Review article

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The neuroprotective function of vascular endothelial growth factor (VEGF)

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Abstract

The vascular endothelial growth factor (VEGF) is known mainly as the potent angiogenic and vascular permeability-enhancing factor. Both processes are very effective in hypoxia. The latest studies show that VEGF has neurotrophic and neuroprotective as well as angiogenic properties. It exerts neuroprotective actions directly through the inhibition of programmed cell death (PCD), or apoptosis and the stimulation of neurogenesis. VEGF is also a mediator of multiple processes including angiogenesis, enhancing blood brain barrier permeability for glucose, antioxidants activation, which indirectly result in neuroprotection. VEGF prevents neurons from death under critical conditions such as hypoxia, glucose deprivation through binding to the specific receptors, which are also expressed on the surface of neuronal cells. The increased expression of VEGFR-2/flk-1/KDR receptors on neurons subjected to hypoxia, glucose deprivation provides evidence that these receptors are mainly involved in neuroprotective effects of VEGF. Furthermore, binding to these receptors triggers the phosphatidyloinositol 3-kinase (PI3K) /Akt signal transduction system and, in consequence, leads to the inhibition of PCD by activating antiapoptotic proteins through the transcription factor NFκB and inhibiting proapoptotic signaling by Bad, caspase-9, caspase-3, and other effectors. Promotion of neuronal cells proliferation by VEGF is also associated with the increased expression of VEGFR-2 receptors and up-regulation of E2F family transcription factors, cyclin D1, cyclin E, and cdc25. It is known that the amount and types of VEGF isoforms influence its action. At least six isoforms of VEGF proteins are formed as a result of alternative mRNA splicing and it is unknown which of them and in what proportion occur in the nervous system in physiology and pathophysiology. It seems to be very essential to find out the mechanisms responsible for specific patterns of VEGF isoforms and their receptors expression in different pathologies of the nervous system. Maybe such knowledge will provide new perspectives in VEGF therapy.

Key words: *neuroprotection, programmed cell death, neurogenesis, caspase-3, VEGFR-2/PI3K/Akt signaling*

Introduction

The vascular endothelial growth factor (VEGF) is a hypoxia-inducible protein with angiogenic and vascular permeability-enhancing properties [8,10,26,32,34,40,52,54]. The latest studies show that vascular endothelial growth factor has neurotrophic

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Table 1. Direct and indirect actions of VEGF

Neuroprotective function of VEGF	
direct actions	indirect actions
1. Inhibition of apoptosis	1. Stimulation of angiogenesis
2. Stimulation of neurogenesis	2. Increase of brain glucose uptake
	3. Antioxidants activation

and neuroprotective as well as angiogenic properties. VEGF acts as an endogenous neuroprotective factor. Under critical conditions for neuronal cells (hypoxia, glucose deprivation, oxidative stress) it becomes a mediator of multiple molecular reactions leading to the inhibition of programmed cell death and the stimulation of neurogenesis [1,19,20,24,41,50,51,55]. Furthermore, VEGF exerts neuroprotective actions indirectly through multiple mechanisms such as stimulation of angiogenesis, enhancing blood brain barrier permeability for glucose, antioxidants activation [2,7,9,18,31,39,43,49] (Table 1).

The amount and types of VEGF isoforms influence VEGF action. These isoforms consist of various numbers of aminoacids and each of them has different biological properties. Until now it has been unknown which of them and in what proportion occur in the nervous system in physiology and pathophysiology. The form and quantity of VEGF isoforms are determined by posttranscriptional and posttranslational modifications. Each isoform consists of 1-5 exons containing required information to recognize VEGF receptors. These exons are present in all described isoforms. The lack or presence of exons 6, 6', 7 or 8 differs isoforms from each other [11]. At least six isoforms of VEGF proteins are formed as a result of alternative mRNA splicing.

