VEGF and Neuronal Survival

Paula M. Calvo, Rosendo G. Hernández,

Angel M. Pastor* and Rosa R. de la Cruz

Departamento de Fisiología, Facultad de Biología, Universidad de Sevilla,
41012-Sevilla, Spain.

*** Corresponding Author:**
Angel M. Pastor
Departamento de Fisiología
Facultad de Biología
Universidad de Sevilla
Avda. Reina Mercedes, 6
41012-Sevilla
Spain

Email: ampastor@us.es

Abstract

Vascular endothelial growth factor (VEGF) is well known by its angiogenic activity, however, recent evidence has revealed a neuroprotective action of this factor on injured or diseased neurons. In the present review, we summarize the most relevant findings that have contributed to establish a link between VEGF deficiency and neuronal degeneration. It should be emphasized that the design of the mutant mice $VEGF^{\delta/\delta}$ has been crucial to establish the neuroprotective role of VEGF. These mice, which develop over time reduced levels of VEGF, show adult-onset muscle weakness and motoneuron degeneration that resemble amyotrophic lateral sclerosis (ALS). VEGF administration through different technical approaches to animal models of ALS has been demonstrated to ameliorate motoneuronal degeneration and improve motor performance. Altogether, the results presented in this review highlight VEGF as an essential motoneuron neurotrophic factor endowed with promising therapeutic potential for the treatment of motoneuron disorders.

Keywords

Neurotrophic factors, neuroprotection, ALS, axotomy, extraocular motoneurons

Introduction

The vascular endothelial growth factor (VEGF) was identified as the result of two parallel lines of research. In 1983, Senger and others carried out the partial purification of a protein derived from a guinea pig tumor cell line, which they named “vascular permeability factor” (VPF), due to its ability of increasing blood vessel permeability in skin. In 1989, Conolly and others isolated and sequenced, from U937 tumor cells, the human VPF molecule. In that same year, Ferrara and Henzel reported the isolation of a diffusible endothelial cell-specific mitogen from medium conditioned by bovine pituitary follicular cells, which they named “vascular endothelial growth factor” (VEGF) to reflect the restricted target-cell specificity of this molecule. The sequence of purified VEGF proved that this protein did not match any known protein in available databases (Ferrara and Henzel 1989). cDNA cloning of VEGF (Leung and others 1989) and VPF (Keck and others 1989), both reported also in 1989, demonstrated that VEGF and VPF were the same molecule. All these findings revealed that VEGF is a potent, diffusible, and specific factor for vascular endothelial cells and led to the hypothesis that this molecule could play a role in the regulation of physiological and pathological growth of blood vessels (Ferrara and Henzel 1989; Ferrara and others 1991; Leung and others 1989). Therefore, VEGF was historically first associated with its potent actions on endothelial cells, inducing vasculogenesis, angiogenesis and increased vascular permeability (Apte and others 2019; Ferrara 1999, 2004; Yancopoulus and others 2000).

Subsequently, different evidence indicated that, in addition to its vascular biological activity, VEGF also acts as a neuroprotective factor. Numerous works have demonstrated that the administration of VEGF exerts trophic effects after different types of lesion and neuronal types. For instance, VEGF protects against seizure-induced neuronal loss in hippocampus (Nicoletti and others 2008) and attenuates status-epilepticus behavioral impairments (Nicoletti and others 2010; Ureña-Guerrero and others 2020). VEGF acts as a neurotrophic factor

improving the damaged hypoxic/ischemic brain (Guo and others 2016; Sun and others 2003; Yang and others 2018), and promotes the anatomical and functional recovery of injured peripheral nerves in the avascular cornea (Pan and others 2013) and after olfactory nerve bulbectomy (Beecher and others 2019). It also rescues hippocampal neurons from glutamate-induced excitotoxicity (Matsuzaki and others 2001). Interestingly, VEGF exerts neurorescue effects in animal models of neurodegenerative diseases, such as Alzheimer and Parkinson, in cerebral cortex and hippocampus (Guo and others 2019; Ureña-Guerrero and others 2020) and in the substantia nigra and striatum (Yasuhara and others 2005).

Strikingly, although VEGF was discovered by its angiogenic activity, this factor has an evolutionarily ancient role as a neurotrophic factor controlling neural cell morphogenesis in invertebrates. Thus, the nematode worm *Caenorhabditis elegans* lacks a vascular system, but VEGF is present as a trophic molecule necessary for the development of its nervous system. In insects, such as *Drosophila melanogaster*, which have a rudimentary vasculature, VEGF controls nervous system development. In vertebrates, which have an elaborated network of vessels, VEGF controls both nervous and vascular development, suggesting that vessels co-opted VEGF from nerves to regulate their development (Zacchigna and others 2008).

VEGF family and receptors

The VEGF family members are dimeric glycoproteins of approximately 40 kDa. In mammals, the VEGF family consists of five members with a high degree of homology, which comprises VEGF-A (the founding molecule of this family, also known as VEGF), VEGF-B, VEGF-C, VEGF-D and the placental growth factor (PlGF). In addition, proteins that are structurally related to the VEGFs are present in parapoxvirus (VEGF-E) and in snake venom (VEGF-F) (Olsson and others 2006). VEGF family members have been implicated in angiogenesis,

lymphangiogenesis, and may play different roles in the nervous system (e.g., neuronal wiring in the CNS, peripheral nerve development and vascularization, neural migration, neurogenesis, neurite outgrowth, neuronal survival, synaptic plasticity, neuroprotection and neuroregeneration) (Lladó and others 2013; Ruiz de Almodovar and others 2009), and has been implicated in the etiology and treatment of various neurological diseases (Lange and others 2016). VEGF-B is a weak angiogenic stimulator, and appears to have a relatively restricted angiogenic activity in the ischemic heart (Li and others 2008). VEGF-B also acts as neuroprotective against neurodegeneration and physiological alterations (Dhondt and others 2011; Poesen and others 2008) The molecules VEGF-C and VEGF-D act on the lymphatic endothelium promoting the process of lymphangiogenesis (Lohela and others 2009). PlGF stimulates angiogenesis selectively under pathological conditions (Carmeliet and others 2001).

The signaling of VEGFs is carried out through binding to three cell surface membrane receptors, which are endowed with tyrosin kinase intracellular activity. Each VEGF factor binds with different affinities and selectivities to these three receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). VEGFRs are formed by two monomers with several extracellular immunoglobulin domains each, which mediate ligand binding, followed by a transmembrane chain and an intracellular effector comprising two regions with tyrosine kinase activity. VEGFRs form homodimers, but also heterodimers of unknown function (Ferrara 2004; Ruiz de Almodovar and others 2009). VEGF binds to VEGFR-1 and VEGFR-2, VEGF-B and PlGF binds to VEGFR-1, and VEGF-C and VEGF-D interact with VEGFR-3 and VEGFR-2 (Lange and others 2016; Fig. 1).

