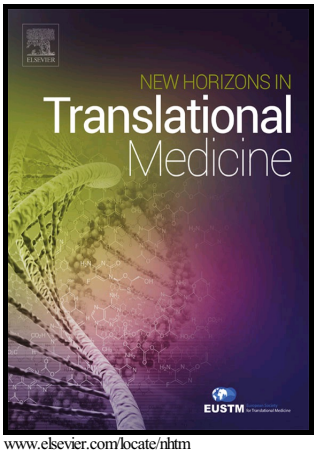

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Luca Domenico D'Andrea, Lucia De Rosa, Chiara Vigliotti, Mauro Cataldi

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Luca Domenico D'Andrea[1], Lucia De Rosa[1], Chiara Vigliotti[2] and Mauro Cataldi[2*]

[1]Institute of Biostructure and Bioimaging, National Research Council, Naples, ITALY and
[2]Division of Pharmacology, Department of Neuroscience, Reproductive and Odontostomatologic Sciences, Federico II University of Naples, ITALY.

Corresponding author: Mauro Cataldi, Division of Pharmacology, Department of Neuroscience, Reproductive and Odontostomatologic Sciences, Federico II University of Naples, ITALY; email: cataldi@unina.it; phone: +39-0817462102; Fax: +39-0817463013

ABSTRACT

VEGF is expressed in central nervous system and its expression increases in hypoxia and in inflammatory brain disorders. A wealth of data suggests that VEGF may exert neuroprotective activities and promote neuroregeneration in disease status. Moreover, the risk of developing certain neurological disorders may be

dependent on dysfunction in the VEGF system. Therefore, a strong rationale does exist to suggest that VEGF-based therapeutics could be implemented in conditions such as stroke or amyotrophic lateral sclerosis and experimental data supporting this hypothesis have been obtained. However, the unfavorable pharmacokinetic profile of this growth factor, and concerns on its safety have limited the development of VEGF as a therapeutic tool for neurological disorders. In this review, we discuss why a new class of VEGF mimic peptides holds promises to become a safer, cheaper and more easily manageable tool for central nervous system therapeutics.

Focal Points:

- **Bench:** The analysis of the effects of small peptides reproducing one or more regions of VEGF may help understanding basic issues on the structure-activity relationship of this growth factor.
- **Bedside:** Small VEGF-mimic peptides could have better pharmacokinetic and/or toxicological properties than VEGF and be, therefore, potentially suitable for use in human diseases.
- **Community:** VEGF-mimic peptides-based therapeutics could help reducing the burden of severe neurodegenerative disorders that cannot be efficiently treated with currently available drugs.

Keywords: VEGF; stroke; Alzheimer disease; Parkinson's disease; Amyotrophic Lateral Sclerosis; Neurodegeneration; Epilepsy; QK peptide; intranasal administration.

Abbreviations: AD: Alzheimer's disease; ALS: Amyotrophic lateral sclerosis; AMD: age-related macular degeneration;ARE: adenine/uridine rich elements; BBB: blood brain barrier; CNS: central nervous system; CSF: cerebrospinal fluid; ECS: electroconvulsive shock; HA: hyaluronic acid; icv: intracerebroventricular; PD: Parkinson's disease; PLGA: poly(lactic-co-glycolic acid); pMCAO: permanent occlusion of the middle cerebral artery; SOD: superoxide dismutase; tMCAO: transient occlusion of middle cerebral artery; VEGF: Vascular Endothelial Growth Factor; VEGFR: Vascular Endothelial Growth Factor receptor.

Vascular Endothelial Growth Factor (VEGF) is the best characterized proangiogenic factor. Its role in neoplastic growth has been firmly established and a large array of drugs inhibiting its biological activity has been developed for the treatment of specific forms of cancer or other diseases, such as age-related macular degeneration (AMD), in which blocking the formation of new blood vessels can be helpful. While in these conditions VEGF activity has to be inhibited, ischemic diseases could benefit of the proangiogenic effects of this growth factor that could, therefore, have specific therapeutic applications. The use of VEGF for therapeutic angiogenesis has been evaluated in cardiovascular disorders such as limb and heart ischemia, with mixed results [1-4]. More recently, it became clear that VEGF could also have potential therapeutic applications in a number of neurological disorders because, besides its beneficial proangiogenic activity, it also has neuroprotective and neuroregenerative effects [5]. This idea that VEGF could have relevant applications in nervous system therapeutics is further supported by the evidence that disturbances in VEGF synthesis and/or release occur in several neurological diseases. This suggests that exogenous supplementation of VEGF could correct the underlying dysfunction in the release or activity of this growth factor. However, several issues complicate the use of VEGF in diseases of the central nervous system including problems in its delivery beyond the blood brain barrier (BBB) and safety concerns. In this paper we will review the main potential clinical application of VEGF therapeutics in central nervous system (CNS), the obstacles to its implementation and how a new class of compounds, the small VEGF-mimic peptides could overcome these problems and prospectically represent a new tool for the treatment of neurological disorders.

1. VEGF AS A TOOL FOR NEUROPROTECTION AND NEUROREGENERATION

A great number of excellent reviews have been published on VEGF structure and biological activity that can help understanding why and how VEGF could help in CNS disorders [5, 6]. Here we will just quickly remember a few points that we believe essential for the issue of this review i.e. the design and potential applications of VEGF-mimic peptides in neurological disorders.

VEGF is a 34-42 kDa homodimeric glycoprotein belonging to the protein family of the cystine-knot growth factors. The VEGF family is composed of several isoforms (Table 1), VEGF-A, VEGF-B, VEGF-C

VEGF-D and PlGF that differ in their ability to bind to VEGF receptors. Most of the biological activity in CNS is exerted by VEGF-A and in the present review we will focus on this isoform.

At least six different VEGF-A isoforms are originated through the differential splicing of the the *vegf* gene: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$. They differ in the C-terminal region where the binding determinants for heparin, encoded for by exons 6 and 7, are located (Figure 1). Consequentely, the different VEGF isoforms differ in their ability to bind heparin and in their mobility in the extracellular space. More specifically, $VEGF_{121}$ is freely diffusible because it does not bind heparan-sulfate whereas $VEGF_{189}$ strongly binds to this molecule and is, therefore, retained in the extracellular matrix and on cell membranes; the main VEGF isoform $VEGF_{165}$ has intermediate properties [7-9]. Splicing isoforms with anti-angiogenic bioactivity have also described ($VEGF_{165b}$ and VEGF-Ax) but their role and activity are still controversial [10-13].

The crystal structure of $VEGF_{8-109}$ has been determined [14,15]. This protein has a cystine knot structure similar to PDGF but, unlike this growth factor, VEGF has a α-elical N-terminus [14] (Fig. 2). VEGF monomers consist of a total of seven β-strand segments (β1 to β7) and two α-helical segments (α1 and α2) [15]. Its molecule has a central portion made by four antiparallel β sheets arranged in two pairs: β1 and β3, and β5 and β6. The cystine knot is made by two disulfide bonds forming a covalently linked ring structure between β3 and β7 plus a third disulfide bond that connects β1 and β4 penetrating the ring. Two monomers assemble in an antiparallel fashion to make a dimer. Two symmetrical disulfide bonds between Cys51 and Cys60 keep the monomer together. Dimeric conformation is stabilized by hydrofobic interactions among loop β1 to β3 and segment β5 to β6 of one monomer, and the N-terminal α helix of the other monomer. The two poles of the dimer contain the cystine knot regions.

Three different VEGF receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1), and VEGFR-3 (Flt-4) have been cloned. VEGFA binds to VEGFR-1 and VEGFR-2, VEGF-B and PIGF to VEGFR-1 whereas VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3 (Table 1). Neuropilin receptors NRP1 and NRP2 act as coreceptors for VEGFR-1 and VEGFR-2 and can bind VEGF-A, VEGF-B and PlGF. The three different receptor types mediate different VEGF biological actions being VEGFR-2 responsible for the mitogenic, angiogenic and permeability-enhancing effects of VEGF and VEGFR-3 involved in lymphoangiogenesis [16]. The role of VEGFR-1 is more controversial because it is believed to act mainly as

a VEGF decoy although it can also cooperate with VEGFR-2 in mediating some of VEGF biological effects [17**]**. VEGFRs are composed of seven immunoglobulin-like extracellular domains joined to the intracellular kinase domains by a short transmembrane region. Mutational and crystallographic studies suggest that the two poles of the dimer represent the portion of the VEGF molecule taking part to receptor binding. More specifically, VEGFR binding to VEGFR-2 receptors involves two identical symmetrical sites each composed by residues of β2 (Ile-46) and β5 (Gln-79, Ile-83, Pro-84, Lys-85) sheets, of the α2-β2 loop (Ile-43) of one monomer, and by residues of the N-terminal helix (Phe-17) and of the β3-β4 loop (Glu-64) of the other [14]. Upon VEGF binding, VEGF receptors dimerize and become auto-phosphorylated at the intracellular kinase domain on specific Tyr residues that act as docking sites for adaptor molecules [18,19]. The assembly of this complex ends with the activation of multiple transduction pathways that include the MEK-MAPK cascade via PLCγ, PI3K-Akt pathway via Shb, Src via TSAd and p38 [20]. An important player in signal transduction through VEGFR-2 is eNOS that is activated through PLC-γ and PI3K-dependent phosphorylation [21]. After activation, VEGFR-2 is internalized and recycled.

VEGF and its receptors are expressed both in prenatal and in postnatal neurons [5,22,23] and their expression greatly increases after injury, for instance after stroke (see the next section). Distinct roles have been identified for VEGF both in the development of central nervous system and in the adult brain but their analysis goes beyond the scope of this review and the interested reader can find details on these issues in several excellent recent reviews [5, 24,25]. Here we will briefly review the biological effects that make VEGF valuable as a potential tool for CNS therapeutics: neuroprotection and neuroregeneration.

As we will review with more details in the next section, VEGF exerts neuroprotective activity in a number of experimental models of neurodegenerative diseases both *in vitro* ed *in vivo* such as, for instance, the exposure to amyloid peptides, to glutamate or the combined deprivation of oxygen and glucose [26-30]. Most of this neuroprotective activity can be explained by the activation via VEGF-R2 of the prosurvival cascade involving PI3-K and Akt [27,28,31]. An additional mechanism that could have a role in VEGF-mediated neuroprotection is the inhibition of voltage gated K^+ channel activity in neurons. It has been demonstrated, indeed, that the efflux of K^+ channels is an early event in neuronal death and that its pharmacological blockade can spare neurons from dying [32,33]. Xu et al. [34] showed that VEGF suppresses K^+ currents in acutely dissociated neurons from rat hippocampal slices. This effect was very fast

and rapidly reversible. Because confocal imaging of the acutely dissociated neurons showed the presence of VEGFR-1 but not of VEGFR-2 it was suggested that this effect was entirely dependent on the activation of VEGFR-1 receptors [34]. VEGF effect on K^+ currents may depend on the phosphorylation of key tyrosine residues on specific K^+ channel subunits. Specifically, an increase in tyrosine phosphorylation of $K_V1.2$ subunits has been observed upon VEGF exposure in SH-SY5Y cells after hypoxia and glucose deprivation [35]. VEGF-induced $K_V1.2$ phosphorylation may involve a PI3-K dependent cascade because it is abolished by the PI3-K inhibitor wortmannin [35]. Interestingly, mutant $K_V1.2$ channels lacking key tyrosine residues have a higher activity than wild type channels suggesting that tyrosines have a role in the negative control of this channel type [36]. Importantly, the enhanced phosphorylation of $K_V1.2$ channels upon VEGF treatment has been observed also *ex vivo* in brain slices obtained from rats in which experimental brain ischemia was induced by the transient occlusion of the middle cerebral artery [37]. Other ion channels are modulated by VEGF in a way that could explain part of the neuroprotection elicited by this growth factor. It has been demonstrated, indeed, that in cultured neurons, VEGF reduces the influx of Ca^{2+} evoked by KCl or glutamate through the inhibition of high voltage activated Ca^{2+} channels [38]. By acting through PLC-γ VEGF also induces a slow and small increase in intracellular Ca^{2+} dependent on the release of this ion from intracellular stores; this effect could have a role both in the physiological effects of this growth factor, such as the promotion of neuronal plasticity, and in neuroprotection [39]. Finally, VEGF shifts leftward the activation curve of voltage gated Na^+ channels hence causing a decrease in their activity and a reduction in neuronal excitability [40].

VEGF beneficial effects in neurological diseases also depend on the ability of this growth factor to promote neuroregeneration. VEGF is indeed the key factor of the so called *neurovascular niche*. This term designes a set of different cell types including neurons, their precursors neuroblasts, astrocytes, endothelial cells and pericytes that cooperate in a highly coordinate way to mediate adult neurogenesis and to repair tissue damage in the CNS [41,42]. Not only it becomes activated and plays a crucial role in repair after ischemic lesions [43-47] but it also involved in the reparative response in inflammatory CNS diseases [48,49]. Angiogenesis has a central role in the activation of the *neurovascular niche*. Both ischemic and inflammatory stimuli trigger the formation of new blood vessels thorough the sprouting of preexisting blood vessels and the homing of circulating endothelial precursor cells and VEGF is the prime responsible of this

response [50,51]. In addition VEGF acts as a chemoattractant for sub-ventricular zone neuroblasts and promotes their differentiation [52]. Moreover, VEGF induces endothelial cells to release other chemoattractant proteins such as Ang-2 and GROα [53]. This process is amplified by the release of more VEGF and of matrix metalloproteinases 2 and 9, BDNF and stromal derived factor 1 by newly formed endothelial cells. By their side recruited neuroblasts further promote the entire process by releasing pro-angiogenic factors like angiopoietin 2 and FGF [50,51].

The data that we just reviewed suggest that VEGF may exert neuroprotective and neuroregenerative effects in the central nervous system. We also mentioned that VEGF synthesis increases after injury and this raises the possibility that this growth factor could be part of an endogenous adaptive system whose activation aims to reduce CNS damage. The major stimulus for the transcription of the VEGF gene in CNS is hypoxia acting through HIF-1 [52,53]. As such, a substantial increase in VEGF synthesis and release will occur in CNS diseases in which tissue perfusion is impaired. Moreover, it will be expected that in patients with an impaired response of VEGF to hypoxia because, for instance, of polymorphysms in the regulatory regions of the *vegf* gene, hypoxia will induce more serious damage than in normal subjects. We will review some examples of this variable susceptibility in the next section. It is very important to metion here that VEGF increase does not occur only in ischemic brain disorders but also in the presence of inflammation that as we mentioned before, is a strong stimulus for the activation of the neurovascular *niche*. This may explain why this growth factor is actually involved in conditions in which tissue hypoperfusion is not the main causative factor.

Several mechanisms that have been extensively reviewed by Szade et al. [56], can explain how the synthesis of VEGF and, more in general, the whole process of angiogenesis are activated by inflammation. Briefly, a certain degree of tissue hypoxia often occur in inflamed tissues, HIF-1 may be activated in an hypoxia-indepent way by NF-kB in response for instance to free oxygen radicals and, possibly, by NO [56].

It has also to be considered that inflammatory cells such as monocytes that penetrate from the blood into the inflammatory focus produce and release VEGF hence contributing to increase the local concentrations of this growth factor [56].

Before moving to the next section where we will examine more in detail the mechanisms of VEGF-mediated neuroprotection in selected neurodegenerative diseases, we would like to mention that several

compounds with proven neuroprotective properties such as polyphenols, may affect VEGF release and activity. Protective effects of polyphenols have been reported in different neurodegenerative diseases including Parkinson's and Alzheimer's disease, and in stroke [57-59]. Multiple mechanisms seem to converge in conferring neuroprotective activity to these natural compounds including the ability to chelate iron, to exert antioxidant and anti-inflammatory effects, to interfere with amyloid, tau and alpha-synuclein aggregation, to prevent of apoptosis and mitochondrial dysfunction, to promote vasodilation in brain vessels and to modulate sirtuin activity [58, 60-63]. The effect of polyphenols on VEGF and, more extensively, on angiogenesis is very complex. Indeed, although there is a great interest on these compounds as tools for cancer chemoprevention [64] because they exert clear antiangiogenic effect in cancer by inhibiting of HIF-1 activation, by blocking metalloproteinase activity and by activating AMPK in endothelial cells [65-68], evidence for polyphenol-induced proangiogenic effects have also been reported. For instance, it has been shown that quercetin activates an angiogenic pathway by inhibiting HIF-prolyl hydroxylase, by stabilizing HIF-1 alpha and by promoting its nuclear localisation [70], that (-)-epicatechin-3-gallate activates HIF-1α and increases VEGF expression in T47D human breast carcinoma cells [71] and that epigallocatechin gallate increases HIF-1α protein level and activates HIF-1- mediated gene transcription even under normoxic conditions in human prostate cancer cells [72]. These effects together with the proven ability to reduce the oxidative stress that is a major factor in causing cell demise in neurodegeneration [73-75] may account for polyphenol-induced neuroprotection.

2. IN WHICH NEUROLOGICAL DISORDERS COULD VEGF BE HELPFUL?

The role of endogenous VEGF and the possible beneficial effects of the administration of exogenous VEGF have been evaluated in many neurological disorders leading to the proposal that a neurovascular approach should be implemented for the treatment of neurodegenerative disease [76]. Excellent reviews on the possible therapeutic applications of VEGF have been recently published [5,6,51,77-79]. We will just remember some issues on a few neurological disorders that we believe salient in the perspective of the present paper, whereas the interested reader may find additional information on these diseases and on others that will be not discussed here such as neurotrauma or demyelinating diseases, in the review paper that we cited before.

2.1 Stroke

After stroke, oxygen tension in the ischemic brain tissue dramatically drops to very low levels and remains low until reperfusion is established. This represents a strong signal for the activation of HIF-1-dependent transcription. Consequently, an increase in VEGF transcription and protein expression is expected to occur as it was observed in different experimental stroke models [80]. Kovács et al. [81] evaluated VEGF expression in the brain after the permanent occlusion of the middle cerebral artery (pMCAO) in the rat. They found a significant increase in both $VEGF_{121}$ and $VEGF_{165}$ isoforms that was already detectable 18 hours after the surgical procedure. A different time course of VEGF expression was observed in macrophages and neurons, where it peaked after 18 hours to rapidity decrease thereafter, and in glial cells where it only peaked 5 days after ischemia. Interestingly, the highest levels of VEGF were found in macrophages. A late expression of VEGF was also observed in endothelial cells where it progressively increased with reparative angiogenesis to peak at about 1 week from surgery. A high expression of VEGFR-1 was observed in endothelial but not in other cell types. After pMCAO, Marti et al. [55] observed in the ischemic penumbra an increase not only in VEGF, and VEGFR-1 but also in VEGFR-2 expression. The evidence that the increase in VEGF was accompanied by a parallel increase in its receptors suggests that, not only, there is more VEGF in the hypoxic brain but tissue responsiveness to this growth factor is potentially also enhanced. VEGF expression also increases after the transient occlusion of middle cerebral artery (tMCAO), an experimental model of brain ischemia that resembles more closely than pMCAO to human stroke because it also reproduces the reperfusion phase. Hayashi et al. [82] observed a very rapid increase in the expression of both $VEGF_{121}$ and $VEGF_{165}$ after tMCAO that was already evident 1 hour after the surgical procedure, peaked after 3 hours and decreased after 1 day. Interestingly, VEGF immunoreactivity was also strongly increased. After bilateral clamping of the carotids, Lee et al. [83] observed a transient increase in VEGF mRNA that occurred earlier in hippocampal neurons starting after 12 hours and decaying after 1 day, and later in hippocampal, striatal and cortical astrocytes. An increase in VEGF expression was also observed in rats after the creation carotid artery-jugular vein fistula [65]. $VEGF_{164}$ was the VEGF isoform preferentially expressed after this procedure whereas the levels of $VEGF_{188}$, and $VEGF_{120}$ were very low; $VEGF_{164}$ expression was detectable 24 hours postoperatively, peaked by 7 days, decreased by 21 days, and returned to basal levels by

90 days [84]. Also in this experimental model of brain hypoxia, the increase in VEGF expression was paralleled by an increase in the expression of VEGFR-1 whereas VEGFR-2 levels did not change [84].

To establish whether the observed increase in VEGF expression after stroke could have any protective role against the ischemic damage, the effect of this growth factor was explored in experimental models in vitro of neuronal ischemia. More specifically, VEGF protected HN33, an immortalized hippocampal neuronal cell line, from the death induced by the combined deprivation of oxygen and glucose [27]. Because these cells only express VEGFR-2 and NP1 but not VEGFR-1, and VEGF effect was not replicated by the neuropilin-1 ligand placenta growth factor-2, it was concluded that neuroprotection was exerted through the activation of VEGFR-2. Pharmacological experiments provided arguments to suggest that the main transduction pathway involved was PI3/Akt [27]. VEGF was also neuroprotective against combined oxygen and glucose deprivation in primary cultures of cortical neurons [26] and protected hippocampal neuronal culture from the excitotoxic damage induced by either glutamate or N-methyl-D-aspartate [29,30]. Moreover, exogenously added VEGF reduced cell death and prevented the impairment of LTP and preserved spontaneous synaptic potentials and excitability in rat CA1 neurons, cultured in vitro and exposed to combined oxygen and glucose deprivation [66]. Importantly, the VEGFR-2 inhibitor SU5416 abolished VEGF-induced neuroprotection suggesting that this class of receptors were involved [85].

