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VEGF-A/VEGFRs system in neuropathies: a crossroad between pain and neuroprotection

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Abstract

Vascular Endothelial Growth Factor (VEGF) is a family of signal proteins produced by different cells, that stimulates the formation of blood vessels. VEGF-A is the most studied member of its family. In addition to its well-known pro-angiogenic properties, it also directly influences neuronal and glial biological processes, exerting trophic and signaling functions in nervous tissue.

In recent years, the involvement of the VEGF family in pain signaling is emerging, highlighting the opportunity of a new possible pharmacological target and making urgent the knowledge of its role in the pathophysiological mechanisms of algic sensitivity.

For these reasons, the purpose of this thesis was to investigate the involvement of VEGF-A and its receptors VEGFR-1 and VEGFR-2 in pain perception, and the possible role of this growth factor in neuroprotection.

In naïve mice, intrathecal infusion of VEGF165b (3- 10 and 30 ng/5 μL- a most representative member of VEGF-A) induced dose-dependent noxious hypersensitivity, assessed by pain threshold measurement, mediated by its VEGFR-1. The involvement of VEGFR-1 was confirmed by both using selective ligands (PlGF and VEGF-E that bind VEGFR-1 and VEGFR-2 respectively) and receptor blockers (mAb D16F7 specific for VEGFR-1 and DC101 for VEGFR-2), and from the silencing of the two VEGFRs by siRNAs in the lumbar spinal cord. In addition, to deepen the molecular mechanism underlying the painful action of VEGF-A, the immunofluorescence analysis showed that VEGFR-1 is more expressed on neuronal rather than astrocytic cells. Consequentially, in the electrophysiological study, VEGF165b stimulated the activity of spinal nociceptive neurons via VEGFR-1.

Furthermore, in the dorsal horn of the spinal cord, immunofluorescence analysis revealed that VEGF-A increased in astrocytes from animals with oxaliplatin-induced neuropathy, compared to microglia and neurons, suggesting that this cell population is the source of the effective pain mediator. In addition, confocal microscopy confirmed the expression of VEGF-A in astrocytes, separately from its vascular component.

To investigate the relevance of this result, we selectively silenced astrocytic VEGF-A via shRNAmir in spinal cord of neuropathic animals, resulting in a block of the development of chemotherapy-induced neuropathic pain. In addition, anti-VEGFR-1 mAb D16F7 effectively relieved neuropathic pain induced by various chemotherapeutic agents.

Following these data, to further investigate the mechanisms of pain modulation and to study the neuroprotective component of VEGF-A in nervous tissue, we used the organotypic spinal cord slice. After fourteen days of cultivation, the slices were analyzed by immunofluorescence analysis with GFAP and NeuN markers to confirm the maintenance of cell morphology and structural organization of the spinal cord; in addition, using RECA-1 as endothelial marker, we highlighted a significant reduction of the normal vascular network.

At this point, we focused our attention on three "key" factors that play important role in the development and maintenance of pain: Calcitonin gene-related peptide (CGRP), widely distributed in peripheral and central nervous system and its receptors are expressed in pain pathways; Substance P, involved in the onset and modulation of different types of pain; Glutamate, that showed a pivotal role in pain sensation and transmission.

Treatment with both oxaliplatin (10 μM) and VEGF65b (100 ng/mL) enhanced the release of CGRP and Substance P in the culture medium of the slices compared to control; co-treatment with D16F7 (300 ng/mL), but not with DC101 (10 ng/mL), prevented the release of both two pro-algic factors. Measuring the mRNA of EAAT1 and EAAT2, the reduction in gene expression of the two glutamate transporters caused by VEGF165b, as well as by oxaliplatin, was improved by VEGFR-1 blocker.

From the toxicity studies, we observed that oxaliplatin causes a dose-dependent neurotoxicity and alteration of neurons morphology expressed as a reduction in fluorescence intensity and in the number of $NeuN^+$ cells, after 24 hours incubation. Moreover, activation of astrocytes (evaluated by immunofluorescence staining) was observed.

The co-treatment with VEGF165b showed neuroprotection of the nervous tissue assessed by PI fluorescence, reduction of astrogliosis and neuronal alterations

caused by oxaliplatin. To investigate the molecular mechanism underlying this neuroprotective effect, we analyzed the role of VEGFR-1 and VEGFR-2 by using PlGF and VEGF-E as their specific ligand and also D16F7 and DC101 as receptor blockers. Quantitative analysis of PI fluorescence showed that VEGFR-2 is involved in VEGF-A-mediated neuroprotection.

Ultimately, since the existence of astrocytic VEGF-A is reported in the literature, as confirmed by our *in vivo* results, we treated the slices with fluorocitrate, a glial metabolism blocker, to evaluate its effect in physiological and pathological conditions. Our results showed that fluoricitrate (80 μM) was able to reduce both VEGF-A baseline release and that induced by oxaliplatin treatment. Moreover, the astrocytic inhibition caused an increase in PI fluorescence at all times considered, as expected, worsening the toxicity due to oxaliplatin after three hours of treatment. The addition of exogenous VEGF165b reduces the toxicity caused by oxaliplatin and fluorocitrate after 24 hours of treatment.

In conclusion, this thesis highlighted that VEGF-A released by astrocytes is a new actor in the complex neuron-glia network that oversees physiological and pathological pain, and mAb D16F7 exerts a potent painkiller action in different models of chemotherapy-induced neuropathic pain. Furthermore, the use of organotypic slices of the spinal cord has allowed to deepen the dichotomy between the proalgic and neuroprotective action of VEGF-A, highlighting that VEGFR-1 could be a promising therapeutic target in the modulation of chemotherapy-induced neuropathic pain, without blocking the protective component of the growth factor.

1. Introduction

1.1. Growth Factors

A growth factor is a natural and soluble substance that can affects growth and division cell by specifically binding to certain cell receptors and transmitting signals within the cell[1]. Growth factors such as "Platelet Derived Growth Factor" (PDGF), "Vascular Endothelial Growth Factor" (VEGF), "Transforming Growth Factor" (TGF-β), "Insulin Like Growth Factor" (IGF) and others, play a central role in:

a) cell survival, through the inhibition of the programmed cell death process called apoptosis;

b) the induction of proliferation or, conversely, the arrest of the cell cycle;

c) the reorganization of the cytoskeleton with consequent morphology change;

d) modulation of adhesion between cell and cell and between cell and extracellular matrix;

e) control of cell migration;

f) regulation of gene expression with consequent definition of the differentiated phenotype[2].

The growth factors, secreted in the extracellular medium, can act on the same cell that produced them (autocrine mechanism), on neighboring cells (paracrine mechanism) or at a distance[3]. Once they reach the target cell, the growth factors bind to one or more types of receptors exposed on the cell surface, with an affinity that can vary up to three orders of magnitude (generally low-affinity receptors bind to growth factors with a nanomolar binding constant and those with high affinity with a picomolar bond constant)[4].

Following the formation of the growth factor-receptor complex, second messengers are generated within the target cell, which in turn control a series of biochemical pathways, regulating the activity of enzymes and transcription factors[5,6]. The specificity of the cellular response to a given growth factor depends on the appropriate combination of the signals activated by the second messengers[6]. While

the same cell responds differently to different growth factors, the same growth factor induces different responses in distinct cell types. Finally, there are cases where different growth factors induce the same response in the same cell[7]. Considering the importance of actions controlled by growth factors, it is not surprising that various mechanisms have developed during evolution that regulate all levels of their production and action[8]. First, their synthesis and maturation are modulated (many growth factors are synthesized in the form of an inactive precursor, which must be processed to be functional). Its secretion is often regulated, and any covalent modifications affect the diffusion and the average life of the growth factors put into circulation[2]. In addition, receptor binding can be regulated by the function of 'carrier' proteins, which transport growth factors within the cell, or by antagonist ligands that compete with them for the binding site on the receptor[9].

1.1.1. The growth factors in neoplastic formation

Considering the importance of cellular responses controlled by growth factors, it is not surprising that their lack of regulation is the cause of serious diseases, first of all cancer[10]. Indeed, uncontrolled proliferation, the ability to survive signals of programmed death, motility and the ability to invade surrounding tissues, typical characteristics of metastatic cancer cells, are all processes under the control of growth factors[11]. Since the mid-1980s, a growing number of scientific publications have shown that growth factors, or their receptors, are improperly expressed in the majority of neoplastic cells, or the proteins that mediate the signal transduction of growth factors are deregulated inside the cell[12]. In fact, cancer cells, from which it is often easy to obtain in culture a homogeneous population capable of unlimited proliferation, have often represented the instrument of choice for the biochemical study of the mechanism of action of growth factors[13]. On the other hand, the role played by particular growth factors in tumor control or progression is not always easy to understand.

1.1.2. Nerve Growth Factors

Neurotrophins are a small family of factors that act primarily, but not exclusively, on nerve cells. They are important regulators of neural survival, development, function, and plasticity[14]. There are four neurotrophins characterized in mammals: NGF (Nerve Growth Factor), BDNF (Brain-Derived Neurotrophic Factor), NT-3 (Neurotrophin-3), and NT-4 (Neurotrophin-4) are derived from a common ancestral gene, are similar in sequence and structure, and are therefore collectively named neurotrophins[15].

NGF was the first growth factor described; was identified in 1951 by Rita Levi Montalcini and Victor Hamburger as a substance, released from a tumor (a mouse sarcoma), capable of stimulating the growth of nerve cells of the sympathetic and sensory system of the chicken embryo[16;17(p4)]. During the development of the nervous system, neurotrophins have three main functions. Primarily they are trophic factors, which allow the survival of target cells[18]. Immature nerve cells, neurotrophin-dependent, compete with each other for these factors, which are produced in limited quantities by target organs[19]. As a result, supernumerary neurons, which fail to bind enough neurotrophins, undergo programmed cell death. In this way the neurotrophins participate in the formation of nerve circuits[20]. A second function of neurotrophins is the trophic action, which determines the growth of nerve fibers in the direction of greater concentration of the growth factor itself. Finally, neurotrophins exert a differentiation function on some immature cells by modulating their repertoire of expressed genes[19].

The activity of neurotrophins is not limited to the immature nervous system; in the adult organism, neurotrophins regulate the function of already formed synaptic circuits, modulating the response to neurotransmitters[21]. From this point of view, the most studied neurotrophin is BDNF, which regulates the process known as 'long term potentiation' (LTP)[22]. Long-term potentiation is the phenomenon that allows a synapse that is repeatedly stimulated over a short period of time to remain, for a certain period, more sensitive to a subsequent stimulation[23]. This mechanism appears to contribute to memory formation. Furthermore, neurotrophins act on non-neuronal cells: for example, they modulate the motility of Schwann cells, glial

cells that form the myelin sheath in the peripheral nervous system[24]. Neurotrophins have also been shown to have an antiapoptotic effect on immune system cells, such as mast cells and lymphocytes B, and are supposed to regulate physiological functions that require a coordinated response between the nervous and immune systems[25].

Neurotrophins, which in the active form are small proteins (about a hundred amino acids with a molecular weight of just over 10,000 Da) are synthesized as inactive precursors, pro-neurotrophins, processed through a proteolytic cut by specific proteases present in the secretion vesicles[26]. This process occurs with less than 100% efficiency and as a consequence both pro-NGF and pro-BDNF can be released into the extracellular space. The maturation of pro-BDNF by extracellular proteases such as plasmin, is a mechanism that modulates the induction of LTP[27]. The pro-NGF exercises its function regardless of the mature form of the NGF; in particular, pro-NGF induces cell death in neuronal subpopulations that express the p75 receptor, a phenomenon that seems relevant in Alzheimer's[28].

There are two types of neurotrophin receptors: p75, which belongs to the family of TNF receptors (Tumor necrosis factor) and binds indiscriminately all neurotrophins with low affinity, and Trk receptors, with tyrosine kinase activity (tyrosine kinases are enzymes that add a phosphate group to the tyrosine residues present in the substrate protein sequence)[29]. Trk receptors have a greater affinity for neurotrophins than p75; in addition, there are three isoforms of Trk receptors. Trk-A which mainly binds NGF, and with lower affinity NT-3; Trk-B capable of binding both BDNF and NT-4/5; Trk-C which specifically binds NT-3[30].

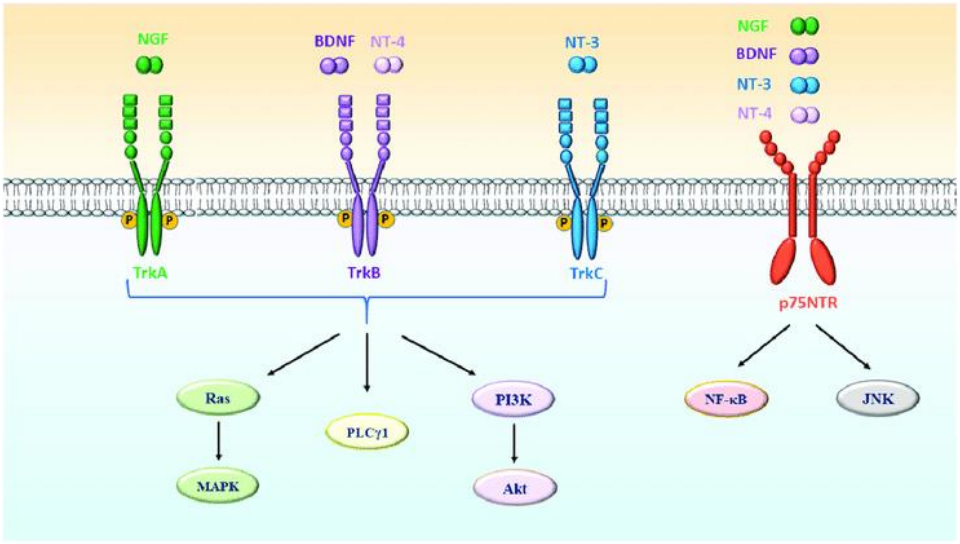


Illustration 1. ***Neurotrophins and their receptors.***

Neurotrophins NGF, BDNF, NT-3 and NT-4 bind as dimers to the TrkA, TrkB and Trkc receptors, as well as to the more common p75. Binding to the extracellular domain of receptors triggers intracellular signaling pathways[31].

Animals in which the Trk receptors have been inactivated by gene knock-out manifest deficits during the development of the nervous system that are much more severe than those present in animals deprived of the p75 receptor[31]. For this reason, p75 is generally considered a co-receptor that modulates the affinity with which neurotrophins bind to the Trk receptor[32]. The mRNAs of Trk receptors are subject to alternative splicing processes, a mechanism whereby more transcripts and consequently different proteins originate from a single gene. Thus, there are variant receptors in the extracellular portion and, consequently, with a different affinity for neurotrophins, and receptors that lack the intracellular portion, with tyrosine kinase activity and therefore unable to generate a biological response[33(p3)]. The binding of neurotrophins to Trk receptors induces the formation of dimers or multimers of the same receptors and stimulates their tyrosine kinase activity[34]. The phosphorylation of tyrosine residues, in the cytoplasmic portion of the receptor, creates high affinity binding sites for a series of molecules with various enzymatic activities or for adapter proteins, which in turn recruit proteins capable of transducing the signal[35(p2)]. In other words, a platform is created on which the molecular signal

transduction machine is assembled. The proteins recruited into a certain and specific set are the basis of the specificity of the cellular response[36].

In polarized cells with complex morphology, such as nerve cells, the receptors may not be uniformly distributed on the cell surface, and therefore the production of second messengers can be localized in specific areas of the cell, with important consequences on the cellular response[16;37]. In the event that neurotrophins bind to Trk receptors present on the cell membrane surrounding the cell body, the distance between the signal transduction machine and the nucleus is short[31]. In the event that the neurotrophins are released from the target organ and bind receptors present at the tip of the axon, the signal must instead travel for great distances with respect to the cell diameter[20]. In this case the neurotrophin-receptor-transduction molecule complex is internalized and transported retrograde from the periphery towards the nucleus[38]. During this process there are large margins to modulate the cellular response by altering the assembly of the proteins that transduce the signal. Neurotrophins are an example of growth factors that act via tyrosine kinase receptors[37]. The list of factors that use a similar signal transduction mechanism also includes the superfamily of EGF (Epidermal Growth Factor), consisting of:

a) 8 EGF-like genes and 4 neuregulins;

b) insulin and insulin-like factors (IGFs);

c) angiogenic factors of the VEGF family (Vascular endothelial growth factor);

d) 23 members of the FGF (Fibroblast growth factor) family;

e) eferins, membrane-anchored growth factors that mediate cell-cell interactions and guide cell migration and directional growth of nerve extensions.

1.1.3. Nerve Growth Factor in pain

NGF plays a leading role in nociception because its selective receptor trkA is mainly expressed on nociceptors[39]. Recent studies have shown that it sensitizes the response to nociceptive stimuli through acute post-transcriptional mechanisms and by modifying the expression of numerous genes[40]. Furthermore, in the inflammatory

process NGF is released in high concentrations by mast cells and induces axonal growth in nociceptive neurons which leads to a greater perception of pain in inflammatory areas[41].

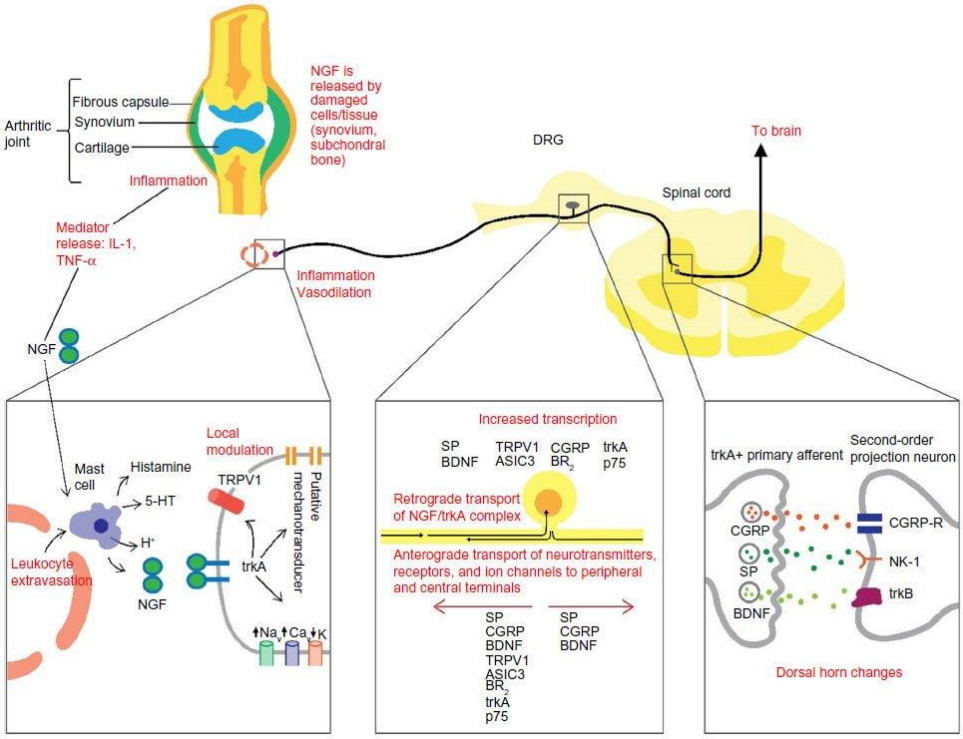


Illustration 2. ***Schematic diagram of the NGF mechanisms involved in the initiation and maintenance of pain***[41]**.**

The importance of NGF has been demonstrated by the numerous cases of congenital insensitivity to pain caused by trkA mutations, the high affinity receptor of NGF[42]. Some of these mutations make trkA receptor insensitive to NGF, which then stops working. Consequently, mice with a mutation in the gene encoding NGF or trkA, show insensitivity to painful stimuli[43]. The reason for the loss of pain sensitivity as a consequence of these NGF/trkA mutations is that NGF is necessary for the peripheral nociceptive system development, so those affected have no way of detecting pain. However, in healthy individuals, NGF continues to play an important role in pain signaling after primary nociceptors have lost their dependence on NGF in the postnatal period. Indeed, nociceptors always express

NGF receptors during adulthood and, consequently, NGF continues to exert significant biological effects on pain for the rest of the individual's life[44].

It has been observed that both in humans[45;46] and in adult animals[47] peripheral injection of NGF induces pain. The mechanism of action identified is: the activation of nociceptors through the binding of NGF to the trkA receptor expressed by the primary peptidergic sensory neurons, the sensitization of nociceptors through phosphorylation of the TRPV1 channels and of the intracellular pathways PI3K and MEK/ERK[48;49;50;51] and the post-translational modification of voltage-dependent ion channels, especially Nav 1.8, which can cause a general increase in nociceptor excitability[52;53].

Furthermore, NGF modulates pain by inducing an increase in the synthesis of neuropeptides and neuromodulators, for example Calcitonin Gene Related Peptide (or CGRP), Substance P and BNDF. In particular, in healthy animals, BDNF is normally expressed in a small number of DRG neurons (about 10%), but following treatment with NGF, the expression of BDNF is induced in the vast majority of sensory neurons expressing trkA[54]. This neurotrophin also acts as a modulator, compromising the efficiency of central nociceptive signals. While neurotrophin-3 and neurotrophin-4/5 appear to play relatively minor roles in pain[44].

As has already been pointed out, NGF has a profound and lasting sensitizing effect on the nociceptive system in both rodents and humans. NGF mRNA and protein levels have been observed to be increased in different types of pain in humans, especially in an inflammatory setting, for example in bladder pain syndrome/interstitial cystitis[55], in inflammatory bowel disease[56], in chronic pancreatitis[57], in osteoarthritis[58], rheumatoid arthritis and spondyloarthritis[59;60] and in cases of burns induced by ultraviolet (UV) radiation[61].

Chronic pain represents a huge health and societal problem, affecting 1 in 5 people. It is associated with multiple comorbidities, such as depression, and has a significant negative impact on quality of life and employment[62]. Current treatments for chronic pain are limited by inadequate efficacy, poor tolerability and abuse potential[63], therefore new analgesics are urgently needed. To this end, there has been a growing interest in targeting NGF and its signaling pathways to provide

analgesia. Data obtained in preclinical pain models have been shown to be encouraging in this regard, suggesting that NGF antagonism may improve the pain condition[64]. Literature data report how the state of thermal and mechanical hypersensitivity evoked by an inflammatory process caused by the administration of Freund's complete adjuvant or by carrageenan in the paw is blocked using anti-NGF antibodies[65;66] or by means of a trkA-IgG molecule to sequester NGF[67].

Anti-NGF therapy produced analgesia but had no effect on tissue edema[66]. This treatment does not affect the inflammatory response *per se*, but rather the sensitization of downstream nociceptors. Anti-NGF has also been shown to reduce hyperalgesia in several preclinical models of chronic pain, including bone cancer pain[68], plantar incision as a postoperative pain model[69], and bone fracture as a model of complex regional pain syndrome[70].

Several mechanisms have been proposed by which anti-NGF can reduce pain. One is the reduction of spontaneous activity and stimulus response of inflammation-evoked nociceptive afferents[71], but it can also normalize the expression of neuromodulators by primary afferents, which is increased in inflammation[67;72].

These encouraging and preclinical data have provided strong motivation for drug development programs aimed at targeting NGF. Thus, several companies have developed humanized monoclonal antibodies that bind to NGF with high specificity and affinity, preventing the latter from interacting with its receptor. These include tanezumab (Pfizer and Eli Lilly), fasinumab (Regeneron), and fulranumab (Janssen). While it is particularly difficult to develop a specific trkA antagonist that does not bind to trkB and trkC, given their high homology. Concern about a non-selective trk receptor inhibitor is related to the potential side effects this could have on the central nervous system. A potential means of avoiding this problem would be the synthesis of compounds with action limited to the periphery only[64].

Several studies concerning the treatment of osteoarthritis have provided an impetus to the use of anti-NGF as an analgesic. All three anti-NGF monoclonal antibodies were shown to be effective in reducing pain and improving function compared to placebo, and tanezumab was shown to be more effective than non-steroidal anti-inflammatory drugs (NSAIDs) and opioid treatment[73]. These results reporting the

potent analgesic effects of anti-NGF are encouraging, however there is concern regarding the long-term safety of these substances as NGF has growth-promoting effects on nociceptive afferents and sympathetic neurons in adults, who would be compromised by anti-NGF therapy. The purpose of anti-NGF therapy should be to normalize NGF levels and signaling and not eliminate it completely, given its role in adulthood. Indeed, adult sensory neurons no longer require NGF for survival, although they remain sensitive to it, which greatly increases the growth of sensory axons *in vitro*[74].

Abnormal sensory phenomena have been reported since Phase I studies with anti-NGF and have been described in multiple patient populations in subsequent Phase II and III studies using different therapies. These sensory changes include burning sensation, paraesthesia, peripheral hypoesthesia and hyperesthesia, and sensory disturbances. Additional symptoms described with anti-NGF therapy versus placebo are myalgia and arthralgia[64]. Furthermore, toxicological studies of anti-NGF in non-human primates have raised the possibility that this may affect the morphology of neurons within sympathetic ganglia, and preclinical studies have shown evidence of reversible shrinkage of sympathetic neurons in adulthood following treatment. anti-NGF[75]. Finally, anti-NGF antibodies downregulate pro-inflammatory neurotransmitters and other mediators such as Substance P or CGRP and thus could potentially induce systemic immunosuppression[64].

1.1.4. Non-nervous Growth Factors

1.1.4.1. Transforming Growth Factor (TGF)

The components of this family, including TGF-β (Transforming growth factor-β), activins and BMP (Bone morphogenic protein) proteins, signal through the activation of serine/threonine kinase receptors[76]. In Mammals the family is made up of 29 members and has two types of receptors: 7 type I and 5 type II receptors[77]. The receptor complex is composed by the association between a homodimer of type I receptors and a homodimer of type II receptors (35 possible combinations). These growth factors exert a plethora of effects during animal differentiation, from induction of the mesoderm to determination of the right-left and anterior-posterior

axis in the embryo, to bone morphogenesis[78;79]. At the cellular level they control processes such as cell proliferation, cell survival, morphology change, cell migration and differentiation. The most studied family member is TGF-β originally isolated from tumor cell culture medium[80;81]. Contrary to what its name suggests, TGF-β often has a cytostatic and apoptotic action, at least on epithelial cells. This phenomenon has been observed both *in vitro* and in various transgenic animal models. For example, TGF-β overexpression in mammary gland cells, pancreatic β cells (those that produce insulin), liver or prostate induces cell division arrest and/or cell death *in vivo*[82;83]. Similarly, TGF-β exerts a cytostatic function on cells of the immune system, in particular on lymphocytes T, so much so that, in mice, the knock-out of the gene for TGF-β causes death in the period immediately following birth, due to severe inflammation resulting from the overactivity of the immune system[84]. More ambiguous is the response of endothelial cells: while TGF-β is clearly cytostatic and pro-apoptotic for endothelial cells *in vitro*, this factor is necessary for vasculature formation and angiogenesis during development and TGF-β injection induces neo-angiogenesis *in vivo* in adult[85;86]. A possible explanation for these findings is provided by the fact that endothelial cells simultaneously express two different type I receptors: ALK1 and ALK5. The former induces a proliferation and migration signal, while the latter induces an antagonist signal[87]. Considering that the receptor complexes bind TGF-β with different affinities, it is possible that the cellular response varies according to the concentration of this factor, in some tumors. Paradoxically, TGF-β is overexpressed in other cancers[88;89]. A possible explanation arises from the fact that high levels of TGF-β can create a microenvironment favorable to tumor development by influencing the growth of different cell types such as fibroblasts, immune system cells and endothelial cells present in the vicinity of the tumor[90(p1)]. For example, inhibition of lymphocytes T and promotion of angiogenesis may alone explain the role of TGF-β in promoting tumor progression and metastasis formation[91].

1.1.4.2. Platelet Derived Growth Factor (PDGF)

PDGF is a peptide growth factor that is secreted by numerous cell types including platelets, fibroblasts and vascular smooth muscle cells and functions as a mitogen for mesenchymal and neuroectodermal cells[92]. PDGF is a dimeric protein consisting of 2 of the 4 known homologous chains (A, B, C and D), which are linked by disulfide bridges and are both homo- (PDGF-AA, PDGF-BB, PDGF-CC, PDGF - DD) and form heterodimers (PDGF-AB)[93]. All PDGFs are synthesized in the endoplasmic reticulum as inactive precursors and must be converted to their active form by proteolytic cleavage. The classical PDGFs, PDGF-A and PDGF-B, are already activated intracellularly during the exocytosis process, while the recently identified PDGF-C and PDGF-D are secreted as latent factors that are activated by extracellular proteases (plasmin, tPA)[94].

Multi-tissue expression analyses showed that the four PDGF chains are ubiquitously expressed; in particular, all the PDGF isoforms are expressed both in the heart and in the vessel wall[94]. The two PDGFR subtypes, α and β, show a non-overlapping expression pattern during embryogenesis, but are drastically regulated during pathological processes.

PDGF induces the proliferation, migration, differentiation and transformation of numerous cell types and is involved in the regulation of gene expression and apoptosis, as well as in the formation of oxygen radicals[93;95]. Ligand binding of PDGF isoforms leads to the dimerization of 2 receptor subunits with a consecutive increase in the intrinsic activity of tyrosine kinase and receptor autophosphorylation. The binding of specific signal transduction molecules to phosphorylated tyrosines leads to the activation of signal transduction cascades, which selectively mediate cellular responses induced by PDGF.

PDGF plays an important role in embryonic development, angiogenesis and physiological processes such as wound healing[96]. However, this growth factor acquires pathophysiological importance in numerous proliferative diseases such as tumorigenesis, as well as in fibrotic and inflammatory diseases.

1.1.4.3. Hepatocyte Growth Factor (HGF)

HGF is among a group of factors possessing angiogenic ability that are described as heparin-binding growth factors[97]. HGF is secreted by fibroblasts and is mitogenic for epithelial and endothelial cells and also melanocytes, but does not affect fibroblasts[98]. HGF induces cell proliferation and invasion by activating PI3K/Akt and ERK signalling pathways, the latter a component of the MAPK system[99]. It induces invasion of cholangiocarcinoma cells in this way. The biological effects of HGF are interfered with or complemented by several interacting signalling systems. The induction of invasion was reported to be accompanied by alteration of membrane-located E-cadherin expression[100]. The ECM proteolytic system of MMPs or PA was unaffected. Inhibition of PI3K abolished the increased invasion. PI3K induced ERK1/2 phosphorylation, but inhibition of this route suppressed invasion only in one of the two cell lines tested[101]. These authors have suggested that different signalling systems might operate in different cell lines. On the other hand, it might possibly be due to the interaction of different effectors with HGF signalling. The cadherins are closely involved with catenin/Wnt signalling. HGF/MET does indeed activate the β-catenin/Wnt pathway[102]. PI3K and MAPK pathways might be activated either independently or in concerted function with other effectors such as EGF or thrombospondin (TSP). EGF has been found to induce MET expression, which might bind to and be activated by HGF and enhance proliferation by ERK1/2 activation[103]. Inhibition of ERK signalling has led to the inhibition of induction of cell proliferation[104].

1.1.4.4. Plasma Rich in Growth Factor (PRGF)

Platelet-rich plasma (PRP) is widely used in regenerative medicine because of its high concentrations of various growth factors and platelets[105]. In addition to PDGF, PRP provides fibrinogen, which is converted into insoluble fibrin fibers, to support cell adhesion and control the delivery of growth factors[106]. Furthermore, PRP provides anti-inflammatory factors and anti-bacterial peptides to optimize the local environment by suppressing inflammatory responses[107;108;109(p2);110].

Plasma rich in growth factors (PRGF), a subtype of P-PRP (pure platelet-rich plasma), is a supernatant enriched in plasma and platelet-derived morphogens,

proteins and growth factors. PRGF represents a complex pool of active mediators that may stimulate and accelerate tissue regeneration, which is generally safe to use and inexpensive to obtain. Indeed, autologous PRGF has been approved for clinical use by the European Community and the U.S. Food and Drug Administration[111], and it is generally employed in ophthalmology as eye drops to treat the ocular surface[112;113].

Retinal ganglion cells (RGCs) are in close contact with Müller glia, the main glial cells in the mammalian retina. These cells that serve to maintain retinal homeostasis, and they are involved in retinal metabolism, in the phagocytosis of neuronal debris, in the release of certain transmitters and trophic factors, as well as in K^+ uptake[114]. Müller cells extend across the thickness of the retina, providing structural stability and maintaining close contact with the majority of retinal neurons[115]. In addition to their involvement in maintaining homeostasis, these cells also provide trophic factors to neurons that potentially promote their survival and repair[116], and they have been seen to enhance RGC survival[117;118]. Therefore, in the CNS, PRGF may interact with these glial cells as it contains growth factors known to accelerate cell proliferation, stimulate differentiation and promote cell survival[119;120]. Among the growth factors present in PRGF, those implicated in proliferation include Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor-beta (TGF-β), Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Insulin-like Growth Factor-I (IGF-I) and Nerve Growth Factor (NGF)[121].

1.1.4.5. Non-nervous Growth Factors in pain

In addition to neurotrophins, there are many other growth factors that play a role in pain modulation. Knowing them is important as they provide new insights into the pathophysiology of nociception and represent promising molecular targets for novel therapeutic agents for pain treatment.

Studies have shown the protective role of TGF-β in nerve injury induced neuropathic pain. TGF-β appears to promote the expression of endogenous opioids and inhibit the neuro-immune responses of glial cells and neurons in the spinal cord

following peripheral injury. Furthermore, there are other indeterminate peripheral mechanisms that contribute to its analgesic effect[122]. Recombinant TGF-β was administered by prolonged intrathecal infusion in sciatic nerve ligated rats and this growth factor was found to significantly attenuate the development of mechanical allodynia and thermal hyperalgesia for 14 days. It has been observed that TGF-β also produces a significant reduction in hyperalgesia with established damage, an important fact from a therapeutic point of view since it has symptomatic as well as preventive properties. These results support the hypothesis that TGF-β exerts peripheral anti-nociceptive effects and helps preventing sensitization of peripheral nociceptors following nerve injury[123]. Furthermore, TGF-β is capable of maintaining the integrity of the blood brain barrier thus carrying out a protective mechanism against the development of pathological pain following inflammatory peripheral[124;125] or neural lesions[126]. This evidence suggests that modulation of TGF-β signaling could be used as a new pharmacological strategy for the control and treatment of chronic pain[123].

Another factor that mediates pain, particularly inflammatory pain, is PDGF. Injection of the PDGF-BB isoform into the rat paw produced thermal and mechanical hyperalgesia, while *in vivo* sequestration of PDGF or inhibition of its receptor alleviated formalin-induced acute inflammatory pain. The proalgic action of PDGF is due to the inhibition of the Kv7/M class of potassium channels which leads to the activation of nociceptive neurons and therefore contributes to inflammatory pain. These findings broaden the knowledge regarding the pathophysiology of inflammatory pain and may have important clinical implications[127].

HGF is a plasminogen-related, mesenchyme-derived pleiotropic growth factor that regulates cell growth, cell motility and morphogenesis in various cell types, including epithelial and endothelial cells[128]. Subsequently, it was found that HGF performs several functions including angiogenesis[129], morphogenesis[130], an anti-inflammatory effect[131], tissue regeneration of different organs[132;133;134] and the enhancement of neurite growth of the dorsal root ganglia[135;136].

Studies have shown that HGF protects neurons from injury and promotes nerve regeneration *in vivo* in central and peripheral nervous system[137]. Furthermore, local intramuscular injection of HGF reverts pain induced by chronic constriction injury (CCI) and tendon injury in rats[138].

HGF therefore emerges as a potential candidate for the analgesic therapy of neuropathies thanks to its multifunctionality that includes angiogenic, neurotrophic and anti-inflammatory actions. Indeed, the release of HGF promotes nerve repair and functional recovery after spinal cord injury, improves neuronal survival and increases the proliferation of oligodendrocyte progenitor cells[139;140]. HGF has been shown to decrease pain induced by tendon injuries, the mechanism of which is associated with an anti-inflammatory effect[141]. In addition, repeated intramuscular injections of non-viral HGF-HVJ liposomes (hemo-agglutinative virus of Japan) attenuated neuropathy induced by loose sciatic nerve ligation (CCI) in rats by preventing sensory nerve degeneration and improving nerve blood flow[142]. However, it must be considered that microglia and astrocytes also play a critical role in the development and maintenance of neuropathy. In fact, the injury of a peripheral nerve leads to the activation of microglia and astrocytes that contribute to the amplification of pain through the production and release of pro-inflammatory cytokines and neuro-excitatory substances[143], while the blockade of activation of microglia and astrocytes at the spinal level attenuates hypersensitivity to pain[144;145]. It was observed that HGF also inhibits LPS-induced activation of microglia cells and decreases the upregulation of LPS-induced IL-1β, IL-6, TNF-α, and iNOS expression *in vitro*. Taken together, these results suggest that HGF may represent a promising therapeutic strategy for the treatment of neuropathy, through the reduction of cytotoxicity products released by activated glial cells and the induction of nerve repair[146].

Some studies have shown that infiltration of plasma rich in growth factors (PRGF-Endoret) effectively led to the reduction of joint pain[147].

