CHRONIC EFFECTS OF VEGF ADENOVIRUS TRANSFECTION INTO ADULT RAT HIPPOCAMPUS

by

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Abstract

CHRONIC EFFECTS OF VEGF ADENOVIRUS TRANSFECTION INTO ADULT RAT HIPPOCAMPUS

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Tsippa F. Ackerman

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Vascular Endothelial Growth Factor (VEGF) is a cytokine growth factor that regulates vasculogenesis and angiogenesis. In addition to its vascular effects, VEGF has more recently been associated with nonvascular effects. We sought to study the effects of continuous expression of VEGF induced via adenoviral vector (AdVEGF) in the rat hippocampus. When brains were examined four weeks following AdVEGF administration, the hippocampi were severely damaged and the lateral ventricles were dramatically enlarged. Neither control adenovirus expression nor VEGF expression induced via adeno-associated viral vector led to degeneration. We sought to characterize and learn about the mechanism of this AdVEGF-induced degeneration. Administration of a control adenovirus followed by infusion of exogenous VEGF protein for four weeks did not lead to hippocampal atrophy or ventricular enlargement, suggesting that the degenerative effect relied on expression of VEGF via adenovirus. Time-course experiments showed that the degeneration takes place largely between seven and twelve days after AdVEGF administration. Adenoviral administration of the anti-leak protein Angiopoietin-1 with VEGF adenovirus did not prevent AdVEGF-induced degeneration, indicating that the degeneration was

not due to vascular leak. Infusion of the corticosteroid anti-inflammatory Dexamethasone for four weeks following AdVEGF administration did not prevent degeneration, indicating that AdVEGF's effects were not due to inflammation. To explore the receptor specificity of AdVEGF-induced degeneration, we induced expression of Placental Growth Factor, a VEGF family member which binds only to VEGFR-1 and NP-1, via adenoviral vector (AdPlGF). Four weeks after adenoviral administration, AdPlGF-treated brains did not show hippocampal degeneration or ventricular enlargement, suggesting that AdVEGF-induced degeneration is VEGFR-2-mediated. We hypothesize that the rapid increase in VEGF expression induced by AdVEGF—and the pattern of VEGF binding that follows—results in a downregulation or dysregulation of VEGFR-2, leading to hippocampal degeneration.

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CHAPTER ONE: INTRODUCTION

The VEGF Family of Proteins

Vascular Endothelial Growth Factor (VEGF) is a cytokine growth factor that was first identified as Vascular Permeability Factor (VPF) in 1983 when its ability to induce vascular leak in guinea pig tissue was discovered (Senger *et al*., 1983). It was discovered a second time in 1989 as a diffusible endothelial cell mitogen (Ferrara & Henzel, 1989), and cloning showed it and VPF to be the same protein. VEGF is now known to regulate the development of blood vessels (vasculogenesis) and the sprouting of capillaries from existing vessels (angiogenesis) during development in every adult tissue tested, including brain (Rosenstein *et al.*, 1998). In addition to VEGF (also termed "VEGF-A"), five other members of the VEGF protein family have been discovered. They are Placental Growth Factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E. The structure, function, distribution, and relative importance of the family members differ.

VEGF

The human VEGF gene is located on chromosome 6p21.3 (Vincenti *et al.*, 1996). It is made up of eight exons separated by seven introns. Six VEGF isoforms are produced through alternative splicing of the VEGF-A gene (for reviews, see Robinson & Stringer, 2001 and Tamella *et al.,* 2004). They are $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$. $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ are the major forms that are secreted by most VEGF-secreting cells. VEGF is a homodimeric glycoprotein of 45 kDa (Ferrara & Henzel, 1989) with heparin-binding properties and bioavailability that vary

according to protein isoform. The mitogenic activity of each individual isoform is related to its degree of heparin binding such that those isoforms with lower heparin affinity demonstrate less potent mitogenic activity (for review, see Ferrara, 2000). $VEGF_{121}$ is acidic and freely soluble, while the highly basic $VEGF_{189}$ and $VEGF_{206}$'s heparin-binding properties are greater, and they are almost completely sequestered in the extracellular matrix. $VEGF_{165}$, the most prevalent isoform of VEGF, possesses an intermediate affinity for heparin, allowing it to diffuse, while a substantial portion remains bound in the extracellular matrix (for review, see Ferrara *et al.,* 2003).

In the developing mouse and rat, VEGF is expressed throughout the organs, including central nervous system, with the highest levels of expression being seen in areas of active angiogenesis and vasculogenesis. The highest levels of expression in the developing brain have been detected in the choroid plexus and ventricular epithelium. In human fetal development, VEGF is widely expressed, with mRNA expression detectable in most tissues. VEGF expression is especially dense in lung, kidney, and spleen and has been detected in myocytes and epithelial cells, but not vascular endothelial cells (for review, see Tammela *et al.,* 2004 and Ferrara, 2004). In the adult animal, VEGF continues to be expressed in areas such as the kidneys and ovaries (Phillips *et al.*, 1990; Ravindranath *et al.*, 1992), but is expressed at negligible levels in the normal brain (Kovacs *et al.*, 1996).

Studies using VEGF isoform-specific knockout mice have been conducted to determine the specific roles and relative importance of the different VEGF-A

isoforms in development and survival. Knockout mice expressing only $VEGF_{120}$ (the murine homologue of human $VEGF_{121}$) or $VEGF_{188}$ (homologue of human $VEGF_{189}$) are not viable; those that do not die soon after birth die later due to impaired arteriolar development, ischemic cardiomyopathy, or multi-organ failure. Knockout mice expressing only $VEGF_{164}$ (homologue of human $VEGF_{165}$), however, are viable and healthy, demonstrating the importance of $VEGF_{164/5}$ in accomplishing VEGF's role in the developing animal (for review, see Tammela *et al.*, 2004).

VEGF monomers dimerize and bind to VEGF receptors in a "head-to-tail" fashion, such that their receptor binding domains are at opposite ends of the VEGF dimer (Keyt *et al.*, 1996; Muller *et al.,* 1997). Though homodimerization is more common, VEGF-A is able to heterodimerize with other members of the VEGF family (DiSalvo *et al.*, 1995). The receptors that bind VEGF are VEGFR-1, VEGFR-2, Neuropilin-1 (NP-1), and Neuropilin-2 (NP-2) (for review, see Ferrara, 2004).

Placental Growth Factor

Placental Growth Factor (PlGF), so named because it was isolated from a term placenta cDNA library, was first described by Maglione, *et al*. (1991). The original PlGF that was isolated was 149 amino acids long and was 53% homologous to the Platelet-Derived Growth Factor-like portion of VEGF. A longer isoform has since been identified. PlGF is dimeric, secreted, and N-glycosylated, and these characteristics, along with its structural homology to

VEGF and its ability to stimulate endothelial cells *in vitro,* led researchers to consider PlGF a member of the VEGF family of proteins.

PlGF is expressed predominantly in heart, placenta, and lungs. PlGF homodimers bind VEGFR-1 and NP-1, but PlGF is also capable of heterodimerizing with VEGF. When PlGF binds to VEGFR-1, the response pattern it induces in the receptor's tyrosine kinase domain, as well as subsequent gene expression, differs from that which follows when VEGF binds to VEGFR-1 (Autiero *et al.*, 2003). While VEGF induces very little DNA synthesis when it binds to VEGFR-1, when PlGF binds to VEGFR-1, MAP-kinase activation, a marker of cell activation and often DNA synthesis, was observed (Landgren *et al.*,1998). VEGF/PlGF heterodimers may bind both VEGFR-1 and VEGFR-2. While PlGF is able to potentiate the effects of low concentrations of VEGF, the potency of a single VEGF homodimer has been found to be 7-fold that of a single VEGF-PlGF heterodimer.

VEGF-B

The precise role of VEGF-B is not known. VEGF-B is also known as VEGF-related factor or VRF. There are two known isoforms of VEGF-B. VEGF-B_{167}, which is the predominant form, is mostly sequestered in the extracellular matrix, and VEGF-B_{186} is freely diffusible.

VEGF-B is highly expressed in striated muscle, brown fat and myocardium. It binds VEGFR-1 and NP-1 and can heterodimerize with VEGF-A (for review, see Olofsson *et al*., 1999).

VEGF-C and VEGF-D

VEGF-C and VEGF-D are involved in inducing lymphangiogenesis, the formation of new lymphatic vessels, both in development and in the adult. During development, they are present mostly in areas where lymphatic vessels develop, but expression then decreases, though they remain high in lymph nodes. VEGF-C binds VEGFR-2 and VEGFR-3 and has been shown to be critical for lymphatic development and animal survival in mouse knockout studies. In humans, VEGF-D binds to both VEGFR-2 and VEGFR-3, but in mice it binds only to VEGFR-3. VEGF-D is present in most tissues and has been found to produce strong angiogenesis in addition to lymphangiogenesis in rabbit hind limb muscle when delivered via adenoviral vector (Rissanen *et al.,* 2003).

VEGF-E

VEGF-E was isolated from the Orf virus, which causes lesions and angiogenesis in its hosts. The precise mechanisms of VEGF-E's actions are not known, though under experimental conditions it has been shown to have mitogenic and angiogenic properties almost as great as those of VEGF. Transgenic mice overexpressing this protein showed dramatically increased angiogenesis without the negative side effects of edema and hemorrhagic spots that have been associated with an excess of VEGF activity. VEGF-E binds only VEGFR-2 and is freely diffusible due to its lack of heparin-binding (Meyer *et al.*, 1999).

The VEGF Protein Family Receptors

The VEGF receptors are differentially distributed throughout the body and brain and differentially bind VEGF family members (see Figure 1). Each appears to play a distinct role in the activities of VEGF (see Table 1). In addition to their individual roles, VEGF receptor subtypes are able to form receptor complexes with each other or otherwise modulate each other's activities. The differential distribution, ligand-binding, and downstream activities of the VEGF receptor subtypes are all factors that contribute to the wide range of downstream effects that VEGF is capable of mediating. Understanding these receptors helps us to understand why and how these different downstream effects of VEGF occur.

VEGFR-1/flt-1

VEGFR-1 (also known as fms-like tyrosine kinase, or flt-1) is composed of seven extracellular immunoglobulin homology domains, a single transmembrane region, and an intracellular tyrosine kinase domain that is interrupted by a kinase-insert domain (de Vries *et al.,* 1992). VEGFR-1 binds VEGF, VEGF-B, and PlGF with high affinity (for review, see Shibuya, 2003). It is expressed throughout the body on various tissues and cell types, and in the brain, in addition to being expressed on vascular endothelial cells, it is also expressed on monocytes (Barleon *et al.,* 1996), and reactive astrocytes (Krum & Rosenstein, 1998). Due to its high affinity, VEGF is sequestered by VEGFR-1, reducing the rate of VEGFR-2 signaling (Fong *et al.,* 1995). It is believed that under certain conditions monocytic inflammation is necessary for VEGF-induced angiogenesis to take place, and it has been proposed that it is because VEGF binds to

VEGFR-1 that monocytic extravasation occurs (Clauss, 1998). VEGFR-1's signaling in response to VEGF binding is much weaker than that of VEGFR-2's (Waltenberger *et al.*, 1994). Despite its low signaling levels, however, VEGFR-1 is necessary in development for the formation of healthy vasculature (Fong *et al.,* 1995; Hiratsuka *et al.,* 1998). Knockout studies conducted with mice have shown that while VEGFR-1 is essential for vascular health and subsequent survival of the organism, the tyrosine kinase domain is not necessary for survival, as mice that express VEGFR-1 that lacks the tyrosine kinase domain do survive (Hiratsuka *et al.,* 1998). These studies demonstrating VEGFR-1's high binding affinity but low signaling activity suggest that VEGFR-1 functions as a decoy receptor (Hiratsuka *et al.*, 1998).

VEGFR-2/flk-1

VEGFR-2 (also known in mice as fetal liver kinase, or flk-1, and KDR in humans) is structurally similar to VEGFR-1. It binds VEGF, VEGF-C, VEGF-D, and VEGF-E (for review, see Shibuya, 2003). It is expressed throughout the body on vascular endothelial cells and in the brain on vascular endothelial cells, as well as on reactive astrocytes (Lennmyr, 1998) and neurons after insult (Croll & Wiegand, 2001). Because of the nature of the VEGF protein's structure and the fact that it has separate binding sites for VEGFR-1 and VEGFR-2, a VEGF dimer may simultaneously bind with VEGFR-1 and VEGFR-2, forming a receptor complex (Kendall *et al*., 1996).

Though VEGFR-2's binding affinity for VEGF is lower than that of VEGFR-1, it is the primary signaling VEGF receptor and is believed to be responsible for

the mitogenic and neuroprotective properties of VEGF through its activation of the PI3 kinase/Akt pathways (Rosenstein *et al*., 2003).

VEGFR-3/flt-4

Though VEGFR-3 (also known as flt-4) is found to be essential for the proper formation of the primary vascular plexus in the developing organism, in adulthood it is expressed almost exclusively in lymphatic endothelial cells and is thought to be involved in lymphangiogenesis (Kukk *et al.,* 1996). It binds VEGF-C and VEGF-D (Joukov *et al.,* 1996; Achen *et al.*, 1998).

Neuropilin 1 (NP-1) and Neuropilin 2 (NP-2)

NP-1 and NP-2 are functionally and structurally different from VEGFR-1 and VEGFR-2. One striking difference between the NP receptors and other VEGF receptors is that the NP receptors have no signaling domain (for review, see Neufeld *et al.*, 2002). Binding all VEGF isoforms except for $VEGF_{121}$, these receptors were first identified as receptors for the class-3 semaphorins that are responsible for axon guidance during development (He and Tessier-Levigne, 1993; Kolodkin *et al*., 1997). NP-1 is expressed mainly on arterial endothelial cells, while NP-2 is expressed primarily on venous and lymphatic endothelial cells (for review, see Eichmann *et al*., 2005). They can also be found on neurons and tumor cells (Soker *et al.,* 1998).

VEGF Receptor Subtype Interactions

As mentioned above, it has been proposed that VEGFR-1, with its high affinity but low signaling, functions as a decoy receptor, thereby decreasing the binding and subsequent activity of VEGFR-2. Zeng *et al.* (2001) have proposed

that VEGFR-1 may exert inhibitory effects in a more direct manner. Their work suggests that VEGFR-1 binding leads to PI3-K dependent inhibition of VEGFR-2-mediated endothelial cell proliferation.

Though VEGFR-1 has the ability to inhibit VEGFR-2, it also appears to enhance VEGFR-2's effect in some cases. Autiero *et al.* (2003) reported that a certain amount of molecular crosstalk can take place between VEGFR-1 and VEGFR-2. They demonstrated *in vitro* that the binding of PlGF to VEGFR-1, but not VEGF to VEGFR-1, leads to transphosphorylation of VEGFR-2.

Gluzman-Poltorak *et al.* (2001) showed that VEGFR-1 and NP-2 form complexes and Karpanen *et al.* (2006) found that NP-2 interacted with VEGFR-3. In studies investigating NP-1 functioning, there was no cellular response to VEGF when no other VEGF receptor subtype was present. However, VEGF binding to VEGFR-2 was found to be more efficient when NP-1 was present as well, and there was a stronger cell migratory response than in cells expressing only VEGFR-2. It is therefore believed that the neuropilin receptors function as co-receptors for VEGF, probably in a modulatory capacity (for review, see Neufeld *et al.*, 1999).

VEGF's Vascular Functions in the Adult Animal

In the years since VEGF was first discovered and identified as Vascular Permeability Factor, its functions have been found to extend beyond stimulating vascular permeability. An endothelial cell mitogen, VEGF also regulates angiogenesis. It does so through its induction of factors that contribute to the

angiogenic process (Wary *et al*., 2003) and possibly through its induction of vascular endothelial cell proliferation and vascular permeability (Dvorak *et al.*, 1995), although it is unclear which effects of VEGF are necessary for angiogenesis to occur. VEGF is also responsible for an immune response characterized by the extravasation of monocytic inflammatory cells (Croll *et al*., 2004b; Proesholdt *et al*., 1999). VEGF-induced inflammation has been observed to be important as one of the components of angiogenesis (Kasselman *et al.*, 2007).

VEGF Signaling

The signaling cascade that follows VEGF binding to its receptors is not yet fully known and understood, but parts of the cascade leading to VEGF's effects have been described. These signaling pathways appear to contribute differentially to its hallmark permeabilizing and angiogenic effects.

Following binding, phosphorylation of phospholipase C-γ (PLC- γ) and phosphatidyl-inositol 3-kinase (PI3-K) occurs, and there is activation of protein kinase C (PKC), an increase in $[Ca^2]_i$ in endothelial cells, and accumulation of inositol-1,4,5-triphosphate (IP3) (Mukhopadhyay *et al.*, 2004). A number of additional signal transduction proteins have been associated with VEGF, including the Src family of tyrosine kinases, Ras GTPase-activating protein, Nck, focal adhesion kinase, Akt/protein kinase B, Raf-1, MEK, extracellular signal regulating kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) (for review, see Matsumoto and Claesson-Welsh, 2001).

As mentioned earlier, though VEGF's affinity is greater for VEGFR-1 than it is for VEGFR-2, VEGFR-2's biological activity is greater than VEGFR-1's, with more signaling taking place upon binding of VEGF. A number of VEGFR-2's tyrosine residues (Tyrs) have been identified as autophosphorylation sites, with some linked to specific downstream signaling pathways. For example, through mutation studies, we know that Tyr^{1059} is essential for VEGF-induced intracellular Ca^{2+} mobilization and ERK phosphorylation (Zeng *et al.*, 2001).

Variations in VEGF-mediated signaling patterns determine the effect that VEGF ultimately has. As seen below, different Tyrs and downstream pathways have been associated with different VEGF-induced effects.

VEGF and Vascular Permeability

VEGF increases vascular permeability by loosening the connections between vascular endothelial cell junction proteins (for review, see Chodobski *et al.,* 2003 and Wang *et al*., 2001). VEGF's permeability-inducing activities have particularly significant implications in the adult brain, where the blood brain barrier (BBB) is in place. VEGF has been shown to induce a break-down of the BBB, increasing the permeability of vessels in the brain within thirty minutes (Dobrogowska *et al.*, 1998; Mayhan, 1998). Studies of receptor specificity suggest that both VEGFR-1 and VEGFR-2 are involved in VEGF-induced vascular hyperpermeability (for review, see Shibuya and Claesson-Welsh, 2006), though opinions differ regarding which plays a more critical role. Recent data suggest that VEGFR-2 may play a larger role than previously thought; VEGF-C, which binds VEGFR-2 but not VEGFR-1, did increase vascular permeability

(Brkovic & Sirois, 2006). VEGF-E, which binds to VEGFR-2, but not VEGFR-1, induced only a small increase in vascular permeability (Kiba *et al.,* 2003). PlGF, which binds VEGFR-1, but not VEGFR-2, similarly induces only a small increase in vascular permeability. The failure of receptor-selective ligands to consistently induce vascular hyperpermeability to a degree comparable to VEGF suggests that both VEGFR-1 and VEGFR-2 participate in VEGF's permeablizing effects. However, the mechanism by which VEGFR-1 and VEGFR-2 contribute to the effect is not yet understood. Receptor heterodimerization or some interaction further downstream of the individual receptors' signaling pathways may be responsible (for review, see Shibuya and Claesson-Welsh, 2006).

VEGF's effects on vascular permeability are dependent upon nitric oxide and appear to be reliant specifically on endothelial nitric oxide synthase (eNOS) expression upregulated by Akt (for review, see Yu *et al.,* 2005 and Cebe-Suarez *et al.,* 2006). Though Spyridopoulos *et al.* (2002) showed that PKC activation is necessary for VEGF-induced angiogenesis, they demonstrated an increase in vascular permeability with inhibition of PKC, which they explain citing PKC's reduction of NOS activity (Bredt *et al*., 1992). Other investigators, however, have noted an increase in vascular permeability with PKC activation in different experimental models (Spyridopoulos *et al.*, 2002).

VEGF and Inflammation

VEGF has been shown to modulate inflammation in brain (Proescholdt *et al.*, 1999). It has also been shown to attract monocytes *in vitro* (Heil *et al.*, 2000), and it is thought that this effect may be mediated directly through VEGFR-1,

which is present on monocytes (Sawano *et al.,* 2001), but this has not been proven.

While VEGF may exert its pro-inflammatory effects directly via VEGFR-1 expressed on monocytes, it may contribute to inflammation more indirectly as well. VEGF induces monocyte chemoattractant protein-1 (MCP-1) in bovine retinal endothelial cells (Marumo *et al.*, 1999) and induces interleukin-8 (IL-8) expression in human brain microvascular endothelial cells *in vitro*, which, in turn, increased neutrophil migration (Lee *et al.,* 2002).

VEGF's potent pro-inflammatory effects can be seen well before VEGF-induced angiogenesis is visible *in vivo* and at doses lower than those necessary to induce angiogenesis (Croll *et al*. 2004b). The relationship between VEGF and inflammation is an interesting one, particularly given the potential of inflammation to lead to damage.

VEGF and Angiogenesis

VEGF is one of the prime regulators of angiogenesis, the sprouting of capillaries from pre-existing blood vessels. It is thought to accomplish this effect by inducing the proliferation of vascular endothelial cells, creating new fenestrations in the vessel, and causing endothelial cells to migrate, forming new capillary tubes (Yancopoulos *et al.*, 2000). These processes appear to occur primarily via VEGFR-2-mediated mechanisms, as has been suggested by studies such as that of Waltenberger *et al.* (1994), in which porcine aortic endothelial cells lacking VEGF receptors displayed chemotaxis and mitogenesis when given a plasmid coding for VEGFR-2, but not when given a plasmid coding for VEGFR-

1. However, monocytes/macrophages, the extravasation of which is induced by VEGF, themselves secrete a number of proangiogenic factors. It may be via this mechanism that VEGFR-1, which is expressed on monocytes/macrophages, contributes to the angiogenic process.

Cell Signaling in Endothelial Cell Proliferation

Endothelial cell proliferation, an important step in angiogenesis, occurs via the MAPK pathway (Yu & Sato, 1999) and is dependent upon PKC, as evident by the decrease in VEGF-induced DNA synthesis and angiogenesis following the blocking of PKC (Spyridopoulos *et al*., 2002). Tyr^{1175} of VEGFR-2, in particular, is involved in endothelial cell proliferation. Phosphorylation of this Tyr leads to activation of PLC- γ and DNA synthesis upon VEGF binding (Takahashi *et al.*, 2001).

Cell Signaling in Endothelial Cell Migration

PI3K activation is necessary for VEGF-induced endothelial cell migration, as demonstrated by experiments utilizing PI3K inhibiters (for review, see Matsumoto and Claesson-Welsh, 2001). Endothelial cell migration is also mediated in some cases by a signaling cascade beginning with phosphorylation of the Y951 site of VEGR-2's kinase insert, which allows binding and phosphorylation of T cell specific adapter (TSAd), which associates with Src, a cytoplasmic tyrosine kinase, thereby regulating the migration of endothelial cells to VEGF (Matsumoto *et al*., 2005).