Reaction of the receptors to VEGF overexpression is not identical. VEGFR-1/Flt-1 receptors are localized on endothelial cells, monocytes and they play an important role in vasculogenesis and angiogenesis as a physiological factor, essential for normal vessels development [14]. VEGFR-2/Flk-1/KDR receptors are localized on endothelial cells and detected in early stages of embryonic development. They are considered a marker of angiogenic phenotype of endothelial cells. VEGFR-2 receptors deficiency leads to disturbed endothelial cells development and hematopoesis [29]. sFlt-1 receptors are the soluble form of VEGFR-1/Flt-1 receptor responsible for negative regulation of angiogenesis [30]. Expression of Flt-4 receptors is detected during embryogenesis, at later developmental stages and in adults and is restricted to the endothelium of lymphatic vessels suggesting participation of angiogenic factors in both angiogenesis and lymphogenesis, which act simultaneously [38].

VEGF preventing neurons from irreversible injury and death under critical conditions exerts its neuroprotecive actions through binding to the specific receptors which are also expressed on the surface of neurons. Experimental studies reveal the increased expression of VEGFR-2 receptors on neuronal cells subjected to hypoxia, glucose deprivation [18,19,20,24,41,55].

Direct neuroprotective actions of VEGF

VEGF exerts its direct neuroprotective effects through the inhibition of programmed cell death and the stimulation of neurogenesis.

VEGF inhibits programmed cell death of neuronal cells under hypoxic conditions

Many factors including VEGF are involved in regulation of programmed cell death (PCD), or apoptosis. VEGF increases neuronal cell survival subjected to hypoxia through the inhibition of apoptosis [19,20,41,50,51].

Cerebral ischemia triggers hypoxia-sensing mechanisms that activate hypoxia-inducible factor-1 (HIF-1), a transcription factor that induces VEGF expression [20,29]. Hypoxic induction of VEGF occurs in neurons, depending on the severity of the injury and is more strongly marked in neurons of the ischemic border zone. The sources of VEGF under hypoxia are both neuronal and nonneuronal cells, including endothelium, astroglia and neurons. VEGF released from these sources might target VEGF receptors on the surface of neurons [20].

Experimental studies indicate that VEGF reduces cell death associated with an in vitro cell culture model of cerebral ischemia. The addition of this factor to cultures leads to an approximately 5-fold increase in cell viability after 24h of hypoxia and glucose deprivation [20]. In order to investigate the mechanism of neuroprotection by VEGF the expression of known target receptors for VEGF was measured. Cultured cells expressed VEGFR-2/Flk-1/KDR receptors and neuropilin-1 (Nrp1), but not VEGFR-1/Flt-1 receptors. Two

phosphatidyloinositol 3'-kinase inhibitors, wortmannin and LY294002, reversed the neuroprotective effect of VEGF. These results provide evidence that VEGF exerts its neuroprotective action through VEGFR-2 receptors and the phosphatidyloinositol 3'- kinase/Akt signal transduction system. The serine-threonine protein kinase Akt contributes to the inhibition of apoptosis by activating antiapoptotic proteins through the transcription factor NFκB and inhibiting proapoptotic signaling by Bad, caspase-9, and other effectors. Several of these proteins are induced or activated in the ischemic brain implicating VEGFR-2/PI3K/Akt signaling in the regulation of hypoxic or ischemic neuronal cell death [20].

Other studies demonstrate that in mouse cortical neuron cultures subjected to hypoxia, the direct neuroprotective effect of VEGF involves suppression of cell-death pathways mediated by caspase-3, which plays a key role in apoptosis. Exposure to hypoxia for 24 h caused the death of about 70% of cultured neurons, this was reduced to 40% by VEGF and to 44% by the caspase-3 inhibitor benzyloxycarbonyl-DEVD-fluoromethyl ketone. An antisense, but not sense, oligodeoxyribonucleotide directed against VEGF increased the proportion of neurons expressing activated caspase-3, and correspondingly reduced the viability of hypoxic neurons by 37%. These results indicate that VEGF exerts its protective action through inhibiting the activation of caspase-3, which is induced by caspase-9 [3,19,51].

In the inhibition of cell-death pathways mediated by caspase-3 VEGFR-2/PI3K/Akt and NFκB signaling is also involved. The serine-threonine protein kinase Akt also denoted as protein B kinase is an essential antiapoptotic factor. Akt inhibits proapoptotic Bcl-2 family member Bad and caspase-9. The proapoptotic protein Bad acts at the surface of the mitochondrial membrane to decrease the mitochondrial transmembrane potential and promotes leakage of cytochrome c. Released cytochrome c complexes with and activates Apaf-1 that binds to procaspase-9, and processes this enzyme into proteolytically active form- caspase-9. Caspase-9 is the initial caspase activating the effector caspases such as caspase-3,6,7, that ultimately lead to apoptosis through nuclear damage (DNA fragmentation, DNA mutations). The inhibition of Bad and caspase-9 by Akt results in proteolytic cascade blockage and prevents caspase-3 mediated DNA fragmentation [3,4,5,16,19,22,51].