A significant clinical motivation for pain relief arises from the need to get patients back to their daily routine and improve their quality of life. An innovative biologically inspired approach to tissue repair is the application of plasma rich in

growth factors (PRGF-Endoret)[148], which has been shown to be an effective treatment for relieving knee and hip pain in patients with osteoarthritis (OA) and to improve their clinical condition by reducing joint pain[149;147].

PRGF-Endoret is an autologous product that carries fibrin incorporated with a pool of growth factors, such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF) and growth factor of nerves (NGF), derived from activated platelets and plasma. It acts as a biological scaffold that determines a prolonged and gradual release of growth factors in dysfunctional and degenerate sites[148].

Inflammation is a term that encompasses clinical, physiological, cellular and molecular phenomena, with pain being the hallmark or tip of the iceberg underlying pro-inflammatory cytokine release, extracellular matrix catabolism and cell death. Therefore, pain and inflammation are two sides of the same coin, namely tissue damage. Data from animal studies strongly suggest that pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 are critical for onset and the maintenance of pain primarily derived from damaged peripheral tissues. In contrast, anti-inflammatory cytokines such as IL-4 and IL-10 have analgesic properties[150]. Some components present in PRGF-Endoret (HGF, LXA4, PF4, IGF-1, PDGF and TGFβ)[151] inhibit the NF-kB signaling pathway in various cell lines including macrophages, chondrocytes, and fibroblasts. Considering that NF-kB plays an important role in mediating the gene expression of pro-inflammatory cytokines such as TNFα, IL-1β, PGE2 and COX-1 and COX-2[152], consequently, it is reasonable to assume that the PRGF-Endoret exerts an anti-apoptotic, protective effect on the extracellular matrix, anti-inflammatory and pain-reducing effectively at the joint level[153;154].

This leads to further emphasis that growth factors play a role in pain signaling and that it is important to know their mechanism of action in order to develop new therapeutic approaches for the treatment of pain.

1.2. Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth, and reproductive functions. VEGF has also been implicated in pathological angiogenesis associated with tumors, intraocular neovascular disorders and other conditions[155].

1.2.1. The discovery

VEGF was discovered in the late 1970s following the observation that fibrin deposits were present in the stroma of animal tumors[156]. Fibrin is generated by the clotting of fibrinogen, a plasma protein normally contained within blood vessels. In order for the fibrinogen to escape from the vessels and thus to coagulate, forming fibrin, the local capillaries had to become hyper-permeable to it.

In 1983, Senger described a protein called vascular permeability factor (VPF) secreted by animal cancer cells (hamster, guinea pig, pig) responsible for the increased permeability of tumor blood vessels to fibrinogen, as well as other plasma proteins, and for development of ascites associated with certain abdominal tumors[157].

In 1989, Ferrara and Da Genentech independently isolated and described the VEGF protein, demonstrating its role in angiogenesis[158]. Subsequently, the two proteins VPF and VEGF turned out to have the same structure[159].

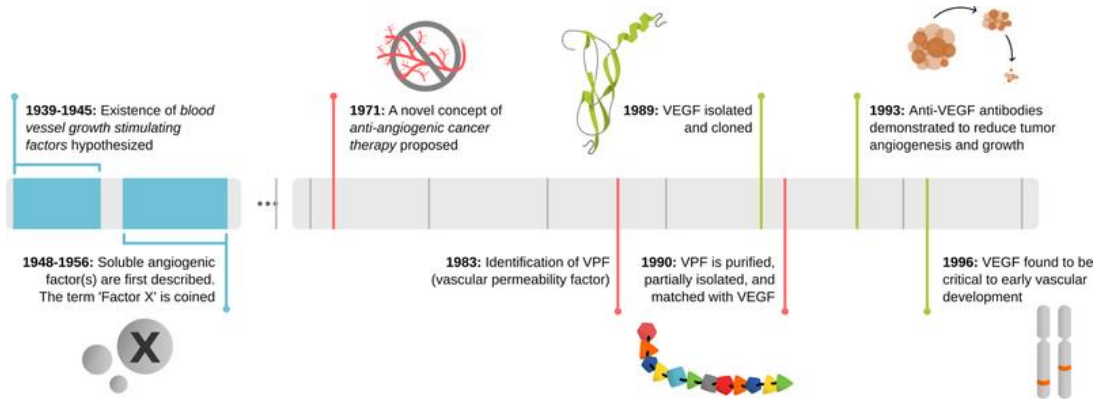


Illustration 3. ***A historical timeline of VEGF discovery***[159]***.***

1.2.2. The VEGF family

The human *VEGF* gene, located on chromosome 6p21.3, is part of the VEGF/platelet-derived growth factor (PDGF) gene family, also called the "Cystine Node (Cys-loop) Growth Factor superfamily"[160].

From a structural point of view, VEGF is a 40 kDa heterodimeric glycoprotein that contains a cystine bridge, characterized by the presence of three disulfide bridges in the protein structure that confer high stability[161].

VEGF family includes several members with various functions: VEGF-A (which has different isoforms), VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral), VEGF- F (present in the venom of some snakes) and placental growth factor (PlGF). More recently, a new member has been added to this family, called endocrine gland-derived vascular endothelial growth factor (EG-VEGF)[162].

VEGF-A, also called VEGF, is the most important and powerful stimulator of angiogenesis, first described as VPF by Senger and colleagues[159]. It plays an important role in vasculogenesis and neoangiogenesis, causing cell proliferation, inhibition of apoptosis, increased vascular permeability, vasodilation, recruitment of inflammatory cells to the injury site[163]. VEGF is secreted, particularly in response to lack of oxygen, not only by endothelial cells, but also by other cells such as tumor cells, macrophages, platelets, keratinocytes, renal mesangial cells, activated T cells, leukocytes, dendritic cells, epithelial cells retinal pigmentary cells, retina Müller cells, astrocytes, osteoblasts, bronchial and alveolar epithelial cells, pericytes. More recently, VEGF has also been found to be expressed in myofibroblasts located in the myocardium, suggesting its implication in post-infarct tissue repair and remodeling[164;165;166;167;168;169].

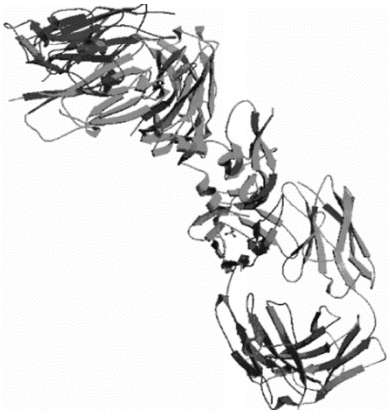


Illustration 4. ***Three-dimensional structure of VEGF-A***[158].

1.2.2.1. VEGF-A isoforms

Alternative splicing of the *Vegfa* gene leads to different VEGF-A isoforms which have been proposed to promote distinct signalling outcomes[170]. Alternative splicing is advantageous in expanding the repertoire of possible VEGF-A isoforms that can be produced from a single gene[171]. These isoforms differ in respect to their length and are designated VEGFxxx, where xxx represents the number of amino acids present in the final protein sequence. To date, 16 distinct VEGFA isoforms have been identified most commonly from six transcripts: VEGF111, VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206[170;172;173]. An additional isoform, VEGF-Ax, was also identified in 2014 that arises from programmed translational read-through (PTR)[173]. VEGF165a was the first isoform characterized and remains the most extensively investigated in respect to its function, signalling, expression and pathological roles[174]. As a potent stimulator of angiogenesis, VEGF165a is considered the prototypical pro-angiogenic VEGF-A isoform. Altered VEGF-A isoform expression has been well documented in tissues during physiological and/or pathological conditions[175;176;177;178;179].

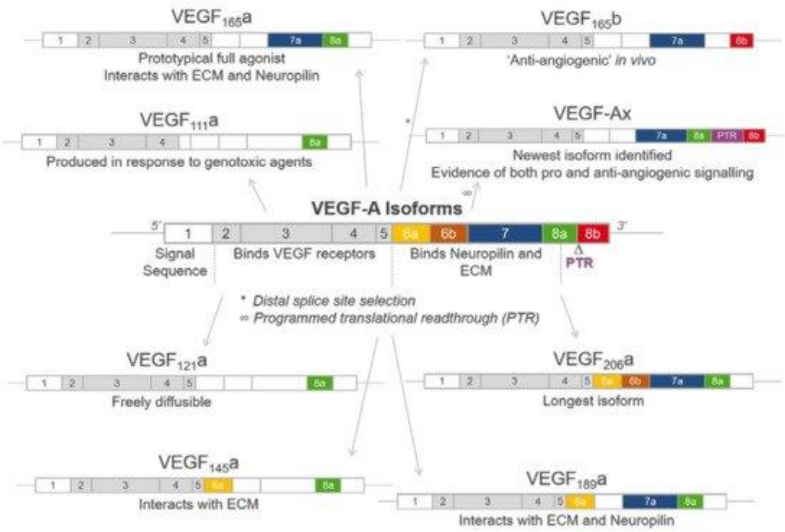


Illustration 5. ***Scheme illustrating the structure of vascular endothelial growth factor A (VEGF-A) isoforms***[176]***.***

A major site of alternative splicing occurs at exon 8, whereby proximal splicing results in the VEGFxxxa forms and distal splicing generates the VEGFxxxb isoforms containing exon 8b[180]. In respect to their sequences, VEGFxxxa and VEGFxxxb

isoforms only differ in the six amino acids found at their C termini; VEGFxxxa isoforms end in the sequence CDKPRR, whereas VEGFxxxb isoforms terminate in SLTRKD[181]. Based on both *in vitro* and *in vivo* experimental evidence, VEGFxxxa isoforms are considered to be "pro-angiogenic" as major mediators of vascular permeability, cell proliferation, survival and migration, and angiogenesis[182]; in contrast, VEGFxxxb isoforms have been reported to have "anti-angiogenic" properties[183;184;185], with evidence that these isoforms may act as regulators and inhibitors of VEGFxxxa-induced pro-angiogenic activity[186;184]. Interestingly, in quiescent vessels, a higher proportion of total VEGF-A is represented by VEGF165b, which is then downregulated in cancer where a switch to pro-angiogenic isoform expression is observed to drive tumour angiogenesis[183;184;187(p2)]. Proximal or distal splicing of exon 8 can be influenced by external stimuli, as proximal splicing has been promoted by insulin like growth factor (IGF1) or tumour necrosis factor alpha (TNFα), whereas stimulation with tumour growth factor beta 1 (TGF-β1) has promoted distal splicing[186]. This bias was governed by the specific SR protein splice factor that was bound to a sequence within exon 8a (SRSF1) or 8b (SRSF6). It is worth noting that this bias may not be consistent in all cell types, however this highlights how isoform expression can be context dependent. There has been some debate as to the existence of VEGFxxxb isoforms physiologically[188;189], with genome wide RNA sequencing data of the human transcriptome questioning whether the relevant exon-exon junctions in the *Vegfa* gene are present[189].

1.2.2.2. VEGF-B

VEGF-B was first isolated in 1996[190], and it is predominantly expressed in the embryonic heart but not in the endocardial cushions[191]. VEGF-B167 and VEGF-B186 are the two isoforms expressed in humans. The VEGF-B167 isoform is mainly expressed in the most tissues including skeletal muscles, myocardium and brown fat and accounts for more than 80% of the total VEGF-B transcripts[192]. The VEGF-B186 isoform is expressed at lower levels and only in a limited number of tissues. VEGF-B is a ligand for VEGFR-1 and Nrp-1, and it can form heterodimers with VEGF-A[193;194]. Neither isoform binds VEGFR-2 or VEGFR-3. VEGF-B167 binds

heparan sulfate proteoglycans and is mostly sequestered in the extracellular matrix while VEGF-B186 is freely diffusible. The precise role of VEGF-B *in vivo* is not precisely known. Study with mice deficient in VEGF-B reported development of smaller hearts and impaired recovery after induced myocardial infarction, suggesting that formation of coronary collaterals might be partly attributed to VEGF-B[195]. Also, VEGF-B has been reported to be weakly angiogenic after adenoviral delivery to periadventitial tissue or hindlimb skeletal muscle[196;197]. Reduced synovial angiogenesis in VEGF-B knockout arthritis models suggest a role of VEGF-B in inflammatory angiogenesis[198].

1.2.2.3. VEGF-C

VEGF-C with the molecular weight of 46.9kDa was purified first by Joukov et al. in 1996[199]. Lee et al. also isolated the corresponding gene, and the protein encoded by this gene was named VEGF-related protein (VRP)[200(p4)]. VEGF-C is produced as a precursor protein and is proteolytically activated in the extracellular space by proteases to generate a homodimeric protein with high affinity for both VEGFR- 2 and VEGFR-3[201]. VEGF-C induces mitogenesis, migration and survival of ECs. VEGF-C is expressed in the heart, small intestine, placenta, ovary and the thyroid gland in adults. Developmental studies, knockout models and gene transfer experiments suggest that VEGF-C is primarily a lymphangiogenic growth factor and its lymphangiogenic effects are mediated by VEGFR-3[201;202]. However, the increase in blood vascular permeability induced by VEGF-C is mediated by VEGFR-2[202]. Disruption of the VEGF-C gene in mice demonstrates that the growth factor is indispensable in embryonic lymphangiogenesis[203]. VEGF-C is also involved in tumor and inflammation associated lymphangiogenesis. Examination of VEGF-C function in a number of assays has also shown an angiogenic activity, presumably via activation of VEGFR-2. VEGF-C gene transfer produced moderate angiogenesis in rabbit skeletal muscle[196] and perivascular tissue[197].

1.2.2.4. VEGF-D

VEGF-D is a secreted glycoprotein and is structurally 48% identical to VEGF-C. It is expressed in many adult tissues including the vascular endothelium, heart, skeletal

muscle, lung, and bowel[204]. The mature form of human VEGF-D binds to and activates VEGFR-2 and VEGFR-3[203]. However, mouse VEGF-D binds only to VEGFR-3. VEGF-D has been shown to be responsible for proliferation of ECs, and it shows angiogenic properties *in vitro* and *in vivo*. Similar to VEGF-C, it also shows lymphangiogenic potential.

1.2.2.5. VEGF-E

VEGF-E is a protein elaborated by the Orf virus, a parapoxvirus that affects sheep, goats and occasionally humans, and has an amino acid homology of about 25% with human VEGF[205]. Infection by this virus causes proliferative skin lesions in which extensive capillary proliferation and dilation are prominent histological features[206]. Several strains of the virus encode different VEGF-E variants, which bind specifically to VEGFR-2 and Nrp-1 and are able to stimulate EC mitogenesis and vascular permeability. Gene expression of VEGF-E induces a strong angiogenic response. Edematous lesions and hemorrhagic spots on the ear which were reported as side effects in VEGF-A transgenic mice were not detectable in VEGF-E transgenic mice[207(p1);206].

1.2.2.6. PlGF

PlGF is a member of the VEGF family which was first identified in placenta but is also known to be present in heart and lungs. Four isoforms–PlGF-1, PlGF-2, PlGF-3 and PlGF-4, have been described[162]. PlGF-1 and PlGF-3 are non-heparin binding diffusible isoforms PlGF-2 and PlGF-4 have heparin binding domains. PlGFs mediate their effects through VEGFR-1[208]. PlGF-2 is also able to bind Nrp-1 and NrP-2 due to the insertion of 21 basic amino acids at the carboxy terminus, while both PlGF-1 and PlGF-3 lack this amino acid insert. PlGF has direct effects on ECs, both by inducing its own signaling and by amplifying VEGF-driven angiogenesis[209]. PlGF-2 overexpression results in the production of significant angiogenesis in different tissues[209;210(p1)]. Various mechanisms by which PlGFs can enhance angiogenesis include: (a) intracellular signal transduction through VEGFRs; (b) Increasing the fraction of VEGF-A available to activate VEGFR-2 by displacing VEGF-A from the 'VEGFR-1 sink'[211(p1)]; (c) Activation of VEGFR-1 by PlGFs results

in intermolecular transphosphorylation of VEGFR-2 that could increase VEGF-A mediated angiogenesis[211(p1)]; (d) PlGF/VEGF-A heterodimer formation, which could act through VEGFR-1/VEGFR-2[211(p1)].

1.2.3. VEGF Receptors

Initially, VEGF binding sites were identified on the cell surface of vascular ECs *in vitro* and *in vivo*. Subsequently, it was clear that VEGF receptors also occur on bone marrow–derived cells[212]. VEGF-A binds two related receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. Both VEGFR-1 and VEGFR-2 have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain[213;214]. VEGFR-3 (fms-like-tyrosine kinase (Flt)-4) is a member of the same family of RTKs but is not a receptor for VEGF-A, binding instead to VEGF-C and VEGF-D8. In addition to these RTKs, VEGF-A interacts with a family of co-receptors, the neuropilins.

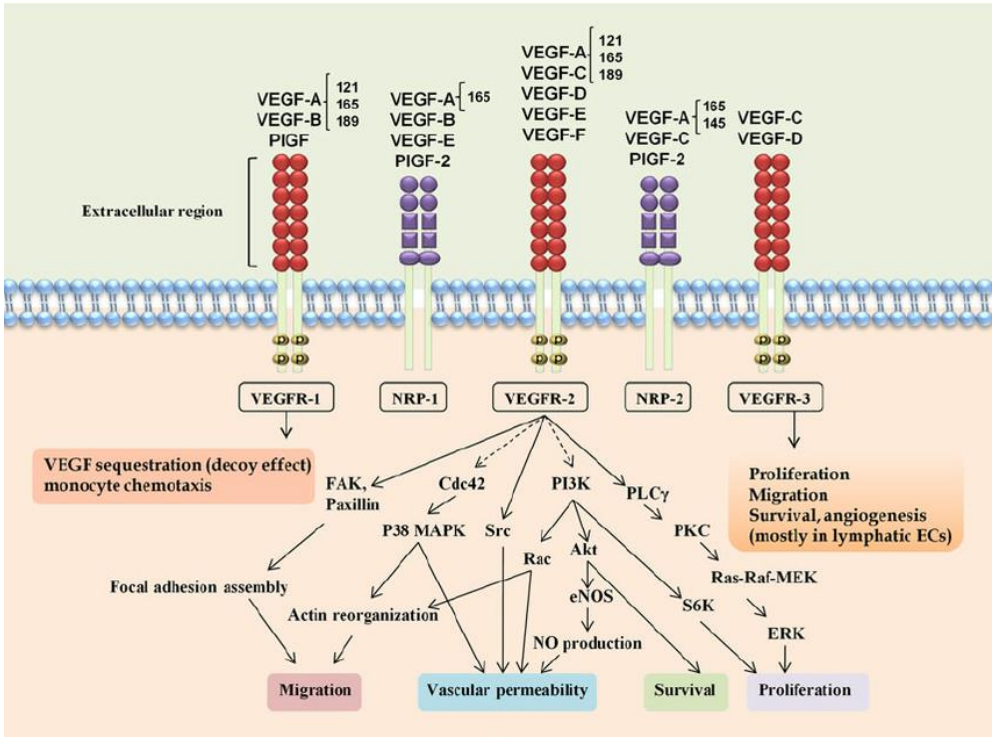


Illustration 6. ***Role of the VEGF receptor tyrosine kinases in different cell types***[155].

1.2.3.1. VEGFR-1 (Flt-1)

It is a member of the receptor tyrosine kinase (RTK) family, with a molecular weight of 180 kDa and binds VEGF-A, VEGF-B, PlGF and VEGF-F, with high affinity. It is expressed on endothelial cells, but also on inflammatory cells, monocytes/macrophages, bone marrow-derived hematopoietic progenitor cells, trophoblastic cells, mesangial kidney cells, tumor cells, vascular smooth muscle cells (VSMC). Furthermore, it has also been identified on myofibroblasts located in the connective tissue of the mouse myocardium in the infarct areas[155]. VEGFR-1 plays an important role in the migration of endothelial cells, monocytes, macrophages and hematopoietic stem cells and is therefore mainly involved in pathological angiogenesis in adult life (tumors, inflammation, ischemia, preeclampsia)[215].

VEGFR-1 has a 10-fold greater affinity for VEGF-A than VEGFR-2 and lower tyrosine kinase activity. Recent data on the biological role of VEGFR-1 in angiogenesis during embryonic development are contradictory. Most authors state that VEGFR-1 plays a role in endothelial cells differentiation and migration, but not in their proliferation[216]. Inactivation of the murine gene that codes for VEGFR-1 (flt-$1^{-/-}$ mice) leads to embryonic death in days 8 or 9 of gestation because, even if the endothelial cells undergo differentiation, vascular channels are formed anarchists and therefore the development and organization of a functional and vital vascular system does not occur. Consequently, according to these studies, VEGFR-1 is only involved in the differentiation of endothelial cells and is not actively involved in the early stages of angiogenesis during embryogenesis[217]. Instead, the molecular mechanisms that refer to the involvement of the VEGFR-1 gene in vasculogenesis are not fully understood, and there are still aspects that need to be clarified. VEGFR-1 appears to inhibit pro-angiogenic signals early in development by preventing the binding of VEGF-A to VEGFR-2, which has a stimulating effect on endothelial cell proliferation and is expressed on newly formed endothelial cells[155].

Some experiments sought to determine which of the three VEGFR-1 receptor domains was involved in vasculogenesis and which in pathological angiogenesis. In mice with the mutation of the segment of the VEGFR-1 gene that codes for the intracellular domain with tyrosine kinase activity (flt-1 $TK^{-/-}$ mice), there is the

differentiation of endothelial cells, but not the migration of macrophages in pathological conditions. The extracellular and transmembrane domains of the receptor, which instead remained unchanged, showed that the effect of VEGFR-1 on vasculogenesis is influenced by these. Thus, the receptor tyrosine kinase activity does not influence the differentiation of endothelial cells during embryogenesis but plays an important role in pathological angiogenesis[218]. In fact, other experiments on mutant mice that do not contain the tyrosine kinase region (flt-1 $TK^{-/-}$ mice), have shown a lower rate of tumor invasion and metastasis and also a lower degree of inflammation (for example in rheumatoid arthritis) than to wild-type [163].

Due to various splicing variants, VEGFR-1 may also produce soluble VEGFR-1 (sVEGFR-1 or sFlt-1). VEGFR-1 and sVEGFR-1 can both bind to VEGF-A with high binding affinity, but VEGFR-1 has low intracellular response compared to VEGFR-2[219;220].

sFlt-1 comprises the extracellular domains of VEGFR-1, and is soluble, being present in the circulation. It acts as an anti-angiogenic protein by antagonizing the actions of both VEGF-A and PlGF. Multiple splice variants of sFlt-1 have significantly different tissue distributions[221], raising the potential for different physiological and pathological roles. For example, in humans, the main sFlt-1 variant, known as sFlt-1 i13, is widely expressed in most tissues, whereas another variant known as sFlt-1 e15a appears to be almost exclusively expressed by the placenta[221].

sFlt-1 results either from alternative splicing of the VEGFR-1 pre-mRNA[222] or through cleavage of the ectodomain of VEGFR-1[223]. Proteolytic cleavage of the extracellular region is thought to occur adjacent to the transmembrane domain[224] and is potentially due to the actions of proteolytic enzymes[225;226]. Proteolytic cleavage produces an sFlt-1 that is identical to the extracellular region of VEGFR-1, while the multiple variants of sFlt-1 that have been identified all have unique C-terminal sequences[227;228].

1.2.3.2. VEGFR-2 (KDR or Flk-1)

It is a member of the tyrosine kinase receptor family, with a molecular weight of 200-230 kDa[229]. It shows higher affinity for VEGF-A and VEGF-E and lower affinity

for VEGF-C and VEGF-D[230]. VEGFR-2 is mainly expressed on blood endothelial cells and lymphatic vessels, but also has a weak expression on hematopoietic cells, megakaryocytes, retinal progenitor cells, neurons, osteoblasts, pancreatic ductal cells, tumor cells[216]. VEGFR-2 is expressed at the beginning of embryonic life (day 7.5 of gestation) on hemangioblasts of mesodermal origin, influencing their migration, differentiation into endothelial cells and the formation of vascular islets in the yolk sac with the onset of vasculogenesis[231].

Inactivation of the murine gene encoding VEGFR-2 in homozygous (-/-) animals leads to embryonic death on days 8 and 9, due to a failure in vascular islet formation. In this case, the differentiation of endothelial cells does not occur and thus the development and organization of the vascular system is blocked. Therefore, VEGFR-2 is essential for the normal course of vasculogenesis during embryonic development[232].

VEGFR-2 has the same domains as the other receptors of this family. The binding of VEGF to the extracellular domain of VEGFR-2 causes the autophosphorylation of tyrosine residues and the activation of some signaling pathways (phospholipase-Cγ (PLCγ)/protein kinase C (PKC) and Ras/Raf/ERK/MAPK) which are involved in the proliferation of endothelial cells. By activating the PI3K/Akt pathway, VEGFR-2 plays a role in endothelial cell survival, mediating an anti-apoptotic effect. Furthermore, it activates some integrins that disrupt cell-cell cohesion and initiate cell migration. The mechanism by which this occurs could depend on the formation of a complex between adhesion molecules and VEGFR-2 which would lead to weakening of the intercellular junctions, destabilization of the cytoskeleton of endothelial cells and formation of endothelial windows. Consequently, there is an increase in vascular permeability and cell migration is favored. Furthermore, by activating the protein kinase Akt, the production of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) in endothelial cells is stimulated, inducing vasodilation and an increase in vascular permeability[187(p2)]. During tumor neoangiogenesis, there are numerous paracrine interactions between endothelial cells and tumor cells. In particular, the binding of VEGF to VEGFR-2 stimulates the secretion of von Willebrand factor by endothelial cells which is an essential event

for tumor progression[233]. Therefore, VEGFR-2 is involved in vasculogenesis, normal angiogenesis and pathological angiogenesis by acting through different mechanisms such as migration of haemangioblasts towards the yolk sac and differentiation into endothelial cells, formation of vascular tubes (tubulogenesis), proliferation of endothelial cells (mitogenic effect), increased vascular permeability, migration of endothelial cells, transmission of signals that promote the survival of endothelial cells by preventing apoptosis and formation of endothelial windows. In pathological processes, VEGFR-2 is often involved in tumor angiogenesis. In fact, it has the strongest pro-angiogenic activity, consequently blocking this receptor could have useful clinical implications. Finally, VEGFR-2 has a higher tyrosine kinase activity than VEGFR-1, but a lower affinity for VEGF[234;235].

1.2.3.3. VEGFR-3 (FLT-4)

VEGFR-3 It belongs to the family of tyrosine kinase receptors, with a molecular weight of 195 kDa. It plays an important role in the morphogenesis of the lymphatic vessel network during embryonic development and is also involved in the formation of new lymphatic vessels in adult life[229]. In adults, lymphangiogenesis, or *de novo* formation of lymphatic vessels from pre-existing postcapillary venules (high endothelial venules), occurs in certain pathological conditions, most frequently in inflammation and tumors[236]. VEGFR-3 has affinity for the growth factors VEGF-C and VEGF-D, it is expressed in the lymphatic endothelium and high endothelial venules, and influences differentiation, proliferation, migration and survival of lymphatic endothelial cells. VEGFR-3 expression has also been observed in other cells, such as osteoblasts, macrophages, neural progenitors; while its presence in cancer cells remains controversial[237]. The signaling pathways that activate lymphangiogenesis, especially during embryonic development, are: activation of kinases regulated by the extracellular signal MAPK (ERK1/2) through the PKC and Ras pathways (important pathways in cell proliferation), as well as the PI3K-Akt pathway/PKB (mainly involved in the survival of lymphatic endothelial cells)[238]. Expression of the murine *VEGFR-3* gene begins on day 8.5 of intrauterine development, resulting in the differentiation of lymphatic endothelial cells and the

formation of lymphatic sac-like structures that will form a dense network of lymphatic vessels. Inactivation of this gene leads to the death of the mouse embryo due to the absence of lymphatic vessel formation and the development of massive edema. Indeed, it is believed that the onset of human hereditary primary lymphedema in adults is related to the activity of VEGFR-3. The binding of VEGF-C to VEGFR-3 is responsible for most of the biological effects of this receptor. The discovery of a soluble form of VEGFR-3 (sVEGFR-3) and experiments on transgenic mice expressing this gene led to the conclusion that sVEGFR-3 inhibits lymphatic vessel development and induces edema by inhibiting VEGF-C and VEGF-D-mediated signals[239(p3)]. Finally, tumors with lymph node metastases have been observed to express high levels of VEGF-C or VEGF-D and, consequently, it has been hypothesized that VEGFR-3 may be involved in the migration of tumor cells through lymphatic vessels.

1.2.3.4. Neuropilins

NRP-1 and NRP-2 are transmembrane receptors located on endothelial cells that function as co-receptors, modulating the activity of tyrosine kinase receptors. Neuropilins selectively bind to some isoforms of the VEGF growth factor, have a low molecular weight of 120-135 kDa and were initially identified as receptors for different types of traffic lights (class 3 traffic lights)[240].

NRP-1 is expressed on endothelial cells of arteries and has an affinity especially for VEGF 165. NRP-1 increases VEGFR-2 activity up to six-fold, affecting angiogenesis and endothelial cell migration. Furthermore, the synergistic action of NRP-1 and VEGFR-2 leads to the intensification of Platelet Activating Factor (PAF) secretion by endothelial cells, promoting inflammation and increasing vascular permeability and migration of endothelial cells. NRP-1 can also be present in other cells such as neurons, smooth muscle cells or cancer cells (it has been detected on the surface of breast, prostate, lung, pancreatic and colon cancer cells, as well as in astrocytomas, glioblastomas and melanomas)[241].

NRP-2 is mainly expressed on endothelial cells of lymphatic vessels and veins where it increases the binding of VEGF-C with VEGFR-3 and consequently its

effects. The role of neuropilins in vasculogenesis was demonstrated using NRP-1 knockout mice (Neuropilin-1$^{-/-}$) which led to the death of mouse embryos due to abnormalities of the vascular system[232].

1.3. Role of VEGF in the Nervous System

In addition to the effects on vascularity, VEGF also regulates the migration of neuronal cells into the CNS. There is a close embryogenetic parallelism between the development of the vascular system and that of the nervous system[242]. Both are composed of a network of afferent and efferent connections: the motor and sensory nerves in the nervous system, the arteries and veins in the circulatory system. These systems are regulated by similar cellular signals and cooperate with each other to coordinate the growth and modeling of neuronal and vascular networks[243]. Recent studies have shown that similar molecular pathways regulate cell differentiation and the development of both systems[244]. In fact, the processes of neurogenesis and angiogenesis are closely related to the function of endothelial cells. On the one hand, the molecules that control axonal growth (netrins, semaphorins, ephrins) also regulate vascular development, on the other hand, the angiogenic molecules regulate the formation of neurons and axons. VEGF is an example of a molecule that participates in the development of both neuronal and vascular cells[128;245].

In the development of retina, astrocytes grow outward thus forming a pattern for the growing vessels. Initially, the developing retina has no vessels and hypoxia stimulates an up-regulation of astrocytic VEGF expression, which promotes vessel growth. Tissue oxygen supply from newly formed vessels produces a down-regulation of VEGF expression in astrocytes and their differentiation[245]. Furthermore, VEGF regulates the migration of nerve cells into the CNS. For example, the migration of facial motor neurons into the developing mouse hindbrain is regulated by VEGF and neuropilin 1[242;245]. Another ligand of the VEGF family, VEGF-C, regulates population expansion of oligodendrocyte precursor (OPC) and neural progenitor cells *in vitro*. VEGF-C acts as a trophic factor for these cells *in vivo*, as VEGF-C deficient mouse embryos show a selective loss of OPC in their optic nerve[246].

The observation that VEGF is involved in the wiring of the developing nervous system is not surprising, given that VEGF and its receptors first appeared in the nervous system of invertebrate species, such as worms and flies, which lack a well-developed vascular network[247].

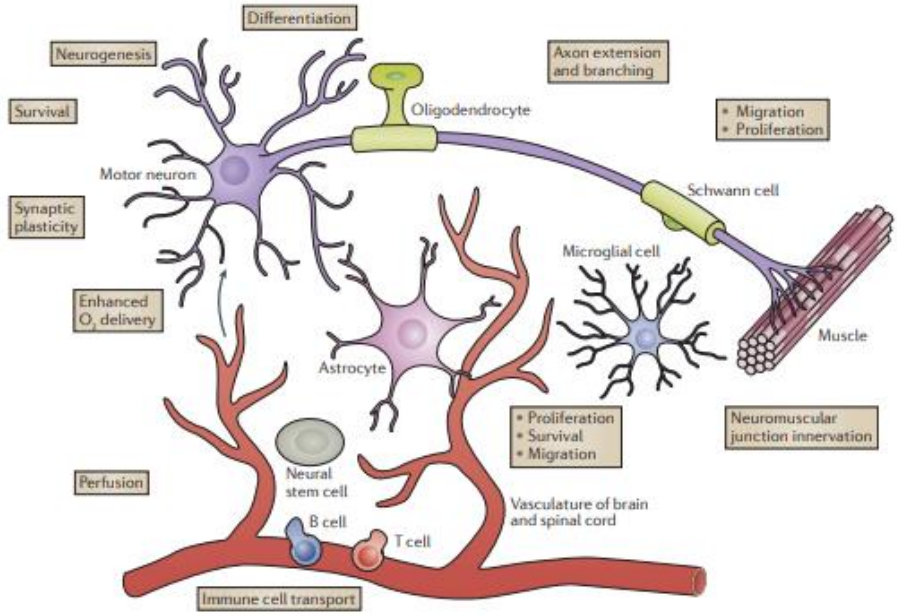


Illustration 7. ***VEGF in the CNS.*** *Vascular endothelial growth factor (VEGF) has multiple roles in the central nervous system, both by directly affecting various neural cells, and by promoting vascular perfusion, immune cell transport, and endothelial cell survival of brain blood vessels. VEGF also stimulates the production of neurogenic growth factors by endothelial cells*[247].

1.3.1. Neurogenesis

VEGF is thought to be involved in both the development of the nervous system as well as the differentiation and formation of vessels in the developing brain[248;249]. VEGF promotes endothelial cell survival and angiogenesis through a paracrine action; it also promotes neuronal survival in the CNS by autocrine and paracrine pathways. Indeed, VEGF regulates vascular growth and directly affects different types of brain cells: neuronal stem cells (NSCs), neurons, microglia and astrocytes[250]. Neurogenesis in an adult person is a dynamic process, regulated by many factors. The neurogenesis of the hippocampus, partially regulated by VEGF, is stimulated by environmental enrichment and physical exercise, learning and antidepressant drugs and is inhibited by aging. The abrogation of neurogenesis by exposure to irradiation induces fear and depressive behaviors[251;252]. On the other hand,

antidepressant drug-induced hippocampal neurogenesis is accompanied by an increase in VEGF expression leading to increased stimulation of VEGFR-2[245]. Many studies in recent years address the problem of the role of neurotrophic factors in the pathogenesis of depression and the effect of antidepressant drugs on neurogenesis and synaptic plasticity[252]. Neuronal stem cells differentiate to form neurons or glial cells. It has been shown in the hippocampus that endothelial cells can influence this process by releasing many factors that induce the differentiation of neuronal precursors. Several studies have shown that VEGF synthesized by ependymal cells (lining the ventricles of the brain and spinal cisterns), acting via VEGFR-2, stimulates the proliferation of neuronal precursors and increases the formation of new neurons in the subventricular (SVZ) and subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus. VEGF subjected to intracerebral stimulated neurogenesis in the SVZ and SGZ of the DG of the hippocampus[253;254]. *In vitro* VEGF promoted the proliferation of neuronal precursors in mouse cortical cell cultures. Integrating these cultures with VEGF increased the diameter of neuronal cells and the number of developing axons[255;256].

VEGF also promotes neurogenesis by stimulating endothelial cells to release neurotrophic factors, such as BDNF which aids neuronal survival and integration into SVZ. Many factors participate in the angiogenic and neurogenic action of VEGF. For example, erythropoietin promoted angiogenesis by increasing the release of VEGF from neuronal precursors and the regulated expression of VEGFR-2 in endothelial cells. The *in vitro* proliferation of neuronal stem cells, mediated by VEGF, was subject to the presence of the basic fibroblast growth factor (bFGF). Granulocyte colony stimulating factor (G-CSF) also stimulated neurogenesis *in vitro* by increasing the expression and release of VEGF from neuronal stem cells. This process requires VEGFR-2 since the integration of cultures with an antagonist of this receptor caused a block of neurogenesis induced by G-CSF[245;245].

Under hypoxic conditions, VEGF stimulates the growth and survival of Schwann cells that form the myelin sheath on the axons of peripheral nerves. On the other hand, experiments with a co-culture of endothelial cells, dorsal root ganglia (DRG) cells and Schwann cells showed that both types of nerve cells, by releasing VEGF,

promoted endothelial cell differentiation. In the peripheral nervous system, VEGF stimulated the proliferation and promoted survival of Schwann cells in superior cervical ganglia (SCG) and DRG explants[257;258;259].

