Vascular Endothelia Growth Factor and its Receptor VEGFR2 Regulate Synaptic Protein Levels in Rat Hippocampal Neurons

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Abstract

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Vascular endothelial growth factor (VEGF) is a well-established angiogenic factor which also elicits protective and stimulatory effects on neuronal function. Recent studies suggest that VEGF signaling plays a critical role in modulating synaptic plasticity and enhances excitatory synaptic transmission in the hippocampus. Other growth factors including brain-derived neurotrophic factor (BDNF) and insulin have been shown to regulate synaptic protein levels to stimulate neural communication but it remains unclear how VEGF participates in synapse function at the molecular level. The notion that VEGF would also modulate synaptic protein levels in differentiated hippocampal neurons has not been explored. Therefore, this work addressed whether VEGF exhibits neurotropic properties in mature rat hippocampal neurons by modulating the postsynaptic protein PSD-95 and protecting against the stress induced by nutritional deprivation. The results show that VEGF signals an increase in cell viability and increases the levels of presynaptic (synaptophysin and synapsin I) and postsynaptic (PSD-95) proteins through its cognate receptor VEGFR2. VEGF signals these events via autocrine and paracrine

mechanisms. Moreover, VEGF regulates PSD-95 protein levels and synapse numbers along dendrites through the PI3K/Akt/mTOR pathway. Additional studies showed that inhibition of the Rho-associated protein kinase (ROCK) increased PSD-95 protein levels which were attenuated by VEGFR2 inhibition. Furthermore, ROCK inhibition enhanced VEGF-mediated synapse formation, survival and neurite extension. Accordingly, these findings suggest that ROCK serves as a negative regulator of VEGF signaling in mature primary hippocampal neurons. Collectively, this study revealed a novel signaling mechanism for VEGF/VEGFR-2 pathway that may function in its reported capacity to stimulate synaptic transmission. These findings implicate VEGF signaling as a potential therapeutic strategy to prevent or hinder synaptic loss in neurodegenerative disorders.

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List of Abbreviations

4E-BP1 eIF-4E binding protein-1

AD Alzheimer's disease

ADDLs amyloid beta peptide-derived diffusible ligands

AGC cAMP-dependent, cGMP dependent and protein kinase C

ALS Amyotrophic lateral sclerosis

AraC cytosine arabinoside

Aβ amyloid β-peptide

BDNF brain-derived neurontrophic factor

BDNF Brain-derived neurontrophic factor

bFGF basic fibroblast growth factor

DIV Days in vitro

DRG dorsal root ganglia

eIF-4E eukaryotic initiation factor 4E

ERK extracellular signal-regulated kinase

FBS fetal bovine serum

Flk-1 fetal liver kinase

Flt-1 fms-like tyrosine- kinase

$GABA_A$ ɣ- Aminobutyric acid A

HBSS Hank's balance salt solution

HRE hypoxia-response element

KDR Kinase domain receptor

LIMKs Lin-11, Isl-1, and mec-3 kinase

LPA lysophosphatidic acid

LTD long-term depression

LTP long-term potentiation

LY294002 PI3K inhibitor

MAGUK membrane-associated guanylate kinase

MAM meprin

MAPK mitogen-activated protein kinase

MEK mitogen-activated protein kinase kinase

MLC myosin regulatory light chain

mTOR mammalian target of rapamycin

mTORC mTOR protein complex

NB Neurobasal medium

NB/B27 neurobasal media supplemented with B27

NP-2 neuropilin-2

P1 1 day postnatal

p70S6K p70 ribosomal protein subunit S6 kinase

PAK P21-activated kinase

PI3K phosphoinositide 3-kinase

PIGF placental growth factor

PLCα phospholipase Cα

PP242 mTORC1 and mTORC2 inhibitor

PSD postsynaptic density

RBD RhoA binding domain

RGCs retinal ganglion cells

ROCK Rho-associated protein kinase

ROS reactive oxygen species

NMDA N-methyl-D-aspartate

NP-1 neuropilin-1

S6 ribosomal protein subunit S6

SCG superior cervical ganglia

Sema 3A semaphoring 3A

siRNA small interfering ribonucleic acid

SOD1 superoxide dismutase

SOD1^{G93A} G93A mutant SOD proteins

SU1498 VEGFR2 inhibitor

SV synaptic vesicles

Trk B tropomyosin-related kinase B

U0126 MEK/ERK1/2 inhibitor

VEGFR VEGF receptor

VPF vascular permeability factor

Y-27623 ROCK inhibitor

Chapter 1

Introduction

1. Introduction

1.1 VEGF family

The VEGFs are a family of highly homologous growth factors including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF), which are physiological subtypes from mammals. The exogenous subtype of VEGF family also includes parapoxviurs genome-encoded VEGF-E and snake venom-derived VEGF-F (Jung et al., 1999; Shen et al., 1999).

Of the VEGF family VEGF-A is the prototypical subtype. It is a homodimeric glycoprotein that was initially discovered as a “vascular permeability factor” (VPF) that increases vascular permeability (Li et al., 1995). VEGF-A was then cloned from pituitary cells (Yeh and Lee, 1999) and it’s pivotal role in angiogenesis has been widely studied both in health and pathological disorders (Carmeliet, 2003). Further studies show that there are 8 exons in VEGF-A mRNA and the alternative splicing produces at least nine spliced transcripts: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{162b}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$, each of which is named on the basis of the number of amino acid residues consisting of the two polypeptide chains forming the homodimeric structure. The variations of the amino acid residues in these VEGF isoforms account for their different heparin and heparin-sulfate binding ability, therefore lead to different solubility, receptor affinity and biological properties (Lee et al., 1999; Neufeld et al., 1999). It is well known that $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ are secreted preferentially in most VEGF-producing cells while the other splice variants are less expressed. With the exception of the shortest form $VEGF_{121}$ all of these isoforms

bind heparin to differing degrees (Neufeld et al., 1996; Tanaka et al., 2008). While $VEGF_{121}$ with the absent exon 6 and exon 7 is a non-heparin-binding acidic protein (Herz et al., 2012; Tischer et al., 1991). $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ are all secreted in form of dimeric proteins however the presence of exon 7 of the VEGF gene in $VEGF_{165}$ and exon 6 in $VEGF_{145}$ distinguish these two isoforms from $VEGF_{121}$. The peptides encoded by exon 7 and 6 allow $VEGF_{165}$ and $VEGF_{145}$ bind to heparin and extracellular matrix. $VEGF_{189}$ and $VEGF_{206}$ are considered to keep sequestered on cell surfaces and in the extracellular matrix upon secreted. As the most abundant and biologically active form, $VEGF_{165}$ normally expressed as a 46-kDa homodimer consisting of two 23-kDa monomers (Kusnoor et al., 2010; Neufeld et al., 1999). It binds selectively to VEGFR-1 and VEGFR-2, however with higher affinity for VEGFR-1 (Jung et al., 1999; Koch et al., 2009; Maes et al., 2010; Page et al., 2000b; Schipani et al., 2009; Yi et al., 1999). Since it has been well established that $VEGF_{165}$ has a protective effect in motor neurons and SK-N-SH neuroblastoma cells against low oxygen tension, oxidative stress or serum deprivation induced apoptosis (Kilic et al., 2006b; Yulug et al., 2006a; Yulug et al., 2006b), this study aim to demonstrate whether $VEGF_{165}$ (hereafter referred to as VEGF) plays a similar protective role in rat hippocampal primary neurons.

1.2 VEGF receptors

There are two types of VEGF receptors (VEGFR) have been identified: tyrosine kinase receptors and nontyrosin kinase receptors. The tyrosine kinase receptors include VEGFR1 (fms-like tyrosine- kinase, Flt-1), VEGFR2 (Kinase domain receptor, KDR in human or fetal liver kinase, Flk-1 in mouse), and VEGFR3 (Flt-4) while the nontyrosine kinase receptors refer to neuropilin-1 (NP-1) and neuropilin-2 (NP-2). The tyrosine

kinase receptors are structurally related, especially VEGFR1 and VEGFR2 share 44% amino acid homology (Neufeld et al., 1999; Spudich et al., 2006; Tanaka et al., 2008; Yi et al., 1999). Both receptors have seven extracellular immunoglobulin-like domains, one hydrophobic transmembrane domain and a cytoplasmic bipartite tyrosine kinase domain splited by an insert sequence. Despite of the similarity of their receptor structure, VEGFR1 has about 10-fold higher affinity for VEGF but much weaker kinase activity comparing with VEGFR2 (Koch et al., 2011; Waltenberger et al., 1994). It is still vague whether VEGFR-1 signals any biological functions but it is considered a decoy receptor that traps VEGF and prevents VEGFR2 activation hence negatively regulates VEGFR2 (Olsson et al., 2006). The extracellular domain of VEGFR1 has been known expressed as a soluble membrane–bound “decoy receptor” that lacks the intracellular transduction domain. It is an endogenous antagonist that plays a negative role in angiogenesis (Fong et al., 1999; Hiratsuka et al., 1998). On the contrary, the role for VEGFR2 is more intensely studied in endothelial cells and neuronal cells. Activated by VEGF ligand binding-stimulated receptor dimerization and transphosphorylation or autophosphorylation of tyrosine residues in intracellular kinase domain, VEGFR2 plays a broad role in cell protection, neurite/axon outgrowth and neurogenesis (Bocker-Meffert et al., 2002; Matsuzaki et al., 2001; Ruiz de Almodovar et al., 2009b; Sondell et al., 2000; Zachary and Gliki, 2001). Unlike VEGFR1 and VEGR2, VEGFR3 is a proteolytic cleaved 195 kDa molecule that yields 120 kDa and 75 kDa polypeptides with a disulphide bridge in the fifth immunoglobulin domain. It is involved in lymphangiogenesis (McColl et al., 2003).

The VEGF family members bind to these receptors with different preference and

binding affinity (**Figure 1.1**): PIGF and VEGF-B only bind to VEGFR1, VEGF-E specifically binds to VEGFR2, VEGF-A activates both VEGFR1 and VEGFR2. VEGF-C and VEGF-D bind to both VEGFR2 and VEGFR3 (Spudich et al., 2006).

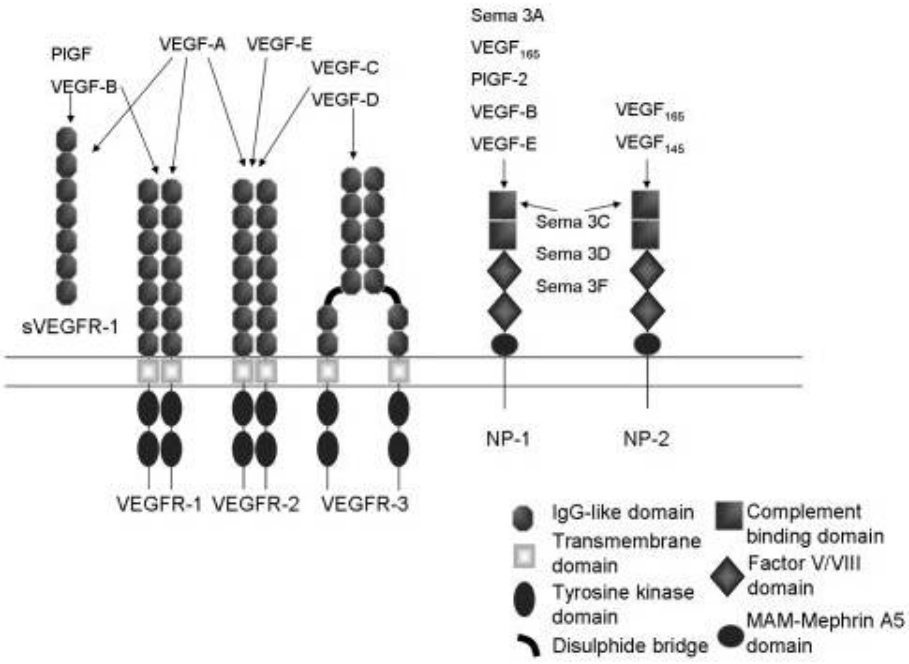

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Figure 1.1 VEGF ligands and receptors. The receptor tyrosine kinases including VEGFR1, VEGFR2 and VEGFR3 possess an extracellular domain with seven immunoglobulin-like domains, a single hydrophobic transmembrane domain and a single kinase cytoplasmic domain which is divided into a bipartite by a non-catalytic kinase insert. VEGFR1 is also expressed independently as a soluble protein (sVEGFR1). VEGF A binds to both VEGFR1 and VEGFR2; VEGFs C, D and E only bind to VEGFR2, while PIGF and VEGF-B bind to VEGFR1 specifically. VEGFR3 is a related receptor for VEGFs C and D that contains 2 polypeptides with a disulphide bridge in the fifth extracellular immunoglobulin domain. NP-1 is nontyrosine kinase receptor for $VEGF_{165}$, the PIGF, VEGF-B and VEGF-E; NP-2 is a receptor for both $VEGF_{165}$ and $VEGF_{145}$.

The VEGF nontyrosine kinase receptors have 2 members: neuropilin-1 (NP-1) and neuropilin-2 (NP-2), which are considered as co-receptors for VEGF isoforms (Jin et al., 2000b; Sun et al., 2002). NPs comprise an extracellular domain that contains MAM (meprin) or C domain, a and b domains, a transmembrane domain and a short cytoplasmic domain (Spudich et al., 2006). They are first identified as receptors for semaphoring 3A (Sema 3A) which is a chemotropic cues responsible for repulsive axonal guidance and development (Page et al., 2000a). Later studies find that NP-1 also is a coreceptor of VEGFR2 for $VEGF_{165}$ binding. In addition, NP-1 is a receptor for PIGF-2, VEGF-B and VEGF-E. NP-2 is primarily for $VEGF_{165}$ and $VEGF_{145}$ binding (Chiu et al., 2010; Loges et al., 2009; Robinet and Pellerin, 2011; Sun et al., 2002).

1.3 The neurotrophic effect of VEGF

VEGF has been well studied as an endothelial-specific growth factor and a potent mitogen that stimulating endothelial cells derived from arteries, veins, and lymphatics (Leung et al., 1989). Recent studies showed that VEGF also plays an important role in nervous system as a neurontrophic factor.

First of all, it has been well established that VEGF protects different types of neuronal cells from various stimuli-induced cell death. Previous studies showed that hypoxia is the best known regulator of VEGF expression for angiogenesis. Cells release angiogenic factors including VEGF to reestablish oxygen supply by vessel formation in response to hypoxia (Ruiz de Almodovar et al., 2009b). Other studies demonstrate that VEGF protect hippocampal neurons from hypoxia or N-methyl-D-aspartate (NMDA)-induced excitotoxicity by a caspase-independent mechanism (Svensson et al., 2002). In mouse cortical neuron cultures, VEGF rescues hypoxia-induced cell death induced by

caspase-3 activation (Jin et al., 2001). VEGF also protect primary motor neurons against serum deprivation-induced cell death in vitro (Van Den Bosch et al., 2004). Additionally, VEGF rescues hippocampal neurons from glutamate toxicity-induced cell death by independently signaling through phosphatidylinositol 3-kinase (PI3K)/AKT pathways and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways (Matsuzaki et al., 2001). The neuroprotective VEGF also prevent retinal neurons, cholinergic neurons and dopaminergic neurons from cell death induced by ischemia, NMDA stimulation and 6-hydroy-dopamine stimulation (Moser and Humpel, 2005; Nishijima et al., 2007; Yasuhara et al., 2004).

Secondly, VEGF has been considered as a neurotrophic factor that promotes neurite outgrowth in nervous system. VEGF stimulates axon outgrowth through VEGFR2 in cultured superior cervical ganglia (SCG) and dorsal root ganglia (DRG) from adult mice (Sondell et al., 2000). Böcker-Meffert S et al demonstrated that VEGF promotes neurite outgrowth in rat retinal ganglion cells (RGCs) in vitro. Furthermore they elucidated the expression of VEGFR2 in the rat retina in vivo and a blockade of VEGFR2 by an anti-VEGFR2 antibody blocked VEGF-induced neurite outgrowth (Bocker-Meffert et al., 2002). The neuritogenic role for VEGF is explored in primary cortical neurons as well. VEGF has been showed to stimulate neurite outgrowth in neonatal cortical neurons and rat cerebral cortical neurons through VEGFR2 and the downstream MAPK and Rho/Rock pathway separately. VEGF-induced neurite extension was blocked by MAPK inhibitor and the Rho/Rock inhibitors (Jin et al., 2006; Khaibullina et al., 2004).

On the other hand, VEGF exerts its neurotrophic function by stimulates

Neurogenesis *in vitro* and *in vivo* (Maurer et al., 2003). Evidences show that VEGF stimulates hippocampal neurogenesis by environmental enrichment and exercise, learning and antidepressant drugs (Fabel et al., 2003; Warner-Schmidt and Duman, 2008). For instance, VEGF expression in hippocampus is found enhanced in rats raised in a physically enriched environment or trained in a Morris water maze task. This increased level of VEGF is accompanied with neurogenesis and improved learning. Intracerebrally overexpression VEGF also promotes hippocampus-dependent neurogenesis associated with improved cognition, while inhibition of VEGF by siRNA blocks neurogenesis in response to environmental enrichment (Cao et al., 2004). Moreover, Ruiz de Almodovar et al. indicated that the antidepressant drug induces neurogenesis by upregulating VEGF expression and hence leads to a greater VEGFR2 activation in hippocampus (Ruiz de Almodovar et al., 2009a). Instead of directly stimulation of hippocampal neurons, VEGF also promotes neurogenesis by stimulating endothelial cells, neuronal precursors and neuronal stem cells to secret various neurotrophic factors, such as BDNF, erythropoietin and basic fibroblast growth factor (bFGF). These factors contribute to the neurogenic effect of VEGF (Ogunshola et al., 2002b; Ruiz de Almodovar et al., 2009a).

In addition of the neurotrophic effect for VEGF in protecting neuronal cells from various stressful stimuli, stimulating neurite outgrowth and enhanced neurogenesis, recently the protective role for VEGF in different neurological disorders has been widely studied. Nicoletti et al. demonstrated that 24 hours after a status epilepticus, VEGF is dramatically upregulated in both neurons and glial cells in the hippocampus and limbic cortex. Intrahippocampal infusion of VEGF protects hippocampal neurons against status

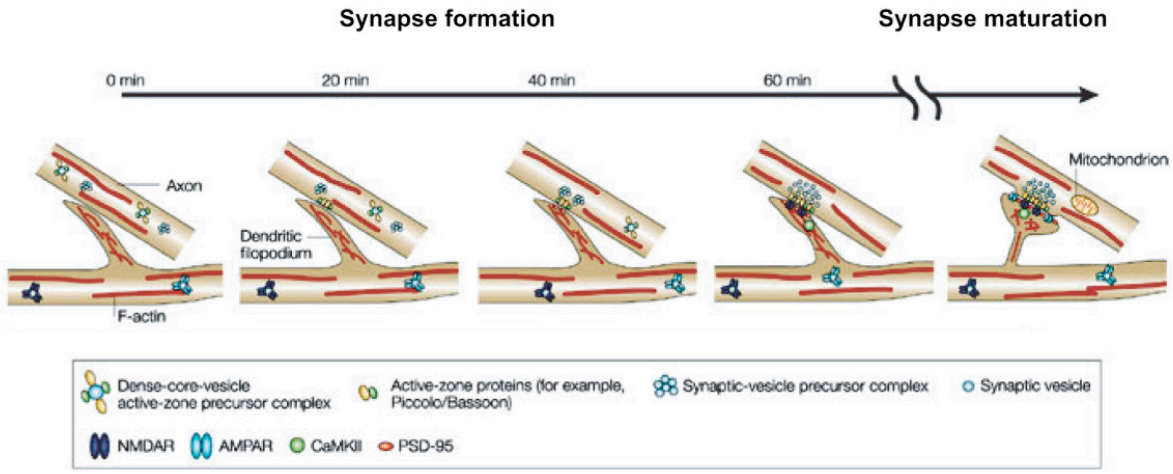
epilepticus-induced cell loss (Nicoletti et. al., 2008). Administration of VEGF to adult rat hippocampal slices suppresses epileptiform activity in epileptic rats (McCloskey et al., 2005a). These studies suggest that VEGF may play a neuroprotective role following status epilepticus. Furthermore, VEGF promotes neurnoal survival against cerebral ischemia in mice through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which makes VEGF a promising candidate for ischemic stroke treatment (Kilic et al., 2006a).