VEGFR-2 is the best characterized signaling receptor, promoting angiogenesis in health and disease; it stimulates endothelial cell proliferation, survival, migration and vascular permeability. VEGFR-2 also signals numerous processes in various neural types (Carmeliet and Ruiz de Almodovar 2013; Ruiz de Almodovar and others 2009). VEGFR-2 has a high tyrosine

kinase activity (Takahashi and Shibuya 2005). Although VEGFR-1 was discovered before VEGFR-2, its role is still unclear. VEGFR-1 has a weak tyrosine kinase activity but binds VEGF with at least 10-fold higher affinity than VEGFR-2 (Takahashi and Shibuya 2005). Consequently, signaling through VEGFR-2 promotes the phosphorylation of a larger number of intracellular effector proteins due to its strong tyrosine kinase activity, and thus VEGFR-2 is considered the principal mediator of VEGF actions. On the other hand, due to the higher affinity of VEGFR1 for VEGF, it has been proposed that VEGFR-1 could act as a "decoy" receptor that traps VEGF and thus prevents excessive activation of VEGFR-2 (Ferrara and Davis-Smyth 1997; Koch and Claesson-Welsh 2012). That is, VEGFR-1 would negatively regulate the activity of VEGFR-2. The third tyrosine kinase VEGFR, VEGFR-3, is present in the endothelial cells of lymphatic vessels, and binds the ligands VEGF-C and VEGF-D (Takahashi and Shibuya 2005).

The members of the VEGF family can also bind selectively two co-receptors named neuropilins (NRP): neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2) (Fig. 1). They were initially identified as semaphorin receptor, proteins involved in axonal guidance. NRPs are cell surface glycoproteins whose intracellular domains are short and lack tyrosine kinase activity. Nevertheless, when coexpress with VEFGRs, NRPs enhance the binding to VEGFRs and increase the effectiveness of VEGFR-mediated signaling transduction (Ferrara and others 2003; Takahashi and Shibuya 2005).

The specific ligands for VEGFRs and NRPs are illustrated in Fig. 1, and are as follows: VEGF (i.e., VEGF-A) binds to VEGFR-1, VEGFR-2, NRP-1 and NRP-2; VEGF-B binds to VEGFR-1 and NRP-1; PlGF binds to VEGFR-1, NRP-1 and NRP-2; and VEGF-C and VEGF-D interact with VEGFR-3, VEGFR-2, NRP-1 and NRP-2 (Ferrara and others 2003; Lange and others 2016; Takahashi and Shibuya 2005).

Regulation of VEGF by hypoxia

Hypoxia is the principal stimulus for VEGF expression. When local oxygen tension decreases, VEGF levels rise promoting angiogenesis, i.e., the formation of new blood vessels from pre-existing ones, which prevents the oxygen deficit in the tissue. Thus, the regulation of VEGF by hypoxia plays an adaptive, homeostatic role in the maintenance of a proper delivery of oxygen and nutrients to areas with low vascular perfusion (Pronto-Laborinho and others 2014). Some studies have shown that the hypoxia-regulated mechanism of the VEGF gene and the erythropoietin gene shares similarities (Pronto-Laborinho and others 2014).

The increase in VEGF protein levels under hypoxic conditions is due to control at both the transcriptional and translational level. At the transcriptional level, the gene sequence involved in hypoxia regulation is termed “hypoxia-response element” (HRE) and has been located in the 5´ promoter of the human and murine VEGF gene (Ferrara 2004). Hypoxia-inducible transcription factors (HIF) bind HRE and thereby upregulate VEGF transcription under hypoxic situations. HIF is a heterodimeric protein formed by two constitutively expressed subunits HIF-1α (120 kDa) and HIF-1β (92 kDa) (Oosthuyse and others 2001; Storkebaum and others 2004; Wang and others 1995).

Under normoxia (Fig. 2), HIF-1α is hydroxylated at two proline residues by the enzymatic action of proline hydroxylase proteins (PHD), which require molecular oxygen to carry out this action. This step is necessary for the interaction with the von Hippel-Lindau (VHL) protein, a component of the E3 ubiquitin ligase complex, tagging HIF-1α for degradation (Pronto-Laborinho and others 2014; Pugh and Ratcliffe 2003; Salceda and Caro 1997). The proteasome degradation of HIF-1α preclude VEGF transcription in normoxia (Fig. 2). However, in hypoxia, PHDs are inactive due to lack of O_2, and consequently HIF-1α are not

hydroxylated and thus not ubiquitinated for proteasome degradation. Therefore, HIF-1α protein levels increase and HIF-1α translocate into the nucleus and associates with HIF-1β and the coactivators P300/CBP. This complex binds to HRE in the VEGF gene promoter, activating VEGF transcription (Semenza 2001, 2010). In this way, VEGF levels increase in response to hypoxia (Fig. 2). HIF-1α can also increase in response to excitotoxicity (Vazquez-Valls and others 2011) and the levels of VEGF consequently rise under this neurotoxic condition (Castañeda-Cabral and others 2017).

VEGF can also be regulated at the translational level. Hypoxia promotes the stabilization of VEGF mRNA through proteins that binds to sequences located in the region UTR (3´untranslated region) of the mRNA such as HuR (Levy and others 1998; Pronto-Laborinho and others 2014; Storkebaum and others 2004). It should be mentioned that VEGF receptors are also increased by hypoxia, as reported in endothelial cells of lung, heart, brain, kidney and liver following systemic hypoxia (Marti and Risau, 1998), and in skeletal muscle in response to chronic ischemia (Milkiewicz and others 2003). The response of receptors to hypoxia enhances VEGF signaling, favoring increased vascular perfusion in ischemic tissues.

VEGF and ALS: evidences form transgenic animals

ALS is a devastating neurodegenerative disease characterized by rapidly progressive degeneration of motoneurons in the spinal cord, brainstem, and premotor neurons in the motor cortex, leading to severe weakness and muscle atrophy. The mean survival of ALS patients is 3-5 years after symptom onset. Most ALS cases are sporadic, but approximately 10% are familial, one fifth of which are caused by mutations in the gene encoding the antioxidant Cu/Zn superoxide dismutase (SOD1). The pathogenesis of ALS appears to be a complex interplay of

several processes, such as oxidative stress, excitotoxicity, protein aggregation, mitochondrial dysfunction, deleterious effects derived from glial cells, abnormal axonal transport, deficit in neurotrophic factors, ultimately leading to motoneuron death. However, the pathophysiological precise mechanisms of motoneuron degeneration in ALS are still unknown. The only drug currently approved worldwide in ALS is riluzole, which acts by decreasing glutamate activity in the CNS, although its impact on survival is modest and ALS is still an incurable disease (Bruijn and others 2004; Dhasmana and others 2022; Kaur and others 2016; Robberech and Philips 2013; Valko and Ciesla 2019). Mice and rats, expressing a mutant SOD1 transgene, develop ALS with clinical, anatomical and pathological features that are highly reminiscent of those found in human ALS patients. SOD1 transgenic mice and rats are a classic animal model for the study of ALS and have been widely used in numerous ALS-related investigations (Tovar-y-Romo and others 2009).

