VEGF signaling mediates neuroprotection against oxidative stress in hippocampal neurons *in vivo* and *in vitro*

by

Tianfeng Hao

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Chair of Examining Committee:

_____ _____

Date **Dr. Patricia Rockwell (Hunter College, CUNY)**

Executive Officer:

_____ _____

Date **Dr. Edward J. Kennelly (Lehman College, CUNY)**

Supervisory Committee:

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Date **Dr. Derrick Brazill (Hunter College, CUNY)**

_____ _____

Date **Dr. Susan Croll (Queens College, CUNY)**

_____ _____

Date **Dr. Peter Werner (Sunovion Pharmaceuticals)**

THE CITY UNIVERSITY OF NEW YORK

Abstract

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Tianfeng Hao

Advisor: Dr. Patricia Rockwell

Vascular endothelia growth factor (VEGF) signaling through its cognate receptor VEGFR-2 has been shown to be neuroprotective against stressful stimuli including oxidative stress. Using a rat pilocarpine model of *status epilepticus* (SE), pilocarpine-induced SE was associated with the induction of the oxidative stress marker Heme oxygenase-1 (HO-1) and increased expression of the pro-inflammatory enzyme Cyclooxygenase-2 (COX-2). Treatments with exogenous VEGF attenuated the induction in HO-1 and the increase in COX-2 levels. Previous studies showed that VEGF protected hippocampal cell death in this model and these results suggest that oxidative stress and inflammation contribute to this neuronal loss. Similarly, in cultured hippocampal neurons, pilocarpine induced neuronal cell death accompanied with increased HO-1 expression, reactive oxygen species (ROS) production, caspase-3 cleavage and a loss of mitochondrial membrane potential ($\Delta\Psi_m$) that were all suppressed by VEGF. To delineate the protective mechanisms associated with VEGF-mediated neuroprotection, VEGFR-2 inhibition was investigated with respect to oxidative stress in primary cultures of hippocampal neurons. Treatments with the pharmacological inhibitor SU1498 elicited a cytotoxicity that was prevented by the antioxidant N-acetyl-cysteine (NAC) and accompanied by induction of HO-1,

cleavage of caspase-3, production of ROS together with a loss of $\Delta\Psi_m$. Knockdown of VEGFR-2 by siRNA generated a similar pattern of ROS and $\Delta\Psi_m$ loss. Treatments with VEGF or VEGF-B prevented neurons from the cell death and mitochondrial dysfunction induced by SU1498 or siRNA, suggesting that a molecular switch occurs where both growth factors may signal through VEGFR-1 when VEGFR-2 activity is blocked. Consistent with a general role for pro-apoptotic BAD and anti-apoptotic Bcl-xL in neuronal cell death and oxidative stress, the phosphorylation of MEK/ERK1/2 and BAD (inactivation) and the protein levels of Bcl-xL that were attenuated by SU1498 were somewhat increased by VEGF. Our findings support a role for VEGF in signaling BAD inactivation and increased Bcl-xL as a specific mechanism that protects hippocampal neurons against oxidative stress. These findings also suggest that VEGF activates VEGFR-2 to protect neurons against oxidative stress but can signal through alternate receptors to serve the same function.

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LIST OF ABBREVIATIONS

ADP adenosine diphosphate

Akt protein kinase B/PKB

ALS amyotrophic lateral sclerosis

ANOVA analysis of variance

Apaf-1 apoptotic protease activating factor 1

ATP adenosine triphosphate

BAD Bcl-2-associated death protein

Bak Bcl-2 homologous antagonist/killer

Bax Bcl-2–associated X protein

Bcl-2 B-cell lymphoma 2

Bcl-xL B-cell lymphoma extra large

Bid BH3 interacting-domain death agonist

COX-2 Cyclooxygenase-2

CREB cyclic AMP-response element-binding protein

$\Delta\Psi_m$ mitochondrial membrane potential

DMEM Dulbecco's Modified Eagle's Minimal Essential Medium

DRG dorsal root ganglia

EC endothelial cell

ERK1/2 extracellular signal-regulated protein kinases 1 and 2

ETC mitochondrial electron transport chain

FBS fetal bovine serum

GCM glia condition medium

HO-1 Heme oxygenase-1

HRE hypoxia-response element

IMM inner mitochondrial membrane

KDR kinase insert domain receptor

MAPK mitogen activated protein kinase

MEK1/2 mitogen-activated protein kinase/ERK kinase 1/2

NAC N-acetyl-cysteine

NP neuropilin

OMM outer mitochondrial membrane

PBS phosphate buffered saline

PD Parkinson's disease

PGs prostaglandins

PI3-kinase phosphoinositide 3-kinase

PlGF placental growth factor

PTP permeability transition pore

ROS reactive oxygen species

SDS Sodium dodecyl sulfate

SE *status epilepticus*

SEM standard error of the mean

SOD superoxide dismutase

SU SU1498, VEGFR-2 inhibitor

sVEGFR-1 soluble VEGFR-1

svVEGF snake venom VEGF

U0126, MEK1/2 inhibitor

VEGF vascular endothelial growth factor

VEGFR vascular endothelial growth factor receptor

Wort Wortmannin PI3K/Akt inhibitor

Chapter I

Introduction

1.1 Vascular endothelial growth factor and VEGF family

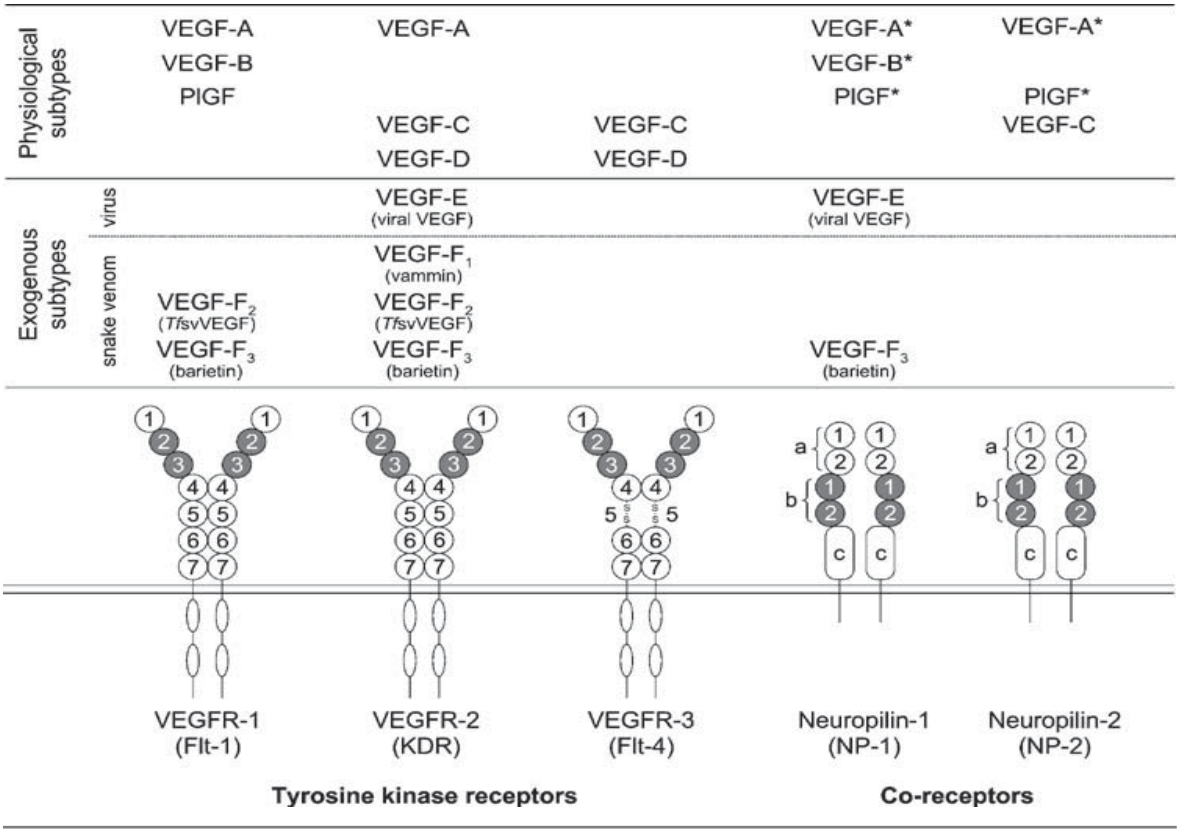
Vascular endothelial growth factor (VEGF) was originally identified in endothelial cells (ECs) as a potent and selective mitogen associated with angiogenesis, vasculogenesis, vascular permeability and cell migration (Ferrara and Davis-Smyth 1997, Neufeld et al. 1999, Zachary and Gliki 2001). VEGF, also known as VEGF-A or $VEGF_{165}$, is a member of the VEGF family proteins that are released from cells as homodimeric glycoproteins. Up to date, the VEGF family consists of VEGF-A, VEGF-B, placenta grow factor (PlGF), VEGF-C and VEGF-D in mammals; viral VEGF (VEGF-E) and snake VEGF (VEGF-F) (Yamazaki and Morita 2006). VEGF-A exerts a universal angiogenic effect under most physiological and pathological conditions. VEGF-B may promote angiogenesis *in vivo* (Silvestre et al. 2003) but its biological function remains debatable. PlGF stimulates endothelial cell migration (Migdal et al. 1998) and promotes VEGF-mediated angiogenesis, EC proliferation and vascular permeability (Luttun et al. 2002, Park et al. 1994). VEGF-C and VEGF-D are primarily involved in lymphangiogenesis (Alitalo et al. 2005).

All VEGF members share a common VEGF homology domain. Alternative splicing of human VEGF-A gene leads to several isoforms, among which $VEGF_{165}$ (hereafter referred as VEGF) is the predominant one and will be the main target of this study.

1.2 VEGF receptors

VEGFs bind to three membrane bound tyrosine kinase receptors to trigger downstream signaling cascades: VEGF receptor-1 (VEGFR-1/fms-like tyrosine kinase-1 (Flt-1)), VEGF receptor-2 (VEGFR-2/kinase insert domain receptor (KDR) /fetal liver kinase-1 (Flk-1)) and VEGF receptor-3 (VEGFR-3/Flt-4). The extracellular domain of VEGFR-1 is also expressed as a

soluble protein which is responsible for negative regulation of angiogenesis (Klagsbrun and D'Amore 1996). Upon binding to ligands, the tyrosine kinase receptors in turn dimerize and become activated through transphosphorylation (autophosphorylation). In addition, the neuropilins (NP-1 and NP-2) are transmembrane non-protein tyrosine kinase co-receptors for the VEGF family and function to modulate VEGFRs activation and signaling.



Yamazaki, 2006

Figure 1. VEGF family proteins and their receptor selectivity. VEGF binds to both VEGFR-1 and VEGFR-2; VEGF-B and PlGF bind to VEGFR-1; VEGF-C and -D bind to VEGFR-2 and VEGFR-3. VEGF-E binds to VEGFR-2. NP-1 is a non-RTK receptor for VEGF, PlGF, VEGF-B and VEGF-E. The ligand-binding domain of each receptor is shaded in *grey*. * binding is observed for some of isoforms.

VEGF isoforms have different preference and affinity regarding to their receptors and co-receptors (Figure 1, (Yamazaki and Morita 2006)). VEGF binds to both VEGFR-1 and VEGFR-2 with a higher affinity (10 fold) to VEGFR-1 (Waltenberger et al. 1994). VEGF-B and PlGF bind exclusively to VEGFR-1 with high affinity. VEGF-C and -D, when initially expressed as pro-peptides, bind to VEGFR-3 and mature VEGF-C and VEGF-D bind to VEGFR-2 with low affinity. VEGF-E binds to VEGFR-2. In addition, all VEGF family members bind to both NP-1 and NP-2 except VEGF-B, which only binds to NP-1. NP-1 stabilizes the binding of VEGF and VEGFR-2 (Holmes D. I. and Zachary 2008) and the presence of NP-1 has been shown to enhance VEGF/VEGFR-2 mediated signaling transduction (Neufeld et al. 2002).

It has been shown that one VEGF dimer binds to two molecules of receptor (Fuh et al. 1998). In ECs, VEGF induces homodimer formation of VEGFR-1, VEGFR-2 and heterodimers of VEGFR-1/2. Signaling capacity of VEGFR-1/2 heterodimers is similar to that of VEGFR-2 homodimers, while VEGFR-1 homodimers signal inefficiently (Cross et al. 2003, Huang K. et al. 2001). In adults, VEGFR-2 expression is mainly on vascular endothelial cells and detectable on neuronal cells (Holmes K. et al. 2007). VEGFR-2 mediates most of the cellular responses to VEGF and will be the main focus of this study; VEGFR-1 signaling is not well defined yet, it may function to segregate excess VEGF in vascular development (Cross et al. 2003).

1.3 VEGF and neuroprotection

The effect of VEGF and its receptors has been well studied in ECs with respect to its ability of regulating angiogenesis, vasculogenesis, mitogenesis, cell permeability as well as migration (Zachary 2003). Recent studies elucidate that VEGF exerts neurotrophic and neuroprotective properties under stressful conditions such as neuronal injury, hypoxia, ischemia

and serum deprivation (Jin et al. 2000a, b, Sondell et al. 2000). In addition, VEGFR-1, VEGFR-2 and NP-1 are expressed on neuronal cells (Jin et al. 2000c, Zachary 2005). VEGF-mediated neuroprotection is mostly regulated through the activation of VEGFR-2 and multiple downstream kinase cascades including PI3K/Akt and/or MEK/ERK pathways (Zachary 2005). However, the mechanisms underlying VEGF-mediated neuroprotection remain unclear.

VEGF has been shown to have a direct neurotrophic effect on several neuronal cell types including autonomic, hippocampal, cerebellar and cortical neurons. For instance, in mouse dorsal root ganglia (DRG), VEGFR-2 is upregulated in response to axonal injury and VEGF stimulates axonal outgrowth and promotes neuronal cell survival through VEGFR-2 (Sondell et al. 2000). MAPK/ERK inhibition blocks VEGF-induced axonal outgrowth but inhibition of MAPK/ERK alone has no significant effect on axonal outgrowth (Sondell et al. 1999). VEGF signaling through VEGFR-2 and PI3K/Akt protects against cell death in cultured mouse cortical neurons exposed to hypoxic-ischemic injury (Jin et al. 2000b) and protects against cell death induced by serum deprivation in HN33 hippocampal cells (Jin et al. 2000a). VEGF-mediated activation of VEGFR-2 protects cultured hippocampal neurons from glutamate-induced toxicity via both PI3K/Akt and MEK/ERK pathways (Matsuzaki et al. 2001). In addition, VEGF stimulates neuronal cells proliferation both *in vivo* and *in vitro*, and VEGF-mediated neurogenesis *in vitro* is blocked by VEGFR-2 inhibitor SU1498 (Jin et al. 2002b). Overexpression of VEGF promotes neurogenesis and enhances motor function after cerebral ischemia in mice (Wang Y. et al. 2007). In cultured cortical neurons, VEGF-mediated cell proliferation is regulated via VEGFR-2 and multiple downstream signal pathways including PI3K/Akt and MEK/ERK (Zhu et al. 2003). Given its neuroprotective effect against cell death, VEGF has been used as a therapeutic

molecule in numerous pathological conditions such as stroke and peripheral nerve damage (Ogunshola et al. 2002).

VEGF can also mediate neuroprotection indirectly through the regulation of neuronal angiogenesis and oxidative stress (Gora-Kupilas and Josko 2005). Ischemic stroke damages nervous tissue due to a reduction of blood flow to the brain region. VEGF and VEGFR-2 has been observed upregulated in the brain tissue after acute ischemic stroke (Issa et al. 1999) and VEGF infusion at 48 hours after ischemia onset promotes angiogenesis and neural recovery (Zhang et al. 2000). Also, VEGF protects against cell loss induced by oxidative stress in differentiated retinal ganglion cells and this protection is blocked by VEGF neutralizing antibody Bevacizumab (Brar et al. 2010). In addition, oxidative stress related to superoxide dismutase (SOD) mutations is responsible for the pathogenesis of amytrophic lateral sclerosis (ALS), a progressive neurodegenerative disorder. VEGF is implicated in ALS and VEGF-mediated neuroprotection may serve a potential role in motoneuron degeneration. In knock-in mice with a deletion of the hypoxia response element (HRE) in the *VEGF* gene promoter ($VEGF^{\partial/\partial}$ mice), reduced VEGF expression causes ALS-like motoneuron degeneration (Oosthuyse et al. 2001). Treatment with VEGF prolonged survival of ALS mice without causing toxic side effects (Azzouz et al. 2004, Takahashi and Shibuya 2005) and overexpression of VEGFR-2 delays the symptoms onset in a mice model of ALS (Lambrechts et al. 2003).

Using SK-N-SH neuroblastoma cells as a neuronal model system, previous studies from our laboratory demonstrated that prolonged serum deprivation induces a dramatic increase in the mRNA levels for VEGF and its cognate receptors, VEGFR-1, VEGFR-2 and NP-1. Also, VEGFR-2 is activated under stressful condition and this activity is enhanced by exogenous VEGF and abolished by VEGFR-2 inhibitor SU1498. Furthermore, the VEGF/VEGFR-2

signaling pathway protects against a caspase-dependent cell death through the activation of MEK/ERK1/2 signaling pathway (Gomes et al. 2007). In the current study, we use both an *in vivo* rat model of *status epilepticus* and an *in vitro* model using hippocampal neurons in culture to investigate the role of VEGF and VEGFR-2 in neuroprotection and the mechanisms underlying.

1.4 VEGF and *status epilepticus*

Epilepsy, characterized by recurrent and unprovoked seizures, is the third most common neurological disorder in the United States after Alzheimer's disease and stroke; and affects approximately 50 million people worldwide (Ngugi et al. 2010). Although about 2-5% of general population will have epilepsy during life time (Neligan et al. 2011), epilepsy remains to be one of the least understood neuronal disorders. Understanding the cause and consequence of epilepsy-induced neurodegeneration and cell death together with the mechanisms underlying will provide information for developing therapeutic strategies against seizures.

Seizures are the results of abnormal electrical activity in the brain. *Status epilepticus* (SE) is generally recognized as over 30 minutes continuous seizure activity or two or more sequential seizures without full recovery in between (Nair et al. 2011). The muscarinic receptor agonist pilocarpine is used to induce limbic seizures and subsequent SE. Upon binding to muscarinic receptors, pilocarpine promotes neuronal cell depolarization and leads to seizure/SE (Klitgaard et al. 2002, Turski W. A. et al. 1983b). The major change after pilocarpine induced SE is hippocampal neuronal death followed by subsequent synaptic reorganization (Turski W. A. et al. 1983a). In rats, the pilocarpine model of SE is well characterized and widely used because it mimics several features of human temporal lobe epilepsy including similarities in pathology, behavioral

abnormalities, and circumstance of both partial and generalized seizures (Turski L. et al. 1987). In cultured rat hippocampal neurons, it has been reported that pilocarpine signals through muscarinic receptors and induces an imbalance between excitatory and inhibitory transmission, which is responsible for the generation of SE in pilocarpine treated animal models (Priel and Albuquerque 2002).

Pilocarpine-induced SE leads to elevated oxidative stress and induces severe neurodegeneration in the hippocampal formation and finally causes neuronal cell death (Muller et al. 2009, Yu et al. 2008). Brain tissue utilizes high levels of oxygen and in turn generates high quantities of free radicals, which makes it susceptible to oxidative damages. The elevated oxidative stress after SE causes early neuronal damages associated with mitochondrial dysfunction and a prolonged neuronal cell death accompanied by caspase activation (Liu et al. 2010). In addition, recent studies indicate that mitochondrial dysfunction and oxidative stress could also trigger seizures and epileptogenesis (Waldbaum and Patel 2010).

Hippocampus, an essential brain region responsible for learning and memory, is particularly sensitive to the deleterious effects of SE such as oxidative stress. More recent studies revealed that seizures were accompanied by induction of the antioxidant enzyme Heme oxygenase-1 (HO-1) and increased levels of the pro-inflammatory enzyme Cyclooxygenase-2 (COX-2) in the hippocampus of pilocarpine-treated rats (Jarvela et al. 2008, Jung et al. 2006, Lee et al. 2007, Yu et al. 2008).

Heme oxygenase (HO) is a stress related enzyme catalyzing the rate limiting step of heme degradation. Three isoforms of HO have been identified: HO-1 (Hsp32), HO-2 and HO-3. HO-1 is induced by various stimuli including oxidative stress, hypoxia and growth factors; and predominantly responsible for HO activity. HO-2 is consistently active under normal conditions.

HO-3 is also consistently present and has very low activity involving in heme binding. HO-1 degrades heme into carbon monoxide (CO), biliverdin/bilirubin and iron. For these degraded products, biliverdin/bilirubin functions as physiological antioxidants; CO has been shown to have a neuroprotective effect through vasodilation (Fiumana et al. 2003); while iron is a very efficient ion in converting hydroxyl peroxide into more active and tissue damaging radical (Halliwell and Gutteridge 1992). Taken together, HO-1 reaction may exert either beneficial effect to cells by its antioxidant ability or aggravate free radical damage. HO-1 expression after seizure is a marker of oxidative stress (Yu et al. 2008) but the role of HO-1 in SE still remains controversial.

Cyclooxygenase (COX) is a rate-limiting enzyme catalyzing the synthesis of prostaglandins (PGs). There are two isoforms of COX, constitutive COX-1 and inducible COX-2. COX-2 is an indicator of inflammation and is the principle isoform of cyclooxygenase in brain. Under normal conditions, COX-2 level is low or undetectable in most organs except in mammalian brain significant levels of COX-2 mRNA are expressed (Feng et al. 1993, Seibert et al. 1994). Normal COX-2 protein is expressed in distinct population of neurons in different brain regions and is enriched in the cortex and hippocampus (Hurley et al. 2002, Yamagata et al. 1993). The expression of inducible COX-2 in brain is associated with acute neurotoxicity such as seizures and ischemia (Koistinaho et al. 1999, Lee et al. 2007) and contributes to neuronal cell death (Kawaguchi et al. 2005).

COX-2 activity has been shown to damage neurons by generating oxidative stress, catalyzing the production of prostaglandins (PGs) and disturbing the ubiquitin-dependent protein processing (Jiang et al. 2004, Li Z. et al. 2004). Accumulated evidence indicates that COX-2 may play a role in epilepsy. For instance, COX-2 expression was upregulated in hippocampus

after seizures induced by pilocarpine and kainate; and seizures induced COX-2 overexpression accelerates hippocampal neuronal cell loss (Hurley et al. 2002, Joseph et al. 2006, Kawaguchi et al. 2005, Voutsinos-Porche et al. 2004). Inhibition of COX-2 with selective inhibitor SC58125 attenuates neuronal cell loss induced by seizures (Kawaguchi et al. 2005).

In addition to its upregulation and neuroprotective effects in pathologic conditions such as hypoxia and ischemia (Hayashi et al. 1997, Ogunshola et al. 2002), VEGF is upregulated after SE and provides protection against hippocampal neuronal cell loss resulted from pilocarpine-induced seizures (Nicoletti et al. 2007). Also in ECs, VEGF regulates expressions of both HO-1 and COX-2 (Bussolati and Mason 2006, Wu et al. 2003). In this study, using a rat pilocarpine model of SE provided by Dr. Susan Croll's lab at Queens College, we further investigated the role of VEGF/VEGFR-2 signaling regarding to HO-1 and COX-2 in SE induced by pilocarpine and the results suggested that VEGF signaling mediated neuroprotection against seizure-induced damage by suppressing inflammation and oxidative stress. Delineating VEGF-mediated signaling pathways that relay protection and oxidative stress during SE would provide valuable insight on the role of VEGF in the neurodegenerative process associated with epilepsy.