Likewise, VEGF plays an important role in neurodegenerative diseases such as Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). AD is a chronic neurodegenerative disorder characterized with the abnormal production and deposition of amyloid β-peptide (Aβ) in the brain, which causes a progressive impaired cognition and memory loss (Murphy and LeVine, 2010). It has been observed that in the brains of AD patients, VEGF is colocalized with Aβ plaques, which in turn traps VEGF in the Aβ complex therefore suppresses the VEGF release which may lead to VEGF deficiency. This observation may contribute to the pathological changes of neurodegeneration and vascular dysfunction in the progression of AD (Yang et al., 2004). The subsequent study shows the protective effect for VEGF on Aβ-induced toxicity by inhibition of Aβ-induced aggregation and reactive oxygen species (ROS) formation (Yang et al., 2005). Moreover, VEGF is involved in the pathogenesis of motor neuron degenerative disorder ALS. Homozygous Deletion of hypoxia-response element (HRE) in the VEGF promoter in the transgenic mice ($VEGF^{\sigma/\sigma}$) leads to a decreased expression of VEGF. As the consequence of vascular abnormality due to the insufficient VEGF level, these knock-in mice demonstrate adult-onset and progressive degeneration of motor neurons, which are strikingly similar to the signs of those seen in ALS patients (Oosthuyse et al., 2001).

In addition to the vascular effect of VEGF on motor neurons in ALS, VEGF also play it's directly neurontrophic role in the pathogenesis of ALS. Based on the fact that 20% of familial ALS patients are caused by mutations in the superoxide dismutase (SOD1) gene (Rosen, 1993), mice overexpressing the G93A mutant SOD proteins ($SOD1^{G93A)}$ have been used as the standard model of familial ALS. Further evidence indicated that reduced levels of VEGF exaggerate the severity of motor neuron degeneration in the double transgenic $VEGF^{\sigma/\sigma}$ /$SOD1^{G93A}$ mice. However, treatment of VEGF protects motor neurons against ischemia-induced cell death. Meanwhile overexpression of the VEGFR-2 in $SOD1^{G93A}$ mice also attenuated the motor neuron degeneration (Lambrechts et al., 2003; Storkebaum et al., 2005). Taken together these studies delineate the neuroprotective role for VEGF in neuronal degenerative disorders.

1.4 Synapse formation precedes synapse loss in neurodegenerative disorders

Neurons are connected to each other through the specialized asymmetric synapses, through which the neurons communicate with each other by transmission of neuron transmitters. Time-lapse imaging in both *in vivo* and *in vitro* studies reveal that the synapse formation is a temporally rapid process (**Figure 1.2**) (Chiu and Cline, 2010). In the early synaptogenesis, the immature form of the dendritic spine known as filopodia, which are thin, long protrusions that are rich of F-actin, dynamically extend and retract along the dendritic shaft to maximize the chance that a developing axon and a target dendrite encounter each other (Ziv and Smith, 1996). Once contacted, a synapse is established and initiates a series of steps of maturation that requires reciprocal signaling pathways on either side of the asymmetric junction (Goda and Davis, 2003).



Chiu, S.L. et al, Neural Dev 5, 7

Figure 1.2 Synapse formation. Synapse formation initiates when highly dynamic dendritic filopodia establish contact with axon. Once contact with a presynaptic axon terminal, the end of the filopodia enlarged and formed a postsynaptic spine followed by the recruitment of presynaptic and postsynaptic specific structures.

A mature excitatory synapse can be structurally identified with three closely related parts: a presynaptic axon terminal filled with synaptic vesicles (SV) and release machinery, the opposite postsynaptic dendritic spine packed with neurotransmitter receptors, scaffold proteins and signaling machinery together with the synaptic cleft that separates the pre- and postsynaptic compartments (**Figure 1.3**) (Kalia et al., 2008; Li and Sheng, 2003). Upon fusion of synaptic vesicle membrane to the plasmalemma of active zone in the presynaptic terminal, the neuronal transmitters are emptied into the synaptic cleft and bind to the postsynaptic receptors therefore the signal is propagated.

Presynaptically speaking, the transmission of synaptic vesicles is a complex process and regulated by different presynaptic proteins (Sudhof and Jahn, 1991). Synaptophysin is one of these proteins and it is used as a presynaptic molecular marker for studies of synapse formation. Synaptophysin is known as an integral membrane

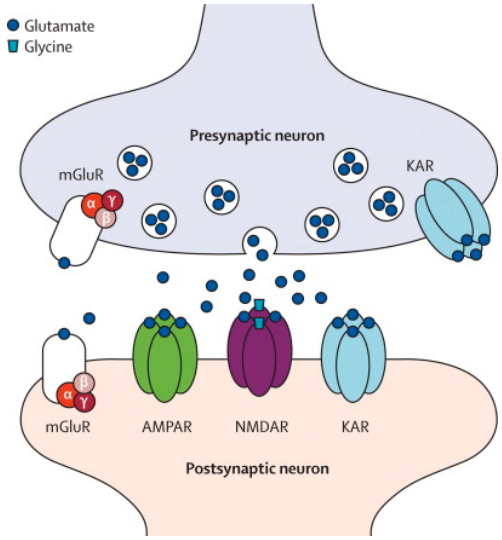


Figure 1.3 Structure of an excitatory synapse in the central nervous system. Synapses are specialized asymmetric junctions between neurons. It consists of three parts: a presynaptic axon terminal filled with synaptic vesicles, a synaptic cleft and a postsynaptic dendritic counterpart packed with neurotransmitter receptors, scaffold proteins and other signaling machinery. The released neurotransmitter from the presynaptic ventricles diffuse through synaptic cleft and bind to the postsynaptic receptors and initiate the subsequent modification of cytoskeletons, scaffold proteins and protein synthesis.

Lorraine V Kalia et al. Lancet Neurol 2008; 7: 742-55

protein of synaptic vesicles, and is expressed ubiquitously throughout all synapses of the central nervous system and peripheral nervous system to modulate the process of synaptic vesicle formation and exocytosis (Wiedenmann and Franke, 1985). Previous studies have demonstrated that synaptophysin contributes in the regulation of activity-dependent synapse formation (Spiwoks-Becker et al., 2001; Tarsa and Goda, 2002). The decreased number of synaptophysin positive boutons found in $SOD1^{G93A\ G1H}$ transgenic mouse, a standard animal model of human ALS, may cause the neruodegeneration in ALS (Zang et al., 2005). Hence synaptophysin immunostaining thus has been commonly used to estimate the changes of synaptic numbers (Jeffrey et al., 2000; Toggas et al., 1996).

Likewise, Synapsin I is also a major protein component of the synaptic vesicles membrane. It is a peripheral protein of the cytoplasmic surface of the synaptic vesicles. Phosphorylation of synapsin I plays a essential role in the regulation of the exo-endocytosis during neurotransmitter release (De Camilli and Greengard, 1986).

Additionally, synapsin I also interacts with cytoskeleton, in particular, F-actin to anchor the synaptic ventricles (De Camilli et al., 1983a; De Camilli et al., 1983b; Huttner et al., 1983). Light- and electron-microscopy immunostaining studies have virtually shown that both synapsin I and synaptophysin are present on small synaptic vesicles at all synapses (De Camilli et al., 1983a; De Camilli et al., 1983b; Navone et al., 1984; Navone et al., 1986). Therefore, these two proteins are regarded as molecular markers for presynaptic specification of synapse, since the presence of synaptic vesicles is a characteristic feature of presynaptic compartments. In this work, I used synaptophysin and synapsin I as the presynaptic markers.

From the postsynaptic point of view, the released neuronal transmitters bind to the cognate neurotransmitter receptors at an electron-dense submembranous region named the postsynaptic density (PSD) and initiate the subsequent postsynaptic signaling transduction. PSD is a thickened disc that organizes neurotransmitter receptors, synaptic adhesion receptors and actin-associated scaffold protein to the postsynaptic membrane (Ethell and Pasquale, 2005b). Its size determines the size of the spine head and is positively related with spine maintenance (Kasai et al., 2003; Trachtenberg et al., 2002). One of the essential scaffold proteins located in PSD is PSD-95, a 95 kDa protein that belongs to the membrane-associated guanylate kinase (MAGUK) protein family (Cho et al., 1992). The function of PSD-95 is still uncertain but accumulated evidences show that PSD-95 interacts with the postsynaptic receptors and cytoskeletons in PSD to modulate the synaptic transmission (El-Husseini et al., 2000), bidirectional synaptic plasticity (Ehrlich and Malinow, 2004; Migaud et al., 1998) and activity-dependent synapse stabilization (Ehrlich et al., 2007). Recently increasing

attention has been paid to the molecular mechanisms that regulate PSD-95. It has been revealed that BDNF stimulates PSD-95 synthesis and transportation to dendrites through PI3K-AKT pathway in cortical neurons (Yoshii and Constantine-Paton, 2007b). Similarly, insulin also up-regulates the local PSD-95 protein synthesis via the PI3K-AKT-mTOR pathway (Lee et al., 2005). However whether VEGF, like BDNF and insulin, regulates PSD-95 in hippocampal neurons has not been studied. Hence in my work, I tested whether VEGF modulates PSD-95 in hippocampal neurons and also delineated the cell signaling pathway underlying these events.

Synaptic connections are the hallmark of neurons and without functional synapses, neurons are in a “solitary” state that leads to a decreased learning ability and declined memory in many neurodegenerative disorders (Lin and Koleske, 2010). Mounting evidences suggest that synapse loss is the best biological standard of the extent of cognition loss in AD (Falke et al., 2003; Terry et al., 1991). Regarding the underlying mechanism of synapses loss in AD, it is believed that accumulation of the soluble amyloid beta peptide-derived diffusible ligands (ADDLs) contributes to the cognitive impairment and the functional synapse loss in AD. Alternatively, ADDLs destroy synapses by reducing the memory-related EphB2 and NMDA receptors and interfering with the actin cytoskeletal stabilization system by reductions in P21-activated kinase (PAK), drebrin and cofilin aggregation (Lacor et al., 2007; Lauren et al., 2009; Ma et al., 2008; Zhao et al., 2006).

Previous studies of neurons from human epilepsy tissue and animal models of focal epilepsy have consistently established a significant loss in postsynaptic dendritic

spine density on hippocampal and neocortical pyramidal neurons as well. Intriguingly, the extent of spine loss correlated with the seizure duration in epilepsy (Isokawa, 1997, 1998; Multani et al., 1994). In the majority of studies the mechanisms for the dendritic spine loss in epilepsy is a consequence of seizure-induced glutamate excitotoxic injury of neurons. Indeed, using glutamate receptor agonists both in vivo and in vitro in hippocampal and cortical neurons led to a decrease in the number of postsynaptic spines (Park et al., 1996; Sloviter and Dempster, 1985). However, emerging evidences suggest that dendritic spine loss may due to an alternative mechanism. One possibility for the spine loss is due to synaptic destruction resulting from an activity-dependent rearrangement of neuronal connection induced by seizure induction. In this regard, John W. et al. demonstrated that persistent epileptiform activity produces long-term depression (LTD) of noncoincidentally active synapse and leads to subsequent synapse elimination, which contributes to the spine loss (Swann et al., 2000). Consistently a recent study showed that mutation of synapsin I, which is a gene essential for neurotransmitter release and synaptogenesis, leads to serious synaptic dysfunction by alteration in postsynaptic integration, excitatory/inhibition balance and activity-dependent synaptic maturation and remodeling (Fassio et al., 2011).

Evidence suggests that synapse loss is consistently attributed to the cognitive decline and memory impairment in neurodegenerative diseases such as Alzheimer's disease and epilepsy. Therefore, therapeutic strategies that increase synaptic plasticity are greatly needed to suppress the synapse loss and neuronal cell death associated with these disorders. However the mechanism underlying the synapse loss and how to protect synapses from the neuronal injury induced cell death is still not very clear. My

work aims to delineate the protective role of VEGF on synapse. This study may shed a light on the potential therapeutic strategy for those neurodegenerative disorders since recovery of the synapse neuronal connections represent a potential therapeutic target under those circumstances.

1.5 Growth factors that regulate synaptic plasticity

Since the activity-induced changes of synaptic connection and the following structural modifications are considered to underlie learning, cognition and memory, the role for neurotrophins as a synaptic modulator has been widely studied (Poo, 2001). Brain-derived neurontrophic factor (BDNF) is known as the prototypic neurontrophic factor that plays a pilot role in synaptic plasticity modulation. Electrical-stimulated long-term potentiation (LTP) of hippocampal synapses is the most well studied form of activity-dependent synaptic plasticity. Evidences showed that BDNF is secreted at glutamatergic synapses in response to the induction of LTP (Aicardi et al., 2004; Gartner et al., 2006; Hartmann et al., 2001). By activation of BDNF receptor tropomyosin-related kinase B (Trk B) in response of LTP-induced synaptic activity, BDNF initiates 3 parallel downstream signaling pathways including the mitogen-activated protein kinase (MAPK), PI3K and phospholipase Cα (PLCα) to modulate synaptic plasticity and synaptic transmission both pre- and postsynaptically (**Figure 1.4**).

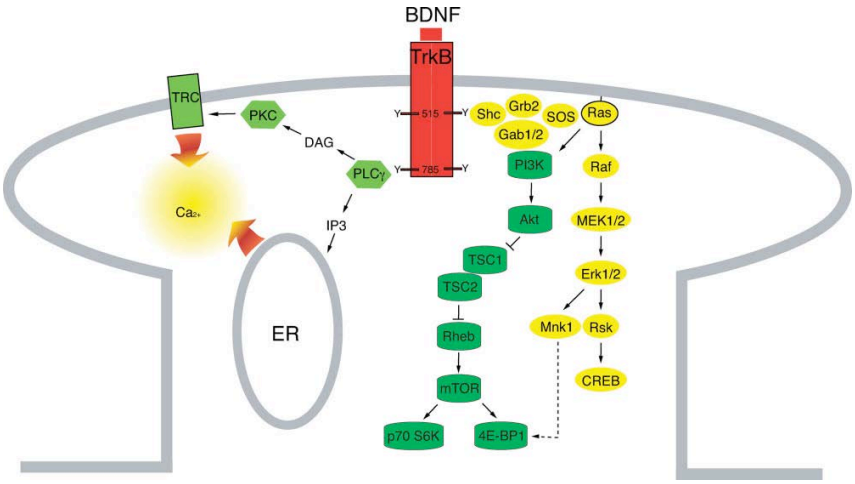


Figure 1.4 The postsynaptic cell signaling pathways activated by BDNF. Binding of BDNF on TrkB receptor on the postsynaptic membrane activates MAPK, PI3K and PLCα pathways to regulate synaptic connection.

It has been showed postsynaptically that MAPK/ERK signaling is involved in BDNF's effect on synaptic plasticity, hippocampal-dependent learning and memory formation (Alonso et al., 2004; Alonso et al., 2002; Blanquet, 2000; Gottschalk et al., 1999). Emerging studies further indicate that the BDNF-activated MAPK/ERK pathway regulates the synaptic plasticity through transcription and translation dependent mechanism. Hence MAPK/ERK contributes to the BNDF stimulated protein synthesis dependent synaptic plasticity (Kelleher et al., 2004; Klann and Dever, 2004; Shaywitz and Greenberg, 1999). Secondly, BDNF activated TrkB-PI3K-AKT pathway initiates the mTOR dependent translation that contributes to the synaptic protein synthesis at potentiated synapses (Tang et al., 2002). Particularly, BDNF not only stimulates PSD-95 protein translation postsynaptically but also transports PSD-95 to dendrites and synaptic puncta through TrkB-PI3K-AKT pathway (Yoshii and Constantine-Paton, 2007b). Thus MAPK and PI3K-AKT pathways are involved in the maintenance of

synaptic plasticity via BDNF-induced protein translation and synaptic protein transport. (Yoshii and Constantine-Paton, 2007b). Thus MAPK and PI3K-AKT pathways are involved in the maintenance of synaptic plasticity via BDNF induced protein translation and synaptic protein transport. On the other hand, coupling of PLCα on TrkB receptor is essential to the hippocampal LTP since mutations in the PLCα-binding sites of TrkB leads to impaired LTP and learning ability (Gruart et al., 2007; Minichiello et al., 2002). Further evidences indicate that the underlying mechanism for BDNF stimulated PLCα pathway to modulate synaptic plasticity is associated with postsynaptic Ca^{2+} release (Lang et al., 2007). Moreover, the PLCα-triggered increased intracellular Ca^{2+} also enhances Ca^{2+} sensitive cAMP activity, which in turn contributes to the trafficking of TrkB receptors to postsynaptic spines and interacts with synaptic scaffolding protein PSD95 (Ji et al., 2005a). Taken together, the BDNF activates the postsynaptic located TrKB receptors to modulate postsynaptic protein synthesis or transport by signaling through different pathways.

In addition of BDNF, insulin and its tyrosine kinase receptor also play diverse role in the CNS, including regulation of synaptic plasticity, consolidation of long-term memory after water maze learning activity. It has been demonstrated that insulin is an important synaptic modulator by trafficking postsynaptic ɣ- Aminobutyric acid A ($GABA_A$) receptors to postsynaptic domains or stimulating local PSD-95 protein translation thorough the PI3K-AKT-mTOR signaling pathway (Dou et al., 2005; Lee et al., 2005; Lin et al., 2000; Wan et al., 1997; Wickelgren, 1998; Zhao et al., 1999).

As a novel neurotrophic factor that also plays multiple roles in the CNS, the role for VEGF in regulating hippocampus-dependent memory and learning has been drawn more attention nowadays. It has been assessed by testing the spontaneous activity and memory formation using both an associative passive voidance task and Morris maze water maze. Rat treated with VEGF shows better performance in the passive avoidance task and enhanced spatial memory in the water maze training. Meanwhile, VEGF expression level in hippocampus is also increased by these two activities via an unknown mechanism (Cao et al., 2004; Greenberg and Jin, 2004). More importantly, VEGF enhances hippocampus-dependent memory independently of its effect on neurogenesis or angiogenesis. Licht, T. et al. finds that overexpression of VEGF promotes hippocampus-dependent memory by increase LTP in the dentate gyrus while suppression of VEGF leads to a completely abrogated LTP. The decreased memory performance caused by blockade of VEGF does not impair neurogenesis and angiogenesis. This study suggests that VEGF stimulates hippocampus-dependent memory probably by reinforcement of the connectivity among existing neurons (Licht et al., 2011a; Welberg, 2011).

The aforementioned underlying mechanism for memory formation and learning process has been broadly studied by exploring the role for BDNF and insulin in regulating synaptic transmission or local synaptic protein synthesis. However, whether VEGF and its tyrosine kinase receptors modulate the synapse formation in mature hippocampal neurons are not known. To address that, my work delineates a neurotrophic role for VEGF and VEGFR2 in promoting synapse maintenance and

stability in post-differentiation hippocampal neurons by modulating the levels of the scaffold protein PSD-95. These findings assign a therapeutic value to VEGF by preventing synapse loss in neurodegenerative disorders.

1.6 mTOR pathway is involved in synaptogenesis and memory formation

Increasing attention has been paid to the important role for new protein synthesis during synapse maintenance and formation of long-term memory. The newly synthesized plasticity-related proteins are showed to stabilize and reinforce synaptic strength after learning events (Davis and Squire, 1984; Frey and Morris, 1998; Izquierdo et al., 2006; Kang and Schuman, 1996; Lamprecht and LeDoux, 2004). There are two proposed mechanisms that explain this phenomenon. The “synaptic tagging” theory emphasized that synaptic activity produces a tag at the activated synapse that directs the subsequent targeting of proteins synthesized in the soma (Frey and Morris, 1997, 1998). The alternative mechanism is dendritic protein synthesis, which claims that synaptic activity stimulates protein synthesis at activated synaptic sites (Kang and Schuman, 1996; Martin et al., 1997). However, little is known about the molecular mechanism underlying the translation of the novel synaptic protein that contributes to the stabilization of synapses and consolidation of memory.

Recently, the serine-threonine protein kinase mTOR has been shown to mediate protein synthesis that modulates cell growth, proliferation and synaptic plasticity in neurons (Hay and Sonenberg, 2004). In mammalian cells, there are 2 forms of mTOR protein complex known as mTORC1 and mTORC2 respectively (Bhaskar and Hay, 2007). The rapamycin-sensitive mTORC1 is involved in a myriad of cellular processes,

such as protein translation, transcription, protein degradation, cell cycle and microtubule dynamics (Harris and Lawrence, 2003; Schmelzle and Hall, 2000). The best known process that mTORC1 is engaged is the protein synthesis by phosphorylation of the repressor protein eukaryotic initiation factor 4E (eIF-4E) binding protein-1 (4E-BP1) and p70S6 kinase (p70S6K). mTOR activation leads to phosphorylation of 4E-BP1, which in turn causes its dissociation from eIF-4E on 4E-BP1 phosphorylation and a subsequent initiation of translation (Beretta et al., 1996; Brunn et al., 1997; Burnett et al., 1998). On other hand, phosphorylation of p70S6 kinase leads to phosphorylation of ribosomal S6 protein. Through this phosphorylation, p70S6 kinase increase the production of regulatory proteins that are involved in the processes of translation hence enhances translational capacity of the cell (Jefferies et al., 1997). Furthermore, evidence shows that mTORC2 primarily regulates actin dynamics and phosphorylate Akt and PKCα (Sarbassov et al., 2004; Sarbassov et al., 2005).

mTOR is activated by various tropic factors, mitogens, amino acids, cell energy status and cellular stress (Avruch et al., 2005; Hay and Sonenberg, 2004; Reiling and Sabatini, 2006) and its activation can be specifically inhibited by the selective inhibitor rapamycin (Ljungberg et al., 2006). In response of stress hippocampal neurons modulate the hippocampal synaptic plasticity by upregulating local translation of PSD-95 through PI3K-AKT-mTOR pathway (Yang et al., 2008).