As described in the previous section, a wealth of experimental data accumulated in the decade after the publication of this first evidence led to the identification of VEGF as the key activating factor of the *neurovascular niche* and provided a clear rational for the neuroprotective effect of this growth factor in stroke. Consistent with the hypothesis that the increase in endogenous VEGF and in its receptors in the ischemic brain could drive a neuroprotective and reparative response is the evidence that VEGF inhibition worsen the ischemic damage. Such a worsening was observed both after the intracerebroventricular administration of antisense oligonucleotides against VEGF-A [86], of anti-VEGF antibodies [87] or after the intraperitoneal administration of the VEGFR-2 inhibitor SU146 [88].

If endogenous VEGF may protect against the ischemic damage, strategies that increases the synthesis of VEGF or of its receptors should be reduce brain damage after stroke. Wei and its collaborators reported an elegant demonstration of this hypothesis. They first showed that Whisker stimulation enhances angiogenesis in the barrel cortex following focal ischemia in mice [89] and later demonstrated that it induces

the expression of VEGFR in neuroblasts that migrated towards the ischemic lesion where they exerted neuroprotective and neuroregenerative effects [90]. Importantly, these effects were VEGF-mediated as indicated by their abrogation by the VEGFR-2 inhibitor SU5416 [71]. The evidence that VEGF dysfunction could be a risk factor for stroke in humans is limited. Two metanalyses on the associations of VEGF-A gene polymorphysms have been recently published [91,92]. Based on the findings of ten different studies Wu et al. [92] concluded that the +936C>T (rs3025039) polymorphism could represent a risk factor for stroke, especially in Asians, whereas the polymorphism -1154G>A (rs1570360) is not associated with this disease. Qiu et al. [91] evaluated five studies with a total of 2904 cases and 2824 controls and concluded that the +1192C>T (rs2305948) and +1719T>A (rs1870377), but not the -604T>C (rs2071559) VEGFR2 polymorphysms could be potential risk factors for stroke.

The evidence reviewed above that VEGF expression increases after stroke and that exogenous VEGF protects neuronal cells from ischemic death in vitro, prompted studies aiming to establish whether the pharmacological administration of exogenous VEGF could reduce brain damage and improve the outcome in experimental stroke. The scenario emerging from the results of the studies performed to address this question was, however, more complicated than one could expect. Briefly, while neuroprotection can be elicited with VEGF it only occurs with the exact timing of its administration and the right choice of its dosage; otherwise, VEGF may actually be detrimental and worsen the ischemic damage. The main reason why VEGF can be dangerous in stroke is that it can increase blood vessel permeability ultimately worsening ischemia-induced brain edema. We will come back to this important point in section 4 because it represents a major obstacle for the implementation of VEGF therapeutics in CNS disorders. However, now we will quickly review the main experimental evidence that led to realize the risks of VEGF therapy in stroke, starting from the seminal paper of Zhang et al. [93]. Because brain edema occurs early after stroke whereas reparative angiogenesis is a later event, they supposed that the risk of increasing blood vessel permeability could be restricted to a short time window after stroke, whereas the proangiogenic effect should last longer. Therefore, VEGF should be dangerous only if given immediately after stroke. To test this hypothesis they compared the effect of the early (1 hour) and late (48 hours) postischemic administration of VEGF. The main finding of the study was that the late VEGF administration reduced ischemic brain damage and improved the neurological recovery in a rat model of focal cerebral embolic ischemia whereas early administration worsened the outcome and

increased edema and hemorrhages. However, the idea that the beneficial effect of VEGF in stroke are only due to the promotion of new vessel formation appears quite limiting. Indeed, Sun et al. [94] demonstrated that when intracerebroventricularly injected 1 or 3 days after stroke, VEGF also promotes the neuronal survival in the penumbra and neurogenesis in the subventricular zone. The evidence that VEGF has neuroprotective effects in brain ischemia in vivo raises the important question whether delaying too much to avoid its permeabilizing effects could actually waste part of its beneficial neuroprotection. Data reported by Harrigan et al. [95] suggest that not only the timing but also the dosage of VEGF injection could determine whether BBB permeabilization will be predominant over neuroprotection. They demonstrated, indeed, that when VEGF was intraventricularly injected at a concentration of 5 μg/ml it promoted neoangiogenesis with no BBB permeabilization in normal rats [95] and reduced the ischemic damage after tMCAO with no worsening in brain edema [96]. Conversely, at the higher dose of 25 μg/ml, VEGF induced ventriculomegaly in normal rats presumably by increasing leakage through BBB [95] The dose dependence of VEGF effect in stroke was also confirmed by Manoonkitiwongsa et al. [97] who infused into the internal carotid artery three different doses of this growth factor (2, 8 and 60 μg) for one week in rats after a 4 hour occlusion of MCAO and foud neuroprotection only for the low and intermediate doses.

In conclusion, available evidence suggests that VEGF could be beneficial in stroke but only if given not too early and at not too high doses. These conditions seriously limit the implementation of VEGF therapeutics for stroke in clinical practice.

2.2 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is one of the neurological diseases in which the involvement of VEGF has been more strongly demonstrated. The first evidence linking ALS and VEGF was reported by the group of Carmeliet that studied the consequences of the genetic ablation of HIF-responsive elements in the VEGF promoter in transgenic mice [98]. These mice (Vegf $^{\delta/\delta}$ mice) had very low viability and only few of them survived till adulthood; those surviving more than 5 months developed symptoms suggestive of a motor neuron disease with severe muscle weakness and atrophy. Pathological analysis showed denervation-like changes in muscles and prominent signs of motor neuron degeneration in ventral horn of the spinal cord. A

decrease in blood vessel density was observed in spinal cord and laser Doppler flowmetry showed a decreased baseline neural blood flow. These data suggest that motor neuron degeneration in Vegf $^{\delta/\delta}$ mice could have been caused by spinal cord hypoperfusion. However, the evidence that primary motor neurons cultures from these animals showed a reduced survival when compared with those from their wild type littermates and that this low in vitro viability was rescued by exogenous $VEGF_{165}$ clearly indicates that the loss of VEGF neuroprotective effect on neurons could also be implicated. VEGFR-2 and NRP1 receptor are probably involved as suggested by the inefficacy of $VEGF_{121}$ in restore to normal motor neuron viability in vitro considering that this VEGF isoform does not bind to NRP1. Moreover, anti-NRP1 and anti-VEGFR-2 antibodies worsened the survival deficit in motor neurons from Vegf $^{\delta/\delta}$ mice.

The finding that the loss of hypoxic VEGF regulation induced an ALS-like phenotype in Vegf $^{\delta/\delta}$ mice raised the important question of VEGF was implicated just in this very unusual ALS model or it could have a more general role in this disease. An important argument in support of this possibility came from the study of G93A SOD1 mice. This is a very popular animal model of ALS that recapitulates the best characterized familial form of the human disease that is caused by inactivating mutations in superoxide dysmutase (SOD) [99,100]. A first evidence of the possible involvement of VEGF in this animal model was the finding of lower levels of VEGF as compared with controls in their spinal cord [101,102]. Moreover, G93A-SOD1 mice have a more severe spinal cord motor neuron loss after experimental spinal cord ischemia than wild type mice that can be rescued by the intraperitoneal administration of $VEGF_{165}$ (2μg) [101]. Evidence has been reported of blood flow-metabolism uncoupling in the spinal cord of these mice even before the appearance of neurological symptoms [103] and, importantly, a significant increase in HIF immunoreactivity in the spinal cord was observed as the disease progressed hence confirming that this structure is chronically hypoxic. This increase in HIF-1 was not paralleled by a similar increase in the level of the target gene VEGF [104] suggesting that somehow the SOD1 G93A mutation affected VEGF synthesis or release. A mechanism that has been proposed to explain this effect of mutant SOD is that it could affect VEGF mRNA stability. It has been shown indeed that different from wild type SOD1, mutant G93A-SOD 1 has a high affinity for adenine/uridine rich elements (ARE) in the 3'-untranslated region (UTR) of the VEGF mRNA [105]. ARE sequences normally regulate mRNA stability through the binding to specific RNA stabilizing proteins such

as members of the embryonic lethal abnormal visual (ELAV). What is supposed to happen in G93A-SOD 1 mice is that mutant SOD prevents the binding of these regulatory proteins hence reducing VEGF mRNA stability and ultimately causing a decrease in VEGF synthesis [106].

An additional evidence of the involvement of VEGF in ALS came from a model in vitro of this disease based on the mouse NSC-34 motor neuron cell line. When CSF from patients with sporadic ALS is added to the culture medium, the expression of VEGFR-2 is downregulated and these cells degenerate and die [107]. This process can be prevented by the addition of exogenous VEGF.

Collectively these data suggest that a defect either in VEGF or in VEGFRs could be responsible for some forms of ALS by causing perfusion deficits and neuronal degeneration in the ventral horn of the spinal cord. Therefore, it is expected that exogenous VEGF could be beneficial in ALS by counteracting these deficits. Consistent with this hypothesis beneficial effects have been observed in G93A-SOD1 transgenic mice upon treatment either with intracerebroventricularly administered $VEGF_{165}$ [108] or with VEGF gene carrying lentiviruses retrogradly transported to motoneurons [109].

PI3-K/Akt activation is probably the primary mechanism responsible for VEGF neuroprotection in ALS. For instance, it was demonstrated that in rat primary motor neuron cultures transfected with an adenoviral vector encoding a mutant SOD1, G93A-SOD1, VEGF attenuated G93A-SOD1-induced caspase-3 cleavage and DNA fragmentation by activating PI3K/Akt signaling via VEGFR2 [110]. Additional mechanisms have been proposed to explain VEGF neuroprotection in ALS. For instance, evidence has been reported that VEGF could decrease the Ca^{2+} permeability of AMPA receptors by promoting the expression of GluR2 subunits [111]. This could represent a neuroprotective mechanism in ALS considering that Ca^{2+} influx through AMPA receptors induces motor neuron degeneration and causes an ALS-like condition in experimental animals [112-115].

The evidence that VEGF is implicated in the genesis of ALS in several animal models raised the question of whether the VEGF system could be impaired in human ALS too. Low levels of VEGF in cerebrospinal fluid (CSF) were observed early in the course of VEGF [116]. In addition, there have been case reports of ALS developing in patients taking anti-VEGF drugs [117]. Several studies evaluated the hypothesis that defect in hypoxic VEGF regulation similar to those induced in the $Vegf^{\delta/\delta}$ mice by genetic manipulation *in vitro* do also occur spontaneously in human ALS because of polymorphisms of the VEGF

gene promoter. Lambrechts et al. [101] performed a metanalysis on over 900 individuals from Sweden and over 1,000 individuals from Belgium and England to investigate the possible association of three common vegf gene ploymorphysms (22578C>A (rs699947), 1154G>A (rs1570360), 2634G>C (rs2010963)) with ALS. They found that people with two haplotypes in the promoter region of VEGF (–2,578A/–1,154A/–634G and –2,578A/–1,154G/–634G) had a 1.8 times greater risk of developing than control subjects. These findings, however, were not confirmed in a later larger metanalysis that provided evidence only of a weak association of the -2578AA genotype with ALS risk in males [118]. Other studies gave conflicting results as some of them reported a possible association between VEGF promoter gene polymorphisms and ALS [119-120] and others did not [121-125]. The search for mutation in the promoter region of VEGFR2 in ALS patients also gave negative results [126].

In conclusion, whereas the evidence that a deficit in VEGF could have a role in several ALS animal models that could also benefit of the treatment with this growth factor, the definite proof of VEGF involvement in human ALS is still missing.

2.3 Alzheimer disease

Even though Alzheimer's disease (AD) has been traditionally considered as a disease that primarily involves neurons, it is now clear that it also has an important vascular component in the form of an ischemic damage that contributes to potentiate and extend the primary neuronal degeneration [127,128]. Alterations in cerebral vascular architecture have been observed in animal model of this disease [129] and perfusion deficits that begin in posterior cingulate and precuneus to later become more widespread and extend to the hippocampus, have been documented in AD patients [130,131]. The basic mechanism of this decrease in perfusion is a specific form of amyloid angiopathy with small sclerotic vessels that accumulate amyloidogenic peptides in their wall [132-134]. Considering that hypoperfusion and the ensuing chronic hypoxia are strong stimuli for the synthesis of VEGF it is not surprising that this growth factor could be somehow involved in AD pathophysiology. Early studies reported high VEGF levels in AD CSF and brain tissue [135-137]. For instance, Tarkowski et al. [136] found high levels of this peptide in CSF of AD patients and in patients with vascular dementia. Immunohistochemical analysis of autoptic samples from AD patients showed higher VEGF levels than in age matched controls mainly localized in clusters of reactive astrocytes in the neocortex

[135]. High VEGF levels were also found in brain microvessels [137] and in the white matter [138] of AD patients. Importantly, evidence was reported for a hypoxic drive of VEGF hyperproduction in AD white matter because the level of this growth factor strongly correlated with the ratio of the myelin-associated glycoprotein- that is highly hypoxia-sensitive- to proteolipid protein 1 –that is hypoxia resistant [138]. Later investigations, however, did not confirm these early data and reported, instead, decreased VEGF levels making the issue of how do VEGF concentration change in AD still somehow controversial [139,140]. The early findings of lower CSF VEGF concentrations in AD patients as compared with normal subjects also prompted larger scale studies to investigate the hypothesis that this growth factor could represent a potential biomarker for AD. These investigations yielded conflicting results. Plasma VEGF concentration was higher than in controls in AD patients in the series of Yu et al. [141] and lower in that of Mateo et al. [142]. Lower than normal VEGF levels in CSF were also found in studies performed with multianalyte platforms for the simultaneous detection of multiple candidate biomarkers [143,144]. In the light of accumulating evidence that VEGF could represent a prognostic marker of disease evolution, more recently the interest moved from differences in CSF VEGF levels between normal subjects and AD patients to differences among different patients. Homan et al. [145] performed a longitudinal study in 130 patients with mild cognitive impairment, in 59 with AD and in 90 age-matched normal controls to evaluate whether CSF VEGF levels could predict the evolution of the disease. They found that higher VEGF levels are consistently associated with a slower progression of hippocampal atrophy and of cognitive decline. Interestingly VEGF interacted both with tau and αβ1-42 in predicting disease progression. Collectively, the data collected in humans seem to suggest a scenario in which AD microangiopathy causes a hypoxic drive for VEGF synthesis and release; the more effective will be the VEGF response to this stimulus the less aggressive will be disease evolution. There have been also reports on the association of specific VEGF polymorphysms and the risk of developing AD [146,147] although they were not confirmed in other studies leaving this question still open [148]. A recent metanalysis by Liu et al. [149] that the risk is related to ApoE status with the -22578C>A (rs699947) polymorphism associated to higher and the -1154G>A (rs1570360) polymorphism to a lower risk of developing AD only in patients with APOE ϵ4 negative status. Conversely, no association between the risk of AD and VEGF promoter polymorphisms was found in another recent metanalysis [150].

Whereas the question of how VEGF concentrations change in AD is still open, consistent evidence suggests that this growth factor does not work as effectively in the AD as in the normal brain. Indeed, AD patients seem to be resistant both to the VEGF angiogenic and vasodilatatory effect. A prevalent hypothesis is that αβ oligomers could directly induce a status of cell unresponsiveness to VEGF. It has been demonstrated, indeed, that they cause a reduction in cerebral blood flow [151] and impair angiogenesis by cerebral endothelial cells *in vivo* and *in vitro* [152,153]. These effects could be the consequence of a direct antagonism of VEGF by αβ oligomers on endothelial VEGF receptors. Patel et al. [154] demonstrated that $\alpha\beta_{1-42}$ inhibits the phosphorylation of VEGFR-2 and Akt-1 in HUVEC cells and that this effect may be reverted by excess VEGF. Moreover, cell-free immunosorbent binding assay experiments showed that $\alpha\beta_{1-42}$ oligomers prevent VEGF binding to VEGFR2 because they bind to the extracellular region of these receptors [154]. The presence of VEGF resistance in AD and the evidence that it can be counteracted by excess VEGF suggest that this growth factor could exert beneficial effects in this disease. Proofs of concept of this hypothesis have been obtained in AD animal models in which an improvement in memory and cognition can be obtained by increasing VEGF levels in the brain through diverse delivery strategies such as the intraperitoneal injection of VEGF [155], transplantation of VEGF overexpressing bone marrow mesenchymal stem cells in the hippocampus [156], or the neuron specific hyperexpression of $VEGF_{165}$ [157].

2.4 Parkinson's disease

VEGF improves the survival of mesencephalic neurons cultured in vitro [158] and of dopaminergic neurons in organotypic cultures of the mesencephalon [159]. This evidence prompted to investigate its possible protective effect in different *in vitro* and *in vivo* Parkinson's disease (PD) models including the exposure to 6-OH-dopamine or to MPP^+. VEGF was protective against both of these neurotoxins: it increased the survival of primary mesencephalic neurons treated *in vitro* with these compounds, reduced dopaminergic neuron loss and promoted reactive astrogliosis *in vivo* and improved neurological symptoms including amphetamine-induced rotational behavior [160-162]. The beneficial effect of VEGF was, however, observed only at low-moderate doses whereas at higher doses it was lost. This was demonstrated both *in vitro* by exposing cultured neurons to different concentrations VEGF (10 or 100 ng/ml) and *in vivo* in rats implanted

with small or big microcapsules containing VEGF-secreting BHK cells. The lower neuroprotective potential *in vivo* of high VEGF doses could be due to blood vessel permeabilization and edema [161].

Other more physiological ways of increasing brain VEGF have been experimented in experimental models of PD. In particular, based on the observation that physical exercise can increase brain concentration of VEGF [163,164], the possible beneficial effect of exercise has been evaluated in rats with experimental PD. In rats the density of microvessels in the substantia nigra decreases with ageing. Villar-Cheda et al. [165] demonstrated that this decrease in vascularization is associated with a parallel decrease in nigral VEGF mRNA levels. Intriguingly, both the deficit in vessel density and in VEGF concentration could be partially corrected by physical exercise. Even though these findings are intriguing, a scenario in which physical exercise could rescue dopaminergic cell from death in human PD appears unlikely mainly because in humans affected with PD, microvessel density and VEGF levels in substantia nigra are actually increased and not decreased as in aged rats [166,167].

A role has been suggested for VEGF also as a mediator of the therapeutic effect of deep brain stimulation of the subthalamic nucleus [168].

Cui et al. [169] investigated the mechanism responsible for VEGF-induced neuroprotection in MPP^+ exposed rat cerebellar granule cells. They showed that VEGF reversed the inhibition of phosphoinositide 3-kinase (PI3-K)/Akt pathway, but further enhanced the activation of ERK induced by MPP(+).

Also in PD the role of VEGF and its receptors as a biomarker begins to be considered of potential value. Janelidze et al. [170] measured these molecules alongside with other angiogenic proteins in CSF of 100 patients with PD and 38 elderly controls. They found higher levels of VEGF and its receptors in PD patients with dementia than in controls. Importantly, high levels of this and other angiogenic factors strongly correlated with the presence of BBB permeabilization as evaluated with the CSF/plasma albumin ratio, white matter lesions, cerebral microbleeds, gait difficulties. The high VEGF levels in PD can be interpreted either as the compensatory response to the repeated brain ischemic insults caused by the severe orthostatic hypothension that may occur in this disease or as the consequence of cytokine-induced release from astrocytes and microglia [171]. VEGF-induced BBB permeabilization could have a role in PD progression by damaging dopaminergic neurons [172]. Among PD patients the highest VEGF levels have been observed in patients with L-dopa induced dyskinesias that were reduced by candesartan [173]. Consistent with the

finding of high VEGF levels in the CSF of PD patients, a high microvessel density and VEGF expression have been observed in the mesencephalon of patients affected with this disease [166,167].

The graft of mesencephalic neurons in the striatum held great promises for the treatment of PD. However, low survival of grafted cells and poor vascularization of the graft may represent a substantial obstacle for the success of this procedure. Based on the evidence that VEGF has both proangiogenic and neuroprotective activity Pitzer et al. [158] investigated the possible use of this growth factor to improve mesencephalic graft survival in the rat. They found that the direct injection of VEGF in the striatum promoted the formation of new vessels and the neovascularization of the graft. However, surprisingly, the survival of grafted dopaminergic neurons was not improved respect to control.

2.5 Epilepsy

One of the first evidence that VEGF could have a role in seizures came from a study not directly related to epilepsy. Newton et al. [174] performed, indeed, a gene microarray analysis to identify the genes whose expression was modulated in the rat brain after an electroconvulsive shock (ECS) with the aim of obtaining information on the mechanism responsible for the beneficial effects of this procedure in psychiatric disorders such as major depression. They found that the expression of genes encoding for neuroptotective and angiogenic factors was strongly increased, and, of note, that VEGF was one of the most sensitive among them. Interestingly, the strongest VEGF induction was observed in the hippocampus and in choroid plexus. This finding was confirmed also at the protein level by Nicoletti et al [175] in the classical pilocarpine experimental model of temporal lobe epilepsy. The well known neuroprotective effect of this growth factor suggested that its induction could represent a mechanism activated to protect hippocampal neurons from the seizure-induced damage that has a major role in causing persistent epilepsy after status epilepticus. In light of this hypothesis exogenous VEGF would be expected to further decrease pilocarpine-induced hippocampal damage as was actually observed in rats with VEGF-containing micropumps that released this growth factor in the dentate hilus [175]. As in the other neurodegenerative diseases considered in the previous paragraphs, also in epilepsy most of the neuroprotective effects of VEGF seem to be mediated by VEGFR-2 and the consequent activation of PI3K/Akt cascade. An elegant demonstration of the role of VEGFR-2 was provided by Nikitidou et al. [176] who showed that transgenic mice overexpressing these receptors develop less

serious epilepsy after electrical kindling, a widely used experimental method to induce epilepsy that is based on the repeated electrical stimulation of specific brain areas such as the amygdala. Importantly, this protective effect occurred in the absence of any change in blood vessel or glia. Additional beneficial effects of VEGF in epilepsy depend on effects directly exerted by this growth factor on neuronal excitability. McCloskey et al. [177] showed that in rat hippocampal slices VEGF reduced the amplitude of responses elicited by Schaffer collateral stimulation. Although this growth factor did not affect the epileptiform activity evoked by GABA receptor blockade with bicuculline in brain slices form normal animals, it significantly reduced the spontaneoud discharges that occur in hippocampal brain slices from pilocarpine-treated animals, an experimental model of temporal lobe epilepsy [177]. Moreover, exogenous VEGF reduced both the ictal and interuictal discharges evoked by the potassium channel blocker 4-aminopyridine or by the removal of Mg^{2+} from the extracellular fluid in mouse hippocampal slices [178].

