Vascular Endothelial Growth Factor Prevents G93A-SOD1-Induced Motor Neuron Degeneration

J. Simon Lunn, Stacey A. Sakowski, Bhumsoo Kim, Andrew A. Rosenberg, Eva L. Feldman

Department of Neurology, University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT: Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by selective loss of motor neurons (MNs). Twenty percent of familial ALS cases are associated with mutations in Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1). To specifically understand the cellular mechanisms underlying mutant SOD1 toxicity, we have established an *in vitro* model of ALS using rat primary MN cultures transfected with an adenoviral vector encoding a mutant SOD1, G93A-SOD1. Transfected cells undergo axonal degeneration and alterations in biochemical responses characteristic of cell death such as activation of caspase-3. Vascular endothelial growth factor (VEGF) is an angiogenic and neuroprotective growth factor that can increase axonal outgrowth, block neuronal apoptosis, and promote neurogenesis. Decreased VEGF gene expression in mice results in a phenotype similar to that seen in patients with ALS, thus linking loss of VEGF to the pathogenesis of MN degeneration. Decreased neurotrophic signals prior to and during disease progression may increase MN susceptibility to mutant SOD1-induced toxicity. In this study, we demonstrate a decrease in VEGF and VEGFR2 levels in the spinal cord of G93A-SOD1 ALS mice. Furthermore, in isolated MN cultures, VEGF alleviates the effects of G93A-SOD1 toxicity and neuroprotection involves phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling. Overall, these studies validate the usefulness of VEGF as a potential therapeutic factor for the treatment of ALS and give valuable insight into the responsible signaling pathways and mechanisms involved. © 2009 Wiley Periodicals, Inc. Develop Neurobiol 69: 871–884, 2009

Keywords: amyotrophic lateral sclerosis; neuroprotection; mechanism; phosphatidylinositol 3-kinase/protein kinase B; vascular endothelial growth factor

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by progressive degeneration of upper and lower motor neurons (MNs) resulting in weakness, paralysis, and subsequent death. It has an incidence of ~ 3–5 cases per 100,000 in the United States and consists of both sporadic and familial forms, which are clinically and pathologically indistinguishable. Familial ALS accounts for $\sim 10\%$ of all cases, one fifth of which are associated with mutations in the Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1) gene, with G93A-SOD1 being the best characterized mutation (Rosen et al., 1993). Mutations in SOD1 result in a toxic gain of unknown function (Valentine et al., 2005).

Vascular endothelial growth factor (VEGF), first characterized as an angiogenic factor, is known to have neurotrophic effects in the nervous system. VEGF can increase axonal outgrowth (Sondell et al.,

© 2009 Wiley Periodicals, Inc.

Published online 11 August 2009 in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/dneu.20747

2000; Rosenstein et al., 2003), block neuronal apoptosis (Matsuzaki et al., 2001; Li et al., 2003; Sun et al., 2003), and promote neurogenesis (Jin et al., 2002; Zhu et al., 2003). Within the nervous system, there are five different isoforms of VEGF (also called VEGF-A). The most commonly studied isoform of VEGF is VEGF165; however, the full biological array of VEGF isoforms may offer increased efficacy (Carmeliet, 2003; Whitlock et al., 2004; Amano et al., 2005). VEGF receptor-2 (VEGFR2) stimulation leads to the activation of multiple signaling pathways including the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and p44/42 mitogen-activated protein kinase (p44/42 MAPK) pathways (Matsuzaki et al., 2001).

VEGF was first linked to ALS pathogenesis in 2001 by Oosthuyse et al. (2001). In the study, mice lacking the hypoxia response element in the VEGF promoter developed adult-onset muscle weakness and neurodegeneration identical to that of ALS. Further studies by the same group later identified two VEGF haplotypes associated with increased risk for developing ALS when VEGF alleles from 600 individuals with ALS and 1000 controls were sequenced (Lambrechts et al., 2003). Since that time, numerous studies have validated the link between VEGF and ALS and reports have begun to examine VEGF as a potential therapy for ALS. In spinal cord cultures, VEGF treatment protects against glutamate-induced MN death (Tolosa et al., 2008). Retrograde delivery of VEGF165 to MNs by gene therapy increases survival of G93A-SOD1 transgenic mice (Azzouz et al., 2004), and the G93A-SOD1 transgenic rat model has also shown evidence of increased survival in response to treatment with intracerebroventricular delivery of VEGF165 (Storkebaum et al., 2005).

While *in vitro* and *in vivo* studies have examined the neuroprotective potential of VEGF, the mechanisms of VEGF neuroprotection against mutant SOD1 toxicity have not been established. This study utilizes primary MN cultures transfected with G93A-SOD1 to evaluate the effects of VEGF on mutant SOD1 toxicity. G93A-SOD1 transfection of primary MNs results in biochemical and morphological defects representative of MN degeneration in ALS. We demonstrate that VEGF attenuates G93A-SOD1-induced caspase-3 cleavage and DNA fragmentation, which is mediated by activation of PI3K/Akt signaling via VEGFR2. This study proposes that VEGF activation of PI3K/Akt signaling constitutes a conserved mechanism that can intervene against cellular insults in ALS and ameliorate disease progression.

METHODS

Animal Care

All animals used for these studies were housed in a pathogen-free environment and cared for following the University of Michigan Committee on the Care and Use of Animals guidelines. Animal care standards described in the National Institute of Health *Guide for Care and Use of Laboratory Animals* are adhered to ensure limited discomfort and distress.

Isolation of Mouse Spinal Cords

End-stage G93A-SOD1 transgenic mice and age-matched controls were euthanized at 120 days of age. Whole spinal cords were collected and fresh tissue was frozen for RNA extraction or mounted in Optimal Cutting Temperature compound (Tissue-Tek, Sakura, Tokyo, Japan) prior to sectioning. Embedded spinal cords were mounted, and 18-μm sections were cut using a Leica CM 1850 cryostat onto glass slides.

Primary Motor Neuron Culture

All culture reagents were obtained from Sigma (St Louis, MO) unless otherwise noted. The primary MN treatment media includes Neurobasal Medium (Gibco BRL, Invitrogen, Carlsbad, CA) with 2.5 mg/mL albumin, 2.5 μg/mL catalase, 2.5 μg/mL SOD, 0.01 mg/mL transferrin, 15 μg/mL galactose, 6.3 ng/mL progesterone, 16 μg/mL putrescine, 4 ng/mL selenium, 3 ng/mL ß-estradiol, 4 ng/mL hydrocortisone, and 1$\times$ penicillin/streptomycin/neomycin (Gibco BRL). For initial plating, 1$\times$ B-27 additives (Gibco BRL) and 2 μM L-glutamine are also included. Growth media contains all the components of the culture media plus 1$\times$ B-27 additives.

Primary MNs were isolated according to our previously published protocol (Vincent et al., 2004). Briefly, spinal cords from E15 Sprague–Dawley rat embryos were collected, perineural membranes were removed, and the tissue was chopped into 2- to 3-mm pieces. Cells were dissociated by incubating the tissue in 0.5% trypsin/EDTA for 10 min at 37°C followed by trituration with a serum-coated glass pipette for 1 min. The resulting cell suspension was layered on a 5.4% Optiprep solution in Leibowitz's L-15 media (Gibco BRL) and centrifuged for 15 min at 2000g. The MNs were collected from the top layer, washed in fresh L-15 media, and resuspended in culture media. MN density was adjusted to between 2 $\times$ 10^4 and 2 $\times$ 10^6 MNs/mL, and 50 μL of the suspension was applied to poly-L-lysine-coated glass coverslips in a 24-well plate or 1 mL of the suspension was applied to poly-L-lysine-coated six-well plates. After 24 h, the MNs on coverslips were fed with 250 μL growth media, and the MNs in six-well plates were fed with 2 mL growth media.

MN Treatment

All MNs spent 3 days in culture prior to treatment. Three adenoviruses, Ad5CMV humanSOD1-G93Amutant (AdmSOD), AdCMV humanSOD1 wildtype (AdwtSOD1) and control Ad5CMVeGFP (AdGFP), were purchased from the Gene Transfer Vector Core at the University of Iowa (Iowa City, IA). Viral transfection of primary MNs was performed as previously described (Vincent et al., 2004). Briefly, virus [0–1000 plaque-forming units (pfu) per MN] was added after 2 days in culture and MNs were incubated for 24 h prior to harvesting cell lysates or fixing for immunostaining or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling. For all VEGF experiments, MNs were changed to treatment media 4 h prior to addition of VEGF165 (Sigma). LY294002, U0126, and SU1498 inhibitors (Calbiochem, La Jolla, CA) were added 1 h prior to VEGF at a concentration of 20 μM.