1.5 Mitochondrial dysfunction and oxidative stress in neurodegeneration

Accumulating evidence demonstrates that mitochondrial dysfunction and oxidative stress involve in different models of both acute and chronic neuronal death and neurodegenerative diseases (Mandemakers et al. 2007, Mattson and Liu 2003). Neurons are particularly sensitive and vulnerable to mitochondrial dysfunction due to their high energy consumption. Mitochondrial dysfunction leads to insufficient ATP generation, ROS production and apoptosis that may contribute to progressive decline in the central nervous system (Mandemakers et al.

2007). For instance, dysfunction of mitochondrial electron transport chain (ETC) is associated with the pathophysiology of Alzheimer's disease (Hirai et al. 2001). Impaired mitochondrial oxidative phosphorylation followed by induced ROS production results in the loss of dopaminergic neurons which is associated with Parkinson's disease pathogenesis (Greenamyre and Hastings 2004). In addition, mitochondria-mediated ROS production contributes to aging which is thought a main cause of neurodegeneration (Marchi et al. 2012).

Mitochondrial cell death is tightly linked to mitochondrial structure and dysfunction. Mitochondrial dysfunction includes the loss of the mitochondrial membrane potential ($\Delta\Psi_m$), production of ROS, opening of the permeability transition pore (PTP) and the release of the cytochrome *c* from the intermembrane space due to outer mitochondrial membrane permeabilization (MMP). Released cytochrome *c* is critical for the initiation of cell death pathway associated with mitochondria (Korsmeyer et al. 2000, Wei et al. 2001).

Mitochondria are double membrane intracellular organelles responsible for ATP generation in almost all eukaryotic cells. The outer mitochondrial membrane (OMM) separates mitochondria from cytosol and contains porins which make it permeable to molecules with molecular weight less than 5-6 kDa (Benz 1994). Larger proteins transport across the membrane using their specific signaling sequences binding to translocase of the OMM (Herrmann and Neupert 2000). OMM permeabilization allows proteins (e.g., cytochrome *c*) leakage from the intermembrane space (space between the out membrane and the inner membrane) into the cytosol and leads to apoptotic cell death (Chipuk et al. 2006). The inner mitochondrial membrane (IMM) is nearly impermeable to all molecules including protons, and specific membrane transporters are required for almost all ions and molecules to across. The IMM contains enzymes associated with the electron transport chain (ETC) and ATP synthesis, which create the

mitochondrial membrane potential ($\Delta\Psi_m$). The mitochondrial matrix space contains metabolic enzymes, mitochondrial DNA (mtDNA) and RNAs (ribosomal and transfer RNA) coding for some proteins involved in oxidative phosphorylation.

The outer mitochondrial membrane integrity and associated cell death is primarily governed by the Bcl-2 family proteins. Bcl-2 family members contain up to four conserved Bcl-2 homology (BH) domains including BH1, BH2, BH3, and BH4. Anti-apoptotic Bcl-2 family members including Bcl-2 and Bcl-xL typically contain four BH domains. Pro-apoptotic members such as Bax and Bak contain at least BH2 and BH3 domains while BAD, Bim and Bid only possess BH3 domain (Akhtar et al. 2004). The pro-apoptotic members function by forming heterodimers with anti-apoptotic members to inhibit their activities. The homodimerization, heterodimerization and ratios between the anti-apoptotic and pro-apoptotic Bcl-2 proteins regulate caspases activation (Korsmeyer 1995, Yang et al. 1995).

Upon activation by apoptotic stimuli, the pro-apoptotic member Bax (Bcl-2-associated X protein) translocates from cytosol to mitochondria and forms oligomers facilitating the permeabilization of outer mitochondrial membrane and the release of cytochrome *c* (Lalier et al. 2007). Released cytochrome *c* activates Apaf-1 (apoptotic protease-activating factor-1), which in turn activates downstream caspases including caspase-9 and caspase-3 and leads to apoptosis (Gross et al. 1999a).

The anti-apoptotic Bcl-xL protein localizes at the OMM (Kaufmann et al. 2003). Bcl-xL can form a heterodimer with Bax and block Bax-induced outer mitochondrial membrane permeabilization (MMP) (Billen et al. 2008). Bcl-xL is expressed in both embryonic and postnatal neuronal tissues and overexpression of Bcl-xL protects against neuronal cell death induced by nerve growth factor (NGF) depletion in cultured rat neurons (Gonzalez-Garcia et al.

1995) and by hypoxia-ischemic insults in adult mouse brain including hippocampus (Parsadanian et al. 1998). Bcl-xL prevents the release of cytochrome *c* (Gross et al. 1999b). Also, Bcl-xL has been shown to bind to Apaf-1 directly and inhibits Apaf-1 dependent caspase-9 activation (Hu et al. 1998).

BAD, the Bcl-2-associated death protein, could initiate or potentiate Bax activation. BAD is expressed in the embryonic nervous system and downregulated postnatally (Shimohama et al. 1998). Under basal conditions, BAD is inactivated through phosphorylation (on serine 112 and serine 136) by growth factor signaling-activated kinases and sequestered by the molecular chaperone 14-3-3 in the cytosol (Zha et al. 1996). Once dephosphorylated, BAD is released from 14-3-3 and becomes constitutively active within cells because of the exposure of the BH3 domain hydrophobic face (McDonnell et al. 1999). Bad then translocates to mitochondria (Cory and Adams 2002) and undergoes dimerization particularly with Bcl-xL and Bcl-2, but not with Bax, Bcl-xs or BAD itself. In mammalian cells, BAD binds to Bcl-xL with higher affinity comparing to Bcl-2 and inhibits the pro-apoptotic function of Bcl-xL but not that of Bcl-2 (Yang et al. 1995). When BAD binds to Bcl-xL through heterodimerization, BAX is released from Bcl-xL leading to MMP which results in the release of cytochrome *c* (Yang et al. 1995).

Several kinase pathways are responsible for the phosphorylation and inactivation of BAD. The PI3K/Akt pathway phosphorylates BAD specifically at Ser 136 (Datta et al. 1997, del Peso et al. 1997). In addition, PI3K/Akt has also been shown to phosphorylate and inactivate caspase-9 to promote cell survival (Cardone et al. 1998) and inhibition of PI3K/Akt exacerbates brain injury after cerebral ischemia (Noshita et al. 2001). The observation that ERK1/2 is distributed in mitochondria of both normal and stressed tissues suggests that ERK1/2 is a potential player for regulating mitochondria function (Horbinski and Chu 2005). ERK1/2

signaling pathway prevents apoptosis by phosphorylating BAD at Ser 112 (Jin et al. 2002a, Zha et al. 1996). Upregulation of ERK1/2 in mitochondria is associated with phosphorylation of both Bcl-2 and BAD; and activated ERK1/2 can translocate to mitochondria to prevent cell death (Deng et al. 2000, Horbinski and Chu 2005). Taken together, it suggests that PI3K/Akt and/or MEK/ERK-mediated phosphorylation (inactivation) of mitochondrial BAD may serve to protect against cell death (Figure 2).

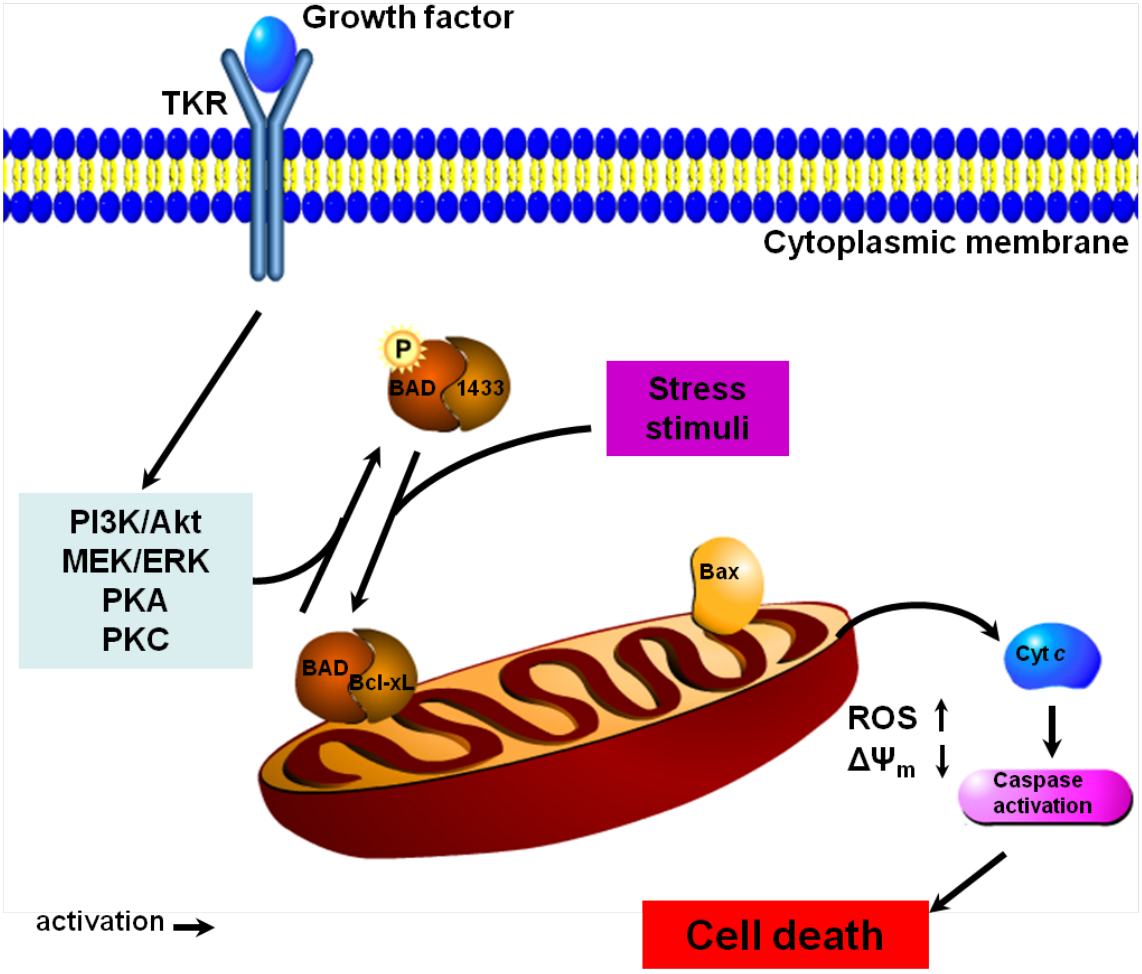


Figure 2. Bcl-2 family-mediated cell death and survival. BAD is inactivated (phosphorylated) by growth factor signaling-activated kinases and sequestered by 14-3-3 in the cytosol. Once dephosphorylated, BAD is released and translocates to mitochondria where it replaces BAX forming heterodimers with Bcl-xL. BAX dissociated from Bcl-xL promotes the release of cytochrome *c*, which triggers caspase activation and leads to cell death.

In apoptosis, accompanied with the MMP, there is typically a rapid reduction in the mitochondrial membrane potential (Goldstein et al. 2000). The inner mitochondrial membrane is impermeable to ions, which allows the electrons transport chain (ETC) to actively build up the proton gradient across IMM that is required for oxidative phosphorylation. The mitochondrial membrane potential ($\Delta\Psi_m$) is predominantly maintained by this proton gradient generated by electron transportation. $\Delta\Psi_m$ is a principle indicator of cell viability, as it reflects the proton flow across the IMM during electron transport and oxidative phosphorylation, providing energy for ATP synthesis. Upon apoptotic stimuli, $\Delta\Psi_m$ is first increased due to the decreased ADP levels in the mitochondrial matrix, followed by a later decrease of $\Delta\Psi_m$ (Vander Heiden et al. 1997). A persist or permanent $\Delta\Psi_m$ dissipation is often associated with cell death (Zamzami et al. 2005).

Mitochondrial reactive oxygen species (ROS) production is one of the very early events preceding the collapse of $\Delta\Psi_m$, release of pro-apoptotic factors, and activation of caspases (Kroemer et al. 2007). ROS are products of normal cellular metabolism of oxygen and consisted of several highly reactive molecular species including free radicals superoxide anion (O_2^-), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2). H_2O_2 is relatively stable but can lead to the production of highly reactive and toxic hydroxyl radical (·OH) under the presence of iron or copper. The toxicity of these free radicals is due to their unpaired electron(s) which cause them highly reactive and have a great tendency to withdraw electrons from non-radical molecules and convert them into newly formed radicals. The newly formed radicals in turn either lose their normal biological functions or they could conduct further radical reactions to other targets and trap cells in a circle of oxidative insults. ROS are generated mainly as by-products of mitochondrial respiration. In addition, cellular production of ROS occurs in multiple cellular compartments including endoplasmic reticulum (ER) and peroxisomes.

Oxidative stress represents the imbalance between ROS production and the ability to immediately remove the reactive intermediates or to repair the resulting damage. Mitochondria are the predominantly intracellular source of ROS and the primary target of oxidative stress. Mitochondria undergo oxidative damage when ROS production exceeds their antioxidant capacity. Oxidative damage impairs mitochondrial ATP synthesis, mitochondrial DNA replication and transcription, which leads to mitochondrial dysfunction and in turn enhances ROS production following further damage to mitochondrial DNA.

It has been shown that VEGF-immunoreactivity was enriched in mitochondria comparing with nucleus, ER and cytosol and the significance of the high level mitochondrial VEGF remains unclear (Fehrenbach et al. 1999). VEGF stimulates the expression of a cluster of nuclear-encoded mitochondrial genes, suggesting a role for VEGF in the regulation of mitochondrial biogenesis (Wright et al. 2008). In addition, it has been reported that both VEGF and VEGF-B protect against cell damage caused by H_2O_2-induced oxidative stress in post-ganglionic sympathetic neurons (Damon 2011).

Using a neuronal model of serum deprived SK-N-SH cells, we showed previously that VEGF signaling through VEGFR-2 prevents cell death (Edelstein et al. 2011, Gomes et al. 2007) that is associated with oxidative stress. This study presents that VEGF signaling-mediated neuroprotection is associated with oxidative stress and mitochondrial dysfunction in cultured hippocampal neurons.

Chapter II

Materials and Methods

2.1 Materials- Recombinant human vascular endothelial growth factor 165 ($VEGF_{165}$) and the anti-human VEGF neutralizing antibody were obtained from PeproTech Inc (Rocky Hill, NJ). The inhibitors of VEGFR-2 (SU1498) and PI3K/Akt (wortmannin) were obtained from EMD Biosciences Inc (San Diego, CA). The inhibitor of MEK1/2 (U0126) was obtained from Promega Corporation (Madison, WI). Pilocarpine was from MP Biomedicals (Solon, OH). Atropine methylbromide and the antioxidant N-acetyl-cysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO). Neutralizing antibody against VEGFR-2 was from R&D (Minneapolis, MN).

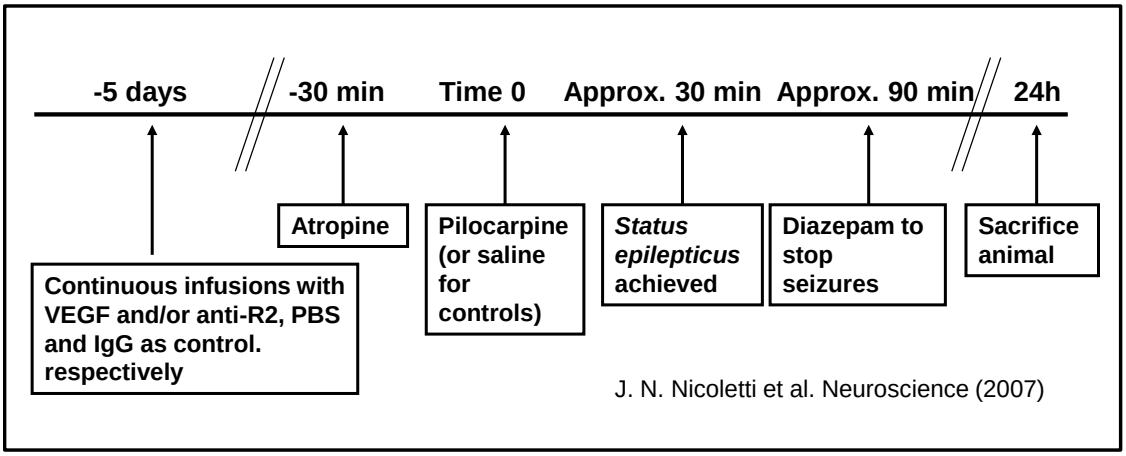


Figure 3. *In vivo* rat model of *status epilepticus*: the experimental timeline.

2.2. Rat Hippocampal Tissues- Rat hippocampal tissues were obtained from Dr. Susan Croll's lab at Queens College. Briefly, VEGF (45 ng/day) and/or VEGFR-2 neutralizing antibody (anti-R2, 20 µg/ml) were infused for 5 days into the rat hippocampus via an Alzet osmotic minipump, saline and/or IgG were used as control, respectively. Five days after, animals were injected s. c. with 1 mg/kg atropine methylbromide 30 min prior to pilocarpine hydrochloride (350mg/kg) IP injection. Seizures were scored and stage 6 was status epilepticus. 60 min later, animals were IP

injected with 10 mg/kg diazepam to stop SE. 24 hours after pilocarpine injection, animals were sacrificed and hippocampal tissues were dissected out 1.5 mm in every direction from the cannula tip for western blotting; in separate animals, brains were removed and sectioned for immunohistochemistry analysis. Figure 3 depicts the timeline for these experiments.

2.3 Protein Extraction from Rat Brain Tissues and Western Blotting- Fresh hippocampal tissues from rats described in 2.2 were collected and immediately frozen in dry ice; protein was extracted using a BioMasher disposable micro homogenizer (Investigen, CA) and protein concentrations were determined with a bicinchoninic acid assay (BCA) according to manufacturer's instructions (Pierce, Rockford, IL). Lysates (50 µg) were subjected to Western analysis by probing with antibodies specific to rat forms of HO-1 (Santa Cruz) and COX-2 (Santa Cruz); actin (Sigma-Aldrich) was probed as a loading control. Immunoreactive bands were detected with corresponding anti-mouse, anti-rabbit and anti-goat secondary antibodies conjugated to horseradish peroxidase and visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Endogen, Rockford, IL). Data were quantified using ImageJ from NIH.

2.4 Immunohistochemistry- Hippocampal tissue sections (40 µm) from rats described in 2.2 were heated in 10 mM sodium citrate (pH 6.0) for 10 min for antigen retrieval. Tissues were then double labeled by immunofluorescence staining with antibodies against phospho-VEGFR-2, total VEGFR-2, HO-1 and COX-2 (all antibodies are from Santa Cruz) and labeled with FITC secondary antibodies (Invitrogen). Neurons were labeled with either NeuN or MAP-2 (Millipore) and illuminated with Texas-Red (Invitrogen). Images were taken at 63X magnification using a

Leica confocal microscope. Data are representative of experiments that were repeated at least three times.

2.5 Primary Cell Culture and Treatment- Hippocampal neuron cultures were prepared from postnatal day 1 (P1) Charles River Sprague Dawley rats. Experiments were carried out under the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by local committee review, and every effort was made to minimize animal suffering and to reduce the number of animals used. Hippocampi was dissected and digested with trypsin (Sigma Chemical) and incubated for 15min at 37°C in Hank's balanced salt solution (HBSS) followed by 3 times wash with HBSS. Cells were transferred to DMEM/FBS containing DNase and suspended by trituration. Cell suspensions were filtered through a 40 µm Falcon nylon cell strainer and plated onto poly-D-lysine coated tissue culture plates or chamber slides in DMEM containing 1% fetal bovine serum. This is DIV 0. Cells were switched to supplemental neurobasal (SNB: neurobasal + B27 + glutamax + antibiotics) after 2 hours and maintained in half SNB and half glia condition medium (GCM) for about 14 days in vitro when >99% of cells were neurons as stained with microtubule-associated protein MAP-2.

On DIV 13, cells were changed to SNB or NB (SNB without B-27) and incubated for 48 hr prior to harvest for western blotting or subject to immunofluoresence staining, cell viability assay, ROS and TMRE detection. All treatments were added during this 48 hr period as follows when applicable: VEGF (100 ng/ml) was treated for 48 hr and replenished after 24 hr. Pilocarpine (10 µM, 100 µM and 1 mM) and SU1498 (10 µM) were treated for 15', 1 hr, 2 hr, 4 hr and 24 hr where indicated. NAC (5 mM) was treated for 24 hr. Wortmannin (100 nM) and

U0126 (10 µM) were treated for 2 hr. DMSO was used as vehicle control for SU1498, Wortmannin and U0126.

2.6 Protein Extraction and Western Blotting- Neurons were plated in pre-coated 6-well plates (5 X 10^5 cells/well) and maintained in half GCM half SNB for about 2 weeks. On DIV 13, cells were changed to SNB or NB (SNB without B-27) in the absence and presence of VEGF (100 ng/ml) for 48 hr prior to harvest. VEGF was replenished after 24 hr. During this 48 hr, all treatments were performed as following when applicable: pilocarpine (10 µM, 100 µM and 1 mM) and NAC (5 mM) were treated for 24 hr. SU1498 (10 µM) was treated for either 2 hr or 24 hr. Wortmannin (100 nM) and U0126 (10 µM) were treated for 2 hr. Total cell lysates were harvested on DIV 15 in a lysis buffer as described previously (Rockwell et al. 2004) except using 0.3% NP40. Protein concentrations were determined using a bicinchoninic acid assay (BCA) according to manufacturer's instructions (Pierce, Rockford, IL). Equal amounts of protein (30 µg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were then blocked and incubated overnight at 4^0C with the following primary antibodies: pVEGFR-2, VEGFR-2, HO-1 and ERK1/2 from Santa Cruz; pERK1/2, pBAD (S112), BAD, Bcl-xL, pAkt (T308, S473) and Akt from Cell Signaling. Actin (Sigma-Aldrich) was used as a loading control. Immunoreactive bands were detected with corresponding anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase and visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Endogen, Rockford, IL). Data were quantified using ImageJ from NIH.

2.7 Double Immunofluorescence Staining and Cell Imaging- Neurons were cultured in pre-coated 8-well chamber slides (1X 10^5 cells/well) and maintained in half GCM half SNB. On DIV 13, cells were changed into NB with the absence and presence of VEGF (100 ng/ml) for 48 hr prior to fixation. VEGF was replenished after 24 hr. On DIV 15, cells were fixed for 20 min with 3.7% formaldehyde in medium at 37°C, permeabilized with 0.1% saponin for 20 min and blocked with 1% BSA in PBST for 30 min. Cells were stained with first primary antibodies against pVEGFR-2(Y-996), VEGFR-2 and VEGFR-1 (Santa Cruz) overnight at 4°C and first secondary antibodies FITC (Invitrogen) for 2 hours at room temperature then followed by second primary antibodies MAP-2 (Millipore) and second secondary antibodies Texas-Red (Invitrogen) staining. Wash with 1X PBS was performed in between. Images were captured at 63X magnification using a Leica confocal fluorescent microscope. Data are representative of experiments that were repeated at least three times.