As one of the important regulators of protein translation, mTOR is involved in long-term synaptic plasticity and memory consolidation. Mounting evidences showed that in hippocampal neurons, mTOR is presented at the synaptic site where it plays a

vital role modulating the synthesis of local synaptic proteins in an activity-dependent manner. In these content, rapamycin also inhibits BDNF or high-frequency electrical induced late LTP, which demonstrates the involvement of a rapamycin-sensitive pathway in the regulation of hippocampal synaptic plasticity (Cammalleri et al., 2003; Tang et al., 2002). In addition, the downstream targets of mTOR, 4E-BP-1 and p70S6 kinase are also present at post-synaptic sites and completely overlay with the postsynaptic protein PSD-95, which confirms the postsynaptic localization of these proteins (Tang et al., 2002). Furthermore, the regulation of mTOR and its best-known substrates p70S6 kinase and 4E-BP1 by different trophic factors such as BDNF and insulin is proved through a PI3K-dependent pathway (Lee et al., 2005; Yoshii and Constantine-Paton, 2007b). mTOR pathway also is important for learning and memory formation. Evidences consistently demonstrated that localized administration of rapamycin around behavior training inhibits consolidation of long-term memory formation in different learning tasks (Bekinschtein et al., 2007; Dash et al., 2006; Helmstetter et al., 2008; Myskiw et al., 2008; Parsons et al., 2006; Tischmeyer et al., 2003).

Despite the important role that mTOR plays in synaptic plasticity and long term memory, little is known about the cellular and molecular mechanisms underlying these events. Previous study in our lab shows that VEGF activates mTOR and its downstream substrate p70S6 kinase and S6 in human SK-N-SH neuroblastoma cells, my work further test whether VEGF links with mTOR pathway to modulate synapses formation in primary hippocampal neurons.

1.7 Rho/ROCK pathway regulates synapse stability

Stable synaptic connectivity is the structural base for our brain to performing learning activity and form memory and reductions in synaptic connectivity are considered to be a major reason for the decreased cognition and impaired perception in neurodegenerative disorders.

Given the importance of synapse stability for normal brain function, regulators of this mechanism have been studied widely. It is commonly believed that the normal synapse function and morphology are modulated by the actin cytoskeleton which is regulated majorly by the Rho family of small GTPases, including RhoA, Rac1 and Cdc42 (Govek et al., 2004a; Nadif Kasri and Van Aelst, 2008). In contrast to Rac and Cdc42, RhoA plays an inhibitory role in synapse maintenance. It has been known that activation of RhoA leads to synapse loss and dendritic regression in mature neurons (Govek et al., 2004a; Lee et al., 2000; Ruchhoeft et al., 1999; Tashiro et al., 2000). The best-known targets of RhoA are Rho-associated protein kinases (ROCKs) (Fujisawa et al., 1996; Lemay et al., 1995), which are members of the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) family of protein kinases. ROCKs include 2 isoforms, ROCK1 and ROCK2. ROCK2 is most abundantly expressed in the brain and hence more specifically involved in spine and synaptic regulation (Suhail A. et al., 2009), hereafter refers as ROCK. ROCK contains a kinase domain, a coiled-coil region with a RhoA binding domain (RBD) and a carboxyl-terminal domain. Pharmacological inhibitors such as Y-27623 inhibits ROCK function by competitive with ATP and

interacting with the kinase domain, therefore this inhibitory probe is very useful for evaluating ROCK functions in cells (Zang et al., 2005).

Leverrier et at., show that an lysophosphatidic acid (LPA), a phospholipid derivative that can act as a potent mitogen, activates Rho which in turn binds to and activates ROCK by disrupting intramolecular inhibitory interactions, which in turn contributes to actomyosin filament assembly and myosin contractile activity by inducing increased phosphorylation of the myosin regulatory light chain (MLC) (Leverrier and Ridley, 2001). Further evidences indicate that the RhoA/ROCK-mediated myosin light chain phosphorylation and actomyosin contractility lead to synapse retraction hence leads to synapse loss (Govek et al., 2004b; Nakayama et al., 2000). On the other hand, increased evidence show that RhoA/ROCK also regulates neurite extension in different neurons via phosphorylation of MLC (Boomkamp et al., 2012; Fuentes et al., 2008; Hirose et al., 1998a). Particularly, ROCK inhibition blocks VEGF-induced neurite extension (Jin et al., 2006). However, whether RhoA/ROCK pathway is involved in VEGF-stimulated synapse formation in mature hippocampal neurons has not been explored. Therefore in this study I test the role for Rho/Rock in regarding to VEGF's stimulatory effects on regulation of neurite outgrowth and synapse formation in primary hippocampal neurons.

Chapter 2

Materials and Methods

2.1 Materials

Recombinant human VEGF 165 was obtained from PeproTech (Rocky Hill, NJ). SU1498, LY294002, U0126, and rapamycin were obtained from LC Labs (Woburn, MA) while PP242 was from ChemDea (Ridgewood, NJ).

2.2 Preparation of hippocampal neurons

All animal studies were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and the approval of the Institutional Animal Care Committee at Hunter College of the City University of New York. Rat hippocampal neurons were prepared from Sprague-Dawley (Charles River Laboratories) 1 day postnatal (P1) rat pups using a modification of a previously described procedure (Buck et al., 2010). Briefly, hippocampi were isolated and dissected aseptically in ice-cold Ca^{2+}/Mg^{2+}-free Hank's balance salt solution (HBSS, Invitrogen/Life Technologies). Following the removal of the meninges, the hippocampal tissue was minced, digested in 2.5% trypsin (10X, Invitrogen/Life Technologies) for 15 minutes at 37°C and then rinsed at 37°C with Ca^{2+}/Mg^{2+}-free HBSS. The tissue was triturated in DMEM/10% FBS (Invitrogen/Life Technologies) through pasteur pipettes and then passed through a 40 µm mesh strainer. Isolated cells were then resuspended in DMEM/10% FBS, and plated into poly-D-lysine (100µg/ml) coated 8-well chamber slides at $5x10^4$/well for immunofluorescence imaging or in 96-well plates at 2 X 10^4/well for cell viability studies. Neurons were plated into 6-well coated plates at 5 X 10^5/ well for Western blotting. The medium is replaced with Neurobasal (Invitrogen/Life Technologies) medium supplemented with B27, L-glutamine (GlutaMAX™ Invitrogen/Life Technologies) and penicillin + streptomycin (Antibiotic-Antimycotic; Invitrogen/Life Technologies)(NB/B27). At day 4,

one-half of the medium was replaced with a culture medium of glial-conditioned medium containing 2 μM cytosine arabinoside (AraC) to inhibit the proliferation of glial cells. Neurons are then cultured for 5 or 12 days in vitro (5 DIV; 12 DIV) as indicated with a replacement of one-half of the culture medium every 4 days. For experiments, neurons were cultured in Neurobasal medium (NB) alone without B27 for 48 hours. Where indicated, VEGF was added at 100ng/ml at 0 and 24 hours. For inhibitor studies, the reagents were added after 42 hours of incubation for a 6 hours treatment with predetermined concentrations of either the VEGFR2 inhibitor SU1498 (10 μM), the PI3K inhibitors LY294002 (20 μM), the mTOR inhibitors rapamycin (1 μM) and PP242 (2.5 μM). Both mTOR inhibitors were also added to cultures after 24 hr to assess their long term effects on viability. ROCK inhibitor Y-27632 (1-10μM) was added for 48 hours.

2.3 SK-N-SH neuroblastoma cell culture and treatment

SK-N-SH neuroblastoma cells were cultured under serum-free conditions with or without the ROCK inhibitor Y-27632 (1-10 μM) for 48 hrs and then treated with the VEGFR2 inhibitor SU1498 (10μM) and 10ng/ml VEGF during the last 6 hrs or 15 minutes, respectively. Cells were subjected to immunofluorescence to analyze extensions and MTS to assay cell viability.

2.4 siRNA

Primary hippocampal were transfected with 50 nM of KDR/Flk-1/VEGFR2 SMARTpool siRNA duplexes or non-specific (scrambled) siRNA duplexes (Dharmacon RNA Technologies) on DIV12 with lipofectamine 2000 according to the manufacturer's directions. Cells were then incubated in supplemented neurobasal medium with deprived B27 in the presence and absence of 100ng/ml VEGF at 37°C for 48 hours.

VEGF was replenished once after 24 hours.

2.5 Protein extraction and immunoblotting

14 DIV primary hippocampal neurons were harvested in lysis buffer and quantified for protein content using the protocol described previous (Rockwell et al., 2004). Equal amounts of protein from each total cell lysate were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were blocked and then incubated overnight at 4 °C with primary antibody. All primary and secondary antibodies were from Cell Signaling (Danvers, MA) except for the antibodies against actin (Sigma) and PSD-95 (Abcam). Immunoblots were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce; Rockford, IL) and quantified where indicated by using ImageJ (NIH, http://rsb.info.nih.gov/ij/). Phosphorylated proteins were normalized to actin and expressed as % of the vehicle control.

2.6 Cell viability assay

Cells were plated in 96-well plates and treated as indicated under B27 deprivation for 48 h at 37°C. 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 or vehicle control.

2.7 Immunofluorescence

Cells were plated in 8-well poly-D-lysine (Sigma)-coated plastic chamber slides and cultured in culture medium for 5 DIV or 12 DIV. Cells were then B27-starved for 48 h in the presence and absence of VEGF. Corresponding inhibitors were added at 42 hours for 6 hours as indicated (ROCK inhibitor Y-27632 was added for 48 hours). Cells were fixed in 3.7% formaldehyde and permeabilized with 0.1% saponin for 20 min and

incubated overnight with primary antibody against rabbit anti-PSD-95 (AB18258, Abcam), mouse anti-PSD-95 (MAI-045, Thermo Pierce), rabbit anti-synaptophysin (S5768, Sigma), rabbit-anti-synapsin (5297,Cell Signaling), rabbit anti-VEGFR2 (sc-504, Santa Cruz), rabbit anti-p-VEGFR2 (sc-16629, Santa Cruz), mouse anti-MAP2 (Millipore) and Rhodamine Phalloidin (Invitrogen) then detected with fluorescein-conjugated FITC and TexRed secondary antibody (Invitrogen). Images were captured at 63X using a Leica TCS SP2 Laser Scanning Spectral Confocal Microscope. Images were quantified where indicated using ImageJ (NIH, http://rsb.info.nih.gov/ij/).

2.8 VEGF analysis by ELISA

Cells were plated in 6-well plates at a density of $0.5x10^6$/well and incubated in 3 different media: 1) culture medium that contains 50% glia conditioned medium and 50% NB/ B27, 2) NB/B27 and 3) NB alone. After 48 hours the supernatant were harvested and processed to determine the secreted level of VEGF165 protein by VEGF immunoassay kit (Quantikine VEGF ELISA Kit, R&D Systems, Minneapolis, MN) according to the manufacturers' manual. VEGF concentration (pg/ml) was measured with using Quantikine Rat VEGF ELISA kit (R&D Systems). The absorbance at 450 nm and 570nm was read by a spectrophotometer and wavelength correction is performed by subtracting readings at 570 nm from the readings at 450 nm.

2.9 Statistical analyses

Data are expressed as the mean ± SEM of cell treatments that were replicated at least three times. Statistical significance was assessed with GraphPad Prism version 5.00 using a one-way or two way analysis of variance (ANOVA) with Bonferroni's posttest; $P < 0.05$ was considered significant (GraphPad Software; San Diego, CA).

Chapter 3

VEGF Stimulates Synaptic Proteins through VEGFR2 in Mature Hippocampal Neurons

3.1 VEGF protects hippocampal neurons from B27 deprivation-induced stress via VEGFR2

Our previous findings showed that VEGF and its cognate receptor VEGFR2 were upregulated in response to serum starvation using neuroblastoma cells as a neuronal model (Edelstein et al., 2011; Gomes et al., 2007). To assess the sensitivity of hippocampal neurons to VEGF-mediated survival, 5 DIV cells were assayed for viability following incubations for 48 hours under two culture conditions, 1) neurobasal media supplemented with B27 (NB/B27) and 2) NB alone (NB). The omission of B27 (NB alone) sensitized neurons to a VEGF-mediated stimulation in viability that was attenuated by the VEGFR2 inhibitor SU1498 (**Figure 3.1A**). SU1498 also reduced viability in the absence of VEGF, suggesting that endogenously released VEGF was an available source of ligand for VEGFR2 activation (Kim et al., 2008). Consistent with these findings, immunofluorescent assays confirmed the presence of phosphorylated VEGFR2 (**Figure 3.1B, p-VEGFR2; a, b**) and total VEGFR2 (**Figure 3.1B, c, d**) in untreated (**a, c**) and VEGF treated (**b, d**) cells in NB. The finding that SU1498 only decreased cell viability in NB treated cells suggested that the neurotrophic factors provided by B27 may activate compensatory pathways that mask the effects of VEGF/VEGFR2. For example, B27 contains the growth factor insulin that signals activation of downstream pathways that are also targeted by VEGF (Schulingkamp et al., 2000; Zachary, 2005).

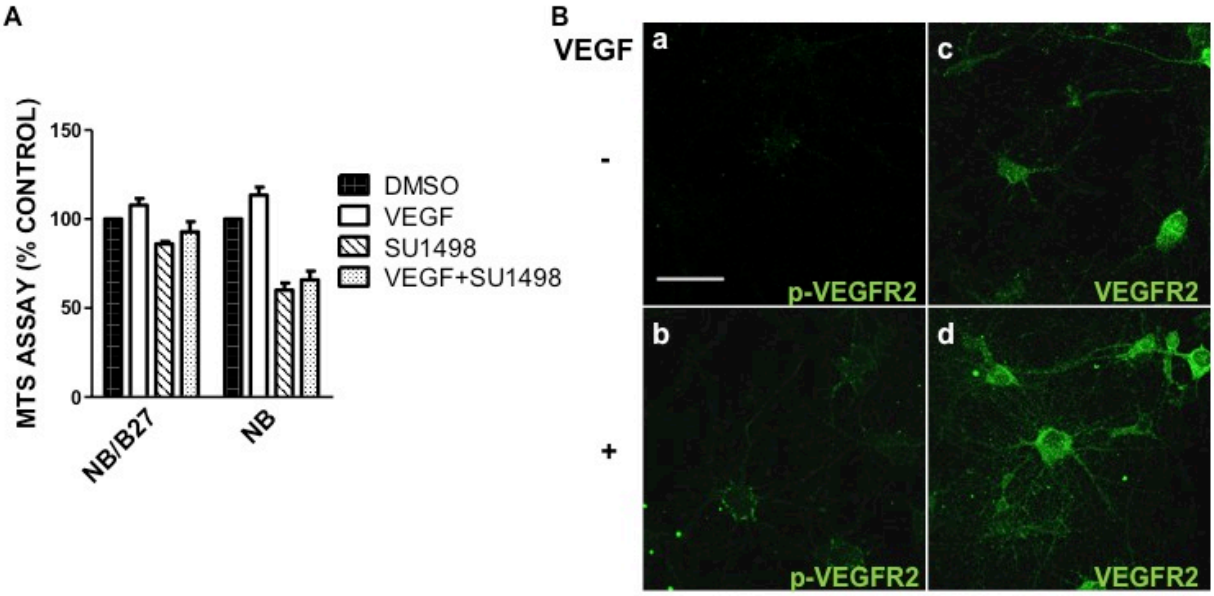
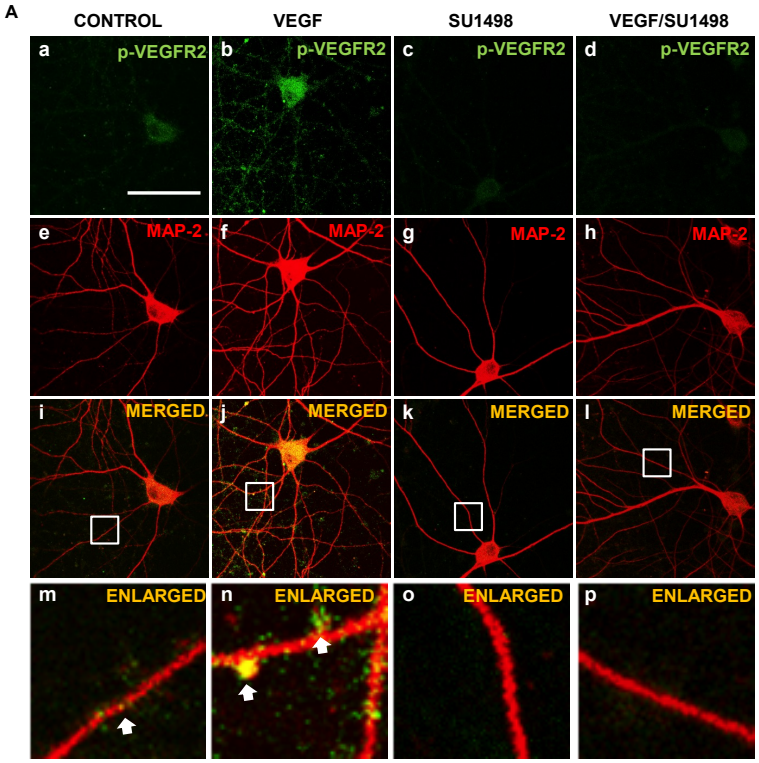


Figure 3.1: VEGF promotes survival through activated VEGFR2 (pVEGFR2) in 7 DIV hippocampal neurons cultured in NB. (A): 5 DIV hippocampal neurons were treated with or without 100 ng/ml VEGF in the presence and absence of 10 μM VEGFR2 inhibitor SU1498 for 48 hours in either neurobasal medium alone (NB) or in neurobasal medium supplemented with B27 (NB/B27). Cell viability was measured as indicated in "Materials and Methods." Results represent the ± S.E.M. of the percent cell viability relative to DMSO treated controls (100%). Data are representatives of one of at least three independent experiments. (B): 5 DIV neurons treated without (**a, c**) and with (**b, d**) 100ng/ml VEGF for 48 hours and stained with anti-pVEGFR-2 **(a, b)** and anti-VEGFR-2 **(c, d)**. Scale bar 20 μm. Data are representative of one of at least three independent experiments.

3.2 VEGF activates VEGFR2 in stressed 14 DIV primary hippocampal neurons in NB

To establish a role for the VEGF signaling pathway in differentiated hippocampal neurons, cells were treated in NB without and with VEGF in the absence and presence of VEGFR2 inhibitor SU1498 at 14 DIV as indicated in "Materials and Methods" and examined by immunofluorescence for receptor phosphorylation (**Figure 3.2 A**). Cells were stained with phosphorylated VEGFR2 (**p-VEGFR2, green; a-d**) and Map2 (**red; e-h**). Insets of a segment of dendrites in the white box in merged image (**i-l**) are enlarged

to show distribution of activated VEGFR2 along dendrites (**m-p**). The result shows that the activation of VEGFR2 by VEGF was detected in soma and dendrites of differentiated neurons (**b, j, n**) and led to an increase in dendritic branching (**f, j**). The detection of activated of VEGFR2 along dendrites and the increased branching induced by VEGF were inhibited by SU1498 (**c, d, k, l, o, p**). In addition, VEGF mediated a significant increase in cell viability that was attenuated by SU1498 (**Figure 3.2B**). The fact that SU1498 diminished viability independent of exogenous VEGF is supported by ELISA assays (**Figure 3.2C**) showing that VEGF is present in NB cultured cells at levels higher than NB/B27 and lower than glia-conditioned media (GCM) . Moreover, SU1498 reduced the levels of VEGF. Together, these results are consistent with evidence that VEGF signals through autocrine and paracrine mechanisms to mediate VEGFR2 activation (Ogunshola et al., 2002a).