The link between VEGF and ALS was established in a fortuitous way. Attempts were made to generate knockout mice that failed to express VEGF, or either of its two principal receptors (VEGFR-1 or VEGFR-2), in studies aimed at investigating the relevance of hypoxic regulation of VEGF-dependent angiogenesis. However, these knockout mice were not viable and they died at embryonic stage due to lack of development of the vascular system (Greenberg and Jin 2004). To overcome this obstacle, Oosthuyse and others (2001) generated mutant mice in which the HRE (i.e., hypoxia-response element) sequence in the VEGF gene promoter was deleted ($VEGF^{\delta/\delta}$ mice). These genetically engineered mice resulted in an unexpected phenotype, unveiling a novel role of VEGF in the survival of adult motoneurons.

$VEGF^{\delta/\delta}$ mice exhibited impaired upregulation of VEGF by hypoxia in neural tissue, so that spinal VEGF levels were reduced by 75%. Strikingly, these $VEGF^{\delta/\delta}$ mice developed symptoms of motoneuron disease beyond five months of age. They became progressively less mobile and showed signs of severe muscle weakness and limb paresis. Behavioral tests revealed

impairment of motor coordination and muscle performance. However, pain threshold was normal in $VEGF^{\delta/\delta}$ mice, indicating that motor functions were primarily affected. Electromyographic recordings showed absence of spontaneous activity in several skeletal muscles in contrast to $VEGF^{+/+}$ mice. The histological examination of muscles revealed signs of atrophy, which seemed to be due to denervation. HRE deletion caused a late-onset progressive degeneration of motoneurons, affecting the ventral horn of the spinal cord and the motor nuclei in the brainstem, with a significant loss of motoneurons and a prominent reactive astrogliosis beyond 7 months of age. The peripheral nerves of $VEGF^{\delta/\delta}$ mice progressively lost 30% of the large myelinated motor axons and showed prominent signs of Wallerian degeneration (Fig. 3A,B). Ultrastructural examination of motoneuron cytoplasm further indicated fewer Nissl bodies, and abnormal mitochondria and cell organelles. At a more advanced stage of degeneration, shrunken motoneurons presented vacuolization, fewer ribosomes and an irregular nucleus with peripheral clumping of chromatin aggregates (Fig, 3C,D). $VEGF^{\delta/\delta}$ mice did not exhibit obvious abnormalities in central structures such as hippocampus, neocortex, cerebellum, thalamus and striatum, indicating that VEGF deficiency selectively impaired motoneurons (Carmeliet and Storkebaum 2002; Lambrechts and others 2004; Oosthuyse and others 2001; Storkebaum and others 2004).

All these findings indicate that $VEGF^{\delta/\delta}$ mice suffer severe adult-onset muscle weakness and motor degeneration. The clinical symptoms and the neuropathological alterations observed in $VEGF^{\delta/\delta}$ mice are similar to those seen in humans suffering from ALS, and also similar to the SOD1 mutant mice which, as mentioned above, is a well-established model of ALS. Altogether, this means that $VEGF^{\delta/\delta}$ mice can be used as an animal model to investigate the etiology of ALS and potential therapeutical strategies (Carmeliet and Storkebaum 2002; Oosthuyse and others 2001). It should be mention that although other neurotrophic factors can

also alleviate symptoms in ALS mouse models such as BDNF, GDNF, CNTF or LIF, however, the loss of these molecules does not cause adult-onset ALS-like motoneuron degeneration

In fact, a molecular relationship has been found between SOD1 and $VEGF^{\delta/\delta}$ mutant mice. It has been reported that in SOD1 mutant mice, the expression of VEGF mRNA in the spinal cord declines significantly early in the course of the disease. Mutant SOD1 impairs post-transcriptional regulation of VEGF mRNA by sequestering key regulatory RNA-binding proteins (HuR and TIAR). In this way, mutant SOD1 destabilizes VEGF mRNA and downregulates VEGF protein production. These findings led to the postulation that the resultant destabilization of VEGF mRNA critically reduces the level of this neuroprotective growth factor and accelerates the neurodegenerative process in ALS that occurs in SOD1 mutant mice (Lu and others 2007, 2009).

Experiments of crossbreeding between transgenic mice have reinforced the role of VEGF as a neuroprotective factor for diseased motoneurons. Thus, crossbreeding of $VEGF^{\delta/\delta}$ with SOD1 mice produce $VEGF^{\delta/\delta}$/SOD1 double mutants that show increased severity of motoneuron degeneration and earlier onset of muscle weakness than mice carrying the SOD1 gene alone (Lambrechts and others 2003). *Vice versa*, when SOD1 mice are crossed with mice overexpressing VEGF, the double transgenic SOD1/$VEGF^{+/+}$ mice show delayed motoneuron degeneration and motor impairment, and prolonged survival compared with SOD1 single transgenics (Wang and others 2007). Thus, if VEGF levels are reduced, the risk of developing ALS-like symptoms increases dramatically.

The deficiency of another neurotrophic factor other than VEGF are not causal to ALS. Although the over- or under-expression of BDNF, GDNF, CNTF or LIF, among others, affect the pre- and postnatal development of motoneurons, and although adenoviral gene transfer of some of these molecules promotes motoneuron survival in animal models of ALS, the lack of

these other neurotrophic factors does not induce motoneuron degeneration as in ALS or paralysis in mice (Lambrechts and Carmeliet 2006). However, it must be emphasized that neurotrophic factors such as neurotrophins, GDNF, IGFs, CNTF, LIF, or CT-1 have important roles in cell survival during development, after injury and in response to disease (reviewed in Gould and Oppenheim 2011 and Tovar-y-Romo and others 2014).

Effects on neurodegeneration of VEGF administration

VEGF delivery has been shown to ameliorate neurodegeneration. Exposure of cell cultures to the combination of hypoxia and hypoglycemia induces consistent cell death, and VEGF treatment significantly protects motoneuron from cell death (Van Den Bosch and others 2004). VEGF protects NSC-34 motoneuron cell line exposed to cerebrospinal fluid from ALS patients (Kulsheshtha and others 2011; Vijayalakshmi and others 2015). Similarly, VEFG protects NSC-34 cells transfected with adenovirus containg the mutan SOD1 protein via the PI3-K/Akt pathway responsible among others for cell survival, growth and angiogenesis (Li and others 2003).