2.8 Cell Viability- Neurons were plated in pre-coated 96-well microtiter plates (2 X 10^4 cells/well) and maintained in half GCM half SNB. On DIV 13, cells were changed into NB or SNB as indicated with the absence and presence of VEGF (100ng/ml) for 48 hr prior to MTS assay. VEGF was replenished after 24 hr. Prior to MTS assay measurement, all treatments were performed as following when applicable: NAC (5 mM) was treated for 24 hr; pilocarpine (10 µM, 100 µM and 1 mM) and SU1498 (10 µM) were treated for 15', 1 hr, 2 hr, 4 hr or 24 hr where indicated. On DIV 15, cell viability was determined using a colorimetric MTS assay (Promega Corp, Madison, WI) and quantified according to manufacturer's instruction. Survival measurements are expressed as the percent of the untreated control.

2.9 Oxidative Stress- ROS was measured using the fluorescent dye Carboxy-H_2DCFCA (Invitrogen) following the manufacture instruction. Briefly, neurons plated in 8-well chamber slides were incubated in NB for 48 hr prior to adding H_2DCFCA. All treatments were performed as following during this 48 hr if applicable: VEGF (100 ng/ml) and VEGF-B (100 ng/ml) were treated for 48 hr and replenished after 24 hr. NAC (5 mM) was treated for 24 hr. SU1498 (10 μM) was treated for 2 hr. THBP (100 μM) was used to induce ROS production as positive control for 90'. On DIV 15, 48 hr after in NB cells were incubated with 25 μM H_2DCFDA following the manufacturer's instructions and images were immediately taken using a Nikon Eclipse TE200 inverted epifluorescence scope. Data are representative of experiments that were repeated at least three times.

2.10 Mitochondrial Membrane Potential- Loss of mitochondrial membrane potential was assessed in living calls labeled with the fluorescent lipophilic cationic dye tetramethylrhodamin methyl (TMRE) (Invitrogen). This dye incorporates into mitochondria with an intact transmembrane potential and its release serves as an indicator of a loss in inner mitochondrial membrane potential. Neurons plated in 8-well chamber slides were incubated in NB for 48 hr prior to adding of TMRE. All treatments were performed as following during this 48 hr if applicable: VEGF (100 ng/ml) and VEGF-B (100 ng/ml) were treated for 48 hr and replenished after 24 hr. Pilocarpine (100 μM) and NAC (5 mM) were treated for 24 hr. SU1498 (10 μM) was treated for 2 hr. FCCP (20uM) was used for 30' to disrupt the mitochondrial membrane potential as a positive control. On DIV 15, 48 hr after in NB, neurons were incubated with 200 nM TMRE for 30 min at 37°C, wash with fresh medium. Both fluorescence and phase images were

immediately taken using a Nikon Eclipse TE200 inverted epifluorescence scope. Data are representative of experiments that were repeated at least three times.

2.11 RNA Interference- Cultured neurons were transfected with 50 nM of KDR/Flk-1/VEGFR-2 SMARTpool® siRNA duplexes (Dharmacon RNA Technologies, Lafayette, CO) using BLOCK-iT Transfection Kit (Invitroen Life Technologies, Grand Island, NY) according to manufacturer's directions. The BLOCK-iT fluorescein-labeled oligo is not homologous to any known genes and was used as negative control and indicator for transfection efficiency. After 4 hr, cells were changed to fresh NB in the absence and presence of VEGF (100 ng/ml) or VEGF-B (100 ng/ml) for 48 hr and then fixed for double immunofluoresence staining probing with anti-VEGFR-2 and anti-VEGFR-1. Neurons were labeled with anti-MAP-2.

2.12 Statistical Analysis- Data are expressed as the mean $\pm$ SEM of experiments, all of which were replicated at least three times. Statistical significance was assessed by a one-way ANOVA followed by pairwise contrasts (Bonferroni analysis). A difference resulting in $P<0.05$ is considered significant.

Chapter III

VEGF protects against cell loss and oxidative stress in hippocampus of rats undergoing seizure

3.1 VEGFR-2 and phosphorylated VEGFR-2 are upregulated in hippocampus of rats undergoing *status epilepticus*

VEGF is upregulated and protects against neuronal cell loss in rat hippocampus after pilocarpine-induced *status epilepticus* (Nicoletti et al. 2007). To further study the mechanism underlying, hippocampal sections from rats with and without seizures were probed with anti VEGFR-2 and anti-phosphorylated VEGFR-2. Immunofluorescence staining showed that VEGFR-2 was increased in rats undergoing seizure (Figure 4A compare A1 with A2) and phosphorylated VEGFR-2 was increased in both CA1 (Figure 4B compare B1 with B7) and CA3 (Figure 4B compare B4 with B10) pyramidal neurons of rats undergoing seizure.

3.2 VEGF increases VEGFR-2 activation in hippocampal pyramidal neurons after 24 and 72 hr seizure induction

To investigate the effect of VEGF in rats with seizure, VEGF was infused 5 days into rat hippocampus prior to seizure induction. Hippocampal sections processed either 24 or 72 hr after pilocarpine injection were probed with phospho-VEGFR-2. Immunofluorescence staining showed that phospho-VEGFR-2 was increased by VEGF infusion in CA1 and CA3 hippocampal pyramidal neurons after 24 hr (Figure 5A compare A2 with A1 for CA1 region; compare A4 with A3 for CA3 region) and 72 hr (Figure 5B compare B2 with B1 for CA1 region; compare B4 with B3 for CA3 region) seizure induction. After 24 hr seizure induction, VEGF infusion stimulated phospho-VEGFR-2 translocation from paranuclear region into nucleus (Figure 5A compare A2 with A1 for CA1 region; compare A4 with A3 for CA3 region).

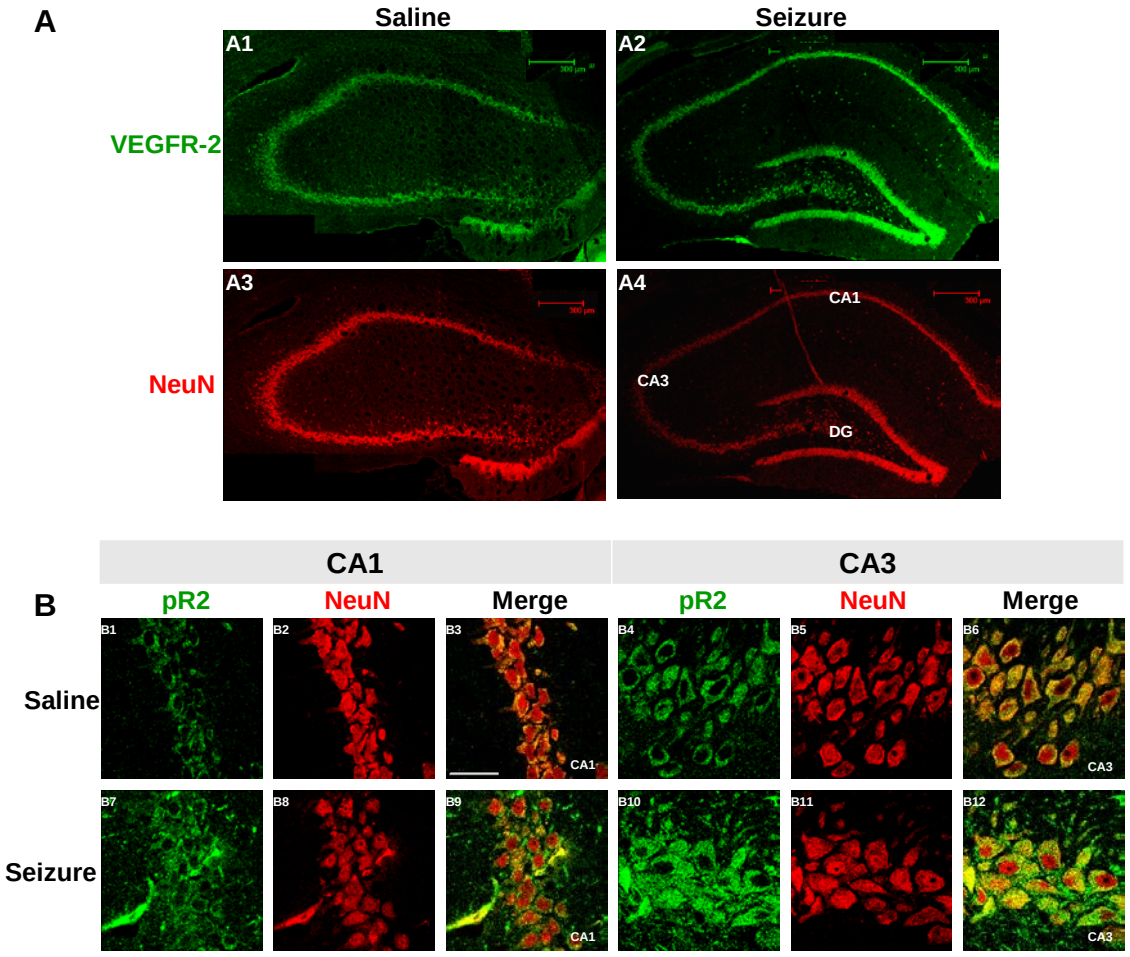


Figure 4. VEGFR-2 and phospho-VEGFR-2 are upregulated 24 hr after pilocarpine-induced SE. Rats were injected with either saline or pilocaprine for 1 hr and animals were scored for seizures. Tissues were obtained after 24 hr and sections were probed with antibodies that specifically recognize (A).VEGFR-2 (green) and (B) phospho-VEGFR-2 (pR2, green). Neurons were labeled with NeuN (red). Scale bar 30 µm if not indicated. Data are representative of experiments repeated at least three times.

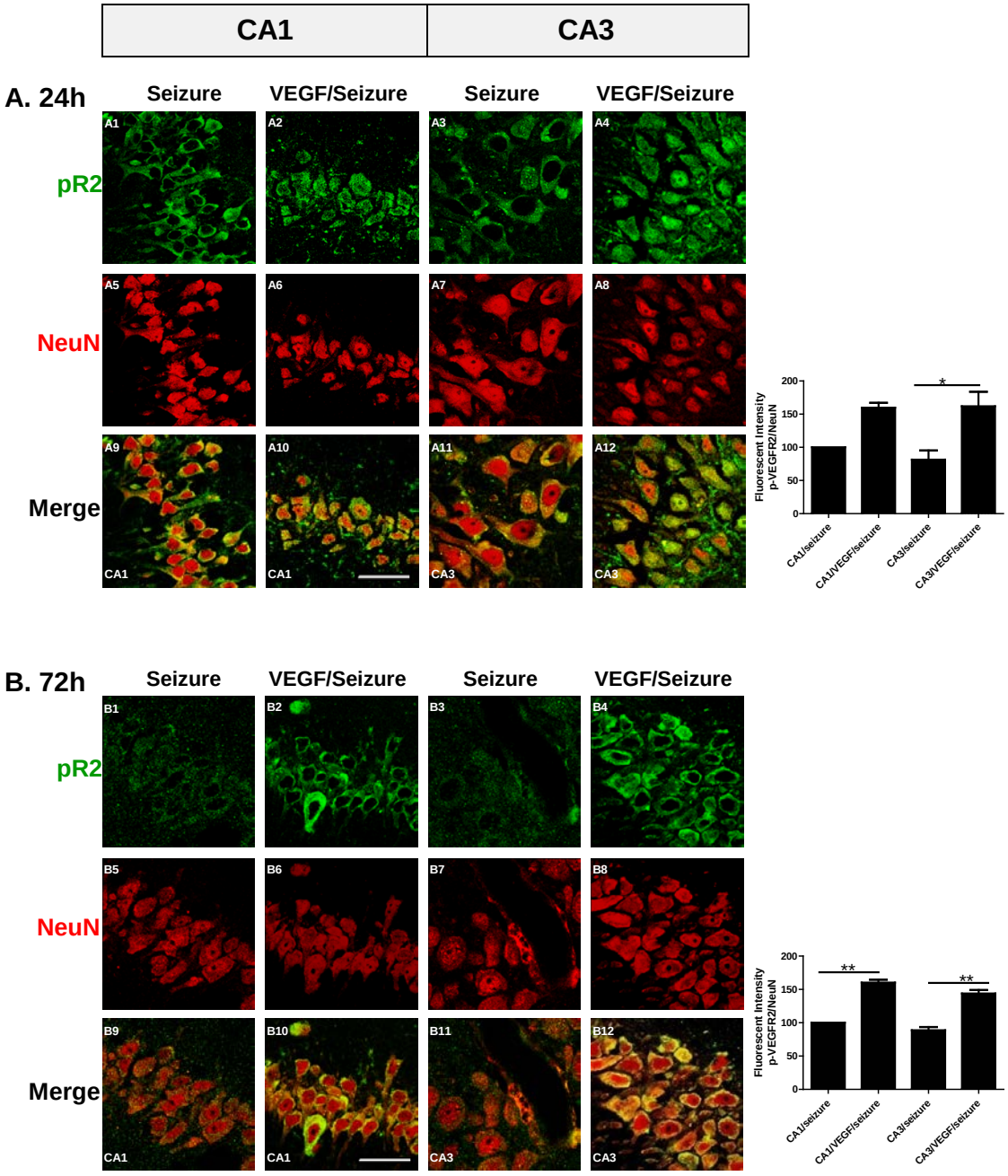


Figure 5. VEGF augments VEGFR-2 activation in hippocampal neurons after 24/72 hr seizure induction. Rats were infused 5 days without and with VEGF followed by pilocarpine injection. Hippocampal sections obtained after either (A). 24 hr or (B.) 72 hr were probed with anti phospho-VEGFR-2 (pR2, green) and NeuN (red). pR2 intensity was quantified using imageJ as a pR2/NeuN ratio relative to seizure alone ± S.E.M. from at least three independent staining. *($P<0.05$) indicates a significant difference between seizure vs. VEGF/seizure for 24 hr; **($P<0.01$) indicates a significant difference between seizure vs. VEGF/seizure for 72 hr. Scale bar 30 µm.

3.3 Seizures induce a transient increase in HO-1 expression that is suppressed by VEGF at 24 hr and attenuated by 72 hr

HO-1 expression after seizures is considered as a marker of oxidative stress which affects neuronal excitability and leads to neuronal damage. To further investigate the mechanism underlying VEGF-mediated neuroprotection, fresh hippocampal tissues were collected 24 hr after seizure induction from rats handled as described in 3.2. Proteins were extracted and subjected to western blotting analysis by probing for HO-1. Results showed that pilocarpine-induced *status epilepticus* induced a transient approximate 2 fold increase in HO-1 expression (Figure 6A compare lane 3 with lane 1). Administration of VEGF into the rat hippocampal region prior to seizure induction diminished HO-1 induction (Figure 6A compare lane 2 and 4 with lane 1 and 3).

To confirm the results, hippocampal sections were collected from the parallel experiment after 24 and 72 hr pilocarpine injection and processed to immunostaining. Tissues were double labeled with antibodies against HO-1 (green) and neuronal marker NeuN (red). Immunostaining confirmed that at 24 hr, HO-1 was present in the hippocampal CA1 and CA3 regions of rats undergoing seizures and attenuated by VEGF infusion (Figure 6B compare B2 with B1 for CA1 region and B6 with B5 for CA3). HO-1 levels didn't persist at 72 hr after seizure induction with or without VEGF administration (Figure 6B, B3 and B4 for CA1, B7 and B8 for CA3).

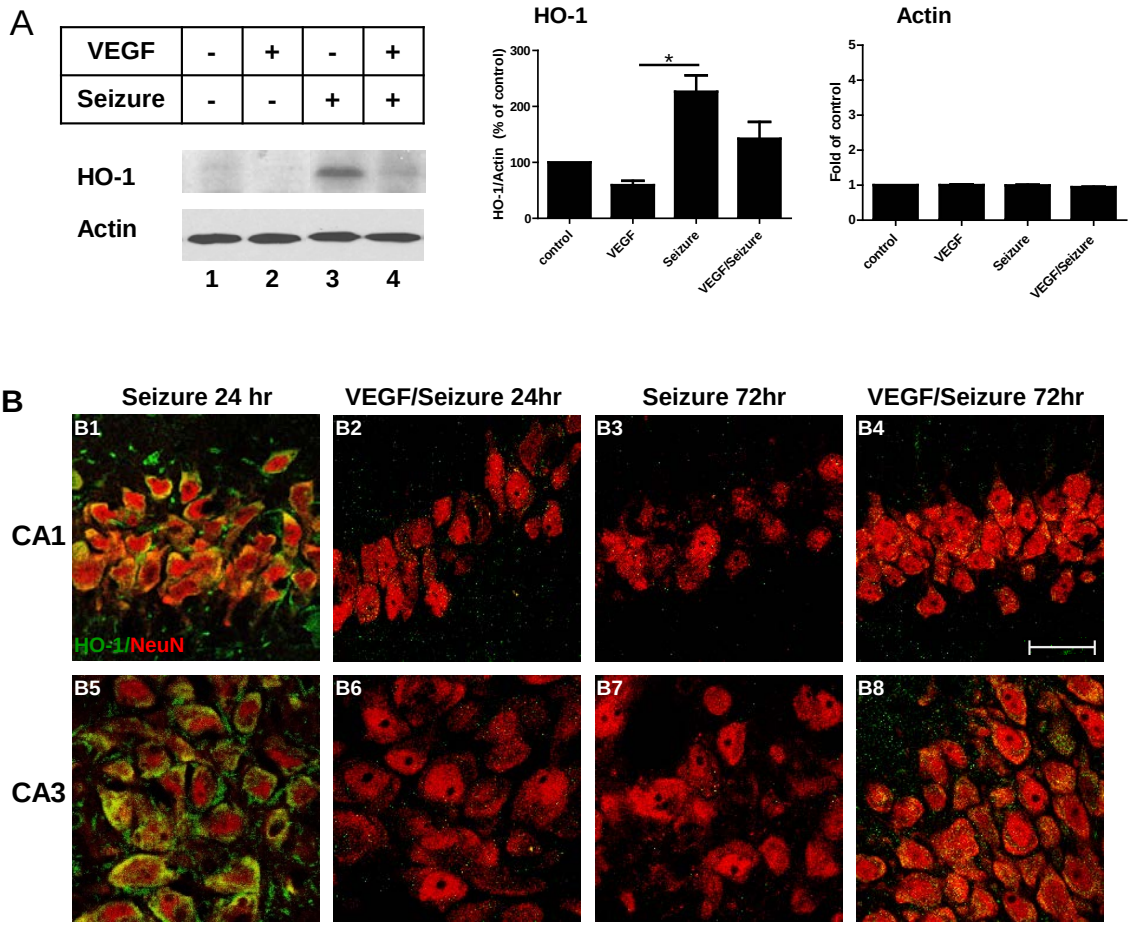


Figure 6. VEGF attenuates HO-1 protein levels in rats undergoing seizure. Rats were infused 5 days without and with VEGF followed by seizure induction with pilocarpine. (A). Proteins were extracted from hippocampal tissue chunks obtained 24 hr after seizure by using BioMaster disposable micro homogenizer and subjected to western analysis by probing with HO-1. Actin was probed as a loading control. Immunobloting was quantified by imageJ and shown as relative to control. *($P<0.05$) indicates a significant difference between seizure vs. VEGF. (B). Hippocampal sections obtained after either 24 hr (B1, 2, 5, 6) or 72 hr (B3, 4, 7, 8) were probed with anti HO-1 (green). Neurons were labeled with NeuN (red). Scale bar 30 µm. Data are representative of experiments repeated at least three times.

3.4 Seizures induce an increase in COX-2 expression persisting for 72 hr that is attenuated by VEGF at 24 and 72 hr

The inflammation indicator COX-2 is present in normal brain and upregulated by seizures (Hurley et al. 2002, Yamagata et al. 1993). COX-2 generates oxidative stress in brain causing further damage to the central nervous system. To explore the role of COX-2 in VEGF-mediated neuroprotection, proteins were extracted as described in 3.3 and subjected to western blotting analysis by probing with COX-2. Results showed that COX-2 was expressed in rat hippocampal regions under basal conditions and upregulated by pilocarpine-induced SE (Figure 7A compare lane 1 with lane 3). VEGF reduced COX-2 protein levels under both basal conditions and SE (Figure 7A compare lane 2 and 4 with lane 1 and 3).

Double immunostaining with COX-2 (green) and NeuN (red) of hippocampal tissue sections collected from the parallel experiment confirmed that COX-2 was present at the hippocampal CA1 and CA3 regions of rats undergoing seizures at 24 hr and persist at 72 hr after seizure onset (Figure 7B, B1 and B3 for CA1, B5 and B7 for CA3.). Infusion of VEGF into rat hippocampus reduced COX-2 expression elevated by seizures at 24 hr and extended to a more dramatic decrease of COX-2 protein levels at 72 hr after seizures (Figure 7B, compare B2 with B1 at 24 hr and B4 with B3 at 72 hr for CA1 region, compare B6 with B5 at 24 hours and B8 with B7 at 72 hr for CA3 region).

Taken together, our results indicate that VEGF mediates neuroprotection against pilocarpine induced SE through the regulation of COX-2 expression in brain.

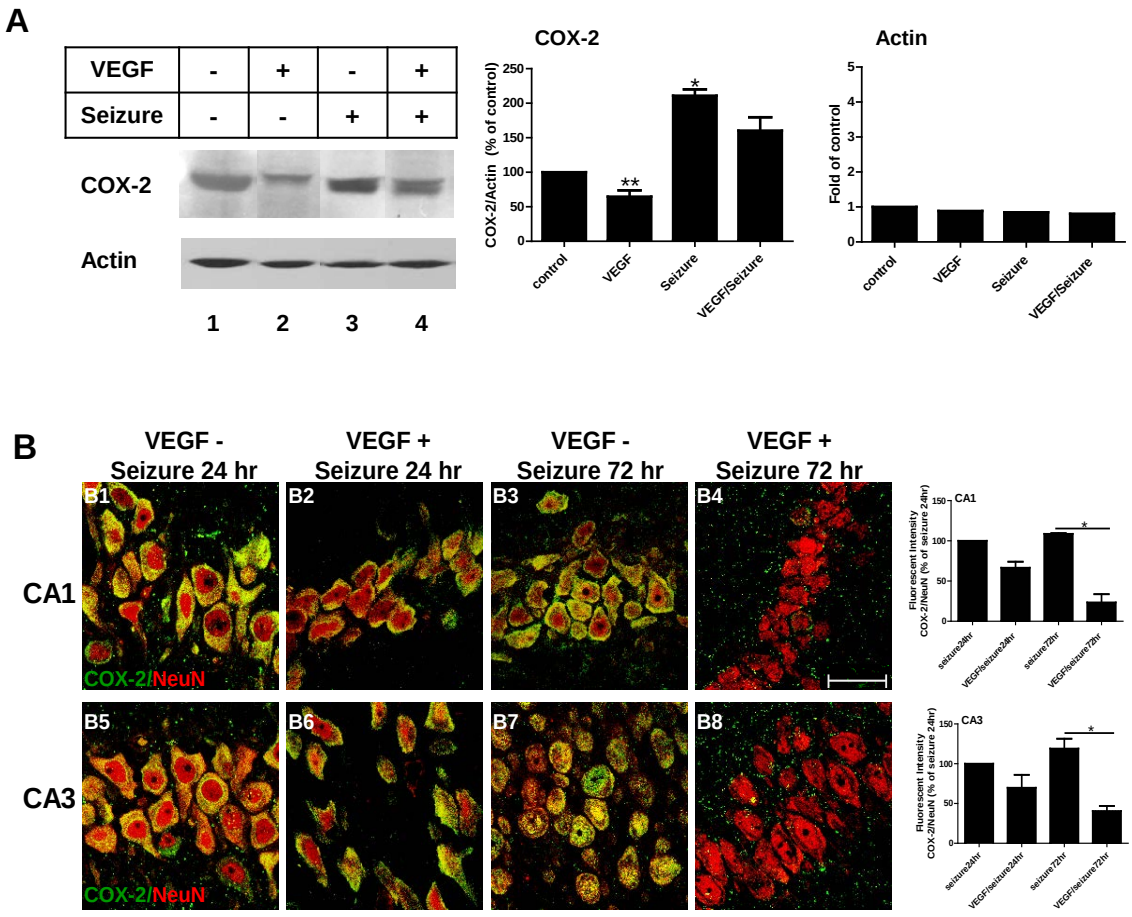


Figure 7. VEGF attenuates COX-2 protein levels in rats undergoing seizure. (A). Proteins as described in Figure 6 were subjected to western analysis by probing with COX-2. Blots was quantified by imageJ and shown as relative to control. * ($P<0.05$) indicates a significant difference between seizure vs. control; **($P<0.01$) indicates a significant difference between VEGF vs. VEGF/seizure. (B). Hippocampal sections obtained after either 24 hr (B1, 2, 5, 6) or 72 hr (B3, 4, 7, 8) were probed with anti COX-2 (green). Neurons were labeled with NeuN (red). Scale bar 30 µm. Fluorescent intensity of COX-2 relative to NeuN was quantified by ImageJ. *($P<0.05$) indicates a significant difference between seizure 72 hr vs. VEGF/Seizure 72 hr in both CA1 and CA3 regions. Data are representative of experiments repeated at least three times.