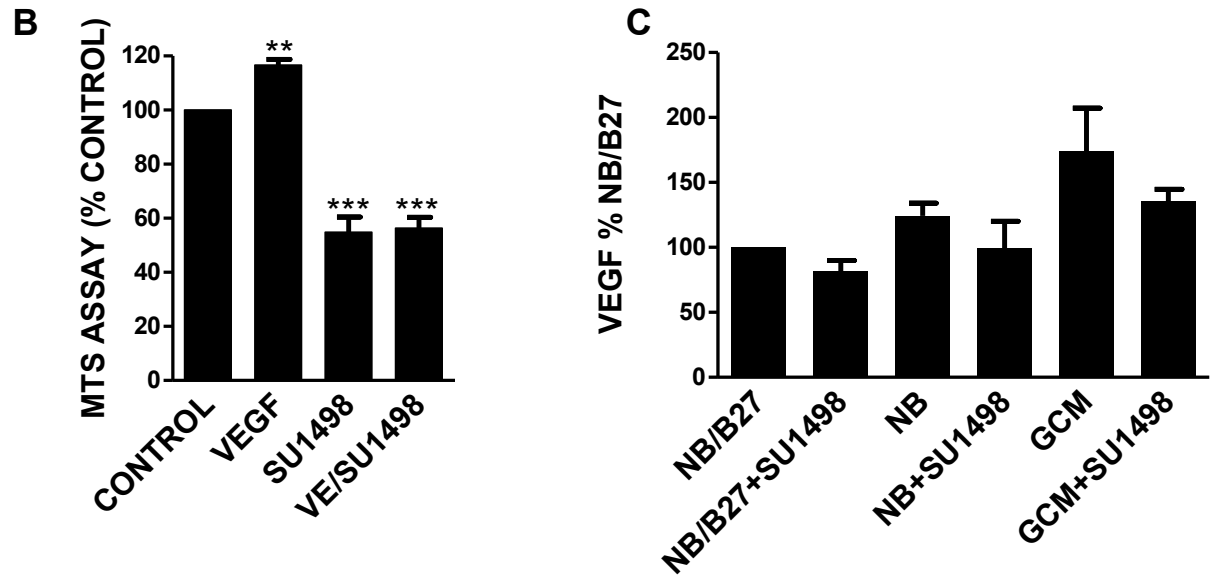
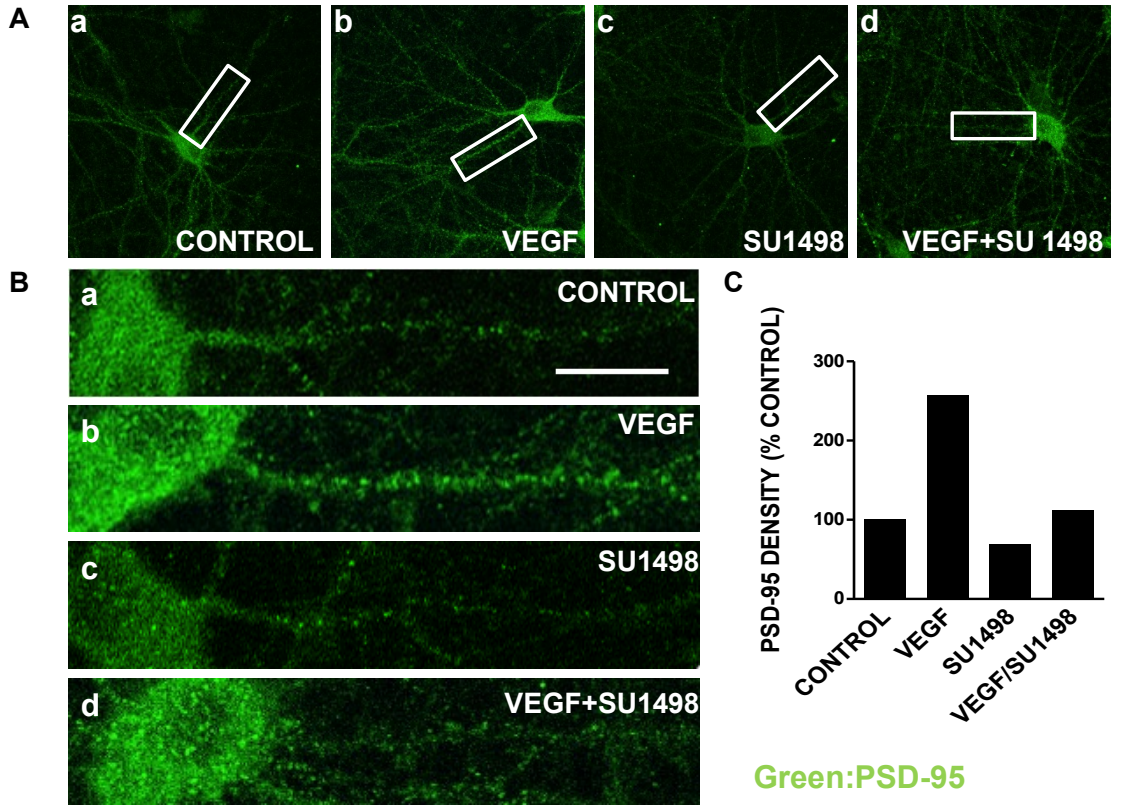


Figure 3.2: VEGF activates VEGFR2 in stressed 14 DIV primary hippocampal neurons in NB. 12 DIV primary neurons were treated with 100 ng/ml VEGF or 10 μM VEGFR2 inhibitor SU1498 alone or in combination and analyzed by (A) confocal microscopy and (B) cell viability assay. (A): Cells are stained with anti-phospho-VEGFR2 **(p-VEGFR2, green; a-d**) and MAP-2 (**red, e-h**,); merged images are shown in **i-l**. Enlargements of merged data in **i and j** show that activated VEGFR2 is distributed alone dendrites (**arrows; m, n**); scale bar 20 μm. (B): Percent cell viability relative to DMSO-treated control cells were measured by the MTS assay, **P<0.01, ***P<0.001. (C): ELISA assay: 12 DIV neurons were treated with and without 10 μM SU1498 under three different conditions: NB supplemented with B27 (**NB/B27**), NB alone (**NB**) and glia conditioned medium (**GCM)**. Supernatants were harvested for ELISA assays to test the % concentration of VEGF (pg/ml) secreted under these circumstances relative to DMSO-treated control in NB/B27. Data are representative of one of at least three independent experiments.

3.3 VEGF increases PSD-95 levels through VEGFR2 and enhances colocalization of PSD-95 with activated VEGFR2 along dendrites

Previous reports demonstrated that BDNF and insulin regulate synaptic protein through their cognate postsynaptic receptors (Lee et al., 2005; Yoshii and Constantine-Paton, 2007). On another hand VEGF stimulates hippocampal-dependent memory formation with an unknown molecular mechanism (Cao et al., 2004; Greenberg and Jin,

2004). These findings suggest that VEGF modulates the levels of synaptic proteins in differentiated neurons. In regard this, immunofluorescent studies with 14 DIV primary cultures of differentiated hippocampal neurons (**Figure 3.3A)** revealed that VEGF-mediated activation of VEGFR2 led to an increase in the protein expression levels of the postsynaptic scaffold protein PSD-95 along dendrites **(a, b)** that was blocked by SU1498 **(c, d**). An enlargement of one dendrite (**in white box**) for each treatment is shown in **Figure 3.3B (a-d)** and the quantification of these data are shown in **Figure 3.3C**. Experiments with siRNA VEGFR2 (**Figure 3.3D)** confirmed the effects of SU1498 on VEGFR2 directed PSD-95 levels. In these experiments, cells were transfected with non-targeting scramble siRNA (**a-d**) and VEGFR2 siRNA (**e-h**) then stained with anti-PSD-95 (**green; a, e**) and VEGFR2 (**red; b, f**). The merged images showed the colocalization of PSD-95 and VEGFR2 in the soma (**merged; c, g**). An enlargement of the merged data showed that the intensity of colocalization staining was greatly reduced by siRNA (**arrows; d and h**). To further examine the colocalization of VEGFR2 with PSD095, mature neurons were treated without (**Figure 3.4 Control**) and with VEGF (**VEGF**) and examined by Immunofluorescence for phosphorylated VEGFR2 (p-VEGFR2, green; **a, b**) and PSD-95 (**c, d**). The merged result (**e, f**) shows that VEGF enhances the colocalizations of PSD-95 with phosphorylated VEGFR2 that appear as merged puncta along dendrites (**arrows, f**).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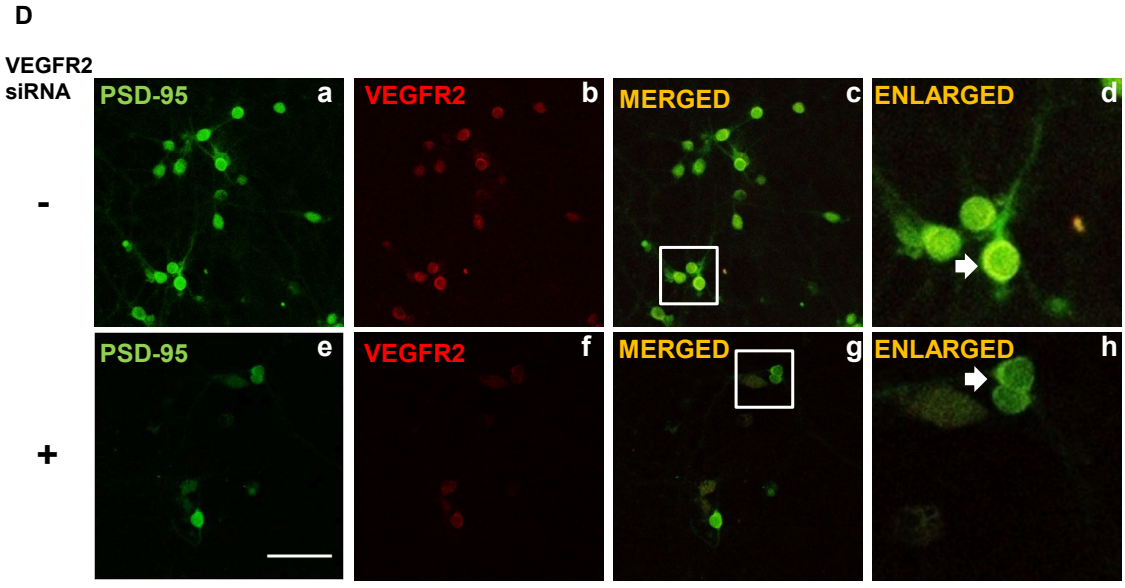
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Figure 3.3

Figure 3.3: VEGF increases PSD-95 levels through VEGFR2. (A): 12 DIV Hippocampal neurons were treated with or without 100ng/ml VEGF in NB for 48 hr. Where indicated 10 μM SU1498 was added at 44 hr for a 6 hr incubation. 14 DIV neurons were fixed in 4% formaldehyde and stained with anti-PSD95 antibody (**green, a-d**). (B): The magnified insets show the dendrites in the white boxes in (**A**). VEGF enhances PSD-95 levels in NB (**b**), which is suppressed by blockade of VEGFR2 with SU1498 (**d**). Scale bar 20 μm. (C): The densities of PSD-95 in the postsynaptic spines in (**B, a-d**) are quantified relative to that of DMSO-treated control (100%). (D): VEGFR2 knockdown by siRNA decreases PSD-95 protein levels. Neurons transfected with non-targeting scrambled siRNA (**a-d)** and VEGFR2 siRNA (**e-h**) were stained with anti-PSD-95 (**green; a, e**) and anti-VEGFR2 (**red; b, f**). Merged images showed the colocalized PSD095 and VEGFR2 in soma (**c, g**). The enlarged insets (white boxes) of **c** and **g** show the decreased level for PSD-95 and pVEGFR-2 by VEGFR-2 knockdown (**d, h**). Arrows in (**d, h**) indicate PSD-95 and VEGFR2 colocalization (**yellow**). Scale bar 20 μm. Data are representative of one of at least three independent experiments.

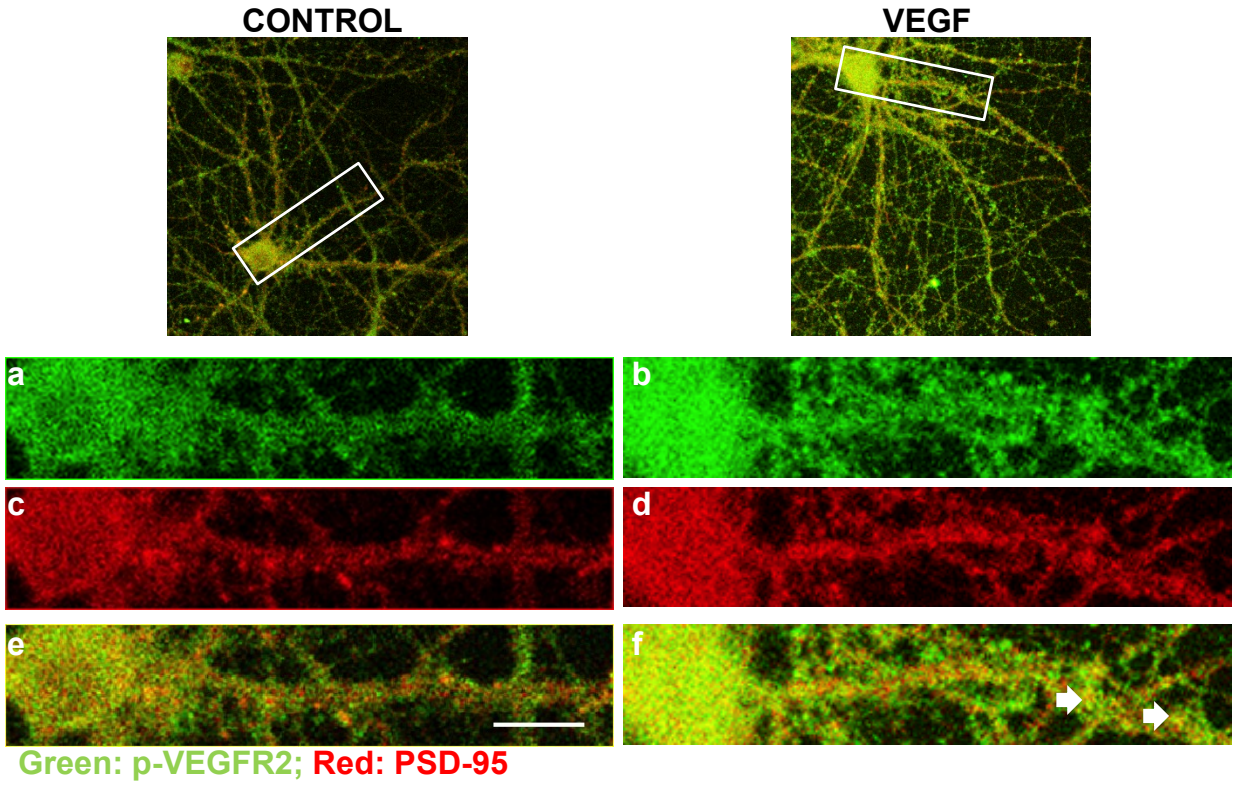


Figure 3.4 Activated VEGFR2 colocalizes with PSD-95 along dendrites. 12 DIV Hippocampal neurons were treated without (**CONTROL**) or with 100 ng/ml VEGF (**VEGF**) for 48 hours. 14 DIV neurons were fixed in 4% formaldehyde and stained with anti-phosphorylated-VEGFR2 (**p-VEGFR2, green; a, b)** and anti-PSD-95 (**red; c, d**). Merged images (**e**, **f)** show the colocalization of PSD-95 with pVEGFR2 on the soma and alone the dendrites (**arrows, f)**. Data are representatives of one of at least three independent experiments. Scale bar 20 μm.

3.4 VEGF/VEGFR2 increases the presynaptic protein synaptophysin levels and synapse numbers

Since PSD-95 is localized at synapses and VEGFR2 regulated its levels of protein expression, we addressed whether VEGF influenced the synapse number in mature hippocampal neurons. BDNF is known to modulate synapse stability by regulating both pre- and postsynaptic protein and data in this study demonstrated that

VEGF stimulated postsynaptic protein PSD-95 levels via VEGFR2. It has been known that synapses are the connecting sites where presynaptic terminal contact the postsynaptic spine to communicate with neighboring neurons. Therefore, we addressed the colocalization of the postsynaptic protein PSD-95 with the presynaptic protein synaptophysin to determine synapse number, cells were treated with or without VEGF alone (**Figure 3.5A, a, b)**or in combination with SU1498 (**c, d**), after 48 hours, cells were then stained with synaptophysin (**green**) and PSD-95 (**red**). Insets (**white boxes**) containing a single dendrite from each treatment in (**A)** are enlarged in (**B)**. Consistent with the results with PSD-95, VEGF/VEGFR2 increased the levels of the pre-synaptic protein synaptophysin in a VEGFR2 dependent manner (**Figure 3.5B, a-d; compare synaptophysin (green) in b and d**). In these data, the colocalization of PSD-95 and synaptophysin represents the number of synapses. Hence these results also showed that the colocalization of presynaptic synaptophysin and postsynaptic PSD-95 at synapses was enhanced by treatments with VEGF (**b**), and an increase in the number of synapses which is suppressed by SU1498 (**d**). The colocalization of synaptophysin (**green**) and PSD-95 (**red**) appear as merged puncta (**arrows, a-d**) that are converted to an image representing synapse numbers (**Figure 3.5C, a-d**). The quantification of the colocalized synapse density is showed in **Figure 3.5D**. Since VEGF activates both VEGFR1 and VEGFR2 while VEGF-B has been shown to activate VEGFR1 alone and be neuroprotective in hippocampal neurons (Li et al., 2008; Poesen et al., 2008), immunofluorescent experiments were performed to test whether the VEGF-modulated synaptophysin expression levels is VEGFR2 dependent in 14 DIV neurons. Cells were treated in the absence and presence of VEGF-B then stained with actin (**red**) and

synaptophysin (**green**). The merged data are shown in **Figure 3.5E**. A branch of dendrites in the white box of each treatment in (**E)** is magnified (**Figure 3.5F, a-f**). There is no significant change between the VEGF-B treated and control group, suggesting that VEGFR1 activated by VEGF-B does not modulate synaptophysin levels. Therefore these findings show that enhancement of synaptophysin is not induced via VEGFR1 activation but specifically through VEGFR2.