1.3.2. Neuronal plasticity

The processes of neuronal plasticity include changes that arise during learning and memory formation, and during developmental and compensatory alterations. Brain plasticity depends on the modification of synaptic function, in particular, on the effectiveness of the transmission of nerve impulses[260]. Research results indicated that VEGF affects neuronal plasticity in the central nervous system of adult animals. In hippocampal neuronal cultures, VEGF enhanced protein synthesis by modulating Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), cAMP-responsive element binding protein (CREB) and target mammalian rapamycin kinase (mTOR). It suggests that VEGF may participate in long-term changes in synaptic efficacy. The mechanism of action of VEGF in synaptic plasticity has not been fully elucidated. At the local level, VEGF plays the role of signal modulator in neurons, increasing the influx of calcium ions and/or activating the transmembrane domains of the tyrosine kinase[261]. Long-term potentiation (LTP) is the most widely used model of synaptic plasticity. High-frequency stimulation of hippocampal axons in different brain regions induces LTP in postsynaptic neurons. Protein kinases, including CaMKII, are indispensable for the induction of LTP[260]. Experiments on hippocampal slices showed that treatment with VEGF prior to high-frequency neuronal stimulation intensified LTP while a VEGFR-2 antagonist reduced this effect. VEGF is released by hippocampal neurons even after activation of the N-methyl-D-aspartate (NMDA) receptor[245]. A single administration of NMDA to hippocampal neuronal precursor cell culture was observed to increase the proliferation and differentiation of these cells. A single injection of NMDA *in vivo* into the hippocampal dentate gyrus increased cell proliferation. NMDA regulated the survival of new hippocampal neurons indirectly by releasing mitogens such as VEGF[262]. Activation of the NMDA receptor in hippocampal neuronal precursor cells, which exhibit VEGF expression, produces an immediate influx of calcium ions and release of VEGF[262;261]. VEGF can

be released from astrocytes by "scattering" the contents of the extracellular vesicles. *In vitro*, astrocytes form extracellular structures containing fibroblast growth factor-2 (FGF-2), VEGF and integrin b1. These compounds are released after the contents of the vesicles "leak"[262;263]. Although the biological significance of VEGF secretion is not yet known, these observations suggest that VEGF release may post-synaptically influence the action of neurotransmitters[245].

1.4. VEGF-A in neuroprotection

VEGF exhibits neurotrophic and neuroprotective activity in the central nervous system and peripheral nervous system. It protects cells of the central and peripheral nervous system from death induced by a variety of damaging factors, such as hypoxia or medium deficiency[245]. *In vitro* studies on hypoxia/ischaemia models indicated that the neuroprotective action of VEGF is mediated by the PI3K/Akt and MAPK/ERK signaling pathways. VEGF inhibited ischemia-induced apoptosis by inhibiting the activity of caspase-3 and promoted the proliferation and migration of neuronal cell precursors[264;265]. The concentration of VEGF and VEGFR-2 and the expression of phosphorylated Akt and ERK was increased in a hypoxia model induced in cultured rat cerebellar granule (CGN) neurons. The neuroprotective action of VEGF has also been demonstrated after exposure of CGN to other harmful influences, such as the elimination of calcium ions from the culture medium or toxic concentrations of glutamate[266]. On the other hand, VEGF stimulated axonal growth and promoted the survival of neurons and satellite cells in dorsal root ganglion cultures. It has also shown a neurotrophic effect in organotypic cell cultures, increasing the survival of midbrain neurons[253]. In many cases VEGF increased neuronal cell survival under conditions of hypoxia, oxidative stress and deprivation of serum or glucose culture medium, by signal transduction via the VEGFR-2 and PI3/Akt pathway[120;267;268;253] (derived from the fusion of mouse hippocampal neuronal cells and neuroblastoma cells). VEGF reduced the cytotoxic effect of glutamate in hippocampal neuronal cell cultures, thereby increasing cell survival. This effect can probably be attributed to VEGF-induced inhibition of glutamate-induced overexpression of caspase-3, which is a key mediator of apoptotic neuronal cell death. The PI3/Akt and MEK/ERK-mediated signaling pathway via VEGFR-2

protects hippocampal neurons from hypoxia-induced damage[244;253;269]. Blockade of VEGF signal transduction in cortical neuronal cultures led to apoptosis[253], while deletion of hypoxic inducible factor (HIF) in the VEGF promoter in mice caused degeneration of motor neurons which it was probably the result of the suspension of an indirect neurotrophic effect of VEGF[120;270]. Thus, VEGF produces *in vitro* an indirect neurotrophic effect on many types of nerve cells, including neurons of the autonomic nervous system, sensory neurons, dopaminergic and hippocampal, cerebellar and cortical nerve cells[259].

In vivo studies in rats exposed to hypoxia through middle cerebral artery occlusion indicated an increase in VEGF mRNA in the hypoxic area. VEGF administered directly to the brain surface resulted in a reduction in the hypoxic area, while its intravenous administration reduced cortical neuronal damage. The exogenous application of VEGF stimulated the maturation of new neurons in the hypoxic area and resulted in the neuroprotective effect. It did not affect angiogenesis and glia proliferation. On the other hand, VEGF at high concentrations was found to be harmful because its proangiogenic action causes edema in the stroke area which worsens the prognosis[271;272]. An increased of *in vivo* VEGF expression was observed mainly in the brains of mice exposed to chronic hypoxia. The increase in VEGF expression was observed in both neurons and glial cells. VEGF had a neuroprotective effect on these cells[266]. VEGF increased the proliferation and migration of astrocytes. Its mitogenic action on these cells has been demonstrated both in cell cultures isolated from the midbrain and *in vivo* after intracerebral administration. Reactive astrocytes have been shown to express VEGFR-1, but not VEGFR-2. This suggests that VEGFR-1 mediates the mitogenic action of VEGF on astrocytes. Furthermore, the activation of VEGFR-1 by VEGF also participates in the stimulation, migration and proliferation of microglia[259]. Thus, the VEGFR-2 signaling pathway is mainly related to the action of VEGF on Schwann cells and peripheral neurons, while VEGFR-1 mediates the effects of VEGF on astrocytes and microglia[120;259]. With its neuroprotective action, VEGF influences hippocampal-dependent processes, such as learning and memory[244]. Changes in VEGF concentration have been shown to occur in diseases such as stroke, amiotropic

lateral sclerosis, Parkinson's disease, and Alzheimer's disease, making VEGF a potential target for neuroprotective drugs[273].

1.5. VEGF-A in pain transmission

Our nervous system is capable of detecting a wide range of stimuli which can evoke pain. These can generate a short-term sensation (acute pain) which usually resolves. However, sometimes this pain becomes persistent. Constant stimulation provokes alterations in nociceptive transmission, enhancing pain signals and increasing sensitivity. If this state persists for more than 3 months, it is defined as chronic pain. It affects over one-quarter of people worldwide and is more prevalent in women than in men. The mechanisms that sustain and drive chronic pain have been comprehensively reviewed[274;275;276]. The current analgesic treatments (opioids or NSAIDs) do not meet patients needs or are inefficient. In addition, their side effects limit their use. Therefore, the development of new drugs is urgently required. Preclinical research studies have identified an array of molecular targets that are involved in the establishment and maintenance of chronic pain and may represent interesting targets for pharmacological intervention. Among these mediators, VEGF has been postulated as a key factor.

1.5.1. VEGF as a therapeutic target

Alterations in the VEGF system, characterized by changes in the expression of its components, have been related to a plethora of diseases. Some of these diseases can occur concomitantly with pain, such as cancer, rheumatoid arthritis or diabetic complications[163;277;278]. There are currently several anti-VEGF-related drugs used in clinical settings for the cancer treatment in combination with chemotherapy. Anti-VEGF drugs are also used to attenuate neovascularization in age-related macular degeneration and diabetic macular edema[163;279]. However, in recent years, the role of the VEGF family in neuroprotection and nociception has received increased attention[280;281;282;283;284;285;286;287]. The involvement of VEGF in the pathophysiology of pain is not fully understood, however, the association between this growth factor and some of the main hallmarks of painful diseases warrants the investigation of VEGF as a therapeutic target for pain treatment.

1.5.2. Involvement of VEGF in several types of pain

Inflammation is a common feature in different painful syndromes and its components sensitize nociceptors which mediate pain sensation. VEGF is one of the most important mediators participating in this pro-inflammatory scenario.

The significance of VEGFR-1 and VEGFR-2 in the pathophysiology of two of the most prevalent chronic inflammatory diseases that are concurrent with pain, namely rheumatoid arthritis (RA) and osteoarthritis (OA), has previously been reported by Hamilton et al. in 2016[288]. Inhibition of VEGF signalling pathways and angiogenesis emerged as a promising approach demonstrating reduced destruction of joints and associated pain in OA[288]. However, the role of each member of the VEGF family, isoforms, or alternative splicing in alleviating chronic inflammatory pain is yet to be clarified.

In osteoarthritis, anomalous VEGF expression in synovial fluids has been associated with higher pain scores[289] and worse prognosis. VEGF seems to mediate cartilage degeneration, bone and neurovascular invasion of articular cartilage, increased migration and/or activity of macrophages, fibroblasts, and neutrophils. These cells, in turn, increase levels of cytokines and VEGF, amplifying the inflammatory response[288;290]. VEGF is able to evoke pain by several pathways in synovium, osteochondral junction and meniscus, through both VEGFR-1 and VEGFR-2[290]. Both signalling axes seem to be directly associated with nociceptor sensitization, and accordingly, VEGF signalling inhibition led to a decreased pain[288]. In addition, other VEGF approaches have been experimentally tested and successfully counteracted pain responses and/or improved cartilage degeneration, synovitis and osteophyte formation[291;288]. Taking all of the aforementioned, it seems plausible that proper VEGF therapies targeting ligands or receptors could counteract osteoarthritis progression and its associated pain. In other painful chronic diseases with an autoimmune component, such as rheumatoid arthritis (RA), one of the most potent factors that seems to be responsible for the typical hypertrophied synovium (pannus), oedema, swelling, and chondrolytic and osteolytic reactions, is VEGF[292;293]. VEGF is expressed in synovial fibroblasts, fibroblasts close to microvessels, vascular smooth muscle and macrophages, but not in endothelial cells of patients with RA[294].

VEGF is augmented in patients' serum and is tightly correlated with TNF-α and some other pro-algesic cytokines (IL-1ß, IL-17, IL-18) which in turn reduce VEGF expression, except in patients who are refractory to TNF-α therapy[186;281]. At experimental level, an increased expression of VEGF, VEGFR-1 and VEGFR-2 was described in an RA animal model and the treatment with an anti-VEGFR-1 efficiently blocked pain. However, the neutralization of either the VEGF ligand or VEGFR-2 did not induce the same anti-nociceptive effect[295]. Contrastingly, other authors suggested that VEGFR-2 acts as a positive transducer in vascular proliferation during RA and its pharmacological blockade reduces mechanical sensitivity in an animal model of RA[281]. While Beazley-Long and colleagues stated that when VEGFR-2 is inhibited allodynia is reduced and/or prevented[281], De Bandt and colleagues showed VEGFR-2 suppression was insufficient for resolution of this type of pain[295]. Further studies aimed to address this discrepancy are needed. The putative role of VEGF in the relief of pain has been most extensively studied in neuropathic pain compared to other types of pain. VEGF-A has been strongly linked with neuroprotection and its neutralization was found to exacerbate neuropathic damage and pain in a retrospective clinical study[296]. Contrary to this, experimental approaches of VEGF blockade have successfully alleviated nociceptive responses in a model of chronic constriction injury, sciatic nerve ligation or diabetic neuropathy. These approaches included the suppression of VEGFR-2 signalling, spinal serine arginine protein kinase 1 (SRPK1) inhibition, and the administration of VEGF-Axxxb. The anti-nociceptive effect derived from VEGFR-2 blockade in painful neuropathies has been reported to be mediated via the interaction with P2X2/3 receptors[295;297], or TRPA1 and/or TRPV1[283;284;298]. *In vitro* studies revealed that in injured peripheral nerves there is an upregulation of VEGF-A in infiltrated cells that seems to mediate angiogenesis, a key component of chronic inflammation and peripheral sensitization[299]. Blocking VEGF-A has been shown to reduce nociception in rodents and to exert a neuroprotective effect by improving neuronal restoration and conduction, decreasing pro-apoptotic Caspase-3 levels in sensory neurons, preventing neural perfusion and epidermal sensory fiber loss[300;283;298]. Another plausible strategy evaluated is SRPK1 inhibition, as this would reduce the pro-

nociceptive and pro-angiogenic forms of VEGF[286]. Several studies indicate that administration of VEGF-A165b (the reported anti-nociceptive form of VEGF-A), could constitute an interesting therapeutic strategy for pain, considering that it also has neuroprotective effects. Contrastingly, a recent study demonstrated in an animal model of oxaliplatin-induced pain, that VEGF-A165b expression is augmented in spinal cord, and the intrathecal administration of bevacizumab or VEGF-A165b antibody reversed the hypersensitivity symptoms[301]. Most studies at an experimental level seem to suggest a pro-nociceptive effect induced by VEGF-A in several types of pain. However, in neuropathic pain due to a partial saphenous nerve ligation injury, Hulse and colleagues focused on the anti-nociceptive effect of VEGF-Axxxb isoform and the relevance of targeting its alternative splicing so as to modulate the balance between the pro- and anti-nociceptive VEGF isoforms. VEGF-A165a (pro-nociceptive) and VEGF-A165b (anti-nociceptive) isoforms have opposing actions on vascular permeability, angiogenesis, and vasodilatation[284]. This had been shown extensively in several papers[282;283;284;286;281]. However, in a model of oxaliplatin-induced pain, Di Cesare Mannelli and colleagues[301] clearly showed the pro-nociceptive role of VEGF-Axxxb isoform. Further studies are urgently needed in order to clarify the role of VEGF-Axxxb and the mechanisms underlying the paradoxical effects reported. These disparate functions raise the possibility that different isoforms may have pro- and anti-nociceptive varying role. Among chronic neurologic diseases, migraine is the third most prevalent and disabling; current treatments are usually unsuccessful. The meningeal and brain mast cells involved can degranulate and release vasoactive substances that can activate trigeminovascular mechanisms inducing pain. Among these mediators, VEGF is one of the most important as it also stimulates nitric oxide synthase and therefore increases nitric oxide levels[302]. Therefore, VEGF plays a direct role in the endothelial cells in the trigeminovascular system. Indeed, increased levels of VEGF have been showed in migraineurs suggesting endothelial alterations[303]. However, decreased serum concentrations of VEGF were found during interictal period[304]. In addition, several VEGF haplotypes have been described to be associated with variable susceptibility to migraine[305]. A better understanding of VEGF fluctuations, genetic

profiling and the potential protective role in migraines could constitute an interesting approach for prophylactic intervention. The importance of VEGF in cancer pathophysiology and therapy has been extensively reported[306;307]. However, the potential anti-nociceptive effect of VEGF in cancer-induced pain is poorly understood. VEGFR-1 is augmented in humans and in an animal model of osteosarcoma-induced pain. The modulation of VEGF/VEGFR-1 axis signalling by an anti-VEGFR-1 antibody or the administration of the VEGFR-1 soluble form (sFlt-1) that decoys VEGF from binding VEGFR-1, effectively counteracted pain[285]. Perturbing expression, activation, or signalling of VEGFR-1, but not of VEGFR-2, in peripheral sensory nerves disrupts attenuated cancer-induced pain and tumor-induced remodelling of nerves in mice *in vivo*[285]. Additional studies using experimental models of cancer-induced pain that address the role of VEGFR-2 are urgently required in order to delineate the role for this integral mediator. This will inform the design and development of new pharmacological strategies.

2. Scope

Vascular Endothelial Growth Factor-A (VEGF-A) is the most studied member of VEGF family. In addition to its well-known pro-angiogenic properties, it also directly influences neuronal and glial biological processes, exerting trophic and signaling functions in nervous tissue. Moreover, the role of VEGF-A in pain has emerged in recent years. About this, the recent data obtained in our laboratory showed that VEGF-A plays an important role in pain modulation both in Peripheral and Central Nervous System[301], highlighting the necessary of an in-depth study about the role of VEGF family in the perception and pathophysiology of pain.

The purpose of the present study was to investigate the role of VEGF-A as a pain and neuroprotective mediator, identifying the VEGF receptors and the nervous cells involved in these signaling pathways.

In the first part of this thesis, we evaluated the pain threshold of naïve mice following intrathecal administration of non-selective and selective ligands for VEGFR-1 and VEGFR-2 and, in parallel, of receptor blockers. Immunofluorescence analysis and the selective silencing of the two VEGFRs by siRNAs in the lumbar spinal cord of naïve mice, confirmed that neuronal VEGFR-1 mediates the pro-algic action of VEGF-A in physiological conditions. The higher expression of VEGF-A on astrocyte cells compared to neurons and microglia, led us to selectively silence VEGF-A in spinal astrocytes by injecting a viral vector into naïve mice. Behavioral tests showed a prevention of hypersensitivity development caused by oxaliplatin treatment. In addition, the selective block of VEGFR-1 by mAb D16F7 showed a pain-relieving action in different models of neuropathic pain.

Following these data in the last part of this study, to deepen pain modulation and the neuroprotection mechanisms of VEGF-A in nervous tissue, we used the organotypic spinal cord slice. We evaluated the molecular mechanism underlying the release of painful factors induced by VEGF-A, confirming the involvement of VEGFR-1. Moreover, like other growth factors, also VEGF in characterized by a dualism between pro-algic and protective effects. Analyzing the effect of VEGF treatment on toxicity induced by oxaliplatin in the slices, we highlighted that VEGF-

A exerts a neuroprotective effect by the interaction with its VEGFR-2.

Ultimately, following the *in-vivo* studies evidences on the involvement of astrocytic VEGF-A in pain signaling, we used fluorocitrate as a glial metabolism blocker, to deepen the involvement of astrocytic VEGF-A both in physiological and oxaliplatin-treatment conditions.

3. Materials and Methods

3.1. Study approval

All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes and with IASP. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 171/2018-PR) and from the Animal Subjects Review Board of the University of Florence and from the Animal Ethics Committee of University of Campania of Naples. Experiments involving animals have been reported according to ARRIVE guidelines[308]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2. Animals

Eight-week-old male CD-1 mice (Envigo, Varese, Italy) weighing 20-25 g at the beginning of the experimental procedure were used. Animals were housed in the Centro Stabulazione Animali da Laboratorio (University of Florence, Italy) and in Stabulario Centralizzato di Ateneo (University of Campania "Luigi Vanvitelli", Naples, Italy) and used at least 1 week after their arrival. Ten mice were housed per cage (size 26 cm x 41 cm); animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12 h light/dark cycle (light at 7 am).

3.3. Treatments

VEGF165b (3 – 30 ng; 5 μl; cat. #3045-VE-025, R&D System, USA), PlGF-2 (3 – 30 ng; 5 μl; cat.465-PL/CF, R&D System, USA), VEGF-E (3 – 30 ng; 5 μl; cat. #CYT-263, Prospec, Israel), D16F7 (10 − 100 ng, 5 μg; 5 μl)[309] and DC101 (100 pg, 1 – 6 ng; 5 μl; catalog. #BE0060 BioCell, Boston, MA, USA) or vehicle (0.9% NaCl 5 μl) were intrathecally (i.t) injected in conscious mice as previously described (Hylden & Wilcox, 1980). Briefly, a 25-μl Hamilton syringe connected to a 30-gauge needle was

intervertebral inserted between the L4 and L5 region, and advanced 6 mm into the lumbar enlargement of the spinal cord. Behavioural measurements were performed before and 30 min, 1 h, 3 h and 6 h after the administration of compounds. DC101 or D16F7 were injected 15 min before the VEGFR-1/2 agonists when administered in the co-treatment experiments.

The scrambled siRNA or the specific VEGFR siRNA (VEGFR-1-VEGFR-2 siRNA, Ambion Life Technologies, Monza, Italy) were i.t. injected twice spaced 24 h apart (3.3 μg/5 μl per mouse) at the lumbar level of the mice spinal cord. On the third day, behavioural measurements were conducted after VEGFRs agonists administration. Mice were sacrificed between the 4th and the 5th days for the Western blot analysis. The target sequences of the anti-mouse VEGFRs siRNAs were: VEGFR-1, sense strand 5′-GCAUCUAUAAGGCAGCGGAtt-3′ and antisense strand UCCGCUGCCUUAUAGAUGCtc-3′; VEGFR-2, sense strand 5′-CCCGUAUGCUUGUAAAGAAtt-3′ and antisense strand 5′-UUCUUUACAAGCAUACGGGct-3′.

3.4. AAV virus infection

An AAV1-GFAP-eGFP-mVEGFA-shRNAmir (1.6 × 1013 GC/ml, Vector Biosystem Inc, Malvern, PA, USA) or scrambled were used. Mice were deeply anaesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg kg-1) (Ketavet, MSD Animal Health, Milan, Italy) and xylazine (10 mg kg-1) (Rompum, 20 mg/ml, Bayer, Milan, Italy) and then were placed in a stereotaxic frame using the mouse spinal adaptor (Stoelting, Wood Dale, IL, USA). The skin was incised at Th12–L5 and the mouse muscles around the left side of the interspace between Th12 − L1 and L4 - L5 vertebrae were removed, and the dura mater and the arachnoid membrane were carefully incised using the tip of a 30G needle to make a small window to allow vector infusion. Four intraspinal injections were done in aech animal using a 5-μl Hamilton syringe connected to a 34G needle. The needle was placed 0.5 mm lateral to the spinal midline at a depth of 0.4 mm from the dorsal surface of the spinal cord and 1 μl of vector or scrambled was bilaterally injected at 0.25 μl/min with a digital microinjector (Stoelting). The needle was left on place for another 3 min to prevent

backflow. The surgical site was then sutured with 3-0 silk and mice were kept on a heating pad until recovery.

3.5. Chemotherapy-induced neuropathic pain (CINP) *in vivo* models

Mice treated with oxaliplatin (Carbosynth, Pangbourne, UK; 2.4 mg kg^{-1}) were administered i.p. for two weeks[310;311]. Oxaliplatin was dissolved in a 5% glucose solution. Control animals received an equivalent volume of vehicle. Behavioural tests were performed on day 15. In mice injected spinally with the viral vector or with the scrambled, oxaliplatin was administered for two weeks (10 total injections) starting 14 days after surgery for the viral vector administration. Control animals received an equivalent volume of vehicle. Behavioural measurements were performed on days 3, 5, 9, 11, 13 and 15.

Mice treated with paclitaxel (Carbosynth; 2.0 mg kg^{-1}) were injected i.p. on four alternate days (days 1, 3, 5 and 8)[312;311]. Paclitaxel was dissolved in a mixture of 10% saline solution and Cremophor EL, a derivative of castor oil and ethylene oxide that is clinically used as paclitaxel vehicle. Control animals received an equivalent volume of vehicle. Behavioural measurements started on day 10.

Mice treated with vincristine (Carbosynth; 0.1 mg kg^{-1}) were injected i.p. for five consecutive days[313]. Vincristine was dissolved in saline solution and control animals received an equivalent volume of vehicle. Behavioural measurements started on day 8.

3.6. Assessment of mechanical hyperalgesia (Paw pressure test)

Mechanical hyperalgesia was determined by measuring the latency in seconds to withdraw the paw away from a constant mechanical pressure exerted onto the dorsal surface[314;315]. A 15 g calibrated glass cylindrical rod (10 mm diameter) chamfered to a conical point (3 mm diameter) was used to exert the mechanical force. The weight was suspended vertically between two rings attached to a stand and was free to move vertically. A single measure was made per animal. A cut-off time of 40 s was used.

3.7. Assessment of thermal allodynia (Cold plate test)

Thermal allodynia was assessed using the Cold-plate test. With minimal animal-handler interaction, mice were taken from home-cages, and placed onto the surface of the cold-plate (Ugo Basile, Varese, Italy) maintained at a constant temperature of 4°C ± 1°C. Ambulation was restricted by a cylindrical plexiglas chamber (diameter: 10 cm, height: 15 cm), with open top. A timer controlled by foot peddle began timing response latency from the moment the mouse was placed onto the cold-plate. Pain-related behaviour (licking of the hind paw) was observed, and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 30 s[316].

3.8. Assessment of mechanical allodynia (Von Frey test)

Mechanical allodynia was measured with the dynamic plantar aesthesiometer (von Frey instrument) (Ugo Basile) as described by Di Cesare Mannelli and colleagues[311] with minor modifications. Briefly, the mice were placed in individual Plexiglas cubicles (8.5 × 3.4 × 3.4 cm) on a wire mesh platform. After approximately 30 min accommodation period, during which exploratory and grooming activity ended, the mechanical paw withdrawal threshold was measured as the hind paw withdrawal responded to von Frey hair stimulation. The mechanical stimulus was delivered to the plantar surface of the hind paw of the mouse from below the floor of the test chamber by an automated testing device. A steel rod (2 mm) was pushed with electronic ascending force (0–5 g in 35 s). When the animal withdrew its hind paw, the mechanical stimulus was automatically withdrawn, and the force recorded to the nearest 0.1 g. Nociceptive response for mechanical sensitivity was expressed as mechanical withdrawal threshold in grams. The mean was calculated from six consecutive trials and averaged for each group of mice.

3.9. Assessment of locomotor activity (Hole-Board test)

The locomotor activity was evaluated by using the hole-board test. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4×4 in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of

5 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice[317].

3.10. Electrophysiological recordings of nociceptive specific (NS) neurons

On the day of electrophysiological recordings, mice were initially anesthetized with tribromoethanol (Avertin, Winthrop laboratories, New York, NY, USA; 1.25%). After tracheal cannulation, a catheter was placed into the right external jugular vein, to allow continuous infusion of propofol (5–10 mg/kg/h, i.v.) and spinal cord segments L4-L6 were exposed by laminectomy, near the dorsal root entry zone, up to a depth of 1 mm[318]. An elliptic rubber ring (about 3×5 mm), sealed with silicone gel onto the surface of the cord, was used for topical spinal drug application and to gain access to spinal neurons. Animals were fixed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) through clamps attached to the vertebral processes. Single unit extracellular activity of dorsal horn NS neurons was performed by using a glass-insulated tungsten filament electrode (3–5 MΩ) (FHC Frederick Haer & Co., ME, USA). Spinal neurons were defined as NS neurons, when they were responding only to high intensity (noxious) stimulation[319]. In particular, to confirm NS response patterns, each neuron was characterized by applying a mechanical stimulation to the ipsilateral hind paw using a von Frey filament with 97.8 mN bending force (noxious stimulation) for 2 s until it buckled slightly[320;321]. Only neurons that specifically responded to noxious hind paw stimulation were considered for recordings. The recorded signals were visualized into a window discriminator, whose output was processed by an interface CED 1401 (Cambridge Electronic Design Ltd., UK) connected to iOS 5 PC. Spike2 software (CED, version 5) was used to create peristimulus rate histograms on-line and to store and analyze digital records of single unit activity off-line. The spontaneous and noxious-evoked neuronal activity was expressed as spikes/sec (Hz) and the effect of drugs was

analyzed as % variation of firing rate, frequency and duration of excitation. After recording a stable basal activity (15 min), topical spinal application of vehicle or drugs was performed, and each extracellular recording was monitored until 45-60 min post-injection. In particular, groups of animals were divided as following: 1) VEGF165b (3 ng/5 µl, pro-nociceptive dose on NS neurons), 2) VEGF165b + DC101 (10 pg/5 µl, the highest non-pro-nociceptive dose) and 3) VEGF165b + D16F7 (100 pg/5 µl). At the end of the experiment, animals were killed with a lethal dose of urethane.

3.11. Preparation of Rat Organotypic Spinal Cord Slice Cultures

Spinal cord was removed from 4- to 6-day old Sprague Dawley (Envigo, Varese, Italy) rat pups and exposed. Transverse slices (420 µm) were prepared using a McIlwain tissue chopper and then transferred onto 30 mm diameter semiporous membranes inserts (Millicell-CM PICM03050; Millipore, Italy), which were placed in six well tissue culture plates containing 1.2 mL medium per well. The culture medium consisted of 50% Eagle's minimal essential medium, 25% heat-inactivated horse serum, 25% Hanks' balanced salt solution, 5 mg/mL glucose, 2 mM L-glutamine, 3.75 mg/mL amphotericin B and 1% of penicillin (100 U/mL) and streptomycin (100 µg/mL). Slices were maintained at 37 °C in an incubator in atmosphere of humidified air and 5% CO_2 for 14 days.

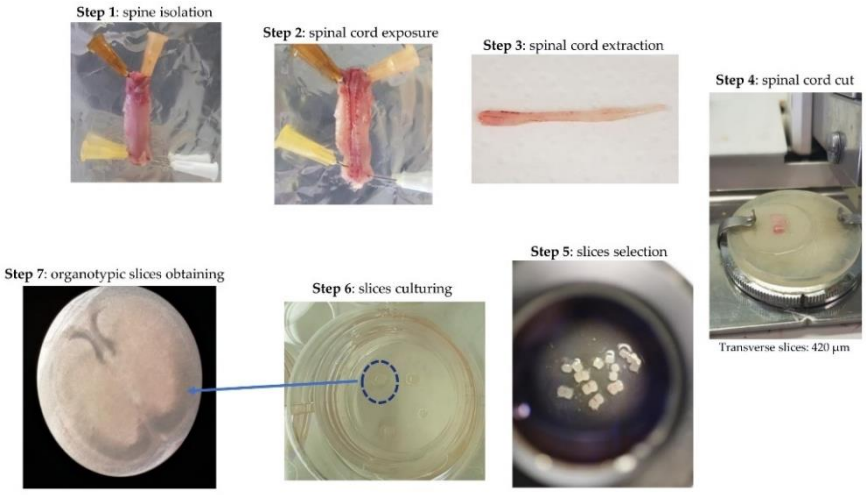


Illustration 8. Schematic illustration of the steps followed to obtain the organotypic culture of the spinal cord, starting from rat pups 4 - 6 PND

3.12. Organotypic Spinal Cord Cultures Treatments

On the 14th day, organotypic spinal cord culture was exposed to: oxaliplatin 1, 10 and 100 μM for 1-3-6 and 24 hours; VEGF165b, VEGF-E and PlGF 30, 100 and 300 ng/mL; DC101 10 ng/mL; D16F7 300 ng/mL; DL-Fluorocitric acid barium salt (Sigma Aldrich- F9634) 80 μM. For toxicity analysis, Propidium iodide (PI) 5 μg/mL (Sigma Aldrich- 81845) was added to the medium and thirty minutes later, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, United Kingdom) equipped with a xenon-arc lamp, a low-power objective (4X) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, United States) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, United States). In order to quantify cell death, the optical density of PI fluorescence was detected using the design function in the image software (ImageJ; NIH, Bethesda, MD, USA).

3.13. Biochemical analysis

At the end of the treatments, the culture medium was collected and used to biochemical analysis. The concentrations of VEGF-A, CGRP and Substance-P were assessed by enzyme-linked immunosorbent assay (ELISA Kit ThermoFisher-ERVEGFA for VEGF-A; BIOMATIK-EKF58049 for CGRP; MyBioSource-MBS703659 for Substance-P), according to manufacturer's instructions. The levels were normalized to cell protein concentrations.

3.14. RNA isolation, Reverse Transcription and Real Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from rat organotypic spinal cord slices using TRI Reagent (Merck, Milan, Italy). One microgram of RNA was retrotranscribed using PrimeScript™ RT reagent Kit with gDNA eraser (Takara Bio cat#RR047A).

RT-PCR was performed using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) following the thermal profile suggested by the kit. The following primers were used: EAAT1: forward 5′- CAGTCATCGTCGGCCTCCTCATTC -3′ and reverse 5′-CTGGTGATGCGTTTGTCCACACCATTG -3′ (Invitrogen); validated primers for

rEAAT2 and rGAPDH were purchased from Biorad (qRnoCED0005967 and qRnoCID0057018).

The differential expression of the transcripts is normalized on the housekeeping gene GAPDH.

3.15. Western blot analysis

The lumbar spinal cord of mice was explanted and immediately frozen with liquid nitrogen. The frozen tissues were homogenized with lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and complete protease inhibitors (Roche, Milan, Italy). The suspensions were sonicated on ice using three high intensity 10s bursts with a cooling period of 10s each burst and centrifuged at 13.000xg for 10 min at 4°C. Protein concentrations were quantified by bicinchoninic acid test. Fifty μg of tissue homogenate were resolved with prefabricated polyacrylamide gel (BOLT 4-12% Bis-Tris Plus gel; Thermo Fisher Scientific, Monza, Italy) before electrophoretic transfer to nitrocellulose membranes (Bio-Rad, Milan, Italy). The membranes were blocked with 1% BSA and 5% fat-free powdered milk in PBS containing 0.1% Tween 20 (PBST) and then probed overnight at 4°C with primary antibodies specific for VEGFR-1, VEGFR-2, VEGF-A, GAPDH or α-Tubulin (Supplementary Table S1). The membranes were then incubated for 1 h in PBST containing the appropriate secondary anti-rabbit or anti-mouse antibody (Supplementary Table S1). ECL (Enhanced Chemiluminescence Pierce, Rockford, IL, USA) was used to visualize peroxidase-coated bands. Densitometric analysis was performed using the "ImageJ" analysis software (ImageJ, NIH, Bethesda, MD, USA). Normalization for α-tubulin or GAPDH content was performed. The values were reported as percentages of controls arbitrarily set at 100%.

3.16. Immunofluorescence staining and confocal imaging

Mice were sacrificed, the L4/L5 segments of the spinal cord were exposed from the lumbovertebral column via laminectomy and identified by tracing the dorsal roots from their respective DRG. Formalin-fixed (and no-fixed, used for VEGFR-1 primary antibody) cryostat sections (7 μm) were washed 3x with phosphate-buffered saline (PBS) and then were incubated, at room temperature for 1 h, in

blocking solution (PBS, 0.3% Triton X-100, 5% albumin bovine serum; PBST). The sections were subsequently incubated with primary antibody, anti-VEGFR-1, anti-VEGF-A, or anti-AQP-4, overnight at 4°C (Supplementary Table S1). The following day, slides were washed 3× with PBS and then sections were incubated in the dark with secondary antibody, goat anti-rabbit or anti-mouse IgG labeled with Alexa Fluor 568, in PBST at room temperature for 2 h. After 3× PBS 0.3% Triton X-100 wash for 10 min, the sections were incubated with DAPI, a nuclei-marker, at room temperature for 5 min and then the slides were mounted using Fluoromount(tm) (Life Technologies-Thermo scientific, Rockford, IL, USA) as a mounting medium.

For double immunofluorescence, on the first day, anti-Iba1 was added, and the slides incubated overnight at 4°C conditions. While the sections to be labelled for GFAP or NeuN were incubated the second day for 2 h in the dark with mouse anti-GFAP Alexa Fluor 488-conjugated or mouse anti-NeuN Alexa Fluor 488-conjugated antibodies (Supplementary Table S1). For triple immunofluorescence, on the first day, anti-RECA-1 was added, and the slides incubated overnight at 4°C conditions, then sections were incubated with the anti-mouse IgG labeled with Alexa Fluor 568 for 2 h. Thereafter, incubation with anti-VEGF-A and anti-GFAP antibodies was allowed overnight in the dark. Finally, anti-mouse IgG labeled with Alexa Fluor 488 and anti-rabbit IgG labeled with Alexa Fluor 647 were added for 2 h in the dark (Supplementary Table S1).

The immunofluorescence analysis on organotypic slices was performed using the free-floating method.

Negative control sections (no exposure to the primary antisera) were processed concurrently with the other sections for all immunohistochemical studies. Images were acquired using a motorized Leica DM6000 B microscope equipped with a DFC350FX camera (Leica, Mannheim, Germany).

The colocalization area was calculated using the "colocalization" plugin of ImageJ (after evaluating the threshold value for each channel) and expressed as percentage relative to the value of the VEGFR-1 or VEGF-A area. The mean fluorescence intensity of VEGF-A in control and oxaliplatin-treated animals was calculated by subtracting the background (multiplied by the total area) from the VEGF-A

integrated intensity. Analyses were performed on three different images for each animal, collected through a 20x objective.

For confocal analysis, images were acquired with a Leica SP2 AOBS confocal microscope using a sequential scan setting (exciting lasers 488 nm and 561nm) to avoid channel bleed-through. Images were acquired though a 63x 1.4NA PL APO objective at voxel size of 232nm (xy) and 121nm (z).

Confocal images were processed and analyzed using Fiji[322]. Briefly, images were deconvolved using Deconvolution Lab2 with a synthetic PSF and ICTM algorithm[323]. Colocalization analysis was performed with JACoP (Fiji plugin)[324] and manually set thresholds. Colocalization parameters were calculated from 8 confocal z-stacks for each analysis and are given as mean ± SEM.

3.17. Statistics

Results were expressed as means ± SEM and the analysis of variance was performed by ANOVA test. A Bonferroni's significant difference procedure was used as post hoc comparison. P values less than 0.05 were considered significant. Data were analysed using "Origin® 10" software.

Electrophysiological data were analysed through one-way ANOVA followed by Dunnet's multiple comparison post-hoc test for statistical significance within groups. Two-way ANOVA followed by Bonferroni post-hoc test for comparison between groups, by using GraphPad Prism 7.0.