Immunostaining

Immunostaining was carried out by standard protocols as previously described (Kim et al., 1997). Briefly, for spinal cords, sections were permeabilized with 0.1% Triton/PBS and then blocked in 5% normal goat serum/0.1% Triton/PBS. VEGFR1, VEGFR2, and VEGFR3 primary antibodies (Cell Signaling, Danvers, MA) were incubated overnight. Sections were then incubated in Alexafluor-594-conjugated secondary antibody (Molecular Probes, Invitrogen, Carlsbad, CA) followed by mounting in ProLong Gold (Molecular Probes, Invitrogen). Images were collected using an Olympus BX-51 microscope. For cultured primary MNs, cells were grown and treated on glass coverslips followed by fixation in 4% PFA for 5 min. Immunostaining was performed as described above for spinal cord sections. Primary antibodies used include phospho-VEGFR2-Y951 (Cell Signaling), phospho-VEGFR2-Y1175 (Cell Signaling), VEGFR2 (Cell Signaling), and ChAT (Calbiochem).

RNA Isolation and PCR

Total RNA was isolated from G93A-SOD1 transgenic and age-matched control mouse spinal cords using the Qiagen (Valencia, CA) RNeasy kit according to the manufacturer's instructions. cDNA was obtained by reverse transcription using random primers followed by PCR using Promega (Madison, WI) PCR MasterMix and the appropriate primer pairs for VEGF family ligands, VEGFRs and actin. Forward and reverse primers were obtained from Integrated DNA Technologies (Coralville, IA) for the following sequences: VEGF-A-forward 5′-ATGCGGATCAAACCT CACCAAAGC-3′; VEGF-A-rev 5′-TTCGTTTAACTCAA GCTGCCTCGC-3′; VEGF-B-forward 5′-AATGCAGATC CTCATGATCC-3′; VEGF-B-rev 5′-TCTGGCTTCACAG CACTCTC-3′; VEGF-C-forward 5′-AACGTGTCAAGAA ATCAGCC-3′; VEGF-C-rev 5′-AGTCCTCTCCCGCAG TAATCC-3′; PlGF-forward 5′-ACAGAAGTGGAAGTGG TG-3′; PlGF-rev 5′-GGCTAATAAATAGAGGGTAGG-3′;

VEGFR1-forward 5′-GAAGCGGTTCACCTGGACTGA GACC-3′; VEGFR1-rev 5′-GGCTTTGCTGGGGGGGATT TCTCTAA-3′; VEGFR2-forward 5′-TGGGAAACCTCCT GCAAGCAAATG-3′; VEGFR2-rev 5′-TTCTGATGCA AGGACCATCCCACT-3′; VEGFR3-forward 5′-CAGACA GACAGCGGGATGGTGC-3′; VEGFR3-rev 5′-AGGCTG TAGTGGGGGTGGGACA-3′; actin-forward 5′-TGAGAG GGAAATCGTGCGTGACAT-3′; actin-rev 5′-ACTCCT GCTTGCTGATCCACATCT-3′. Results of at least four independent experiments were analyzed by densitometry using Quantity One 1-D Analysis software (BioRad Laboratories) and normalized to actin.

Western Blotting

Western blotting was performed as previously described (Vincent et al., 2004). MN lysates were prepared by scraping cells in RIPA buffer (20 mM Tris, pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1 mM Na deoxycholate, 1% Triton X-100, 0.1 trypsin units/μL aprotinin, 10 mg/mL leupeptin, and 50 mg/mL PMSF. Equal amounts of protein were loaded in each lane of a polyacrylamide gel, with gel percentages (either 7.5 or 12.5%) dependent on the size of the protein of interest. Nitrocellulose membranes were incubated with primary antibody overnight at 4°C and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Primary antibodies used are from Cell Signaling unless otherwise indicated: phospho-p44/42 (Thr202/Tyr204) MAPK; phospho-Akt (Ser473); cleaved caspase-3 (Asp175); GAPDH (Chemicon, Temecula, CA); antihuman SOD1 (Medical and Biological Laboratories, Nagoya, Japan); GFP. Antibody binding was developed with LumiGLO Reagent and Peroxide (Cell Signaling) and exposed to Kodak BioMax XAR film (Sigma). Each experimental paradigm was tested on three separate occasions using different cultures or tissue samples. Membranes were reprobed for GAPDH to confirm equal protein loading, and densitometry results were normalized to GAPDH.

TUNEL

MNs were grown and treated on glass coverslips followed by fixation in 4% PFA for 5 min. TUNEL was used to detect DNA fragmentation as an indication of apoptotic death. MN plating densities were equal between treatment conditions and were adjusted to 50 MNs/mm^2. TUNEL analysis included blinded counting of at least 10 representative fields per condition in at least three independent experiments per our published protocol for an average total of $\sim$300 MNs per condition (Russell et al., 2002; Sullivan et al., 2007). Samples were labeled with digoxygenin-dUTP and then stained with horseradish peroxidase-conjugated anti-digoxygenin antibody using the ApopTag Plus In Situ Apoptosis Peroxidase Detection Kit (Chemicon). Alternatively, for detection of fluorescent TUNEL signal, MNs were grown and treated in 96-well culture plates. After fixa-

tion, TUNEL processing was carried out as described above with a Flurorecein-labeled conjugate using the ApopTag Plus In Situ Apoptosis Fluorescein Detection Kit (Chemicon). Fluorescent signal was detected and recorded using a Fluoroskan Ascent FC microplate reader (Labsystems, Helsinki, Finland) (Vincent et al., 2007).

Statistical Analysis

Results are representative of at least three independent experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test to compare individual data points using Prism software version 3.0 (GraphPad Software, La Jolla, CA).

RESULTS

VEGF and VEGFRs in the Spinal Cord

To confirm the potential of VEGF to affect MNs in ALS, MNs from G93A-SOD1 and wildtype mice were first analyzed for the expression of VEGFRs. Spinal cord tissue collected from endstage G93A-SOD1 transgenic mice at 120 days of age [Fig. 1(B,D,F)] and age-matched control mice [Fig. 1(A,C,E)] was immunostained for VEGFR1, VEGFR2, and VEGFR3. VEGFR expression is evident in ventral horn MNs in control mice and is also evident in G93A-SOD1 transgenic mice, despite the decreased number of MNs present. To quantify the relative levels of VEGF family ligands and VEGFR expression, total RNA was isolated from spinal cords and analyzed by PCR [Fig. 1(G)]. Equal levels of total RNA from spinal cords of transgenic and control mice were confirmed by PCR for actin (data not shown). There is ∼50% reduction in the levels of both VEGF-A and VEGFR2 in G93A-SOD1 transgenic mouse spinal cords compared to control spinal cords following quantification by densitometry. PCR results also confirm that expression of other VEGF family ligands and VEGFRs did not demonstrate a compensatory upregulation to the decreased levels of VEGF-A and VEGFR2. These data indicate a decrease in VEGF and VEGFR2 levels in G93A-SOD1 transgenic mice and confirm the expression of VEGFRs on ventral horn MNs in ALS mice.

VEGF Signaling

Immunostaining of MNs isolated from E15 rat spinal cord confirms expression of VEGFR2 in cultured primary MNs, as indicated by ChAT co-staining to identify MNs [Fig. 2(A–C)]. Exogenous addition of VEGF promotes phosphorylation of VEGFR2 at Y951 and Y1175 [Fig. 2(D–G)]. These data demonstrate that VEGFR2 is expressed and capable of VEGF-induced phosphorylation and activation in our model system.

VEGF activates multiple signaling pathways in MNs. To determine which pathways are activated by VEGF in primary MNs, we treated cultures with VEGF (0–500 ng/mL) for 30 min. Dose-dependent phosphorylation of Akt and p44/42 MAPK was observed that reached maximal activation at a concentration of 250 ng/mL VEGF, while no alterations in JNK or p38 MAPK signaling were detected (data not shown). The temporal activation of PI3K/Akt and p44/42 MAPK signaling was also examined. Addition of VEGF (250 ng/mL) resulted in sustained phosphorylation of Akt after 5 min, which persisted for at least 60 min, while p44/42 MAPK phosphorylation peaked at 15 min [Fig. 3(A)]. Activation of both PI3K/Akt and p44/42 MAPK signaling is prevented by inhibition of VEGFR2 using SU1498 [20 μM; Fig. 3(B)]. The pathway-specific inhibitors LY294002 and U0126 prevent VEGF-induced phosphorylation of Akt and p44/42 MAPK, respectively [Fig. 3(B)].