The beneficial effects played by VEGF on neurons could be partially counteracted by its vascular effects. As mentioned in section 4 exogenous VEGF induces blood vessel permeabilization and increases the permeability of the blood brain barrier as well. This could be highly unwanted in epilepsy also considering that, according to a prevalent pathogenetic model, the loss of blood brain barrier tightness could be one of the triggering factor of epilepsy [179, 180]. In this perspective, Morin-Brureau et al. [181] investigated whether also the endogenous VEGF that is released during seizures may increase blood brain barrier permeability. To this aim they studied the blood vessels of organotypic hippocampal cultures that were exposed to 25 μM kainate to induce epileptic events in vitro and looked to blood vessel density and to the expression of blood brain markers such as zonulin-1, claudin and occludin. They found that seizure induction by kainate causes an increase in vascular density and decreased and disorganized the expression of ZO-1.

In conclusion, whereas VEGF seems to be beneficial in epilepsy it could also worsen this disease by inducing BBB permeabilization.

3. PHARMACOKINETICS ISSUES COMPLICATING THE IMPLEMENTATION OF VEGF THERAPEUTICS AND POSSIBLE SOLUTIONS

The data that we quickly reviewed in the previous section provide arguments to suggest that VEGF could have potential therapeutic applications for several neurological disorders. However, VEGF pharmacokinetics hinders the implementation of its use in clinical practice. Some of these problems appeared already evident when VEGF was considered as a potential drug for therapeutic angiogenesis in cardiology [1] and are similar to those that emerged when other growth factors were considered for CNS therapeutics [182].

As mentioned in the introduction VEGF is a glycoprotein and, as such, a potentially unfavorable pharmacokinetic profile is expected. As a general rule, protein drugs have indeed a problematic pharmacokinetics: they have to be administered by parenteral route, may be degraded in plasma or tissues and may have significant difficulties in distributing to peripheral organs. Because of the interest in implementing its use as a pharmacological tool for cardiac revascularization after myocardial ischemia, the pharmacokinetics of exogenous recombinant VEGF has been studied with some detail. Eppler et al. [183] performed a pharmacokinetic noncompartmental analysis in patients receiving rhVEGF by intracoronary and intravenous injection. The main findings were that this growth factor has dose-dependent nonlinear pharmacokinetics. Terminal half life was 13.4±8.3min after the administration of a low dose (17 ng/kg/min) and 33.7±13 min when a high dose (50 ng/kg/min) was given, whereas the steady state distribution volume was 1520±900 and 960±260 ml/kg, respectively. Clearance ranged around 39.1±17 ml/min/kg after the low dose and decreased to 19.1±5.7 after the high. These findings were explained assuming that VEGF removal from plasma depends on two different mechanisms: -the interaction with high affinity receptors that are believed to be mainly represented by endothelial VEGFR and by –low affinity high capacity elimination systems that could be contributed for both by low affinity receptor sites or by classical elimination organs such as the kidney. According to these data, VEGF rapidly disappears from plasma because it is removed in a diffuse and “untargeted” way by endothelial cells through their receptors. A crucial role of clearance by endothelial cells was also demonstrated in modeling studies that addressed the pharmacokinetics of endogenously released VEGF, an issue of major practical interest for rational dose adjustment of anti-VEGF drugs. These models assume that VEGF is released by parenchimal cells in the interstitium. Then, it, in part, passively crosses the endothelium in a way predicatable on the basis of its Stokes-Einstein radius, i.e. of the radius of an ideal sphere that diffuses at the same rate, and, in part, is cleared by the endothelial cells through

abluminal VEGFRs [184]. Once that it reaches the plasma it is cleared by peripheral endothelial cells through luminal VEGFR [185]. Therefore, the basic model involves two compartments, blood and tissues, separated by an endothelial barrier that bilaterally operates a significant clearance. Interestingly, according to this model changes in tissue permeability do not modify the concentration of tissue VEGF but can significantly affect the concentration in plasma of this growth factor [184]. To describe VEGF pharmacokinetics in patients with solid tumors, the model was further complicated introducing a third high permeability compartment made by the tumor [186]. An additional factor that can modify plasma and tissue VEGF concentration is its degradation both in plasma and in peripheral tissues. The evidence that when incubated in vitro with plasma in the absence of any cellular element, VEGF has a short half-life ($\tau_{1/2}$~40 min) suggested that it is degraded with a rate constant of 2.3-2.8·$10^{-4}s^{-1}$ [187]. Most of this degradation is operated by plasmin that cleaves VEGF165 at the Arg110/Ala111 residues yielding to two fragments VEGF1-110 and VEGF111-165. The former retains the binding regions for VEGFR-1 and -2 but has a low biological activity as compared to native VEGF [188]. Interestingly, modified forms of VEGF of higher stability and bioactivity were obtained by substitution of Arg110 with Ala110 or Gln110, and Ala111 with Pro111 [189]. VEGF can also be proteolytically processed by tissue metalloproteinases in multiple different ways that differentally contribute to the fine tuning of its biological activity in tissues [187]. Collectively, the available evidence on VEGF pharmacokinetics that we just reviewed, suggests that once given intravenously this growth factor will evenly distribute to peripheral tissues being, however, largely captured by the endothelial cell lining that sequester it and prevent its further diffusion deep in these tissues. Given this pharmacokinetic profile a low CNS penetration for peripherally administered VEGF is expected similar to what has been described for other growth factors such as LIF or NGF [190]. The poor penetration of VEGF in CNS was confirmed by Yang et al. [191] who observed that after the intravenous injection of ^{125}I-VEGF in rats the majority of radioactivity localized to peripheral tissues whereas it was very low in the brain. Considering this unfavorable pharmacokinetic profile in most of the studies that used VEGF as a neuroprotective agent the growth factor was given by invasive admistration routes allowing its direct penetration in the brain. The simplest strategy that has been used is the intracerebroventricular administration in which the drug is directly injected in the ventricular system, usually in lateral ventricular, through cannulae that are stereotaxically positioned after drilling a hole in the skull. Such an administration route has

been used in studies exploring, among the others, the effect of exogenous VEGF on stroke, PD, AD, ALS that we reviewed already in the previous section. The intracerebroventricular (icv) administration has several important limitations that include its invasiveness, the risk of serious neurosurgical complications and, importantly, the need of repeated administration when short lived drugs are administered. Indeed, icv administered drugs are expected to rapidly disappear from cerebrospinal fluid and, therefore, if a prolonged pharmacological effect is required, repeated injections or the implantation of reservoirs that slowly release the molecule through a stably implanted cannula. This strategy proved successful, for instance, in animal models of ALS. Storkebaum et al. [108] implanted in $SOD1^{G93A}$ rats a minipump that released VEGF in the lateral ventricles for more than 100 days and were able to document both the transportation of the growth factor to the spinal cord and a significant amelioration of the symptoms of the disease. Quantitative data are missing to firmly establish how much of the administered VEGF really reaches the neurons or brain endothelial cells but it is commonly believed that a large part of what is injected is actually lost because it is sequestered by the ependimal cells lining the ventricles [192]. To overcome the limitation of the short duration of action and, possibly, of the low intracerebral bioavailability several strategies have been developed and tested in animal models of neurodegenerative diseases [182]. Among them of special interest appears encapsulation. VEGF, as other growth factors, may be included in microspheres that once implanted in the brain slowly release their content. VEGF has been encapsulated in alginate microgels obtaining its sustained release [193,194]. However, this formulation was not used for the intracerebral administration that was, inteatd, experimented with poly(lactic-co-glycolic acid) (PLGA) microspheres. These microspheres appear well suited for sustained drug relase because once that they are injected they are gradually hydrolyzed and slowly release their content; in addition, they seem reasonably safe and well tolerated [195]. Moreover, the speed of their hydrolysis may be finely adjusted by modifying the ratio of of poly(lactic acid) to poly(glycolic acid). VEGF was originally incapsulated in PLGA nanoparticles to obtain a sustained release in cardiovascular disorders. For instance, Golub et al. [196] showed that they promoted revascularization in a mouse model of femoral artery ischemia and Formiga et al. [197] observed a substantial improvement in vasculaogenesis and tissue remodeling in a rat model of cardiac ischemia-reperfusion. VEGF-containing PLGA nanoparticles have been also used in animal models of neurological disorders. Herrán et al. [198] instilled VEGF containing PLGA nanospheres on the cortical surface of APP/Ps1 mice, an experimental

model of AD, through a craniotomy window, after opening the dura mater. Animals treated with the nanospheres performed much better than untreated controls in behavioral tests showing no difference from wild type littermates. Moreover, the histopathological analysis of their brains collected three months after the procedure showed a much smaller amyloid plaque load, a reduced neuronal loss and an increase in angiogenesis. Evidence for enhanced neurogenesis was also obtained [199]. The same research group also evaluated the efficacy of VEGF plus GDNF containing PLGA nanospheres in rats made parkinsonian with the intrastriatal injection of 6-OH dopamine showing both an improvement in amphetamine-induced rotation behavior and an increase in the density of dopaminergic neurons in substantia nigra [200,201]. A surgical approach based on the implantation in the core of the ischemic lesion of a scaffold made by hyaluronic acid (HA)-based biodegradable hydrogel, mixed with PLGA microspheres containing VEGF and angiopoietin-1, and antibodies against Nogo receptor has been evaluated in experimental stroke [202].

Encapsulation techniques have a crucial relevance also for cellular therapies of neurodegenerative diseases that are based on the surgical transplantation of cells genetically engineered to stably release VEGF. However, these therapeutic modalities are beyond the scopes of the present review. We will, instead, examine in more detail another delivery strategy that can be used to direct VEGF in a noninvasive way, the intranasal administration.

3.1 Intranasal VEGF administration

Intranasal administration represents a promising alternative to the invasive approaches that we just described. This delivery strategy uses the nasal cavities to get a direct access to the brain bypassing the general circulation [203]. This is made possible by the anatomy of nasal cavities. Briefly, they consist of two main regions: a smaller region, the vestibule, and the main nasal cavity. Vestibule is the outer part and receives the air crossing the nostrils. The main nasal cavity consists of two different portions: the respiratory and the olfactory regions. The respiratory region is lined by a mucus secreting epithelium with cilia. Because of the presence of turbinates and of microvilli the total surface of this portion of nasal cavities is larger than expected. The cilia move the mucus towards the pharynx and, remarkably, the speed of mucus flux is much slower than in the respiratory tree. This epithelium is highly vascularized which makes it potentially suitable for systemic absorption of drugs. However, the respiratory region of the nasal cavities is also innervated by

fibers of the trigeminal nerves that can be used by inhaled drugs as paths to reach the brain [190-192]. This process can also take place in the olfactory region of the nasal cavity. It is made by the cribriform plate that is crossed by the fibers of the olfactory nerves that make contact with olfactory receptors in the olfactory mucosa. This is a pseudostratified nonbeating mucous epithelium. Drugs may cross the olfactory epithelium and be transported perineurally along the olfactory fibers towards the olfactory bulbs hence crossing the BBB. Additional mechanisms such as perivascular transportation or direct diffusion to the cerebrospinal fluid may also contribute to drug transportation to CNS. Centrally-acting drugs such as antiepileptic drugs, general anesthetics, analgesic and antipsychotic drugs have been administered through the intranasal route and in some cases this is also a relevant route of administration in clinical practice [204-217] because drugs given intranasally bypass, at least in part, the general circulation and get direct access to the brain, this administration route appears as an interesting option for the administration of neuroprotective growth factors [218-221]. The advantage of directing therapeutics to the brain avoiding peripheral distribution and degradation does not seem to be limited to small drugs and peptides and, intriguingly, the use of the intranasal route has been also explored for the administration of umbilical stem cells to promote neurovascular repair in the ischemic brain [222,223]. The ability of VEGF to penetrate into the brain after intranasal administration was demonstrated Yang et al. [191]. They gave ^{125}I-VEGF to rats by the intranasal route and looked for the appearance of radioactivity in the brain 30 minutes thereafter. A strong signal was observed especially in the trigeminal nerve, the optic nerve, olfactory bulbs, olfactory tubercle, striatum, medulla, frontal cortex, midbrain, pons, appendix cerebri, thalamus, hippocampus, cerebellum. Conversely peripheral distribution of intranasally administered VEGF was negligible was and substantial if it was given intravenously.

It was shown that when it was given by this route, VEGF promoted the formation of new blood vessels in the boundary of the ischemic lesion after MCAO in rats and reduced the neurological impairment [224]. An unusual bell-shaped dose dependence curve was observed for the neuroprotective effects of VEGF after tMCAO with the highest protection after the intranasal administration of 200 μg/ml and the lowest after either 100 or 500 μg/ml [205]. Intranasal VEGF was also effective in a rat model of global cerebral ischemia reducing cognitive impairment as evaluated in behavioral tests performed 15 days after stroke [85]. Whereas these data demonstrate that intranasally administered VEGF is effective in eliciting neuroprotection, high

doses of this growth factor were required suggesting that this administration route is probably highly inefficient. Although, at the best of our knowledge, the nasal bioavailability of VEGF has not been determined yet, values between 0.01 to 0.1% have been suggested as a general rule, for intranasally administered drugs [226]. Several factors could contribute to lowering the bioavailability of intranasally administered drugs including, for instance, their transfer in the farynx where they are swallowed, their degradation by enzymes of the nasal mucus and of the nasal epithelium and the short residency time on the absorbing surface of the mucosa [227]. It remains to be established whether and to which extent permeabilizing effects of VEGF on nasal mucosa could compensate for these factors. It has been reported, indeed, that this growth factor permeabilizes the nasal mucosa about 1.10^6 more potently than histamine on a molar base [228].

4. SAFETY CONCERNS ON VEGF THERAPEUTICS AND POSSIBLE SOLUTIONS

Potential concerns can be raised on the use of VEGF in CNS therapeutics. Some of them are not related to the specific indication for neurological disorders and are similar to those that could take place if VEGF was used for the treatment of heart or limb ischemia, namely hypotension, whereas, others are specifically observed in the context of CNS diseases.

Because of the well established role of VEGF in tumor growth, an increased risk of cancer has been considered as a likely, seriously worring potential complication of VEGF treatment. However, the clinical evidence accumulated up to date in trials evaluating therapeutic angiogenesis with VEGF did not substantiate this worry [229-234]. Moreover, in experimental animals hyperexpressing VEGF, an high prevalence in hemangiomas but not of cancers was observed [235-239].

The most serious concern about the use of VEGF in CNS therapeutics is related to the effect of this growth factor on BBB permeability. In normal conditions the tightness of BBB is strictly regulated and its loss occurs in many neurological disorders including stroke, traumatic brain disease, AD, PD, ALS, multiple sclerosis and viral encephalitis [240-247]. The loss of the integrity of BBB has an important role in the progression of the aforementioned disorders because it contributes to extend brain damage by multiple mechanisms that include, among the others, the impairment of the clearance of toxic products such as amyloid peptides and the influx into brain parenchyma of toxic substances, of cytokines and blood proteases

such as coagulation factors, and of inflammatory cells [247,248]. VEGF increases blood vessel permeability and it was actually initially identified because of this biological activity and not because of its proangiogenic activity [249]. As such it is a well known BBB permeabilizing agent. Different mechanism contributes to VEGF BBB-permeabilizing activity. Specifically, this growth factor promotes transcytosis [250,251]. In addition, it induces the dissociation of tight junction by downregulating the expression of zonulin-1, claudin and occluding [252-255].

Current evidence suggests that endogenous VEGF could be one of the causative factors of BBB permeabilization in CNS diseases. It has been demonstrated, indeed, that reactive astrocytes release this growth factor that can diffuse from the brain parenchyma to the endothelium and permeabilize the BBB [256]. Mice with targeted disruption of VEGF in astrocytes showed a reduced BBB breakdown in response to the injection of recombinant adenovirus expressing IL-1 and milder neuropathology after induction of experimental allergic encephalomyelitis [256]. Interestingly, in control mice, most of the detrimental effects of VEGF on BBB integrity were lost after the pharmacological blockade of eNOS suggesting that this growth fact was acting by inducing the synthesis of NO. An additional mechanism that could increase VEGF levels in the damaged brain is activated in brain ischemia and involves the tissue plasminogen activator (tPA) [257,258]. It has been demonstrated, indeed, that tPA induces the expression of VEGF in neurons, endothelial cells and bone marrow-derived myeloid cells by a mechanism that could involve the activation of HIF-1 dependent transcription [257,258]. A convincing argument of the involvement of endogenous VEGF in BBB dysfunction after stroke is the evidence that it can be reduced with either anti-VEGF antibodies or blockers of VEGFR-2 [257, 259]. The hypothesis that not only VEGF levels but also the sensitivity to this growth factor increased after stroke, was evaluated in diabetic mice and in normal controls. After stroke, a marked increase in the expression of VEGFR-2 was observed in the periinfartual area in both these groups although it was much stronger in diabetic than in normal mice [260]. These data suggested that a heightened responsiveness to endogenous VEGF could have a role in BBB permeabilization after stroke. This hypothesis was confirmed by the evidence that shutting down the expression of VEGFR-2 in conditional adult knock-out mice or pharmacologically inhibiting their activity with the VEGFR-2 inhibitor SU5416 reduces BBB leakage in experimental stroke. Surprisingly, however, the protective effects were observed

only in diabetic and not in normal mice suggesting that the potentiation of VEGFR response is probably a causative factor of BBB dysfunction only in diabetes.

As a whole the data that we reviewed show that in several neurological diseases endogenous VEGF actually contributes to brain damage by inducing BBB dysfunction. This raises serious concerns about the rationale of using this growth factor to treat CNS disorders because its beneficial effect could be reverted by the detrimental effects on BBB leakage. As a matter of fact there is a rich scientific literature showing that VEGF can actually act a double edge sward in many neurological disorders. Probably stroke is the condition in which this issue appeared as more urgent.

5. VEGF-MIMIC PEPTIDES AS A NEW TOOL FOR VEGF THERAPEUTICS IN DISORDERS OF THE CENTRAL NERVOUS SYSTEM

The data reviewed in the previous sections show that although there is a solid rationale and convincing evidence that VEGF could be helpful in several neurological disorders, this growth factor is far from having a good pharmacological profile for its development in clinical practice. Indeed, as detailed before, it has an unfavorable pharmacokinetics and toxicological profile. In addition, it is not a molecule easy to prepare on large scale for pharmaceutical purposes considering the stringent requirements with respect to impurity identification and reproducibility of the batch. A first structural characteristic that complicates its preparation is that the main VEGF isoform is constituted by two monomers, each 165 amino acids long, covalently linked by a two intermolecular disulfide bonds. Furthermore, the VEGF structure is characterized by a cystine knot motif which is formed by three intramolecular and interconnected disulfide bonds. The presence of these specific disulfide bonds make the preparation of VEGF, in a reproducible and standardize protocol, challenging. VEGF expression in bacteria and eukaryotic cells has been reported [261-264] as well as the chemical synthesis by native chemical ligation [265]. All these techniques present advantage and drawbacks. For example, VEGF expression in bacteria and chemical synthesis require a refolding step respectively from inclusion body and after assembly of the full length protein. The refolding procedure is time and reagent consuming, reduce the yield of final protein, and introduce variability in the preparation. Collectively these considerations suggest that an additional factor besides the unfavorable pharmacokinetics and toxicology, complicating VEGF implementation in CNS therapeutics is its complicated and expensive synthesis.