4. Results

4.1. Nociceptive effect of VEGFRs selective ligands infused in spinal cord

To study the spinal impact of VEGF-A signalling modulation on pain threshold in mice, we firstly evaluated the effect of the most expressed isoform VEGF165b. After i.t. administration, pain sensitivity was measured as latency response to a cold stimulus (Cold plate test). As shown in Fig. 1A, VEGF165b (3, 10 and 30 ng, in bolus in a total volume of 5 μl) dose-dependently reduced pain threshold with a long-lasting effect starting 30 min after injection that completely disappeared only after 6 h, resembling its effect observed in rats[301]. To note, VEGF165a (1, 3 and 30 ng, i.t.) evoked similar dose-dependent nociceptive effects (Supplementary Fig. S1). Since VEGF165 isoforms may interact with both VEGFR-1 and VEGFR-2, in order to explore the implications of the receptor types in pain modulation, we also tested the effect of placental growth factor 2 (PlGF-2) and VEGF-E, which are specific VEGFR-1 and VEGFR-2 agonists, respectively[325]. As shown in Fig. 1B and 1C, both PlGF-2 and VEGF-E (3, 10 and 30 ng, i.t.) significantly reduced the licking latency of animals challenged on a cold surface (Cold Plate test), even if PlGF-2 showed a profile similar to VEGF165b while VEGF-E exhibited a lower efficacy. Interestingly, the selective VEGFR-1 blockade by the anti-VEGFR-1 mAb D16F7 (1 μg, i.t.), in the absence of VEGF165b, did not significantly alter pain threshold at microgram dose (Fig. 1D). On the contrary, nanogram dose of the anti-murine VEGFR-2 mAb DC101 (1 and 6 ng, i.t.) induced hypersensitivity (Fig. 1E) and this effect was blocked by D16F7 mAb (10 and 100 ng; Fig. 1F). In this test, non-specific mouse IgG (1 μg), used as control, was inactive. These findings suggested that the nociceptive effects evoked by VEGF165b were the result of VEGFR-1 stimulation. Furthermore, algesic effects induced by the DC101 mAb were likely due to the antibody-dependent displacement of the endogenous VEGF-A from VEGFR-2, thus making it available for binding to VEGFR-1; this hypothesis was further demonstrated by the loss of the effect when the anti-VEGFR-1 mAb D16F7, was administered together with DC101.

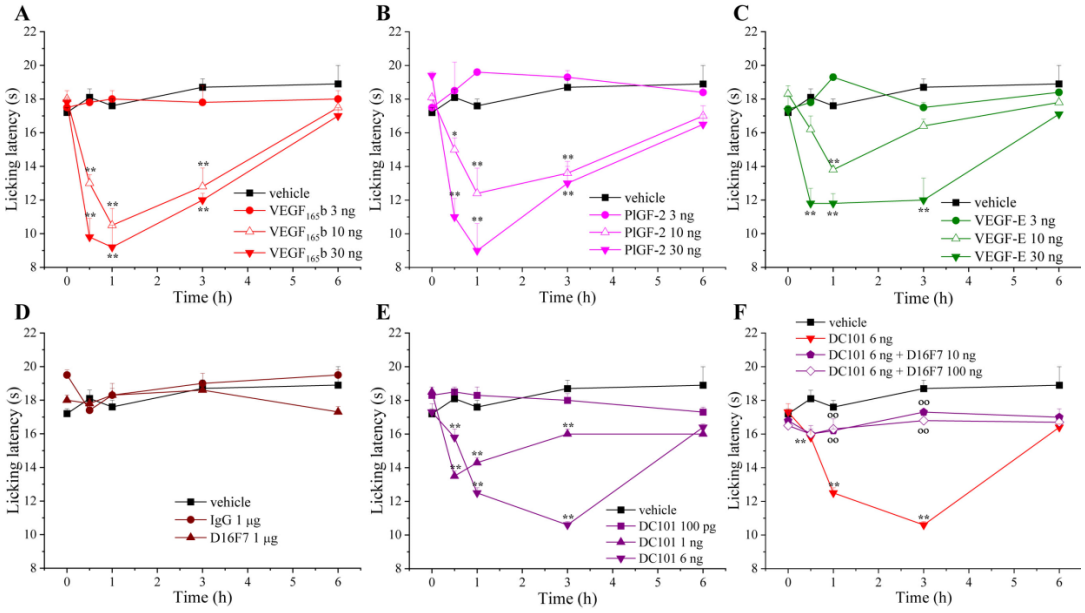


Figure1. Nociceptive effect of VEGFRs selective ligands infused in spinal cord. *The pain threshold was measured by the Cold plate test over time after the i.t. injection of compounds. Effect of (A) VEGF165b (n=7), (B) the selective VEGFR-1 agonist PlGF-2 (n=5), (C) the selective VEGFR-2 agonist VEGF-E (n=5), (D) the selective anti-VEGFR-1 antibody D16F7 (n=7) or a murine control IgG (n=5) and (E) the selective anti-VEGFR-2 antibody DC101 (n=5). (F) Effect of DC101 in mice pre-treated (15 min before) with D16F7. Each value represents the mean ± SEM. *P<0.05 and **P<0.01 vs vehicle-treated animals; °°P<0.01 vs DC101 6 ng treated animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

4.2. Hypersensitivity-induced by VEGF-A signalling modulators is due to VEGFR-1 activation

The hypothesis that VEGFR-1 activation is required for VEGF165b-mediated nociception was demonstrated by crossing the combinations of receptor agonists and antagonists. Both selective agonists, PlGF-2 and VEGF-E[326;327;328;329], share the same binding sites of VEGF-A on the corresponding receptors. At variance with DC101 mAb which is a competitive antagonist of VEGF-A and VEGF-E for VEGFR-2 binding[330], D16F7 mAb is a non-competitive antagonist since it interacts with VEGFR-1 at a site different from that used by the receptor ligands[309;331]. Consistently with our hypothesis, the algesic effects of VEGF165b are blocked by D16F7 mAb (Fig. 2A). A similar profile was obtained also for the VEGFR-1 ligand PlGF-2 (Fig. 2B) as well as for the VEGFR-2 ligand VEGF-E (Fig. 2C). DC101 mAb used at the

highest non-algesic dose (but able to selectively block VEGFR-2)[330] did not block the effect of both VEGF165b and PlGF-2, but further exacerbated VEGF-E hypersensitivity (Supplementary Fig. S2). These findings confirmed the pivotal role of VEGFR-1 in pain signalling which is directly activated by the selective agonist PlGF-2 or by the exogenously added (Fig. 2A) or endogenously present VEGF-A (Fig. 2C) displaced from VEGFR-2. Moreover, the selective knockdown of VEGFR-1 or VEGFR-2 by siRNA further validated the specificity of the VEGFR-1-mediated mechanism (Fig. 2D-F). The silencing of VEGFR-1 completely blocked the effects of VEGF165b, PlGF-2 and VEGF-E (Fig. 2E) whereas the silencing of VEGFR-2 did not alter the algesic properties of the ligands (Fig. 2F).

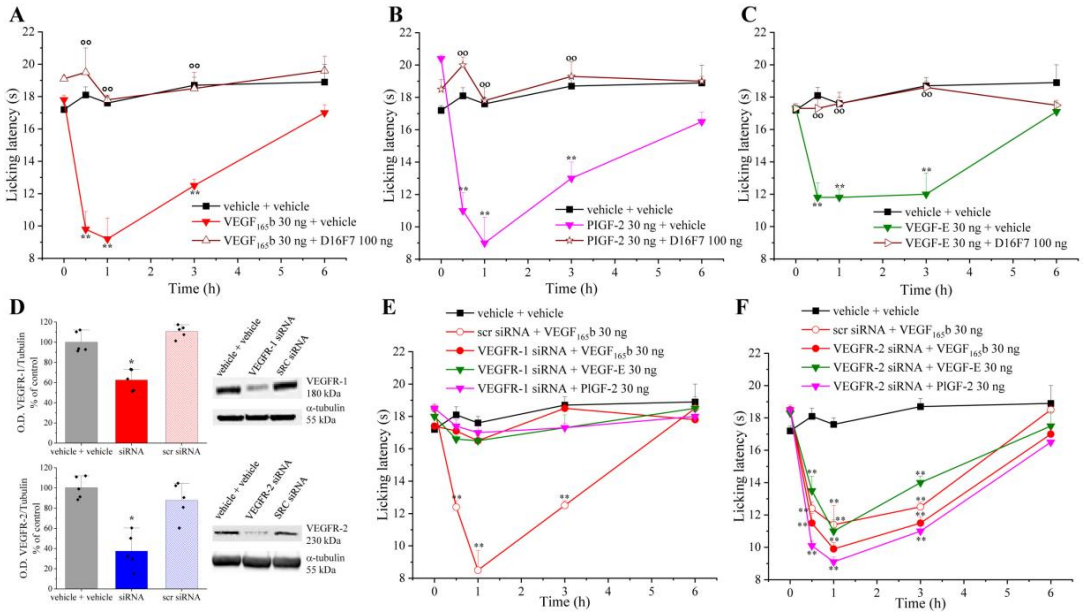


Figure2. Hypersensitivity-induced by VEGF-A signalling modulators is due to its interaction with VEGFR-1. *The response to a thermal stimulus (Cold plate test) was recorded after i.t. infusion of different VEGFR ligands (30 ng) preceded (15 min before) or not by the anti-VEGFR-1 mAb D16F7 (100 ng) or vehicle: (A) VEGF165b ± D16F7 (n=5), (B) PlGF-2 ± D16F7 (n=5), (C) VEGF-E ± D16F7 (n=5). (E − F) Effects of VEGFRs ligands (i.t.) in mice undergone a selective knockdown of VEGFR-1 (D, n=5) or VEGFR-2 (E, n=5) at the lumbar level of the spinal cord by siRNA. (D) Representative Western blot images and densitometric analysis showing the expression of VEGFR-1 or VEGFR-2 in the lumbar section of the spinal cord after the siRNAs administration (n=5). Each value represents the mean ± SEM. **P<0.01 vs vehicle + vehicle-treated animals; °°P<0.01 vs vehicle + VEGFRs ligands-treated animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

4.3. VEGF165b increases the activity of spinal nociceptive neurons by VEGFR-1 activation

To investigate the effect of the spinal application of VEGF165b on the hyperexcitability of spinal nociceptive specific (NS) neurons, *in vivo* electrophysiological experiments were performed. The results relate to NS neurons (one cell recorded from each animal per treatment) localized at a depth of 0.7-1.0 mm from the surface of the spinal cord. This cell population was characterized by a mean rate of basal firing of 0.015 ± 0.002 spikes/sec and only cells showing this pattern were chosen for the experiment. Spontaneous and noxious-evoked (mechanical stimulation) activity of NS neurons was measured after spinal application of VEGF165b preceded or not by treatment with the anti-VEGFR-1 mAb D16F7 to investigate the involvement of VEGF-A receptor subtype. Representative ratematers of the results obtained with VEGF165b in the absence or presence of D16F7 mAb are shown in Fig. 3A and 3B, respectively. In mice pre-treated with vehicle (DMSO in 0.9% NaCl), VEGF165b (3 ng/5 μl) spinal application induced an increase in spinal electrical activity as compared to baseline levels (100%). In particular, NS neurons showed a variation of spontaneous activity compared to baseline of 217.05 ± 29.2% as well as a noxious-evoked activity with frequency of 234 ± 30.9% and duration of 316.2 ± 27.2%, starting from 25 min post VEGF165b (Fig. 3A, C-E). The spinal VEGF165b-induced hypersensitivity was mainly mediated by VEGFR-1 rather than VEGFR-2 activation. Indeed, electrophysiological recordings revealed that spinal pre-application of D16F7 mAb (100 pg/5 μl) significantly prevented the increase of spontaneous and noxious-induced activity of NS neurons resulting in a pattern similar to basal (Fig. 3B, C-E). D16F7 (100 pg/5 μl) alone was not able to affect either spontaneous or evoked activity of NS neurons (Fig. 3B). On the contrary, DC101 at 30 and 100 pg, showed a pro-nociceptive effect on NS spinal activity per se (Supplementary Fig. S3, representative ratematers). In fact, post-injection level of either spontaneous (187.3 ± 17.7% at 100 pg and 151.1 ± 6.9% at 30 pg) or noxious pressure-evoked firing rates (frequency: 212.6 ± 27% at 100 pg and 152.9 ± 6.9% at 30 pg; duration: 235.7 ± 25.3% at 100 pg and 119.7 ± 8.6% at 30 pg) were significantly higher respect to the baseline, in a dose-dependent manner.

Overall, these results further confirmed the involvement of VEGFR-1 in VEGF-A-induced electrophysiological changes of NS.

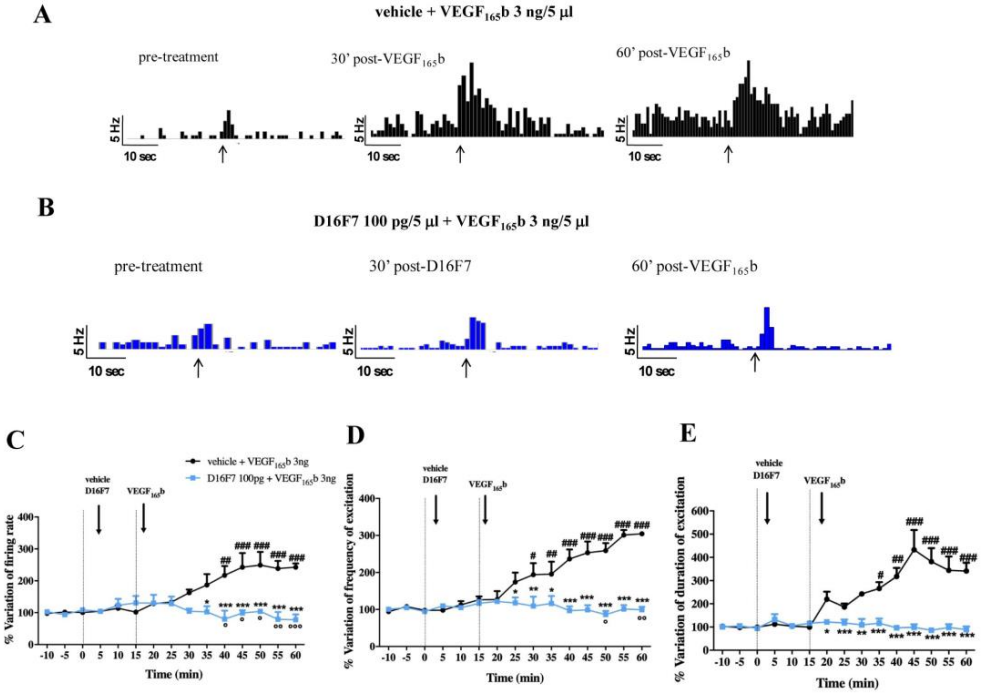
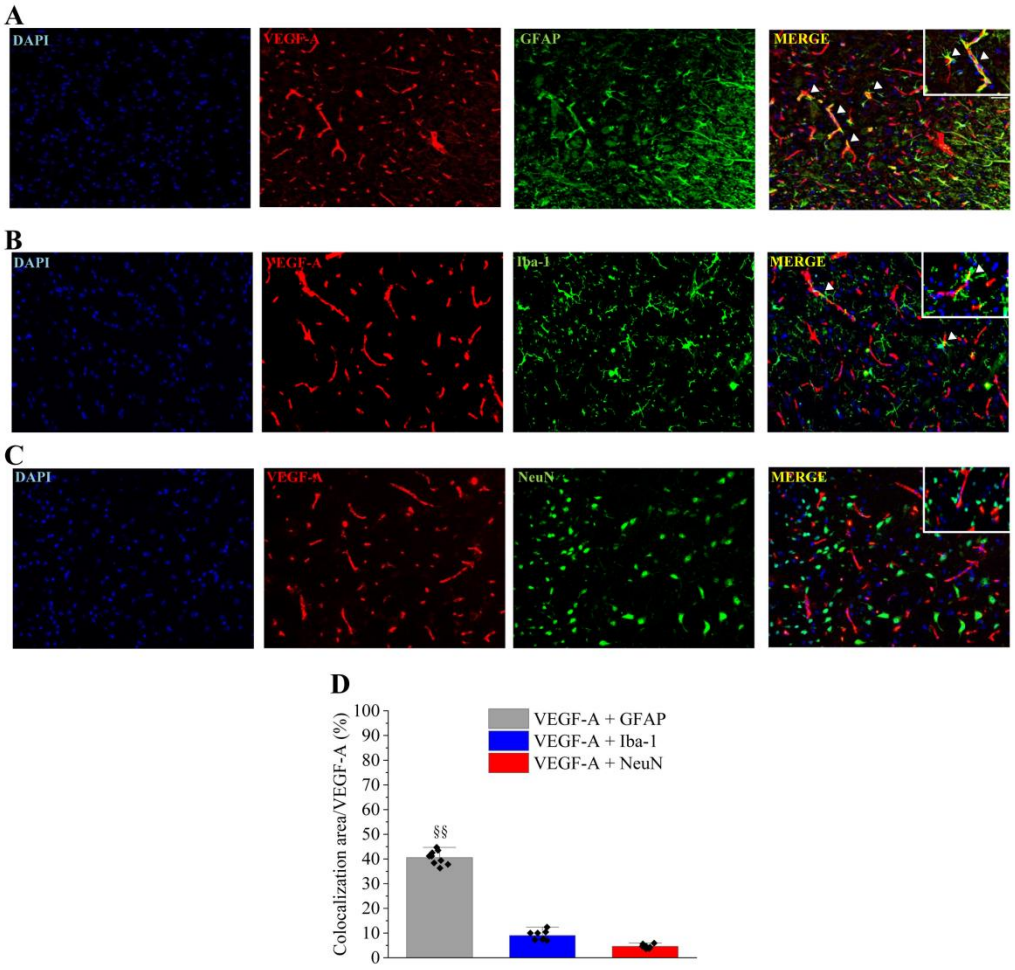


Figure3. VEGF165b increases spontaneous and noxious-evoked activity of NS neurons by VEGFR-1.

Representative ratematers showing spontaneous and noxious-evoked activity of NS neurons after spinal application of VEGF165b alone or in combination with D16F7 mAbs (A and B, respectively); black arrows indicate the noxious stimulation on the mouse hind-paw. Mean ± S.E.M. population data of spinal cord application of VEGF165b (3 ng/5 □l) in the presense of vehicle (DMSO in 0.9% NaCl), or D16F7 (100 pg/5 □l) on firing rate of spontaneous activity (C), frequency (D) and duration of evoked activity (E) of NS neurons in CD1 mice. Black arrows indicate vehicle, D16F7 or VEGF165b spinal application. Each point represents the mean of 5 different mice per group (one neuron recorded per each mouse). #P<0.05, ##P<0.01 and ###P<0.001 indicate statistically difference vs baseline; *P<0.05, **P<0.01 and ***P<0.001 indicate statistically difference vs vehicle + VEGF165b. One-way ANOVA followed by Dunnet's multiple comparison post-hoc test was performed for statistical significance within groups. Two-way ANOVA followed by Bonferroni post-hoc test was used for comparison between groups.

4.4. VEGF-A and VEGFR-1 localization in the spinal cord of naïve mice

Immunofluorescence analysis was performed in the dorsal horn of the spinal cord to study VEGF-A and VEGFR-1 expression profile in the nervous cells. VEGF-A immunoreactivity in astrocytes (as colocalization with GFAP; Fig. 4A and 4D) was significantly higher in comparison to microglia (Iba1 positive cells) and neurons (NeuN positive cells) (Fig. 4B, 4C and 4D). As expected, VEGF-A staining is strictly related to vessel structure (Fig. 4) since its expression was observed both on endothelial cells and astrocyte end-feet[332].



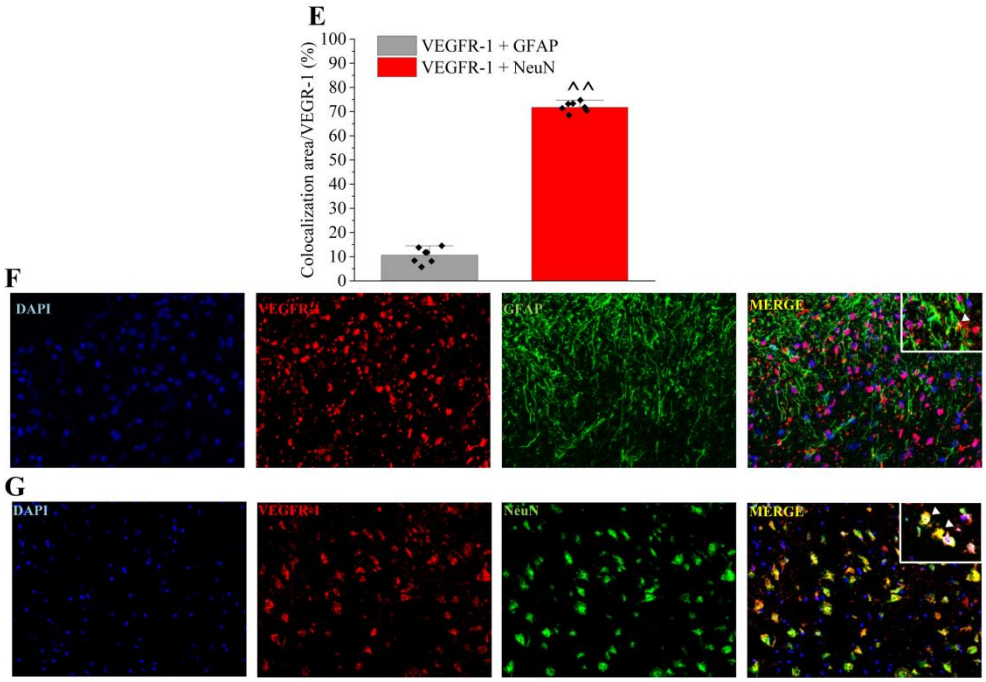
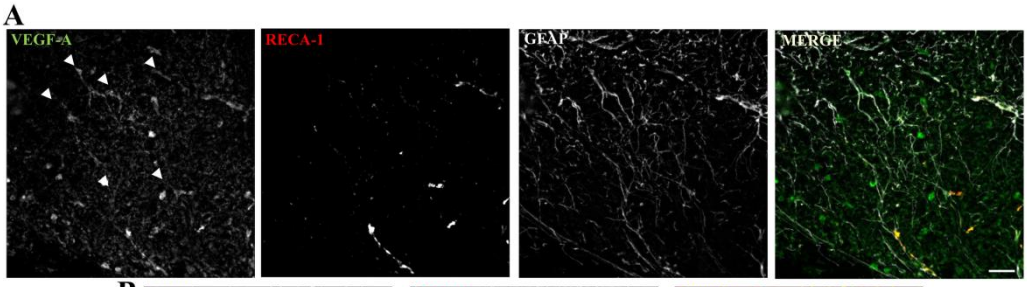


Figure4. Cellular localization of VEGF-A and VEGFR-1 in the spinal cord of naïve mice.

VEGF-A immunoreactivity was analysed in the spinal cord dorsal horn of naïve mice. Colocalization with GFAP-positive astrocytes (A, n=9), Iba1 positive microglia (B, n=8) and NeuN-positive neurons (C, n=7) was evaluated and quantified (D). Immunofluorescence co-staining of VEGFR-1 in the dorsal horn with GFAP (F, n=7) or NeuN (G, n=8) positive cells, and quantitative analysis (E). Scale bar: 100 μm. Each value represents the mean ± SEM. §§P<0.01 vs VEGF-A + Iba1 and VEGF-A + NeuN. ^^P<0.01 vs VEGFR1 + GFAP.

To better investigate this aspect, we compared the co-localization of VEGF-A with GFAP and RECA-1, a marker of endothelial cells. As shown in Fig. 5A, it is possible to identify separate areas of VEGF-A/GFAP and VEGF-A/RECA-1 colocalization. Furthermore, VEGF-A expression in astrocytes was also confirmed by confocal microscopy. Results shown in Fig. 5B and 5C confirm the colocalization of VEGF-A with GFAP and Aquaporin 4 (AQP4, a marker of astrocytic end-feet). Indeed, the Van Steensel's cross-correlation function (CCF) clearly shows that VEGF-A co-localizes with GFAP and AQP4 in cellular structures with an estimated diameter of 1.00 ± 0.11 μm and 1.28 ± 0.12 μm (CCF at FWHM, mean ± SD, Supplementary Figure S4C and S4F), respectively, which are compatible with the size of astrocytic processes. Collectively, these analyses demonstrate the presence of a VEGF-A pool in astrocytes.

As regards the expression of VEGFR-1, it was more prominent in neurons than in astrocytes (Fig. 4E, 4F and 4G).



Staining	PCC	M1	M2	Li's ICQ
GFAP/VEGF-A	0.38 ± 0.04	0.42 ± 0.06	0.33 ± 0.04	0.20 ± 0.01
AQP4/VEGF-A	0.78 ± 0.01	0.62 ± 0.08	0.87 ± 0.03	0.35 ± 0.01

Figure5. Colocalization analysis of VEGF-A and RECA-1, GFAP or AQP4 in the spinal cord of naïve mice.

A) VEGF-A immunoreactivity was analysed in the spinal cord dorsal horn of naïve mice in comparison to RECA-1-positive endothelial cells and GFAP-positive astrocytes; arrows indicate the presence of VEGF-A in astrocytes; scale bar: 100 μm. B and C). Deconvolved confocal z-stacks shown as maximum intensity projection. Arrows indicate points of interest. B) Representative GFAP and VEGF-A z-stack. C) Representative GFAP and Aquaporin-4 z-stack. Table). Colocalization parameters are given as mean ± SEM (n=8), PCC= Pearson's Correlation Coefficient; M1=Mander's M1; M2= Mander's M2; Li's ICQ= Li's Intensity Correlation Quotient. Colocalization graph are shown in Supplementary Figure S4.

4.5. VEGF-A is increased in spinal astrocytes of mice with oxaliplatin-induced neuropathy

A painful neuropathy was reproduced in mice by a repeated treatment with oxaliplatin[310;311]. After 2 weeks of treatment, when hypersensitivity was developed, VEGF-A immunoreactivity significantly increased in dorsal horns of the spinal cord in comparison to control animals (Fig. 6A and Supplementary Fig. S5). The increment was specifically confirmed in astrocytes when colocalization of VEGF-A expression in GFAP-positive cells was measured (Fig. 6B, 6C and 6D). As regards VEGFRs, VEGFR-2 expression increased in the spinal cord of oxaliplatin-treated mice as revealed by Western blot, on the contrary VEGFR-1 was unaffected by chemotherapy (Supplementary Fig. S6).

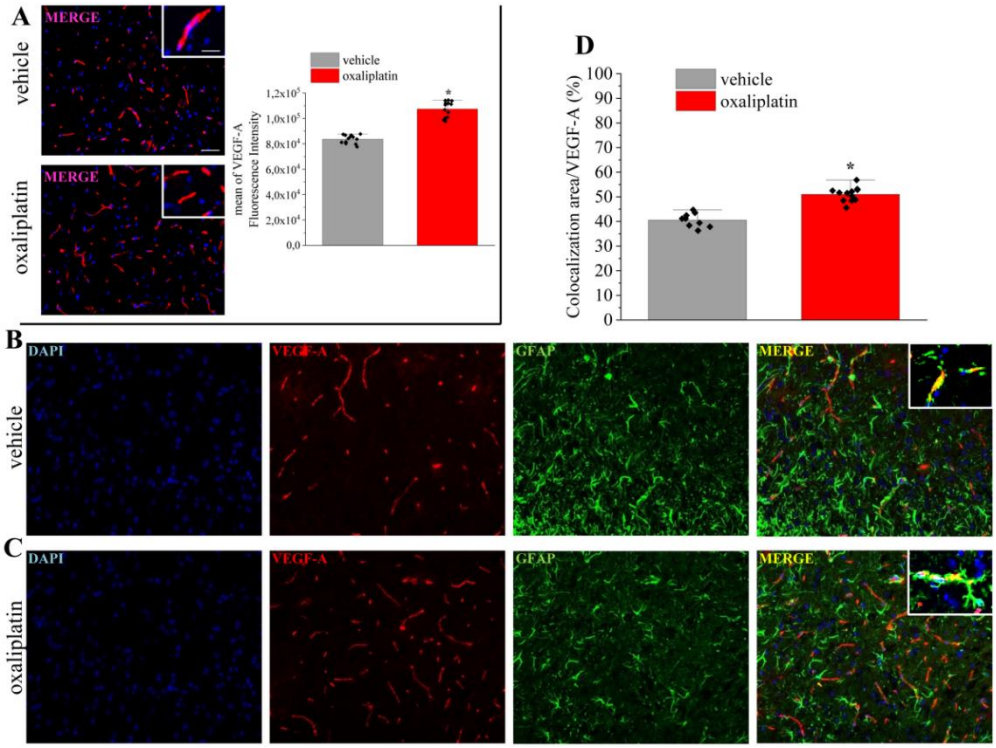


Figure6. VEGF-A is increased in spinal astrocytes of mice with oxaliplatin-induced neuropathy.

*(A) Representative images and quantitative analysis of mean VEGF-A fluorescence intensity in the dorsal horn of oxaliplatin-treated mice in comparison to control (n=13). (B-D) Colocalization analysis of VEGF-A and GFAP in the different groups, a quantitative analysis was reported (D) (vehicle + vehicle, n=13; oxaliplatin + vehicle, n=12). Scale bar: 100 μm; insert: 50 μm. Each value represents the mean ± SEM. *P<0.05 vs vehicle + vehicle group. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

4.6. VEGF-A silencing in astrocytes prevents neuropathic pain

To study the influence of astrocytic VEGF-A modulation on pain signaling, we selectively silenced VEGF-A in spinal astrocytes by injecting an AAV1-GFAP-eGFP-VEGFA-shRNAmir. The vector was bilaterally injected at the lumbar and thoracic levels of the spinal cord 2 weeks before the first oxaliplatin treatment. As shown in Fig. 7A, 4 weeks after injection, the vector fluorescence colocalized with GFAP-positive cells inducing a significant decrease of VEGF-A expression (Fig. 7B). The pain threshold measurements by employing thermal (Cold plate test) and mechanical (Von Frey test) non-noxious stimuli over time showed a significant prevention of hypersensitivity development during the 2 weeks of oxaliplatin treatment in the group that received the VEGF-A specific shRNAmir in comparison to scrambled- and vehicle-treated mice (Fig. 7C and 7D). To verify the lack of neurological and motor alterations which could interfere with pain behavior recordings, VEGF-A shRNAmir and scrambled-treated mice motor functionality and exploratory activity were evaluated by the Hole board test. No alterations were highlighted with the exception of a higher exploratory activity on day 3 of oxaliplatin protocol (Supplementary Table S2).

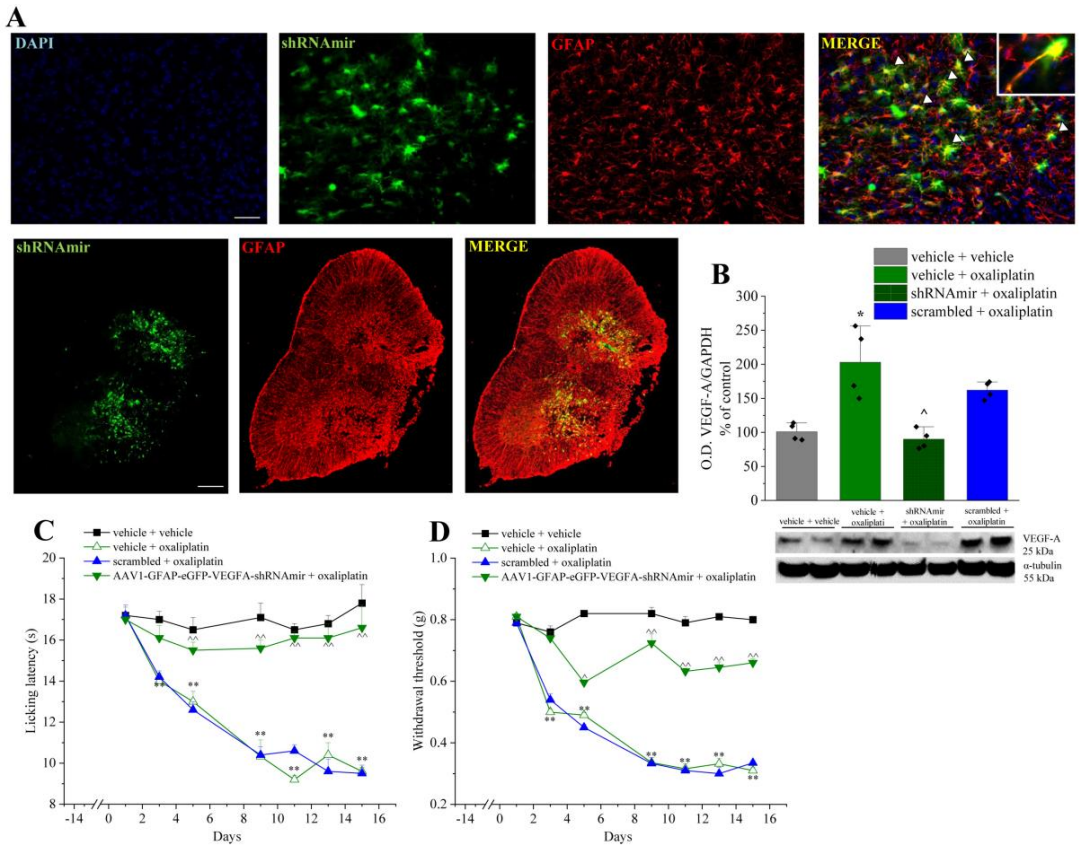


Figure7. VEGF-A silencing in astrocytes prevents neuropathic pain.

*AAV1-GFAP-eGFP-VEGFA-shRNAmir was injected in the spinal cord to decrease VEGF-A expression in astrocytes. (A) Representative image of eGFP and GFAP fluorescence in a whole section at lumbar level, scale bar: 100 μm. Higher magnifications were reported to visualize the colocalization, scale bar: 50 μm (n=4). (B) Representative Western blot images and densitometric analysis showing the expression of VEGF-A in the lumbar section of the spinal cord after the vector administration (n=4, blot of samples obtained from 2 animals of each group are shown). Pain threshold was evaluated by (C) Cold plate and (D) Paw pressure test (n=5). Each value represents the mean ± SEM. *P<0.05 and **P<0.01 vs vehicle + vehicle; ^P<0.05 and ^^P<0.01 vs scrambled + oxaliplatin group. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

4.7. The anti VEGFR-1 mAb D16F7 relieves pain in different models of CINP

To evaluate the anti-hypersensitivity potential of D16F7, we tested its activity against neuropathic pain induced by anticancer drugs. In the already described oxaliplatin model, D16F7 was infused i.t. (100 ng, 1 μg and 5 μg) showing a significant, dose-dependent, increase of the pain threshold both after thermal and

mechanical non-noxious and noxious stimulation, respectively. Hypersensitivity was fully counteracted (up to control values) from 30 min to 3 h after treatment (Fig. 8A and 8B). On the contrary, the anti VEGFR-2 antibody DC101 (100 pg i.t.) was ineffective (data not shown). Interestingly, D16F7 mAb maintained its efficacy also when systemically injected by the i.p. route (1, 5, 15 and 25 mg kg^{-1}). It was active starting from the dose 5 mg kg^{-1}, the onset of the analgesic effect was observed at 60 min and efficacy maintained up to 120 min (Supplementary Fig. S7A and S7B). The pain-relieving properties of D16F7 mAb seems to be not limited to the oxaliplatin neurotoxicity since it was also effective in mice become hypersensitive after treatment with the neurotoxic anticancer drugs vincristine and paclitaxel. In both models, D16F7 mAb (1 and 5 μg, i.t.) was active between 30 min and 3 h (Fig. 8C-F) in the Cold plate and Paw pressure tests with a particular efficacy when the pain response was evoked by thermal stimuli (Fig. 8C and 8E). In paclitaxel-treated mice 15 μg D16F7 mAb dosed i.t. was effective up to 5 h (Fig. 8E).