In vivo studies demonstrated neuroprotection by VEGF. When a lentiviral vector expressing VEGF was injected in several muscles of the SOD1 mouse model of ALS, both neuroprotection and increased life expectancy occurred (Azzouz and others 2004). Intrathecal spinal cord transplantation of immortalized human -VEGF expressing- neural stem cells delayed onset and prolonged the survival of SOD transgenic mice by a downregulation of proapoptotic proteins and upregulation of antiapoptotic proteins in the spinal cord tissue (Hwang and others 2009). VEGF delivered via an adeno-associated virus 9 in the SOD1 mice resulted beneficial via the activation of the PI3-K/Akt survival pathway. In addition, VEGF

reduced the expression of the toxic M1 microglia and enhanced the expression of neuroprotective M2 microglial phenotype (Wang and others 2016).

The direct administration of VEGF in ALS and excitotoxic models also resulted beneficial by a molecular mechanism that would require activation of VEGFR-2 (Storkebaum and others 2005). The intraperitoneal administration of VEGF reduced astrogliosis in the ventral horn of the spinal cord, preserves neuromuscular junctions, increases motor performance, delays disease progression, and prolongs survival in ALS transgenic mice (Zheng and others 2004, 2007). Interestingly, in a rat model of excitotoxic spinal cord neurodegeneration, implantation of osmotic minipumps into the dorsal spinal cord for VEGF administration prevents hindlimb paralysis and motoneuron death induced by AMPA through VEGFR-2 and activation of PI3-K pathway and inhibition of p38MAPK (Tovar-y-Romo and Tapia 2010).

Evidence between VEGF deficiency and ALS in humans

ALS literature shows contradictory evidences with that on animal models. Spontaneous mutations of the HRE in the promoter region of VEGF gene have not been detected in ALS individuals, as is the case for $VEGF^{\delta/\delta}$ mice. A meta-analysis of about 2000 human subjects from Sweden, Belgium and England detected that subjects homozygous for the ‑2578A/‑1154A/‑634G or ‑2578A/‑1154G/‑634G haplotypes in the VEGF gene promoter were more common in the population of ALS patients than in healthy individuals. These “at-risk” haplotypes reduced the levels of VEGF in plasma and decreased VEGF gene transcription (Lambrechts and others 2003; Terry and others 2004). Other studies reported no association between the “at-risk” haplotypes and ALS in various populations from other countries (Del Bo and others 2008; Van Vught and others 2005; Zhang and others 2006). Subsequently, a meta-

analysis was carried out to clarify the different results including over 7000 subjects from eight European and three American populations. The results did not support the original conclusion that VEGF haplotypes increase the risk of ALS in humans, but the significant association of the low-VEGF -2578AA genotype with increased susceptibility to ALS in males reconsiders again the link between reduced VEGF concentrations and ALS (Lambrechts and others 2009).

Reports on the levels of VEGF found in plasma, serum and cerebro-spinal fluid are also contradictory. Plasma VEGF levels were shown to be about 50% lower in individuals with ALS than in unaffected spouses (Lambrechts and others 2003). However, other studies have not found a difference in serum or plasma VEGF levels between ALS patients and healthy individuals (Devos and others 2004), or even VEGF have been found to be increased in serum from patients with ALS (Nygren and others 2002). Disease side effects such as plateled release of VEGF or hypoxia due to respiratory dysfunction might explain the variety of data reported. Therefore, plasma VEGF levels might initially be lower but subsequently increase due to hypoxia at later stages of the disease complicating data interpretation (Lambrechts and others 2004).

VEGF levels in the cerebro-spinal fluid of patients at an early stage of the disease have been reported to be low (Devos and others 2004). However, other studies report that VEGF is increased in the cerebro-spinal fluid from ALS patients (Gupta and others 2011; Ilzecka 2004). These contradictory results could be due to the small groups of patients and controls studied and to methodological issues.

In an interesting study evaluating the spinal cord of *post-mortem* ALS and control individuals, immunohistochemistry procedures demonstrated a reduction in the staining for VEGF and VEFGR-2 in the neuropil of the anterior horn in ALS cases. A reduced expression of VEGF and VEGFR-2 was confirmed by Western blotting and quantitative PCR. These

results would support the hypothesis that reduced VEGF signaling may play a role in the pathogenesis of ALS (Brockington and others 2006).

Mechanisms of action of VEGF on ALS animal models

The presence of ALS-like symptoms due to the decreased VEGF levels in $VEGF^{\delta/\delta}$ mutant mice has led to the proposal of two hypotheses, non-mutually exclusive, to explain the mechanism of action by which the deficiency of this factor produces motoneuron death: the vascular and the neurotrophic insufficiency hypotheses. In the SOD1 transgenic mice, the mutant SOD1 protein competes with HuR for binding, destabilizing VEGF mRNA and thereby downregulating VEGF synthesis. Thus, SOD1 mice also present low VEGF levels and the motoneuron degeneration found in this model might be also explained, at least in part, by the same mechanisms proposed for $VEGF^{\delta/\delta}$ mice as schematized in Fig. 4.

The possibility of vascular abnormalities was investigated in $VEGF^{\delta/\delta}$ mice. Capillary densities and lumen size were normal in peripheral nerves and spinal cords, with no signs of leakiness, obstruction or microangiopathy. However, reduced spinal cord perfusion seemed specific as renal perfusion and muscle oxygenation were normal (Oosthuyse and others 2001). Chronic neuronal ischemia leads to oxidative stress and excitotoxicity, and motoneurons are especially vulnerable to oxidative damage, due to their large size, high metabolism and distinct profile of glutamate receptors and calcium binding proteins (Shaw and Eggett 2000). Alternatively, the lack of sufficient supply of oxygen and nutrients to motoneurons due to reduced vascular perfusion in mutant $VEGF^{\delta/\delta}$ mice can be enough to trigger a neurodegenerative process.

The second mechanism could be that motoneurons in $VEGF^{\delta/\delta}$ mice degenerate because there is insufficient VEGF-dependent neuroprotection, but independent of its angiogenic

activity (Fig. 4). Oosthuyse and others (2001) studied the possible VEGF neuroprotective effect on cultured primary motoneurons of $VEGF^{+/+}$ embryos, which lack vascular irrigation. The addition of VEGF to the culture increased survival of motoneurons by 20% in baseline conditions and by 50% after serum deprivation. VEGF also protected the mouse motoneuron NSC-34 cells against apoptosis induced by tumor necrosis factor-α, hypoxia, oxidative stress (H_2O_2), or serum deprivation. The survival effect of VEGF required the activation of both VEGFR-2 and NRP-1, since the percentage of survival partially dropped by adding either anti-NRP-1 antibody or anti-VEGFR-2 antibody to the culture medium. A combination of anti-VEGFR-2 and anti-NRP-1 antibodies was required to completely neutralize the VEGF-dependent motoneuron survival activity (Oosthuyse and others 2001). Altogether, it is possible that motoneurons in $VEGF^{\delta/\delta}$ mice degenerate because of insufficient VEGF-dependent neuroprotection. Since VEGF is strongly and rapidly induced by hypoxia and other insults (e.g., acidosis), VEGF may represent a neuroprotective factor for motoneurons exposed to stress conditions. Therefore, motoneuronal VEGF-mediated neuroprotection may be relevant not only during hypoxia but also under other conditions that can represent a damage to motoneurons. For instance, excitotoxicity due to an excess of glutamate has been reported as one of the possible causes of ALS (Dhasmana and others 2022; Valko and Ciesla 2019) and VEGF administered *in vivo* protects motoneurons against excitotoxicity (Tovar-y-Romo and others 2007; Tovar-y-Romo and Tapia 2010).

