A ROLE OF ANGIOGENIN IN MODELS OF PARKINSON’S DISEASE

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ABSTRACT

Parkinson’s Disease (PD) is the most common neurodegenerative movement disorder and is characterized by the loss of dopaminergic neurons in the substantia nigra. Gene multiplication of alpha-synuclein causes an autosomal dominant inheritance pattern of PD. A mouse model overexpressing alpha-synuclein demonstrates some of the phenotype seen in PD. In a microarray of these mice, a downregulation of angiogenin was observed prior to symptom onset, demonstrating a potential link of angiogenin to pathology in this model. Angiogenin has also been associated with the onset of Amyotrophic Lateral Sclerosis (ALS), and is neuroprotective in models of ALS through activation of the Akt pathway, a pro-survival signaling pathway.

Based on these findings we hypothesized that angiogenin is protective in models of PD through activation of the Akt pathway. The goals of these studies was to determine whether 1) angiogenin was protective in cellular models of PD, 2) angiogenin required the Akt pathway to be protective, and 3) angiogenin was protective in a mouse model of PD. We first demonstrate that angiogenin is protective in two dopaminergic cell lines against toxins used to model PD. This finding supports angiogenin’s function in neurons to be important for cell survival. We then demonstrate that angiogenin induces Akt phosphorylation in a dopaminergic cell line, but inhibition of Akt signaling did not inhibit angiogenin’s protective response. We next investigated a mutant of angiogenin, K40I, for its ability to be protective in cellular models of PD. This mutant
has previously been shown not to be protective in motoneurons and fails to activate Akt phosphorylation. Our findings revealed that K40I does not phosphorylate Akt in a dopaminergic cell line. We further demonstrate K40I still has the capacity to be protective, indicating angiogenin to promote survival of dopaminergic neurons in a different manner than motoneurons. Lastly, we investigated angiogenin’s ability to be protective in a mouse model of PD. Our results demonstrate that in our experimental paradigm angiogenin was not protective, and that other delivery options to investigate angiogenin’s protective effect may be necessary.

These studies demonstrate that angiogenin provides a neuroprotective effect in cellular models of PD and with further investigation may be a potential therapeutic agent for PD.
DEDICATION

My ability to reach this point is through the countless prayers, endless encouragement, and assured confidence and support given by my parents and family. Your resolve to keep my eyes looking forward when I wanted to look back, or at other paths, has brought me to this summit. I am indebted to your investment in my life with the numerous cards, regular e-mails, encouraging calls, and an ever supply of food - all done with a generous and joyful disposition. This life has given me the greatest of blessings by having you as my family, my support team, and an example of a life lived in fullness. Thus, with great honor I dedicate this dissertation to my parents, Thomas and Dianne Steidinger, and my siblings, Beth Ann & Corey Steffen, Rebecca Biggerstaff, Richard and Kimberly Steidinger, Bruce & Beverly Young, Rodney & Denise Steidinger, Troy & Bobbie Jo Steidinger.
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Thanks to each of you for carrying me through!!
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Figure 3: Angiogenin overexpression does not reduce loss of dopaminergic neurons of the substantia nigra in response to MPTP.

**DISCUSSION AND SUMMARY**

1. Angiogenin overexpression does not reduce loss of dopaminergic neurons of the substantia nigra in response to MPTP.
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<tr>
<td>α-syn</td>
<td>Alpha-synuclein</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 associated death promoter protein</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DBS</td>
<td>Deep Brain Stimulation</td>
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<tr>
<td>ECG</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellularly signal-regulated kinase</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O</td>
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<tr>
<td>GADPH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<tr>
<td>LB</td>
<td>Lewy Body</td>
</tr>
<tr>
<td>L-dopa</td>
<td>Levadopa</td>
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<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>Abbreviation</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PDGFB</td>
<td>Platelet-derived growth factor beta</td>
</tr>
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<td>PDK-2</td>
<td>Pyruvate dehydrogenase kinase isoform 2</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>SAPK/JNK</td>
<td>Stress-activated protein kinase/c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF attachment protein receptor</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER ONE

INTRODUCTION

History

Parkinson Disease (PD) is the second most common neurodegenerative disease, affecting about 1 million Americans and about 1-2% of the population over 60 years of age (de Lau & Breteler 2006, Olanow et al. 2009b). Identification and an initial characterization of Parkinson Disease was in 1817 when Dr. James Parkinson noted a repeated characteristic observed in some of his patients that he coined “shaky palsy” (Parkinson 2002). In 1912 a pathological hallmark of PD was discovered by Frank Lewy and was thus named Lewy bodies (Rodrigues e Silva et al. 2010). The role of dopamine (DA) was not recognized until forty years later when Arvid Carlsson identified a reduction of dopamine in basal ganglia in PD (Carlsson 1971, Carlsson 1988). In 1959, Bertler and Rosengred, recognized the role of dopamine in the basal ganglia and its role in motor movement disorders (Goetz 2011).

Dr. Parkinson’s descriptions of PD remain accurate today, with the predominant clinical findings including: bradykinesia, resting tremor, gait instability, and rigidity (Dauer & Przedborski 2003). However, in conjunction with motor symptoms, non-motor symptoms commonly identified in patients include: cognitive impairment, sleep disturbances, sensory abnormalities, depression, anxiety, and disturbances of autonomic function. Following diagnosis PD continues to progress as demonstrated in a longitudinal analysis of 149 idiopathic patients over a 20 year period. At the end point of
this study 74% were deceased and 48% of the remaining were in nursing homes, 83% had dementia, and 74% had hallucinations (Reid et al. 2011).

**Epidemiology**

The mean age of onset for PD is 55 years, and approximately 1-2% population over 65 are affected (Eriksen et al. 2003). Individuals with a family history of PD have double the risk for developing PD and patients with onset of disease under 50 years of age demonstrate a strong potential for a genetic link to the disease (Tanner et al. 1999). The economical burden of PD is considered to be upwards of $23 billion, and individual costs of drugs and medical care can be up to $10,000 a year (Huse et al. 2005). Projection analysis of 15 countries perceive a doubling of PD patients from 4.5 million in 2005 to 9 million in 2030, further increasing the medical and societal costs of PD (Dorsey et al. 2007).

**Pathology**

A diagnostic hallmark for PD, Lewy bodies (LB) contains a variety of components including lipids, neurofilaments, synphilin, ubiquitin, and α-syn. The predominant clinical feature of PD stems from the degeneration of substantia nigra pars compacta that presents with motor impairments; however, non-motor symptoms may precede this with symptoms such as gastrointestinal dysfunction, anosmia, and sleep disorders (Abbott 2003, Gagnon 2002, Muller 2002). PD demonstrates a 70-80% reduction in DA depletion of the basal ganglia and a 50% dopaminergic cell death of SN upon the presentation of the disease (Dunnet & Bjorklanin 1999). Although not the only
reduced neurotransmitter in PD, dopamine is the primary source of the motor symptoms associated with PD (Chase 1998).

The PD pathology and progression has been well defined by Braak et al. In 2004 his group described the progression of the α-syn pathology and its related progression to disease severity. They describe six stages to PD. In stages 1 and 2 inclusion bodies and pathology are restricted to the dorsal motor nucleus and anterior olfactory nucleus. In stage 3 the beginnings of substantia nigra pathology with LB presence is observed. Stage 4 is identified by the loss of substantia nigra neurons and the expansion of pathology into the magnocellular nuclei of the basal forebrain, the amygdala, and the thalamus. By stage 5 olfactory areas are severely affected and pathology spreads into sensory association areas and prefrontal areas. At stage 6 there is the development of pathology throughout the entire neocortex including primary motor field and first order association area. Their findings demonstrate a non-simultaneous degeneration of nigral and extranigral cells with the initial LB being found in non-catecholaminergic neurons (Braak 2004, Del Tredici 2002).

**Current Treatment Options**

Treatment options currently available to PD patients are only effective in limiting the presenting symptoms. Levadopa (L-dopa), despite being the oldest, remains today as the most effective therapy available for PD symptoms. L-Dopa is an immediate precursor to DA in the biosynthetic pathway of catecholamines and is able to cross the blood brain barrier, unlike dopamine. L-dopa is able to restore depleted DA levels in the striatum limiting motor symptoms; however, over time L-dopa patients develop motor
fluctuations and dyskinesias, which greatly limit the quality of life in the patient and compromise the efficacy of the treatment (Poewe et al. 1986, Schrag & Quinn 2000). L-dopa has been considered to potentially augment disease progression by the formation of free radicals during DA catabolism (Fahn 1996). One study, the Earlier versus Later L-DOPA trial, investigated whether L-dopa treatment could inhibit disease progression. A 40-week study with a two week washout at the end, demonstrated that at multiple doses L-dopa improved the functional abilities of the patients, implying a neuroprotective response by L-dopa. However, functional improvement in L-dopa treated patients could have reflected residual effects of L-dopa due to a short washout period (Fahn 1999). Furthermore examination of patients treated with L-dopa, by positron emission tomography (PET) and single-photon emission computed tomography (SPECT) imaging, demonstrated a reduction in dopamine transporter binding in the striatum following L-dopa treatment, suggestive of increased dopaminergic cell loss (Guttman et al. 2001, Fahn 1999). However, these imaging findings could alternatively reflect changes in dopamine transporters without changes in DA cell counts (Fahn et al. 2004).

Another means of treating symptoms from depleted DA of the striatum is to use an agonist of the dopamine receptor, such as pramipexole and ropinole. These both have demonstrated conflicting results in their neuroprotective potential. With both, imaging demonstrated a reduced decline in DA uptake following treatment; however, no improvement on clinical scales was observed compared to L-dopa (Whone et al. 2003, Anon. 1993). Deep brain stimulation (DBS) is another option found to be effective in treating PD symptoms. A five year study of patients treated with DBS in the subthalamic nucleus demonstrated a significant improvement of symptoms and a reduction
in the intake of dopaminergic drugs (Gervais-Bernard et al. 2009). Detriments to DBS include the risk of surgical implantation as well as an increased tendency for depression, mania, and dysarthria in DBS patients (Krack 2002). The major downfall in all of the mentioned treatments is that they are restricted to treating symptoms manifesting from the disease progression, rather than stopping the underlying pathological process.

Pathogenic Mechanisms

The pathogenesis of PD is not fully elucidated. Multiple factors including environment and genetics are known to play a role in the disease. Distinct mechanisms have been associated with the cause of PD including protein aggregation and misfolding, mitochondrial dysfunction, and apoptosis (Dauer & Przedborski 2003).

Protein aggregation and formation of Lewy bodies is a hallmark of PD. Multiple proteins have been associated with protein aggregation in PD including Parkin and α-syn. Brain tissues of Parkin-associated PD patients fail to have consistent evidence of Lewy body pathology. However Parkin, an E3 ubiquitin ligase, substrates have been associated with protein turnover and degradation and therefore it is considered a putative role in aggregation (Moore et al. 2008). The link of α-syn aggregation and neuronal toxicity is not completely understood. It is postulated that α-syn mediates toxicity through gene regulation, cell signaling, dopamine signaling, and inflammation (Abeliovich et al. 2000, Masliah et al. 2000).

Mitochondrial dysfunction results in the formation of reactive oxygen species. Complex I of the mitochondria is found to be diminished in activity in post-mortem tissue of PD patients (Schapira et al. 1989). Furthermore inhibitors of complex I, 1-methyl-4-
phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, treated in animal models replicate some of the findings seen in PD (Przedborski & Jackson-Lewis 1998). These inhibitors also induce reactive oxygen species causing damage to proteins, nucleic acids, and lipids. This damage in turn can lead to cell death. An increase in damaged proteins reflective of reactive oxygen species has been demonstrated in the PD patients (Schapira et al. 1989).

Apoptosis has been revealed in animal models and in the substantia nigra of PD brains. The role of apoptosis in inducing cell death reflects a response to disease progression rather than the primary cause of the disease. However, its induction of cellular pathways that lead to cell death has become a target for treatment options that limit neurodegeneration. Clinical trials using anti-apoptotic factors have been investigated as a potential neuroprotective agent in PD (Olanow et al. 2006, Wang & Johnson 2008).

**Neuroprotective Therapies**

Multiple agents have been investigated for their effectiveness in inhibiting disease progression, based on the mechanisms implicated in the pathogenesis of PD. Potential neuroprotective agents include anti-oxidants, anti-apoptotic factors, and neurotrophic agents.

Several antioxidants have been investigated for PD. An inhibitor of DOPA oxidation, selegiline demonstrated an effect at delaying the need for L-Dopa treatment; however, its effect on pathological progression was confounded due to its innate ability to provide symptomatic relief in PD (Anon. 1996). Antioxidant studies using creatine, a
promoter of ATP production in mitochondria, demonstrated a neuroprotective effect in animal models, but have failed to show significant effect in PD patients (Bender et al. 2006). Rasagiline, an inhibitor of the enzyme that degrades dopamine (monoamine oxidase B), demonstrated in cell culture and mouse models to be protective to dopaminergic neurons (Akao et al. 2002), and in PD patients rasagiline has demonstrated strong evidence as a potential disease modifier (Olanow et al. 2009a). However these results were difficult to interpret due to increase dosage demonstrating a reduced effect. A systematic review of randomized clinical trials demonstrated an effect of the antioxidant Coenzyme Q10 using two different methods of scoring. However recently a phase III clinical trial was ended as it was determined Coenzyme 10 is not likely to provide a significant benefit (Liu et al. 2011). These studies only exemplify the complexity of identifying a clinically relevant and an effective agent to inhibit progressive pathology.

Anti-apoptotic agents have also been hopeful avenues of research and have included glyceraldehyde-3-phosphate dehydrogenase inhibitor, TCH346, and an inhibitor of JNK pathway, CEP-1347. TCH346 inhibits an initiator of apoptosis, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). CEP-1347 inhibits several kinases known to be involved in the cell death pathway. Both these agents failed to be effective in PD during the clinical stage of development (Olanow et al. 2006, Wang & Johnson 2008).

Neurotrophic factors have repeatedly been demonstrated to support survival of neurons and to be protective. Glial cell line-derived neurotrophic factor (GDNF) demonstrated strong evidence of protection in cell culture, monkeys, and rats; however, its clinical studies have not demonstrated an effect in patients (Lang et al. 2006, Nutt et
al. 2003). Likewise a structurally related protein to GDNF, Neurturin, also failed to provide a neuroprotective effect to patients after twelve months (Marks et al. 2010). Although multiple approaches and molecular targets have been investigated to slow the pathological progression in PD, it still remains elusive.

Genetics

Though the etiology is largely unknown for most of the PD cases, the past twenty years has uncovered multiple genetic links associated with PD. Greater than ten forms of familial PD exist, accounting for 5-10% of PD cases. At least twenty genes have demonstrated an association with PD and five of these have conclusively been linked to PD: α-syn, LRRK2, PINK1, Parkin, DJ1 (Martin et al. 2011). Alpha-synuclein is the most abundant protein in, and best biomarker of Lewy Bodies. Inclusions of α-syn, however, are not restricted to PD as they are also prevalent in Dementia with Lewy Bodies, and Multiple System Atrophy. Alpha-synuclein function is thought to play a role in mediating the assembly of SNARE complexes, which directly affect neurotransmitter release. LRRK2’s function is not fully understood, but it has kinase activity considered to be the driving force of its toxicity. PINK1 and Parkin play a role in mitochondrial turnover. PINK1 is a mitochondrial targeted kinase, and Parkin is E3 ubiquitin ligase that functions in protein degradation. Both have been linked to autosomal recessive PD. DJ1 plays a mitochondrial role by maintaining the function and integrity of the available mitochondrial pool [see reviews (Martin et al. 2011, Schapira & Jenner 2011)]. These genes are important targets of research due to their genetic links to PD.
Modeling PD

Multiple models have been developed to replicate the clinical and pathological features of PD, although none have provided the ideal representation. One approach for investigating PD is to use toxins that replicate some of the pathology and phenotype of PD, such as MPTP and rotenone. Another is to replicate known genetic causes of PD in human disease, such as the point mutations of \(\alpha\)-syn or multiplication of wild type \(\alpha\)-syn.