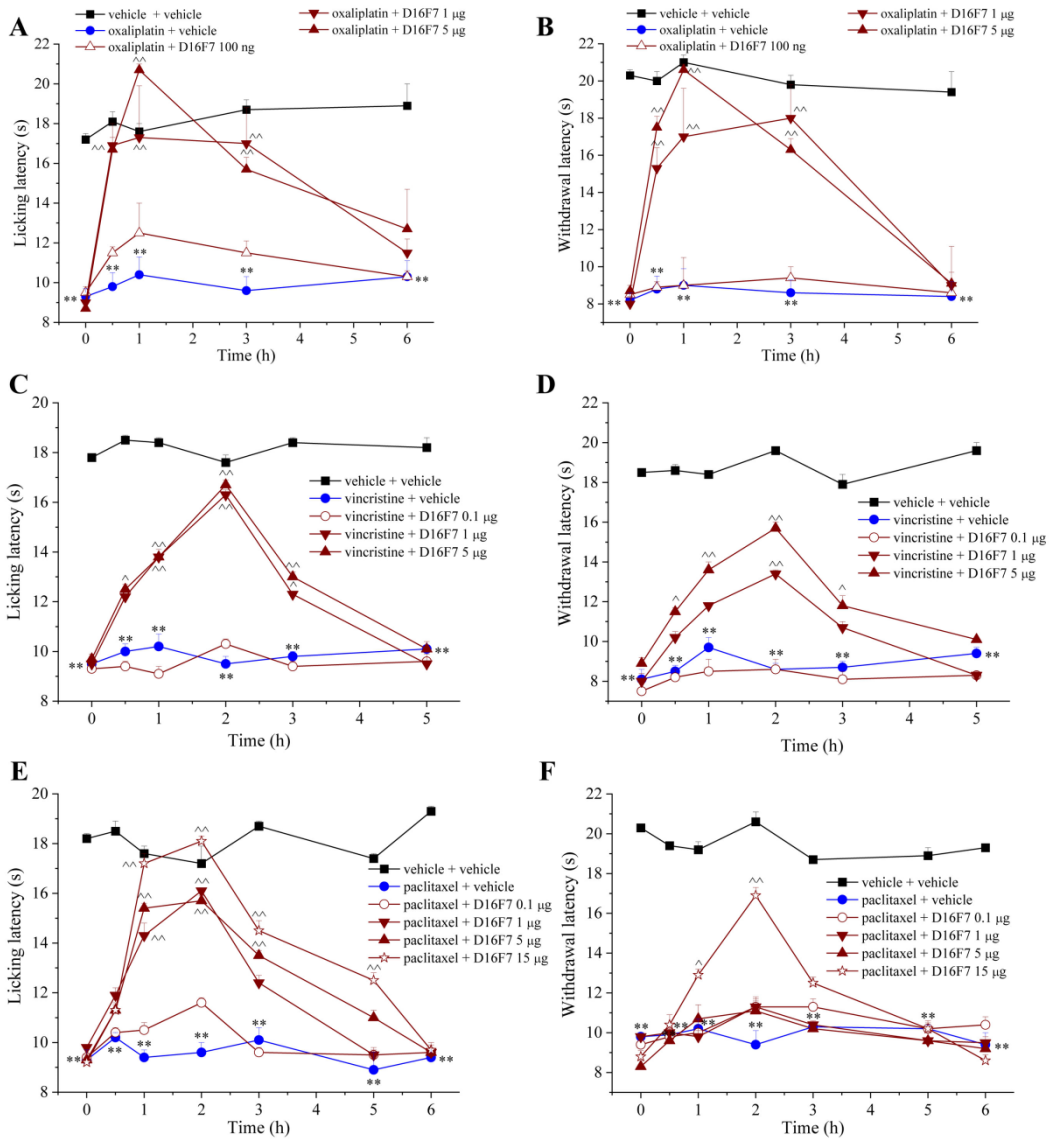


Figure8. D16F7 mAb reduces pain in different models of chemotherapy-induced neuropathy.

*Effect of D16F7 mAb evaluated by (A) Cold plate and (B) Paw pressure tests in a mouse model of oxaliplatin-induced neuropathy after i.t. injection (A, B, n=6). (C, D) Effect of D16F7 mAb after i.t. administration in vincristine-treated mice stimulated with thermal (C) or mechanical (D) stimuli (n=6). (E, F) Effect of D16F7 after i.t. administration in paclitaxel-treated mice stimulated with thermal (E) or mechanical (F) stimuli (n=6). Each value represents the mean ± SEM. **P<0.01 vs vehicle + vehicle-treated animals; ^P<0.05 and ^^P<0.01 vs chemotherapeutic drugs + vehicle-treated animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

4.8. Organotypic Spinal Cord Slices maintain their structural organization and show a reduction of vascular network

To deepen the non-vascular role of VEGF-A in nervous tissue and to further investigate its functions, we isolated its "nerve component" from the vascular one. For this reason, we used organotypic tissue culture as a study system, which represent an important crossroad between *in vivo* and *in vitro* studies. After obtaining the organotypic spinal cord culture, as previously described in the "Materials and Methods" section, we evaluated the structural organization of the tissue. During the two weeks of cultivation, the slices reduce their initial thickness (as shown in the images reported in Fig. 9A, taken at several days), but always maintaining their original structure. After 14 days, we analyzed the slices by immunofluorescence, using GFAP (for astrocyte cells) and NeuN (for neuronal cells) markers. Fig. 9B shows that the cell morphology and the structural organization of the spinal cord are maintained in the slices. Furthermore, using the endothelial marker RECA-1, we highlighted a significant reduction of the normal vascular network (Fig. 9C), a fundamental aspect that allowed us to be able to use this system for our study.

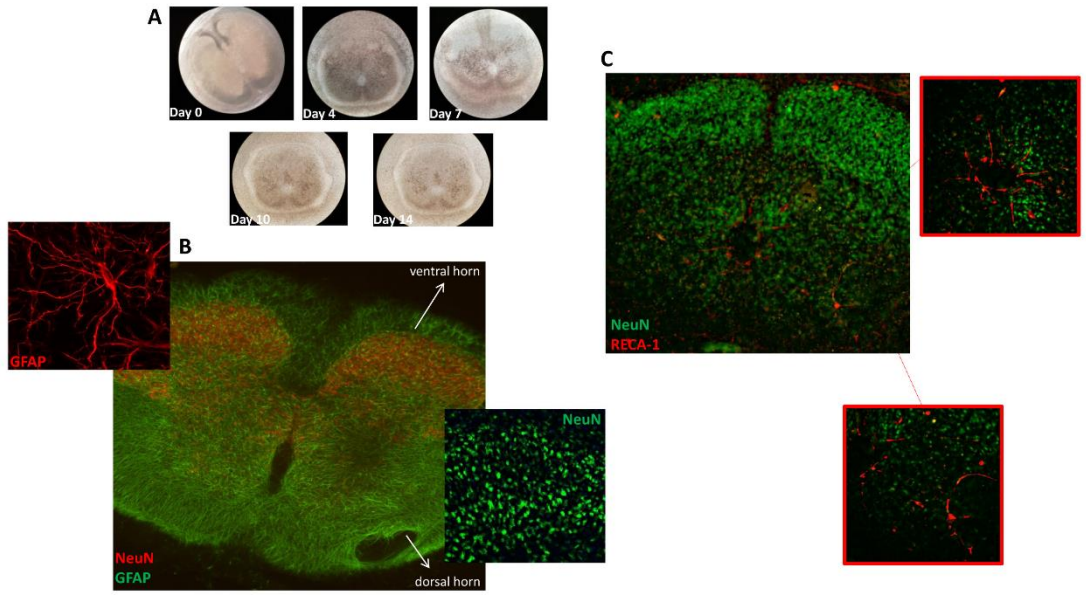


Figure9. The slices maintain their structural organization with a reduction of vascular network.

During the two weeks of culture, pictures of the slices were taken at several days to evaluate their structural integrity (A), and after 14 days an immunohistochemical analysis was performed with GFAP, NeuN (B) and RECA-1 (C) as markers.

4.9. Oxaliplatin induces time and dose-dependent toxicity in organotypic sections of the spinal cord

Having evaluated in the results shown above the involvement of VEGF-A in chemotherapy-induced neuropathy *in vivo* models, here we investigated the effect of oxaliplatin treatment on organotypic slices. After 14 days of cultivation, we treated them at different times (1- 3- 6- 24h) and with increasing doses of oxaliplatin (1- 10- 100 μM), evaluating its toxicity by analyzing the fluorescence of Propidium Iodide (5 μg/mL, fluorescent agent, commonly used to evaluate cell viability).

Quantitative analysis of PI fluorescence in Fig. 10B showed that oxaliplatin 10 μM after 24h of treatment, induces neurotoxicity increasing PI fluorescence intensity in a statistically significantly manner compared to the control. Moreover oxaliplatin 10 μM did not induce the complete tissue death, which happens with 100 μM (as is shown in Fig. 10A). For this reason, oxaliplatin 10 μM will be the dose used for all subsequent experiments.

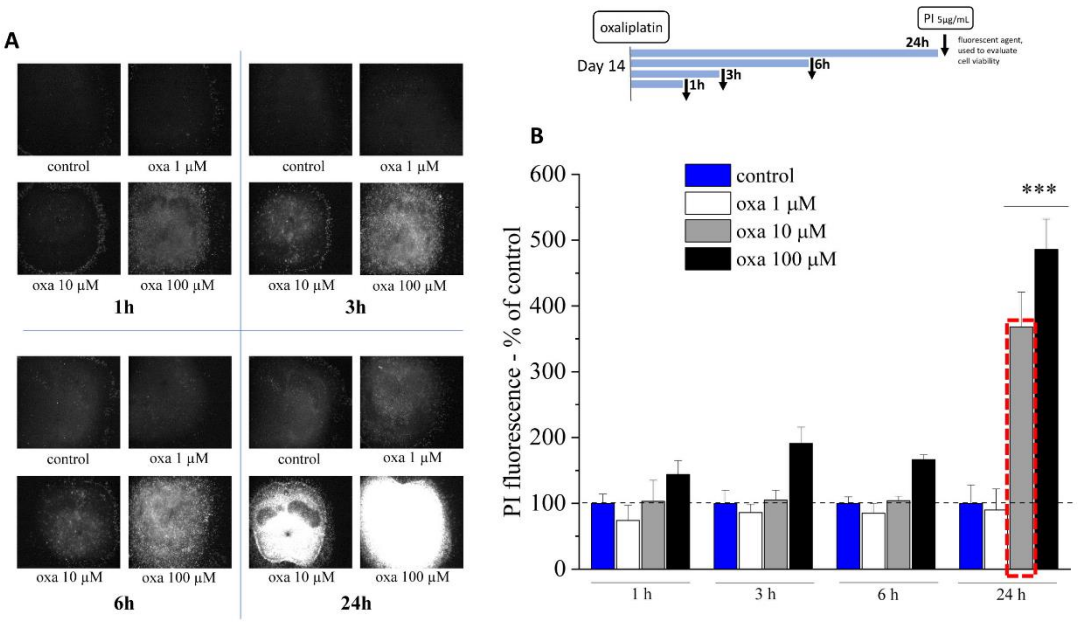


Figure10. Oxaliplatin 10 µM induces neurotoxicity in spinal cord slices.

After 14 days of cultivation, qualitative (A) and quantitative (B) analysis of Propidium Iodide (5 µg/mL) fluorescence intensity were evaluated to investigate the toxicity of treatment at different times (1- 3- 6 and 24 hours) and with increasing doses (1- 10 and 100 µM) of oxaliplatin.

*Each value represents the mean ± SEM. ***P<0.001 vs control. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

From the immunofluorescence experiments reported in Fig. 11A and from the quantitative analyses in Fig. 11B and 11C, it is shown that oxaliplatin 10 µM induces a time-dependent and statistically significant increase of the fluorescence intensity and of the number of $GFAP^+$ cells compared to the control. Moreover, from a morphological point of view, the astrocytes treated with oxaliplatin appear less defined cells and with a less evident cell body than the control.

In addition, oxaliplatin 10 µM also causes an alteration of neuronal morphology, with a reduction in the number and fluorescence intensity of $NeuN^+$ cells (Fig. 11D and E).

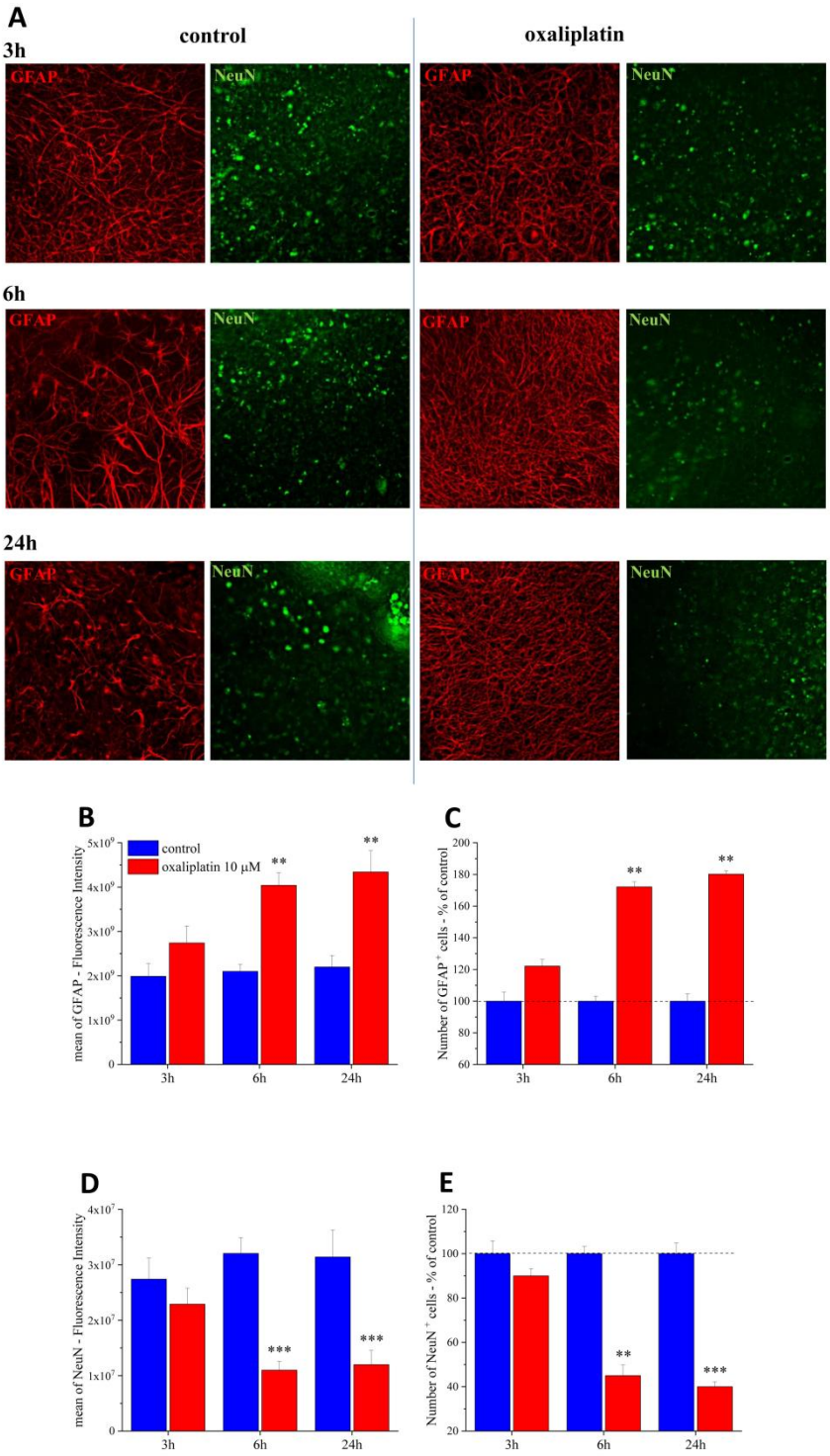


Figure11. Morphological alteration induced by oxaliplatin.

*(A) Representative images of GFAP and NeuN staining on organotypic spinal cord slices, after 14 days of cultivation, treated with oxaliplatin 10 µM for 3- 6 and 24 hours. The mean of GFAP fluoresce intensity (B), number of GFAP positive cells (C), NeuN fluorescence intensity (D) and positive cells (E) were reported. Each value represents the mean ± SEM. **P<0.01 and ***P<0.001 vs control. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

4.10. mAb D16F7 modulates the painful factor release caused by VEGF165b and oxaliplatin

To elucidate the mechanisms of VEGF-A mediated hypersensitivity, we focused on three "key" factors that play an important role in the development and maintenance of pain. Calcitonin gene-related peptide (CGRP), widely distributed in the peripheral and central nervous system and its receptors are expressed in pain pathways; Substance P (SP), found involved in the onset and modulation of different types of pain; Glutamate, the most abundant excitatory neurotransmitter in the brain. Over the past few decades, the accumulation of evidence has shown a pivotal role for glutamate in pain sensation and transmission. In our study, we decided to pay attention to glutamate transporters, which contribute to the maintenance of synaptic homeostasis. In particular, EAAT1 and EAAT2 are mainly expressed in glial cells. EAAT2 is responsible for over 90% of glutamate transport in the brain, with the exception of some regions, including the cerebellum, circumventricular organs, and the retina, where EAAT1 is the main transporter. EAAT1 is also expressed in neurons and oligodendrocytes.

Therefore, we decided to evaluate the quantity of CGRP and SP released in the culture medium of organotypic slices, after treatment with oxaliplatin (10 μM) or VEGF165b (100 ng/mL) for 3 and 6 hours; we also evaluated whether treatment with selective blockers for VEGFR-1 (D16F7 300ng/mL) and VEGFR-2 (DC101 10ng/mL) prevented the release of these factors. In Fig. 12A is shown that treatment with both oxaliplatin and VEGF16b caused a statistically significant increase in CGRP release compared to control, at both times considered. Only co-treatment with D16F7, and not with DC101, reversed the increase in CGRP release mediated by both oxaliplatin and VEGF165b.

The same trend is shown in figure 12B for Substance P, but only at 6 hours of treatments.

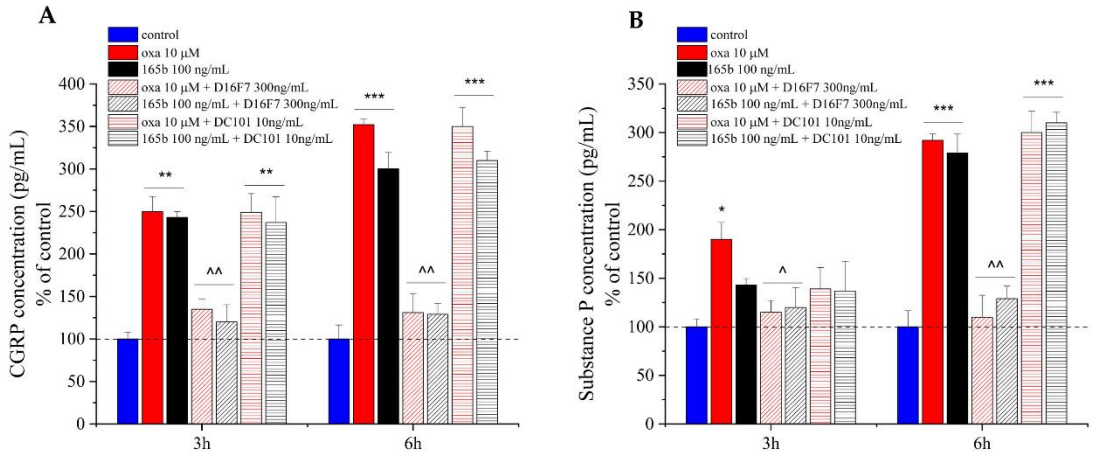


Figure12. D16F7 prevents the release of CGRP and SP, mediated by both oxaliplatin and VEGF165b.

The quantity of CGRP (A) and Substance P (B) released into the medium of the organotypic slices was measured by enzyme-linked immunosorbent assay, after treatment for 3 and 6 hours with oxaliplatin (10 µM) or VEGF165b (10 ng/mL) alone and in co-treatment with D16F7 (100 ng/mL) and DC101 (10 ng/mL). The levels were normalized to cell protein concentrations.

*Each value represents the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 vs control; ^P<0.05 and ^^P<0.01 vs oxaliplatin treatment. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

Unlike CGRP and SP, it was not possible to measure the quantity of glutamate released in the culture medium of the organotypic slices for experimental reasons. For this reason, we measured the mRNA expression of EAAT1 and EAAT2 genes after the same treatments seen above. In Fig. 13A and 13B it is shown that treatments with both oxaliplatin and VEGF165b reduced the gene expression of EAAT1 and EAAT2 by about half compared to the control group, only after 6 hours. Co-treatment with oxaliplatin and D16F7 returned the gene expression of EAAT1 and EAAT2 to control levels, while co-treatment with D16F7 and VEGF165b showed a statistically significant increase in the expression of two genes not only compared to the control but also compared to VEGF165b alone.

We therefore interpreted these data as an increase in glutamate in the extracellular space caused by treatment with oxaliplatin as well as with VEGF165b, while only the selective blockade of VEGFR-1 restores the glutamate levels to the control values.

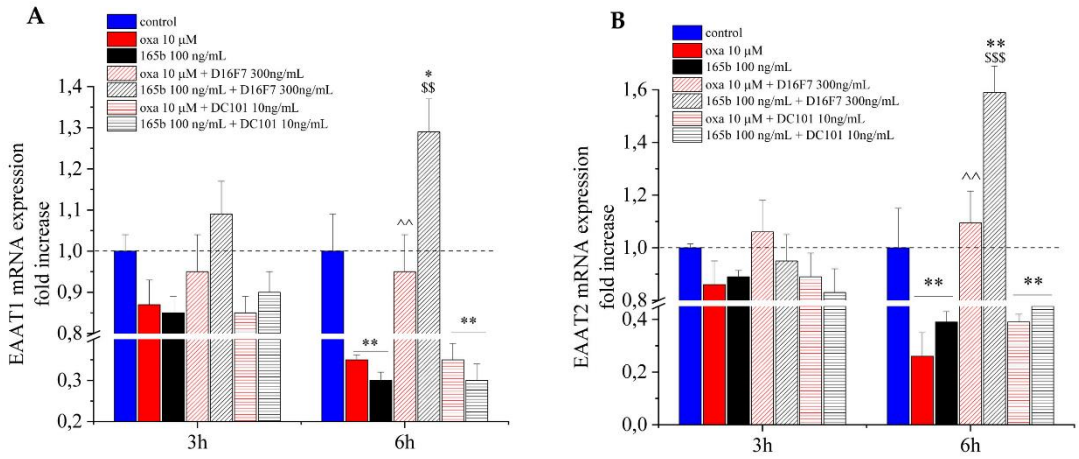


Figure13. D16F7 upregulates the gene expression of "glial" Glutamate Transporters, reduced by both oxaliplatin and VEGF165b.

The mRNA expression of EAAT1 (A) and EAAT2 (B) genes of the slices was measured by RT-PCR, after treatment for 3 and 6 hours with oxaliplatin (10 µM) or VEGF165b (10 ng/mL) alone and in co-treatment with D16F7 (100 ng/mL) and DC101 (10 ng/mL).

Each value represents the mean ± SEM. $^{*}P<0.05$ and $^{**}P<0.01$ vs control; ^^P<0.01 vs oxaliplatin treatment; $^{\$\$\$}P<0.001$ vs 165b treatment. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.

4.11. VEGF165b performs neuroprotective effect on oxaliplatin-induced neurotoxicity

To evaluate the effect of VEGF-A on toxicity induced by oxaliplatin, we co-treated the slices with oxaliplatin 10 µM and VEGF165b (300-100-30 ng/mL) for 24 hours: qualitative images in Fig. 14A and quantitative analysis of the fluorescence of the Propidium iodide in Fig. 14B showed that co-treatment with VEGF165b reduced oxaliplatin-induced toxicity in a dose-dependent manner.

Furthermore, treatment with VEGF165b alone produced no effect.

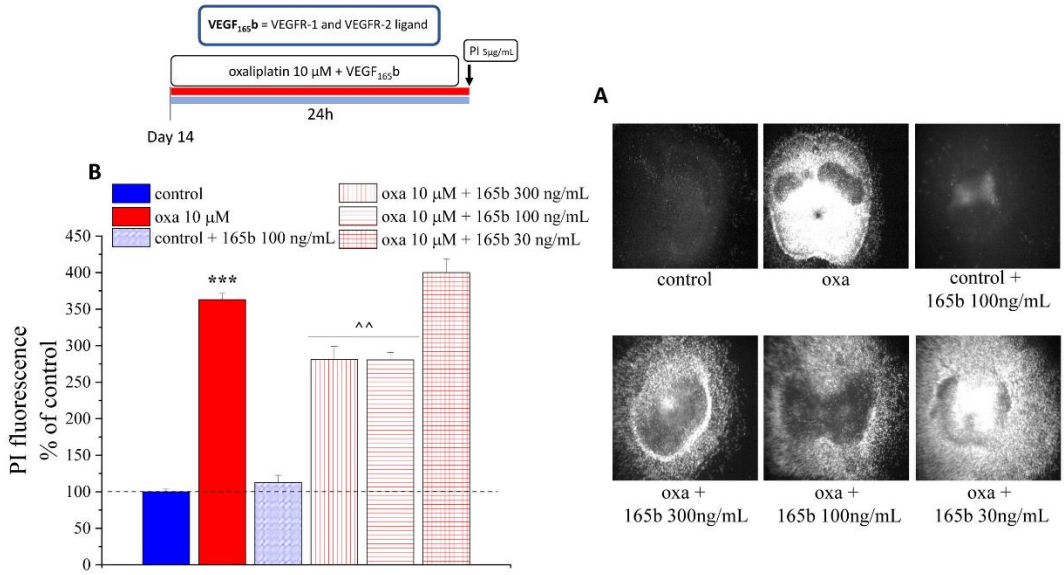


Figure14. VEGF165b prevented the neurotoxicity induced by oxaliplatin.

After 14 days of cultivation, qualitative (A) and quantitative (B) analysis of Propidium Iodide (5 µg/mL) fluorescence intensity were evaluated to study the effect of VEGF165b treatment (30- 100 and 300 ng/mL) on oxaliplatin-induced toxicity.

*Each value represents the mean ± SEM. ***P<0.001 vs control; ^^ P<0.01 vs oxaliplatin treatment. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

Moreover, from the immunofluorescence experiments reported in Fig. 15A and from the quantitative analyses in Fig. 15B and 15C, we observe a statistically significant reduction in the fluorescence intensity and in the number of $GFAP^+$ cells in the slices co-treated with oxaliplatin and VEGF165b 100 ng/mL, compared to those treated with oxaliplatin alone; co-treatment with VEGF165b 100 ng/mL was also able to reduce the neuronal alterations caused by oxaliplatin (Fig. 15D and E).

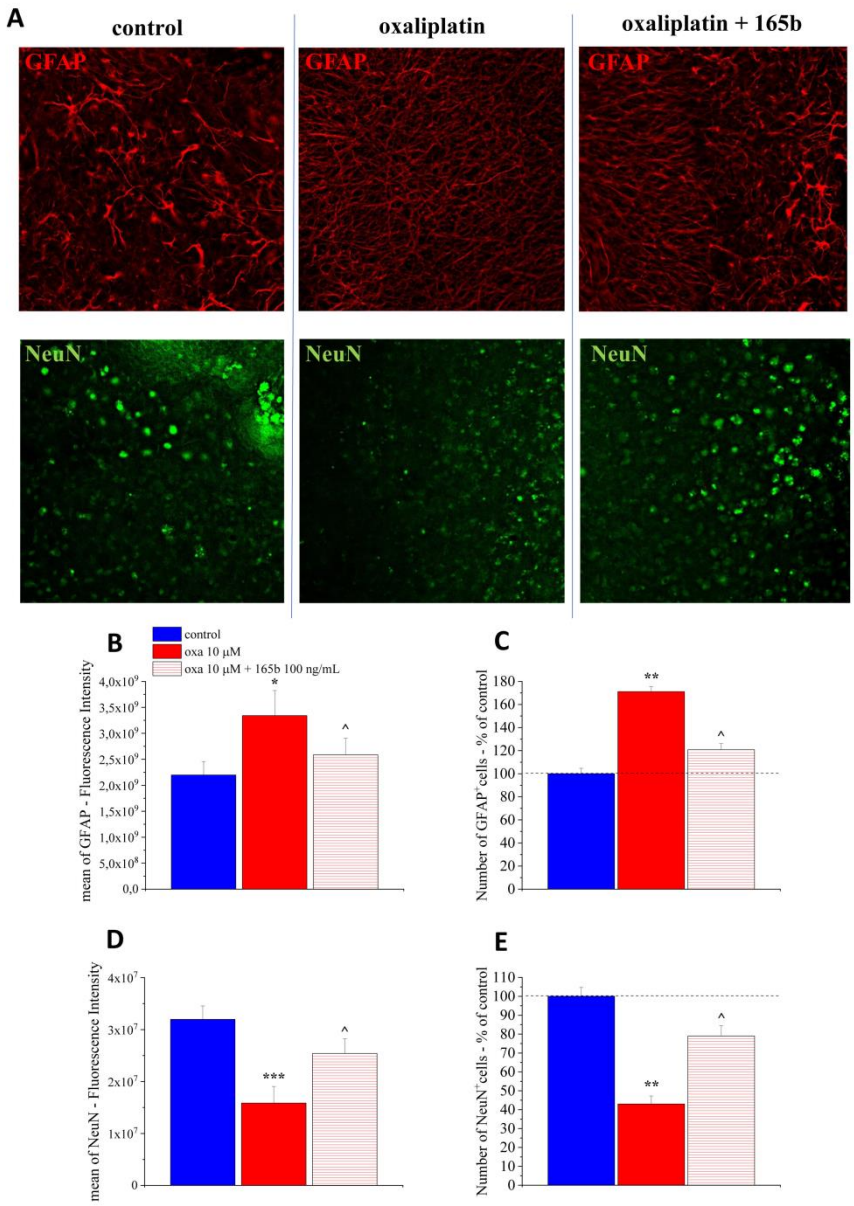


Figure15. VEGF165b reduces the morphological alterations caused by oxaliplatin.

(A) Representative images of GFAP and NeuN staining on organotypic spinal cord slices, after 14 days of cultivation, co-treated with oxaliplatin (10 µM) and VEGF165b (100 ng/mL) for 24 hours. The mean of GFAP fluoresce intensity (B), number of GFAP positive cells (C), NeuN fluorescence intensity (D) and positive cells (E) were reported.

*Each value represents the mean ± SEM. *P<0.05 and **P<0.01 vs control; ^P<0.05 vs oxaliplatin treatment. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

4.12. Molecular mechanism underlying VEGF-mediated neuroprotection

To investigate the molecular mechanism underlying VEGF-A-mediated neuroprotection, we decided to treat the slices with two selective ligands for VEGFR-1 (PlGF 300-100-30 ng/mL) and VEGFR-2 (VEGF-E 300-100-30 ng/mL), respectively.

From the representative images in Fig. 16A and from the quantitative analysis of PI fluorescence in Fig. 16B, it is observed that the selective binding of VEGF-E with VEGFR-2 reduced the oxaliplatin-induced toxicity in a dose-dependent manner.

Therefore, from these results we hypothesized that the neuroprotection of VEGF-A could be mediated by its binding with VEGFR-2.

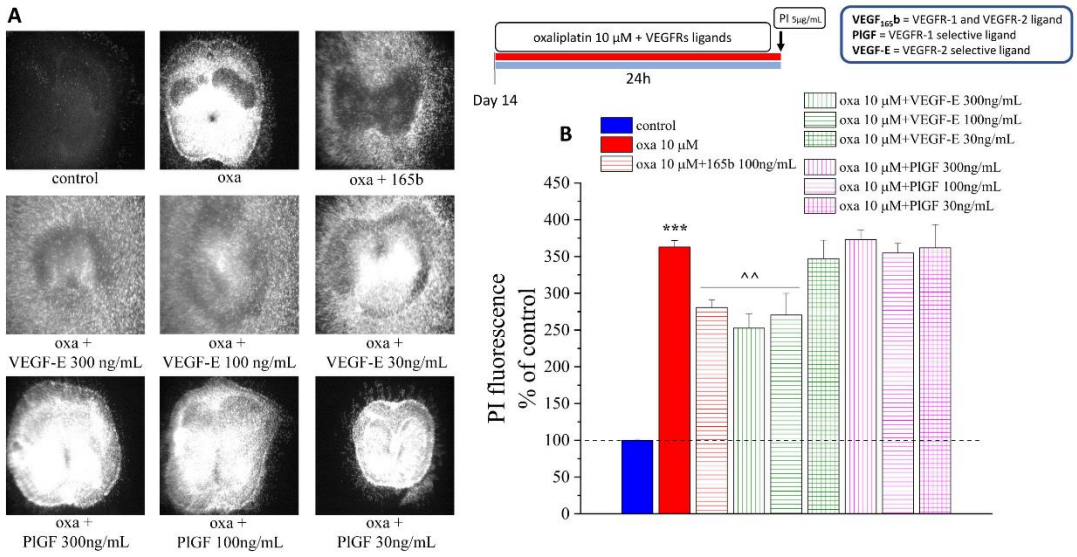


Figure16. VEGF-E reduces the oxaliplatin-induced toxicity.

After 14 days of cultivation, qualitative (A) and quantitative (B) analysis of Propidium Iodide (5 µg/mL) fluorescence intensity were evaluated to study the effect of VEGF-E (300- 100 and 30 ng/mL) and PlGF (300-100 and 30 ng/mL) treatment on oxaliplatin-induced toxicity.

*Each value represents the mean ± SEM. ***P<0.001 vs control; ^^ P<0.01 vs oxaliplatin treatment. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

To confirm this hypothesis, we evaluated whether this neuroprotective effect mediated by VEGF165b was preserved or not using two receptor antagonists: D16F7 (300 ng/mL), a selective antagonist for the VEGFR-1, and DC101 (10 ng/mL) selective for VEGFR-2.

Based on the quantitative analysis in Fig. 17B, co-treatment with DC101 and VEGF165b, in toxicity condition, blocked the neuroprotective effect mediated by VEGF165b; on the contrary, the blockade of VEGFR-1 by D16F7 continues to preserve the neuroprotective effect of VEGF165b.

Treatment with DC101 and D16F7, alone or in combination, does not worsen the toxicity of oxaliplatin (Fig. 17B). While treatment of control slices with DC101 causes toxicity; this result suggests that VEGF-A plays a neuroprotective role in toxic conditions but also has important physiological roles, mediated by the VEGFR-2 receptor.

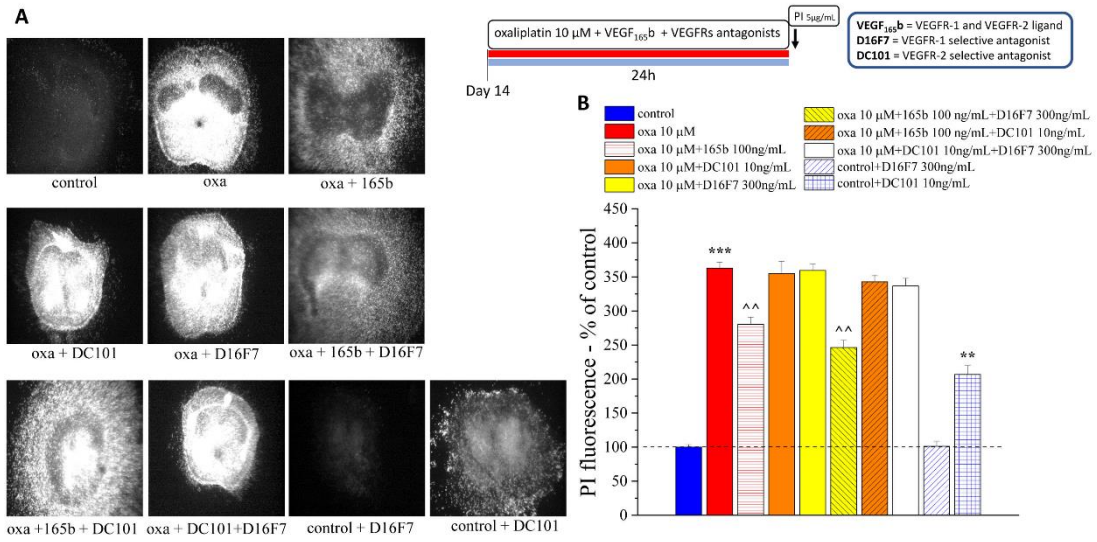


Figure17. VEGFR-2 mediates the extra-vasal VEGF-A neuroprotective and physiological effects.

After 14 days of cultivation, qualitative (A) and quantitative (B) analysis of Propidium Iodide (5 µg/mL) fluorescence intensity were evaluated to study the effect of D16F7 (300 ng/mL) and DC101 (10 ng/mL) treatment on oxaliplatin-induced toxicity.

Each value represents the mean ± SEM.***P<0.01 and ***P<0.001 vs control; ^^ P<0.01 vs oxaliplatin treatment. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.

4.13. Fluorocitrate blocks oxaliplatin-mediated release of VEGF-A, causing toxicity

From the results previously shown, we demonstrated that in CNS an extra-endothelial component of VEGF-A exists in astrocyte cells, with a relevant role in pain signaling. So, as the last point of this thesis, we evaluated the quantity of VEGF-A released in the culture medium of the organotypic spinal cord slices, after treatment with Fluorocitrate both in control and oxaliplatin treatment conditions.

Fluorocitrate, a derivative of fluoroacetate, is a compound that blocks the Krebs Cycle at the level of aconitase, causing ultrastructural alterations only in glial cells and inhibiting the tricarboxylic cycle[333;334]. For this reason, it is usually used as an inhibitor of glial metabolism; in particular for our purpose, it was chosen to investigate the "source" of VEGF-A in our non-vascularized system of organotypic spinal cord slices.

Fig. 18 shows that treatment with oxaliplatin 10 μM resulted in a statistically significant and time-dependent increase of VEGF-A released in the culture medium, compared to the control; while co-treatment with fluorocitrate reduced the released VEGF-A; moreover, treatment with only fluorocitrate, reduced the release of VEGF-A, compared to the basal quantity (Fig. 18).

This *in-vitro* result confirms our hypothesis that VEGF-A is also released from astrocyte cells.