Proof of direct effect of VEGF in neuroprotection is the lack of angiogenesis caused by VEGF delivery (Azzouz and others 2004). Moreover, the intracerebrospinal administration of VEGF in SOD1 rats is also beneficial without inducing changes to blood vessel density or permeability, suggestive also of a direct neurotrophic effect of VEGF (Storkebaum and others 2005). In another work, VEGF was administered using osmotic minipumps in a rat model of spinal cord excitotoxicity, and the factor was demonstrated to rescue motoneurons from

384 excitotoxic cell death with no changes in the vascular architecture of the spinal cord (Tovar-y-
385 Romo and others 2007). Interestingly, a dose-dependent study of the effects of VEGF
386 administration on ischemic rat brains demonstrated that doses not inducing angiogenesis were
387 the only neuroprotective. On the contrary, the high dose of VEGF induced angiogenesis and
388 showed absence of neuroprotection perhaps due to the deleterious effects of brain edema and
389 inflammation (Manoonkitiwongsa and others 2004).

390

391 *Neurotrophic action of VEGF in extraocular motoneurons*

392 Extraocular motoneurons are involved in the generation of eye movements and lay in three
393 distinct brainstem nuclei: the oculomotor, the trochlear and the abducens nuclei. The
394 physiological actions of VEGF have been studied in detail in the abducens motoneurons of the
395 cat following lesion and exogenous VEGF administration, as well as after anti-VEGF antibody
396 treatment in undamaged motoneurons. The abducens nucleus offers several advantages for the
397 study of lesion-induced plasticity: the discharge pattern of abducens motoneurons is well
398 characterized, and both their afferents and the signals they carry have been described in detail
399 (Büttner-Ennever 2006; Davis-López de Carrizosa and others 2011; Delgado-García and others
400 1986; Escudero and Delgado-García 1988; Escudero and others 1992; Horn and Straka 2021).
401 In addition, the firing activity of abducens motoneurons can be recorded using the alert chronic
402 animal preparation, which allows a direct correlation of motoneuronal firing with eye
403 movements under different experimental conditions (e.g., lesion, VEGF or anti-VEGF antibody
404 treatment).

405 Extraocular motoneurons are less vulnerable than other cranial or spinal motoneurons
406 to degeneration in ALS (Haenggeli and Kato 2002; Reiner and others 1995). However, it is
407 important to note that they also exhibit some signs of degeneration but at very late stages of the

disease (Kimura and others 2014; Sharma and others 2011; Takahashi and others 1993; Tjust and others 2017).

Peripheral administration of VEGF to axotomized abducens motoneurons

Extraocular eye muscles contain VEGF (Calvo and others 2018; Silva-Hucha and others 2020) and thus the axotomy of abducens motoneurons leaves these cells deprived of their retrograde source of VEGF. The recording of the discharge of these motoneurons in alert cats has revealed that (control) abducens motoneurons show a typical tonic-phasic firing pattern that correlates with eye position and eye velocity, respectively, during the different types of eye movements (Delgado-García and others 1986). When these motoneurons are axotomized, they exhibit an overall decrease in firing rate and a significant reduction in neuronal eye position and velocity sensitivities due, at least in part, to the loss of synaptic inputs (a process known as "synaptic stripping", reviewed in Alvarez and others 2020), as first described by Delgado-García and others (1988) and later confirmed by other authors (Calvo and others, 2018, 2020; Davis-López de Carrizosa and others, 2009, 2010).

The chronic administration of VEGF to the proximal stump of the transected VIth (abducens) nerve prevents and recovers the synaptic loss and the firing alterations induced by axotomy. These findings were obtained after using two different protocols of administration. In the immediate administration protocol, VEGF first dose was applied just immediately after lesion, preventing the appearance of axotomy-induced alterations. In the delayed administration protocol (VEGF first dose 20 days after lesion), VEGF recovered the synaptic and physiological changes already present in severed motoneurons (Calvo and others 2018). It was also demonstrated that the tyrosine kinase receptors of VEGF, i.e., VEGFR-1 and VEGFR-2, mediate different roles in the maintenance of synaptic afferents to abducens motoneurons. It

should be mentioned that, in contrast to VEGF, the administration to axotomized abducens motoneurons of the neurotrophins BDNF, NT-3 or NGF induces only partial recovery of synaptic inputs and discharge activity (Davis-López and others 2009, 2010).

Interestingly, the administration of VEGF-B, using the immediate administration protocol, also prevents the modifications induced by injury in abducens motoneurons (Calvo and others 2018). Previous results have also reported a neuroprotective role for VEGF-B in spinal motoneurons. Thus, VEGF-B protects cultured primary motoneurons against degeneration. Moreover, mice lacking VEGF-B also develop a more severe form of motoneuron degeneration when intercrossed with mutant SOD1 mice. When delivered intracerebroventricularly, VEGF-B prolongs the survival of mutant SOD1 rats (Poesen and others 2008). Altogether, these results may have an important therapeutic value, because VEGF-B is virtually non-angiogenic and, therefore, its administration in the case of motoneuronal disorders could produce beneficial effects without the side-effects of a high dose of VEGF, such as tissue edema and inflammation due to excessive angiogenesis and vascular permeability (Calvo and others 2018; Manoonkitiwongsa and others 2004; Poesen and others 2008).

Several types of brainstem and spinal motoneurons downregulate the expression of choline acetyltransferase when axotomized (reviewed by Navarro and others 2007; see also Morcuende and others 2005, 2013). Thus, peripheral administration of VEGF to axotomized extraocular motoneurons in adult rats also prevents the loss of their cholinergic phenotype. (Acosta and others 2018). The immunostaining, enzymatic activity, and mRNA levels of choline acetyltransferase are also reduced in spinal motoneurons of ALS patients (Nagata and others 1982; Oda and others 1995; Virgo and others 1992). All these findings suggest that VEGF administered to diseased motoneurons not only rescues them from cell death (as

456 described above) but also maintains their neurotransmissive phenotype, synaptic inputs, and
457 discharge pattern (Calvo and others 2018; Acosta and others 2018).

458

459 Intraventricular delivery of a single dose of VEGF to axotomized abducens motoneurons

460 The administration of VEGF into the IVth ventricle, located dorsal to the abducens nucleus,
461 just after the section of the VIth nerve, also prevents the axotomy-induced alterations in the
462 discharge pattern and synaptic inputs of damaged abducens motoneurons, as demonstrated by
463 single-unit extracellular recordings and immunocytochemistry (Calvo and others 2020).