MPTP

The emergence of MPTP as a neurotoxin was in 1982 when young drug addicts developed PD symptoms following the use of street synthesized meperidine which had been contaminated with MPTP. MPTP is a by-product in the chemical synthesis of a meperidine analog (Langston et al. 1983). Both in human and non-human primates it causes an irreversible Parkinsonian syndrome, replicating nearly all of the features seen in PD patients. Following MPTP entering the brain it is converted by monoamine oxidase B in glial cells to its active metabolite MPP+ (Chiba et al. 1984). MPP+ is selectively taken up by the dopamine transporter on neurons, leading to dopaminergic cell loss (Javitch et al. 1985, Javitch & Snyder 1984). Its mechanism in the cell involves inhibition of the complex 1 in the electron transport chain, causing decreasing ATP and increasing reactive oxygen species. A limitation of MPTP models is the acute nature of the pathology, whereas in patients develops over several years to decades the MPTP can occur in hours and days (Jackson-Lewis et al. 1995). Furthermore MPTP treatment has no consistent evidence of Lewy body formation (Beal 2010, Przedborski et al. 2001).
Rotenone

Rotenone is found in roots and stems of several tropical plants. However, it is commonly used as a commercial agent to eliminate insects at home gardens, lice and ticks in pets and humans, and fish in water management (Betarbet et al. 2000). Epidemiological studies have demonstrated an elevated risk of PD for individuals in rural environments and at risk of herbicides and pesticide exposure, suggesting rotenone as a potential model for PD (Tanner et al. 1999). Similar to MPTP, rotenone’s mechanism of toxicity is through the inhibition of complex I of the electron transport chain in the mitochondria. Chronic low doses of rotenone leads to selective nigrostriatal degeneration and α-syn rich inclusions. Behaviorally rats treated with rotenone demonstrate rigidity, decline in locomotors activities, and reduced spontaneous motor activity. Limitations of rotenone include variability in rotenone effect and technical challenges in its administration (Betarbet et al. 2000, Ferrante et al. 1997).

Alpha-synuclein

Alpha-synuclein is expressed throughout the brain at high levels and is the most predominant protein in Lewy bodies. Alpha-synuclein’s role in brain function is not fully elucidated but it is known to localize to presynaptic terminals, bind to lipid membranes, and be involved in vesicle fusion and release of neurotransmitters. Its function has been postulated to negatively regulate activity dependent release of dopamine (Lee & Trojanowski 2006, Volles & Lansbury 2003).

Duplication and triplication of α-syn in humans causes an autosomal dominant form of PD, and a direct relationship with increased α-syn to increased risk of PD and
reduced levels to reduced risk of PD has been observed (Singleton et al. 2003). Certain polymorphisms in the α-syn promoter region have been associated with an increase in α-syn expression and increased susceptibility to idiopathic PD (Pals et al. 2004). Evidence points to 3% of PD cases having polymorphisms in the promoter of α-syn. Furthermore, three point mutations (A30P, E46K, and A53T) of α-syn have been linked as a causative factor for familial PD (Kruger et al. 1998, Polymeropoulos et al. 1997, Zarranz et al. 2004).

Pathologically α-syn is found increased and aggregated in substantia nigra of sporadic PD cases (Spillantini et al. 1997). Although the exact mechanism of α-syn toxicity is not fully understood, it is postulated to be through aggregation, inflammation, cell signaling, and transcriptional dysregulation. Rats and primates overexpressing α-syn by viral vector show dopaminergic cell loss in the substantia nigra (Hashimoto et al. 2003).

**Alpha-synuclein mouse models**

Mouse models of Parkinson’s disease have been developed that overexpress α-syn or express point mutations associated with human PD. Despite the development of several α-syn mouse models, none of them recapitulate all of the PD pathological features (Dawson et al. 2002). One α-syn model by Masliah et al. involves wild type human α-syn overexpression using the PDGFβ promoter. Dopaminergic neurons in the substantia nigra and neocortex show abnormal accumulation of α-syn, consistent with findings in human Lewy body disease. Similarly, α-syn neuritic processes were also observed, along with evident staining of ubiquitin, an accumulated protein seen in LB. Density of the
substantia nigra tyrosine hydroxylase-positive neurons remained the same between overexpressing and non-overexpressing lines; however, a significantly reduced number of tyrosine hydroxylase-positive nerve terminals were observed in the striatum of overexpressing mice. Investigation of the functional deficit using the rotarod revealed deficits in motor performances for overexpressing mice (Masliah et al. 2000).

Our lab investigated the potential effects of α-syn overexpression in this α-syn mouse via gene microarray. Using laser capture microdissection, RNA from the substantia nigra was obtained from transgenic and control mice. A microarray analysis was performed investigating gene expression alterations at 3 months of age, when only a few pathological findings are present. This array revealed over 200 genes altered in expression, with most sharing modest changes. One gene, mouse angiogenin1 (mAng1) was, however, substantially altered compared to the others. A 7.5-fold reduction was seen in expression of mAng1 in overexpressing transgenic mice compared to control mice (Yacoubian et al. 2008).

**Angiogenin**

Angiogenin was first isolated from HT-29 colon adenocarcinoma cell line. It has since been classified in the family of RNases, the only family of vertebrate specific enzymes. The angiogenin gene has a single exon coding for a 14kda protein that is, in its mature form, a 123 a.a. sequence. The functions of angiogenin require three distinct sites: a receptor-binding site, a nuclear localization signal, and a catalytic site. Its functions includes cell migration, invasion, proliferation, angiogenesis, and recently neuroprotection (Osorio et al. 2007).
Angiogenesis initiates with basement membrane degradation, which provides an environment for vasculogenesis. This includes the formation of tubular structures that become covered with pericytes and smooth muscle cells and requires migration and proliferation of endothelial and smooth muscle cells, both of which have been shown to be activated by angiogenin (Hu et al. 1994, Jimi et al. 1995). Angiogenin is known to bind to a cell surface receptor followed by internalization and trafficking to the nucleus. Following localization into the nucleus, angiogenin induces rRNA transcription. This induction of rRNA is a major mechanism by which angiogenin stimulates angiogenesis as it is a rate-limiting step for ribosome biogenesis, a requirement for endothelial cell growth and proliferation (Li & Hu 2011).

Apart from its angiogenic activity, angiogenin is found to be a mediator of stress response in cells. Angiogenin’s catalytic activity cleaves the anti-codon region off tRNAs, producing two tiRNA products a 5’-tiRNA and 3’-tiRNA. The 5’-tiRNAs inhibits protein translation thereby facilitating a conservation of energy for cellular repair. The tiRNAs also stimulate stress granule formations, cytoplasmic foci induced in stressful conditions consisting of untranslated mRNA, as a means of translation inhibition (Emara et al. 2010). These granules sequester mRNA, inducing global suppression of protein translation. Prosurvival and anti-apoptotic gene expression, are not repressed (Li et al. 2011).

**Angiogenin in CNS**

The role of angiogenin in the CNS has been a recent discovery following research performed on ALS and PD patients. In 2004 Greenway et al. did a genetic screening on
169 ALS patients of Irish Scottish descent and identified increased frequency of the G allele of rs11701 SNP in the ALS population compared to controls. This group also identified two ALS patients with an A to T mutation of angiogenin leading to a substitution of lysine to isoleucine at amino acid 40 (Greenway et al. 2004). Genotyping was further done in 2006 on 1629 patients of ALS and 1264 controls, wherein identification of 15 individual with mutations of angiogenin had ALS, four of them familial and eleven were sporadic. Seven missense mutations were associated with ALS in this screening: Q12L, K17E, K17I, R31K, C39W, K40I, and I46V. This study became the first noted association of angiogenin to a disease (Greenway et al. 2006). Based on previous evidence of a potential shared genetic susceptibility with ALS and PD as well as findings that ALS patients with angiogenin variants also demonstrated signs of PD, a broad systemic analysis was performed on PD patients for angiogenin variants (Majoor-Krakauer et al. 1994, van Es et al. 2011). This analysis demonstrated an association between angiogenin variants and PD and noted angiogenin as a genetic link between ALS and PD (van Es et al. 2011).

Angiogenin has been demonstrated to play a role in neuronal survival. In 2007 Subramanian & Feng demonstrated that pharmacological inhibition of angiogenin in mouse neuronal cultures caused a compromise in neurite projections and an increase in neuron clustering. Subramanian et al. investigated the ALS variants of angiogenin and their effect on neurite extension and survival and demonstrated the variant K40I had significant reduction in neurite outgrowth. Differentiated motoneurons treated with angiogenin had increased neuronal survival over control conditions, but variants C39W and K40I had reduced survival. Wild type angiogenin showed a neuroprotective effect to
motoneurons in hypoxic conditions, however, K40I caused an increase in toxicity. The K40I variant revealed the most significant functional effect of the variants studied (Subramanian et al. 2008). The K40I mutation in the catalytic site of the protein is associated with abolishment of ribonucleolytic activity of angiogenin, as well as reduced angiogenic and mitogenic properties (Crabtree et al. 2007). A clear relationship of the ribonucleolytic activity of angiogenin to its function has been demonstrated, as mutagenesis (Shapiro & Vallee 1989), RNase inhibitor (Shapiro & Vallee 1987), or chemical modification (Shapiro et al. 1987) of the enzymatic activity of angiogenin has demonstrated inhibition of its activity including the formation of tiRNAs (Emara et al. 2010). Furthermore mutations enhancing the catalytic activity have demonstrated an increase in angiogenesis (Harper & Vallee 1988).

More evidence for the role of angiogenin in neuronal survival has been demonstrated in multiple systems. Motoneurons placed in hypoxic conditions demonstrate an increase in angiogenin expression that may induce stress granule formation from tiRNA production (Emara et al. 2010, Sebastia et al. 2009). In primary cultures of spinal cord motor neurons treated with AMPA or tunicamycin, two commonly used ALS cellular models, angiogenin co-treatment induced a significant increase in cell survival (Kieran et al. 2008). Primary motoneuron cultures treated with angiogenin also showed reduced cell death in response to hypoxia (Sebastia et al. 2009). Knockdown of angiogenin by siRNA demonstrated an increase in sensitivity to AMPA and hypoxia in a motoneuron cell line (Kieran et al. 2008, Sebastia et al. 2009). The K40I variant, however, was not protective against tunicamycin, serum deprivation, or hypoxia induced toxicity (Kieran et al. 2008).
An ALS mouse model was also analyzed for angiogenin’s ability to alter disease progression. Beginning at both pre-symptomatic (50 days) and post symptomatic (90 days) conditions mice were injected daily with angiogenin. The treated mice in both conditions revealed increased longevity over non-treated SOD1 mice. Furthermore significant protective effect against stride length was observed following treatment. Sciatic motoneuron cell count revealed in both treatments, beginning at 50 days and 90 days, a significant increase in cell survival (Kieran et al. 2008).

Angiogenin’s potential in clinical therapy for disease has been investigated for its safety, tolerability, and effectiveness. In 2008 Zavalishia et al. injected recombinant adenovirus, Ad-VEFG+Ang, bilaterally into quadriceps, deltoid, and trapezius muscles of sporadic ALS patients over 2 year period with injections occurring every 4 weeks. The safety of the virus was evaluated during this time by examination of blood pressure, heart rate, body temperature, body weight, ECG and lab tests of blood and urine. The data demonstrated angiogenin as a safe agent for clinical treatment with no undesirable events observed. The effectiveness of the angiogenin virus on ALS was mitigated by virus neutralizing antibodies in the blood that was significantly increased in Adv-VEGF+ANG treated patients. Although the severity of neurological deficits and vital lung capacity was not improved with treatment, patient survival under hypoxic conditions was significantly improved with angiogenin viral injections (Zavalishin et al. 2008). These results coincide with previous findings of angiogenin overexpression increasing survival of mesenchymal stem cells under hypoxic conditions (Liu et al. 2008). Exogenously treated and angiogenin overexpressing motoneurons have also demonstrated increased survival following hypoxic injury (Sebastia et al. 2009).
Mechanism for Neuroprotection

The mechanism of angiogenin’s cellular function is not fully elucidated and dependent on the cell type, as several pathways have been identified. The function of angiogenin requires three distinct sites: a receptor-binding site (residues 58-70), a nuclear localization sequence (residues 31-35), and a catalytic site (residues 12, 41, 119) (Osorio et al. 2007). Cellular binding of angiogenin has been discovered to be mediated through a 42kda actin receptor, syndecan 4, and an unknown cell-density dependent 170kda receptor (Hu et al. 1997, Hu et al. 1993, Skorupa et al. 2012). Downstream activation of several signaling pathways has been identified as well as endocytosis and trafficking to the nucleus. In human umbilical vein endothelial cells, smooth muscle cells, and motoneurons, angiogenin activates the ERK1/2 and Akt pathway (Kieran et al. 2008, Kim et al. 2007, Liu et al. 2001). In human umbilical artery smooth muscle cells, the SAPK/JNK pathway is also activated (Xu et al. 2001). Each of the above pathways has been considered important for cellular proliferation and angiogenesis (Gao & Xu 2008), but have also been linked to pro-survival signaling (Zeng et al. 2011, Scuteri et al. 2010, Dudek et al. 1997). Pulmonary artery, umbilical vein, and capillary endothelial cells have all demonstrated activation of phospholipase C, which was dependent on angiogenin’s ribonucleolytic activity (Bicknell & Vallee 1988). Angiogenin activity has been found necessary for other angiogenic factors to be effective in cell signaling, as knockdown of angiogenin in endothelial cells resulted in insensitivity to growth factors such as VEGF and bFGF (Kishimoto et al. 2005).

The mechanism of angiogenin’s protective response in neurons was postulated to be through the Akt-P or Erk1/2-P pathway (Kieran et al. 2008). Intracellular signaling of
angiogenin in motoneurons demonstrated an increase in Akt-P and ERK1/2-P. Inhibition of Akt-P pathways by Wortmannin, a PI3K inhibitor, causes an inhibition of protection demonstrating a direct link of Akt to survival induced by angiogenin in motoneurons (Kieran et al. 2008).

**Akt**

The Akt protein is expressed in the brain and plays a cell survival role (Yang et al. 2004). The Akt pathway has been implicated in many diseases and processes including diabetes and cancer (Manning & Cantley 2007, Sen et al. 2003). It is involved in multiple signaling cascades through its activity as a Ser/Thr kinase. Akt is activated by phosphatidylinositol 3-kinase (PI3K). Upon cell surface activation, tyrosine kinase receptors activate PI3K to add a phosphate group to phosphatidylinositol resulting in PI 3,4-bisphosphate (PIP2) becoming PI3,4,5-trisphosphate (PIP3). These products remain in the membrane lipid bilayer to serve diverse functions, including binding of Akt and PDK-1 and recruiting both to the membrane. Upon recruitment PDK-1 activates Akt at Thr308 site with a conformational change that allows phosphorylation of Akt by PDK-2 at Ser 473 site. Phosphorylation of both sites of Akt makes it fully active. Activated Akt then phosphorylates a number of proteins involved in apoptosis and cell survival roles. Akt substrates include several proteins that play a role in cell survival or death: Bax, Bad, GSK3B, Bcl-2, and caspase 9. Phosphorylation of pro-caspase 9 by Akt prevents cleavage of pro-caspase 3, which when cleaved induces DNA fragmentation and cell death [see reviews (Franke et al. 1997, Manning & Cantley 2007)].
Akt’s role in neurological diseases has been well documented. The CNS role of Akt was first demonstrated in cerebellar neurons where it was shown that insulin caused an inhibition of cell death, and mutant Akt inhibited this protective effect (Dudek et al. 1997). Angiogenin has demonstrated activation of the PI3K/Akt pathway in human umbilical vein endothelial cells, as an inhibitor of PI3K prevented endothelial cell migration \textit{in vitro} and angiogenesis \textit{in vivo} (Kim et al. 2007). Models of ALS have demonstrated that Akt activation by angiogenin inhibits AMPA, tunicamycin, and hypoxia toxicity to motoneurons and this is prevented when inhibitors of PI3K/Akt pathway are present. SOD mice, an ALS mouse model, demonstrate an increase in Akt phosphorylation and have increased longevity and sciatic motoneuron cell survival following angiogenin treatment (Kieran et al. 2008). In a neuroblastoma cell line caffeine has demonstrated a prosurvival effect against MPP+ and rotenone through activation of the Akt pathway (Nakaso \textit{et al.} 2008). A constitutively active form of Akt virally transduced into the substantia nigra of mice demonstrated near complete protection against a model PD toxin, 6-OHDA (Ries \textit{et al.} 2006). Post-mortem tissue of PD patients demonstrates a reduction in phosphorylated Akt but not total Akt (Malagelada \textit{et al.} 2008). The exact mechanism of depleted Akt phosphorylation in PD is not elucidated; however, factors associated with the onset of PD may induce the expression of genes responsive to stressful stimuli, such as RTP801, which in turn reduce Akt phosphorylation. RTP801, an inducer of neuronal cell death, is increased in expression in multiple cellular models of PD and suppresses Akt phosphorylation. RTP801 has also been demonstrated to be increased in post-mortem PD brains (Malagelada \textit{et al.} 2008). This suggests that reduced Akt phosphorylation may be a down
stream factor associated with neuronal death in PD, and with increased activation may mitigate the rate of substantia nigra cell loss.

