storkebaum, 2002; Rosenstein & Krum, 2004). While VEGF has traditionally been classified as a trophic factor for endothelial cells, recent research has demonstrated that VEGF administration in brain results in the proliferation of smooth muscle actin-positive cells, astrocytes, and microglia (Krum et al., 2002; Croll et al., 2004b). Further, application of VEGF to retinal cells in vitro caused the proliferation of photoreceptor and amacrine cells (Yourey et al., 2000). Silverman and colleagues (1999) demonstrated that VEGF application to organotypic fetal ventral mesencephalic explants stimulated angiogenesis, vessel sprouting, and lumen enlargement. A similar effect was found by Sondell and colleagues (1999, 2000) in which VEGF application to adult dorsal root and superior cervical ganglia explants resulted in significant axonal outgrowth and enhanced neuronal survival. Sondell et al.

(1999, 2000) further demonstrated that these effects are the result of VEGF binding VEGFR2 since the application of SU5416, a VEGFR2 inhibitor, blocked these effects.

VEGF has also been found to have a neuroprotective role in pathological conditions. More specifically, in an in vitro cell culture model of cerebral ischemia, VEGF reduced cell death which often results from hypoxia and glucose deprivation (Jin et al., 2000, 2001). Matsuzaki et al. (2001) demonstrated a similar neuroprotective effect in primary neuronal cultures where application of VEGF protected against glutamate-induced neurotoxicity. As previously described, these effects are mediated via VEGFR2.

VEGF has also been reported to protect against ischemic neuronal damage *in vivo*. Hayashi et al. (1998) topically applied VEGF to the cortex of rats that underwent a middle cerebral artery occlusion for ninety minutes. Results revealed a significant reduction in infarct volume, brain edema, and blood-brain barrier breakdown. TUNEL staining, which stains for apoptotic processes, was significantly reduced at 24 and 48 hours after reperfusion. Sun et al. (2003) demonstrated that local intracerebroventricular delivery of VEGF in a rat model of transient focal ischemia resulted in a reduction of infarct size as well as enhanced neurogenesis within the dentate gyrus and subventricular zone. Bellomo and colleagues (2003) found similar results utilizing an adeno-associated virus transferring gene for VEGF (rAAV-VEGF) in a gerbil model of transient brain ischemic injury. Specifically, they found that rAAV-VEGF significantly improved brain edema and delayed CA1 neuronal death.

VEGF's neuroprotective role has been explored more recently in the context of neurodegenerative disorders. Oosthuyse et al. (2001) selectively deleted the hypoxia response element in the VEGF promoter region of embryonic stem cells. The hypoxia

response element, which is the binding site for hypoxia inducible factors, is responsible for VEGF upregulation and resultant angiogenesis during hypoxic conditions. They found that this deletion reduced hypoxic VEGF expression in the spinal cord and resulted in severe adult-onset muscle weakness stemming from progressive degeneration of lower motor neurons that occurs in amyotrophic lateral sclerosis (ALS). They postulated that this degeneration may result from insufficient levels of VEGF resulting in chronic ischemia due to insufficient neural vascular perfusion or lack of neuroprotection. Further in vitro studies revealed that VEGF protected cultured primary motor neurons against hypoxia-induced apoptosis. This protection was demonstrated through activation of VEGFR2 and neuropilin-1. Zheng and colleagues (2004) further investigated the role of VEGF in an animal model of ALS and found that administration of VEGF delayed the onset of symptoms and prolonged survival. In addition to replicating these results, Storkebaum et al. (2005) found that overexpression of VEGFR2 was necessary for this outcome.

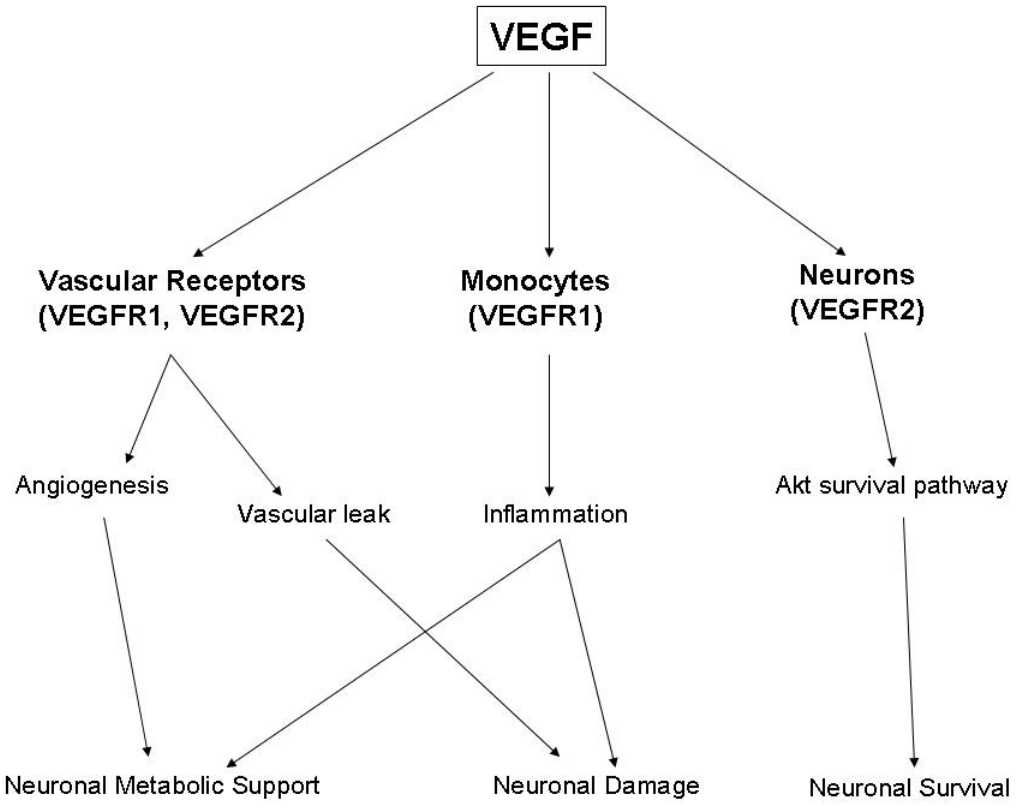

Figure 2. VEGF mediates various effects based on its receptor localization. During adverse conditions in which the system has become compromised, binding to specific receptors leads to beneficial effects while binding to other receptors leads to detrimental consequences (adapted from Croll et al., 2006).

VEGF and Seizures

VEGF has been studied in the context of many pathological disorders. VEGF inhibition as a therapeutic strategy has been investigated in disorders characterized by pathological vasculature including proliferative retinopathies, tumors, and psoriasis (for review, see Storkebaum & Carmeliet, 2004). VEGF's angiogenic actions have also been

of interest as a target therapeutic for relieving chronic ischemic conditions (Bauters et al., 1994; Isner et al., 1996; Isner et al., 1998; Losordo et al., 2002). VEGF as a neurotrophic factor in neurological diseases, however, has received less attention and has only recently become of interest given the accumulating evidence for its neuroprotective role. The most extensive research to date regarding VEGF's role has been conducted in models of ischemic stroke and hypoxic damage.

Severe epileptic seizures, like stroke, are characterized by the breakdown of the blood-brain barrier, vascular leak, and inflammation to a lesser degree (Ates et al., 1999; Cornford, 1999; Roch et al., 2002). Several inflammatory cytokines, including IL-1 and TNF-α, are upregulated after seizures and research suggests that these cytokines increase the potential for seizures (Vezzani et al., 2000, 2002). It has also been demonstrated that these cytokines upregulate VEGF. Further, VEGF has been shown to induce inflammation, which has been documented to contribute to decreased seizure thresholds and increased seizure damage (Vezzani et al., 1999). Taken together, these data suggest that VEGF could potentially have a detrimental effect on seizures and seizure-related sequelae. In fact, VEGF has been shown to worsen post-ischemic edema (Zhang et al., 2000). Further, inhibition of VEGF following stroke significantly decreases edema (van Bruggen et al., 1999). Hence, it is possible that VEGF may worsen edema after seizures.

In spite of its inflammatory and edemic effects, accumulating evidence suggests that VEGF plays a neuroprotective role in pathological conditions such as stroke, cerebral ischemia, hypoxia, neurodegenerative disorders, and in vitro models which mimic the pathological processes that underlie seizures (Hayashi et al., 1998; Jin et al., 2000, 2001; Matsuzaki et al., 2001; Oosthuyse et al., 2001; Bellomo et al., 2003; Sun et al., 2003;

Zheng et al., 2004; Storkebaum et al., 2005). The dual role of VEGF in inducing detrimental and beneficial effects may arise from its actions at two different receptors, and renders any conclusions about the net effect of VEGF after seizures non-obvious. Studies have shown that other VEGFR1 agonists, such as PlGF, also induce inflammation and vascular leak, which indicates that VEGFR1 may mediate these processes (Luttun et al., 2002; Autiero et al., 2003). VEGFR2, on the other hand, has clearly been established as the receptor that mediates the protective effects of VEGF (Sondell et al., 1999, 2000; Matsuzaki et al., 2001; Storkebaum et al., 2005).

Although VEGF has been found to play a role in various pathological disorders, whether it is detrimental or beneficial, it is currently unclear if VEGF plays a role in epilepsy. Microarray analysis and in situ hybridization revealed neuronal expression and regulation of VEGF mRNA following seizures induced by electroconvulsive shock treatments in rat (Newton et al., 2003). Thus, it is possible that VEGF may play a role in epilepsy.

Specific Aims

As previously mentioned, the fact that current AEDs fail to effect change in the underlying pathophysiology of epilepsy has shifted the current focus towards neurotrophic factors. Research has demonstrated that VEGF, a current neurotrophic factor of interest, is often involved in pathological conditions, including seizures, and that it has the potential to exert positive and negative effects. While it is involved in the induction of vascular permeability, inflammation, vascular leak, and angiogenesis, it also

functions to protect neurons. These various functions likely occur through binding at different receptors.

The role of VEGF in seizures is currently unclear. Studies have shown that VEGF mRNA is increased after seizures (Newton et al., 2003), but no work has shown that this increase in mRNA translates to an increase in VEGF protein. Further, the consequences of any increase in VEGF protein after seizures remain unstudied. Thus, the following aims were employed to address this question as well as to characterize the role of VEGF in seizures.

Specific Aim 1: To determine if VEGF protein is upregulated after seizures.

Specific Aim 2: To determine if increases in vascular density in the dorsal hippocampus occur concomitant with increases in VEGF after seizures.

Specific Aim 3: To determine if blockade of endogenous VEGF worsens seizure-related cell damage, and if infusion of exogenous VEGF attenuates seizure-related cell damage.

Specific Aim 4: To determine if infusion of exogenous VEGF during status epilepticus and for three weeks thereafter will reduce the severity of the functional consequences of seizures.

Specific Aim Rationales

Specific Aim 1: To determine if VEGF protein is upregulated after seizures.

Epileptic seizures are characterized by vascular leak and inflammation. In animal models of cerebral ischemia, VEGF expression is upregulated in a time course that correlates with that of vascular leak and inflammation. In view of the fact that they are both characterized by similar sequelae and given that VEGF mRNA was found to be

increased after seizures induced by electroconvulsive shock (Newton et al., 2003), we first wanted to explore whether VEGF protein was increased after pilocarpine-induced status epilepticus and if it was expressed in neurons and glia within the central nervous system. We used an acute model of epilepsy because it tends to produce considerably greater pathology than the chronic model and also allows for the investigation of the latent period.

While we investigated various areas within the central nervous system, our primary focus was on the hippocampus for several reasons. First, the hippocampus is highly susceptible to seizures as it has the lowest seizure threshold of any brain region (Green, 1964). Second, cell structures are readily identifiable at both the gross and histological levels. Finally, the hippocampus plays a pivotal role in learning and memory and as one of the structures within the limbic system, it is also involved in emotional functioning. Therefore, we could also explore functional consequences of VEGF's role in seizures.

Specific Aim 2: To determine if increases in vascular density in the dorsal hippocampus occur concomitant with increases in VEGF after seizures.

It has been well documented that one of VEGF's functions is the induction of new vasculature from pre-existing vessels. Therefore, based on our findings of increased VEGF protein after seizures, we investigated if there was an increase in vascular density in the dorsal hippocampus following pilocarpine-induced status epilepticus. We also investigated the time course of this process since it has been documented that angiogenesis occurs two to three days after administration of exogenous VEGF (Croll et al., 2004). Therefore, we looked at three different time points, 24 hours, 3 days, and 7

days after seizures, to determine if and when upregulated endogenous VEGF increased vascular density. Since it has been postulated that angiogenesis is dependent upon the increased permeability of brain vasculature and resultant edema, which also happen to be effects mediated by VEGF, we also investigated these processes in tissue taken at the three time points. To further investigate VEGF's angiogenic role after seizures, we conducted studies in which we inhibited endogenous VEGF by infusing Flt-Fc, an immunoadhesin, which functions to bind VEGF ultimately preventing VEGF from binding to its endogenous receptors.

Specific Aim 3: To determine if blockade of endogenous VEGF worsens seizure-related cell damage, and if infusion of exogenous VEGF attenuates seizure-related cell damage.

Accumulating evidence suggests that VEGF directly protects neurons from excitotoxic death and that this function is mediated through actions at VEGFR2. Neurons constitutively contain neuropilins and have been shown, under conditions including development, ischemia, and in cell culture, to express VEGFR2 (Lennmyr et al., 1998; Sondell et al., 1999, 2000; Croll & Wiegand, 2001; Ogunshola et al., 2002). Glial cells have also been shown to contain VEGF receptors. Based on these findings, we first explored whether endogenous VEGF played a protective role in cell loss after status epilepticus. We then conducted studies in which animals received continuous infusions of exogenous VEGF prior to and during status epilepticus to determine the amount of VEGF needed to protect cells from damage.

If VEGF does prove useful as a neuroprotective agent in this model of epilepsy, its administration in its current form would not be optimal in humans due to the fact that

it is a large protein which needs to be delivered directly and continuously to the brain via an indwelling cannula. Administration of the protein could be impeded by complications with the cannula, such as a blockage, or the cannula could become dislodged. Further, an indwelling cannula leaves the brain susceptible to infection. Thus, in addition to the protein studies, we conducted a preliminary gene therapy experiment to see if we would get similar results to our protein studies. We used an adeno-associated viral vector as it allows one to induce long-term expression of physiological levels of VEGF with a single microinjection. Additionally, adenoviral delivery allows for a wider range of protein expression than protein infusions.

Finally, if exogenous VEGF protects neurons from damage after severe seizures, it would be interesting to determine if endogenous VEGF subserves the same role. This information could elucidate mechanisms of endogenous neuroprotection which could be relevant in multiple disease states, including ALS, in which mutations that lower endogenous VEGF levels have been found. Endogenous VEGF was blocked with continuous infusion of the VEGF receptor body Flt-Fc to evaluate its endogenous protective effects after severe seizures.

Specific Aim 4: To determine if infusion of exogenous VEGF during status epilepticus and for three weeks thereafter will reduce the severity of the functional consequences of seizures.

As discussed previously, epilepsy often results in functional impairments such as deficits in intellectual functioning, learning and memory, speed of processing, and executive skills. It also underlies some psychiatric disorders including anxiety and depression. The hippocampus plays a fundamental role in learning and memory and

emotional functioning. Based on our findings that VEGF preserved neurons after status epilepticus and decreased damage in the hippocampus, we hypothesized that VEGF may also attenuate behavioral impairments often seen in epilepsy. In order to investigate functional preservation, animals were continuously treated with VEGF before, during, and after pilocarpine-induced status epilepticus. Behavioral testing to evaluate learning, memory, and emotional functioning was conducted during the latent period when it is hypothesized that physiological changes take place which result in chronic epilepsy.

GENERAL METHODS

1. Subjects, Proteins, and Surgeries

Subjects

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

Proteins

The VEGF used for protein infusions was human recombinant $VEGFA_{165}$. VEGF was stored frozen until used and then diluted in sterile phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) to attain doses of 15ng, 30ng, 45ng, and 60ng/day in a 12µl volume delivered .5µl/hour via osmotic minipump. PBS was autoclaved before being used as a diluent for protein reagents. These doses were chosen based on pilot data (not shown) demonstrating no significant effect of VEGF in our model when infused at a dose of 15ng/d or lower, as well as data demonstrating that infusion of more than 30ng/d of mouse VEGF (Croll et al., 2004a) or 60ng/d of human VEGF (unpublished data) resulted in overt angiogenesis.

An adeno-associated mVEGF viral vector (AAV 2/1) was used for microinjections. For these procedures, VEGF was tagged with an EGFP (enhanced green fluorescent protein) fluorescent marker prior to placement within a replication-deficient adeno-associated viral vector. The virus was injected into the dorsal hippocampus. This

virus functioned to infect cells within the hippocampus, causing them to upregulate endogenous VEGF.

Flt-Fc, an immunoadhesin designed to sequester endogenous VEGF, was used at a dose of 12µg/d to interfere with endogenous VEGF receptor binding. This reagent is a forced dimer of regions 1-3 of Flt (VEGF receptor 1) fused to the Fc domain of human IgG (hFc) and was dissolved in sterile PBS.

BowAng1, a fusion of four molecules of the vascular growth factor angiopoietin-1 with two molecules of hFc (Davis et al., 2003), was used at a dose of 3µg/d. Angiopoietin-1 has been shown to block VEGF's vascular permeabilizing effects (Thurston et al., 2000) but not its angiogenic effects in brain (Croll et al., unpublished observations).

PBS was purchased in powder form, mixed with distilled water, sterilized, and used as a control. Additional controls were used in some cohorts, which include the protein controls BSA (bovine serum albumin, to control for protein load), hFc (a recombinant human control protein), and inactivated VEGF. VEGF was inactivated by repeated freeze-thaw cycles, which has previously been shown to eliminate VEGF's bioactivity (unpublished data), rather than by heat, which results in a precipitate. Proteins were a generous gift of Regeneron Pharmaceuticals.

Pump implantation and protein infusion

Animals were anesthetized using 6mg/kg chlorpromazine injected intraperitoneally followed by 210mg/kg ketamine (Sigma-Aldrich) administered intramuscularly or 65mg/kg sodium pentobarbital (Henry Schein, Melville, NY). The scalp was shaved, cleaned with alcohol, and treated with iodine. Animals were placed in

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

One week following cannula implantations, animals were re-anesthetized following the same procedure and an incision was made at the nape of the neck. The heat-sealed tip of the catheter was snipped and an Alzet osmotic minipump (Durect Corporation, Palo Alta, CA), containing rhVEGF$_{165}$, control proteins, or sterile PBS, infusing 0.5 µl per hour, was attached to the catheter or catheters and glued. The pump was inserted into the subcutaneous space at the nape of the neck and the incision was closed with nylon sutures. Animals were placed under a heat lamp to recover.