An interesting strategy that is emerging in the last years to overcome these limitations is to prepare smaller and simpler peptides that could interact with VEGF receptors and trigger their activation hence reproducing VEGF biological activity. These peptides are known as *VEGF-mimic* or *VEGF-mimetic* peptides. The rationale behind their synthesis is that the interaction of VEGF with its receptors does not involve the whole VEGF molecule but only part of it that can be reproduced in isolation and will, hopefully, retain the ability to stimulate the receptor itself. Because there is the need of maintaining the intrinsic activity at the receptor, it is much more challenging to design such molecules than peptides that simply bind to the receptors but do not have agonistic properties. As a matter of fact, a larger number of VEGF-antagonist peptides has been synthesized and tested as potentially useful tools to inhibit pathological angiogenesis in tumors [266-270] whereas only few peptides with VEGF biological properties have been synthesized [271]. The first of them was reported in 2005 by D'Andrea et al.[272] It was named QK and it is a 15 mer α-helical peptide that was designed to reproduce the α-helical region of VEGF 17-25 using a structure-based approach. The choice of this region derived from the analysis of the published crystal structure of VEGF bound to domain 2 of VEGFR1 [273]. This domain together with domain 3 is known to be the only of the seven extracellular domain that is really critical for VEGF binding [243]. This crystal study showed that the interaction between VEGF and VEGFR1 involves three discontinuous portions of the VEGF molecule: the N-terminal helix (residues 17–25), the loop joining strand β3 and β4 (residues 61–66) of one VEGF monomer, and the β-hairpin encompassing strand β5 and β6 (residues 79–93) of the other VEGF monomer. The QK peptide was, therefore, designed to reproduce a portion of the VEGF molecule critically involved in receptor binding as it includes 5 (Phe-17, Met-18, Tyr-21, Gln-22, and Tyr-25) out of 21 located at a distance less than 4.5 Å from the receptor surface [272]. Specific residues of the 17-25 VEGF sequence were substituted in QK with the aim of either increase the stability of the α-helix conformation in water or of the increase the activity at VEGFR. For instance, Trp was substituted for Phe-17 to increase hydrophobic interactions, Gln was substituted for Met-18 to enhance hydrogen bond interactions at the receptor site, Asp-19 was replaced by Glu, and Ser-24 with Lys to increase helix propensity [272]. The whole structure was further stabilized by introducing N- and C-capping extra-sequences. Circular Dichroism analysis and NMR spectroscopy showed that peptide assumes in water a high populated helical conformation (Figure 3). The structural determinants of QK structure were studied in water by NMR and molecular dynamics revealing an

unusual thermodynamic stability [274]. The molecular determinants of peptide stability were also characterized [275,276]. The ability of QK to bind to VEGFR was verified in a radioassay experiments on isolated EC membranes [272], and by flow cytometric analysis on A375 cells [276]. The VEGF agonistic activity of the QK peptide was demonstrated using both models *in vitro* and *in vivo*. More specifically, it was observed that, in cultured endothelial cells, QK activates VEGFR2 inducing the phosphorylation of its intracellular kinase domain [242], and the activation of Akt, Erk1/2, eNOS [277]. Moreover, like VEGF, QK also induced the upregulation of *vegfr2* gene in these cells [277]. QK was also able to reproduce the major biological effects of VEGF on endothelial cells inducing their proliferation, migration and survival [272,277]. QK retained VEGF proangiogenic properties as demonstrated ex vivo on aortic rings [277] and in vivo in matrigel plugs [272, 277]. Although we cannot conclusively exclude that part of the effect of QK of other VEGF-mimic peptides could be exerted intracellularly, this appears very unlikely and current evidence all convege in suggesting that it acts at the level of VEGF plasmamembrane receptors.

Because of its ability to promote new vells formation, the possibility that QK could be effective as a tool for therapeutic angiogenesis appeared likely and was experimentally tested. The first disease models in which it was evaluated were hindlimb ischemia and wound healing [278]. Hindlimb ischemia was induced by the permanent closure of the right common femoral artery was isolated and QK or VEGF or a scrambled peptide as control, were infused intraperitoneally for two weeks. Not differently from $VEGF_{165}$, QK improved the perfusion of the ischemic hindlimb as evaluated at angiography and increased the density of capillaries in the tibialis anterior muscle of the ischemic hindlimb [278] (Figure 4). To test the effect on wound healing a 20 mm in diameter open wound was surgically made on the dorsum of anesthetized rats and QK, $VEGF_{165}$ or a scrambled peptide were applied for 5 days as a pluronic acid gel on the wound. Both QK and VEGF accelerated wound healing as compared with the scramble peptide [278]. QK showed regenerative properties also in an experimental model of gastric ulcer accelerating healing in a iNOS-dependent manner [279] and improved the course of experimental diabetic peripheral neuropathy [280]. The chemical and biological properties of peptide QK stimulated the interest of several groups working on biomaterial for applications in regenerative medicine [281-290] ratifying the importance of this molecule as VEGF substitute for applications were a proangiogenic response is required.

Collectively the accumulated data suggested that QK could be a reasonable alternative to VEGF in proangiogenic and regenerative therapies. The effect of QK was tested in a pMCAO model of experimental brain ischemia. QK was administered intracerebrovetricularly using a microinfusion pump that released a solution containing a concentration of 3 μg/ml QK at the rate of 1μl/hr the peptide and that was implanted before surgery [291]. Ischemic damage evaluated three days after surgery was significantly smaller in rats given QK than in controls. In particular, QK should easily distribute in tissues and reach the ischemic tissue even when given intravenously. This hypothesis was tested by intravital pial microvessel videoimaging, a technique that visualized pial vessels after the intravascular administration of fluorescent probes in living animals through a window made in the skull. Using this technique, we observed that after intravenous administration QK rapidly adhered to the endothelial lining of pial vessels and thereafter extravasated in brain parenchyma on the venular side (Figure 5). This finding suggests some important difference from VEGF (that remains, however, to be quantitatively tested) because, as we mentioned in section 3, this growth factor is in great part captured inside endothelial cells and this capture limits its diffusion beyond vessel endothelial lining. The ability to diffuse outside the capillary lumen into the brain parenchyma, suggested that QK could be effective even if given intravenously. This hypothesis was tested giving different QK doses (1.2, 12, 40, 120, 360 and 1200 μg/kg) through an indwelling femoral catheter to different groups of rats one hour after pMCAO. A significant reduction of the infarct area was observed in rats receiving 40 or 120 μg/kg QK whereas higher or lower doses were ineffective. Because of its small size, QK was also expected to easily diffuse into the brain when given intranasally. We tested this hypothesis using a fluorescent derivative of this peptide and performing confocal imaging on brain slices obtained three hours after its intranasal administration. A strong fluorescence was observed in several brain areas including the cerebellar cortex, the hippocampus and striatum and the olfactory bulbs whereas the signal was fainter in cerebral cortex. This distribution profile was similar to what has been described before for other intranasally administred peptides. The evidence that QK diffuses well into the brain parenchyma suggested that this administration route could be used in brain ischemia. This hypothesis was confirmed by experiments performed in rats that received QK intranasally three hours after pMCAO. The ischemic damage evaluated after three days was 40% lower in these animals that in controls receiving vehicle and the beneficial effect of the treatment persisted also in animals sacrificed 7 days after surgery. Interestingly, in experiments performed with all of the three

administration routes that we used we noticed that there was no apparent increase in the volume of the brain of QK-treated rats as compared with controls. This important findigs stes a major difference as compared with VEGF because it suggests that different from VEGF, this peptide could not induce edema formation. To further investigate this point QK and VEGF were compared for their ability to permeabilize pial vessels as evaluated from FITC-dextran extravasation in intravital pial microvessel videoimaging experiments. The results obtained showed that fluorescent dextran significantly extravasated after the administration of VEGF given either by intracarotid injection or by topical application but remained inside blood vessesl when QK was give by either these routes (Figure 6). Collectively the results of our study showed that QK could be an extremely interesting option for CNS therapeutics because it has neuroprotective properties but boes not permeabilize the BBB. Much work remains to be done with this promising peptide. For instance, its possible activity in experimental model of other CNS disorders remains to be tested. Moreover, a mechanistic explanation for the lack of BBB permeabilization after QK administration remains to be established although several hypotheses could explain this property of the peptide. For instance, we have mentioned in section 4 that different transduction mechanisms are responsible for the proangiogenic and prosurvival effect of VEGF on one side and its vessel permeabilizing activity on the other. Specifically, vessel permeabilization seems to be largely dependent on the activation of the src cascade. Therefore, it is tempting to speculate that, somehow, the QK peptide could selectively activate the beneficial VEGF transduction cascades but not those leading to vessel permeabilization. Interestingly, a similar divergence in the ability to activate postreceptor events has been described for VEGF121 and VEGF165 that in the skin differentially activate src and the MAPK cascades [292]. Another possible explanation is in the different structure of QK and VEGF. As we mentioned at the beginning of this section, the binding site of VEGF to VEGFR has a multimodular structure with different components from different portions of the VEGF dimer and the 17-25 sequene is only part of it. It is conceivable therefore that the QK peptide could be less potent than VEGF on a molar base and that, therefore, it could be safer than VEGF as far as BBB permeabilization is concerned simply because this effect is observed at high VEGF doses [95-97].

Under many respect the QK peptide can be considered as a prototypical neuroprotective peptide that showed the feasibility of a therapeutic approach using only part and not the whole VEGF molecule. After its synthesis further efforts have been directed to synthetise novel and possibly better molecules. We just

pointed out that QK only reproduces one of the different portions of the VEGF monomer that take part to VEGF biding site to its receptors. The formal demonstration that VEGF receptors can also be activated by synthetic peptides reproducing these other interaction modules was obtained with the synthesis of a 17 mer peptide that was designed on the β-hairpin region 87-101 of PlGF [293]. When dissolved, the designed peptide, HPLW, assumes in water a well formed β-hairpin conformation, which fold following a two-step folding mechanism [294]. The peptide HPLW is able to bind to VEGFRs as demonstrated by NMR studied on purified domain 2 of VEGFR-1 [293,295] and in presence of ECs overexpressing VEGFRs [296]. The binding studied were also confirmed in vitro by citofluorimetry using ECs [293]. The biological activity of peptide HPLW was determined in vitro and in vivo. HPLW activates VEGF-dependent intracellular pathways, induces proliferation and rescue from apoptosis in ECs, and promotes capillary formation in an in vivo angiogenesis assay [293].

Because both QK and HPLW reproduce only one interaction module of VEGF with its receptors it is conceivable that more potent peptides could be obtained by the combination of multiple modules in the same molecule. The design of such multimodular molecules raises specific problems because these different regions have to maintain their relative spatial arrangement to interact with the receptors. De Rosa et al. [297] were successful in designing a series of second generation VEGF mimic peptides that include not only the α -helix 17–25 of QK but also a region similar to the β -hairpin 79–93, joined by variable linkers. The effect of most interesting of these peptides, named EP3, EP5 and EP6 were tested in vitro for their ability to induce the phosphorylation of VEGF receptor and the activation of the ERK1/2 pathway, to promote endothelial cell migration and proliferation *in vitro* and angiogenesis both in vitro and *in vivo*. The results showed that these peptides have biological effects similar to VEGF. In particular, the most active of them, EP6, showed a biological activity comparable to QK [297]. It will be interesting to test in future experiments whether these peptides yield any advantage respect to QK in experimental models of neurodegenerative diseases.

6. CONCLUSION

In conclusion, the data that we reviewed in the present paper show that VEGF has a great potential as a therapeutic tool for neurodegenerative diseases: However, the development of such a VEGF-based therapeutics for practical clinical applications is hampered by the unfavorable pharmacokinetics profile of

this molecule and by concerns in its safety. New VEGF-mimic peptides could represent a cheaper and easier to administer alternative to VEGF. Only QK, a prototype of first generation compounds that reproduce only one of the VEGF binding molecules to VEGFR, has been tested in neurological disorders and the data obtained suggested that it could be safer than VEGF. New, second generation peptides, designed to reproduce multiple VEGFR interaction modules hold promises of being more effective than QK and studies with these molecule in CNS disorders are eagerly awaited.

7. REFERENCES

1. I. Baumgartner, Therapeutic angiogenesis: theoretic problems using vascular endothelial growth factor. Curr. Cardiol. Rep. 2 (2000) 24-28.

2. R.C. Hendel, T.D. Henry, K. Rocha-Singh, J.M. Isner, D.J. Kereiakes, F.J. Giordano, M. Simons, R.O. Bonow, Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion: evidence for a dose-dependent effect. Circulation 101 (2000) 118-121.

3. T.D. Henry, B.H.Annex, G.R.McKendall, M.A.Azrin, J.J Lopez., F.J.Giordano, P.K.Shah, J.T Willerson., R.L.Benza, D.S.Berman, C.M.Gibson, A.Bajamonde, A.C.Rundle, J.Fine, E.R.McCluskey, VIVA Investigators, The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation 107 (2003) 1359-1365.

4. S. Takeshita, L.P. Zheng, E. Brogi, M. Kearney, L.Q. Pu, S. Bunting, N. Ferrara, J.F. Symes, J.M. Isner, Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. J. Clin. Invest. 93 (1994) 662-670.

5. C. Ruiz de Almodovar, D. Lambrechts, M. Mazzone, P. Carmeliet, Role and therapeutic potential of VEGF in the nervous system. Physiol. Rev. 89 (2009) 607-648.

6. C. Lange, E. Storkebaum, C.R. de Almodóvar, M. Dewerchin, P. Carmeliet, Vascular endothelial growth factor: a neurovascular target in neurological diseases. Nat. Rev. Neurol. 12 (2016) 439-454

7. K.A. Houck, D.W. Leung, A.M. Rowland, J. Winer, N. Ferrara, Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J. Biol. Chem. 267 (1992) 26031-26037.

8. J.E. Park, G.A. Keller, N. Ferrara, The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol. Biol. Cell. 4 (1993) 1317-1326.

9. N. Ortéga, F.E. L'Faqihi, J. Plouët, Control of vascular endothelial growth factor angiogenic activity by the extracellular matrix. Biol. Cell. 90 (1998) 381-390.

10. E.M. Amin, S. Oltean, J. Hua, M.V. Gammons, M. Hamdollah-Zadeh, G.I. Welsh, M.K. Cheung, L. Ni, S. Kase, E.S. Rennel, K.E. Symonds, D.G. Nowak, B. Royer-Pokora, M.A. Saleem, M. Hagiwara, V.A. Schumacher, S.J. Harper, D.R. Hinton, D.O. Bates, M.R. Ladomery, WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. Cancer Cell. 20 (2011) 768-780.

11. S.M. Eswarappa, A.A. Potdar, W.J. Koch, Y. Fan, K. Vasu, D. Lindner, B. Willard, L.M. Graham, P.E. DiCorleto, P.L. Fox, Programmed translational readthrough generates antiangiogenic VEGF-Ax. Cell 157 (2014) 1605-1618.

12. S. Harris, M. Craze, J. Newton, M. Fisher, D.T. Shima, G.M. Tozer, C. Kanthou, Do anti-angiogenic VEGF (VEGFxxxb) isoforms exist? A cautionary tale. PLoS One. 7 (2012) e35231.

13. H. Xin, C. Zhong, E. Nudleman, N. Ferrara, Evidence for Pro-angiogenic Functions of VEGF-Ax. Cell 167 (2016) 275-284.

14. Y.A. Muller, B. Li, H.W. Christinger, J.A. Wells, B.C. Cunningham, A.M. de Vos, Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proc. Natl. Acad. Sci. U. S. A. 94 (1997a) 7192-7197.

15. Y.A. Muller, H.W. Christinger, B.A. Keyt, A.M. de Vos, The crystal structure of vascular endothelial growth factor (VEGF) refined to 1.93 Å resolution: multiple copy flexibility and receptor binding. Structure 5 (1997b) 1325-1338.

16. N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors. Nat, Med. 9 (2003) 669-676.

17. Y. Cao, Positive and negative modulation of angiogenesis by VEGFR1 ligands.Sci. Signal. 2 (2009) re1.

18. M.J. Cross, J. Dixelius, T. Matsumoto, L. Claesson-Welsh, VEGF-receptor signal transduction. Trends Biochem. Sci. 28 (2003) 488-494.

19. M. Shibuya, L. Claesson-Welsh, Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp. Cell. Res. 312 (2006) 549-560.

20. A.K. Olsson, A. Dimberg, J. Kreuger, L. Claesson-Welsh, VEGF receptor signalling in control of vascular function. Nat. Rev. Mol. Cell. Biol. 7 (2006) 359-371.

21. D.S. Gélinas, P.N. Bernatchez, S. Rollin, N.G. Bazan, M.G. Sirois, Immediate and delayed VEGF-mediated NO synthesis in endothelial cells: role of PI3K, PKC and PLC pathways. Br. J. Pharmacol. 137 (2002) 1021-1030.

22. J.M. Rosenstein, J.M. Krum, C. Ruhrberg, VEGF in the nervous system. Organogenesis 6 (2010) 107-114.

23. K. Jin, Y. Zhu, Y. Sun, X.O. Mao, L. Xie, D.A. Greenberg, Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 11946-11950.

24. T. Licht, E.Keshet, Delineating multiple functions of VEGF-A in the adult brain. Cell. Mol. Life Sci. 70 (2013) 1727-1737.

25. P. Carmeliet, C. Ruiz de Almodovar, VEGF ligands and receptors: implications in neurodevelopment and neurodegeneration. Cell. Mol. Life Sci. 70 (2013) 1763-1778.

26. K. Jin, X.O. Mao, S.P. Batteur, E. McEachron, A. Leahy, D.A. Greenberg, Caspase-3 and the regulation of hypoxic neuronal death by vascular endothelial growth factor. Neuroscience 108 (2001) 351-358.

27. K.L. Jin, X.O. Mao, D.A. Greenberg, Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. Proc Natl Acad Sci U S A 97 (2000a) 10242-10247.

28. K.L. Jin, X.O. Mao, D.A. Greenberg, Vascular endothelial growth factor rescues HN33 neural cells from death induced by serum withdrawal. J. Mol. Neurosci. 14 (2000b) 197-203.

29. H. Matsuzaki, M. Tamatani, A. Yamaguchi, K. Namikawa, H. Kiyama, M.P. Vitek, N. Mitsuda, M. Tohyama, Vascular endothelial growth factor rescues hippocampal neurons from glutamate-induced toxicity: signal transduction cascades. FASEB J. 15 (2001) 1218-1220.

30. B. Svensson, M. Peters, H.G. König, M. Poppe, B. Levkau, M. Rothermundt, V. Arolt, D. Kögel, J.H. Prehn, Vascular endothelial growth factor protects cultured rat hippocampal neurons against hypoxic injury via an antiexcitotoxic, caspase-independent mechanism. J. Cereb. Blood. Flow Metab. 22 (2002) 1170-1175.

31. H.P. Gerber, A. McMurtrey, J. Kowalski, M. Yan, B.A. Keyt, V. Dixit, N. Ferrara, Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J. Biol. Chem. 273 (1998) 30336-30343.

32. S. Yu, Y.P. Liu, Y.H. Liu, S.S. Jiao, L. Liu, Y.J. Wang, W.L. Fu, Diagnostic utility of VEGF and soluble CD40L levels in serum of Alzheimer's patients. Clin. Chim. Acta. 453 (2016) 154-159.

33. S.P. Yu, Z.S. Farhangrazi, H.S. Ying, C.H. Yeh, D.W. Choi, Enhancement of outward potassium current may participate in beta-amyloid peptide-induced cortical neuronal death. Neurobiol. Dis. 5 (1998) 81-88.

34. J.Y. Xu JY, P. Zheng, D.H. Shen, S.Z. Yang, L.M. Zhang, Y.L. Huang, F.Y. Sun, Vascular endothelial growth factor inhibits outward delayed-rectifier potassium currents in acutely isolated hippocampal neurons. Neuroscience 118 (2003) 59-67.

35. M.H. Qiu, R. Zhang, F.Y. Sun, Enhancement of ischemia-induced tyrosine phosphorylation of Kv1.2 by vascular endothelial growth factor via activation of phosphatidylinositol 3-kinase. J. Neurochem. 87 (2003) 1509-1517.

36. X.Y. Huang, A.D. Morielli, E.G. Peralta, Tyrosine kinase-dependent suppression of a potassium channel by the G protein-coupled m1 muscarinic acetylcholine receptor. Cell 75 (1993) 1145-1156.

37. K.W. Wu, P. Yang, S.S. Li, C.W. Liu, F.Y. Sun, VEGF attenuated increase of outward delayed-rectifier potassium currents in hippocampal neurons induced by focal ischemia via PI3-K pathway. Neuroscience 298 (2015) 94-101.

38. Y.Y. Ma, K.Y. Li, J.J. Wang, Y.L. Huang, Y. Huang, F.Y. Sun, Vascular endothelial growth factor acutely reduces calcium influx via inhibition of the Ca2+ channels in rat hippocampal neurons. J Neurosci Res. 87 (2009) 393-402.

39. B.W. Kim, M. Choi, Y.S. Kim, H. Park, H.R. Lee, C.O. Yun, E.J. Kim, J.S. Choi, S. Kim, H. Rhim, B.K. Kaang, H. Son, Vascular endothelial growth factor (VEGF) signaling regulates hippocampal neurons by elevation of intracellular calcium and activation of calcium/calmodulin protein kinase II and mammalian target of rapamycin. Cell. Signal. 20 (2008) 714-725.

40. G.C. Sun, Y.Y. Ma, Vascular endothelial growth factor modulates voltage-gated Na+channel properties and depresses action potential firing in cultured rat hippocampal neurons. Biol. Pharm. Bull. 36 (2013) 548-555.

41. T.D. Palmer, A.R. Willhoite, F.H. Gage, Vascular niche for adult hippocampal neurogenesis. J. Comp. Neurol. 425 (2000) 479-494.

42. N.L. Ward, J.C. Lamanna, The neurovascular unit and its growth factors: coordinated response in the vascular and nervous systems. Neurol. Res. 26 (2004) 870-883.

43. M.A. Font, A. Arboix, J. Krupinski, Angiogenesis, neurogenesis and neuroplasticity in ischemic stroke. Curr. Cardiol. Rev. 6 (2010) 238-244.

44. D.A. Greenberg, K. Jin, Vascular endothelial growth factors (VEGFs) and stroke. Cell. Mol. Life Sci. 70 (2013) 1753-1761.

45. J.A. Madri, Modeling the neurovascular niche: implications for recovery from CNS injury. J. Physiol. Pharmacol. 60 Suppl 4 (2009) 95-104.

46. J.J. Ohab, S. Fleming, A. Blesch, S.T. Carmichael, A neurovascular niche for neurogenesis after stroke. J. Neurosci. 26 (2006) 13007-13016.

47. R.L. Zhang, M. Chopp, C. Roberts, X. Liu, M. Wei, S.P. Nejad-Davarani, X. Wang, Z.G.Zhang, Stroke increases neural stem cells and angiogenesis in the neurogenic niche of the adult mouse. PLoS One. 9 (2014) e113972.