Central Hypothesis

In summary, much evidence points to the neuroprotective potential of angiogenin in PD. In an α-syn overexpressing mouse model of PD, angiogenin mRNA is substantially reduced compared to wild type mice (Yacoubian et al. 2008). Angiogenin variants have been found to be significantly increased in PD patients and ALS patients (van Es et al. 2011, Greenway et al. 2006). Angiogenin is protective in several models of ALS via activation of the PI3K/Akt pathway (Kieran et al. 2008). Based on these data, our overall hypothesis is that angiogenin is protective in models of PD via the activation of the PI3K/Akt pro-survival signaling pathway.

In this dissertation, I examine the potential neuroprotective effects of angiogenin in several models of PD. In chapter one, I describe our findings in two neurotoxin-based in vitro models. In chapter two, I discuss possible mechanisms involved in angiogenin’s effect on these in vitro models of PD. In chapter three, I explain our findings of angiogenin treatment to an in vivo model of PD. Lastly, in the Discussion and Summary I discuss the implications of our findings to angiogenin function and PD.
A NEUROPROTECTIVE ROLE FOR ANGIOGENIN IN MODELS OF PARKINSON’S DISEASE

by

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CHAPTER TWO

A NEUROPROTECTIVE ROLE FOR ANGIOGENIN IN MODELS OF PARKINSON’S DISEASE

Abstract

We previously observed marked downregulation of the mRNA for angiogenin, a potent inducer of neovascularization, in a mouse model of Parkinson’s disease (PD) based on overexpression of alpha-synuclein. Angiogenin has also been recently implicated in the pathogenesis of amyotrophic lateral sclerosis. In this study, we confirmed that mouse angiogenin-1 protein is dramatically reduced in this transgenic alpha-synuclein mouse model of PD, and examined the effect of angiogenin in cellular models of PD. We found that endogenous angiogenin is present in two dopamine-producing neuroblastoma cell lines, SH-SY5Y and M17, and that exogenous angiogenin is taken up by these cells. Applied angiogenin protects against the cell death induced by the neurotoxins MPP+ and rotenone and reduces the activation of caspase-3. Together our data supports the importance of angiogenin in protecting against dopaminergic neuronal cell death and suggests its potential as a therapy for PD.
Introduction

Parkinson’s Disease (PD) is the most common neurodegenerative movement disorder, with approximately 1-2% of the population over 65 years of age affected (Eriksen et al. 2003). While the causes of PD are not well understood, recent studies have implicated the protein alpha-synuclein (α-syn): point mutations or gene multiplication of α-syn cause inherited forms of PD (Singleton et al. 2003, Polymeropoulos et al. 1997, Kruger et al. 1998, Athanassiadou et al. 1999), while in sporadic cases α-syn is found aggregated in the substantia nigra and other brain regions (Spillantini et al. 1997).

We have previously investigated how α-syn may cause toxicity through gene microarray analysis of transgenic mice overexpressing human α-syn under the PDGFβ promoter (Yacoubian et al. 2008). We found over 200 genes with alterations in gene expression at three months, but most of the changes were modest. However, one gene, mouse angiogenin1 (mAng1), was dramatically reduced by 7.5-fold compared to wildtype. In mice six isotypes of angiogenin exist, with mAng1 being the predominantly expressed form and a homolog to the only angiogenin gene in humans (Ibaragi et al. 2009). Angiogenin’s biological roles include cell migration, invasion, proliferation, angiogenesis, and neuroprotection (Kieran et al. 2008, Hu 1998, Hu et al. 1994, Gao & Xu 2008).

Angiogenin has been recently linked to the neurodegenerative disorder, amyotrophic lateral sclerosis (ALS). Several point mutations in angiogenin have been discovered in both sporadic and familial ALS (Greenway et al. 2004, Greenway et al.
Wildtype angiogenin has been shown to reduce neuronal death in *in vitro* models of ALS, and knockdown of angiogenin expression by siRNA promotes cell death (Kieran et al. 2008). In contrast, mutant angiogenin inhibits neurite extension and promotes hypoxia-induced cell death in motoneurons (Subramanian & Feng 2007, Subramanian *et al.* 2008, Sebastia *et al.* 2009). *In vivo*, systemic treatment with wildtype angiogenin increases motoneuron survival, delays motor dysfunction, and increases lifespan of mutant superoxide dismutase 1 mice, a model for ALS (Kieran et al. 2008). This neuroprotective effect appears to be mediated by activation of the PI3K/Akt pathway, as inhibition of this pathway disrupts angiogenin’s neuroprotection *in vitro* (Kieran et al. 2008).

Because angiogenin levels are reduced in a transgenic α-syn mouse of PD and angiogenin is protective in models for ALS, we hypothesized that increasing angiogenin may also provide protection in PD. Here we examine whether exogenous angiogenin protects against cell death in cellular models for PD. We found that angiogenin reduces cell death in response to both rotenone and 1-methyl-4-phenylpyridinium (MPP+) in dopaminergic cell lines, and this protection appears to be mediated through inhibition of apoptosis via the PI3K-Akt signaling pathway.

**Methods**

*Cell Culture.* SH-SY5Y cells were a gift from J. Zhang (Birmingham, AL) and were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SK-N-BE(2)-M17(M17) cells (ATCC, Manassas, VA) were cultured in a 1:1 mix of Eagle’s minimum essential media and F12K media supplemented
with 10% FBS. Both cell lines were cultured at 37°C with 5% CO2 and 95% air atmosphere in a humidified incubator.

Animals. α-Syn transgenic animals originally created by Masliah et al. (2000) were bred at Charles River Laboratories (Wilmington, MA). Three-month-old wildtype and transgenic mice were sacrificed by CO2 inhalation. The use of animals was supervised by the Massachusetts General Hospital and University of Alabama at Birmingham Animal Resources Programs in accordance with the PHS policy on the Humane Care and Use of Laboratory Animals. Cortical tissue from three-month-old α-syn knockout mice and wildtype littermates were a gift from Robert Nussbaum (Cabin et al. 2002).

Western Blot Analysis. SH-SY5Y cells were sonicated in lysis buffer (150nM NaCl, 10mm Tris-HCl (pH 7.4), 1mM EGTA, 1mM EDTA, 0.5%NP-40, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)) and centrifuged at 16000g for 10 min at 4°C. Protein concentrations of supernatants were determined using the bicinchoninic assay (Pierce, Rockford, IL). Each sample was boiled for 5 min in 4 x DTT sample loading buffer (8% SDS, 0.25M Tris-HCl, 200mM DTT, 30% glycerol, and Bromophenol Blue), resolved on 12% SDS-polyacrylamide gels, and transferred electrophoretically to 0.45-μm nitrocellulose membranes at 100V for 1 hour. Following transfer, membranes were incubated for 30 min in 5% non-fat dry milk in TBST (25mM Tris-HCl pH 7.6, 137mM NaCl, 0.1% Tween 20) and then incubated overnight at 4°C in primary goat polyclonal antibody against angiogenin (1:500; R&D Systems, Minneapolis, MN), rabbit or mouse monoclonal antibody against α-tubulin (1:10000; Sigma, St. Louis, MO). After three washes in TBST, blots were incubated for two hours with HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:2000;
Jackson ImmunoResearch, West Grove, PA) and then washed in TBST six times for 10 minutes each. Super signal chemiluminescence (Pierce, Rockford, IL) was used to detect protein bands. Quantification of bands was performed using densitometry, and each band was normalized to the average of all bands on each blot.

For mouse brain samples, cortex was homogenized in TEVP buffer (10mM Tris-HCl, pH 7.4, 5mM sodium fluoride, 1mM sodium orthovanadate, 1mM EDTA, 1mM EGTA) with 320mM sucrose and centrifuged at 15000g for 16 minutes. Pellets were resuspended and sonicated in TEVP buffer supplemented with protease inhibitors (Roche). Immunoblotting against angiogenin was performed as described above, using a goat polyclonal antibody against angiogenin.

**Immunocytochemistry:** SH-SY5Y cells were washed and fixed in 4% paraformaldehyde for 30 min. After permeabilization in 0.1% Triton X-100 and blocking with 1% normal donkey serum, cells were incubated with goat polyclonal antibody against angiogenin overnight at 4C. After three washes in TBS, cells were incubated in cy3-conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch) for two hours at room temperature. Cells were rinsed with TBS for two times and once with distilled water prior to cover slipping. Internalization of angiogenin was visualized using a confocal scanning microscope (Leica Microsystems, Exton, PA).

**LDH Assay:** Cells were plated in 24-well plates for 8 hours, and then pretreated with angiogenin (100nM) for 12 hours. Rotenone (0.2μM) or MPP+ (1mM) was then added with angiogenin into serum-free media for another 24 hours. Cell death was measured by
lactate dehydrogenase (LDH) release into media using a LDH assay kit (Roche). LDH release was normalized to maximal LDH release for each well.

**Statistical Analysis:** All analysis of experiments were performed with GraphPad Prism5 (GraphPad Inc., LaJolla, CA). LDH assay experiments were analyzed with one-way ANOVA followed by post-hoc Bonferonni’s test. Protein blot analysis was performed using either unpaired t-test, one group t-test with Bonferroni correction, or one-way ANOVA with a post-hoc Tukey’s test.

**Results**

*Angiogenin expression is reduced in α-syn transgenic mice*

Our previous microarray analysis of α-syn transgenic mice revealed a substantial reduction in the mRNA for mAng1, the mouse homolog for human angiogenin, at three months (Yacoubian et al. 2008). Among all genes evaluated in this study, mAng1 was the most altered and was reduced by 7.5-fold (Fig. 1a). Tyrosine hydroxylase expression was not significantly altered in these mice at three months of age (Yacoubian et al. 2008). We extended this analysis by studying the expression of angiogenin protein in the same mouse model. To investigate this we used the cortices of the mice investigated in the gene microarray, as they also represent pathology of the substantia nigra with diffuse α-syn inclusion formations. Cell lysates from the cortex of both transgenic and wildtype littermates were run on a SDS-polyacrylamide gel and immunoblotted with an antibody against angiogenin (Fig. 1b). We found that protein levels of angiogenin were reduced by 70% in transgenic mice at three months by Western blotting (Fig. 1c). To further examine a relationship between α-syn and angiogenin, we analyzed angiogenin
expression in the cortices of α-syn knockout mice. As our interest was in angiogenin protein levels, we investigated angiogenin protein levels by western blot. We found no significant differences in angiogenin protein between α-syn knockout mice and wildtype littermates (Fig. 1d, e).

**SH-SY5Y and M17 cells express and respond to angiogenin**

To evaluate the potential neuroprotective role of angiogenin in cell culture models of PD, we first examined whether commonly used dopaminergic (DA) cell lines can synthesize and respond to angiogenin. Using both quantitative PCR and Western blotting, we found that both the mRNA and the protein for angiogenin were detectable in SH-SY5Y and SK-N-BE(2)-M17 (M17) dopamine-producing neuroblastoma cell lines (Fig. 2a). Immunostaining of native SH-SY5Y cells revealed a low level of intracellular angiogenin protein with a punctate distribution (Fig. 2b).

We next examined if the SH-SY5Y dopaminergic cell line could take up exogenous angiogenin. Recombinant angiogenin was applied at 100nM to SH-SY5Y cells in culture, and the cells were then fixed and immunostained for angiogenin at varying time points. We detected a clear increase in angiogenin staining at two hours in these cells, and the intensity of the intracellular angiogenin staining continued to increase for up to 36 hours (Fig. 2b). Mild increase in angiogenin staining was detected as early as 15 minutes.
Angiogenin is protective against the neurotoxin MPP+

Having shown that SH-SY5Y cells can respond to angiogenin, we next investigated whether angiogenin can reduce toxicity in response to the neurotoxin MPP+, the active metabolite of MPTP which induces a Parkinsonian-like syndrome in animals and humans (Przedborski et al. 2001). SH-SY5Y cells were first pretreated with angiogenin for 12 hours and then co-treated with MPP+ and angiogenin for another 24 hours. Cell death was determined by LDH release into the media. Angiogenin reduced cell death in response to MPP+ in a dose-dependent manner (Fig. 3a).

Prior research has demonstrated an inverse relationship between angiogenin’s biological activity and cell density. Biological activity is reduced with increasing cell density, an effect which may be related to alterations in angiogenin receptor levels (Moroianu & Riordan 1994, Hatzi et al. 2000). Here we tested the relationship of cell density to angiogenin’s neuroprotective effect in SH-SY5Y cells. Angiogenin (100nM) reduced cell death induced by MPP+ at all densities tested; however, the effect was significantly greater at lower cell densities. At the lowest density tested (30,000 cells/well), cell death induced by MPP+ was blocked almost entirely by the addition of angiogenin (Fig. 3b).

Since MPP+ induces apoptosis in SH-SY5Y cells (Gomez et al. 2001), we next tested whether angiogenin alters the apoptotic cascade by evaluating caspase-3 activation. SH-SY5Y cells were pretreated with angiogenin for 12 hours followed by treatment with 1mM MPP+ with or without angiogenin for 24 hours, and cleaved caspase-3 levels were...
determined by Western blotting of cell lysates (Fig. 3a). Cells pretreated with angiogenin showed a significant reduction in the induction of cleaved caspase-3 by MPP+ (Fig. 4b).

**Angiogenin is also protective against rotenone**

We next asked whether angiogenin’s neuroprotective effect in SH-SY5Y cells was limited to MPP+ or whether it could protect against other neurotoxins. We turned to the pesticide rotenone, which has been shown to induce a Parkinsonian syndrome in rodents (Betarbet *et al.* 2000). In SH-SY5Y cells, we found that pretreatment with 100nM angiogenin markedly reduced cell death in response to rotenone (0.2μM), as determined by LDH release. Protection was seen at both cell densities tested, with more complete protection at the lower cell density (Fig. 5a). A similar protective effect of angiogenin against rotenone toxicity was found in M17 cells (Fig. 5b).

**Discussion**

In this study, we provide evidence that angiogenin plays a protective role in PD models. We demonstrate that mouse angiogenin levels are reduced in a transgenic mouse model in which α-syn is overexpressed. Dopaminergic cell lines can take up exogenous angiogenin *in vitro*, and application of angiogenin to these cell lines reduces rotenone and MPP+ toxicity in a dose and cell density-dependent manner. This reduction in toxicity is associated with a reduction in apoptosis, as determined by caspase-3 cleavage. The increased dose of MPP+ used to demonstrate the caspase-3 cleavage, may reflect the different passage number of SH-SY5Y and their sensitivity to MPP+, as well as the technical issues relating to the sensitivity between lactate dehydrogenase release as compared to western blotting of caspase-3 cleavage. Although not investigated in the
present study, rotenone has been associated with caspase-3 cleavage, and angiogenin’s effect in rotenone toxicity is also likely mediated through inhibition of this pathway (Li et al. 2003). Treatment of cells with angiogenin is also associated with Akt phosphorylation, suggesting that angiogenin may reduce apoptotic signaling through activation of this survival pathway.