G93A-SOD1 Effects on Primary MN

Expression of G93A-SOD1 in primary MNs results in biochemical and morphological characteristics resembling ALS. Transfection with AdmSOD (0–1000 pfu/MN) promotes expression of the mutant protein and a corresponding cleavage of caspase-3 [Fig. 4(A)], indicative of apoptotic cell death, while transfection with AdGFP does not promote caspase-3 cleavage, eliminating any toxic effects due to viral titer alone [Fig. 4(B)]. Activation of apoptotic death by G93A-SOD1 in primary MNs is further confirmed using fluorescent TUNEL labeling, which results in a dose-dependent increase in DNA fragmentation [Fig. 4(C)]. Furthermore, comparable overexpression of wtSOD1 followed by TUNEL analysis indicates that there is no apparent toxicity associated with overexpression of SOD1 itself [Fig. 5(A)].

Axonal degeneration is associated with ALS pathogenesis (Fischer et al., 2004; Fischer and Glass, 2007); therefore, we next examined MN morphology. Expression of G93A-SOD1 in primary MNs promotes dose-dependent axonal degeneration represented by decreased axon length and aberrant branching [Fig. 4(D–G)], while transfection with AdGFP does not promote axonal degeneration, again elimi-
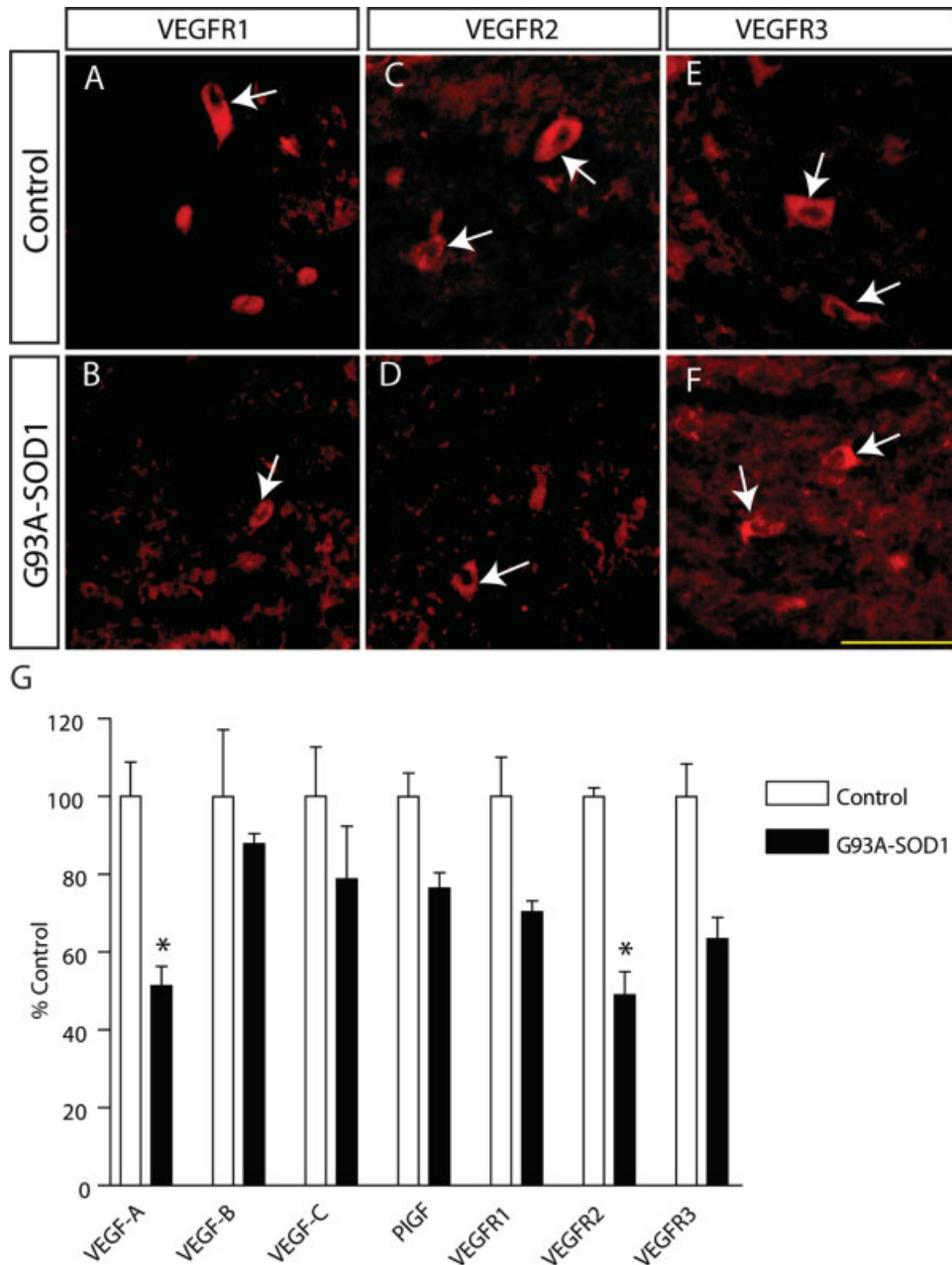
Figure 1 VEGFR expression in G93A-SOD1 and nontransgenic mouse spinal cords. Immuno-staining for VEGFR1 (A, B), VEGFR2 (C, D), and VEGFR3 (E, F) in nontransgenic (A, C, E) and G93A-SOD1 (B, D, F) mouse spinal cord sections. Arrows point to VEGFR expression in MNs in the ventral horn. (G) PCR on RNA isolated from G93A-SOD1 and nontransgenic control mouse spinal cords for VEGF family ligands and VEGFR RNA. Densitometry followed by one-way ANOVA and Tukey's multiple comparison test demonstrates significant decreased expression of VEGF-A and VEGFR2 RNA in G93A-SOD1 spinal cords compared to nontransgenic control spinal cords (*$p < 0.01$). Results are normalized to actin. Scale bar 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nating any toxic effects due to viral titer alone (data not shown). These data validate AdmSOD transfection of primary MNs as a suitable model system for examining neuroprotection in ALS.