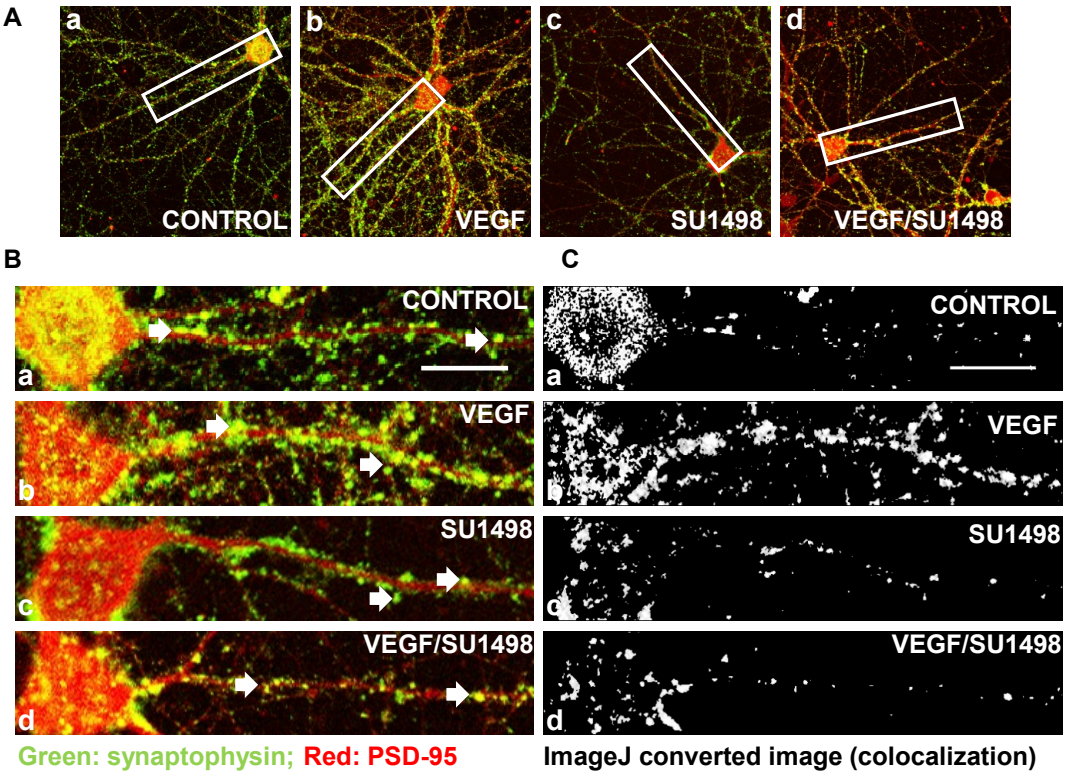


Figure 3.5

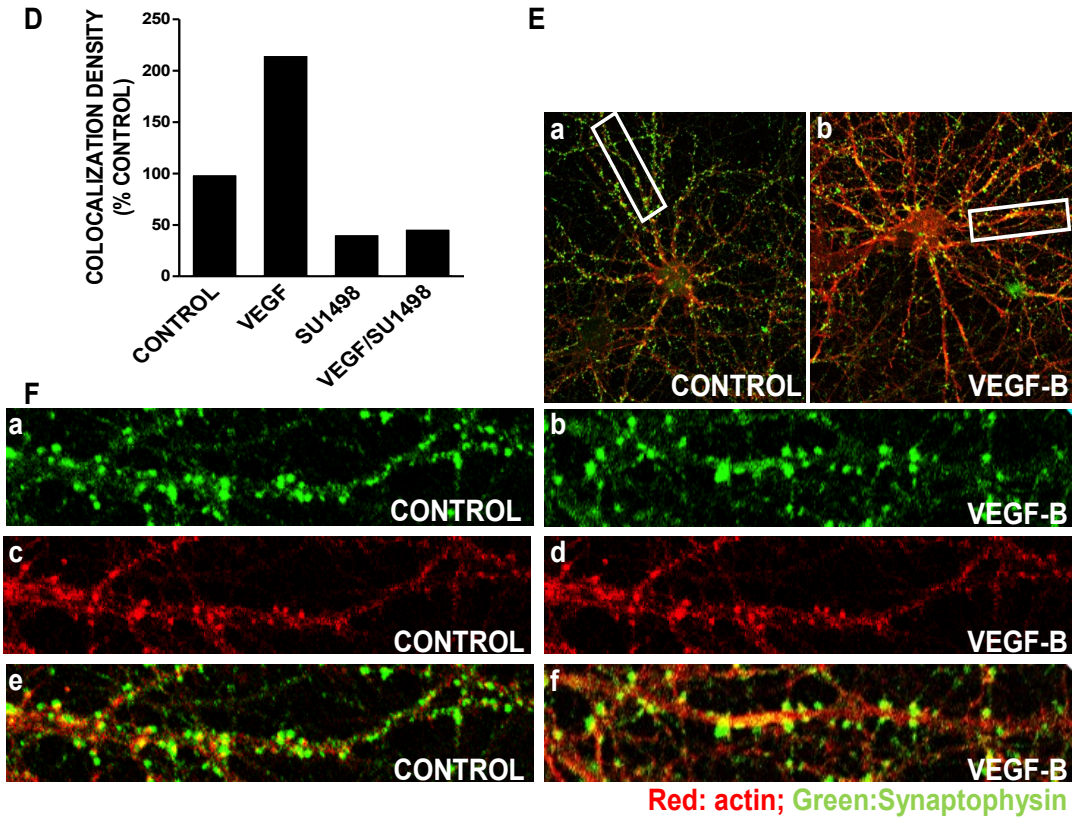
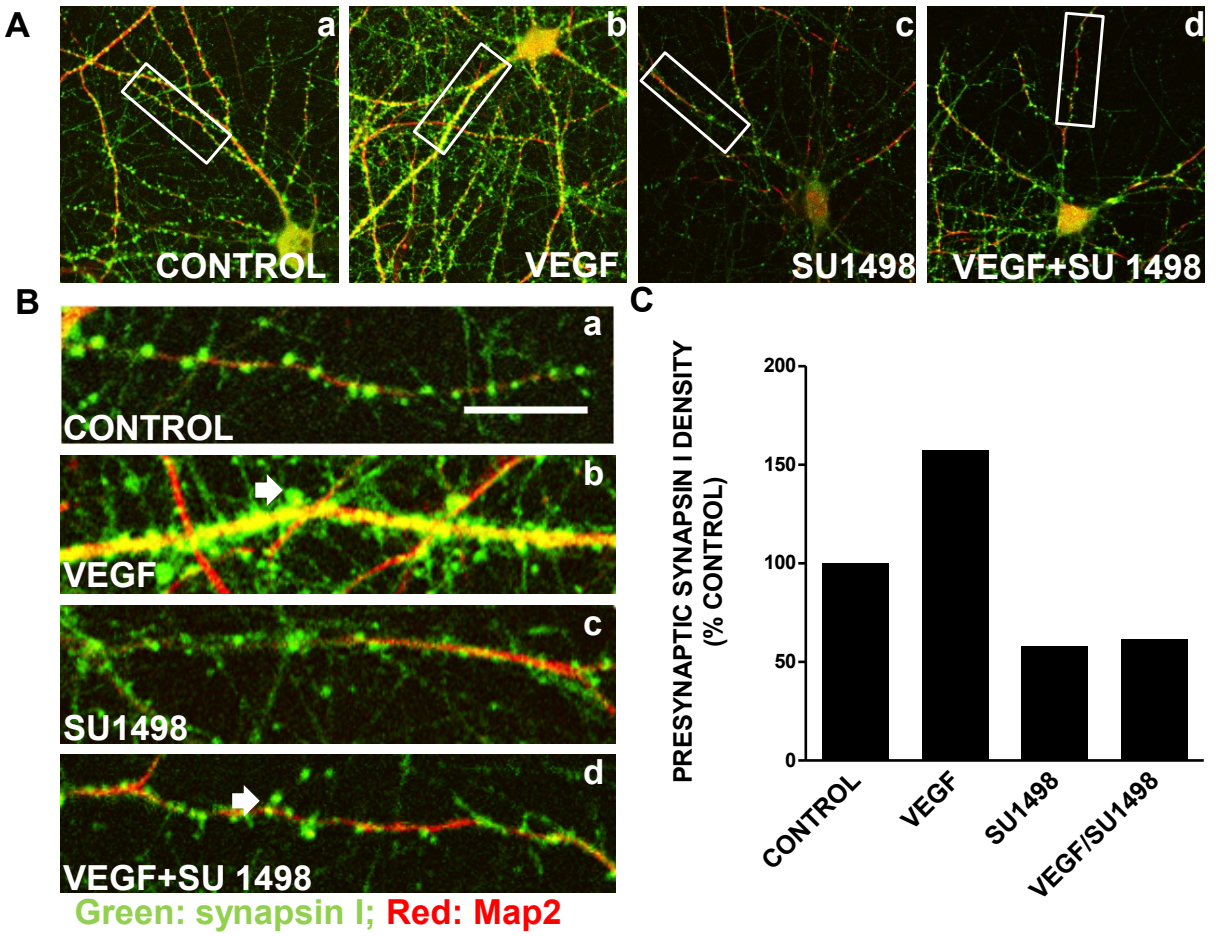


Figure 3.5 VEGF/VEGFR2 signals synaptophysin and PSD-95 colocalization to increase synapse number. (A) 12 DIV hippocampal neurons were treated with or without 100ng/ml VEGF in NB for 48 hr. Where indicated 10 μM SU1498 was added at 44 hr for a 6 hr incubation. Immunofluoresent imaging of 14 DIV neurons shows the distribution of presynaptic protein synaptophysin (**green**), postsynaptic protein PSD-95 (**red**) in mature neurons. (B): Magnified insets (**white boxes**) from (**A**) show a segment of one dendrite from each treatment. The colocalization of PSD-95 and synaptophysin represents synapses (arrows pointed yellow puncta, **a-d**) that are converted to white and black images (**C**). The synapse density is quantified relative to % of vehicle control (**D**). (E): Cells were treated without or with 50ng/ml VEGF-B for 48 hours followed by immunofluorescent imaging. Cells were stained with anti-synaptophysin (**green; a, b**) and actin rhodamine phalloidin (**red; c, d**). Data are representatives of one of at least three independent experiments. Scale bar 20 μm.

3.5 VEGF/VEGFR2 stimulates presynaptic protein synapsin I in 14 DIV hippocampal neurons

To further explore the stimulatory effects of VEGF on presynaptic proteins, immunofluorescent and western blotting assay were used to test whether VEGF influenced the levels of the synapsin I, another presynaptic protein. Synaptophysin and synapsin I both are presynaptic proteins that are widely used in immunochemistry staining as presynaptic markers. Cell were treated as the same as in **Figure 3.3A.** Cells were then stained with anti-synapsin I (**green**) and Map2 (**red**) (**Figure 3.6A, a-d).** A branch of dendrites for each treatment is enlarged (**white box, Figure 3.6B**) to show the synapsin I (**green**) which is located within the synaptic vesicle of dendrites (**red**). Like PSD-95, VEGF increases synapsin I levels through VEGFR2. The levels of synapsin I are quantified (**Figure 3.6C**). The stimulation of VEGF on synaptic proteins are confirmed by western blotting. Results show that the protein level of PSD95 and synapsin I are both increased by VEGF and inhibited by SU1498 (**Figure 3.6D**). Data are quantified relative to % of the vehicle control.


A
a
b
c
d
CONTROL
VEGF
SU1498
VEGF+SU 1498
B
a
CONTROL
b
VEGF
c
SU1498
d
VEGF+SU 1498
Green: synapsin I; Red: Map2
C
PRESYNAPTIC SYNAPSIN I DENSITY
(% CONTROL)
200
150
100
50
0
CONTROL
VEGF
SU1498
VEGF/SU1498

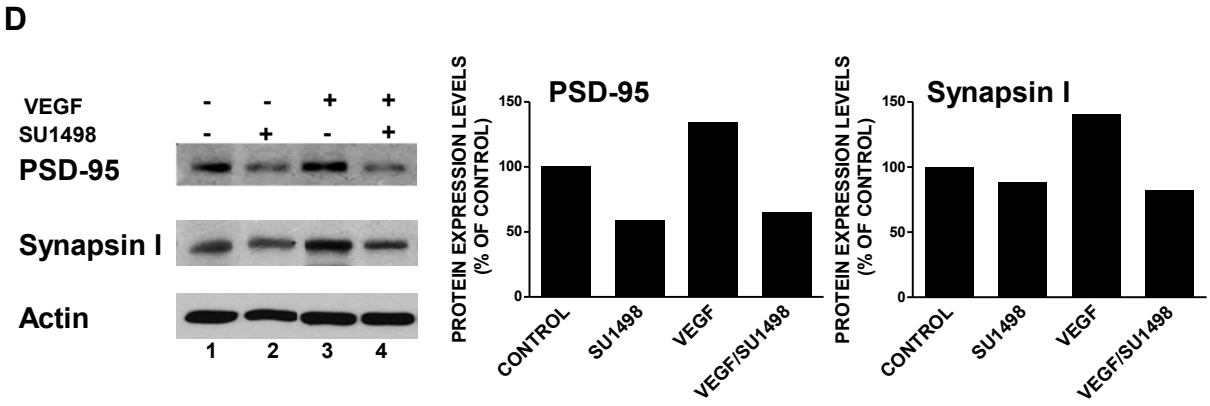

D
VEGF
SU1498
PSD-95
Synapsin I
Actin
1 2 3 4
PROTEIN EXPRESSION LEVELS
(% OF CONTROL)
PSD-95
150
100
50
0
CONTROL
SU1498
VEGF
VEGF/SU1498
PROTEIN EXPRESSION LEVELS
(% OF CONTROL)
Synapsin I
150
100
50
0
CONTROL
SU1498
VEGF
VEGF/SU1498

Figure 3.6

Figure 3.6 VEGF/VEGFR2 stimulates presynaptic protein synapsin I in 14 DIV hippocampal neurons. (A): Cells were treated with or without 100ng/ml VEGF in NB for 48 hr. Where indicated 10 μM SU1498 was added at 44 hr for a 6 hr incubation. (A): 14 DIV neurons were fixed and stained with anti-synapsin I (**green**) and anti-Map2 (**red**). (B): Magnified dendrites from insets (**white boxes in A, a-d**) show that the VEGF stimulates presynaptic synapsin I puncta that are inhibited by SU1498 (**arrows; b, d**). The density of presynaptic synapsin I is quantified with Image J (**C**). (D): Western blotting analysis was performed for detection of PSD-95 and synapsin I under the same experimental condition. Lysates were analyzed by immunoblotting Control group was treated with vesicle DMSO. Actin is used as the loading control. The density of each band was quantified relative to % of the control. Data are representative of one of at least three independent experiments.

Chapter 4

The PI3K/mTOR Pathway is a Downstream Target for VEGFR2-Mediated Stimulation of PSD-95 in Mature Hippocampal Neurons

4.1 VEGF signals PSD95 upregulation through the PI3K/Akt but not MEK/ERK1/2

VEGFR2 has been show to protect hippocampal neurons against the damage induced by stressful stimuli by signaling through the PI3K/Akt and MEK/ERK1/2 pathways (Khaibullina et al., 2004; McCloskey et al., 2005a; Ogunshola et al., 2002a). Therefore these experiments addressed whether VEGF increased synaptic protein levels by signaling through PI3/Akt and MEK/ERK1/2 pathway. Cells were treated without or with VEGF in the absence or presence of SU1498 and lysates were subjected to western blotting for detection of phosphorylated and total protein levels of Akt and ERK1/2 as well as PSD-95 protein levels. Data show that VEGF stimulated Akt and ERK1/2 phosphorylation that were parallel by an increase in the levels of PSD-95 through VEGFR2 (**Figure 4.1A; compare PSD-95 in lane 3 with lane 4**). To address whether MEK/ERK1/2 pathway is involved in VEGF-stimulated PSD-95, VEGF treated cells were treated without or with MEK/ERK1/2 inhibitor U0126 at time frame indicated in "Materials and Methods". The data showed that the enhanced phosphorylation of MEK/ERK1/2 pathway VEGF (**Figure 4.1B, compare p-ERK1/2 in lane 2 with lane 1)** was inhibited by the MEK selective inhibitor U0126 without affecting PSD-95 expression levels (compare PSD-95 in lane **4** with lane **2**). These findings suggest VEGF-mediated stimulation of PSD-95 is not ERK1/2 dependent. Therefore, Akt appears to regulate PSD-95 protein expression levels in response to VEGF. To test whether Akt is regulated by PI3K, cells were treated without or with the PI3K inhibitor LY294002. Lysates were subjected to western blotting for detection of phosphorylated and total protein levels of Akt and PSD-95 levels **(Figure 4.1C)**. Indeed, PSD-95 protein levels were blocked by inhibition of the PI3K, an upstream activator of Akt (**compare PSD-95 in lane 3 with**

lane 1). This concept is supported by evidence that BDNF and insulin signal PSD-95 through the PI3K/Akt pathway (Lee et al., 2005; Yoshii and Constantine-Paton, 2007b). The phosphorylation levels of ERK1/2, Akt and the protein level of PSD95 are quantified relative to % of vehicle control in the right panels.

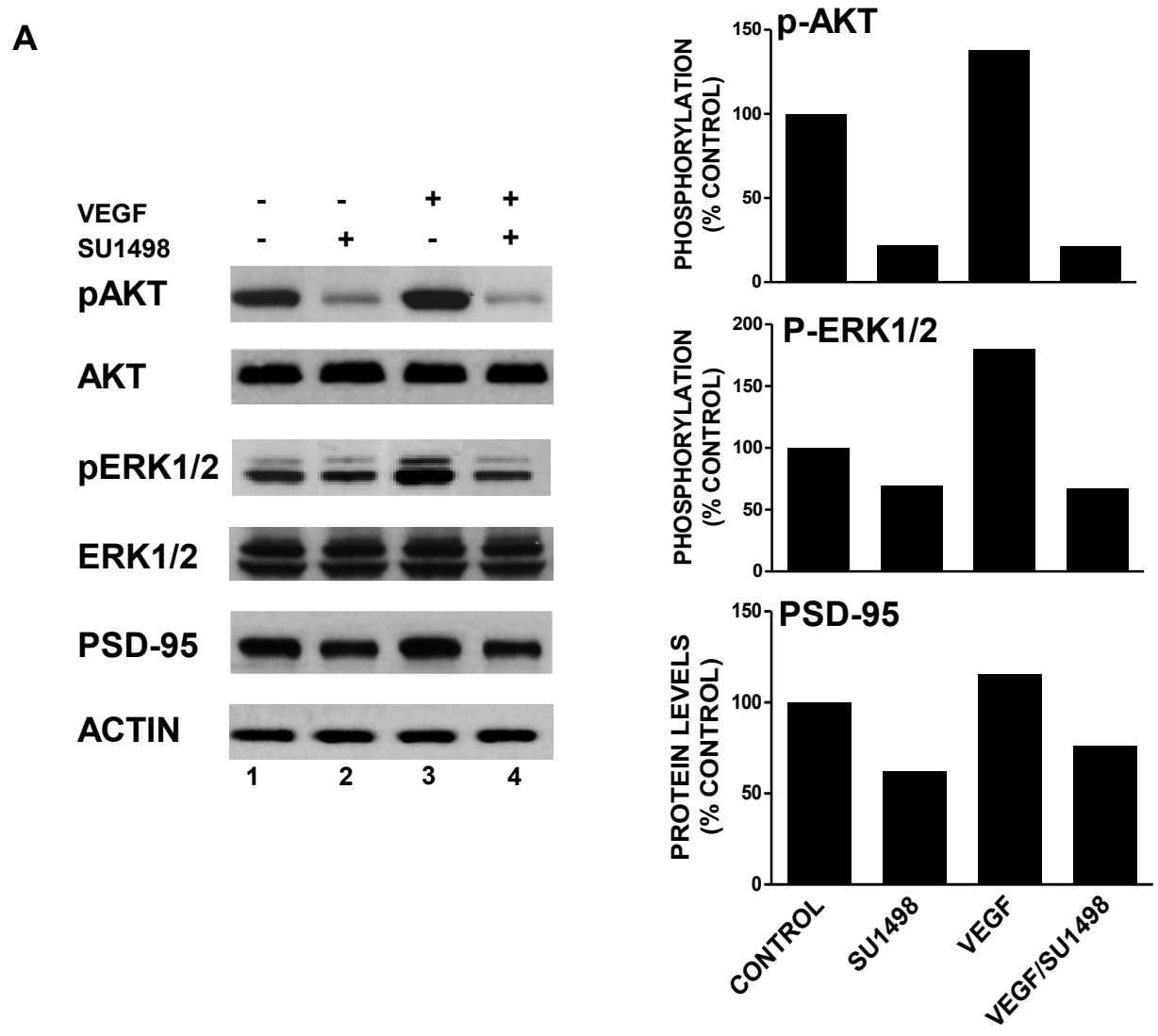


Figure 4.1

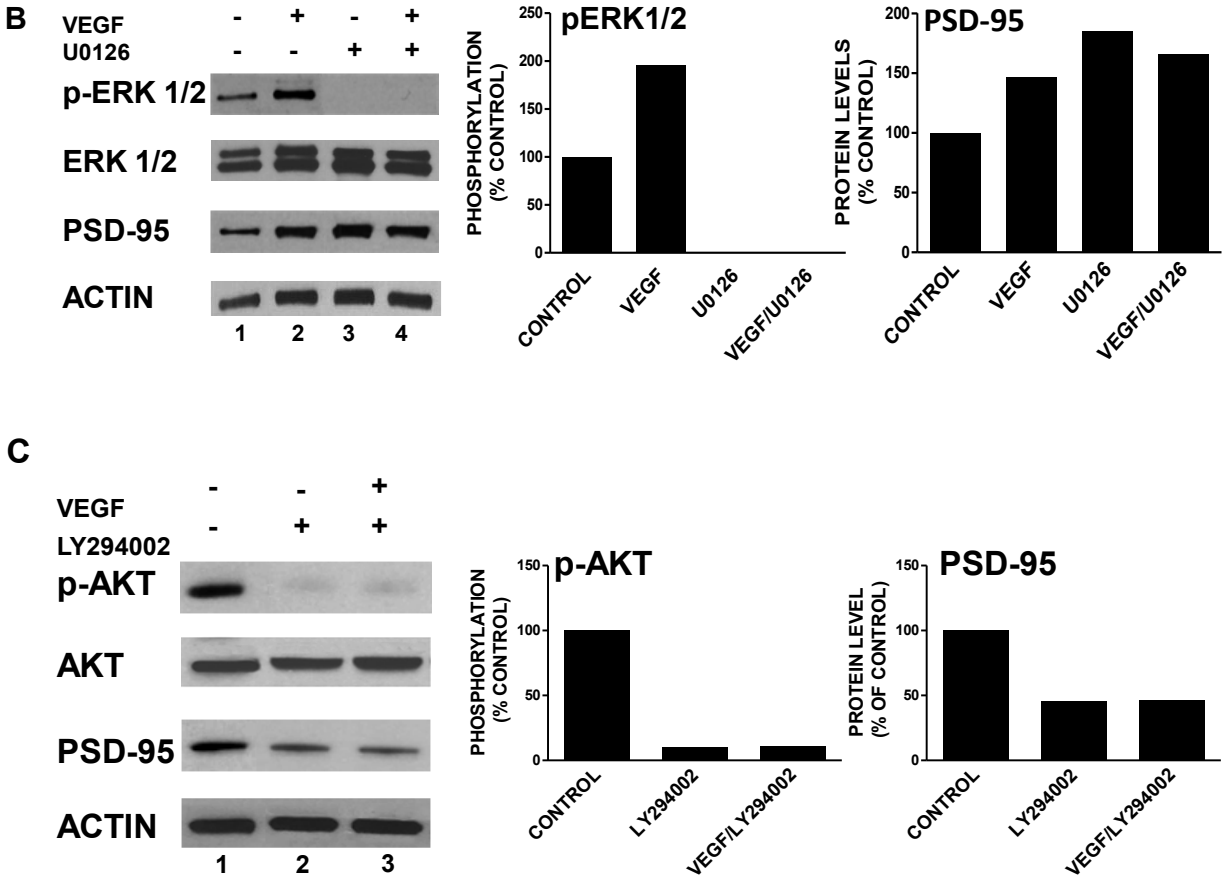


Figure 4.1 VEGF signals PSD95 upregulation through the PI3K/Akt but not MEK/ERK1/2. (A): 12 DIV primary hippocampal neurons were treated without or with 100 ng/ml VEGF in the absence and presence of 10 μM SU1498 at time frame indicated in "Materials and Methods". Lysates were subject to western blotting to detect the phosphorylated and total protein level of Akt, ERK1/2 and protein level of PSD-95. Actin was used as loading control. The phosphorylation level of Akt and ERK1/2 as well as protein level of PSD-95 were quantified relative to DMSO treated control (100%) (right panel). (B): Cells were treated without or with 100 ng/ml VEGF in the absence and presence of 10 μM MEK/ERK1/2 inhibitor U0126 as indicated in "Materials and Methods". Western blotting was performed and blots were probed with anti-phospho-ERK1/2 (**p-EKK1/2**), anti-ERK1/2 and anti-PSD-95. Actin was used as loading control. The phosphorylation level of ERK1/2 and protein expression level of PSD-95 were quantified relative to the vehicle control (100%) in the right panel. (C): Neurons were treated with VEGF as in (A) without and with 20 μM LY294002 as described in "Materials and Methods". Lysates were subjected to western blotting to detect PSD-95 level and phosphorylated Akt level (**p-AKT**) under these conditions, which is quantified and shown in the right panel. Data are representative of one of at least three independent experiments.