We first discovered a potential link of angiogenin to PD through our gene microarray study which investigated alterations in gene expression in a transgenic α-syn mouse. As determined by in situ hybridization, mAng1 is normally expressed at highest levels in the granule cell layer of the cerebellum, the dentate granule cells and CA1 and CA3 pyramidal neurons of the hippocampus, and the main olfactory bulb in mouse brain, while lower levels are detected ubiquitously in the brain, including the substantia nigra and cortex (Lein et al. 2007). In the rat brain, angiogenin protein has been detected in the cortex and subcortical regions (Huang et al. 2009). In the microarray study, we found that mAng1 was the gene most reduced in expression in the substantia nigra of three-month-old transgenic mice (Yacoubian et al. 2008). Here we confirm that angiogenin protein expression is also reduced in the cortex of α-syn transgenic mice. At three months of age these transgenic mice show minimal pathological or behavioral changes but with age develop α-syn inclusions, reduction in striatal dopamine levels, and motor deficits (Masliah et al. 2000). Since the loss of angiogenin expression is an early feature of this transgenic model, it is possible that the reduction in this protein contributes to the subsequent degenerative changes. This view, while speculative, is consistent with our finding that exogenous angiogenin promotes cell survival in the face of PD toxins such as
MPP+ and suggests that endogenous angiogenin may play a role in dopaminergic cell function and survival in vivo.

It is unclear how overexpression of α-syn causes decreased angiogenin expression in this mouse model. One possibility is that α-syn normally negatively regulates angiogenin expression in the brain. Indeed, α-syn has been shown to affect transcription via inhibition of histone acetylation (Kontopoulos et al. 2006). Therefore, α-syn overexpression could cause a reduction in angiogenin levels via this mechanism. We did evaluate whether a reciprocal increase in angiogenin levels is seen in an α-syn knockout mouse, but we did not detect any differences between wildtype and knockout mice. This finding suggests that α-syn does not regulate angiogenin levels under normal circumstances. Instead, an alteration in angiogenin expression is likely a pathological consequence of α-syn overexpression. Consistent with this, functional genomic analysis of our microarray study has shown that the main consequence of α-syn overexpression in the α-syn mouse is alteration of genes involved in transcriptional processes (Yacoubian et al. 2008).

Regardless whether or not reduced angiogenin levels play a role in the pathogenesis of PD pathology, the ability of exogenous angiogenin to reduce cell death in PD models suggests that increasing angiogenin levels could serve as a means to slow degeneration in PD. The mechanism for angiogenin’s neuroprotective effects in this and other model systems is unclear, but the PI3K/Akt pathway may play an important role. In ALS models blockade of the PI3K/Akt pathway prevents the neuroprotective effects of angiogenin (Kieran et al. 2008). Activation of this pathway has been previously shown to be protective in the 6-OHDA mouse model (Ries et al. 2006), and interestingly
postmortem tissue from PD patients demonstrates depletion of phosphorylated Akt (Malagelada et al. 2008). Akt signaling can result in downstream inhibition of apoptosis through phosphorylation of its substrates, such as Bad, caspase-9, and FOXO (Burke 2007, Nakaso et al. 2008, Woodgett 2005, Zhou et al. 2000). We demonstrate that caspase-3 cleavage, a marker of apoptosis, was increased in SH-SY5Y cells following MPP+ treatment and that angiogenin inhibited this effect. Caspase-3 activation has been linked to PD. Adult mice show increased caspase-3 cleavage in the substantia nigra following MPTP administration (Turmel et al. 2001, Yu et al.). Similarly, post-mortem PD brains demonstrate apoptotic cell death with increased caspase-3 cleavage in nigral neurons (Hartmann et al. 2001). Our results indicate the protective effect of angiogenin may be mediated through inhibition of apoptosis.

In summary our findings demonstrate a neuroprotective role of angiogenin in dopaminergic cell lines, similar to findings in motoneurons. It is possible that angiogenin may act as a general pro-survival factor, yet our findings of the decrease in angiogenin levels in a mouse model of PD suggest that alterations in angiogenin may play a specific role in disease pathophysiology. The neuroprotective mechanism includes inhibition of the apoptotic cascade. In clinical trials of ALS, angiogenin has been delivered by gene therapy approaches (Zavalishin et al. 2008). Systemic delivery may also be feasible: in vivo studies show that angiogenin administered by intraperitoneal injections can cross the blood-brain barrier and have therapeutic effects (Kieran et al. 2008). Either of these approaches might be used to deliver angiogenin to PD patients, making it a potentially promising approach as a new therapy to slow neurodegeneration.
**Conflicts of Interest:**

Trent Steidinger, Talene Yacoubian, and David Standaert declare no potential conflict of interest.

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Figures

a. 

Fold Reduction in Expression

b. 

α-syn transg | wildtype

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20kD | 50kD

c. 

Relative ang expression (normalized to tubulin)

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d. 

α-syn KO | wildtype

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Relative ang expression (normalized to tubulin)

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Figure 1. Mouse angiogenin 1 levels are reduced in transgenic mice overexpressing α-syn.

a. mAng1 is the most downregulated gene in a gene microarray study evaluating gene expression in the substantia nigra of transgenic α-syn mice compared to wildtype littermates. The top 20 most downregulated genes are shown from three-month-old mice. This data was extracted from the supplemental data of our previous publication (Yacoubian et al. 2008).

b. mAng1 protein levels are also reduced in the cortex of three-month-old transgenic mice. Representative Western blot from lysates from transgenic and wildtype mouse cortices.

c. Quantification of Western blotting against angiogenin by densitometry. Tubulin was used as a loading control, and angiogenin levels were normalized to tubulin. **p<0.01 (unpaired t-test). Error bars reflect SEM.

d. Angiogenin expression is not altered in the cortex of three-month-old α-syn knockout mice. Representative Western blot from lysates of knockout and wildtype mouse cortex homogenate.

e. Quantification of Western blotting against angiogenin by densitometry. Tubulin was used as a loading control, and angiogenin levels were normalized to tubulin. ns = no significant difference (unpaired t-test). Error bars reflect SEM.
Figure 2. Dopaminergic cell lines express and take up angiogenin.

a. Western blotting reveals endogenous levels of angiogenin produced by both M17 and SH-SY5Y dopaminergic cell lines. Tubulin was used as loading control.

b. A robust increase in intracellular angiogenin staining is observed after treatment with exogenous angiogenin. SH-SY5Y cells were immunostained using a goat polyclonal antibody against angiogenin and a cy3-conjugated donkey anti-goat antibody at A) control, B) 2 hours, C) 12 hours, and D) 36 hours after treatment with 100nM recombinant angiogenin. Scale bar represents 25µm.
Figure 3. Exogenous angiogenin reduces MPP+ toxicity in SH-SY5Y cells.

a. Angiogenin demonstrates a concentration-dependent protective response to MPP+ induced cell death. Cells were plated 150,000 cells/well and pretreated with varying concentrations of angiogenin for 12 hours prior to treatment with 0.5mM MPP+. Cell death was assayed by LDH release into media. n=6. ***p<0.001 (one-way ANOVA followed by post-hoc Bonferroni’s test). Error bars reflect SEM.

b. Angiogenin’s neuroprotective effect against MPP+ varies with cell density. SH-SY5Y cells were plated at cell densities ranging from 30,000 cells/well up to 125,000 cells/well and pretreated with 100nM angiogenin for 12 hours prior to treatment with 1 mM MPP+. Cell death was assayed by LDH release. n=4. ***p<0.001 (one-way ANOVA followed by post-hoc Bonferroni’s test). Error bars reflect SEM.
Figure 4. Exogenous angiogenin reduces caspase-3 cleavage in response to MPP+ treatment.

a. Representative Western blot against cleaved caspase-3 of SH-SY5Y cells treated with 1mM MPP+ with or without 100nM angiogenin. Tubulin was used as a loading control.

b. Densitometric quantification of Western blots against cleaved caspase-3. Results reflect three independent experiments. Each condition was normalized to MPP+-treated condition for the given blot. **p<0.01 (one group t-test with Bonferroni correction). Error bars reflect SEM.
Figure 5. Angiogenin reduces cell death induced by rotenone.

a. Exogenous angiogenin reduces rotenone-induced toxicity in SH-SY5Y cells in a cell-density-dependent manner. Cells were plated at either 30,000 or 50,000 cells/well and pretreated with angiogenin (100nM) prior to treatment with 0.2μM rotenone. n=4.

b. Angiogenin reduces rotenone toxicity in a different dopaminergic cell line, M17. n=4. *p<0.05 (one-way ANOVA followed by post-hoc Tukey’s test). Error bars reflect SEM.
CHAPTER THREE

ANGIOGENIN’S ACTIVATION OF AKT IS NOT A REQUIREMENT FOR NEUROPROTECTION IN DOPAMINERGIC NEURONS

Abstract

Angiogenin, an angiogenic factor, has recently been linked to Amyotrophic Lateral Sclerosis (ALS) and Parkinson Disease (PD). We have previously shown that angiogenin is protective in a neurotoxin model of PD. Here we examine whether Akt activation is required for angiogenin’s protective effect in this model, as has been demonstrated for ALS models. We observed a rapid induction of Akt phosphorylation in SH-SY5Y cells with angiogenin treatment, but not the K40I mutant. However, inhibition of Akt with a dominant negative Akt did not reduce the protective effects of angiogenin against MPP+. The angiogenin mutant, K40I, was similarly protective as wildtype angiogenin against MPP+. This study demonstrated that Akt phosphorylation is not a critical component in angiogenin’s protective response in the MPP+ model.
Introduction

Angiogenin, originally identified as a potent angiogenic factor, has recently been associated with neurodegenerative diseases, such as ALS and PD (Greenway et al. 2006, van Es et al. 2011). Research has since revealed that wildtype angiogenin provides protection against hypoxia, serum deprivation, ER stress, and excitotoxicity in motoneurons (Kieran et al. 2008, Subramanian & Feng 2007, Sebastia et al. 2009). Our lab previously demonstrated a robust down-regulation of angiogenin expression in a mouse model of PD (Yacoubian et al. 2008, Steidinger et al. 2011). This discovery, in combination with recent evidence of angiogenin’s neuroprotective role in ALS (Kieran et al. 2008), led us to investigate the neuroprotective role of angiogenin in PD models. We demonstrated that exogenous angiogenin reduced toxicity by rotenone and 1-methyl-4-phenylpyridine (MPP+) in two different dopaminergic cell lines in a cell density-dependent manner (Steidinger et al. 2011).

Angiogenin, having ribonucleolytic and angiogenic activities, acts in multiple capacities including cell migration, invasion, proliferation, angiogenesis, and recently neuroprotection (Gao & Xu 2008). Angiogenin is known to localize to the nucleus where it induces rRNA translation and pro-survival protein expression (Xu et al. 2002b, Li et al. 2010). In endothelial and smooth muscle cells, angiogenin is linked to the induction of several pathways including stress-associated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), phospholipase C (PLC), extracellular signal-related kinase 1/2 (ERK1/2), and Akt (Liu et al. 2001, Kim et al. 2007, Kieran et al. 2008, Xu et al. 2002a, Bicknell & Vallee 1988). Akt activation has been linked to angiogenin’s neuroprotective effects in ALS models. In motoneurons and in SOD1 mice angiogenin induced Akt
phosphorylation and was found to be neuroprotective. In contrast, the ALS-associated mutant K40I failed to induce Akt phosphorylation and failed to be protective in motoneurons. Furthermore, the PI3K inhibitor wortmannin reduced angiogenin’s neuroprotective effect against AMPA-induced toxicity in motoneurons (Kieran et al. 2008).

Parkinson disease has been associated with alterations of Akt. Post-mortem tissue of PD patients has revealed reduced levels of phosphorylated Akt, but not total Akt (Malagelada et al. 2008, Hashimoto et al. 2004). Furthermore, a constitutively active form of Akt which was virally transduced into mice, demonstrated near complete protection against 6-hydroxydopamine, a toxin used to model PD (Ries et al. 2006).

Based on these findings, we investigated whether activation of the PI3K-Akt pathway is required for angiogenin’s protective effect in PD models. We first tested whether angiogenin induces Akt phosphorylation. We then investigated whether inhibition of this pathway reduces the protective response of angiogenin seen in cellular models of PD.

Methods

Cell Culture

SH-SY5Y cells were a gift from J. Zhang (Birmingham, AL) and were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SK-N-BE(2)-M17(M17) cells (ATCC, Manassas, VA) were cultured in a 1:1 mix of Eagle’s minimum essential media and F12K media supplemented with 10%
FBS. Both cell lines were cultured at 37C with 5% CO2 and 95% air atmosphere in a humidified incubator.

Transfection

DN-Akt (K179M) construct, in a pCMV5 construct, along with an empty vector was obtained from Addgene (Cambridge, MA). SH-SY5Y cells were transfected with the DN-Akt or an empty pCMV5 plasmid using the Amaxa nucleofector (Lonza, Walkersville, MD) under the D-017 protocol. Two million cells were used for each transfection with 5ug of plasmid.

Western Blot Analysis

SH-SY5Y cells were sonicated in a lysis buffer (150nM NaCl, 10mm Tris-HCl (pH 7.4), 1mM EGTA, 1mM EDTA, 0.5%NP-40, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)) and centrifuged at 16000g for 10 min at 4C. Protein concentrations of supernatants were determined using the bicinchoninic assay (Pierce, Rockford, IL). Each sample was boiled for 5 min in a 4 x DTT sample loading buffer (8% SDS, 0.25M Tris-HCl, 200mM DTT, 30% glycerol, and Bromophenol Blue), resolved on 12% SDS-polyacrylamide gels, and transferred electrophoretically to 0.45-
\( \mu \)m nitrocellulose membranes at 100V for 1 hour. Following transfer, membranes were incubated for 30 min in 5% non-fat dry milk in TBST (25mM Tris-HCl pH 7.6, 137mM NaCl, 0.1% Tween 20) and then incubated overnight at 4C in mouse monoclonal antibody against HA (1:1000 Covance, Emeryville, CA), rabbit polyclonal antibody against Akt phosphorylated at serine 473 (1:1500 Cell Signaling, Danvers, MA), rabbit polyclonal antibody against total Akt (1:1500; Cell Signaling, Danvers, MA), or mouse
monoclonal antibody against α-actin (1:10000; Sigma, St. Louis, MO). After three washes in TBST, blots were incubated for two hours with HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:2000; Jackson ImmunoResearch, West Grove, PA) and then washed in TBST six times for 10 minutes each. Super signal chemiluminescence (Pierce, Rockford, IL) was used to detect protein bands. Quantification of bands was performed using densitometry, and each band was normalized to the average of all bands on each blot.

*LDH Assay*

Transfected cells were plated in 24-well plates for 6-8 hours, and then pretreated with angiogenin (100nM) for 12 hours. MPP+ (0.75mM) was then added with angiogenin into serum-free media for another 24 hours. Cell death was measured by lactate dehydrogenase (LDH) release into media using a LDH assay kit (Roche). LDH release was normalized to maximal LDH release for each well.

*Statistical Analysis*

All analysis of experiments was performed with GraphPad Prism5 (GraphPad Inc., LaJolla, CA). LDH assay and protein blot analysis was performed using a one-way ANOVA with a post-hoc Tukey’s test.

*Results*

*Angiogenin induces Akt phosphorylation in the SH-SY5Y cell line*

We examined the effect of exogenous angiogenin on Akt phosphorylation in SH-SY5Y cells. Cells were treated with 100nM angiogenin and cell lysates were collected at
30 seconds, 1 minute, 15 minutes, 6 hours, and 12 hours. Levels of phosphorylated (Ser473) Akt were determined by western blotting (Fig. 1a). As early as 30 seconds after application, angiogenin increased phospho-Akt significantly, and the level of phospho-Akt remained elevated for several hours (Fig. 1b). The increase in phospho-Akt was not secondary to changes in total Akt levels, as levels of total Akt remained stable at all evaluated time points.

Akt inhibition does not inhibit angiogenin protective effects

To investigate if angiogenin’s protective effect against MPP⁺ is mediated through the PI3K/Akt pathway, we tested whether inhibition of this pathway would impede the protective response. We attempted to disrupt this pathway through three different mechanisms: 1) pharmacological agents, 2) siRNA knockdown, and 3) dominant negative Akt.