Mechanisms of VEGF Neuroprotection

To examine VEGF neuroprotection in ALS, we treated primary MNs with 500 or 1000 pfu/MN AdmSOD in
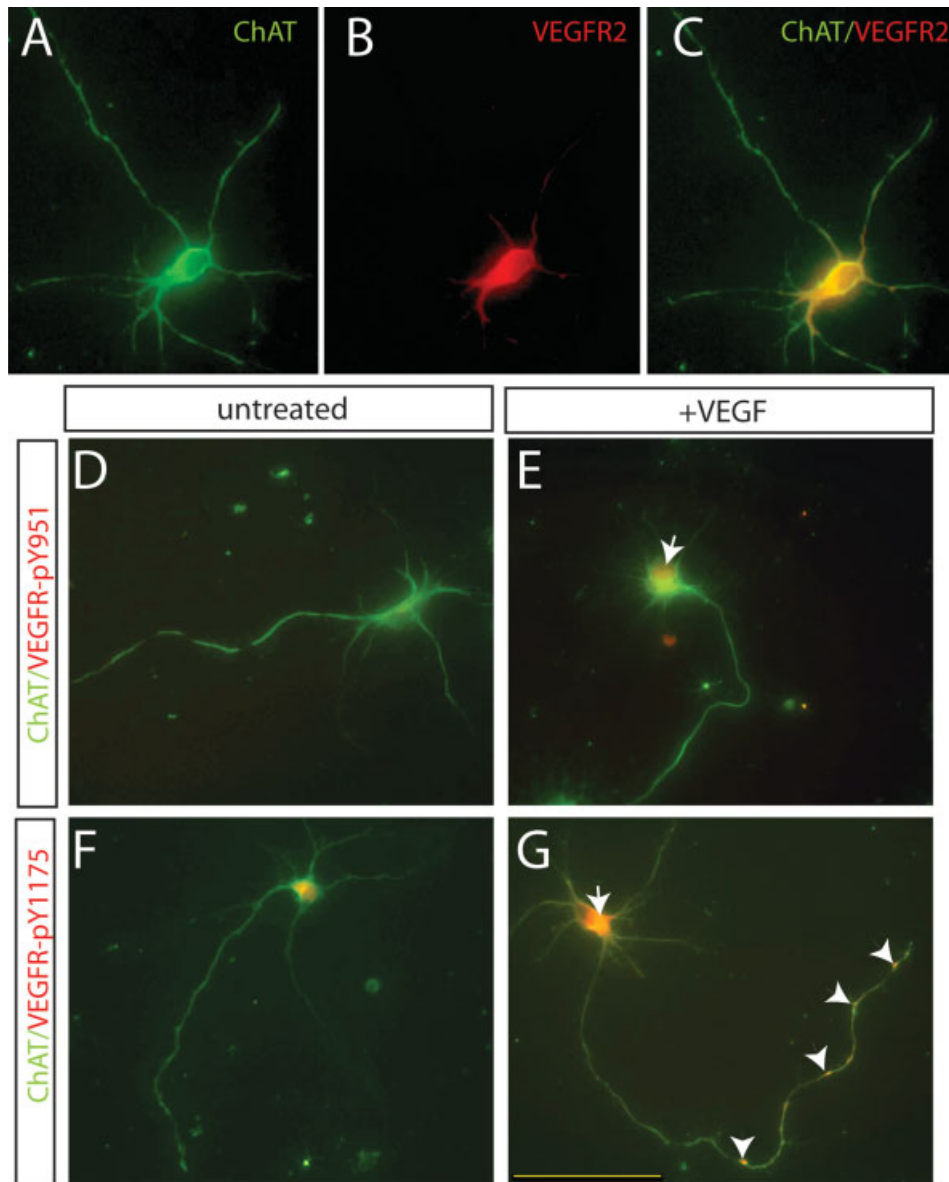
Figure 2 VEGFR expression and activation by VEGF in cultured primary MNs. Immunostaining of primary MNs for VEGFR2 (B) and the MN marker ChAT (A) demonstrates expression of VEGFR2 in MNs (C). Treatment with VEGF (250 ng/mL, 30 min) promotes activation of VEGFR2 as indicated by phosphorylation of Y951 (E) and Y1175 (G) throughout the MN cell body and axon (ChAT positive; arrows) upon VEGF stimulation. Phosphorylation is not seen in the absence of VEGF treatment (D, F). Scale bar 50 μm.

the absence or presence of VEGF and measured DNA damage using the TUNEL assay [Fig. 5(A)]. VEGF significantly decreased the level of DNA damage associated with G93A-SOD1 expression. To determine the temporal efficacy of VEGF in AdmSOD-treated MN, VEGF was administered to MN cultures at the time of AdmSOD transfection or added 4 h pretransfection or 18 h posttransfection and caspase-3 activation was examined by Western blotting [Fig. 5(B)]. All VEGF

treatments decrease caspase-3 cleavage associated with G93A-SOD1, with pretreatment conferring the greatest efficacy. These data confirm that VEGF is neuroprotective against G93A-SOD1 caspase-3 cleavage and DNA damage in primary MNs.

To determine what signaling pathways activated by VEGF are important in neuroprotection, we utilized pathway-specific inhibitors for PI3K/Akt and p44/42 MAPK signaling pathways and assessed the
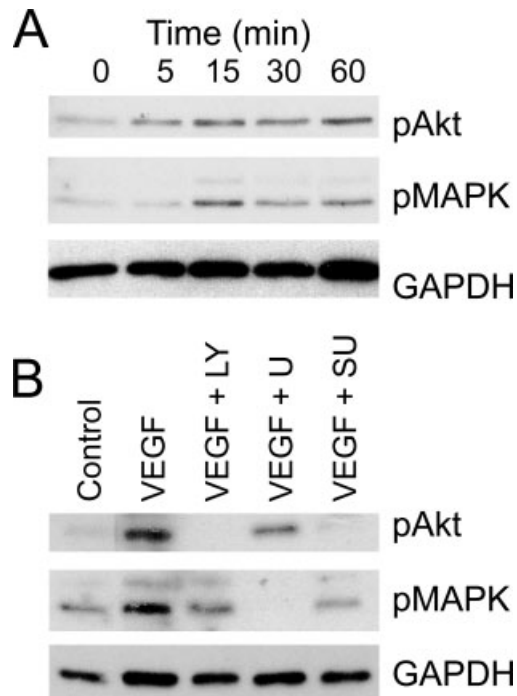
Figure 3 VEGF signaling in cultured primary MNs. Western blotting of primary MNs treated with VEGF. (A) Time course of Akt and p44/42 MAPK phosphorylation in response to VEGF (250 ng/mL) treatment. (B) Pretreatment (1 h) with inhibitors to the PI3K/Akt pathway (LY294002; 20 μM), p44/42 MAPK pathway (U0126; 20 μM), and VEGFR2 (SU1948; 20 μM) prevents activation of the respective signaling pathways in response to VEGF treatment.

ability of VEGF to attenuate G93A-SOD1 DNA damage using the TUNEL assay (see Fig. 6). Inhibition of the VEGFR2 with SU1498 completely prevented the neuroprotective effects of VEGF. LY294002, which prevents signaling via the PI3K/Akt pathway, also completely prevented VEGF neuroprotection, while inhibition of p44/42 MAPK signaling using U0126 did not impact the neuroprotective potential of VEGF. These data indicate that VEGF neuroprotection against G93A-SOD1 is mediated by activation of the PI3K/Akt pathway via VEGFR2 activation.

DISCUSSION

To date there are no effective treatments for ALS and the mechanisms of disease onset and progression have not been determined. In addition to the toxic gain-of-function mechanism associated with SOD1 mutations, genetic mutations have also been identified in Alsin, dynactin, angiogenin, senataxin, and vesicle-associated membrane protein/synaptobrevin-

associated membrane protein B (Pasinelli and Brown, 2006; Gonzalez de Aguilar et al., 2007; Valdmanis and Rouleau, 2008). Additional proposed mechanisms of ALS include autoimmune and inflammatory responses (Appel et al., 1991; Appel et al., 1994), neurofilament aggregation (Lin et al., 2004; Ge et al., 2005), mitochondrial impairment (Dupuis et al., 2004; Bacman et al., 2006; Vande Velde et al., 2008), oxidative stress (Yim et al., 1996; Liu et al., 2007), glutamate toxicity (Rothstein et al., 1990; Coyle and Puttfarcken, 1993), and altered neurotrophism (Ekestern, 2004). The combination of multiple insults and attenuated protective responses, which are all reflected in gene profiling data from patients with ALS (Wang et al., 2006), compounds the neurodegenerative phenotype of the MNs. The development of therapies that can target multiple facets of the disease will play a crucial role in understanding and treating ALS. The current study investigates the therapeutic potential of a neurotrophic factor, VEGF, using the ALS model system created by expressing G93A-SOD1 in primary MNs. Expression of G93A-SOD1 in cultured primary MNs induces biochemical and morphological characteristics of cell death resembling human ALS (see Fig. 4). Overexpression of wildtype SOD1, on the other hand, does not promote DNA damage [Fig. 5(A)]. In agreement with this, the literature strongly supports that only expression of mutant SOD1 promotes disease (Gurney et al., 1994; Reaume et al., 1996), confirming that the observed toxicity is due to expression of mutant SOD1 and not overexpression of SOD1 itself.