VEGF's Non-vascular Effects

For a decade after it was discovered, VEGF was known and studied for its vascular effects in both normal and pathological states. The focus expanded when Yang and Cepko (1996) proposed that VEGF's importance may extend beyond blood vessels by demonstrating that VEGFR-2 is expressed on progenitor cells during retinal neurogenesis. A great deal of investigation into VEGF's non-vascular effects followed, spreading from the retina to other areas of the nervous system, and there is increasing evidence in support of a neurotrophic and mitogenic role of VEGF in the nervous system.

VEGF in Normal Neural Development

Following Yang and Cepko's (1996) discovery that VEGFR-2 is expressed on retinal progenitor cells, Yourey *et al.* (2000) showed that application of VEGF to developing retina resulted in an increased number of photoreceptors and amacrine cells. Meanwhile, Sondell *et al.* (1999) demonstrated *in vitro* that application of VEGF to dorsal root and superior cervical ganglia induces axonal outgrowth and preservation of neuronal and Schwann cells, with VEGFR-2 expression present on growth cones of regenerating axons as well as Schwann cells. Silverman *et al.* (1999) showed that neuritic outgrowth and astroglial proliferation are induced by VEGF administration in fetal ventral mesencephalic explants, and Bagnard *et al.*'s (2001) demonstration that VEGFR-1 and NP-1 are co-localized on developing neurites suggested the possibility that VEGF-induced neuritic outgrowth is a direct effect, rather than an effect secondary to VEGF's vascular effects. Rosenstein *et al.* (2003) demonstrated that VEGF-induced

neuritic outgrowth can indeed occur via a direct, non-vascular mechanism by first showing that VEGF stimulated neuritic outgrowth in primary cortical neuronal cultures. They then proceeded to block VEGFR-1 and VEGFR-2 individually in separate experiments. Blocking VEGFR-2 prevented nearly all VEGF-induced neuritic outgrowth, whereas blocking VEGFR-1 had no effect. Sondell *et al.* (2000), too, found that their VEGF-induced axonal outgrowth in dorsal root ganglia was dependent on VEGFR-2 activity.

VEGF's ability to bind to NP-1 seems to be an important part of VEGF's purported non-vascular role in development. NP-1 is primarily a receptor for the semaphorins, a family of molecules heavily involved in axonal guidance during embryonic development (for review, see Bagri *et al.*, 2002). In a series of experiments, Bagnard *et al.* (2001) explored the relationship among the actions of Semaphorin 3A (Sema3A), VEGF, NP-1, and VEGFR-1 using a line of neuroectodermal progenitor cells expressing VEGFR-1. Sema3A, which competes with VEGF for NP-1-binding, functions as a repellent guidance cue for progenitor cells and can also induce NP-1-mediated apoptosis with prolonged activity. When VEGF was added to the cell culture, however, the apoptotic and repellent effects of Sema3A were blocked. The authors were also able to prevent the cellular actions of Sema3A with anti-VEGFR-1 treatment of the cell cultures. Along with the fact that NP-1 acts as a VEGF co-receptor (Fuh *et al.,* 2000) and that VEGFR-1, in addition to NP-1, is expressed on developing neurons, the directly competitive and divergent neuronal effects of Sema3A and

VEGF in progenitor cell cultures provide more evidence for a non-vascular role of VEGF during development.

VEGF in the Normal Adult

VEGF and its signal-transducing receptors are almost completely absent in the healthy adult brain (Soker *et al.,* 1998), with VEGF expression normally detected only in the choroid plexus, area postrema, (Breier *et al.*, 1992) and cerebellar granule cells (Monacci *et al*., 1993). However, *in vitro* and *in vivo* studies have shown that there are circumstances under which VEGF can have non-vascular effects in the adult brain.

VEGF in Normal Neural Tissue

When applied to cultivated microglial cells, VEGF induced proliferation and chemotaxis (Forstreuter *et al.*, 2002). When VEGF was delivered to the brain via osmotic minipump or subdural gelatin sponge for three days, a proliferative effect on astrocytes and microglia was seen in addition to the expected angiogenic response (Krum *et al.,* 2002; Croll *et al.*, 2004b). Conversely, inhibition of endogenous VEGF by infusion of anti-VEGF via osmotic minipump decreased trauma-induced astroglial proliferation (Krum and Khaibullina, 2003).

VEGF has been implicated in neurogenesis as well. Jin *et al.* (2002) looked at the neuronal effects of VEGF both *in vitro*, using cerebral cortical cultures, and *in vivo,* by intracerebroventricular (icv) infusion of VEGF via osmotic minipump. In both models, VEGF induced neuronal proliferation, and proliferation of astroglia and endothelial cells was present as well following VEGF

infusion *in vivo*. Fabel *et al.* (2003) demonstrated the importance of endogenous VEGF in normal, exercise-induced neurogenesis in the adult rat hippocampus by blocking VEGF and thereby blocking running-induced neurogenesis.

Cellular Mechanisms of VEGF's Non-vascular Effects

VEGF likely exerts its non-vascular effects through a number of separate and interacting mechanisms. Some of the effects may, in fact, arise indirectly as a result of VEGF's vascular effects. Louissaint *et al.* (2002), for example, suggest that the new endothelial cells that are present following VEGF-induced angiogenesis produce BDNF, which induces neuronal migration and neurogenesis.

VEGF's pro-survival effects may be related to its ability to inhibit caspase-3 activity (Jin et al., 2001), thereby inhibiting apoptosis. VEGF's ability to block Sema3A-induced apoptosis in Bagnard *et al.*'s (2001) line of progenitor cells as described above also points in the direction of an anti-caspase-3 mechanism of neuroprotection.

Some of VEGF's neuroprotective effects appear to rely on activation of the phosphatidylinositol 3'-kinase (PI3K)/Akt and MAPK pathways. Aside from showing that VEGF-induced neuritic outgrowth was prevented by blocking VEGFR-2, Rosenstein *et al.* (2003) selectively blocked the PI3K/Akt and MAPK pathways individually. In both cases, VEGF-induced neuritic outgrowth was prevented; blocking the MAPK pathway completely abolished the outgrowth and blocking the PI3K/Akt pathway nearly completely abolished it. The PI3K/Akt pathway's importance in VEGF-induced neuroprotection was demonstrated *in*

vivo as well. Kilic *et al.* (2006) used a line of mutant mice that overexpressed VEGF in the brain to demonstrate that VEGF is neuroprotective following MCAo. When they blocked the PI3K/Akt pathway, infarct volume and neurological deficits matched those of the wildtype controls.

The PI3K/Akt pathway affects many downstream processes. Blocking VEGF-induced neuroprotection through this pathway, therefore, provides a limited amount of information regarding the mechanisms of VEGF's actions. Sun and Guo (2005) observed that along with upregulation of VEGF following ischemic and hypoxic events, there is increased expression and phosphorylation of Kv1.2, a voltage-gated potassium channel protein, both *in vivo* and *in vitro*. VEGF antisense resulted in less tyrosine phosphorylation of Kv1.2 and increased cell death. They also demonstrated that administration of VEGF further reduced cell death and increased phosphorylation of Kv1.2. In addition, the authors were able to block VEGF-enhanced phosphorylation of Kv1.2 by using a PI3K inhibitor, showing that VEGF-induced Kv1.2 phosphorylation is a product of the PI3K pathway.

VEGF-induced neuroprotection and phosphorylation of Kv1.2 may be related to previous findings by Xu *et al.* (2003) that VEGF inhibits the amplitude of outward delayed-rectifier potassium currents (I_K). This effect occurred only in cells expressing VEGF receptors, and this study was the first demonstration of potential electrophysiological effects of VEGF on neurons.

McCloskey *et al.* (2005) also looked at physiological mechanisms that may play a role in VEGF-induced neuroprotection. VEGF applied to hippocampal

slices reduced the peak amplitude of depolarization in CA1 pyramidal cells upon stimulation to the Shaffer collateral pathway. This effect did not appear to be GABA-mediated. VEGF also reduced the amplitude and frequency of spontaneous bursts in CA3 pyramidal cells in hippocampal slices taken from rats one to four months after status epilepticus, when chronic epileptiform activity had developed. These findings may help explain how VEGF protects neurons against glutamate-induced excitotoxicity and seizures, as demonstrated by Matsuzaki *et al.* (2001) and Croll *et al.* (2004a).

Which of VEGF's actions accounts for its protective effects likely depends on the source of damage in any given experimental model or clinical situation. The receptor signaling patterns that rescue neurons in one set of physiological circumstances may fail to do the same in another or may actually be harmful, as will be introduced in this chapter and further explored later in "Chapter Five: Discussion."

VEGF as a Protective Factor in Pathological States

VEGF has emerged as a factor in a number of pathological states. In many of these states, VEGF upregulation, whether as a natural response or induced by researchers, plays an important protective role and provides clues about VEGF's beneficial effects.

VEGF in Hypoxia and Ischemia

VEGF has been shown to be upregulated in response to hypoxia and ischemic injuries in the Central Nervous System (CNS) (for review, see Rosenstein and Krum, 2004; Kovacs *et al.* 1996; Lennmyr *et al.* 1998). In

normal, healthy tissue, hypoxia-inducible transcription factor 1-α (HIF-1 α) is degraded and kept from dimerizing with HIF-1β and binding to the VEGF gene's hypoxia-response element (HRE). In the hypoxic brain, the proteins that keep HIF-1 α at its low basal activity level are no longer able to do so, allowing dimerization with HIF-1β and binding to the VEGF gene's HRE to take place, thereby inducing VEGF transcription (for review, see Brockington *et al., 2004* and Storkebaum *et al.*, 2004).

Studies of VEGF upregulation in the ischemic brain provide evidence for its neurotrophic properties. Sun *et al.* (1995) first showed that VEGF is upregulated by cerebral ischemia. Since then, a number of studies have demonstrated this effect using a variety of models. Strong VEGF expression can be seen in the ischemic core within one to three hours after focal cerebral ischemia in rats. Expression surrounding the infarct can be seen starting six to twenty-four hours after the ischemic event and continues for as long as twenty-eight days. Intravenous administration of VEGF within forty-eight hours results in enhanced neovascularization of the area surrounding the infarct as well as improved neural recovery. Hayashi *et al.* (1998) demonstrated VEGF-induced protection in the brain as well by topically administering VEGF following transient middle cerebral artery occlusion (MCAo). Infarct volume, edema, and neuronal damage were significantly reduced.

Blocking, rather than administering, VEGF in the ischemic brain provides corroborating evidence in support of its neuroprotective properties. When VEGF upregulation is prevented using the antisense knockdown technique following

ischemic stroke, the results are a larger infarct volume and a higher number of damaged neurons (Yang *et al.* 2002). The neuronal effects may be secondary to VEGF's pro-angiogenic effects; however, VEGF receptor expression patterns following ischemia hint at a role for VEGF in repairing or protecting cells from further damage. VEGF receptors are located on neurons and glia in addition to endothelial cells, and their expression is upregulated in neurons as a result of ischemia (Croll & Wiegand, 2001). Following transient focal ischemia caused by (MCAo) in adult rats, VEGF mRNA can be detected (Lee *et al.,* 1999), and when VEGF is trapped or VEGFR-2 blocked in cortical neurons exposed to hypoxia *in vitro*, there is a decrease in neuronal survival (Ogunshola *et al.,* 2002).

Much of the groundbreaking work in the area of VEGF and neuroprotection was done in the spinal cord and peripheral nervous system. Mutant mice with a VEGF-deficiency are more susceptible to paralysis after spinal cord ischemia, and treatment of the mice with VEGF protected them from motoneuron death (Lambrechts *et al.*, 2003). Schratzberger *et al*. (2000) induced ischemia in the rabbit hind-limb and then restored motor and sensory function with intramuscular gene transfer of a plasmid encoding VEGF. Apart from the enhanced vascularization that the VEGF gene transfer induced, there was increased Schwann cell migration and improved survival. Meanwhile, Oosthuyse *et al.* (2001) created a line of mutant mice in which the VEGF gene was missing the hypoxia-response element ($VEGF^{\delta/\delta}$.) These mice showed motor neuron pathology and muscle atrophy. In addition, VEGFR-2 and NP-1 were expressed on the motor neurons and peripheral axons of wild-type controls

but not mutant mice in their study. In complementary *in vitro* experiments using primary cultures of motor neurons, VEGF increased neuronal survival following serum deprivation. This effect was prevented when the cultures were treated with anti-NP-1 and anti-VEGFR-2 antibodies. These findings support the suggestion that VEGF's neurotrophic effects are, at least in part, independent of its vascular effects.

ALS/Motor Neuron Disease

The work done in the area of VEGF and the spinal cord and peripheral nervous system led to the exploration of the idea that a dysregulation or dysfunction of the VEGF system may play a role in motor neuron disease. In particular, Oosthuyse *et al.*'s (2001) work, described above, suggested a link between VEGF and Amyotrophic Lateral Sclerosis (ALS), an adult-onset disease characterized by the degeneration of large motor neurons in the cortex, brainstem, and spinal cord, which eventually leads to muscle atrophy and paralysis and, ultimately, death. The motor neuron pathology and muscle atrophy that develop in adulthood in his $VEGF^{\delta/\delta}$ mutant mice resemble the pathology seen in other ALS animal models and in ALS patients (for review, see Storkebaum *et al.,* 2004).

While $VEGF^{\delta/\delta}$ and ALS pathology resemble each other in a number of ways, neither the mechanism of neurodegeneration in $VEGF^{\delta/\delta}$ mice nor ALS is perfectly understood. One possibility is that lack of VEGF-dependent neuroprotection is responsible in both cases. Evidence in support of this possibility comes from the study of a mutation of the Cu/Zn superoxide

dismutase (SOD1) gene, which has been linked to the 10% of ALS cases that are familial (Rosen *et al.*, 1993). SOD^{G93A} mice overexpress the ALS-associated mutant human Cu/Zn superoxide dismutase, and they develop progressive motor neuron degeneration like that seen in ALS patients and $VEGF^{\delta/\delta}$ mice (for review, see Carmeliet and Storkebaum, 2002). Intraperitoneal and icv injections of VEGF delay the progression of motor symptoms and increase survival in SOD1 mice (Zheng *et al.*, 2004; Storkebaum *el al*., 2005). Oosthuyse *et al.*'s finding (2001) that VEGR-2 and NP-1 are co-expressed on motor neurons, mentioned above, implicate a direct neuronal mechanism of VEGF-dependent motor function preservation. Furthermore, Brockington *et al.* (2006) showed that VEGF and VEGFR-2 expression are reduced in the anterior horn cells of ALS patients.

The resemblance between the pathology seen in $VEGF^{\delta/\delta}$ mice and ALS patients led researchers in the direction of exploring how chronic vascular insufficiency might play a role in ALS. Baseline neural blood flow is reduced by 50% in $VEGF^{\delta/\delta}$ mice, and PET scans of ALS patients' brains show decreased regional cerebral blood flow, even in patients not exhibiting cognitive impairment (Waldemar *et al.,* 1992). Additional evidence for the contribution to ALS pathology by vascular abnormalities comes from further research done with the SOD1 gene in mice. When expression of mutant SOD1 is restricted to only astrocytes or neurons, it is not sufficient to lead to the onset of motor neuron degeneration (Gong *et al.*, 2000; Pramatarova *et al.,* 2001). This finding suggests that vascular cells might be required to express mutant SOD1 in order for the degenerative effect to take place.

Another possibility is that a dysregulation or dysfunction of VEGF exists in ALS independent of some other abnormality that triggers the cascade leading to motor degeneration. An excess of glutamate has been found in the nervous systems of ALS patients, and Riluzole, the only drug approved for treatment of ALS's symptoms, has anti-excitotoxic properties. It is therefore believed that the motor neurodegeneration that takes place in ALS can be attributed to excitotoxicity (for review, see Heath and Shaw, 2002). VEGF is known to protect cultured hippocampal neurons from glutamate-induced excitotoxic death (Matsuzaki *et al.*, 2001; Svennson *et al.*, 2002). Perhaps it is via the same mechanism that VEGF conveys neuroprotection in SOD1 mice and perhaps it is the reduced levels of VEGF and VEGFR-2 in ALS that allow the motor system to succumb to the glutamatergic stress and excitotoxic death.

VEGF and Seizures

As mentioned above in regard to ALS, VEGF is known to reduce excitotoxic damage to hippocampal neurons *in vivo* (Matsuzaki *et al.*, 2001; Svennson *et al.*, 2000). Pretreatment of VEGF infused via osmotic minipump protects hippocampal neurons from death following pilocarpine-induced seizure (Croll *et al.*, 2004a), and VEGF expression increases following seizure (Newton *et al.,* 2003; Croll *et al.*, 2004a). It is therefore thought that VEGF upregulation following seizure is an endogenous mechanism of neuroprotection.

VEGF as a Potential Contributor to Pathology

A number of disease states have come to be associated with elevated levels of VEGF and its potentially deleterious effects, inflammation and edema.

One challenge has been in trying to determine for each of these disease states whether VEGF is elevated in response to a primary dysfunction and resulting pathology or if VEGF itself is contributing to the damage. In some cases, the answer seems to be the former, but there are other diseases for which the latter is clearly supported by clinical studies. Yet in even in the cases where increased VEGF appears to be an epiphenomenon or compensatory mechanism, there is reason to suspect that it may contribute to the problem more than it helps alleviate it.

VEGF and the Ischemic Brain

As mentioned earlier in regard to VEGF's protective role in the hypoxic or ischemic brain, VEGF is upregulated following hypoxic and ischemic events in the brain (Sun *et al.*, 1995; Lee *et al.,* 1999). In a number of studies, the presence of VEGF following such events was shown to be beneficial (Hayashi *et al.*, 1998; Zhang *et al.*, 2000; Sun & Guo, 2005). However, VEGF also has the potential to be harmful when increased in the ischemic brain. Because of its permeabilizing effects, there is a risk of cerebral edema formation, which would raise the intracranial pressure and lead to further damage. When mice received regular injections of a protein that sequesters murine VEGF, there was a reduction in volume of edematous brain tissue one day after exposure to transient cortical focal ischemia, and there were significantly smaller infarct sizes eight to twelve weeks later (van Bruggen *et al.*, 1999).

VEGF and Multiple Sclerosis

Multiple Sclerosis (MS) is known primarily as a demyelinating disease of the central nervous system. However, in addition to neuronal degeneration, vascular changes take place in MS. Most notably, a breakdown of the BBB precedes symptoms or other visible MRI signs of the disease's progression (Kermode *et al*., 1990). When serum levels of a number of vascular growth factors were tracked over relapsing and remitting phases of MS patients with spinal cord lesions, VEGF was the only one tracked that showed a sharp rise at the time of relapse (Su *et al.*, 2006). Proescholdt *et al*. (2002) found that VEGF is upregulated in MS plaques compared to healthy white matter. They also found that VEGF was upregulated in the spinal cords of rats during the onset of experimental allergic encephalomyelitis. When they infused VEGF in the brains of rats that had been immunized with myelin basic protein, there was an inflammatory response that was not present in immunized rats that received control infusions nor in control, non-immunized rats that received VEGF infusions.

VEGF and Alzheimer's Disease

Enhanced VEGF immunoreactivity has been observed in the brains of patients with Alzheimer's Disease (AD) compared with normal elderly controls, with increased levels of VEGF co-localized with amyloid plaques in cortex. Furthermore, VEGF has been shown to bind to Amyloid-ß (Aß), the peptide which accumulates to form amyloid plaques, with higher affinity than any other known binding partner of Aß *in vitro.* At the same time, it remains able to bind

with receptors on endothelial cells and exercise its mitogenic effects (Yang *et al.*, 2004). This elevated presence of VEGF in the AD brain may interact with Aß-induced effects in a number of ways, as explored below.

Immune Activation in AD

Immune activation has been observed in AD and a key question has been what precise role the immune system plays in the cell death associated with AD (for review see Eikelenboom & van Gool, 2004). The use of nonsteroidal anti-inflammatory drugs (NSAIDs) to prevent or delay the development of AD has shown promising results in both animal models of AD and post-mortem human studies. Correlative studies using arthritis patients who were treated with NSAIDs show an inverse relationship between the use of these drugs and development of AD. Post-mortem studies have shown reduced inflammation in the brains of AD patients who had been taking NSAIDs. It is believed that the NSAIDs' effectiveness in preventing AD is through their ability to inhibit cyclooxygenase (COX). NSAIDs may work through other pathways as well to activate a class of hormones that inhibit the transcription of pro-inflammatory genes (for review, see Tuppo & Arias, 2005). In a transgenic mouse model of AD, a diet including ibuprofen for 6 months decreased inflammation, amyloid deposition, and dystrophic neurite formation (Lim *et al.*, 2000).

Microglia play a central role in the response to Aß in the AD brain. Microglia demonstrate chemotaxis and activation in response to Aß deposits, leading to increased secretion of pro-inflammatory cytokines such as interleukin-1ß (IL-1 ß) and interleukin-6 (IL-6) and the chemokines interleukin-8 (IL-8),

macrophage inflammatory protein-1α (MIP-1α) and monocyte chemo-attractant protein-1 (MCP-1). Aß also causes peripherally circulating macrophages to cross the blood brain barrier, leading to an even greater inflammatory burden in the brain. IL-1, in turn, promotes the synthesis of Aß precursor protein, further microglial and astrocyte activation, and continued expression of IL-1. In addition, it enhances acetylcholinesterase activity, contributing to the acetylcholine deficiency observed in the AD brain. And, though Aß–production is primarily neuronal, microglia activated by pro-inflammatory stimuli may secrete Aß as well, contributing to an inflammatory and destructive cycle. In addition to their inflammatory and phagocytitic responses, activated microglia secrete reactive oxygen species that may lead to further damage of neurons via the free radical oxidative damage pathway (for review, see Tuppo & Arias, 2005).

Though microglia play a major role in the inflammatory response observed in AD, astrocytes are suspected to play a role as well. Astrocytes are seen clustering at the sites of Aß deposits in the brain and have been shown to secrete pro-inflammatory molecules. In addition to being able to cause cell damage via reactive oxygen species in a manner similar to that of microglia, astrocytes have been found to over-express the cytokine S100B, a protein associated with neuronal death as well as the neuronal expression of Aβ precursor protein. Astrocytes also produce neuron-damaging levels of nitric oxide (NO) in response to IL-1. Another protein that is elevated in the AD brain is intercellular adhesion molecule-1 (ICAM-1), a transmembrane glycoprotein involved in cell adhesion. ICAM-1 leads to astrocyte production of

proinflammatory cytokines such as IL-1α, IL-1β, and IL-6 (Lee *et al.*, 2000). Finally, chemokines that are released by astrocytes attract microglia, which in turn attract more circulating macrophages and secrete more pro-inflammatory proteins, perpetuating the neurodegenerative cycle surrounding Aß. Neurons themselves are also able to produce several cytokines such as IL-1 and may do so in AD as well (Tuppo & Arias, 2005).