48. A.J. Brumm, S.T. Carmichael, Not just a rush of blood to the head. Nat. Med. 18 (2012) 1609-1610.

49. R. Muramatsu, C. Takahashi, S. Miyake, H. Fujimura, H. Mochizuki, T. Yamashita, Angiogenesis induced by CNS inflammation promotes neuronal remodeling through vessel-derived prostacyclin. Nat. Med. 18 (2012) 1658-1664.

50. D.M. Hermann, A. Zechariah, Implications of vascular endothelial growth factor for postischemic neurovascular remodeling. J. Cereb. Blood Flow Metab. 29 (2009) 1620-1643.

51. Y. Ma, A. Zechariah, Y. Qu, D.M.Hermann, Effects of vascular endothelial growth factor in ischemic stroke. J. Neurosci. Res. 90 (2012) 1873-1882.

52. N.O. Schmidt, W. Przylecki, W. Yang, M. Ziu, Y. Teng, S.U. Kim, P.M. Black, K.S. Aboody, R.S.Carroll, Brain tumor tropism of transplanted human neural stem cells is induced by vascular endothelial growth factor. Neoplasia. 7 (2005) 623-629.

53. N.O. Schmidt, D. Koeder, M. Messing, F.J. Mueller, K.S. Aboody, S.U. Kim, P.M. Black, R.S. Carroll, M. Westphal, K. Lamszus, Vascular endothelial growth factor-stimulated cerebral microvascular endothelial cells mediate the recruitment of neural stem cells to the neurovascular niche. Brain Res. 1268 (2009) 24-37.

54. J.C. LaManna, N.T. Kuo, W.D. Lust, Hypoxia-induced brain angiogenesis. Signals and consequences. Adv. Exp. Med. Biol. 454 (1998) 287-293.

55. H.J. Marti, M. Bernaudin, A. Bellail, H. Schoch, M. Euler, E. Petit, W. Risau, Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. Am. J. Pathol. 156 (2000) 965-976.

56. A. Szade, A. Grochot-Przeczek, U. Florczyk, A. Jozkowicz, J. Dulak, Cellular and molecular mechanisms of inflammation-induced angiogenesis. IUBMB Life. 67 (2015) 145-159.

57. M. Caruana, R. Cauchi, N. Vassallo, Putative Role of Red Wine Polyphenols against Brain Pathology in Alzheimer's and Parkinson's Disease. Front. Nutr. 3 (2016) 31.

58. S.A. Mandel, O. Weinreb, T. Amit, M.B. Youdim, Molecular mechanisms of the neuroprotective/neurorescue action of multi-target green tea polyphenols. Front. Biosci. (Schol Ed). 4 (2012) 581-598.

59. J. Pérez-Hernández, V.J. Zaldívar-Machorro, D. Villanueva-Porras, E. Vega-Ávila, A. Chavarría A Potential Alternative against Neurodegenerative Diseases: Phytodrugs. Oxid. Med. Cell Longev. 2016 (2016) 8378613.

60. M. Ajami, H. Pazoki-Toroudi, H. Amani, S.F. Nabavi, N. Braidy, R.A. Vacca, A.G. Atanasov, A. Mocan, S.M. Nabavi, Therapeutic role of sirtuins in neurodegenerative disease and their modulation by polyphenols. Neurosci. Biobehav. Rev. (2016) pii: S0149-7634(16)30495-X. doi: 10.1016/j.neubiorev.2016.11.022. [Epub ahead of print]

61. M. Donà, I. Dell'Aica, F. Calabrese, R. Benelli, M. Morini, A. Albini, S. Garbisa, Neutrophil restraint by green tea: inhibition of inflammation, associated angiogenesis, and pulmonary fibrosis. J. Immunol. 170 (2003) 4335-4341.

62. S. Malhotra, M. Tavakkoli, N. Edraki, R. Miri, S.K. Sharma, A.K. Prasad, L. Saso, C. Len, V.S. Parmar, O.Firuzi Neuroprotective and Antioxidant Activities of 4-Methylcoumarins: Development of Structure-Activity Relationships. Biol. Pharm. Bull. 39 (2016) 1544-1548.

63. D. Lapi, S. Vagnani, G. Pignataro, E. Esposito, M. Paterni, A. Colantuoni, Protective Effects of Quercetin on Rat Pial Microvascular Changes during Transient Bilateral Common Carotid Artery Occlusion and Reperfusion. Front. Physiol. 3 (2012) 32.

64. A. Albini, F. Tosetti, V.W. Li, D.M. Noonan, W.W. Li Cancer prevention by targeting angiogenesis. Nat. Rev. Clin. Oncol. 9 (2012) 498-509.

65. R. Benelli, R. Venè, D. Bisacchi, S. Garbisa, A. Albini, Anti-invasive effects of green tea polyphenol epigallocatechin-3-gallate (EGCG), a natural inhibitor of metallo and serine proteases. Biol Chem. 383 (2002) 101-105.

66. W. Fernando, H.P. Rupasinghe, D.W. Hoskin, Regulation of Hypoxia-inducible Factor-1α and Vascular Endothelial Growth Factor Signaling by Plant Flavonoids. Mini Rev Med Chem. 15 (2015) 479-489.

67. B. Fu, J. Xue, Z. Li, X. Shi, B.H. Jiang, J.Fang, Chrysin inhibits expression of hypoxia-inducible factor-1alpha through reducing hypoxia-inducible factor-1alpha stability and inhibiting its protein synthesis. Mol Cancer Ther. 6 (2007) 220-226.

68. C. Gallo, K. Dallaglio, B. Bassani, T. Rossi, A. Rossello, D.M. Noonan, G. D'Uva, A Bruno, A. Albini, Hop derived flavonoid xanthohumol inhibits endothelial cell functions via AMPK activation. Oncotarget. (2016) Aug 1. doi: 10.18632/oncotarget.10990. [Epub ahead of print]

69. P. García-Maceira, J. Mateo, Silibinin inhibits hypoxia-inducible factor-1alpha and mTOR/p70S6K/4E-BP1 signalling pathway in human cervical and hepatoma cancer cells: implications for anticancer therapy. Oncogene 28 (2009) 313-324.

70. H. Jeon, H. Kim, D. Choi, D. Kim, S.Y. Park, Y.J. Kim, Y.M. Kim, Y. Jung, Quercetin activates an angiogenic pathway, hypoxia inducible factor (HIF)-1-vascular endothelial growth factor, by inhibiting HIF-prolyl hydroxylase: a structural analysis of quercetin for inhibiting HIF-prolyl hydroxylase. Mol. Pharmacol. 71 (2007) 1676-1684.

71. Y.D. Zhou, Y.P. Kim, X.C. Li, S.R. Baerson, A.K. Agarwal, T.W. Hodges, D. Ferreira, D.G. Nagle, Hypoxia-inducible factor-1 activation by (-)-epicatechin gallate: potential adverse effects of cancer chemoprevention with high-dose green tea extracts. J. Nat. Prod. 67 (2004) 2063-2069.

72. R. Thomas, M.H. Kim, Epigallocatechin gallate inhibits HIF-1a degradation in prostate cancer cells. Biochem Biophys. Res. Commun. 334 (2005) 543-548.

73. S. Amoroso, A. Gioielli, M. Cataldi, G. Di Renzo, L. Annunziato, In the neuronal cell line SH-SY5Y, oxidative stress-induced free radical overproduction causes cell death without any participation of intracellular Ca^{2+} increase. Biochim. Biophys. Acta. 1452 (1999)151-160.

74. L. Annunziato, A. Pannaccione, M. Cataldi, A. Secondo, P. Castaldo, G. Di Renzo, M. Taglialatela, Modulation of ion channels by reactive oxygen and nitrogen species: a pathophysiological role in brain aging? Neurobiol. Aging. 23 (2002) 819-834.

75. L Annunziato, M Cataldi, G Pignataro, A Secondo, P. Molinaro, Glutamate-independent calcium toxicity: introduction. Stroke 38(2 Suppl) (2007) 661-664.

76. S. Zacchigna, D. Lambrechts, P. Carmeliet, Neurovascular signalling defects in neurodegeneration. Nat. Rev. Neurosci. 9 (2008) 169-181.

77. T.M. Hansen, A.J. Moss, N.P.Brindle, Vascular endothelial growth factor and angiopoietins in neurovascular regeneration and protection following stroke. Curr. Neurovasc. Res. 5 (2008) 236-245.

78. M.M. Nowacka, E.Obuchowicz, Vascular endothelial growth factor (VEGF) and its role in the central nervous system: a new element in the neurotrophic hypothesis of antidepressant drug action. Neuropeptides 46 (2012) 1-10.

79. I. Zachary, Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. Neurosignals 14 (2005) 207-221.

80. H. Beck, K.H. Plate, Angiogenesis after cerebral ischemia. Acta Neuropathol. 117 (2009) 481-496.

81. Z. Kovács, K. Ikezaki, K. Samoto, T. Inamura, M. Fukui, VEGF and flt. Expression time kinetics in rat brain infarct. Stroke 27 (1996) 1865-1872.

82. T. Hayashi, K. Abe, H. Suzuki, Y. Itoyama, Rapid induction of vascular endothelial growth factor gene expression after transient middle cerebral artery occlusion in rats. Stroke 28 (1997) 2039-2044.

83. M.Y. Lee, W.K. Ju, J.H. Cha, B.C. Son, M.H. Chun, J.K. Kang, C.K. Park, Expression of vascular endothelial growth factor mRNA following transient forebrain ischemia in rats. Neurosci. Lett. 265 (1999) 107-110.

84. J. Hai, S.T. Li, Q. Lin, Q.G. Pan, F. Gao, M.X. Ding, Vascular endothelial growth factor expression and angiogenesis induced by chronic cerebral hypoperfusion in rat brain. Neurosurgery 53 (2003) 963-970.

85. J. Yang, Y. Yao, T. Chen, T. Zhang, VEGF ameliorates cognitive impairment in in vivo and in vitro ischemia via improving neuronal viability and function. Neuromolecular Med. 16 (2014) 376-388.

86. Z.J. Yang, W.L. Bao, M.H. Qiu, L.M. Zhang, S.D. Lu, Y.L. Huang, F.Y. Sun, Role of vascular endothelial growth factor in neuronal DNA damage and repair in rat brain following a transient cerebral ischemia. J. Neurosci. Res. 70 (2002) 140-149.

87. W.L. Bao, S.D. Lu, H. Wang, F.Y. Sun, Intraventricular vascular endothelial growth factor antibody increases infarct volume following transient cerebral ischemia. Zhongguo Yao Li Xue Bao 20 (1999) 313-318.

88. J. Shimotake, N. Derugin, M. Wendland, Z.S. Vexler, D.M. Ferriero, Vascular endothelial growth factor receptor-2 inhibition promotes cell death and limits endothelial cell proliferation in a neonatal rodent model of stroke. Stroke 41 (2010) 343-349.

89. V.R. Whitaker, L. Cui, S. Miller, S.P. Yu, L. Wei, Whisker stimulation enhances angiogenesis in the barrel cortex following focal ischemia in mice. J. Cereb. Blood Flow Metab. 27 (2007) 57-68.

90. W.L. Li, J.L. Fraser, S.P. Yu, J. Zhu, Y.J. Jiang, L. Wei, The role of VEGF/VEGFR2 signaling in peripheral stimulation-induced cerebral neurovascular regeneration after ischemic stroke in mice. Exp Brain Res. 214 (2011) 503-513.

91. S. Qiu, T. Wu, P. Wang, J. Li, Q. Li, J. Du, The Association between VEGFR Gene Polymorphisms and Stroke: A Meta-Analysis. PLoS One. 11 (2016) e0151371.

92. T. Wu, S. Qiu, P. Wang, J. Li, Q. Li, J. Du, The association between vascular endothelial growth factor gene polymorphisms and stroke: a meta-analysis. Brain Behav. 6 (2016) e00482.

93. Z.G. Zhang, L. Zhang, Q. Jiang, R. Zhang, K. Davies, C. Powers, N.V. Bruggen, M. Chopp, VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. J. Clin. Invest. 106 (2000) 829-838.

94. Y. Sun, K. Jin, L. Xie, J. Childs, X.O. Mao, A. Logvinova, D.A. Greenberg , VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J. Clin. Invest. 111 (2003) 1843-1851.

95. M. Harrigan, S. Ennis, T. Masada,R. Keep, Intraventricular infusion of vascular endothelial growth factor promotes cerebral angiogenesis with minimal brain edema. Neurosurgery 50 (2002) 589-598.

96. M.R. Harrigan, S.R. Ennis, S.E. Sullivan, R.F. Keep, Effects of intraventricular infusion of vascular endothelial growth factor on cerebral blood flow, edema, and infarct volume. Acta Neurochir (Wien). 145 (2003) 49-53.

97. P.S. Manoonkitiwongsa, R.L. Schultz, D.B. McCreery, E.F. Whitter, P.D. Lyden, Neuroprotection of ischemic brain by vascular endothelial growth factor is critically dependent on proper dosage and may be compromised by angiogenesis. J. Cereb. Blood Flow Metab. 24 (2004) 693-702.

98. B. Oosthuyse, L. Moons, E. Storkebaum, H. Beck, D. Nuyens, K. Brusselmans, J. Van Dorpe, P. Hellings, M. Gorselink, S. Heymans, G. Theilmeier, M. Dewerchin, V. Laudenbach, P. Vermylen, H. Raat, T. Acker, V. Vleminckx, L. Van Den Bosch, N. Cashman, H. Fujisawa, M.R. Drost, R. Sciot, F. Bruyninckx, D.J. Hicklin, C. Ince, P. Gressens, F. Lupu, K.H. Plate, W. Robberecht, J.M. Herbert, D. Collen, P. Carmeliet, Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat. Genet. 28 (2001) 131-138.

99. S.L. Green, R.J. Tolwani, Animal models for motor neuron disease. Lab. Anim. Sci.49 (1999) 480-487.

100. J.M. Shefner, A.G.Reaume, D.G. Flood, R.W. Scott, N.W. Kowall, R.J. Ferrante, D.F. Siwek, M. Upton-Rice, R.H. Jr. Brown, Mice lacking cytosolic copper/zinc superoxide dismutase display a distinctive motor axonopathy. Neurology 53 (1999) 1239-1246.

101. D. Lambrechts, E.Storkebaum, M. Morimoto, J. Del-Favero, F. Desmet, S.L. Marklund, S. Wyns, V. Thijs, J. Andersson, I. van Marion, A. Al-Chalabi, S. Bornes, R. Musson, V. Hansen, L. Beckman, R. Adolfsson, H.S. Pall, H. Prats, S. Vermeire, P. Rutgeerts, S. Katayama, T. Awata, N. Leigh, L. Lang-Lazdunski, M. Dewerchin, C. Shaw, L. Moons, R. Vlietinck, K.E. Morrison, W. Robberecht, C. Van Broeckhoven, D. Collen, P.M. Andersen, P.Carmeliet, VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat. Genet. 34 (2003) 383-394.

102. J.S. Lunn, S.A. Sakowski, B. Kim, A.A. Rosenberg, E.L. Feldman, Vascular endothelial growth factor prevents G93A-SOD1-induced motor neuron degeneration. Dev. Neurobiol. 69 (2009) 871-884.

103. K.Miyazaki, K.Masamoto, N. Morimoto, T. Kurata, T. Mimoto, T. Obata, I. Kanno, K. Abe, Early and progressive impairment of spinal blood flow-glucose metabolism coupling in motor neuron degeneration of ALS model mice. J. Cereb. Blood Flow Metab. 32 (2011a) 456-467.

104. K. Sato, N. Morimoto, T. Kurata, T. Mimoto, K. Miyazaki, Y. Ikeda, K. Abe, Impaired response of hypoxic sensor protein HIF-1α and its downstream proteins in the spinal motor neurons of ALS model mice. Brain Res. 1473 (2012) 55-62.

105. X. Li, L. Lu, D.J. Bush, X. Zhang, L. Zheng, E.A. Suswam, P.H. King, Mutant copper-zinc superoxide dismutase associated with amyotrophic lateral sclerosis binds to adenine/uridine-rich stability elements in the vascular endothelial growth factor 3'-untranslated region. J. Neurochem. 108 (2009) 1032-1044.

106. L. Lu, L. Zheng, L. Viera, E. Suswam, Y. Li, X. Li, A.G. Estévez, P.H. King, Mutant Cu/Zn-superoxide dismutase associated with amyotrophic lateral sclerosis destabilizes vascular endothelial growth factor mRNA and downregulates its expression. J. Neurosci. 27 (2007) 7929-7938.

107. K. Vijayalakshmi, P. Ostwal, R. Sumitha, S. Shruthi, A.M. Varghese, P. Mishra, S.G. Manohari, B.C. Sagar, T.N. Sathyaprabha, A. Nalini, T.R. Raju, P.A. Alladi, Role of VEGF and VEGFR2 Receptor in Reversal of ALS-CSF Induced Degeneration of NSC-34 Motor Neuron Cell Line. Mol. Neurobiol. 51 (2015) 995-1007.

108. E. Storkebaum, D. Lambrechts, M. Dewerchin, M.P. Moreno-Murciano, S. Appelmans, H. Oh, P. Van Damme, B. Rutten, W.Y. Man, M. De Mol, S. Wyns, D. Manka, K. Vermeulen, L. Van Den Bosch, N. Mertens, C. Schmitz, W. Robberecht, E.M. Conway, D. Collen, L. Moons, P. Carmeliet, Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. Nat. Neurosci. 8 (2005) 85-92.

109. M. Azzouz, G.S. Ralph, E. Storkebaum, L.E. Walmsley, K.A. Mitrophanous, S.M. Kingsman, P. Carmeliet, N.D. Mazarakis, VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. Nature 429 (2004) 413-417.

110. J.S. Lunn, S.A. Sakowski, B. Kim, A.A. Rosenberg, E.L. Feldman, Vascular endothelial growth factor prevents G93A-SOD1-induced motor neuron degeneration. Dev. Neurobiol. 69 (2009) 871-884.

111. E. Bogaert, P. Van Damme, K. Poesen, J. Dhondt, N. Hersmus, D. Kiraly, W. Scheveneels, W. Robberecht, L. Van Den Bosch, VEGF protects motor neurons against excitotoxicity by upregulation of GluR2. Neurobiol. Aging. 31 (2010) 2185-2191.

112. R.J. Bridges, D.R. Stevens, J.S. Kahle, P.B. Nunn, M. Kadri, C.W. Cotman, Structure-function studies on N-oxalyl-diamino-dicarboxylic acids and excitatory amino acid receptors: evidence that beta-L-ODAP is a selective non-NMDA agonist. J. Neurosci. 9 (1989) 2073-2079.

113. R.A. Chase, S. Pearson, P.B. Nunn, P.L.Lantos, Comparative toxicities of alpha- and beta-N-oxalyl-L-alpha, beta-diaminopropionic acids to rat spinal cord. Neurosci. Lett. 55 (1985) 89-94.

114. P.S. Spencer, D.N. Roy, A. Ludolph, J. Hugon, M.P. Dwivedi, H.H.Schaumburg, Lathyrism: evidence for role of the neuroexcitatory aminoacid BOAA. Lancet 2-8515- (1986) 1066-1067.

115. J. Hugon, A. Ludolph, D.N. Roy, H.H. Schaumburg, P.S. Spencer , Studies on the etiology and pathogenesis of motor neuron diseases. II. Clinical and electrophysiologic features of pyramidal dysfunction in macaques fed Lathyrus sativus and IDPN. Neurology 38 (1988) 435-442.

116. D. Devos, C. Moreau, P. Lassalle, T. Perez, J. De Seze, V. Brunaud-Danel, A. Destée, A.B. Tonnel, N. Just, Low levels of the vascular endothelial growth factor in CSF from early ALS patients. Neurology 62 (2004) 2127-2129.

117. A. Canosa, A. Calvo, M. Barberis, M. Brunetti, G. Restagno, S. Cammarosano, A. Ilardi, M.C. Vigliani, A. Chiò, C. Moglia, Amyotrophic lateral sclerosis onset after prolonged treatment with a VEGF receptors inhibitor. Amyotroph Lateral Scler Frontotemporal Degener. 16 (2015) 129-130.

118. D. Lambrechts, K. Poesen, R. Fernández-Santiago, A. Al-Chalabi, R. Del Bo, P.W. Van S Vught, S. Khan, SL Marklund, A. Brockington, I. van Marion, J. Anneser, C. Shaw, A.C. Ludolph, N.P. Leigh, G.P. Comi, T. Gasser, P.J. Shaw, K.E. Morrison, P.M. Andersen, L.H. Van den Berg, V. Thijs, T. Siddique, W. Robberecht, P. Carmeliet, Meta-analysis of vascular endothelial growth factor variations in amyotrophic lateral sclerosis: increased susceptibility in male carriers of the -2578AA genotype. J. Med. Genet. 46 (2009) 840-846.

119. D. Chen, L. Shen, L. Wang, A. Lu, H. Zhang, X. Zhang, Y. Zhang, W. Shui, L. Li, D. Fan, J. Zhang, Association of polymorphisms in vascular endothelial growth factor gene with the age of onset of amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. 8 (2007) 144-149.

120. R. Fernández-Santiago, M. Sharma, J.C. Mueller, H. Gohlke, T. Illig, J. Anneser, C. Münch, A. Ludolph, C. Kamm, T. Gasser, Possible gender-dependent association of vascular endothelial growth factor (VEGF) gene and ALS. Neurology 66 (2006) 1929-1931.

121. W. Chen, M. Saeed, H. Mao, N. Siddique, L. Dellefave, W.Y. Hung, H.X. Deng, R.L. Sufit, S.L. Heller, J.L. Haines, M. Pericak-Vance, T. Siddique, Lack of association of VEGF promoter polymorphisms with sporadic ALS. Neurology 67 (2006) 508-510.