Utilizing cultured E15 rat primary MNs enables us to examine the effects of VEGF directly on G93A-SOD1-expressing MNs. While some studies have examined MNs isolated from transgenic rodent models of ALS expressing various forms and levels of mutant SOD1 (Raoul et al., 2002, 2006; Spalloni et al., 2004; Lobsiger et al., 2007), utilizing rat primary MNs allows for control over the expression levels and toxicity of G93A-SOD1 (Howland et al., 2002; Alexander et al., 2004; Goos et al., 2007). This system results in the morphological and biochemical alterations associated with ALS MN degeneration including axonal degeneration, activation of caspase-3 and DNA damage indicative of cell death (Fig. 4; Martin, 1999; Li et al., 2000; Fischer et al., 2004; Fischer and Glass, 2007). The observed severity of alterations follow a dose-dependant association with mutant SOD1 expression levels in cultures from both transgenic animal models expressing different copy numbers of the mutant SOD1 protein and wildtype animals transfected with the mutant SOD1 protein (Howland et al., 2002; Alexander et al., 2004). Taken
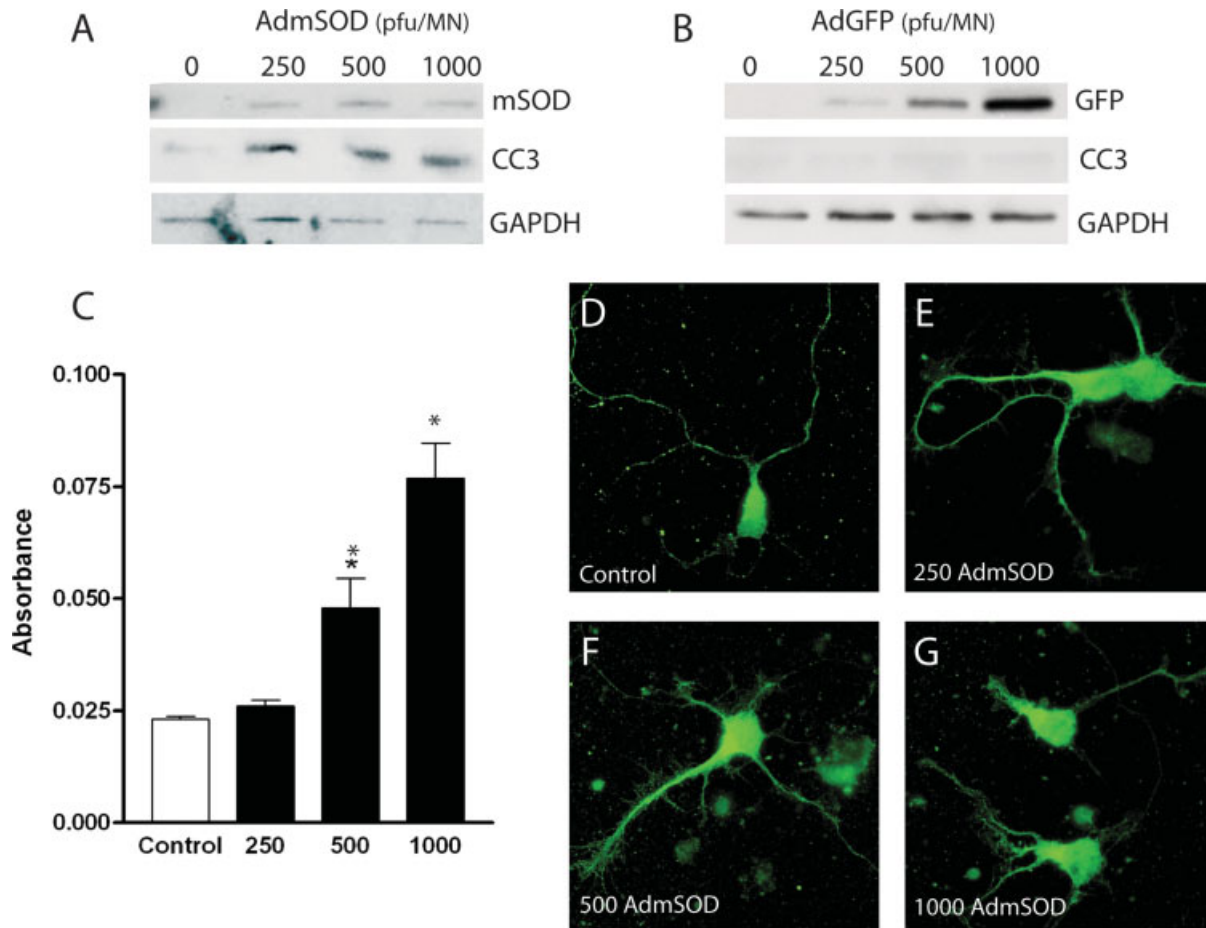
Figure 4 Effects of G93A-SOD1 on cultured primary MNs. Caspase-3 cleavage in response to adenoviral transfection of cultured primary MNs with (A) AdmSOD (0–1000 pfu/MN) or (B) AdGFP (0–1000 pfu/MN). Western blotting demonstrates dose-dependent caspase-3 cleavage in response to G93A-SOD1 expression in cultured primary MNs, while expression of comparable amounts of GFP do not, ruling out toxic effects of viral titer alone. (C) Fluorescent TUNEL staining of AdmSOD-transfected primary MNs. Results are averaged from three independent experiments and analyzed for statistical significance using one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.01$). (D–G) Immunostaining of MNs transfected with AdmSOD for the MN marker ChAT demonstrates dose-dependent effects of G93A-SOD1 on MN morphology. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
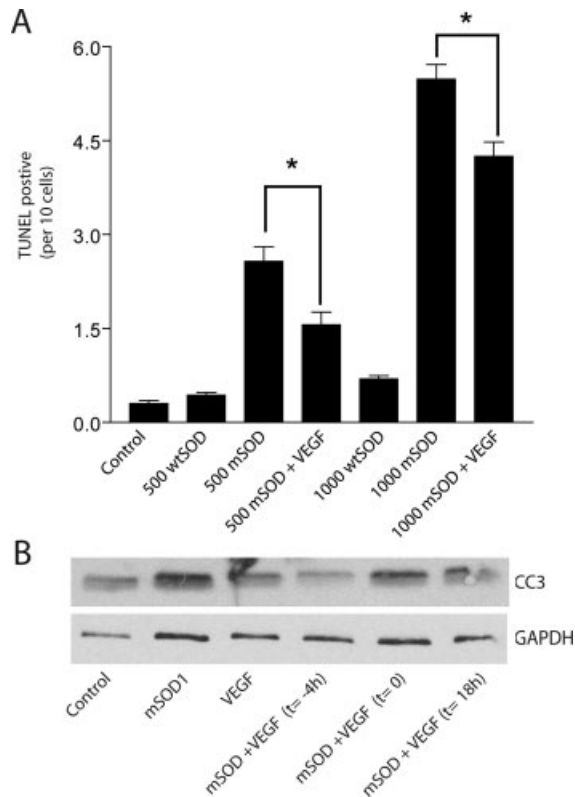
together, while other models exist to study neuroprotection in ALS, primary rat MNs provide an ideal controllable system for the mechanistic studies undertaken here.

Recently, the role of mutant SOD1 in ALS pathogenesis has been examined in both MNs and glia. In agreement with our findings in MN cultures, studies indicate that expression of G93A-SOD1 exclusively in MNs is sufficient to promote protein aggregation and onset of MN degeneration (Jaarsma et al., 2008). Expression of G93A-SOD1 in glia, however, appears to play a more important role in disease progression (Clement et al., 2003; Di Giorgio et al., 2007; Yama-

naka et al., 2008). These focused studies are in agreement with the current understanding of the role of G93A-SOD1 in MNs and enable us to examine the contribution and mechanisms of VEGF neuroprotection at the level of the MN.

Loss of VEGF was first associated with ALS pathogenesis in 2001 when the deletion of the hypoxia response element in the VEGF promoter in transgenic mice resulted in a phenotype essentially identical to that of human ALS (Oosthuyse et al., 2001). Within the G93A-SOD1 mouse spinal cord, we observe a decrease in VEGF and VEGFR2 levels concurrent with loss of large MNs (see Fig. 1). This

For VEGF treatment to be efficacious in ALS, remaining MNs in the spinal cord must be capable of responding to VEGF. Immunostaining for VEGFRs in G93A-SOD1 mouse spinal cords demonstrates that the remaining MNs express all three VEGFRs [Fig. 1(B,D,F)], and expression of VEGFR2 is also demonstrated in cultured primary MNs [Fig. 2(A–C)]. Furthermore, cultured primary MNs respond to VEGF treatment, as evidenced by phosphorylation and activation of VEGFR2 [Fig. 2(D–G)]. These data confirm that MNs in the spinal cord and in our culture system are capable of responding to exogenous VEGF application.