A Potential Role of VEGF in the AD Degenerative Process

As described above, microglia activated by Aβ cause peripherally circulating macrophages to cross the BBB. VEGF, may contribute to the ability of peripherally circulating macrophages to reach the brain in response to activated microglia because it increases the permeability of the BBB (Dobrogowska *et al.*, 1998; Croll *et al.,* 2004a). In some of the more recent studies of its potential to be neuroprotective, VEGF has been shown to lead to glial hypertrophy (Ackerman *et al.*, 2003) and proliferation (Krum *et al.,* 2002; Croll *et al.,* 2004b). Under the pro-inflammatory conditions present in the AD brain, in which glial cells are activated and probably contribute to cell death in the manner described above, this product of VEGF's actions may contribute to the neurodegenerative cascade of events in AD.

As mentioned above, elevated levels of ICAM-1 are found in the AD brain. VEGF has been shown to increase ICAM-1 both *in vitro* (Kim *et al.*, 2001; Melder *et al.*, 1996) and *in vivo* (Croll *et al.*, 2004b; Miyamoto *et al.*, 2000; Proescholdt *et al.*, 1999). ICAM-1 appears to be necessary for migration of monocytes across the endothelium in response to VEGF (Heil *et al.*, 2000; Kasselman *et al.,* 2007).

While there is no evidence that the elevated level of VEGF observed in the AD brain is the catalyst that results in elevated ICAM-1 in the AD brain, it is conceivable that VEGF contributes to the increase in ICAM-1 and subsequent astrocyte production of IL-1α, IL-1β, and IL-6 which it induces. In addition, as mentioned above, inflammatory proteins such as those seen to be induced by VEGF have been shown to lead to elevated expression of amyloid precursor protein and production of Aß from astrocytes and neurons (for review, see Ringheim *et al.*, 2004).

Perhaps VEGF, a potentially neuroprotective protein that induces inflammation, is upregulated to help protect cells from the damage brought on by Aß and vascular compromise, only to ultimately contribute to the problem it was upregulated to help alleviate.

Understanding the Influence of Physiological Environment on VEGF's Effects

As the study of VEGF's role in both vascular and nonvascular effects continues, research reinforces the idea that VEGF's actions are varied and complex. Though the majority of research so far has suggested that VEGF is a good candidate for treatment or prevention of various pathological states, we can not ignore the cases in which VEGF may actually contribute to damage. The findings that VEGF may contribute to infarct size following ischemic events in the brain, that VEGF upregulation is associated with and may precede symptoms of MS, and that VEGF and its actions have been linked with AD demonstrate that whether VEGF is ultimately beneficial or harmful may depend on the precise

environment in which it is present. The research presented in this dissertation is aimed at describing and understanding one particular condition in which VEGF appears to be harmful.

CHAPTER TWO: SPECIFIC AIMS

Based on what is known about VEGF, we were interested in seeing how continuous expression of physiological levels of VEGF would affect the hippocampus. Our preliminary data showed that VEGF administered in the hippocampus via adenovirus can be damaging, suggesting that VEGF's effects may be even more complex than previously realized. We sought to learn more about this degenerative process in order to understand when VEGF may contribute to deleterious conditions rather than mediate beneficial effects.

Specific Aim 1: To determine the structural effects of long-term expression and secretion of VEGF via adenoviral vector on rat hippocampus

Specific Aim 2: To study the specificity of AdVEGF's degenerative effect

Specific Aim 3: To establish a time course of AdVEGF's effects on the hippocampus and lateral ventricles

Specific Aim 4: To determine the role of inflammation and edema in hippocampal degeneration and ventricular enlargement following AdVEGF administration

Specific Aim 5: To examine the role of VEGF receptor subtypes in the degenerative and edemic process following AdVEGF administration

Specific Aim Rationales

Specific Aim 1: Effects of Long-term Expression and Secretion of VEGF via Adenoviral Vector on Rat Hippocampus

Because of the mitogenic and protective properties of VEGF, we are interested in the effect of continuous expression of VEGF in the rat brain at low physiological levels in the range seen when VEGF is naturally upregulated. We chose to focus our study on the hippocampus because it is a well-defined structure with functions that are easy to test behaviorally, allowing us to study not only the neuroanatomical effects of VEGF, but functional effects as well. Adenovirus was our chosen method of administration. An adenovirus is a vector that delivers the genetic code of a protein to the nuclei of individual cells, causing them to express the protein. Injecting adenovirus coding for VEGF (AdVEGF) allows us to induce long-term expression of physiological levels of VEGF with a single microinjection.

Specific Aim 2: Specificity of AdVEGF's Degenerative Effect

Administration of AdVEGF results in the expression and secretion of VEGF by the cells themselves (in this case, most VEGF is expressed by astrocytes surrounding the neurons). In this specific aim we asked whether adenovirus must be combined with the effects of endogenously-secreted VEGF in order for hippocampal degeneration and lateral ventricular enlargement to occur, or if these effects will occur when control adenovirus is combined with

infused exogenous VEGF protein as well. If infusion of VEGF protein concurrent with injection and expression of control adenovirus causes hippocampal atrophy, we will have more information regarding the specificity of our observed degeneration.

Specific Aim 3: Time Course of AdVEGF's Effects on the Hippocampus and Lateral Ventricles

In pilot studies, when AdVEGF was allowed to express for seven days, it induced the normal angiogenic effects associated with VEGF and caused no visible damage to the hippocampus and no visible enlargement of the lateral ventricles. When brains were taken four weeks after adenoviral administration, however, dramatic hippocampal atrophy and ventricular enlargement were observed. In this specific aim, we sought to address the nature of these effects. Specifically, we attempted to establish a time course of the observed hippocampal degenerative effects and ventricular enlargement in order to determine at what point after adenovirus delivery the degeneration begins and at what rate it progresses.

There are a number of mechanisms of cell death, some of which are known to be characteristic of different pathological states. Knowing more about the type of cell death that occurs following administration of AdVEGF can provide clues about what factors are contributing to the degeneration observed in this phenomenon. Cleaved caspase-3 is one of the most common markers of apoptotic cell death. Therefore, as part of the time course experiment, we asked

whether the cell death occurred via caspase 3-dependent apoptosis by staining for cleaved caspase-3 during what our preliminary data led us to believe was the critical time period in the degenerative process.

Specific Aim 4: Role of Inflammation and Edema in Hippocampal Degeneration and Ventricular Enlargement Following AdVEGF Administration

Because some of VEGF's effects, including pathological angiogenesis, have been shown to depend on inflammation (Kasselman *et al.*, 2007), we asked whether inflammation plays a role in AdVEGF-induced hippocampal and ventricular effects. In addition to the strong monocytic immune-response VEGF induces, edema caused by increased vascular permeability is another early effect of VEGF's actions. In the brain, edema can be damaging, and we were therefore interested in investigating the role of edema in the hippocampal damage and ventricular enlargement following AdVEGF expression.

To investigate the roles of edema and inflammation in the AdVEGF-induced degenerative effect, we chose to inhibit these two processes in separate experiments using Angiopoietin-1 delivered via adenovirus and Dexamethasone, respectively. Angiopoietin-1 is a protein shown to inhibit vascular leak when co-administered with VEGF or other leak-inducing treatments (Thurston *et al.*, 2000), and Dexamethasone is a potent corticosteroid with anti-inflammatory actions which inhibits both inflammation and edema.

Specific Aim 5: The Role of VEGF Receptor Subtypes in the Degenerative and Ventricular Enlargement Phenomena Following AdVEGF Administration

As described in the Introduction, VEGF binds to multiple receptors, most notably the signal-transducing receptors flt-1 (VEGFR-1) and flk-1 (VEGFR-2), and the non-transducing receptors neuropilin-1 (NP-1) and neuropilin-2 (NP-2). However, other ligands may bind to one or both of these receptors. Placental Growth Factor (PlGF), another member of the VEGF family of proteins, is one such ligand. PlGF binds to VEGFR-1 and NP-1 but not VEGFR-2, and has been associated with a monocytic inflammatory response similar to that of VEGF (Pipp *et al.,* 2003). Determining whether or not AdPlGF causes degeneration to occur helps us determine whether AdVEGF-induced degeneration is mediated by VEGFR-1.

The question of receptor specificity was particularly interesting to us being that we suspected edema was involved in some or all of the effects observed following AdVEGF injection, and, as mentioned in the introduction, there are differing opinions regarding which of VEGF's receptors is more important in increasing vascular permeability.

CHAPTER THREE: METHODS

General Methods

1. Subjects and Surgery

Subjects: Adult female Sprague-Dawley rats (250-350g, Charles River Laboratories) were double-housed in a temperature-stabilized animal facility with food (Rat LabDiet 1500) and water available *ad libitum*. Animals were maintained on a 12-hour light/dark cycle (lights on 0700) and acclimated to their colony room for at least one week prior to any experiment.

Microinjections: Rats were sedated with chlorpromazine (3mg/kg) followed ten minutes later by anesthesia with Ketamine (120mg/kg). Incision sites were shaved, cleaned, and treated with topical povidone iodine. Animals were secured in a stereotaxic device (Kopf Instruments, Tujunga, CA) and a small burr hole was drilled through the skull using the following coordinates: -3.8mm posterior to bregma; -2.6mm lateral from bregma; incisor bar set at -3.5mm. Microinjections of adenovirus and adeno-associated virus ($1x10^8$ PFU; Regeneron Pharmaceuticals, in total volume of 1.6µl to 3.7µl) were administered using a Hamilton microsyringe lowered into the dorsal hippocampus (-4.0mm ventral from skull, angled at 0 degrees) at a rate of 0.2 microliters/minute. Differences in total volume of injections among experiments are due to variations in the concentration of different lots of adenovirus and adeno-associated virus. Wounds were sutured with polyamid nylon suture thread (CP Medical) and

treated with antibacterial ointment, and rats were kept under heat lamps until recovered.

Pump Implantation: For experiments requiring continuous infusion, we used Alzet mini-osmotic pumps (model 2002, .5 µl/hr) attached by a polyethylene catheter to cannulae (28 gauge, 4mm from pedestal, Plastics One). Immediately after adenoviral microinjection, the cannulae were lowered into the hippocampus through the same burr hole. The cannulae were secured in the skull using two anchor screws and dental acrylic. The osmotic pump was slid into the subcutaneous space in the nape of the neck. The skin over the scalp was pulled over the dental acrylic cap and the wound closed with sutures. Wounds were treated with antibacterial ointment, and rats were kept under heat lamps until they recovered. In experiments that required four weeks of continuous protein infusion, animals were re-anesthetized with isoflurane after two weeks of infusion. An inch-long incision was made allowing access to the catheter and pump, and the old pump was replaced with a new one containing fresh protein. The wound was sutured and antibacterial ointment was applied.

2. Sacrifices, Histology, and Staining

Tissue Collection: Animals were administered an overdose of a pentobarbital-based euthanasia solution (Euthasol, Del Marva Laboratories, Henry Schein, Long Island, New York) and perfusion-fixed as follows. Once deeply anesthetized, the chest cavity was opened, and a needle was inserted into the left ventricle of the heart and positioned in the ascending aorta. An incision was

made in the right atrium for drainage of blood, and heparinized isotonic (0.9%) saline was perfused through the heart to exsanguinate the animal. The animal were then perfused with 200ml of 4% paraformaldehyde solution in acetate buffer (pH 6.5) followed by 200ml of 4% paraformaldehyde solution in borate buffer (pH 9.5). All solutions were pumped through the animal using an electric variable speed mini-pump (VWR Scientific) at an approximate rate of 20ml/minute. Brains were removed and placed in a 30% buffered sucrose solution for 3-7 days.

Sectioning and Staining: After 3-7 days in the buffered sucrose solution, brains were sectioned coronally at a thickness of 40μm using a sliding microtome (American Optical Company, Buffalo, New York). Sections were stored in an ethylene glycol based cryoprotectant solution at -20 degrees Celsius until they were mounted and stained. Some sections were stained with cresyl violet and cover-slipped with ShurMount (Triangle Biomedical Sciences, Durham, North Carolina) for evaluation of brain volume. Other sections were immunostained with the following antibodies: anti-OX-1 to detect all leukocytes (1:10,000, Harlan Bioproducts, Cincinnati, OH); anti-active caspase-3 for apoptosis (0.2 μg/ml, Chemicon); and anti-IgG for vascular leak (1:1500, Vector Laboratories). For immunostaining, a biotinylated secondary antibody-avidin-peroxidase reaction (1:1500, Vectastain Elite kit, Vector Laboratories) was used, followed by peroxidase visualization with diaminobenzidine (DAB) and nickel (II) sulfate in a chromagen solution. Tissue from control and experimental conditions were stained concurrently to ensure equivalent immunostaining and optimal

comparisons. After staining, sections were counterstained with methylene blue or cresyl violet, mounted, and cover-slipped with ShurMount.

3. Data Collection and Analysis

Calculation of Hippocampal and Ventricular Volume: Every twelfth brain section was mounted and used for evaluation. The outlines of the hippocampus and lateral ventricle in each section were traced using the Neurolucida software system (MicroBrightField, Inc). The software derives the hippocampal and ventricular volume calculations from the areas calculated for each individual section, taking into account the sections that were not mounted and measured and calculating the volume using estimated areas for those sections.

Subjective Rating of Hippocampal Damage: Subjective rating of hippocampal damage was performed by a blind rater using a damage rating scale of 0 to 4 (Rudge *et al.*, 1998). On this scale, a score of 0 is given when no damage is observed. A score of 1 represents slight but definite damage, with a loss of less than 10% of neurons; 2 represents a loss of ~10%--40%; 3 represents a loss of ~40%--80%; and 4 represents more than 80% loss in neurons. We advanced our scale in increments of 0.5 for more precise assessment of damage.

Statistical Analysis: To determine if there are any statistical differences between groups, volumetric data was analyzed with a one-way or factorial ANOVA, depending on the design of the particular experiment. If statistical significance was attained, a Tukey LSD post-hoc test was performed to determine which groups were statistically different from each other. For analysis

of subjective ratings of hippocampal damage, data were analyzed with a Mann-Whitney Test in the case of two comparison groups and a Kruskal-Wallis in the case of more than two groups. All statistical analyses were conducted using SPSS software (version 11.5). On all graphs included, error bars represent the standard error of the mean (SEM).

Specific Aim Methods

Specific Aim One

Administration of AdVEGF: Effects of Long-term Expression and Secretion of VEGF via Adenoviral Vector on Rat Hippocampus

In this specific aim we sought to describe the effects of long-term expression and secretion of VEGF in the hippocampus when induced by administration of AdVEGF.

Experiment 1A:

Animals received a single unilateral microinjection of adenoviral VEGF (AdVEGF) ($1x10^8$ PFU in 1.6µl) in the hippocampus (see General Methods). An equal dose and volume of Adenoviral hFc (AdhFc) was used to control for adenoviral expression as well as the effects of adenovirally-induced increased protein secretion, because hFc has few effects on brain and is a protein which is secreted from the cell (Croll *et al.*, 2004). An experiment with two groups was conducted, as shown in the table below.

Group 1	**n=11**	**AdVEGF**
Group 2	**n=13**	**AdhFc**

Animals were sacrificed four weeks after microinjection and brains were sectioned (40µm), mounted, stained with cresyl violet, and coverslipped. Hippocampal volume was measured using the Neurolucida system (see General Methods).

Experiment 1B:

Experiment 1A was repeated using an adeno-associated viral vector as an additional control to further explore whether the neurodegenerative effects of AdVEGF are due to continuous secretion of VEGF or to some interaction between VEGF and its adenoviral vector. Adeno-associated viral vectors also induce cells to continuously express a protein, but their structure and mechanism of action are different from adenoviral vectors' (for review, see Osten *et al.*, 2007).

In addition to groups of animals receiving AdVEGF and AdhFc as they did in Experiment 1A, a group received VEGF via adeno-associated virus (AAVVEGF), as shown in the table below.

Group 1	**n=8**	**AdVEGF**
Group 2	**n=8**	**AAVVEGF**
Group 3	**n=4**	**AdhFc**

Animals were sacrificed four weeks after microinjection and brains were sectioned (40μm), mounted, stained with cresyl violet, and coverslipped. Hippocampal volume was measured.

Specific Aim Two

The specificity of AdVEGF's Degenerative Effect: Co-administration of Control Adenovirus and VEGF Protein

In this specific aim we sought to investigate the specificity of AdVEGF effects when administered in the rat hippocampus. Our aim was to determine if

the effects are due to the combination of adenovirus and VEGF protein, regardless of whether it is endogenous or exogenous, or the adenovirus must be combined with the effects of endogenously-secreted VEGF in order for hippocampal degeneration to occur.

Experiment 2:

We injected two groups of rats with AdhFc. Immediately following adenoviral injection, we implanted a cannula in the hippocampus and attached an osmotic minipump (see General Methods) filled with either VEGF (30 ng/day) or control protein; in one cohort, the control protein was BSA, and in the other, the control protein was freeze-thaw inactivated VEGF. The two groups are shown in the table below.

Groups:	Treatment	n
1	AdhFc + Control	12
2	AdhFc + VEGF protein	14

Animals were sacrificed and brains collected after 30 days. Brains were sectioned (40 μm), mounted, stained with cresyl violet, coverslipped, and hippocampal volume was measured using the Neurolucida system.

Specific Aim Three

Time Course of AdVEGF's Effects on the Hippocampus and Lateral Ventricles

In pilot studies, neither hippocampal damage nor ventricular enlargement occurred within one week of AdVEGF-administration. When animals were examined four weeks after AdVEGF-administration, however, there was

extensive hippocampal damage, as well as ventricular enlargement. In this specific aim we addressed the time and rate at which the observed damage takes place.

Experiment 3A:

Animals in the experimental condition received a single unilateral microinjection of AdVEGF ($1x10^8$ PFU in 1.6µl) into the hippocampus. Control animals received an equal dose and volume of AdhFc. The experiment's groups and sacrifice time points are shown in the table below.

	2 Weeks	3 Weeks	4 Weeks
AdVEGF	**n=5**	**n=4**	**n=4**
AdhFc	**n=6**	**n=3**	**n=5**

Animals were sacrificed at two, three, or four weeks after injection. Brains were sectioned (40 µm), mounted, stained with cresyl violet, and coverslipped. Hippocampus and ventricles were grossly examined and photographed to determine at which point in this broad time course the degeneration is taking place in order to know between which time points a tighter time-course experiment would be useful.

Experiment 3B:

In pilot studies, neither hippocampal damage nor ventricular enlargement was observed after one week of AdVEGF expression. After two weeks of expression, however, we observed nearly the full extent of hippocampal atrophy and ventricular enlargement that we observed four weeks after AdVEGF administration. In Experiment 3B, we sought to determine at what point between

weeks one and two post AdVEGF administration the damage occurs, and we evaluated levels of markers that might suggest mechanism of damage.

Experimental animals received a single unilateral microinjection of AdVEGF ($1x10^8$ PFU in 2.5µl) into the hippocampus. Control animals received an equal dose and volume of AdhFc. Animals were sacrificed and brains removed at seven, ten, or twelve days after injection. The subject groups and sacrifice time points are shown in the table below.

	Day 7	Day 10	Day 12
AdVEGF	**n=5**	**n=7**	**n=7**
AdhFc	**n=5**	**n=3**	**n=9**

Brains were sectioned at 40 µm, mounted, Nissl stained with cresyl violet, and coverslipped. Hippocampal and ventricular volume were measured using the Neurolucida system. Tissue was stained for cleaved caspase-3 (see General Methods), a marker of apoptosis, to learn whether AdVEGF-induced cell death is apoptotic in nature.

Specific Aim Four

The Role of Inflammation and Edema in Hippocampal Degeneration and Ventricular Enlargement Following AdVEGF Administration

Because inflammation and increased vascular permeability are hallmark effects of VEGF, we explored whether either of these effects played a role in the hippocampal degeneration and ventricular enlargement following AdVEGF administration and expression. We tried to answer these questions using

Angiopoietin-1, an angiogenic factor that inhibits vascular leak, and Dexamethasone, a corticosteroid anti-inflammatory agent.

Experiment 4A:

To evaluate the role of vascular permeability in the destructive process, we co-administered Angiopoietin-1 via adenovirus (AdAng-1) along with AdVEGF. AdhFc was co-administered with AdVEGF in the control condition. The four groups are shown in the table below.

	AdhFc	AdVEGF
AdAng-1	**n=6**	**n=9**
AdhFc	**n=7**	**n=7**

This experiment was performed in two cohorts. In the first, animals received $1x10^8$ PFU of AdVEGF and either AdAng-1 or AdhFc in a total volume of 3.7µl. In the second cohort, animals received $1x10^8$ PFU of AdVEGF and either AdAng-1 or AdhFc in a total volume of 3.2µl).

Animals were sacrificed four weeks after co-administration to evaluate the extent of the hippocampal atrophy. Brains were sectioned at 40µm, mounted in a 1:12 series, Nissl stained with cresyl violet, and coverslipped, and ventricular and hippocampal volume were measured.

Experiment 4B:

To determine if inhibition of inflammation prevents the hippocampal degeneration and ventricular enlargement observed in our model, Dexamethasone (24 µg/day) or its vehicle (Excipients, Exc) were infused into the hippocampus via cannula and osmotic pump (see General Methods) beginning

immediately following microinjection of AdVEGF ($1x10^8$ PFU in 1.6µl), as shown in the table below.

	AdhFc	AdVEGF
Exc Pump	**n=2**	**n=2**
Dex Pump	**n=2**	**n=4**

Animals were sacrificed and brains collected after 30 days to evaluate the extent of hippocampal atrophy. Brains were sectioned at 40 µm. Tissue was stained for OX-1, a marker for inflammation. Additional sections were mounted in a 1:12 series, Nissl stained with cresyl violet, coverslipped, and ventricular volume was measured using the Neurolucida system. Hippocampal damage was assessed using a subjective rating score of damage (Rudge *et al.*, 1998).

Specific Aim Five

The Role of VEGF Receptor Subtypes in the Degenerative and Edemic Process Following AdVEGF Administration

VEGF binds to VEGFR-1 and VEGFR-2, its two main signal-transducing receptors, as well as the neuropilins. VEGFR-1 and VEGFR-2 have different distribution patterns and are associated with different VEGF-induced effects. In this specific aim, we asked what role the individual VEGF receptors play in the hippocampal degeneration and ventricular enlargement observed following AdVEGF injection. We compared the effects of adPlGF to AdVEGF because PlGF has been seen to have some effects similar to VEGF's but binds only to VEGFR-1 and NP-1.

Experiment 5:

Animals received microinjections of AdPlGF, AdVEGF, or AdhFc, as shown in the table below.