We first attempted to inhibit the PI3K pathway with pharmacological agents: wortmannin and LY294002. Both of these agents are commonly employed inhibitors of the PI3K/Akt pathway, and they function through inhibition of PI3K activity by irreversible (wortmannin) and reversible (LY294002) mechanisms (Powis et al. 1994, Vlahos et al. 1994). We first demonstrated an inhibition of Akt phosphorylation in SH-SY5Y cells with wortmannin treatment; however, due to the short half-life of wortmannin (Holleran et al. 2003), we saw a lack of inhibition to Akt phosphorylation after four hours in culture (Fig 2a). Due to our experimental paradigm being over 36 hours, this approach was not feasible. LY294002 was then investigated because of its longer half-life (Okkenhaug & Vanhaesebroeck 2001). We demonstrated a strong
inhibition of Akt phosphorylation with LY294002 at 30uM and higher, but not at 10uM (Fig 2b). Using this agent, we demonstrated a reduction of angiogenin protection against MPP+; however, the addition of LY294002 to MPP+ increased the baseline toxicity response. Therefore, whether the increase in cell death was from inhibition of Akt phosphorylation or increased toxicity from the combination of LY294002 and MPP+ was unclear. Despite lowering doses of LY294002 to 10uM, an increase in MPP+ toxicity was still present (Fig 2c).

Next, we attempted inhibition of the Akt pathway by RNAi. Using Amaxa nucleofector, we transfected 2uM Akt siRNA into SH-SY5Y cells. Akt knockdown was seen by western blot as early as 72 hours following transfection. However, similar to the pharmacological approach, increased toxicity was seen when MPP+ and siRNA were combined together, as opposed to MPP+ alone (Fig 3).

Lastly, we investigated whether a dominant-negative Akt construct would inhibit angiogenin protection. The kinase defective mutant of Akt (K179M) has been widely used to inhibit the Akt signaling pathway (Dudek et al. 1997, Franke et al. 1997, Tanno et al. 2002), and it has demonstrated effectiveness in our cell line of interest, SH-SY5Y (Kang et al. 2003). Following transfection, we saw expression of the K179M Akt protein by western blot in as early as twelve hours. This dominant negative blocks Akt signaling in SH-SY5Y cells as demonstrated by a lack of Akt phosphorylation in response to cells treated with Insulin Growth Factor (Fig 4a). Angiogenin (100nM) was applied 24 hours following transfection and 12 hours later cells were treated MPP+. Twenty-four hours following MPP+ treatment cells were collected and analyzed for caspase-3 cleavage by western blot (Fig 4b). Our results indicate that angiogenin is still protective against
MPP+ in the presence of DN-Akt, suggesting that Akt activation is not a critical mechanism for angiogenin’s effect in the SH-SY5Y dopaminergic cell line (Fig 4c).

**K40I mutant fails to induce Akt phosphorylation but remains neuroprotective**

The ALS-associated mutant K40I has demonstrated an inability to be protective in multiple ALS models (Kieran et al. 2008). Furthermore, K40I has been shown to be ineffective at providing protection against other neuronal-toxicity models including hypoxia and serum deprivation (Subramanian et al. 2008, Sebastia et al. 2009). While the mechanism whereby K40I is ineffective compared to wild-type angiogenin is not fully understood, it has been postulated to be a result of its inability to phosphorylate Akt (Kieran et al. 2008). Therefore we first tested whether K40I mutant could induce Akt phosphorylation. Angiogenin (100nM) and K40I (100nM) was applied to SH-SY5Y cells for 30 seconds. Cells were then collected and analyzed for phosphorylated Akt Ser437 by western blot, where it was observed that K40I failed to induce Akt phosphorylation (Fig 5a). We next investigated whether K40I was neuroprotective against MPP+ toxicity. SH-SY5Y cells were pretreated with wildtype angiogenin and K40I for 12 hours and then co-treated with MPP+ and wildtype or K40I for another 24 hours. Cell death was determined by LDH release into the media. Surprisingly, our results indicate that K40I provides no significant difference compared to wildtype angiogenin against MPP+ induced toxicity in the SH-SY5Y cell line (Fig 5b).

**Discussion**

In this study we have investigated the role of Akt phosphorylation in angiogenin’s neuroprotective response. We show that angiogenin phosphorylates Akt in a
dopaminergic cell line, but this is not critical for angiogenin’s neuroprotective effect against MPP+ toxicity. We found that angiogenin induces Akt phosphorylation very rapidly, within thirty seconds, and Akt phosphorylation remains present for several hours. Furthermore, we demonstrated that inhibition of Akt phosphorylation does not significantly alter angiogenin’s neuroprotective effect. Additionally, the K40I mutant failed to induce Akt phosphorylation, but was still protective against MPP+.

The mechanism for angiogenin’s neuroprotective effect is not fully elucidated, but the PI3K/Akt pathway has been implicated for its effect in ALS models. In a model of ALS, inhibition of the PI3K/Akt pathway blocked the neuroprotective response of angiogenin (Kieran et al. 2008). Furthermore, the ALS-associated mutant (K40I), which is unable to phosphorylate Akt, is not protective in multiple neuronal assays (Kieran et al. 2008, Subramanian et al. 2008, Sebastia et al. 2009). Consistent with these observations, we find that angiogenin induces phosphorylation of Akt at serine 473 in SH-SY5Y cells (Fig. 1), and that Akt phosphorylation is not induced by the ALS-associated mutant, K40I (Fig 5a). Angiogenin could activate Akt phosphorylation by binding an extracellular receptor, or it may become endocytosed followed by activation of an intracellular signaling cascade. We see Akt phosphorylation within seconds as measured by western blot, whereas we observed angiogenin uptake after minutes by immunocytochemistry (Steidinger et al. 2011). Investigation of angiogenin and K40I uptake into the cell was not investigated in relation to Akt phosphorylation. Previous findings have demonstrated uptake of K40I in SH-SY5Y cells (Cho et al. 2010b), demonstrating uptake is not a factor limiting Akt phosphorylation. The rapid activation of Akt indicates a potential extracellular receptor-mediated signaling response, rather than an induction of Akt
phosphorylation after endocytic uptake of angiogenin. It is proposed that an unknown 170 kDa receptor that binds angiogenin is capable of activating PI3K/Akt signaling in endothelial cells and to respond in a cell density-dependent manner (Hu et al. 1997, Kim et al. 2007, Trouillon et al. 2010), similar to our previous findings with neuroprotection (Hu et al. 1997, Kim et al. 2007, Trouillon et al. 2010, Steidinger et al. 2011). Akt signaling can result in downstream inhibition of apoptosis through phosphorylation of its substrates, such as Bad, caspase-9, and FOXO (Burke 2007, Nakaso et al. 2008, Woodgett 2005, Zhou et al. 2000). We previously demonstrated that caspase-3 cleavage, a marker of apoptosis, was increased in SH-SY5Y cells following MPP+ treatment and that angiogenin inhibited this effect (Steidinger et al. 2011).

Based on the above findings, we initially proposed Akt to be a critical and necessary signaling pathway for angiogenin’s protective effect in a dopaminergic cell line. Contrary to our expectations we demonstrate that Akt may mediate pro-survival signaling in SH-SY5Y cells, but is not a necessary signaling component for protection by angiogenin. Limitations to our experimental paradigm include the lack of certainty of a complete inhibition to the Akt signaling cascade. To identify full inhibition of Akt, full analysis of Akt substrates would be necessary. Akt has been identified to have numerous substrates making this method not a feasible approach (Manning & Cantley 2007). However, we believe our findings with angiogenin mutant, K40I, validates the lack of Akt as a required signaling pathway for protection in SH-SY5Y cell line. We demonstrated that contrary to wild type angiogenin, K40I did not induce Akt phosphorylation and yet showed no significant difference to angiogenin’s affect on
toxicity. Together we believe our findings support Akt as not being a required factor for neuroprotection of SH-SY5Y cells to MPP+ toxicity.

Other components of angiogenin’s prosurvival function, not investigated here, include activation of NF-κB to promote cellular survival as well as induction of expression of the anti-apoptotic factor Bcl-2. In a P19 cell line, angiogenin’s anti-apoptotic effect was determined dependent on Bcl-2, as knockdown of Bcl-2 demonstrated in increase in apoptosis (Li et al. 2011). NF-κB, a widely expressed transcriptional factor known to play a role in cell survival, is another protein that angiogenin has been found to increase in expression as well as increase its nuclear localization (Li et al. 2010). Another pathway activated by angiogenin is the Erk1/2 pathway (Liu et al. 2001, Kieran et al. 2008). Angiogenin treated to SH-SY5Y cells prevented hydrogen peroxide induced cell death and increased ERK1/2 phosphorylation (Cho et al. 2010a). Furthermore, angiogenin treated SOD1 mice demonstrated an increase of ERK1/2 phosphorylation and had increased longevity, stride length, and survival of motoneurons (Kieran et al. 2008). Of more interest ERK1/2 phosphorylation, and its downstream affect on up-regulating 14-3-3 proteins, was previously found to be a mechanism of protection against MPP+ (Su et al. 2008). The family of 14-3-3 was down regulated in the α-syn mouse model of PD, and has recently been identified in our lab to have a neuroprotective affect against rotenone and MPP+ (Yacoubian et al. 2010). These results have been determined to be through 14-3-3’s interaction with Bax, leading to the inhibition of Bax’s induction of cytochrome c release and caspase-3 cleavage (Slone et al. 2011). Ribosomal RNA is also increased in expression following angiogenin
expression, indicating a role of angiogenin in protein production which may be required following cellular insult (Zhu et al. 2007)

Further investigation is necessary to elucidate whether a single pathway is required for angiogenin’s protective effect in the SH-SY5Y cell line, or whether multiple pathways functioning in parallel act to promote survival such that inhibition of one pathway is not sufficient to block neuroprotection by angiogenin. K40I is able to localize to the nucleus similar to wild-type angiogenin in our cell line of interest (Cho et al. 2010a). This ability of K40I to localize to the nucleus implies that the expression of pro-survival genes induced by both wild type angiogenin and K40I may be augmented and a means of protection.

In summary we demonstrate Akt to be activated by angiogenin, but that it is not a necessary component of angiogenin’s neuroprotective effect in our dopaminergic cell line. Angiogenin provides a rapid induction of Akt phosphorylation upon treatment, but this activation is not required for neuroprotection against MPP+, as inhibition by DN-Akt fails to significantly inhibit this protective effect (Fig 4). Likewise an ALS-associated mutant of angiogenin which is unable to phosphorylate Akt has been shown not to be protective in cellular models of ALS, but remains protective here in our cellular model of PD (Fig 5). Our findings suggest that in our SH-SY5Y cell line, angiogenin and K40I activity induces multiple prosurvival signaling pathways, and this potentially could include transcriptional activation of ribosomal RNA, NF-κB, and/or Bcl-2. Further investigation is needed to elucidate the critical components of angiogenin protection.
References


Figure 1. Angiogenin induces Akt phosphorylation at serine 473.

a. Representative western blot demonstrates increased Akt phosphorylation at serine 473 within 30 seconds following treatment of SH-SY5Y cells with recombinant angiogenin (100nM). This Akt phosphorylation demonstrates a trend of remaining increased for several hours. Total Akt levels are unchanged with angiogenin treatment. Actin is used as a loading control.

b. Densitometric quantification of Western blots against phosphorylated Akt. Results reflect three independent experiments. **p<0.01 (One-way ANOVA with Tukey’s post-hoc test). Error bars reflect SEM.
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**Figure 2.** Wortmannin has a short half life and LY294002 is toxic.

a. Western blot of Akt phosphorylation at serine 473 following SH-SY5Y cell treatment with wortmannin. Wortmannin (100nM) was applied at multiple time points to investigate its length of effectiveness. Results demonstrate a short half life as phosphorylation of Akt returned starting at 2.5 hours after treatment. Total Akt and Actin was unchanged at lower doses. n=1

b. Western blot of Akt phosphorylation following LY294002 treatment to SH-SY5Y cells. LY294002 was applied for 50 min at 10, 30, 50 and 100uM to investigate the optimal dose to inhibit Akt phosphorylation. Inhibition of Akt phosphorylation by LY294002 is present starting at a 30uM concentration in SH-SY5Y cells. n=1

c. Inhibition of angiogenin’s protective response is seen at 10uM as measured by the LDH cell death assay. LY294002 (10uM) was applied as a pretreatment for 40 minutes prior to recombinant angiogenin (100nm) being applied. Twelve hours following angiogenin treatment, MPP+ (0.75mM) was applied with fresh LY294002 and angiogenin. Twenty four hours following MPP+ treatment LDH assay was performed. LY294002 demonstrated a potential inhibition of angiogenin’s protective effect. Total Ak and actin was used as controls. n=2

d. The combined treatment of MPP+ and LY294002 increases toxicity at 10uM of LY294002. LY294002 was applied for 30 minutes followed by angiogenin 100nM. Twelve hours after MPP+ (1mM) was applied with fresh LY294002 and angiogenin. Twenty four hours after LDH assay was performed. Results indicate that LY294002 increases toxicity at doses as low as 10uM. n=2
Figure 3. Akt siRNA knocks down Akt protein and increases toxicity.

a. Western blot demonstrating a knockdown of total Akt after transfection in SH-SY5Y cells. Akt siRNA (2uM) and scrambled (Scr) siRNA (2uM) was transfected into SH-SY5Y cells using a nucleoporator and collected at 72 hrs and 92 hrs. Knockdown of Akt is seen at 72 hrs but not as robust at 92 hrs. n=1

b. Western blot revealing increased caspase-3 cleavage by MPP+ (0.75mM) in siRNA transfected cells as compared to a scrambled sequence. Treatment of MPP+ was given at 48 hours after transfection and collected 22 hours later. Increased caspase-3 cleavage was demonstrated with Akt siRNA and MPP+ as compared to scrambled siRNA and MPP+ at this time point, demonstrating increased toxicity by siRNA. n=1
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Figure 4. Dominant negative Akt does not inhibit angiogenin protection.

a. Western blot demonstrating reduced Akt phosphorylation in SH-SY5Y cells transfected with DN-Akt construct. Twenty-four hours following transfection of empty pCMV5 vector or DN-Akt vector, SH-SY5Y cells were treated with Insulin Growth Factor (50ng/mL). Thirty seconds after Insulin Growth Factor application cell lysates were collected for western blotting. Actin was used as a loading control.

b. Representative western blot demonstrating reduced caspase-3 cleavage following angiogenin treatment to empty plasmid (pCMV5) and a HA-tagged DN-Akt plasmid transfected into SH-SY5Y cells. DN-Akt construct and pCMV5 was transfected using nucleoporator. Twenty-four hours following transfection angiogenin (100nM) was applied for twelve hours. MPP+ (0.75mM) was then applied with fresh angiogenin for an additional 24 hours prior to collection. DN-Akt expression as demonstrated by the HA band did not inhibit angiogenin’s protective effect. Actin was used as loading control.

c. Densitometric quantification of Western blots against caspase-3 cleavage. Results reflect three independent experiments. *p<0.05, ***p<0.001 (One-way ANOVA with Tukey’s post-hoc test). Error bars reflect SEM.
Figure 5. K40I fails to activate Akt phosphorylation.

a. Western blot of Akt phosphorylation after 30 sec treatment with recombinant angiogenin (100nM) and K40I (100nM) on SH-SY5Y cells. n=1

b. LDH cell death assay of MPP+ induced toxicity on SH-SY5Y cells with angiogenin or K40I treatments. Angiogenin (100nM) or K40I (100nM) was applied for 12 hrs prior to MPP+ (0.75uM) and fresh angiogenin or K40I. Twenty-four hours following MPP+ the LDH cell death assay was performed. Angiogenin demonstrated a protective effect that was not significantly different from K40I. n=4 *p<0.05 (One-way ANOVA with Tukey’s post-hoc test). Error bars reflect SEM.
CHAPTER FOUR
OVEREXPRESSION OF ANGIOGENIN IN THE SUBSTANTIA NIGRA DOES NOT INHIBIT MPTP INDUCED TOXICITY

Abstract

Angiogenin variants have been linked to both amyotrophic lateral sclerosis (ALS) and Parkinson’s disease (PD). Angiogenin has been shown to be neuroprotective in models of ALS and PD. We have previously demonstrated that angiogenin reduces cell loss in response to the toxins rotenone and MPP+ in dopaminergic cell lines. Here we examined whether angiogenin overexpression provides increased survival of dopaminergic (DA) neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. Using adeno-associated virus (AAV) vector, angiogenin was delivered into the substantia nigra of eight week old male mice, and mice were subsequently treated with 30 mg/kg MPTP per day intraperitoneally for five days. Our findings demonstrate that angiogenin does not reduce the MPTP induced degeneration of dopaminergic cells in the substantia nigra, nor limit the depletion of DA and its metabolites in the striatum. These results indicate that viral-mediated overexpression of angiogenin may not be a viable approach to reduce toxicity in PD models, yet other forms of delivery may be effective.
Introduction

Angiogenin, an inducer of angiogenesis, has recently been linked to ALS and PD (Greenway et al. 2004, van Es et al. 2011). Previously our lab demonstrated a reduction of angiogenin expression in transgenic mice expressing human alpha-synuclein (α-syn), a mouse model of Parkinson disease (Yacoubian et al. 2008). In cellular models of PD, we demonstrated angiogenin to be neuroprotective against MPP+ and rotenone toxicity, two toxins that inhibit electron transport chain in the mitochondria and develop pathology in animal models reflective of PD (Steidinger et al. 2011, Alam & Schmidt 2002, Miwa et al. 2004). Angiogenin has also been found to be protective in cellular models of ALS. Furthermore, SOD1 mutant mice, a model for ALS, had increased lifespan, stride length, and motoneuron survival following daily intraperitoneal angiogenin injections (Kieran et al. 2008).