122. R. Del Bo, M. Scarlato, S. Ghezzi, F. Martinelli-Boneschi, S. Corti, F. Locatelli, D. Santoro, A. Prelle, C. Briani, M. Nardini, G. Siciliano, M. Mancuso, L. Murri, N. Bresolin, G.P. Comi, Absence of angiogenic genes modification in Italian ALS patients. Neurobiol. Aging 29 (2008) 314-316.

123. F. Gros-Louis, S. Laurent, A.A. Lopes, J. Khoris, V. Meininger, W. Camu, G.A. Rouleau, Absence of mutations in the hypoxia response element of VEGF in ALS. Muscle Nerve 28 (2003) 774-775.

124. P.W. Van Vught, N.A. Sutedja, J.H. Veldink, B.P. Koeleman, G.J. Groeneveld, C. Wijmenga, B.M. Uitdehaag, J.M. de Jong, F. Baas, J.H. Wokke, L.H.Van den Berg, Lack of association between VEGF polymorphisms and ALS in a Dutch population. Neurology 65 (2005) 1643-1645.

125. Y. Zhang, H. Zhang, Y. Fu, H. Song, L. Wang, J. Zhang, D. Fan, VEGF C2578A polymorphism does not contribute to amyotrophic lateral sclerosis susceptibility in sporadic Chinese patients. Amyotroph Lateral Scler. 7 (2006) 119-122.

126. A. Brockington, B. Wokke, H. Nixon, J. Hartley, P.J..Shaw, Screening of the transcriptional regulatory regions of vascular endothelial growth factor receptor 2 (VEGFR2) in amyotrophic lateral sclerosis. BMC Med. Genet. 8 (2007) 23.

127. M. Cataldi, The changing landscape of voltage-gated calcium channels in neurovascular disorders and in neurodegenerative diseases. Curr. Neuropharmacol. 11 (2013) 276-297.

128. R.N. Kalaria, Small vessel disease and Alzheimer's dementia: pathological considerations. Cerebrovasc. Dis. 13 Suppl 2 (2002):48-52.

129. E.P. Meyer, A. Ulmann-Schuler, M. Staufenbiel, T. Krucker, Altered morphology and 3D architecture of brain vasculature in a mouse model for Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 3587-3592.

130. A. Bozzao, R. Floris, M.E. Baviera, A. Apruzzese, G. Simonetti, Diffusion and perfusion MR imaging in cases of Alzheimer's disease: correlations with cortical atrophy and lesion load. AJNR Am. J. Neuroradiol. 22 (2001) 1030-1036.

131. H. Matsuda, Cerebral blood flow and metabolic abnormalities in Alzheimer's disease. Ann. Nucl. Med. 15 (2001) 85-92.

132. R.J. Ellis, J.M. Olichney, L.J. Thal, S.S. Mirra, J.C. Morris, D. Beekly, A. Heyman, Cerebral amyloid angiopathy in the brains of patients with Alzheimer's disease: the CERAD experience, Part XV. Neurology 46 (1996) 1592-1596.

133. M.M. Esiri, G.K.Wilcock, Cerebral amyloid angiopathy in dementia and old age. J. Neurol. Neurosurg. Psychiatry 49 (1986) 1221-1226.

134. S. Love, J.S.Miners, Cerebral Hypoperfusion and the Energy Deficit in Alzheimer's Disease. Brain Pathol. 26 (2016) 607-617.

135. R.N. Kalaria, D.L. Cohen, D.R. Premkumar, S. Nag, J.C. LaManna, W.D. Lust, Vascular endothelial growth factor in Alzheimer's disease and experimental cerebral ischemia. Brain Res. Mol. Brain Res. 62 (1998) 101-105.

136. E. Tarkowski, R. Issa, M. Sjögren, A. Wallin, K. Blennow, A. Tarkowski, P. Kumar, Increased intrathecal levels of the angiogenic factors VEGF and TGF-beta in Alzheimer's disease and vascular dementia. Neurobiol. Aging 23 (2002) 237-243.

137. L. Thirumangalakudi , P.G. Samany, A. Owoso, B. Wiskar, P. Grammas, Angiogenic proteins are expressed by brain blood vessels in Alzheimer's disease. J, Alzheimers Dis. 10 (2006) 111-118.

138. R. Barker, E.L. Ashby, D. Wellington, V.M. Barrow, J.C. Palmer, P.G. Kehoe, M.M. Esiri, S. Love, Pathophysiology of white matter perfusion in Alzheimer's disease and vascular dementia. Brain 137 (2014) 1524-1532.

139. J. Provias, B. Jeynes, Reduction in vascular endothelial growth factor expression in the superior temporal, hippocampal, and brainstem regions in Alzheimer's disease. Curr. Neurovasc. Res. 11 (2014) 202-209.

140. J. Provias, B.Jeynes, Neurofibrillary tangles and senile plaques in Alzheimer's brains are associated with reduced capillary expression of vascular endothelial growth factor and endothelial nitric oxide synthase. Curr. Neurovasc. Res. 5 (2008) 199-205.

141. S. Yu, Y.P. Liu, Y.H. Liu, S.S. Jiao, L. Liu, Y.J. Wang, W.L.Fu, Diagnostic utility of VEGF and soluble CD40L levels in serum of Alzheimer's patients. Clin. Chim. Acta 453 (2016)154-159.

142. I. Mateo, J. Llorca, J. Infante, E. Rodríguez-Rodríguez, C. Fernández-Viadero, N. Peña, J. Berciano, O. Combarros Low serum VEGF levels are associated with Alzheimer's disease. Acta Neurol Scand. 116 (2007) 56-58.

143. R. Craig-Schapiro, M. Kuhn, C. Xiong, E.H. Pickering, J. Liu, T.P. Misko, R.J.Perrin, K.R. Bales, H. Soares, A.M. Fagan, D.M. Holtzman K.R., H. Soares, R.J. Perrin, Bales, Fagan AM, Holtzman DM. Multiplexed immunoassay panel identifies novel CSF biomarkers for Alzheimer's disease diagnosis and prognosis. PLoS One 6 (2011) e18850.

144. Y.Y. Leung, J.B. Toledo, A. Nefedov, R. Polikar, N. Raghavan, S.X. Xie, M. Farnum, T. Schultz, Y. Baek, V.V. Deerlin, W.T. Hu, D.M. Holtzman, A.M. Fagan, R.J. Perrin, M. Grossman, H.D. Soares, M.A. Kling, M. Mailman, S.E. Arnold, V.A. Narayan, V.M. Lee, L.M. Shaw, D. Baker, G.M. Wittenberg, J.Q. Trojanowski, L.S. Wang, Identifying amyloid pathology-related cerebrospinal fluid biomarkers for Alzheimer's disease in a multicohort study. Alzheimers Dement. (Amst). 1 (2015) 339-348.

145. T.J. Hohman, S.P. Bell, A.L. Jefferson,; Alzheimer's Disease Neuroimaging Initiative. The role of vascular endothelial growth factor in neurodegeneration and cognitive decline: exploring interactions with biomarkers of Alzheimer disease. JAMA Neurol. 72 (2015) 520-529.

146. J. Chapuis, J. Tian, J. Shi, F. Bensemain, D. Cottel, C. Lendon, P. Amouyel, D. Mann, J.C.Lambert, Association study of the vascular endothelial growth factor gene with the risk of developing Alzheimer's disease. Neurobiol. Aging. 27 (2006) 1212-1215.

147. R. Del Bo, M. Scarlato, S. Ghezzi, F. Martinelli Boneschi, C. Fenoglio, S. Galbiati, R. Virgilio, D. Galimberti, G. Galimberti, M. Crimi, C. Ferrarese, E. Scarpini, N. Bresolin, G.P.Comi, Vascular endothelial growth factor gene variability is associated with increased risk for AD. Ann. Neurol. 57 (2005) 373-380.

148. S. Landgren, M.S. Palmér, I. Skoog, L Minthon, A. Wallin, N. Andreasen, M. Zetterberg, K. Blennow, H. Zetterberg, No association of VEGF polymorphims with Alzheimer's disease. Neuromolecular Med. 12 (2010) 224-228.

149. S.Y. Liu, F.F. Zeng, Z.W. Chen, C.Y. Wang, B Zhao, K.S. Li, Vascular endothelial growth factor gene promoter polymorphisms and Alzheimer's disease risk: a meta-analysis. CNS Neurosci. Ther. 19 (2013) 469-476.

150. D. He, W. Lu, K. Chang, Y. Liu, J. Zhang, Z. Zeng Vascular endothelial growth factor polymorphisms and risk of Alzheimer's disease: a meta-analysis. Gene. 518 (2013) 296-302.

151. N.A. Johnson, G.H. Jahng, M.W. Weiner, B.L. Miller, H.C. Chui, W.J. Jagust, M.L. Gorno-Tempini, N. Schuff, Pattern of cerebral hypoperfusion in Alzheimer disease and mild cognitive impairment measured with arterial spin-labeling MR imaging: initial experience. Radiology 234 (2005) 851-859.

152. D.Paris, N. Patel, A. DelleDonne, A. Quadros, R. Smeed, Mullan M. Impaired angiogenesis in a transgenic mouse model of cerebral amyloidosis. Neurosci. Lett. 366 (2004a) 80–85.

153. D. Paris, K. Townsend, A. Quadros, J. Humphrey, J. Sun, S. Brem, M. Wotoczek-Obadia, A. DelleDonne, N. Patel, D.F. Obregon, R. Crescentini, L. Abdullah, D. Coppola, A.M. Rojiani, F. Crawford, S.M. Sebti, M.Mullan, Inhibition of angiogenesis by Abeta peptides. Angiogenesis. 7 (2004) 75-85.

154. N.S. Patel, V.S. Mathura, C. Bachmeier, D. Beaulieu-Abdelahad, V. Laporte, O. Weeks, M. Mullan, D.Paris, Alzheimer's beta-amyloid peptide blocks vascular endothelial growth factor mediated signaling via direct interaction with VEGFR-2. J. Neurochem. 112 (2010) 66-76.

155. Wang P, Xie ZH, Guo YJ, Zhao CP, Jiang H, Song Y, Zhu ZY, Lai C, Xu SL, Bi JZ. VEGF-induced angiogenesis ameliorates the memory impairment in APP transgenic mouse model of Alzheimer's disease. Biochem. Biophys. Res. Commun 411 (2011) 620-626.

156. K.O. Garcia, F.L. Ornellas, P.K. Martin, C.L. Patti, L.E. Mello, R. Frussa-Filho, S.W. Han, B.M. Longo, Therapeutic effects of the transplantation of VEGF overexpressing bone marrow mesenchymal stem cells in the hippocampus of murine model of Alzheimer's disease. Front. Aging Neurosci. 6 (2014) 30.

157. P. Religa, R. Cao, D. Religa, Y. Xue, N. Bogdanovic, D. Westaway, H.H. Marti, B. Winblad, Y.Cao, VEGF significantly restores impaired memory behavior in Alzheimer's mice by improvement of vascular survival. Sci. Rep. 3 (2013) 2053.

158. M.R.Pitzer, C.E.Sortwell, B.F.Daley, S.O.McGuire, D.Marchionini, M.Fleming, T.J. Collier, Angiogenic and neurotrophic effects of vascular endothelial growth factor (VEGF165): studies of grafted andcultured embryonic ventral mesencephalic cells, Exp. Neurol. 182 (2003) 435-445.

159. W.F. Silverman, J.M. Krum, N. Mani, J.M. Rosenstein, Vascular, glial and neuronal effects of vascular endothelial growth factor in mesencephalic explant cultures, Neuroscience 90 (1999) 1529–1541.

160. T. Yasuhara, T. Shingo, K. Kobayashi, A. Takeuchi, A. Yano, K. Muraoka, T.Matsui, Y.Miyoshi, H.Hamada, I. Date, Neuroprotective effects of vascular endothelial growth factor (VEGF) upon dopaminergic neurons in a rat model of Parkinson's disease, Eur. J. Neurosci. 19 (2004) 1494-1504.

161. T. Yasuhara, T. Shingo, K. Muraoka, Y. wen Ji, M. Kameda, A. Takeuchi, A. Yano, S. Nishio, T. Matsui, Y. Miyoshi, H. Hamada, I. Date, The differences between high and low-dose administration of VEGF to dopaminergic neurons of in vitro and in vivo Parkinson's disease model. Brain Res. 1038 (2005) 1-10.

162. Y.Y. Tian, C.J. Tang, J.N. Wang, Y. Feng, X.W. Chen, L. Wang, X. Qiao, S.G. Sun, Favorable effects of VEGF gene transfer on a rat model of Parkinson disease using adeno-associated viral vectors. Neurosci. Lett. 421 (2007) 239-244.

163. Y.H. Ding, X.D. Luan, J. Li, J.A. Rafols, M. Guthinkonda, F.G. Diaz, Y. Ding, Exercise-induced overexpression of angiogenic factors and reduction of ischemia/reperfusion injury in stroke. Curr. Neurovasc. Res. 1 (2004) 411-420.

164. Y.H. Ding, J. Li, Y. Zhou, J.A. Rafols, J.C. Clark, Y. Ding, Cerebral angiogenesis and expression of angiogenic factors in aging rats after exercise. Curr. Neurovasc. Res. 3 (2006) 15-23.

165. B. Villar-Cheda, D. Sousa-Ribeiro, J. Rodriguez-Pallares, A.I. Rodriguez-Perez, M.J. Guerra, J.L. Labandeira-Garcia, Aging and sedentarism decrease vascularization and VEGF levels in the rat substantia nigra. Implications for Parkinson's disease. J. Cereb. Blood Flow Metab. 29 (2009) 230-234.

166. B.A. Faucheux, A.M. Bonnet, Y. Agid, E.C. Hirsch, Blood vessels change in the mesencephalon of patients with Parkinson's disease. Lancet 353 (1999) 981-982.

167. K. Wada, H. Arai, M. Takanashi, J. Fukae, H. Oizumi, T. Yasuda, Y. Mizuno, H. Mochizuki, Expression levels of vascular endothelial growth factor and its recept ors in Parkinson's disease. Neuroreport 17 (2006) 705-709.

168. I.S. Pienaar, C.H. Lee, J.L. Elson, L. McGuinness, S.M. Gentleman, R.N. Kalaria, D.T.Dexter, Deep-brain stimulation associates with improved microvascular integrity in the subthalamic nucleus in Parkinson's disease. Neurobiol. Dis. 74 (2015) 392-405.

169. W. Cui, W. Li, R. Han, S. Mak, H. Zhang, S. Hu, J. Rong, Y. Han, PI3-K/Akt and ERK pathways activated by VEGF play opposite roles in MPP+-induced neuronal apoptosis. Neurochem. Int. 59 (2011) 945-953.

170. S Janelidze, D Lindqvist, V Francardo, S Hall, H Zetterberg, K Blennow, CH Adler, TG Beach, GE Serrano, D van Westen, E Londos, MA Cenci, O. Hansson, Increased CSF biomarkers of angiogenesis in Parkinson disease. Neurology 85 (2015) 1834-1842.

171. Y. Yao, S.E.Tsirka, Monocyte chemoattractant protein-1 and the blood-brain barrier. Cell. Mol. Life Sci. 71 (2014) 683-697.

172. I. Rite, A. Machado, J. Cano, J.L.Venero, Blood-brain barrier disruption induces in vivo degeneration of nigral dopaminergic neurons. J. Neurochem, 101 (2007) 1567-1582.

173. A. Muñoz, P. Garrido-Gil, A. Dominguez-Meijide, J.L.Labandeira-Garcia, Angiotensin type 1 receptor blockage reduces l-dopa-induced dyskinesia in the 6-OHDA model of Parkinson's disease. Involvement of vascular endothelial growth factor and interleukin-1β. Exp. Neurol. 261 (2014) 720-732.

174. S.S. Newton, E.F. Collier, J. Hunsberger, D. Adams, R. Terwilliger, E. Selvanayagam, R.S. Duman, Gene profile of electroconvulsive seizures: Induction of neurotrophic and angiogenic factors. J. Neurosci 23 (2003)10841-10851.

175. J.N. Nicoletti, S.K. Shah, D.P. McCloskey, J.H. Goodman, A. Elkady, H. Atassi, D. Hylton, J.S. Rudge, H.E. Scharfman, S.D.Croll, Vascular endothelial growth factor is up-regulated after status epilepticus and protects against seizure-induced neuronal loss in hippocampus. Neuroscience 151 (2008) 232-241.

176. L. Nikitidou, I. Kanter-Schlifke, J. Dhondt, P. Carmeliet, D. Lambrechts, M.Kokaia, VEGF receptor-2 (Flk-1) overexpression in mice counteracts focal epileptic seizures. PLoS One. 7 (2012) e40535.

177. D.P. McCloskey, S.D. Croll, H.E. Scharfman, Depression of synaptic transmission by vascular endothelial growth factor in adult rat hippocampus and evidence for increased efficacy after chronic seizures. J Neurosci. 25 (2005) 8889-8897.

178. M. Cammalleri, D. Martini, C. Ristori, A.M. Timperio, P. Bagnoli, Vascular endothelial growth factor up-regulation in the mouse hippocampus and its role in the control of epileptiform activity. Eur. J. Neurosci. 33 (2011) 482-498.

179. A. Vezzani, A. Friedman, R.J. Dingledine, The role of inflammation in epileptogenesis. Neuropharmacology 69 (2013) 16-24.

180. T. Shimada, T. Takemiya, H. Sugiura, K.Yamagata, Role of inflammatory mediators in the pathogenesis of epilepsy. Mediators Inflamm. 2014 (2014) 901902.

181. M. Morin-Brureau, A. Lebrun, M.C. Rousset, L. Fagni, J. Bockaert, F. de Bock, M. Lerner-Natoli, Epileptiform activity induces vascular remodeling and zonula occludens 1 downregulation in organotypic hippocampal cultures: role of VEGF signaling pathways. J Neurosci. 31 (2011) 10677-10688.

182. T. Rhim, D.Y. Lee, M. Lee, Drug delivery systems for the treatment of ischemic stroke. Pharm Res. 30 (2013) 2429-2444.

183. SM Eppler, DL Combs, TD Henry, JJ Lopez, SG Ellis, JH Yi, BH Annex, ER McCluskey, TF Zioncheck,: A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clin. Pharmacol. Ther. 72 (2002) 20-32.

184. M.O. Stefanini, F.T. Wu, F Mac Gabhann, A.S.Popel, A compartment model of VEGF distribution in blood, healthy and diseased tissues. BMC Syst. Biol. 2 (2008) 77.

185. M.O. Stefanini, F.T. Wu, F. Mac Gabhann, A.S. Popel The presence of VEGF receptors on the luminal surface of endothelial cells affects VEGF distribution and VEGF signaling. PLoS Comput. Biol. 5 (2009) e1000622.

186. M.O. Stefanini, F.T. Wu, F. Mac Gabhann, Popel A.S. Increase of plasma VEGF after intravenous administration of bevacizumab is predicted by a pharmacokinetic model. Cancer Res. 70 (2010) 9886-9894.

187. P. Vempati, A.S. Popel, F. Mac Gabhann, Extracellular regulation of VEGF: isoforms, proteolysis, and vascular patterning. Cytokine Growth Factor Rev. 25 (2014) 1-19.

188. B.A. Keyt, L.T. Berleau, H.V. Nguyen, H. Chen, H. Heinsohn, R. Vandlen, N. Ferrara The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. J. Biol. Chem. 271 (1996) 7788-7795.

189. G. Lauer, S. Sollberg, M. Cole, T. Krieg, S. A. Eming, Generation of a novel proteolysis resistant vascular endothelial growth factor165 variant by a site-directed mutation at the plasmin sensitive cleavage site. FEBS Lett. 531 (2002) 309-313.

190. R.G. Thorne, G.J. Pronk, V. Padmanabhan, W.H. 2nd. Frey, Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. Neuroscience 127 (2004) 481-496.

191. J.P. Yang, H.J. Liu, S.M. Cheng, Z.L. Wang, X. Cheng, H.X. Yu, X.F. Liu, Direct transport of VEGF from the nasal cavity to brain. Neurosci. Lett. 449 (2009) 108-111.

192. R.G.Thorne, W.H. II Frey, Delivery of neurotrophic factors to the central nervous system: pharmacokinetic considerations. Clin. Pharmacokinet. 40 (2001) 907-946.

193. F. Gu, B. Amsden, R. Neufeld, Sustained delivery of vascular endothelial growth factor with alginate beads. J. Control. Release 96 (2004) 463-472.

194. K.W. Lee, J.J. Yoon, J.H. Lee, S.Y. Kim, H.J. Jung, S.J. Kim, J.W. Joh, H.H. Lee, D.S. Lee, S.K. Lee, Sustained release of vascular endothelial growth factor from calcium-induced alginate hydrogels reinforced by heparin and chitosan. Transplant. Proc. 36 (2004) 2464-2465.

195. B. Semete, L. Booysen, Y. Lemmer, L. Kalombo, L. Katata, J. Verschoor, H.S.Swai, In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. Nanomedicine 6 (2010) 662-671.

196. J.S. Golub, Y.T. Kim, C.L. Duvall, R.V. Bellamkonda, D. Gupta, A.S. Lin, D. Weiss, W. Robert Taylor, R.E.Guldberg Sustained VEGF delivery via PLGA nanoparticles promotes vascular growth. Am. J. Physiol.Heart Circ. Physiol. 298 (2010) H1959-H1965.

197. F.R. Formiga, B. Pelacho, E. Garbayo, G. Abizanda, J.J. Gavira, T. Simon-Yarza, M. Mazo, E. Tamayo, C. Jauquicoa, C. Ortiz-de-Solorzano, F. Prósper, M.J.Blanco-Prieto, Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model. J. Control. Release. 147 (2010) 30-37.