Group 1	**n=12**	**AdPlGF**
Group 2	**n=7**	**AdVEGF**
Group 3	**n=4**	**AdhFc**

This experiment used two cohorts. In the first cohort, animals received an adenoviral load of $1x10^8$ PFU in a total volume of 1.6µl. In the second cohort, animals received an adenoviral load of $1x10^8$ PFU in a total volume of 3.2µl). This dose of AdPlGF had previously been shown to express well *in vivo* (personal communication, Ella Ioffe, Regeneron Pharmaceuticals).

Animals were sacrificed after four weeks after co-administration to evaluate the extent of the hippocampal atrophy. Brains were sectioned at 40 µm, mounted in a 1:12 series, Nissl stained with cresyl violet, coverslipped, and ventricular and hippocampal volume were measured.

CHAPTER FOUR: RESULTS

Specific Aim One

Effects of Long-term Expression and Secretion of VEGF via Adenoviral Vector on Rat Hippocampus

Pilot Data

We induced expression of VEGF in the rostral portion of the dorsal hippocampus by administering VEGF via adenoviral vector (AdVEGF) at a dose of 1 x 10^8 PFU in 1.6 µl total volume. Animals were sacrificed one, three, and seven days following microinjection of the adenovirus in order to examine the pattern of adenoviral expression and ascertain the adequacy of the dose.

Control animals received an equal volume of phosphate buffered saline (PBS). Enhanced green fluorescent protein (EGFP) co-expressed along with VEGF was used as a marker of adenoviral expression. On Day 3, only low, basal levels of fluorescence were detected in the brains of control animals. Brains of animals that received AdVEGF showed increased fluorescence, indicating that the adenovirus worked properly in hippocampal tissue and successfully induced protein expression (Figure 2). This fluorescence was observed primarily in cells of glial or endothelial morphology, though occasional cells of endothelial and neuronal morphology did also express EGFP.

We tested a range of AdVEGF doses that were selected based on their success at inducing vascular effects in tissue such as muscle (personal communication, Ella Ioffe, Regeneron Pharmaceuticals). Our chosen dose was

evaluated based on its ability to induce the expected VEGF-induced vascular effects that have been previously established to occur with VEGF administration in the brain (Rosenstein *et al.*, 2003, Proescholdt *et al.*, 1999, Croll *et al.,* 2004). Figure 3 depicts the effects of our chosen dose of AdVEGF at Days 1, 3, and 7. By Day 3, brains of animals that had received AdVEGF showed increased vascular diameter and mild monocytic extravasation in the area around the microinjection site. This was our desired effect, as it has previously been shown that it takes three days of VEGF protein administration to induce VEGF-induced gross vascular changes in this tissue (Croll *et al.*, 2004b). At Day 7, vascular density was elevated in AdVEGF-treated hippocampi.

Experiment 1A

To study the effects of long-term exposure of the hippocampus to VEGF, we administered 1 x 10^8 PFU AdVEGF in 1.6 µl into the rat hippocampus, as described in General Methods. Control animals received an equal dose of AdhFc as a control for secreted protein. After four weeks, the animals were sacrificed and the volume of their hippocampi was measured.

As illustrated in Figure 4, the hippocampi of animals that had received AdVEGF were significantly smaller than those of animals that had received AdhFc ($t(22) = 3.407$, $p < .003$).

Figure 5 shows tracings of brain sections, made using the Neurolucida software, from animals that received AdVEGF versus AdhFc and that were sacrificed four weeks post microinjection. The aim of our initial experiments was

to study the effects of continuous expression of AdVEGF in the hippocampus, but upon examining the brains of animals sacrificed four weeks post microinjection, we discovered that the lateral ventricles of animals that had received AdVEGF appeared dramatically larger than those of animals that had received the control adenovirus (Figure 6). In later experiments we further investigate and analyze the ventricular effects that are observed but not quantified here due to limited availability of sections containing lateral ventricles.

Experiment 1B

Because research has shown that continuous administration of VEGF can be beneficial (Matsuzaki *et al.*, 2001; Croll *et al.*, 2004a) and the results of Experiment 1A were therefore unexpected, we sought to further explore whether it was the continuous expression and secretion of VEGF in the hippocampus that directly caused the observed hippocampal atrophy or whether it was some interaction of the adenoviral vector and the VEGF protein it induces. To do so, we performed an experiment with an additional group of animals. Aside from animals receiving AdVEGF or the control adenovirus, AdhFc, we injected a group of animals with an adeno-associated viral vector coding for VEGF (AAVVEGF). Adeno-associated viral vectors, like adenoviral vectors, induce cells to express the protein for which they are carrying the genetic code. However, the precise mechanism by which they do so differs (Osten *et al.,* 2007). Animals were sacrificed four weeks later and brains taken for histological analysis.

As in the previous experiment, there was a significant effect of adenoviral treatment on hippocampus ($F(2, 17) = 7.696$, $p < .004$) (Figure 7). A Tukey LSD post hoc test shows that the hippocampi of animals that had been injected with AdVEGF were significantly smaller than that of control animals that had been injected with AdhFc ($p < .002$) but that the hippocampi of animals that had been injected with AAVVEGF were not significantly smaller than that of animals that had been injected with AdhFc ($p = .25$). In addition to the significant difference between the hippocampal volume of AdVEGF animals and AdhFc animals, the hippocampus of animals that received AdVEGF were also significantly smaller than that of animals that received AAVVEGF ($p < .024$). The normal, undamaged appearance of an AAVEGF-treated hippocampus can be seen in Figure 8C, along with an AdVEGF-treated hippocampus (8B) and a control AdhFc-treated hippocampus (8A).

Summary of Results: Specific Aim One

AdVEGF did not cause hippocampal damage within one week of administration, but when brains were removed and examined four weeks after AdVEGF administration, significant hippocampal degeneration had taken place. Damage was not observed in control animals that received an adenovirus coding for a control protein. The induction of VEGF expression with adeno-associated virus also did not lead to degeneration, showing that AdVEGF-degeneration was not caused by the adenovirus nor by continuous expression of VEGF by hippocampal cells.

Specific Aim Two

The specificity of AdVEGF's Degenerative Effect: Co-administration of Control Adenovirus and VEGF Protein

The results of our initial experiments demonstrated that neither four weeks of adenovirus expression nor four weeks of VEGF expression alone are enough to cause hippocampal degeneration. The effect we were dealing with appeared to be very specific, and we sought to further explore the specificity of the degeneration brought on by the combination of adenovirus and VEGF.

Experiment 2

Administration of AdVEGF results in the expression and secretion of VEGF by the cells themselves. To determine whether adenovirus must be combined with the effects of endogenously-secreted VEGF in order for hippocampal degeneration to occur, we continuously infused VEGF protein via a cannula attached to an osmotic pump implanted immediately following microinjection of AdhFc, our control adenovirus. Control animals were infused with either BSA or freeze-thaw inactivated VEGF protein. Pumps were replaced after two weeks, and animals were sacrificed after four weeks total of adenoviral expression and protein infusion.

Gross hippocampal atrophy was not observed for any group in this experiment. Therefore, we measured hippocampal cell loss using a previously-established and validated subjective rating scale of damage (Rudge *et al.*, 1998). On this scale, the minimum rating of 0 indicates no cell loss, and the maximum

rating of 4 indicates very severe damage. The scale advances in increments of .5 and allows for detection of less severe degeneration, insufficient to caused marked atrophy.

As shown in Figure 9, hippocampi of animals that had received VEGF protein infusion following AdhFc administration were not significantly more damaged than those of animals that had received control infusion, either in the CA3 region of the hippocampus ($p=.297$; Mann-Whitney), which is the site of our adenoviral microinjections, or in the CA1 region of the hippocampus ($p=.347$; Mann-Whitney), to which a small amount of the adenovirus is seen to diffuse.

Summary of Results: Specific Aim Two

When VEGF protein or control protein was continuously infused into the hippocampus for four weeks following administration of AdhFc (the control adenovirus), VEGF-treated hippocampi did not show significantly more damage than hippocampi that were treated with control protein. These results suggest that the degenerative effect observed with AdVEGF was specific to this route of administration. That is, the presence of VEGF and adenovirus together was not sufficient to cause the degenerative phenomenon observed in the hippocampus four weeks after AdVEGF administration.

Specific Aim Three

Time Course of AdVEGF's Effects on the Hippocampus and Lateral Ventricles

From our pilot study, we knew that the hippocampal and ventricular effects described in Experiment 1 did not occur within the first seven days following microinjection of AdVEGF into the hippocampus. At four weeks following microinjection, however, the effects were striking. The goal of Experiment 3A, therefore, was to determine when the hippocampal damage and ventricular enlargement observed four weeks following AdVEGF administration first become noticeable.

Experiment 3A

Animals received hippocampal microinjections of AdVEGF or AdhFc ($1x10^8$ PFU in 1.6µl). Sacrifices were performed at the two-week, three-week, and four-week time points.

Figure 10 depicts hippocampal sections of control animals and animals that received AdVEGF. Both controls and experimental animals looked grossly normal at the Day 3 and Day 7 time points in the pilot study described earlier. At the two-week time point, however, many of the AdVEGF-treated brains already showed dramatic hippocampal damage, while the AdhFc-treated brains appeared normal. AdhFc-treated brains continued to look grossly normal at the four-week time point.

Experiment 3B

Based on the results of our pilot studies and Experiment 3A, in which we found that no hippocampal damage was visible at Day 7 following AdVEGF administration but that some animals showed dramatic hippocampal atrophy by the two-week time point, we conducted an additional experiment, aimed at "catching" the hippocampal atrophy when it was taking place.

Animals in the experimental condition received a microinjection of AdVEGF ($1x10^8$ PFU in 2.5µl) into the hippocampus. Control animals received an equal dose and volume of AdhFc. Animals were sacrificed and brains taken at seven, ten, or twelve days after injection.

Within this tighter time-course of twelve days, the degenerative effect of AdVEGF on the hippocampus was already significant ($F(1, 28) = 7.052$, $p < .013$) (Figure 11). The significance lies between AdVEGF and AdhFc at the Day 10 time-point, with the Day 12 time-point showing a trend toward significance.

With this experiment, we began to more carefully study the lateral ventricles, being sure to section tissue far more rostrally in the brain, beginning before the lateral ventricles. There was a statistical trend toward larger lateral ventricles when comparing all AdVEGF-treated and all control animals ($F(1, 30) = 3.959$, $p=.056$), with the AdVEGF-treated animals' ventricles' volume increasing across the three sacrifice time points (Figure 12). By the Day 12 time point, the lateral ventricles of AdVEGF-treated animals were larger than those of control animals ($t(14) = 3.502$, $p < .009$) (Figure 13).

One of the questions we were interested in answering was what type of cell death was occurring and leading to the observed hippocampal atrophy. Though there are a number of distinct mechanisms via which cells in the brain may die, the two main umbrella categories of cell death are apoptotic and necrotic. Caspase-3 is a central mediator of apoptotic cell death and cleaved caspase-3 is therefore often used as a marker of apoptosis (for review, see Lavrik *et al.,* 2005). When we immunostained sections of brains taken seven, ten, or twelve days after AdVEGF microinjection, the time period during which the observed degenerative process is likely taking place, all sections were caspase-3 negative (data not shown). We did notice that cells appear swollen and morphologically resemble neurons undergoing necrosis (Figure 14).

Summary of Results: Specific Aim Three

Brains were removed and examined two, three, and four weeks after administration of AdVEGF. As early as two weeks after AdVEGF administration extensive hippocampal damage had occurred. We therefore conducted a tighter time course experiment with the aim of studying the progression of damage before the two-week time point. When brains were removed and examined at seven, ten, and twelve days after administration of AdVEGF, a significant decrease in hippocampal volume could already be observed at Day 10. By the Day 12 time point, the lateral ventricles of AdVEGF-treated animals were significantly enlarged.

Specific Aim Four

The Role of Inflammation and Edema in Hippocampal Degeneration and Ventricular Enlargement Following AdVEGF Administration

Because increased vascular permeability and inflammation are two hallmark processes that occur along with VEGF activity (see Introduction), we sought to inhibit these effects in order to see if doing so would prevent the observed degenerative effects that occur following microinjection of AdVEGF. Because both of these effects can, when present in large degrees, be damaging in the brain, and because adenoviral vectors are known to induce an immune response of their own (for review, see Osten *et al.*, 2007), we considered the possibility that it was one or both of these effects of VEGF combined with the inflammatory effect of the adenovirus that resulted in enlarged lateral ventricles and hippocampal atrophy.

Experiment 4A

To evaluate the role of increased vascular permeability—or edema as a result thereof—in the destructive process, we co-administered Angiopoietin-1, a protein shown to inhibit vascular leak (Thurston *et al.*, 2000), via adenovirus (AdAng-1, $1x10^8$ PFU) along with $1x10^8$ PFU AdVEGF. Previous research has validated the ability of adenovirally administered Ang-1 to potently inhibit vascular leak (Thurston *et al.*, 2000). In the control condition, $1x10^8$ PFU AdhFc was co-administered with $1x10^8$ PFU AdVEGF, in order to balance the viral load. Animals were sacrificed after four weeks.

Once again, AdVEGF administration resulted in significantly smaller hippocampal volume ($F(1, 25) = 17.642$, $p=.000$). As can be seen in Figure 15, there is a significant interaction effect ($p< .028$), such that there was a greater difference between AdVEGF-treated animals that were co-injected with AdAng-1 versus those that were not than there was between AdhFc animals that were treated with AdAng-1 and those that were not. Unexpectedly, as can be seen from the graph, AdAng-1 co-administration with AdVEGF resulted in smaller hippocampal volume than did co-administration with control adenovirus, rather than larger. AdAng-1-treated hippocampi appeared visibly less edematous than those treated with AdVEGF alone.

Analysis of lateral ventricular volume revealed that there was a statistical trend toward enlarged ventricles in the animals that received AdVEGF ($F(1, 26) = 3.055$, $p=.092$). There is not a statistically significant interaction effect between AdVEGF treatment and AdAng-1 treatment, but as can be seen on the graph in Figure 16, ventricles were less enlarged in those AdVEGF-treated animals that were co-administered AdAng-1. There was a mean ventricular volume of 18. 24 mm^3 for AdVEGF-treated animals and 9.33 mm^3 for AdVEGF-treated animals co-administered AdAng-1, suggesting some role for vascular leak in the hypertrophy.

Experiment 4B

To determine if inhibition of inflammation prevents the hippocampal degeneration and ventricular enlargement observed in our model,

Dexamethasone or its vehicle were infused into the hippocampus via cannula and osmotic pump, beginning immediately following microinjection of $1x10^8$ PFU AdVEGF. Animals were sacrificed after 4 weeks.

Figure 17 shows an AdVEGF-treated hippocampus infused with control excipients compared with an AdVEGF-treated hippocampus infused with Dexamethasone. These sections were immunostained with an anti-OX-1 antibody for leukocytes. The dark background staining in the excipients-infused brain reflects the higher degree of immunoglobulin leak in that brain due to compromise of the blood brain barrier, and more immune cells are visible in control brain than in the Dexamethasone-infused brain, as would be expected.

For this experiment, we once again measured hippocampal degeneration using the subjective rating scale of damage described earlier, due to the absence of overt hippocampal atrophy.

In our initial analysis of this experiment, which included two cohorts, Cohort 1 and Cohort 2, there was no significant effect of Dexamethasone on hippocampal damage (p=.859; Kruskal-Wallis). However, we noticed that on average, the AdhFc-treated animals showed an unexpected degree of damage Cohort 1. This degree of damage in the AdhFc-treated animals prevented a significant effect of adenoviral treatment in this experiment in either CA3 (p=.238; Mann-Whitney) or CA1 (p=.194; Mann-Whitney), with the mean damage ratings for AdhFc-treated and AdVEGF-treated animals being very close to each other. As mentioned above, there was no significant effect of Dexamethasone, but as

can be seen on the graph in Figure 18, whatever effect there was appears to lie within the AdhFc-treated animals only.

We found the implications of the results of our first analysis interesting, but we decided to do an additional analysis including only Cohort 2, the cohort in which the AdhFc-treated brains appeared normal, as in Cohort 1 it appeared that perhaps the sterility of the cannulae or surgical environment had been compromised.

As shown in Figure 19, in the CA3 region of the hippocampus, which is the main site of our adenoviral microinjections, AdVEGF-treated animals showed significantly more damage than AdhFc-treated animals, which showed no damage at all ($p < .016$; Mann-Whitney). In the CA1 region of the hippocampus as well, to which a small amount of the adenovirus diffuses, AdVEGF-treated animals showed more damage than AdhFc-treated animals, which showed no damage at all ($p < .048$; Mann-Whitney).

There was no significant difference between hippocampal damage in Dexamethasone-treated animals and controls, either in CA3 ($p = .099$; Kruskal-Wallis) or in CA1 ($p = .174$; Kruskal-Wallis) (Figure 20).

There was a trend toward larger ventricles in AdVEGF-treated animals when both cohorts were analyzed together ($F(1, 26) = 3.492$, $p = .074$). There was no significant effect of Dexamethasone infusion on ventricular volume ($F(1, 26) = .037$, $p = .849$) (Figure 21).

When Cohort 1, the cohort with damaged AdhFc-treated hippocampi was removed, the lateral ventricles were significantly larger in the AdVEGF-treated

animals than in AdhFc-treated controls (F (1, 6) = 6.701, p=.041) (Figure 22). Dexamethasone's effect on lateral ventricular volume was not significant (F (1, 6) = .069, p=.801).

Summary of Results: Specific Aim Four

AdAng-1, which inhibits vascular leak, did not prevent AdVEGF-induced hippocampal damage. The hippocampal volume of animals co-treated with AdVEGF and AdAng-1 was, in fact, smaller than that of animals that received AdVEGF without AdAng-1, probably because of decreased edema in those animals. AdAng-1 appeared to have a preventative effect on AdVEGF-induced ventricular enlargement, but these results were not significant. These results suggest that AdVEGF-induced hippocampal damage is not caused by edema but that edema may contribute to AdVEGF-induced ventricular enlargement.

Continuous infusion of the anti-inflammatory corticosteroid Dexamethasone following AdVEGF administration did not have a significant preventative effect on AdVEGF-induced hippocampal damage or ventricular enlargement, suggesting that inflammation is not the cause of AdVEGF-induced degenerative effects.

Specific Aim Five

The Role of VEGF Receptor Subtypes in the Degenerative and Edemic Process Following AdVEGF Administration

VEGF binds to multiple receptors, and each is associated with different patterns of cell signaling and different VEGF-induced effects (see Introduction). VEGF's main receptors are VEGFR-1 and VEGFR-2, though it also binds to the non-transducing neuropilin receptors. Other members of the VEGF family bind to these VEGF receptors as well, but there is no single VEGF family member that binds to exactly the same receptors as VEGF. PlGF is a member of the VEGF family of proteins that binds to VEGFR-1 and NP-1 but not VEGFR-2. Like VEGF, it has been associated with a monocytic inflammatory response (Pipp *et al.*, 2003). In the following experiment, our aim was to determine whether administration of AdPlGF would cause a degenerative effect like the one we have seen following AdVEGF administration.

Experiment 5

To learn about the receptor specificity of AdVEGF-induced degeneration, animals received microinjections of $1x10^8$ PFU AdPlGF, AdVEGF, or AdhFc. All animals were sacrificed after four weeks.

As shown in Figure 23, there was a significant difference among the three groups' hippocampal volumes ($F(2, 20) = 3.693$, $p < .043$). AdVEGF's degenerative effect in the hippocampus was just short of significant in this experiment when compared with the AdhFc controls (Tukey LSD, $p=.053$), but

hippocampi that received AdVEGF were significantly smaller than those of hippocampi that received AdPlGF (Tukey LSD, $p < .019$).

There was a significant effect of treatment on the lateral ventricles as well ($F(2, 21) = 3.744$, $p < .041$) (Figure 24). The lateral ventricles of AdVEGF-treated animals were significantly larger than those of AdPlGF-treated animals (Tukey LSD, $p = .015$). As with the hippocampal measurements in this experiment, there is not a significant difference between the lateral ventricles of AdVEGF-animals and AdhFc-treated animals (Tukey LSD, $p = .068$), but with means of 3.057 mm^3 for AdhFc and 10.065 mm^3 for AdVEGF, as can be seen in Figure 24, statistical significance appears to be prevented by high variability within the AdVEGF subject group.

Summary of Results: Specific Aim Five

AdPlGF, a VEGF protein family member which binds to VEGFR-1 and NP-1, but not VEGFR-2, did not cause hippocampal damage or ventricular enlargement in brains that were examined four weeks after adenovirus administration, suggesting that the degenerative effects observed four weeks after AdVEGF administration are not mediated by VEGFR-1 or NP-1.

CHAPTER FIVE: DISCUSSION

Based on the pre-existing body of evidence supporting a protective and beneficial role of VEGF in a variety of circumstances, our original goal in beginning this study was to examine the effects of VEGF continuously administered at low, physiological doses over a long period of time. We chose the hippocampus as our main site of interest for two reasons. The first is that the hippocampus is a well-defined neuroanatomical structure with much-studied and fairly well-understood behavioral and cognitive processes associated with it. A number of validated behavioral tests of hippocampally-mediated learning and memory exist, which would allow us to characterize not only the neuroanatomical effects of chronic VEGF administration, but cognitive effects as well. The second reason that we chose the hippocampus as our site of interest is that this structure and its surrounding cortex are prone to damage in common pathological states such as epilepsy, global ischemia, and certain types of dementia. Therefore, we were interested in studying the effect of VEGF administration to as a step toward evaluating the value of VEGF administration as a treatment or preventative measure.

Because we had hoped to administer low doses of VEGF over the course of periods as long as six months or a year, we were limited in our choices of administration methods. Our lab has a great deal of experience using osmotic minipumps attached to a cannula to deliver protein to the brain, but this approach was not realistic for our intended goal. The first problem with using pumps is the prohibitive cost and higher degree of trauma to the animals; we would have had

to surgically replace the pumps regularly, due not only to the limitation on the volume that can be held by a pump, but to the VEGF protein's limited stability of two weeks maximum once inside the pump. The second problem with using pumps is the risk of damage caused by cannulae, which are likely to become loose and unstable if left for many months.

Our choice of administration method, then, was VEGF gene delivery via adenoviral vector. This would allow us to attain continuous expression of VEGF by the hippocampal cells themselves with a single microinjection of the adenovirus. An added benefit of this approach is that sometimes the blood vessels formed following administration of exogenous VEGF protein are abnormal. With AdVEGF, we hoped to induce a more normal angiogenesis comparable to that which occurs when the cells themselves produce and secrete VEGF. In addition, the field of gene therapy is an exciting one, and if continuous VEGF administration were found to be beneficial, there was a good chance that research would, in any case, eventually arrive at the use of VEGF gene delivery.

