VEGFR2 and PKA Converge at the MEK/ERK1/2 Pathway to Promote Survival in Serum Deprived Neuronal Cells

by

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Abstract

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Evan Gomes

Advisor: Dr. Patricia Rockwell

Identifying prosurvival mechanisms in stressed neuronal cells would provide protective strategies to hinder neurodegeneration. Recent evidence shows that vascular endothelial growth factor (VEGF), a well established endothelial cell mitogen, can mediate neuroprotection through activation of its cognate receptor VEGFR2 in response to stressful stimuli. In addition, growth factor receptor signaling pathways have been shown to crosstalk with cAMP-dependent Protein Kinase A (PKA) to protect neuronal cells against harmful insults. Whether VEGFR2 and PKA cooperate to mediate neuronal cell survival under stress conditions is unknown. We show that serum deprivation induces an upregulation in VEGF and VEGFR2 that concomitantly serves as a signaling pathway to promote survival through activation of the extracellular signal-regulated protein kinases (ERK1/2). Studies revealed that the PKA and VEGFR2 signaling pathways converge at MEK/ERK1/2 as a protective mechanism against caspase-3/7 activation and a cell death that can be prevented by caspase inhibition or overexpression of ERK1. The loss in survival resulting from VEGFR2 inhibition was also accompanied by depleted levels of the antiapoptotic protein Bcl-xL; however, overexpression of Bcl-xL also prevented cell death. Nevertheless, treatments with an inhibitor of mitogen-

activated protein kinase kinase (MEK), the upstream kinase that activates ERK1/2, reversed the protection elicited by caspase inhibition or overexpression of ERK1 or Bcl-xL. Furthermore, evidence suggests that VEGF mediates neuroprotection against a caspase activation that may involve regulation by the p38 mitogen activated protein kinase (MAPK) pathway. Herein, we show that inhibition of p38 MAPK augments the survival, the phosphorylation of Akt and ERK1/2 and the extent of caspase inhibition mediated by VEGF in serum starved neuronal cells. Inhibition of the VEGF receptor, VEGFR2, abrogated the protective effects induced by p38 MAPK inhibition in serum starved cells and re-established caspase activation, suggesting that p38 MAPK negatively regulates VEGF-mediated signaling of neuroprotection through VEGFR2. Collectively, these findings suggest that neuronal cell fate during starvation involves crosstalk between a prosurvival pathway directed by VEGFR2 and PKA that converge at MEK/ERK1/2 to prevent stressed neuronal cells from a caspase-dependent cell death. Additionally, VEGF signals protection against the activation of caspase-mediated cell death pathways induced by p38 MAPK-dependent and -independent mechanisms.

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LIST OF ABBREVIATIONS

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 promoter

Bak Bcl-2 homologous antagonist/killer

Bax Bcl-2–associated X protein

Bcl-2 B-cell lymphoma 2

BDNF brain-derived neurotrophic factor

cAMP cyclic adenosine monophosphate

CPT 8-CPT-cAMP, cAMP analog that activates protein kinase A

CREB cyclic AMP-response element-binding protein

DMEM Dulbecco's Modified Eagle's Minimal Essential Medium

ECM extracellular matrix

EGF epidermal growth factor

EGFR epidermal growth-factor receptor

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

ETC mitochondrial electron transport chain

FAK focal adhesion kinase

FBS fetal bovine serum

FGF fibroblast growth factor

GSK-3 glycogen synthase kinase 3

H89 Protein Kinase A inhibitor

HIF hypoxia-inducible transcription factors

HRE hypoxia-response element

Hsp27 heat shock protein 27

IAP inhibitors of apoptosis proteins

ICAD inhibitor of caspase-activated DNase

IGF-I insulin-like growth factor I

JNK c-Jun NH2-terminal kinases

LY LY294002, PI3K inhibitor

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

MAPK mitogen activated protein kinase

NAC N-acetylcysteine

NADH Nicotinamide adenine dinucleotide

NGF nerve growth factor

NRP neuropilin

PBS phosphate buffered saline

PD Parkinson's disease

PDGF platelet-derived growth factor

PDGFR platelet-derived growth-factor receptor

PI3-kinase phosphoinositide 3-kinase

PKA cAMP-dependent protein kinase A

PKC protein kinase C

PKI PKI 14-22 Amide, protein kinase A inhibitor (PKI) peptide

PLC phospholipase C

PlGF placental growth factor

ROS reactive oxygen species

RT PCR reverse transcriptase polymerase chain reaction

SB SB202190, p38 MAP Kinase inhibitor

SBMA X-linked spinobulbar muscular atrophy

SDS Sodium dodecyl sulfate

SEM standard error of the mean

Sema 3A semaphorin 3A

SOD1 superoxide dismutase

SU SU1498, VEGFR2 inhibitor

sVEGFR1 soluble VEGFR1

svVEGF snake venom VEGF

TNF tumor necrosis factor

TNFR TNF receptor

U U0126, MEK1/2 inhibitor

VEGF vascular endothelial growth factor

VEGFR vascular endothelial growth factor receptor

VRAP VEGFR-associated protein

XIAP X-chromosome-linked IAP

z-VAD z-VAD-FMK, pan-caspase inhibitor

Chapter I

Introduction

Neuronal cell death in selective areas of the brain is a hallmark of neurodegenerative disorders. Identifying mechanisms that mediate neuronal cell death or can promote survival under pathological conditions provides useful information for developing strategies to halt disease progression. Treating degenerating neurons with protein growth factors is one method for reducing death. Vascular endothelial growth factor (VEGF) is a well established endothelial cell mitogen that has been shown to protect neurons through activation of its cognate receptor VEGFR2. Increasing evidence shows that VEGF exerts protection in several paradigms of neuronal cell damage caused by stressful stimuli. In addition, growth factor receptor signaling pathways have been shown to crosstalk with cAMP-dependent Protein Kinase A (PKA) to protect neuronal cells against harmful insults. Whether VEGFR2 and PKA cooperate to mediate neuronal cell survival under stress conditions is unknown. This study demonstrates that the VEGF/VEGFR2 and PKA pathways converge at the MEK/ERK1/2 pathway to protect neuronal cells against a caspase-dependent cell death induced by serum deprivation. Our studies also suggest that VEGFR2 signaling may alleviate cell death through ERK1/2 by modulating the antiapoptotic effects of Bcl-xL. Furthermore, our results suggest that p38 MAPK serves dual functions playing a role as a prodeath effector negatively regulating VEGFR2-mediated survival and a regulator of neurite extension in serum deprived neuronal cells. This study is supported by reports showing that VEGF-mediated activation of VEGFR2 signals protection through ERK1/2 in neuronal cells, and that inhibition of p38 enhances VEGF-mediated survival. Our work is significant since the cell death mechanisms associated with VEGF-mediated survival in neuronal cells have not been fully elucidated. Therefore, this study aimed to delineate VEGF-mediated

signaling cascades that protect human neuronal cells from stress-induced cell death. The rationale for these studies is that a dissection of the molecular pathways that mediate neuronal cell survival in response to stress would provide a mechanism to prevent neuronal cell loss.

1.1 Vascular Endothelial Growth Factor (VEGF)

VEGF was initially identified as having an essential role in regulating endothelial cell progenitor differentiation leading to neovascularization (vasculogenesis) and the sprouting of new blood vessels from pre-existing ones (angiogenesis) [1,2]. VEGF is not only crucial for physiological processes during development and differentiation of new organs, postnatal angiogenesis during pregnancy, and wound healing but also plays a role in pathological diseases including cancer, rheumatoid arthritis, and cardiovascular diseases [3-7]. In endothelial cells, VEGF signals cellular responses that include cell survival, proliferation, migration, and differentiation in addition to its role in angiogenesis [8]. Although much initial evidence attributed VEGF to be exclusive to vascular endothelial cells it is now apparent that it is a pleiotropic factor exerting effects on diverse cell types and tissues. VEGF has also been shown to have trophic effects on non-endothelial cell types such as haematopoietic, epithelial, mesenchymal, germ, schwann, glial, and neuronal cells (neural stem cells and many peripheral and central nervous system cells) [9-12]. Interestingly, many reports have now revealed that VEGF possesses neurotrophic properties protecting various neuronal cell types from stress-induced death [13, 14]. Studies have demonstrated that under conditions of hypoxic, excitotoxic, or oxidative stress, VEGF increases the survival of hippocampal, cortical,

cerebellar granule, dopaminergic, autonomic, and sensory neurons [15-18]. In addition, abnormal regulation of VEGF expression has been implicated in several neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS) in which reduced VEGF expression leads to a loss in motor neurons [19-21]. These effects have created increasing interest in assessing the therapeutic potential of VEGF as a neuroprotective factor in neurodegenerative disorders. However, the signaling mechanisms associated with the neuroprotective effects of VEGF have yet to be fully elucidated.

1.2 VEGF Family

Vascular endothelial growth factor (VEGF), also known as VEGFA or $VEGF_{165}$, is the prototypical member of a family of closely-related growth factors, which includes VEGFs B, C, D, and placenta growth factor (PlGF) all of which are endogenously expressed in mammals [22]. In addition, proteins that are structurally related to the VEGFs exist, including the sheep parapoxvirus orf (VEGFE) [23] and in snake venom (svVEGF) [24]. The VEGF family members are secreted glycoproteins generally as covalently linked homodimers of approximately 40 kDa. Alternative splicing of the human VEGFA mRNA from a single 14kb gene containing eight exons separated by seven introns give rise to nine major transcripts, some of which are shown in Figure 1, encoding isoforms of 121, 145, 148, 162, 165, 165b (an endogenous inhibitory isoform that binds to VEGF receptor 2 with similar affinity as $VEGF_{165}$ but does not activate it or stimulate downstream signaling pathways) 183, 189 and 206 amino acid residues after

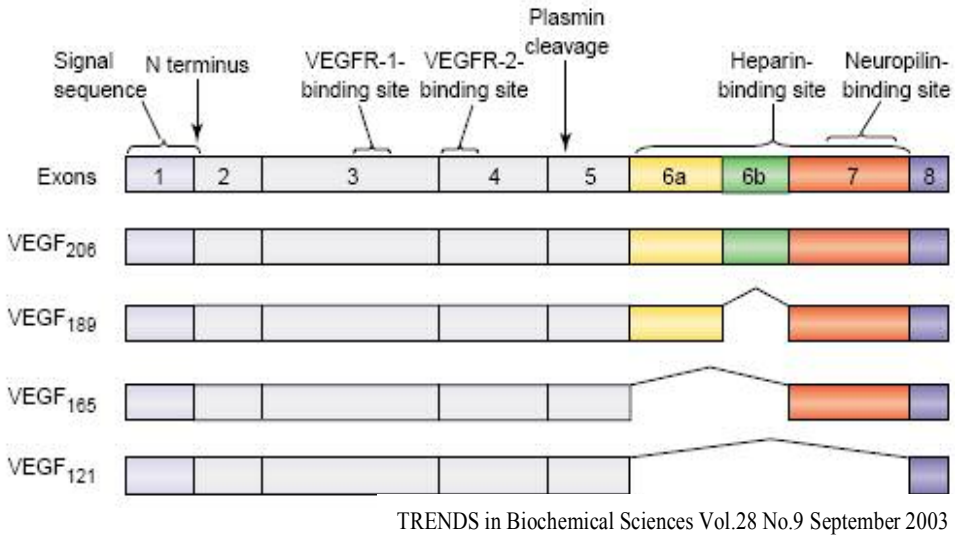

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Figure 1. Exon structure of the vascular endothelial growth factor (VEGF)-A mRNA splice variants. The VEGFA gene consists of eight exons that encode several different structural motifs and alternative splicing of a single pre-mRNA species.

signal sequence cleavage [19, 25]. $VEGF_{165}$ has been determined to be the most predominant and biologically active isoform. All transcripts contain exons 1–5, encoding the signal sequence and VEGF receptor-binding domain, and exon 8, with the diversity in the generated isoforms through the alternative splicing of exons 6 and 7. Exon 6 encodes a heparin-binding domain, while exons 7 and 8 encode a domain that mediates binding to both heparin and the receptor neuropilin-1 (NRP1). Most VEGF-producing cells appear to preferentially express $VEGF_{121}$, $VEGF_{165}$, $VEGF_{183}$ and $VEGF_{189}$, while the other splice variants are less abundant. All isoforms, with the exception of $VEGF_{121}$ which is freely diffusible because of its nonheparin-binding acidic nature, bind heparin to differing degrees based on the difference of their heparin-binding domain. $VEGF_{165}$ and $VEGF_{121}$ are secreted as covalently linked homodimeric proteins, whereas the larger isoforms, $VEGF_{189}$ and $VEGF_{206}$ are not readily diffusible and are almost completely sequestered in the extracellular matrix (ECM). $VEGF_{165}$ has intermediary properties, as it is secreted but

a significant fraction remains bound to the cell surface and ECM. Loss of the heparin-binding domain results in a significant loss of the mitogenic activity of $VEGF_{165}$.

All VEGF family members are able to regulate angiogenesis. However, in contrast to VEGFA the precise biological roles of the other VEGF family members are not yet fully understood. VEGFB has been associated with regulation of extracellular matrix degradation, cell adhesion and migration [26]. Knockout studies have demonstrated a role of VEGFB in pathological vascular remodeling in inflammatory arthritis [27] and protection of the brain from ischaemic injury [28]. VEGFC and VEGFD are both implicated as biologically important mediators of lymphangiogenesis, and VEGFC may also be involved in wound healing [29, 30]. PlGF is hypothesized to potentiate VEGFA-induced endothelial cell proliferation through heterodimerization with VEGFA, since on its own exerts only weak mitogenicity [31]. However, studies have shown that a loss in PlGF impairs not only angiogenesis but results in plasma extravasation (vascular leakage) and collateral artery growth during ischemia, inflammation, wound healing and cancer [32]. VEGFE of the parapoxvirus orf has been shown to have similar activities as the human VEGFA inducing the proliferation, migration and sprouting of cultured vascular endothelial cells *in vitro* and angiogenesis *in vivo* [23]. The snake venom VEGF family member also resembles VEGFA in that it shows vascular permeability activity, although significantly enhanced, and is thought to increase the toxic effects of the venom [24].

Since $VEGF_{165}$ (hereafter referred to as VEGF) has been well established as promoting cellular responses that include survival and proliferation in endothelial cells, the present studies aimed to establish whether VEGF exhibited similar effects in neuronal

cells. This notion is supported by various studies showing that VEGF confers neurotrophic properties and protect neuronal cells from stress-induced damage [13, 19]. Delineating the downstream survival cascades and cell death mechanisms associated with VEGF-mediated signaling in neuronal cells would be useful in assessing the therapeutic potential of VEGF. This study presents evidence for a neuroprotective mechanism mediated by VEGF in stressed neuronal cells.

1.3 VEGF Receptors

The biological effects of the VEGF ligands are mediated through interaction with their three primary protein tyrosine kinase receptors VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1) and VEGFR3 (Flt-4) either alone or with its co-receptors the neuropilin (NRP) family of class 3 semaphorin receptors, NRP1 and NRP2 [19, 33]. The VEGF ligands bind in an overlapping pattern, as shown in figure 2, inducing receptor dimerization and transphosphorylation of the tyrosine kinase domain creating docking sites for downstream transduction of signals that direct cellular function [19, 34]. Similar to other receptor tyrosine kinases such as platelet-derived growth-factor receptor (PDGFR) and epidermal growth-factor receptor (EGFR), VEGF receptors are structurally characterized by a conserved intracellular tyrosine kinase catalytic domain that is interrupted by a non-catalytic kinase insert sequence, a single hydrophobic transmembrane domain and seven immunoglobulin(Ig)-like domains in the extracellular regions [19]. While VEGFR2 is considered to be the major mediator of several physiological and pathological effects such as proliferation, survival, migration and permeability in endothelial cells, VEGFR1 function is still unclear. An alternatively

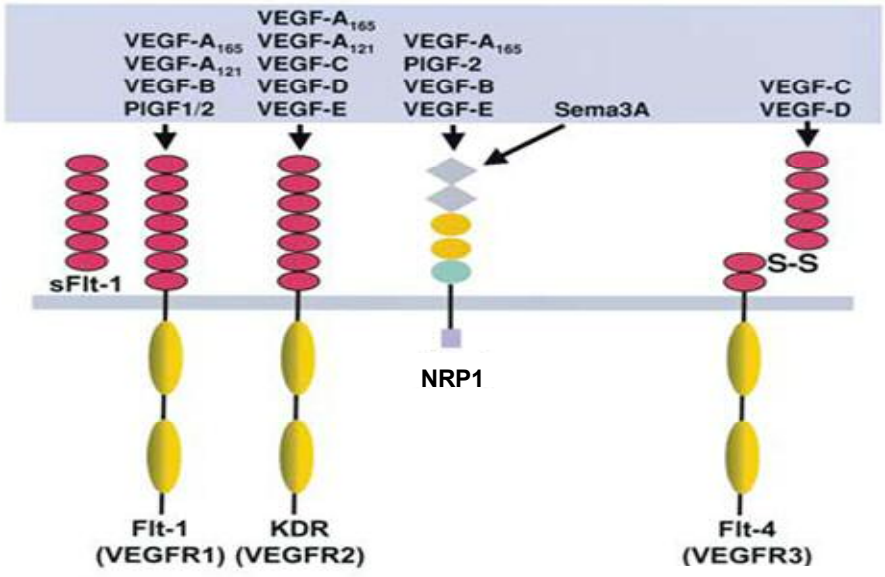

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Figure 2. VEGF receptor structure and ligand specificity. The VEGF ligand family all bind in a specific manner to three different receptor tyrosine kinases (RTK), VEGFR1, 2 and 3 either alone or with its co-receptor, neuropilin-1 (NRP1). The extracellular domain of VEGFR1 is also expressed as a soluble protein. VEGFA, B and PLGF bind to VEGFR1, VEGFA, C, D and E bind to VEGFR2, and VEGFC and D bind to VEGFR3. The RTKs possess an extracellular domain containing seven Ig-like loops (red ovals), a single hydrophobic membrane-spanning domain, and a cytoplasmic domain comprising a single kinase domain (yellow ovals) that is interrupted by a non-catalytic region, called the kinase insert. NRP1 is a non-RTK receptor for VEGF165, the PlGF-2 isoform, VEGF-B and VEGF-E. NRP1 comprises an extracellular region with MAM (meprin, AS, μ tyrosine phosphatase) (or C), a and b domains, a transmembrane region and a short cytoplasmic domain.

spliced truncated soluble extracellular form (sVEGFR1) has been shown to act as a ligand-binding molecule sequestering VEGF from VEGFR2 and thereby preventing its activation and subsequent downstream signaling [35, 36]. Its role as a decoy receptor negatively regulating VEGF activity is further confirmed by the observation that VEGFR1 undergoes weak autophosphorylation in response to VEGF and does not exhibit a mitogenic response [37]. Reports have demonstrated that the sequestering of VEGF by sVEGFR1 negatively affects angiogenesis during early development and under pathological conditions such as in cancer, ischemia**,** and pre-eclampsia [38-40]. Increased levels of sVEGFR1 in patients with pre-eclampsia are associated with decreased

circulating levels of VEGF resulting in general endothelial dysfunction. VEGFR3 is primarily expressed in lymphatic endothelial cells and its activation induces their proliferation, migration and survival [41]. A study showed that blocking VEGFR3 function by overexpression of a soluble recombinant VEGFR3 in the skin of transgenic mice leads to regression of lymphatic vessels and features of lymphedema [42].