Hypoxia causes the generation of reactive oxygen species and free radicals substrates that lead to cell damage. The effect of free radicals action is lipid membrane peroxidation which is a permanent source of free radicals and may lead to inflammation, aging, neoplasms, degenerations, atherosclerosis etc. [12,13,27]. Besides, reactive oxygen species stimulate multiple transcription factors such as NFκB, HIF1α and AP-1, which increase expression of some genes including the gene for VEGF [6,27,30,35]. VEGF backwards through Akt activates NFκB that mediates multiple molecular reactions resulting in the increase of neuronal cell survival. This transcription factor enhances the expression of inhibitor of apoptosis protein (IAP) family members such as c-IAP-1, c-IAP-2, XIAP involved in the inhibition of cell death pathways mediated by caspases 3,7,9. Moreover, NFκB stimulates directly activity of antiapoptotic Bcl-2 homologs such as Bfl-1/A1, Bcl-Xl, which influence cell survival under stress conditions (hypoxia, glucose deprivation, oxidative stress) [20,33].

Experimental studies conducted *in vivo* on mice with the deletion of the hypoxia-response element in the vascular endothelial growth factor promoter provide evidence that VEGF exerts its neuroprotective effects also in the peripheral nervous system. The described mutation causes reduction of hypoxic VEGF expression in the spinal cord and results in adult – onset progressive motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (ALS). In more than 95% of patients with sporadic ALS the pathogenetic mechanisms of this neurodegenerative disease remain unknown. Experimental studies indicate a previously unknown function for VEGF in the pathogenesis of motor neuron degeneration. Reduced hypoxic VEGF expression leads to decreased neural vascular perfusion and to the insufficiency of VEGF-dependent neuroprotective mechanisms. In vitro studies demonstrate $VEGF_{165}$ as the isoform involved in neuroprotection. $VEGF_{165}$ mediates survival of motor neurons under hypoxia through binding to VEGFR-2/KDR receptor and Nrp1.

Both anti-VEGFR-2 and anti-Nrp antibodies are required to completely block the VEGF-dependent survival effect. Nrp might be a co-receptor for VEGFR-2, as in endothelial cells. Moreover, VEGFR-2 receptors are also expressed *in vivo* on the surface of motor neurons in the spinal cord [41]. The question is whether chronic vascular insufficiency and insufficient

VEGF-dependent neuroprotection lead to motor neuron degeneration in human.

Vascular endothelial growth factor stimulates neurogenesis

Neurogenesis, or the production of new neurons is regulated by physiological and pathological processes including aging, stress, and brain injury. Many mitogenic and trophic factors that regulate proliferation of nonneuronal cells are also involved in neurogenesis. These include the vascular endothelial growth factor [20,46,50,55].

VEGF stimulates proliferation and migration of endothelial cells resulting in an increase of angiogenesis [46]. VEGF exerts this action through the binding to the specific receptors and activating multiple intracellular signaling pathways which contribute to changes in expression of many genes responsible for regulation of cell divisions [37,42,44]. Furthermore, experimental studies have indicated that VEGF up-regulates the expression of the intracellular adhesion molecule-1 (ICAM-1) through a pathway including phosphatidylinositol 3 OH-kinase (PI3K), AKT, and nitric oxide (NO), which leads to brain microvessel endothelial cell migration [46]. The above described effects of VEGF are not restricted to the vascular endothelial cells.