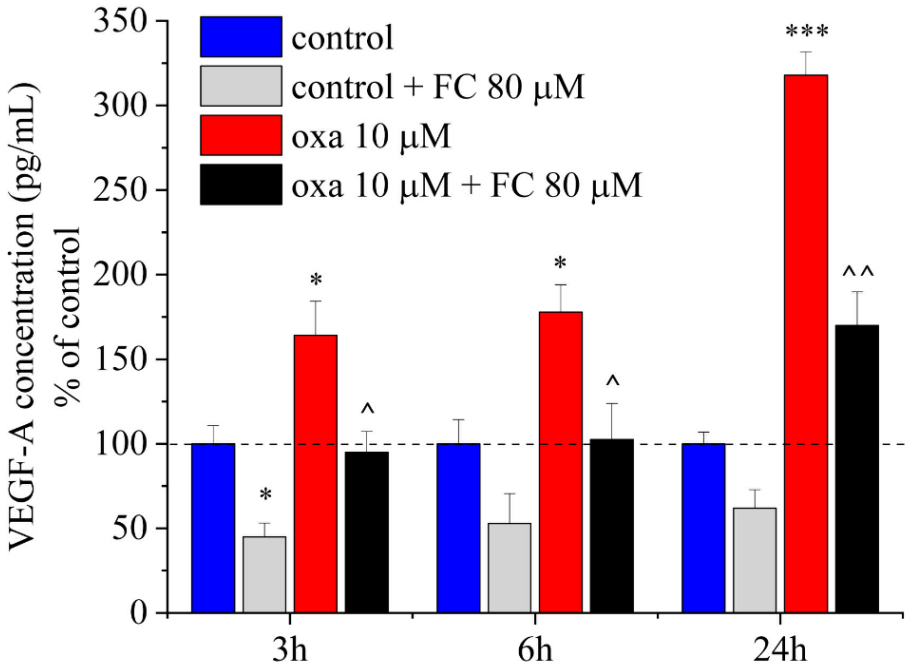


Figure18. Fluorocitrate blocks the VEGF-A release.

The quantity of VEGF-A released into the medium of the organotypic slices was measured by enzyme-linked immunosorbent assay, after treatment for 3, 6 and 24 hours with fluorocitrate (80 µM) alone or in co-treatment with oxaliplatin (10 µM). The levels were normalized to cell protein concentrations.

Each value represents the mean ± SEM. $^{*}P<0.05$ and $^{***}P<0.001$ vs control; ^P<0.05 and ^^P<0.01 vs oxaliplatin treatment. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.

Considering the neuroprotective effects of VEGF-A on oxaliplatin-induced toxicity seen above, we investigated the consequences of fluorocitrate treatment. Fig. 19B shows that treatment with fluorocitrate alone causes toxicity compared to the control group at any time considered, while co-treatment with fluorocitrate and oxaliplatin worsens the toxicity induced by oxaliplatin in a statistically significant manner after 3 hours of treatment. The addition of exogenous VEGF165b reduces the toxicity caused by oxaliplatin and fluorocitrate, after 24 hours of treatment.

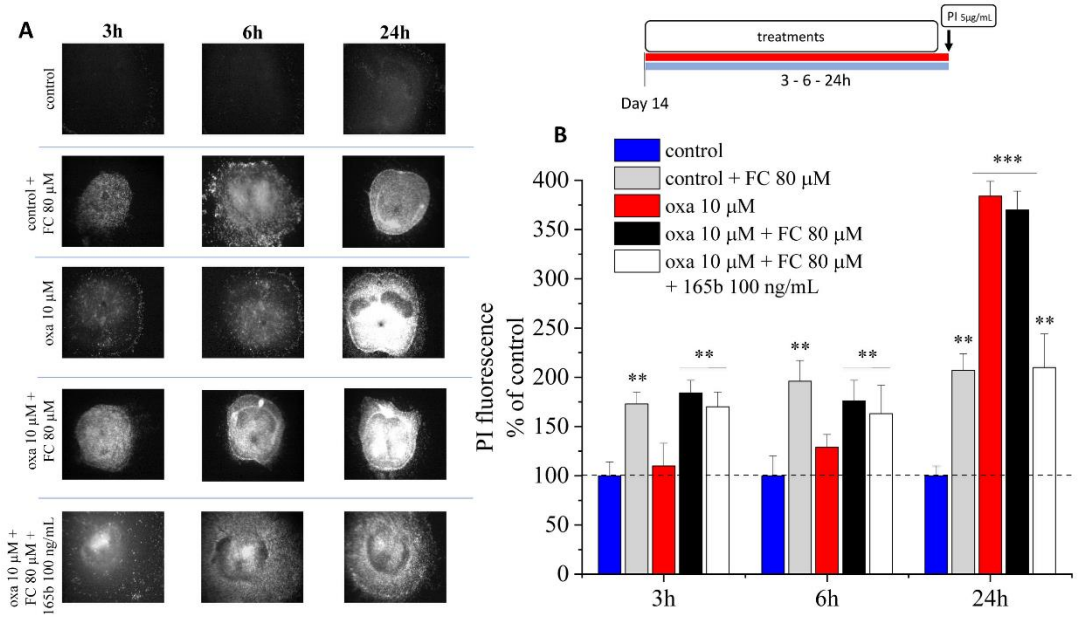


Figure19. Inhibition of astrocytic VEGF-A by fluorocitrate causes toxicity.

After 14 days of cultivation, qualitative (A) and quantitative (B) analysis of Propidium Iodide (5 µg/mL) fluorescence intensity were evaluated to study the effect of fluorocitrate (80 µM) treatment on oxaliplatin-induced toxicity.

Each value represents the mean ± SEM. $^{**}P<0.01$ and $^{***}P<0.001$ vs control. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.

5. Discussion

Vascular Endothelial Growth Factor-A (VEGF-A) is a member of a larger family of signalling ligands, with a variety of effects on different cell types. VEGF-A is mainly known as a pro-angiogenic factor mediating blood vessel formation, vascular permeability, endothelial cell proliferation, differentiation, leakage, migration, survival and motility[335]. Furthermore, its role in various forms of pain has been recognized in recent years, although conflicting data are present in literature suggesting both proalgic and analgesic effects of this growth factor. The aim of this thesis was to deepen the role of VEGF-A in pain perception, as well as its possible function in neuroprotection, and to investigate the involvement of its main VEGFR-1 and VEGFR-2 receptors in these pathways.

The first part of our data indicate that VEGF-A evokes pain through VEGFR-1 activation at the CNS site in physiological and pathological conditions. In particular, CINP is sustained by a spinal VEGF-A release from astrocytes that can be counteracted by the anti-VEGFR-1 mAb D16F7.

In naïve mice the intrathecal administration of VEGF165b (the most expressed endogenous isoform of VEGF-A, acting as a non-selective ligand for VEGFR-1 and VEGFR-2 receptors) induces a significant reduction in the pain threshold in a dose-dependent manner. Even the administration of the same doses of PlGF-2, a selective ligand for VEGFR-1[336], and VEGF-E, a selective ligand for VEGFR-2[155], evokes a similar proalgic effect.

In literature there are numerous preclinical studies supporting the hyperalgesic effect induced by VEGF-A[288]. Following intraplantar injection of VEGF-A, Selvaraj and colleagues observed significant hypersensitivity to mechanical (by Von Frey test) and thermal stimuli (by Plantar test) in mice, within 30 minutes of VEGF-A administration[285]. Also in this case, VEGF-A

induced a proalgic effect in a dose-dependent manner. The same evaluations were carried out following intraplantar administration of PlGF-2 showing that treatment with this ligand also induced thermal and mechanical hypersensitivity in a dose-dependent manner[285]. Another study reported that VEGF165 injection in rats with spinal cord injury (SCI) significantly increased not only the incidence of pain, but also mechanical hypersensitivity[337]. These results indicated that exogenous VEGF165 likely amplified the processes underlying the development of SCI neuropathic pain[337]. Still in support of VEGF-A-induced hyperalgesia, there are preclinical studies in which is reported that VEGF can act directly on sensory neurons to produce pain sensitization. VEGF-A has been shown to primarily potentiate P2X2/3 receptor-mediated pain responses on dorsal root ganglion (DRG) neurons[297]. In fact, the administration of anti-VEGF antibodies in rats with chronic sciatic nerve constriction injiury (CCI) led to the blocking of primary afferent nociceptive transmission mediated by VEGFR-2 and P2X2/3 receptors, determining the reduction of hyperalgesia in the states of chronic pain[297].

On the contrary, there are studies supporting the analgesic effect of VEGF-A. VEGF receptor inhibitors, SU5416 and DC101, were administered intraperitoneally in mice at doses that successfully inhibit tumor angiogenesis[338;339]. The development of tactile allodynia and thermal hyperalgesia was observed. The same treatment was carried out in mice with painful paclitaxel neuropathy, which developed a more marked tactile hypersensitivity than in animals treated with only paclitaxel[340].

Our studies also focused on understanding which of VEGFR-1 and VEGFR-2 receptors was most involved in VEGF-A proalgic signalling. We intrathecally administered D16F7 (VEGFR-1 blocker) and DC101 (VEGFR-2 blocker) in naïve mice. D16F7 is an anti-VEGFR-1 monoclonal antibody that does not prevent the interaction of VEGFR-1 with VEGF-A and PlGF-2, but inhibits

the cellular response following ligand binding to the receptor[309]. In fact, D16F7 does not increase the amount of VEGF-A available to interact and activate VEGFR-2. Furthermore, it does not hinder the ability of sVEGFR-1 to act as a decoy receptor for VEGF-A and PlGF-2[341]. This is particularly important considering the role of VEGFR-1 in pathological angiogenesis, its up-regulation in a variety of tumours and consequently its contribution to tumor progression[342]. In our experiments, D16F7 did not cause a significant reduction in the pain threshold compared to the control group, while the administration of DC101 induced hyperalgesia. We hypothesized that the proalgic effect observed for DC101, as well as that induced by VEGF-E administration, is not given by VEGFR-2 activation, but is due to the displacement of the endogenous VEGF-A from VEGFR-2 receptor by these ligands, which make it available for binding to VEGFR-1. In fact, pre-treatment with D16F7 blocks the hyperalgesia induced by both DC101 and the VEGF165b, PlGF-2 and VEGF-E agonists. These data have revealed VEGFR-1 as the receptor mainly involved in mediating the pro-algic effect induced by VEGF-A. To further confirm this data, the knockdown of VEGFR-1 prevented VEGF165b, PlGF-2 and VEGF-E effects, strongly indicating the pivotal role of this receptor in the spinal pain pathway. These data agree with those described by Selvaraj and colleagues[285] in the peripheral nervous system where VEGF-A induced nociceptive sensitization via VEGFR-1.

Consistently with behavioral data, electrophysiological experiments revealed that VEGF165b spinal application, caused a strongly increase of both spontaneous and evoked activity of NS neurons in naïve animals. In particular, the increased responsiveness to mechanical noxious stimuli of NS neurons induced by VEGF165b spinal microinjection suggests that low doses of this compound were able to induce a central sensitization, similarly to the neuropathic pain condition induced by nerve injury. In this context, the pre-

application of D16F7 prevented the VEGF-A-induced neuronal hyperexcitability, ruling out the contribution of this receptor in VEGF-A-mediated painful effects.

To investigate which cells are involved in VEGF-A signaling in pain, an immunohistochemical analysis was performed in the dorsal horn of the spinal cord of naïve mice to identify in which nerve cells VEGF-A and VEGFR-1 are more expressed. Although VEGF is known mainly for the fundamental role it plays in physiological and pathological vasculogenesis and angiogenesis, it is also important to highlight the functions it performs in the CNS. Indeed, it is believed that VEGF may be involved in the development of the nervous system, both in vessels differentiation and formation in the developing brain, as well as in neurogenesis and neuron growth control[248;249]. Furthermore, in pathological conditions, VEGF-A safeguards stressed neurons, induces axons extension and branching, promotes synaptic plasticity, triggers astrocytes proliferation, survival and migration and stimulates the expression of trophic factors by astrocytes and microglia[245;332]. We searched VEGF-A and its VEGFR-1 receptor in glial cells as these play a crucial role in maladaptive plasticity of the nervous system in pain, particularly chronic and neuropathic[343]. In fact, the glia is activated by neuronal damage or periphery signals and participates in the development and chronicization of pain[344;345(p1)] by increasing the release of soluble factors, such as cytokines and growth factors, which possess a direct nociceptive effect[346].

The present results show, as expected[332], a relevant spinal VEGF-A concentration in the vessel structure; nevertheless, the existence of an extra-endothelial component was verified and confirmed. In comparison to microglia and neurons, astrocytes of healthy mice showed the highest amount of VEGF-A, which was clearly distinguishable from the vascular

component. In addition, its VEGFR-1 appears clearly expressed on neuronal cells.

The repeated treatment with oxaliplatin up to the development of painful neuropathy significantly increased the presence of the growth factor in astroglia. The selective VEGF-A knockdown in dorsal horn astrocytes at the lumbar and thoracic levels of the spinal cord strongly reduced oxaliplatin-dependent neuropathic pain, suggesting astrocytic VEGF-A as a relevant component of the pain signalling orchestrated by glia. In support of this, the inhibition of astrocytic metabolism by fluorocitrate, in our rat organotypic slices *in-vitro* model, showed a notable reduction in VEGF-A levels in both physiologic and oxaliplatin treatment conditions, also causing an increase of toxicity. It is well known in literature that astrocytic activation in CIPN is involved in the development and maintenance of pain[347;348], releasing pro-inflammatory cytokines (e.g., IL-1β), chemokines (e.g., monocyte chemoattractant protein-1, or CCL2), and also growth factors (e.g., EGF, TGF-α, PDGF, VEGF) to enhance and prolong persistent pain states[349]. In particular, the hypoxia inducible factor-1 driven by IL-1 promotes VEGF-A release from astrocytes that induces down-regulation or loss of endothelial tight proteins claudin-5 and occludin, determining a loss of BBB function[350;351] by mechanisms involving VEGFR-1[352]. On the other hand, the increase in VEGF-A levels in neurotoxic conditions is generally related to hypoxia, clearly demonstrated in diabetic- as well as in chemotherapy-induced neuropathies[353;354;301], suggesting the need of improving vascular functions[332]. In addition, in our *in-vitro* model, VEGF-A treatment positively influenced the expression of mediators with a pivotal role in the development and maintenance of pain, such as Calcitonin gene-related peptide (CGRP), Substance P (SP) and Glutamate (the latter evaluated as a reduction in the expression of its two glial transporters EAAT1 and EAAT2). In the spinal

cord, high densities of CGRP receptor binding sites are found at all segmental levels in lamina I as well as the medial portion of laminae III–V while lower levels were found in lamina II[355]. Ma and colleagues reported the presence of immunoreactivities for the CGRP receptor components, CLR, RAMP1 and RCP, in neurons of lamina I to III of the dorsal horn in the rat spinal cord[356;356], suggesting the existence of functional CGRP receptors in the dorsal horn and their possible role in nociception. Data in literature suggest that CGRP not only directly activates nociceptive dorsal horn neurons to induce nociception, but also interacts with other pain-inducing excitatory neurotransmitters in this region[356]. CGRP has been shown to enhance the release of excitatory neurotransmitters such as glutamate, aspartate and SP in the spinal cord[357;358]. The alterations in CGRP and SP levels, and downregulation of EAAT1 and EAAT2 genes, caused by VEGF-A and oxaliplatin, were restored by D16F7 receptor blocker demonstrating once again the involvement of VEGFR-1 in pain modulation.

The experiments carried out by Oosthuyse and colleagues were the first to suggest that VEGF acted as a neurotrophic factor at the CNS, as the reduction of VEGF function induced a specific degeneration of motoneurons in the adult mice[359]. Our data obtained on organotypic spinal cord slices are perfectly in line with these results. Treatment with VEGF165b reduces in a dose-dependent manner not only the toxicity caused by oxaliplatin, but also the alterations in astrocytes and neurons. The rescue role of VEGF-A is based on its extra-vascular neuroprotective and neurodegenerative properties mainly due to the activation of the VEGFR-2[280]. Treatments with the selective ligands PlGF and VEGF-E, and with mAb D16F7 and DC101 blocking VEGFR-1 and VEGFR-2 respectively, has highlighted the main role of the VEGFR-2 receptor in VEGF-A-mediated neuroprotection.

VEGF-A stimulates the migration and survival of Schwann cells[258], it protects neurons against chemotherapy-induced cytotoxicity via activation of VEGFR-2 and MEK1/2 and inhibition of caspase-3[280]. VEGF-A-signalling through VEGFR-2 leads to the protection of dorsal root ganglion sensory neurons in models of drug (paclitaxel) or hyperglycaemia-induced neuropathies, through induction of Heat Shock Protein 90 deacetylation and increase of Bcl-2[340;360]. The loss of endothelial VEGFR-2 signalling leads to tissue alteration in the dorsal horn and the development of hyperalgesia whereas neuronal overexpression of VEGFR-2 in mice reduced the sensitivity to paclitaxel-induced peripheral neuropathy[340]. This outcome seems to be related to neuroprotective effects and, accordingly, we also showed *in-vivo* an increase of VEGFR-2 spinal expression in oxaliplatin-treated mice that could be considered an adaptive response to the damage. On the contrary, the acute stimulation of VEGFR-2 does not directly interfere with pain.

6. Conclusions

Our data show that VEGF-A induces pain by selectively activating the VEGFR-1, which is expressed on spinal sensory neurons. In this view, the selective anti-VEGFR-1 mAb D16F7 induced a potent pain-relieving effect against nociception triggered by VEGF-A or PlGF-2 as well as against neuropathic pain evoked by the neurotoxic adverse reactions of different anticancer drugs like oxaliplatin, paclitaxel and vincristine. In addition, the pain-relieving effect of D16F7 was demonstrated after local (i.t.) and systemic (i.p.) administration.

Using a complex *in-vitro* system such as rat organotypic spinal cord slices, we highlighted a dichotomy between the pro-algesic VEGFR-1-signaling and the protective VEGFR-2-signaling, offering the possibility to relieve pain through a target that conserves the neuroprotective effects of the endogenous VEGF-A.

References

1. Hunzelmann N, Eming S, Rosenkranz S. [Growth factors]. *Z Rheumatol*. 2007;66(4):290, 292-296. doi:10.1007/s00393-007-0175-3

2. Raja null, Sivamani K, Garcia MS, Isseroff RR. Wound re-epithelialization: modulating keratinocyte migration in wound healing. *Front Biosci*. 2007;12:2849-2868. doi:10.2741/2277

3. Lind M. Growth factors: Possible new clinical tools: A review. *Acta Orthopaedica Scandinavica*. 1996;67(4):407-417. doi:10.3109/17453679609002342

4. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen*. 2008;16(5):585-601. doi:10.1111/j.1524-475X.2008.00410.x

5. Pikuła M, Langa P, Kosikowska P, Trzonkowski P. [Stem cells and growth factors in wound healing]. *Postepy Hig Med Dosw (Online)*. 2015;69:874-885. doi:10.5604/17322693.1162989

6. Hsu EL, Stock SR. Growth Factors, Carrier Materials, and Bone Repair. *Handb Exp Pharmacol*. 2020;262:121-156. doi:10.1007/164_2020_371

7. Clark R, ed. *The Molecular and Cellular Biology of Wound Repair*. Springer US; 1988. doi:10.1007/978-1-4899-0185-9

8. Smythe G. Role of Growth Factors in Modulation of the Microvasculature in Adult Skeletal Muscle. *Adv Exp Med Biol*. 2016;900:161-183. doi:10.1007/978-3-319-27511-6_7

9. Adzick NS, Lorenz HP. Cells, matrix, growth factors, and the surgeon. The biology of scarless fetal wound repair. *Ann Surg*. 1994;220(1):10-18. doi:10.1097/00000658-199407000-00003

10. Cohen S, Levi-Montalcini R, Hamburger V. A NERVE GROWTH-STIMULATING FACTOR ISOLATED FROM SARCOM AS 37 AND 180*. *Proc Natl Acad Sci U S A*. 1954;40(10):1014-1018.

11. Todaro GJ, De Larco JE, Cohen S. Transformation by murine and feline sarcoma viruses specifically blocks binding of epidermal growth factor to cells. *Nature*. 1976;264(5581):26-31. doi:10.1038/264026a0

12. Waterfield MD, Scrace GT, Whittle N, et al. Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. *Nature*. 1983;304(5921):35-39. doi:10.1038/304035a0

13. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61(5):759-767. doi:10.1016/0092-8674(90)90186-i

14. Dechant G, Neumann H. Neurotrophins. *Adv Exp Med Biol*. 2002;513:303-334. doi:10.1007/978-1-4615-0123-7_11

15. Hallböök F. Evolution of the vertebrate neurotrophin and Trk receptor gene families. *Curr Opin Neurobiol*. 1999;9(5):616-621. doi:10.1016/S0959-4388(99)00011-2

16. Levi-Montalcini R. The nerve growth factor 35 years later. *Science*. 1987;237(4819):1154-1162. doi:10.1126/science.3306916

17. Bothwell M. NGF, BDNF, NT3, and NT4. *Handb Exp Pharmacol*. 2014;220:3-15. doi:10.1007/978-3-642-45106-5_1

18. Thoenen H. The changing scene of neurotrophic factors. *Trends Neurosci*. 1991;14(5):165-170. doi:10.1016/0166-2236(91)90097-e

19. Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. *Nat Rev Neurosci*. 2005;6(8):603-614. doi:10.1038/nrn1726

20. Reichardt LF. Neurotrophin-regulated signalling pathways. *Philos Trans R Soc Lond B Biol Sci*. 2006;361(1473):1545-1564. doi:10.1098/rstb.2006.1894

21. Ahmed S, Reynolds BA, Weiss S. BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors. *J Neurosci*. 1995;15(8):5765-5778.

22. Minichiello L. TrkB signalling pathways in LTP and learning. *Nat Rev Neurosci*. 2009;10(12):850-860. doi:10.1038/nrn2738

23. Lachyankar MB, Condon PJ, Quesenberry PJ, Litofsky NS, Recht LD, Ross AH. Embryonic precursor cells that express Trk receptors: induction of different cell fates by NGF, BDNF, NT-3, and CNTF. *Exp Neurol*. 1997;144(2):350-360. doi:10.1006/exnr.1997.6434

24. Shetty AK, Turner DA. In vitro survival and differentiation of neurons derived from epidermal growth factor-responsive postnatal hippocampal stem cells: inducing effects of brain-derived neurotrophic factor. *J Neurobiol*. 1998;35(4):395-425. doi:10.1002/(sici)1097-4695(19980615)35:4<395::aid-neu7>3.0.co;2-u

25. Jones KR, Fariñas I, Backus C, Reichardt LF. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell*. 1994;76(6):989-999. doi:10.1016/0092-8674(94)90377-8

26. Benraiss A, Chmielnicki E, Lerner K, Roh D, Goldman SA. Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. *J Neurosci*. 2001;21(17):6718-6731.

27. Scharfman H, Goodman J, Macleod A, Phani S, Antonelli C, Croll S. Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. *Exp Neurol*. 2005;192(2):348-356. doi:10.1016/j.expneurol.2004.11.016

28. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2000;103(2):211-225. doi:10.1016/s0092-8674(00)00114-8

29. Allsopp TE, Robinson M, Wyatt S, Davies AM. TrkA mediates an NGF survival response in NGF-independent sensory neurons but not in parasympathetic neurons. *Gene Ther*. 1994;1 Suppl 1:S59.

30. Barrett GL, Bartlett PF. The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc Natl Acad Sci U S A*. 1994;91(14):6501-6505. doi:10.1073/pnas.91.14.6501

31. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci*. 2003;4(4):299-309. doi:10.1038/nrn1078

32. Strohmaier C, Carter BD, Urfer R, Barde YA, Dechant G. A splice variant of the neurotrophin receptor trkB with increased specificity for brain-derived neurotrophic factor. *EMBO J*. 1996;15(13):3332-3337.

33. Clary DO, Reichardt LF. An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. *Proc Natl Acad Sci U S A*. 1994;91(23):11133-11137. doi:10.1073/pnas.91.23.11133

34. Wiesmann C, Ultsch MH, Bass SH, de Vos AM. Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. *Nature*. 1999;401(6749):184-188. doi:10.1038/43705

35. Baxter GT, Radeke MJ, Kuo RC, et al. Signal transduction mediated by the truncated trkB receptor isoforms, trkB.T1 and trkB.T2. *J Neurosci*. 1997;17(8):2683-2690.

36. Hapner SJ, Boeshore KL, Large TH, Lefcort F. Neural differentiation promoted by truncated trkC receptors in collaboration with p75(NTR). *Dev Biol*. 1998;201(1):90-100. doi:10.1006/dbio.1998.8970

37. Barde YA. Trophic factors and neuronal survival. *Neuron*. 1989;2(6):1525-1534. doi:10.1016/0896-6273(89)90040-8

38. Frade JM, Rodríguez-Tébar A, Barde YA. Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature*. 1996;383(6596):166-168. doi:10.1038/383166a0

39. Chartier SR, Mitchell SA, Majuta LA, Mantyh PW. Immunohistochemical localization of nerve growth factor, tropomyosin receptor kinase A, and p75 in the bone and articular cartilage of the mouse femur. *Mol Pain*. 2017;13:1744806917745465. doi:10.1177/1744806917745465

40. Hiltunen JO, Laurikainen A, Väkevä A, Meri S, Saarma M. Nerve growth factor and brain-derived neurotrophic factor mRNAs are regulated in distinct cell populations of rat heart after ischaemia and reperfusion. *J Pathol*. 2001;194(2):247-253. doi:10.1002/path.878

41. de Azevedo Cardoso T, Mondin TC, Wiener CD, et al. Neurotrophic factors, clinical features and gender differences in depression. *Neurochem Res*. 2014;39(8):1571-1578. doi:10.1007/s11064-014-1349-4

42. Indo Y. Molecular basis of congenital insensitivity to pain with anhidrosis (CIPA): mutations and polymorphisms in TRKA (NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. *Hum Mutat*. 2001;18(6):462-471. doi:10.1002/humu.1224

43. McMahon S, Bennett D, Bevan S. Inflammatory mediators and modulators of pain. *Wall and Melzack's textbook of pain*. Published online 2006:49-72.

44. Pezet S. [Neurotrophins and pain]. *Biol Aujourdhui*. 2014;208(1):21-29. doi:10.1051/jbio/2014002

45. Petty BG, Cornblath DR, Adornato BT, et al. The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. *Ann Neurol*. 1994;36(2):244-246. doi:10.1002/ana.410360221

46. Svensson P, Cairns BE, Wang K, Arendt-Nielsen L. Injection of nerve growth factor into human masseter muscle evokes long-lasting mechanical allodynia and hyperalgesia. *Pain*. 2003;104(1-2):241-247. doi:10.1016/s0304-3959(03)00012-5

47. Lewin GR, Ritter AM, Mendell LM. Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. *J Neurosci*. 1993;13(5):2136-2148.

48. Khasar SG, Lin YH, Martin A, et al. A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron*. 1999;24(1):253-260. doi:10.1016/s0896-6273(00)80837-5

49. Chuang HH, Prescott ED, Kong H, et al. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature*. 2001;411(6840):957-962. doi:10.1038/35082088

50. Ji R-R, Samad TA, Jin S-X, Schmoll R, Woolf CJ. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron*. 2002;36(1):57-68. doi:10.1016/s0896-6273(02)00908-x

51. Zhuang Z-Y, Xu H, Clapham DE, Ji R-R. Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. *J Neurosci*. 2004;24(38):8300-8309. doi:10.1523/JNEUROSCI.2893-04.2004

52. Kerr BJ, Souslova V, McMahon SB, Wood JN. A role for the TTX-resistant sodium channel Nav 1.8 in NGF-induced hyperalgesia, but not neuropathic pain. *Neuroreport*. 2001;12(14):3077-3080. doi:10.1097/00001756-200110080-00019

53. Hoheisel U, Unger T, Mense S. Excitatory and modulatory effects of inflammatory cytokines and neurotrophins on mechanosensitive group IV muscle afferents in the rat. *Pain*. 2005;114(1-2):168-176. doi:10.1016/j.pain.2004.12.020

54. Priestley JV, Michael GJ, Averill S, Liu M, Willmott N. Regulation of nociceptive neurons by nerve growth factor and glial cell line derived neurotrophic factor. *Can J Physiol Pharmacol*. 2002;80(5):495-505. doi:10.1139/y02-034

55. Chen W, Ye D-Y, Han D-J, et al. Elevated level of nerve growth factor in the bladder pain syndrome/interstitial cystitis: a meta-analysis. *Springerplus*. 2016;5(1):1072. doi:10.1186/s40064-016-2719-y

56. di Mola FF, Friess H, Zhu ZW, et al. Nerve growth factor and Trk high affinity receptor (TrkA) gene expression in inflammatory bowel disease. *Gut*. 2000;46(5):670-679. doi:10.1136/gut.46.5.670

57. Friess H, Zhu ZW, di Mola FF, et al. Nerve growth factor and its high-affinity receptor in chronic pancreatitis. *Ann Surg*. 1999;230(5):615-624. doi:10.1097/00000658-199911000-00002

58. Iannone F, De Bari C, Dell'Accio F, et al. Increased expression of nerve growth factor (NGF) and high affinity NGF receptor (p140 TrkA) in human osteoarthritic chondrocytes. *Rheumatology (Oxford)*. 2002;41(12):1413-1418. doi:10.1093/rheumatology/41.12.1413

59. Aloe L, Tuveri MA, Carcassi U, Levi-Montalcini R. Nerve growth factor in the synovial fluid of patients with chronic arthritis. *Arthritis Rheum*. 1992;35(3):351-355. doi:10.1002/art.1780350315

60. Barthel C, Yeremenko N, Jacobs R, et al. Nerve growth factor and receptor expression in rheumatoid arthritis and spondyloarthritis. *Arthritis Res Ther*. 2009;11(3):R82. doi:10.1186/ar2716

61. Dawes JM, Calvo M, Perkins JR, et al. CXCL5 mediates UVB irradiation-induced pain. *Sci Transl Med*. 2011;3(90):90ra60. doi:10.1126/scitranslmed.3002193

62. Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D. Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *Eur J Pain*. 2006;10(4):287-333. doi:10.1016/j.ejpain.2005.06.009

63. Mitra R. The utilization of opiates in pain management: use or abuse. *Eur J Phys Rehabil Med*. 2013;49(1):93-96.

64. Denk F, Bennett DL, McMahon SB. Nerve Growth Factor and Pain Mechanisms. *Annu Rev Neurosci*. 2017;40:307-325. doi:10.1146/annurev-neuro-072116-031121

65. Lewin GR, Rueff A, Mendell LM. Peripheral and central mechanisms of NGF-induced hyperalgesia. *Eur J Neurosci*. 1994;6(12):1903-1912. doi:10.1111/j.1460-9568.1994.tb00581.x

66. Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neuroscience*. 1994;62(2):327-331. doi:10.1016/0306-4522(94)90366-2

67. McMahon SB, Bennett DL, Priestley JV, Shelton DL. The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. *Nat Med*. 1995;1(8):774-780. doi:10.1038/nm0895-774

68. Sevcik MA, Ghilardi JR, Peters CM, et al. Anti-NGF therapy profoundly reduces bone cancer pain and the accompanying increase in markers of peripheral and central sensitization. *Pain*. 2005;115(1-2):128-141. doi:10.1016/j.pain.2005.02.022

69. Zahn PK, Subieta A, Park SS, Brennan TJ. Effect of blockade of nerve growth factor and tumor necrosis factor on pain behaviors after plantar incision. *J Pain*. 2004;5(3):157-163. doi:10.1016/j.jpain.2004.02.538

70. Sabsovich I, Wei T, Guo T-Z, et al. Effect of anti-NGF antibodies in a rat tibia fracture model of complex regional pain syndrome type I. *Pain*. 2008;138(1):47-60. doi:10.1016/j.pain.2007.11.004

71. Koltzenburg M, Bennett DL, Shelton DL, McMahon SB. Neutralization of endogenous NGF prevents the sensitization of nociceptors supplying inflamed skin. *Eur J Neurosci*. 1999;11(5):1698-1704. doi:10.1046/j.1460-9568.1999.00590.x

72. Woolf CJ. Phenotypic modification of primary sensory neurons: the role of nerve growth factor in the production of persistent pain. *Philos Trans R Soc Lond B Biol Sci*. 1996;351(1338):441-448. doi:10.1098/rstb.1996.0040

73. Schnitzer TJ, Marks JA. A systematic review of the efficacy and general safety of antibodies to NGF in the treatment of OA of the hip or knee. *Osteoarthritis Cartilage*. 2015;23 Suppl 1:S8-17. doi:10.1016/j.joca.2014.10.003

74. Lindsay RM. Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J Neurosci*. 1988;8(7):2394-2405.

75. Mullard A. Drug developers reboot anti-NGF pain programmes. *Nat Rev Drug Discov*. 2015;14(5):297-298. doi:10.1038/nrd4612

76. Morikawa M, Derynck R, Miyazono K. TGF-β and the TGF-β Family: Context-Dependent Roles in Cell and Tissue Physiology. *Cold Spring Harb Perspect Biol*. 2016;8(5):a021873. doi:10.1101/cshperspect.a021873

77. Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc Natl Acad Sci U S A*. 1981;78(9):5339-5343. doi:10.1073/pnas.78.9.5339

78. Derynck R, Jarrett JA, Chen EY, et al. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature*. 1985;316(6030):701-705. doi:10.1038/316701a0

79. Mathews LS, Vale WW. Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell*. 1991;65(6):973-982. doi:10.1016/0092-8674(91)90549-e

80. Raftery LA, Twombly V, Wharton K, Gelbart WM. Genetic screens to identify elements of the decapentaplegic signaling pathway in Drosophila. *Genetics*. 1995;139(1):241-254. doi:10.1093/genetics/139.1.241

81. Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH, Gelbart WM. Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. *Genetics*. 1995;139(3):1347-1358. doi:10.1093/genetics/139.3.1347

82. Lagna G, Hata A, Hemmati-Brivanlou A, Massagué J. Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. *Nature*. 1996;383(6603):832-836. doi:10.1038/383832a0

83. Kawabata M, Inoue H, Hanyu A, Imamura T, Miyazono K. Smad proteins exist as monomers in vivo and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. *EMBO J*. 1998;17(14):4056-4065. doi:10.1093/emboj/17.14.4056

84. Zhang Y, Feng X, We R, Derynck R. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature*. 1996;383(6596):168-172. doi:10.1038/383168a0

85. Massagué J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell*. 2000;103(2):295-309. doi:10.1016/s0092-8674(00)00121-5

86. Ten Dijke P, Goumans M-J, Itoh F, Itoh S. Regulation of cell proliferation by Smad proteins. *J Cell Physiol*. 2002;191(1):1-16. doi:10.1002/jcp.10066

87. Groppe J, Greenwald J, Wiater E, et al. Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. *Nature*. 2002;420(6916):636-642. doi:10.1038/nature01245

88. Greenwald J, Fischer WH, Vale WW, Choe S. Three-finger toxin fold for the extracellular ligand-binding domain of the type II activin receptor serine kinase. *Nat Struct Biol*. 1999;6(1):18-22. doi:10.1038/4887

89. Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massagué J. The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell*. 2001;8(3):671-682. doi:10.1016/s1097-2765(01)00332-x

90. Cheng SK, Olale F, Bennett JT, Brivanlou AH, Schier AF. EGF-CFC proteins are essential coreceptors for the TGF-beta signals Vg1 and GDF1. *Genes Dev*. 2003;17(1):31-36. doi:10.1101/gad.1041203

91. Derynck R, Gelbart WM, Harland RM, et al. Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell*. 1996;87(2):173. doi:10.1016/s0092-8674(00)81335-5

92. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev*. 1999;79(4):1283-1316. doi:10.1152/physrev.1999.79.4.1283

93. Rosenkranz S, Kazlauskas A. Evidence for distinct signaling properties and biological responses induced by the PDGF receptor alpha and beta subtypes. *Growth Factors*. 1999;16(3):201-216. doi:10.3109/08977199909002130

94. Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev*. 2004;15(4):197-204. doi:10.1016/j.cytogfr.2004.03.007

95. Vantler M, Caglayan E, Zimmermann WH, Bäumer AT, Rosenkranz S. Systematic evaluation of anti-apoptotic growth factor signaling in vascular smooth muscle cells. Only phosphatidylinositol 3′-kinase is important. *J Biol Chem*. 2005;280(14):14168-14176. doi:10.1074/jbc.M413310200

96. Wang J, Dai H, Yousaf N, et al. Grb10, a positive, stimulatory signaling adapter in platelet-derived growth factor BB-, insulin-like growth factor I-, and insulin-mediated mitogenesis. *Mol Cell Biol*. 1999;19(9):6217-6228. doi:10.1128/MCB.19.9.6217

97. Gherardi E, Gray J, Stoker M, Perryman M, Furlong R. Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interactions and movement. *Proc Natl Acad Sci U S A*. 1989;86(15):5844-5848. doi:10.1073/pnas.86.15.5844

98. Higashio K, Shima N, Goto M, et al. Identity of a tumor cytotoxic factor from human fibroblasts and hepatocyte growth factor. *Biochem Biophys Res Commun*. 1990;170(1):397-404. doi:10.1016/0006-291x(90)91287-3

99. Kozlow W, Guise TA. Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy. *J Mammary Gland Biol Neoplasia*. 2005;10(2):169-180. doi:10.1007/s10911-005-5399-8

100. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nat Rev Cancer*. 2002;2(4):289-300. doi:10.1038/nrc779

101. Menakongka A, Suthiphongchai T. Involvement of PI3K and ERK1/2 pathways in hepatocyte growth factor-induced cholangiocarcinoma cell invasion. *World J Gastroenterol*. 2010;16(6):713-722. doi:10.3748/wjg.v16.i6.713

102. Previdi S, Maroni P, Matteucci E, Broggini M, Bendinelli P, Desiderio MA. Interaction between human-breast cancer metastasis and bone microenvironment through activated hepatocyte growth factor/Met and beta-catenin/Wnt pathways. *Eur J Cancer*. 2010;46(9):1679-1691. doi:10.1016/j.ejca.2010.02.036

103. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol*. 2003;4(12):915-925. doi:10.1038/nrm1261

104. Accornero P, Miretti S, Starvaggi Cucuzza L, Martignani E, Baratta M. Epidermal growth factor and hepatocyte growth factor cooperate to enhance cell proliferation, scatter, and invasion in murine mammary epithelial cells. *J Mol Endocrinol*. 2010;44(2):115-125. doi:10.1677/JME-09-0035

105. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: Growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 1998;85(6):638-646. doi:10.1016/s1079-2104(98)90029-4

106. Kawase T, Okuda K, Wolff LF, Yoshie H. Platelet-rich plasma-derived fibrin clot formation stimulates collagen synthesis in periodontal ligament and osteoblastic cells in vitro. *J Periodontol*. 2003;74(6):858-864. doi:10.1902/jop.2003.74.6.858

107. El-Sharkawy H, Kantarci A, Deady J, et al. Platelet-rich plasma: growth factors and pro- and anti-inflammatory properties. *J Periodontol*. 2007;78(4):661-669. doi:10.1902/jop.2007.060302

108. Cieslik-Bielecka A, Dohan Ehrenfest DM, Lubkowska A, Bielecki T. Microbicidal properties of Leukocyte- and Platelet-Rich Plasma/Fibrin (L-PRP/L-PRF): new perspectives. *J Biol Regul Homeost Agents*. 2012;26(2 Suppl 1):43S-52S.