Neuropilins (NRPs) are non-tyrosine kinase transmembrane receptors that bind VEGF but lack established VEGF-induced catalytic function due to a short intracellular domain insufficient for the independent transduction of biological signals, but act as co-receptors enhancing the binding of VEGF to VEGFR2 [43]. NRP1 was first identified as a receptor for semaphorin 3A (Sema 3A) implicated in axonal guidance and patterning. Interestingly, NRPs have also been shown to play essential roles in angiogenesis, one study demonstrating that overexpression of NRP1 in mice results in increased capillary formation, vasodilatation and malformation of the heart [44], whereas mice deficient in NRP1 exhibit defects in embryonic axonal patterning and an array of vascular abnormalities including defective development of large vessels and impaired neural and yolk sac vascularization [45, 46]. In contrast, NRP2 seems to be involved in lymphangiogenesis where mice deficient in NRP2 show reductions in small lymphatic vessel and capillary formation [47]. The complexity of VEGF/VEGF receptor interactions and the processes they mediate emphasize the diversity of VEGF signaling in physiological and pathological conditions.

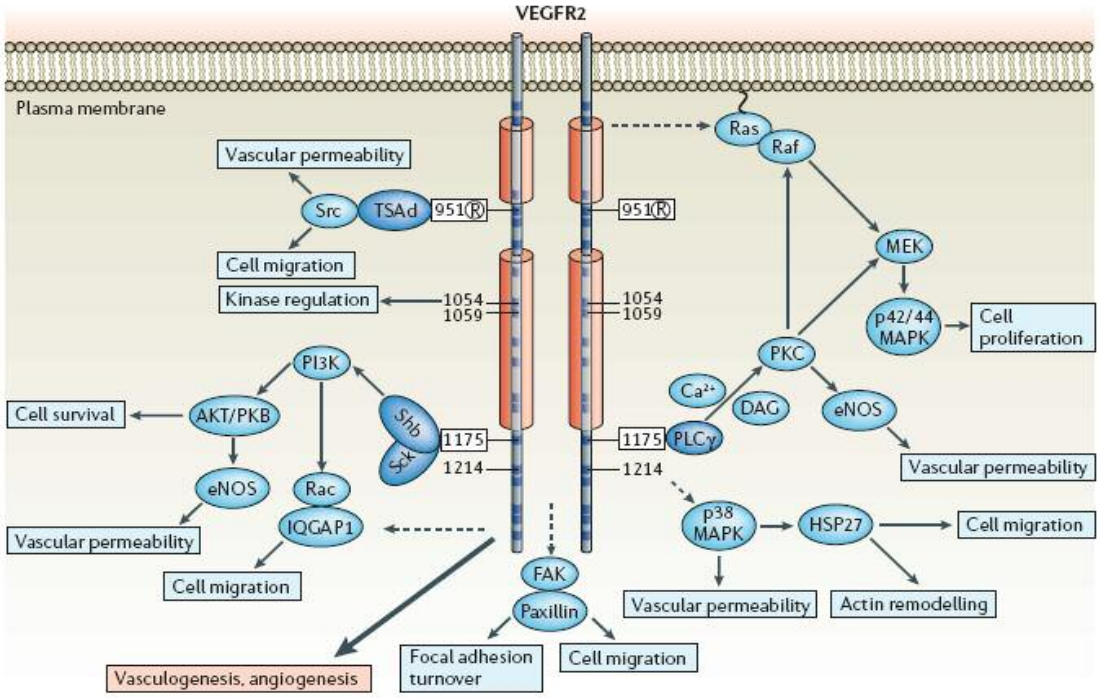
Evidence presented in this study further supports the notion that VEGFR2 is a major mediator of the mitogenic effects of VEGF, here exhibiting protective effects in neuronal cells subjected to harmful stimuli. Many reports have provided evidence

demonstrating that activation of VEGFR2 by VEGF promotes neuroprotection [13] but the mechanisms associated with VEGF-mediated survival in neuronal cells have not been fully elucidated. Therefore, this study sought to delineate the downstream cascades activated by VEGF/VEGFR2 to prevent neuronal cell death.

1.4 VEGF Signaling in Endothelial Cells

In endothelial cells, VEGF is secreted to elicit autocrine and paracrine types of signaling through VEGFR2 [49], where it can activate cell surface receptors on the cell of its origin (autocrine) or receptors on target cells in the extracellular environment that reside in close proximity (paracrine). Binding of VEGF to the extracellular domains of VEGFR2 leads to phosphorylation and induction of tyrosine kinase activity with subsequent activation of several cytoplasmic proteins, as shown in Figure 3, thereby signaling cellular responses that include cell survival, proliferation, migration, permeability, and angiogenesis [49]. The complexity of VEGF signaling is increased by crosstalk between activated pathways downstream of VEGFR2 activation. Studies have associated a number of signal transduction molecules that are activated or modified in response to VEGF stimulation downstream to VEGFR2 activation, including the Src family of tyrosine kinases, the Ras GTPase-activating protein, protein kinase C (PKC), PLCγ, phosphoinositide 3-kinase (PI3-kinase) and its downstream substrate the serine/threonine kinase Akt/protein kinase B (PKB), and members of the mitogen activated protein kinase (MAPK) family such as ERK1/2 [48]. MAP kinases comprise a highly conserved cascade of proline-directed serine/threonine kinases that connects growth factor-receptor induced signaling pathways to regulatory targets in response to

various stimuli [50, 51]. The MAPK members include the extracellular signal-regulated protein kinase (ERK)-1/2 and the stress-activated protein kinases c-Jun NH2-terminal kinases (JNK) and p38 MAPK. ERK1/2 normally plays a role in promoting proliferation and differentiation and is considered to be a major mediator of survival, while p38 MAPK and JNK are both activated to induce stress-related pathways, such as inflammation or apoptosis, and are more involved in control of cell cycle checkpoints,



Nat Rev Mol Cell Biol. 2006 May; 7(5):359-71

Figure 3. VEGFR2 intracellular signaling. Ligand binding induces dimerization and the autophosphorylation of intracellular tyrosine residues indicated by numbers. Dark blue squares in the receptor molecules indicate positions of tyrosine residues. Binding of signaling molecules (dark blue ovals) to phosphorylation sites (boxed numbers), initiates signaling cascades (light blue ovals), leading to biological responses (pale blue boxes). The initiation of certain signaling chains is unclear (dashed arrows).

and differentiation [52]. Evidence has demonstrated VEGF as being a strong activator of ERK1/2 via VEGFR2 in stimulating endothelial cell proliferation and angiogenesis. This is supported by the finding that specific inhibitors of MEK1/2, the kinase responsible for ERK activation, reduce endothelial tubulogenesis (capillary tube formation creating a

lumen which allows the transport of substances throughout an organ) *in vitro* [53]. The antiapoptotic phosphatidylinositol 3'-kinase/Protein Kinase B (PI3K/Akt) serine/threonine kinase pathway is another critical mediator of cell survival in response to growth factor stimulation [54], having been shown to suppress a number of pro-apoptotic proteins including glycogen synthase kinase 3 (GSK-3), BAD, and caspase-9 [55-58]. VEGF-dependent endothelial cell survival has been shown to be mediated in part via PI3-kinase (phosphoinositide 3′-kinase)-dependent activation of Akt/PKB [54, 59] by inhibiting apoptotic effectors. This is supported by reports suggesting that VEGF mediated survival occurs through up-regulation of anti-apoptotic proteins, including Bcl-2 and A1 [60], and the IAP (inhibitors of apoptosis) proteins, survivin and XIAP (X-chromosome-linked IAP) [61]. Based on these findings in endothelial cells, our studies sought to assess whether VEGF/VEGFR2 conferred similar protection of neuronal cells under stress through activation of the MEK/ERK1/2 pathway or PI3K/Akt. VEGF-mediated mechanisms in nonendothelial cells, such as in neuronal cells, still remain unclear and require further delineation. This study presents evidence for VEGF/VEGFR2 directed neuroprotection mediated through activation of both the PI3K/Akt and ERK1/2 downstream pathways, and antagonizes survival induced by p38 MAPK.

1.5 Neuroprotective Effects of VEGF

Whether VEGF exerts similar effects on neuronal cells as it does on endothelial cell proliferation and survival is an emerging area of research in neurobiology. Increasing evidence suggests that VEGF possesses neurotrophic properties and protects neurons from stress-induced cell death through autocrine and paracrine mechanisms [12, 16, 62,

63], observed both *in vitro* and *in vivo*. VEGF stimulates proliferation of astrocytes [16], Schwann cells [12, 64], microglia [65] and cortical neurons [66, 67], and its neuroprotective effects are observed in its ability to protect hippocampal, cortical, granule, dopaminergic, autonomic and peripheral sensory neurons [64-66] against cell death induced under various conditions of stress including serum withdrawal, hypoxia, glucose deprivation, ischemia, and oxidative and excitotoxic stimuli [12, 13, 17, 68, 69]. Its neurotrophic effects were also demonstrated by its ability to stimulate axonal outgrowth and stimulate proliferation and survival of neural stem cells (NSCs) [12, 15, 70]. *In vivo*, VEGF has been observed to promote both neurogenesis and neuroprotection in animal models of cerebral ischemia, neurodegenerative diseases and traumatic nerve injury [71-73].

However, the exact pathways associated with VEGF mediated signaling events in neuronal cells have not been completely elucidated. Identifying the events associated with VEGF signaling is complicated by the fact that the biological effects of VEGF are mediated through interaction with its tyrosine kinase receptors VEGFR1 and VEGFR2 either alone or with its co-receptors, NRP1 and NRP2 [13]. VEGF-mediated survival relies on its tyrosine kinase receptors and several downstream signaling cascades. Its protective effects were demonstrated by a study in HN33 cells, where VEGF signaling through VEGFR2 promoted a survival effect mediated by PI3K/Akt, increased phosphorylation of IκB-α and nuclear translocation of the transcription factor NF-κB [17, 74]. In addition, VEGFR2 signaling via the PI3K/Akt and the MEK/ERK1/2 pathways protected hippocampal neurons from glutamate-induced death [69] while in another study VEGF promoted a protective effect in cerebellar granular neurons that was dependent on

the PI3K/Akt pathway but independent of MEK/ERK1/2 activation [75]. Evidence also suggests that VEGF may exert protection by suppressing the stress-induced activation of caspase-3 through PI3K/Akt [54, 75, 76] and/or MEK/ERK1/2 [62]. Caspase-3 is essential for the execution step in the apoptotic program of cell death and would be an appropriate target for VEGF mediated survival. Whereas the MEK/ERK1/2 cascade is involved in cell proliferation and survival, the p38 MAPK pathway is preferentially activated by stressful stimuli. In neuronal cells, activation of p38 MAPK is implicated as a mediator of apoptosis in response to glutamate, β-amyloid, 6-hydroxydopamine and Fas receptor activation [77-80]. Consequently, activated p38 MAPK is implicated as a mediator of caspase activation induced by the extrinsic (death receptor) and intrinsic (mitochondrial) pathways of cell death during the neurodegenerative process [81, 82]. Although studies in neuronal cells implicate p38 MAPK as a regulator of apoptosis, the exact mechanism associated with this event remains unclear. VEGF has been shown to mediate survival through PI3K/Akt and MEK/ERK1/2 pathways in stressed neuronal cells by suppressing caspase activation [83, 84] and in certain cases, p38 MAPK activation [85]. Consequently, these findings link VEGF with protection against a p38 MAPK-mediated neuronal cell death. However, the relationship between p38 MAPK activation and VEGF signaling in neuronal cells is unclear since VEGF was shown to stimulate p38 MAPK after brain hypoxia [86] and inhibit its activation after retinal ganglion cell axotomy [18]. Therefore, the present study involved delineating the VEGF-mediated signaling cascades activated to promote survival in stressed neuronal cells and the cell death mechanisms associated with the stress induced death. Our findings suggest

a model in which VEGF signaling through VEGFR2 protects serum deprived neuronal cells from a caspase-dependent cell death that is mediated in part by p38 MAPK.

Cell survival is dependent on the appropriate balance of both positive signals, such as growth factor induced stimulation of proliferation, and negative signals, such as increased intracellular oxidant levels or the presence of misfolded proteins. Perturbations in this critical balance may lead to the triggering of the apoptotic cell death program. Apoptosis is a necessary physiological process and is an important mechanism in both development and homeostasis in adult tissues for the elimination of infected, transformed, or damaged cells that represent a threat to the integrity of the organism [87]. Deregulation of apoptosis may result in the onset of diseases such as cancer, immune diseases, and neurodegenerative disorders [88-90]. The major functional groups of effectors involved in triggering and affecting the apoptotic process include caspases, a group of cysteine proteases that carry out the degradation of the cell by cleaving vital cellular proteins, adaptor proteins, which control the activation of the initiator caspases, members of the tumor necrosis factor receptor (TNFR) superfamily, and the pro-apoptotic members of the Bcl-2 family of proteins [87]. Within the apoptotic program, death may result from caspase-dependent or -independent events. The present study supports a model for a caspase-dependent rather than a caspase-independent cell death demonstrated by results showing that a blockade of caspases using a caspase pharmacological inhibitor promotes survival under our paradigm of cell death. Caspase-dependent cell death is further divided into two death signals comprised of the intrinsic and extrinsic pathways [91]. The intrinsic pathway implicates a mitochondria-mediated apoptotic death [92] where membrane integrity is compromised leading to the release of

mitochondrial proteins that activate caspases, while the extrinsic pathway is induced by death ligands Fas or TNF that engage death receptors that contain intracellular regions providing direct receptor-triggered signaling into caspase activation and apoptosis [91]. However, the present study provides evidence suggesting a stress induced cell death through a mitochondrial mediated mechanism although we cannot rule out the possibility that the death receptors are also contributing to the caspase activation and cell death observed under our conditions of stress.

The integrity of the mitochondria is tightly regulated by the Bcl-2 family of proteins that reside upstream of the mitochondria where a balance in the ratio of antiapoptotic (e.g., Bcl-2 and Bcl-xL) and proapoptotic (e.g., Bax and Bak) members protect against mitochondrial outer membrane permeabilization, that would lead to impairment of the electron transport chain (ETC), ATP generation and lipid biogenesis, and the release of mitochondrial proteins into the cytosol, such as cytochrome c, ultimately resulting in caspase activation and an apoptotic cell death [87]. The antiapoptotic members bind prodeath family members prior to their activation inhibiting them and thus maintaining mitochondrial membrane integrity. Damage or stress to cells may cause disruption of the balance in anti- versus pro-apoptotic Bcl-2 family members and thus the loss of mitochondrial functions essential for cell survival [92]. Once cytochrome c is released into the cytoplasm it forms an active complex with the apoptotic protease activating factor (Apaf-1), pro-caspase-9 and dATP, a complex designated as the apoptosome. The apoptosome facilitates the self-activation of pro-caspase-9 which activates other caspases, such as the executioner caspase caspase-3, leading to an expanding cascade of proteolytic activity within the cell. Caspase-3 then mediates

cleavage of the caspase-activated DNase (ICAD) inhibitor allowing it to enter the nucleus [92]. These events ultimately result in cell death due to the digestion of proteins essential for cell survival, structural proteins in the cytoplasm, chromosomal DNA degradation and phagocytosis of the cell. Cells also exhibit cell shrinkage, plasma membrane asymmetry exhibited by bubble-like blebs on their surface, detachment from the cellular matrix, fragmentation of the cell into membrane-bound apoptotic bodies, cell breakage into small membrane-wrapped fragments, and the phospholipid, phosphatidylserine, becomes exposed on the surface from within the plasma membrane, which is then bound by receptors on phagocytic cells like macrophages and dendritic cells which then engulf the cell fragments and secrete cytokines that inhibit inflammation [87].

Extrinsic signals such as growth factors, cytokines, or hormones may protect neuronal cells from death by suppressing the apoptotic program. Reports suggest that perturbations of VEGFR2 in stressed neuronal cells trigger an apoptotic cell death that is mediated through caspase activation [84]. Also, the ERK1/2 pathway has been shown to prevent mitochondrial dysfunction induced by stress by modulating the expression levels of Bcl-xL [93]. Overexpression studies of anti-apoptotic Bcl-2 and Bcl-xL in neurons of transgenic mice and in direct studies of human tissues have demonstrated significant neuroprotective functions, implicating the Bcl-2 family as key regulators of neuronal cell fate [94]. The present study supports previous reports by providing evidence of a VEGF/VEGFR2 mediated mechanism to promote neuroprotection from a caspase-dependent death by activating ERK1/2 and possibly suppressing mitochondrial dysfunction by promoting the anti-apoptotic effects of Bcl-xL.

1.6 VEGF and Neurodegenerative Disorders

Abnormal regulation of VEGF expression has been implicated in several neurodegenerative disorders, including the pathogenesis of motor neuron degeneration of amyotrophic lateral sclerosis (ALS), X-linked spinobulbar muscular atrophy (SBMA), and degeneration of dopaminergic neurons in Parkinson's disease (PD). ALS causes adult-onset, progressive motor neuron degeneration in the spinal cord, brainstem, and cerebral cortex, leading to muscle atrophy, paralysis and death [95]. ALS1, an autosomal dominant form of adult ALS is associated with mutations in the gene encoding superoxide dismutase (SOD1), believed to cause motor neuron degeneration by gaining a toxic property [96]. I*n vitro* studies have shown that VEGF has direct neuroprotective effects in cultured motor neurons against death in conditions of hypoxia, oxidative stress, serum deprivation, glutamate-induced excitotoxicity, or mutant SOD-1-induced toxicity [97, 98]. In mice where the hypoxia-response element (HRE) sequence in the VEGF promoter was deleted, to which the hypoxia-inducible transcription factors HIF-1α and HIF-2α bind inducing expression of VEGF, an impaired potential to upregulate VEGF levels in conditions of stress was observed. These mice developed an ALS-like neuropathology, associated with decreased circulating levels of VEGF and reduced VEGF gene expression, suggesting that motor neurons are particularly sensitive to reductions in the levels of VEGF [99]. Insufficient VEGF may deprive motor neurons of critical survival and neuroprotective signals, especially in conditions of hypoxic or excitotoxic stress, and thereby cause motor neuron degeneration. Breakthrough experiments demonstrated that a single injection of a VEGF-expressing lentiviral vector into various muscles delayed onset and slowed progression of ALS in mice engineered to

overexpress the gene coding for mutated SOD1, increasing the life expectancy by 30 percent [99]. Most importantly, VEGF retained its protective efficacy even when VEGF-expressing lentiviral vector was delivered after half of the motor neurons had already died. These observations highlight that deregulation of VEGF expression is directly implicated in the pathogenesis of a neurodegenerative disease, thus reinforcing the neuroprotective potential of VEGF.

1.7 Protein Kinase A (PKA) Signaling in Neuronal Cells

Growth factor receptor signaling pathways have been shown to crosstalk with cAMP-dependent protein kinase A (PKA) to protect neuronal cells against harmful insults. The cAMP-inducible PKA is a well established pathway in neuronal cells that has been shown to be neuroprotective and promote survival of dorsal root ganglion neurons, retinal ganglion neurons, and cerebellar granule neurons under several experimental stresses, including growth factor withdrawal [100-103]. PKA is an effector system that responds functionally to changes in intracellular cAMP. Adenyl cyclase is activated by extracellular ligands increasing intracellular concentrations of cAMP which subsequently binds to and induces phosphorylation of PKA [104]. One of the hallmarks of cAMP is its ability to stimulate cell growth in many cell types while inhibiting cell growth in others [104]. Its proliferative effects are mainly associated with the RAS/RAF/MEK/ERK1/2 pathway. ERK1/2 usually signals cell proliferation when stimulated by growth factors through GTP activated Ras that activates the MAP kinase kinase kinase (MAPKKK) Raf-1, a serine/threonine kinase which phosphorylates and activates the dual-specificity threonine/tyrosine kinase MEK (MAPKK), which in turn phosphorylates ERK1/2.