Our pilot studies, in which we allowed AdVEGF to express for one, three, or seven days in the rostral hippocampus, indicated that a dose of $1x10^8$ PFU was sufficient to begin inducing the normal, expected vascular effects of VEGF by Day 3, as well the mild monocytic extravasation that is known to occur along with VEGF's vascular effects. At Day 7 vascular density in the treated hippocampi was elevated, and the tissue appeared grossly normal and healthy. These effects are similar to those observed after the same amount of time of low dose VEGF protein delivery in studies. We therefore chose this dose for our

planned experiments to study VEGF's long-term neuroanatomical and cognitive effects.

Before beginning our study of AdVEGF's effects when allowed to express for the long time periods we were originally interested in, we first allowed the adenovirus to express for four weeks in a few animals in order to confirm that the hippocampal tissue would continue to appear normal and that the adenovirus was continuing to work as expected. As a control, we used AdGFP, an adenovirus coding only for the green fluorescing protein that is co-expressed by all adenoviruses in our use. To our surprise, when we sacrificed the animals, a single look at a cross-section of the brain at the level of the hippocampus revealed that a great deal of atrophy had taken place in animals that had received AdVEGF, but not AdGFP. After finding this effect with additional animals, we chose to pursue this issue and attempt to characterize our observed effect and attempt to understand its cause.

Specific Aim One

Experiment 1A

In our initial pilot studies, control animals had received vehicle microinjections of PBS or AdGFP. The use of AdGFP allowed us to control for the activity of an adenoviral vector, but an important difference between GFP and VEGF is that GFP is not secreted by a cell once expressed, whereas VEGF is. Therefore, we could not be sure that the observed AdVEGF-induced degeneration was not due to forcing the cells to express and secrete protein

continuously for four weeks, or if it was due to something more specific about the AdVEGF. To answer this question, we chose as our new control an adenoviral vector coding for human Fc (AdhFc), a protein which is secreted when expressed by cells but is not demonstrated to have many effects in the hippocampus, except for a mild microglial activation (Croll *et al.,* 2004).

After four weeks of adenoviral expression, the hippocampi of animals that had received AdVEGF were smaller than those of animals that had received AdhFc, indicating that this degenerative effect was not due to our inducing continuous expression of a secreted protein. We could also conclude from this that it was not the adenoviral vector itself that causes the hippocampal atrophy.

Though the results would have seemed to suggest that the damage to the hippocampus we observed following four weeks of AdVEGF expression was due to four weeks of VEGF administration, past work in our lab indicated that the explanation was more complicated. When our lab had delivered VEGF protein to the hippocampus continuously for four weeks using osmotic minipumps, there was no degenerative effect at all (unpublished data). Animals that were receiving VEGF performed well on behavioral tests of learning and memory, and when their brains were examined following sacrifice, they appeared grossly normal (unpublished data). These findings, bolstered by mounting evidence for a protective and beneficial role of VEGF, particularly in the Central Nervous System (see Introduction), led us to pursue an alternate explanation of the observed hippocampal atrophy in our experiment. We sought to determine

whether it was something specific about VEGF delivered via adenovirus that results in degeneration.

Experiment 1B

In order to answer the question posed above, we introduced an additional control group that received VEGF administered via adeno-associated virus rather than adenovirus. Adeno-associated viruses are structurally different from adenoviruses, but they accomplish the same goal of delivering a gene to targeted tissue and causing the cells to express and secrete a particular protein.

Four weeks following microinjection, the hippocampi of AdVEGF-treated animals were significantly smaller than the hippocampi of AdhFc-treated animals, as expected, and they were also significantly smaller than the hippocampi of AAVVEGF-treated animals. Because the onset of adeno-associated virus expression takes place more slowly than does the onset of adenovirus, peaking at around three weeks, we waited until eight weeks after microinjection before sacrificing a sub-group of AAVVEGF animals. The hippocampi of these animals did not exhibit any more damage than the four-week AAVVEGF animals' (Figure 25), and there was no apparent difference between the brains of control animals that had received AdhFc and those that had received AAVVEGF. These results suggest that the degenerative effect observed following four weeks of AdVEGF expression is, indeed, due to some interaction between an adenoviral vector and the VEGF it encodes.

Specific Aim Two

In our model, VEGF is expressed and secreted by the cells themselves. We wondered if the effects of an adenoviral vector must be combined with endogenously secreted VEGF in order for degeneration to occur or if the interaction between adenovirus and VEGF that results in degeneration would occur when the adenovirus is brought together with exogenous, infused VEGF protein.

Experiment 2

All animals in this experiment received microinjections of AdhFc followed immediately by implantation of a cannula attached to an osmotic minipump. The osmotic minipumps infused either VEGF protein or control protein. Adenoviral expression and protein infusion were allowed to continue for four weeks, with pump replacements being performed at the two-week time-point in order to ensure that active VEGF was being infused for all four weeks in the experimental animals.

In analyzing the results of this experiment, we measured hippocampal degeneration using a previously validated subjective rating scale of damage. The reason for this is that in our experiments in which osmotic minipumps were implanted following adenoviral microinjection, we did not see the same degree of AdVEGF-induced damage as in the non-pump experiments. We believe that this is because the infusion from the pump diffuses the adenovirus farther from the infusion site, resulting in a lower concentration of viral activity in the immediate site of adenoviral microinjection. However, when we administer AdVEGF

followed by infusion of fluid from a pump, while the degree of damage is not as great as when AdVEGF is allowed to express without the interference of infusion from a pump, the hippocampal tissue does appear compromised, albeit not to the degree that it is reflected in volumetric measurements. By blindly rating each hippocampus, we are able to assess this damage.

After four weeks of AdhFc expression along with VEGF protein infusion, there was not a significant amount of hippocampal damage nor was there enlargement of the lateral ventricles, suggesting that the interaction between adenovirus and VEGF that leads to hippocampal damage and the observed ventricular enlargement is, at least in part, reliant on the way VEGF is compartmentalized.

Because of the VEGF receptors' differential distribution, the way VEGF is compartmentalized could result in very different patterns of receptor binding and, thus, a very different set of downstream effects. When exogenous VEGF is infused, the tissue is bathed in the protein, and the pattern of receptor binding and subsequent downstream processes are, in all probability, different from that which occurs with endogenous VEGF upregulation. When VEGF protein is infused, a large amount is probably bound to the brain vasculature's dense distribution of high affinity VEGF receptors. It would bind to the vasculature's VEGFR-1 and VEGFR-2, which are both expressed by vascular endothelial cells. Less VEGF would therefore be available to bind to the brain's neurons, which have been seen expressing modest amounts of NP-1 and VEGFR-2 (only under conditions of trauma), and glia, which have been purported by different

researches to express both VEGFR-1 and VEGFR-2 (Krum & Rosenstein, 1998; Lennmyr, 1998; Forstreuter *et al.,* 2002) though also in low amounts. Neuronal binding would be limited not only by the decreased amount of circulating VEGF following passing of the infused protein into the vascular sink, but by the relatively low binding affinity of VEGFR-2. VEGF that is left to bind to non-vascular cells is most likely to bind the high-affinity VEGFR-1, which is located on infiltrating monocytes and possibly on microglia and astrocytes.

When VEGF encoded by adenovirus is expressed and secreted by the cells themselves, on the other hand, it is released on a very local level, directly onto the VEGF receptors on nearby cells. While some protein may be reaching blood vessels, it will be far less than in the case of infused exogenous protein. Without the abundance of vascularly-located VEGF receptors to compete for binding, VEGF will have a chance to bind to the VEGFR-2 and NP receptors that are likely being expressed by neurons in response to the presence of an adenovirus and perhaps to the microinjection itself. More VEGF will be available to bind to the neighboring glia as well. In this way, VEGF administered and compartmentalized differently would have different results in the brain and these different patterns of binding and activity may account for their different effects when combined with an adenoviral vector.

Specific Aim Three

The hippocampal atrophy observed following four weeks of AdVEGF-expression seemed interesting to us in that while it had a delayed onset following microinjection, the degenerative process itself appeared to occur rapidly in a

fairly short period of time. While brains in our pilot study appeared normal and healthy at seven days following microinjection, the treated hippocampi in some of our animals were completely gone by four weeks. We sought to learn more about the nature of AdVEGF-induced hippocampal degeneration. Specifically, we wanted to get a better idea of the time-frame of the degeneration as well as to investigate the type of cell death that is occurring.

Experiment 3A

We performed an initial time-course study in which animals were sacrificed two, three, or four weeks following microinjection of AdVEGF or, as our control, AdhFc. Even at the two-week time-point, some of the AdVEGF-treated animals showed dramatic hippocampal damage greater than what we would have expected given how healthy they appeared seven days after AdVEGF microinjection in our pilot studies. These results indicate that the visible degeneration occurs even more rapidly than we had originally suspected.

Experiment 3B

Based on the results of the previous experiment, in which we demonstrated that a great deal of the AdVEGF-induced hippocampal degeneration takes place between seven days and two weeks following microinjection, we performed a second time-course study, this time with a tighter series of time-points aimed at acquiring tissue from brains that are at the height of whatever cellular process it is that results in hippocampal atrophy.

Volumetric Results: In our tighter time-course, we sacrificed animals at seven, ten, and twelve days after microinjection of either AdVEGF or AdhFc.

Though the hippocampal damage was not as extreme as in previous experiments, in which AdVEGF had been allowed to express for longer, the hippocampi of AdVEGF-treated animals were already significantly smaller than the hippocampi of AdhFc-treated animals.

The lateral ventricles of AdVEGF-treated rats increased across the three sacrifice time-points and were significantly larger than the lateral ventricles of control animals by the Day 12 time-point.

Immunohistochemical Results: Cell death mechanisms are traditionally categorized as apoptotic or necrotic, though recent work has shown that mechanisms of cell death can not always be so neatly parceled (Lockshin *et al.,* 2000). However, apoptosis and necrosis remain as two categories that are useful starting points with which to begin with when exploring the cause of cell death. Most instances of apoptosis are mediated by the caspase family of proteases, and cleaved caspase-3 is the most common marker of apoptosis. We immunostained all the tissue from the Day 7, Day 10, and Day 12 time-points for cleaved caspase-3. As a positive control, we used tissue from another study in our lab in which seizures had been induced in rats and cells are known to have undergone apoptosis. All AdhFc and AdVEGF sections stained negative, suggesting that AdVEGF-induced degeneration is not occurring through one of the many caspase-3-dependent mechanisms of apoptotic cell death.

It is important to once again note that a negative cleaved caspase-3 immunostain is not definitive evidence that the observed cell death is necrotic rather than apoptotic in nature. However, because most instances of cell death

in which cleaved caspase-3 is not present are, in fact, necrotic, this seems to be the more likely interpretation in this case as well. In addition, many cells around the infusion site in AdVEGF-treated animals appear swollen (Figure 14), as commonly occurs preceding necrotic cell death.

There are a number of causes and mechanisms of necrotic cell death. Based upon what we know about VEGF and adenoviral vectors, immune-mediated or edema-mediated necrosis was an appealing explanation of AdVEGF-induced hippocampal degeneration. As described in the Introduction and shown earlier in the results of our pilot studies, VEGF is known to induce vascular leak and edema as well as an immune response, specifically, inflammation characterized by monocytic extravasation. At the same time, adenoviral vectors, while very useful for gene delivery in many cases, are known to trigger an acute immune reaction. Adeno-associated viral vectors, on the other hand, do not trigger the same degree of acute immune reaction seen with adenoviral vectors. Our AAVVEGF-treated animals in Experiment 1A did not show hippocampal damage. We wondered, therefore, if the AdVEGF-induced degeneration could be explained by the combined edematous and immune responses of VEGF and the adenoviral vector. While neither is known on its own to cause the kind of damage we have shown, perhaps combining the two results in a destructive inflammatory event.

Specific Aim Four

We hypothesized that one of VEGF's two potentially harmful effects, edema, may be creating an environment that makes the brain more susceptible

to harm caused by the other, inflammation. Under normal circumstances, the blood brain barrier helps shield the brain from peripherally circulating immune cells. VEGF has been shown to increase the permeability of blood vessels even within the brain, causing a breakdown of the blood brain barrier (Dobrogowska *et al.,* 1998). In a state of already-elevated immune activation, this could allow even more immune cells to enter the brain. In this way, perhaps VEGF's edematous effects are contributing to AdVEGF-induced degeneration.

Experiment 4A

To evaluate the roll of increased vascular permeability as a contributing factor to the AdVEGF-induced destructive process, we co-administered AdAng-1 with AdVEGF, as AdAng-1 inhibits vascular leak but does not interfere with VEGF's other effects (Thurston *et al*, 2000).

Our analysis of hippocampal volume following four weeks of adenovirus expression revealed that co-administration with AdAng-1 did not prevent AdVEGF-induced hippocampal atrophy. In fact, animals that were co-administered AdAng-1 and AdVEGF had smaller hippocampi than animals that had been treated with AdVEGF alone. Our hypothesis as to why this may be is that intercellular fluid could cause the structure to expand, inflating the volumetric measurements. Bringing down vascular leak would bring down the hippocampal volume. Indeed, we have noticed that AdVEGF-treated hippocampal tissue that is still remaining after four weeks of adenoviral expression often appear swollen and less densely packed with cells.

While AdAng-1 does not appear to be sufficient for preventing AdVEGF-induced hippocampal degeneration, it does seem to prevent the accompanying lateral ventricular enlargement described and demonstrated earlier. Though the lateral ventricles of AdVEGF-treated animals that were co-treated with AdAng-1 were not statistically significantly smaller than the lateral ventricles of AdVEGF-treated animals that were co-treated with control adenovirus, the lack of statistical significance is likely due to extremely high variability, particularly within the AdVEGF subject groups.

Experiment 4B

To determine if inhibition of inflammation would prevent degeneration from occurring following administration of AdVEGF, we continuously infused either Dexamethasone or its excipients as a control into the hippocampus for the entire four weeks of AdVEGF expression, via an osmotic minipump that was implanted immediately following microinjection of the adenovirus.

In analyzing the results of this experiment, we once again measured hippocampal degeneration using the subjective rating scale of damage. One of this experiment's cohorts appeared abnormal in that the AdhFc-treated animals appeared more damaged than AdhFc-treated brains typically are. Because there was hippocampal damage across both adenovirus treatment groups, there was no significant effect of AdVEGF on hippocampal damage in this cohort. However, though it was not statistically significant, it appeared that there was an effect of Dexamethasone on the damage in AdhFc-treated animals but not in AdVEGF-treated animals. This suggests that the nature of the hippocampal

damage differs between the AdhFc and AdVEGF-treated hippocampi in this cohort, and that while the damage in the AdhFc-treated brains was, in fact, probably due to the sort of inflammation and edema one would get with a contaminated surgical environment, the damage observed in the AdVEGF-treated brains was of a different nature, as it did not appear to be affected by Dexamethasone, the way traditional inflammation and edema would be.

We repeated the analysis with just the cohort that included normal AdhFc subjects. AdVEGF-treated animals showed significantly more damage to CA3 and, to a lesser degree, CA1, than AdhFc-treated animals. There was no apparent difference between AdVEGF-treated brains that had been infused with Dexamethasone versus control excipients. Thus, our results suggest that inflammation is not, as we had hypothesized, responsible for the hippocampal damage that occurs following administration of AdVEGF.

Our present findings lead us back to attempting to explain the results of Experiment 1B, in which we compared the effects of AAVVEGF to AdVEGF. One of the major differences between adenoviral vectors and adeno-associated viral vectors is the acute immune response that the former, but not the latter, induces. We demonstrated that AAVVEGF does not cause hippocampal degeneration. Upon seeing those results, we believed at first that they supported our initial suspicion that it was the combined inflammatory effects of adenovirus and VEGF, possibly paired with VEGF-induced vascular leak, that resulted in hippocampal degeneration. We must consider, now, other differences between

AdVEGF and AAVVEGF that may account for why, of the two, only AdVEGF's activity would cause degenerative effects.

As mentioned earlier, the onset and peak of AAVVEGF's expression is later than AdVEGF's. We allowed the AAVVEGF to express for eight weeks in some of the subjects so that there would be peak levels of expression for approximately the same amount of time as in AdVEGF animals. However, this accounts only for the amount of time during which expression is taking place, and not the speed of expression onset. With AdVEGF, peak levels of VEGF are reached more quickly, perhaps overwhelming the population of VEGF receptors. If this is the case, the sudden upregulation of VEGF in the hippocampus may cause an extreme downregulation of VEGF receptor number or activity in response. It may be the initial bombarding of the receptors or the subsequent receptor downregulation that operates as the catalyst and results in damage. If VEGF receptors are, indeed, suddenly downregulated, there may be an insufficient number left to carry out the necessary protective effects of either the adenovirally-induced VEGF or VEGF that is being naturally upregulated in response to the normal trauma associated with a microinjection and the recovery thereof. With the ongoing activity of the adenovirus and the VEGF system in a state of imbalance, the hippocampus may be unable to protect itself. Even if protected by Dexamethasone from the full strength of an inflammatory attack, without the trophic support provided by VEGF, the hippocampus may still be compromised.

Specific Aim Five

As described earlier (see Introduction), VEGF's receptors are differentially distributed and associated to different degrees with its various hallmark effects. VEGFR-1 and VEGFR-2 are VEGF's two main signal transducing receptors, with NP-1 and NP-2 functioning mainly as co-receptors. Because of VEGFR-1's expression on monocytes, it has been proposed that VEGF-induced monocytic inflammation is mediated primarily through this receptor, though this has not been directly demonstrated. VEGF's protective and neurotrophic effects, on the other hand, have been attributed to VEGFR-2. We wondered if we could isolate the receptor primarily responsible for the AdVEGF-induced degenerative effects. We attempted to do so by administering PlGF via adenovirus, as PlGF is a member of the VEGF family of proteins that binds only to VEGFR-1 and NP-1.

Experiment 5

After four weeks of adenoviral expression, the hippocampi of AdPlGF-treated animals remained significantly larger than the atrophied hippocampi of AdVEGF-treated animals. Furthermore, AdPlGF-expression did not result in enlargement of the lateral ventricles. It should be noted that PlGF is not the perfect control for VEGF when attempting to isolate VEGFR-1's and VEGFR-2's contributions to VEGF's effects. Though PlGF, like VEGF, binds VEGFR-1, its precise downstream effects upon doing so have been seen to differ from the effects observed following VEGF binding to VEGFR-1. When administered *in vitro*, PlGF's effects on VEGFR-2 signaling differ from those of $VEGF^{Flt1}$, a VEGF variant engineered to bind specifically to VEGFR-1 (Autiero, *et al.,* 2003).

However, Autiero *et al.*'s experimental conditions were very specific and do not provide a great deal of information about how PlGF's VEGFR-1-mediated downstream signaling effects differ from VEGF's *in vivo* in a normal animal. The use of PlGF was the most readily-available and least problematic tool at our disposal to approach our question about receptor specificity in AdVEGF-induced degeneration, and the information provided is useful.

AdPlGF's failure to induce hippocampal atrophy suggests that the damage we observe following administration of AdVEGF is due, at least in part, to some role of VEGFR-2. These results do agree with our finding that inflammation is not the primary cause of AdVEGF-induced hippocampal damage. Because VEGF-induced inflammation is believed to be mediated by VEGFR-1, the VEGF receptor to which PlGF binds, the lack of hippocampal degeneration following AdPlGF microinjection supports our previous findings that AdVEGF-induced hippocampal degeneration is not inflammation-dependent.

Yet, at the same time, the idea that a ligand of VEGFR-2, but not VEGFR-1, would lead to degeneration was surprising to us, keeping in mind the large body of evidence in support of VEGFR-2's role in neuroprotection and neurogenesis. Consideration of this unexpected finding leads us to once again ask what difference exists between adenoviral vectors and adeno-associated viral vectors such that AAVVEGF, which also induces the expression of VEGFR-2-binding VEGF, does not lead to degeneration. As before, we arrive at the speed at which peak levels of VEGF expression are reached. If, as we speculate may be the case, a sudden surge in VEGF expression leads to an extreme

downregulation of VEGFR-2 receptors or dysregulation of VEGFR-2 signaling, the hippocampus could be deprived of the protective effects of VEGF. Without the benefit of VEGF, the trauma of a microinjection, ordinarily not a very damaging procedure, may set off a degenerative process from which the hippocampus is no longer equipped to protect itself.

A Proposed Model

To summarize our findings, the AdVEGF-induced degeneration we have observed was not induced by adenoviral vectors themselves, nor was it induced by adeno-associated virus coding for VEGF. Yet while it is clear that the effect was a result of adenovirus and VEGF together, it did not occur when the two were given separately by injecting control adenovirus and infusing exogenous VEGF protein. Co-treatment with AdAng-1 or Dexamethasone did not prevent damage from occurring following microinjection of AdVEGF but did appear to prevent the full extent of AdVEGF-induced ventricular enlargement, suggesting that neither vascular leak nor inflammation is solely responsible for AdVEGF-induced hippocampal degeneration but that one or both contribute to the ventricular effect. Finally, administration of AdPlGF, which induces expression of PlGF, a ligand of VEGFR-1 and NP, did not result in degeneration. This finding rules out VEGFR-1 as the VEGF receptor critical in mediating the degenerative effects of AdVEGF.

One thing we know for certain about AdVEGF versus AAVVEGF is that the onset of AdVEGF-induced protein expression is earlier than AAVVEGF's, and

peak expression rates are reached more rapidly. Though we allowed AAVVEGF to express for longer so as to ensure that the same level of VEGF expression and secretion was attained as in AdVEGF-treated animals, this did not control for the difference in onset and rate of expression. Because this is where a salient difference between AdVEGF and AAVVEGF lies, we turn our attention to this factor and draw together all our earlier points of speculation in attempting to construct a theoretical model that explains AdVEGF-induced degeneration and takes into account the results of all our experiments.

Following microinjection of AdVEGF, there is a rapid increase in VEGF in the hippocampus. The adenovirus-induced VEGF is expressed primarily by glial cells and, to a lesser extent, neurons, and released on a local level. Most binding likely takes place on receptors of neighboring cells. In the case of surrounding microglia, the receptor bound would be VEGFR-1; with neurons, the receptors bound would be VEGFR-2 and NP; and with astrocytes, either or both VEGFR-1 and VEGFR-2 would be present and bound. As proposed earlier, this burst in VEGF activity may result in downregulation of VEGF's receptors, either physically or by a dampening of VEGF signaling. We hesitate to speculate as to whether this suspected downregulation occurs with all of VEGF's receptors or just VEGFR-2. However, we do know that following injection of AdPlGF, there is a rapid increase in PlGF in the hippocampus, and the surrounding receptors which the PlGF would bind are VEGFR-1, located on microglia and possibly reactive astrocytes, and NP-1, located on neurons. Because we do not observe hippocampal damage or ventricular enlargement with AdPlGF, we can deduce

regarding VEGFR-1 that even if it is downregulated in response to a sudden burst in binding, no obviously pathological state ensues. Therefore, we conclude that it is probably a dysregulation of VEGFR-2, not VEGFR-1, that causes the observed pathology.