464 In addition, the study of the vasculature after VEGF intraventricular delivery revealed
465 absence of angiogenesis in the abducens nucleus as well as in other brainstem nuclei (Calvo
466 and others 2020). Absence of angiogenesis and vascular leakage has also been shown after the
467 peripheral administration of VEGF in the three extraoculomotor nuclei after axotomizing all
468 extraocular motoneurons in adult rats (Acosta and others 2018). These findings indicate that
469 the beneficial effects obtained with VEGF result likely from a direct action of the factor on the
470 motoneurons themselves rather than by an indirect action on the vascular system (Acosta and
471 others 2018; Calvo and others 2020). A recent study using an anti-VEGF-antibody, to reduce
472 the retrograde delivery and transport of this factor, has shown that control, treated extraocular
473 motoneurons turn into a lesioned state, both in terms of firing and synaptic coverage (Calvo and
474 others 2022)

475 A schematic drawing is illustrated in Fig. 5 to compare the changes in synaptic inputs
476 and firing of abducens motoneurons in the three conditions, i.e., control (Fig. 5A), axotomy
477 (Fig. 5B), and anti-VEGF antibody treatment (Fig. 5C). The data obtained in extraocular
478 motoneurons demonstrating the neurotrophic effects of VEGF (Acosta and others 2018; Calvo
479 and others 2018, 2020, 2022) are consistent with all the evidence summarized in the present

review supporting the neuroprotective effects of VEGF studied mainly in spinal motoneurons from animal models of motoneuron disease.

Conclusions

Vascular endothelial growth factor (VEGF) was discovered by its angiogenic activity, but strikingly it appears first in evolution as a neurotrophic factor, necessary for the appropriate development of the nervous system in invertebrates lacking a vascular system or having a rudimentary vasculature. Neuroscience interest in VEGF has been growing remarkably in recent years due to numerous evidence showing that VEGF is neuroprotective. Mutant mice characterized by low levels of VEGF ($VEGF^{\delta/\delta}$) suffer severe adult-onset muscle weakness and neurodegeneration, symptoms that resemble ALS. Experiments of crossbreeding between transgenic mice have reinforced the role of VEGF as an essential factor for motoneuron survival. Thus, when SOD1 mice (a classical animal model of ALS) are crossed with mice overexpressing VEGF ($VEGF^{+/+}$), the double transgenic animals show reduced motoneuron degeneration and a longer life expectancy. The opposite occurs when $VEGF^{\delta/\delta}$ and SOD1 mice are crossed, as the double mutants show earlier and more severe motoneuron degeneration and muscle weakness.

Recent results in extraocular motoneurons also point to a role of VEGF in neuroprotection since the administration to the proximal stump of the sectioned nerve or intraventricularly prevents and recovers all structural and functional changes induced by lesion, returning axotomized motoneurons into a normal operation mode. A striking result has recently been shown in this model consisting in the administration of VEGF neutralizing antibody to intact, undamaged extraocular motoneurons. Results have shown that antibody treated

motoneurons resemble injured motoneurons in that they discharge abnormally at low frequencies and also experience synaptic loss.

All the findings summarized in the present review indicate that VEGF is an essential neurotrophic factor for the survival and physiology of motoneurons and suggest that this factor might have a high potential therapeutic value in the treatment of motor neuron disorders.

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The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iDs

Angel M. Pastor: https://orcid.org/0000-0002-6213-7454

Rosa R de la Cruz: https://orcid.org/0000-0001-5301-5108

Paula Martín Calvo https://orcid.org/0000-0001-9817-8728

Rosendo García Hernández https://orcid.org/0000-0001-7098-8630

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Figure legends

Figure 1. VEGF family and receptors. The figure is a representation of the members of the vascular endothelial growth factor (VEGF) family and the specific binding of each member to the different VEGF receptors. In addition to VEGF-A (also known as VEGF), the other isoforms of the VEGF family in mammals are VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGF receptors include the tyrosine kinases VEGFR-1, VEGFR-2 and VEGFR-3 and the co-receptors neuropilin-1 (NRP-1) and NRP-2.

Figure 2. Regulation of VEGF by hypoxia. Under normoxia, HIF-1α is hydroxylated by proline (P) hydroxylase domain-containing proteins (PHD), which requires O_2 for their enzymatic activity. Hydroxylation of HIF-1α allows its interaction with the von Hippel-Lindau (VHL) protein, a component of the E3 ubiquitin ligase complex. This interaction results in ubiquitination (Ub) and degradation by the proteasome pathway of HIF-1α and, consequently, prevents VEGF transcription in normoxia. Under hypoxic conditions, PHDs are inactive due to lack of O_2. Therefore, HIF-1α cannot be hydroxylated and thus not ubiquitinated for proteasome degradation. This allows HIF-1α to enter the nucleus, dimerizes with HIF-1β and, in association with the p300/CBP coactivators, this complex binds to HRE in the VEGF gene promoter activating VEGF transcription.

Figure 3. Motoneuronal degeneration in $VEGF^{\delta/\delta}$ mice. (A, B) Images of sciatic nerve semi-thin sections from wild type (A) and $VEGF^{\delta/\delta}$ mice (B). In contrast to the wild type (A), the sciatic nerve of the $VEGF^{\delta/\delta}$ mice shows signs of axonal loss and Wallerian degeneration (B). (C, D) Transmission electron microscopy images of spinal cord motoneurons from wild type (C) and $VEGF^{\delta/\delta}$ mice (D). The motoneuron in C shows typical ultrastructural characteristics of this cell type such as a large round nucleus, with homogeneous chromatic and a conspicuous nucleolus, and a cytoplasm containing a prominent rough endoplasmic reticulum

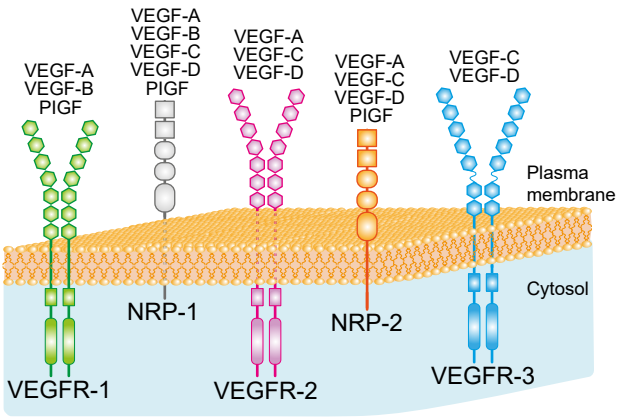
and mitochondria. The ultrastructure of the VEGF$^{\delta/\delta}$ motoneuron (D) shows signs of clear degeneration. Note cell shrinkage, an irregular nucleus with aggregates of peripheral chromatin, and a reduced cytoplasm with vacuolized mitochondria and endoplasmic reticulum. Reprinted from Carmeliet P, Storkebaum E. 2002. Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. Semin Cell Dev Biol 13(1):39-53. Copyright (2002), with permission from Elsevier.