However, cAMP-PKA has been shown to modulate the ERK1/2 cascade of signaling events either directly or via stimulatory or inhibitory interactions with growth factor-mediated mechanisms [104].

ERK can be activated or inhibited by cAMP to dictate the growth effects of cAMP. The mechanisms explaining cAMP inhibition of ERKs involve the uncoupling of Raf-1 from Ras activation, either by direct actions of PKA on Raf-1 or Rap1. In support of this, PKA was shown to block Ras-dependent signals to ERKs by blocking Raf-1 activation. *In vitro* studies showed that PKA phosphorylates Raf-1, reducing the affinity of Raf-1 to Ras GTP, interfering with Raf-1 activation [105]. Another possible mechanism involves the GTPase Rap1, which antagonizes Ras activation of Raf-1 and ERKs by binding and sequestering Raf-1 away from Ras, an event triggered by PKA [106]. In contrast to these findings, nerve growth factor (NGF) and cAMP analogues were shown to stimulate ERK1/2 activation to induce neuronal cell differentiation [107] while ERK1/2 activation by cAMP alone was shown to protect against the damaging effects of excitotoxicity [108] and oxidative stress [109] in neuronal cells. Stimulation of ERK1/2 by cAMP may occur through PKA-dependent signaling through Rap1 to B-Raf Rap1 can also activate B-Raf independently of Ras and provides another pathway for cAMP to activate ERKs. Interestingly, in cells that do not express B-Raf, transfection with B-Raf converts cAMP from an inhibitor to an activator of ERKs [110, 111].

More recently, the neuroprotective properties of cAMP-dependent PKA were shown to resemble that elicited by VEGF in that PKA can also activate the ERK1/2 and PI3K/Akt pathways and suppress caspase-3 to promote neuronal cell survival [83, 112]. In support of this, a study suggested a neuroprotective role for pituitary adenylate cyclase

activating polypeptide (PACAP) against neurotoxicity that was associated with both activation of MAPK by PKA and inhibition of caspase-3 activity [83]. Also, cAMP-PKA signaling has been shown to inhibit the proapoptotic factor BAD, inhibiting caspase activation and apoptosis [113]. Therefore**,** the present study sought to investigate whether the PKA pathway functioned concurrently with the VEGF signaling pathway in stressed neuronal cells to promote survival through similar downstream targets. The existence of crosstalk between the PKA and VEGF-mediated survival pathways has not been explored in neuronal cells. Whether VEGFR2 and PKA cooperate to mediate neuronal cell survival under stress conditions is unknown.

1.8 Cell Death and Oxidative Stress

There is increasing evidence that oxidative stress accompanies caspase activation resulting from perturbations in mitochondrial function [114]. The present study investigated whether overproduction of reactive oxygen species (ROS) was associated with an increase in caspase-3/7 activity and cell death of neuronal cells exposed to stress. Furthermore, both VEGF and PKA have been shown to protect against stress-induced cell death by suppressing caspase activation [83, 112]. Therefore, we investigated whether the neuroprotective effects elicited by the VEGF/VEGFR2 and PKA signaling pathways includes a suppression of a possible ROS overproduction induced by stress. Excessive ROS production can be cytotoxic and is implicated in a wide variety of diseases including cancer and neurological disorders [115, 116]. Oxidative stress results when the critical oxidant and antioxidant balance is compromised because of uncontrollable levels of ROS, antioxidant depletion, or both. Excessive ROS can trigger a

loss in the mitochondrial membrane potential leading to mitochondrial electron transport chain impairment and subsequent release of caspases resulting in cell death [115]. ROS can cause damage to DNA and cause oxidation of polydesaturated fatty acids in lipids and amino acids. Since ROS overproduction is a key mediator of cellular dysfunction in a wide variety of diseases [115, 116], the present study investigated if excessive ROS contributed to the harmful effects observed in stressed neuronal cells.

1.9 VEGF and Neuronal Differentiation

VEGF has also been associated with exerting direct effects on neuronal cells promoting differentiation characterized by neurite extension. Reports have demonstrated that VEGF stimulates neurite outgrowth from cultured retinal ganglion cells [117] and cerebral cortical neurons [118, 119] through VEGFR2. However, neuronal differentiation is a complex process involving many different signaling pathways and the signal transduction pathways associated with VEGF-mediated neuritogenesis is incompletely understood. Neurite extensions are critical for interneuronal communication and aberrant neurite formation and growth may contribute to the pathogenesis of neurological disorders [120]. In addition, the cAMP/PKA pathway has also been shown to promote differentiation. Hormones and neurotransmitters that increase intracellular levels of cAMP have been shown to induce neuronal differentiation mediated by PKA activation that leads to growth of neuronal extensions or creates new synaptic connections [121, 122]. Members of the MAPK family, in addition to playing central roles in neuronal cell death and survival, have been shown to participate in neuronal differentiation downstream of growth factor and hormone signaling cascades [117, 123]. Recent

evidence suggests that neurite outgrowth is mediated by p38 MAPK [124, 125] and in endothelial cells, p38 MAPK can be activated by VEGF [126]. These reports suggest that VEGF induced neuronal differentiation is regulated by p38 MAPK mediated functions. In support of these findings our results show that both VEGF/VEGFR2 and cAMP/PKA signaling leads to neuronal differentiation characterized by neurite extensions regulated by p38 MAPK mediated functions. Our studies demonstrate a functional relationship between VEGF/VEGFR2 signaling and p38 MAPK in mediating neuronal differentiation.

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 VEGFR2 (SU1498), p38 MAPK (SB202190) PI3K/Akt (LY294002), and PKA (H89 and PKI 14-22 Amide) were obtained from EMD Biosciences Inc (San Diego, CA). The inhibitor of MEK1/2 (U0126) was obtained from Promega Corporation (Madison, WI). The pan-caspase inhibitor, z-VAD-fmk, and cAMP analog mediator of PKA activation, 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP), were obtained from Biomol International (Plymouth Meeting, PA).

2.2 Cell Culture- Human neuroblastoma SK-N-SH cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained at 37^0C in DMEM-F10 (1:1) supplemented with 5% fetal bovine serum (FBS) (Invitrogen Corporation, Carlsbad, CA). For each experiment, cells were grown to 80% confluency followed by 48 hr of serum

2.3 Transient Transfection- SK-N-SH cells were transfected with 0.05 μg of pcDNA3-Bcl-xL (gift of Dr. Shigemi Matsuyama, Case Comprehensive Cancer Center, Cleveland, OH) and pCEP4-Erk1 (gift of Dr. Melanie H. Cobb, University of Texas Southwestern Medical Center, TX) using siIMPORTER transfection reagent (Upstate Cell Signaling Solutions, Charlottesville, Virginia), in 96-well or 10-cm plates according to the manufacturer's instructions. An empty pcDNA3 vector served as the control. For each

transfection, cells were incubated for 4 hr in the transfection medium at 37^0C followed by 48 hr treatments as indicated under serumfree conditions.

2.4 RNA Interference- Cells were transfected with 20 μM of KDR/Flk-1/VEGFR2 SMARTpool® siRNA duplexes or non-specific (control) siRNA duplexes (Dharmacon RNA Technologies, Lafayette, CO) according to the manufacture's directions. Cells were then treated as indicated and incubated for 48 hr under serumfree conditions.

2.5 Reverse Transcriptase PCR- Total RNA was purified from cells cultured in 10 cm plates and treated as indicated using the RNeasy RNA isolation Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed at 50^0C for 30 min and amplified for 35 cycles using the QIAGEN OneStep RT-PCR Kit according to the manufacturer's instructions using the following primers: $VEGF_{165}$, forward 5'-AGCCTTGCCTTGCTGCTCTA-3'; reverse 5'-GTGCTGGCCTTGGTGAGG-3'; VEGFR2, forward 5'-GCATCTCATCTGTTACAGC-3'; reverse 5'-CTTCATCAATCTTTACCCC-3'; VEGFR1, forward 5'-GACGTCTAGAGTTTGACACGAAGC-3'; reverse 5'-GCATGCAACACTGAGTAACATGAC-3'; NRP1, forward 5'-AAAAGCCCACGGTCATAG-3'; reverse 5'-TGTCATCCACAGCAATCC-3'; NRP2, forward 5'-CAAGTTGCTGTGGGTCATC-3'; reverse 5'-AATTGCTCCAGTCCACCTC-3'; and actin, forward 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'; reverse 5'-

CTAGAAGCATTTGCGGTGGACGATGGAGGG-3.' PCR products were visualized by electrophoresis on 2% agarose gels.

2.6 Cell Viability- Cells were plated in 96-well microtiter plates and treated as indicated under serumfree conditions for 48 hr at 37^0C. 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.

2.7 Caspase-3/7 Activities- Caspase activity was measured in cells plated in a 96-well format using the fluorescence cell-based Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega) according to manufacturer's directions. Caspase activity was quantified using the Molecular Dynamics Typhoon^{TM} 9410 Imaging System with ImageQuant software (Amersham Pharmacia Biotech, Piscataway, NJ). Data were normalized as fluorescent units/μg protein.

2.8 Oxidative Stress- ROS was measured using the fluorescent dye 2,7'-dichlorofluorescein-diacetate (H_2DCF-DA), (Molecular Probes, Eugene, OR), as described previously [129]. Briefly, cells were plated in a 96-well format and treated as indicated under serumfree conditions for 48 hr. Cells were then incubated with 10 μM H_2DCFDA and ROS was quantified using the Molecular Dynamics Typhoon^{TM} 9410 Imaging System. Data were normalized as fluorescent units/μg protein.

2.9 Protein Extraction and Western Blotting- Total cell lysates were obtained from cells cultured in 10 cm plates and treated for 48 hr at 37^0C under serum deprivation and harvested in a lysis buffer as described previously [129]. Protein concentrations were determined with a bicinchoninic acid assay (BCA) according to manufacturer's instructions (Pierce, Rockford, IL). Equal amounts of protein (25 μg) from each lysate 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: rabbit antibodies that recognized both phosphorylated and total forms of ERK1/2, p38 MAPK and Akt or the pro- and cleaved forms of caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA) and mouse antibodies against Hsp27 (1:1000; Upstate Cell Signaling Solutions, Charlottesville, Virginia) and Bcl-xL (1:500; BD Biosciences, San Jose CA). A mouse antibody against β-actin (1:1000; Sigma-Aldrich) was used as a loading control. Immunoreactive bands were detected with anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase and visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Endogen, Rockford, IL).

2.10 Protein Immunoprecipitation- VEGFR2 was selectively immunoprecipitated from equal concentrations (500 μg) of total protein lysates using a rabbit antibody raised against VEGFR2 (1:200; Upstate Cell Signaling Solutions). Antibody-antigen complexes were selectively removed by binding to protein A sepharose beads (Sigma-Aldrich) in a final volume of 200 μl overnight at 4^0C. Beads were then collected by centrifugation, washed, resuspended in 25 μl of 2x SDS loading buffer and subjected to Western blotting

analysis. To detect protein expression of VEGFR2, blots were probed with a rabbit antibody raised against VEGFR2 (1:1000; Santa Cruz Biotechnology, Inc, Santa Cruz CA). To detect activated VEGFR2, blots were probed with a mouse antibody that specifically recognizes phosphotyrosine (1:1000; Santa Cruz Biotechnology, Inc, Santa Cruz CA) and immunoreactive bands were detected as described in the protocol for Western Blotting.

2.11 Immunofluorescent Staining and Cell Imaging- Cells were plated in 8-well glass chamber slides and treated as indicated under serumfree conditions for 48 hr at 37^0C. Brightfield images of control and treated cells were captured at 20X magnification using a Nikon ECLIPSE TE200 microscope prior to immunofluorescent staining. Cells were then washed with PBS, fixed for 20 min with 3.7% formaldehyde in PBS, permeabilized with 0.1% saponin for 20 min and incubated at room temperature with 1 μg/ml of the chromatin-specific dye Hoechst 33342 (Sigma) for 20 min according to the manufacturers' instructions (Molecular Probes, Eugene, OR). Stained nuclei were assessed for chromatin condensation and images were captured at 60X magnification on a Nikon OPTIPHOT-2 fluorescent microscope.

2.12 Statistical Analyses- Data are expressed as the mean $\pm$ SEM of experiments that 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

VEGFR2 and PKA Converge at the MEK/ERK1/2 Pathway to Promote Survival in Serum Deprived Neuronal Cells

3.1 The mRNA expression levels of VEGF and its receptors, VEGFR1, VEGFR2, and NRP1 are upregulated in serum deprived neuronal cells- There is increasing evidence that VEGF elicits its neuroprotective effects under stress conditions through interactions with cognate receptors that are expressed on neuronal cells [13]. An RT-PCR analysis revealed that 48 hr of serum deprivation in the human neuroblastoma cell line, SK-N-SH, upregulated the expression levels of VEGF, VEGFR1, VEGFR2 and NRP1 and downregulated NRP2 (Fig. 4A). Since VEGF signals most biological functions through VEGFR2, we examined whether the upregulation in VEGFR2 gene expression was coincident with increased levels of protein. Immunoprecipitations of the receptor showed that VEGFR2 protein was detected only in serum starved cells independent of the inclusion of exogenous VEGF (Fig. 4B). Subsequent immunoprecipitation experiments (Fig. 4C) revealed that the endogenous level of receptor was phosphorylated only in serum starved cells (Fig. 4C, lane 2) and this event was augmented 2-fold by the addition of exogenous VEGF (Fig. 4C, lane 3). Conversely, cotreatments with a selective inhibitor of VEGR2 activation**,** SU1498, attenuated VEGFR2 phosphorylation (Fig. 4C) to 28% and 26% of the activation levels induced by exogenous (Fig. 4C, compare lanes 2 and 4) and endogenous (Fig. 4C, compare lanes 3 and 5) VEGF, respectively. The concentration of exogenous VEGF employed in these studies was determined from pretested titrations in serum starved cells where 10-100 ng/ml of the growth factor mediated a 30-35% increase in survival (data not shown). Given these findings, we postulated that VEGF functions as an autocrine and paracrine factor in our neuronal model of serum deprivation.

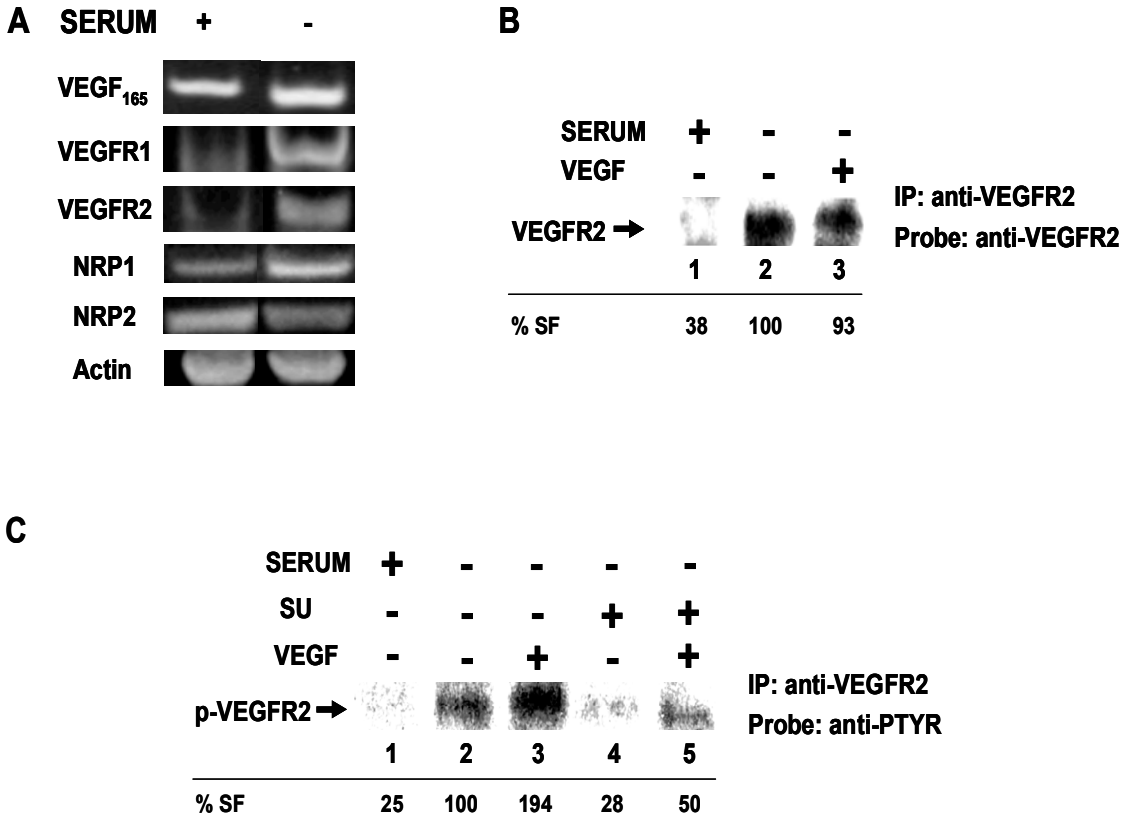


Figure 4. VEGF and its receptors are upregulated by serum deprivation in neuronal cells. *A*, RT-PCR analyses for VEGF and receptor expression were performed with RNA isolated from SK-N-SH cells cultured for 48 hr at 37^0C under serum and serumfree conditions as described in "Materials and Methods." *B*, VEGFR2 protein was immunoprecipitated from total lysates of cells grown as described in *A* with the addition of serumfree cells treated with 10 ng/ml of exogenous VEGF. *C*, VEGFR2 protein was immunoprecipitated from total lysates of cells grown as described in *B* with the addition of serumfree cells treated with 10 μM of the inhibitor SU1498 (SU) without and with VEGF. Immunoprecipitated VEGFR2 was subjected to Western blots and analyzed for total protein in *B* and phosphorylated receptor in *C* using an anti-VEGFR2 or anti-phosphotyrosine antibody (1:1000). The numbers below each lane in *B* and *C* refer to densitometry readings normalized to serumfree (SF) treatments.

3.2 VEGF signals through VEGFR2 to promote survival in stressed neuronal cells- To establish whether VEGF or VEGFR2 mediate neuronal cell survival, SK-N-SH cells were incubated for 48 hr with VEGF in the presence and absence of serum and then assayed for cell survival following treatments with a neutralizing antibody against VEGF or SU1498 (Figs. 5A and 5B). Whereas no changes were observed in the viability of serum treated cells, VEGF increased survival by 20% in serum deprived cells while inhibition of either VEGF or VEGFR2 incubated without and with VEGF elicited a significant ($p<0.001$) loss in viability. Moreover, VEGF had no effect on cell viability when VEGFR2 activation was blocked, suggesting that VEGF mediated its protective effects mainly through VEGFR2. Nevertheless, we cannot rule out the possibility that VEGFR2 can heterodimerize with other receptors such as Nrp-1 or VEGFR1 to signal protection against the cell death induced by serum deprivation. To confirm that the effects of the pharmacological inhibitor SU1498 were specific for VEGFR2 in serum starved SK-N-SH neuronal cells, cells were transfected with siRNA that specifically silence VEGFR2 gene expression (Fig. 5C). Consistent with the loss in survival observed with the pharmacological inhibitor, siRNA also decreased cell viability to levels that mimicked SU1498 (Fig. 5D). Taken together, these results suggest that endogenous VEGF can be secreted to function in an autocrine manner to signal survival through VEGFR2 in serum starved neuronal cells. Alternatively, the fact that exogenous VEGF increases survival suggests that VEGF activates VEGFR2 in a paracrine mechanism on nearby neuronal cells to provide protection.