The lateral ventricular effect is a bit harder to interpret, as it is difficult to determine what the direct cause of the enlargement is. It may be due in part to VEGF's permeabilizing effects but worsened as an effect secondary to the hippocampal damage that takes place. Another possibility is that the ventricular enlargement is caused by leak resulting from damage or dysregulation of astrocytes. Astrocytes have been found to be a critical player in maintenance of the blood-brain barrier. Their relationship with blood vessels is comprised not only of molecular modulation (Willis *et al.,* 2004), but physical contact as well (Ramon y Cajal, pp. 230−252). The endfeet of perivascular astrocytes wrap around microvasculature in the brain, regulating the blood brain barrier through various channels and signaling pathways. The loss of astrocytes during AdVEGF-induced hippocampal atrophy may, in this way, trigger a large amount of vascular leak, resulting in ventricular enlargement.

The idea that a downregulation or dysregulation of VEGFR-2's functioning would result in hippocampal damage makes sense, as it is VEGFR-2 that is responsible for VEGF's trophic effects. Should VEGFR-2 be downregulated, especially following an invasive procedure, withdrawal of these trophic effects could potentially result in damage. The possibility that VEGFR-2 signaling is dysregulated rather than downregulated as a result of AdVEGF-administration is

an interesting alternative to consider. VEGF binding to VEGFR-2 leads to ERK 1/2 phosphorylation (for review, see Matsumoto and Claesson-Welsh, 2001). ERK 1/2 phosphorylation is ordinarily associated with pro-survival effects, but mounting evidence suggests that under some conditions, ERK 1/2 can actually be pro-death instead (Zhuang and Schnellmann, 2006; Pat Rockwell, personal communication). Both caspase-3-dependent and caspase-3-independent mechanisms of pro-death ERK 1/2 activity have been described, and while no examples of VEGF-mediated pro-death ERK 1/2 activity have yet been published, in recent unpublished work by a collaborator, it was found that under certain circumstances of aberrant VEGFR-2 signaling, ERK 1 and 2 become pro-death (Pat Rockwell, personal communication). This work is in its early stages, and the mechanism of the effect is not yet understood, but it is an intriguing finding to consider in light of our own discoveries.

Implications

In looking at the broader picture encompassing our work, the suggestion that an upregulation of VEGF can result in degeneration is a new and interesting idea. Conventional wisdom tells us that VEGF is especially beneficial following trauma to the brain, but we have found a situation where VEGF upregulation appears to be particularly *damaging* following a traumatic event. Our discovery has prompted us to investigate under what circumstances VEGF can be damaging and which receptor is predominantly responsible for the effect. Our results have pointed in the direction of VEGFR-2 as a critical factor, and the next step is to investigate what occurs with this receptor following AdVEGF

microinjection. In understanding this, we hope to know more about what VEGF can do in different physiological states and anticipate when its upregulation would be beneficial and when it would be a hazard.

Understanding VEGF's role in different physiological states will help us to determine not only when it would be beneficial to upregulate it as a treatment approach, but to understand how VEGF may be contributing to pathology in certain states in which VEGF has been shown to be upregulated such as stroke, Multiple Sclerosis, or Alzheimer's Disease (AD).

VEGF's role in ischemia is a matter of some controversy. VEGF has been found to be beneficial in the ischemic brain in a number of studies (Hayashi *et al.*, 1998; Zhang *et al.*, 2000; Sun & Guo, 2005), but, as mentioned in the Introduction, Van Bruggen *et al.* (1999) found that using a protein to sequester VEGF upregulated in response to a transient focal ischemic in the mouse brain resulted in a significantly smaller infarct size eight to twelve weeks later. One day following induction of the ischemia, significantly less edematous tissue was seen in the animals that received the VEGF-sequestering protein. It was hypothesized that inhibiting VEGF resulted in smaller infarct size because of this prevention of edema. This may, in fact, be the case, but we wonder if there may be additional damage caused by other activities of VEGF.

In the case of other pathological states such as Multiple Sclerosis and AD, there is no direct evidence that VEGF is contributing to the degeneration or that inhibiting VEGF would slow or prevent further degeneration. However, in both these states, VEGF's role in the damage is not understood. Indeed, it is not

known whether VEGF is upregulated in response to the damage or if it is a contributing cause. However, in the case of MS, there is reason to suspect that VEGF upregulation may be more than a response to the disease. A breakdown of the blood brain barrier precedes MS's visible progression, as assessed using MRI (Kermode *et al*., 1990). Since serum levels of VEGF rise at the time of MS relapses (Su *et al.*, 2006) and is upregulated in MS plaques (Proescholdt *et al*., 2002), it is a fair guess that the blood brain barrier's breakdown preceding MS signs and symptoms may be attributed to an upregulation of VEGF before a significant amount of degeneration has taken place. It is possible that VEGF is upregulated in response to the very earliest stages of MS pathology and continues to increase as the degeneration becomes visible and progresses. However, it is also possible that VEGF is a contributor to MS pathology beginning in the early stages of the disease. One study in support of this possibility is Proescholdt *et al.*'s (2002) work in which they demonstrated that VEGF triggers a stronger inflammatory response in rats with experimental allergic encephalomyelitis as a model for MS. This provides more support for the idea that VEGF's effects can vary and be potentially harmful depending on the physiological state in which it is upregulated.

AD is another disease which has, in recent years, come to be associated with elevated levels of VEGF, with the areas of greatest VEGF concentration being co-localized with amyloid plaques (Yang et al., 2004). Again, VEGF upregulation in AD may be a result of the damage caused by amyloid plaques or the disease's underlying pathology. However, as discussed in the Introduction,

VEGF may ultimately be contributing to the degeneration initiated by other processes. It may be doing so through microglial activation and inflammatory effects, two phenomena which appear to play a role in AD pathology (for review, see Tuppo & Arias, 2005). Furthermore, ICAM-1, which has been shown to be elevated by VEGF both *in vitro* (Kim *et al.*, 2001; Melder *et al.*, 1996) and *in vivo* (Croll *et al.*, 2004b; Miyamoto *et al.*, 2000; Proescholdt *et al.*, 1999) has also been seen to be elevated in the AD brain, where it leads to astrocyte production of proinflammatory cytokines such as IL-1α, IL-1β, and IL-6 (Lee *et al.*, 2000).

The connections between AD and inflammation and between VEGF and inflammation are strong, and it is therefore a logical hypothesis that this is where the connection between the two lies. However, it is interesting to consider other possibilities. Perhaps in the environment of an AD brain, VEGF's effects differ from the more commonly-observed situations. Just as VEGF's effects in experimental allergic encephalomyelitis rats differ from its effects in normal control rats, and just as ERK 1 and 2, pathways associated with VEGF that are ordinarily pro-survival, can become pro-death under circumstances of aberrant VEGFR-2 signaling, perhaps there are similarly aberrant mechanisms in place that contribute to AD pathology.

Conclusion

The degenerative phenomenon we have described and begun to investigate is intriguing in its complexity. We have shown that VEGF, a protein which is sometimes protective, can be damaging given the right set of circumstances. PlGF, a VEGF-family member that does not bind to VEGFR-2,

does not cause degeneration. VEGF administered via a method which causes the protein to reach peak levels more gradually does not cause this degeneration either, and we have therefore formed the hypothesis that AdVEGF-induced degeneration may be attributed to a downregulation or disruption of VEGFR-2 signaling caused by a sudden burst of VEGF expression. We have yet to demonstrate this directly or to elucidate the mechanisms in place, but we hope that in doing so, we will come closer to explaining some currently inexplicable forms of cell death and degenerative states.

Table 1. Differential Roles of VEGF Receptor Subtypes		
Vascular Permeability	VEGFR-1 VEGFR-2	*Shibuya & Claesson-Welsh, 2006; Brkovic & Sirois, 2006; Kiba* et al*., 2003*
Inflammation	VEGFR-1	*Sawano* et al*., 2001*
Angiogenesis	VEGFR-2 VEGFR-1 (indirectly)	*Waltenberger* et al*., 1994*
Neuroprotection	VEGFR-2	*Sondell* et al*., 2000; Rosenstein, 2003*

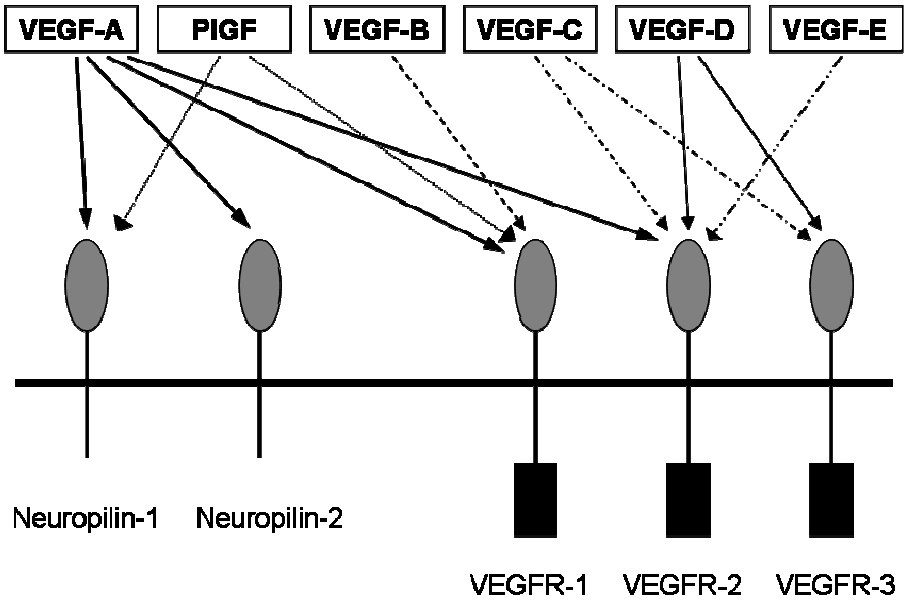


Figure 1: The VEGF family members and their receptors, the receptor tyrosine kinases VEGR-1, VEGFR-2, and VEGFR-3 and co-receptors Neuropilin-1 and Neuropilin-2

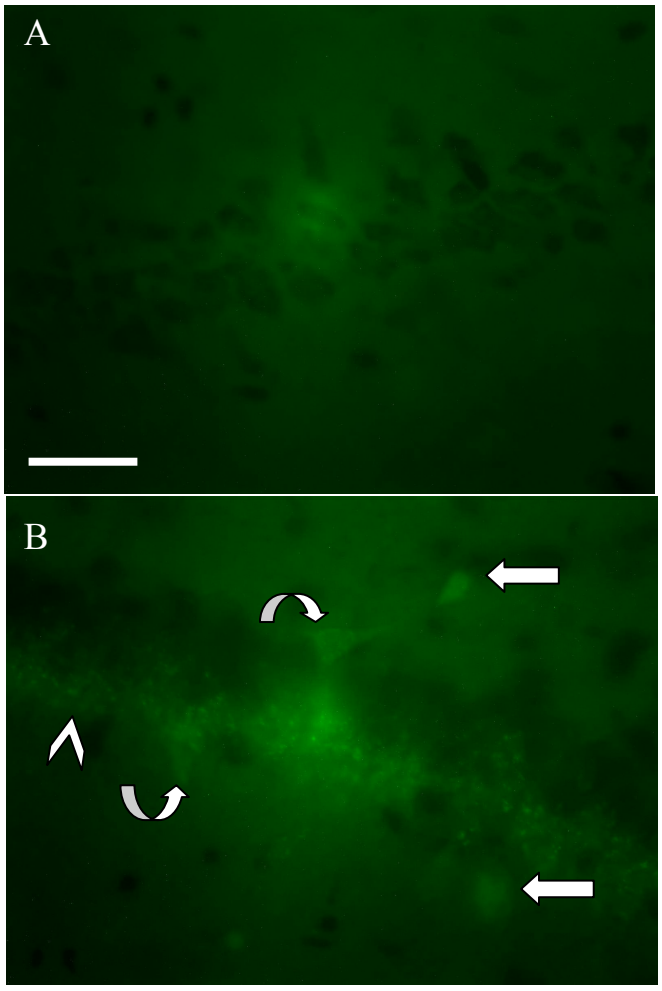


Figure 2. Expression of EGFP protein at 3 days after injection of adenovirus coding for VEGF and EGFP. The first panel shows a control animal in which no specific expression was observed. The second is from a rat injected with AdVEGF and shows specific cellular fluorescence across many cell morphologies including those with an astroglial morphology (most common, examples indicated by straight arrows), neuronal morphology (examples indicated by curved arrows), and endothelial cell morphology (least common, not shown). Scale bar=50μm.

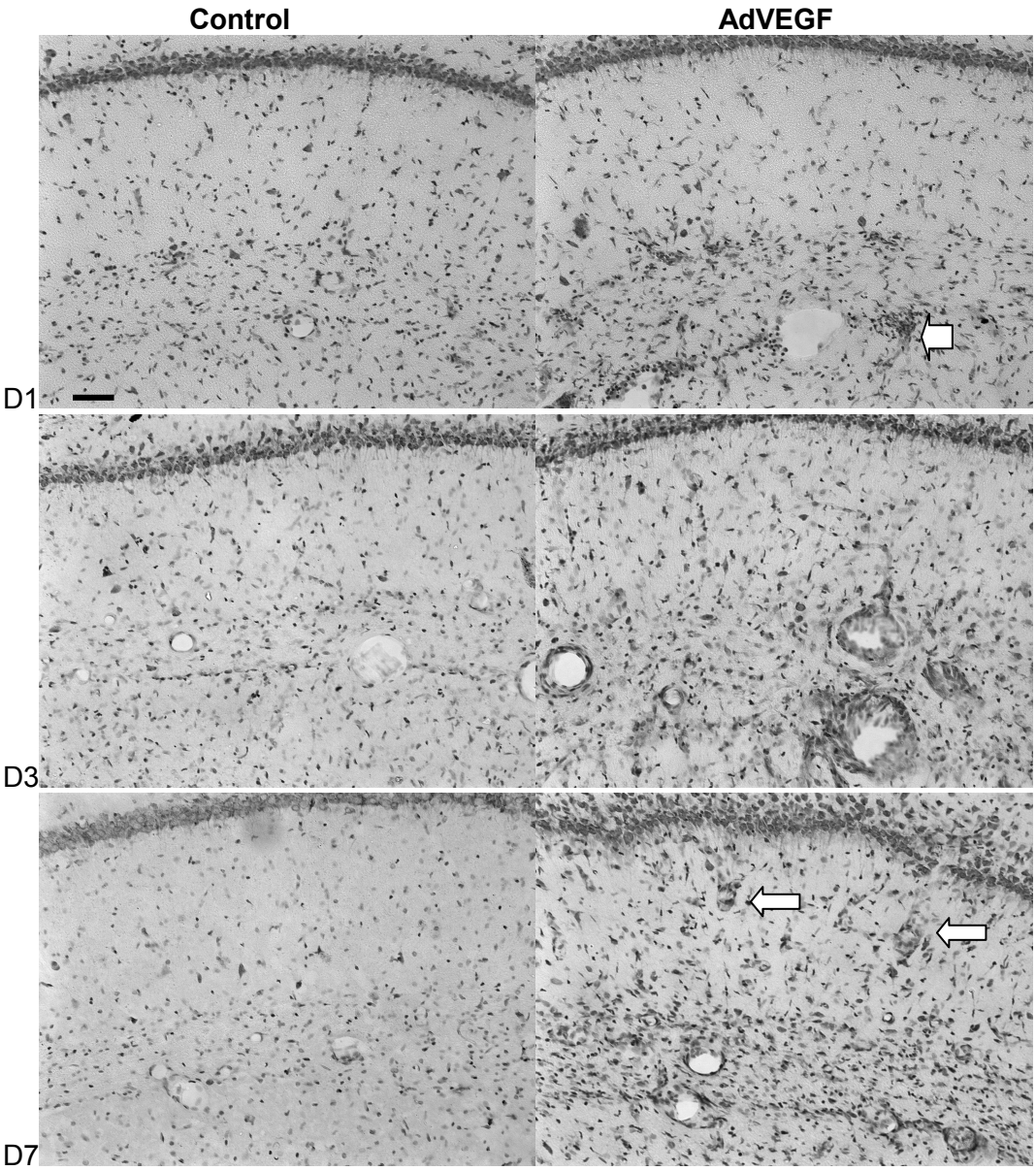


Figure 3. Nissl staining of AdVEGF-treated brains shows an early monocytic infiltrate followed by angiogenesis. The small, round, dark cells surrounding the vasculature in VEGF animals, but not controls, are monocytic in morphology (arrow in Day 1 VEGF diagram points to a cluster of monocytes). In Day 1 animals, increased vascular diameter is apparent. At Day 3, angiogenesis is not definitive, but more vasculature appears to be present, with some putative sprouts discernable, and increased vascular diameter is marked. By Day 7, angiogenesis is apparent, as vasculature is observed in the area between CA1 and the hippocampal fissure in regions not normally occupied by vessels of the type observed (see arrows in the Day 7 VEGF diagram). Scale bar=100μm.

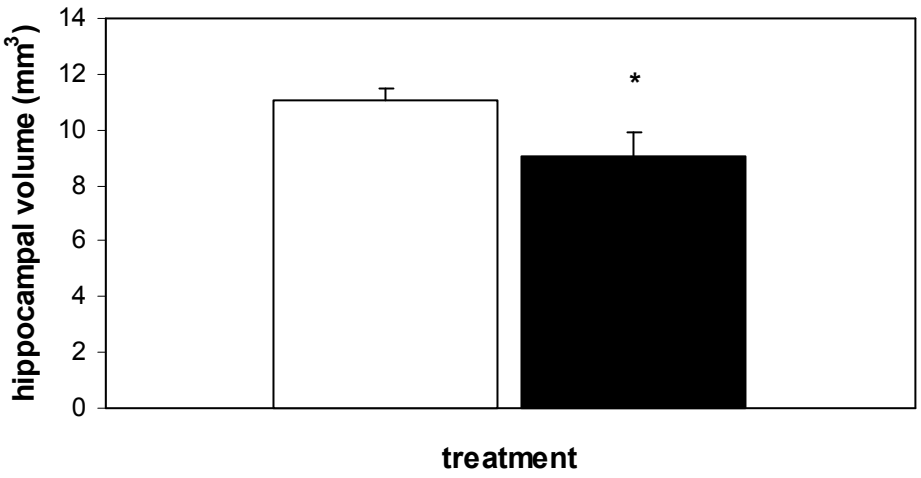
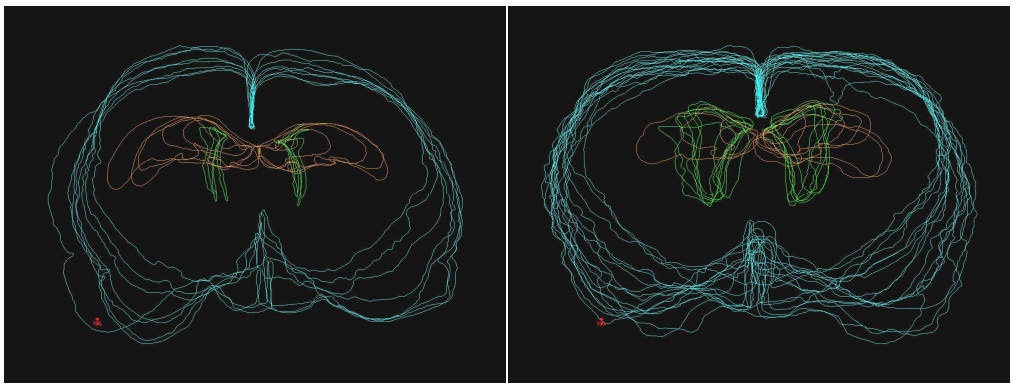


Figure 4. Animals that received AdVEGF (n=11) showed significantly smaller hippocampal volume ($p < .003$) than animals that received AdhFc (n=13).



AdhFc **AdVEGF**

Figure 5. Reconstructions of the brain made by tracing sections in a 1:12 series. The first panel is a reconstruction of an AdhFc-treated brain. The second panel is a reconstruction of an AdVEGF-treated brain. The hippocampi traced in orange, appear smaller in animals treated with AdVEGF. The lateral ventricles, in green, appear larger in AdVEGF-treated brains. Tracings were done at 4x magnification.

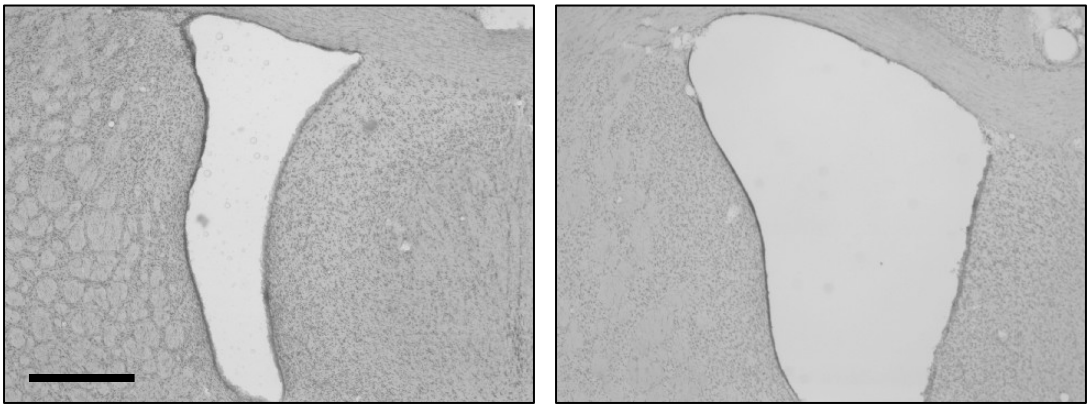


Figure 6. Lateral ventricles four weeks after adenovirus injection. The first panel is from an AdhFc-treated control brain. The second panel is from an AdVEGF-treated experimental brain, and is markedly hypertrophied. Scale bar=500μm.