109. Tohidnezhad M, Varoga D, Wruck CJ, et al. Platelets display potent antimicrobial activity and release human beta-defensin 2. *Platelets*. 2012;23(3):217-223. doi:10.3109/09537104.2011.610908

110. Burnouf T, Chou M-L, Wu Y-W, Su C-Y, Lee L-W. Antimicrobial activity of platelet (PLT)-poor plasma, PLT-rich plasma, PLT gel, and solvent/detergent-treated PLT lysate biomaterials against wound bacteria. *Transfusion*. 2013;53(1):138-146. doi:10.1111/j.1537-2995.2012.03668.x

111. Anitua E, Pascual C, Antequera D, et al. Plasma rich in growth factors (PRGF-Endoret) reduces neuropathologic hallmarks and improves cognitive functions in an Alzheimer's disease mouse model. *Neurobiol Aging*. 2014;35(7):1582-1595. doi:10.1016/j.neurobiolaging.2014.01.009

112. López-Plandolit S, Morales M-C, Freire V, Etxebarría J, Durán JA. Plasma rich in growth factors as a therapeutic agent for persistent corneal epithelial defects. *Cornea*. 2010;29(8):843-848. doi:10.1097/ICO.0b013e3181a81820

113. López-Plandolit S, Morales M-C, Freire V, Grau AE, Durán JA. Efficacy of plasma rich in growth factors for the treatment of dry eye. *Cornea*. 2011;30(12):1312-1317. doi:10.1097/ICO.0b013e31820d86d6

114. Vecino E, Rodriguez FD, Ruzafa N, Pereiro X, Sharma SC. Glia-neuron interactions in the mammalian retina. *Prog Retin Eye Res*. 2016;51:1-40. doi:10.1016/j.preteyeres.2015.06.003

115. Bringmann A, Reichenbach A. Role of Muller cells in retinal degenerations. *Front Biosci*. 2001;6:E72-92. doi:10.2741/bringman

116. Reichenbach A, Bringmann A. New functions of Müller cells. *Glia*. 2013;61(5):651-678. doi:10.1002/glia.22477

117. García M, Forster V, Hicks D, Vecino E. Effects of müller glia on cell survival and neuritogenesis in adult porcine retina in vitro. *Invest Ophthalmol Vis Sci*. 2002;43(12):3735-3743.

118. Ruzafa N, Pereiro X, Lepper MF, Hauck SM, Vecino E. A Proteomics Approach to Identify Candidate Proteins Secreted by Müller Glia that Protect Ganglion Cells in the Retina. *Proteomics*. 2018;18(11):e1700321. doi:10.1002/pmic.201700321

119. O'Kusky JR, Ye P, D'Ercole AJ. Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *J Neurosci*. 2000;20(22):8435-8442.

120. Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc Natl Acad Sci U S A*. 2002;99(18):11946-11950. doi:10.1073/pnas.182296499

121. Orive G, Anitua E, Pedraz JL, Emerich DF. Biomaterials for promoting brain protection, repair and regeneration. *Nat Rev Neurosci*. 2009;10(9):682-692. doi:10.1038/nrn2685

122. Böttner M, Krieglstein K, Unsicker K. The transforming growth factor-betas: structure, signaling, and roles in nervous system development and functions. *J Neurochem*. 2000;75(6):2227-2240. doi:10.1046/j.1471-4159.2000.0752227.x

123. Lantero A, Tramullas M, Díaz A, Hurlé MA. Transforming growth factor-β in normal nociceptive processing and pathological pain models. *Mol Neurobiol*. 2012;45(1):76-86. doi:10.1007/s12035-011-8221-1

124. Ronaldson PT, Demarco KM, Sanchez-Covarrubias L, Solinsky CM, Davis TP. Transforming growth factor-beta signaling alters substrate permeability and tight junction protein expression at the blood-brain barrier during inflammatory pain. *J Cereb Blood Flow Metab*. 2009;29(6):1084-1098. doi:10.1038/jcbfm.2009.32

125. Ronaldson PT, Finch JD, Demarco KM, Quigley CE, Davis TP. Inflammatory pain signals an increase in functional expression of organic anion transporting polypeptide 1a4 at the blood-brain barrier. *J Pharmacol Exp Ther*. 2011;336(3):827-839. doi:10.1124/jpet.110.174151

126. Echeverry S, Shi XQ, Rivest S, Zhang J. Peripheral nerve injury alters blood-spinal cord barrier functional and molecular integrity through a selective inflammatory pathway. *J Neurosci*. 2011;31(30):10819-10828. doi:10.1523/JNEUROSCI.1642-11.2011

127. Barkai O, Puig S, Lev S, et al. Platelet-derived growth factor activates nociceptive neurons by inhibiting M-current and contributes to inflammatory pain. *Pain*. 2019;160(6):1281-1296. doi:10.1097/j.pain.0000000000001523

128. Nakamura T, Sakai K, Nakamura T, Matsumoto K. Hepatocyte growth factor twenty years on: Much more than a growth factor. *J Gastroenterol Hepatol*. 2011;26 Suppl 1:188-202. doi:10.1111/j.1440-1746.2010.06549.x

129. Cui S, Guo L, Li X, et al. Clinical Safety and Preliminary Efficacy of Plasmid pUDK-HGF Expressing Human Hepatocyte Growth Factor (HGF) in Patients with Critical Limb Ischemia. *Eur J Vasc Endovasc Surg*. 2015;50(4):494-501. doi:10.1016/j.ejvs.2015.05.007

130. Harada K, Negishi M, Katoh H. HGF-induced serine 897 phosphorylation of EphA2 regulates epithelial morphogenesis of MDCK cells in 3D culture. *J Cell Sci*. 2015;128(10):1912-1921. doi:10.1242/jcs.163790

131. Ohda Y, Hori K, Tomita T, et al. Effects of hepatocyte growth factor on rat inflammatory bowel disease models. *Dig Dis Sci*. 2005;50(5):914-921. doi:10.1007/s10620-005-2664-z

132. Matsumoto K, Nakamura T. Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. *Biochem Biophys Res Commun*. 1997;239(3):639-644. doi:10.1006/bbrc.1997.7517

133. Ishikawa H, Jo J-I, Tabata Y. Liver Anti-Fibrosis Therapy with Mesenchymal Stem Cells Secreting Hepatocyte Growth Factor. *J Biomater Sci Polym Ed*. 2012;23(18):2259-2272. doi:10.1163/156856211X614761

134. Flaquer M, Franquesa M, Vidal A, et al. Hepatocyte growth factor gene therapy enhances infiltration of macrophages and may induce kidney repair in db/db mice as a model of diabetes. *Diabetologia*. 2012;55(7):2059-2068. doi:10.1007/s00125-012-2535-z

135. Caton A, Hacker A, Naeem A, et al. The branchial arches and HGF are growth-promoting and chemoattractant for cranial motor axons. *Development*. 2000;127(8):1751-1766.

136. Maina F, Klein R. Hepatocyte growth factor, a versatile signal for developing neurons. *Nat Neurosci*. 1999;2(3):213-217. doi:10.1038/6310

137. Esaki S, Kitoh J, Katsumi S, et al. Hepatocyte growth factor incorporated into herpes simplex virus vector accelerates facial nerve regeneration after crush injury. *Gene Ther*. 2011;18(11):1063-1069. doi:10.1038/gt.2011.57

138. Yoshida S, Harada T, Mitsunari M, et al. Hepatocyte growth factor/Met system promotes endometrial and endometriotic stromal cell invasion via autocrine and paracrine pathways. *J Clin Endocrinol Metab*. 2004;89(2):823-832. doi:10.1210/jc.2003-030874

139. Thompson J, Dolcet X, Hilton M, Tolcos M, Davies AM. HGF promotes survival and growth of maturing sympathetic neurons by PI-3 kinase- and MAP kinase-dependent mechanisms. *Mol Cell Neurosci*. 2004;27(4):441-452. doi:10.1016/j.mcn.2004.07.007

140. Ohya W, Funakoshi H, Kurosawa T, Nakamura T. Hepatocyte growth factor (HGF) promotes oligodendrocyte progenitor cell proliferation and inhibits its differentiation during postnatal development in the rat. *Brain Res*. 2007;1147:51-65. doi:10.1016/j.brainres.2007.02.045

141. Zhang J, Middleton KK, Fu FH, Im H-J, Wang JH-C. HGF mediates the anti-inflammatory effects of PRP on injured tendons. *PLoS One*. 2013;8(6):e67303. doi:10.1371/journal.pone.0067303

142. Tsuchihara T, Ogata S, Nemoto K, et al. Nonviral retrograde gene transfer of human hepatocyte growth factor improves neuropathic pain-related phenomena in rats. *Mol Ther*. 2009;17(1):42-50. doi:10.1038/mt.2008.214

143. Tiwari V, Guan Y, Raja SN. Modulating the delicate glial-neuronal interactions in neuropathic pain: promises and potential caveats. *Neurosci Biobehav Rev*. 2014;45:19-27. doi:10.1016/j.neubiorev.2014.05.002

144. Mika J, Osikowicz M, Rojewska E, et al. Differential activation of spinal microglial and astroglial cells in a mouse model of peripheral neuropathic pain. *Eur J Pharmacol*. 2009;623(1-3):65-72. doi:10.1016/j.ejphar.2009.09.030

145. Gao Y-J, Ji R-R. Light touch induces ERK activation in superficial dorsal horn neurons after inflammation: involvement of spinal astrocytes and JNK signaling in touch-evoked central sensitization and mechanical allodynia. *J Neurochem*. 2010;115(2):505-514. doi:10.1111/j.1471-4159.2010.06946.x

146. Hu C-T, Wu J-R, Cheng C-C, Wu W-S. The Therapeutic Targeting of HGF/c-Met Signaling in Hepatocellular Carcinoma: Alternative Approaches. *Cancers (Basel)*. 2017;9(6):E58. doi:10.3390/cancers9060058

147. Anitua E, Sánchez M, Aguirre JJ, Prado R, Padilla S, Orive G. Efficacy and safety of plasma rich in growth factors intra-articular infiltrations in the treatment of knee osteoarthritis. *Arthroscopy*. 2014;30(8):1006-1017. doi:10.1016/j.arthro.2014.05.021

148. Anitua E, Sánchez M, Orive G. Potential of endogenous regenerative technology for in situ regenerative medicine. *Adv Drug Deliv Rev*. 2010;62(7-8):741-752. doi:10.1016/j.addr.2010.01.001

149. Sánchez M, Fiz N, Azofra J, et al. A randomized clinical trial evaluating plasma rich in growth factors (PRGF-Endoret) versus hyaluronic acid in the short-term treatment of symptomatic knee osteoarthritis. *Arthroscopy*. 2012;28(8):1070-1078. doi:10.1016/j.arthro.2012.05.011

150. Uçeyler N, Schäfers M, Sommer C. Mode of action of cytokines on nociceptive neurons. *Exp Brain Res*. 2009;196(1):67-78. doi:10.1007/s00221-009-1755-z

151. van Buul GM, Koevoet WLM, Kops N, et al. Platelet-rich plasma releasate inhibits inflammatory processes in osteoarthritic chondrocytes. *Am J Sports Med*. 2011;39(11):2362-2370. doi:10.1177/0363546511419278

152. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol*. 2004;25(6):280-288. doi:10.1016/j.it.2004.03.008

153. Anitua E, Sánchez M, Orive G, Padilla S. A biological therapy to osteoarthritis treatment using platelet-rich plasma. *Expert Opin Biol Ther*. 2013;13(8):1161-1172. doi:10.1517/14712598.2013.801450

154. Kirchner F, Anitua E. Intradiscal and intra-articular facet infiltrations with plasma rich in growth factors reduce pain in patients with chronic low back pain. *J Craniovertebr Junction Spine*. 2016;7(4):250-256. doi:10.4103/0974-8237.193260

155. Ferrara N, Gerber H-P, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9(6):669-676. doi:10.1038/nm0603-669

156. Dvorak HF, Orenstein NS, Carvalho AC, et al. Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. *J Immunol*. 1979;122(1):166-174.

157. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*. 1983;219(4587):983-985. doi:10.1126/science.6823562

158. Ferrara N. From the discovery of vascular endothelial growth factor to the introduction of avastin in clinical trials - an interview with Napoleone Ferrara by Domenico Ribatti. *Int J Dev Biol*. 2011;55(4-5):383-388. doi:10.1387/ijdb.103216dr

159. Senger DR. Vascular endothelial growth factor: much more than an angiogenesis factor. *Mol Biol Cell*. 2010;21(3):377-379. doi:10.1091/mbc.e09-07-0591

160. Apte RS, Chen DS, Ferrara N. VEGF in Signaling and Disease: Beyond Discovery and Development. *Cell*. 2019;176(6):1248-1264. doi:10.1016/j.cell.2019.01.021

161. Yamazaki Y, Morita T. Molecular and functional diversity of vascular endothelial growth factors. *Mol Divers*. 2006;10(4):515-527. doi:10.1007/s11030-006-9027-3

162. Tjwa M, Luttun A, Autiero M, Carmeliet P. VEGF and PlGF: two pleiotropic growth factors with distinct roles in development and homeostasis. *Cell Tissue Res*. 2003;314(1):5-14. doi:10.1007/s00441-003-0776-3

163. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev*. 2004;25(4):581-611. doi:10.1210/er.2003-0027

164. Rosen LS. Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers. *Cancer Control*. 2002;9(2 Suppl):36-44. doi:10.1177/107327480200902S05

165. Duffy AM, Bouchier-Hayes DJ, Harmey JH. *Vascular Endothelial Growth Factor (VEGF) and Its Role in Non-Endothelial Cells: Autocrine Signalling by VEGF*. Landes Bioscience; 2013. Accessed October 18, 2021. https://www.ncbi.nlm.nih.gov/books/NBK6482/

166. Klettner A, Westhues D, Lassen J, Bartsch S, Roider J. Regulation of constitutive vascular endothelial growth factor secretion in retinal pigment epithelium/choroid organ cultures: p38, nuclear factor κB, and the vascular endothelial growth factor receptor-2/phosphatidylinositol 3 kinase pathway. *Mol Vis*. 2013;19:281-291.

167. Wang J-J, Zhu M, Le Y-Z. Functions of Müller cell-derived vascular endothelial growth factor in diabetic retinopathy. *World J Diabetes*. 2015;6(5):726-733. doi:10.4239/wjd.v6.i5.726

168. Maloney JP, Gao L. Proinflammatory Cytokines Increase Vascular Endothelial Growth Factor Expression in Alveolar Epithelial Cells. *Mediators Inflamm*. 2015;2015:387842. doi:10.1155/2015/387842

169. Raza A, Franklin MJ, Dudek AZ. Pericytes and vessel maturation during tumor angiogenesis and metastasis. *Am J Hematol*. 2010;85(8):593-598. doi:10.1002/ajh.21745

170. Woolard J, Bevan HS, Harper SJ, Bates DO. Molecular diversity of VEGF-A as a regulator of its biological activity. *Microcirculation*. 2009;16(7):572-592. doi:10.1080/10739680902997333

171. Guyot M, Pagès G. VEGF Splicing and the Role of VEGF Splice Variants: From Physiological-Pathological Conditions to Specific Pre-mRNA Splicing. *Methods Mol Biol*. 2015;1332:3-23. doi:10.1007/978-1-4939-2917-7_1

172. Gu F, Li X, Kong J, et al. VEGF111b, a new member of VEGFxxxb isoforms and induced by mitomycin C, inhibits angiogenesis. *Biochem Biophys Res Commun*. 2013;441(1):18-24. doi:10.1016/j.bbrc.2013.09.144

173. Eswarappa SM, Potdar AA, Koch WJ, et al. Programmed translational readthrough generates antiangiogenic VEGF-Ax. *Cell*. 2014;157(7):1605-1618. doi:10.1016/j.cell.2014.04.033

174. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun*. 1989;161(2):851-858. doi:10.1016/0006-291x(89)92678-8

175. Pritchard-Jones RO, Dunn DBA, Qiu Y, et al. Expression of VEGF(xxx)b, the inhibitory isoforms of VEGF, in malignant melanoma. *Br J Cancer*. 2007;97(2):223-230. doi:10.1038/sj.bjc.6603839

176. Bates DO, Mavrou A, Qiu Y, et al. Detection of VEGF-A(xxx)b isoforms in human tissues. *PLoS One*. 2013;8(7):e68399. doi:10.1371/journal.pone.0068399

177. Dehghanian F, Hojati Z. Comparative insight into expression of recombinant human VEGF111b, a newly identified anti-angiogenic isoform, in eukaryotic cell lines. *Gene*. 2014;553(1):57-62. doi:10.1016/j.gene.2014.10.002

178. Ye X, Abou-Rayyah Y, Bischoff J, et al. Altered ratios of pro- and anti-angiogenic VEGF-A variants and pericyte expression of DLL4 disrupt vascular maturation in infantile haemangioma. *J Pathol*. 2016;239(2):139-151. doi:10.1002/path.4715

179. Barratt SL, Blythe T, Jarrett C, et al. Differential Expression of VEGF-Axxx Isoforms Is Critical for Development of Pulmonary Fibrosis. *Am J Respir Crit Care Med*. 2017;196(4):479-493. doi:10.1164/rccm.201603-0568OC

180. Stevens M, Oltean S. Modulation of VEGF-A Alternative Splicing as a Novel Treatment in Chronic Kidney Disease. *Genes (Basel)*. 2018;9(2):E98. doi:10.3390/genes9020098

181. Ladomery MR, Harper SJ, Bates DO. Alternative splicing in angiogenesis: the vascular endothelial growth factor paradigm. *Cancer Lett*. 2007;249(2):133-142. doi:10.1016/j.canlet.2006.08.015

182. Olsson A-K, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol*. 2006;7(5):359-371. doi:10.1038/nrm1911

183. Bates DO, Cui T-G, Doughty JM, et al. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res*. 2002;62(14):4123-4131.

184. Woolard J, Wang W-Y, Bevan HS, et al. VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. *Cancer Res*. 2004;64(21):7822-7835. doi:10.1158/0008-5472.CAN-04-0934

185. Catena R, Larzabal L, Larrayoz M, et al. $VEGF_{121}b$ and $VEGF_{165}b$ are weakly angiogenic isoforms of VEGF-A. *Mol Cancer*. 2010;9:320. doi:10.1186/1476-4598-9-320

186. Nowak DG, Woolard J, Amin EM, et al. Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *J Cell Sci*. 2008;121(Pt 20):3487-3495. doi:10.1242/jcs.016410

187. Cébe Suarez S, Pieren M, Cariolato L, et al. A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. *Cell Mol Life Sci*. 2006;63(17):2067-2077. doi:10.1007/s00018-006-6254-9

188. Harris S, Craze M, Newton J, et al. Do anti-angiogenic VEGF (VEGFxxxb) isoforms exist? A cautionary tale. *PLoS One*. 2012;7(5):e35231. doi:10.1371/journal.pone.0035231

189. Bridgett S, Dellett M, Simpson DA. RNA-Sequencing data supports the existence of novel VEGFA splicing events but not of VEGFAxxxb isoforms. *Sci Rep*. 2017;7(1):58. doi:10.1038/s41598-017-00100-3

190. Paavonen K, Horelli-Kuitunen N, Chilov D, et al. Novel human vascular endothelial growth factor genes VEGF-B and VEGF-C localize to chromosomes 11q13 and 4q34, respectively. *Circulation*. 1996;93(6):1079-1082. doi:10.1161/01.cir.93.6.1079

191. Aase K, Lymboussaki A, Kaipainen A, Olofsson B, Alitalo K, Eriksson U. Localization of VEGF-B in the mouse embryo suggests a paracrine role of the growth factor in the developing vasculature. *Dev Dyn*. 1999;215(1):12-25. doi:10.1002/(SICI)1097-0177(199905)215:1<12::AID-DVDY3>3.0.CO;2-N

192. Enholm B, Paavonen K, Ristimäki A, et al. Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene*. 1997;14(20):2475-2483. doi:10.1038/sj.onc.1201090

193. Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. *Cardiovasc Res*. 2005;65(3):550-563. doi:10.1016/j.cardiores.2004.12.002

194. Nash AD, Baca M, Wright C, Scotney PD. The biology of vascular endothelial growth factor-B (VEGF-B). *Pulm Pharmacol Ther*. 2006;19(1):61-69. doi:10.1016/j.pupt.2005.02.007

195. Bellomo D, Headrick JP, Silins GU, et al. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ Res*. 2000;86(2):E29-35. doi:10.1161/01.res.86.2.e29

196. Rissanen TT, Markkanen JE, Gruchala M, et al. VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circ Res*. 2003;92(10):1098-1106. doi:10.1161/01.RES.0000073584.46059.E3

197. Bhardwaj S, Roy H, Gruchala M, et al. Angiogenic responses of vascular endothelial growth factors in periadventitial tissue. *Hum Gene Ther*. 2003;14(15):1451-1462. doi:10.1089/104303403769211664

198. Mould AW, Tonks ID, Cahill MM, et al. Vegfb gene knockout mice display reduced pathology and synovial angiogenesis in both antigen-induced and collagen-induced models of arthritis. *Arthritis Rheum*. 2003;48(9):2660-2669. doi:10.1002/art.11232

199. Joukov V, Pajusola K, Kaipainen A, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J*. 1996;15(2):290-298.

200. Lee J, Gray A, Yuan J, Luoh SM, Avraham H, Wood WI. Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci U S A*. 1996;93(5):1988-1992. doi:10.1073/pnas.93.5.1988

201. Enholm B, Karpanen T, Jeltsch M, et al. Adenoviral expression of vascular endothelial growth factor-C induces lymphangiogenesis in the skin. *Circ Res*. 2001;88(6):623-629. doi:10.1161/01.res.88.6.623

202. Karkkainen MJ, Haiko P, Sainio K, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol*. 2004;5(1):74-80. doi:10.1038/ni1013

203. Jeltsch M, Kaipainen A, Joukov V, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science*. 1997;276(5317):1423-1425. doi:10.1126/science.276.5317.1423

204. Farnebo F, Piehl F, Lagercrantz J. Restricted expression pattern of vegf-d in the adult and fetal mouse: high expression in the embryonic lung. *Biochem Biophys Res Commun*. 1999;257(3):891-894. doi:10.1006/bbrc.1999.0562

205. Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M. A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. *J Biol Chem*. 1998;273(47):31273-31282. doi:10.1074/jbc.273.47.31273

206. Shibuya M. Vascular endothelial growth factor receptor-2: its unique signaling and specific ligand, VEGF-E. *Cancer Sci*. 2003;94(9):751-756. doi:10.1111/j.1349-7006.2003.tb01514.x

207. Wise LM, Veikkola T, Mercer AA, et al. Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. *Proc Natl Acad Sci U S A*. 1999;96(6):3071-3076. doi:10.1073/pnas.96.6.3071

208. Ylä-Herttuala S, Alitalo K. Gene transfer as a tool to induce therapeutic vascular growth. *Nat Med*. 2003;9(6):694-701. doi:10.1038/nm0603-694

209. Ahmed A, Dunk C, Ahmad S, Khaliq A. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen--a review. *Placenta*. 2000;21 Suppl A:S16-24. doi:10.1053/plac.1999.0524

210. Luttun A, Tjwa M, Moons L, et al. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med*. 2002;8(8):831-840. doi:10.1038/nm731

211. Autiero M, Waltenberger J, Communi D, et al. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med*. 2003;9(7):936-943. doi:10.1038/nm884

212. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev*. 1997;18(1):4-25. doi:10.1210/edrv.18.1.0287

213. Shibuya M, Yamaguchi S, Yamane A, et al. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene*. 1990;5(4):519-524.

214. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB. Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene*. 1991;6(9):1677-1683.

215. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J*. 1999;13(1):9-22.

216. Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev*. 2004;56(4):549-580. doi:10.1124/pr.56.4.3

217. Shibuya M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. *Genes Cancer*. 2011;2(12):1097-1105. doi:10.1177/1947601911423031

218. Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb Perspect Med*. 2012;2(7):a006502. doi:10.1101/cshperspect.a006502

219. Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Oncogene*. 1995;10(1):135-147.

220. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem*. 1994;269(43):26988-26995.

221. Jebbink J, Keijser R, Veenboer G, van der Post J, Ris-Stalpers C, Afink G. Expression of placental FLT1 transcript variants relates to both gestational hypertensive disease and fetal growth. *Hypertension*. 2011;58(1):70-76. doi:10.1161/HYPERTENSIONAHA.110.164079

222. He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol*. 1999;13(4):537-545. doi:10.1210/mend.13.4.0265

223. Rahimi N, Golde TE, Meyer RD. Identification of ligand-induced proteolytic cleavage and ectodomain shedding of VEGFR-1/FLT1 in leukemic cancer cells. *Cancer Res*. 2009;69(6):2607-2614. doi:10.1158/0008-5472.CAN-08-2905

224. Raikwar NS, Liu KZ, Thomas CP. Protein kinase C regulates FLT1 abundance and stimulates its cleavage in vascular endothelial cells with the release of a soluble PlGF/VEGF antagonist. *Exp Cell Res*. 2013;319(17):2578-2587. doi:10.1016/j.yexcr.2013.07.005

225. Raikwar NS, Liu KZ, Thomas CP. N-terminal cleavage and release of the ectodomain of Flt1 is mediated via ADAM10 and ADAM 17 and regulated by VEGFR2 and the Flt1 intracellular domain. *PLoS One*. 2014;9(11):e112794. doi:10.1371/journal.pone.0112794

226. Han K-Y, Dugas-Ford J, Lee H, Chang J-H, Azar DT. MMP14 Cleavage of VEGFR1 in the Cornea Leads to a VEGF-Trap Antiangiogenic Effect. *Invest Ophthalmol Vis Sci*. 2015;56(9):5450-5456. doi:10.1167/iovs.14-16248

227. Sela S, Itin A, Natanson-Yaron S, et al. A novel human-specific soluble vascular endothelial growth factor receptor 1: cell-type-specific splicing and implications to vascular endothelial growth factor homeostasis and preeclampsia. *Circ Res*. 2008;102(12):1566-1574. doi:10.1161/CIRCRESAHA.108.171504

228. Heydarian M, McCaffrey T, Florea L, et al. Novel splice variants of sFlt1 are upregulated in preeclampsia. *Placenta*. 2009;30(3):250-255. doi:10.1016/j.placenta.2008.12.010

229. Takahashi H, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clin Sci (Lond)*. 2005;109(3):227-241. doi:10.1042/CS20040370

230. Niu G, Chen X. Vascular endothelial growth factor as an anti-angiogenic target for cancer therapy. *Curr Drug Targets*. 2010;11(8):1000-1017. doi:10.2174/138945010791591395

231. Shibuya M. Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J Biochem Mol Biol*. 2006;39(5):469-478. doi:10.5483/bmbrep.2006.39.5.469

232. Lanzer P, Topol EJ, eds. *Pan Vascular Medicine: Integrated Clinical Management*. Springer-Verlag; 2002. doi:10.1007/978-3-642-56225-9

233. Zanetta L, Marcus SG, Vasile J, et al. Expression of Von Willebrand factor, an endothelial cell marker, is up-regulated by angiogenesis factors: a potential method for objective assessment of tumor angiogenesis. *Int J Cancer*. 2000;85(2):281-288. doi:10.1002/(sici)1097-0215(20000115)85:2<281::aid-ijc21>3.0.co;2-3

234. Esser S, Wolburg K, Wolburg H, Breier G, Kurzchalia T, Risau W. Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J Cell Biol*. 1998;140(4):947-959. doi:10.1083/jcb.140.4.947

235. Heloterä H, Alitalo K. The VEGF family, the inside story. *Cell*. 2007;130(4):591-592. doi:10.1016/j.cell.2007.08.012

236. Paupert J, Sounni NE, Noël A. Lymphangiogenesis in post-natal tissue remodeling: lymphatic endothelial cell connection with its environment. *Mol Aspects Med*. 2011;32(2):146-158. doi:10.1016/j.mam.2011.04.002

237. Smith GA, Fearnley GW, Tomlinson DC, Harrison MA, Ponnambalam S. The cellular response to vascular endothelial growth factors requires co-ordinated signal transduction, trafficking and proteolysis. *Biosci Rep*. 2015;35(5):e00253. doi:10.1042/BSR20150171

238. Mäkinen T, Norrmén C, Petrova TV. Molecular mechanisms of lymphatic vascular development. *Cell Mol Life Sci*. 2007;64(15):1915-1929. doi:10.1007/s00018-007-7040-z

239. Mäkinen T, Jussila L, Veikkola T, et al. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med*. 2001;7(2):199-205. doi:10.1038/84651

240. Nakamura F, Goshima Y. Structural and functional relation of neuropilins. *Adv Exp Med Biol*. 2002;515:55-69. doi:10.1007/978-1-4615-0119-0_5

241. Herzog Y, Kalcheim C, Kahane N, Reshef R, Neufeld G. Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. *Mech Dev*. 2001;109(1):115-119. doi:10.1016/s0925-4773(01)00518-4

242. Autiero M, De Smet F, Claes F, Carmeliet P. Role of neural guidance signals in blood vessel navigation. *Cardiovasc Res*. 2005;65(3):629-638. doi:10.1016/j.cardiores.2004.09.013

243. Eichmann A, Simons M. VEGF signaling inside vascular endothelial cells and beyond. *Curr Opin Cell Biol*. 2012;24(2):188-193. doi:10.1016/j.ceb.2012.02.002

244. McCloskey DP, Hintz TM, Scharfman HE. Modulation of vascular endothelial growth factor (VEGF) expression in motor neurons and its electrophysiological effects. *Brain Res Bull*. 2008;76(1-2):36-44. doi:10.1016/j.brainresbull.2007.11.018

245. Ruiz de Almodovar C, Lambrechts D, Mazzone M, Carmeliet P. Role and therapeutic potential of VEGF in the nervous system. *Physiol Rev*. 2009;89(2):607-648. doi:10.1152/physrev.00031.2008

246. Le Bras B, Barallobre M-J, Homman-Ludiye J, et al. VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. *Nat Neurosci*. 2006;9(3):340-348. doi:10.1038/nn1646

247. Theis V, Theiss C. VEGF - A Stimulus for Neuronal Development and Regeneration in the CNS and PNS. *Curr Protein Pept Sci*. 2018;19(6):589-597. doi:10.2174/1389203719666180104113937

248. Storkebaum E, Lambrechts D, Dewerchin M, et al. Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. *Nat Neurosci*. 2005;8(1):85-92. doi:10.1038/nn1360

249. Brockington A, Wharton SB, Fernando M, et al. Expression of vascular endothelial growth factor and its receptors in the central nervous system in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol*. 2006;65(1):26-36. doi:10.1097/01.jnen.0000196134.51217.74

250. Maurer MH, Tripps WKC, Feldmann RE, Kuschinsky W. Expression of vascular endothelial growth factor and its receptors in rat neural stem cells. *Neurosci Lett*. 2003;344(3):165-168. doi:10.1016/s0304-3940(03)00407-5

251. Fabel K, Fabel K, Tam B, et al. VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci*. 2003;18(10):2803-2812. doi:10.1111/j.1460-9568.2003.03041.x

252. Warner-Schmidt JL, Duman RS. VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants. *Proc Natl Acad Sci U S A*. 2007;104(11):4647-4652. doi:10.1073/pnas.0610282104

253. Ogunshola OO, Antic A, Donoghue MJ, et al. Paracrine and autocrine functions of neuronal vascular endothelial growth factor (VEGF) in the central nervous system. *J Biol Chem*. 2002;277(13):11410-11415. doi:10.1074/jbc.M111085200

254. Wada T, Haigh JJ, Ema M, et al. Vascular endothelial growth factor directly inhibits primitive neural stem cell survival but promotes definitive neural stem cell survival. *J Neurosci*. 2006;26(25):6803-6812. doi:10.1523/JNEUROSCI.0526-06.2006

255. Rosenstein JM, Mani N, Khaibullina A, Krum JM. Neurotrophic effects of vascular endothelial growth factor on organotypic cortical explants and primary cortical neurons. *J Neurosci*. 2003;23(35):11036-11044.

256. Zhu Y, Jin K, Mao XO, Greenberg DA. Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. *FASEB J*. 2003;17(2):186-193. doi:10.1096/fj.02-0515com

257. Mukouyama Y, Shin D, Britsch S, Taniguchi M, Anderson DJ. Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell*. 2002;109(6):693-705. doi:10.1016/s0092-8674(02)00757-2

258. Schratzberger P, Schratzberger G, Silver M, et al. Favorable effect of VEGF gene transfer on ischemic peripheral neuropathy. *Nat Med*. 2000;6(4):405-413. doi:10.1038/74664

259. Storkebaum E, Lambrechts D, Carmeliet P. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. *Bioessays*. 2004;26(9):943-954. doi:10.1002/bies.20092

260. Kułak W, Sobaniec W, Kuzia J-S, Boćkowski L. Neurophysiologic and neuroimaging studies of brain plasticity in children with spastic cerebral palsy. *Exp Neurol*. 2006;198(1):4-11. doi:10.1016/j.expneurol.2005.11.014

261. Kim BW, Choi M, Kim Y-S, et al. 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*. 2008;20(4):714-725. doi:10.1016/j.cellsig.2007.12.009

262. Joo J-Y, Kim B-W, Lee J-S, et al. Activation of NMDA receptors increases proliferation and differentiation of hippocampal neural progenitor cells. *J Cell Sci*. 2007;120(Pt 8):1358-1370. doi:10.1242/jcs.002154

263. Proia P, Schiera G, Mineo M, et al. Astrocytes shed extracellular vesicles that contain fibroblast growth factor-2 and vascular endothelial growth factor. *Int J Mol Med*. 2008;21(1):63-67.

264. Holmes K, Roberts OL, Thomas AM, Cross MJ. Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. *Cell Signal*. 2007;19(10):2003-2012. doi:10.1016/j.cellsig.2007.05.013

265. Li B, Xu W, Luo C, Gozal D, Liu R. VEGF-induced activation of the PI3-K/Akt pathway reduces mutant SOD1-mediated motor neuron cell death. *Brain Res Mol Brain Res*. 2003;111(1-2):155-164. doi:10.1016/s0169-328x(03)00025-1

266. Wick A, Wick W, Waltenberger J, Weller M, Dichgans J, Schulz JB. Neuroprotection by hypoxic preconditioning requires sequential activation of vascular endothelial growth factor receptor and Akt. *J Neurosci*. 2002;22(15):6401-6407. doi:20026672

267. Lee E, Son H. Adult hippocampal neurogenesis and related neurotrophic factors. *BMB Rep*. 2009;42(5):239-244. doi:10.5483/bmbrep.2009.42.5.239

268. Mani N, Khaibullina A, Krum JM, Rosenstein JM. Vascular endothelial growth factor enhances migration of astroglial cells in subventricular zone neurosphere cultures. *J Neurosci Res*. 2010;88(2):248-257. doi:10.1002/jnr.22197

269. Rosenstein JM, Krum JM. New roles for VEGF in nervous tissue--beyond blood vessels. *Exp Neurol*. 2004;187(2):246-253. doi:10.1016/j.expneurol.2004.01.022

270. Namiecińska M, Marciniak K, Nowak JZ. [VEGF as an angiogenic, neurotrophic, and neuroprotective factor]. *Postepy Hig Med Dosw (Online)*. 2005;59:573-583.