4.2 VEGF/VEGFR2 regulates PSD95 through the mTOR pathway

Since Akt signals the activation of the mTOR pathway and it plays a critical role in synaptic function (Lee et al., 2005; Yang et al., 2008; Yoshii and Constantine-Paton, 2007b), and VEGF stimulated PSD-95 levels via Akt, subsequent experiments addressed whether mTOR plays a role in the regulation of PSD-95 expression by VEGF. To answer this question, cells were treated with or without VEGF in the absence and presence of SU1498 as indicated. Lysates were collected for western blotting to detect the phosphorylated and total protein levels of mTOR substrates as well as the protein levels of PSD-95. The results in **Figure 4.2A** show that PSD-95 expression and the phosphorylation levels of the mTOR substrates S6K, S6 and 4EBP1 were enhanced by treatments with VEGF (**compare lane 2 with lane 1**) and blocked by SU1498 (**compare lane 4 with lane 2**). These findings suggest that VEGF/VEGFR2 pathway mediated its increased expression of PSD-95 through mTOR. To further confirm these data, cells were treated with the allosteric and active site inhibitors of mTOR, rapamycin and PP242 respectively and assessed for PSD-95 expression. Results show that mTOR inhibition blocked the activation of S6K, S6, 4EBP1, Akt and the VEGF mediated increase in PSD-95 protein levels (**Figure 4.2B, compare lane 3, 4 with lane 2)**. Interestingly, rapamycin elicited a partial inhibition of 4EBP1 phosphorylation as reported for various cancer cell lines (Choo et al., 2008; Hsieh et al., 2010). Furthermore, the dramatic decreased phosphorylation level of Akt, 4EBP1 correlates with a complete blockade of PSD-95, suggesting VEGF regulates PSD-95 via activation of mTORC2 that is inhibited by PP242. Immnofluorescent assays also showed that mTOR inhibition attenuated the levels of PSD-95 along dendrites relative to a DMSO-

treated control (**Figure 4.2C).** In this experiment, cells were treated without or with mTOR inhibitor rapamycin and PP242 and then stained with anti-PSD-95 (**red, a-c**). Insets (**white boxes**) from **a-c** were magnified in **d-f** and quantified in **g** to show that mTOR inhibition suppressed PSD-95 density. Cell viability was also measured in cells treated with or without VEGF in the absence and presence of mTOR inhibitors rapamycin and PP242 for 6 hours or 24 hours (**Figure 4.2D**). The results demonstrated that both short (**6 hr**) and long term (**24 hr**) mTOR inhibition decreased the stimulation of cell viability by VEGF. Together, these findings support the notion that VEGF/VEGFR2 pathway promotes survival and synapse numbers through the PI3K/mTOR pathway in differentiated hippocampal neurons.

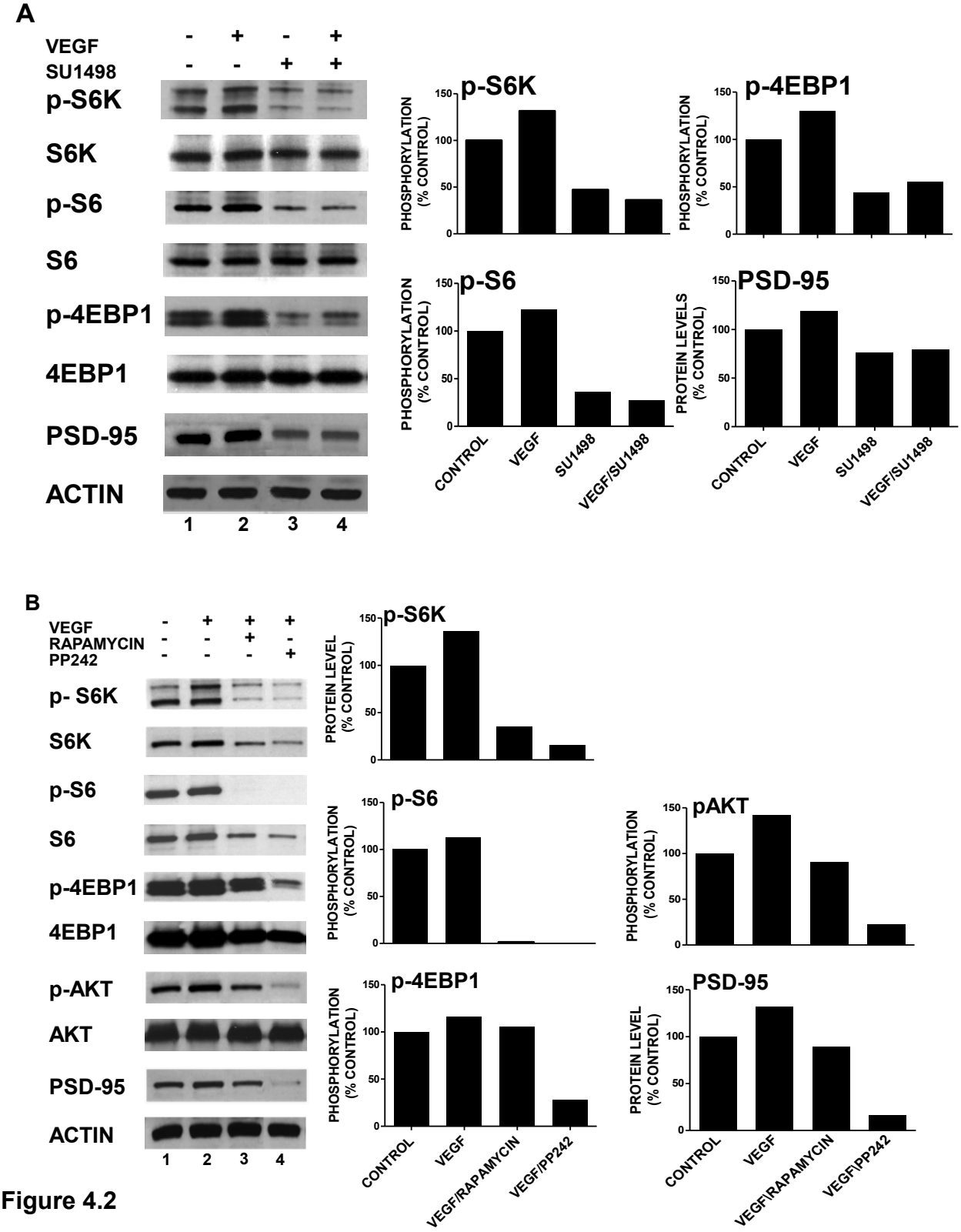
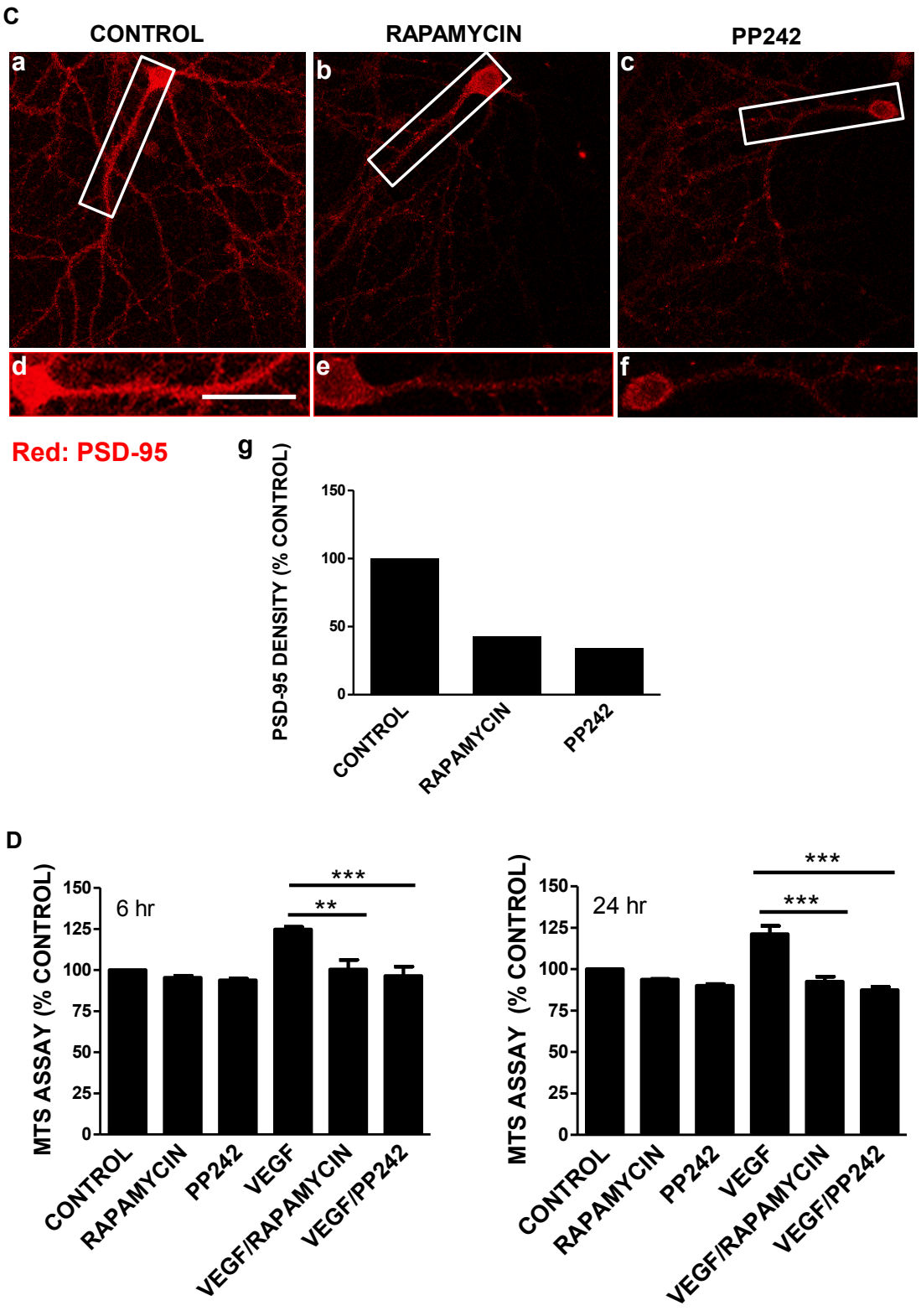

A
VEGF
SU1498
p-S6K
S6K
p-S6
S6
p-4EBP1
4EBP1
PSD-95
ACTIN
1 2 3 4
p-S6K
p-4EBP1
p-S6
PSD-95
PHOSPHORYLATION (% CONTROL)
PROTEIN LEVELS (% CONTROL)
CONTROL
VEGF
SU1498
VEGF/SU1498
B
VEGF
RAPAMYCIN
PP242
p- S6K
S6K
p-S6
S6
p-4EBP1
4EBP1
p-AKT
AKT
PSD-95
ACTIN
1 2 3 4
p-S6K
p-S6
pAKT
p-4EBP1
PSD-95
PROTEIN LEVEL (% CONTROL)
PHOSPHORYLATION (% CONTROL)
CONTROL
VEGF
VEGF/RAPAMYCIN
VEGF/PP242

Figure 4.2


C
CONTROL
RAPAMYCIN
PP242
a
b
c
d
e
f
Red: PSD-95
g
PSD-95 DENSITY (% CONTROL)
150
100
50
0
CONTROL
RAPAMYCIN
PP242
D
MTS ASSAY (% CONTROL)
6 hr
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150
125
100
75
50
25
0
CONTROL
RAPAMYCIN
PP242
VEGF
VEGF/RAPAMYCIN
VEGF/PP242
MTS ASSAY (% CONTROL)
24 hr
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150
125
100
75
50
25
0
CONTROL
RAPAMYCIN
PP242
VEGF
VEGF/RAPAMYCIN
VEGF/PP242

Figure 4.2

Figure 4.2 VEGF/VEGFR2 regulates PSD-95 through the mTOR pathway. (A): Neurons were treated with or without 100 ng/ml VEGF in the absence and presence of 10 μM SU1498 in NB at time frame indicated in "Materials and Methods". Lysates were subjected to western blotting to detect the phosphorylated and total protein level of mTOR substrates S6K, S6 and 4EBP1 as well as protein level of PSD-95. Actin was used as loading control. The phosphorylation level of S6K, S6 and 4EBP1 as well as protein level of PSD-95 were quantified relative to % of DMSO-treated control (**right panel**). (B): Cells were treated without and with 100 ng/ml VEGF alone or in the presence of 1 μM mTORC1 inhibitor rapamycin (**lane 3**) or 2.5 μM mTORC1 and mTORC2 inhibitor PP242 (**lane 4**). Actin is used as loading control. The phosphorylation and total level for mTOR pathway substrates and protein expression level for PSD-95 were quantified relative to DMSO treated control (**right panel**). (C) Cells were treated without or with mTOR inhibitors rapamycin and PP242 in NB at time frame described in "Materials and Methods". Cells were fixed and stained with anti-PSD-95 (**red**) for immunofluorescent imaging. Insets (**white boxes**) in **a-c** are magnified in **d-f** to show PSD-95 localization in the soma and dendrite. Quantification of PSD-95 density alone dendrites in **d-f** relative to % vehicle control is shown in panel **g**. (D) Neurons were treated with 1 μM rapamycin or 2.5 μM PP242 alone or in combination with 100 ng/ml VEGF at 6 hours or 24 hours as indicated. Cells were then assayed for viability as described in "Materials and Methods". Cell survival was expressed as the percent viability relative to the vehicle-treated control (100%) ±S.E.M from at least three independent experiments. ** $P<0.01$, *** $P<0.001$.

Chapter 5

ROCK is a negative inhibitor of VEGF-stimulated neurite outgrowth and synaptic connection

It has been well documented that VEGF promotes neurite extension and neuroprotection through its cognate receptor, VEGFR2 (Bocker-Meffert et al., 2002; Jin et al., 2001; Jin et al., 2006; Matsuzaki et al., 2001; Sondell et al., 2000) . ROCK was also shown to enhance VEGF-mediated neurite extension in cortical neurons (Jin et al., 2006) but increase dendrite length and branching in hippocampal neurons (Nakayama et al., 2000). To delineate the regulatory role of ROCK in VEGF signaling, the effects of ROCK inhibition were examined on 1) VEGF-mediated neurite outgrowth and viability in serum-deprived human SK-N-SH neuroblastoma cells and on 2) VEGF-mediated dendritic length, cell viability and synaptic protein levels in rat primary hippocampal neurons.

5.1 ROCK inhibition stimulates a concentration dependent increase in neurite outgrowth and survival in serum starved SK-N-SH neuroblastoma cells

To examine a role for ROCK on VEGF's neuroprotective and neurotrophic effect serum starved SK-N-SH neuroblastoma cells were treated with 1-10 μM ROCK inhibitor Y-27632 with and without 10 ng/ml VEGF and assayed for cell viability (**Figure 5.1A**) as described in "Material and Methods". The results showed that ROCK inhibition enhanced the cell viability in a concentration dependent manner that was further increased by VEGF. Since 7.5 μM Y-27632 produced the optimal cell viability hence subsequent experiments were performed with this concentration of inhibitor. To assess whether ROCK is involved in VEGF-stimulated neurite extension, SK-N-SH cells were treated without (**Figure 5.1 B; a, b**) or with 7.5 μM Y-27632 (**c, d**) in serum free medium for 48 hours in the absence (**a, c**) and presence (**b, d**) of 10 ng/ml VEGF as indicated in "Material and Methods". Cells were stained with actin (**red**) and Map2 (**green**) to show

the neurites. Unlike previous findings (Jin et al., 2006), immunofluoresent staining showed that both VEGF and ROCK inhibition dramatically promotes neurite extension outgrowth in SK-N-SH neuroblastoma cells (**arrows; b, c, d**).

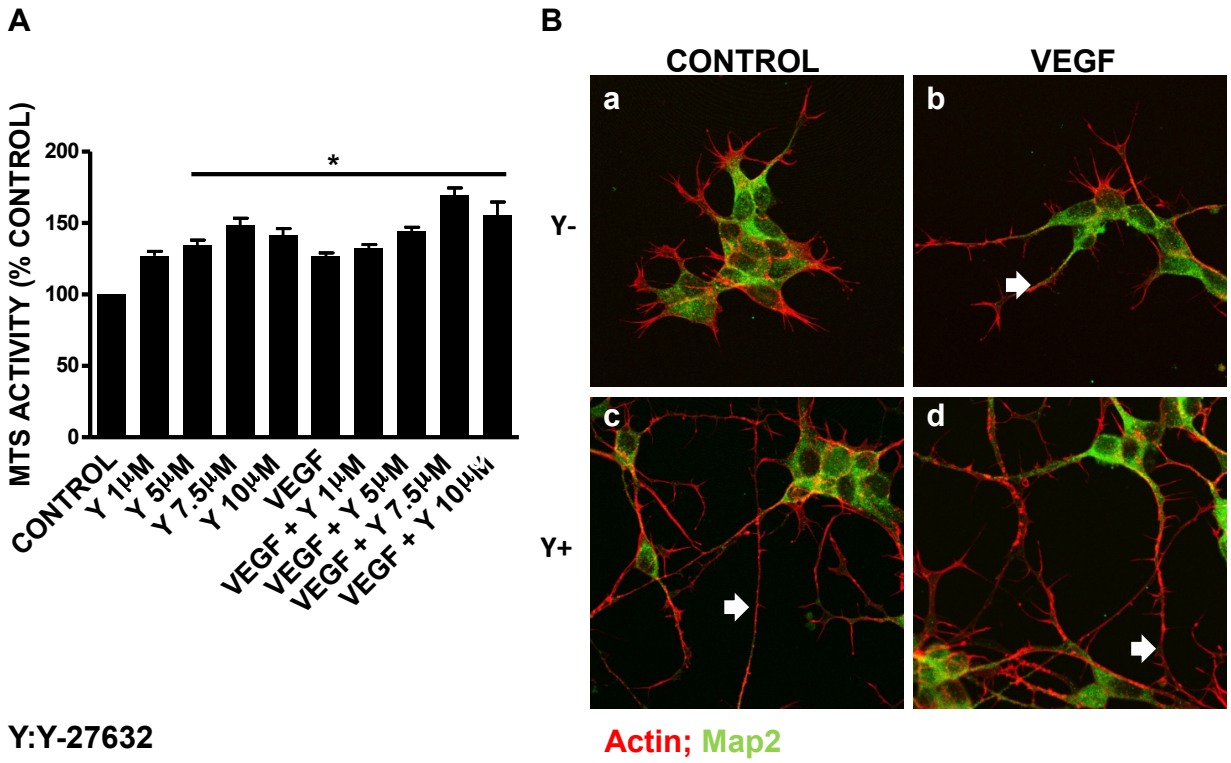

Figure 5.1 ROCK inhibition stimulates a concentration dependent increase in neurite outgrowth and survival in serum starved SK-N-SH neuroblastoma cells. (**A**) SK-N-SH neuroblastoma cells were cultured, and treated with 1-10 μM Y-27632 without and with VEGF as described in "Material and Methods". ROCK inhibition promotes cell survival in a concentration dependent way, which is exaggerated with cotreatment of VEGF. Results represent the percent cell viability relative to the 100% control value ± S.E.M from at least three independent experiments, *P<0.05. (**B**) Cells were treated without (**a, b**) or with (**c, d**) 7.5 μM Y-27632 and 10 ng/ml VEGF alone (**b**) or in combination (**d**). Neurite extensions (**arrows**) were analyzed using double immunofluorescence by staining cells with rhodamine phalloidin for actin (**red**) and the neuronal marker Map2 bound to a FITC-labeled antibody (**green**). Data are representative of one of at least three independent experiments.