3.5 Neutralizing antibody against VEGFR-2 (anti-VEGFR-2) blocks VEGFR-2 phosphorylation in VEGF infused rats undergoing seizure

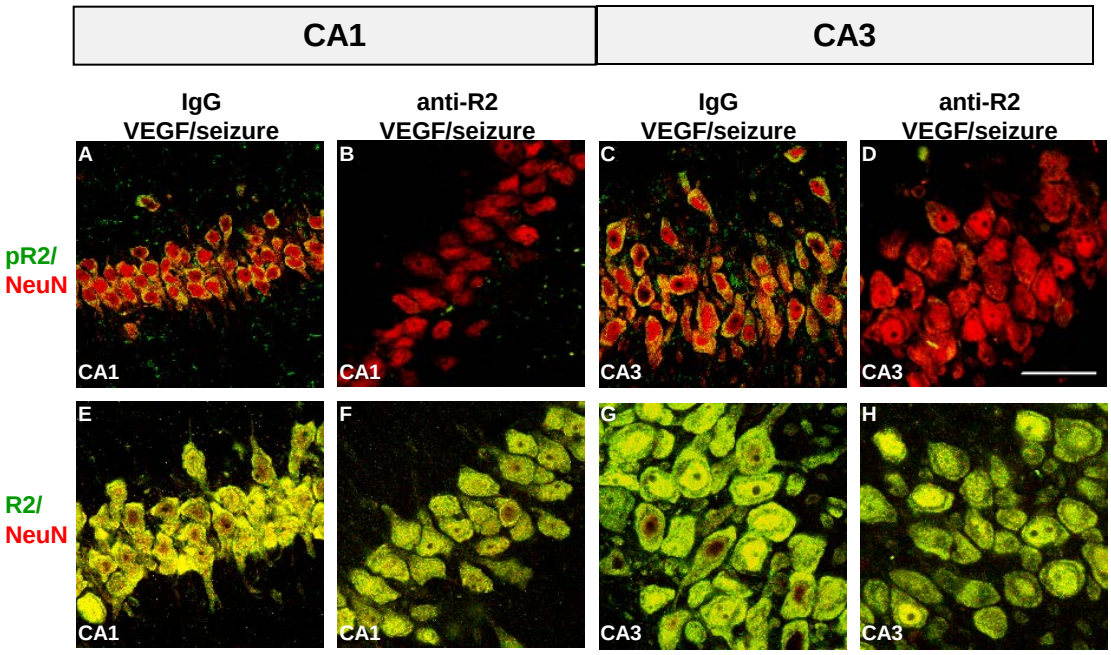


Figure 8. Anti-VEGFR-2 (anti-R2) blocks VEGFR-2 phosphorylation. Rats were infused 5 days with VEGF and/or anti-R2 followed by seizure induction with pilocarpine. Hippocampal tissue sections were obtained after 24 hr and probed with anti-phospho-VEGFR-2 (pR2, green, A-D) and VEGFR-2 (R2, green, E-H). Neurons were labeled with NeuN (red). Scale bar 30 µm. Data are representative of experiments repeated at least three times.

To verify that VEGF functions through VEGFR-2 to exert its neuroprotective effects, a neutralizing antibody against VEGFR-2 (anti-VEGFR-2, anti-R2) was used to block VEGF signaling. VEGF and/or anti-R2 were infused 5 days into rat hippocampus with PBS and IgG as control, respectively. All animals were injected with pilocarpine to induce seizure. Double immunofluorescence staining with hippocampal sections using specific antibodies showed that anti-VEGFR-2 antibody blocked VEGFR-2 activation with or without the presence of VEGF in

both hippocampal CA1 (Figure 8, compare B with A) and CA3 regions (Figure 8, compare D with C) in rats developed seizure without affecting the total VEGFR-2 levels (Figure 8, E-H).

3.6 VEGF protects against hippocampal neuronal cell loss and attenuates HO-1 protein levels induced by anti-VEGFR-2 in rats undergoing seizure

To determine the effect of anti-VEGFR-2 on VEGF-mediated neuroprotection, neuronal density of hippocampal sections from rats described in 3.5 was quantified using a stereological software package (performed by Elisa Saleni in Dr. Susan Croll's lab at Queens College). The results showed that VEGFR-2 blockade led to a more severe neuronal cell loss compared to seizure alone (Figure 9A, compare column #3 with #1). Surprisingly, anti-VEGFR-2 completely inhibited VEGFR-2 phosphorylation but only partially blocked VEGF-mediated neuroprotection against seizure (Figure 9A, compare column #4 with #3), suggesting that VEGF promotes neuronal survival against seizure not only through VEGFR-2; other receptors binding to VEGF such as VEGFR-1 or NP-1 which are also expressed in neurons may be involved.

Double immunofluorescence staining with hippocampal tissue sections using specific antibody showed that VEGFR-2 blockade induced elevated HO-1 expression compared to seizure alone (Figure 9B, compare B with A). And the elevated HO-1 levels were downregulated by VEGF (Figure 9B, compare D with C) to the similar HO-1 levels induced by seizure alone (Figure 9B, compare D with A), which was correlated with the neuronal cell counts (Figure 9A, compare column #4 with column #1), suggesting that HO-1 expression is associated with neuronal cell loss.

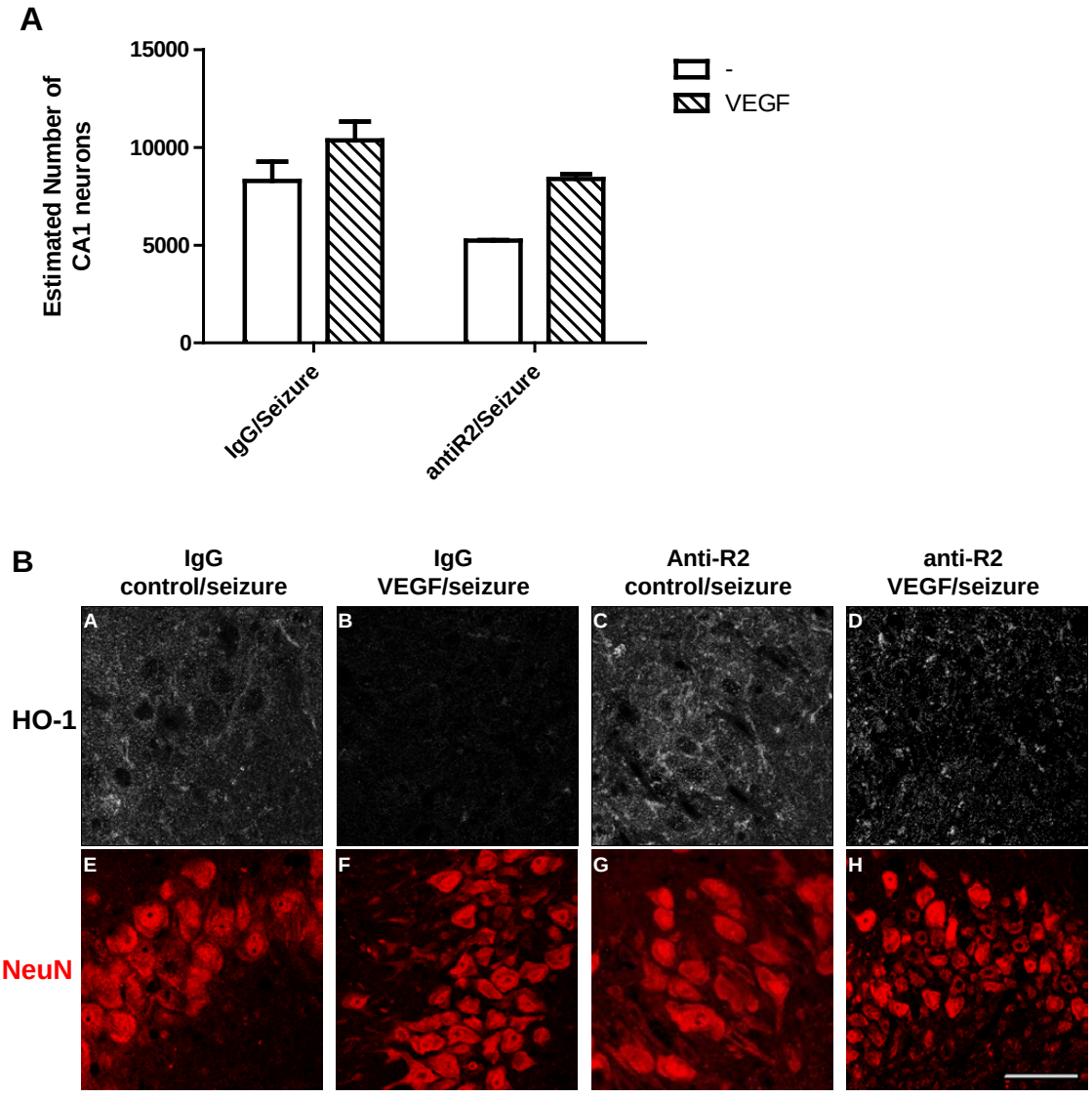


Figure 9. VEGF protects against anti-VEGFR-2 (anti-R2) induced neuronal damage in rats undergoing seizure. Rats were infused 5 days with VEGF and/or anti-R2 followed by seizure induction with pilocarpine. Hippocampal tissue sections were obtained after 24 hr. (A). Stereology counts of hippocampal neurons, cell counts provided by Elisa Saleni in Dr. Susan Croll's lab at Queens College. (B). Tissue sections were probed with anti HO-1 (A-D). Neurons were labeled with NeuN (red). Scale bar 30 µm. Data are representative of experiments repeated at least three times.

Taken together, our *in vivo* studies indicate that VEGF-mediated neuroprotection is regulated through the activation of VEGFR-2 and other VEGF receptors such as VEGFR-1 or/and NP-1 may be involved. VEGF protects against neuronal cell loss induced by SE through the regulation of seizure-related COX-2 and HO-1 expression.

Chapter IV

VEGF protects against cell death and oxidative stress induced by pilocarpine in primary culture of rat hippocampal neurons

We next use primary culture of hippocampal neurons to further assess VEGF-mediated neuroprotection and the signaling mechanisms underlying.

4.1 VEGF protects against cell loss and attenuates HO-1 expression induced by pilocarpine in NB cultured neurons

In our *in vitro* studies, the concentration of pilocarpine was optimized as 100 μM comparing with 10 μM and 1 mM (data not shown). The same concentration has been shown to depolarize isolated neurons in primary cultures (Turrigiano and Marder 1993, Yavorsky and Lukyanetz 1997).

To investigate the effects of pilocparpine *in vitro*, MTS assay was performed to assess cell viability. Primary hippocampal neurons were maintained in culture with half GCM and half SNB for approximately 2 weeks. Cells were then incubated for 48 hr in SNB (remove GCM alone from culture medium) or NB (remove both GCM and B-27) in the absence and presence of 100 ng/ml VEGF and treated with 100 μM pilocarpine for 24 hr prior to MTS assay, with VEGF being replenished after 24 hr. The results showed that neurons were more stressful in NB than in SNB and pilocarpine only reduced cell viability in NB when B-27 was further removed; VEGF was stimulatory in both culture conditions and recued the cell loss induced by pilocarpine in NB (Figure 10).

In addition, pilocarpine exerted a time dependent effect on cell viability *in vitro*: pilocarpine induced loss of cell viability requiring at least 4 hr treatment and 24 hr treatment further reduced cell viability (data not shown). In our *in vivo* studies, almost all tissues used were collected 24 hr after pilocarpine injection except one set of animals was processed 72 hr after pilocarpine injection as presented in chapter 3.

Our *in vivo* results showed that pilocarpine-induced seizures were accompanied with increased HO-1 expression that was suppressed by VEGF (Figure 6). Using cultured hippocampal neurons, western blotting results showed that *in vitro* HO-1 was present in cells in NB (Figure 10B, lane 1); 24 hr pilocarpine treatment induced a 2 fold HO-1 expression that was suppressed by VEGF administration (Figure 10B, compare lane l and 2 with lane 1 and 3).

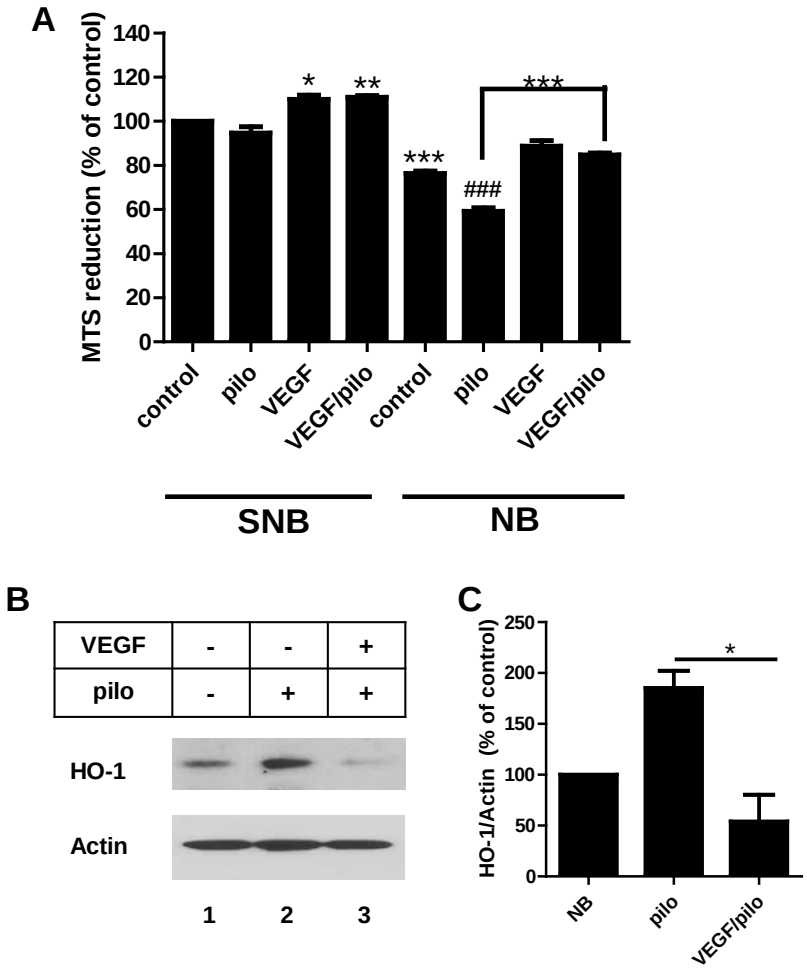


Figure 10. VEGF protects against cell loss and attenuates HO-1 expression induced by pilocarpine in primary hippocampal neurons. Cells were incubated for 48 hr in SNB or NB without and with 100 ng/ml VEGF and treated with 100 µM pilocarpine for 24 hr. VEGF was replenished after 24 hr. (A). Cell viability was determined using a MTS assay as described in Materials and Methods section. Results represent the percent cell viability relative to the vehicle treated control ± S.E.M. from at least three independent experiments. *($P<0.05$) indicates a significant difference between VEGF vs. control and **($P<0.01$) indicates a significant difference between VEGF/pilo vs. control in SNB; ***($P<0.001$) indicates a significant difference between control NB vs. control SNB; a significant difference between pilo vs. VEGF/pilo in NB; $^{###}$($P<0.001$) indicates a significant difference between pilo vs. control in NB. (B). Lysates of cells in NB were analyzed by western blotting probing with anti HO-1. (C). Blots were quantified by imageJ and shown as relative to control ± S.E.M. from at least three independent experiments. *($P<0.05$) indicates a significant difference between pilo vs. VEGF/pilo.

4.2 Pilocarpine-induced cell loss *in vitro* is associated with oxidative stress

Pilocarpine increased HO-1 expression in NB (Figure 10B), which is an indicator of cellular oxidative stress and is induced under stressful conditions. Previous studies in our lab using SK-N-SH neuroblastoma cells also demonstrated that pilocarpine induces cell death involving oxidative stress and caspase cleavage (Edelstein et al. 2011). In addition, pilocarpine reduced viability of cultured neurons only in NB without the presence of B-27 (Figure 10), which contains antioxidant cocktail, suggesting that oxidative stress may be involved in pilocarpine-induced cell loss *in vitro*. Antioxidant NAC was used to confirm the presence of oxidative stress. Western blotting showed that NAC suppressed HO-1 expression stimulated by pilocarpine to nearly the basal levels of cells cultured in NB (Figure 11A, lane 1, 2, and 4). MTS assay also showed that NAC prevented cell loss induced by pilocarpine (Figure 11C). Taken together, our observations *in vitro* are consistent with those *in vivo*: pilocarpine-induced cell death is associated with oxidative stress.

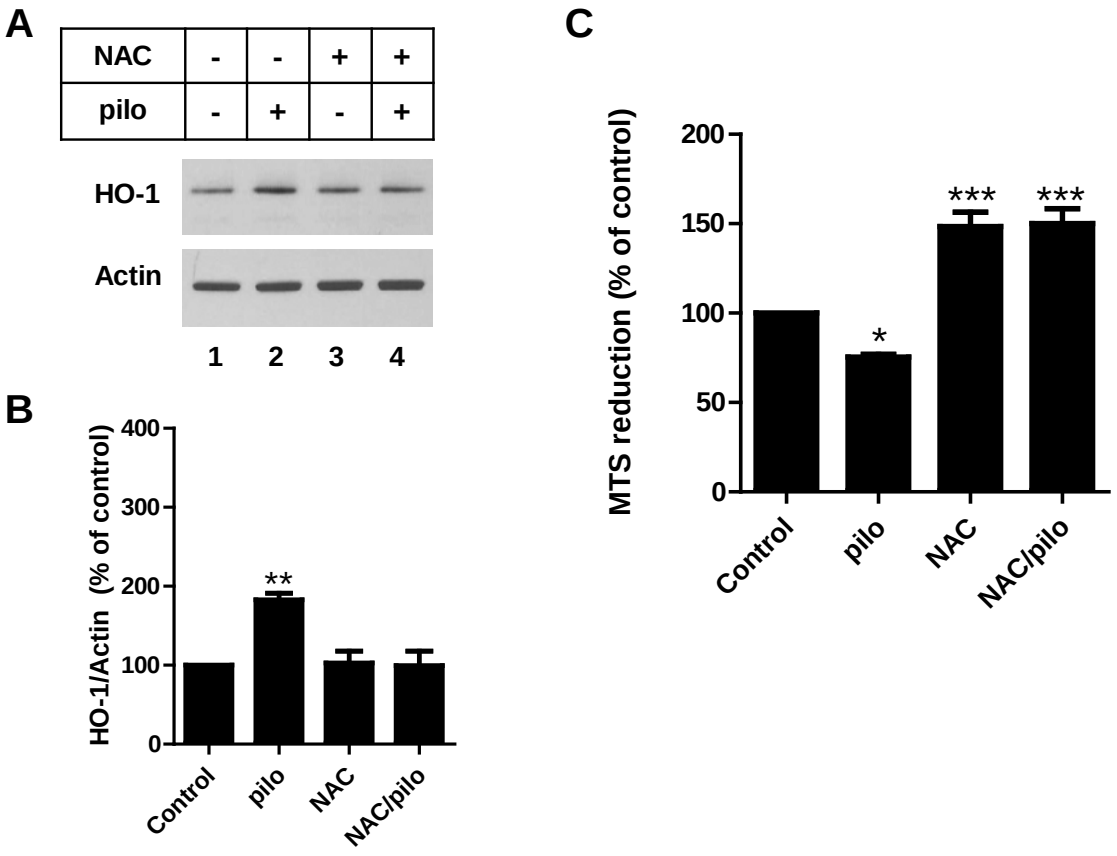


Figure 11. HO-1 upregulation accompanies pilocarpine-induced neuronal loss and both events are diminished by NAC. Cells were incubated in NB for 48 hr and treated with pilocarpine (100 µM) with or without NAC (5 mM) for 24 hr. (A). Lysates were analyzed by western blotting probing with anti HO-1; Acting was probed as a loading control. (B). Blots were quantified by imageJ and shown as relative to control ± S.E.M. **($P<0.01$) indicates a significant difference between pilo vs. control, NAC or NAC/pilo. (C). Cell viability was determined using a MTS assay as described in Materials and Methods section. Results represent the percent cell viability relative to the vehicle treated control ± S.E.M. *($P<0.05$) indicates a significant difference between pilo vs. control; ***($P<0.001$) indicates a significant difference between NAC vs. control and pilo vs. NAC/pilo.

4.3 VEGF/VEGF-B prevents ROS production induced by piloparpine *in vitro*

We next assessed oxidative stress by measuring ROS levels upon pilocarpine treatment. Living cells were stained with Carboxy-H_2DCFCA. Fluorescent images showed that pilocarpine induced ROS production after 24 hr treatment (Figure 12A, compare A6 with A2).

Cellular ROS is primarily generated in mitochondria and is associated with the collapse of $\Delta\Psi_m$ (Kroemer et al. 2007). We then evaluated the $\Delta\Psi_m$ of living cells by labeling with TMRE, a positive charged dye retained in healthy mitochondria due to high $\Delta\Psi_m$. Results showed that 24 hr pilocarpine treatment had no effect on $\Delta\Psi_m$ in cultured hippocampal neurons (Figure 12B, compare B6 with B2).

Since our *in vivo* studies showed that VEGF protected against neuronal cell loss and attenuated HO-1 expression induced by anti-VEGFR-2 in rats of SE, we hypothesized that VEGFR-2 might not be the only receptor regulating VEGF signaling under this circumstances. *In vitro* ROS labeling showed that exogenous VEGF or VEGF-B decreased ROS production induced by pilocarpine (Figure 12A, compare A10, A14 with A2). Since VEGF binds to both VEGFR-1 and VEGFR-2 while VEGF-B only binds to VEGFR-1, our results suggested that VEGFR-2 and VEGFR-1 both exerted protective effect against pilocarpine upon ligand stimulation.