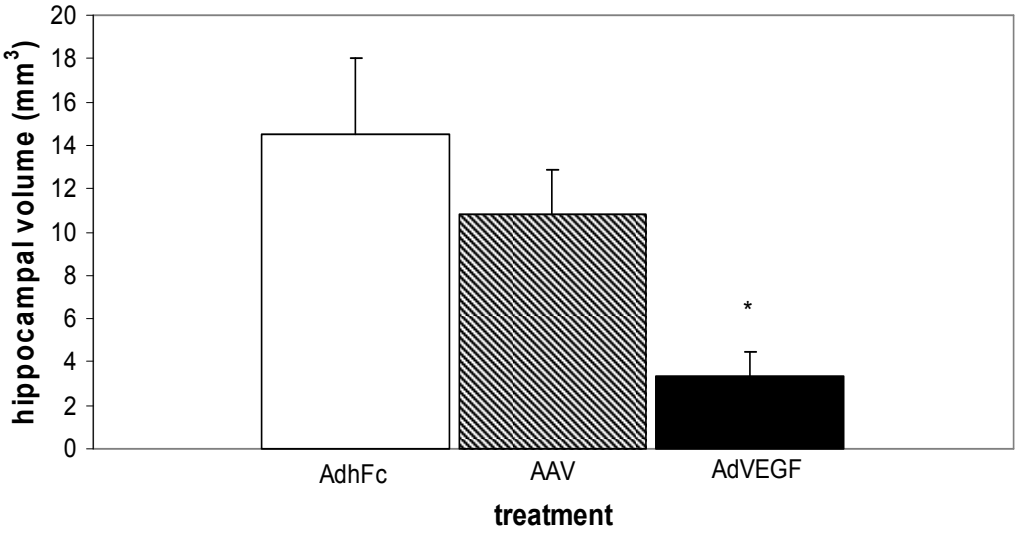


Figure 7. Graph showing significant effect of treatment ($p < .004$) on hippocampal volume four weeks after injection of AdhFc (n=4), AAVVEGF (n=8), or AdhFc (n=8). AdVEGF-treated hippocampi were significantly smaller than AdhFc-treated ($p < .006$) and AAVVEGF-treated ($p < .024$) hippocampi. There was no significant difference between hippocampi treated with AAVVEGF and AdhFc ($p = .25$).

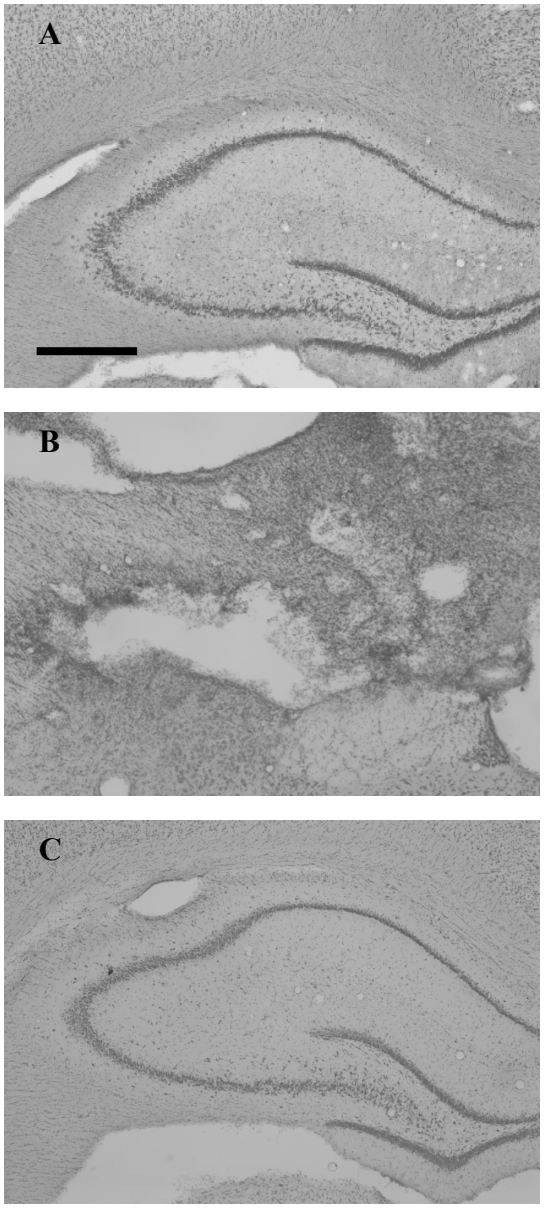


Figure 8. Hippocampus from animal treated with (A) AdhFc, (B) AdVEGF, and (C) AAVVEGF. Animals were sacrificed four weeks after microinjection. Scale bar=500μm.

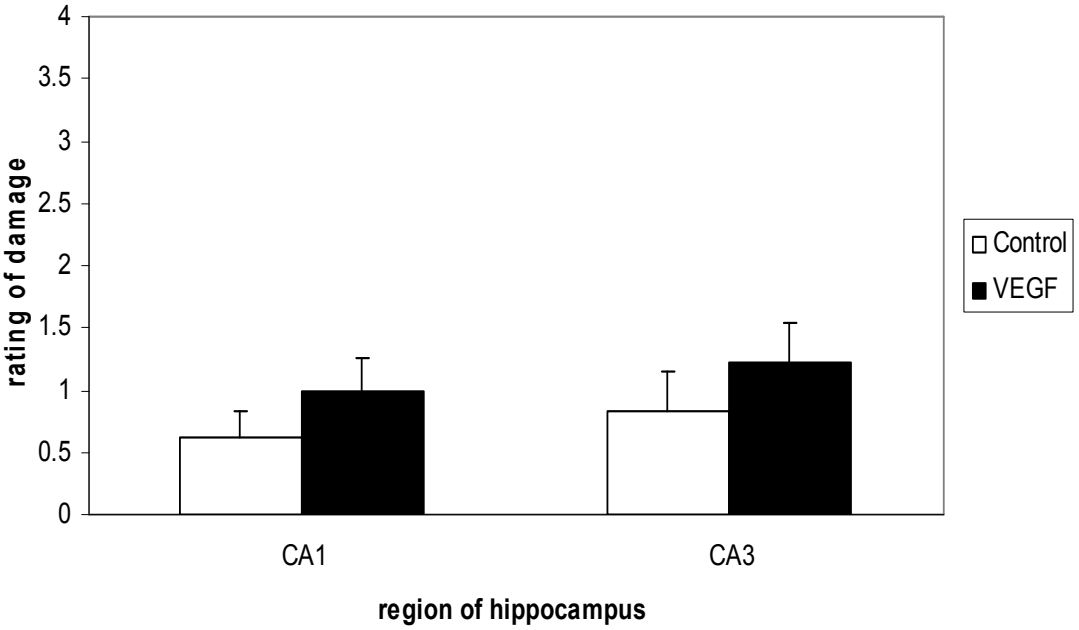


Figure 9. Graph of mean subjective ratings of damage for hippocampi that received AdhFc followed by infusion of either VEGF protein (n=14) or control protein (n=12). There is no significant difference between groups in either the CA3 region of the hippocampus (p=.297) or in the CA1 region of the hippocampus (p=.347).

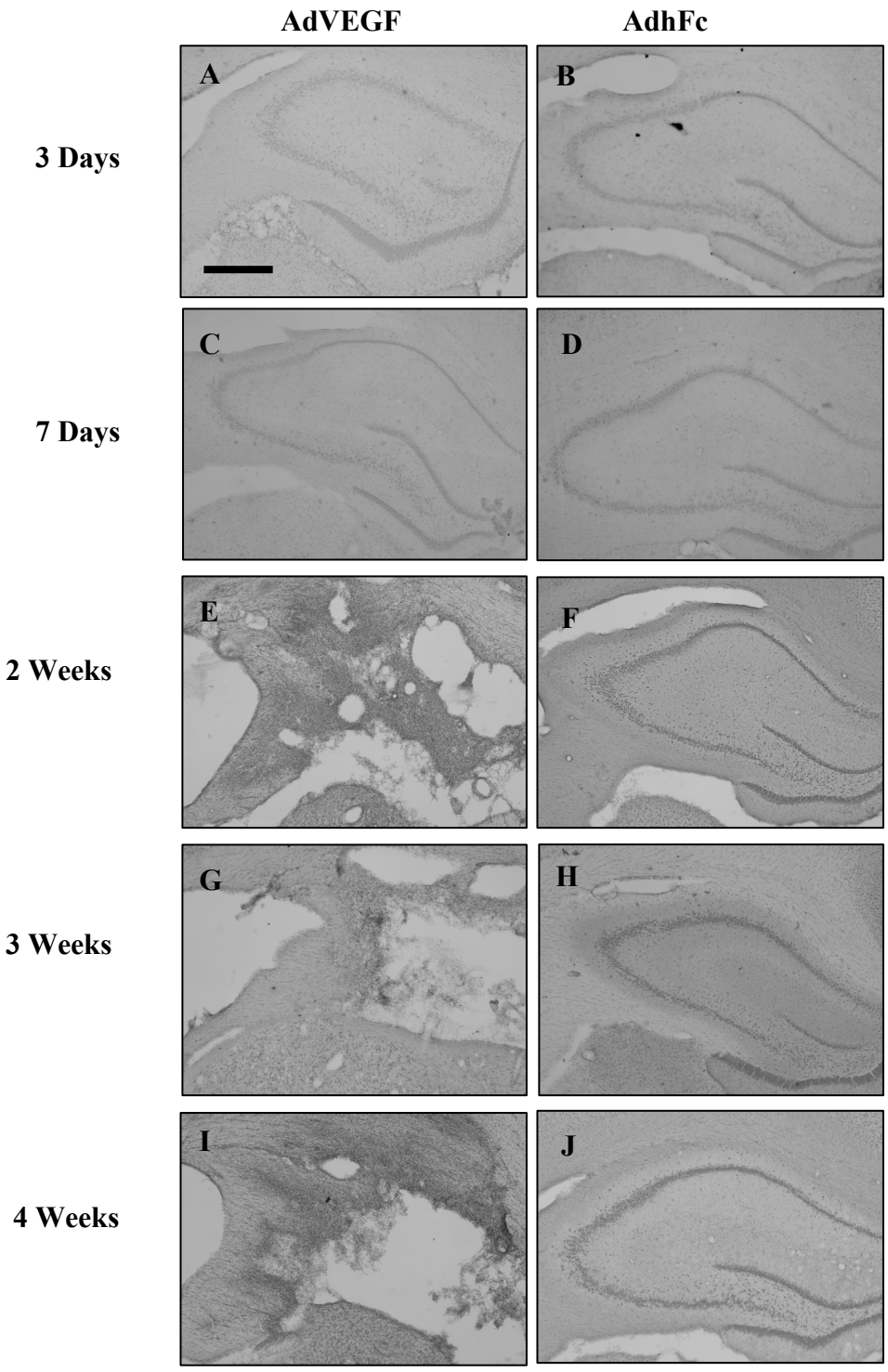


Figure 10. AdVEGF brains at 3 days (A), 7 days (C), 2 weeks (E), 3 weeks (G), and 4 weeks (I) after microinjection compared to control (B), (D), (F), (H), and (J). Extensive damage is visible as early as 2 weeks after microinjection in AdVEGF-treated brains. Scale bar=500μm.

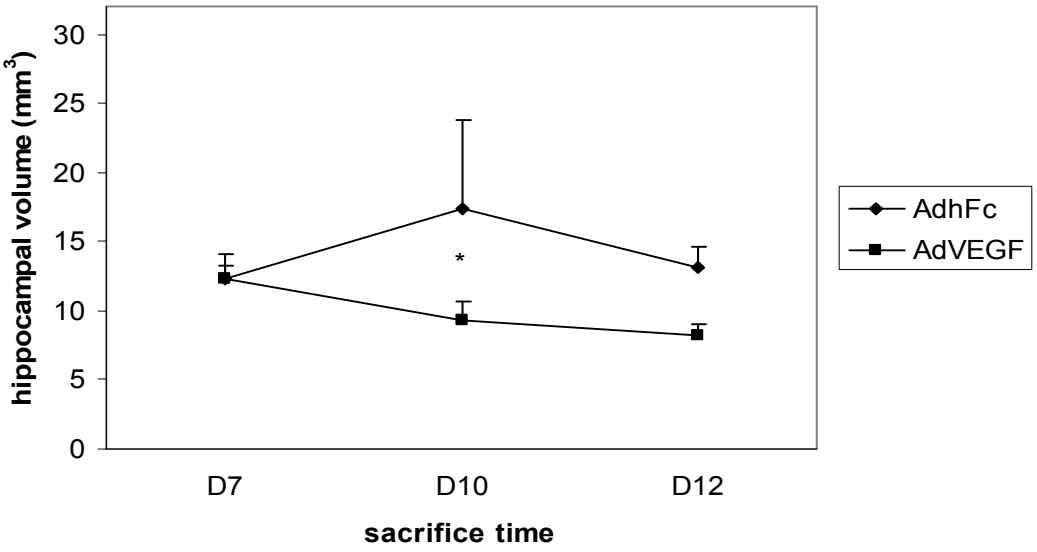


Figure 11. Graph of hippocampal volume for animals treated with AdVEGF (n=17) or AdhFc (n=17) and sacrificed after 7 (n=10), 10 (9), or 12 (n=15) days. Hippocampal volume was significantly smaller for AdVEGF-treated animals ($p < .013$), with significance achieved at the Day 10 time-point and a trend toward significance at Day 12.

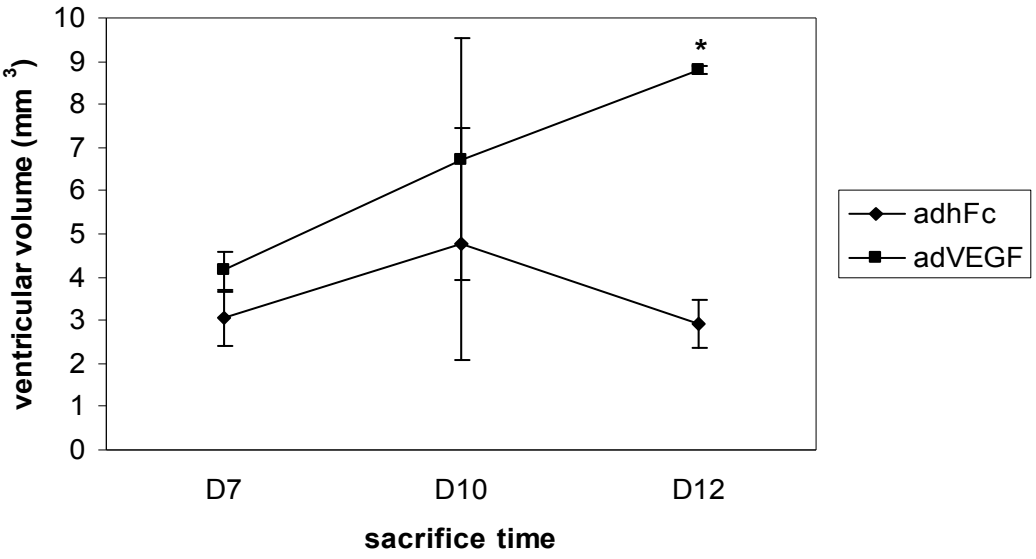


Figure 12. Graph showing lateral ventricular volume for animals treated with AdVEGF (n=17) or AdhFc (n=17) and sacrificed after 7 (n=10), 10 (9), or 12 (n=15) days. There was a statistical trend toward larger lateral ventricles in AdVEGF-treated animals (p=.056). By the Day 12 time-point, the lateral ventricles of AdVEGF-treated animals are larger than those of control animals ($t(14) = 3.502$, $p < .009$).

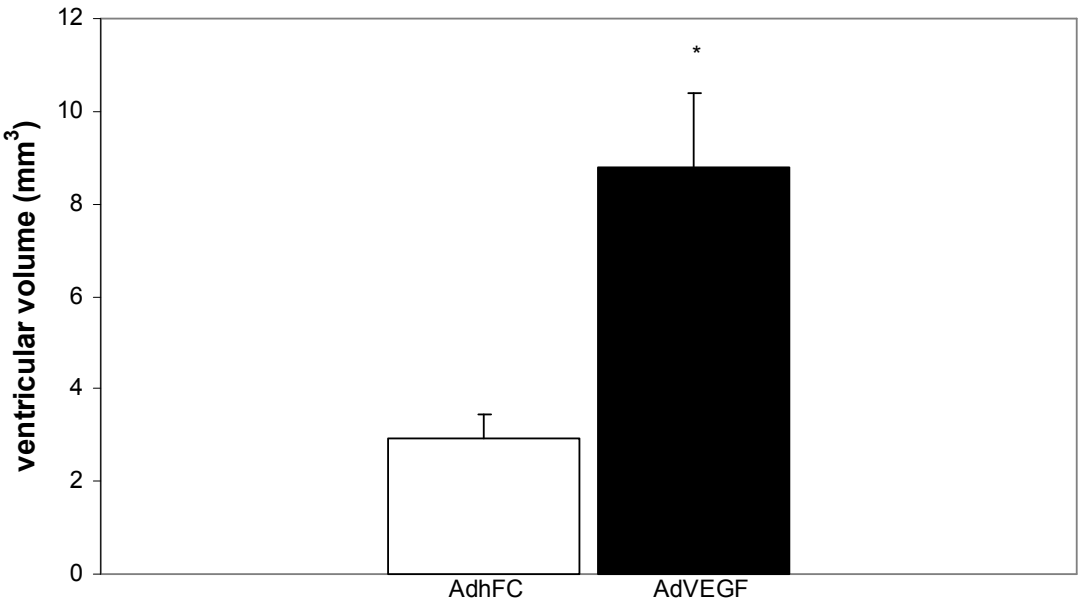


Figure 13. By the Day 12 time-point, the lateral ventricles of AdVEGF-treated animals (n=7) are larger than those of control animals (n=9) ($t(14) = 3.502$, $p < .009$).

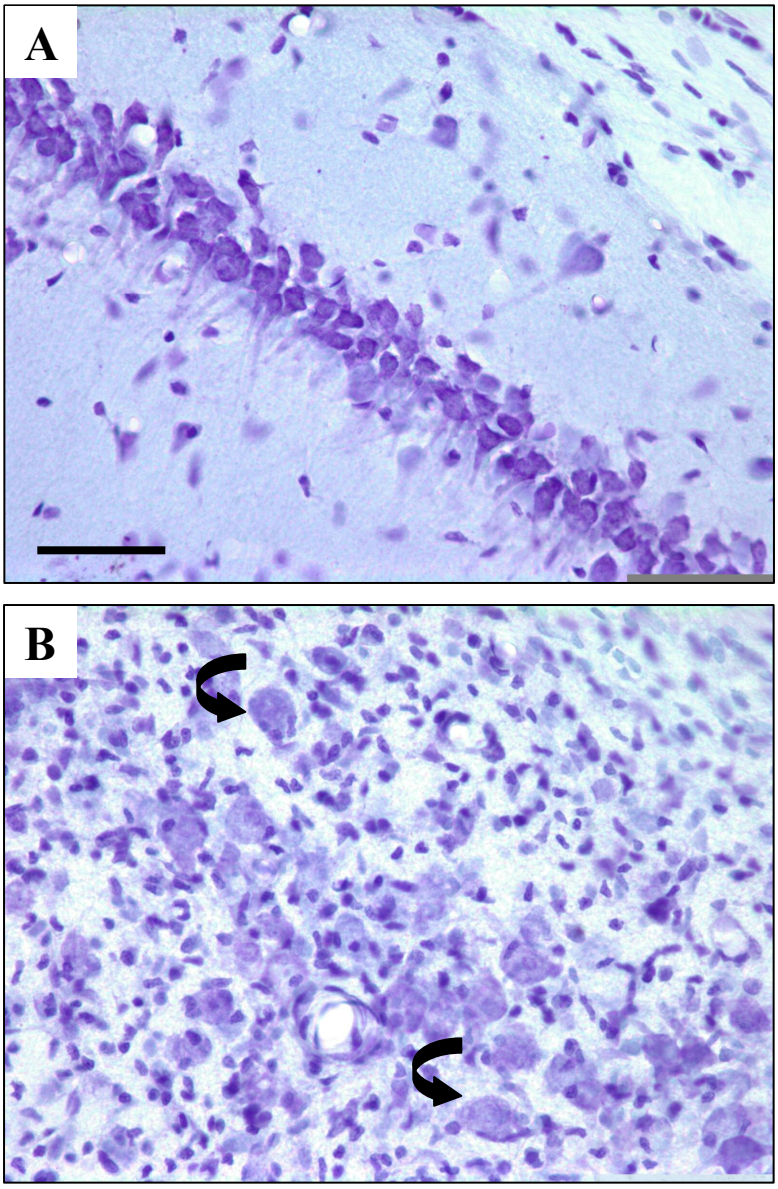


Figure 14. Panel A is CA1 of the hippocampus of an AdhFc-treated animal 10 days after microinjection. The cells appear normal in size, shape, and organization. Panel B is CA1 of the hippocampus of an AdVEGF-treated animal 10 days after microinjection. The neurons appear large, abnormal in shape, and disorganized. Surrounding the neurons is a large number of immune cells. Sections are Nissl stained. Scale bar=50μm.

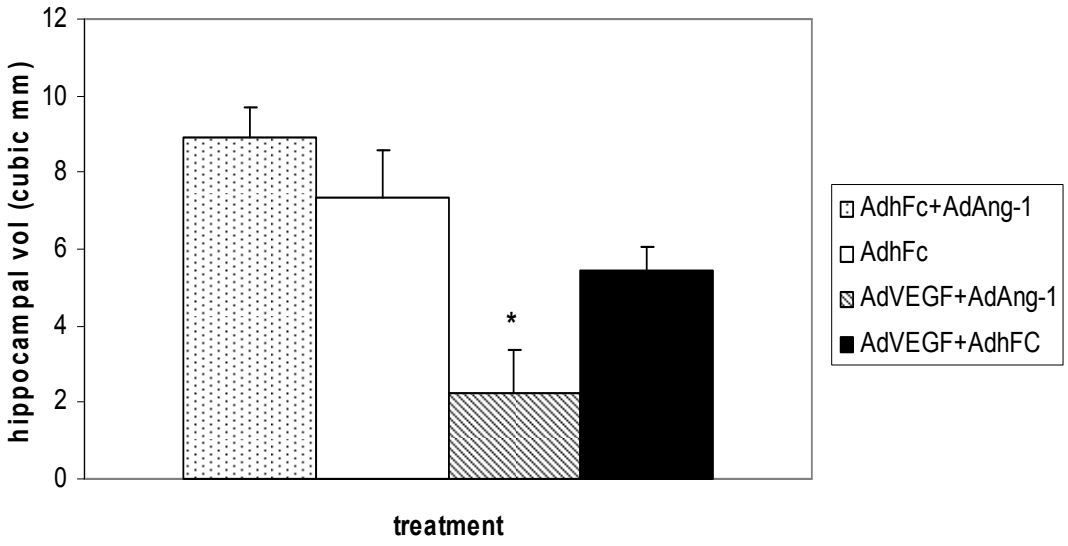


Figure 15. Graph of hippocampal volume for animals treated with AdVEGF+AdAng-1 (n=9), AdVEGF+AdhFc (n=7), AdhFc+AdAng-1 (n=6), or AdhFc alone (n=7). AdVEGF administration resulted in significantly smaller hippocampal volume (p=.000). There is a significant interaction effect (p< .028), such that there was a greater difference between AdVEGF-treated animals that were co-injected with AdAng-1 and those that were not than there is between AdhFc animals that were treated with AdAng-1 and those that were not.