The current studies demonstrate that VEGF can protect MNs from G93A-SOD1-induced damage. Addition of VEGF significantly prevents G93A-SOD1-induced DNA fragmentation [Fig. 5(A)] and caspase-3 cleavage [Fig. 5(B)]. While VEGF provides lower levels of neuroprotection when administered concurrent with or following G93A-SOD1 transfection compared to pretreatment, the ability to
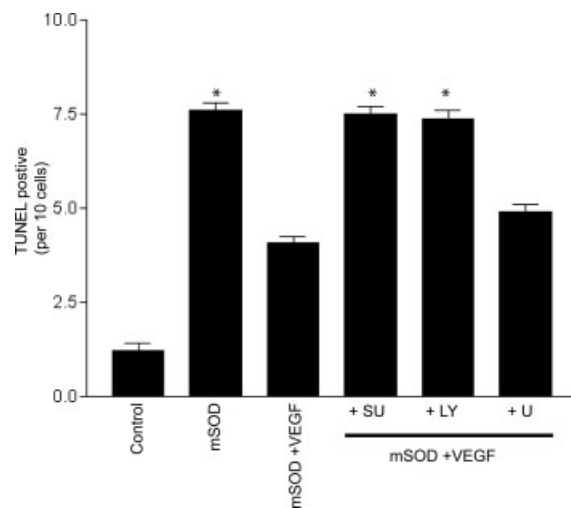

Figure 5 VEGF neuroprotection against G93A-SOD1 in cultured primary MNs. (A) TUNEL labeling demonstrates increased DNA damage associated with 500 and 1000 pfu/MN AdmSOD transfection, which is significantly attenuated in the presence of VEGF (250 ng/mL). Transfection with equal concentrations of AdwtSOD1 does not result in increased DNA damage. MN densities are equivalent between treatments groups, and results are averaged over at least 10 fields of three independent experiments and analyzed for statistical significance using one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$). (B) Western blotting demonstrates decreased levels of G93A-SOD1-induced caspase-3 cleavage with VEGF treatment. Pretreatment (4 h pre) is the most efficacious, with decreased efficacy associated with VEGF cotreatment or posttreatment (18 h post).

decrease in VEGF and VEGFR2 expression is not associated with a compensatory upregulation of other VEGF family members [Fig. 1(G)]. Reductions in VEGF and VEGFR2 are also evident in patients with ALS (Brockington et al., 2006), and the role of VEGF in ALS is further supported by evidence that mutant SOD1 destabilizes VEGF mRNA (Lu et al., 2007). Reduced VEGFR2 levels can potentially result in decreased responsiveness to VEGF neurotrophism, or reduced VEGF may increase MN susceptibility to additional cellular insults promoting disease progression.

Figure 6 Identification of VEGF signaling pathways involved in neuroprotection against G93A-SOD1. TUNEL labeling to identify signaling pathways responsible for VEGF neuroprotection against G93A-SOD1-induced DNA fragmentation. Treatment with AdmSOD (1000 ng/mL) results in DNA damage and is significantly attenuated by VEGF treatment (250 ng/mL). Inhibition of VEGFR2 signaling with SU1498 (20 μM; 1 h pretreatment) prevents VEGF neuroprotection. Inhibition of PI3K/Akt signaling with LY294002 (20 μM; 1 h pretreatment) also prevents VEGF neuroprotection, while inhibition of p44/42 MAPK signaling with U0126 (20 μM; 1 h pretreatment) has no effect on VEGF neuroprotection. Results are averaged over at least 10 fields of three independent experiments and analyzed for statistical significance using one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.01$ compared to mSOD + VEGF).

decrease caspase-3 cleavage in this *in vitro* system that represents an endstage disease phenotype implies that VEGF treatment efficacy, when translated into the human MN environment, could be effective throughout the disease course. These findings are congruent with previous reports supporting the neuroprotective effects of VEGF in ALS. Retrograde delivery of VEGF to MNs by gene therapy increases survival of G93A-SOD1 transgenic mice (Azzouz et al., 2004). Systemic delivery of VEGF also decreases astrogliosis and increases neuromuscular junctions in these mice (Zheng et al., 2007). Double transgenic mice expressing G93A-SOD1 and increased VEGF levels demonstrate delayed MN loss, delayed motor impairment, and increased survival (Wang et al., 2007). G93A-SOD1 transgenic rats also show evidence of increased survival in response to treatment with intracerebroventricular delivery of VEGF (Storkebaum et al., 2005). G93A-SOD1 expression in a zebrafish model of ALS also demonstrates attenuation of G93A-SOD1-induced neuronal defects with increased VEGF levels and exacerbated defects with decreased VEGF concentrations (Lemmens et al., 2007).

Our findings demonstrate that VEGF is neuroprotective against G93A-SOD1 toxicity (see Fig. 5). This data suggests that the decreased toxicity and increased MN survival in response to VEGF treatment, which is supported in additional reports (Li et al., 2003; Zheng et al., 2004, 2007; Dewil et al., 2007), may indicate a role for VEGF neuroprotection in ALS. In fact, VEGF is protective against glutamate toxicity in spinal cord cultures, one of the main non-neuronal mechanisms of MN damage in ALS (Tovar et al., 2007; Tolosa et al., 2008). These data, along with the finding of reduced VEGF levels in transgenic ALS mouse spinal cords [Fig. 1(G)], suggest that the loss of VEGF neurotrophism may be a contributing factor to MN survival in ALS. Restoration of VEGF levels, therefore, is supported as a viable approach to treating MN degeneration in ALS.

VEGF activates many signaling pathways in MNs including PI3K/Akt and p44/42 MAPK (see Fig. 3). We have previously demonstrated that PI3K/Akt and p44/42 MAPK signaling are involved in the neuroprotective effects of insulin-like growth factor-I (IGF-I) against glutamate toxicity (Vincent et al., 2004). We are able to address the contribution of these signaling pathways to VEGF neuroprotection in our model system using the pathway-specific inhibitors LY294002 and U0126 to prevent PI3K/Akt and p44/42 MAPK signaling, respectively (Vlahos et al., 1994; Favata et al., 1998). Work in

our laboratory and others (Kim et al., 1998; Leinninger et al., 2004; Vincent et al., 2004, 2007) have extensively examined and optimized these inhibitors for dosing regimens and their selective effectiveness in neuronal cultures, and at the concentrations used for these studies, inhibitor concentrations are sufficient to prevent activation of the respective pathways [Fig. 3(B)] with minimal toxicity. Unlike results from studies examining IGF-I neuroprotection (Vincent et al., 2004), our results demonstrate that VEGF-induced PI3K/Akt signaling is neuroprotective against G93A-SOD1 toxicity, with no apparent involvement of p44/42 MAPK signaling (see Fig. 6). The role of PI3K/Akt in ALS has been previously examined in various systems with conflicting results. Familial and sporadic ALS patients have decreased levels of phosphorylated Akt in MNs, as also evidenced in G93A-SOD1 mice prior to symptom onset (Dewil et al., 2007). Examination of phosphorylated Akt levels in G93A-SOD1 mouse spinal cords in an alternate study, however, demonstrated no change in phosphorylated Akt (Peviani et al., 2007). Despite these discrepancies, constitutive activation of Akt in G37R-SOD1-expressing N2a cells provides neuroprotection (Dewil et al., 2007). Furthermore, neuroprotective effects of intracerebroventricular VEGF treatment in G93A-SOD1 rats result in increased phospho-Akt levels (Dewil et al., 2007). Therefore, VEGF specifically mediates its protective effects via PI3K/Akt signaling, which may constitute a conserved mechanism for VEGF neuroprotection in ALS.

VEGF efficacy has also been previously examined in models of sporadic ALS. Familial ALS and sporadic ALS are clinically and pathologically indistinguishable, and it is suggested that both forms of the disease potentially result from a common mechanism. Reports on sporadic ALS demonstrate an involvement of non-neuronal glutamate toxicity in MN degeneration and disease progression (Rothstein et al., 1990; Coyle and Puttfarcken, 1993; Clement et al., 2003; Boillee et al., 2006; Di Giorgio et al., 2007; Yamanaka et al., 2008). In fact, VEGF decreases MN loss by over 75% in a rat model of excitotoxic MN degeneration (Tovar et al., 2007). Activation of PI3K/Akt signaling by IGF-I is also associated with neuroprotection against glutamate toxicity (Vincent et al., 2004). Tolosa et al. (2008) demonstrated direct neuroprotection by VEGF via PI3K/Akt signaling against excitotoxic stress.