5.2 ROCK inhibition stimulates neurite outgrowth via VEGFR2 in primary DIV 7 hippocampal neurons

To test whether ROCK inhibition stimulated neurite extension in rat primary hippocampal neurons in a VEGFR2-dependent manner, 5 DIV hippocampal neurons were treated without (**Figure 5.2 A, a-d**) and with VEGF (**e-h**) in the absence (**a, b, e, f**) and presence of Y-27632 (**c, d, g, h**) in NB for 48 hours. Recent studies showed that neurite extension was VEGF and VEGFR2-dependent (Bocker-Meffert et al., 2002; Jin et al., 2006; Sondell et al., 2000). To address whether ROCK inhibition influenced VEGFR2 inhibition cells were also treated with SU1498 for the last 6 hours of the 48 hours incubation (**b, f, d, h**). 7 DIV neurons were stained with actin (**red**) and Map2 (**green**) to show the neurite extensions. Immunofluoresent image show that both VEGF and Y-27632 stimulates neurite extension (**arrow; e, c**) which was blocked by VEGFR2 inhibition (**arrow; d, h**). Quantification of neurite length is plotted as the fold change in length relative to % of vehicle control (**Figure 5.2B).**

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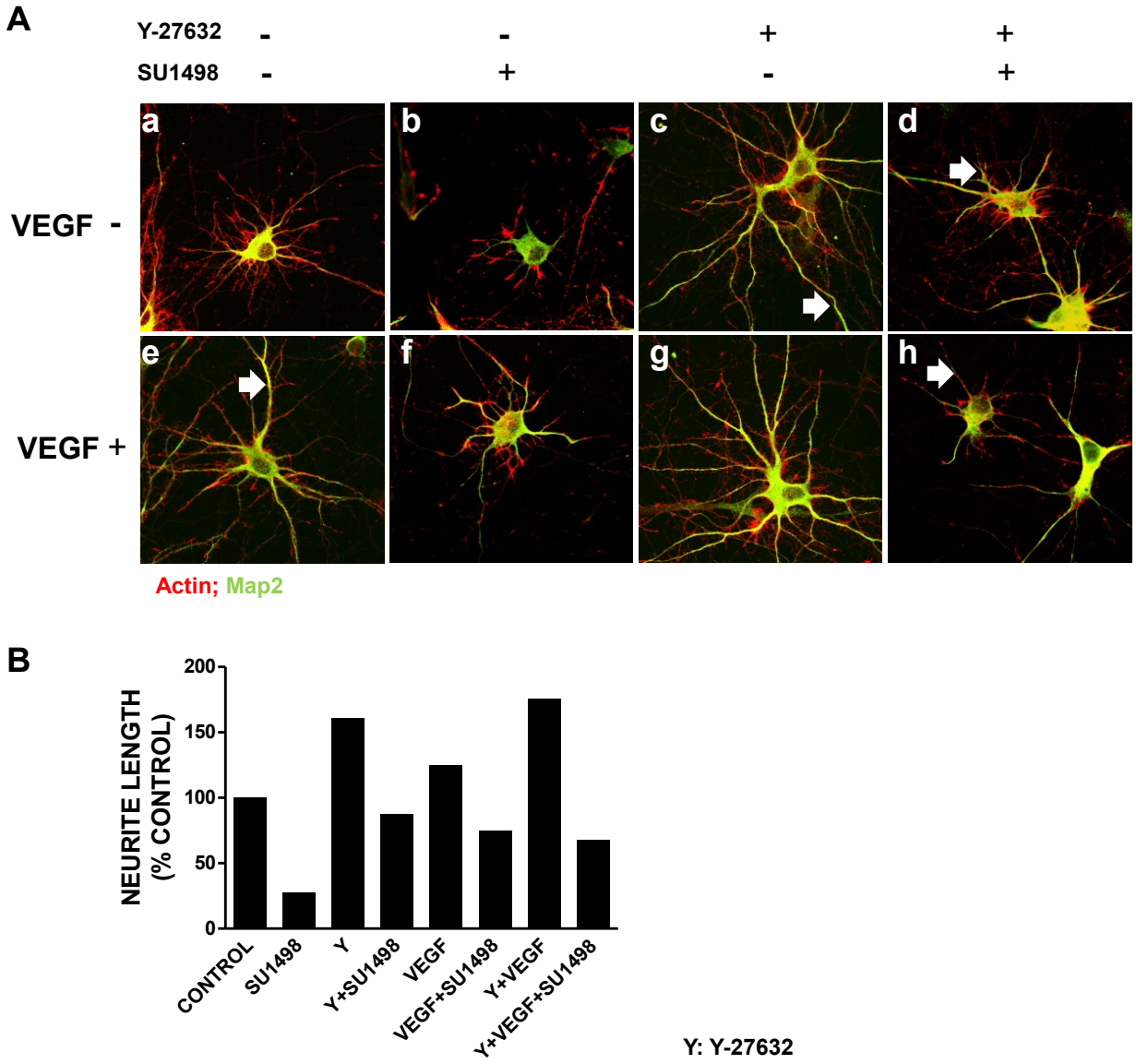

Figure 5.2 ROCK inhibition stimulates neurite outgrowth via VEGFR2 in primary 7 DIV hippocampal neurons. Rat primary hippocampal neurons were isolated and treated without and with 7.5 μM Y-27632 (**Y**), 100 ng/ml VEGF and/or 10 μM SU1498 as described in "Materials and Methods". (A) Cells were stained for actin (**red**) and the neuronal marker Map2 (**green**) to assess neurite outgrowth (**arrows**). (B) Neurite length from A was quantified. Results are typical of the neurite length observed in at least 3 independent experiments.

5.3 ROCK inhibition stimulates cell survival and the levels of the presynaptic protein synaptophyin in 14 DIV rat hippocampal neurons through VEGFR2

Actin is the most abundent cytoskeletal componet of in dendritic spines (Fischer et al., 1998; Matus, 2000). Dynamic polarization and depolarization of actin contributes to the formation of dendritic spines and the morphological changes of synapses (Ethell and Pasquale, 2005a; Lippman and Dunaevsky, 2005). More evidences show tht In addition, RhoA/ROCK-mediated myosin light chain phosphorylation and actomyosin contractility leads to synapse retraction and loss (Fukata et al., 1998; Hirose et al., 1998b; Jalink et al., 1994).

To assess the role for ROCK on synapses, 12 DIV rat primary hippocampal neurons were untreated (**Figure 5.3A, a**) or treated with 7.5 μM Y-27632 (b) or 100 ng/ml VEGF (c) either alone or both in combination (d) or each with 10 μM SU1498 (e, f) in NB for 48 hours. 14 DIV neurons were fixed and stained with anti-synaptophysin (**green**) and rhodamine phalloidin actin (**red**). A segment of the dendrite (**white box**) from the each treatment is magnified (**Figure 5.3B, a-f)**. Results show that Y-27632 mimics VEGF in increasing the protein levels of synaptophysin (**compare c with d**) and this stimulatory effect was blocked by VEGFR2 inhibition (**e, f**). A quantification of the presynaptic synaptophysin density for these treatments is shown in **Figure 5.3C**. These results show that ROCK inhibition is more stimulatory than VEGF in increasing synaptophysin levels. Furthermore, the increased level of synaptophysin by VEGF or Y-27632 correlated with an increase in cell viability (**Figure 5.3D**) that was greater when neurons were treated with both in combination.

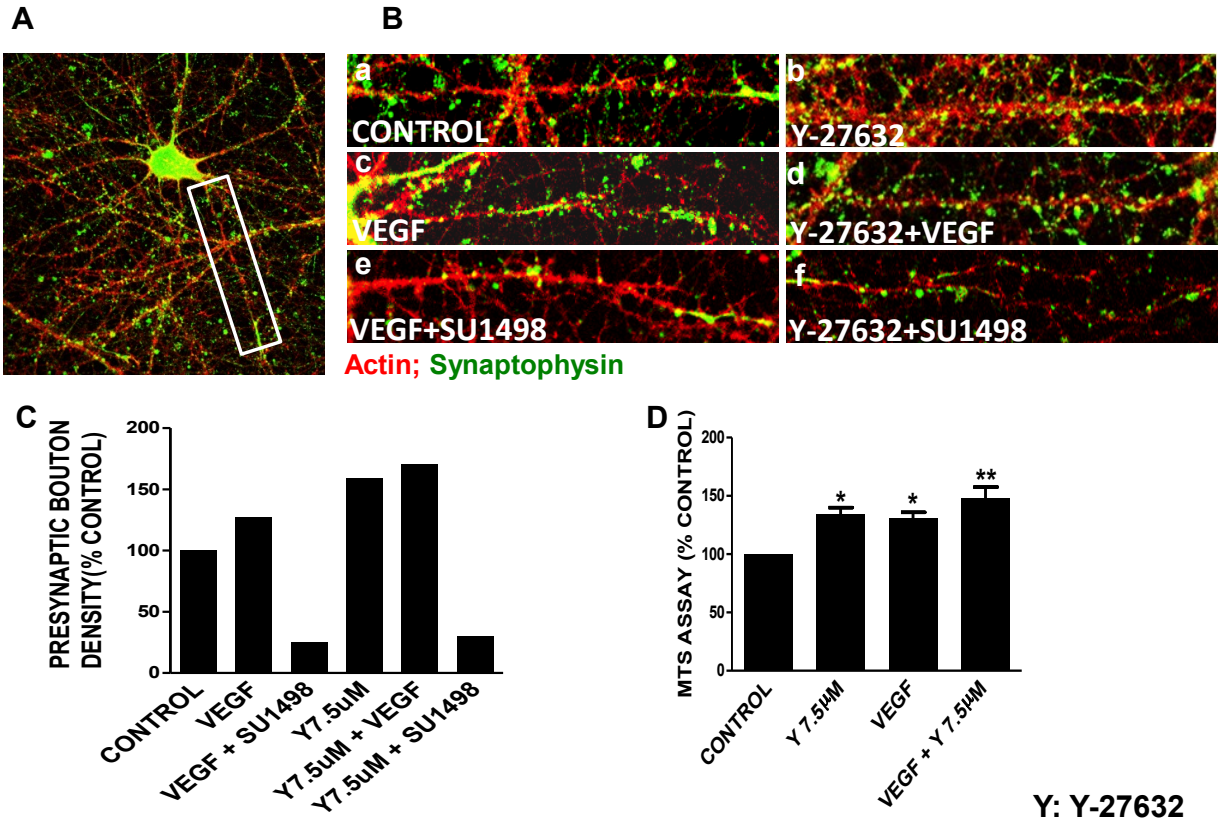

Figure 5.3 ROCK inhibition stimulates cell survival and the levels of the presynaptic protein synaptophysin in 14 DIV rat hippocampal neurons through VEGFR2. Primary hippocampal neurons cells were isolated, cultured and treated with Y-27632 (Y), VEGF and/or SU1498 as described in the "Materials and Methods". (A): Dendrites from 14 DIV hippocampal neurons were detected by staining for actin (**red**) and for FITC-labeled synaptophysin in presynaptic boutons (**green**). One dendrite (**white box in A**) from each treatment is enlarged in (**B**) and a quantification of its presynaptic bouton density is shown in (**C**). Results are typical of 3 independent experiments. (D) Cells were assessed for viability. Results represent the corresponding percent cell viability measurements relative to the 100% control value ± S.E.M from at least three independent experiments. *P<0.05; **P<0.01.

5.4 ROCK inhibition increases the levels of PSD-95 and the activation of Akt and the mTORC1 substrates S6K, S6 and 4EBP1 in a VEGFR2 dependent manner

My previous work demonstrated that VEGF stimualtes synaptic connection by upregulation of the postsynaptic protein PSD-95 level via PI3K/Akt/mTOR pathway. To

further examine whether ROCK is involved in VEGF-stimulated synapse number, 12 DIV rat hippocampal neurons were treated with 7.5 μM Y-27632 in the absence and presence of 100 ng/ml VEGF (**Figure 5.4 A**) or 10 μM SU1498 (**Figure 5.4 B**) in NB as indicated in "Materials and Methods". Lysates from these treatments were harvested for western blotting to detect the phosphorylated and total protein levels of mTOR downstream targets S6K, S6 and 4EBP1, and PSD95 protein levels as well as phosphorylated and total Akt levels. Results showed that ROCK inhibition enhances the activation of Akt, PSD95 and mTORC1 substrates S6K, S6 as well as 4EBP1 to almost the same extent as VEGF (**Figure 5.4 A, compare lane 2 and lane 3**), which is blocked by SU1498 (**Figure 5.4 B, compare lane 3 with lane 2**). Together these data indicate that ROCK plays a negative role in VEGF-stimulated neurite extension, viability and PSD-95 protein levels through Akt/mTOR pathway. It stimulates PSD95 as well as mTORC1 substrates and Akt in a VEGFR2 dependent way.

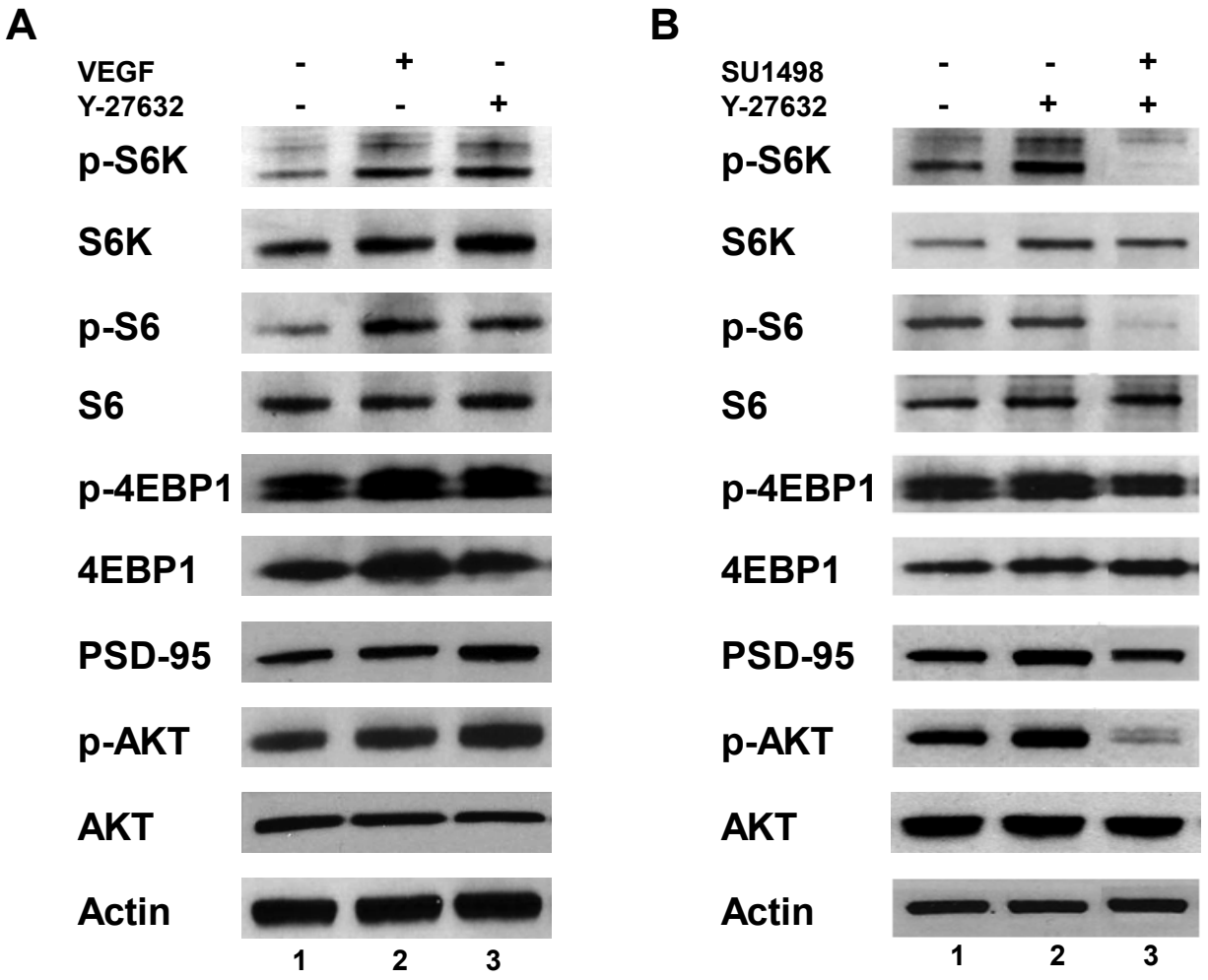

Figure 5.4 ROCK inhibition increases the levels of PSD-95 and the activation of Akt and the mTORC1 substrates S6K, S6 and 4EBP1 in a VEGFR2 dependent manner. 14 DIV primary hippocampal neurons were treated with 7.5 μM ROCK inhibitor Y-27632 in the absence and presence of 100 ng/ml VEGF for 48 hours (**A**) or 10 μM SU1498 for 6 hours (**B**) in NB. Lysates from (**A**) and (**B**) were subjected to western blot analysis for detection of the phosphorylated protein and total protein levels of mTORC1 substrates (S6K, S6 and 4EBP1), PSD-95 and the phosphorylated and total Akt. Actin served as the protein loading control. Data are representative of one of at least three independent experiments.

Chapter 6

Discussion

Whereas increasing evidence shows that VEGF regulates synapse transmission in addition to neuroprotection, the signaling events underlying this finding have not been elucidated. This work provides evidence that VEGF modulates the protein expression levels of the post-synaptic protein PSD-95, synapse numbers, dendritic branching and survival through its cognate receptor VEGFR2 in differentiated (14 DIV) primary hippocampal neurons (**Figure 3.2, Figure 3.3, Figure 3.5** and **Figure 3.6**). These findings are in line with evidence that VEGF can enhance synapse transmission in addition to its angiogenic and neuroprotective properties. Under the experimental conditions in this work, VEGF is secreted from NB cultured cells to mediate VEGFR2 phosphorylation in an autocrine fashion that is further enhanced by the inclusion of exogenous VEGF (**Figure 3.2C**). When compared to incubations with NB/B27, cells cultured in NB show higher levels of released VEGF which I attribute to a stress response induced by omitting B27. These findings are supported by evidence that VEGF and VEGFR2 activation is upregulated under various stress conditions (Ruiz de Almodovar et al., 2009b) and studies showing that VEGF is released from rat hippocampal neurons follows membrane depolarization (Kim et al., 2008). The loss in cell viability by VEGFR2 (**Figure 3.2B**) is consistent with previous studies showing that VEGFR2 protects hippocampal neurons from damage induced by cerebral ischemia (Jin et al., 2000b) and hypoxia and glucose deprivation (Jin et al., 2000a). Notably, the neuroprotection elicited by VEGF is accompanied by increased levels of PSD-95 that are VEGFR2-dependent (**Figure 3.3** and **Figure 3.6**). Moreover, VEGF stimulated a colocalization of VEGFR2 with PSD-95 (**Figure 3.4**) as well as an increase in synapse numbers as determined by the colocalization of PSD-95 with the presynaptic protein

synaptophysin (**Figure 3.5**) and synapsin I (**Figure 3.6**). The fact that these events were attenuated by VEGFR2 inhibition implicates VEGF/VEGFR2 as an important regulatory mechanism of PSD-95 localization in synapses. In support of this finding, treatments with BDNF were shown to increase PSD-95 levels dendritic spines (Hu et al., 2011; Yoshii and Constantine-Paton, 2007a) while PSD-95 was shown to colocalize with the BDNF receptor TrkB in the postsynaptic density of hippocampal synapses (Ji et al., 2005b). Similarly, the insulin receptor localizes within the postsynaptic density of synapses of primary hippocampal neurons as a regulator of neurotransmitter receptor trafficking and synapse number (Chiu and Cline, 2010).