Microinjections

Animals were anesthetized and prepared using the same procedures as for pump implantations. Four small burr holes were drilled through the skull using the following

coordinates: -2.7 posterior to bregma, -3.8 lateral to bregma; -2.7 posterior to bregma, -4.6 lateral to bregma; -2.7 posterior to bregma, +3.8 lateral to bregma; -2.7 posterior to bregma, +4.6 lateral to bregma; incisor bar set at -3.5mm. Microinjections were administered using a Hamilton micro-syringe lowered into the dorsal hippocampus (-3.0mm ventral from skull and -4.0mm ventral from the skull, angled at 0 degrees) at a rate of 0.25 microliters/minute. Time between injections was a maximum of three minutes. Wounds were sutured with polyamid nylon suture thread and treated with topical antimicrobial ointment, and rats were kept under heat lamps until recovered.

Acute seizure induction

Five days following pump implantations for protein infusions, animals were pre-treated with 1mg/kg atropine methylbromide (Sigma-Aldrich) injected subcutaneously 30 minutes prior to receiving either 350mg/kg pilocarpine hydrochloride (Sigma-Aldrich) or an equivalent volume of saline intraperitoneally. Seizures were scored from stages 1-4 based on Racine's scale (1972) and modified to include stage 5, defined as sudden but transient, whole-body tonus, stage 6, defined as status epilepticus, stage 7, defined as status with a period of tonus, and stage 8, death occurring during status epilepticus (Rudge et al., 1998). Status epilepticus was defined as seizures with no intervening return to normal behavior for greater than five minutes. Status epilepticus was truncated with 10mg/kg diazepam (Henry Schein) after 60 minutes. Animals not achieving status epilepticus received diazepam 90 minutes after pilocarpine. Status epilepticus is typically induced 30 minutes after pilocarpine administration, therefore, animals that achieved status epilepticus and those that did not received diazepam injections in approximately the same time frame. Animals were hydrated immediately following diazepam injections

with 3cc of a glucose and saline solution and received apple slices for further hydration. Animals received hydration injections daily for one week or until sacrificed.

The above protocol was instated two weeks following virus microinjections for designs in which animals received virally-administered VEGF instead of protein infusions via pump administration.

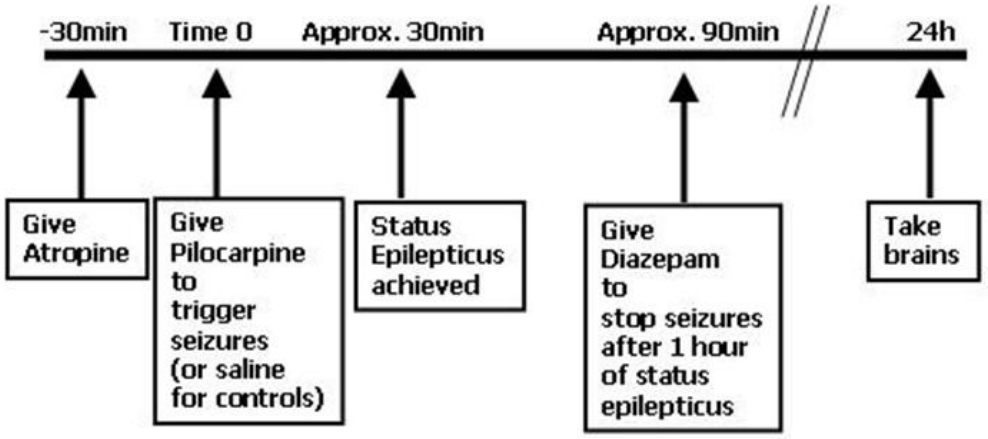

Figure 3. Timeline of seizure induction.

2. Behavioral Analyses

Morris Water Maze: Each animal was placed in a 130cm diameter water maze, made opaque with white, non-toxic paint, back-end first to avoid stress and facing the pool-side to avoid bias. The animal was placed in the pool for three trials per day with an inter-trial interval of one minute. Each trial ended when the animal escaped onto the submerged, hidden goal platform or when the animal had been in the maze for two minutes. Any animal that had not located the platform within two minutes was guided to the platform by hand. Each animal was tested daily until acquisition of the memory was achieved. Acquisition was defined as naïve or control animals in each cohort locating the

platform in less than 10 seconds. This typically occurred by the fourth day (twelfth trial) although acquisition trials in some cohorts were extended to the fifth day if the acquisition was not achieved. Following the acquisition trials, the goal platform was removed for a spatial probe trial in which each animal was placed in the maze for 30 seconds, and the proportion of time spent in the goal and other quadrants was recorded. Additionally, swim speed was estimated using mean quadrant crossing time to ensure that increased latencies to escape did no reflect decreased swim speed.

Social Interaction: Each animal was placed on an 86cm square open field with a novel rat of similar strain, age, and gender. Each animal was observed for five minutes to assess the number of times that the experimental rat initiated face-to-face contact with the novel rat, number of times that the experimental rat initiated face-to-body contact with the novel rat, number of times that the experimental rat displayed aggression toward the novel rat, and the amount of time spent in non-exploratory physical contact with the novel rat.

Grid Locomotor Activity: Each animal was placed in the center of an 86cm square open field on which six 29cm squares were formed using white masking tape. Each animal was observed for six minutes, divided into two-minute bins, to measure the number of grid crossings. Measurement of the total number of grid crossings, habituation across the time bins, and number of fecal eliminations were recorded.

Light-Dark Exploration: Each animal was placed into a 43cm x 86cm box in which one side was covered and painted black and the other side was open and painted white. The animal was placed on the white side of the box. The amount of time spent in the black chamber versus the white chamber was recorded for a trial total of five minutes.

3. Tissue Collection and Processing

Tissue Collection

Fresh Tissue Collection for ELISAs: Animals were deeply anesthetized with an overdose of a pentobarbital-based euthanasia solution (Euthasol, Del Marva Laboratories, Henry Schein). For analysis of VEGF protein levels by enzyme-linked immunosorbent assay (ELISA), animals were immediately decapitated and the brains were removed and placed on ice for 3 minutes until firm. They were then placed in an acrylic brain matrix (MyNeuroLab, Inc., www.myneurolab.com) and cut into 1.5mm slabs with a thin razor. Two slabs containing the full medial-lateral extent of dorsal hippocampus were selected, and a 3mm wide sample was cut from the center of dorsal hippocampus and the overlying cortex (selected to match the region of VEGF infusion in pump studies). Tissue samples were frozen in Eppendorf tubes on dry ice. Tissue remained frozen at -80° until ready for analysis.

Fixed Tissue Collection for Histology: For other assays, animals were perfusion fixed as follows. The chest cavity was opened, a needle was inserted into the left ventricle of the heart, and an incision was made in the right auricle for release of fluids. The animals were exsanguinated with heparinized isotonic (0.9%) saline perfused through the heart. Following exsanguination, animals were perfusion-fixed first with 4% paraformaldehyde in acetate and then 4% paraformaldehyde in borate buffer, as previously described (Croll et al., 1999). The brains were removed and placed in 30% sucrose borate buffer at 4°C until sectioned.

Sectioning

After 3-7 days in the buffered sucrose solution, brains were sectioned coronally at 40µm using a sliding microtome (American Optical Company, Buffalo, New York). Sections were placed in a 24-well plate and stored in an ethylene glycol-based cryoprotectant solution (Watson et al., 1986) at -20 degrees Celsius until stained.

Histology

Cell loss: Some sections were stained with cresyl violet for subjective evaluation of cell damage. Additional sections were stained with methylene blue for quantitative evaluation of cell damage. Sections were hydrated through graded ethanols and then stained with a 1.6%/1% methylene blue/azure II solution following exposure to 1% periodic acid.

Immunocytochemistry: Sections were immunostained for VEGF as previously described (Scharfman et al., 2000) using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and an anti-VEGF (goat polyclonal, 1:1,000, R & D Systems, Minneapolis, MN) antibody; other sections were additionally immunostained as previously described (Croll et al., 2004a) with a secondary antibody, glial fibrillary acidic protein (GFAP monoclonal, 1:1,000, Sigma). Before staining brain tissue, we verified the specificity of the VEGF antibody by staining adjacent sections using in situ hybridizations for VEGF mRNA in developing embryos and adult rat ovaries (data not shown). In addition, the primary antibody was not added to some sections. Additional sections were immunostained with anti-rat endothelial cell antigen (RECA) for vasculature (mouse monoclonal, 1:250, Serotec, Raleigh, NC), anti-OX-1 for leukocytes (mouse monoclonal, 1:10,000, Serotec), and anti-VEGFR2 for receptor verification

(mouse monoclonal, 1:150, 1:300, 1:450, Serotec). All tissues were exposed using a nickel sulfate-intensified diaminobenzidene (DAB) chromagen reaction.

VEGF ELISA

Rat

An Immuno Maxisorp plate (VWR Scientific, West Chester, PA) was coated with 100µl per well $rhVEGF_{165}$ (Regeneron Pharmaceuticals, Tarrytown, NY) at 2µg/ml in carbonate/bicarbonate buffer (Sigma-Aldrich) and incubated overnight at 4°C. The plate was then washed with KPL buffer followed by 300µl 0.2% I-blocking buffer (Tropix, Foster City, CA) at room temperature for 1 hour. The standard was diluted to 100ng/ml and serially diluted in diluent with normal mouse serum. Samples were diluted and 100µl was placed in each well, in duplicate, and incubated for 2 hours at room temperature. The plate was then washed 4 times with 300µl wash buffer. Goat anti-human IgG Fc conjugated to HRP (Sigma-Aldrich) at 1:20,000 was added in diluent and incubated for 1 hour at room temperature. The plate was washed again 4 times, followed by 100µl per well of TMB substrate (Sigma-Aldrich), and developed at room temperature for 30 minutes. Development was stopped by adding 100µl per well 2N H_2SO_4. The plate was read at 450-570nm, and samples were normalized to standards where the range of the standard curve was 0.14 to 100 ng/ml.

Human

Tissue samples were removed from the -80° freezer, weighed, placed in a homogenization buffer solution (1:10), and ground with an Ultra-Turrax T 25 Basic homogenizer (IKA Works Inc., Wilmington, NC). The ELISA was performed using a kit obtained from R&D Systems following manufacturer's instructions. Briefly, assay diluent RD1W (50µL) was added to each well before tissue samples were placed in the

wells. The plate was mixed by gently tapping for one minute, covered with adhesive strip, and incubated for two hours at room temperature. Wells were then aspirated and the plate underwent three washes by adding 400µL wash buffer to the wells during each wash. Following the final wash, the plate was inverted and blotted dry. This was followed by the application of 200µL of VEGF conjugate to each well. The plate was then covered with new adhesive strip and incubated for two hours at room temperature. Following this incubation period, VEGF conjugate was aspirated and the plate underwent three washes following the same procedure above. After the last wash, 200µL of substrate solution was added to each well and was allowed to incubate for 20 minutes at room temperature. The plate was protected from light during this incubation period. Development was stopped by adding stop solution (50µL) to each well. The plate was read at 450nm.

4. Anatomical Quantification

Vasculature: Vascular density and vascular diameters were measured in RECA-immunostained tissue sections as previously described (Croll et al., 2004a). Briefly, images were viewed under a Nikon Eclipse E400 microscope (Morrell Instruments, Melville, NY) captured with a digital video camera into SPOT software and imported into the public access image analysis program, NIH Image (National Institutes of Health, Bethesda, MD). Vascular density was measured as the proportion of area occupied by RECA-positive lumens in equatorial sections by point-count stereology using a randomly-oriented acetate-grid overlay. Vascular diameters were measured by taking the smallest diameter across cross-sectional vascular profiles, and the perpendicular distance across longitudinally-oriented vessels. Both measures were taken using the NIH Image

length function on those vessels randomly selected by the grid used in point-count stereology.

Rating of inflammation: Inflammation was assessed in OX-1-stained sections using a subjective rating scale from 0 to 4 (0: no infiltrated inflammatory cells; 1: sparse inflammatory infiltrate; 2: mild inflammatory infiltrate; 3: either mild but widespread inflammation or dense local inflammation; 4: dense widespread inflammation). Scores were determined by two trained evaluators blind to the treatment of the animals. Four sections were quantified per animal and the average score was used to represent inflammation for each animal.

Rating of neuronal damage: Neuronal loss was initially assessed using a previously published subjective rating scale (Rudge et al., 1998). Two evaluators blind to the treatment of the animals evaluated loss of CA1 pyramidal neurons in the dorsal hippocampus on a scale of 0-4 (with 0 representing no damage and 4 representing an estimated loss of greater than 80%). Four sections were quantified per animal and the average score was used to represent the damage score for the animal.

Neuronal Density: Estimates of status epilepticus-related cell loss were made using the optical fractionator method (West et al., 1991). The total number of neurons was determined within a region of interest in area CA1 that was within the diffusion range of the cannula tip (i.e., 1.5mm radius). The region of interest was defined as the area of the pyramidal cell layer between area CA2 and the subiculum in the medial-lateral axis, and from the initial appearance of the CA1 pyramidal cell layer to the portion of the hippocampus where the dorsal and ventral portions of area CA3 united in the rostral caudal axis. Coronal sections in a one-in-six series, with a randomly-determined starting

point, were mounted on slides and stained with a 1.6%/1% methylene blue/azure II solution following exposure to 1% periodic acid as previously described.

Sections were viewed with an Olympus BX-51 microscope and Optronics video camera. Using a stereological software package (Stereo Investigator, Microbrightfield Inc.), a pre-determined counting frame ($25um^2$) was systematically moved along a randomly placed grid ($125um^2$), and the number of cell nucleoli that came into focus within a portion of the section (excluding 4um upper and lower guard zones) were counted. Only cells that had a darkly stained nucleolus surrounded by a lightly stained nucleus and cytoplasm were counted. In the event that two nucleoli could not be distinguished as belonging to two separate neurons, only one neuron was counted. Neurons that were pyknotic were not considered viable neurons and were excluded from the analysis. The total number of neurons within the region of interest were estimated with the formula: N = sum Q- x 1/tsf x 1/asf x 1/ssf, where the number of neurons counted (sum Q-) were 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).

Hippocampal Volumes: Sections were taken in a 1:6 series and utilized for evaluation of hippocampal volume with the Neurolucida system (MicroBrightField, Inc). Immunostained areas were quantified using the contrast threshold measurement in NIH Image. Volumetric measurements were adjusted to account for differences in overall brain size before being statistically analyzed.

5. Data Analysis

To determine if there were any statistical differences between groups, quantitative data were analyzed with Student's independent groups t-test, a one-way analysis of variance (ANOVA), or a factorial ANOVA, depending on the design of the particular experiment. All statistical analyses were conducted using SPSS software (version 11.5) using an alpha value of .05. If statistical significance was attained, a Tukey LSD post-hoc test was performed when appropriate to determine which groups were statistically significantly different from each other. The Tukey LSD test was set at alpha value of .05.

All behavioral tasks were assessed for outliers, which were defined as scores greater than two standard deviations from the mean. Outliers were subsequently removed and data re-analyzed. For the Morris water maze, if an animal's data on two or more days was an outlying score, the animal was considered an overall outlier and was completely removed from the analysis for that task. If an animal's datum was an outlier on only one day of the maze, the animal's datum was interpolated by transforming all scores for that animal on the specific task to z-scores, obtaining the mean z-score, and then transforming the z-score for the outlying day back to a raw score.

SPECIFIC AIM METHODS AND RESULTS

Specific Aim One

VEGF Protein Upregulation after Seizures

Previous research has demonstrated that VEGF mRNA is increased after severe seizures (Newton et al., 2003). Furthermore, during pathological events such as cerebral ischemia, VEGF is upregulated and functions to permeate vasculature causing a breakdown of the blood brain barrier, vascular leak, and the resultant inflammatory response. Since seizures result in similar sequelae, it was hypothesized that VEGF protein would be upregulated in neurons and glia after pilocarpine-induced status epilepticus.

Experiment 1A:

In order to determine if VEGF is upregulated after seizures, animals received injections of pilocarpine to induce status epilepticus or saline as a control as previously described (see General Methods). The experiment consisted of two groups as shown in the table below.

Group 1	n = 4	Pilocarpine
Group 2	n = 3	Saline

Animals were sacrificed 24 hours after injections and fresh tissue was obtained for VEGF mRNA analysis in the hippocampus and temporal neocortex via ELISA.

VEGF ELISA

VEGF ELISAs were used to quantify changes in VEGF protein in tissue of animals that had pilocarpine compared to saline controls (n=3 per group). ELISA data

revealed a statistically significant doubling of VEGF protein 24 hours after pilocarpine-induced status epilepticus in both cortex and hippocampus (treatment effect $F(1,8)=50.344$, $p<.05$, see Figure 4). Therefore, VEGF protein was significantly upregulated in temporal cortex and hippocampus 24 hours after status epilepticus.

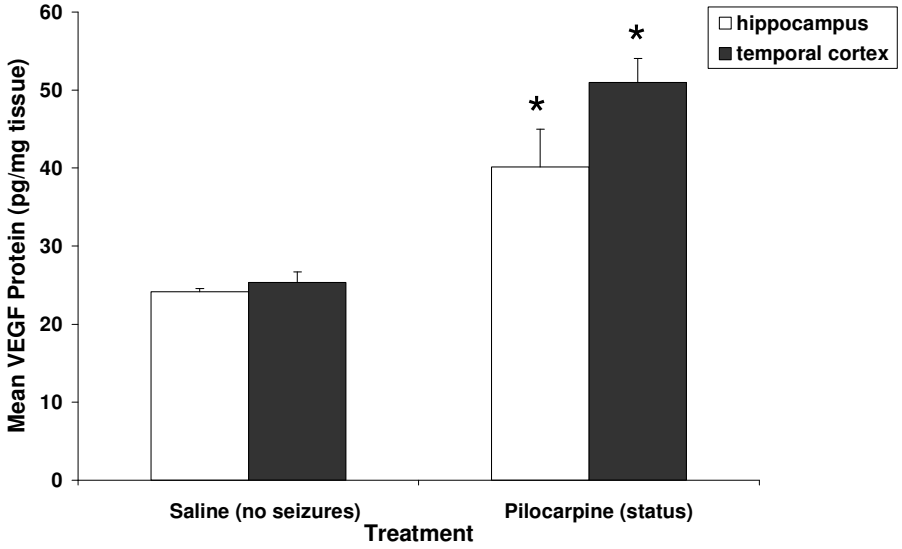

Figure 4. VEGF protein as measured by ELISA 24 hours after pilocarpine-induced status epilepticus.
VEGF protein was doubled in both neurons and glia 24 hours after pilocarpine-induced seizures (n=3), *significantly different than saline (n=3), $p<.05$.

Experiment 1B:

To ascertain the localization of VEGF upregulation because ELISA does not provide information about cellular localization of increased VEGF protein, the above experiment was repeated except tissue was prepared for immunostaining. The groups were as follows:

Group 1	n = 4	Pilocarpine
Group 2	n = 4	Saline

Twenty-four hours following injections, animals were sacrificed and perfusion fixed as previously described (see General Methods). Tissue was immunostained with an antibody to VEGF (see General Methods) and analyzed for VEGF upregulation.