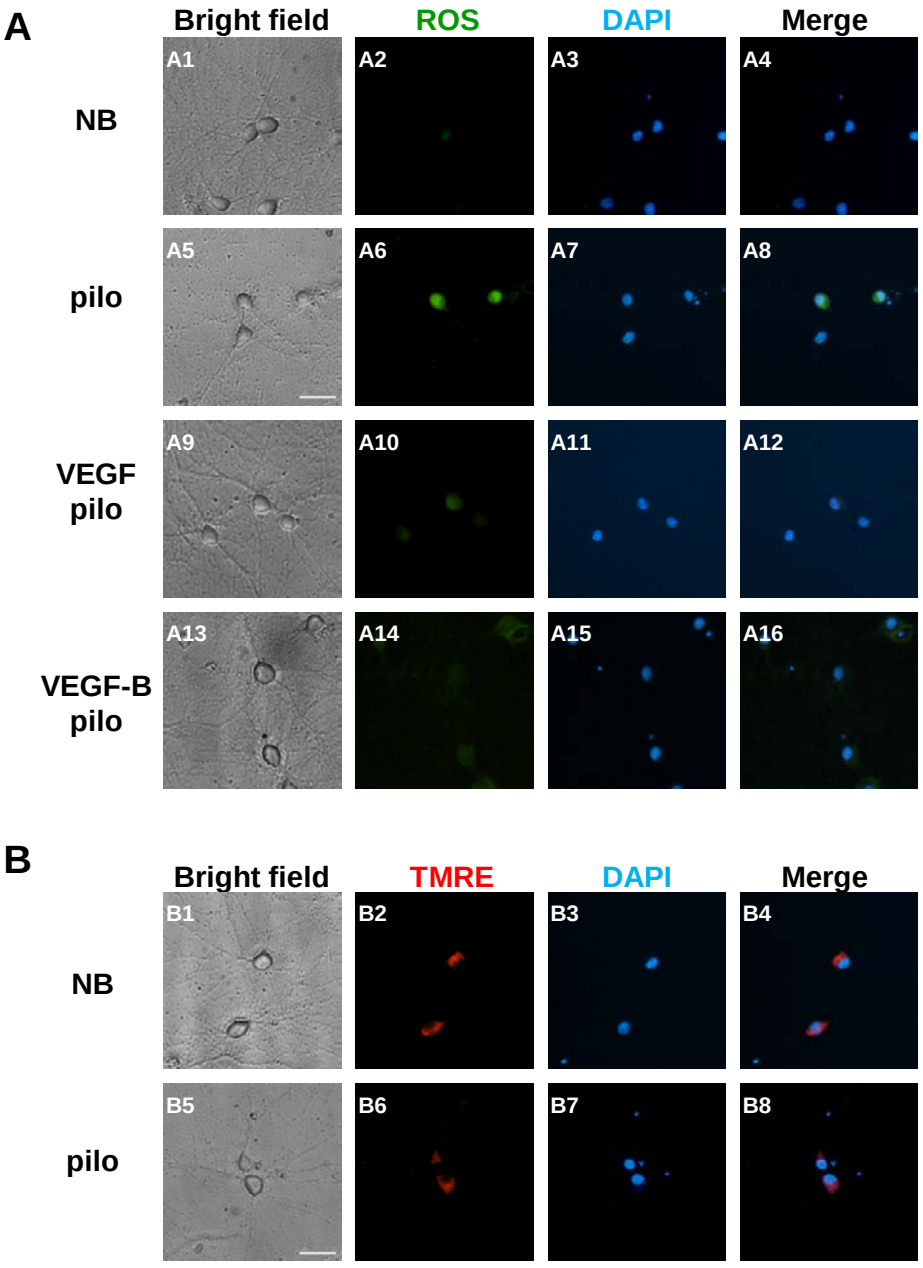


Figure 12. Pilocarpine induces ROS production attenuated by VEGF/VEGF-B but has no effect on mitochondrial membrane potential. Cells were incubated for 48 hr in NB without and with VEGF (100 ng/ml) and treated with pilocarpine (100 µM) for 24 hr. Living cells were labeled with (A). ROS (green) and (B). TMRE (red) as described in Materials and Methods section. Nuclei were counterstained with DAPI. Scale bar: 50 µm. Data are representative of experiments repeated at least three times.

Chapter V

VEGF-mediated neuroprotection is associated with oxidative stress and mitochondrial dysfunction in primary hippocampal neurons

5.1 VEGF stimulates VEGFR-2 phosphorylation in primary hippocampal neurons

VEGFR-2 is the major VEGF receptor expressed in neurons. To assess the effect of VEGF on VEGFR-2 activation, cells in NB with the absence and presence of VEGF for 48 hr were treated with SU1498 for 24 hr. Western blotting showed that phospho-VEGFR-2 was present in neurons cultured in NB (control) (Figure 13A, lane 1) and exogenous VEGF increased VEGFR-2 activation (Figure 13A, compare lane 3 with lane 1); 24 hr SU1498 treatment completely blocked VEGFR-2 activation (Figure 13A, lane 2 and lane 4), suggesting that VEGF signals through VEGFR-2 activation in primary neurons. Immunostaining with antibody against phospho-VEGFR-2 confirmed that phospho-VEGFR-2 was present under control conditions (NB) and exogenous VEGF increased VEGFR-2 activation (Figure 13B, compare B4 with B1). VEGF also stimulated phospho-VEGFR-2 distribution along the dendrites (Figure 13B, B4).

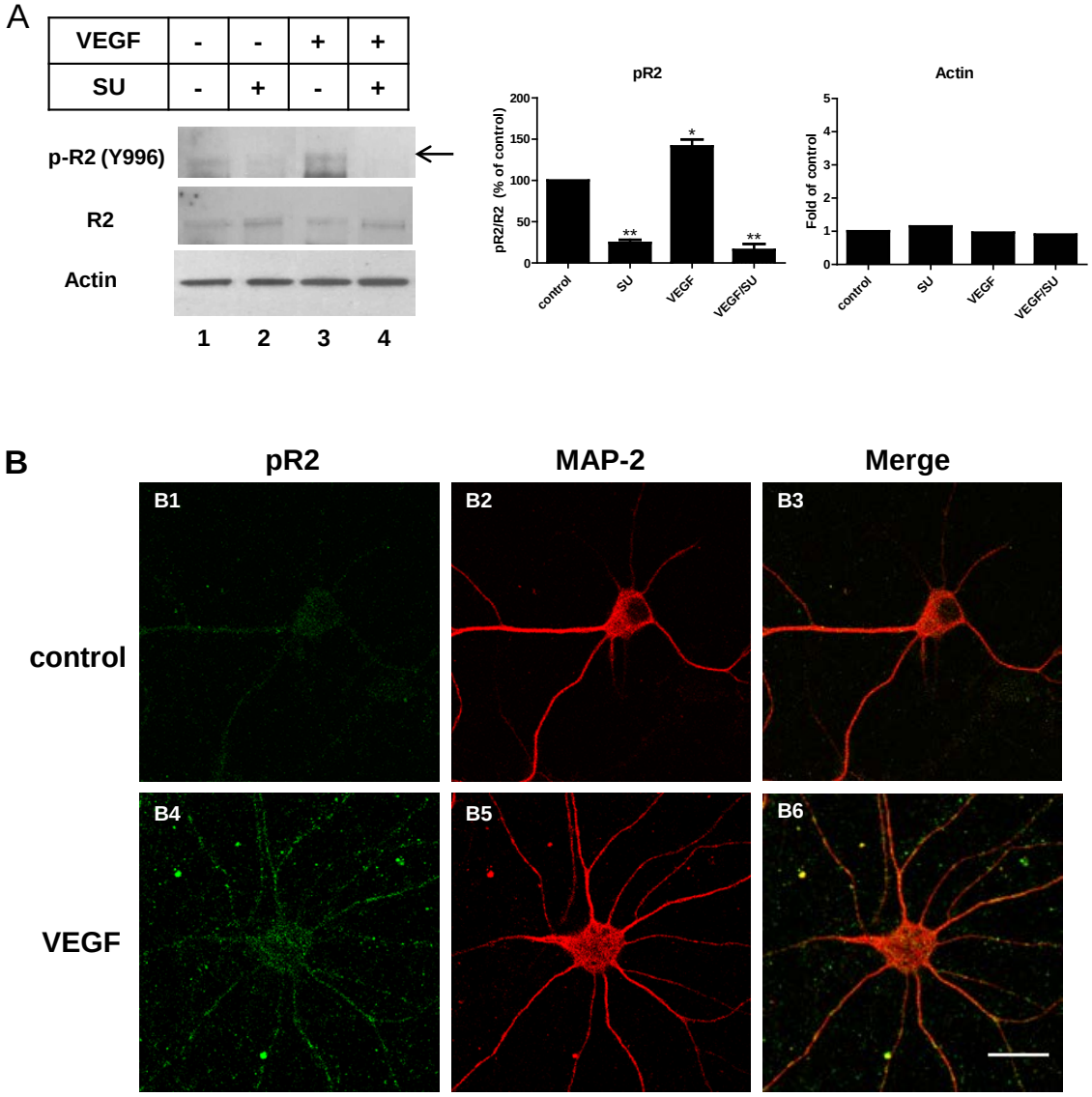


Figure 13. VEGF stimulates VEGFR-2 activation in primary hippocapmal neurons. Cells in NB with the absence and presence of VEGF (100 ng/ml) for 48hr were (A). treated with SU1498 (10 µM) for 24 hr. Lysates were analyzed by western blotting probing with antibodies for phospho-VEGFR-2 (p-R2) and R2. Blots were quantified using ImageJ. *($P<0.05$) indicates a significant difference between VEGF vs. control; **($P<0.01$) indicates a significant difference between SU or VEGF/SU vs. control for pR2. (B). double labeled with antibodies against p-R2 (green) and MAP-2 (red). Scale bar 100 µm. Data are representative of experiments repeated at least three times.

5.2 Inhibition of VEGFR-2 by SU1498 induces cell death in SNB and NB in different patterns

To investigate the effects of VEGFR-2 inhibition *in vitro*, MTS assay was performed to assess cell viability. Primary hippocampal neurons were kept in culture for approximately 2 weeks. Cells were then incubated for 48 hr in SNB (removing GCM alone) or NB (removing both GCM and B-27). SU1498 (10 μM) was added at 15 min, 1, 2, 4 or 24 hr prior to MTS assay. The results showed that inhibition of VEGFR-2 by SU1498 induced cell death in both SNB and NB but with distinct patterns. In SNB where B-27 is present, SU1498 induced a time dependent neuronal cell death starting after 1 hr inhibitor treatment and cell viability gradually dropped over time to about 40% at 24 hr (Figure 14A). However, in NB when B-27 is removed, SU1498 immediately reduced cell viability to about 50% after 15 min treatment and prolonged inhibitions up to 24 hr causing no further significant damages regarding to cell viability (Figure 14B). B-27 supplement consists of antioxidant cocktail, Vitamin A and Insulin. In addition, our studies in chapter 3 and chapter 4 demonstrated that VEGF-mediated neuroprotection against pilocarpine is associated with oxidative stress. Taken together, our results indicated that SU1498-induced cell death may be associated with oxidative stress.

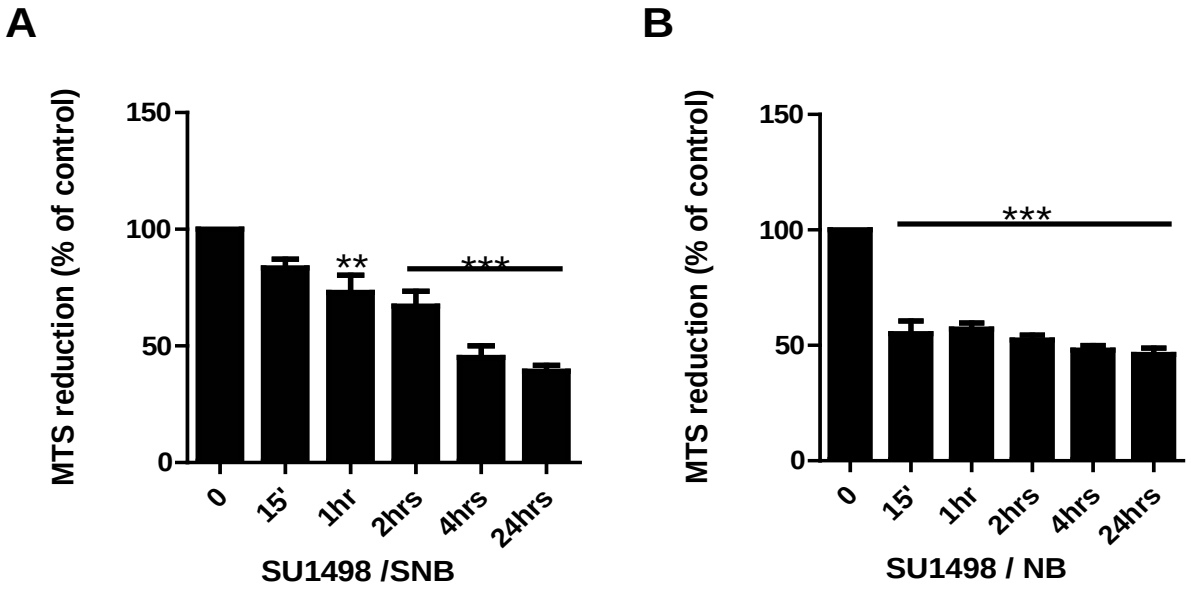


Figure 14. VEGFR-2 inhibition by SU1498 induces cell death in SNB and NB. Cells were treated with SU1498 (10 µM) for 15', 1, 2, 4 and 24 hr. Cell viability was determined using a MTS assay as described in Materials and Methods section. Results represent the percent cell viability relative to the vehicle treated control ± S.E.M. from at least three independent experiments. **($P<0.01$) indicates a significant difference between 1 hr SU1498 vs. control (SNB). ***($P<0.001$) indicates a significant difference between 2, 4, and 24 hr SU1498 vs. the SNB control and between 15', 1, 2, 4, 24 hr SU1498 vs. the NB control.

5.3 SU1498 exerts direct oxidative stress causing cell death accompanied with caspase-3 cleavage and ROS production

To further determine whether the cell death induced by VEGFR-2 inhibition is associated with oxidative stress and apoptosis, cells in NB for 48 hr were treated with SU1498 in the absence and presence of NAC for 24 hr. Western blotting showed that the oxidative indicator HO-1 was expressed in neurons cultured in NB (Figure 15A, lane 1). SU1498 induced a 3 fold HO-1 expression and promoted caspase-3 cleavage that were both suppressed by NAC to nearly the basal levels (Figure 15A, compare lane 2 & 3 with lane 1). Correspondingly, MTS assays showed that NAC protected against the loss of cell viability induced by SU1498 (Figure 15B), suggesting that cell death induced by VEGFR-2 inhibition is associated with oxidative stress.

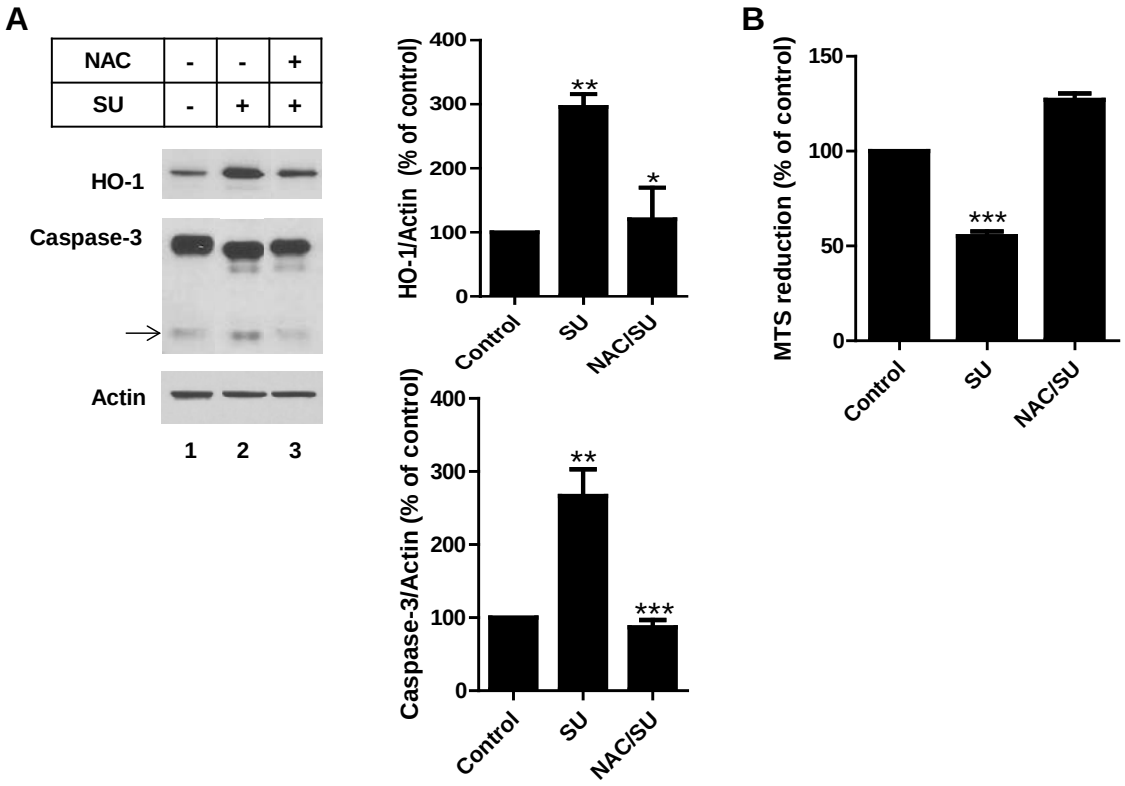


Figure 15. NAC reduces HO-1 levels, casepase-3 cleavage and protects against neuronal cell loss induced by SU1498. Cells in NB for 48 hr were treated with SU1498 (10 µM) in the absence and presence of NAC (5 mM) for 24 hr. (A). Cell lysates were analyzed by western blotting probing with anti HO-1 and Caspase-3. Acting was probed as a loading control. Blots were quantified using ImageJ. *($P<0.05$) indicates a significant difference between SU vs. NAC/SU for HO-1 and **($P<0.01$) indicates a significant difference between SU vs. control for HO-1 and Caspase-3. ***($P<0.001$) indicates a significant difference between SU vs. NAC/SU for Caspase-3. (B). Cell viability was determined using a MTS assay as described in Materials and Methods section. ***($P<0.001$) indicates a significant difference between SU vs. control or NAC/SU.

5.4 VEGF reduces HO-1 levels, protects against caspase-3 cleavage and neuronal cell loss induced by short-term SU1498 treatment

In NB, SU1498-induced cell death occurred after 15 min treatment and the decrease in cell viability reached a plateau at 2 hr that was unchanged after 24 hr of inhibition (Figure 14). Therefore, the effects of VEGF on cell viability were evaluated at 2 (short term) and 24 hr (long term) after cells were treated with SU1498. MTS assay with cells pretreated with VEGF showed that VEGF protected against neuronal cell loss induced by 2 hr SU1498 treatment but had no protective effect when VEGFR-2 was blocked by SU1498 for 24hr (Figure 16A).

Nevertheless, the protective effect of VEGF against SU1498 is consistent with our *in vivo* findings that VEGF protects against cell loss induced by VEGFR-2 blockade by VEGFR-2 neutralizing antibody in rats with SE (Figure 9A).

We then assessed the effect of SU1498 treatment on oxidative stress related mechanisms. Similar to 24 hr SU1498 treatment (Figure 15), Western blotting showed that SU1498 treatment for 2 hr induced a 2 fold HO-1 expression and stimulated caspase-3 activation. Both events were attenuated by exogenous VEGF (Figure 16B, compare lane 2 with lane 1 and 4). Furthermore, western blotting confirmed that VEGFR-2 phosphorylation was completely blocked by 2 hr SU1498 without and with exogenous VEGF (data now shown). These findings suggest that VEGF might signal through an alternate receptor such as VEGFR-1 or NP-1 to exert protection when VEGFR-2 activity is blocked.

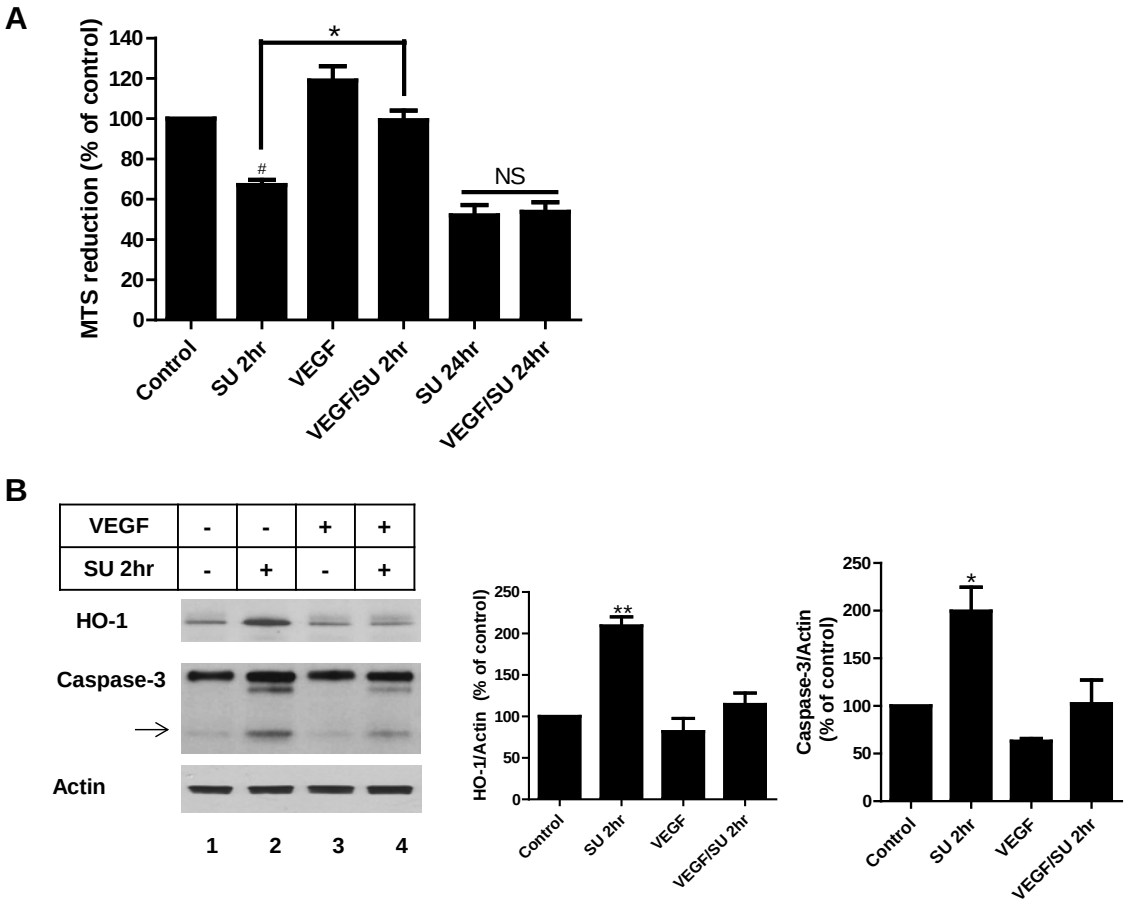


Figure 16. VEGF reduces HO-1 levels, protects against caspase-3 cleavage and neuronal cell loss induced by 2 hr SU1498 treatment. (A). Cells were treated without and with VEGF (100 ng/ml) for 48 hr in NB. Cell viability was measured after either 24 hr or 2 hr SU1498 (10 µM) treatment as described in Materials and Methods section. *($P<0.05$) and $^{\#}$($P<0.05$) indicate a significant difference between SU 2 hr vs. VEGF/SU 2 hr or control, respectively. (B). Cells in NB were treated for 48 hr without and with VEGF (100 ng/ml) and SU1498 (10 µM) for 2 hr. Lysates were analyzed by western blotting probing with anti HO-1 and Caspase-3. Blots were quantified by imageJ. **($P<0.01$) and *($P<0.05$) indicate a significant difference between SU vs. control or VEGF/SU1498 for HO-1 and caspase-3, respectively.