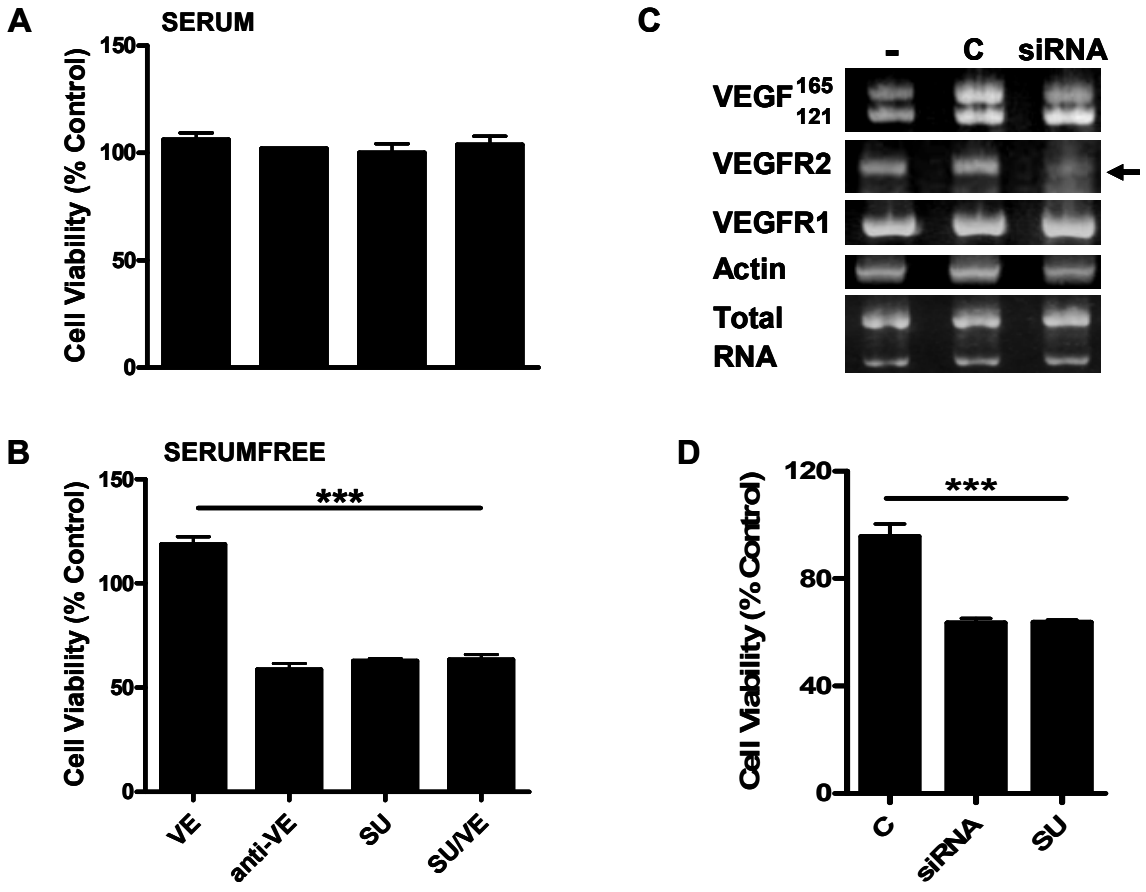


Figure 5. VEGF signals VEGFR2 activation to promote survival. *A,* and *B,* Cell viability was determined using a MTS assay as described in "Materials and Methods." Cells incubated with (A*)* and without (B*)* serum were stimulated for 48 hr with VEGF (VE) alone or in combination with 0.5 μg/ml of neutralizing antibody against human VEGF (anti-VE), or SU1498 (SU). C, RT-PCR analyses of VEGF and its receptors and D, cell viability assays were performed with cells treated with and without SU and transfected with 20 μM of siRNA duplexes silencing gene expression of VEGFR2 or a non-specific siRNA duplex control (C) and incubated for 48 hr under serumfree conditions. Results represent the $\pm$ S.E.M. of the percent cell viability relative to serumfree or C control cells (100%) from at least three independent experiments. (*** $P<0.001$) indicates significance between VE and inhibitor treated cells in *B*, and between C and siRNA and SU in *D*.

3.3 VEGF activates Akt and ERK1/2 through VEGFR2- Several studies have shown that VEGF signals neuroprotection by activating the PI3K/Akt and MEK/ERK1/2 pathways both *in vitro* and *in vivo* [18, 69]. Consistent with these reports, Western blot analyses revealed that treating serum deprived cells with the pharmacological inhibitor SU1498 or VEGFR2 siRNA was coincident with a loss in the phosphorylation of Akt and ERK1/2 (Fig. 6A). Treatments with the PI3K/Akt (LY294002) or MEK (U0126) inhibitors alone had little effect on survival while a simultaneous blockade of both pathways led to a significant ($p<0.001$) loss in cell viability (Fig. 6B). These findings suggest that MEK/ERK1/2 and PI3K/Akt signaling cascades act as distinct pathways that function to mediate neuroprotection in response to VEGF [69].

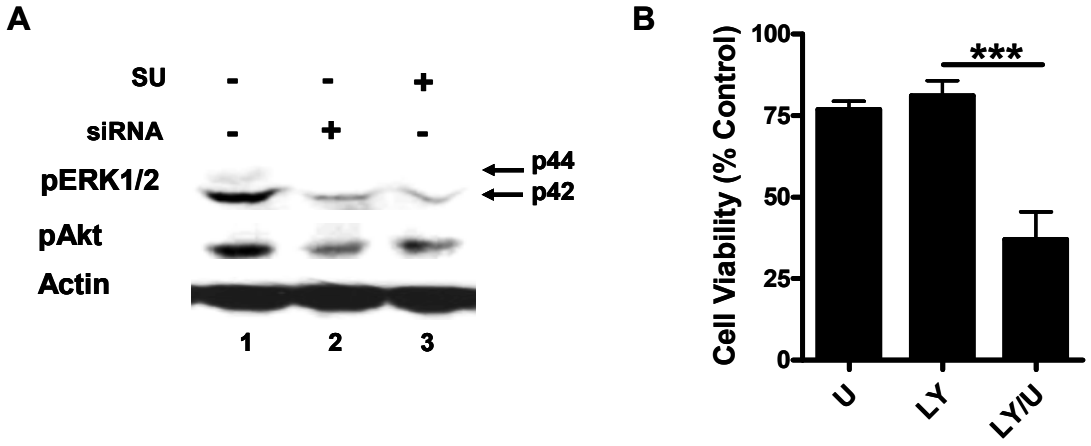


Figure 6. Gene silencing of VEGFR2 prevents activation of ERK1/2 and Akt. A, Total lysates were prepared from serum deprived cells treated with and without 10 μM SU1498 (SU) or transfected with VEGFR2 siRNA and subjected to immunoblot analyses for the detection of the phosphorylated forms of ERK1/2 (p44/p42) and Akt, as described in "Materials and Methods." Actin serves as a protein loading control. *B*, Cell viability was measured in cells treated with inhibitors for MEK1/2 (U0126; U) and PI3K/Akt (LY294002; LY) alone and in combination. Results in *A* and *B* represent the $\pm$ S.E.M. of the percent cell viability relative to their respective control cells (100%) from at least three independent experiments. (*** $P<0.001$) indicates significance between VEGFR2 siRNA (*A*) or U and LY in combination (*B*) and their respective controls.

3.4 PKA contributes to survival and ERK1/2 activation in response to serum deprivation- There is increasing evidence that the cAMP-dependent PKA pathway mediates protection induced by nerve growth factor (NGF) and can prevent drug-induced neurotoxicity through ERK1/2 activation [83, 112]. For this reason, we investigated the effects of the cAMP analogue 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP), a PKA activator, on ERK1/2 phosphorylation relative to that obtained with VEGF. Whereas 8-CPT-cAMP increased the endogenous levels of ERK phosphorylation in serum starved SK-N-SH cells (Fig. 7A, compare lanes 1 and 3), maximal induction was achieved with VEGF independent of 8-CPT-cAMP (Fig. 7A, lanes 2 and 4). The signaling of ERK1/2 phosphorylation through VEGFR2 was abolished with U0126 (U) to block the function of MEK the upstream activator of ERK1/2 (Fig. 7B, lanes 3 and 4).

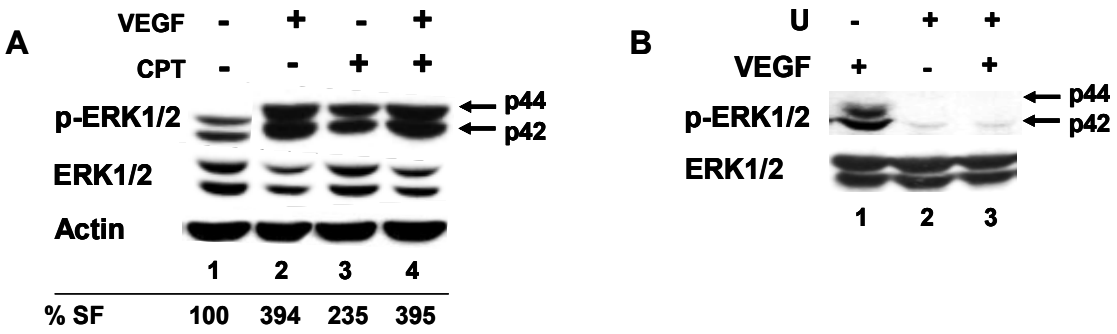


Figure 7. VEGF stimulates MEK-dependent activation of ERK1/2. *A*, Lysates were prepared from cells treated in the absence and presence of 10 ng/ml VEGF and 250 μM 8-cAMP-CPT (CPT) alone or in combination and then analyzed by Western blotting as described in "Materials and Methods" for phosphorylated and total forms of ERK1/2 (p44/p42). Actin serves as a protein loading control. *B*, Cell lysates from treatments with and without 10 ng/ml VEGF in the absence and presence of 10 μM U0126 (U) were analyzed for ERK1/2 phosphorylation as described in *A*. The numbers below each lane in *A* refer to densitometry readings normalized to serumfree (SF) treatments.

To test whether PKA mediated protection in response to serum deprivation, SK-N-SH cells were treated with the selective inhibitor of PKA, H89, and assayed for cell viability following 48 hr of incubation in the presence and absence of serum. Consistent with the results in Fig. 5A and 5B, treatments with H89 induced a significant decrease in cell viability only in serum deprived SK-N-SH cells (Fig. 8A). To insure that the inhibitory effects of H89 were specific for PKA, cell viability measurements were confirmed in comparative assays with a highly selective peptide inhibitor PKI 14-22 Amide (PKI) (Fig. 8B). Together, these findings raised the possibility that the PKA and VEGFR2 pathways converge at the MEK/ERK1/2 pathway to promote survival in response to serum deprivation.

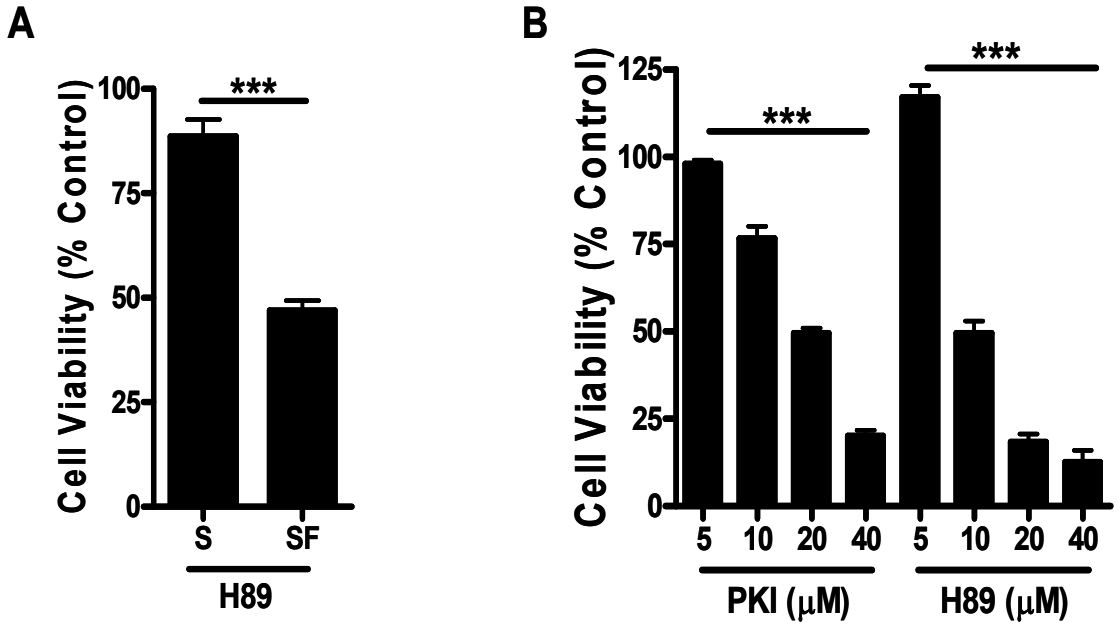


Figure 8. VEGFR2 and PKA function as independent pathways in mediating survival. *A*, Cells were incubated with and without serum for 48 hr in the absence and presence of 10 μM H89 and assayed for viability as described in "Materials and Methods." Results represent the ± S.E.M. of the percent cell viability of H89 treated cells relative to serum and serumfree controls (100%) from at least three independent experiments. (*** $P<0.001$) indicate significance between serum and serumfree cells treated with H89. *B*, The effects of increasing concentrations (5 μM to 40 μM) of the PKA inhibitors H89 and PKI 14-22 Amide (PKI) were assessed on cell viability in serum starved cells. Results represent the ± S.E.M. of the percent cell viability relative to serumfree control cells (100%) from at least three independent experiments. (*** $P<0.001$) indicate significance between cells treated with 5 μM H89 and PKI versus those treated with concentrations of 10 μM to 40 μM.

Moreover, Western blot analyses showed that PKA inhibition reduced ERK1/2 activation observed in the absence and presence of VEGF (Fig. 9A compare lanes 1 with 5 and 2 with 6) to 24% and 32% respectively, while ERK1/2 phosphorylation was abrogated by VEGFR2 inhibition alone or in combination with H89 (Fig. 9A lanes 3, 4, 7, and 8). These findings suggested that PKA contributes to ERK1/2 activation downstream from VEGFR2. Interestingly, PKA inhibition had only a minimal effect on the phosphorylation of Akt mediated through VEGFR2 (Fig. 9A lanes 5 and 6), suggesting that the PI3K/Akt prosurvival pathway is activated mainly in response to VEGF. In addition, treatments with H89 potentiated the loss in cell viability induced by VEGFR2 siRNA, suggesting that VEGFR2 and PKA serve as distinct pathways to promote survival (Fig. 9B). Taken together, these findings suggest that VEGFR2 and PKA cooperate to promote survival in serum starved SK-N-SH cells through the MEK/ERK1/2 pathway.

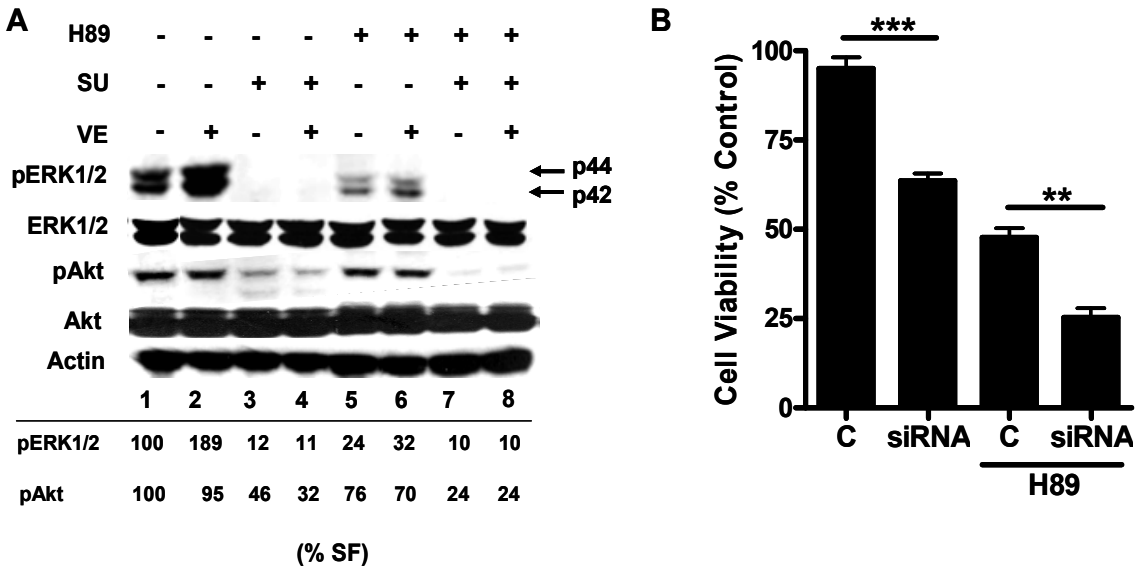


Figure 9. VEGR2 and PKA differentially activate ERK1/2 and Akt in serum deprived neuronal cells. *A*, Cells incubated with and without serum for 48 hr and treated in the presence and absence of exogenous VEGF (VE) alone or in combination with 10 μM SU1498 (SU) and H89 were analyzed by Western blotting for ERK1/2 (p44/p42) and Akt activation. Total ERK1/2, Akt and actin serve as protein loading controls. *B,* Cells transfected with 20 μM of siRNA to VEGFR2 alone and in combination with an inhibitor of PKA (H89) for 48 hr under serumfree conditions were assayed for viability as described in "Materials and Methods." 20 μM of a non-specific siRNA duplex (C) was used as a negative control. Results represent the $\pm$ S.E.M. of the percent cell viability relative to serumfree control (100%) from at least three independent experiments. (*** $P<0.001$ and ** $P<0.01$) indicates significance between VEGFR2 siRNA alone or in combination with H89 and their respective controls. The numbers below each lane in *A* refer to densitometry readings normalized to serumfree (SF) treatments for both pERK1/2 and pAkt.

3.5 The VEGFR2 and PKA signaling pathways suppress caspase activation- There is increasing evidence that VEGF and PKA protect neuronal cells from stress-induced cell death by suppressing caspase-3 activation [83, 84]. In our neuronal model, serum deprivation induced over a 2-fold increase in caspase-3/7 activation (Fig. 10A) that was reduced significantly by treatments with VEGF or 8-CPT-cAMP (Fig. 10B). Conversely, VEGFR2 or PKA inhibition induced a 2-fold increase in caspase-3/7 activation in serum

starved cells that was also observed when both inhibitors were administered simultaneously (Fig. 10C). Silencing VEGFR2 gene expression with siRNA elicited a similar increase in caspase activation (data not shown). Interestingly, treatments with the inhibitors of MEK or PI3K, U0126 and LY294002, respectively, indicated that inhibition of ERK1/2, but not Akt, induced a significant increase in caspase activation in serum starved cells (Fig. 10D). These data are consistent with the demonstration that inhibition of ERK1/2 activation by VEGF sensitizes neuronal cells toward apoptosis [62].