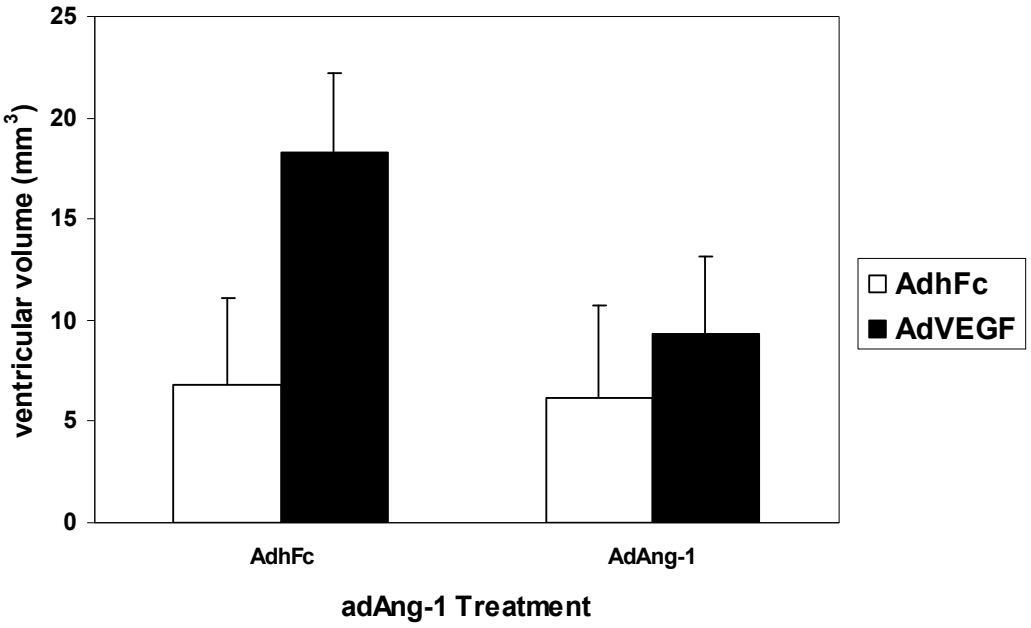


Figure 16. Graph of lateral ventricular volume of animals that received AdVEGF+AdAng-1 (n=9), AdVEGF+AdhFc (n=7), AdhFc+AdAng-1 (n=6), or AdhFc alone (n=7). There is a statistical trend toward enlarged ventricles in the animals that received AdVEGF (p=.092). The mean ventricular volume of 18.24 mm^3 for AdVEGF-treated animals is not significantly different from the mean volume of 9.33 mm^3 for animals co-treated with AdVEGF and AdAng-1.

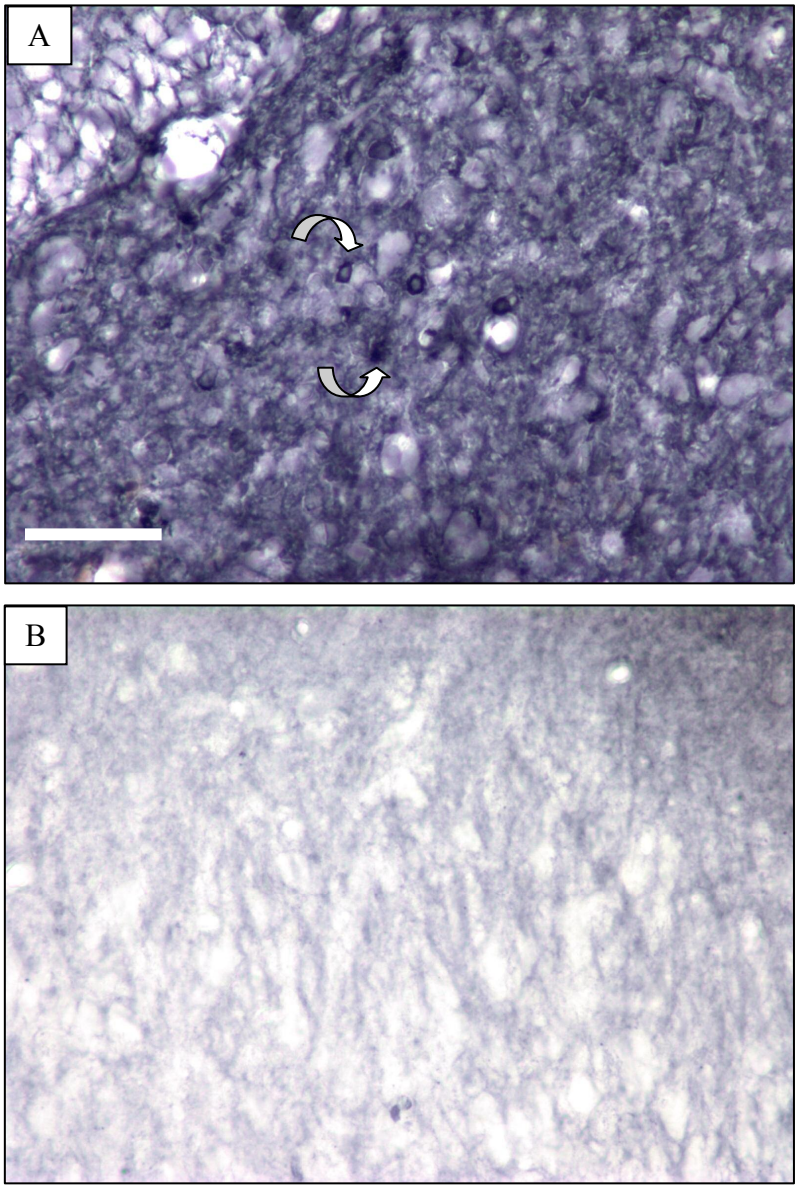


Figure 17: Sections immunostained with anti-OX-1 antibody for leukocytes. Panel A is an AdVEGF-treated hippocampus infused with control excipients. The dark background staining reflects the higher degree of vascular leak in the brain, and a number of immune cells can be seen (examples indicated by arrows). Panel B is an AdVEGF-treated hippocampus infused with Dexamethasone, showing only one immune cell and little leak. Scale bar=50μm.

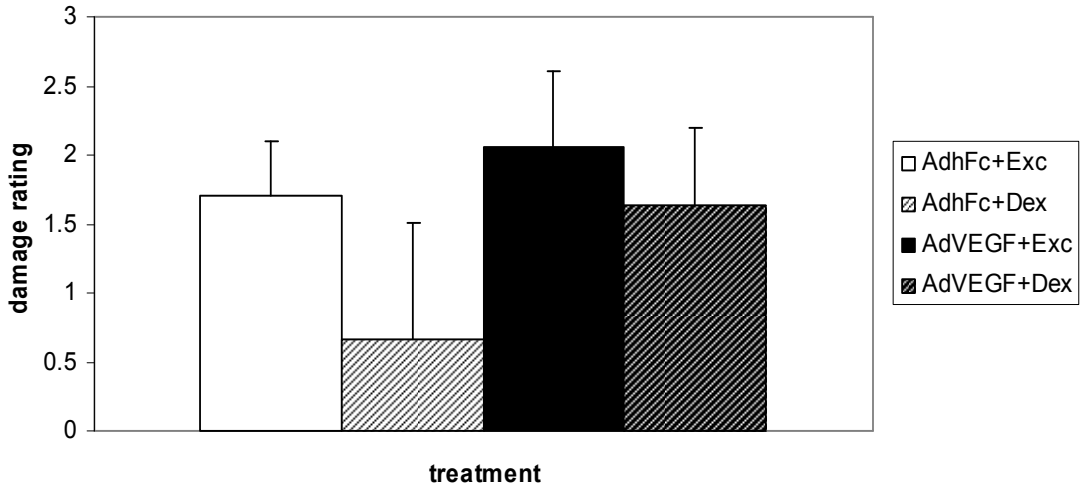


Figure 18. Graph comparing CA3 hippocampal damage ratings of brains treated with AdhFc+Exc (n=5), AdhFc+Dex (n=4), AdVEGF+Exc (n=7), and AdVEGF+Dex (n=8). There was no significant difference among groups (p=.859), but there appears to be a trend toward an effect of Dexamethasone on hippocampal damage in the AdhFc-treated animals.

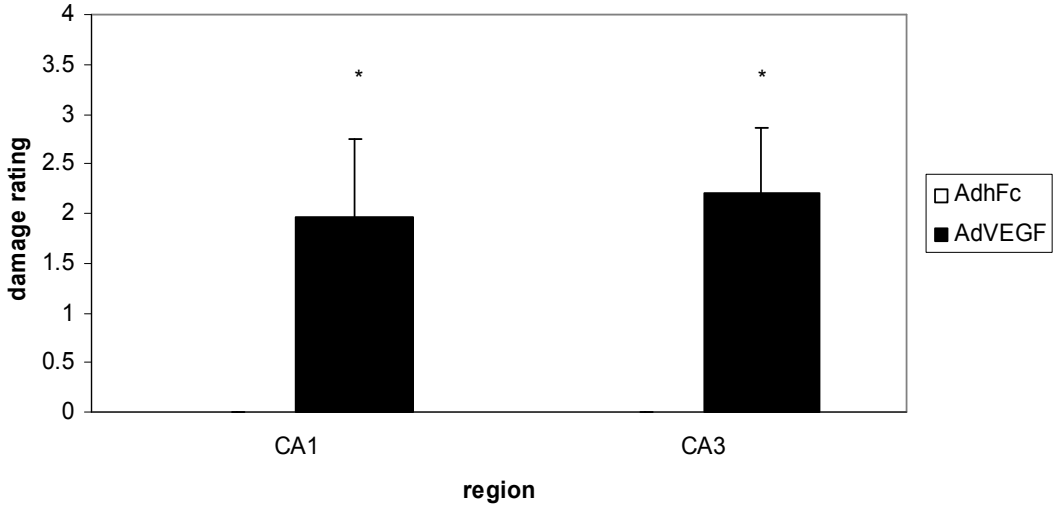


Figure 19. Graph comparing subjective ratings of damage for hippocampi of Cohort 2 animals treated with AdhFc (n=4) or AdVEGF (n=6). Mean ratings for AdhFc animals were 0 and bars are therefore not visible for those animals. AdVEGF-treated animals showed significantly more damage than AdhFc-treated animals (in CA3, $p<.016$; in CA1, $p<.048$).

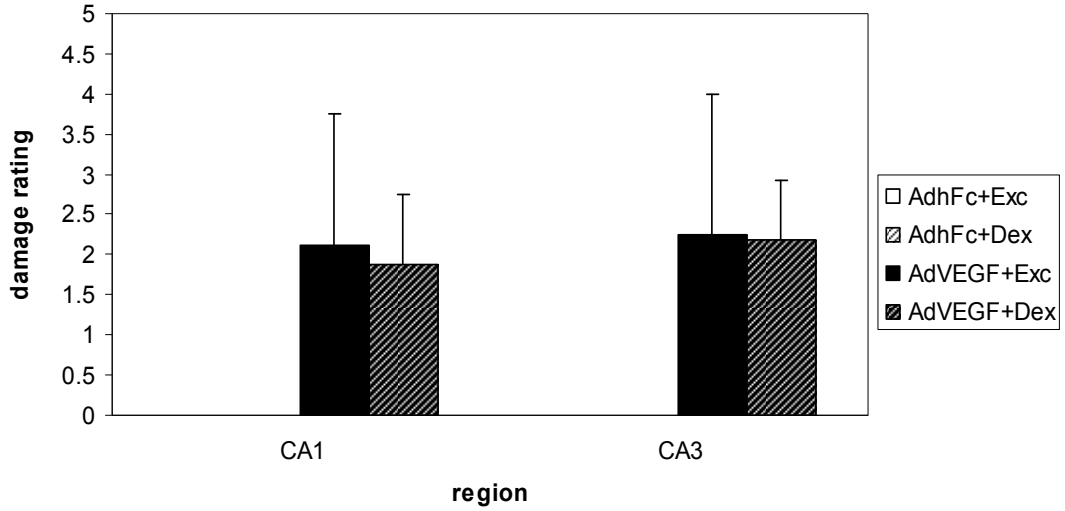


Figure 20. Graph showing hippocampal damage of Cohort 2 animals that were treated with AdVEGF+Exc (n=2), AdVEGF+Dex (n=4), AdhFc+Exc (n=2), or AdhFc+Dex (n=2). There was no significant difference between hippocampal damage in Dex-treated animals and controls, either in CA3 (p=.099) or in CA1 (p=.174).

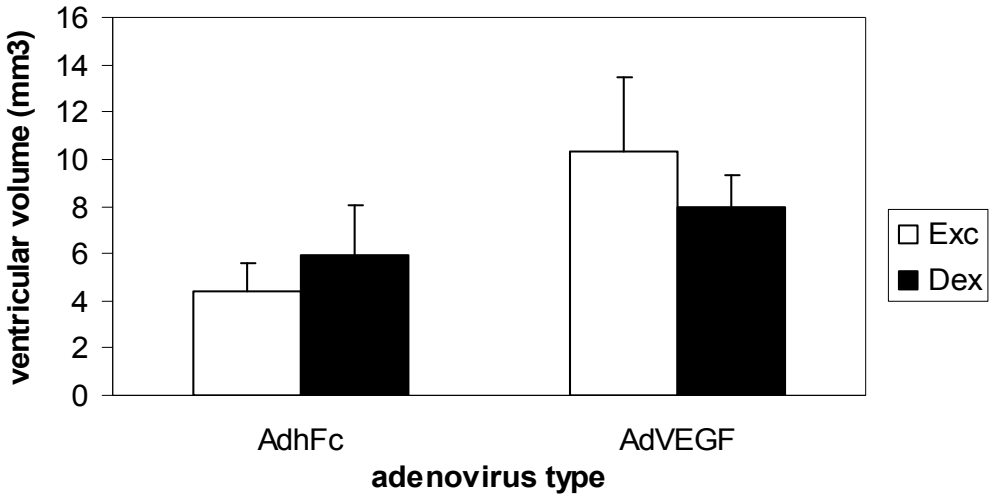


Figure 21. Graph comparing lateral ventricular volume of animals treated with AdhFc+ Exc (n=6), AdhFc+Dex (n=5), AdVEGF+Exc (n=7), or AdVEGF+Dex (n=9). There is a statistical trend toward effect of adenovirus on ventricular volume (p=.074) and no significant effect of Dexamethasone treatment (p=.849).

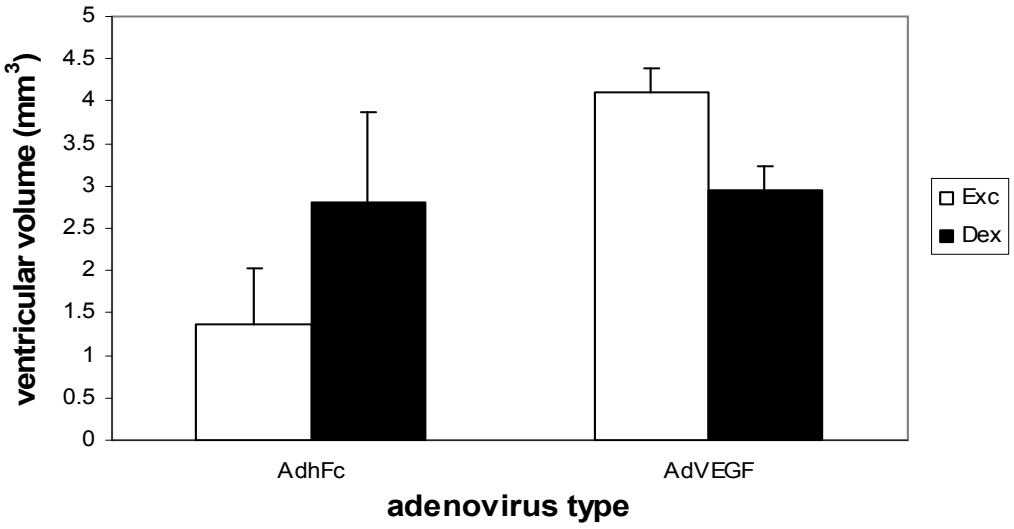


Figure 22. Graph comparing the ventricular volume of Cohort 2 animals that received either AdVEGF+Exc (n=2), AdVEGF+Dex (n=4), AdhFc+Exc (n=2), or AdhFc+Dex (n=2). The lateral ventricles were significantly larger in AdVEGF-treated animals than in AdhFc-treated animals ($p < .041$), but Dexamethasone did not significantly alleviate this hypertrophy.

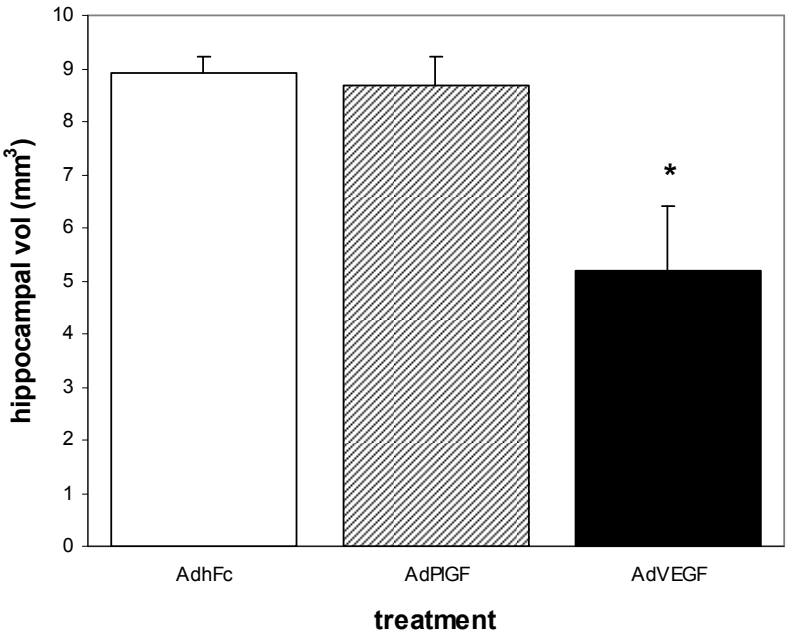


Figure 23. Graph showing hippocampal volume of animals treated with AdhFc (n=4), AdPlGF (n=12), or AdVEGF (n=7). Hippocampi of animals that received AdVEGF were significantly smaller than those of animals that received AdPlGF (Tukey LSD, $p < .019$). AdVEGF's degenerative effect in the hippocampus was just short of significant in this experiment when compared with the AdhFc controls (Tukey LSD, $p = .053$).

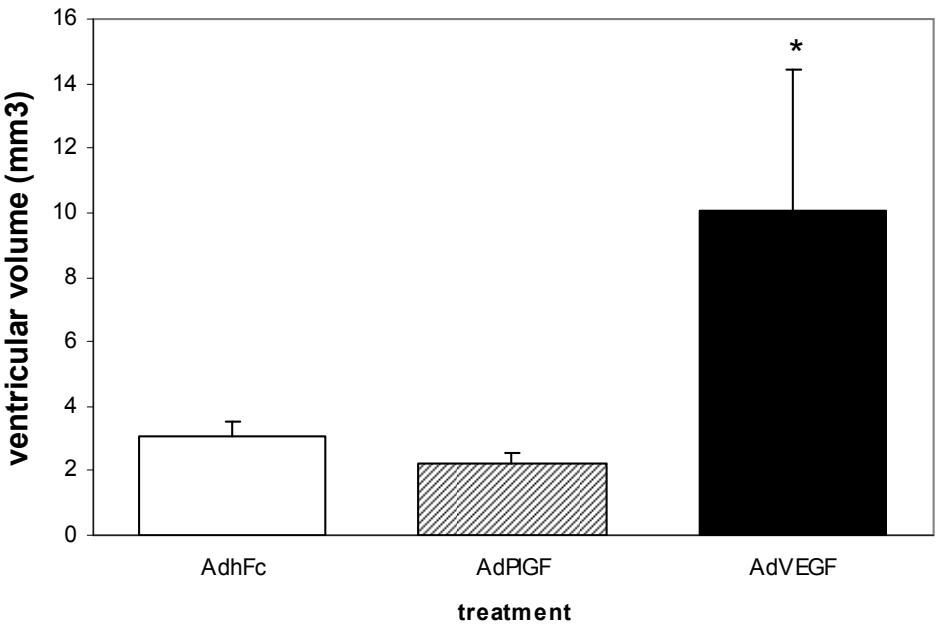


Figure 24. Graph showing lateral ventricular volume of animals treated with AdhFc (n=4), AdPlGF (n=12), or AdVEGF (n=7). AdVEGF-treated animals had significantly larger lateral ventricles than AdPlGF-treated animals (Tukey LSD, $p<.015$). The difference between the mean ventricular volume of AdhFc-treated animals and AdVEGF-treated animals approaches significance ($p=.068$).

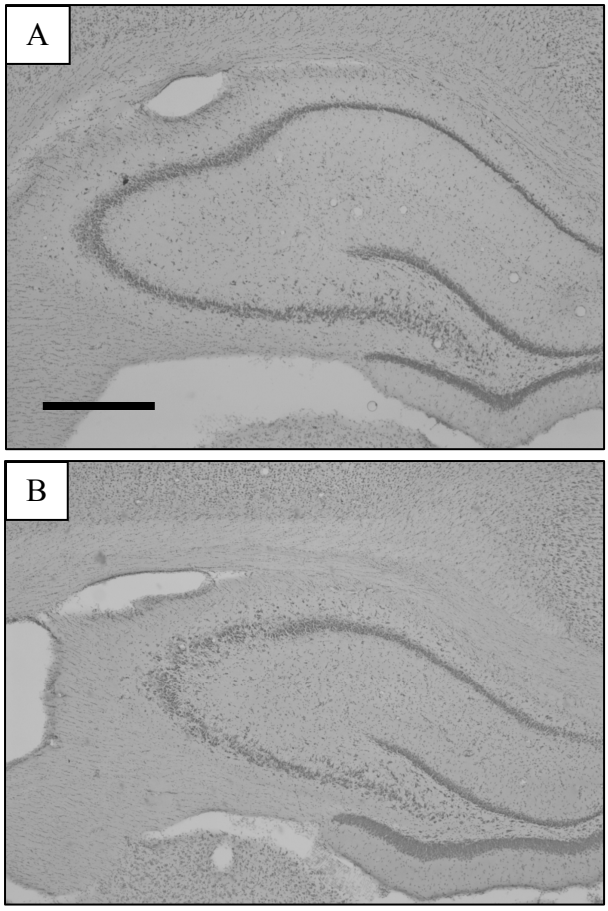


Figure 25 : Panel A is a picture of an AAVVEGF-treated hippocampus 4 weeks after microinjection. Panel B is an AAVVEGF-treated hippocampus 8 weeks after microinjection. Even after 8 weeks, the hippocampus does not resemble a degenerated AdVEGF-treated hippocampus. Photos taken at 4x magnification. Scale bar=500μm.

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