In vitro and *in vivo* studies conducted on rats have demonstrated that VEGF promotes also neuronal cells proliferation [24,50,55]. This factor stimulated bromodeoxyuridine incorporation into cells that expressed immature neuronal marker proteins and increased cell number in cultures by 20-30%. The increase in BrdUrd labeling after administration of VEGF confirms its proliferative properties. Both in vitro and in vivo cells labeled by BrdUrd expressed VEGFR2/flk-1, but not VEGFR1/flt-1 receptors, and the effect of VEGF was blocked by the VEGFR2/flk-1 receptor tyrosine kinase inhibitor SU1498 [24]. The VEGFR2/flk-1 receptors expression was colocalized with the immature neuronal marker, doublecortin (Dcx). Using BrdUrd labeling as an index of cell proliferation shows that the in vitro neuroproliferative effect of VEGF is associated with up-regulation of E2F family transcription factors, cyclin D1, cyclin E, and cdc25. VEGF also increases nuclear expression of E2F1, E2F2, and E2F3, consistent with regulation of the G1/S phase transition of the cell cycle. The described action of VEGF is inhibited by the extracellular signal-regulated (MEK) inhibitor PD98059, the phospholipase C (PLC) inhibitor U73122, the protein kinase C (PKC) inhibitor GF102390X, and the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, indicating involvement of multiple signaling pathways [55].

The increase of neurogenesis appears also after cerebral ischemia caused by occlusion of the middle cerebral artery in the rat. Besides, several studies demonstrate that newborn neurons migrate toward ischemic lesions, where they are in a position to participate in brain repair and functional recovery. These effects depend on the number and the delayed survival of newborn neurons. VEGF affects both the number and the survival of new neuronal cells through multiple mechanisms including angiogenesis, inhibition of apoptosis, and stimulation of neuroproliferation. VEGF may, therefore, improve histological and functional outcome from stroke through described mechanisms [21,23,50].

Indirect neuroprotective actions of VEGF

The neuroprotective function of VEGF includes also indirect actions which affect neuronal survival under critical conditions.

VEGF and heme oxygenases – antioxidative function

Hypoxia of the nervous tissue, VEGF and many other substances induce heme oxygenases 1 (HO-1) and 2 (HO-2) [39,42]. HO-2 is a constitutional enzyme with strongly marked activity in olfactory epithelium, olfactory bulb, hippocampus, cerebellum, and pons. HO-1 is an induced enzyme that plays a key role in hypoxia. HO-1 activity raises significantly under hypoxic conditions, particularly in the brain. Macrophages infiltrating the hypoxic region of the brain are together with endothelial cells the main source of HO-1. Thus, HO-1 is used in diagnostic tests as a marker of infiltrating macrophages [39]. Both HO-2 and HO-1 participate in heme degradation. Carbon monoxide (CO), ferrum ions (Fe^{2+}) and biliverdin are the products of this reaction, which prevent cells from oxidative damage caused by free radicals [45]. Carbon monoxide acts as an essential intracellular messenger in both central and peripheral nervous system. It mediates in vessels dilatation through the activation of cyclic guanylate cyclase (cGC), which increases the concentration of cyclic guanylate monophosphate (cGMP). CO is a physiological regulator of cellular

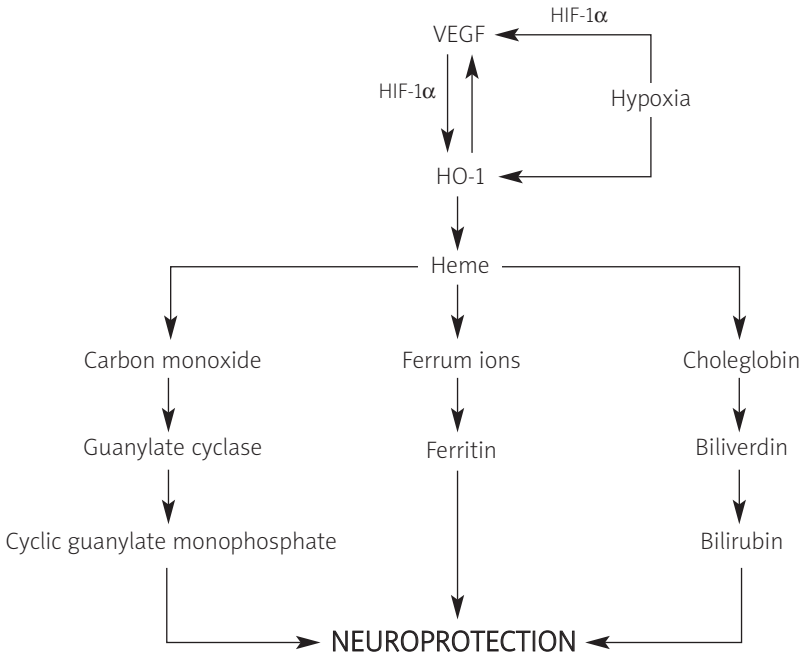

Fig. 1. Neuroprotective properties of VEGF and heme oxygenase-1 (HO-1)