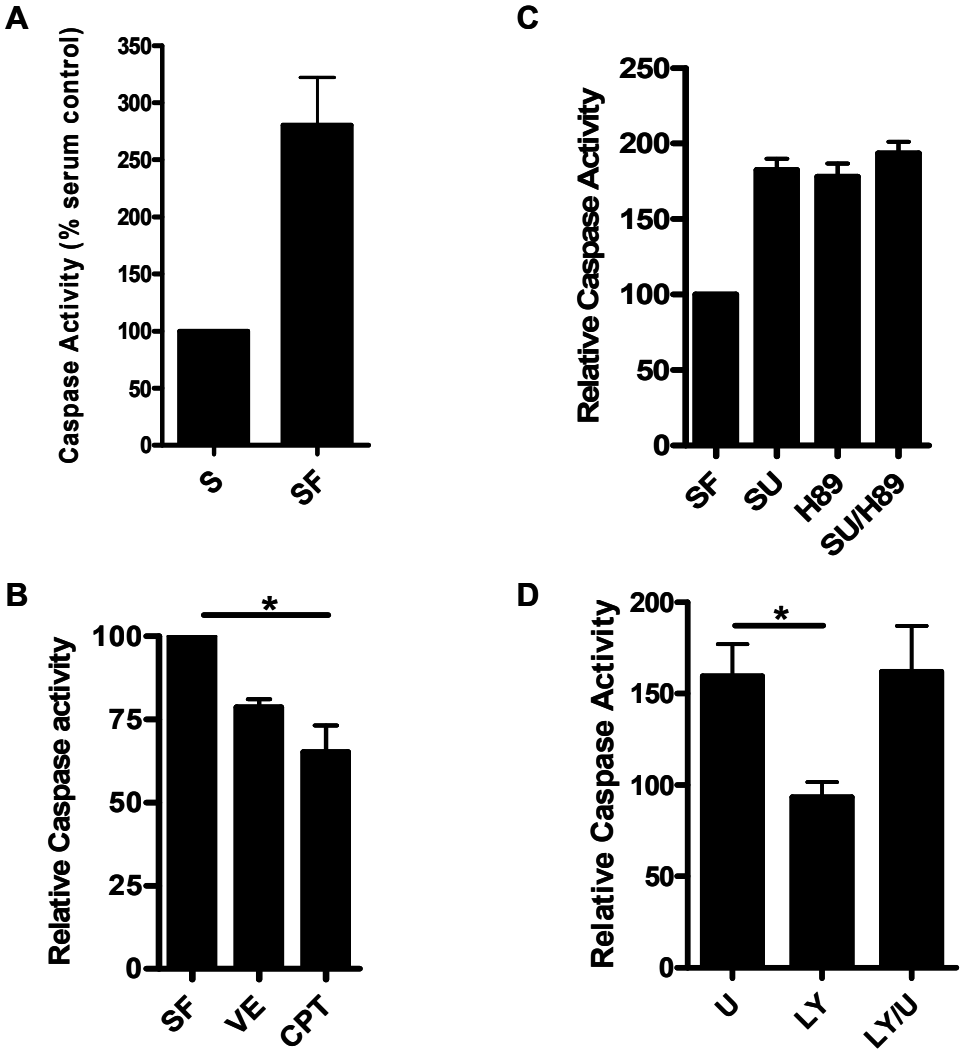


Figure 10. VEGFR2 and PKA reduce the caspase activation observed under serum deprivation. Caspase-3/7 was measured in cells cultured with (S) and without (SF) serum in *A*, with the addition of serum deprived (SF) cells treated with 10 ng/ml VEGF (VE) or 250 μM of CPT in *B*, 10 μM SU1498 (SU) or 10 μM H89 alone or in combination in C, and 10 μM U0126 (U) and 20 μM LY294002 (LY) alone or in combination in *D*, as described in "Materials and Methods." Results represent the ± S.E.M. of the caspase activity (units/μg protein) relative to serumfree control (100%) from at least three independent experiments. (* $P<0.05$) indicates significance for caspase activities between the SF and VE and CPT in *A* and between U and LY in *D*.

3.6 VEGFR2 or PKA inhibition induces a caspase-dependent cell death that is prevented by treatments with z-VAD-fmk- To determine whether caspase-3/7 mediated cell death, cells were pretreated with the pan caspase inhibitor z-VAD-fmk followed by exposure to SU1498 or H89 under serum free conditions. As shown in Fig. 11A, z-VAD-fmk induced a significant decrease in caspase activity while promoting a concomitant increase in survival for cells incubated with SU1498 or H89 alone but not in combination (Fig 11B). Hoechst staining to assess nuclear morphology by fluorescent microscopy showed that SU1498, H89 or PKAI induced the nuclear condensation and chromatin fragmentation that are typical of apoptosis (Fig. 11C; left panel, indicated by *arrows*) and these events were diminished by pretreatments with z-VAD-fmk (Fig. 11C; right panel). Whereas these data indicate that VEGFR2 or PKA inhibition induces a caspase-dependent cell death, the finding that z-VAD-fmk failed to provide protection when PKA and VEGFR2 were inhibited simultaneously suggests that these pathways regulate additional factors to maintain neuronal cell homeostasis. Together, these data suggested that perturbations of either the VEGFR2 or PKA pathway alone in serum deprived neuronal cells trigger an apoptotic cell death that is mediated through caspase activation.

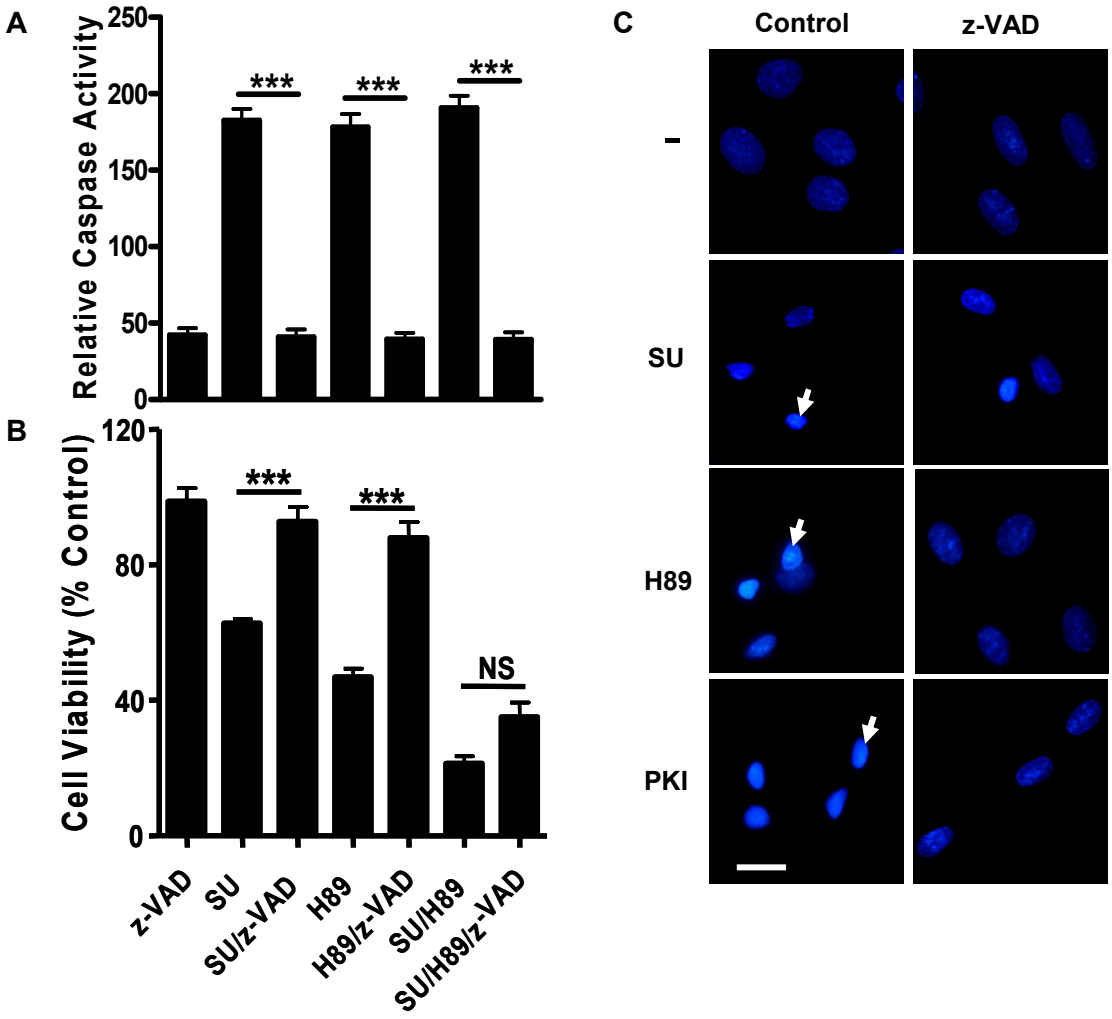


Figure 11. Caspase inhibition prevents the cell death induced by a blockade of VEGFR2 or PKA function. *A*, Caspase-3/7 activation was measured in cells pretreated with 20 μM of the caspase inhibitor z-VAD-fmk (z-VAD) followed by incubations with 10 μM SU1498 (SU) and H89 alone or in combination. *B*, Cell viability of cells treated as described in *A*. Results represent the ± S.E.M. of the percent cell viability or caspase activity (units/μg protein) relative to serumfree control (100%) from at least three independent experiments. (*** $P<0.001$) indicates significance between cells pretreated with and without z-VAD alone and in combination with SU, H89 or cotreatments of SU with H89. NS, not significant. *C*, Nuclear morphology was analyzed for chromatin condensation (*arrows*) by staining with Hoechst 33342 as described in "Materials and Methods." Scale bar is 20 μm in length.

3.7 PKA prevents against a caspase-dependent increase in oxidative stress- There is increasing evidence that caspase activation also induces oxidative stress as a result of perturbations in mitochondrial function [114]. We therefore examined whether overproduction of reactive oxygen species (ROS) was associated with the increase in caspase-3/7 activity in cells treated with SU1498 and H89. As shown in Fig. 12A, inhibition of PKA (H89) but not VEGFR2 (SU1498) induced a significant increase in ROS that was attenuated by pretreatments with z-VAD-fmk (Fig. 12B). However, pretreatments with the antioxidant N-acetylcysteine (NAC) attenuated the excessive ROS induced by PKA inhibition (data not shown) but failed to promote survival (Fig. 12C), suggesting that ROS resides downstream from caspase activation. Together, these findings suggest that PKA prevents serum deprived neuronal cells from a caspase-dependent increase in oxidative stress.

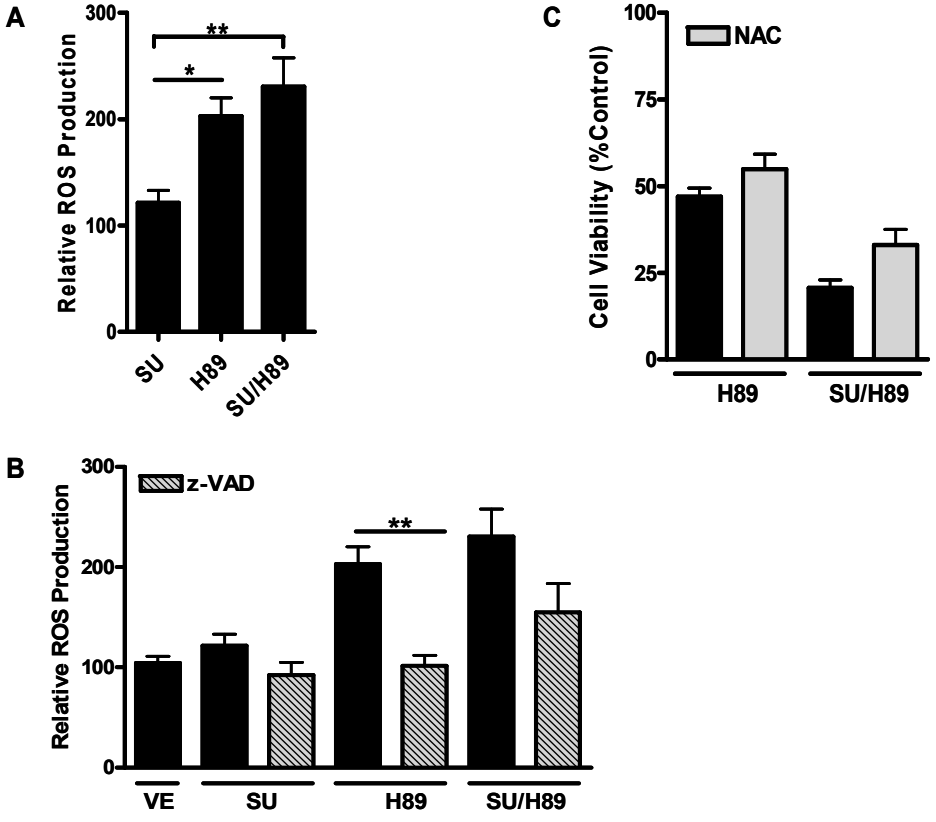


Figure 12. PKA but not VEGFR2 inhibition induces ROS overproduction. *A*, ROS was measured in cells treated with 10 μM SU1498 (SU) and H89 alone or in combination using the fluorescent dye 2', 7'-dichlorofluorescein-diacetate as described in "Materials and Methods." *B*, ROS was measured in cells pretreated with and without z-VAD-fmk (z-VAD) followed by incubations with 10 ng/ml VEGF (VE) and the inhibitor treatments in *A*. *C*, Cell viability was measured on cells treated with H89 alone or in combination with SU in the presence and absence of the antioxidant N-acetylcysteine (NAC). Results represent the $\pm$ S.E.M. of the mean fluorescence units (units/μg protein) or percent cell viability relative to serumfree control cells (100%) from at least three independent experiments. (*** $P<0.001$) indicate significance between serumfree controls and treatments. (* $P<0.05$ and ** $P<0.01$) IN A IS SU vs H89 and SU vs SU/H89 and IN B (** $P<0.01$) H89 vs z-VAD/H89

3.8 VEGFR2 inhibition induces Bcl-xL depletion- In many paradigms of cellular stress, death can result from a mitochondrial dysfunction involving decreased levels of antiapoptotic proteins. The ERK1/2 pathway has been shown to prevent the mitochondrial dysfunction induced by stressful stimuli in different cell types by modulating the expression levels of the antiapoptotic protein Bcl-xL [93]. Bcl-xL maintains mitochondrial function by preventing the membrane permeabilization that leads to cytochrome c release, caspase activation and cell death [130]. Therefore, we examined whether the differential effects of VEGFR2 or PKA inhibition on ERK1/2 phosphorylation were paralleled by similar changes in the protein levels of Bcl-xL. Consistent with the results in Fig. 6A, the loss in ERK1/2 phosphorylation induced by SU1498 or VEGFR2 siRNA was coincident with a nearly complete depletion in the protein levels of Bcl-xL independent of VEGF (Fig. 13A, lanes 3 and 4). However, the levels of Bcl-xL were only reduced by 53% in H89 treated cells while cotreatments with VEGFR2 siRNA abrogated Bcl-xL completely (Fig. 13A, lanes 5 and 6). To address whether Bcl-xL depletion played a direct role in the cell death induced by VEGFR2

inhibition, SU1498 treated cells were transfected with a construct that overexpresses Bcl-xL (Fig. 13B). Indeed, Bcl-xL overexpression decreased caspase activation (Fig. 13C) and promoted survival (Fig. 13D) indicating that the protection provided by VEGFR2 is also Bcl-xL dependent.

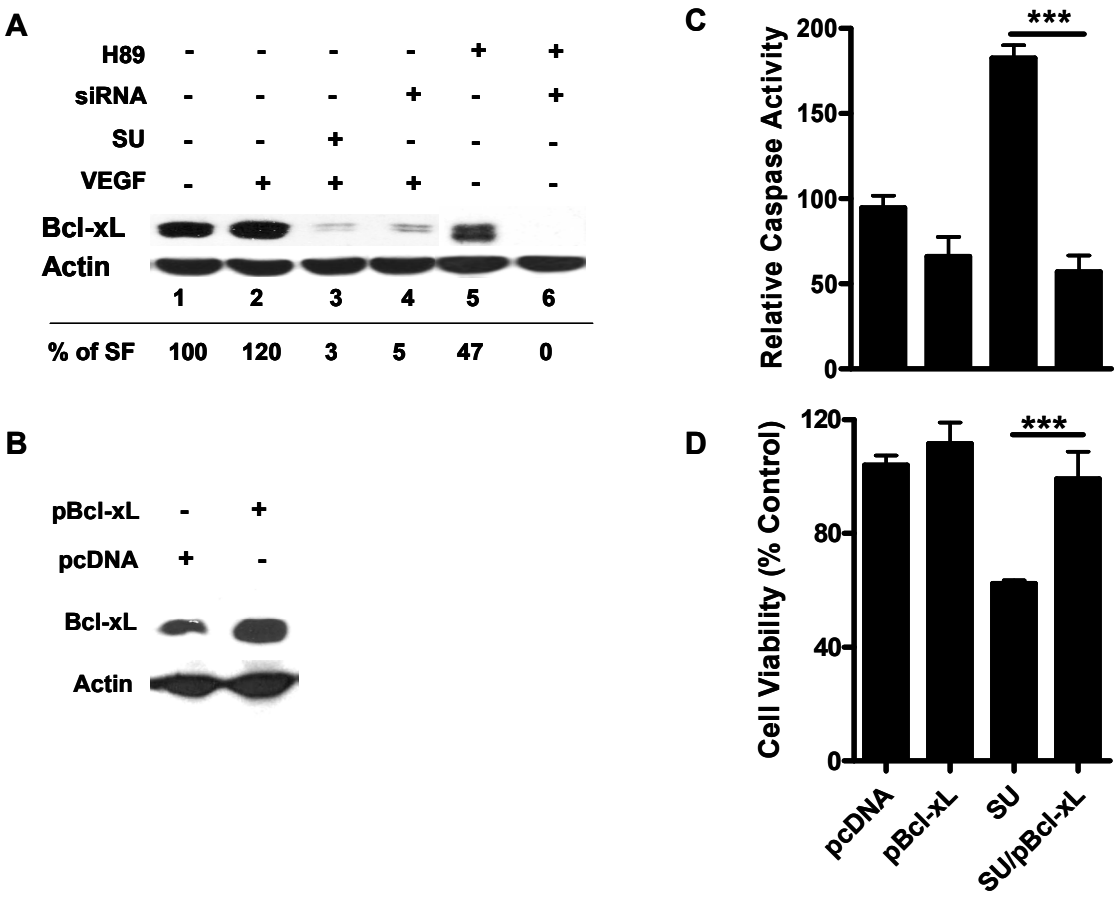


Figure 13. Overexpression of Bcl-xL prevents the cell death induced by VEGFR2 inhibition. Lysates from cells *A*, treated with and without 10 ng/ml VEGF alone or VEGF in combination with 20 μM VEGFR2 siRNA or 10 μM SU1498 (SU) or H89 alone or in combination with VEGFR2 siRNA or *B*, transfected with a construct that overexpresses Bcl-xL (pBcl-xL) or with a pcDNA empty vector that served as a control, were assayed and subjected to immunoblot analysis for detection of Bcl-xL with an actin as a loading control. *C*, Caspase activation and *D*, cell viability was also measured in cells transfected with pBcl-xL and then incubated with and without SU1498 (SU). Results represent the $\pm$ S.E.M. of the percent cell viability or caspase activity (units/μg protein) relative to serumfree controls (100%) from at least three independent experiments. (*** $P<0.001$) denote significance between SU and SU/Bcl-xL in (*C*) and (*D*).