VEGF Immunostaining

In order to determine which cells upregulate VEGF after seizures and because ELISA does not provide information about cellular localization of increased VEGF protein, immunostaining for VEGF was conducted (n=4 per group) in collaboration with the Scharfman lab (Helen Scharfman and Jeffrey Goodman). In saline controls, VEGF immunostaining was very light, and not observed in neurons. In contrast, staining revealed a marked expression of VEGF protein in neurons of hippocampal CA1 and CA3 (Figure 5), as well as the temporal neocortex (Figure 6) 24 hours after status epilepticus, which resolved by 7 days after status (data not shown). This immunostaining appeared to be cytosolic, particularly in CA3 (Figure 5D), given that the darkest staining was observed between the outer membrane and the nucleus (which was unstained).

Therefore, it appears that hippocampal CA1 and CA3 neurons upregulate VEGF after status epilepticus.

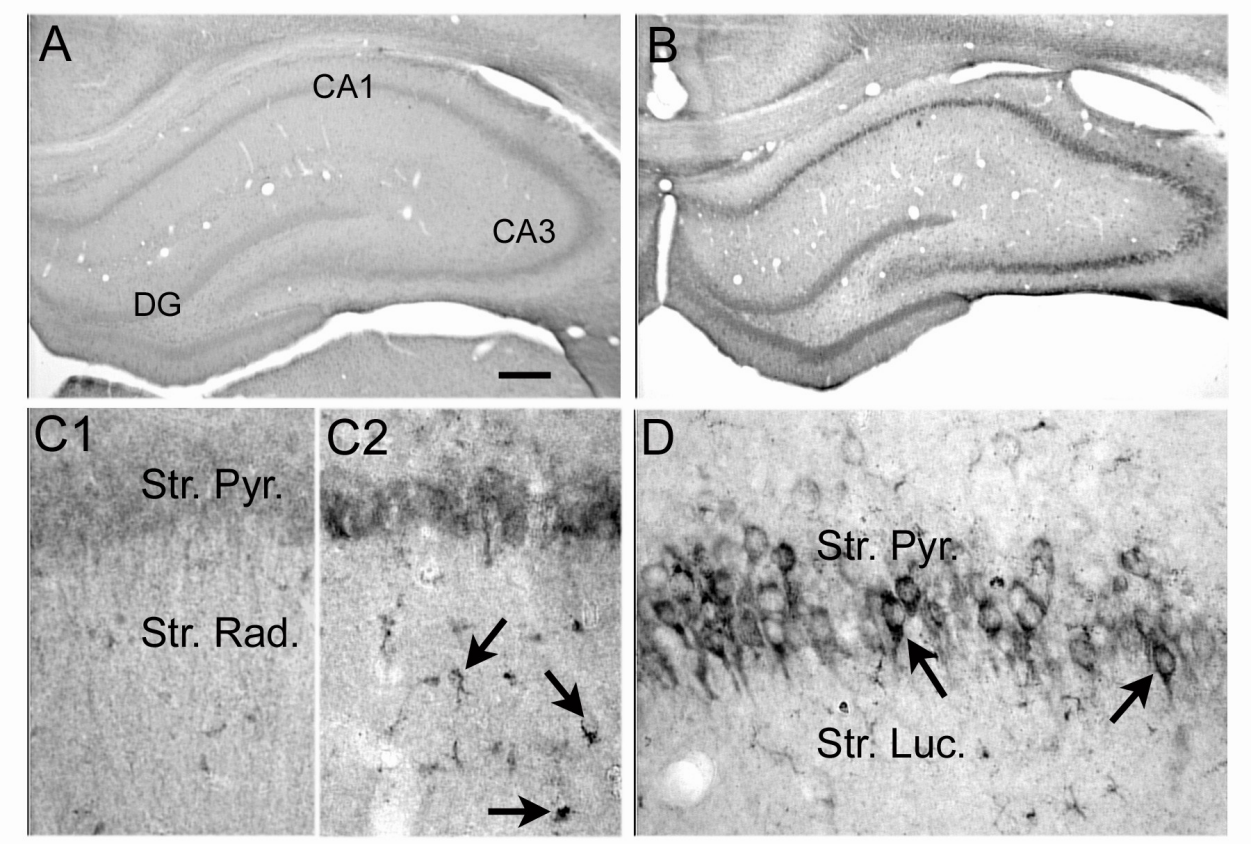

Figure 5. Increased VEGF protein expression in hippocampus 24 hours after pilocarpine-induced status epilepticus.

A-B. Sections from a saline- (**A**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**B**) were processed together using an antibody to VEGF. Increased VEGF protein was evident in the cell layers. DG = dentate gyrus. Calibration = 200μm.

C. Increased magnification of area CA1 from part **A** (**C1**) and **B** (**C2**) shows that glial-like structures were associated with increased VEGF immunoreactivity after pilocarpine-induced status compared to controls. Scale bar from panel A = 50 μm for panels C(1) and C(2).

D. A different section from a pilocarpine-treated rat that had status epilepticus, showing increased VEGF immunoreactivity in area CA3 pyramidal cell somata. Scale bar shown in panel A = 100 μm for panel D.

Similar patterns of VEGF protein upregulation after status epilepticus were observed in other regions of the brain commonly implicated in seizures, including the dorsal midline thalamus and amygdala (Figure 6), with the neuronal upregulation particularly striking in amygdala (Figure 6D2).

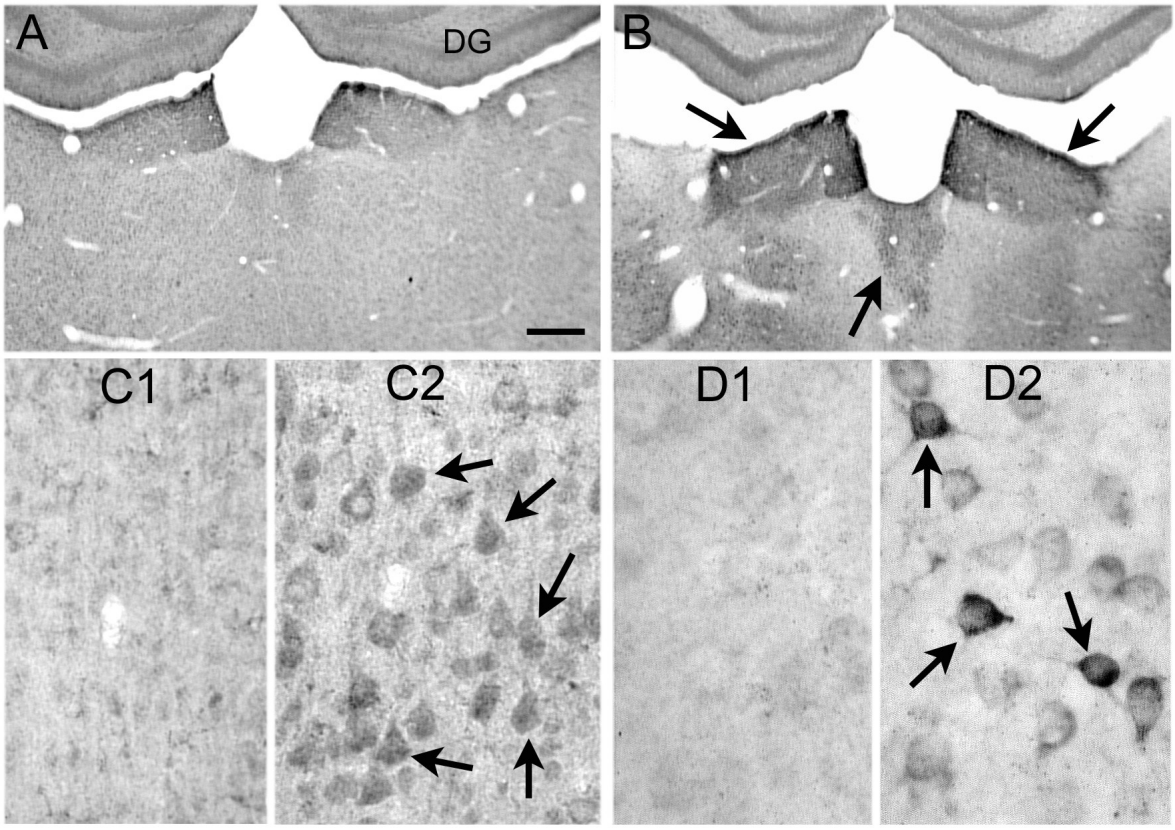

Figure 6. Increased VEGF protein expression in thalamus, neocortex, and amygdala 24 hours after pilocarpine-induced status epilepticus.

A-B. Sections from a saline- (**A**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**B**) were processed together using an antibody to VEGF. Increased VEGF protein was particularly evident in the dorsal midline thalamic nuclei. DG=dentate gyrus, Calibration = 200µm.

C. Sections from a saline- (**C1**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**C2**). Increased VEGF protein was evident in pyramidal cell somata (arrows) in temporal neocortex. Scale bar shown in panel A = 50 µm for panels C1 and C2.

D. Sections from a saline- (**D1**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**D2**). Increased VEGF protein was evident in somata of amygdala neurons (arrows). Scale bar shown in panel A = 25 µm for panels D1 and D2.

In addition to VEGF expression in neurons, VEGF protein was consistently evident in cells throughout the hippocampus and cortex that appeared to have a glial morphology. While both saline and pilocarpine-treated animals showed this staining pattern, it was much more pronounced in tissue from the pilocarpine-treated rats (Figure 5C2 versus 5C1). On these cells, staining was punctate and marginal, suggesting the possibility of cell-surface staining. To verify that these cells were indeed astrocytes, tissue sections treated with the VEGF antibody were also processed using an antibody to GFAP, a marker of mature astrocytes. The glia-like cells that expressed VEGF also expressed GFAP, confirming that the cells were in fact astroglia (Figure 7). Thus, glial cells are also affected by VEGF upregulation.

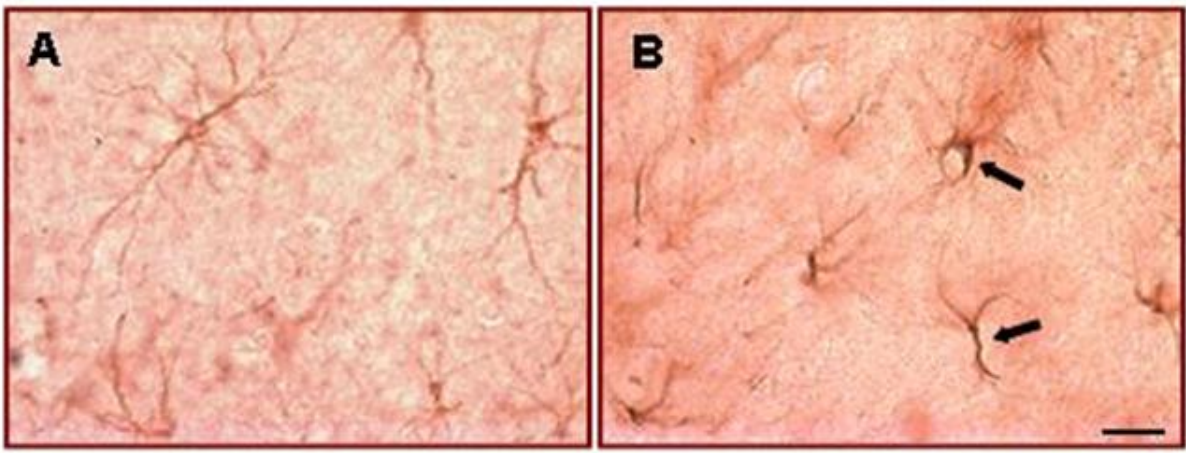

Figure 7. Increased VEGF protein expression in astrocytes 24 hours after pilocarpine-induced status epilepticus.
A-B. Sections from a saline- (**A**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**B**) were processed together using antibodies to VEGF and glial fibrillary acidic protein. Increased VEGF immunoreactivity was evident in glial profiles after status. Calibration = 25μm.

Experiment 1C:

The primary receptors for VEGFA are VEGFR1 and VEGFR2. Previous research has demonstrated that these receptors are upregulated in neurons or glia after perturbation of brain. Further, it has been demonstrated that VEGFR2 is localized to neurons after cerebral ischemia. Since convincing antibodies for VEGFR1 immunohistochemistry are not available, the following experiment was conducted to assess if VEGFR2, for which convincing antibodies exist, was upregulated following pilocarpine-induced status epilepticus. The experiment consisted of two groups as follow:

Group 1	n = 14	Pilocarpine
Group 2	n = 6	Saline

Twenty-four hours following injections, animals were sacrificed and perfusion fixed first with 2% paraformaldehyde in acetate and then 2% paraformaldehyde in borate buffer. A lower concentration of paraformaldehyde was utilized to enhance the likelihood of staining. Tissue was immunostained with the antibody to VEGFR2 (see General Methods) and analyzed for VEGFR2 upregulation.

VEGFR2 Immunostaining

Previous research has demonstrated that VEGFR2, one of VEGF's primary receptors, is upregulated after neuronal disruption. Further, VEGFR2 has reportedly been localized to neurons after an insult within the central nervous system. Immunostaining for VEGFR2 was therefore conducted to explore whether this receptor was upregulated after pilocarpine-induced status epilepticus. In saline controls (n=6), VEGFR2

immunostaining was very light, and not observed in neurons. In contrast, staining revealed a marked expression of VEGFR2 protein in neurons of hippocampal CA2 and CA3 (Figure 8) as well as the thalamus (Figure 9) 24 hours after status epilepticus (n=14).

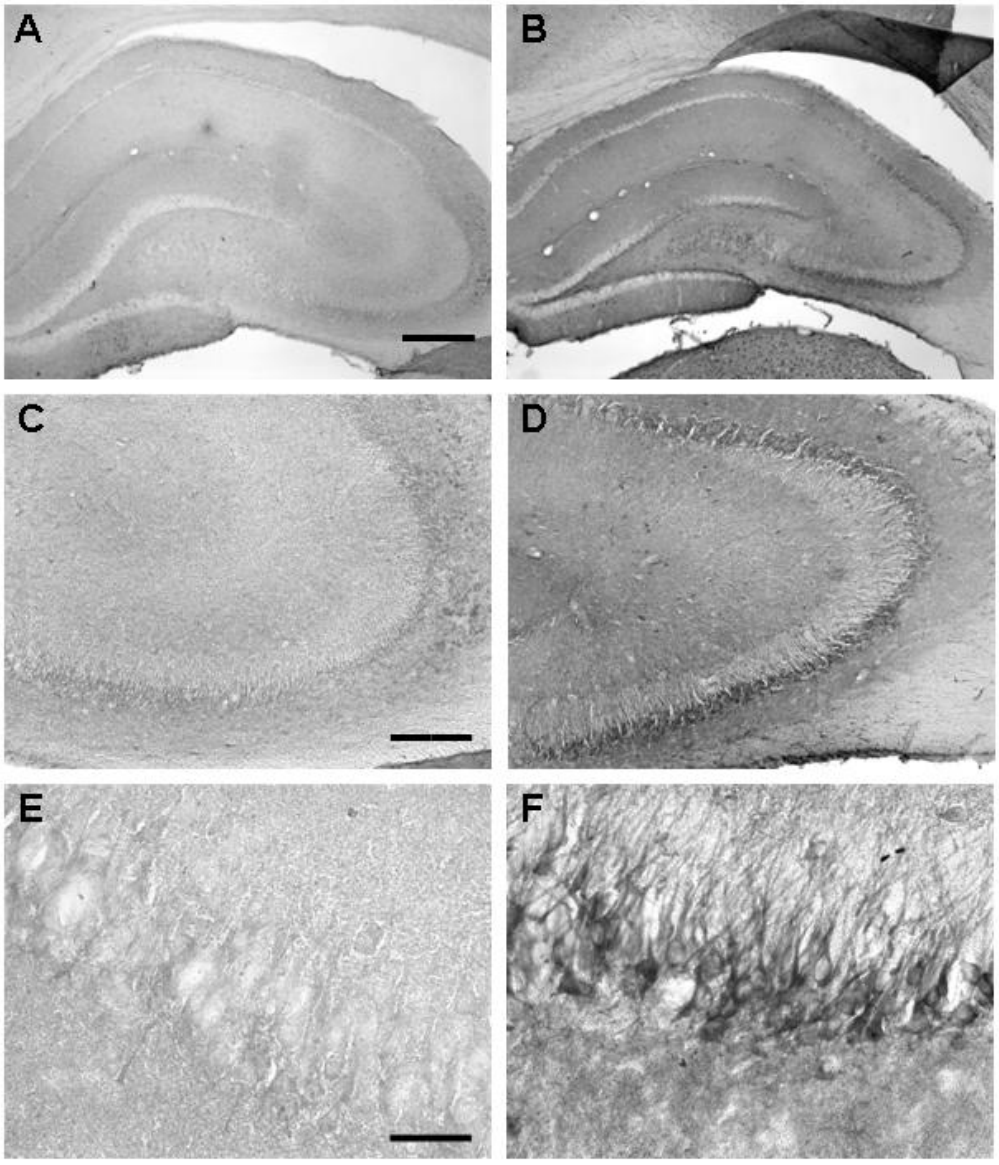

Figure 8. Increased VEGFR2 protein expression in the hippocampus 24 hours after pilocarpine-induced status epilepticus.

A-B. Sections from a saline- (**A**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**B**) were processed together using antibodies to VEGFR2. Increased VEGFR2 protein was evident in CA2/3 of the hippocampus after status. Calibration = 500µm.

C-D. Increased magnification of area CA2 from part **A** (**C**) and **B** (**D**) shows that neurons were associated with increased VEGFR2 immunoreactivity after pilocarpine-induced status compared to controls. Calibration = 200µm.

E-F. Increased magnification of area CA3 part **A** (**E**) and **B** (**F**) shows that neurons were associated with increased VEGFR2 immunoreactivity after pilocarpine-induced status compared to controls. Calibration =50 µm.

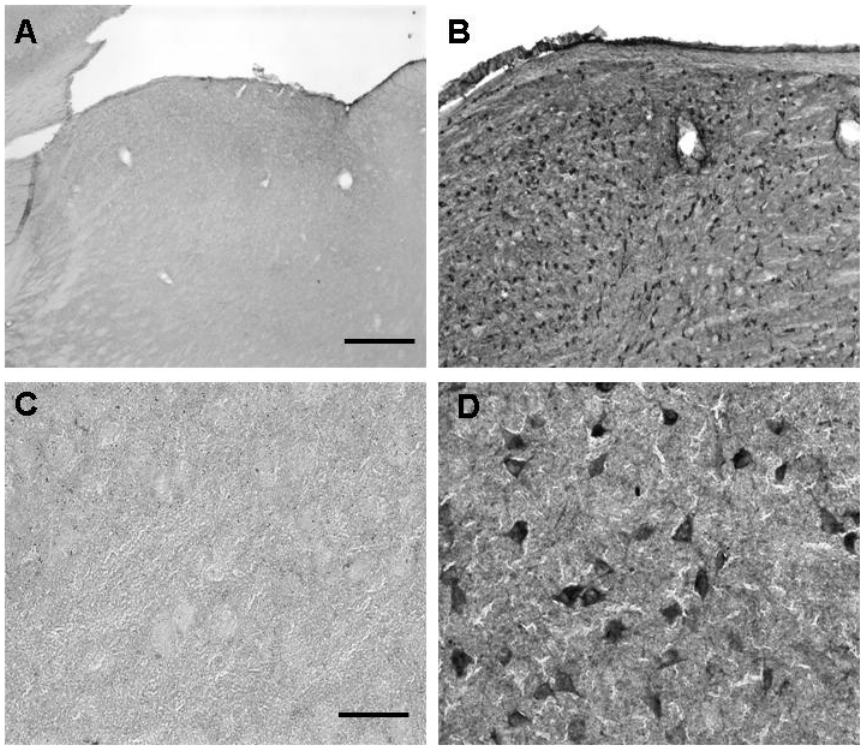

Figure 9. Increased VEGFR2 protein expression in the thalamus 24 hours after pilocarpine-induced status epilepticus.
A-B. Sections from a saline- (**A**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**B**) were processed together using antibodies to VEGFR2. Increased VEGFR2 protein was evident in the thalamus after status compared to controls. Calibration = 100µm.
C-D. Increased magnification from part **A** (**C**) and **B** (**D**) shows that neurons were associated with increased VEGFR2 immunoreactivity after pilocarpine-induced status compared to controls. Calibration = 50µm.