PSD-95 is a marker of synaptic strength through its function as a scaffold protein that couples postsynaptic receptors to downstream effector signaling pathways. For example, PSD-95 anchors NMDA receptors within the PSD through a direct interaction with its NR2B subunit (Kornau et al., 1995). The finding that VEGFR2 also interacts with the NR2B receptor (Meissirel et al., 2011) and data in this study showing that VEGF stimulates activated VEGFR2 to colocalize with PSD-95 and increase synapse number in dendritic spines provide a plausible role for VEGF signaling in regulating synaptic transmission and stability. In support of this concept, a blockade of TrkB signaling was shown to result in the disassembly of postsynaptic acetylcholine receptor clusters at neuro-muscular junctions (Gonzalez et al., 1999) and reduce the number of NMDA receptor clusters at hippocampal synapses (Elmariah et al., 2004). In a similar fashion, VEGFR2 was shown to interact with NMDA receptor subunits NR1 and NR2B before synapse formation, an event that enhances NMDA stimulation of immature granule cells in response to VEGF (Meissirel et al., 2011). Since NMDA receptor plays a critical role

in synaptic plasticity in hippocampal neurons and can interact with PSD-95 and VEGFR2, these data raise the possibility that VEGFR2, NMDA and PSD-95 interact within the postsynaptic density synapses as a complex that modulates synaptic transmission.

The VEGF-mediated increase in dendritic PSD-95 (**Figure 3.4**) and its reduction by VEGFR2 inhibition (**Figure 3.3**) also implicates VEGF as a possible regulator of PSD-95 localization from the soma to dendrites and synapses as shown for the BDNF/TrkB pathway in developing neurons (Yoshii and Constantine-Paton, 2007a). The observation that the decrease in PSD-95 and synapse number induced by SU1498 (**Figure 3.5**) is accompanied by a loss in cell viability (**Figure 3.2B**) links VEGFR2-directed neuroprotection with synapse stability in hippocampal neurons. This notion is supported by evidence that VEGF and VEGFR2 upregulation can mediate neuroprotection in the hippocampus by modulating synaptic transmission resulting from seizure-induced excitotoxicity in both in vitro and in vivo models of epilepsy (Cammalleri et al., 2011; McCloskey et al., 2005b).

To show further similarities between the VEGF/VEGFR2 and BDNF/TrkB pathways, these experiments show that whether VEGF, like BDNF, signals the activation of the PI3K/Akt and MEK/ERK1/2 pathways in hippocampal neurons (Almeida et al., 2005). However, inhibitor studies revealed that a blockade of VEGFR2, PI3K but not MEK activation decreased PSD-95 levels (**Figure 4.1**), suggesting that VEGF/VEGFR2 regulates PSD-95 through the PI3K/Akt pathway. This finding is consistent with reports that the PI3K/Akt pathway regulates the translation and trafficking of PSD-95 in response to BDNF and insulin in neurons (Yoshii and

Constantine-Paton, 2007b) and stimulates hippocampal synaptic plasticity (Lee et al., 2005; Yang et al., 2008). In addition, PI3K/Akt was shown to serve as a downstream effector of neuroprotection mediated by BDNF, insulin and VEGF (Jin et al., 2000b; Kilic et al., 2006b; Zheng and Quirion, 2004).

The mTOR pathway regulates neuron development and survival and plays a critical role in synaptic plasticity, learning and memory formation in the adult brain (Swiech et al., 2008). The demonstration that VEGF activates the mTOR substrates S6K, S6 and 4EBP1 in a VEGFR2-dependent fashion (**Figure 4.2**) is supported by previous studies in hippocampal neurons (Kim et al., 2008). However, my studies are unique by linking activation of the mTOR pathway through VEGFR2 with the increase in PSD-95 protein levels (**Figure 4.2A**, **compare PSD-95 levels in lane 2 with lane 4**) and enhanced viability mediated by VEGF (**Figure 3.2**). In this context, rapamycin blocks the increase of PSD-95 (**Figure 4.2B**) and viability (**Figure 4.2D**) augmented by VEGF in hippocampal neurons. Therefore, these data implicate the PI3K/Akt/mTOR pathway in mediating the stimulation of synaptic stability and neuroprotection by VEGF in differentiated hippocampal neurons. This notion is supported by evidence that BDNF and insulin signal protein synthesis through mTOR in neuronal dendrites (Takei et al., 2004) as well as the protein levels of PSD-95 in spines (Hu et al., 2011). Similarly, insulin stimulates translation of local protein synthesis of PSD-95 in neuronal dendrites through the PI3K/Akt/mTOR pathway (Lee et al., 2005) . Given these findings, it is conceivable that the signaling of increased PSD-95 protein levels directed through VEGFR2 involves mTOR-mediated protein synthesis in hippocampal neuronal dendrites.

Evidence suggests that the activation of VEGFR2 by VEGF mediates learning through increased neurogenesis in adult hippocampal neurons (Cao et al., 2004). Moreover, recent studies show that VEGF can regulate synaptic plasticity independently of its role in neurogenesis and angiogenesis (Licht et al., 2011b). Therefore, these findings provide a molecular mechanism for these reported roles of VEGF in enhancing neuronal plasticity. It is unclear from my studies whether VEGFR2 inhibition reduces PSD-95 and synapse levels along dendrites by inducing a disassembly of preformed synapses or by blocking the dendritic localization of PDS-95 required for synapse formation. In either case, the synapse loss induced by VEGFR2 inhibition would disrupt signaling pathways that maintain neural connections required for optimal functioning of hippocampal processes of memory and learning. In support of this notion, a decrease in synapse number correlates with the cognitive decline and memory loss in neurological disorders such as AD (Penzes et al., 2011). Moreover, synapse loss precedes amyloid plaque accumulation and neuronal cell death in AD. Therefore, factors that maintain synapse function would serve as an effective strategy for preventing neurodegeneration. The findings in this work suggest that the VEGFR2 pathway elicits neurotrophic effects that overlap those mediated by BDNF and insulin. Thus, my studies reveal a novel VEGF-mediated signaling mechanism that has therapeutic potential for preventing synapse loss in neurodegenerative diseases.

On other hand, VEGF and ROCK are all intensely characterized for their regulatory role to stimulate neurite extension (Bocker-Meffert et al., 2002; Jin et al., 2006; Khaibullina et al., 2004; Sondell et al., 2000). Evidence shows that VEGF regulates neurite extension via Rho/ROCK pathway in cortical neurons. Blockade of

VEGFR2 or ROCK both diminish VEGF-stimulated neurite extension (Jin et al., 2006). Consistent with these findings, VEGF promote neurite outgrowth both in SK-N-SH neuroblastoma cells (**Figure 5.1**) and primary rat hippocampal neurons (**Figure 5.2**). However, in contrast to previous finding, my work shows that ROCK inhibition enhances neurite length and cell viability alone and in combination with VEGF in SK-N-SH cells and hippocampal neurons (**Figure 5.1**, **Figure 5.2**). Although this result is conflict with Jin et al's previous study which shows VEGF regulates neurite extension through RhoA/ROCK pathway (Jin et al., 2006), this data are consistent with many studies showing that believe ROCK is a negative regulator of neurite extension (Boomkamp et al., 2012; Fuentes et al., 2008; Hirose et al., 1998a). Since activation of RhoA/ROCK pathway phosphorylates the myosin regulatory light chain (MLC), which causes growth cone collapse and processes retraction (Govek et al., 2005; Linseman and Loucks, 2008), inhibition of ROCK with Y-27632 blocks this inhibitory effect of ROCK hence leading to elongated extensions.

The actin cytoskeleton provides essential structural support for synapse maintenance, as one of the most important Rho-GTPases administrators of the cytoskeleton, RhoA and its downstream target ROCK are found as crucial players in synapse regulation (Govek et al., 2004a; Lee et al., 2000; Ruchhoeft et al., 1999; Sfakianos et al., 2007; Tashiro et al., 2000). Since the work in Chapter 3 shows that VEGF stimulates synapse formation, I tested whether ROCK is associated with this event. Consistent with the stimulatory effects on VEGF-mediated neurite extension, ROCK inhibition also increases synaptophysin which resides in presynaptic terminal numbers and cell viability that are further enhanced by VEGF (**Figure 5.3B, C**).

Moreover, a blockade of VEGFR2 decreases Y-27632-induced presynaptic bouton numbers. The result indicates that ROCK inhibition enhances presynaptic synaptophysin level through VEGFR2. These data are supported by the concept that ROCK plays an inhibitory role in dendritic spine formation and maintenance as well as in Long term potentiation (LTP) (O'Kane et al., 2004; Tashiro and Yuste, 2004). Increasing evidence shows that activation of RhoA stimulates ROCK and actomyosin contractility by controlling the phosphorylation level of MLC hence causes spine collapse and synapse loss (Govek et al., 2004b; Nakayama et al., 2000). Actomyosin contractility is essential to long-term spine maintenance and myosins are the major cytoskeletal motor proteins that enhance actomyosin contractility in nonmuscle cells. Interestingly, myosin IIB has been localized in the PSD. It plays an essential role to maintain synaptic morphology by binding to and contracting actin (Ryu et al., 2006). Therefore blocking ROCK inhibits MLC phosphorylation hence enhances dendritic spine stability by preventing spine collapse and synapse loss. Alternatively, the inhibitory role for ROCK on synaptic connection could likely due to ROCK-dependent cofilin regulation via (Lin-11, Isl-1, and mec-3 kinase) LIMKs. LIMK can be directly phosphorylated by ROCK at Thr508 and inactivate the actin depolymerization factors cofilin, which localizes in both developing and mature synapses and catalyzes the severing of actin cytoskeleton to modulate actin cytoskeletal reorganization and maintain normal synaptic connection and activity (Hotulainen et al., 2009; Racz and Weinberg, 2006).

Since ROCK negatively regulate presynaptic protein synaptophysin levels via VEGFR2 and VEGF/VEGFR2 enhances PSD-95 by signaling through PI3K/AKT/mTOR pathway, subsequent experiments addressed whether ROCK regulates postsynaptic

protein PSD-95 levels by modulated PI3K/AKT/mTOR pathway. Western blotting data shows that ROCK inhibition increases the postsynaptic protein level of PSD95, phosphorylated AKT and the phosphorylation levels of mTORC1 substrates S6K, S6 as well as 4EBP1 to almost the same level as that stimulated by VEGF (**Figure 5.4A**). Furthermore, this event is VEGFR2-dependent (**Figure 5.4B**). The increased PSD-95 level by ROCK inhibition is consistent with the notion that Y-27632 increases the number of newly formed postsynaptic spines in cultured mouse hippocampal slices (Tashiro and Yuste, 2004). Taken together, this study suggests that ROCK serves as a negative regulator of VEGF-mediated increases in PSD95 protein levels via PI3K/AKT/mTOR pathway.

Chapter 7

Conclusions and Future Directions

7.1 Conclusions

This study delineates a neurotrophic role for VEGF in mature rat hippocampal neurons by modulating the postsynaptic protein PSD-95 and providing protection against the stress induced by nutritional deprivation. I provide evidence that VEGF carries out these events by signaling through VEGFR-2 via autocrine and paracrine mechanisms. A diagram summarizing these signaling interactions is presented in Figure 6.1. The VEGF induced increase in cell viability is coincident with an upregulation of presynaptic (synaptophysin and synapsin I) and postsynaptic protein (PSD-95) levels. I also provide evidence that VEGF mimics the growth factors BDNF and insulin in signaling synapse stability in the presynaptic and postsynaptic compartments through similar downstream targets. Notably, I found that VEGF increases PSD-95 protein level and synapse numbers along dendrites via PI3K/Akt/mTOR pathway. Furthermore, I found that ROCK plays an inhibitory role in VEGF-induced, neurite extension, synapse formation and cell survival in primary hippocampal neurons. In particular, ROCK negatively regulates the VEGF-mediated stimulation of PSD-95 through the VEGFR2/Akt/mTOR pathway. Collectively, my study provides a novel role for VEGF/VEGFR-2 signaling pathway in its reported capacity to stimulate synaptic transmission. This work provides insight for developing potential therapeutic strategies to prevent or delay synapse loss in neurodegenerative diseases.

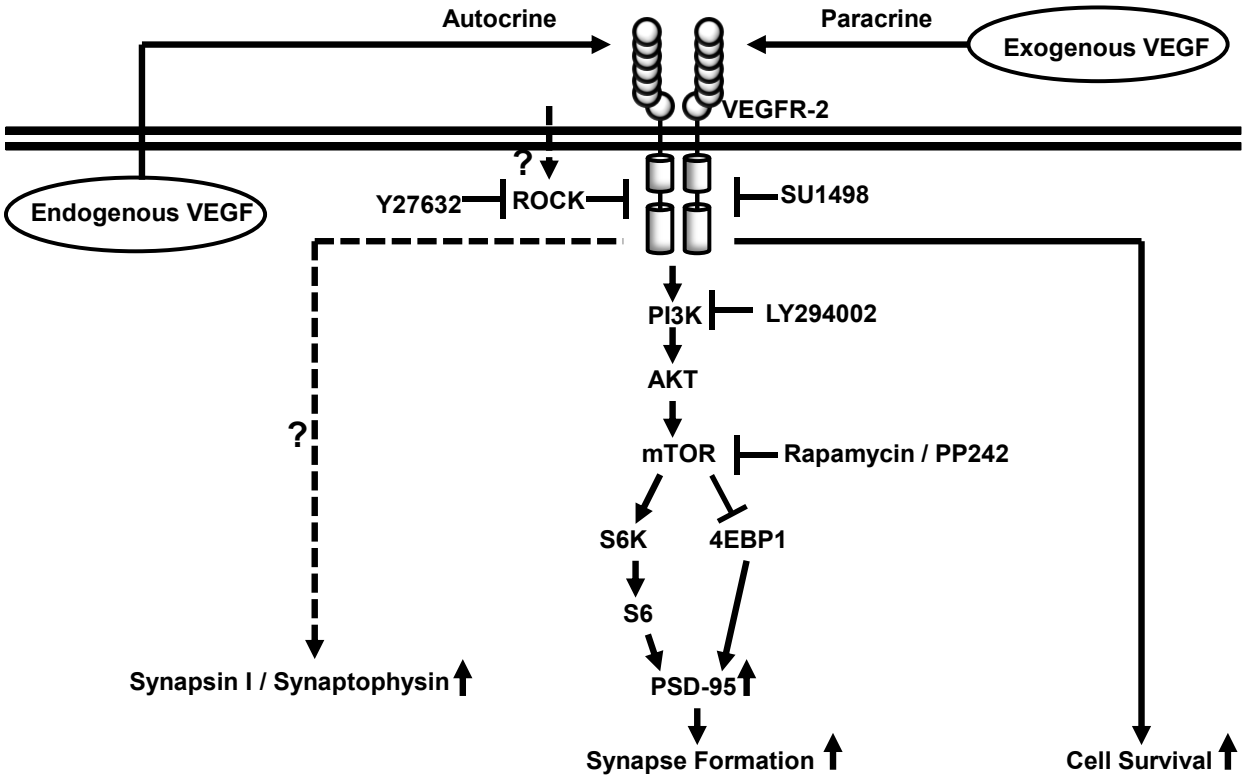

Figure 7.1 VEGF/VEGFR2 regulates synaptic protein levels in rat primary hippocampal neurons. In primary hippocampal neurons VEGF/VEGFR2 stimulates synapse formation by increasing the levels synapsin I, synaptophysin and PSD-95. PSD-95 is regulated through the PI3K/AKT/mTOR pathway. The increased PSD-95 levels are coincident with increased cell survival. ROCK negatively regulates PSD-95 via VEGFR2/Akt/mTOR pathway. Additionally, ROCK inhibition augments VEGF-stimulated synaptophysin in a VEGFR2 dependent manner.

7.2 Future studies

The results of this study demonstrated that VEGF regulates synaptic stability in mature hippocampal neurons by modulating PSD-95 levels through the VEGFR2/PI3K/AKT/mTOR pathway. The colocalization of VEGFR2 with PSD-95 by immunofluorescence suggested that both proteins may directly interact as a complex within the postsynaptic compartment. To address this possibility, immunoprecipitation

experiments would be performed to see if PSD-95 co-precipitates with VEGFR2.

The results that the increased protein levels of PSD-95 parallel the cell survival mediated by the VEGF/VEGFR2 pathway implicates a role for PSD-95 in cell survival. To test this possibility, cells would be transfected with a vector that overexpresses PSD-95 to test whether the protein can rescue neurons from the loss in cell viability induced by VEGFR2 inhibition with SU1498. Since PSD-95 protein levels are coincident with the activation levels of Akt, similar experiment would be performed by overexpressing Akt when VEGFR2 signaling is blocked. For these experiments, western blot analyses would be performed to see whether overexpression of Akt also increases the levels of PSD-95. These experiments would provide a link between VEGFR2 and Akt-mediated signaling of PSD-95 and cell viability.

Whereas the results show that VEGF increases activated VEGFR2 and PDS-95 along dendrites, the mechanism associated with this event requires further delineation. To determine whether VEGF stimulates the transport of PSD-95 along dendrites over time, fluorescent recovery after photobleaching (FRAP) and live imaging would be performed to visualize the process in real time. For these experiments, neurons are transfected with green fluorescent protein (GFP)–tagged PSD-95 in the absence and presence of VEGF. A specific region of the dendrite is subjected to photobleaching followed by imaging over time (0, 5, 10, 30 and 60 minutes) to detect the recovery (translocation) of the PSD-GFP fluorescence to the same region. The recovered PSD-GFP signal intensities in the bleached field are measured using ImageJ. If a greater level of fluorescence will be detected relative to untreated cells, which suggests VEGF stimulates PSD-95 translocation, then whether this event signals through the

VEGFR2/PI3K/Akt/mTOR pathway will be further addressed.

The results of this project also indicated that VEGF stimulates synaptic protein PSD-95 levels via the PI3K/Akt/mTOR pathway. However, the regulation of PSD-95 levels through a mTOR-dependent translation was not determined. Therefore neurons will be treated without and with VEGF in the absence and presence of translation inhibitors such as anisomycin and cycloheximide. Lysates from these cells will be examined by western blotting to detect PSD-95 levels. If VEGF-stimulated PSD-95 level is suppressed by adding translation inhibitors, these experiments would show that the VEGF increased PSD-95 levels is translation dependent via mTOR pathway.

The results also demonstrated that VEGF stimulates presynaptic proteins synaptophysin and synapsin I via VEGFR2. Since BDNF and its receptor TrKB are both expressed in pre- and postsynaptic compartments of neurons, then the possibility exists that VEGFR2 may reside in presynaptic terminals. Hence, neurons will be examined by immunofluorescence for the colocalization of VEGFR2 with synapsin I or synaptophysin. If colocalization is detected, immunoprecipitation experiments would be performed to test whether VEGFR2 interacts with presynaptic protein synaptophysin and synapsin I by cooimmunoprecipitation. Moreover, since data in this study showed that mTOR regulates the VEGF-mediated increase in postsynaptic protein PSD-95. Since evidence suggests that rapamycin blocks local presynaptic protein synthesis (Beaumont et al., 2001), experiments would address if mTOR also modulates the levels presynaptic protein synaptophysin and synapsin I in response to VEGF. To this end, VEGF-treated cells would be incubated with or without mTOR inhibitor rapamycin and PP242 and examined by western blotting and immunofluorescence for detection of synaptophysin

and synapsin I protein levels. A decrease in presynaptic protein levels caused by mTOR inhibition would suggest that the VEGF also modulate presynaptic protein levels via mTOR pathway.

Furthermore, this work indicated a negative regulatory role for ROCK in modulation of synaptic protein levels in mature hippocampal neurons. In this regard, ROCK inhibition augments a VEGFR2-mediated increase in the levels of synaptophysin and PSD-95 as well survival. To further address this mechanism, neurons will be transfected with a vector that overexpressing an inactive form of ROCK to replace the pharmacological inhibitor Y-27623 in VEGF-treated cells. Immunofluoresent imaging and western blotting would be performed to detect whether the level of synaptophysin and PSD-95 are increased when ROCK is constitutively inactivated.

My current studies delineated a neurotrophic and neuroprotective role for VEGF in modulating synaptic protein levels via VEGFR2. Further experiments are needed to characterize the specific mechanisms that VEGF signals at both pre- and postsynaptic terminals to determine the full extent of its regulation of synapse function. Collectively these experiments would provide valuable information regarding the contribution of VEGF/VEGFR2 signaling pathways to synaptic plasticity.

Chapter 8

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