198. E. Herrán, R. Pérez-González, M. Igartua, J.L. Pedraz, E Carro, R.M. Hernández, VEGF-releasing biodegradable nanospheres administered by craniotomy: a novel therapeutic approach in the APP/Ps1 mouse model of Alzheimer's disease. J, Contro,l Release 170 (2013a) 111-119.

199. E. Herran, R. Perez-Gonzalez, M. Igartua, J.L. Pedraz, E. Carro, R.M. Hernandez, Enhanced Hippocampal Neurogenesis in APP/Ps1 Mouse Model of Alzheimer's Disease After Implantation of VEGF-loaded PLGA Nanospheres. Curr. Alzheimer Res. 12 (2015) 932-940.

200. E. Herrán, J.Á. Ruiz-Ortega, A. Aristieta, M. Igartua, C. Requejo, J.V. Lafuente, L. Ugedo, J.L. Pedraz, R.M. Hernández, In vivo administration of VEGF- and GDNF-releasing biodegradable polymeric microspheres in a severe lesion model of Parkinson's disease. Eur J. Pharm. Biopharm. 85(3 Pt B) (2013b) 1183-1190.

201. E. Herrán, C. Requejo, J.A. Ruiz-Ortega, A. Aristieta, M. Igartua, H. Bengoetxea, L. Ugedo, J.L. Pedraz, J.V. Lafuente, R.M. Hernández, Increased antiparkinson efficacy of the combined administration of VEGF- and GDNF-loaded nanospheres in a partial lesion model of Parkinson's disease. Int. J. Nanomedicine 9 (2014) :2677-2687.

202. R. Ju, Y. Wen, R. Gou, Y. Wang, Q. Xu, The experimental therapy on brain ischemia by improvement of local angiogenesis with tissue engineering in the mouse. Cell Transplant. 23 Suppl 1 (2014) S83-S95.

203. J. Zhu, Y. Jiang, G. Xu, X. Liu, Intranasal administration: a potential solution for cross-BBB delivering neurotrophic factors. Histol. Histopathol. 27 (2012) 537-548.

204. C. Andrade, Intranasal drug delivery in neuropsychiatry: focus on intranasal ketamine for refractory depression. J. Clin. Psychiatry. 76 (2015) 628-631.

205. C.J. Derry, S. Derry, R.A.Moore, Sumatriptan (intranasal route of administration) for acute migraine attacks in adults. Cochrane Database Syst. Rev. 2 (2012) CD009663.

206. E. Dietrich, J.G.Gums, Intranasal fentanyl spray: a novel dosage form for the treatment of breakthrough cancer pain. Ann. Pharmacother. 46 (2012) 1382-1391.

207. S. Grassin-Delyle, A. Buenestado, E. Naline, C. Faisy, S. Blouquit-Laye, L.J. Couderc, M. Le Guen, M. Fischler, P.Devillier, Intranasal drug delivery: an efficient and non-invasive route for systemic administration: focus on opioids. Pharmacol. Ther. 134 (2012) 366-379.

208. D. Kerr, P. Dietze, A.M. Kelly Intranasal naloxone for the treatment of suspected heroin overdose. Addiction 103 (2008) 379-386.

209. R.H.Kronenberg, Ketamine as an analgesic: parenteral, oral, rectal, subcutaneous, transdermal and intranasal administration. J. Pain Palliat. Care Pharmacother. 16 (2002) 27-35.

210. A. Murphy, R. O'Sullivan, A. Wakai, T.S. Grant, M.J. Barrett, J. Cronin, S.C. McCoy, J. Hom, N. Kandamany, Intranasal fentanyl for the management of acute pain in children. Cochrane Database Syst. Rev. 10 (2014) CD009942.

211. A. Pires, A. Fortuna, G. Alves, A.Falcão, Intranasal drug delivery: how, why and what for? J. Pharm. Pharm. Sci. 12 (2009) 288-311.

212. A.M. Rapoport, M.E. Bigal, S.J. Tepper, F.D.Sheftell, Intranasal medications for the treatment of migraine and cluster headache. CNS Drugs. 18 (2004) 671-685.

213. A. Robinson, D.P.Wermeling, Intranasal naloxone administration for treatment of opioid overdose. Am. J. Health Syst. Pharm. 71 (2014) 2129-2135.

214. B, Santangelo, F, Micieli, F, Marino, F, Reynaud, P, Cassandro, A, Carfora, R, Petrella, R, Borriello, M, Cataldi, G, Vesce,. Plasma concentrations and sedative effects of a dexmedetomidine, midazolam, and butorphanol combination after transnasal administration in healthy rabbits. J, Vet, Pharmacol, Ther. 39 (2016) 408-411.

215. T. Sibley, R. Jacobsen, J. Salomone, Successful administration of intranasal glucagon in the out-of-hospital environment. Prehosp. Emerg. Care. 17 (2013) 98-102.

216. D.P.Wermeling, Intranasal delivery of antiepileptic medications for treatment of seizures. Neurotherapeutics 6 (2009) 352-358.

217. T.R. Wolfe, T. Bernstone, Intranasal drug delivery: an alternative to intravenous administration in selected emergency cases. J. Emerg. Nurs. 30 (2004) 141-147.

218. M. Guardia Clausi, P.M. Paez, L.A. Pasquini, J.M.Pasquini, Inhalation of growth factors and apo-transferrin to protect and repair the hypoxic-ischemic brain. Pharmacol. Res. 109 (2016) 81-85.

219. C.L. Graff, G.M.Pollack, Nasal drug administration: potential for targeted central nervous system delivery. J Pharm Sci. 94 (2005) 1187-1195.

220. S.K. Tayebati, I.E. Nwankwo, F. Amenta, Intranasal drug delivery to the central nervous system: present status and future outlook. Curr. Pharm. Des. 19 (2013) 510-526.

221. Spetter MS, Hallschmid M. Intranasal Neuropeptide Administration To Target the Human Brain in Health and Disease. Mol Pharm. 2015 Aug 3;12(8):2767-80.

222. J. Sun, Z.Z. Wei, X. Gu, J.Y. Zhang, Y. Zhang, J. Li, L.Wei, Intranasal delivery of hypoxia-preconditioned bone marrow-derived mesenchymal stem cells enhanced regenerative effects after intracerebral hemorrhagic stroke in mice. Exp. Neurol. 272 (2015) 78-87.

223. Q Zhao, J Hu, J Xiang, Y Gu, P Jin, F Hua, Z Zhang, Y Liu, K Zan, Z Zhang, J Zu, X Yang, H Shi, J Zhu, Y Xu, G Cui, X.Ye, Intranasal administration of human umbilical cord mesenchymal stem cells-conditioned medium enhances vascular remodeling after stroke. Brain Res. 1624 (2015) 489-496.

224. J.P. Yang, H.J. Liu, X.F. Liu, VEGF promotes angiogenesis and functional recovery in stroke rats. J. Invest. Surg. 23 (2010) 149-155.

225. J.P. Yang, H.J. Liu, Z.L. Wang, S.M. Cheng, X. Cheng, G.L. Xu, X.F.Liu, The dose-effectiveness of intranasal VEGF in treatment of experimental stroke. Neurosci. Lett. 461 (2009) 212-216.

226. L. Illum Transport of drugs from the nasal cavity to the central nervous system. Eur. J. Pharm. Sci. 11 (2000) 1-18.

227. M. Kapoor, J.C. Cloyd, R.A. Siegel A review of intranasal formulations for the treatment of seizure emergencies. J. Control. Release 237 (2016) 147-159.

228. S. Matsune, J. Ohori, K. Yoshifuku, Y.Kurono, Effect of vascular endothelial growth factor on nasal vascular permeability. Laryngoscope 120 (2010) 844-848.

229. Henry T, Annex B, Azrin M, et al. Double blind placebo controlled trial of recombinant human vascular endothelial growth factor: the VIVA trial. J Am Coll Cardiol. 1999;33:384A. Abstract.

230. Laham RJ, Sellke FW, Edelman ER, et al. Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial. Circulation. 1999;100:1865–1871.

231. Laitinen M, Hartikainen J, Hiltunen MO, et al. Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. Hum Gene Ther. 2000;11:263–270.

232. Losordo DW, Vale PR, Symes JF, et al. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation. 1998;98:2800–2804.

233. Rosengart TK, Lee LY, Patel SR, et al. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. Circulation. 1999;100:468–474.

234. Schumacher B, Pecher P, von Specht B, et al. Induction of neoangiogenesis in ischemic myocardium by human growth factors. Circulation. 1998;97:645–650.

235. Springer, M.L., Chen, A.S., Kraft, P.E., Bednarski, M. and Blau, H.M. (1998) VEGF gene delivery to muscle: potential role for vasculogenesis in adults. Mol. Cell 2, 549–558

236. Lee, R.J., Springer, M.L., Blanco-Bose, W.E., Shaw, R., Ursell, P.C. and Blau, H.M. (2000) VEGF gene delivery to myocardium: deleterious effects of unregulated expression. Circulation 102, 898–901

237. Pettersson, A., Nagy, J.A., Brown, L.F., Sundberg, C., Morgan, E., Jungles, S., Carter, R., Krieger, J.E., Manseau, E.J., Harvey, V.S. et al. (2000)Heterogeneity of the angiogenic response induced in

different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. Lab. Invest. 80, 99–115

238. Sundberg, C., Nagy, J.A., Brown, L.F., Feng, D., Eckelhoefer, I.A., Manseau, E.J., Dvorak, A.M. and Dvorak, H.F. (2001) Glomeruloid microvascular proliferation follows adenoviral vascular permeability factor/vascular endothelial growth factor-164 gene delivery. Am. J. Pathol. 158, 1145–1160

239. Schwarz, E.R., Speakman, M.T., Patterson, M., Hale, S.S., Isner, J.M., Kedes, L.H. and Kloner, R.A. (2000) Evaluation of the effects of intramyocardial injection of DNA expressing vascular endothelial growth factor (VEGF) in a myocardial infarction model in the rat: angiogenesis and angioma formation. J. Am. Coll. Cardiol. 35, 1323–1330

240. B. Chen, B. Friedman, Q. Cheng, P. Tsai, E. Schim, D. Kleinfeld, P.D. Lyden, Severe blood-brain barrier disruption and surrounding tissue injury. Stroke 40 (2009) e666-e674.

241. J. Keaney, M. Campbell, The dynamic blood-brain barrier. FEBS J. 282 (2015) 4067-4079.

242. A. Montagne, D.A. Nation, J. Pa, M.D. Sweeney, A.W. Toga, B.V. Zlokovic, Brain imaging of neurovascular dysfunction in Alzheimer's disease. Acta Neuropathol. 131 (2016) 687-707.

243. B.O. Popescu, E.C. Toescu, L.M. Popescu, O. Bajenaru, D.F. Muresanu, M. Schultzberg, N. Bogdanovic, Blood-brain barrier alterations in ageing and dementia. J. Neurol. Sci. 283 (2009) 99-106.

244. R. Prakash, S.T. Carmichael, Blood-brain barrier breakdown and neovascularization processes after stroke and traumatic brain injury. Curr. Opin. Neurol. 28 (2015) 556-564.

245. H.B. Stolp, K.M. Dziegielewska, Review: Role of developmental inflammation and blood-brain barrier dysfunction in neurodevelopmental and neurodegenerative diseases. Neuropathol. Appl. Neurobiol. 35 (2009) 132-146.

246. M. Ueno, Y. Chiba, K. Matsumoto, R .Murakami, R. Fujihara, M. Kawauchi, H. Miyanaka, T. Nakagawa, Blood-brain barrier damage in vascular dementia. Neuropathology 36 (2016) 115-124.

247. B.V. Zlokovic, Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. Nat. Rev. Neurosci.12 (2011) 723-738.

248. B.V. Zlokovic, The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 57 (2008) 178-201.

249. D.R. Senger, S.J. Galli, A.M. Dvorak, C.A. Perruzzi, V.S. Harvey, H.F. Dvorak Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219 (1983) 983-985.

250. A. Horowitz, H.R. Seerapu, Regulation of VEGF signaling by membrane traffic. Cell Signal. 24 (2012) 1810-1820.

251. M. Nakayama, P. Berger, Coordination of VEGF receptor trafficking and signaling by coreceptors. Exp Cell Res. 319 (2013) 1340-1347.

252. A.T. Argaw, B.T. Gurfein, Y. Zhang, A. Zameer, G.R. John, VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 1977-1982.

253. S. Fischer, M. Wobben, H.H. Marti, D. Renz, W. Schaper, Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. Microvasc. Res. 63 (2002) 70-80.

254. R. Ghassemifar, C.M. Lai, P.E. Rakoczy, VEGF differentially regulates transcription and translation of ZO-1alpha+ and ZO-1alpha- and mediates trans-epithelial resistance in cultured endothelial and epithelial cells. Cell Tissue Res. 323 (2006) 117-125.

255. W. Wang, W.L. Dentler, R.T. Borchardt, VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly. Am. J. Physiol. Heart Circ. Physiol. 280 (2001) H434-H440.

256. A.T. Argaw, L. Asp, J. Zhang, K. Navrazhina, T. Pham, J.N. Mariani, S. Mahase, D.J. Dutta, J. Seto, E.G. Kramer, N. Ferrara, M.V. Sofroniew, G.R.John, Astrocyte-derived VEGF-A drives blood-brain barrier disruption in CNS inflammatory disease. J. Clin. Invest. 122 (2012) 2454-2468.

257. Y. Suzuki, N. Nagai, K. Yamakawa, Y. Muranaka, K. Hokamura, K. Umemura, . Recombinant tissue-type plasminogen activator transiently enhances blood-brain barrier permeability during cerebral ischemia through vascular endothelial growth factor-mediated endothelial endocytosis in mice. J. Cereb. Blood Flow Metab. 35 (2015) 2021-2031.

258. Y. Suzuki, N. Nagai, K.Umemura, A Review of the Mechanisms of Blood-Brain Barrier Permeability by Tissue-Type Plasminogen Activator Treatment for Cerebral Ischemia. Front. Cell. Neurosci. 10 (2016) 2.

259. M. Kanazawa, H. Igarashi, K. Kawamura, T. Takahashi, A. Kakita, H. Takahashi, Inhibition of VEGF signaling pathway attenuates hemorrhage after tPA treatment. J. Cereb. Blood Flow Metab. 31 (2011), 1461-1474.

260. P. Reeson, K.A. Tennant, K. Gerrow, J. Wang, S. Weiser Novak, K. Thompson, K.L. Lockhart, A. Holmes, P.C. Nahirney, C.E. Brown, Delayed inhibition of VEGF signaling after stroke attenuates blood-brain barrier breakdown and improves functional recovery in a comorbidity-dependent manner. J. Neurosci. 35 (2015) 5128-5143.

261. I.L. Lee, P.S. Li, W.L. Yu, H.H. Shen, Prokaryotic expression, refolding, and purification of functional human vascular endothelial growth factor isoform 165: purification procedures and refolding conditions revisited. Protein Expr. Purif. 76 (2011) 54-58.

262. T. Cohen, H. Gitay-Goren, G. Neufeld, B.Z. Levi, High levels of biologically active vascular endothelial growth factor (VEGF) are produced by the baculovirus expression system. Growth Factors. 7 (1992) 131-138.

263. S.A. Pizarro, J. Gunson, M.J. Field, R. Dinges, S. Khoo, M. Dalal, M. Lee, K.A. Kaleas, K. Moiseff, S. Garnick, D.E. Reilly, M.W. Laird, C.H. Schmelzer, High-yield expression of human vascular endothelial growth factor VEGF(165) in Escherichia coli and purification for therapeutic applications. Protein Expr. Purif. 72 (2010) 184-193.

264. P. Scheidegger, W. Weiglhofer, S. Suarez, B. Kaser-Hotz, R. Steiner, K. Ballmer-Hofer, R. Jaussi, Vascular endothelial growth factor (VEGF) and its receptors in tumor-bearing dogs. Biol. Chem. 380 (1999) 1449-1454.

265. K. Mandal, S.B. Kent, Total chemical synthesis of biologically active vascular endothelial growth factor. Angew. Chem. Int. Ed. Engl. 50 (2011) 8029-8033.

266. D.G. Bae, T.D. Kim, G. Li, W.H. Yoon, C.B.Chae, Anti-flt1 peptide, a vascular endothelial growth factor receptor 1-specific hexapeptide, inhibits tumor growth and metastasis. Clin. Cancer Res. 11 (2005) 2651-2661.

267. A. Basile, A. Del Gatto, D. Diana, R. Di Stasi, A. Falco, M. Festa, A. Rosati, A. Barbieri, R. Franco, C. Arra, C. Pedone, R. Fattorusso, M.C. Turco, L.D. D'Andrea, Characterization of a designed vascular endothelial growth factor receptor antagonist helical peptide with antiangiogenic activity in vivo. J. Med. Chem. 54 (2011) 1391-1400.

268. K.C. Foy, Z. Liu, G. Phillips, M. Miller, P.T.Kaumaya, Combination treatment with HER-2 and VEGF peptide mimics induces potent anti-tumor and anti-angiogenic responses in vitro and in vivo. J. Biol. Chem. 286 (2011) 13626-13637.

269. L. Rastelli, M.L. Valentino, M.C. Minderman, J. Landin, U.M. Malyankar, M.K. Lescoe, R. Kitson, K. Brunson, L. Souan, S. Forenza, R.H. Goldfarb, S.A.Rabbani, A KDR-binding peptide (ST100,059) can block angiogenesis, melanoma tumor growth and metastasis in vitro and in vivo. Int. J. Oncol. 39 (2011) 401-408.

270. D. Vicari, K.C. Foy, E.M. Liotta, P.T. Kaumaya, Engineered conformation-dependent VEGF peptide mimics are effective in inhibiting VEGF signaling pathways. J. Biol. Chem. 286 (2011) 13612-13625.

271. L.D. D'Andrea, A. Del Gatto, L. De Rosa, A. Romanelli, C. Pedone, Peptides targeting angiogenesis related growth factor receptors. Curr. Pharm. Des. 15 (2009) 2414-2429.

272. L.D. D'Andrea, G. Iaccarino, R. Fattorusso, D. Sorriento, C. Carannante, D. Capasso, B. Trimarco, C. Pedone, Targeting angiogenesis: structural characterization and biological properties of a de novo engineered VEGF mimicking peptide. Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 14215-14220.

273. C. Wiesmann, G. Fuh, H.W. Christinger, C. Eigenbrot, J.A. Wells, A.M. de Vos, Crystal structure at 1.7 Å resolution of VEGF in complex with domain 2 of the Flt-1 receptor. Cell 91 (1997) 695-704.

274. D. Diana, B. Ziaco, G. Colombo, G. Scarabelli, A. Romanelli, C. Pedone, R. Fattorusso, L.D. D'Andrea, Structural determinants of the unusual helix stability of a de novo engineered vascular endothelial growth factor (VEGF) mimicking peptide. Chemistry 14 (2008) 4164-4166.

275. D. Diana, B. Ziaco, G. Scarabelli, C. Pedone, G. Colombo, L.D. D'Andrea, Fattorusso R., Structural analysis of a helical peptide unfolding pathway. Chemistry 16 (2010) 5400-5407.

276. B. Ziaco, D. Diana, D. Capasso, R. Palumbo, V. Celentano, R. Di Stasi, R. Fattorusso, L.D. D'Andrea, C-terminal truncation of Vascular Endothelial Growth Factor mimetic helical peptide preserves structural and receptor binding properties. Biochem. Biophys. Res. Commun. 424 (2012) 290-294.

277. F. Finetti, A. Basile, D. Capasso, S. Di Gaetano, R. Di Stasi, M. Pascale, C.M. Turco, M. Ziche, L. Morbidelli, L.D. D'Andrea, Functional and pharmacological characterization of a VEGF mimetic peptide on reparative angiogenesis. Biochem. Pharmacol. 84 (2012) 303-11.

278. G. Santulli, M. Ciccarelli, G. Palumbo, A. Campanile, G. Galasso, B. Ziaco, G.G. Altobelli, V. Cimini, F. Piscione, L.D. D'Andrea, C. Pedone, B. Trimarco, G. Iaccarino, In vivo properties of the proangiogenic peptide QK. J. Transl. Med. 7 (2009) 41.

279. G.K. Dudar, L.D. D'Andrea, R. Di Stasi, C. Pedone, J.L. Wallace, A vascular endothelial growth factor mimetic accelerates gastric ulcer healing in an iNOS-dependent manner. Am. J. Physiol. Gastrointest. Liver Physiol. 295 (2008) G374- G381.

280. A. Verheyen, E. Peeraer, D. Lambrechts, K. Poesen, P. Carmeliet, M. Shibuya, I. Pintelon, J.P. Timmermans, R. Nuydens, T. Meert, Therapeutic potential of VEGF and VEGF-derived peptide in peripheral neuropathies. Neuroscience 244 (2013) 77-89.

281. T.R. Chan, P.J. Stahl, S.M. Yu, Matrix-Bound VEGF Mimetic Peptides: Design and Endothelial Cell Activation in Collagen Scaffolds. Adv. Funct. Mater. 21 (2011) 4252-4262.

282. V.A. Kumar, N.L. Taylor, S. Shi, B.K. Wang, A.A. Jalan, M.K. Kang, N.C. Wickremasinghe, J.D. Hartgerink, Highly angiogenic peptide nanofibers. ACS Nano 9 (2015) 860-868.

283. J.S. Lee, A.J. Wagoner Johnson, W.L. Murphy, A modular, hydroxyapatite-binding version of vascular endothelial growth factor. Adv. Mater. 22 (2010) 5494-5498.