Specific Aim Two

VEGF and Vascular Density

VEGF plays a pivotal role in angiogenesis. During pathological conditions, especially states of absolute or relative hypoxia, VEGF induces new vascular sprouts, however, these vessels are typically leaky and poorly differentiated. Since one of VEGF's many functions is the induction of new vasculature, it was hypothesized that an increase in vascular density would occur concurrently with an increase in VEGF following pilocarpine-induced seizures.

Experiment 2A:

To determine the time course of VEGF's vascular effects, status epilepticus was induced as previously described (see General Methods) and animals were sacrificed at three different time points as depicted in the table below. These time points were chosen because we found that VEGF is upregulated 24 hours after seizures and we wanted to see if vascular density occurred immediately after the upregulation or whether it took a few days to occur, as previous research has demonstrated it takes two to three days (Croll et al., 2004a). We also looked at the 7 day time point to see if vascular density settles down after cell death has ended because previous work has suggested that once cell death has stopped, increased vascular density that has occurred as a result subsides (Manoonkitiwongsa et al., 2001).

Group	24 hours	3 days	7 days
Pilocarpine	N = 16	n = 16	n = 18
Saline	N = 9	n = 9	n = 9

Animals were sacrificed and perfusion fixed as previously described (see General Methods) 24 hours, three days, or seven days following injections. Tissue was immunostained with RECA, a selective vascular endothelial marker in rats, for point-count stereological measurements of vascular density and diameter. Further, hippocampal volumes were assessed using the Neurolucida system (MicroBrightField, Inc). Sections taken in a 1:6 series were used for evaluation. Volumetric measurements that were attained were used to adjust vascular measurements for differences in overall brain size. Other sections were immunostained to assess inflammation while additional sections were Nissl-stained to assess concomitant neuronal damage.

Vascular Quantification

One of VEGF's best-described biological effects is the induction of new blood vessels from previously existing blood vessels (i.e., angiogenesis). Since VEGF has been found to be upregulated after pilocarpine-induced status epilepticus, VEGF's role as an angiogenic factor was investigated in this context. Tissues were immunostained with RECA and vascular density was investigated in saline- and pilocarpine-treated animals that were sacrificed 1 day (saline n=5, pilocarpine n=9), 3 days (saline n=6, pilocarpine n=10), or 7 days (saline n=6, pilocarpine n=7) after status epilepticus. We conducted a 2(treatment) x 3(region) x 3(day) ANOVA, where treatment is saline vs. pilocarpine, region is CA1 vs. CA3 vs. fissure, and day is day 1 vs. day 3 vs. day 7. Quantification of vascular density by point-count stereology revealed a significant increase in vascular density for pilocarpine-treated animals in areas CA1 and CA3 (effect of treatment: $F(1, 37)=6.40$, $p<.05$) (Figure 10A and 10B), with the effect accounted for by an increase in

vascular density at the 3-day time point (Tukey HSD post hoc test, $p<.05$). This increase in vascular density was limited to dorsolateral microvasculature in CA1 and CA3 but not the larger vessels of the hippocampal fissure (effect of region, $F(2, 74)=102.714$, $p<.05$). No significant differences were found for the 24 hour or 7-day time points (Tukey HSD, $p>.05$). In addition, there were no statistically significant differences for vascular diameter over time ($F(2,34)=1.051$, $p>.05$), suggesting that the increased vascular density was more likely due to the sprouting of new blood vessels than to vasodilation. Further, hippocampal volumes were measured to assess vasculature per volume area. Results revealed there were no significant differences in hippocampal volumes over time ($F(2, 36) = 1.663$, $p>.05$).

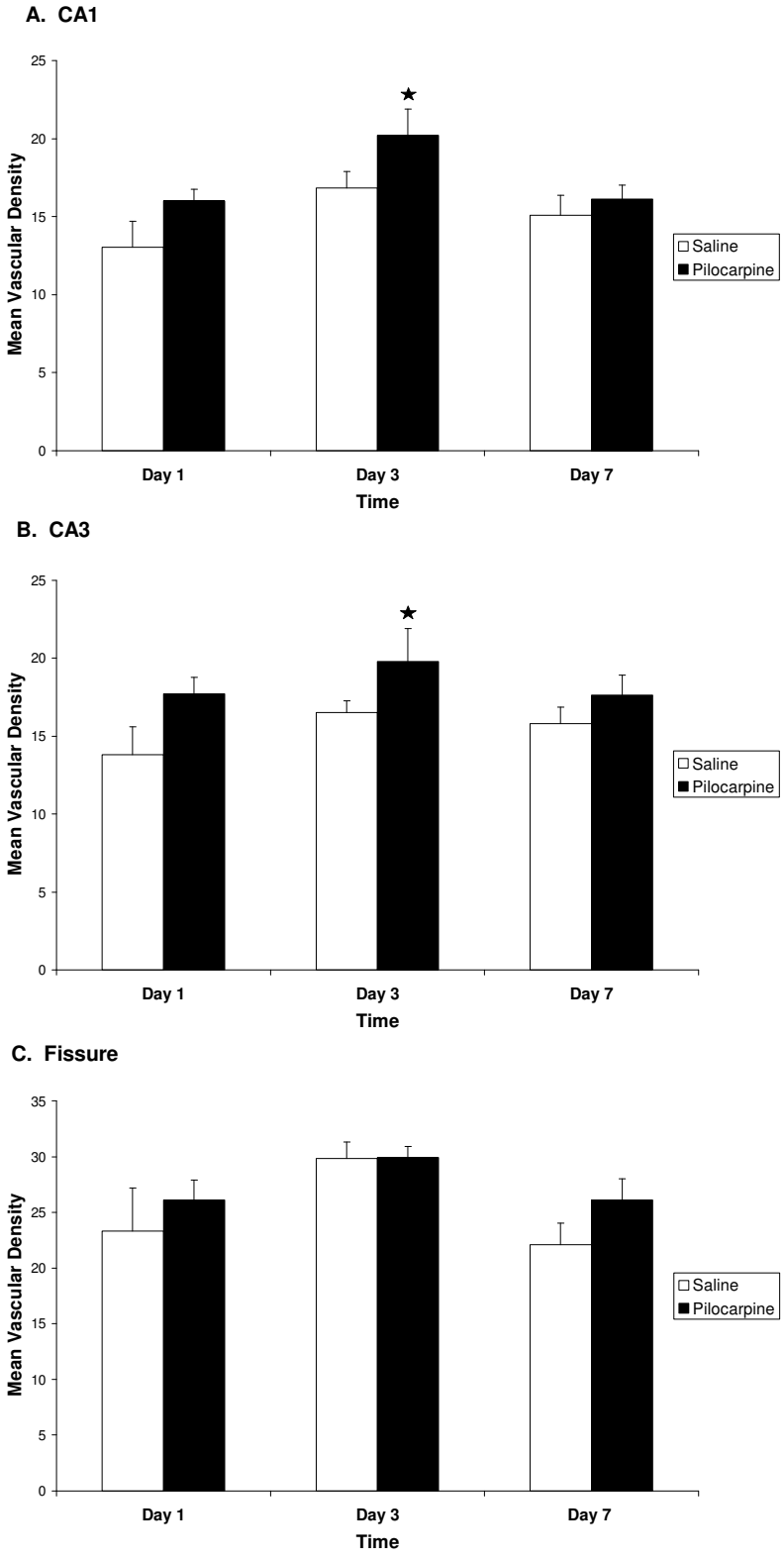

Figure 10. Timecourse data for vascular density after pilocarpine-induced status epilepticus.

A. Graph illustrates a significant increase in vascular density in CA1 at the 3 day time point, *significantly different than saline, $p<.05$.

B. Graph illustrates a significant increase in vascular density in CA3 at the 3 day time point*significantly different than saline, $p<.05$.

C. Graph illustrates no significant difference in vascular density in the hippocampal fissure ($p>.05$).

Neuronal Damage and Inflammation

In addition to its angiogenic properties, VEGF also induces inflammation. It has been postulated that this inflammation precedes or facilitates the angiogenic process. Both Nissl and OX-1 immunostaining revealed an increase in inflammatory cells in animals achieving status epilepticus (see Figure 11). This increased inflammation could be observed in all of the same regions as the increases in VEGF immunostaining, although the most striking increases were observed in hippocampus. Nissl staining also revealed neuronal damage, particularly in CA1, three days after status epilepticus (see Figure 11B). In contrast, no cell damage was observed in animals that did not achieve status epilepticus (see Figure 11A).

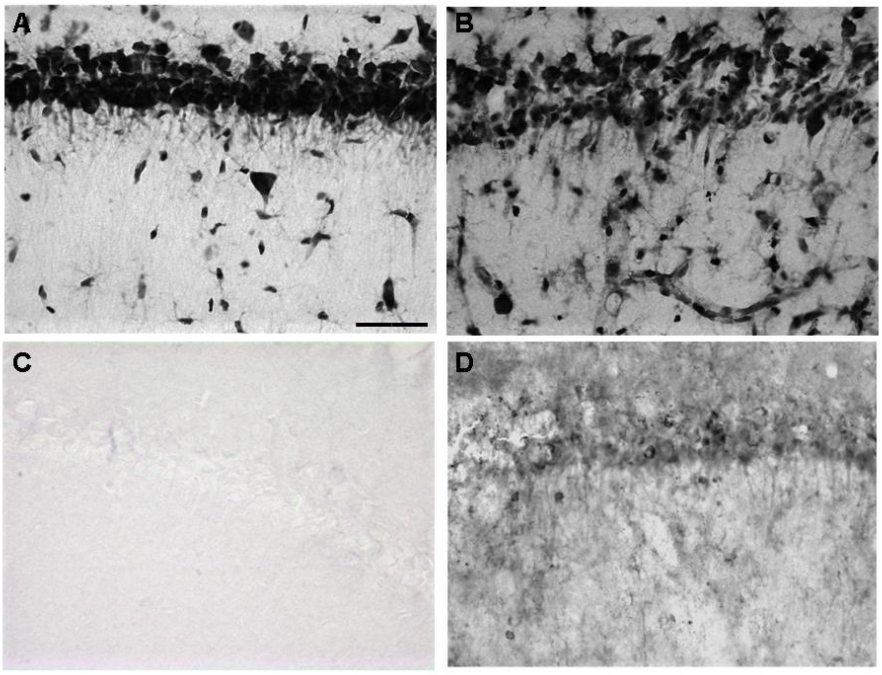

Figure 11. Photomicrographs of CA1 of the hippocampus 3 days after saline or pilocarpine-induced status epilepticus.
A-B. Sections from a saline- (**A**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**B**) were stained with cresyl violet. A greater cell loss was evident after pilocarpine-induced status epilepticus. Calibration = 200μm.
C-D. Sections from a saline- (**C**) and pilocarpine-treated rat that had 60 minutes of status epilepticus (**D**) were processed together using an antibody to OX-1. A greater number of inflammatory cells were evident after pilocarpine-induced status epilepticus. Calibration = 50μm.

Regression Analysis

Regression analyses were conducted to determine if increased vascular density could be predicted by seizure severity, neuronal damage, or inflammation. Significant correlations between vascular density and seizure score ($r=.449$, $p<.05$), neuronal damage ($r=.509$, $p<.05$), and inflammation ($r=.615$, $p<.05$) were detected (see Table 2). The best predictor of vascular density after pilocarpine-induced seizures was inflammation score ($r^2=.378$, $p<.05$). However, regression analyses also revealed that neuronal damage score ($r^2=.259$, $p<.05$) and seizure score ($r^2=.202$, $p<.05$) significantly predicted vascular density. However, significant correlations were also found between seizure score and damage ($r^2=.473$, $p<.05$), seizure score and inflammation ($r^2=.329$, $p<.05$), and damage and inflammation ($r^2=.511$, $p<.05$), suggesting that all of these factors co-vary.

Predictors	R	r^2	P
Seizure score	.449	.202	.003*
Neuronal damage	.509	.259	.001*
Inflammation	.615	.378	.001*
Relationship between Predictors	**R**	**r^2**	**P**
Seizure score vs. Damage	.688	.473	.001*
Seizure score vs. Inflammation	.574	.329	.001*
Damage vs. Inflammation	.715	.511	.001*

Table 2. Predictors of vascular density and intercorrelations after pilocarpine-induced status epilepticus.

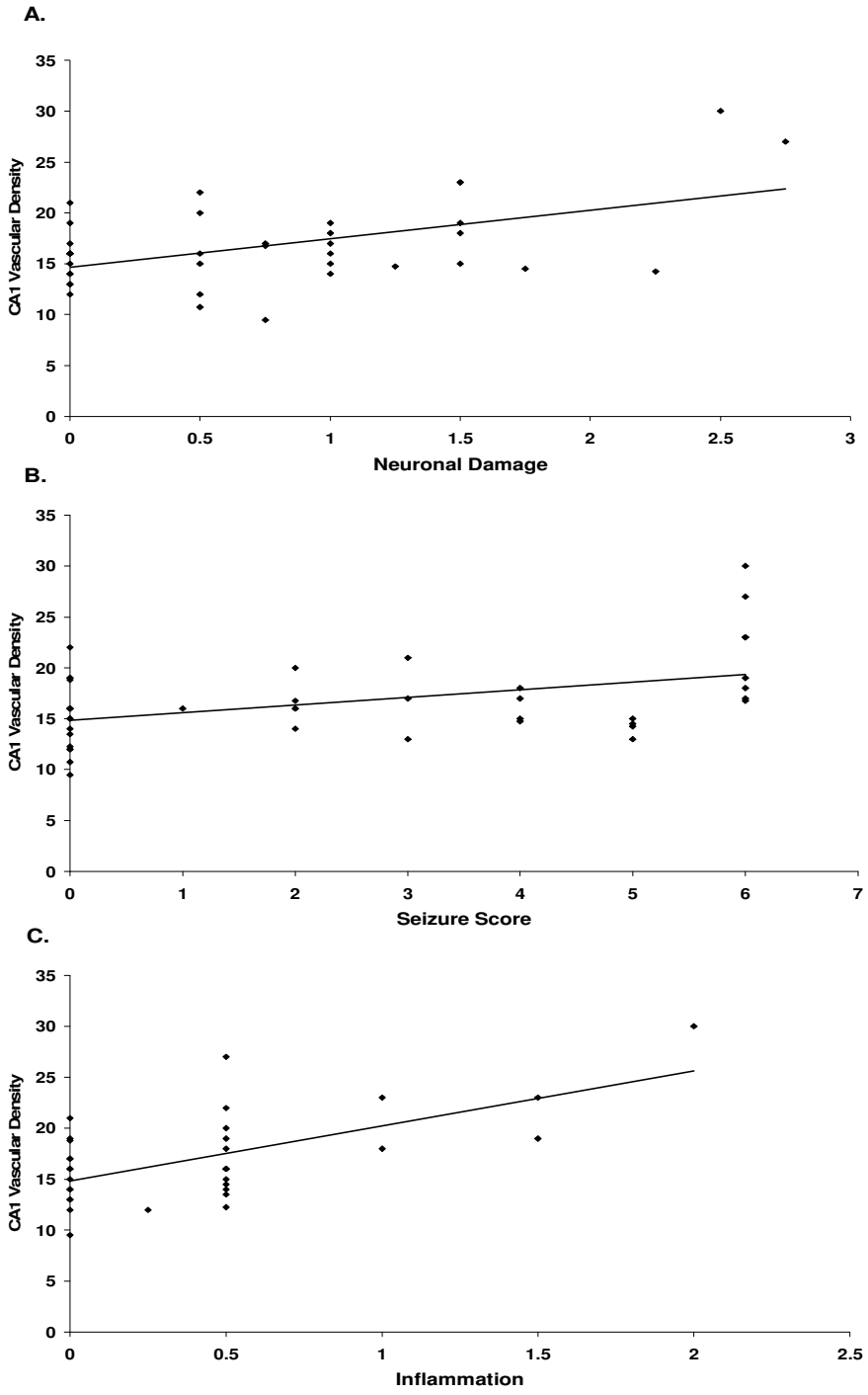

Figure 12. Scatterplots depicting hippocampal CA1 vascular density as a function of (A) neuronal damage, (B) seizure score, and (C) inflammation after pilocarpine-induced status epilepticus.

A. There is a significant relationship between vascular density and neuronal damage ($r=.509$, $p<.05$).

B. There is a significant relationship between vascular density and seizure score ($r=.449$, $p<.05$).

C. There is a significant relation between vascular density and inflammation ($r=.615$, $p<.05$).

Experiment 2B:

To determine if endogenous VEGF might contribute to increased vascular density, Flt-Fc, an inhibitor that functions to prevent VEGF from binding to its endogenous receptors, or its control, hFc, was infused into the hippocampus via cannula and an osmotic minipump (see General Methods). Status epilepticus was induced as previously described (see General Methods) and animals were sacrificed and perfusion fixed (see General Methods) three days after status epilepticus as depicted below.

Group	3 days
Flt-Fc	n = 15
hFc	n = 15

Tissue was immunostained to assess inflammation, vascular density, and vascular diameter.

Flt-Fc Infusion

To determine if endogenous VEGF contributes to increased vascular density, the VEGF blocker Flt-Fc was continuously infused by an osmotic minipump for 5 days before and during status epilepticus. The pump continued to infuse for 3 days thereafter (see Methods). Other animals were infused with hFc as a control protein. Tissues were immunostained with RECA and quantification of vascular density revealed no significant differences in vasculature between Flt-Fc- (n=2) and hFc- (n=3) treated animals after status epilepticus ($t(3)=-.063$, $p>.05$, see Figure 13A), suggesting that Flt-Fc did not block seizure-induced increases in vascular density.. However, Flt-Fc did eradicate the relation between neuronal damage and vascular density ($r=.268$, $p>.05$, see Figure 13B).

Thus, neuronal damage no longer predicted vascular density when VEGFR1 ligands were prevented from binding to their receptors.