Given our findings from in vitro models of PD, we here examined whether angiogenin could protect against dopaminergic cell loss in the MPTP mouse model. MPTP induces dopaminergic neuron loss in the substantia nigra and striatal dopamine loss in mice, primates, and humans. MPTP was discovered accidently in 1982 when a group of young adults self-administered a synthetic analog of heroin, MPTP, and developed acute onset of Parkinsonism associated with nigral cell loss (Langston et al. 1983). In our study, we overexpressed human angiogenin in the substantia nigra of mice by adeno-associated viral (AAV) injection. Our findings indicate that angiogenin overexpression by this method did not reduce MPTP induced toxicity.
Methods

_Virus Injection_

Mice were used in accordance with the guidelines of the National Institute of Health (NIH) and University of Alabama Institutional Animal Care and Use Committee (IACUC). Eight-week-old male C57BL/6 mice were anesthetized with 3% isoflurane and then placed into a stereotactic frame (Kopf, Tujunga, CA). Anesthesia was maintained using 1.5-2% isoflurane in oxygen through a nose tip built into the stereotactic frame. The tip of 5.0-μl syringe (Hamilton Company Reno, Nevada) was inserted using stereotactic coordinates of anterior-posterior, -3.2mm from bregma, medio-lateral, -1.2mm from mid-line and dorso-ventral, -4.6 from the dura. Injections were performed using 2μl of AAV2-Angiogenin (7.5 x 10^{11} viral genome/ml) or AAV2-Green Fluorescent Protein (GFP) (4.5 x 10^{10} viral genome/ml) at a rate of 0.25μl/min. Two minutes following injection the needle was removed.

_MPTP_

MPTP handling and safety measures were in accordance with the University of Alabama IACUC guidelines. Four weeks following rAAV injection, mice were subjected to a subacute MPTP treatment. MPTP was administered intraperitoneally every day at a dose of 30mg/kg of body weight for five days. Three weeks following the last injection mice were sacrificed. As control, some AAV-Ang and AAV-GFP mice were injected with saline intraperitoneally.
Tissue Preparation

Animals were sacrificed three weeks following MPTP injections. Striatum was dissected and immediately placed on ice for high pressure liquid chromatography (HPLC) analysis. These samples were shipped on dry ice to the Neurochemistry Core Lab at Vanderbilt University Medical Center, Nashville, TN for HPLC analysis of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in the striatum. The midbrains were rinsed in PBS and fixed by immersion in 4% paraformaldehyde for 3 days, then placed in 30% sucrose for 2 days, and frozen in 2-methyl-butane.

Immunohistochemistry

Using a microtome brains were sliced at 40um and stored in 50% glycerol in PBS. Free floating SN slices were treated with 3% hydrogen peroxide to quench endogenous peroxidases. Sections were then blocked in 10% normal goat serum (NGS) for 30 min, and incubated in primary rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (1:1000 Pelfreeze Biologicals, Rogers, AR) in 2% NGS overnight at 4°C. Sections were rinsed in PBS, blocked again in 10% NGS, and incubated in goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:500 Jackson Immunoresearch, West Grove, PA) in 2% NGS for an hour at room temperature. Chromogen staining was displayed with the use of diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). To identify localization and expression of virally injected cells, free floating sections were incubated with primary rabbit anti-green fluorescent protein (1:1000 Abcam, Cambridge, MA) or rabbit anti-V5 (1:1000 Sigma, St. Louis, MO) overnight followed by Alexa-488 conjugated goat anti-rabbit (1:500 Molecular Probes,
Eugene, OR) or CY3 conjugated goat ant-rabbit (1:500 Jackson ImmunoResearch, West Grove, PA)

**Stereological Analysis**

For each animal, every fourth section through the substantia nigra (SN) on both viral-injected and uninjected sides was analyzed. Using the Olympus BX51 brightfield microscope, SN regions were scanned and contours traced at low power objective. TH-positive cells were counted with an optical fractionator from the Stereoinvestigator 7.0 software from MBF Biosciences (Microbrightfield Inc, Williston, VT). The number of TH-positive neurons was determined in each counting frame using the optical dissector method, and section thickness was determined to correct for variation between tissue thicknesses.

**Statistical Analysis**

All statistical analysis of data was performed using GraphPad Prism5 (GraphPad Inc., LaJolla, CA). A one-way ANOVA followed by post-hoc Tukey’s test was used for analysis of HPLC data and stereology cell counts.

**Results**

*Angiogenin does not modify MPTP-induced dopaminergic degeneration*

Eight-week-old male mice were stereotactically injected with either AAV-GFP or AAV-human angiogenin tagged at the C-terminal end with the V5 epitope tag into the right substantia nigra. To verify that these viruses do express, a subset of mice were sacrificed
and their brains stained for GFP or V5. Control brains injected with AAV-GFP did not demonstrate V5 staining. Mice injected with AAV-Ang demonstrated modest angiogenin expression colocalized with tyrosine hydroxylase in the substantia nigra at two weeks. Intracellularly overexpressed angiogenen demonstrated localization in a perinuclear pattern and within vesicular puncta (Fig 1).

To test whether human angiogenin can reduce MPTP toxicity, a total of 60 mice were injected sterotactically with either AAV-GFP or AAV-angiogenin. Mice were then treated with either saline or MPTP (30mg/kg/day x 5 days) four weeks following AAV injection. Four weeks after MPTP treatment, mice were sacrificed and brains analyzed for dopamine metabolites in the striatum and for dopaminergic neuronal counts in the SN. The striatal DA levels in the MPTP-treated mice injected with AAV-GFP were significantly reduced by 67% (p<0.001) as compared to saline-treated mice injected with AAV-GFP. Similarly, 3,4-Dihydroxyphenylacetic acid and homovanillic acid levels were also reduced by 48% (p<0.001) and 50% (p<0.001) respectively in the MPTP-treated mice injected with AAV-GFP compared to saline-injected controls. The AAV-Ang mice treated with saline did not demonstrate significant reduction in dopamine metabolites compared to AAV-GFP, indicating angiogenin did not affect dopamine metabolite levels at baseline. MPTP treated mice injected with AAV-Ang showed a similar decrease in striatal DA and its metabolites homovanillic acid (HVA) and 3, 4-dihydroxyphenylacetic acid (DOPAC), as compared to MPTP treated AAV-GFP mice (Fig 2).

We next examined nigral dopamine cell counts using stereology. TH-positive cell counts were reduced by 28% (p<0.05) in the AAV-GFP mice following MPTP
administration compared to AAV-GFP treated with saline, as revealed by stereological
counts of the AAV injected side of the substantia nigra (Fig 3). TH-positive cell counts
in AAV-Ang mice treated with MPTP were similar to MPTP-treated mice injected with
AAV-GFP.

Discussion

Here we tested whether angiogenin is protective against MPTP toxicity in mice.
Angiogenin has been shown to be protective in an in vivo ALS model (Kieran et al.
2008), and our lab has previously demonstrated protection by angiogenin in
dopaminergic cell lines against rotenone and MPP+ (Steidinger et al. 2011), two
commonly employed toxins to model PD. Using AAV-Ang to drive overexpression in
the substantia nigra of MPTP treated mice, our results here indicate angiogenin does not
inhibit dopaminergic cell death of the substantia nigra nor the depletion of striatal DA
following MPTP treatment.

Possible explanations for our findings can be understood in two respects. One
conclusion is that angiogenin does not promote dopaminergic cell survival in vivo and is
not a viable agent for neuroprotection in PD. Alternatively, angiogenin failed to be
protective in the experiments described here due to technical issues. Considerations of
the influences in our experimental paradigm that may have altered angiogenin’s effect
include: 1) the level of angiogenin overexpression attained by our method was not
sufficient, 2) AAV-mediated overexpression of angiogenin has a detrimental affect on
localization and trafficking of angiogenin, 3) angiogenin produced by viral transfection is
unable to activate the necessary signaling cascades for neuroprotection which can be
activated by exogenous angiogenin, and 4) angiogenin’s protection in vivo is not through an autocrine response.

We turned to stereotactic AAV2 injection into the substantia nigra for angiogenin delivery, as this method has worked well for overexpressing other proteins in the substantia nigra (Paterna et al. 2007, Kim et al. 2012, Nihira et al. 2011). Furthermore, the AAV2 serotype remains the preferred vector platform used in human cases of gene therapy. This is a result of its low inflammatory and immune response, as well as its high transduction rates into neurons (Feng & Maguire-Zeiss 2010). Although quantification of angiogenin expression was not analyzed in this study, our findings by immunostaining demonstrated only modest levels of angiogenin overexpression in the substantia nigra. As a result, it could be that the therapeutic effect of angiogenin in the substantia nigra was not obtained using our AAV-Ang construct due to insufficient angiogenin levels. Other viral vectors could be used to attempt to augment expression levels. In this regard, we injected two mice with lentivirus also expressing angiogenin. Our findings of angiogenin expression in these two mice also demonstrated only modest levels of expression. Other vector platforms that could be considered to investigate this further would be other serotypes of AAV including AAV1 and AAV5, which have demonstrated higher transduction rates into neurons than the AAV2 used here (Paterna et al. 2004, Mason et al. 2010, Blits et al. 2010). These may increase expression levels of angiogenin and provide a protective effect against MPTP.

The viral method of angiogenin overexpression may have led to inappropriate cellular processing of the angiogenin protein. In AAV-Ang injected mice we identified angiogenin to be distributed throughout the cytoplasm in vesicular-like puncta with a
higher localization in the perinuclear region. As angiogenin is a secreted protein, it may be that with overexpression by an AAV construct angiogenin is not properly post-translationally modified, trafficked, and/or secreted. For identification of angiogenin we used a V5 tag fused to angiogenin. This addition to the angiogenin protein, though small in size, could have induced alterations in trafficking and/or impact angiogenin’s activity (Desplantez et al. 2011). Angiogenin secretion is known to occur through the classic secretory pathway, and dysfunction in either endoplasmic reticulum or golgi may inhibit secretion of angiogenin from our AAV-Ang transfected cells. Providing angiogenin’s effect of protection is mediated through an extracellular receptor, failure to have significant secretion would prevented the signaling needed for angiogenin’s protective response. If inappropriate processing of angiogenin did occur, angiogenin may not have been able to activate the appropriate signaling pathways involved in protection, such as ERK1/2, Akt, and SAPK/JNK. These pathways have been associated with angiogenin signaling and linked to pro-survival signaling (Zeng et al. 2011, Scuteri et al. 2010, Kieran et al. 2008, Xu et al. 2001). Similarly angiogenin has been identified to have ribonucleolytic activity to tRNA (Tsuji et al. 2005, Shapiro & Vallee 1989), which is considered to play a pro-survival role intracellularly following cellular stress (Yamasaki et al. 2009) and may provide a protective role in dopaminergic neurons. Providing angiogenin was aberrantly trafficked or sequestered in vesicles preventing it from binding and activating its appropriate pro-survival target, its protective effect through these pathways would have been lost.

Angiogenin as a neuroprotective agent may be ineffective upon cells that are overexpressing angiogenin. Angiogenin’s receptor has been determined to be
downregulated in confluent populations of endothelial cells where angiogenin is not in demand for proliferation and/or migration (Gao & Xu 2008, Hu et al. 2000, Hatzi & Badet 1999). We similarly identified angiogenin’s neuroprotective effect to be dependent on cell density in a dopaminergic cell line (Steidinger et al. 2011). This response of neuronal downregulation upon increased density reflects feedback mechanisms in cells based on the requirements for angiogenin. Findings in our lab where overexpression of angiogenin in a cell line did not result in a reproducible protective effect further support a feedback mechanism that may modulate angiogenin’s protective effect and may be the cause of our findings here.

Lastly, angiogenin’s protective effect in dopaminergic cells may not be occurring through autocrine signaling. Recently angiogenin overexpressed and secreted from motoneurons was found to provide protection in a paracrine manner, as no ribonucleolytic products or evidence of uptake was observed in neurons expressing angiogenin, but was evident in neighboring astrocytes. It may be that angiogenin’s influence on astrocytes limits the toxicity they have on motoneurons in the ALS mouse model (Skorupa et al. 2012). Providing dopaminergic neurons have a similar signaling response, our method of using AAV to overexpress angiogenin in neurons may not be a viable method for protecting the MPTP induced toxicity on these neurons.

Our findings here demonstrate that angiogenin overexpression in the substantia nigra does not inhibit MPTP toxicity and this may be due to angiogenin not being a viable agent of protection or that our paradigm is not conducive for angiogenin to activate its signaling pathway. Based on earlier data in ALS models and in PD cellular models it seems possible that angiogenin can induce protection against dopaminergic neurons in
vivo. Our in vivo data here is in conflict with our in vitro findings that found angiogenin to be protective against rotenone and MPP+ (Steidinger et al. 2011). Differences between our in vitro and in vivo findings include the method of delivery of angiogenin. Our in vitro protective response was mediated through exogenous angiogenin treatment, whereas our in vivo method involved AAV driven overexpression. We propose that other means of delivering angiogenin to the substantia nigra should be considered to elucidate its effectiveness in animal models of PD. In an ALS mouse model angiogenin was protective through intraperitoneal injections (Kieran et al. 2008), and this should investigated in an in vivo models of PD as well. These results reflect a need for further investigation of angiogenin’s potential as a neuroprotective agent in an in vivo model of PD.
References


Figure 1. Angiogenin is localized and expressed in the substantia nigra.

a) An AAV-Ang injected mice treated with saline was sacrificed eight weeks after injection and stained for the V5 tag fused to angiogenin. Expression is localized in the substantia nigra with expression at modest levels. b) Staining of the substantia nigra using tyrosine hydroxylase antibody (green) to identify dopaminergic neurons. c) A merge of V5 staining (red) and tyrosine hydroxylase staining (green) demonstrates expression was localized in the substantia nigra. Scale bar 100μm. d) V5 staining (red) identifies angiogenin’s localization to be perinuclear and in vesicular puncta of the cell. Angiogenin was not noticeably present in the nucleus. Scale bar 10μm. e) Control staining for V5 in an AAV-GFP injected substantia nigra. f) Tyrosine hydroxylase antibody (green) to identify substantia nigra of a control injected brain. Scale bar 100μm.
a. Dopamine

![Dopamine Graph]

b. DOPAC

![DOPAC Graph]

c. HVA

![HVA Graph]
**Figure 2.** Angiogenin does not prevent MPTP induced depletion of DA and its metabolites in the striatum. Mice sacrificed had striatal tissue immediately removed and frozen for HPLC. DA and its metabolites, DOPAC and HVA, were analyzed by HPLC. GFP-Saline n= 10, Ang-Saline n=10, GFP-MPTP n=9, Ang-MPTP n=6

a. Striatal dopamine levels as determined by HPLC. AAV-GFP mice treated with MPTP had a 67% reduction in DA levels in the striatum as compared to AAV-GFP mice treated with saline as control. AAV-Ang mice treated with MPTP showed a comparable loss in striatal DA. ***p<0.001 (One-way ANOVA with Tukey’s post-hoc test). Error bars reflect SEM.