284. J.E. Leslie-Barbick, J.E. Saik, D.J. Gould, M.E. Dickinson, J.L.West, The promotion of microvasculature formation in poly(ethylene glycol) diacrylate hydrogels by an immobilized VEGF-mimetic peptide. Biomaterials 32 (2011) 5782-5789.

285. X. Liu, X. Wang, A. Horii, X. Wang, L. Qiao, S. Zhang, F.Z. Cui, In vivo studies on angiogenic activity of two designer self-assembling peptide scaffold hydrogels in the chicken embryo chorioallantoic membrane. Nanoscale 4 (2012) 2720-2727.

286. W. Mulyasasmita, L. Cai, Y. Hori, S.C. Heilshorn, Avidity-controlled delivery of angiogenic peptides from injectable molecular-recognition hydrogels. Tissue Eng. Part A. 20 (2014) 2102-2114.

287. P.J. Stahl, T.R. Chan, Y.I. Shen, G. Sun, S. Gerecht, S.M. Yu, Capillary Network-Like Organization of Endothelial Cells in PEGDA Scaffolds Encoded with Angiogenic Signals via Triple Helical Hybridization. Adv. Funct. Mater. 24 (2014) 3213-3225.

288. D. Suárez-González, K. Barnhart, F. Migneco, C. Flanagan, S.J. Hollister, W.L. Murphy, Controllable mineral coatings on PCL scaffolds as carriers for growth factor release. Biomaterials 33 (2012) 713-721.

289. X. Wang, A. Horii, S. Zhang, Designer functionalized self-assembling peptide nanofiber scaffolds for growth, migration, and tubulogenesis of human umbilical vein endothelial cells. Soft Matter 4 (2008), 2388-2395.

290. M.J. Webber, J. Tongers, C.J. Newcomb, K.T. Marquardt, J. Bauersachs, D.W. Losordo, S.I. Stupp, Supramolecular nanostructures that mimic VEGF as a strategy for ischemic tissue repair. Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 13438-13443.

291. G. Pignataro, B. Ziaco, A. Tortiglione, R. Gala, O. Cuomo, A. Vinciguerra, D. Lapi, T. Mastantuono, S. Anzilotti, L.D. D'Andrea, C. Pedone, G. di Renzo, L. Annunziato, M. Cataldi, Neuroprotective Effect of VEGF-Mimetic Peptide QK in Experimental Brain Ischemia Induced in Rat by Middle Cerebral Artery Occlusion. ACS Chem. Neurosci. 6 (2015) 1517-1525.

292. Y. Zhang, M. Furumura, E. Morita, Distinct signaling pathways confer different vascular responses to VEGF 121 and VEGF 165. Growth Factors. 26 (2008) 125-131.

293. D. Diana, A. Basile, L. De Rosa, R. Di Stasi, S. Auriemma, C. Arra, C. Pedone, M.C. Turco, R. Fattorusso, L.D. D'Andrea, β-hairpin peptide that targets vascular endothelial growth factor (VEGF) receptors: design, NMR characterization, and biological activity. J. Biol. Chem. 286 (2011) 41680-41691.

294. D. Diana, L. De Rosa, M. Palmieri, A. Russomanno, L. Russo, C. La Rosa, D. Milardi, G. Colombo, L.D. D'Andrea, R.Fattorusso, Long range Trp-Trp interaction initiates the folding pathway of a pro-angiogenic β-hairpin peptide. Sci. Rep. 5 (2015) 16651.

295. R. Di Stasi, D. Diana, D. Capasso, R. Palumbo, A. Romanelli, C. Pedone, R. Fattorusso, L.D. D'Andrea, VEGFR1(D2) in drug discovery: Expression and molecular characterization. Biopolymers. 2010;94(6):800-809.

296. D. Diana, A. Russomanno, L. De Rosa, R. Di Stasi, D. Capasso, S. Di Gaetano, A. Romanelli, L. Russo, L.D. D'Andrea, R. Fattorusso, Functional binding surface of a β-hairpin VEGF receptor targeting peptide determined by NMR spectroscopy in living cells. Chemistry 21 (2015) 91-95.

297. L. De Rosa, F. Finetti, D. Diana, R. Di Stasi, S. Auriemma, A. Romanelli, R. Fattorusso, M. Ziche, L. Morbidelli, L.D. D'Andrea, Miniaturizing VEGF: Peptides mimicking the discontinuous VEGF receptor-binding site modulate the angiogenic response. Sci. Rep. 6 (2016) 31295.

8. FUNDING

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9. CONFLICT OF INTEREST

The authors do not have any conflict of interest to disclose.

10. LEGENDS TO FIGURES

Figure 1. Schematic representation of VEGF-A isoform generates by alternative splicing mechanism. Exons number are indicated. VEGF-Ax is generated by programmed readthrough translation. 8a and 8b sequence are six amino acids long. 8 sequence is 22 amino acids long.

Figure 2. Ribbon structure of the VEGF dimer. Note that the two monomers assemble in a antiparallel manner and that the cystine knots are located at opposite poles of the dimer. Note also that regions of the molecule critically relevant for the interaction with VEGFR, namely the β-hairpin between β5 and β6 strands of one VEGF monomer and the α1 helix and the loop joining strand β 3 and β 4 of the other are very close at the poles of the dimer **(**Reproduced from Muller et al. [15] with permission).

Figure 3. Structure of the VEGF mimic peptide QK. A) NMR structure of the QK peptide. Note the α-helix conformation and the two caps at the N- and C- terminals of the peptide. B) Model reconstruction of QK binding to VEGF receptors (Flt-1D2) **(**Reproduced from D'Andrea et al., [242], no permission needed).

Figure 4. The QK peptide promotes a regenerative response in hindlimb ischemia. Panel a shows representative sections of the tibialis anterior muscle obtained from animals treated with the inactive peptide $VEGF_{15}$, with VEGF or with QK that were continuously infused in the femoral artery for for 14 days all at the concentration of 10^{-7} Mol/L (Magnification ×40; bar = 10 μm). The sections were stained with lectin GBS-I to visualize the capillaries. Panel b shows the quantification of the data as mean±SEM. Note that muscle capillary density is significantly higher in rats treated with either VEGF or QK than in animals that received $VEGF_{15}$ (Reproduced from Santulli et al. 2009 [248], no permission needed).

Figure 5. The QK peptide freely diffuses in brain parenchyma after intracarotid injection. The images were obtained by acquiring sequential images by pial vessel intravital videomicroscopy through a window opened in the skull of a rat before (A) and after (B,C and D) the intracarotid injection of fluorescent QK. Note that after injection fluorescence quickly moves from the arteriola (B) to the venular compartment (C) to completely translocate, thereafter, into the brain parenchyma (Reproduced from Pignataro et al., [261], no permission needed).

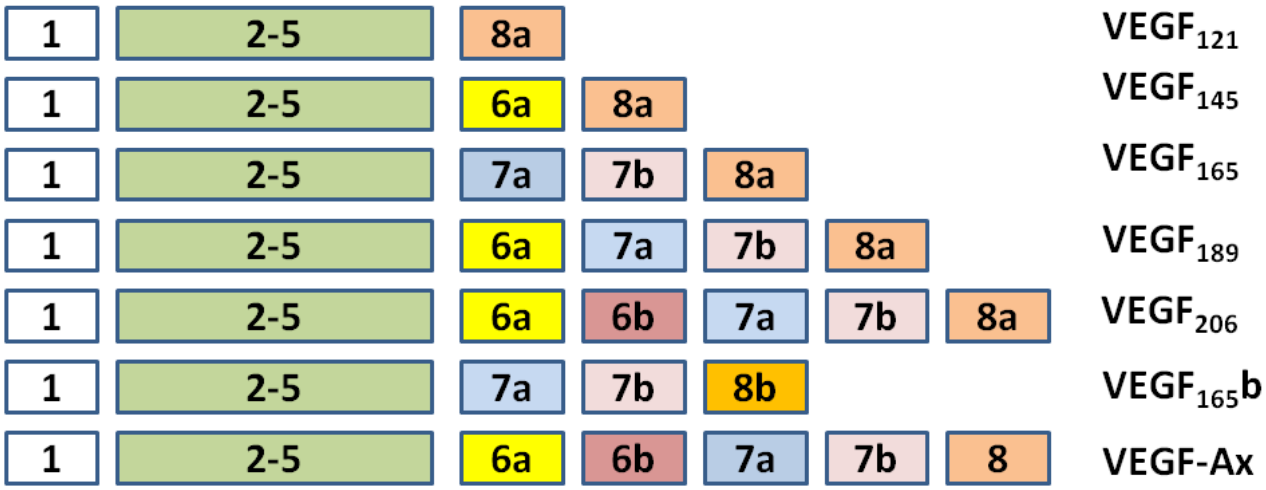
Figure 6. The QK peptide does not induce BBB permeabilization. The tightness of BBB was evaluated by measuring the leakage of 70 kDa fluorescent dextran, a high molecular weight fluorescent probe that is retained in the capillary lumen unless the endothelium is permeabilized. Panel A shows representative images obtained before and after the intracarotid injection of QK (top row) or VEGF (bottom row). Note that the parenchyma become much brighter after VEGF than after QK injection. The bar graphs in panel B show the mean values of the fluorescence of brain parenchyma after the administration of different doses of QK (on the left) or VEGF (on the right). Note that fluorescence becomes significantly higher after VEGF than after QK injection. (Reproduced from Pignataro et al., [261], no permission needed).

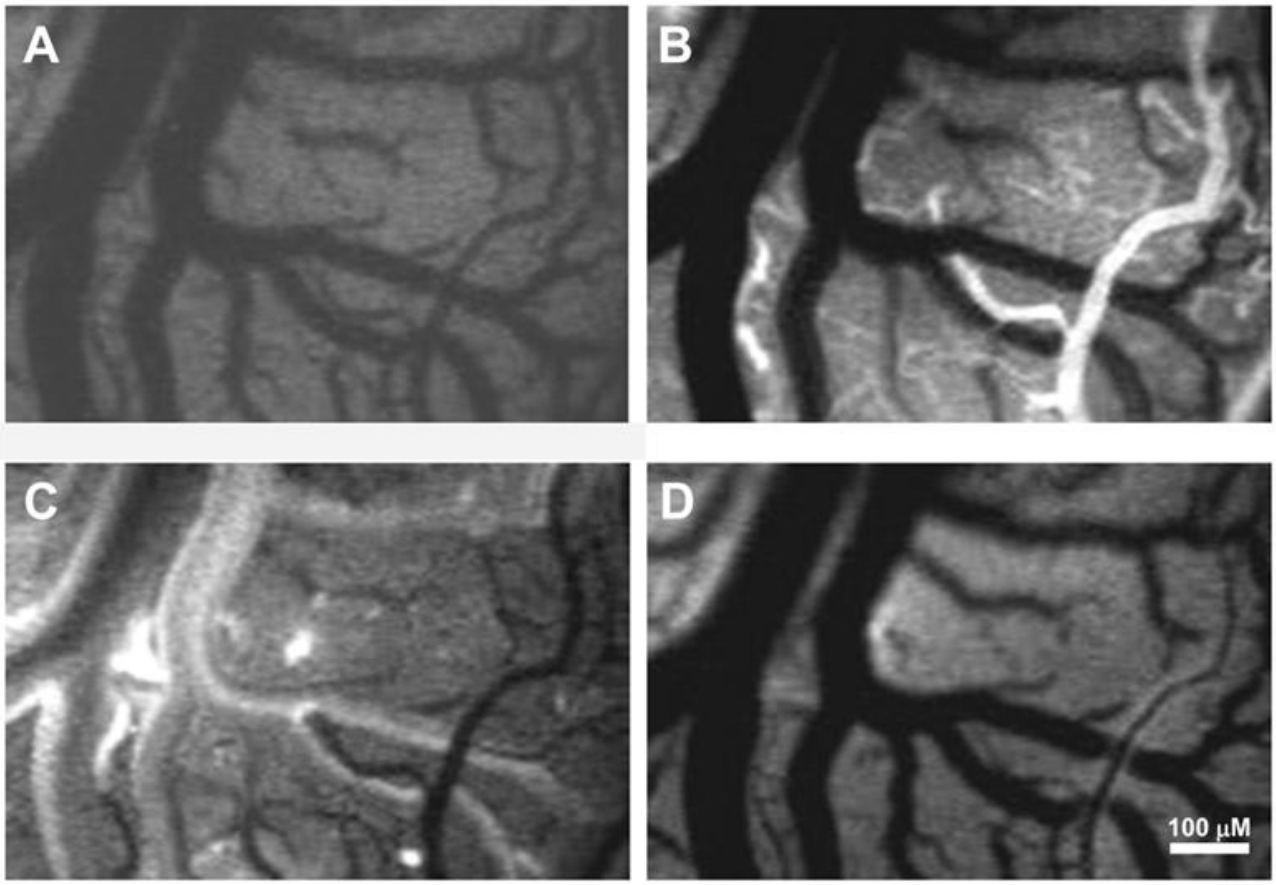
Table 1: List of mammalian proteins belonging to the VEGF family

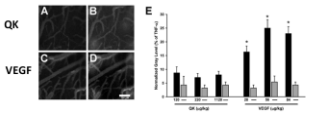
LIGAND	TARGET	ISOFORMS
VEGF-A	VEGFR1 VEGFR2 NRP1 NRP2	$VEGF_{121}$ $VEGF_{145}$ $VEGF_{165}$ $VEGF_{183}$ $VEGF_{189}$ $VEGF_{206}$ VEGF-Ax $VEGF_{165}b$
VEGF-B	VEGFR1 NRP1	$VEGF\text{-}B_{167}$ $VEGF\text{-}B_{186}$
VEGF-C	VEGFR2[a] VEGFR3 NRP2	
VEGF-D	VEGFR2[a] VEGFR3	

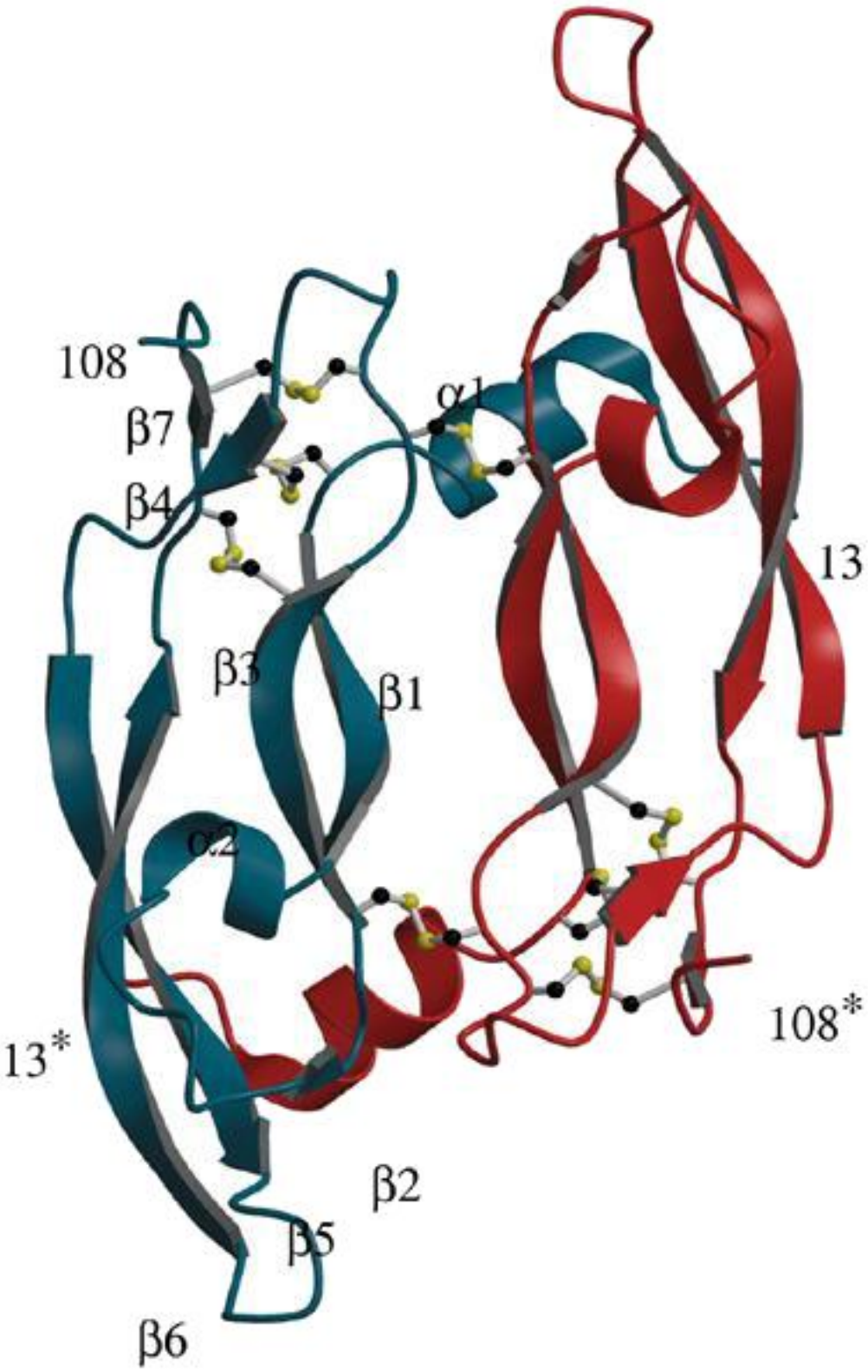
PlGF	VEGFR1	PlGF-1
		PlGF-2
	NRP1	PlGF-3
	NRP2	PlGF-4

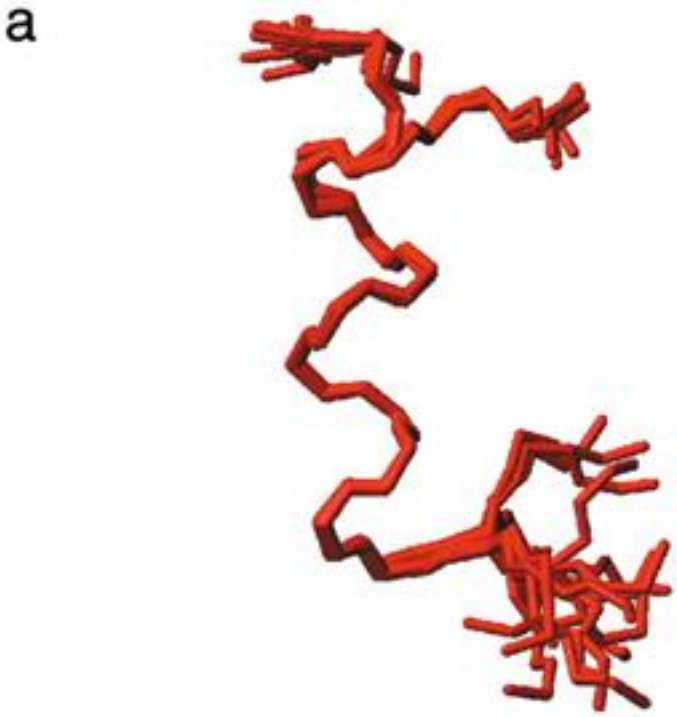
[a]only a proteolitically processed form of the ligand binds to the receptor

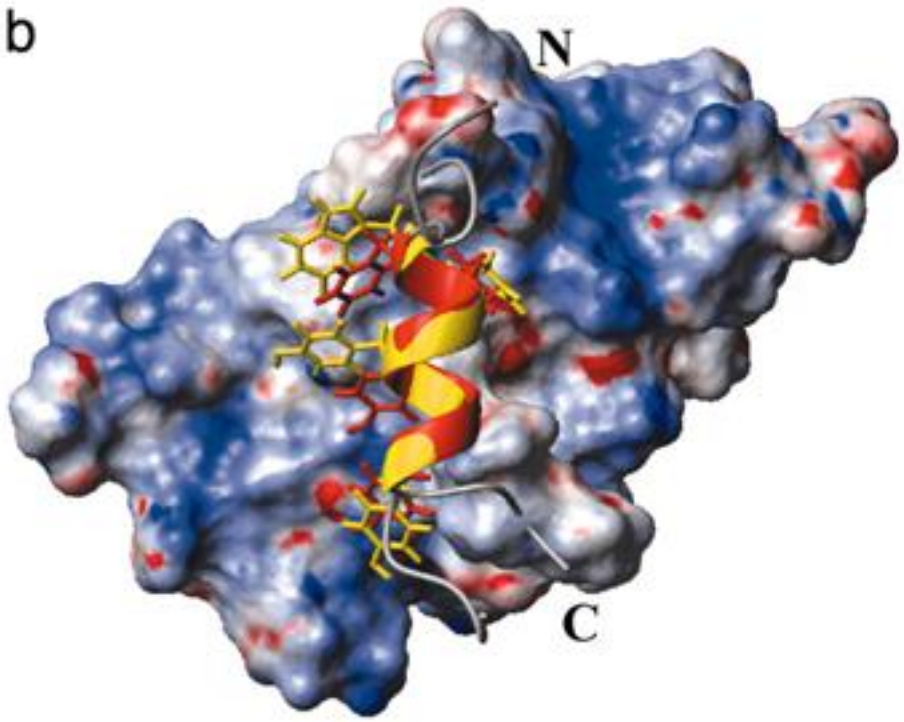


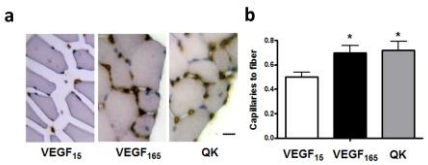





108
β7
β4
α1
13
β3
β1
α2
13*
108*
β2
β5
β6


a


b
N
C


a
b
Capillaries to fiber
VEGF15
VEGF165
QK
VEGF15
VEGF165
QK