cGMP concentration. Ferrum ions induce synthesis of ferritin – protein responsible for ferrum storing in cells. Ferritin, biliverdin, and bilirubin are physiological antioxidants in serum and extravascular space, which protect neurons from damage due to production of reactive oxygen species. Hypoxia by inducing heme oxygenases contributes to the increase of heme degradation products concentration. These products exert their neuroprotective actions through vessels dilatation and antioxidation. HO-1 induces expression hypoxia-inducible factor 1α (HIF-1α), including the gene encoding VEGF. VEGF induces backwards HO-1 and through this positive biofeedback it mediates in antioxidation [36,47] (Fig. 1).

VEGF enhances glucose transport across blood brain barrier (BBB)

Glucose, the most important energetic substrate for the brain must pass the blood brain barrier to obtain access to the brain. Glucose is transported by carrier mechanisms through microvessel endothelial cells and the plasma membranes of neurons and glia. The carrier protein specific for glucose transport across the BBB is GLUT-1 [31,48]. Reduced availability of blood glucose (i.e. during hypoglycemia) enhances uptake of glucose into the brain by reducing uptake of glucose into the peripheral tissues. The hormonal mediator that directly enhances brain glucose uptake under critical conditions has not been identified yet. However, counterregulatory hormones such as epinephrine, glicocortycoides, somatotropin released during acute hypoglycemia are indirectly responsible for this effect by reducing uptake of glucose into the peripheral tissues. Release of these hormones and sympathetic activation induce adrenergic symptoms, such as trembling, and neuroglycopenic symptoms, such as sweating, blurred vision, and sleepiness [2,15]. The absence of the described symptoms in systemic hypoglycemia suggests that the brain can adapt to low levels of blood glucose by increasing cerebral blood flow and glucose transport across the BBB [7,31].

There are studies indicating VEGF as a candidate for directly regulating brain glucose uptake under critical conditions [7]. The highest density of VEGF binding receptors on the brain microvessel endothelium forming the BBB may suggest its influence on the blood brain barrier function. VEGF mediates enhancement of glucose transport through BBB by increasing GLUT-1 gene expression and translocation of cytosolic GLUT-1 to the plasma membrane surface. Furthermore, VEGF mediates

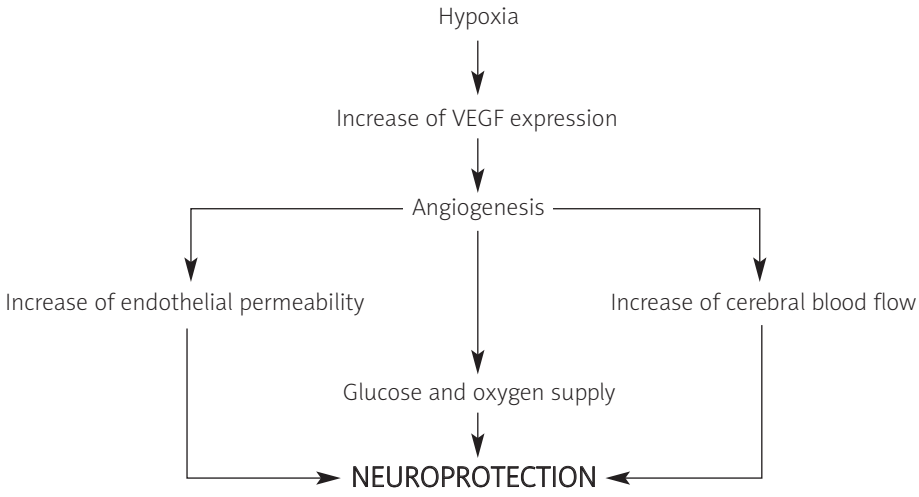

Fig. 2. The role of VEGF-induced angiogenesis on neuronal cells function

induction of endothelial fenestrations, thereby increasing the transport of small molecules [7,43,49].