b. Striatal DOPAC levels as determined by HPLC. Striatal DOPAC levels were reduced by 53% in AAV-GFP MPTP injected mice compared to AAV-GFP Saline treated mice. A similar reduction in DOPAC was seen in MPTP treated AAV-Ang injected mice as compared to AAV-Ang MPTP. ***p<0.001 (One-way ANOVA with Tukey’s post-hoc test). Error bars reflect SEM.

c. Striatal HVA levels as determined by HPLC. Striatal HVA content was reduced by 50% in MPTP treated mice of AAV-GFP as compared to Saline treated AAV-GFP mice. AAV-Ang injected mice treated with MPTP had a similar decrease in HVA as compared to AAV-GFP mice treated with MPTP. ***p<0.001 (One-way ANOVA with Tukey’s post-hoc test). Error bars reflect SEM.
Figure 3. Angiogenin overexpression does not reduce loss of dopaminergic neurons of the substantia nigra in response to MPTP. Four weeks following AAV injection, MPTP was administered subacutely (30mg/kg/day x 5). Mice were sacrificed four weeks following MPTP and stained for tyrosine hydroxylase to identify DA neurons in the substantia nigra. Stereology counts of tyrosine hydroxylase-positive nigral neurons in the viral-injected side are shown for each of the four conditions: GFP-Saline (n=7), Ang-Saline (n=4), GFP-MPTP (n=17), and Ang-MPTP (n=16). MPTP induced a 28% reduction in tyrosine hydroxylase stained neurons in the substantia nigra of GFP injected mice, and angiogenin did not increase the survival of dopaminergic neurons from MPTP toxicity. *p<0.05, **p<0.01 (One-way ANOVA with Tukey’s post-hoc test). Error bars reflect SEM.
CHAPTER FIVE
DISCUSSION AND SUMMARY

Here we investigated the role of angiogenin as a neuroprotective agent in models of Parkinson Disease. Previously it has been demonstrated that angiogenin provides a neuroprotective effect in models of Amyotrophic Lateral Sclerosis (ALS), a neurodegenerative disease. Our lab previous demonstrated that angiogenin is significantly down-regulated in a mouse model of Parkinson Disease (PD) (Steidinger et al. 2011). Given that alterations in angiogenin is associated with another neurodegenerative disease and that models of PD have reduced levels of angiogenin, we theorized that angiogenin is neuroprotective in models of PD.

**Angiogenin in CNS**

Evidence of angiogenin having a role in CNS function is a recent finding, as originally angiogenin was best understood for its role as an angiogenic factor. Angiogenin has been known to function on endothelial and smooth muscle cells to induce proliferation, migration, and tubular formation [see (Vallee & Riordan 1997, Gao & Xu 2008) for review]. However, recent investigations of angiogenin’s role in the nervous system demonstrated that until mid-gestation angiogenin is more highly expressed in the mouse nervous system than any other organ and mutations in angiogenin detrimentally affect neurite extension and path finding in vitro (Subramanian et al. 2008, Subramanian & Feng 2007). Furthermore, angiogenin is expressed in endothelial and motor neurons within the spinal cord of adult humans, and demonstrates a ubiquitous pattern of expression in mouse brain tissue with the highest levels observed in the cerebellum,
hippocampus, and olfactory bulb (Wu et al. 2007, Lein et al. 2007). These results indicate a critical role of angiogenin in neuronal function.

Angiogenin has also recently been linked to neurodegenerative diseases. This was first identified following a screen of familial and sporadic ALS patients where mutations of angiogenin were identified (Greenway et al. 2004). The link to angiogenin’s function, and its relationship to ALS pathology has not been elucidated; however, angiogenin has been identified to signal several cellular pathways associated with survival and cellular maintenance (Kieran et al. 2008, Li & Hu 2010, Li et al. 2011). It may be that aberrant forms of angiogenin lacking proper signaling leave neuronal cells less resistant to toxic stimuli. This is supported by the demonstration that knockdown of angiogenin in motoneurons increases cell death induced by AMPA. Furthermore, the protective function of angiogenin was demonstrated in in vitro toxicity models of ALS, where angiogenin demonstrated an inhibition of cell death to motoneurons (Kieran et al. 2008, Sebastia et al. 2009); this protective role of angiogenin was also demonstrated in an in vivo model of ALS (Kieran et al. 2008). Recently, a clinical screen identified angiogenin variants to be associated with PD as well as ALS (van Es et al. 2011). Additionally, several ALS patients with angiogenin variants displayed symptoms representing PD, (Seilhean et al. 2004, van Es et al. 2011, van Es et al. 2009). A neuropathological investigation of a 46-year old woman with a considered mutant identified in ALS (K17I), demonstrated an atrophy of corticospinal fibers as well as neuronal loss of motor cortex and substantia nigra (Seilhean et al. 2004). These findings demonstrate that altered forms of angiogenin may be related to degeneration in both motoneurons and dopaminergic neurons. These findings of alterations in angiogenin
correlated with previous findings in our laboratory of angiogenin’s link to degeneration in PD models.

Through a gene microarray study, our lab demonstrated significant reduction of angiogenin expression in the substantia nigra of an overexpressing α-syn mouse model of PD (Yacoubian et al. 2008, Yacoubian et al. 2010). This finding was of great interest, as α-syn gene multiplication causes familial PD, and α-syn is the most significant protein associated with Lewy bodies, a pathological hallmark of PD (Spillantini et al. 1997, Singleton et al. 2003). Furthermore, at later stages in the disease process these mice reflect some of the phenotype associated with PD, including reduced dopamine levels in the striatum, α-syn inclusions, and motor deficits (Masliah et al. 2000). The findings of the microarray indicated a potential role of angiogenin in the early stages of pathogenesis in this PD model, and were to our knowledge the first link of angiogenin to PD. Data from this microarray also revealed a downregulation of a family of binding proteins, 14-3-3, which have demonstrated to have a prosurvival role in neurons (Yacoubian et al. 2010, Slone et al. 2011), and have been associated with activation of the extracellular signal-regulated kinase (ERK1/2) pathway (Su et al. 2008). As angiogenin has demonstrated activation of ERK1/2 signaling it could be that angiogenin is an upstream molecule leading to pathology in this PD model.

Here we demonstrated that angiogenin protein expression is reduced in the cortex of overexpressing α-syn mice. The mechanism for α-syn’s effect in reducing angiogenin expression is not fully understood, but α-syn has been shown to inhibit histone acetylation and thereby affect transcription (Kontopoulos et al. 2006). To further elucidate α-syn’s relationship to angiogenin expression we investigated the protein levels
of angiogenin in a knockout mouse model of α-syn. Levels of angiogenin was not significantly altered in wild-type compared to knockout mice brains, indicating that endogenous α-syn does not normally regulate angiogenin expression but that the effect on angiogenin when α-syn was over-expressed suggests a pathological process. One of the predominant effects of α-syn overexpression is dysregulation of transcriptional processes (Yacoubian et al. 2008), and the change in angiogenin expression may reflect an indirect pathological process of α-syn on transcription.

In agreement with the association of angiogenin to PD and its protective role in motoneuron cell survival, we believed angiogenin to be protective in models of PD. We investigated whether angiogenin would be protective against two PD toxins, rotenone and MPP+, in dopaminergic cell lines. SH-SY5Y cells pretreated with angiogenin did provide a protective response against MPP+. This effect was also present in another dopaminergic cell line, M17, and against another PD model toxin, rotenone (Steidinger et al. 2011). These findings directed us to examine the mechanism of this response.

**Angiogenin activity is cell density dependent**

Prior research on endothelial cells and smooth muscle cells demonstrated angiogenin to have a 170kda unknown receptor which had an inverse relationship of expression to cell density. Angiogenin’s induction of proliferation in endothelial cells and its activation of SAPK/JNK in smooth muscle cells were found be reduced with increased cell density, indicative of reduced receptor expression (Hu et al. 1997, Hu et al. 2000, Xu et al. 2001). Our data supports these findings, as angiogenin’s protective response was inversely related to density in two dopaminergic cell lines (Steidinger et al.
Together these findings indicate angiogenin’s effect may be mediated through an extracellular receptor or that its endocytic uptake occurs in a cell density dependent manner. Another explanation could be that angiogenin’s mechanism of uptake is not altered in a cell density dependent manner, but the volume of angiogenin available for uptake is increased at lower cell densities. This could lead to an increase in the concentration of angiogenin endocytosed per vesicle and an increase in intracellular signaling response mediating the protective effect. Furthermore, as an increase in toxicity of baseline is observed at lower cell densities, it could be that the sensitivity to toxicity of these cells is modified at lower cell densities. This may be a result of adhesion molecules and cell-to-cell interactions being increased at higher cell densities providing an increase in cellular resilience to toxicity. The cellular changes induced at lower cell densities which increase toxicity may be more responsive to angiogenin and thereby demonstrate a greater protective response to these cells. Our findings that angiogenin activates Akt rapidly, whereas uptake was observed after minutes indicates this signaling to be through an extracellular receptor. However, this extracellular signaling response may not be a required factor for angiogenin’s protective effect.

**Angiogenin can induce Akt phosphorylation**

Research in motoneurons demonstrated the Akt pathway as a necessary pathway for angiogenin’s neuroprotective effect. Inhibition of this pathway demonstrated a reduction in angiogenin’s ability to be protective against AMPA-induced toxicity in motoneurons (Kieran et al. 2008). Akt’s role was further supported in a mouse model of ALS (SOD1) treated with angiogenin, in which increased Akt phosphorylation was
associated with increased survival (Kieran et al. 2008). These studies led us to investigate if angiogenin’s effect of protection was being mediated through the Akt pathway. Using the same dose previously demonstrating a protective response, angiogenin had a rapid and robust increase in Akt phosphorylation of the SH-SY5Y cell line (Steidinger et al. 2011). This directed us to further determine if this pathway was necessary component of neuroprotection.

We first investigated the necessity of Akt by using a pharmacological approach. Two commonly used inhibitors for this pathway include wortmannin and LY294002. Wortmannin in our model demonstrated a short half life not conducive to our paradigm. LY294002 inhibited Akt phosphorylation, but increased the toxicity induced by MPP+, making results difficult to interpret. We next investigated knocking down Akt by siRNA. Similar to the pharmacological approach, we observed an increase in toxicity when siRNA transfected cells were treated with MPP+, and therefore this approach also did not provide a viable method of investigating this pathway.

The last approach to inhibit Akt signaling was with the use of a dominant negative Akt. The results of this approach suggested that Akt activation is not required for angiogenin’s protective effects, as angiogenin remained protective despite DN-Akt expression. In addition, our finding that the mutant K40I, which does not induce Akt phosphorylation, is also protective is consistent with this interpretation. These results were unexpected based on previous literature where inhibition of Akt inhibited angiogenin’s protective effect (Kieran et al. 2008). Although angiogenin has been determined to signal through Akt and to be a necessary component of protection in
motoneurons, other pro-survival signaling pathways are activated through angiogenin which may explain our findings.

**Alternate Mechanisms for Angiogenin’s Protection**

Apart from Akt phosphorylation angiogenin has been identified as signaling through multiple other pathways. Angiogenin is known to signal through the phospholipase C and stress-activated protein kinase/c-Jun NH2-terminal kinase, both found to have a role in pro-survival signaling of neurons (Li *et al.* 2008, Zeng *et al.* 2011). In endothelial cells and in motoneurons the (ERK1/2) pathway has been identified to be increased following angiogenin treatment (Liu *et al.* 2001, Kieran *et al.* 2008).

As angiogenin signaling of ERK1/2 has been demonstrated in multiple cell lines, it is a potential signaling pathway necessary for angiogenin protection. ERK1/2 is a member of the MAP kinase family, known to regulate a variety of biological functions through a phosphorylation signaling cascade. This signaling cascade activates specific molecules, such as kinases and transcription factors which regulate cellular activity including proliferation, differentiation, and apoptosis [see review (Seger & Krebs 1995)]. Angiogenin treated to a mouse model of ALS (SOD1) demonstrated an increase of ERK1/2 phosphorylation and had increased survival of motoneurons (Kieran *et al.* 2008). Angiogenin treatment of SH-SY5Y cells prevented hydrogen peroxide induced cell death and increased ERK1/2 phosphorylation (Cho *et al.* 2010). ERK1/2 signaling has also been demonstrated to have a pro-survival signaling effect in dopaminergic neurons (Alavian *et al.* 2009), and to inhibit caspase activation (Erhardt *et al.* 1999), a mechanism of cell death activated in our SH-SY5Y cell line following MPP+ toxicity. Of more
interest, ERK1/2 phosphorylation demonstrated an upregulation of 14-3-3 proteins that provided inhibition of cell death induced by MPP+ toxicity (Su et al. 2008). Our lab previously demonstrated 14-3-3 proteins to have a neuroprotective effect against MPP+ induced toxicity in a dopaminergic cell line, as well as a reduction of α-syn toxicity in a C. elegans PD model (Yacoubian et al. 2010). Therefore, ERK1/2 signaling on 14-3-3 may be a mechanism for angiogenin protection (Fig. 1). Furthermore, as 14-3-3 is a family of proteins significantly down-regulated in the α-syn overexpressing mouse model (Yacoubian et al. 2008), it is possible that angiogenin is a potential upstream target for protection against the pathogenesis of PD.

To investigate the potential role of ERK1/2 in angiogenin’s response, a pharmacological inhibitor of the ERK1/2 pathway could be used. In endothelial cells inhibition of the upstream kinases to ERK1/2, MEK1 and MEK1, by PD98059 revealed inhibition of angiogenin mediated cellular proliferation (Liu et al. 2001). As MEK1 and MEK2 are known to only have one or two substrates downstream, a tightly controlled effect can be seen from this inhibition. Furthermore, the use of a PD98059 is advantageous as its inhibition of MEK1 and MEK2 has low off target effect on other kinases, due to its lack of being competitive with ATP (Hoshino et al. 1999, Alessi et al. 1995). The use of a pharmacological approach is also advantageous as it would provide the ability to adjust for concentration and incubation time and therefore alleviate potential issues of toxicity. The use of a MEK inhibitor is advantageous as it is known to have lower off target effects than other kinase inhibitors. As our paradigm requires a twelve hour pretreatment of angiogenin prior to application of MPP+, the half life of the inhibitor could be a technical challenge in this approach. Other means of investigating
the ERK1/2 signaling pathway could include the use of RNAi to MEK1/2. Providing inhibition of ERK1/2 reduces the protective effect of angiogenin, it would be important to investigate the potential role of angiogenin on 14-3-3.

Our laboratory previously demonstrated that several 14-3-3 isoforms provide a protective effect against MPP+ induced toxicity (Yacoubian et al. 2010). Potentially, angiogenin activates increased expression of 14-3-3 through ERK1/2 signaling (Su et al. 2008). Further studies to investigate this could include measuring protein and mRNA levels of 14-3-3 following angiogenin treatment at determined time points. Furthermore, to elucidate if angiogenin signals through 14-3-3 an inhibitor of 14-3-3, difopein, could be used. Difopein has previously been demonstrated to inhibit 14-3-3 in a dopaminergic cell line (Yacoubian et al. 2010). Results from this experiment, if demonstrating a reduction in protection, would indicate 14-3-3 as a required downstream target of angiogenin signaling for protection. To further specify angiogenin’s response specific isoforms of 14-3-3 could be targeted. The 14-3-3θ isoform was found to have a significant protective effect against MPP+ (Yacoubian et al. 2010), and would be a choice to investigate. To elucidate the effects of this isoform on angiogenin’s protective response, shRNA targeted to this isoform could give insight into whether this isoform is required. The association of angiogenin to ERK1/2, and ERK1/2’s association to 14-3-3 demonstrates a potential link between angiogenin and 14-3-3 dysregulation that may underly the development of pathology seen in the α-syn transgenic mouse model of PD. It is possible that α-syn overexpression induces angiogenin downregulation, which in turn causes reduced levels of 14-3-3 that induces toxicity and pathology in this mouse model.
Angiogenin’s function also has been determined to induce mRNA expression of multiple prosurvival genes including Bcl-2, NF-kappaB and Ripk1 (Li et al. 2010). In particular angiogenin signaling through Bcl-2 has resulted in an inhibition of caspase 3 cleavage. This increase in Bcl-2 expression, an anti-apoptotic protein, identifies a method of which angiogenin provides a protective response that is not mediated through Akt (Fig. 1) In contrast to results identified in motoneurons where co-treatment of angiogenin with AMPA was protective (Kieran et al. 2008), our dopaminergic cells required an additional twelve hour pretreatment window to limit MPP+ toxicity. This pretreatment period may be a necessary time frame for the anti-apoptotic protein to be expressed and activated prior to MPP+ treatment. This form of protective signaling requires uptake of angiogenin which we have previously demonstrated in a dopaminergic cell line, and has not been observed in motoneurons (Skorupa et al. 2012).