3.9 ERK1 overexpression protects against a caspase-dependent cell death- The demonstration that MEK inhibition increased caspase activation (Fig. 10D) suggested that ERK1/2 activation played a critical role in protecting neuronal cells from the damaging effects induced by serum deprivation. We therefore examined whether ERK1 activation alone would protect against the cell death induced by VEGFR2 and PKA inhibition. Overexpression of ERK1 (Fig 14C) in SU1498 or H89 treated cells diminished the caspase-3/7 activity (Fig. 14A) and rescued cells from the loss in cell viability (Fig. 14B) induced by VEGFR2 and PKA inhibition to levels that resembled the cells pretreated with z-VAD-fmk (Fig. 11A and 11B) or those overexpressing Bcl-xL (Figs. 13C and 13D). While ERK1 overexpression also diminished caspase-3/7 activity in cells cotreated with SU1498 and H89, the kinase failed to prevent cell death.

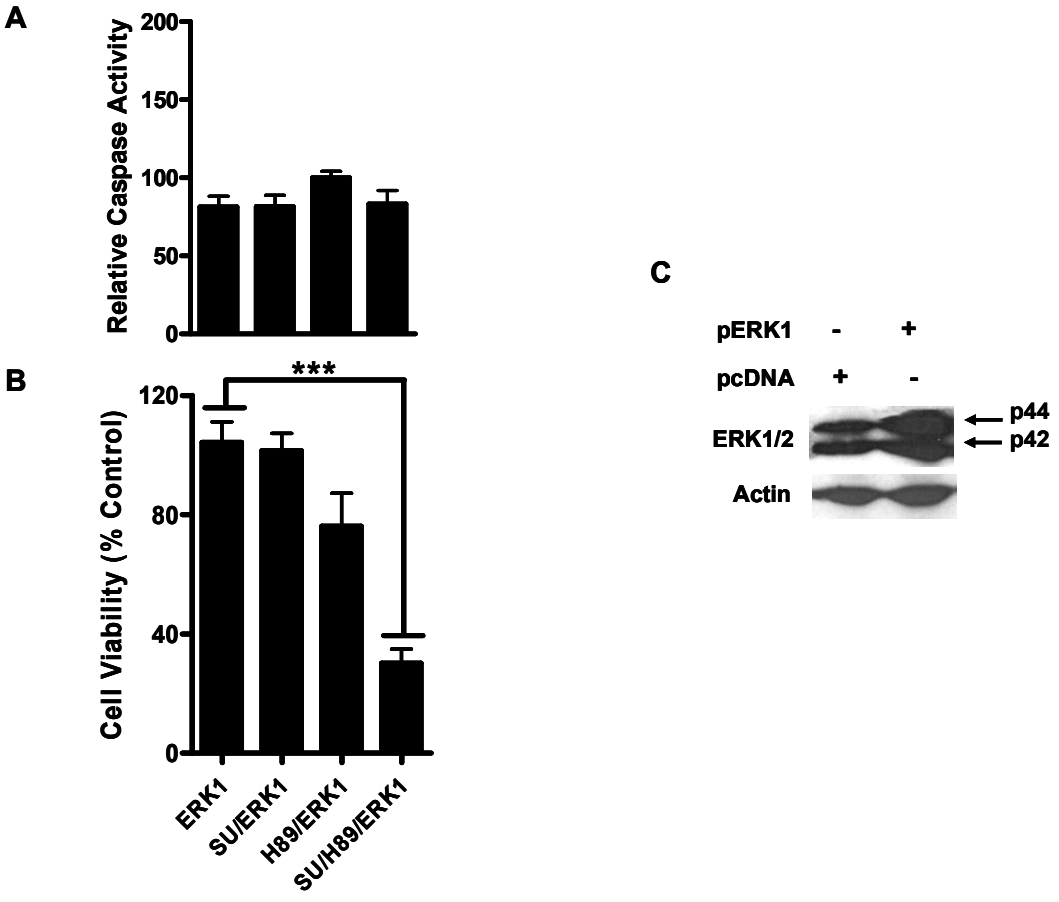


Figure 14. Overexpression of ERK1 protects against the cell death induced by both VEGFR2 and PKA inhibition. *A*, Caspase activity was measured in cells transfected with a construct that overexpresses ERK1 and incubated with 10 μM SU1498 (SU) or 10 μM H89 alone or in combination. *B*, Cell viability was also determined for treatments described in *A*. Results represent the ± S.E.M. of the percent cell viability or caspase activity (units/μg protein) relative to serumfree controls (100%) from at least three independent experiments. (** $P<0.01$) denotes significance between ERK1 and ERK1/SU/H89. *C,* Lysates from cells transfected with a construct that overexpresses ERK1 (pERK1) or with a pcDNA empty vector that served as a control, were subjected to immunoblot analysis for detection of ERK1/2 (p44/p42) with an actin as a loading control.

3.10 The protection elicited by caspase inhibition and overexpression of Bcl-xL and ERK1 is MEK-dependent- In addition, the protection elicited by ERK1 and Bcl-xL overexpression as well as z-VAD-fmk in SU1498 treated cells was abrogated with the inclusion of U0126, indicating that MEK/ERK1/2 activation by VEGFR2 is required for survival (Fig. 15). Blocking MEK function also prevented the protection induced by caspase inhibition and ERK1 overexpression in H89 treated cells (data not shown). Collectively, these findings indicate that protection due to activated VEGFR2 or PKA in serum starved neuronal cells is dependent upon activation of the MEK/ERK1/2 signaling cascade. Taken together, these results support a role for the MEK/ERK1/2 pathway as a critical mediator of neuronal cell survival by VEGF/VEGFR2 and PKA under stress conditions.

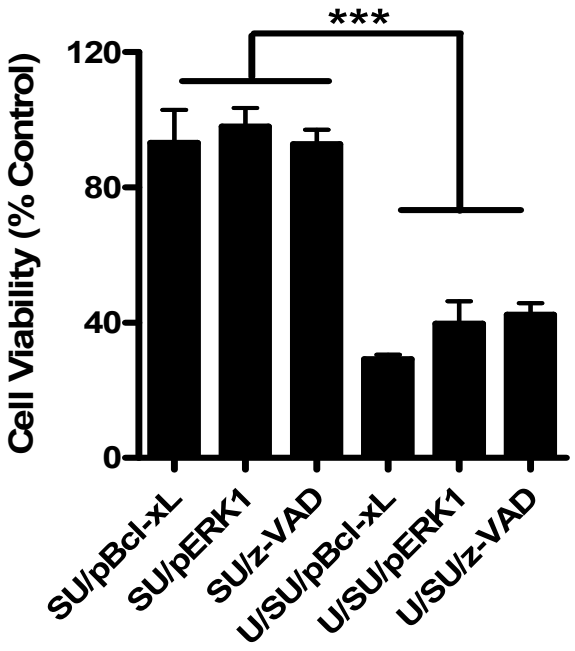


Figure 15. MEK inhibition abrogates the protection elicited by caspase inhibition and overexpression of ERK1 and Bcl-xL in SU1498 treated cells. *A*, Cell viability was measured on cells treated with 20 μM z-VAD-fmk (z-VAD) or transfected with the pBcl-xL or pERK1 construct and incubated with 10 μM SU1498 (SU) in the absence and presence of U0126 (U). Results represent the + S.E.M. of the percent cell viability relative to serumfree control (100%) from at least three independent experiments. (*** $P<0.001$) denotes significance between SU/pBcl-xL or SU/pERK1 or SU/z-VAD with and without U in combination.

Chapter IV

VEGFR2 Signaling Prevents a Caspase-dependent Cell Death Regulated in Part by p38 MAPK

4.1 VEGFR2 regulates the survival induced by p38 MAPK inhibition in serum starved neuronal cells- In neuronal cells, activated p38 MAPK is implicated as a critical contributor to the apoptosis induced by various harmful insults [142, 143, 145]. Several reports have suggested that VEGF protects against stress-induced neuronal cell death by decreasing the activation levels of caspase-3 and the p38 MAPK pathway [84**,** 85]. We and others have shown that VEGF can signal neuroprotection against a caspase-mediated cell death that is regulated by unknown mechanisms [84, 85]. In the present study, we examined whether a relationship exists between p38 MAPK and the caspase activation and cell death suppressed by VEGF in serum deprived SK-N-SH neuronal cells. As shown in Fig. 16A, treatments with a selective inhibitor of p38 MAPK, SB202190, increased viability only in serum starved cells and this effect was significantly enhanced by the inclusion of exogenous VEGF. Since these data suggested that VEGF signals survival from a p38 MAPK-mediated cell death, we examined the effects of VEGFR2 inhibition on the protection induced by p38 MAPK inhibition. Consistent with the results in Figure 16A, a blockade of VEGFR2 function with a selective inhibitor, SU1498, abrogated survival induced by p38 MAPK inhibition in the presence (Fig. 16B) and absence (data not shown) of VEGF only in serum deprived cells. Similarly, survival was diminished in SB202190-treated cells transfected with siRNA that specifically silence VEGFR2 gene expression (Fig. 16C). These findings indicated that SU1498 or VEGFR2 siRNA abolished VEGFR2 signaling by endogenous or exogenous VEGF. Unlike previous reports [11,13,16], VEGF or p38 MAPK inhibition did not reduce the p38 MAPK phosphorylation in serum deprived cells (Fig. 16D). Taken together, these data

suggest that p38 MAPK serves as a negative regulator of VEGFR2-directed survival in serum deprived neuronal cells.

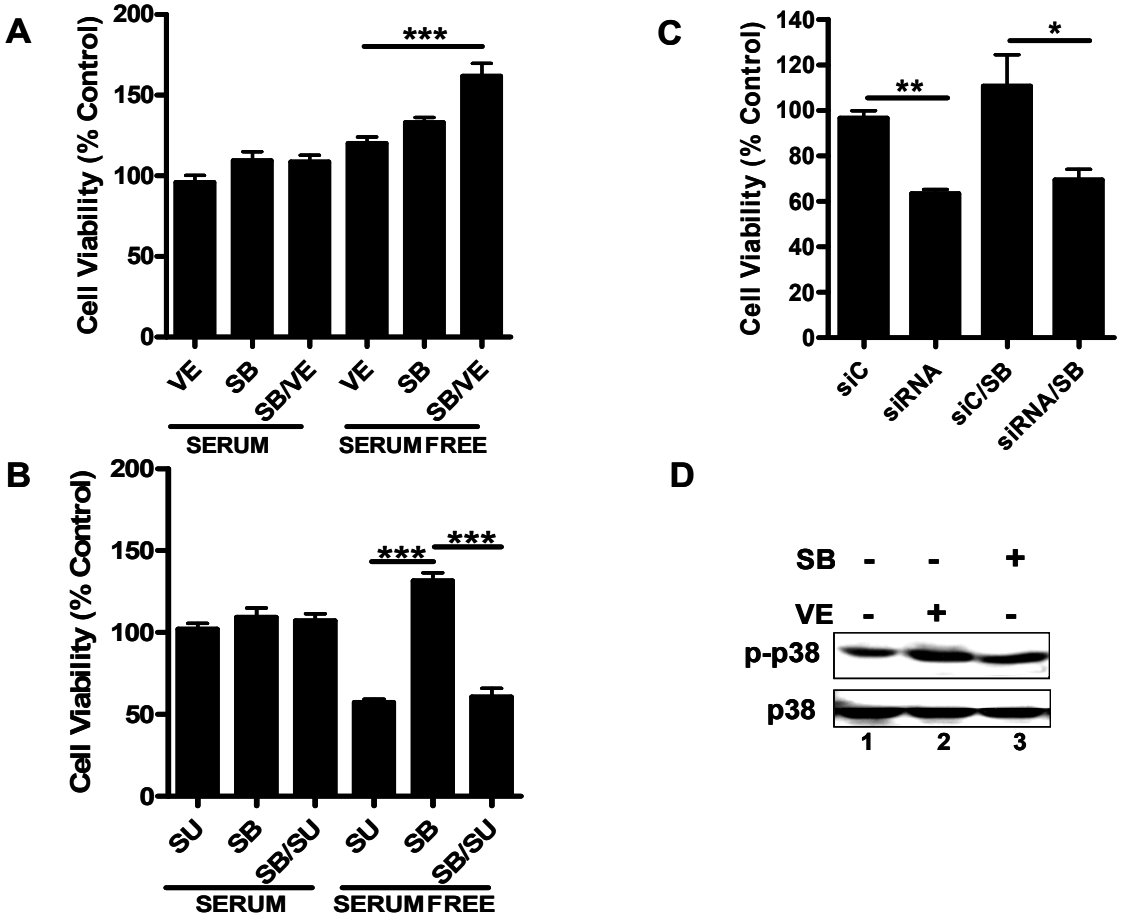


Figure 16. VEGF and p38 MAPK have opposing effects on the survival of serum deprived neuronal cells. Cell viability was measured as described in "Materials and methods" following incubation for 48 hr in the presence and absence of serum with *A*, 10 ng/ml VEGF (VE) and 10 μM SB202190 (SB) alone or in combination (SB/VE), or 10 ng/ml VEGF with *B*, 10 μM SU1498 (SU) and SB202190 (SB) alone and in combination (SB/SU) or *C*, 20 μM of nonspecific siRNA duplexes (control;siC) and 20 μM of VEGFR2 silencing duplexes (siRNA) alone and in combination with SB202190 (siRNA/SB). *D*, Total lysates from serum starved cells treated without or with 10 ng/ml VEGF (VE) or 10 μM SB202190 (SB) were analyzed by Western blotting for the phosphorylated and total forms of p38 MAPK. Results represent the $\pm$ S.E.M. of the percent cell viability relative to untreated control cells (100%), respectively, from at least three independent experiments. (*** $P<0.001$) indicates significance for cell viability between SB/VE versus VE or SB in *A*; SB versus SU or SB/SU in *B*. (** $P<0.01$) indicates significance between siC versus siRNA and (* $P<0.05$) indicates significance between siC/SB versus siRNA/SB in *C*.

4.2 VEGFR2 and p38 MAPK exert opposing effects on activation of ERK1/2 in serum starved neuronal cells- Increasing evidence suggests that VEGF signals neuroprotection through the activation of downstream kinase intermediates, the PI3K/Akt and MEK/ERK1/2 pathways [18]. Thus, in order to gain a better understanding of the interplay between p38 MAPK and VEGF signaling we evaluated the effects of p38 MAPK on Akt and ERK1/2 activation in serum starved neuronal cells. Consequently, we evaluated the phosphorylation levels of Akt and ERK1/2 in cells treated with SB202190 alone and in combination with SU1498. Western blot analyses revealed that treatments with SB202190 alone increased the phosphorylation of Akt and ERK1/2 to levels that were, respectively, 2 and 50 fold greater than serumfree controls (Fig. 17A, compare lanes 1 and 3) and 2-fold greater than VEGF treated cells (compare lanes 2 and 4) with no changes in the levels of total protein expression occurred. Unlike Akt, ERK1/2 phosphorylation levels in untreated cells were increased 2.5-fold by exogenous VEGF (Fig. 17A, lanes 1 and 2). Treatments with exogenous VEGF did not change the phosphorylation of Akt and ERK in SB202190 treated cells, suggesting that endogenous VEGF induced maximal levels of activation in response to p38 MAPK inhibition. However, Akt and ERK1/2 activation were abrogated by VEGFR2 inhibition in VEGF treated cells irrespective of p38 MAPK inhibition (Fig. 17A, lanes 5 and 6). Consistent with these data, ERK1/2 phosphorylation was attenuated in SB202190 treated cells transfected with VEGFR2 siRNA (Fig. 17B, lane 4). Collectively, these data indicate that VEGF/VEGFR2 signals protection against a caspase-dependent cell death that precludes induction by activated p38 MAPK in serum starved neuronal cells.

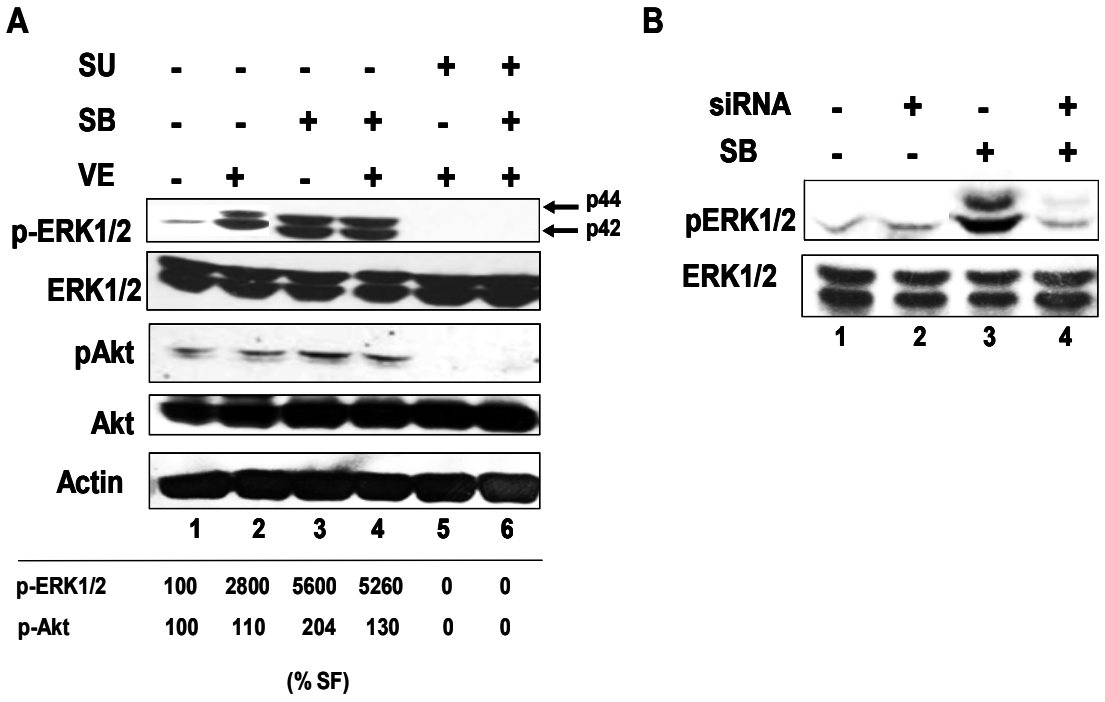


Figure 17. p38 MAPK inhibition augments VEGF-mediated activation of ERK1/2 and Akt in serum deprived SK-N-SH neuronal cells. *A,* Western Blot analyses were performed with total lysates prepared from cells treated without and with 10 ng/ml VEGF (VE) alone and with 10 μM SB202190 (SB), 10 μM SU1498 (SU) and both inhibitors in combination, or *B*, without and with VEGFR2 siRNA and 10 μM SB202190 (SB) alone and in combination. Blots were probed for detection of the phosphorylated and total forms of Akt (*A*), ERK1/2 (p44/p42) (*A* and *B*) with actin in *A* or total ERK1/2 in *B* as the protein loading controls as described in "Materials and Methods."