Since VEGF only prevents neuronal cell loss against 2 hr but not 24 hr SU1498 treatment (Figure 16A), cell in all the following experiments were treated with a short period (2 hr) of SU1498 if not indicated.

5.5. VEGF and NAC decrease ROS accumulation and prevent the loss of $\Delta\Psi_m$ induced by SU1498

The HO-1 induction and caspase-3 cleavage suggested that mitochondrial dysfunction was associated with the cell death induced by SU1498. ROS production is tightly associated with mitochondrial dysfunction. To address this possibility, cellular ROS and mitochondrial dysfunction were investigated upon VEGFR-2 inhibition.

Living cell images with Carboxy-H_2DCFCA showed that VEGFR-2 inhibition by SU1498 was accompanied by a dramatically increased ROS production, which was abrogated by NAC (Figure 17, compare A4 with A2) and VEGF (Figure 19A, compare A5 with A11).

To address the mitochondrial dysfunction associated with these events, $\Delta\Psi_m$ was measured by labeling living cells with TMRE. The results showed that inhibition of VEGFR-2 by SU1498 caused a loss of $\Delta\Psi_m$ that was prevented by NAC (Figure 18, compare B4 with B2) and VEGF (Figure 19B, compare B5 with B11).

Taken together, our findings demonstrate that the increased HO-1 expression, ROS production, caspase-3 activation and loss of mitochondrial membrane potential is associated with increased oxidative stress caused by VEGFR-2 inhibition. In addition, VEGF may signal through an alternative receptor when VEGFR-2 function is blocked.

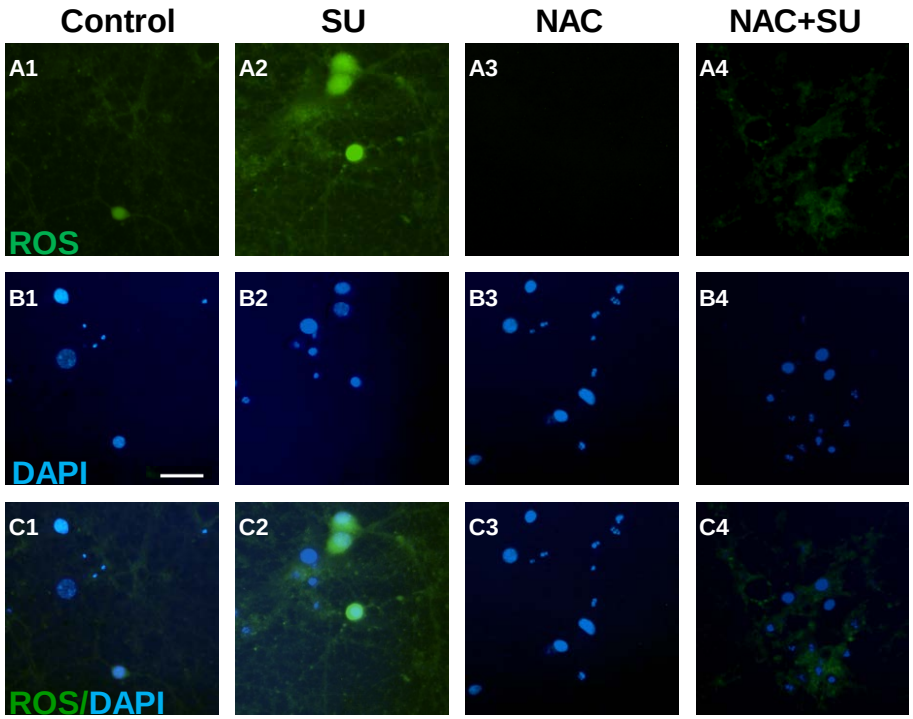


Figure 17. NAC diminishes ROS accumulation induced by SU1498. Cells in NB for 48 hr were treated with NAC (5 mM) for 24 hr. SU1498 (10 µM) was added 2 hr before ROS labeling. ROS in living cells was labeled by Carboxy-H_2DCFCA (green) as described in Materials and Methods section. Nuclei were counterstained with DAPI. Scale bar: 50 µm. Data are representative of experiments repeated at least three times.

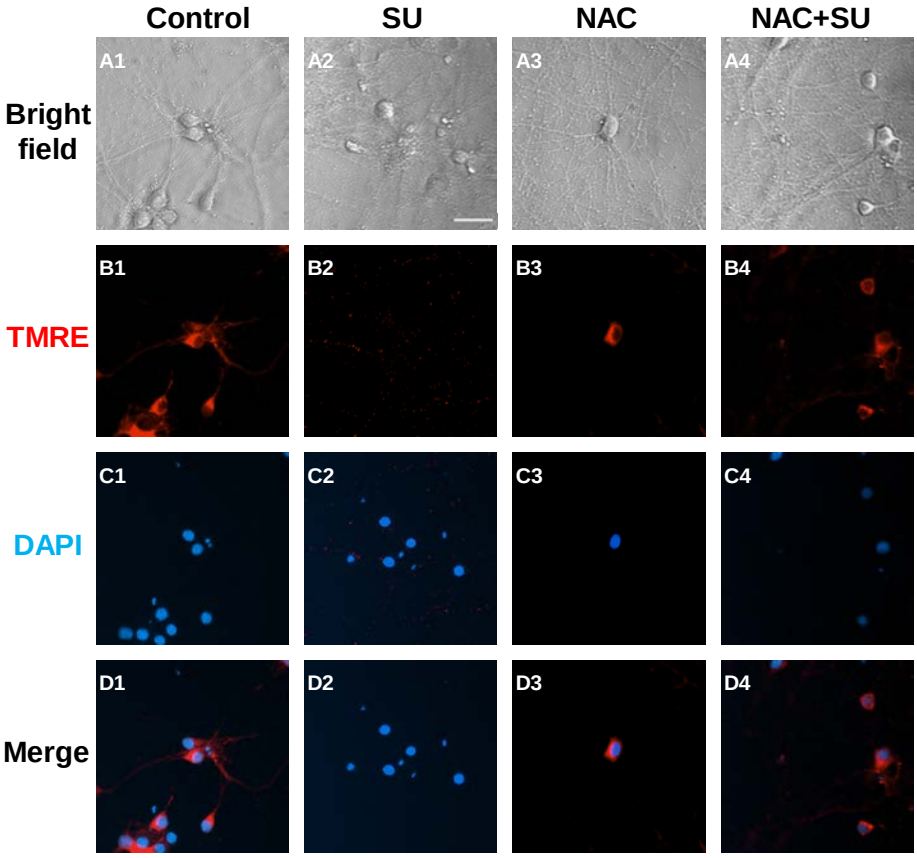


Figure 18. NAC prevents the loss of mitochondrial membrane potential induced by SU1498. Cells in NB for 48 hr were treated with NAC (5 mM) for 24 hr. SU1498 (10 µM) was added 2 hr prior to TMRE labeling. Living cells were labeled with TMRE as described in Materials and Methods section. Nuclei were counterstained with DAPI. Scale bar: 50 µm. Data are representative of experiments repeated at least three times.

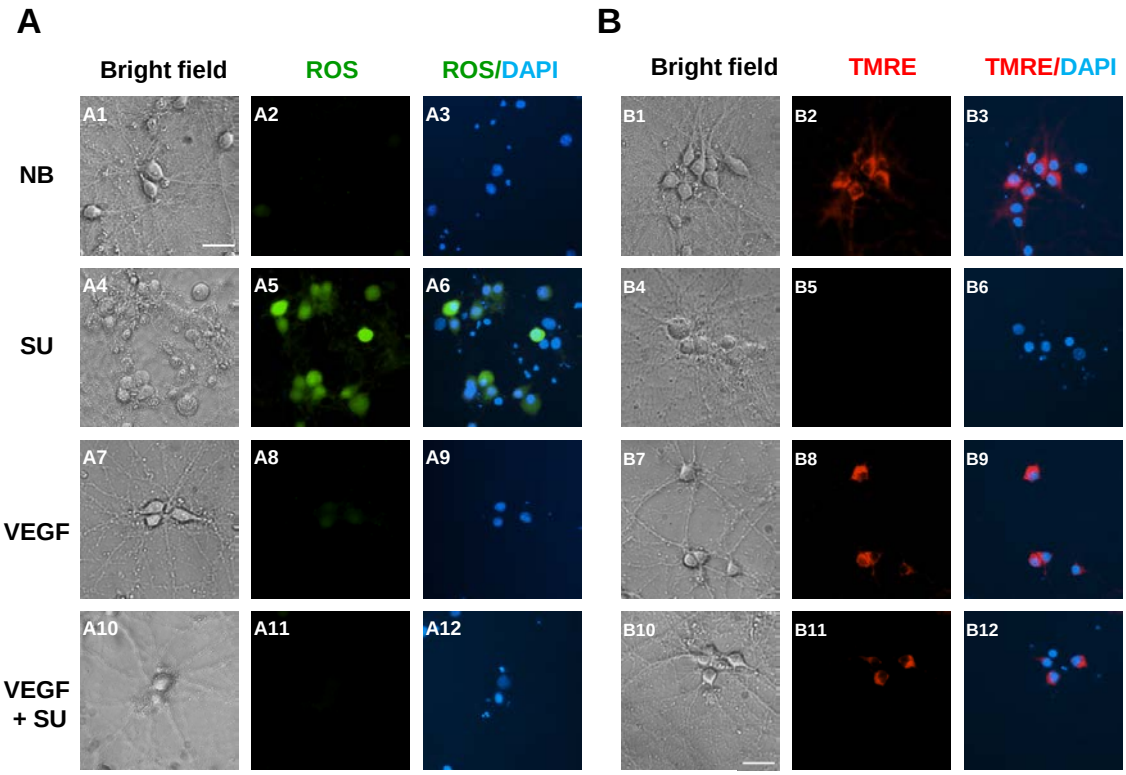


Figure 19. VEGF reduces ROS accumulation and prevents the loss of mitochondrial membrane potential induced by SU1498. Cells in NB were treated without and with 100 ng/ml VEGF for 48 hr and 10 µM SU1498 for 2 hr and then labeled with (A). ROS (green) and (B). TMRE (red) as described in Material and Methods section. Nuclei were counterstained with DAPI. Scale bar: 50 µm. Data are representative of experiments repeated at least three times.

5.6 VEGF signals through the activation of PI3K/Akt and MEK/ERK1/2 pathways in primary hippocampal neurons

To further delineate the VEGFR-2 mediated protective signaling mechanisms that are blocked by SU1498, cells were treated with selective inhibitors of PI3K/Akt and/or MEK/ERK1/2 pathways and analyzed by western blotting and cell viability. Both pathways are involved in VEGF-mediated neuroprotection against harmful stimuli including serum deprivation, ischemia and glutamate-induced excitotoxicity (Jin, 2000a, b; Matsuzake, 2001). Our results from western blotting showed that VEGF stimulated AKT and ERK1/2 phosphorylation and VEGFR-2 inhibition by 2 hr SU1498 treatment totally blocked AKT activation at both Thr308 and Ser473 sites with or without the presence of VEGF; however, ERK1/2 activation was attenuated by SU1498 that was slightly recovered by exogenous VEGF (Figure 20A, compare lane 2 with lane 1, 3 and 4), suggesting that VEGF may stimulate ERK1/2 activation through other receptor(s) when VEGFR-2 is blocked. Wortmannin and U0126, specific inhibitors for PI3K/Akt and MEK/ERK1/2 respectively, only inhibited its own target without affecting the other in the presence of VEGF (Figure 20A, compare lane 5 with 2 for wortmannin and lane 6 with 2 for U0126). MTS assay showed that inhibition of either pathway hindered the protective effect of VEGF regarding to cell viability (Figure 20B). Taken together, our results indicate that VEGF signals through the activation of the PI3K/Akt and MEK/ERK1/2 pathways to promote survival.

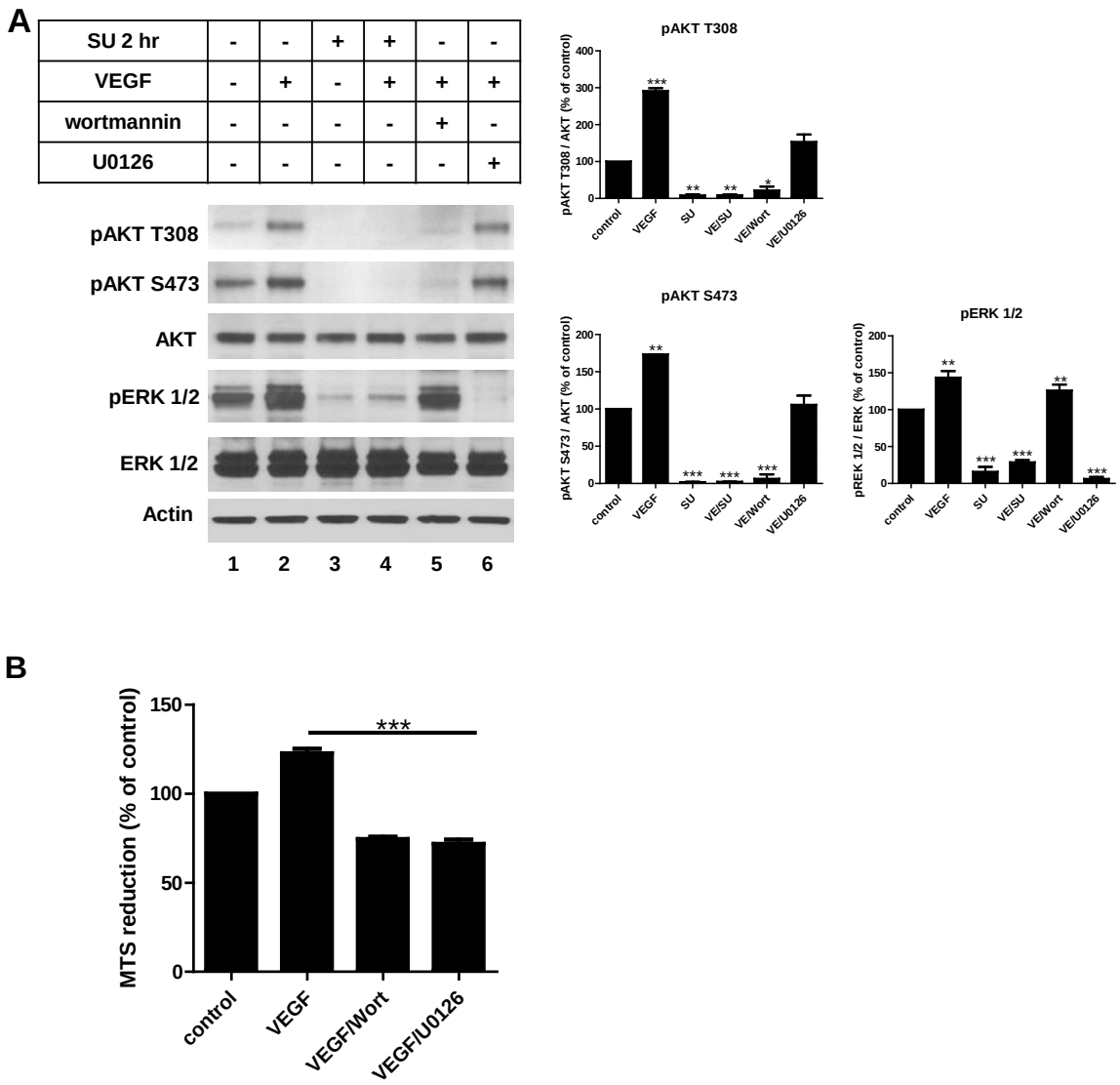


Figure 20. VEGF signals through the activation of PI3K/Akt and MEK/ERK1/2. Cells were incubated without and with VEGF (100 ng/ml) for 48 hr and treated with SU1498 (10 µM), Wortmannin (Wort) or U0126 for 2 hr. (A). Lysates were analyzed by western blotting probing with pAkt and pERK. Blots were quantified relative to the vehicle control. ***(P<0.001) indicates a significant difference between VEGF vs. control for pAkt (T308); a significant difference between SU, VE/SU or VE/Wort vs. control for pAkt (S473); a significanct difference between SU, VE/SU or VE/U0126 vs. control or VEGF for pERK. **(P<0.01) indicates a significant difference between SU or VE/SU vs. control for pAkt (T308); a significant difference between VEGF or VE/Wort vs. control for pERK. *(P<0.05) indicates a significant difference between VE/Wort vs. control for pAkt (T308). (B). Cell viability was determined in VEGF cultured cells without or with 2 hr incubations with Wort or U0126. ***(P<0.001) indicates a significant difference between VEGF, VEGF/Wort or VEGF/U0126 vs. control.

5.7 VEGF stimulates BAD phosphorylation and Bcl-xL expression

VEGF decreases ROS accumulation and prevents the loss of $\Delta\Psi_m$ induced by SU1498 (Figure 19). Both events are associated with mitochondrial dysfunction that is tightly regulated by the Bcl-2 family proteins. The pro-apoptotic protein BAD can be inactivated by PI3K/Akt and/or MEK/ERK1/2 through phosphorylation at Ser136 and Ser112, respectively. Western blotting showed that VEGF stimulated BAD phosphorylation and Bcl-xL expression, and SU1498 treatment for 2 hr attenuated but could not completely block this VEGF effect (Figure 21, compare lane 2 with lane 1 and 3). VEGF partially recovered BAD phosphorylation and Bcl-xL expression when VEGFR-2 was blocked by SU1498 (Figure 21, compare lane 4 with lane 3), which could be due to the partial recovery of ERK1/2 phosphorylation (Figure 20, lane 4). Together with the MTS assay showing that VEGF protects against cell death only against short-term SU1498 treatment, our results indicated that VEGF may activate MEK/ERK1/2 or other signaling cascades to promote cell survival by inactivating the pro-apoptotic protein BAD and stimulating the anti-apoptotic protein Bcl-xL expression through other receptor(s) when VEGFR-2 function is blocked.

In addition, NAC, just like VEGF, stimulated ERK1/2 and BAD phosphorylation and Bcl-xL expression (Figure 22, compare lane 2 with lane 1), which may contribute to NAC-mediated protection against oxidative stress and mitochondrial dysfunction induced by SU1498.

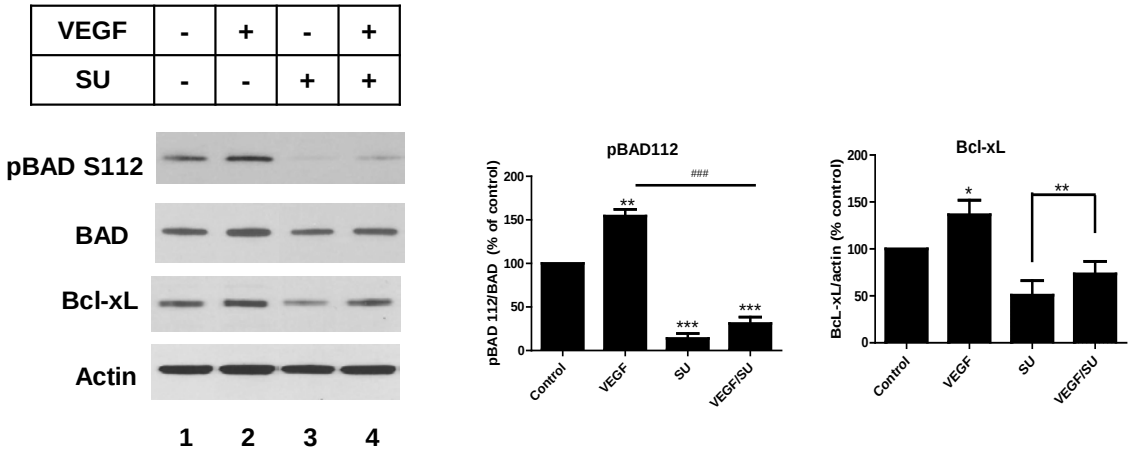


Figure 21. VEGF increases BAD phosphorylation and Bcl-xL expression. Cells were incubated in the absence and presence of VEGF (100 ng/ml) for 48 hr in NB and treated with SU1498 (10 µM) for 2 hr. VEGF was replenished after 24 hr. Cell lysates were analyzed by western blotting probing with pBAD and Bcl-xL with Actin as a loading control. Blots were quantified by imageJ. $^{###}$ ($P<0.001$) indicates a significant difference between VEGF vs. SU or VEGF/SU and ***($P<0.001$) indicates a significant difference between SU or VEGF/SU vs. control for pBAD; **($P<0.01$) indicates a significant difference between VEGF vs. control for pBAD and a significant difference between SU vs. VEGF/SU for Bcl-xL; *($P<0.05$) indicates a significant difference between VEGF vs. control for Bcl-xL.

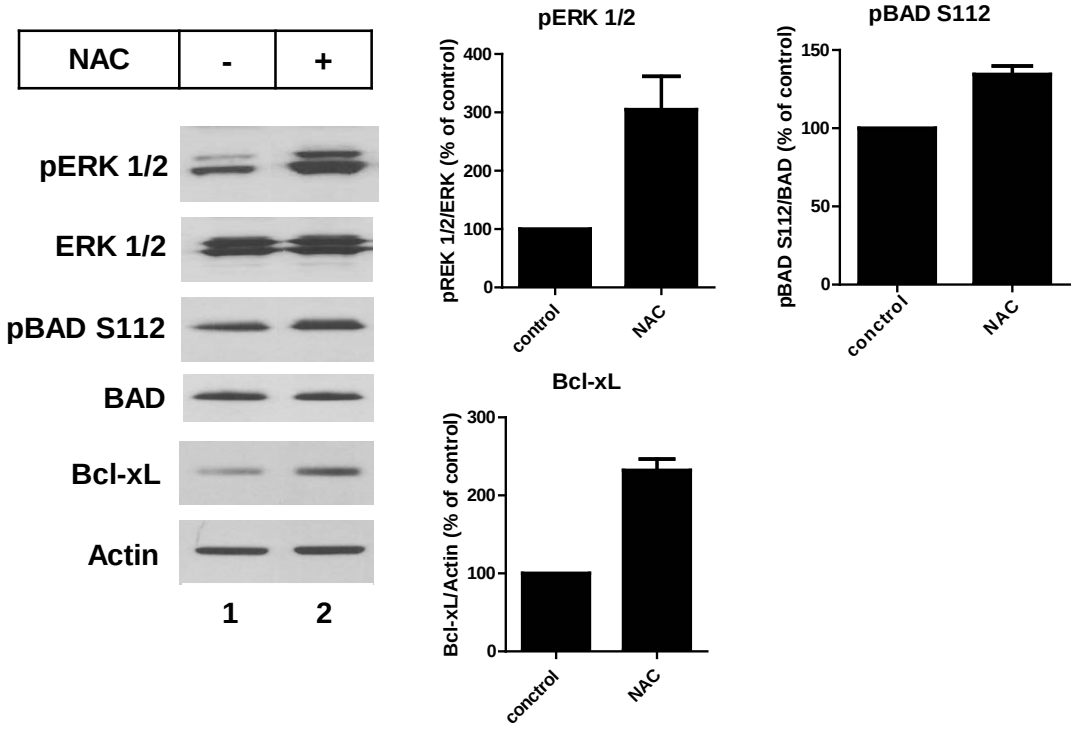


Figure 22. NAC mimics VEGF stimulating ERK1/2, BAD phosphorylation and Bcl-xL expression. Cells in NB for 48 hr were treated with NAC (5 mM) for 24 hr. Lysates were analyzed by western blotting probing with pERK, pBAD and Bcl-xL with Actin as a loading control. Blots were quantified by imageJ.