Therefore, investigation of angiogenin’s effect on Bcl-2 would further elucidate the mechanism of angiogenin protection seen in our in vitro models of PD. Investigating this would include verifying angiogenin’s ability to increase Bcl-2 in our cell line. Providing angiogenin does increase Bcl-2 expression, it would be insightful to know whether overexpression of Bcl-2 inhibits MPP+ toxicity, indicating a sufficiency of Bcl-2 for protection in our model. This result would be advantageous in understanding the potential of Bcl-2 in protection, but would not identify whether it is the mechanism of angiogenin’s protection. Determination of a direct effect of Bcl-2 for protection could be determined by transfecting shRNA targeted to Bcl-2 to see if a reduction in angiogenin’s protective effect against MPP+ is observed. Previously Li et al. demonstrated that with knockdown of Bcl-2 the protective effect of angiogenin on serum starved cells was
inhibited (Li et al. 2011). This approach could however provide technical challenges in our model as reduction in Bcl-2 may increase the toxicity of MPP+. To circumvent this, subcloning the shRNA into a lentivirus under an inducible promoter could limit the time of knockdown and reduce toxicity.

Another mechanism whereby angiogenin may be protective is through its ribonuclease activity, which has been directly associated with stress induced translational repression. Under stress angiogenin expression is known to produce stress-induced small RNA (tiRNA), a novel class of RNA induced by stress, by cleavage of tRNA at the anticodon loop. This cleavage produces two fragments, 5’-tiRNA and 3’-tiRNA (Yamasaki et al. 2009, Emara et al. 2010). The function of 5’-tiRNA includes reprogramming of protein translation with suppression of global translation. This inhibition of translation provides a conservation of anabolic energy which can be used for the repair of damage induced by the stressor, and thereby potentially increase cell survival (Yamasaki et al. 2009). This mechanism may provide a protective response against MPP+, but our results indicate it is not a necessary mechanism for protection. In our studies we found the angiogenin mutant, K40I, to have a similar neuroprotective effect to angiogenin in protection. Although our recombinant K40I protein had not been investigated for its lack of ribonucleolytic activity, this mutant has previously been demonstrated to have less than 1% of angiogenin’s ribonucleolytic activity (Crabtree et al. 2007). Therefore, we believe that our findings indicate that the ribonucleolytic activity is not a requirement for protection.
**Angiogenin mutation does not inhibit protection**

Our finding that inhibition of Akt phosphorylation was not sufficient to prevent angiogenin from having a protective effect was further supported by our results with the mutant K40I. One of the mutants identified in ALS patients is the lysine 40 residue conversion to isoleucine (Greenway et al. 2006). Previous findings demonstrate that this mutant is unable to phosphorylate Akt in motoneurons (Kieran et al. 2008, Cho et al. 2010). We demonstrated a similar finding as angiogenin provided a robust increase in Akt phosphorylation, but K40I did not induce Akt phosphorylation. In cell death assays using tunicamycin or hydrogen peroxide to induce cell death, the K40I mutant of angiogenin was not able to provide a protective response (Kieran et al. 2008). Despite the lack of Akt phosphorylation and based on our dominant negative Akt results, K40I still could provide a response in our toxicity model. Using MPP+ to induce cell death, angiogenin was found to be protective and the K40I mutant did not show a significant difference from angiogenin’s response. Unlike previous results in motoneurons, the K40I appears to still induce prosurvival signaling in our dopaminergic cell line.

Furthermore, in a screen of ALS and PD patients, the K40I was associated multiple times with ALS, but in none of the PD cases (van Es et al. 2011), further indicating divergent mechanism of signaling between these two populations of neurons.

**Angiogenin is not protective in a mouse model of PD**

As a result of our in vitro findings, we investigated angiogenin’s ability to be protective in an in vivo model of PD. As a result of α-syn mice having a mild phenotype and no reduction of dopaminergic neurons we used another mouse model, MPTP
Masliah et al. 2000). MPTP provides a means of modeling PD that is reflective of pathogenesis occurring in humans, and is the most commonly used animal model for PD (Jackson-Lewis & Przedborski 2007). We tested angiogenin’s protective effect against MPTP, by cloning angiogenin into an adeno-associated virus serotype 2 (AAV2) and injecting this into the substantia nigra for overexpression. Our results following MPTP treatment demonstrated that angiogenin overexpression in the substantia nigra did not provide a robust or significant reduction in dopamine depletion following MPTP treatment. Similarly, our counts of dopaminergic neurons in the substantia nigra further reflected the findings in the striatum with no increase in survival following angiogenin overexpression.

These results were of particular interest as we had identified angiogenin to be protective against a dopaminergic cell line in vitro and angiogenin has been demonstrated to be protective in an in vivo model of ALS (Kieran et al. 2008, Steidinger et al. 2011). Although it could be that angiogenin is not protective against dopaminergic neurons of the substantia nigra, it is very probable that factors relating to our paradigm resulted in angiogenin’s protective response being ineffective.

Potential factors limiting angiogenin’s effect include the level of overexpression seen from our AAV2 viral construct, altered folding and/or trafficking of angiogenin, or feedback mechanisms following overexpression. We observed only a modest level of angiogenin expression following AAV-Ang injection in our mice. It could be that our findings reflect a need for a higher threshold of angiogenin’s presence than obtained in our model, to induce a protective response.
Another factor effecting our result is that angiogenin overexpression by AAV in these cells may alter its trafficking and secretion. Observation of AAV-Ang transduced cells demonstrated vesicular like puncta in the cytoplasm with predominance in the perinuclear region. It is probable that angiogenin’s processing through the ER and Golgi may be hindered through altered folding, glycosylation and/or packaging, which could prevent angiogenin from activating its appropriate signaling cascades important for protection. Similarly, failure to properly secrete angiogenin would confound any extracellular receptor signaling of angiogenin. Angiogenin has been identified to have an unknown receptor that has a direct effect on angiogenin uptake and angiogenesis in endothelial cells (Gao & Xu 2008, Hu et al. 2000, Hatzi & Badet 1999). Providing angiogenin’s neuroprotective effect is mediated through an extracellular receptor, lack of secretion would prevent proper induction for protection. Furthermore, overexpression of angiogenin may provide feedback to reduce receptor expression or intracellular processes important for angiogenin’s protective response. Angiogenin’s receptor has been identified in endothelial cells and in our dopaminergic cell line to be modulated based on density (Hatzi & Badet 1999). It may be that increased angiogenin levels over time induce downregulation of this receptor.

Although our findings of investigating angiogenin protection in the substantia nigra was not effective, we believe with modification in our experimental paradigm the effectiveness of angiogenin to protect against dopaminergic cell death in vivo will be demonstrated. In our approach we used the AAV2 platform. This vector platform is the preferred choice of delivery of gene therapy in human trials as a results of its long-term expression, low immune response, and efficient transduction (Feng & Maguire-Zeiss
As angiogenin expression from our AAV2 injected mice appeared to be only modest, we believe that other means of delivery would be a better choice for overexpression. To this end, we injected a lentivirus that overexpressed angiogenin into the substantia nigra of two mice. Two weeks later, we sacrificed and stained these mice to determine angiogenin expression levels. Our findings with the lentiviral injection were similar to AAV2 injected mice, with only modest level of angiogenin expression. To further pursue overexpression in the substantia nigra, a different serotype of AAV could be used. In particular, AAV1 and AAV5, have demonstrated higher levels of transduction into neurons than AAV2 (Paterna et al. 2004, Mason et al. 2010, Blits et al. 2010).

Despite the possibility that other forms of overexpression may increase the level of angiogenin in the substantia nigra, we believe this is not the best approach to test protection of angiogenin in vivo.

The ideal form of delivery for angiogenin may be by an exogenous approach. This form of delivery will circumvent issues of processing, trafficking, and secretion that may have been a problem in our model. Furthermore, research done in ALS models, demonstrate that daily intraperitoneal injections of angiogenin provided a protective effect to motoneurons in the spinal cord (Kieran et al. 2008). Intraperitoneal injections would be advantageous as it would alleviate the need for a major surgery to the mice. Furthermore, it would provide a method of modifying the dose and therefore optimize angiogenin’s response. One technical challenge in this approach is the uncertainty of angiogenin to cross the blood-brain barrier (BBB). Kieran et al. used fluorescein-conjugated angiogenin to identify uptake of angiogenin into the spinal cord, which was visualized two hours following injection (Kieran et al. 2008). Although this supports
angiogenin’s ability to cross the BBB, the animal model used (SOD1) has been demonstrated to develop leakage of the blood-brain barrier as part of the disease process (Zhong et al. 2008), therefore further investigation should be performed before this form of delivery is attempted. Another potential pitfall of this approach is the possibility that angiogenin may cause a deleterious effect when administered at high levels in the blood, as angiogenin has been associated with tumor formation (Kawada et al. 2007, Yoshioka et al. 2006). Kieran et al. injected angiogenin over 75 day period, and noted that no effect on breeding behavior, mouse viability, or motoneuron survival was observed (Kieran et al. 2008). However, analysis of brain tissue in their treated mice was not discussed. As higher dosing of angiogenin may be required to obtain sufficient levels in the substantia nigra, this could be an issue with this approach.

Intracerebroventricular delivery is another option of delivery of angiogenin to the substantia nigra (Karlsson et al. 2004, Zhao & Janson Lang 2009, Yoshimura et al. 2003). Infusion into the ventricles may be performed with the use of a miniosmotic pump attached to a cannula (Chang et al. 2008, Wernicke et al. 2010). Previously, Zhao & Janson Lang demonstrated that two weeks following infusion of BrdU into the right cerebral ventricle, diffusion into substantia nigra occurred without any toxicity to the region demonstrating the effectiveness of this approach (Zhao & Janson Lang 2009). As the spatial diffusion of a specific protein in the brain following intraventricular infusion is dependent on its physicochemical properties, angiogenin’s localization by diffusion would need to be investigated (Sendelbeck & Urquhart 1985). This delivery method would have the advantage of circumventing the BBB, as well as reducing potential side effects associated with the need for high doses of angiogenin in the periphery. A
technical challenge with this approach, however, is that it requires surgical placement of
the cannula into the ventricle. Implantation of a foreign object in the brain may induce
inflammation and/or an infection in this region, and thereby could alter the findings of
angiogenin’s protection. Furthermore, due to the timeframe of our model, follow-up
surgeries with replenishment of the osmotic pump would be required, depending on the
dosing and infusion rate used. This approach, though more technically challenging, may
be the best method for angiogenin treatment to the substantia nigra.

Our findings demonstrate that the effectiveness of angiogenin for protection may
be particular to the method of administration and the factors they include. In both the
intraperitoneal and intracerebroventricular approaches of delivery, angiogenin is
delivered to substantia nigra by an exogenous manner. This method was used both in our
in vitro models of PD and in vivo models of ALS and demonstrated a neuroprotective
effect. Here we did not find a protective response in vivo following overexpression
through the AAV2 construct, and this discrepancy between the ALS mouse model and
our PD model may be due to our form of delivery. This difference also reflects findings
in our lab where exogenous application of angiogenin was protective, but overexpressing
angiogenin did not provide a reliable response. As results from the exogenous approach
demonstrated protection, it is apparent that this should be the next approach for an in vivo
model of PD.

Conclusions

In conclusion, this dissertation demonstrates that angiogenin provides a protective
response in in vitro models of PD, but its required mechanism of action and its ability to
be therapeutic in an in vivo model of PD are not fully elucidated. The previous findings in our lab that angiogenin was the most significantly reduced gene in a mouse model of PD identified angiogenin as a target molecule for drug development (Yacoubian et al. 2008). This thesis investigated angiogenin’s role as a potential agent of neuroprotection in PD. Our findings demonstrated:

- Angiogenin is protective against MPP+ and rotenone induced cell death in dopaminergic cell lines, and this protective effect is mediated in a cell-density dependent manner.
- Angiogenin activates the Akt pro-survival signaling pathway, but inhibition of the Akt pathway does not inhibit angiogenin’s protective effect.
- Overexpression of angiogenin by the AAV2 construct does not reduce MPTP induced depletion of dopamine and its metabolites in the striatum, nor does it inhibit MPTP induced substantia nigra cell loss.

Further research is necessary to elucidate angiogenin’s potential and this should include investigating role of ERK1/2 pathway and expression of anti-apoptotic genes as a mechanism of protection in vitro. Furthermore, these findings should not give the conclusion that angiogenin is not a viable protective agent for animal models of PD. Additional testing of angiogenin’s potential in vivo should include an alterative form of delivery than overexpression. Such options include intraperitoneal injection or intracerebroventricular infusion.

We believe angiogenin should be further investigated as a neuroprotective agent in animal models of PD and potentially clinical cases of PD. Recently, a twenty year
longitudinal study on PD patients investigated the rate of dementia, non-responsive levodopa symptoms, and mortality. Results indicated 73% of surviving PD patients had struggles with hallucinations requiring change in medications, 83% had dementia, 71% had urinary incontinence, and for 54% PD was regarded as significant cause of mortality (Reid et al. 2011). These findings underscore the need for novel agents to target the overall degenerative process in these patients. While current treatments available for PD are effective in providing reduced motor symptoms and in turn an improvement in patients’ lifestyle (Fahn 1999), there remains no agent available that prevents degeneration. Angiogenin’s altered expression in a mouse model of PD and its evidence of protection to both motoneurons and dopaminergic neurons identify it as a novel agent that with further investigation may be a means of inhibiting disease progression in PD.
Figure 1. Potential mechanisms of protection mediated by angiogenin to MPP+ and Rotenone toxicity in dopaminergic cells. Inhibition of complex 1 of the electron transport chain in the mitochondria by MPP+ or rotenone induces cytochrome c release and caspase-3 cleavage. The protective response of angiogenin may be mediated through two mechanisms. One mechanism could include activation of the MAPK kinase pathway (solid arrow heads) and the induction of ERK1/2 phosphorylation. ERK1/2 activation increases 14-3-3 protein levels which mediates protection through inhibition of Bax and its induction of cytochrome c release. Another potential mechanism for angiogenin’s protective response includes endocytic uptake of angiogenin, nuclear localization, and induced expression of anti-apoptotic proteins, such as Bcl-2 (dashed arrowheads), which can act as an inhibitor of caspase-3 cleavage and apoptosis.


plasminogen activator in bovine endothelial cells. *Biochem Biophys Res Commun, 211*, 476-483.


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NOTICE OF RENEWAL

DATE: December 22, 2011

TO: TALENE ALENE YACOUBIAN, PhD
    CIRC-560D 0021
    FAX: (205) 996-6580

FROM: Judith A. Kapp, Ph.D., Chair
       Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title. Neuroprotective Role of Angiogenin in Models of Parkinson Disease
         Sponsor: NIH
         Animal Project Number: 120108979

As of January 14, 2012, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>B</td>
<td>80</td>
</tr>
</tbody>
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Animal use must be renewed by January 13, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120108979 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.
MEMORANDUM

DATE: December 22, 2011

TO: TALENE ALENE YACOUBIAN, PhD
    CIRC-560D 0021
    FAX: (205) 996-5580

FROM: Judith A. Kapp, Ph.D., Chair
      Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 22, 2011.

Title of Application: Neuroprotective Role of Angiogenin in Models of Parkinson Disease
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)