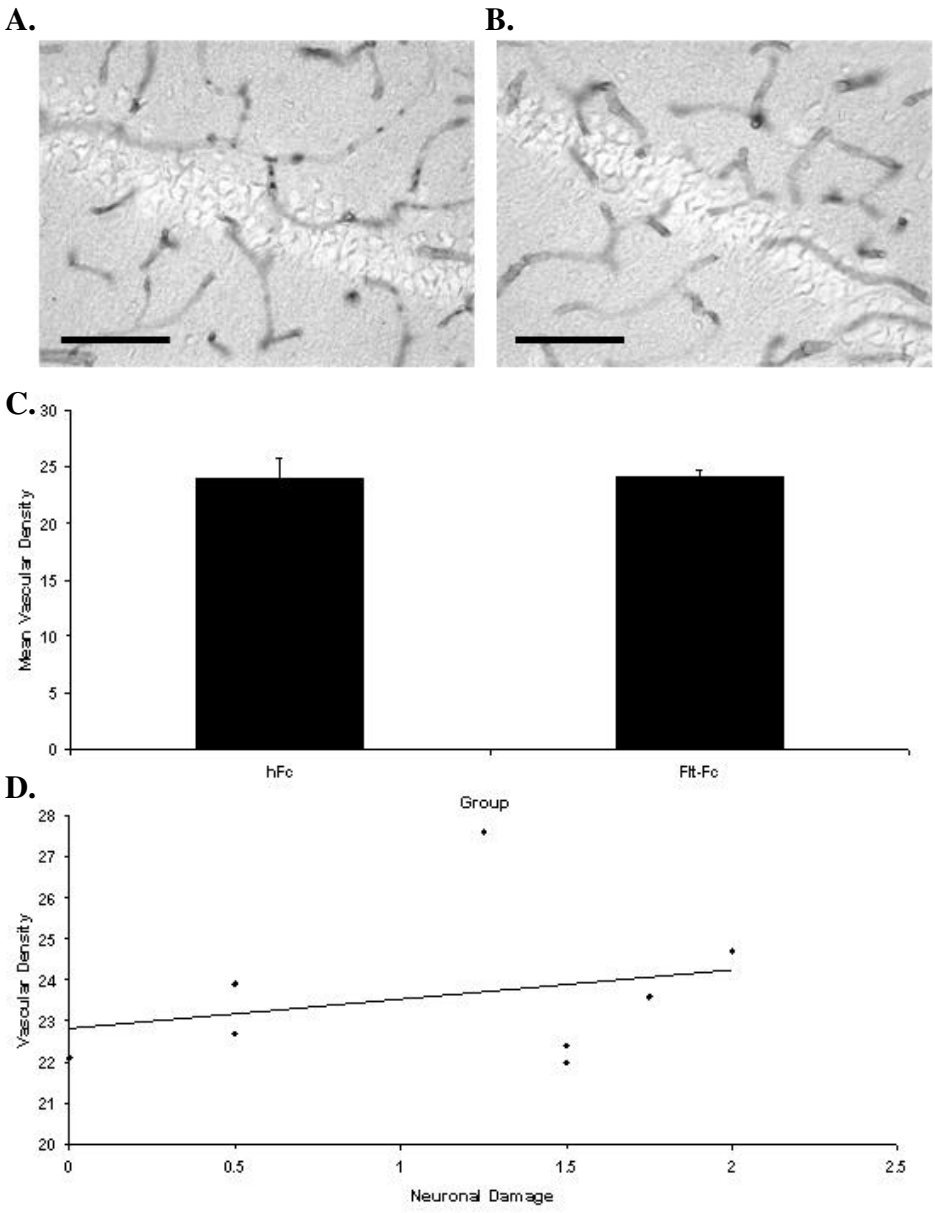

Figure 13. Vasculature after treatment with Flt-Fc, which sequesters endogenous VEGF, during pilocarpine-induced status epilepticus.
A-B. Sections from a hFc- **(A)** and Flt-Fc-treated rat that had 60 minutes of status epilepticus **(B)** were processed together using an antibody to RECA. No differences were detected. Calibration = 50μm.
C. Bar graph showing no difference in vascular density between animals treated with hFc versus Flt-Fc after status epilepticus ($t(3)=-.063$, $p>.05$).
D. Scatterplot showing a breakdown in the ability of cell loss to predict vascular density with Flt-Fc ($r=.27$, $p>.05$).

Specific Aim Three

VEGF and Neuronal Loss

During development, VEGF is involved in supporting sensory cells in the retina and dorsal root ganglia. In addition to this supportive role, VEGF has been found to protect neurons during pathological conditions such as ischemia and in animal models of amyotrophic lateral sclerosis. Based on this evidence, it was hypothesized that endogenous VEGF as well as infusion of exogenous VEGF before and after pilocarpine-induced status epilepticus would attenuate hippocampal neuronal loss.

Experiment 3A:

To determine whether endogenous VEGF serves a protective role in cell loss following status epilepticus, animals were unilaterally infused with either Flt-Fc, to block endogenous VEGF, or hFc as a control. Status epilepticus was induced as previously described (see General Methods) and animals were sacrificed and perfusion fixed (see General Methods) 24 hours after status epilepticus as depicted below.

Group	24 hours
Flt-Fc	n = 14
hFc	n = 14

Tissue was stained with methylene blue and estimates of status epilepticus-related cell loss were made using the optical fractionator method (see General Methods).

Flt-Fc Infusion

To determine whether endogenous VEGF serves a protective role in cell loss following status epilepticus, animals were treated with Flt-Fc or hFc. Protein was

continuously infused into the hippocampus by an osmotic minipump for 5 days before and during status epilepticus. The pump continued to infuse for 1 day thereafter (see Methods). After observing an apparent increased cell loss in Flt-Fc treated animals using the subjective neuronal loss rating scale [($t(4)=3.536$, $p<.05$)], stereological estimates of neuronal density were performed. These analyses confirmed those of the initial ratings and revealed a statistically significant decrease in neuronal density in animals treated with Flt-Fc (n=5) relative to their hFc controls (n=4) [($t(7)=3.482$, $p<.05$] (Figure 14).

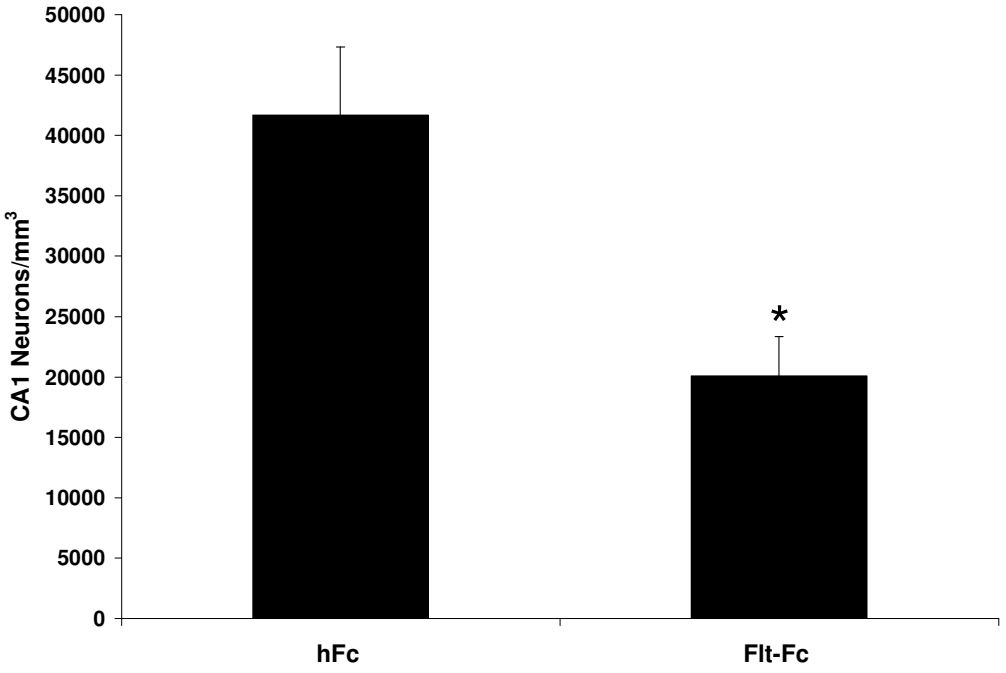

Figure 14. Neuronal density estimates after treatment with Flt-Fc, which sequesters endogenous VEGF, 24 hours after pilocarpine-induced status epilepticus.
Bar graph shows significantly worse cell loss in animals treated with Flt-Fc (n=5) relative to those treated with the hFc (n=4) control protein, *significantly different than hFc, $p<.05$.

Experiment 3B:

During protein infusions by osmotic minipumps, cannulae may become blocked as a result of scar tissue and may therefore fail to infuse properly. To assess the amount of VEGF protein that animals receive through infusions by an osmotic minipump prior to status epilepticus, animals were unilaterally infused with 60ng/d VEGF or saline as a control as shown below.

Group	5 days
VEGF	n = 4
Saline	n = 4

Animals were sacrificed five days following protein infusions and fresh tissue was obtained for quantitative analysis of VEGF protein in the hippocampus via ELISA.

VEGF ELISA

VEGF ELISAs were used to quantify the amount of VEGF protein that animals received after infusions of VEGF (60ng/d) compared to saline controls (n=2 per group). This preliminary analysis revealed that VEGF treatment resulted in 7.21ng VEGF protein per mg tissue weight while PBS-treated tissue had .8ng VEGF protein per mg tissue weight. The fact that PBS-treated animals demonstrated a trace amount of VEGF protein likely reflects an artifact of the assay.

Experiment 3C:

To assess neuronal loss subsequent to status epilepticus, animals received 15, 30, 45, or 60ng/d VEGF or a control protein via unilateral infusion through cannula and an osmotic minipump (see General Methods). Five days following protein infusions, status epilepticus was induced as previously described (see General Methods). The experimental groups are illustrated below.

Group 1	Control	n = 27
Group 2	15ng/d	n = 15
Group 3	30ng/d	n = 18
Group 4	45ng/d	n = 13
Group 5	60ng/d	n = 21

Animals were sacrificed and perfusion fixed (see General Methods) 24 hours after status epilepticus. Tissue was stained with methylene blue and estimates of status epilepticus-related cell loss were made using the optical fractionator method (see General Methods).

CA1 Cell Loss After Protein Infusions

Because infusions with Flt-Fc protein caused significantly more cell loss after seizures, we hypothesized that VEGF plays a neuroprotective role after status epilepticus. To further investigate this hypothesis, as well as to determine whether exogenous VEGF would provide additional protection to neurons, animals were infused with $rhVEGF_{165}$ protein during status epilepticus. Comparison of seizure severity of VEGF- (n=104) versus vehicle- (n=66) infused animals across studies revealed no significant difference

in behavioral rating of status (i.e., seizure score of 1-8; see Methods; t(155.771)=1.647, p>.05, see Figure 15).

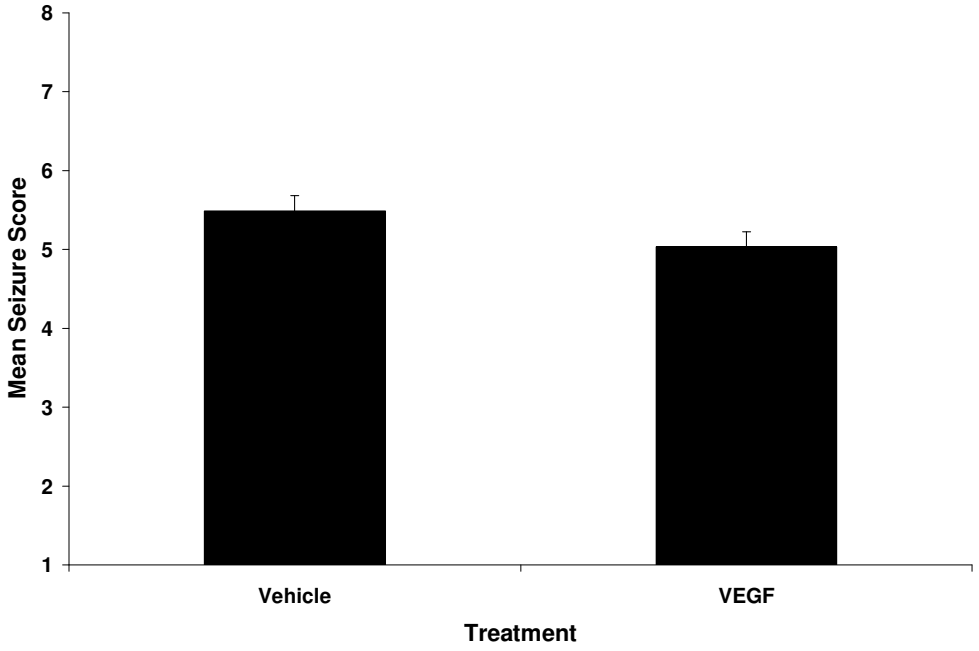

Figure 15. Seizure severity scores for VEGF- and vehicle-infused animals. There is no significant difference in seizure severity between VEGF- (n=104) and vehicle- (n=66) treated animals, t(155.771)=1.647, p>.05).

Neuronal loss was investigated only in animals that achieved status epilepticus. Nissl staining revealed a loss of neurons 24 hours after status epilepticus in CA1 of the hippocampus (Figure 16A). Animals infused with VEGF appeared to have less neuronal loss and fewer neurons with pyknotic profiles (Figure 16B). Neuronal density estimates, quantified stereologically, revealed that animals infused with 30ng/d (n=10) or 60ng/d (n=11) VEGF had significantly higher neuronal densities than animals given pilocarpine and infused with PBS or control proteins (n=14) ($F(4,47)=2.577$, $p<.05$, see Figure 16C).

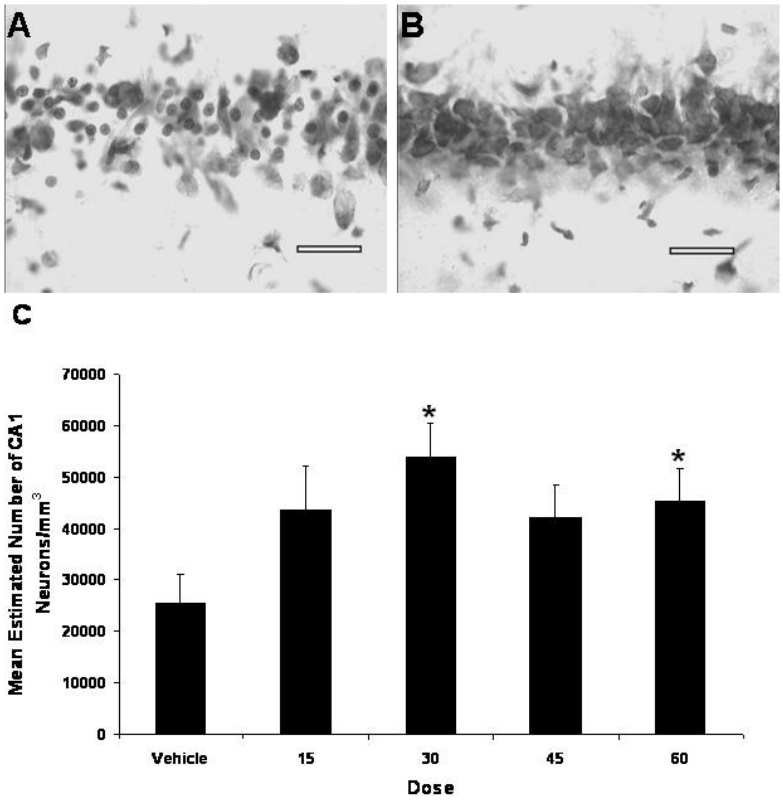

Figure 16. Neuronal density estimates after treatment with VEGF 24 hours after pilocarpine-induced status epilepticus.
A-B. Representative photomicrograph of the CA1 region following status epilepticus in (**A**) PBS infused hippocampus and (**B**) $rhVEGF_{165}$ infused hippocampus. Scale bar = 50µm.
C. Neuronal density estimates calculated using the optical dissector method revealed that infusion of $rhVEGF_{165}$ (15ng/d (n=6), 30ng/d (n=10), 45ng/d (n=11), and 60ng/d (n=11)) 5 days prior to and 1 day following pilocarpine-induced status epilepticus significantly attenuated pyramidal cell loss compared to infusion of PBS Vehicle (n=14), *significantly different than PBS by Tukey HSD post hoc test, $p<.05$.

Experiment 3D:

Although the difference was not significant, we observed a tendency toward less severe seizures in animals infused with VEGF. Because the relationship between seizure severity and activity in the hippocampus may be non-linear, it is possible that increased vascular permeability caused by VEGF could have increased distance between cells in the region of VEGF diffusion thus reducing excitotoxic transmission. To investigate this possibility, animals were unilaterally infused with 3 μg/d BowAng1 to block VEGF's vascular permeabilizing effects, along with VEGF or hFc as a control. Status epilepticus was induced as previously described (see General Methods) and animals were sacrificed and perfusion fixed (see General Methods) 24 hours after status epilepticus as depicted below.

	hFc	BowAng1
hFc	n=6	n=7
VEGF	n=7	n=7

Tissue was stained with methylene blue and estimates of status epilepticus-related cell loss were made using the optical fractionator method (see General Methods).

Angiopoietin-1 Infusion

Although not significant, slightly less severe seizures were observed in animals treated with VEGF. Increased vascular permeability could have increased distance between cells in the region of VEGF diffusion, hence reducing excitotoxic transmission. To address this possibility, animals were co-infused with both VEGF and BowAng1, a

potent form of angiopoietin-1, an inhibitor of VEGF-induced vascular permeability. Animals co-treated with BowAng1 showed no reduction in neuroprotection relative to those receiving VEGF alone ($F(1,20)=.528$, $p>.05$). In addition, there was no difference in seizure severity between the groups ($p>.05$).

Experiment 3E:

Use of VEGF protein as a therapeutic agent in human epilepsy is not a clinically feasible option due to the fact that VEGF is a large protein which does not cross the blood-brain barrier and which also has detrimental consequences. Therefore, an alternate delivery method was considered. It was hypothesized that microinjections of adeno-associated virus containing VEGF gene fragments would result in similar effects to those observed with exogenously-infused VEGF.

Animals received microinjections of either mVEGF AAV 2/1 or control (see General Methods). Status epilepticus was induced two weeks following microinjections as pilot studies have demonstrated this time frame is necessary for VEGF expression in virally-infected cells. The experimental groups are illustrated below.

Group	Pilocarpine	Saline
AAV VEGF 2/1	n = 6	n = 4
AAV Blank	n = 6	n = 4

Animals were sacrificed and perfusion fixed (see General Methods) 24 hours after status epilepticus. Tissue was stained with methylene blue and estimates of status epilepticus-related cell loss were made using the optical fractionator method (see General Methods).