5.8 VEGF-B reduces ROS accumulation and prevents the loss of mitochondrial membrane potential against VEGFR-2 inhibition in NB

Our results *in vivo* and *in vitro* both indicated that VEGF protected against neuronal cell loss when VEGFR-2 was blocked (Figure 9, Figure 16), suggesting that VEGF-mediated neuroprotection is not only regulated through the activation of VEGFR-2 but other VEGF receptors may be involved. VEGF-B, unlike VEGF, only binds to VEGFR-1 but not VEGFR-2 and is expressed in neurons. To this end, we investigated the role of VEGF-B in ROS production and mitochondrial dysfunction. Our results showed that VEGF-B reduced ROS accumulation (Figure 23A, compare A14 to A6) and prevented the loss of $\Delta\Psi_m$ induced by VEGFR-2 inhibition (Figure 23B, compare B14 to B6), suggesting that when VEGFR-2 is blocked, VEGF ligands can signal through VEGFR-1.

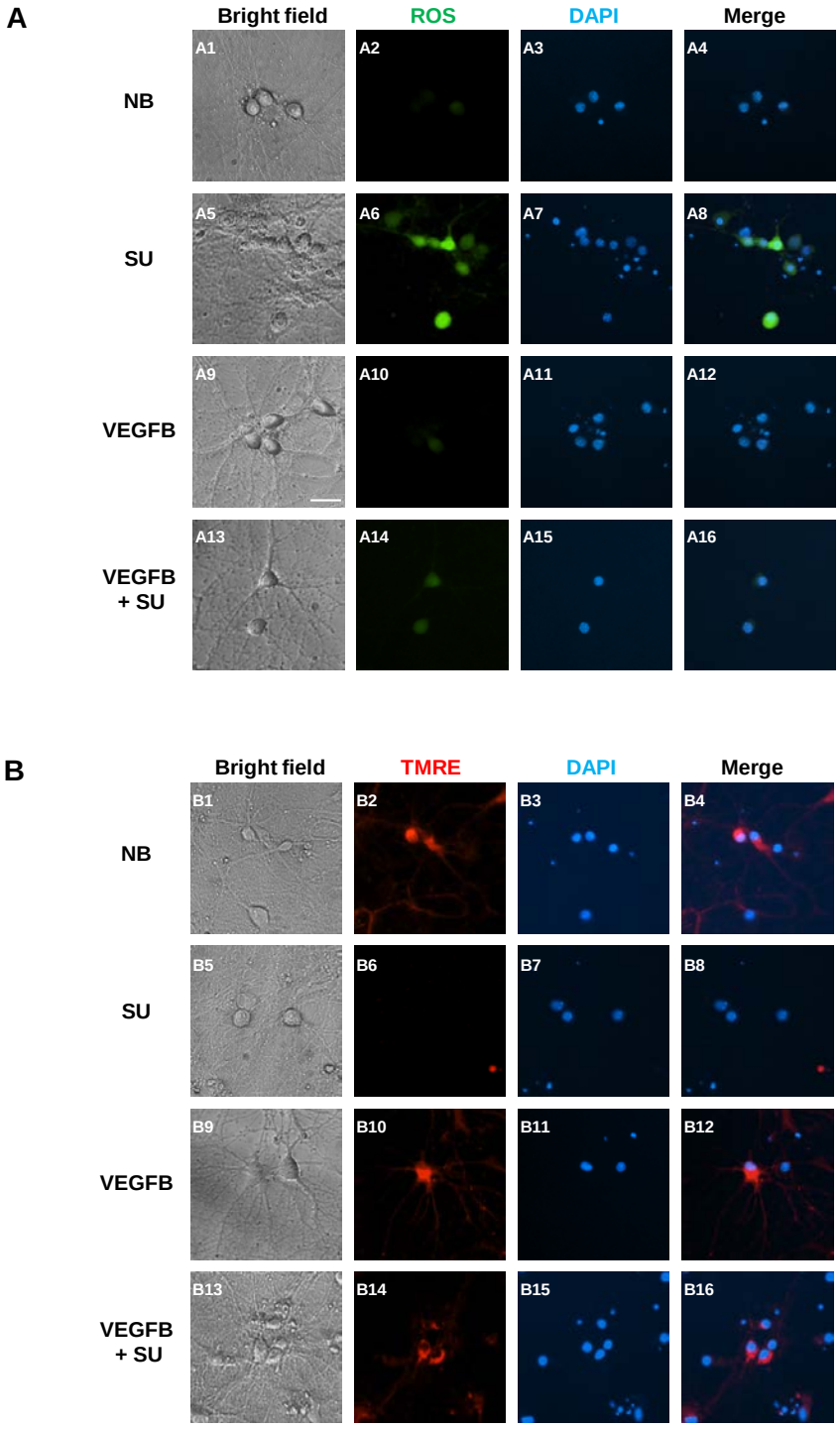


Figure 23. VEGF-B reduces ROS accumulation and prevents the loss of mitochondrial membrane potential induced by SU1498. Cells were incubated without and with VEGF (100 ng/ml) for 48 hr and treated with SU1498 (10 µM) for 2 hr. Cells were labeled with (A.). ROS (green) and (B.). TMRE (red) as described in Materials and Methods section. Nuclei were counterstained with DAPI. Scale bar: 50 µm. Data are representative of experiments repeated at least three times.

5.9 VEGF-B protects against cell loss induced by VEGFR-2 inhibition

MTS assay showed that although VEGF-B treatment alone had no significant protective effect comparing with control cells in NB, VEGF-B, like VEGF, protected against neuronal cell loss induced by 2 hr SU1498 treatment (Figure 24), suggesting that VEGF-B/VEGFR-1 functions to protect against neuronal cell loss.

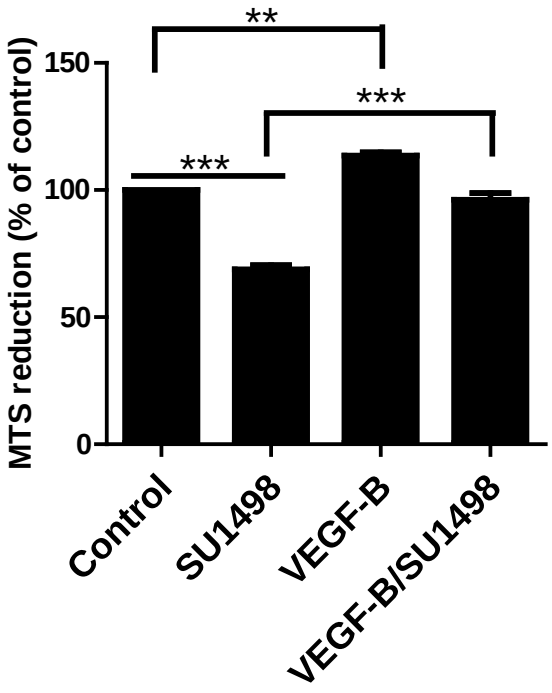


Figure 24. VEGF-B protects against cell loss induced by 2 hr SU1498 treatment. Cells were incubated in the absence and presence of VEGF-B (100 ng/ml) in NB for 48 hr and treated with SU1498 (10 μM) for 2 hr. VEGF-B was replenished after 24 hr. Cell viability was determined using a MTS assay as described in Materials and Methods section. **($P<0.01$) indicates a significant difference between VEGF-B vs. control and ***($P<0.001$) indicates a significant difference between SU1498 vs. control or VEGF-B/SU1498.

5.10 VEGF/VEGF-B prevents ROS production and mitochondrial dysfunction induced by VEGFR-2 siRNA

To confirm the inhibitory effect of SU1498 on ROS production and the loss of $\Delta\Psi_m$ and the protection by VEGF and VEGF-B, siRNA against VEGFR-2 (siR2) was performed. Immunostaining results showed that siR2 downregulated VEGFR-2 expression in NB (Figure 25A, compare A2 with A1). VEGF-stimulated VEGFR-2 expression was suppressed by siR2 to the basal levels compared with control (Figure 25A, compare A3 and A4 with A1). Also, VEGFR-1 was expressed in neurons and siR2 has no effect on VEGFR-1 expression (Figure 25B, compare B2 with B1). VEGFR-1 was present primarily in the nuclei of neurons. In addition, exogenous VEGF stimulated VEGFR-2 localization along the dendrites (Figure 25, A3 for VEGFR-2 and A11 for VEGFR-2 along the dendrites) and this localization was blocked by siR2 (Figure 25, A4 and A12). Instead, VEGF stimulated VEGFR-1 localization along the dendrites when VEGFR-2 was blocked by siRNA (Figure 25, B4 and B12).

Labeling living cells with ROS and TMRE also confirmed that inhibition of siR2 exerted the similar effect on VEGFR-2 comparing with SU1498. Cellular ROS production was induced by siR2 and elevated ROS production was diminished by either VEGF or VEGF-B (Figure 26, compare C2, D2 with B2). Correspondingly, VEGFR-2 inhibition by siR2 resulted in a loss of $\Delta\Psi_m$, which is prevented by the administration of either VEGF or VEGF-B (Figure 27, compare C2, D2 with B2).

Taken together, our results indicated that VEGF/VEGF-B signals through VEGFR-1 to protect against neuronal damages induced by VEGFR-2 blockade.

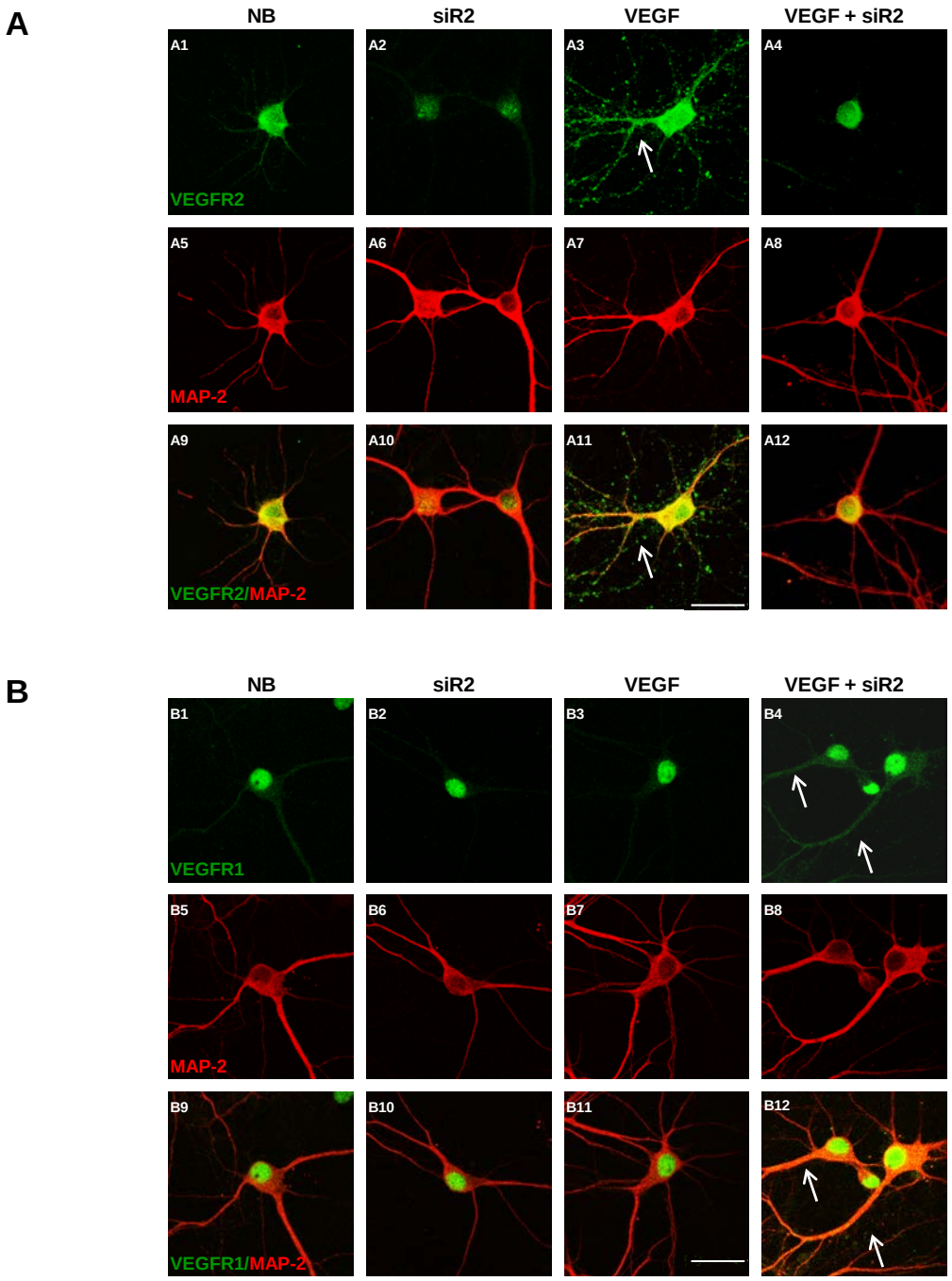


Figure 25. VEGFR-2 siRNA knocks down VEGFR-2 expression without affecting VEGFR-1. Cells were transfected with siVEGFR-2 (siR2) as described in Materials and Methods section and incubated with the absence and presence of VEGF (100 ng/ml) in NB for 48 hr. Cells were double labeled with (A). VEGFR-2 (green), (B). VEGFR-1 (green) and MAP-2 (red). Scale bar: 100 µm. Data are representative of experiments repeated at least three times.

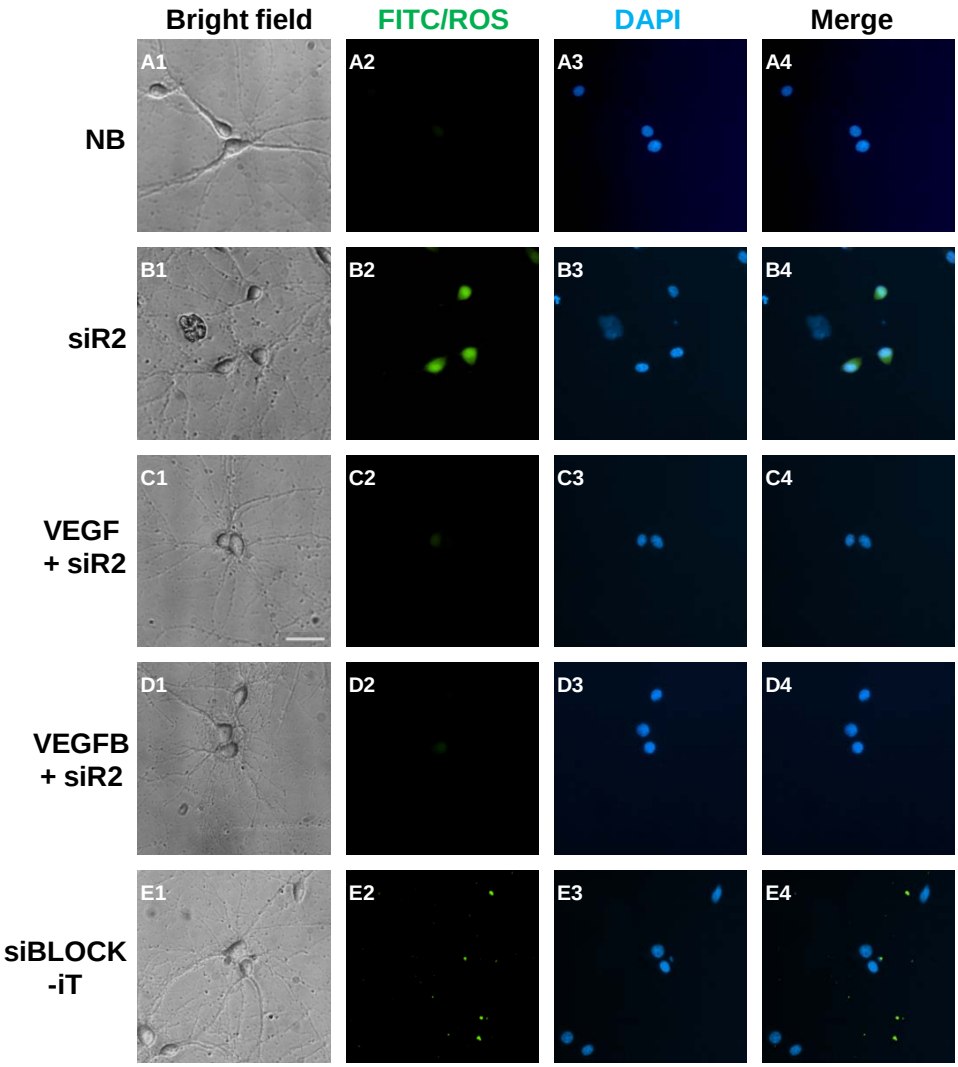


Figure 26. VEGF/VEGF-B reduces ROS accumulation induced by siVEGFR-2 (siR2). Cells were transfected with siR2 as described in Materials and Methods section and incubated with the absence and presence of VEGF or VEGF-B (100 ng/ml) for 48 hr in NB. ROS in living cells was labeled by Carboxy-H_2DCFCA (green) as described in Materials and Methods section. Nuclei were counterstained with DAPI. siBLOCK-iT (FITC labeled) was used as a negative control and transfection efficiency indicator. Scale bar: 50 µm. Data are representative of experiments repeated at least three times.

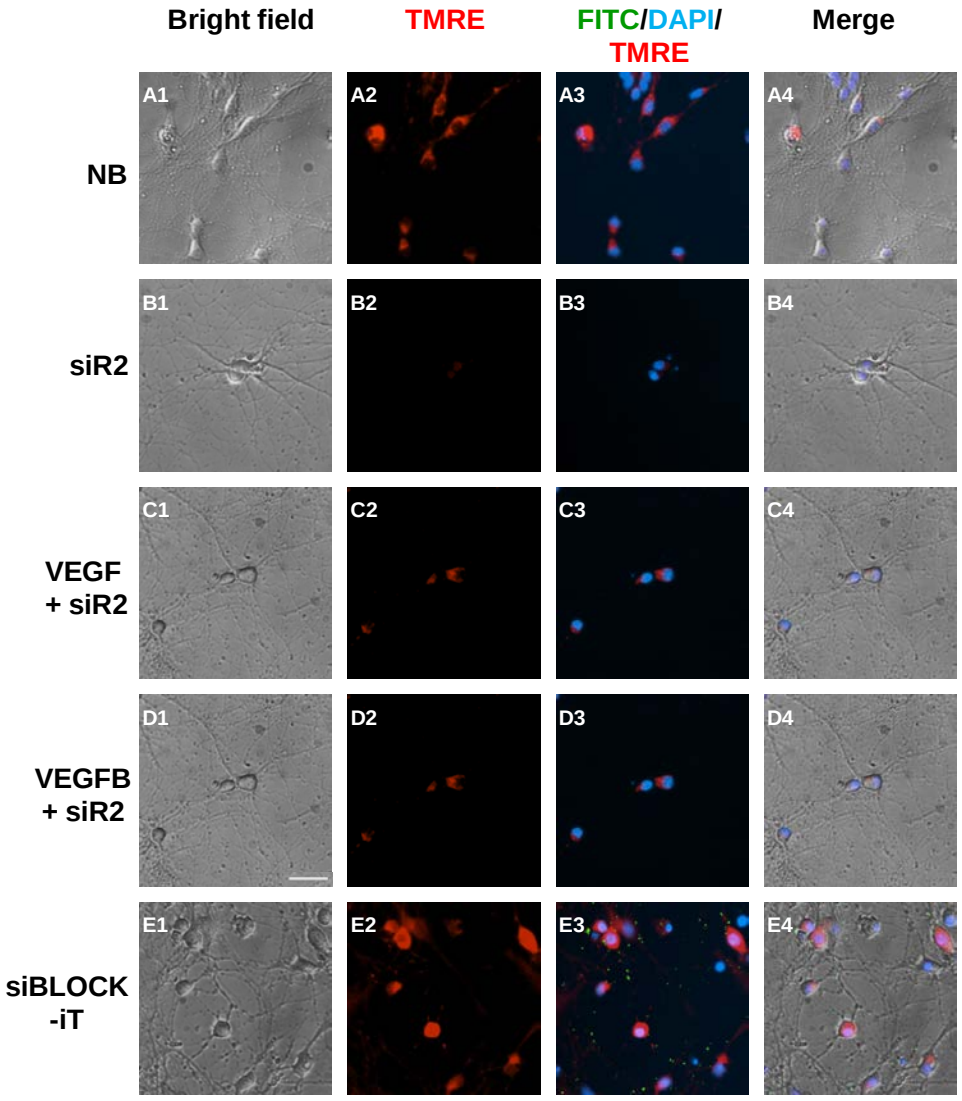


Figure 27. VEGF/VEGF-B prevents the loss of mitochondrial membrane potential impaired by siVEGFR-2 (siR2). Cells were transfected with siR2 as described in Materials and Methods section and incubated with the absence and presence of VEGF or VEGF-B (100 ng/ml) for 48 hr in NB. Living cells were labeled with TMRE as described in Materials and Methods section. Nuclei were counterstained with DAPI. siBLOCK-iT (FITC labeled) was used as negative control and transfection efficiency indicator. Scale bar: 50 µm. Data are representative of experiments repeated at least three times.

Chapter VI

Discussion

6.1 VEGF protects neuronal cell damage against pilocarpoine *in vivo* and *in vitro*

Status epilepticus (SE) is a neurological disorder with persistent morbidity and high mortality, which induces brain injury leading to neuronal cell death (Araki et al. 2002, Henshall et al. 2002). VEGF have been shown to be upregulated and protective against the neuronal loss that results from pilocarpine-induced SE (Nicoletti et al. 2007). These findings implicate VEGF as a factor that can alleviate seizure-induced damages. In this study, we show that VEGFR-2 activation is upregulated in the hippocampal pyramidal region during SE with or without exogenous VEGF (Figure 4). A neutralizing antibody for VEGFR-2 completely inhibits VEGFR-2 phosphorylation but only partially blocked VEGF-mediated neuroprotection against seizure (Figure 8 and Figure 9A), suggesting that VEGFR-2 is not the only receptor through which VEGF signals to exert its neuroprotective effects against pilocarpine-induced SE.