Taken together, the current studies strengthen the role of VEGF as a therapy for ALS. We have established that VEGF-mediated PI3K/Akt signaling
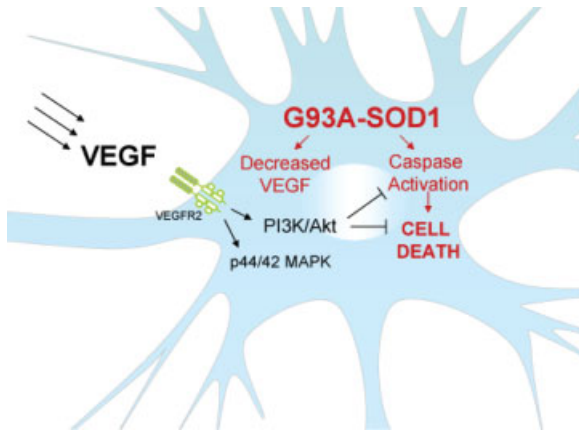
Figure 7 Model of G93A-SOD1 and VEGF effects on MNs. Expression of G93A-SOD1 in MNs results in decreased endogenous production of VEGF and the activation of caspase-3 ultimately resulting in cell death. Addition of exogenous VEGF activates VEGFR2 and subsequent PI3K/Akt and p44/42 MAPK signaling. Activation of PI3K/Akt signaling prevents G93A-SOD1-induced caspase-3 activation and cell death. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

is an important mechanism for protection against G93A-SOD1 in MNs. Induction of PI3K/Akt signaling by VEGF may constitute a conserved neuroprotective mechanism for MNs in response to a number of cellular insults in ALS pathogenesis including mutant SOD1 and glutamate toxicity. These data support the idea that G93A-SOD1 promotes destabilization of VEGF mRNA and decreased protein levels. The reduced protein level propagates susceptibility onto the MNs to additional cellular insults, contributing to disease onset. MNs remaining in the ALS spinal cord, however, are capable of responding to VEGF stimulation and thus can respond to therapeutic exogenous VEGF administration via activation of PI3K/Akt signaling resulting in neuroprotection (see Fig. 7).

Determining the most efficacious delivery mechanism for VEGF is important for achieving neuroprotection in ALS, and gene-based therapies offer much potential for effective delivery of therapeutics to MNs (Federici and Boulis, 2006). Achieving the maximal impact of VEGF neurotrophism is also important to consider. The current studies investigate the efficacy of VEGF165, one of the five VEGF splice variants normally expressed in the nervous system. Induction of the full biological array of VEGF isoforms, however, may promote an additive effect on neuroprotection (Whitlock et al., 2004; Amano et al., 2005) and increased potency and longevity of VEGF neuroprotection may be observed.

Studies are currently underway to establish a more efficacious delivery mechanism using gene-based treatment strategies to increase PI3K/Akt activation in ALS using the full biological array of VEGF splice variants.

REFERENCES

Alexander GM, Erwin KL, Byers N, Deitch JS, Augelli BJ, Blankenhorn EP, Heiman-Patterson TD. 2004. Effect of transgene copy number on survival in the G93A SOD1 transgenic mouse model of ALS. Brain Res Mol Brain Res 130:7–15.

Amano H, Hackett NR, Kaner RJ, Whitlock P, Rosengart TK, Crystal RG. 2005. Alteration of splicing signals in a genomic/cDNA hybrid VEGF gene to modify the ratio of expressed VEGF isoforms enhances safety of angiogenic gene therapy. Mol Ther 12:716–724.

Appel SH, Engelhardt JI, Garcia J, Stefani E. 1991. Autoimmunity and ALS: A comparison of animal models of immune-mediated motor neuron destruction and human ALS. Adv Neurol 56:405–412.

Appel SH, Smith RG, Engelhardt JI, Stefani E. 1994. Evidence for autoimmunity in amyotrophic lateral sclerosis. J Neurol Sci 124 (Suppl):14–19.

Azzouz M, Ralph GS, Storkebaum E, Walmsley LE, Mitrophanous KA, Kingsman SM, Carmeliet P, et al. 2004. VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. Nature 429:413–417.

Bacman SR, Bradley WG, Moraes CT. 2006. Mitochondrial involvement in amyotrophic lateral sclerosis: Trigger or target? Mol Neurobiol 33:113–131.

Boillee S, Vande Velde C, Cleveland DW. 2006. ALS: A disease of motor neurons and their nonneuronal neighbors. Neuron 52:39–59.

Brockington A, Wharton SB, Fernando M, Gelsthorpe CH, Baxter L, Ince PG, Lewis CE, et al. 2006. Expression of vascular endothelial growth factor and its receptors in the central nervous system in amyotrophic lateral sclerosis. J Neuropathol Exp Neurol 65:26–36.

Carmeliet P. 2003. Blood vessels and nerves: Common signals, pathways and diseases. Nat Rev Genet 4:710–720.

Clement AM, Nguyen MD, Roberts EA, Garcia ML, Boillee S, Rule M, McMahon AP, et al. 2003. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. Science 302:113–117.

Coyle JT, Puttfarcken P. 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science 262:689–695.

Dewil M, Lambrechts D, Sciot R, Shaw PJ, Ince PG, Robberecht W, Van den Bosch L. 2007. Vascular endothelial growth factor counteracts the loss of phospho-Akt preceding motor neurone degeneration in

amyotrophic lateral sclerosis. Neuropathol Appl Neurobiol 33: 499–509.

Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K. 2007. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. Nat Neurosci 10:608–614.

Dupuis L, Gonzalez de Aguilar JL, Oudart H, de Tapia M, Barbeito L, Loeffler JP. 2004. Mitochondria in amyotrophic lateral sclerosis: A trigger and a target. Neurodegener Dis 1:245–254.

Ekestern E. 2004. Neurotrophic factors and amyotrophic lateral sclerosis. Neurodegener Dis 1:88–100.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, et al. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273:18623–18632.

Federici T, Boulis NM. 2006. Gene-based treatment of motor neuron diseases. Muscle Nerve 33:302–323.

Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez A, Khan J, et al. 2004. Amyotrophic lateral sclerosis is a distal axonopathy: Evidence in mice and man. Exp Neurol 185:232–240.

Fischer LR, Glass JD. 2007. Axonal degeneration in motor neuron disease. Neurodegener Dis 4:431–442.

Ge WW, Wen W, Strong W, Leystra-Lantz C, Strong MJ. 2005. Mutant copper-zinc superoxide dismutase binds to and destabilizes human low molecular weight neurofilament mRNA. J Biol Chem 280:118–124.

Gonzalez de Aguilar JL, Echaniz-Laguna A, Fergani A, Rene F, Meininger V, Loeffler JP, Dupuis L. 2007. Amyotrophic lateral sclerosis: All roads lead to Rome. J Neurochem 101:1153–1160.

Goos M, Zech WD, Jaiswal MK, Balakrishnan S, Ebert S, Mitchell T, Carri MT, et al. 2007. Expression of a Cu,Zn superoxide dismutase typical for familial amyotrophic lateral sclerosis increases the vulnerability of neuroblastoma cells to infectious injury. BMC Infect Dis 7:131.

Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, et al. 1994. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 264:1772–1775.

Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, Erickson J, et al. 2002. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). Proc Natl Acad Sci USA 99:1604–1609.

Jaarsma D, Teuling E, Haasdijk ED, De Zeeuw CI, Hoogenraad CC. 2008. Neuron-specific expression of mutant superoxide dismutase is sufficient to induce amyotrophic lateral sclerosis in transgenic mice. J Neurosci 28:2075–2088.

Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. 2002. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci USA 99:11946–11950.

Kim B, Cheng HL, Margolis B, Feldman EL. 1998. Insulin receptor substrate 2 and Shc play different roles in insulin-like growth factor I signaling. J Biol Chem 273: 34543–34550.

Kim B, Leventhal PS, Saltiel AR, Feldman EL. 1997. Insulin-like growth factor-I-mediated neurite outgrowth in vitro requires MAP kinase activation. J Biol Chem 272:21268–21273.

Lambrechts D, Storkebaum E, Morimoto M, Del-Favero J, Desmet F, Marklund SL, Wyns S, et al. 2003. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat Genet 34:383–394.

Leinninger GM, Backus C, Uhler MD, Lentz SI, Feldman EL. 2004. Phosphatidylinositol 3-kinase and Akt effectors mediate insulin-like growth factor-I neuroprotection in dorsal root ganglia neurons. FASEB J 18: 1544–1546.

Lemmens R, Van Hoecke A, Hersmus N, Geelen V, D'Hollander I, Thijs V, Van Den Bosch L, et al. 2007. Overexpression of mutant superoxide dismutase 1 causes a motor axonopathy in the zebrafish. Hum Mol Genet 16:2359–2365.

Li B, Xu W, Luo C, Gozal D, Liu R. 2003. VEGF-induced activation of the PI3-K/Akt pathway reduces mutant SOD1-mediated motor neuron cell death. Brain Res Mol Brain Res 111:155–164.