4.3 VEGFR2 suppresses caspase activation by p38 MAPK-dependent and -independent pathways- Evidence suggests that VEGF and p38 MAPK exert opposing effects on neuronal cell survival by modulating the activity of caspase-3 (69, 84). Therefore we examined whether the increase in viability by SB202190 or VEGF in serum starved cells (Fig. 16A) was paralleled by changes in caspase-3/7 activation. Indeed,

treatments with SB202190 or VEGF induced a decrease in caspase activity that was augmented significantly when both treatments were administered in combination (Fig. 18A). Conversely, the cell death that accompanied VEGFR2 inhibition (Fig. 16B) correlated with a significant increase in caspase-3/7 activity independent of p38 inhibition (Fig. 18B) and the inclusion of VEGF (data not shown). SB202190 treated cells transfected with VEGFR2 siRNA generated similar results (data not shown). As a further assessment of the effects of p38 MAPK inhibition on caspase activation, we evaluated the processing of the proform (35 kDa) of casapse-3 into 17 and 19 kDa fragments in cells incubated with SU1498 (Fig. 18B) or VEGFR2 siRNA (data not shown) in the absence and presence of SB202190. Interestingly, VEGFR2 inhibition induced a cleavage of caspase-3 that was partly reduced by blocking p38 MAPK function (Fig. 18B, compare lanes 2 and 4). The observation that VEGFR2 inhibition induced caspase activation and cell death when p38 MAPK was inhibited, suggests that signaling through VEGFR2 prevents apoptosis independent of p38 MAPK function. Although cell death proceeded under these conditions, pretreatments with the pan caspase inhibitor z-VAD-fmk restored viability in SU1498 treated cells to levels that resembled the untreated (100%) control irrespective of p38 MAPK inhibition (Fig. 19A). Interestingly, z-VAD-fmk did not restore cell viability in SU1498 treated cells to the enhanced levels observed when p38 MAPK activity is blocked alone. Consistent with these findings, Hoechst staining in SU1498 treated cells incubated with and without SB202190 showed apoptotic nuclei containing chromatin condensation while cells treated with SB202190 resembled that of control cells (Fig. 19B; top panel, indicated by *arrows*). However, pretreatments with z-VAD-fmk (Fig. 19B; bottom panel) attenuated the nuclear condensation,

suggesting that caspase-3/7 mediated neuronal cell death. Collectively, these data suggest that the activation of VEGFR2 by VEGF in serum starved neuronal cells suppresses caspase-mediated cell death mechanisms induced by p38 MAPK-dependent and -independent pathways.

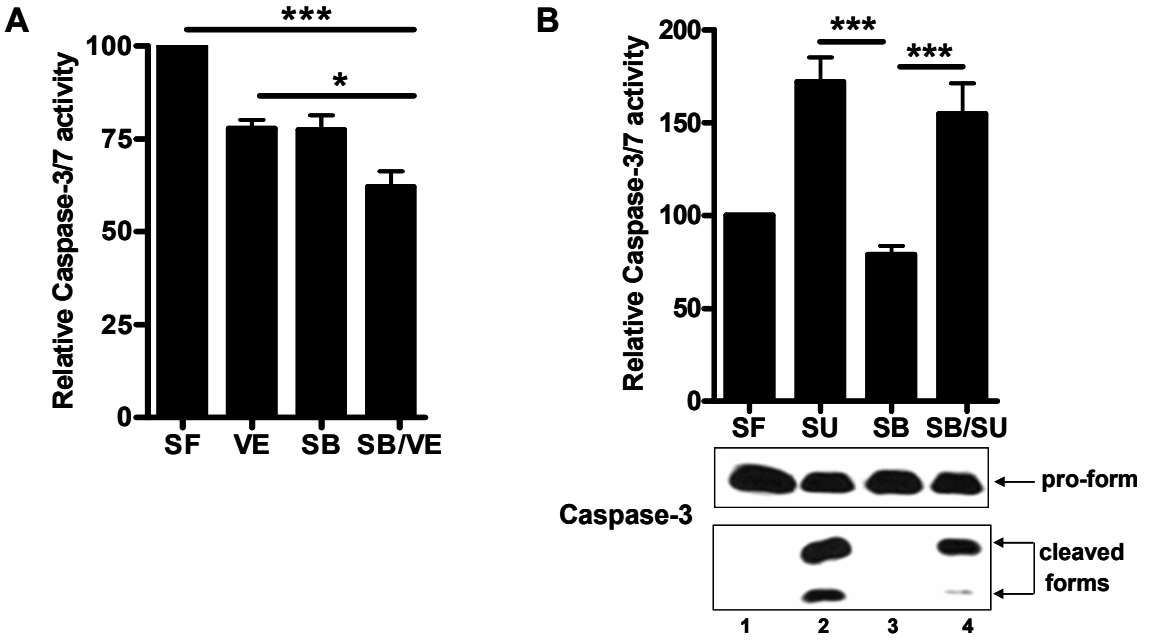


Figure 18. VEGF and p38 MAPK inhibition regulate caspase activation in serum starved neuronal cells. *A*, Serum starved cells were treated without (SF) and with 10 μM SB202190 (SB) and 10 ng/ml VEGF (VE) alone and in combination (SB/VE) or *B*, 10 μM SU1498 (SU) and SB202190 (SB) alone and in combination (SB/SU) and then assayed for caspase-3/7 activity as described in "Materials and Methods." Total lysates from serum starved cells treated under the same conditions were analyzed by Western blotting for caspase-3 cleavage. Results represent the $\pm$ S.E.M. of the caspase activity (units/μg protein) relative to untreated control cells (100%) from at least three independent experiments. (*** $P<0.001$) indicates significance for caspase activities between SF versus VE, SB, or SB/VE; (* $P<0.05$) indicates significance between SB/VE versus VE or SB in *A*. (*** $P<0.001$) indicates significance between SB versus SU or SB/SU in *B*.

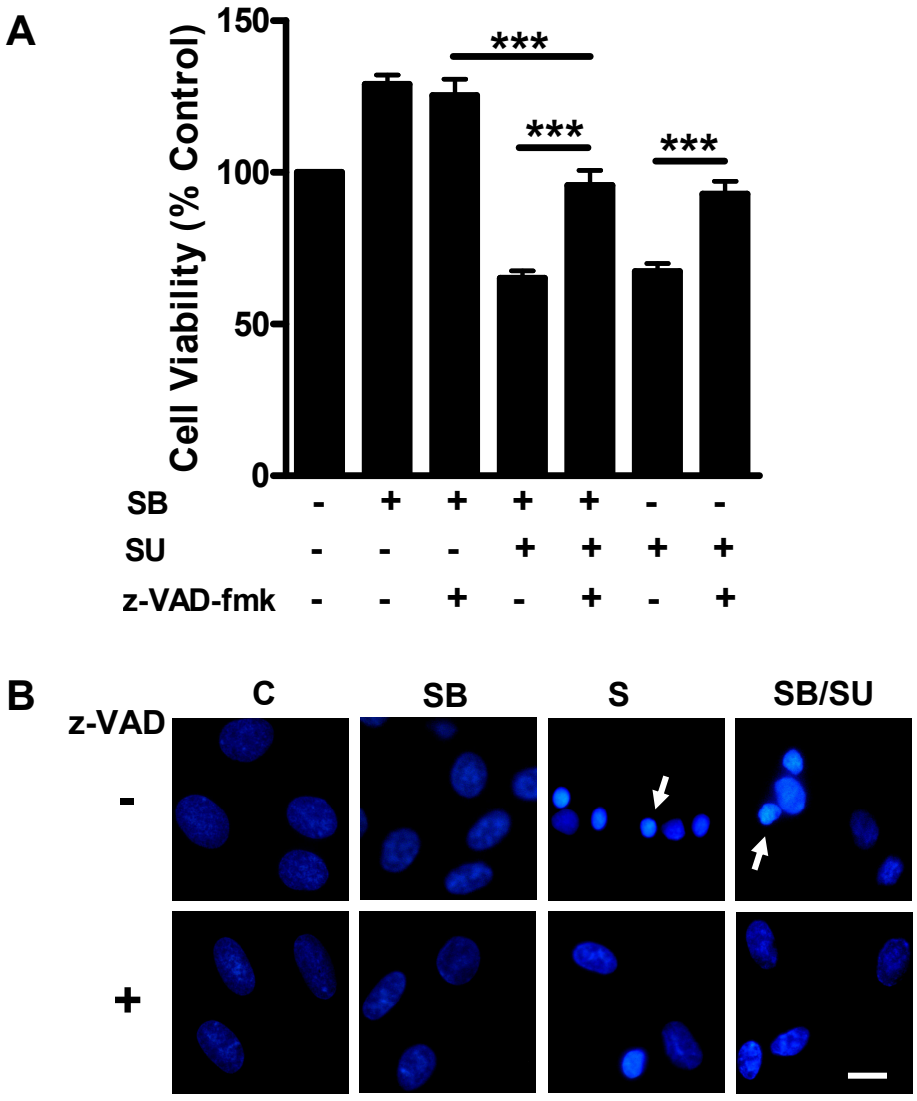


Figure 19. VEGFR2 inhibition induces a caspase-dependent cell death. Serum starved cells were pretreated without and with 20 μM of the caspase inhibitor z-VAD-fmk (z-VAD) and then incubated with 10 μM SB202190 (SB) and 10 μM SU1498 (SU) alone and in combination (SB/SU). *A*, Cells were then analyzed by a MTS assay for cell viability or *B*, by Hoechst 33342 staining for nuclear chromatin condensation (*arrows*) as described in "Materials and Methods." Scale bar is 20 μm. Results represent the ± S.E.M. of the percent cell viability relative to untreated control cells (100%) from at least three independent experiments. (*** $P<0.001$) indicates significance for cell viability between z-VAD/SB versus SB/SU or z-VAD/SB/SU, SB/SU versus z-VAD/SB/SU and SU versus z-VAD/SU in *A*.

Chapter V

VEGFR2 Promotes Neurite Extension Mediated by p38 MAPK in Serum Deprived Neuronal Cells

5.1 An onset of neuronal differentiation is observed under serum deprivation that is enhanced by VEGF- Since reports have shown that VEGF promotes neurite outgrowth [117-119], our studies sought to determine whether these events are also coincident in neuronal cells under serum deprived conditions. Results show that exposure of SK-N-SH neuroblastoma cells to serum deprivation induces an onset of neuronal differentiation characterized by neurite extensions (Fig. 20, compare panel A with C). Interestingly, treatment with exogenous VEGF promoted an increase in neurite extension outgrowth (Fig. 20, compare C and D) while a blockade of VEGFR2 function with a selective inhibitor of VEGFR2 activation SU1498 (SU) caused the disassembly of neurite extension even in the presence of exogenous VEGF (Fig. 20E and F) under serum deprivation. These observations are coincident with the upregulation of the VEGF2 under serumfree conditions and with the opposing effect on cell survival elicited by VEGFR2 inhibition. These results suggest that VEGF signaling through VEGFR2 may play an important role in neuronal differentiation under serum starved conditions

5.2 VEGF-mediated neurite extension is regulated by p38 MAPK- Members of the mitogen-activated protein kinase (MAPK) family have been shown to play central roles in neuronal cell death and survival but also in differentiation [124]. Reports have demonstrated that p38 MAPK plays a role in VEGF-induced actin reorganization, stress fiber formation and cell migration in human endothelial cells [124, 125]. Our results show that in serum deprived cells, incubation with the selective inhibitor of p38 MAPK SB202190 (SB) caused an unusual cell morphology characterized by cell aggregates that lacked neurite extensions (Fig. 20G) suggesting that p38 MAPK plays a distinct role in

neuronal differentiation. This change in morphology was not rescued by addition of exogenous VEGF (Fig. 20H). These results suggest that p38 MAPK may be involved in the regulation of neurite extensions.

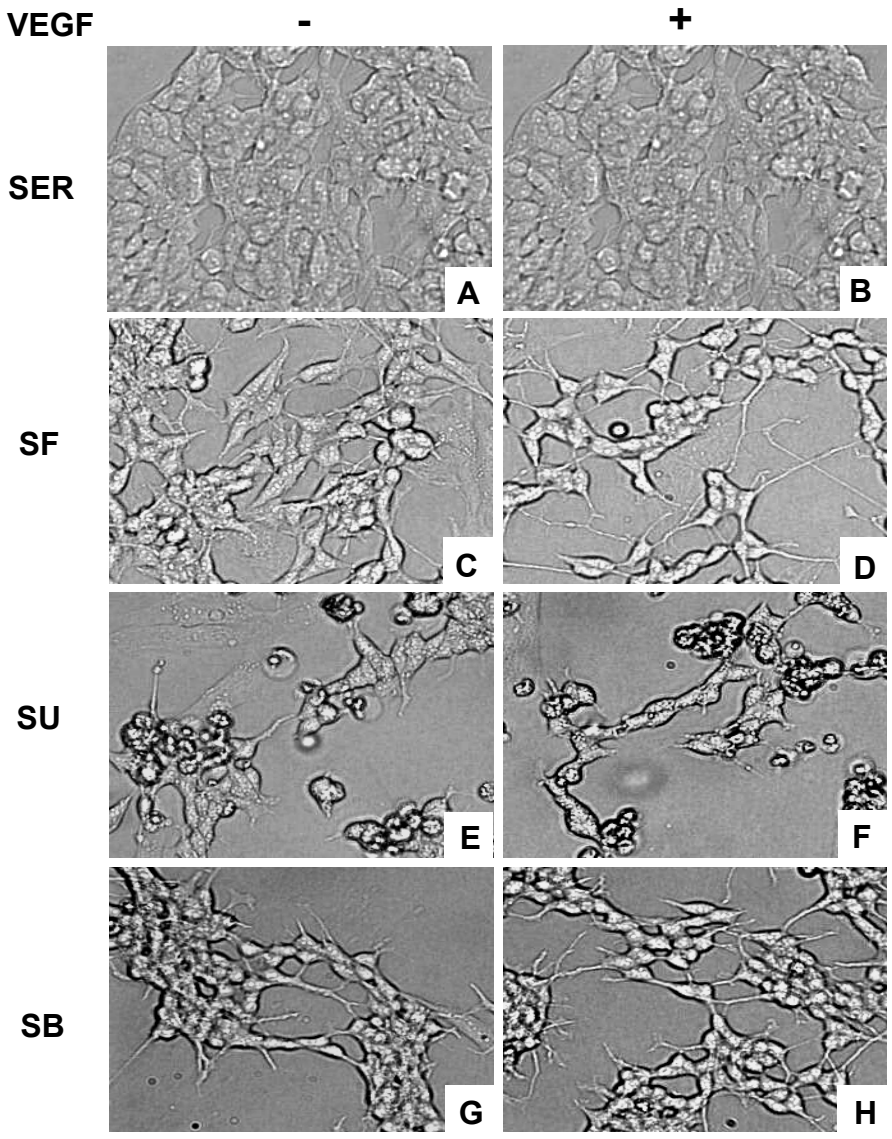


Figure 20. Neurite extension mediated by VEGF activation of VEGFR2 is abrogated by inhibition of p38 MAPK. SK-N-SH cells were cultured for 48 hr with (SER) and without serum (SF) in the absence and presence of 10 ng/ml of exogenous VEGF with the addition of serum deprived cells also treated with 10 μM of the selective inhibitors of VEGFR2 (SU1498; SU), in *E* and *F* or p38 MAPK (SB202190; SB), in *G* and *H*. Brightfield images were captured as described in "Materials and Methods."

5.3 PKA promotes neurite extension independent of p38 MAPK- Since cAMP/PKA signaling has been associated with neuronal differentiation [121, 123], we investigated the effects of PKA activation by the cAMP analogue 8-CPT-cAMP (CPT) on neurite extension in serum deprived neuronal cells. Stimulation of PKA with CPT alone promoted neurite extension (Fig. 21B). Interestingly, after 7 days of serum deprivation CPT not only rescued the neurite extension abolished by inhibition of p38 MAPK but promoted extensive neurite outgrowth only when p38 MAPK function was abolished (Fig. 21C). Our results show that PKA can promote neuronal differentiation independent of p38 MAPK function and that after prolonged nutrient starvation a loss in p38 MAPK exacerbates the PKA mediated neurite outgrowth. These results suggest that p38 MAPK negatively regulates PKA mediated neurite extension.

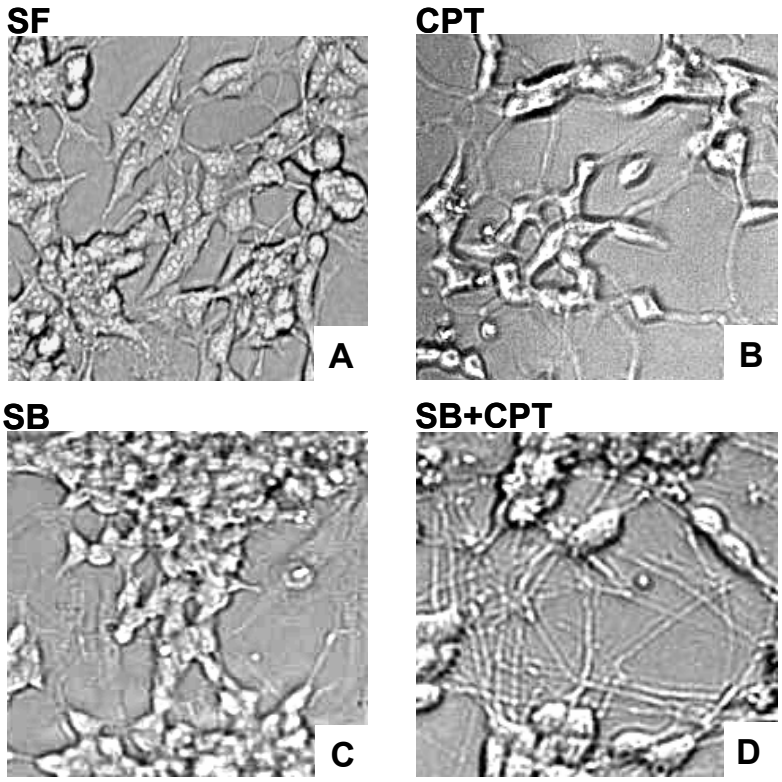


Fig. 21. PKA opposes the p38 MAPK driven inhibition of neurite extension. Cells cultured under serum deprived conditions for 7 days were treated in the absence and presence of 250 μM of 8-CPT-cAMP (CPT), *A* and *B*, alone or in combination with SB or 10 μM SU1498 (SU) in *C* and *D*. Brightfield (left column) images were captured as described in "Materials and Methods."

Chapter VI

Discussion

In these studies we show that prolonged serum deprivation in neuronal cells upregulates the gene expression of VEGF and its cognate receptors VEGFR1, VEGFR2 and NRP1 and downregulates NRP2. While several of these receptors may function under these conditions, our findings are consistent with previous reports showing that upregulated VEGF and VEGFR2 serve concomitantly as a prosuvival pathway that signals protection through the activation of the downstream targets PI3K/Akt and MEK/ERK1/2 [18, 69]. The demonstrations that exogenous VEGF increased the phosphorylation levels of VEGFR2 (Fig. 4C) and that inhibition of VEGF or VEGFR2 leads to cell death (Fig. 5B) is in agreement with the concept that VEGF can function as an autocrine and paracrine factor to prevent neuronal cell death under stress conditions.