Experimental studies conducted on rats have demonstrated that local administration of VEGF into cerebral microcirculation increases BBB permeability and contributes to cerebral arterioles dilatation. Importantly, this response is rapid and occurs within 10 minutes after the administration of VEGF [7].

In humans acute hypoglycemia is associated with an increase of VEGF in serum (at plasma glucose concentrations about 4.2 mmol/liter). Enhanced VEGF secretion during hypoglycemic conditions is positively correlated to the maintenance of neurocognitive function and it may suggest rapid adaptation of neurons to this state. There is no such relation for epinephrine, cortisol, or other counterregulatory hormones and it may indicate that VEGF is a mediator of brain adaptation to hypoglycemia by increasing uptake of glucose into the brain. A putative mechanism of VEGF release in response to hypoglycemia relies on the stimulatory effect of the specific glucose-responsive neurons of the ventromedial hypothalamus on the release of neuropeptides from neurons of the paraventricular nucleus (PVN). Cells of the PVN synthesize VEGF and other neuropeptides such as CRH, vasopressin, and the pituitary adenylate cyclase-activating polypeptide etc. VEGF is also released from the pituitary, where it is detected in folliculostellate cells located in the anterior lobe. VEGF from hypothalamic nuclei and the pituitary may have direct access to the systemic blood circulation. The hypothalamus-pituitary system is the major source of the increase in serum VEGF [7].

Because VEGF mRNA is diffusely expressed throughout the brain we may consider the increase of VEGF production a general response of neurons to glucose deprivation.

In conclusion, VEGF can increase brain glucose uptake by enhancing the transport via

GLUT-1, dilating cerebral arterioles, inducing endothelial fenestrations in conditions of brain glucose deprivation.

Neuroprotective role of angiogenesis

Many studies on cerebral ischemia in animals reveal that hypoxia induced by occlusion of the cerebral middle artery contributes to overexpression of VEGF and angiogenesis [11,17,18,40,54]. VEGF as the most potent angiogenic factor promotes differentiation, proliferation and migration of endothelial cells. It is a major mitogen for vascular endothelial cells derived from arteries, veins and lymphatics. Other investigations conducted on rats show that overexpression of VEGF occurs also after subarachnoid hemorrhage (SAH). Increased expression of this factor is particularly detected in the following regions of the brain hippocampus, thalamus, lateral ventricles, the fourth ventricle, smooth muscle cells of subarachnoid vessels and cerebellar cortex [25,28]. These findings provide the evidence that endothelial

cells hypoxia stimulates directly VEGF expression and, in consequence, promotes angiogenesis.

Experimental investigations demonstrate that angiogenesis stimulated mostly by

VEGF in a region of cerebral ischemic tissue surrounding an acute cerebral infarct (penumbra) that is dysfunctional but potentially viable, restores perfusion and improves neurons function [18,53] (Fig. 2).

Together with up-regulated production of VEGF vascular permeability is increased and cerebral edema is formed [29]. These dual actions of VEGF lead to favorable effects such as restoration or increase of neuronal perfusion but, on the other hand, to unfavorable induction of cerebral edema. The potential, therapeutic usefulness of VEGF in cerebral ischemia is also limited by the fact that its angiogenic effect is delayed in onset, and therefore presumably is too late to rescue many vulnerable neurons. The possibility of direct neuroprotective VEGF actions in ischemic tissue in the interval preceding angiogenesis may help prolong cell survival until angiogenesis can occur [20].

New aspects of VEGF actions raise new questions

VEGF is involved in very complicated mechanisms. Thus, it exerts combined effects that make difficulties in finding proper and selective therapeutic methods in the nervous system disorders related to hypoxia. Besides, the antiapoptotic actions of VEGF in the nervous system put it in the new position as a factor which abnormal expression may lead to many diseases caused by dysregulation of programmed cells death (neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease).

Concluding, it seems to be very essential to find out the mechanisms responsible for specific patterns of VEGF isoforms and their receptors expression in different pathologies of nervous system. Maybe such knowledge will provide the possibility of selective blocking or stimulating particular VEGF isoforms and receptors and, in consequence, pathological processes mediated by VEGF.

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