CA1 Cell Loss After Adeno-Associated Viral Vector Treatment

Because VEGF was found to significantly preserve neurons after pilocarpine-induced status epilepticus, a preliminary study investigating an alternate delivery method was conducted because infusion of VEGF into human brain is not optimal for two reasons. First, VEGF is a large protein that cannot cross the blood-brain barrier and therefore would need to be continuously infused into brain in order to obtain its beneficial effects. Second direct application of pharmacological amounts of VEGF to brain could mediate some detrimental consequences such as edema and monocytic inflammation. To circumvent these problems, a viral vector was utilized to upregulate endogenous VEGF two weeks prior to the induction of seizures. Neuronal density estimates revealed that animals that received AAV VEGF (n=2) microinjections had higher neuronal densities than animals that received vehicle microinjections (n=3). These differences, however, did not achieve statistical significance ($F(2,4)=3.740$, $p>.05$, see Figure 17). Because this was a preliminary study, we had a small number of animals in each group which likely accounts for the fact that we failed to reach significance. Based on the means, however, neuronal densities of AAV VEGF animals were similar to those of non-seized animals. Therefore, it appears that AAV VEGF may preserve neurons after status epilepticus although additional studies will need to be conducted.

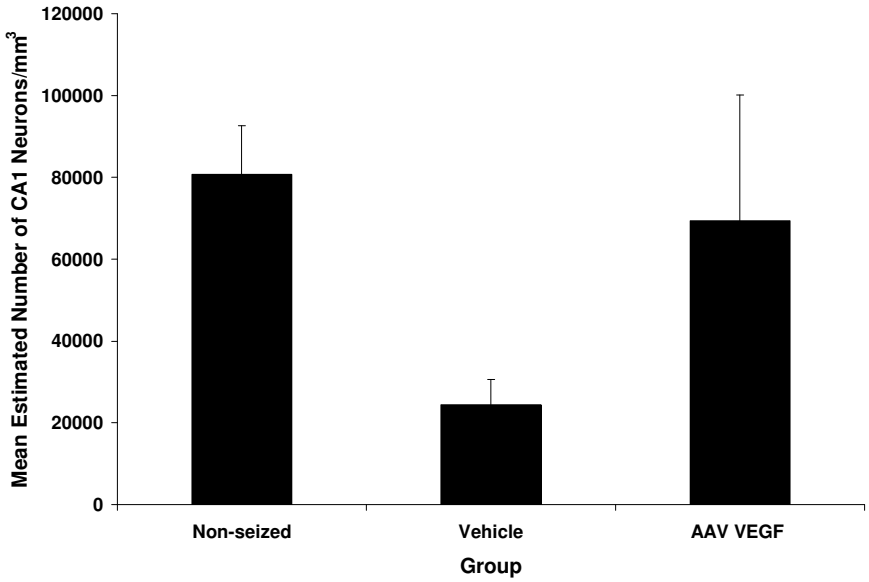

Figure 17. Neuronal density estimates after treatment with AAV VEGF 24 hours after status epilepticus.
Neuronal density estimates calculated using the optical dissector method revealed that microinjections of AAV VEGF (n=2) two weeks prior to pilocarpine-induced status epilepticus attenuated pyramidal cell loss compared to vehicle (n=3), p>.05.

Specific Aim Four

VEGF and Long-Term Functional Consequences

In addition to neuronal loss, seizures often result in functional impairments. Animal models of epilepsy have demonstrated impairments in hippocampally-mediated memory tasks as well as an anxiolytic effect on anxiety tasks. Since VEGF has been previously demonstrated to function as a neuroprotective agent and may preserve neurons after seizures, it was hypothesized that infusion of exogenous VEGF before and after pilocarpine-induced status epilepticus would result in functional preservation in terms of memory, behavior, and emotional functioning.

Experiment 4A:

To assess functional preservation effects of VEGF, animals received infusions of 60ng/d VEGF or a control protein (see General Methods). Five days following protein

infusions, status epilepticus was induced as previously described (see General Methods). Animals received a total of two weeks of infusion through the attached pumps, beginning five days prior to seizure induction. After the two weeks, pumps were exchanged for fresh pumps that infused 60ng/d VEGF or control for an additional two weeks. The experimental groups are illustrated below.

Group	Pilocarpine
VEGF	n = 12
Vehicle	n = 12

To assess cognitive functioning during weeks two through six after seizure induction, animals underwent behavioral testing to evaluate the effects of VEGF. These tests included the Morris water maze, light-dark exploration, social interaction, and grid locomotor testing on an open field. Animals were sacrificed six weeks after seizure inductions. Tissue was stained with methylene blue and estimates of status epilepticus-related cell loss were made using the optical fractionator method (see General Methods).

Experiment 4B:

To maximize our chance of detecting functional preservation, the above experiment was replicated in animals that were implanted with bilateral hippocampal cannulae. We chose to use the highest dose of VEGF found to preserve neurons to avert possible complications with pump infusions as previously described. Pumps infused 60 ng/d VEGF or a control protein. In addition, a saline control group for each condition and a naïve control group were included as follows:

Group	Pilocarpine	Saline	No Treatment
VEGF	n = 9	n = 4	xxx
Control	n = 9	n = 4	xxx
Naïve	xxx	xxx	n = 6

Behavioral and Emotional Functioning

After status epilepticus, animals experience neuronal loss in the hippocampus and other limbic structures, which results in functional impairments in learning, memory, and emotional functioning. Since VEGF was found to preserve neurons 24 hours after status epilepticus, we explored whether or not this neuronal preservation translated to functional preservation. It is well known that damage to the hippocampus results in behavioral and emotional impairments. Although it has been proposed that only one hippocampus needs to remain intact to preserve function, we conducted experiments in which VEGF was infused either unilaterally or bilaterally to maximize our chances of detecting functional preservation. We found a significant cohort effect in reference to learning and memory but no significant difference between cohorts regarding emotional functioning. In neither case did we find an interaction between cohort and treatment, so we combined the data in order to obtain more power. We found no difference between saline controls (i.e. non-seized animals) and naïve animals across measures. Therefore, we also combined these groups to represent a sole group of "non-seized" animals.

Learning and Memory

Learning and memory were evaluated in the Morris water maze. Learning is represented by the median latency to find the escape platform for each trial block of 3

trials for each animal. For this analysis, two animals were removed as outliers and one animal's datum was interpolated as previously described (see Methods). We conducted a mixed 3(treatment) x 4(day) ANOVA, where treatment is non-seized vs. vehicle vs. VEGF and day is day 1 vs. day 2 vs. day 3 vs. day 4. Results revealed an overall significant difference in learning between groups ($F(2,17)=21.437$, $p<.05$, see Figure 18A). Animals that did not experience status epilepticus (n=10), learned significantly better in the Morris water maze than either VEGF- (n=4) ($p<.05$) or vehicle-treated (n=6) animals ($p<.05$) that experienced status epilepticus. Post hoc analyses revealed that VEGF-treated animals were not statistically better than vehicle-treated animals although there was a statistical trend toward them being better ($p>.05$). These differences were not likely to be accounted for by differences in estimated swim speed, as analyses on these measures revealed no significant difference between groups on this measure ($F(2,17)=2.905$, $p>.05$, see Figure 18B). Although swim speed was not significantly different between the groups, if anything the seized groups swam more quickly than non-seized groups and therefore their delay in finding the platform could not be accounted for by slow swim speed. It should be noted that animals receiving bilateral infusions learned significantly better than animals that received unilateral infusions ($F(1,15)=11.969$, $p<.05$). Therefore, partial preservation of one hippocampus may not be enough to preserve learning in our model of epilepsy. Their differences also were not accounted for by differences in swim speed ($F(1,14)=2.355$, $p>.05$).

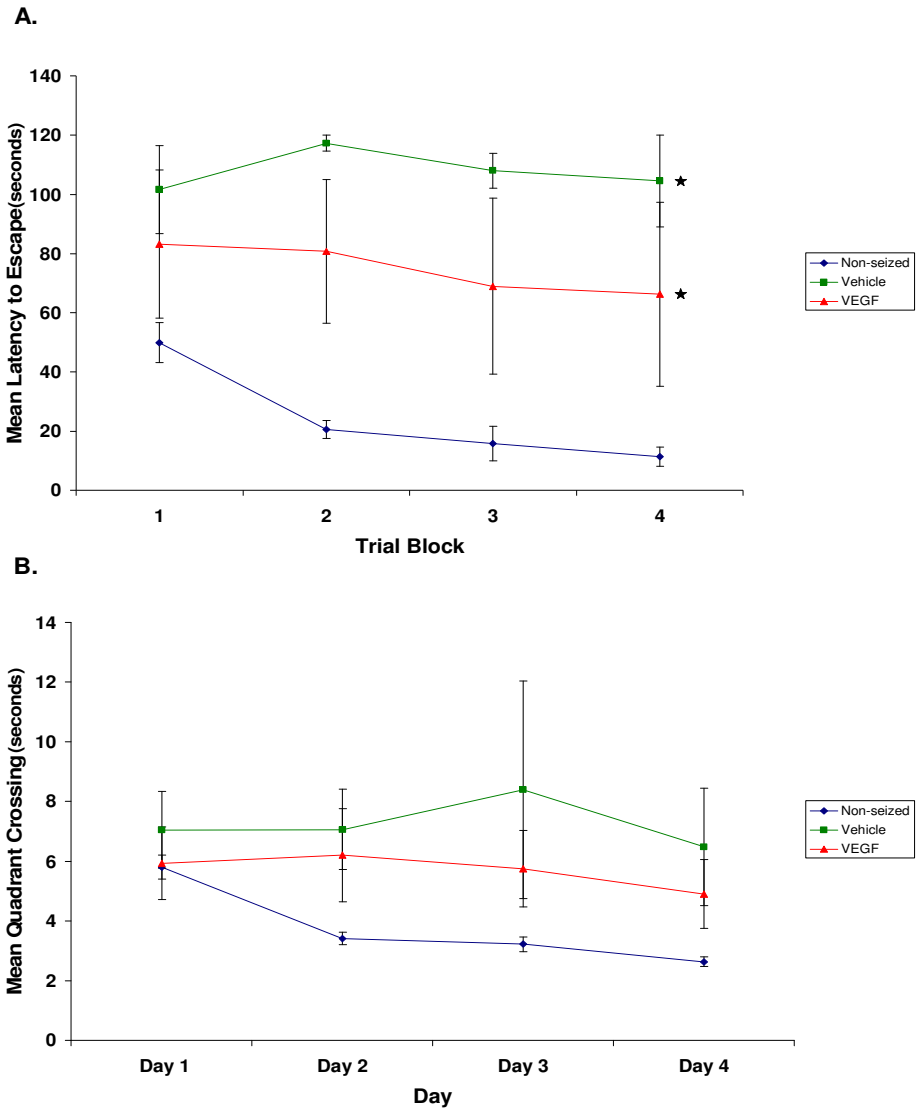

Figure 18. Learning based on the Morris water maze 3 weeks after pilocarpine-induced status epilepticus.
A. Non-seized animals learned significantly better than vehicle- ($p<.05$) and VEGF- ($p<.05$) treated animals. VEGF-treated animals learned better than vehicle-treated animals although this result was not significant ($p>.05$).
B. There was no significant difference in estimated swim speed between non-seized (n=10), VEGF- (n=4), and vehicle- (n=6) treated animals, $p>.05$).

A terminal retention trial, which is expressed as the mean proportion of time spent swimming in the goal quadrant during a spatial probe trial, was used to assess memory. Results revealed an overall significant difference between groups ($F(2,19)=12.68$, $p<.05$,

see Figure 19). Post hoc analyses revealed that there was no significant difference in our measure of memory based on the Morris water maze between VEGF- (n=4) and vehicle- (n=7) treated animals (p>.05). There was, however, a significant difference in our measure of memory between animals that did not experience status epilepticus (n=11) and VEGF- (p<.05) and vehicle-treated (p<.05) animals that experienced status epilepticus. Specifically, non-seized animals spent significantly more time swimming in the goal quadrant compared to seized animals.

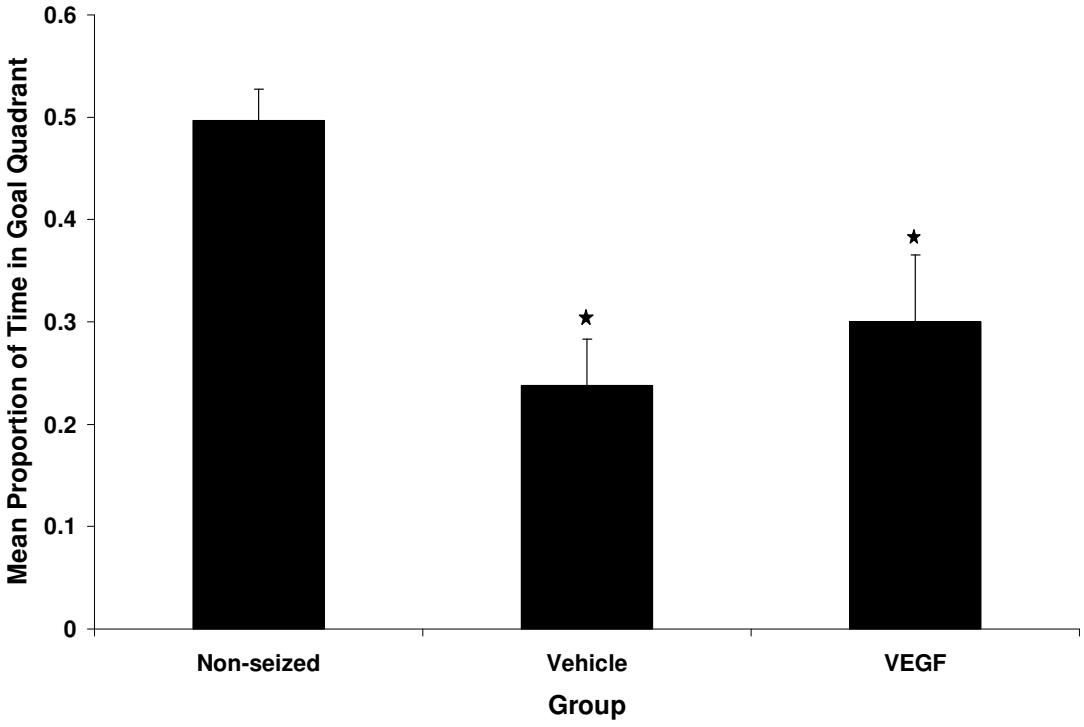

Figure 19. Memory based on the Morris water maze 3 weeks after pilocarpine-induced status epilepticus.
Non-seized animals spent a significantly longer amount of time in the goal quadrant than vehicle- and VEGF-treated animals, *significantly different at .05. There was no significant difference between VEGF and vehicle animals, p>.05.

Social Interaction

To assess preservation of social functioning, animals were placed on a platform with a novel animal and the total number of social initiations was recorded. Results revealed that there was no significant difference in the number of interactions between non-seized (n=11), vehicle- (n=7), and VEGF- (n=4) treated animals ($F(2,19)=2.193$, $p>.05$, see Figure 20). Although there were no significant differences, vehicle-treated animals made the fewest attempts to initiate contact with a novel rat while VEGF-treated animals showed a more normal tendency to initiate contact. No significant differences in the total number of social interactions were found for unilateral versus bilateral infusions ($F(1,17)=1.566$, $p>.05$).

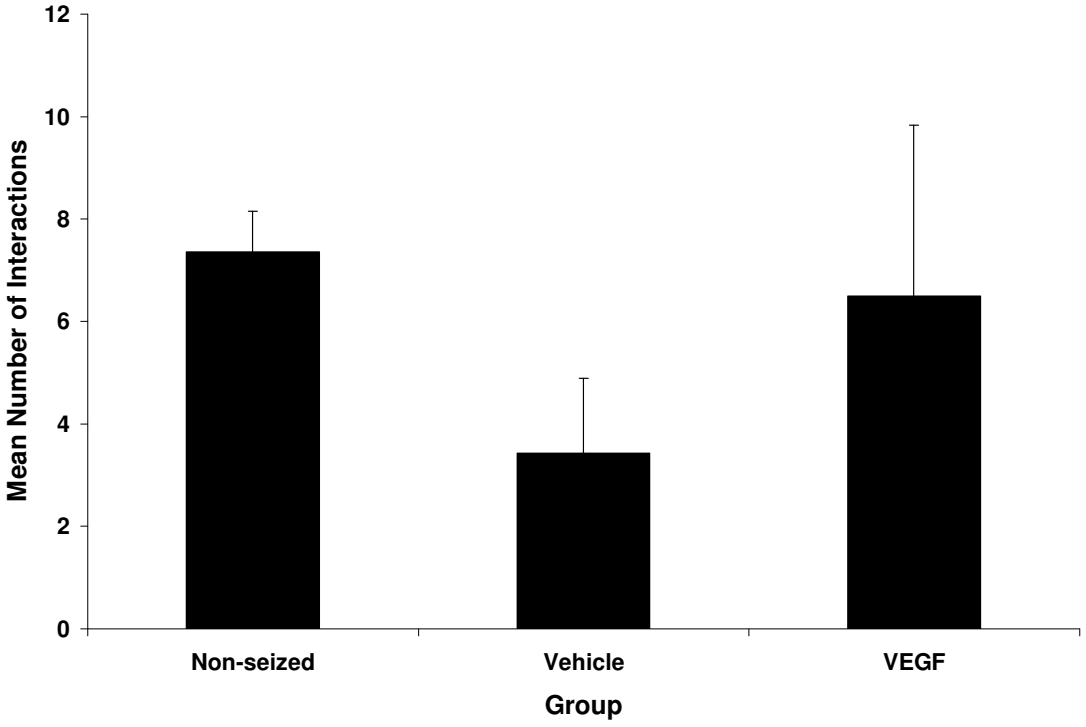

Figure 20. Social interaction 3 weeks after pilocarpine-induced status epilepticus. VEGF-treated animals demonstrate a more normal tendency to initiate contact with a novel rat than vehicle-treated animals.

Exploratory Locomotor Behavior

A task which assesses exploratory locomotor behavior was conducted as an additional control for speed of movement. Results demonstrated no significant difference in locomotor behavior between non-seized (n=10), vehicle- (n=7), and VEGF- (n=4) treated animals ($F(2,18)=2.027$, $p>.05$, see Figure 21). For this analysis, one outlier was removed. While there were no significant differences, VEGF-treated animals exhibited the greatest mean number of grid crossings. No significant differences in total grid crossings were found for unilateral versus bilateral infusions ($F(1,16)=3.875$, $p>.05$).