Li M, Ona VO, Guegan C, Chen M, Jackson-Lewis V, Andrews LJ, Olszewski AJ, et al. 2000. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. Science 288:335–339.

Lin H, Zhai J, Canete-Soler R, Schlaepfer WW. 2004. 3′ untranslated region in a light neurofilament (NF-L) mRNA triggers aggregation of NF-L and mutant superoxide dismutase 1 proteins in neuronal cells. J Neurosci 24:2716–2726.

Liu D, Bao F, Wen J, Liu J. 2007. Mutation of superoxide dismutase elevates reactive species: Comparison of nitration and oxidation of proteins in different brain regions of transgenic mice with amyotrophic lateral sclerosis. Neuroscience 146:255–264.

Lobsiger CS, Boillee S, Cleveland DW. 2007. Toxicity from different SOD1 mutants dysregulates the complement system and the neuronal regenerative response in ALS motor neurons. Proc Natl Acad Sci USA 104:7319–7326.

Lu L, Zheng L, Viera L, Suswam E, Li Y, Li X, Estevez AG, et al. 2007. Mutant Cu/Zn-superoxide dismutase associated with amyotrophic lateral sclerosis destabilizes vascular endothelial growth factor mRNA and downregulates its expression. J Neurosci 27:7929–7938.

Martin LJ. 1999. Neuronal death in amyotrophic lateral sclerosis is apoptosis: Possible contribution of a programmed cell death mechanism. J Neuropathol Exp Neurol 58:459–471.

Matsuzaki H, Tamatani M, Yamaguchi A, Namikawa K, Kiyama H, Vitek MP, Mitsuda N, et al. 2001. Vascular endothelial growth factor rescues hippocampal neurons from glutamate-induced toxicity: Signal transduction cascades. FASEB J 15:1218–1220.

Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, et al. 2001. Deletion of the hypoxia-response element in the vascular endothelial

growth factor promoter causes motor neuron degeneration. Nat Genet 28:131–138.

Pasinelli P, Brown RH. 2006. Molecular biology of amyotrophic lateral sclerosis: Insights from genetics. Nat Rev Neurosci 7:710–723.

Peviani M, Cheroni C, Troglio F, Quarto M, Pelicci G, Bendotti C. 2007. Lack of changes in the PI3K/AKT survival pathway in the spinal cord motor neurons of a mouse model of familial amyotrophic lateral sclerosis. Mol Cell Neurosci 34:592–602.

Raoul C, Buhler E, Sadeghi C, Jacquier A, Aebischer P, Pettmann B, Henderson CE, et al. 2006. Chronic activation in presymptomatic amyotrophic lateral sclerosis (ALS) mice of a feedback loop involving Fas, Daxx, and Fas L. Proc Natl Acad Sci USA 103:6007–6012.

Raoul C, Estevez AG, Nishimune H, Cleveland DW, deLapeyriere O, Henderson CE, Haase G, et al. 2002. Motoneuron death triggered by a specific pathway downstream of Fas. potentiation by ALS-linked SOD1 mutations. Neuron 35:1067–1083.

Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, et al. 1996. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. Nat Genet 13:43–47.

Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, et al. 1993. Mutations in Cu/Zn superoxide dismutase are associated with familial amyotrophic lateral sclerosis. Nature 362:59–62.

Rosenstein JM, Mani N, Khaibullina A, Krum JM. 2003. Neurotrophic effects of vascular endothelial growth factor on organotypic cortical explants and primary cortical neurons. J Neurosci 23:11036–11044.

Rothstein JD, Tsai G, Kuncl RW. 1990. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. Ann Neurol 28:18–25.

Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, Feldman EL. 2002. High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. FASEB J 16:1738–1748.

Sondell M, Sundler F, Kanje M. 2000. Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. Eur J Neurosci 12:4243–4254.

Spalloni A, Albo F, Ferrari F, Mercuri N, Bernardi G, Zona C, Longone P. 2004. Cu/Zn-superoxide dismutase (GLY93→ALA) mutation alters AMPA receptor subunit expression and function and potentiates kainate-mediated toxicity in motor neurons in culture. Neurobiol Dis 15:340–350.

Storkebaum E, Lambrechts D, Dewerchin M, Moreno-Murciano MP, Appelmans S, Oh H, Van Damme P, et al. 2005. Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. Nat Neurosci 8:85–92.

Sullivan KA, Hayes JM, Wiggin TD, Backus C, Su Oh S, Lentz SI, Brosius F, 3rd, et al. 2007. Mouse models of diabetic neuropathy. Neurobiol Dis 28:276–285.

Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA. 2003. VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest 111:1843–1851.

Tolosa L, Mir M, Asensio VJ, Olmos G, Llado J. 2008. Vascular endothelial growth factor protects spinal cord motoneurons against glutamate-induced excitotoxicity via phosphatidylinositol 3-kinase. J Neurochem 105:1080–1090.

Tovar YRLB, Zepeda A, Tapia R. 2007. Vascular endothelial growth factor prevents paralysis and motoneuron death in a rat model of excitotoxic spinal cord neurodegeneration. J Neuropathol Exp Neurol 66:913–922.

Valdmanis PN, Rouleau GA. 2008. Genetics of familial amyotrophic lateral sclerosis. Neurology 70:144–152.

Valentine JS, Doucette PA, Zittin Potter S. 2005. Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis. Annu Rev Biochem 74:563–593.

Vande Velde C, Miller TM, Cashman NR, Cleveland DW. 2008. Selective association of misfolded ALS-linked mutant SOD1 with the cytoplasmic face of mitochondria. Proc Natl Acad Sci USA 105:4022–4027.

Vincent AM, Feldman EL, Song DK, Jung V, Schild A, Zhang W, Imperiale MJ, et al. 2004. Adeno-associated viral-mediated insulin-like growth factor delivery protects motor neurons in vitro. Neuromol Med 6:79–86.

Vincent AM, Mobley BC, Hiller A, Feldman EL. 2004. IGF-I prevents glutamate-induced motor neuron programmed cell death. Neurobiol Dis 16:407–416.

Vincent AM, Perrone L, Sullivan KA, Backus C, Sastry AM, Lastoskie C, Feldman EL. 2007. Receptor for advanced glycation end products activation injures primary sensory neurons via oxidative stress. Endocrinology 148:548–558.

Vincent AM, Russell JW, Sullivan KA, Backus C, Hayes JM, McLean LL, Feldman EL. 2007. SOD2 protects neurons from injury in cell culture and animal models of diabetic neuropathy. Exp Neurol 208:216–227.

Vlahos CJ, Matter WF, Hui KY, Brown RF. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002). J Biol Chem 269:5241–5248.

Wang XS, Simmons Z, Liu W, Boyer PJ, Connor JR. 2006. Differential expression of genes in amyotrophic lateral sclerosis revealed by profiling the post mortem cortex. Amyotroph Lateral Scler 7:201–210.

Wang Y, Mao XO, Xie L, Banwait S, Marti HH, Greenberg DA, Jin K. 2007. Vascular endothelial growth factor overexpression delays neurodegeneration and prolongs survival in amyotrophic lateral sclerosis mice. J Neurosci 27:304–307.

Whitlock PR, Hackett NR, Leopold PL, Rosengart TK, Crystal RG. 2004. Adenovirus-mediated transfer of a minigene expressing multiple isoforms of VEGF is more effective at inducing angiogenesis than comparable vectors expressing individual VEGF cDNAs. Mol Ther 9:67–75.

Yamanaka K, Chun SJ, Boillee S, Fujimori-Tonou N, Yamashita H, Gutmann DH, Takahashi R, et al. 2008. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. Nat Neurosci 11:251–253.

Yim MB, Kang JH, Yim HS, Kwak HS, Chock PB, Stadtman ER. 1996. A gain-of-function of an amyotrophic lateral sclerosis-associated Cu, Zn-superoxide dismutase mutant: An enhancement of free radical formation due to a decrease in Km for hydrogen peroxide. Proc Natl Acad Sci USA 93:5709–5714.

Zheng C, Nennesmo I, Fadeel B, Henter JI. 2004. Vascular endothelial growth factor prolongs survival in a transgenic mouse model of ALS. Ann Neurol 56:564–567.

Zheng C, Skold MK, Li J, Nennesmo I, Fadeel B, Henter JI. 2007. VEGF reduces astrogliosis and preserves neuromuscular junctions in ALS transgenic mice. Biochem Biophys Res Commun 363:989–993.

Zhu Y, Jin K, Mao XO, Greenberg DA. 2003. Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. FASEB J 17:186–193.