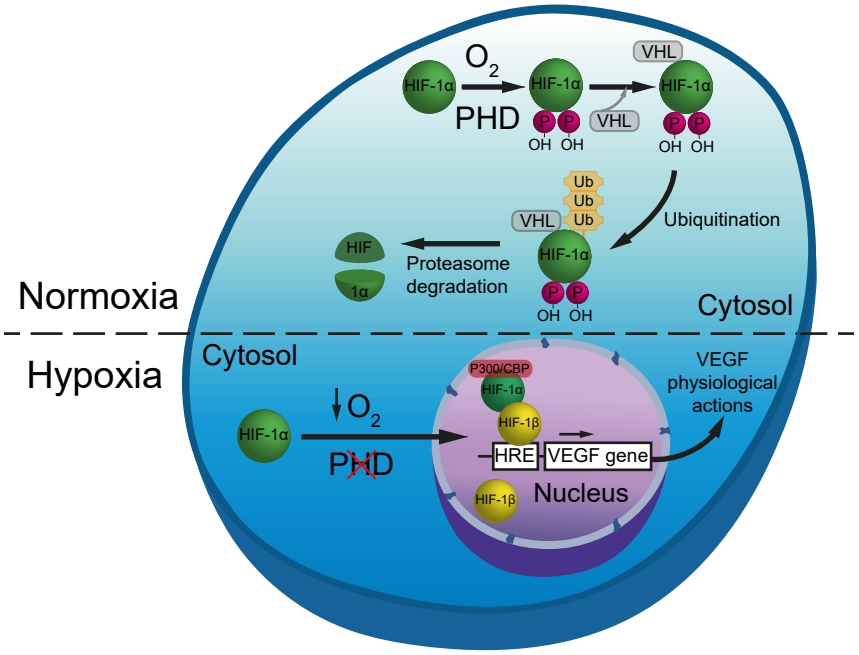
Figure 4. Proposed mechanisms of action of VEGF in ALS motoneurons. (A) In healthy conditions, VEGF is adequately synthesized and acts on motoneurons by two likely mechanisms. First, due to its effects on the vasculature, motoneurons are provided by appropriate levels of oxygen and nutrients. Second, VEGF acts also as a neurotrophic factor for motoneurons. (B) In ALS, the synthesis of VEGF is reduced. The effects of low levels of VEGF on blood vessels produces a deficit in oxygen and nutrient supply to motoneurons. In addition, there is insufficient VEGF neurotrophic support. These two processes leads to motoneuron degeneration. (C) The potential therapeutic value of VEGF can be used as a strategy to promote motoneuron recovery. VEGF delivery (through different routes and methods) reestablishes normal levels of VEGF that would act on diseased motoneurons enhancing the supply of oxygen and nutrients via the restored vascular function and by providing the required neurotrophic support and thus preventing motoneurons from degeneration.

Figure 5. Schematic representation summarizing the effects of the administration of anti-VEGF antibody to abducens motoneurons on their discharge activity and synaptic inputs. (A) Control motoneurons receive abundant synaptic boutons contacting their cell body, of both excitatory (in red) and inhibitory (in blue) nature. They exhibit a typical tonic-phasic discharge pattern of action potentials (AP) in correlation with eye movements (EP, eye position). (B) After axotomy, the motoneuron experiences a significant loss of afferent synaptic boutons (excitatory and inhibitory), a phenomenon known as synaptic stripping. In congruence, the

911 discharge pattern of axotomized motoneurons is markedly reduced. (C) Uninjured motoneurons
912 treated with the neutralizing antibody against VEGF (VEGF ab) applied in the muscle lack their
913 retrograde source of VEGF. Treated motoneurons also show synaptic stripping and a reduced
914 firing pattern in correlation with eye movements, resembling the axotomy situation.

915


VEGF-A
VEGF-B
PIGF
VEGF-A
VEGF-B
VEGF-C
VEGF-D
PIGF
VEGF-A
VEGF-C
VEGF-D
VEGF-A
VEGF-C
VEGF-D
PIGF
VEGF-C
VEGF-D
Plasma
membrane
Cytosol
NRP-1
NRP-2
VEGFR-1
VEGFR-2
VEGFR-3