271. Manoonkitiwongsa PS, Schultz RL, McCreery DB, Whitter EF, Lyden PD. 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*. 2004;24(6):693-702. doi:10.1097/01.WCB.0000126236.54306.21

272. Sun Y, Jin K, Xie L, et al. VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J Clin Invest*. 2003;111(12):1843-1851. doi:10.1172/JCI17977

273. Nowacka MM, Obuchowicz E. 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*. 2012;46(1):1-10. doi:10.1016/j.npep.2011.05.005

274. Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science*. 2000;288(5472):1765-1769. doi:10.1126/science.288.5472.1765

275. Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. *Cell*. 2009;139(2):267-284. doi:10.1016/j.cell.2009.09.028

276. von Hehn CA, Baron R, Woolf CJ. Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron*. 2012;73(4):638-652. doi:10.1016/j.neuron.2012.02.008

277. Maharaj ASR, D'Amore PA. Roles for VEGF in the adult. *Microvasc Res*. 2007;74(2-3):100-113. doi:10.1016/j.mvr.2007.03.004

278. Rosenstein JM, Krum JM, Ruhrberg C. VEGF in the nervous system. *Organogenesis*. 2010;6(2):107-114. doi:10.4161/org.6.2.11687

279. Kim LA, D'Amore PA. A brief history of anti-VEGF for the treatment of ocular angiogenesis. *Am J Pathol*. 2012;181(2):376-379. doi:10.1016/j.ajpath.2012.06.006

280. Beazley-Long N, Hua J, Jehle T, et al. VEGF-A165b is an endogenous neuroprotective splice isoform of vascular endothelial growth factor A in vivo and in vitro. *Am J Pathol*. 2013;183(3):918-929. doi:10.1016/j.ajpath.2013.05.031

281. Beazley-Long N, Moss CE, Ashby WR, et al. VEGFR2 promotes central endothelial activation and the spread of pain in inflammatory arthritis. *Brain Behav Immun*. 2018;74:49-67. doi:10.1016/j.bbi.2018.03.012

282. Hulse RP, Beazley-Long N, Hua J, et al. Regulation of alternative VEGF-A mRNA splicing is a therapeutic target for analgesia. *Neurobiol Dis*. 2014;71:245-259. doi:10.1016/j.nbd.2014.08.012

283. Hulse RP, Beazley-Long N, Ved N, et al. Vascular endothelial growth factor-A165b prevents diabetic neuropathic pain and sensory neuronal degeneration. *Clin Sci (Lond)*. 2015;129(8):741-756. doi:10.1042/CS20150124

284. Hulse RP, Drake RAR, Bates DO, Donaldson LF. The control of alternative splicing by SRSF1 in myelinated afferents contributes to the development of neuropathic pain. *Neurobiol Dis*. 2016;96:186-200. doi:10.1016/j.nbd.2016.09.009

285. Selvaraj D, Gangadharan V, Michalski CW, et al. A Functional Role for VEGFR1 Expressed in Peripheral Sensory Neurons in Cancer Pain. *Cancer Cell*. 2015;27(6):780-796. doi:10.1016/j.ccell.2015.04.017

286. Hulse RP. Role of VEGF-A in chronic pain. *Oncotarget*. 2017;8(7):10775-10776. doi:10.18632/oncotarget.14615

287. Lai HH, Shen B, Vijairania P, Zhang X, Vogt SK, Gereau RW. Anti-vascular endothelial growth factor treatment decreases bladder pain in cyclophosphamide cystitis: a Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network animal model study. *BJU Int*. 2017;120(4):576-583. doi:10.1111/bju.13924

288. Hamilton JL, Nagao M, Levine BR, Chen D, Olsen BR, Im H-J. Targeting VEGF and Its Receptors for the Treatment of Osteoarthritis and Associated Pain. *J Bone Miner Res*. 2016;31(5):911-924. doi:10.1002/jbmr.2828

289. Takano S, Uchida K, Inoue G, et al. Vascular endothelial growth factor expression and their action in the synovial membranes of patients with painful knee osteoarthritis. *BMC Musculoskelet Disord*. 2018;19(1):204. doi:10.1186/s12891-018-2127-2

290. Nagao M, Hamilton JL, Kc R, et al. Vascular Endothelial Growth Factor in Cartilage Development and Osteoarthritis. *Sci Rep*. 2017;7(1):13027. doi:10.1038/s41598-017-13417-w

291. Nagai T, Sato M, Kobayashi M, Yokoyama M, Tani Y, Mochida J. Bevacizumab, an anti-vascular endothelial growth factor antibody, inhibits osteoarthritis. *Arthritis Res Ther*. 2014;16(5):427. doi:10.1186/s13075-014-0427-y

292. Afuwape AO, Kiriakidis S, Paleolog EM. The role of the angiogenic molecule VEGF in the pathogenesis of rheumatoid arthritis. *Histol Histopathol*. 2002;17(3):961-972. doi:10.14670/HH-17.961

293. Malemud CJ. Growth hormone, VEGF and FGF: involvement in rheumatoid arthritis. *Clin Chim Acta*. 2007;375(1-2):10-19. doi:10.1016/j.cca.2006.06.033

294. Nagashima M, Yoshino S, Ishiwata T, Asano G. Role of vascular endothelial growth factor in angiogenesis of rheumatoid arthritis. *J Rheumatol*. 1995;22(9):1624-1630.

295. De Bandt M, Ben Mahdi MH, Ollivier V, et al. Blockade of vascular endothelial growth factor receptor I (VEGF-RI), but not VEGF-RII, suppresses joint destruction in the K/BxN model of rheumatoid arthritis. *J Immunol*. 2003;171(9):4853-4859. doi:10.4049/jimmunol.171.9.4853

296. Matsuoka A, Maeda O, Mizutani T, et al. Bevacizumab Exacerbates Paclitaxel-Induced Neuropathy: A Retrospective Cohort Study. *PLoS One*. 2016;11(12):e0168707. doi:10.1371/journal.pone.0168707

297. Liu S, Xu C, Li G, et al. Vatalanib decrease the positive interaction of VEGF receptor-2 and P2X2/3 receptor in chronic constriction injury rats. *Neurochem Int*. 2012;60(6):565-572. doi:10.1016/j.neuint.2012.02.006

298. Zeng Y, Han H, Tang B, Chen J, Mao D, Xiong M. Transplantation of Recombinant Vascular Endothelial Growth Factor (VEGF)189-Neural Stem Cells Downregulates Transient Receptor Potential Vanilloid 1 (TRPV1) and Improves Motor Outcome in Spinal Cord Injury. *Med Sci Monit*. 2018;24:1089-1096. doi:10.12659/msm.905264

299. Kiguchi N, Kobayashi Y, Kadowaki Y, Fukazawa Y, Saika F, Kishioka S. Vascular endothelial growth factor signaling in injured nerves underlies peripheral sensitization in neuropathic pain. *J Neurochem*. 2014;129(1):169-178. doi:10.1111/jnc.12614

300. Taiana MM, Lombardi R, Porretta-Serapiglia C, et al. Neutralization of schwann cell-secreted VEGF is protective to in vitro and in vivo experimental diabetic neuropathy. *PLoS One*. 2014;9(9):e108403. doi:10.1371/journal.pone.0108403

301. Di Cesare Mannelli L, Tenci B, Micheli L, et al. Adipose-derived stem cells decrease pain in a rat model of oxaliplatin-induced neuropathy: Role of VEGF-A modulation. *Neuropharmacology*. 2018;131:166-175. doi:10.1016/j.neuropharm.2017.12.020

302. Bussolati B, Dunk C, Grohman M, Kontos CD, Mason J, Ahmed A. Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide. *Am J Pathol*. 2001;159(3):993-1008. doi:10.1016/S0002-9440(10)61775-0

303. Rodríguez-Osorio X, Sobrino T, Brea D, Martínez F, Castillo J, Leira R. Endothelial progenitor cells: a new key for endothelial dysfunction in migraine. *Neurology*. 2012;79(5):474-479. doi:10.1212/WNL.0b013e31826170ce

304. Michalak S, Kalinowska-Lyszczarz A, Wegrzyn D, Thielemann A, Osztynowicz K, Kozubski W. The Levels of Circulating Proangiogenic Factors in Migraineurs. *Neuromolecular Med*. 2017;19(4):510-517. doi:10.1007/s12017-017-8465-7

305. Gonçalves FM, Martins-Oliveira A, Speciali JG, et al. Vascular endothelial growth factor genetic polymorphisms and haplotypes in women with migraine. *DNA Cell Biol*. 2010;29(7):357-362. doi:10.1089/dna.2010.1025

306. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology*. 2005;69 Suppl 3:4-10. doi:10.1159/000088478

307. Goel HL, Mercurio AM. VEGF targets the tumour cell. *Nat Rev Cancer*. 2013;13(12):871-882. doi:10.1038/nrc3627

308. McGrath JC, Lilley E. Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Br J Pharmacol*. 2015;172(13):3189-3193. doi:10.1111/bph.12955

309. Graziani G, Ruffini F, Tentori L, et al. Antitumor activity of a novel anti-vascular endothelial growth factor receptor-1 monoclonal antibody that does not interfere with ligand binding. *Oncotarget*. 2016;7(45):72868-72885. doi:10.18632/oncotarget.12108

310. Cavaletti G, Tredici G, Petruccioli MG, et al. Effects of different schedules of oxaliplatin treatment on the peripheral nervous system of the rat. *Eur J Cancer*. 2001;37(18):2457-2463. doi:10.1016/s0959-8049(01)00300-8

311. Di Cesare Mannelli L, Lucarini E, Micheli L, et al. Effects of natural and synthetic isothiocyanate-based H2S-releasers against chemotherapy-induced neuropathic pain: Role of Kv7 potassium channels. *Neuropharmacology*. 2017;121:49-59. doi:10.1016/j.neuropharm.2017.04.029

312. Polomano RC, Mannes AJ, Clark US, Bennett GJ. A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. *Pain*. 2001;94(3):293-304. doi:10.1016/S0304-3959(01)00363-3

313. Weng H-R, Cordella JV, Dougherty PM. Changes in sensory processing in the spinal dorsal horn accompany vincristine-induced hyperalgesia and allodynia. *Pain*. 2003;103(1-2):131-138. doi:10.1016/s0304-3959(02)00445-1

314. Russo R, D'Agostino G, Mattace Raso G, et al. Central administration of oxytocin reduces hyperalgesia in mice: implication for cannabinoid and opioid systems. *Peptides*. 2012;38(1):81-88. doi:10.1016/j.peptides.2012.08.005

315. Lucarini E, Pagnotta E, Micheli L, et al. Eruca sativa Meal against Diabetic Neuropathic Pain: An H2S-Mediated Effect of Glucoerucin. *Molecules*. 2019;24(16):E3006. doi:10.3390/molecules24163006

316. Baptista-de-Souza D, Di Cesare Mannelli L, Zanardelli M, et al. Serotonergic modulation in neuropathy induced by oxaliplatin: effect on the 5HT2C receptor. *Eur J Pharmacol*. 2014;735:141-149. doi:10.1016/j.ejphar.2014.04.028

317. Ghelardini C, Galeotti N, Calvani M, Mosconi L, Nicolai R, Bartolini A. Acetyl-l-carnitine induces muscarinic antinocieption in mice and rats. *Neuropharmacology*. 2002;43(7):1180-1187. doi:10.1016/s0028-3908(02)00225-3

318. McGaraughty S, Chu KL, Perner RJ, Didomenico S, Kort ME, Kym PR. TRPA1 modulation of spontaneous and mechanically evoked firing of spinal neurons in uninjured, osteoarthritic, and inflamed rats. *Mol Pain*. 2010;6:14. doi:10.1186/1744-8069-6-14

319. Telleria-Diaz A, Schmidt M, Kreusch S, et al. Spinal antinociceptive effects of cyclooxygenase inhibition during inflammation: Involvement of prostaglandins and endocannabinoids. *Pain*. 2010;148(1):26-35. doi:10.1016/j.pain.2009.08.013

320. Boccella S, Vacca V, Errico F, et al. D-aspartate modulates nociceptive-specific neuron activity and pain threshold in inflammatory and neuropathic pain condition in mice. *Biomed Res Int*. 2015;2015:905906. doi:10.1155/2015/905906

321. Simone DA, Khasabov SG, Hamamoto DT. Changes in response properties of nociceptive dorsal horn neurons in a murine model of cancer pain. *Sheng Li Xue Bao*. 2008;60(5):635-644.

322. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9(7):676-682. doi:10.1038/nmeth.2019

323. Sage D, Donati L, Soulez F, et al. DeconvolutionLab2: An open-source software for deconvolution microscopy. *Methods*. 2017;115:28-41. doi:10.1016/j.ymeth.2016.12.015

324. Bolte S, Cordelières FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*. 2006;224(Pt 3):213-232. doi:10.1111/j.1365-2818.2006.01706.x

325. Cudmore MJ, Hewett PW, Ahmad S, et al. The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. *Nat Commun*. 2012;3:972. doi:10.1038/ncomms1977

326. Cai M, Wang K, Murdoch CE, Gu Y, Ahmed A. Heterodimerisation between VEGFR-1 and VEGFR-2 and not the homodimers of VEGFR-1 inhibit VEGFR-2 activity. *Vascul Pharmacol*. 2017;88:11-20. doi:10.1016/j.vph.2016.11.007

327. Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem*. 1994;269(41):25646-25654.

328. Persico MG, Vincenti V, DiPalma T. Structure, expression and receptor-binding properties of placenta growth factor (PlGF). *Curr Top Microbiol Immunol*. 1999;237:31-40. doi:10.1007/978-3-642-59953-8_2

329. Meyer M, Clauss M, Lepple-Wienhues A, et al. A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J*. 1999;18(2):363-374. doi:10.1093/emboj/18.2.363

330. Falcon BL, Chintharlapalli S, Uhlik MT, Pytowski B. Antagonist antibodies to vascular endothelial growth factor receptor 2 (VEGFR-2) as anti-angiogenic agents. *Pharmacol Ther*. 2016;164:204-225. doi:10.1016/j.pharmthera.2016.06.001

331. Lacal PM, Graziani G. Therapeutic implication of vascular endothelial growth factor receptor-1 (VEGFR-1) targeting in cancer cells and tumor microenvironment by competitive and non-competitive inhibitors. *Pharmacol Res*. 2018;136:97-107. doi:10.1016/j.phrs.2018.08.023

332. Lange C, Storkebaum E, de Almodóvar CR, Dewerchin M, Carmeliet P. Vascular endothelial growth factor: a neurovascular target in neurological diseases. *Nat Rev Neurol*. 2016;12(8):439-454. doi:10.1038/nrneurol.2016.88

333. Szerb JC, Issekutz B. Increase in the stimulation-induced overflow of glutamate by fluoroacetate, a selective inhibitor of the glial tricarboxylic cycle. *Brain Res*. 1987;410(1):116-120. doi:10.1016/s0006-8993(87)80030-6

334. Hassel B, Paulsen RE, Johnsen A, Fonnum F. Selective inhibition of glial cell metabolism in vivo by fluorocitrate. *Brain Res*. 1992;576(1):120-124. doi:10.1016/0006-8993(92)90616-h

335. Iyer S, Acharya KR. Tying the knot: the cystine signature and molecular-recognition processes of the vascular endothelial growth factor family of angiogenic cytokines. *FEBS J*. 2011;278(22):4304-4322. doi:10.1111/j.1742-4658.2011.08350.x

336. Fischer C, Mazzone M, Jonckx B, Carmeliet P. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nat Rev Cancer*. 2008;8(12):942-956. doi:10.1038/nrc2524

337. Nesic O, Sundberg LM, Herrera JJ, Mokkapati VUL, Lee J, Narayana PA. Vascular endothelial growth factor and spinal cord injury pain. *J Neurotrauma*. 2010;27(10):1793-1803. doi:10.1089/neu.2010.1351

338. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest*. 2000;105(8):R15-24. doi:10.1172/JCI8829

339. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest*. 2003;111(9):1287-1295. doi:10.1172/JCI17929

340. Verheyen A, Peeraer E, Nuydens R, et al. Systemic anti-vascular endothelial growth factor therapies induce a painful sensory neuropathy. *Brain*. 2012;135(Pt 9):2629-2641. doi:10.1093/brain/aws145

341. Kerber M, Reiss Y, Wickersheim A, et al. Flt-1 signaling in macrophages promotes glioma growth in vivo. *Cancer Res*. 2008;68(18):7342-7351. doi:10.1158/0008-5472.CAN-07-6241

342. Li C, Liu B, Dai Z, Tao Y. Knockdown of VEGF receptor-1 (VEGFR-1) impairs macrophage infiltration, angiogenesis and growth of clear cell renal cell carcinoma (CRCC). *Cancer Biol Ther*. 2011;12(10):872-880. doi:10.4161/cbt.12.10.17672

343. Scholz J, Woolf CJ. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci*. 2007;10(11):1361-1368. doi:10.1038/nn1992

344. Stockstill K, Doyle TM, Yan X, et al. Dysregulation of sphingolipid metabolism contributes to bortezomib-induced neuropathic pain. *J Exp Med*. 2018;215(5):1301-1313. doi:10.1084/jem.20170584

345. Di Cesare Mannelli L, Marcoli M, Micheli L, et al. Oxaliplatin evokes P2X7-dependent glutamate release in the cerebral cortex: A pain mechanism mediated by Pannexin 1. *Neuropharmacology*. 2015;97:133-141. doi:10.1016/j.neuropharm.2015.05.037

346. Sommer C, Leinders M, Üçeyler N. Inflammation in the pathophysiology of neuropathic pain. *Pain*. 2018;159(3):595-602. doi:10.1097/j.pain.0000000000001122

347. Hald A. Spinal astrogliosis in pain models: cause and effects. *Cell Mol Neurobiol*. 2009;29(5):609-619. doi:10.1007/s10571-009-9390-6

348. Gao Y-J, Ji R-R. Targeting astrocyte signaling for chronic pain. *Neurotherapeutics*. 2010;7(4):482-493. doi:10.1016/j.nurt.2010.05.016

349. Salhia B, Angelov L, Roncari L, Wu X, Shannon P, Guha A. Expression of vascular endothelial growth factor by reactive astrocytes and associated neoangiogenesis. *Brain Res*. 2000;883(1):87-97. doi:10.1016/s0006-8993(00)02825-0

350. Argaw AT, Asp L, Zhang J, et al. Astrocyte-derived VEGF-A drives blood-brain barrier disruption in CNS inflammatory disease. *J Clin Invest*. 2012;122(7):2454-2468. doi:10.1172/JCI60842

351. Chapouly C, Tadesse Argaw A, Horng S, et al. Astrocytic TYMP and VEGFA drive blood-brain barrier opening in inflammatory central nervous system lesions. *Brain*. 2015;138(Pt 6):1548-1567. doi:10.1093/brain/awv077

352. Lee GW, Son JY, Lee AR, Ju JS, Bae YC, Ahn DK. Central VEGF-A pathway plays a key role in the development of trigeminal neuropathic pain in rats. *Mol Pain*. 2019;15:1744806919872602. doi:10.1177/1744806919872602

353. Ved N, Da Vitoria Lobo ME, Bestall SM, et al. Diabetes-induced microvascular complications at the level of the spinal cord: a contributing factor in diabetic neuropathic pain. *J Physiol*. 2018;596(16):3675-3693. doi:10.1113/JP275067

354. Rojas DR, Tegeder I, Kuner R, Agarwal N. Hypoxia-inducible factor 1α protects peripheral sensory neurons from diabetic peripheral neuropathy by suppressing accumulation of reactive oxygen species. *J Mol Med (Berl)*. 2018;96(12):1395-1405. doi:10.1007/s00109-018-1707-9

355. Jacques D, Dumont Y, rossum DV, Quirion R. Chapter viii calcitonin gene-related peptide (cgrp), amylin and adrenomedullin: anatomical localization and biological functions in the mammalian and human brains. In: Quirion R, Björklund A, Hökfelt T, eds. *Handbook of Chemical Neuroanatomy*. Vol 16. Peptide Receptors. Elsevier; 2000:301-374. doi:10.1016/S0924-8196(00)80010-X

356. Ma W, Chabot J-G, Powell KJ, Jhamandas K, Dickerson IM, Quirion R. Localization and modulation of calcitonin gene-related peptide-receptor component protein-immunoreactive cells in the rat central and peripheral nervous systems. *Neuroscience*. 2003;120(3):677-694. doi:10.1016/s0306-4522(03)00159-3

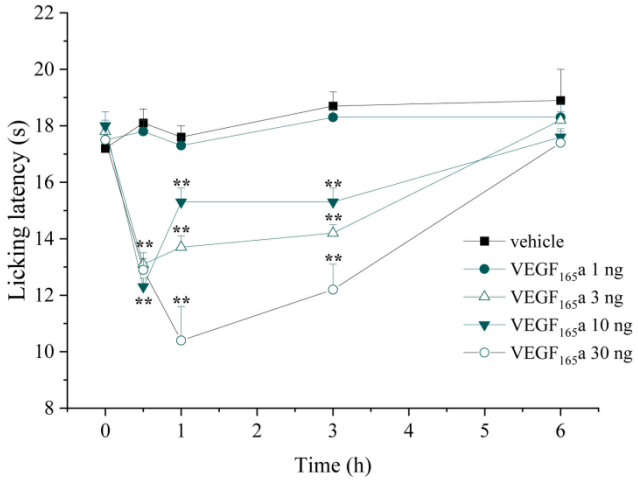
357. Kangrga I, Randic M. Tachykinins and calcitonin gene-related peptide enhance release of endogenous glutamate and aspartate from the rat spinal dorsal horn slice. *J Neurosci*. 1990;10(6):2026-2038.

358. Oku R, Satoh M, Fujii N, Otaka A, Yajima H, Takagi H. Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. *Brain Res*. 1987;403(2):350-354. doi:10.1016/0006-8993(87)90074-6

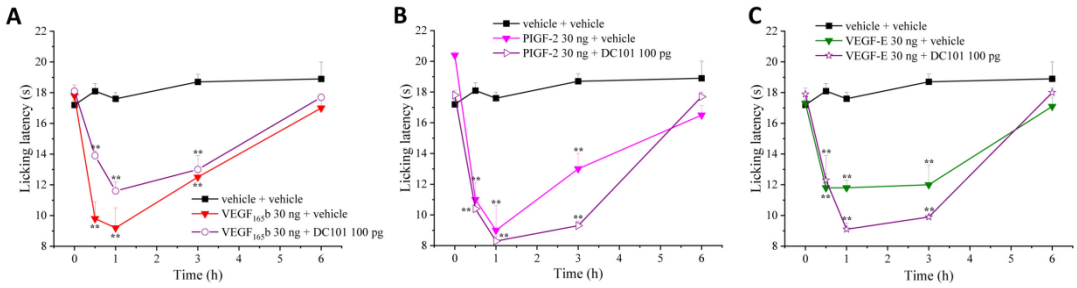
359. Oosthuyse B, Moons L, Storkebaum E, et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet*. 2001;28(2):131-138. doi:10.1038/88842

360. Verheyen A, Peeraer E, Lambrechts D, et al. Therapeutic potential of VEGF and VEGF-derived peptide in peripheral neuropathies. *Neuroscience*. 2013;244:77-89. doi:10.1016/j.neuroscience.2013.03.050

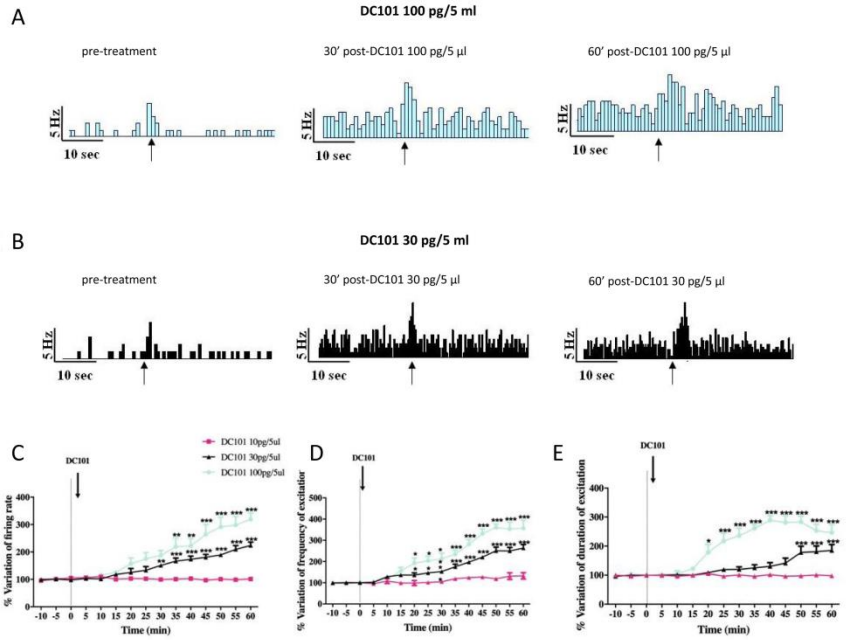
Supplementary Materials and Tables



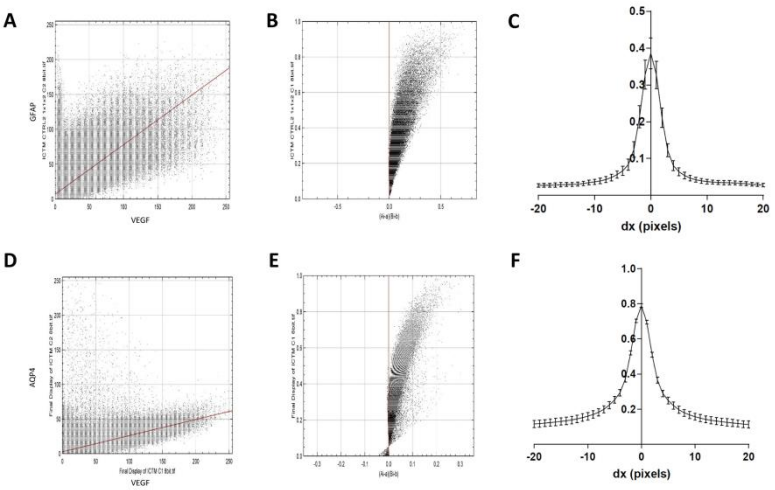
Supplementary Figure S1. Nociceptive effect of VEGF165a. *The pain threshold was measured by the Cold plate test over time after the intrathecal injection of VEGF165a (n=5). Each value represents the mean ± SEM. **P<0.01 vs vehicle-treated animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*



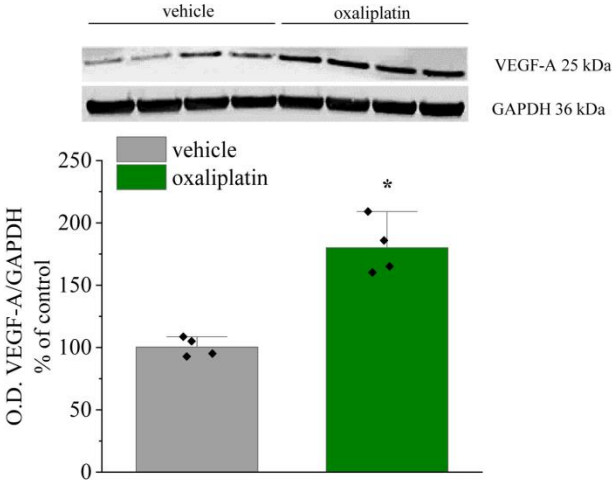
Supplementary Figure S2. Hypersensitivity-induced by VEGF-A signalling modulators is not due to its interaction with VEGFR-2. *The response to a thermal stimulus (Cold plate test) was recorded after intrathecal infusion of (A) VEGF165b 30 ng pretreated (15 min before) with DC101 (n=5), (B) PlGF-2 pretreated with DC101 (n=5), (C) VEGF-E pretreated with DC101 (n=5). Each value represents the mean ± SEM. **P<0.01 vs vehicle + vehicle-treated animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*



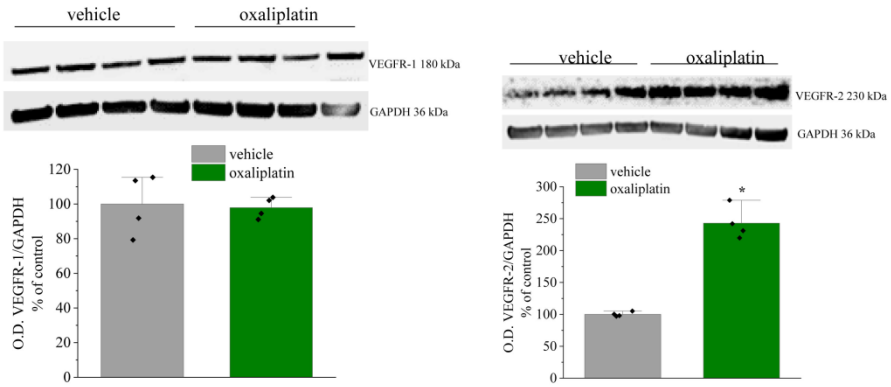
Supplementary Figure S3. DC101 increases spontaneous and noxious-evoked activity of NS neurons. *Representative ratematers showing spontaneous and noxious-evoked activity of NS neurons after spinal application of DC101 antibodies at 100 pg (A) and 30pg (B), black arrows indicate the noxious stimulation on the mouse hind-paw. Mean ± SEM population data of spinal cord application of DC101 (30 pg and 100 pg) on % variation of firing rate (C), % variation of frequency of excitation (D) and % variation of duration of evoked activity (E) of NS neurons in CD1 mice. Black arrows indicate vehicle, DC101 spinal application. Each point represents the mean of 5 different mice per group (one neuron recorded per each mouse). *P<0.05, **P<0.01 and ***P<0.001 indicate statistically difference vs pre-drug. One- way ANOVA followed by Dunnet's multiple comparison post-hoc test was performed for statistical significance within groups.*



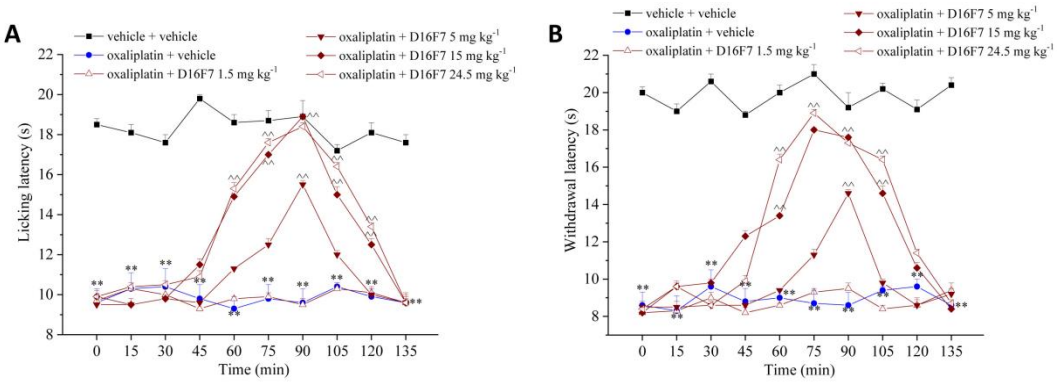
Supplementary Figure S4. Analyses of GFAP and VEGF co-localization in confocal z-stacks. *A) Cytofluorogram relative to images in Fig. 5 B) Li's Intensity Correlation Analysis relative to images in Fig. 5. C) Van Steensel's Cross-Correlation Function (CCF), relative to all datasets (n=8, mean ± SEM). Pearson's correlation coefficient (PCC) is given by the CCF value corresponding to x =0. CCF at FWHM = 1.00 ± 0.04 μm (mean ± SEM, n=8). D, E)* ***Analyses of AQP4 and VEGF co-localization in confocal z-stacks.*** *D) Cytofluorogram relative to images in Fig. 5 E) Li's Intensity Correlation Analysis relative to images in Fig. 5. CF Van Steensel's Cross-Correlation Function (CCF), relative to all datasets (n=8, mean ± SEM). Pearson's correlation coefficient (PCC) is given by the CCF value corresponding to x =0. CCF at FWHM =1.28±0.04 μm (mean ± SEM, n=8)*



Supplementary Figure S5. VEGF-A is increased in the spinal cord of mice with oxaliplatin-induced neuropathy. *Representative Western blot images and densitometric analysis of VEGF-A expression in the lumbar section of the spinal cord of oxaliplatin-treated mice in comparison to control (n=4). Each value represents the mean ± SEM. *P<0.05 vs vehicle + vehicle group. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*



Supplementary Figure S6. VEGFR-2 is increased in the spinal cord of mice with oxaliplatin-induced neuropathy. *Representative Western blot images and densitometric analysis of VEGF-R1 and VEGFR-2 expression in the lumbar section of the spinal cord of oxaliplatin-treated mice in comparison to control (n=4). Each value represents the mean ± SEM. *P<0.05 vs vehicle + vehicle group. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*



Supplementary Figure S7. D16F7 mAb reduced oxaliplatin-induced pain after systemic administration. *Effect of D16F7 mAb evaluated by (A) Cold plate and (B) Paw pressure tests in a mouse model of oxaliplatin-induced neuropathy after i.p. injection (A, B, n=6). Each value represents the mean ± SEM. **P<0.01 vs vehicle + vehicle-treated animals; ^^P<0.01 vs oxaliplatin + vehicle-treated animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.*

Supplementary Table S1. List of antibodies used for immunohistochemistry and Western blot assays

Antigen	Supplier	Catalog#	Antibody	Host	Usage	Conc.	Analysis
GFAP	Merck Millipore	MAB3402X	Monoclonal conj. 488	Ms	Primary	1:500	IF
GFAP	Dako	ZO334	Polyclonal	Rb	Primary	1:500	IF
NeuN	Merck Millipore	MAB377X	Monoclonal conj. 488	Ms	Primary	1:500	IF
Iba-1	Wako	016-20001	Polyclonal	Rb	Primary	1:200	IF
VEGFR1	Bioss	bs-0170R	Polyclonal	Rb	Primary	1:100	IF
VEGF-A	Santa Cruz Biotechnology	sc-7269	Monoclonal	Ms	Primary	1:100	IF
VEGF	BD Pharmigen	555036	Monoclonal	Ms	Primary	1:1000	WB
VEGFR-1	Abcam	32152	Monoclonal	Rb	Primary	1:1000	WB
VEGFR2/DC101	Bio X Cell	BE0060	Monoclonal	Ms	Primary	1:5000	WB
AQP-4	Santa Cruz Biotechnology	sc-32739	Monoclonal	Ms	Primary	1:100	IF
RECA-1	Santa Cruz Biotechnology	Sc-52665	Monoclonal	Ms	Primary	1:100	IF
GAPDH	Santa Cruz Biotechnology	sc-32233	Monoclonal	Ms	Primary	1:2500	WB
Rabbit FC	Life technologies	A-11011	Polyclonal	Rb	Secondary Alexa Fluor 568	1:500	IF
Mouse FC	Life technologies	A-11004	Polyclonal	Ms	Secondary Alexa Fluor 568	1:500	IF
Mouse FC	Life technologies	A-11001	Polyclonal	Ms	Secondary Alexa Fluor 488	1:500	IF
Rabbit FC	Life technologies	A-21443	Polyclonal	Rb	Secondary Alexa Fluor 647	1:200	IF
DAPI	Thermo scientific	62248	N.A.	N.A.	N.A.	1:2000	IF
r-IgG-h	Bethyl	A120-201P	Polyclonal	Rb	Secondary conj. HRP	1:5000	WB
m-IgGk BP-HRP	Santa Cruz Biotechnology	Sc-516102	N.A.	Ms	Secondary conj. HRP	1:5000	WB
α-4a	Sigma-Aldrich	T6074	Monoclonal	Ms	Secondary	1:5000	WB

Supplementary Table S2. Hole Board test

	Day 3		Day 5		Day 9	
Treatments	hole	board	hole	board	hole	board
vehicle + vehicle	46.8 ± 10.1	71.2 ± 11.1	27.4 ± 5.2	56.5 ± 6.0	21.0 ± 1.0	37.0 ± 1.5
vehicle + oxaliplatin	57.5 ± 4.1	66.4 ± 6.6	33. 8 ± 7.5	44.6 ± 5.8	18.0 ± 1.4	39.2 ± 4.0
scrambled + oxaliplatin	49.2 ± 6.0	64.3 ± 8.5	25.9 ± 6.3	47.3 ± 3.8	24.4 ± 3.6	36.8 ± 2.6
VEGFA-shRNAmir + oxaliplatin	65.8 ± 4.8	174.4 ± 18.3**	38.5 ± 6.6	69.6 ± 6.7	22.8 ± 2.7	40.4 ± 3.6

*The Hole board test was performed 3, 5 and 9 days after the beginning of oxaliplatin treatment (n=5). Each value represents the mean ± SEM. **P<0.01 vs vehicle + vehicle treated animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant procedure was used as post hoc comparison.*