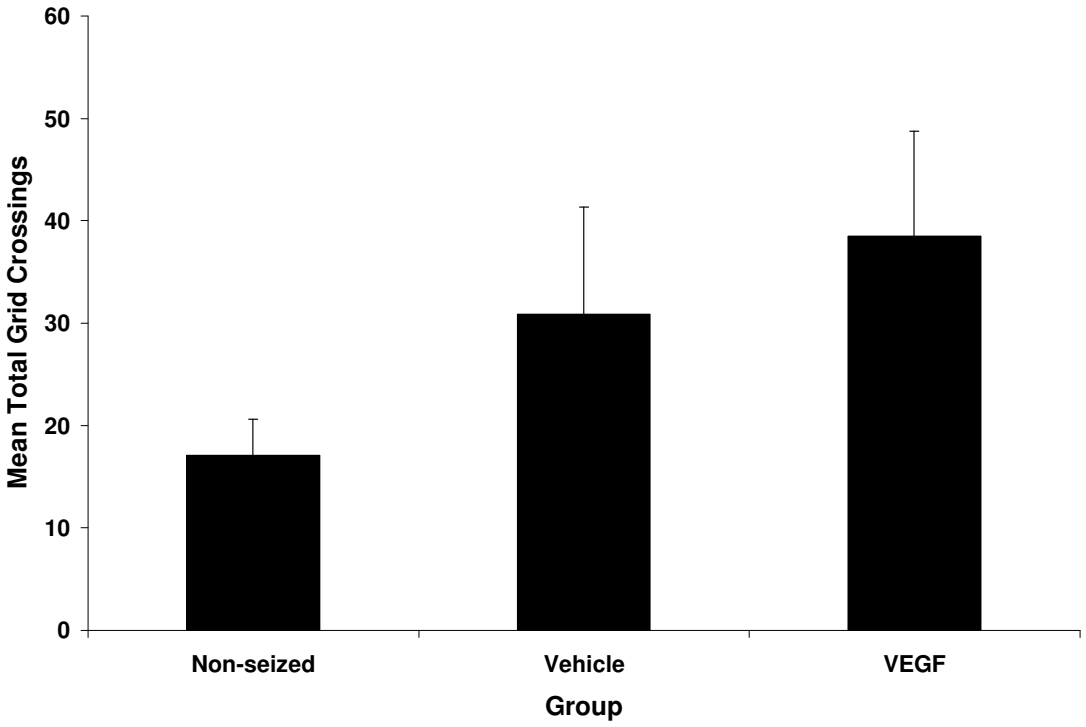

Figure 21. Exploratory locomotor behavior 3 weeks after pilocarpine-induced status epilepticus.
VEGF-treated animals show greater exploratory locomotor behavior than vehicle-treated and non-seized animals.

Anxiety

A light-dark exploration task, a well-validated and commonly used test of anxiety, was used to assess anxiety in our animals. For this analysis, one outlier was removed. Results revealed an overall significant difference in the amount of time spent in the dark compartment between groups ($F(2,18)=4.955$, $p<.05$, see Figure 22). Post hoc analyses revealed no significant difference in the amount of time non-seized (n=10) animals and VEGF-treated (n=4) animals that experienced status epilepticus spent in the dark compartment ($p=.711$). Thus, animals treated with VEGF showed the normal preference for the dark compartment. Vehicle-treated (n=7) animals, however, spent significantly more time in the light compartment compared to non-seized and VEGF-treated animals ($p<.05$), indicating that the vehicle-treated animals exhibited less anxiety. No significant differences in time spent in the dark compartment were found for unilateral versus bilateral infusions ($F(1,16)=2.394$, $p>.05$).

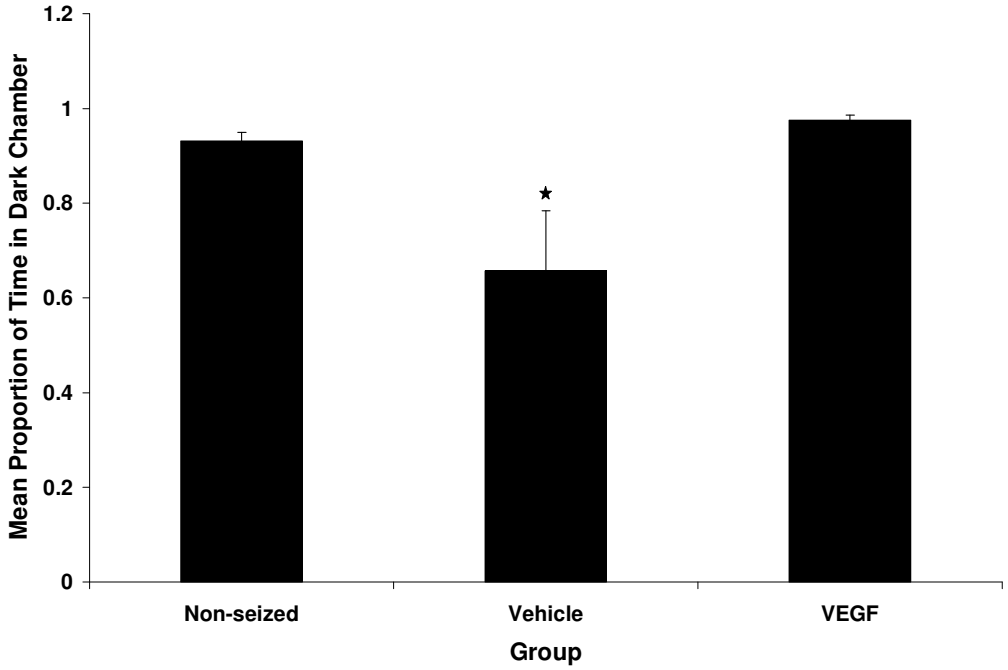

Figure 22. Anxiety 3 weeks after pilocarpine-induced status epilepticus. Vehicle-treated animals that experienced status epilepticus spent significantly more time in the light compartment compared to VEGF-treated animals that experienced status epilepticus and non-seized animals ($p<.05$). There was no significant difference between VEGF-treated and non-seized animals, *significantly different than both non-seized and VEGF-treated groups

CA1 Cell Loss

Because treatment with VEGF prior to a pilocarpine-induced status epilepticus event was found to preserve neurons during the acute period, we investigated whether this neuronal preservation translated to functional preservation. Behavioral analyses of intellectual and emotional functioning yielded inconsistent results and therefore we cannot state with certainty whether or not function is preserved after an acute insult. One possible explanation underlying the results found for learning and memory, social interaction, and exploratory behavior is that although neurons were preserved immediately after a status epilepticus event, they may not have been preserved long-term. Neuronal density estimates, quantified stereologically, revealed a significant difference in neuronal densities between groups ($F(2,6)=6.834$, $p<.05$, see Figure 23). Specifically, naïve animals (n=3) had significantly more neurons than VEGF- (n=3) ($p<.05$) or

vehicle-infused (n=3) ($p<.05$) animals 30 days after status epilepticus, suggesting that the neuronal preservation observed 24 hours after status epilepticus was not sustained for the first month after status.

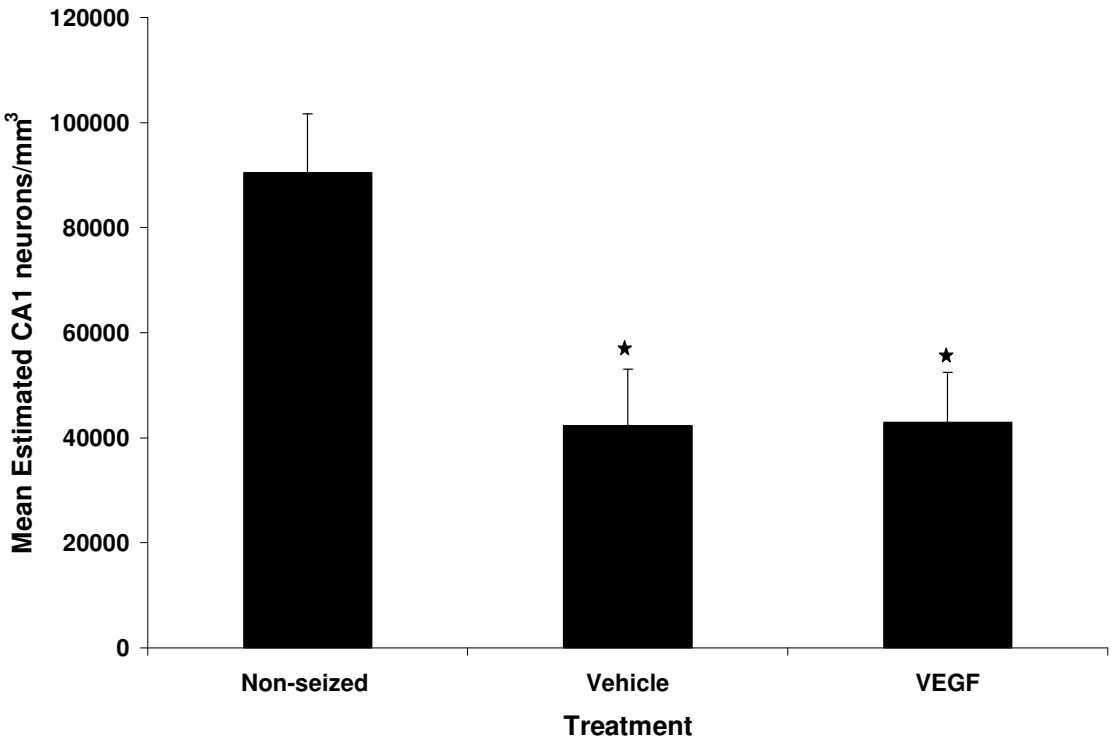

Figure 23. Neuronal density estimates after treatment with VEGF one month following status epilepticus.
Both VEGF- and vehicle-treated animals had significantly less neurons 4 weeks after status epilepticus than non-seized animals, *significantly different at .05. There was no significant difference between VEGF and vehicle animals, $p>.05$.

Discussion

Summary

In the present experiments, we studied the role of VEGF in epilepsy using a well-established animal model of status epilepticus. We first found that VEGF protein was dramatically upregulated in neurons and on glia in the hippocampus, thalamus, neocortex, and amygdala 24 hours after pilocarpine-induced status epilepticus. The purpose of this upregulation of endogenous VEGF following status was unclear. As previously discussed (see Introduction), one of VEGF's primary roles is the induction of new vasculature from previously existing vasculature, also known as angiogenesis. Thus, one possible explanation for the upregulation of VEGF was to bring new vasculature to the area to support cells which have undergone trauma or relative hypoxia as a result of the insult and increased metabolic activity. Our studies revealed that vascular density was indeed increased after status epilepticus, with the most significant increase occurring three days after status. However, further studies in which animals were treated with a VEGF-blocker, Flt-Fc, revealed no significant attenuation of the increase in vascular density after pilocarpine-induced status epilepticus. Therefore, VEGF was not likely the key mediator of increased vasculature in this paradigm.

Another possibility was that VEGF was upregulated in an effort to directly protect cells from damage and death. Studies in which endogenous VEGF was inhibited with Flt-Fc revealed a significant increase in neuronal loss in Flt-Fc-infused animals compared to controls after status epilepticus. Further, when exogenous VEGF was infused into hippocampus prior to and during status epilepticus, neurons were significantly preserved. These findings suggest the possibility that VEGF operated as a neuroprotective factor

after status epilepticus. In fact, we found that VEGFR2, which has been demonstrated to be involved in the neuroprotective signal transduction cascade that activates phosphatidylinositol 3-kinase(PI3-K)/Akt and mitogen-activated protein kinase kinase (MEK)/extracellular-signal-regulated kinase (ERK), was upregulated in neurons in the hippocampus and thalamus 24 hours after pilocarpine-induced status epilepticus. Therefore, the VEGF receptor system has potential as a novel therapeutic pathway for the development of exogenous ligands to prevent cell loss after severe seizures.

VEGF Upregulation

Brain regions often compromised after severe seizures include the hippocampus, thalamus, and amygdaloid body (Watson, 1991). Accordingly, our data revealed that VEGF protein was upregulated in neurons and on glia in these same areas after pilocarpine-induced status epilepticus. More specifically, VEGF protein was observed between the outer membrane and the nucleus of neurons in hippocampal CA1 and CA3 and the temporal neocortex. A recent study using in situ hybridization demonstrated that neurons increase the synthesis of VEGF mRNA after seizures induced via electroconvulsive shock (Newton et al., 2003), suggesting that the neurons themselves could be the source of the increased VEGF. Taken together, these data suggest that neurons may upregulate VEGF when they are at risk from an insult. Upregulation of VEGF in neurons at physiologically-relevant levels could result in an increase of VEGF in the local microenvironment, thereby causing autocrine and paracrine effects on local cells. In our studies, we also observed VEGF protein on the cell surface of glia. Hence, the finding that VEGF immunostaining appeared both in neurons and at the surface of glia suggests the possibility that multiple cell types are affected by this upregulation. It

has been well-documented that a significant regulator of VEGF production is hypoxia (Minchenko et al., 1994; Stone et al., 1995; Pierce et al., 1995; Neufeld et al., 1999; Krum et al., 2002). During status epilepticus, animals may experience states of relative hypoxia and therefore VEGF may be upregulated in an effort to induce new vasculature to increase blood flow into the metabolically active area.

Vascular Effects of VEGF

VEGF is a potent angiogenic factor. When administered to a wide variety of tissues, VEGF induces the development of new blood vessels (Krum et al., 2002; Bauters et al., 1994; Takeshita et al., 1994; Pearlman et al., 1995; Rosenstein et al., 1998; Springer et al., 1998; Croll et al., 2004a). Therefore, it is possible that the upregulation of VEGF after seizures leads to increases in vascular density. Indeed three days after seizures, we noted a significant increase in vascular density in the hippocampus. Previous work has shown that it takes two to three days of VEGF exposure before brain vasculature will exhibit increased density (Croll et al., 2004a). Because we found increases in VEGF protein by 1 day after seizures, the timecourse of vascular density increase is consistent with the possibility that endogenous VEGF upregulation led to the enhanced vascular investment of the hippocampal microvasculature. Previous work with VEGF has consistently shown that VEGF not only increases vascular density but also increases vascular diameter (for review, see Carmeliet & Storkebaum, 2002; Croll et al., 2004a). Therefore, if VEGF were the factor mediating this increase in vascular density, we might have expected a concomitant increase in vascular diameter. We were surprised, therefore, to find no significant increase in vascular diameter in our model.

To better understand whether VEGF could be responsible for the increases in vascular density observed after status epilepticus, we infused Flt-Fc, which functions to bind any reagent that would normally bind to VEGFR1. If VEGF were responsible for the angiogenic response after seizures, we would have expected a significant attenuation of the increase in vascular density after status epilepticus in Flt-Fc-treated animals. Instead, results revealed no significant difference in vascular density between Flt-Fc- and hFc-infused animals. Thus, VEGF did not appear to be the primary mediator of vascular density in this paradigm. Because Flt-Fc will "trap" any VEGFR1 agonist, our experiment also suggested no role for VEGFB or PlGF, other VEGFR1 agonists, in the angiogenesis observed after status. However, research has demonstrated that other growth factors, such as fibroblast growth factor (FGF) and hepatocyte growth factor (HGF), mediate angiogenesis (Baffour et al., 1992; Bussolino et al., 1992; Yang et al., 1996; Yang & Feng, 2000; Zhang et al., 2003; Asano et al., 2007). Moreover, previous research has shown a dramatic increase in FGF after seizures (Riva et al., 1992; Follesa et al., 1994; Gall et al., 1994; Van Der Wal et al., 1994).

The fact that VEGF was not involved in the post-status angiogenic response may be explained by the compartmentalization of VEGF protein upregulation and VEGF receptor changes following status epilepticus. It is neurons which increase the synthesis of VEGF mRNA after seizures induced via electroconvulsive shock (Newton et al., 2003). In our experiments, VEGF protein was found in higher levels in neurons and on glia after status epilepticus. In neurons, staining was darkest between the outer membrane and the nucleus, suggesting that neurons manufacture VEGF, while in glia, staining appeared to be on the cell surface, suggesting that glia may not manufacture

VEGF in this context. Therefore, secreted VEGF may be compartmentalized specifically within the neuronal microenvironment, which may not always be closely apposed to vasculature. While high affinity VEGF receptors are predominantly localized to vascular endothelium in normal brain, research has demonstrated that VEGF receptors are upregulated on neurons and glia following disruption of the central nervous system (Lennmyr et al., 1998; Jin et al., 2000; Sondell et al., 2000; Sawano et al., 2001; Krum et al., 2002). Our finding that VEGFR2 is dramatically upregulated in neurons after status epilepticus is consistent with these findings. Given the fact that VEGF is found in neurons and that the VEGFR2 receptor is upregulated in neurons after status, it is possible that secreted VEGF is more likely to encounter high affinity receptors in the neuronal microenvironment after status than constitutively. That is, if neuronally-produced VEGF can reach vascular VEGF receptors in normal animals, it may not necessarily be able to do so in seized animals, where increased neuronal VEGF receptors may serve as a VEGF sink. Under these circumstances, when the brain has been exposed to an insult, it is possible that neuronally-secreted VEGF binds to receptors on neurons and glia rather than on receptors on vascular endothelium.

Because most biological processes are adaptive and evolve to fit a need, we next wondered what conditions were conducive to the development of increased vascular density. To look at predictive relationships between some of these factors and vascular density, we conducted a series of regression analyses to determine which parameters of the post-status hippocampus would best predict increases in vascular density. Although seizure severity, inflammation, and neuronal damage all proved to be significant predictors of increased vascular density, the best predictor of the three was inflammation.

This finding is consistent with the supposition that inflammation precedes or facilitates pathological angiogenesis (Proescholdt et al, 1999; Croll et al, 2004a; Kasselman et al., 2007). However, because inflammation is also significantly related both to seizure severity and to neuronal damage, it is difficult to attribute the increases in vascular density to any one factor, particularly given the correlational nature of regression analyses.

The precise function of the increased vascular density after status epilepticus is unclear. During status epilepticus, cells are exposed to increased levels of glutamate resulting in excitotoxic damage and cell death. Thus, a possible explanation for the increase in vascular density is to bring in new vasculature to attract immune cells to the area to clear damage and debris. In fact, Manoonkitiwongsa et al. (2001) have found that damage consistently attracts blood vessels in stroke models. Their hypothesis is that the blood vessels are synthesized in an adaptive response to deliver immune cells which can then destroy and remove necrotic tissue and debris. After this process is complete, the microvessels are no longer needed and therefore degrade. This hypothesis is consistent with our finding of damage 24 hours after status followed by vascular density increases at 3 days and a subsequent decrease in vascular density at 7 days.

Another possible explanation for increased vascular density is that neurons involved in the status epilepticus event are hyperexcitable and have substantially elevated metabolic needs as a consequence of increased glutamate signaling. A failure of cells to meet their metabolic needs leads to a relative state of hypoxia and hypoglycemia. Hypoxia-related factors such as HIF-1α are potent inducers of angiogenic factors (Marti et al., 2000; Chiarugi et al., 1999; El Awad et al., 2000; Tsuzuki et al., 2000; Yuan et al.,

2000; Bergeron et al., 1999). Thus, the additional vasculature could have grown into the area to meet the increased metabolic demands of the tissue. It has been found that even small increases in the metabolic needs of brain tissue, such as results from exposure of animals to complex environments, are sufficient to result in increased vascular density (Sirevaag & Greenough, 1987).

Because inflammation increases after status epilepticus, and VEGF is known to attract inflammatory cells, it is still possible that VEGF plays a role in driving this process, regardless of whether it triggers the increases in vascular density. Increased inflammation and enhanced vascular density might or might not be dissociable events, as it is the vasculature that delivers immune cells into tissue. The exact role of inflammation after status epilepticus is unknown but, as suggested previously for increased vasculature, it could serve to clean up debris in damaged tissue. The strong relationship between cell loss and inflammation in our experiments illustrates the correspondence of the two processes. Immune cells such as macrophages, which we have observed in hippocampal tissue after status epilepticus, subserve a major clean-up function in the body. However, the full range of consequences of this inflammation is not yet fully understood. While cleaning up debris might be a beneficial function of immune cells, recent evidence suggests that the presence of immune cells in the hippocampus during and after seizures also results in detrimental effects (De Simoni et al., 2000: Vezzani et al., 1999, 2000, 2002).