Normoxia
Hypoxia
Cytosol
Cytosol
HIF-1α
O2
PHD
HIF-1α
P P
OH OH
VHL
VHL
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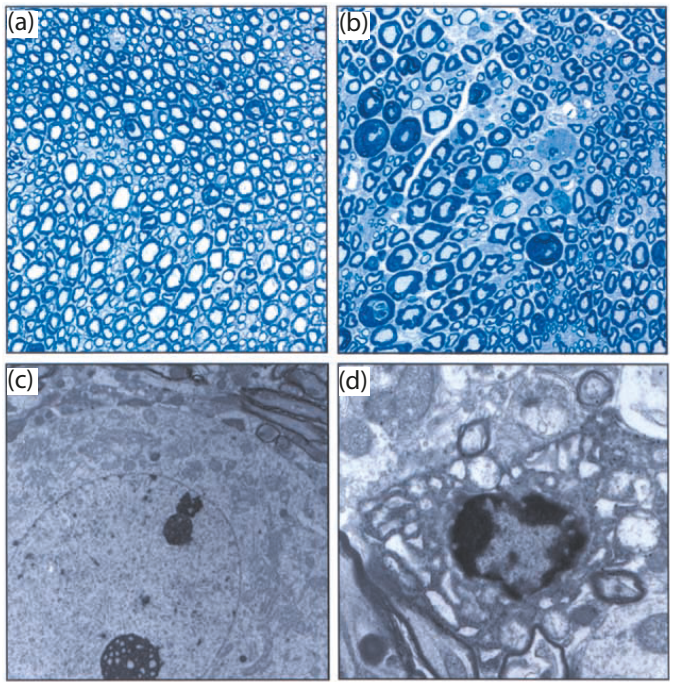
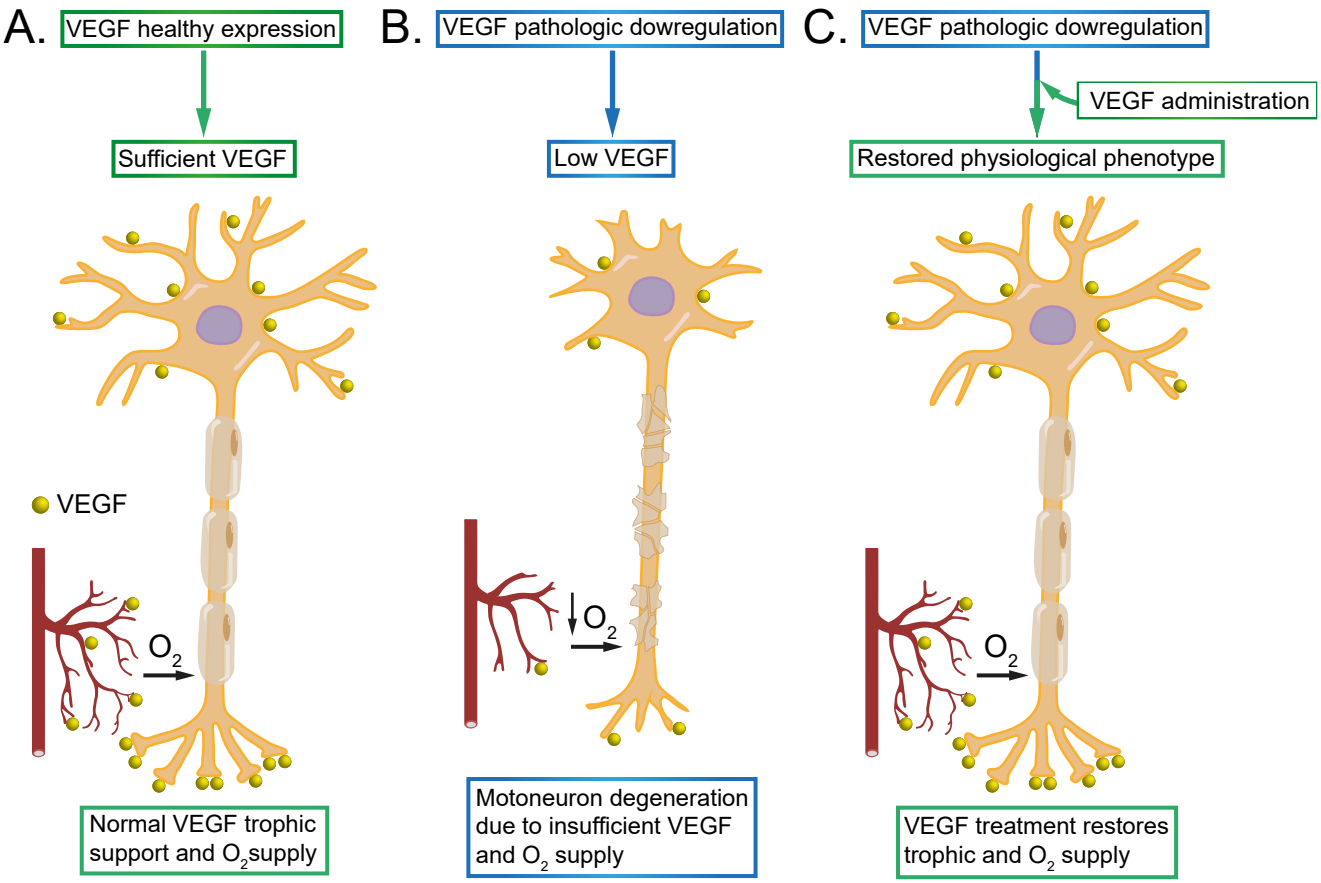
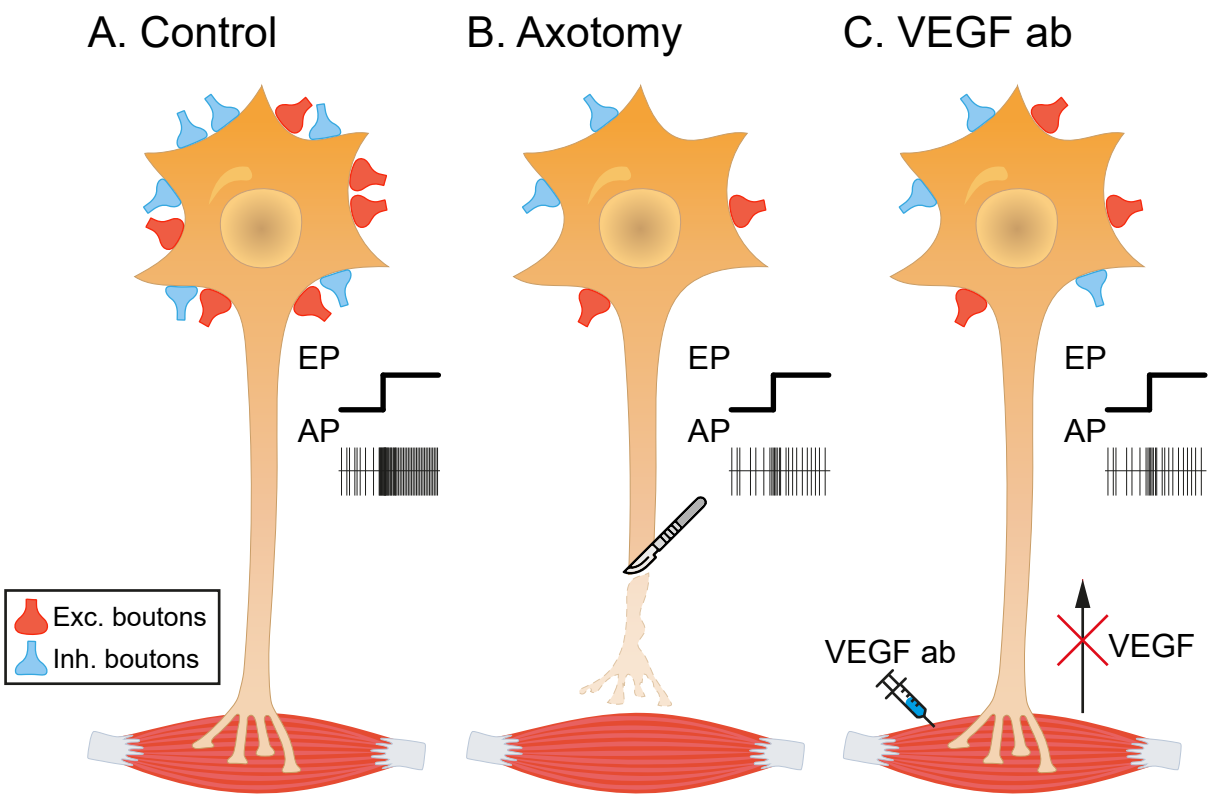
P P
OH OH
Ubiquitination
Ub
Ub
VHL
Ub
HIF-1α
P P
OH OH
HIF
1α
Proteasome
degradation
HIF-1α
O2
PHD
P300/CBP
HIF-1α
HIF-1β
HRE
VEGF gene
Nucleus
HIF-1β
VEGF
physiological
actions


(a)
(b)
(c)
(d)


A.
VEGF healthy expression
Sufficient VEGF
VEGF
O2
Normal VEGF trophic support and O2 supply
B.
VEGF pathologic dowregulation
Low VEGF
O2
Motoneuron degeneration due to insufficient VEGF and O2 supply
C.
VEGF pathologic dowregulation
VEGF administration
Restored physiological phenotype
O2
VEGF treatment restores trophic and O2 supply


A. Control
B. Axotomy
C. VEGF ab
EP
AP
EP
AP
EP
AP
Exc. boutons
Inh. boutons
VEGF ab
VEGF