One important aspect of these studies is the demonstration that the cAMP-inducible PKA pathway cooperates with VEGF-directed signaling through VEGFR2 to stimulate activation of the MEK/ERK1/2 pathway to promote survival. This finding is in contrast to a previous report showing that PKA inhibits VEGF-directed proliferation of endothelial cells by blocking Raf-1, an upstream activator of ERK1/2 [131]. Nevertheless, our results are consistent with growing evidence that PKA stimulates ERK1/2 signaling to mediate growth factor-induced proliferation and differentiation in neuronal cells [107, 110]. Our observation that VEGF and the cAMP analogue, 8-cAMP-CPT, induce an activation of ERK1/2 (Fig. 7A) that is concomitant with a decrease in caspase-3/7 activation (Fig. 10B) implicates ERK1/2 as a critical mediator of VEGFR2 or PKA-mediated protection against caspase activation and cell death induced by serum deprivation. These findings are consistent with reports showing that PKA and VEGF protect neuronal cells from cell death by suppressing caspase-3 activation [83, 84]. While

our studies support a role for the PI3K/Akt pathway in promoting neuronal cell survival, the observation that inhibition of MEK but not PI3K induced caspase activation (Fig 10D) and that caspase inhibition (Fig. 11B) and ERK1 overexpression (Fig. 14B) prevented the cell death induced by VEGFR2 and PKA inhibition suggests that the MEK/ERK1/2 pathway is both necessary and sufficient for the antiapoptotic activity of VEGF or PKA. This notion is further supported by the demonstration that MEK inhibition suppressed the survival induced by ERK1 overexpression and caspase inhibition when signaling through PKA or VEGFR2 is blocked (Fig. 15). In agreement with these findings, brain-derived neurotrophic factor (BDNF) was shown to protect neurons from the damaging effects of hypoxia through ERK1/2 but not PI3K [132]. Similarly, blocking VEGF-mediated signaling of MEK but not PI3K in cortical neurons sensitized neuronal cells toward an apoptotic cell death [62], suggesting that ERK1/2 plays a more direct role in preventing the activation of cell death pathways. The concept that PKA or VEGFR2 pathways cooperate to provide protection through the MEK/ERK1/2 cascade is consistent with studies showing that EGF, insulin-like growth factor I (IGF-I) and forskolin-induced PKA promote the survival of prostate cancer cells through the stimulation of signaling pathways that converge at the level of ERK1/2 [133]. Collectively, these data implicate ERK1/2 as a downstream effector of VEGFR2 and PKA-mediated protection against a caspase-dependent cell death in serum deprived neuronal cells.

Although VEGFR2 and PKA share ERK1/2 as a downstream target, several observations indicate that both pathways cooperate in a differential manner to promote survival in our neuronal paradigm of serum deprivation. First, a quantification of ERK1/2

activation (Fig. 7A) showed that VEGF induced maximal levels of stimulation that were nearly 2-fold greater than that induced by 8-cAMP-CPT. In accordance with these findings, gene silencing or direct inhibition of VEGFR2 resulted in a coordinated loss in ERK1/2 activation (Fig. 6A) and Bcl-xL protein levels (Fig 13A) while PKA inhibition (Fig. 9A) reduced these events by 62% and 53%, respectively. In addition, treatments with SU1498 but not H89 prevented Akt phosphorylation, indicating that VEGFR2 alone regulates Akt and PKA only contributes to VEGF-mediated activation of ERK1/2. The demonstration that cell death and caspase activation is suppressed by Bcl-xL overexpression in SU1498 treated cells also implicates a role for VEGFR2 in protecting mitochondria function. In contrast, Bcl-xL overexpression had no effect on the cell death induced by H89 (data not shown). Furthermore, the observation that inhibition of PKA (H89) but not VEGFR2 (SU1498) induced a significant increase in ROS (Fig. 12A), that paralleled the increase in caspase-3/7 activity (Fig. 10C), and both events were attenuated by pretreatments with z-VAD-fmk (Figs. 11B and 12B), suggested that PKA protects from a caspase-dependent increase in oxidative stress in serum deprived neuronal cells. Finally, the observation that a simultaneous inhibition of VEGFR2 and PKA increased cell death (Fig. 8B) and failed to be rescued by caspase inhibition (Fig. 11B) or ERK1 overexpression (Fig. 14B) indicates that both pathways are distinct in function and signal survival through additional as yet to be identified mechanisms.

The observation that maximal levels of phosphorylated ERK1/2 are induced by VEGF (Fig. 7A) and inhibition of VEGFR2 but not PKA abrogates this event (Fig. 9A, lanes 3, 4, 5, 6) suggests that signaling through VEGFR2 resides upstream from PKA in activating the MEK/ERK1/2 pathway. However, the fact that VEGF fails to restore ERK

phosphorylation to peak levels and promote survival in H89 treated cells (Figs. 9A, lane 6; data not shown) also suggests that VEGFR2 and PKA may depend upon common regulatory points to fully activate the MEK/ERK1/2 cascade. One possible regulatory mechanism is through the small GTPase binding proteins, such as Ras, Rac and Rap1 which together with the Raf kinases, A-Raf, B-Raf, and Raf-1 are key elements in regulating the MEK/ERK1/2 pathway by receptor tyrosine kinases and cAMP analogues [134]. In this context, VEGF-mediated activation of VEGFR2 is known to signal ERK1/2 phosphorylation through the sequential activations of Ras/Raf-1 [34] while Rap-1-mediated activation of B-Raf links PKA activation by cAMP analogues to MEK1 stimulation in neuronal cells [110]. However, PKA activation has also been shown to require Ras or PKC to stimulate ERK1/2 activation [104, 135, 136]. While further experimentation is needed, it is tempting to speculate that the Raf-1 and B-Raf isoforms mediating VEGFR2 and PKA signaling, respectively, require Ras as a common activator to fully stimulate the MEK/ERK1/2 pathway in response to serum deprivation.

Although the exact roles for enhanced ERK1/2 activation under pathological conditions are currently unclear, emerging evidence suggests that activated ERK1/2 can protect against mitochondrial dysfunction in neuronal cells through transcriptional-dependent and -independent mechanisms [130, 138]. The integrity of the mitochondria is tightly regulated by the Bcl-2 family of proteins where a balance in the ratio of antiapoptotic (e.g., Bcl-2 and Bcl-xL) and proapoptotic (e.g., Bax and Bak) members protects against outer membrane permeabilization that leads to cytochrome c release and caspase activation [93]. In our studies, the observation that Bcl-xL depletion is coincident with a loss in ERK1/2 activation when VEGFR2 signaling is blocked raises the

possibility that VEGF modulates Bcl-xL protein expression through the MEK/ERK1/2 pathway. In support of this concept, activated ERK1/2 was shown to regulate the expression levels of Bcl-xL to prevent apoptosis in various cancer cell lines and in particular through ERK1/2-mediated transcriptional activation of cAMP-responsive element binding protein (CREB) [138-140]. In addition, the observation that Bcl-xL or ERK1 overexpression requires MEK to rescue SU1498 treated cells from a caspase-dependent cell death also suggests that activated ERK1/2 may target cytoplasmic proteins that would prevent mitochondrial dysfunction. In accordance with these findings, phosphorylation of ERK1/2 by VEGF in serum-deprived nonneuronal cells was shown to suppress apoptosis by modulating the Bax/Bcl-2 ratio and by preventing cytochrome c release and caspase-3 activation [42].

Several lines of evidence also link activation of the stress kinase p38 MAPK in promoting an apoptotic cell death since treatments with inhibitors of p38 MAPK attenuate caspase activation and promote survival [85**,** 142, 143]. In addition, VEGF appears to confer neuroprotection by inhibiting both caspase-3 as well as p38 MAPK activation [84**,** 85]. In this report, we establish a functional link between VEGF-mediated survival and the regulation of caspase activation by p38 MAPK in serum deprived neuronal cells. We base this conclusion on the demonstration that an inhibitor of p38 MAPK, SB202190, decreased caspase activity (Fig. 18A) and enhanced the cell viability (Fig. 16A) and activation levels of ERK1/2 and Akt mediated by VEGF (Fig. 17A). The demonstration that the pharmacological inhibitor SU1498 or VEGFR2 siRNA (Figs. 1, 2, 3 and data not shown) abolished SB202190-mediated effects on caspase activation, viability and ERK1/2 and Akt phosphorylation also suggests that the survival induced by

p38 MAPK inhibition is signaled through the VEGF/VEGFR2 pathway. The fact that p38 MAPK inhibition reduces caspase-3 cleavage in SU1498 treated cells (Fig. 18B) further suggests that p38 MAPK and VEGFR2 signal opposing effects on caspase activation in response to serum deprivation. Accordingly, these results implicate p38 MAPK as a negative regulator of VEGF-mediated neuroprotection and are consistent with studies showing that activated p38 MAPK attenuates survival signaled by VEGF and FGF in endothelial cells [85, 146]. However, the observation that VEGFR2 inhibition induces caspase-3/7 activation and cell death despite a SB202190-mediated reduction in caspase-3 cleavage and chromatin condensation (Figs. 18B and 19B) suggest that a loss in VEGFR2 signaling triggers induction of different apoptotic pathways that preclude regulation by activated p38 MAPK. However, we attribute the difference in caspase-3/7 activity versus caspase-3 cleavage due to the fact that the caspase-3/7 assay is a more highly sensitive measurement of apoptosis than western blotting. These findings, together with the demonstration that caspase inhibition maintains viability under these conditions, raise the possibility that VEGF mediates a global neuroprotection through VEGFR2 against caspase-dependent cell death pathways when neuronal cells are exposed to apoptotic stimuli such as serum deprivation. Consequently, caspase-3 activation may result from a dual induction by caspase-8 (non-mitochondrial) and mitochondrial-mediated mechanisms in which p38 MAPK would participate by regulating either the translocation of the proapoptotic protein Bax to the mitochondria, death receptor activation or caspase-8 activation directly [79, 80, 144].

Increasing evidence from *in vivo* and *in vitro* studies suggests that PI3K/Akt and MEK/ERK1/2 are critical downstream effectors of VEGF-mediated

survival in response to harmful insults [13, 18, 69]. The demonstration that the survival induced by p38 MAPK inhibition coincides with an enhanced phosphorylation of ERK1/2 and Akt that is signaled through VEGFR2 (Fig. 17A) provides further evidence that crosstalk exists between VEGFR2 and p38 MAPK in signaling the antiapoptotic properties of VEGF in serum deprived neuronal cells. In this context, a loss in p38 MAPK function would allow VEGF to signal VEGFR2 to promote neuronal cell survival through high activation levels of ERK1/2 and Akt. Interestingly, caspase inhibition promotes survival when VEGFR2 signaling is blocked but not to the enhanced levels observed with p38 MAPK inhibition alone (Fig 19A). It is tempting to speculate that this observation reflects a need for VEGFR2-directed activation of ERK1/2 or Akt to relay the additional increase in viability.

The 50-fold increase in ERK1/2 activation by p38 MAPK inhibition (Fig. 17A) suggests that ERK1/2 alone is highly sensitive to perturbations by p38 MAPK activity. In accordance with these findings, a blockade of p38 MAPK function was shown to enhance both VEGF-dependent survival and ERK1/2 phosphorylation in endothelial cells [86]. The fact that VEGFR2 inhibition induces a loss in ERK1/2 phosphorylation (Fig. 17A) that correlates with increased caspase activity (Fig. 18B) and cell death (Fig. 16B) is consistent with reports showing that a downregulation in ERK1/2 activation accompanies p38 MAPK-mediated apoptosis in both neuronal and nonneuronal cells [147, 148, 149]. Our results provide new insight on the protection signaled by the VEGF/VEGFR2 pathway when neuronal cells are exposed to apoptotic stimuli. These findings implicate VEGFR2-directed activation of its downstream targets Akt and ERK1/2 in protecting serum starved neuronal cells from a caspase-mediated cell death

induced by p38 MAPK-dependent and -independent mechanisms. Our findings suggest that VEGFR2 protects against cell death that is dually regulated by p38 MAPK and a yet to be identified pathway. Thus, the cell death mechanisms associated with caspase activation in our neuronal model of serum deprivation requires further elucidation.

In addition to its mitogenic and protective functions, VEGF has also been implicated in exerting direct effects on neural tissues by stimulating neurite extension. Reports have demonstrated that VEGF stimulates neurite outgrowth from cultured retinal ganglion cells [117] and cerebral cortical neurons [118, 119]. Our results show that serum deprivation promotes neurite extension (Fig. 20, compare A with C) that is potentiated by VEGF (Figs 20D) and abrogated when VEGFR2 was inhibited (Figs. 20E) even in the presence of VEGF (Fig. 20F), suggesting an important role of VEGFR2 in promoting neuronal differentiation. Furthermore, reports have also implicated the cAMP/PKA pathway in driving differentiation by stimulating neurite elongation [121-123]. Interestingly, treatment with the cAMP agonist, CPT, mimicked VEGF in that it stimulated neurite extension (Fig. 21B). Although, the mechanisms governing neuronal differentiation are unclear studies have demonstrated that the stress kinase, p38 MAPK, serves as a downstream target of VEGF mediated differentiation. In our studies, we show that a blockade of p38 MAPK activity inhibited neurite extension (Fig. 20G). Although VEGF failed to restore neurite outgrowth under these conditions (Fig. 20H), CPT surprisingly opposed the effects by p38 MAPK inhibition not only restoring but potentiating neurite extension outgrowth (Fig. 21C). Collectively, our findings suggest that while VEGF/VEGFR2 signals p38 MAPK to modulate neurite extension in cells exposed to serum deprivation, PKA mediated neuronal differentiation is negatively

regulated by p38 MAPK function. In this study, we identify a novel signaling pathway that is crucial for VEGF and PKA-induced neurite extension in neuronal cells. Our findings suggest that a coordinated balance between p38 MAPK and VEGF and PKA is critical for modulation of differentiation in the neuronal stress response to serum deprivation.

Chapter VII

Conclusion

In conclusion, the present data demonstrate that the VEGF/VEGFR2 and PKA signaling pathways crosstalk in neuronal cells to protect against a caspase-dependent cell death induced by serum starvation. Both pathways cooperate to promote neuronal cell survival through differential stimulation of the MEK/ERK1/2 pathway with maximal activation requiring VEGF-mediated signaling through VEGFR2. These findings suggest that the MEK/ERK1/2 pathway is a critical downstream effector of survival mediating protection in neuronal cells by preventing the activation of prodeath mechanisms. Consequently, our findings also suggest a molecular mechanism in which the VEGF/VEGFR2 signaling pathway protects neuronal cells from a caspase-dependent death that involves dual regulation by p38 and a yet to be identified p38-independent pathway. Thus, the cell death mechanisms associated with caspase activation in our neuronal model of serum deprivation requires further elucidation. In addition, p38 MAPK serves dual roles exerting opposing effects that separate survival from differentiation in the neuronal response to serum deprivation. While VEGF-mediated neuronal differentiation is regulated by p38 MAPK, PKA can promote neurite extension independent of p38 MAPK function.

This study is innovative because it focused on identifying downstream effectors of the VEGF/VEGFR2 signaling pathway that mediate neuroprotection under stressful stimuli. Understanding the cell death mechanisms associated with stress and the signaling cascades regulated by VEGF that protect against these harmful insults would be desirable for developing therapeutic strategies that would augment the activity of specific molecular intermediates that enhance neuronal cell survival. Herein, we investigated the signaling mechanisms underlying VEGF neuronal protection using an *in vitro* model of

serum deprivation. This study identified critical molecular intermediates linking prodeath and prosurvival mechanisms associated with VEGF signaling in neuronal cells. These studies would consequently lead to assess whether these mechanisms function in a similar context *in vivo*. Identification of similar mechanisms *in vivo* could potentially lead to a therapeutic intervention to delay or halt the neurodegenerative process. These studies demonstrate the potential of the VEGF/VEGFR2 signaling pathway to mitigate neurodegeneration.

Chapter VIII

Model

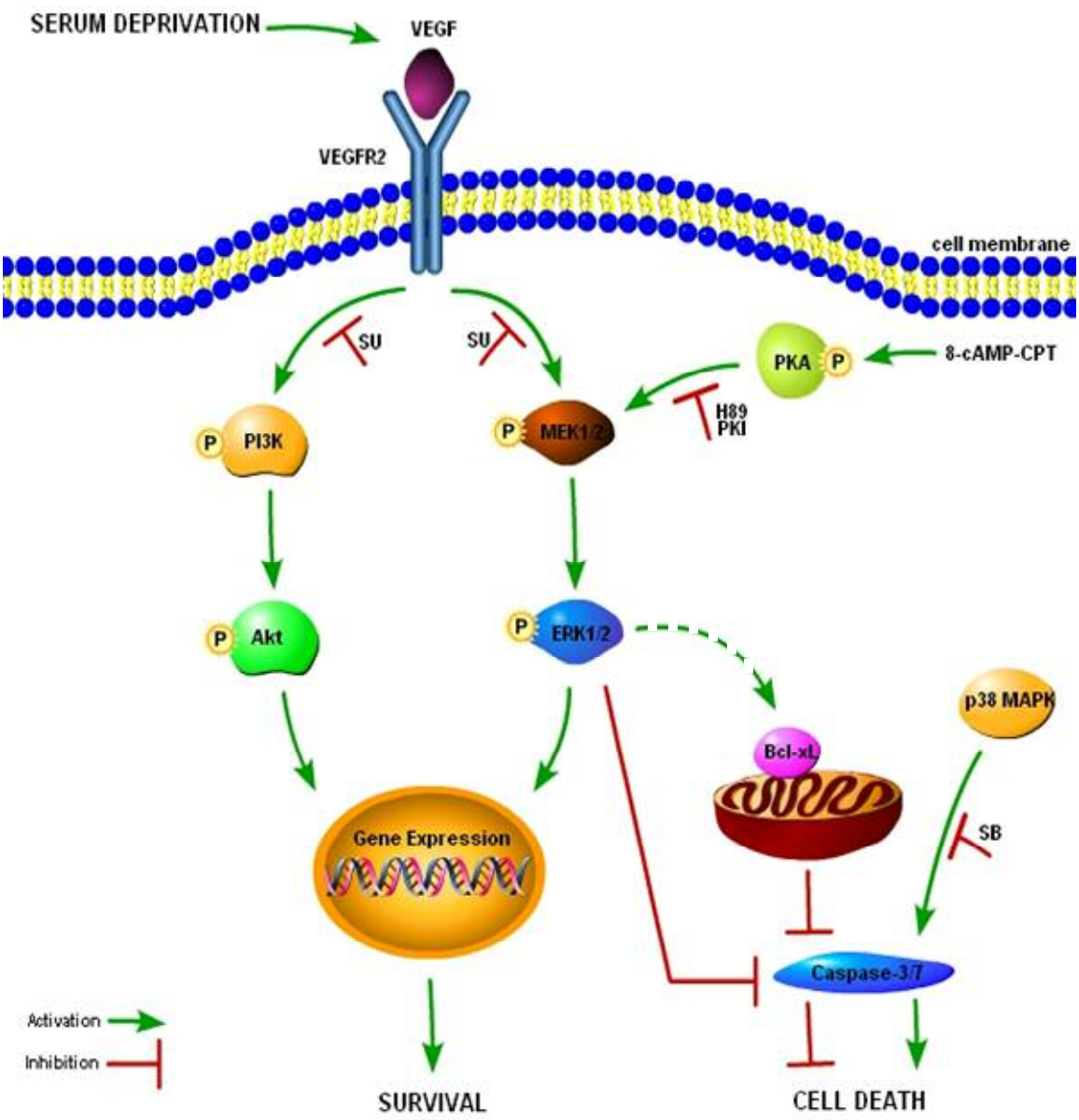


Figure 22. Model for protective mechanism by VEGF and PKA in neuronal cells after serum deprivation- Serum deprivation induces increased levels of VEGF and VEGFR2 which together with PKA signal protection through the MEK/ERK1/2 pathway. The PKA pathway was also evaluated by treatments with the cAMP analog mediator of PKA activation, 8-cAMP-CPT. VEGFR2 alone activates the PI3K/Akt pathway. Activated ERK1/2 (or ERK1 overexpression) mediates a decrease in caspase-3/7 activation and cell death to provide survival perhaps through transcriptional-dependent and -independent mechanisms. VEGFR2 also regulates Bcl-xL protein expression levels possibly through ERK1/2 (hatched arrow) to protect against caspase activation. In addition, p38 MAPK induces a caspase-mediated prodeath mechanism that is abrogated by treatments with SB202190 (SB).

Chapter IX

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