Although the principal VEGF receptor expressed in neurons is VEGFR-2, VEGFR-1 and NP-1 are also expressed in neurons and would serve as potential alternate receptors for VEGF to mediate neuroprotection. This notion is supported by the demonstration that VEGF binds to VEGFR-1 with higher affinity compared to VEGFR-2 (Holmes K. et al. 2007). Whereas VEGF protects against 6-hydroxydopamine (6-OHDA)-induced cell death in mesencephalic dopaminergic neurons through an unidentified receptor in an *in vitro* model of Parkinson's disease; and VEGFR-1 but not VEGFR-2 expression is upregulated in astrocytes and neurons in the brains of patients with the disease (Wada et al. 2006, Yasuhara et al. 2004). VEGF co-receptor NP-1, when co-expressed with VEGFR-2, can function to promote VEGFR-2 activation and signaling (Geretti et al. 2008). In addition, NP-1 is a co-receptor for both VEGF and Semaphorin 3A. VEGF competes with Semaphorin 3A for the binding of NP-1 and may antagonize the detrimental effects caused by Semaphorin 3A/NP-1 signaling in neurons. For

instance, in DRGs, VEGF stimulates the growth of sensory neuron axons; while Semaphorin 3A promotes axon repulsion and growth cone collapse (Sondell et al. 2000). It also has been reported that VEGF competes with Semaphorin 3A for the binding to NP-1 and antagonizes Semaphorin 3A/NP-1-induced apoptosis in a neural progenitor cell line; and anti-VEGFR-1 antibody partially blocked the VEGF-mediated neuroprotection under this circumstance (Bagnard et al. 2001), suggesting that VEGF-mediated neuroprotection involves VEGFR-1 and NP-1, which is in agreement with our findings.

SE generates various damages including excitotoxicity, oxidative stress, mitochondrial dysfunction and cell death to the forebrain, especially in the hippocampal pyramidal cells (Fujikawa 1996). HO-1, as an indicator of oxidative stress, its role in the CNS under pathological conditions is controversial. For instance, HO-1 is upregulated after brain injury, but HO-1 knockout mice exhibit less brain injury with decreased ROS production, suggesting HO-1 accelerates brain damage *in vivo* (Wang J. and Dore 2007). In contrast, *in vitro* studies suggest that HO-1 may protect against neuronal damage and cell death induced by oxidative stress (Chen et al. 2000, Scapagnini et al. 2006). This may be due to the biological products of Heme degradation catalyzed by HO-1, among which CO and biliverdin/bilirubin may be protective through vasodialtion or anti-oxidation while iron is capable of enhancing free radical damage (Abraham and Kappas 2008, Fiumana et al. 2003, Halliwell and Gutteridge 1992).

HO-1 has been observed to be upregulated for 72 hr after SE with a peak at 24 hr. The activation of cerebral peroxisome proliferator-activated receptors gamma (PPARγ) protects against neuronal cell loss and suppresses HO-1 expression and ROS production induced by SE (Yu et al. 2008). Our studies are consistent with these findings in that the protection elicited by VEGF against anti-VEGFR-2-induced cell loss is accompanied by a concomitant decrease in

HO-1 expression in rats undergoing seizure (Figure 9). The function of HO-1 expression under these circumstances needs to be further addressed.

In addition to oxidative stress, inflammation is also responsible for the pathogenesis of neurodegenerative disorders (Gonzalez-Scarano and Baltuch 1999). Increased COX-2 expression during seizure together with associated inflammatory processes may contribute to neuronal cell death in postnatal rat hippocampus (Jarvela et al. 2008). It has been demonstrated that in adult rats, COX-2 expression in the perinuclear region of hippocampal neurons was upregulated by pilocarpine induced seizure and the enhanced COX-2 expression sustained up to 2 weeks with a peak between 1 day and 4 days after SE (Jung et al. 2006). COX-2 inhibitors attenuate COX-2 expression in the rat brain, reduce hippocampal cell death and seizure frequencies in different seizure models and have emerged as new therapeutic strategies against seizure (Baran et al. 1994, Jung et al. 2006, Kunz and Oliw 2001, Paoletti et al. 1998). Our results confirm that COX-2 expression is perinuclear in the hippocampal CA1 and CA3 region and is upregulated 24 hr after SE onset and persisted for at least 72 hr afterwards. We further demonstrate that exogenous administration of VEGF prior to SE induction reduced COX-2 expression at both time points with a more dramatic decrease at 72hr (Figure 7), suggesting that VEGF protects against neuronal cell loss after SE through the regulation of COX-2 expression. These results implicate VEGF as a potential candidate for suppressing COX-2 expression other than COX-2 inhibitors in anti-seizure therapies.

Taken together, our *in vivo* studies indicate that in our rat pilocarpine model, VEGF protects against *status epilepticus*-induced neuronal cell loss accompanied with decreased HO-1 and COX-2 expression; and VEGF-mediated neuroprotection after *status epilepticus* is regulated

through the activation of VEGFR-2 and may involve the activation of VEGFR-1 and/or NP-1 when VEGFR-2 is not available.

Previous studies in our laboratory showed that pilocarpine induced a ROS dependent cell death in SK-N-SH neuroblastoma cells that is rescued by treatments with NAC (Edelstein et al. 2011). The studies herein show that pilocarpine exerts similar effect in cultured hippocampal neurons by inducing a cell death that is accompanied by increased HO-1 expression that is suppressed by NAC (Figure 10 and Figure 12). In addition, these studies show that VEGF and VEGF-B both attenuate pilocarpine induced ROS expression (Figure 12). The mitochondrial electron-transport chain (ETC) is the main source of ROS production under both normal and pathological conditions including ischemia, hypoxia and aging (Becker et al. 1999, Lesnefsky et al. 2001, Moghaddas et al. 2003). Production of ROS accompanied with decreased $\Delta\Psi_m$ contribute to cell death (Kroemer et al. 2007). Notably, pilocarpine induces ROS production but has no effect on $\Delta\Psi_m$. One possible explanation could be that mitochondrial ROS production occurs prior to the collapse of $\Delta\Psi_m$. For instance, in cardiomyocytes, the loss of $\Delta\Psi_m$ starts after 24 hr inhibition of erbB2 receptor when ROS production has been already accumulated to significant levels (Gordon et al. 2009). In future studies, ROS production and $\Delta\Psi_m$ could be measured with a time-dependent pilocarpine treatment in this respect. Also, pilocarpine increased NADPH oxidase levels in cultured hippocampal neurons (Di Maio et al. 2011) which could be the source of ROS production in our *in vitro* model.

Collectively, our *in vivo* and *in vitro* results are consistent and demonstrate that VEGF-mediated neuroprotection against pilocarpine is associated with oxidative stress. ROS production and mitochondrial dysfunction have been recognized as the critical factors in the causes and

consequences of temporal lobe epilepsy (Waldbaum and Patel 2010). Our findings may provide insightful information for the development of strategies to prevent seizure.

6.2 VEGF promotes neuronal cell survival against oxidative stress and mitochondrial dysfunction *in vitro*

Over the last decade, studies have demonstrated that VEGF acts as a neurotrophic and neuroprotective factor in addition to its angiogenic properties (Gora-Kupilas and Josko 2005, Storkebaum and Carmeliet 2004). CNS neurons especially those from cortical and hippocampal regions are particularly sensitive to oxidative stress and mitochondrial dysfunction due to their high demand of oxygen and energy consumption (Mandemakers et al. 2007, Satoh et al. 1998). Oxidative stress and mitochondrial dysfunction have been associated with neuronal cell death induced by ischemia, hypoxia or serum deprivation and implicated in multiple neurodegeneration diseases including Alzheimer's disease, Parkinson's disease and Huntington's disease (Almeida et al. 2002, Greenamyre and Hastings 2004, Huang X. et al. 1999, Niizuma et al. 2009, Satoh et al. 1996, Stack et al. 2008, Zhou et al. 2008). However, the connections between VEGF-mediated neuroprotection and oxidative stress and mitochondrial dysfunction need to be elucidated.

Previous studies in our lab indicate that VEGF promotes survival through VEGFR-2 in serum deprived SK-N-SH cells (Gomes et al. 2007). In this project, using primary hippocampal neurons, we showed that activated VEGFR-2 was present in cells cultured in NB and exogenous VEGF increased VEGFR-2 activation (Figure 13); VEGFR-2 inhibition by SU1498 blocked VEGFR-2 phosphorylation and induced cell death (Figure 13 & 14), indicating that VEGF functions through VEGFR-2 in cultured hippocampal neurons. These studies also showed that

neurons cultured in NB alone were more sensitive to the oxidative insult induced by VEGFR-2 inhibition at early time frames of exposure when compared to cells cultured in the supplemented media NB/B27 containing antioxidant cocktail (Figure 14). In this regard, these studies serve as a model for neurons predisposed to oxidative insults that would participate in the neurodegenerative process. In addition, VEGFR-2 inhibition by SU1498 induced neuronal cell death was accompanied with increased ROS production, caspase-3 cleavage and the loss of $\Delta\Psi_m$ and all these events were prevented by NAC (Figure 15, 17 & 18), suggesting that SU1498-induced cell death mimics the oxidative stress, mitochondrial dysfunction and caspase-3 activation observed in many neurodegenerative disorders.

VEGF exerts direct neuroprotective effects by inhibiting programmed cell death under stressful conditions including hypoxia, serum deprivation and oxidative stress (Baek et al. 2000, Jin et al. 2001, Oosthuyse et al. 2001). In this study, exogenous VEGF promoted neuronal cell survival against 2 hr SU1498 treatment but not 4hr (data not shown) or 24 hr (Figure 16). It is important to note that VEGF failed to protect against cell death induced by SU1498 treatment when cells were cultured in the supplemented media NB/B27 (data not show). Therefore, VEGFR-2 inhibition has a damaging effect on mature hippocampal neurons independent of culture media. Notably, the inclusion of exogenous VEGF prevented ROS production, caspase-3 cleavage and the loss of $\Delta\Psi_m$ induced by 2 hr SU1498 treatment (Figure 16 & 19). These results suggest that VEGF, when administered to cells where VEGFR-2 is inhibited for a short-term of exposure (2hr), has the capacity to protect against the oxidative stress and mitochondrial dysfunction leading to neuronal cell death. After that point, cells treated with SU1498 undergo a death path of no return, which may be due to continuous ROS accumulation and persistent $\Delta\Psi_m$

dissipation. Moreover, this neuroprotection appears to involve other VEGF receptors binding to VEGF.

VEGF/VEGFR-2-mediated neuroprotection is regulated through multiple downstream kinase cascades including PI3K/AKT, PLC-γ/PKC and MEK/ERK1/2 (Zachary 2005). All three pathways are capable of inactivating pro-apoptotic protein BAD by phosphorylation, which in turn replaces BAX forming heterodimers with Bcl-xL neutralizing its anti-apoptotic property. PI3K/Akt phosphorylates BAD at Ser136 and PLC-γ/PKC and MEK/ERK1/2 phosphorylates BAD at Ser112 (Baines et al. 2002, Datta et al. 1997, Zha et al. 1996). Our results showed that exogenous VEGF stimulated activation of both PI3K/Akt and MEK/ERK1/2 pathways; and inhibition of VEGFR-2 by SU1498 blocked PI3K/Akt and MEK/ERK1/2 phosphorylation. In addition, blockade of either PI3K/Akt or MEK/ERK1/2 pathway impaired VEGF-mediated neuroprotection, suggesting that VEGF signals through both PI3K/Akt and MEK/ERK1/2 pathways in cultured hippocampal neurons in NB (Figure 20).

However, the PI3K/Akt remained completely blocked by the presence of exogenous VEGF at the 2 hr SU1498 treatment while the activation of MEK/ERK1/2 phosphorylation was partially restored (Figure 20A, lane 4). Similarly, the blockade of BAD phosphorylation at Ser112 and the reduction in Bcl-xL expression by 2 hr SU1498 treatment was partially restored by exogenous VEGF (Figure 21), suggesting that VEGF involved in protection against the mitochondrial dysfunction induced by VEGFR-2 inhibition. This notion is further supported by the findings that VEGF suppressed caspase-3 activation and protects against neuronal cell death induced by 2hr SU1498. Together, these results are consistent with the demonstration that overexpression of VEGF increases Bcl-xL expression, decreases caspase-3 expression and promotes survival in transgenic ALS mice (Hwang et al. 2009). Our results also suggested that

VEGF mediated neuroprotection against short-term VEGFR-2 inhibition through the regulation of MEK/ERK1/2 and BAD phosphorylation and Bcl-xL expression. Since MEK/ERK1/2 phosphorylation was only partially rescued by VEGF in SU1498 treated cells, it is possible that other signaling cascades may be involved in providing protection against cell death under this scenario. One possible candidate is ERK5, which has been shown to be activated by VEGF to suppress apoptosis by regulating BAD phosphorylation, Bcl-2 expression and caspase-3 activity (Roberts et al. 2010). Mitochondrial anchored PKA is also capable of phosphorylating and inactivating BAD (Harada et al. 1999). Notably, NAC exerted the same effect comparing with VEGF stimulating ERK1/2 phosphorylation and Bcl-xL expression (Figure 22), suggesting that ERK1/2 activation and Bcl-xL involve in the regulation of oxidative stress.

The finding that VEGF is protective against VEGFR-2 inhibition suggests that VEGF may signal through other receptor(s) such as VEGFR-1 or NP-1, which are both expressed in neurons (Jin et al. 2000c, Zachary 2005). VEGF-B only binds to VEGFR-1 and has been reported to protect against motor neuron and sensory neuron degeneration through VEGFR-1 (Dhondt et al. 2011, Poesen et al. 2008). VEGF-B stimulates VEGFR-1 and MEK/ERK1/2 activation in cultured cortical neurons and inhibits apoptosis through VEGFR-1 by suppressing the expression of BH3 only proteins including BAD (Li Y. et al. 2008). Our results showed that VEGF-B protected against SU1498 (2 hr)-induced neuronal cell death accompanied with reduced ROS production and prevented the loss of $\Delta\Psi_m$ (Figure 24 & 23, respectively), suggesting that VEGFR-1 is functional to promote survival by regulating oxidative stress and mitochondrial dysfunction in cultured hippocampal neurons.

The demonstration that siRNA specific to VEGFR-2 downregulated VEGFR-2 expression and induced ROS production and the loss of $\Delta\Psi_m$ without affecting the protein levels

of VEGFR-1, confirmed the specificity of the effects of SU1498 on VEGFR-2 inhibition (Figure 25). In addition, the rescue of these effects by VEGF or VEGF-B (Figure 26 & 27, compare C2 with B2) and the finding that VEGF stimulated VEGFR-1 but not VEGFR-2 to localize along the dendrites in siVEGFR-2 treated neurons (Figure 25) suggest that VEGF signaling switches from VEGFR-2 to VEGFR-1 when VEGFR-2 is blocked.

In this study, we provide evidence that VEGF-mediated neuroprotection is associated with oxidative stress and mitochondrial dysfunction in mature hippocampal neurons. A blockade of VEGFR-2 by pharmacological inhibitor or siRNA leads to HO-1 induction, ROS over-production, caspase-3 cleavage and the loss of $\Delta\Psi_m$. We speculate that when VEGFR-2 is blocked, VEGF may signal through an alternate receptor such as VEGFR-1 to promote neuronal survival. Our findings also implicated that VEGF-mediated neuroprotection involves a VEGFR-2-regulated phosphorylation of the pro-apoptotic protein BAD and the protein expression levels of the anti-apoptotic protein Bcl-xL. These mechanisms are consistent with those reported for tyrosine kinase receptors that mediate survival in non-neuronal cells (Gordon et al. 2009, Grazette et al. 2004).

Chapter VII

Conclusion

In conclusion, the present data demonstrates that signaling through the VEGF receptor mediates neuroprotection against oxidative stress and mitochondrial dysfunction.

VEGF protects against pilocarpine-induced neuronal cell loss in both a rat model of SE and primary hippocampal neurons by suppressing oxidative stress including HO-1 expression and ROS production. VEGF also protects against inflammation *in vivo* through the regulation of COX-2 expression.

In primary hippocampal neurons, VEGFR-2 inhibition induced a neuronal cell death that is accompanied by increased ROS production, caspase-3 cleavage and the loss of mitochondrial membrane potential which are a direct consequence of oxidative stress. VEGFR-2 mediates neuroprotection in part through the downstream activation of the PI3K/Akt and MEK/ERK1/2 signaling pathways. In addition, exogenous VEGF protects against oxidative stress and mitochondrial dysfunction by activating an alternate receptor such as VEGFR-1 to regulate the phosphorylation (inactivation) of the pro-apoptotic protein BAD and expression of anti-apoptotic protein Bcl-xL in part through MEK/ERK1/2. Taken together, our results suggest that VEGFR-2 signals neuroprotection through downstream mechanisms that prevent oxidative stress and mitochondrial dysfunction. In this regard, VEGF or VEGF-B signaling would protect against the oxidative damage induced by ROS overproduction and the loss in mitochondrial function as critical mechanisms that maintain the high energy demand required for neuronal function. In addition, these findings reveal a unique interplay among VEGF receptors where they serve compensatory functions to promote neuronal survival in response to stressful stimuli.

Chapter VIII

Future Studies

8.1 The role of HO-1

HO-1 was upregulated by pilocarpine both *in vivo* and *in vitro* and by SU1498 in cultured neurons. However, the role of HO-1 in these paradigms of oxidative stress is unknown. To address whether the increased expression of HO-1 is protective or deleterious to neurons, cells will be treated with the HO-1 antagonist protoporphyrin (SnPP) together with pilocarpine or SU1498 and analyzed for cell viability.

8.2 Does pilocarpine-induced ROS production precede a loss of $\Delta\Psi_m$?

In this study, 24 hr treatment of pilocarpine induced cell death and ROS production without affecting $\Delta\Psi_m$. To investigate whether ROS production induced by pilocarpine precedes a loss of $\Delta\Psi_m$, cells will be treated with pilocarpine for 24, 36 and 48 hr and imaged for ROS and $\Delta\Psi_m$. MTS assay will be performed to assess cell viability.

8.3 Dose pilocarpine or SU1498 induce a caspase dependent cell death in hippocampal neurons?

Pilocarpine and SU1498 induce cell death accompanied with increased caspase-3 cleavage. To further investigate if both events are caspase-dependent, cells will be treated with the general caspase inhibitor z-VAD-fmk in the absence and presence of either pilocarpine or SU1498. Cell viability will be measured.

8.4 Confirm that VEGF-B signals through VEGFR-1 and determine whether PI3K/AKT and/or MEK/ERK1/2 are downstream pathway(s) of this event

To assess that VEGF-B stimulates VEGFR-1 activation, cells treated with VEGF-B will be subjected to immunoprecipitation with anti-VEGFR-1 and probed with an anti-phospo-Tyrosine antibody by western blotting for VEGFR-1 activation.

Our results show that VEGF and VEGF-B protects against oxidative stress induced by SU1498 and VEGF signals neuroprotection through the activation of both PI3K/AKT and MEK/ERK1/2. To investigate the signaling pathway downstream of VEGF-B, lysates from cells treated with VEGF-B will be analyzed by western blotting for pAKT, pERK, pBAD in the absence and presence of the PI3K/Akt inhibitor wortmannin or MEK/ERK1/2 inhibitor U0126. VEGFB treated cells will also be examined for the levels Bcl-xL and cell viability under these conditions.

8.5 Is overexpression of Bcl-xL, MEK/ERK1/2 or PI3K/AKT capable of rescuing neurons from the cell death induced by SU1498?

Our findings suggest that Bcl-xL and an ERK1/2-mediated phosphorylation of pBAD might be responsible for VEGF-mediated neuroprotection against SU1498. To confirm this possibility, cells will be transfected with a vector that overexpresses Bcl-xL, ERK or AKT followed by treatment with SU1498. Cell transfected with an empty vector serves as a control. Cells from these experiments will be analyzed for ROS production, a loss in the mitochondrial membrane potential (TMRE assay) and cell viability. Cell lysates will also be analyzed by Western blotting for the phosphorylation of BAD.

Chapter IX

Model

Model of VEGF-mediated neuroprotection against oxidative stress

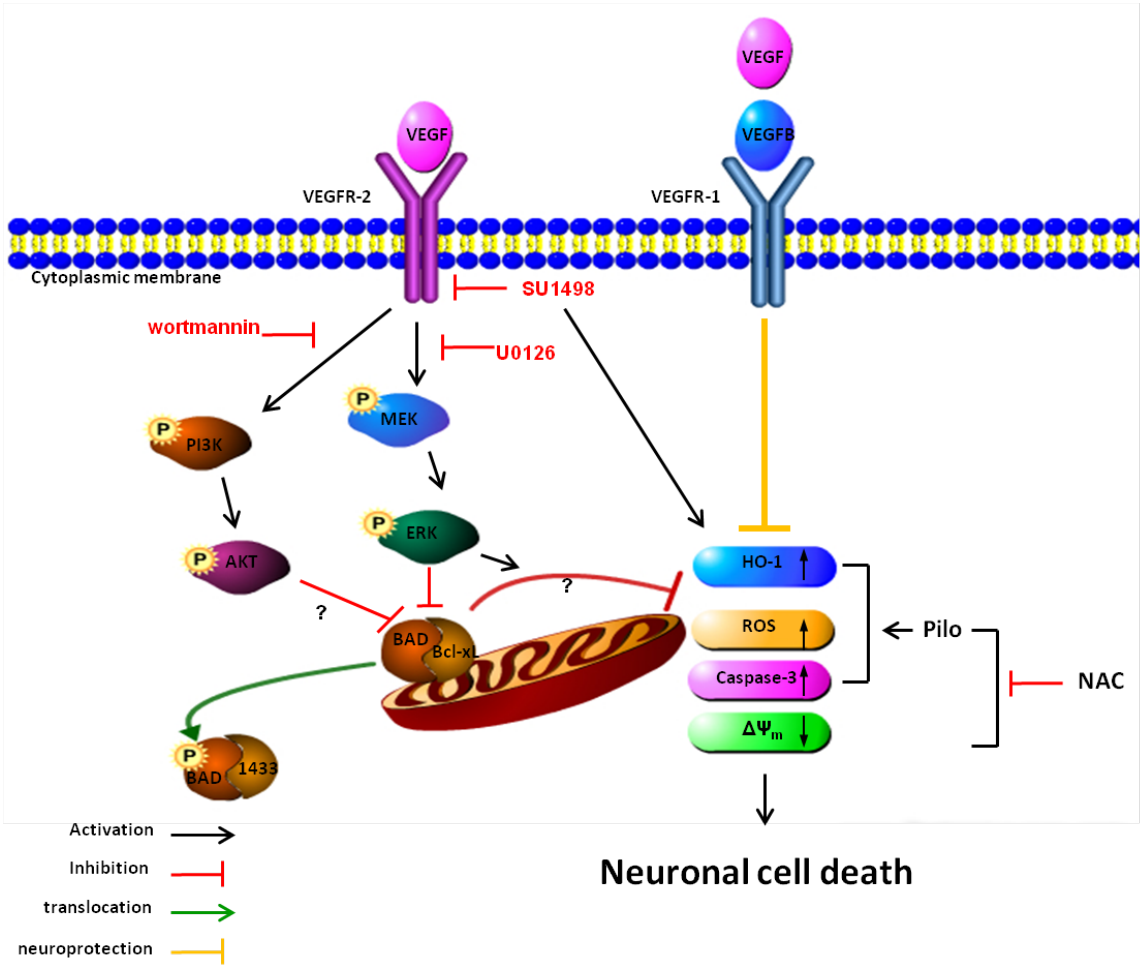


Figure 28. Model of VEGF-mediated neuroproteciton against oxidative stress. VEGF signaling protects against cell death induced by pilocarpine or SU1498 by suppressing HO-1, ROS production, caspase-3 cleavage and preventing the loss of $\Delta\Psi_m$. The phosphoylation of BAD and expression of Bcl-xL are involved in VEGF-mediated neuroproteion and possibly through the regulation of MEK/ERK. Both VEGFR-1 and -2 are involved in VEGF-mediated neuroprotecition.

Chapter X

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