The increase in vascular density may result in an effort to protect neurons from increased seizure susceptibility. That is, it is possible that increased vascular leak, which often accompanies angiogenesis, could result in a larger distance between synapses which

would result in less excitability and therefore a decrease in seizure susceptibility. This possibility, however, is unlikely given the fact that treatment with Angiopoietin-1 does not affect seizure behavior (Nicoletti & Croll, unpublished data), although it blocks the increases in vascular leak induced by VEGF (Thurston et al., 2000). Because the lack of change in seizures was evaluated by behavioral assessment, rather than by EEG, the possibility remains that subtle changes in firing and excitability contributed to the ultimate excitotoxic demise of some neurons. This seems like a particularly attractive explanation given the recent finding that VEGF "quiets" neurons (McCloskey et al., 2005).

Neuroprotection

Studies in rodents (for example, see Borges et al., 2003) and in humans (Jutila et al., 2001: Bernasconi et al., 2003) have shown that structures including hippocampus, amygdala, entorhinal, and perirhinal cortices are compromised as a result of severe seizures. Given that VEGF has been shown to have neuroprotective effects across a wide variety of manipulations (for review, see Carmeliet & Storkebaum, 2002; Storkebaum et al., 2004; Rosenstein & Krum, 2004), we suspected that VEGF upregulation reflected compensatory mechanisms of neurons to protect themselves from death.

To examine whether or not endogenous VEGF was upregulated in an effort to protect neurons after status epilepticus, we infused Flt-Fc (which sequesters endogenous VEGF) or hFc, as a control, prior to inducing status. We found significantly more neuronal damage in animals treated with Flt-Fc compared to controls. Thus, either endogenous VEGF or other endogenous VEGFR1 ligands did appear to play a role in neuroprotection after seizures. However, whether the levels of upregulated VEGF would

be sufficient to provide maximal protection remained to be determined. To address this issue, we infused exogenous VEGF into brain prior to inducing status epilepticus and found that only doses of 30ng/d or higher of VEGF significantly preserved neurons after status. Thus, it seems unlikely that endogenous VEGF levels alone are sufficient to optimize VEGF's neuroprotective potential.

Mechanisms of Neuroprotection

The exact mechanism of VEGF's neuroprotection after status epilepticus is currently unknown. As previously described (see Introduction), VEGFA binds VEGFR1, VEGFR2, neuropilin-1, and neuropilin-2. While neuronal VEGFR1 has never been reported in adults, neurons constitutively express neuropilin-1 and have been shown to express VEGFR2 receptors after insults to brain. Elegant studies have shown that the neuroprotective effects of VEGF are generally mediated via VEGFR2 (Sun et al., 2003; Jin et al., 2001: Matsuzaki et al., 2001; Wick et al., 2002). VEGFR2 signals through the PI3-K/Akt and MEK/ERK cell survival pathways, and hence could be causing its protective effects through this activation (Sun et al., 2003; Mazure et al., 1997; Gerber et al., 1998). While VEGFR2 is not localized to neurons constitutively, its mRNA or protein has been consistently observed in neurons in culture (Sondell et al., 2000; Matsuzaki et al., 2001) as well as in adult brain after insults such as cerebral ischemia (Croll & Wiegand, 2001). We found that VEGFR2 was dramatically upregulated in the soma and processes of hippocampal CA3 neurons, and mildly upregulated in the processes of CA1 neurons, after status epilepticus. Thus, VEGFR2 could have transduced pro-survival signals in neurons upon exposure to VEGF protein in our model.

Another possible route through which VEGF may exert its neuroprotective effects is through activation of neuropilin-1. As previously described (see Introduction), neuropilin-1 is most densely expressed on neurons and may act as a co-receptor to the VEGF family of receptors, particularly VEGFR2, to enhance VEGF's activities. Oosthuyse and colleagues (2001) demonstrated that VEGF protected cultured primary motor neurons against hypoxia-induced apoptosis through activation of VEGFR2 and neuropilin-1. Therefore, it is possible that neuropilin-1 may act in concert with VEGFR2 after seizures to preserve neurons. Neuropilin-1 is expressed more highly in hippocampal pyramidal cells than almost any other cell in the adult brain, with especially high levels in CA1 (Kawakami et al., 1996; Chedotal et al., 1998).

Since VEGFA binds VEGFR1, VEGFR2, neuropilin-1, and neuropilin-2, we cannot conclude with any certainty through which receptor VEGF exerts its neuroprotective effects. To elucidate the precise role of each of these receptors in relation to VEGF's neuroprotective effects, future studies should employ $VEGF_{121,}$ which does not bind neuropilin-1, PlGF, which binds neuropilin-1 and VEGFR1, and VEGFC or VEGFE, which only bind VEGFR2 in brain. If PlGF but not $VEGF_{121}$ treatment mimics the effects of VEGF, the results would strongly suggest that VEGFR1 but not neuropilin-1 play a role in VEGF-mediated neuroprotection. If VEGFC or VEGFE treatment, but not PlGF treatment, mimics the effects of VEGF, the results would strongly suggest a role for VEGFR2 in VEGF-mediated neuroprotection after status epilepticus. Ideally, future studies could also take advantage of selective pharmacological blockers of these receptors. Currently, only selective reagents for

VEGFR2 are available, and these reagents are largely insoluble in buffers considered safe for direct administration to brain tissue.

It is also possible that VEGF exerted its neuroprotective effects through glial activation. As previously mentioned, McCloskey et al. (2005) evaluated the ability of VEGF to depress epileptiform activity in the hippocampi of rats with chronic spontaneous seizures. They found that VEGF decreased epileptiform activity when administered to hippocampal slices from epileptic rats, which was not mediated by intrinsic changes. They also found that VEGF decreased synaptic transmission in slices from normal rats. Since they failed to establish presynaptic and postsynaptic actions of VEGF, they postulated that the glia invaginate the synaptic cleft and sequester glutamate, ultimately decreasing excitability and excitotoxicity within the system. We observed increased punctate VEGF staining on the cell surfaces of glia after status epilepticus, suggesting that VEGF protein was binding to glial VEGF receptors. Further, Ackerman et al. (2003) found that VEGF significantly hypertrophies and activates astroglia in adult brain. Thus, it is possible that VEGF exerts is protective effects through decreasing neuronal excitability indirectly via glial activation. One important role of astrocytes is glutamate uptake (Rothstein et al., 1996; Tanaka et al., 1997; Danbolt, 2001), so one potential mechanism by which glia could be modulating neuronal excitability would be by upregulating their uptake function. Whatever the mechanism, a "quieting" effect of VEGF may be expected to result in lower seizure scores and hence a decrease in cell loss. We did not, however, observe any significant decrease in seizure behaviors in our VEGF-infused animals. Nevertheless, we still cannot rule out the possibility that subtle "quieting" effects of VEGF led to decreased excitotoxicity in the absence of observable

decreases in seizure behaviors. Additional research will be necessary to fully elucidate the mechanism of VEGF-induced neuroprotection after status epilepticus.

Delivery of VEGF

Since we were able to demonstrate that infusion of VEGF protein significantly protects neurons after status epilepticus, we next sought to establish an alternate route of delivery. Because VEGF protein does not cross the blood-brain barrier, therapeutic VEGF would need to be continuously infused into brain via cannulae. This method, while possible, presents a number of potential complications. For example, over time cannulae can become loose or dislodge, ultimately causing further physical trauma to the brain and possibly delivery of VEGF to another area if dislodged. Cannulae can also become clogged and therefore infused doses of VEGF could be insufficient to provide protection. Significant scar tissue can build up around cannulae causing an infiltration of monocytes and inflammatory cells, and limiting the diffusion of the therapeutic VEGF. Perhaps most importantly, indwelling cannulae leave the brain vulnerable to infection. Given the multiple complications regarding clinical utility, we piloted an adeno-associated viral vector to infect cells which would function to upregulate endogenous VEGF. AAV VEGF could be administered once, or infrequently, into epileptic brain and accomplish long-lasting upregulation of VEGF. We found that animals which received AAV VEGF had higher neuronal densities than animals which received a control viral vector. Due to the small number of animals in this preliminary study, we failed to find a statistically significant increase in AAV VEGF versus blank AAV. However, it should be noted that AAV VEGF animals had neuronal densities that were very similar to those in the non-seized animals. Further studies will need to be conducted to determine if this

route of administration significantly protects neurons after status epilepticus. VEGF protein should also be assayed with ELISA after AAV VEGF to quantify the amount of protein available with this method of delivery.

Functional Consequences

Since VEGF significantly preserves neurons 24 hours after status epilepticus, we wondered if these neurons remained functional. To assess whether neuronal preservation translated to functional preservation, we evaluated behavior during the latent period, two to four weeks after status epilepticus. As previously described (see Introduction), neuroanatomical and neurophysiological changes, such as neuronal loss, mossy fiber sprouting, gliosis, and synaptic re-organization, which are believed to underlie spontaneous recurrent seizures, occur during the latent period (Cavazos & Cross, 2006). These changes mainly occur in the hippocampus, thalamus, and amygdala, which are structures that have been implicated in behaviors such as learning, memory, and anxiety (Jarrard, 1993; Davis, 1992). Given that VEGF significantly preserved neurons 24 hours after status epilepticus, we expected to see a relative preservation of learning and memory and anxiety after treatment with VEGF.

Animals that received infusions of VEGF for 4 weeks demonstrated better learning and memory than animals that received control infusions. VEGF-treated animals, however, still performed significantly worse than non-seized animals on this task. While previous experiments have demonstrated that as little as 26% of the dorsal hippocampus was necessary to support spatial memory (Moser et al., 1995), it may be the case that in our model of epilepsy, partial preservation of one hippocampus was not enough to preserve learning and memory. It is unclear if this significant difference is due

to a difference in unilateral versus bilateral infusions or if the difference is due to the fact that the two cohorts were different in some other way. To address this dilemma, future studies should employ both unilateral and bilateral infusions within the same cohorts.

In relation to emotional functioning, VEGF-treated animals exhibited more anxiety than vehicle-treated animals as demonstrated by their normal preference for the dark compartment in a light-dark exploration task. In fact, VEGF-treated animals did not significantly differ from non-seized animals in their preference for the dark compartment. In contrast, control seized animals failed to show a normal anxiety response to the novel context of the task, an observation frequently seen in hippocampally-damaged rats (Kalynchuk et al., 1998; Mortazavi et al., 2005; Detour et al, 2005; Szyndler et al., 2002). VEGF-treated animals also initiated more contact with a novel rat than vehicle-treated animals. Their tendency to initiate contact reflects the similar tendency at contact initiation by non-seized rats. VEGF-treated animals exhibited more exploratory behavior than vehicle-treated animals and non-seized animals. There were no significant differences in social or emotional functioning between animals receiving unilateral versus bilateral infusions.

Although there was significant improvement in anxiety after status epilepticus with VEGF treatment, VEGF-treated animals still exhibited significant impairments in relation to non-seized animals, particularly on a task of learning and memory. There are several explanations for the selective profile of our functional recovery. First, we had a small number of animals in each group and the relatively large variability in some measures could account for the fact that some tendencies in the data failed to achieve significance. Sources of variability could include variable response to seizures and

variable sensitivity to environmental stimuli such as colony conditions and experimenter handling. While all animals were subjected to similar colony conditions and handling procedures, we cannot rule out the possibility that these confounds interacted with treatment group. In addition, while all seized animals in the behavioral experiments achieved status epilepticus, the extent of the status was sometimes anecdotally different from animal to animal.

Perhaps the most parsimonious explanation regarding our behavioral results is that they really do reflect a relationship between functioning and the number of neurons preserved. VEGF protein administration never accomplished full preservation of hippocampal neurons in our experiments. Anxiety functioning was normal in VEGF treated animals in our experiments, but it may be that this behavioral function is less sensitive to mild to moderate neuronal loss than learning. Another possible explanation for our incomplete behavioral protection could be that while many neurons were preserved 24 hours after status epilepticus, they may not have been preserved long-term. Neuronal density estimates revealed no significant difference in neuronal densities in animals infused with VEGF compared to vehicle-infused animals at the one month time point. Given the fact that our behavioral and stereological analyses were based on the relatively limited number of animals which both achieved status epilepticus and were healthy enough to survive for a month after this insult, we cannot make definitive conclusions regarding VEGF's ability to preserve function until additional studies are conducted.

While neuronal loss may be one explanation for our lack of functional preservation, there are several other mechanisms which may account for the lack of

functional preservation in our model. Liu and colleagues (2003) found that rats who experienced status epilepticus, regardless of when the insult was experienced during their life-span, demonstrated impaired learning and memory on a spatially-mediated task. They also found defective place cells at the network level and abnormal connectivity at the structural level. Specifically, place cells in rats who experienced status epilepticus were less coherent, that is, the local smoothness of their firing fields was lower, and their fields were less stable (Liu et al., 2003). Further, place cell discharge frequency was not significantly different between groups. Therefore, abnormal electrical activity could not account for the memory deficits. Brun et al. (2001) investigated the place-cell network within the hippocampus and whether place-related firing resulted from intrahippocampal computations. By effectively isolating CA1, they demonstrated that direct connections from the entorhinal cortex into CA1 were sufficient for maintaining the fundamental properties of place cells in area CA1, ultimately preserving spatial recognition memory. They also demonstrated that an intact CA3-CA1 associate network was necessary for recall. Leung and Shen (2006) observed changes in the medial perforant path-evoked dentate gyrus population spike and medial perforant path-evoked polysynaptic wave in CA1 kindled rats. They suggested that these changes in synaptic transmission underlie the deficits in spatial performance. Given these findings, it is possible that both place cell abnormalities and disrupted connectivity play a role in impaired spatial ability after status epilepticus, regardless of degree of neuronal protection.

Another possible mechanism which may have contributed to behavioral deficits after status epilepticus were the biochemical changes related to glutamate. During a seizure, extracellular glutamate levels increase, bind to NMDA receptors, and cause an

influx of calcium which ultimately leads to excitotoxic cell death (Scott et al., 1998; Chapman, 2000; Fujikawa, 2005). While it is clear that glutamate plays a role in the neuroanatomical changes after status epilepticus, it is also possible that it directly affects behavior after seizures. It has been well-established that glutamate, and in particular its NMDA receptor, plays a role in modulating hippocampally-mediated cognitive processes such as learning, memory, and long-term potentiation (Morris et al., 1986; Davis et al., 1992; Miyamoto, 2006; Shukla et al., 2007). Thus, changes in signaling mediated by this amino acid could contribute to the deficits in learning and memory often seen after seizures, regardless of whether or not neurons are preserved.

Szyndler et al. (2006) investigated the relationship between several amino acids, including glutamate, and behavior after kindling in rats. They found that learning and memory deficits were associated with a decrease in brain concentrations of glutamate, glycine, an inhibitory neurotransmitter that functions as a co-agonist of the NMDA receptor (Johnson & Ascher, 1987), and alanine, an important precursor of glutamate. Although neuronal loss is a likely explanation for decreased glutamate levels, this decrease may also be the result of an upregulation of glutamate transporters (Bruhn et al., 1997; Nonaka et al., 1998). In fact, Ghijsen and colleagues (1999) found an increase in glial and neuronal glutamate transporters after seizures. We earlier proposed the possibility that VEGF's enhancement of glial phenotype could have increased glutamate transporters even more. Despite the cause, dysfunction of the glutamatergic system within the brain could possibly account for the cognitive deficits observed after seizures.

In sum, we observed significant neuronal preservation 24 hours after status epilepticus which led us to believe that we might see some preservation of cognitive and

emotional functioning. When we investigated behavioral functioning, we found a selective profile of preservation. That is, VEGF preserved normal anxiety functioning but did not preserve learning and memory. One possibility which may account for these deficits is a lack of neuronal preservation long-term. When we assessed neuronal densities at four weeks post-status epilepticus, we found no significant difference in neuronal densities between VEGF- and vehicle-treated animals. Since we only looked at two time points post-status, we cannot be certain about neuronal densities during the exact time when behavior was tested. Regardless of whether the neurons rescued by VEGF were still present during behavioral testing, research has demonstrated that there are many physiological changes that occur after seizures. Therefore, it may also be the case that these physiological changes contributed to the behavioral results in our model. That is, even if there were no longer any rescued neurons when testing was performed, VEGF may have partially protected against abnormal functioning in the remaining neurons. As previously described, McCloskey et al. (2005) found that VEGF decreased synaptic activity and excitability, which may function to ultimately preserve some functions (such as anxiety) while failing to significantly preserve others (such as memory) in our model.

Since we observed discrete deficits in learning and memory and a relative preservation of anxiety after status epilepticus, it may be the case that learning and memory are more sensitive to these neuroanatomical and neurophysiological changes than anxiety. Anxiety, which can be considered an expression of fear, is a natural, adaptive reaction to the environment (Charney & Bremner, 1999). Animals typically experience fear and anxiety when faced with a life threatening situation. Since this basic

biological response has been developed to ensure survival, it may be the case that alterations in this behavior are more resistant to impairment by damage after status epilepticus than learning and memory would be. At this point, we cannot be sure of the mechanisms responsible for the selective profile of functional preservation observed after status epilepticus in our model. Further studies which include a timecourse evaluation of neuronal density as well as measures of pathological physiological functioning in remaining neurons will be needed to elucidate this process.

Conclusions

Our finding of increased endogenous VEGF protein after status epilepticus suggested that VEGF might play a role in seizures or their sequelae. While VEGF is a potent angiogenic factor, our finding that increased vascular density after pilocarpine-induced status epilepticus is not mediated by upregulated endogenous VEGF suggests that its primary role in this system is not angiogenic. Rather, it appears that endogenous VEGF is upregulated in an effort to protect cells after seizures. Endogenous levels of VEGF, however, are insufficient to provide complete neuroprotection as demonstrated by the finding that infusion of at least 30ng/d exogenous VEGF resulted in significantly more neuronal preservation after status epilepticus than endogenous VEGF alone. Based on previous research, it is likely that VEGF exerted its neuroprotective effect through VEGFR2, which we found to be upregulated in hippocampal neurons after status epilepticus. Although VEGF preserved neurons 24 hours after seizures, we were unable to demonstrate that VEGF protected neurons long-term. Consequently, the extent to which VEGF preserves function after seizures is still unclear, though it appears that some

functions (anxiety) are better preserved by VEGF than others (memory). Further studies need to be conducted to elucidate the long-term implications of VEGF treatment.

Although exogenous VEGF is neuroprotective in this paradigm, it is unlikely that exogenous VEGF will be useful as a clinically therapeutic agent to protect neurons during severe seizures. As a large protein with multiple effects, issues of delivery and specificity of effect will be significant barricades to its use as a drug. Preliminary data with an adeno-associated viral vector demonstrated that this may be a promising route of administration. Further, if the receptor systems underlying these effects could be elucidated, small molecule neuroprotective reagents could be developed with specificity for the relevant receptors. Therefore, the finding that exogenous VEGF protein significantly protected hippocampal neurons from cell death after status epilepticus could lead the way to novel approaches to cell protection in epilepsy.

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