

Diverse roles for VEGF-A in the nervous system

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Summary

Vascular endothelial growth factor A (VEGF-A) is best known for its essential roles in blood vessel growth. However, evidence has emerged that VEGF-A also promotes a wide range of neuronal functions, both in vitro and in vivo, including neurogenesis, neuronal migration, neuronal survival and axon guidance. Recent studies have employed mouse models to distinguish the direct effects of VEGF on neurons from its indirect, vessel-mediated effects. Ultimately, refining our knowledge of VEGF signalling pathways in neurons should help us to understand how the current use of therapeutics targeting the VEGF pathway in cancer and eye disease might be expanded to promote neuronal health and nerve repair.

Key words: VEGF-A, *Vegfa*, Axon guidance, Neurogenesis, Neuron, Neuronal migration and survival

Introduction

During embryonic development of vertebrates, a multitude of growth factors and guidance cues cooperate to regulate the formation of the vascular and neuronal networks that are essential for tissue homeostasis. The most potent inducer of developmental blood vessel growth known to date is the dimeric glycoprotein vascular endothelial growth factor A (VEGF-A, or VEGFA) (reviewed by Ruhrberg, 2003). VEGF-A is a member of the cysteine knot family of growth factors, which in mammals also includes VEGF-B, VEGF-C, VEGF-D (VEGFB, VEGFC, FIGF, respectively) and placental growth factor (PIGF, or PGF) (Tammela et al., 2005). In adults, VEGF-A is also important for blood vessel growth, most notably during organ remodelling and in pathologies with vascular involvement. For example, it plays a central role in wound healing, tumour angiogenesis (see Glossary, Box 1) and in the eye diseases diabetic retinopathy and age-related macular degeneration (see Glossary, Box 1) (reviewed by Andreoli and Miller, 2007; Goel et al., 2011). More recently, evidence has emerged that VEGF-A also plays key roles in neurons. Here, we discuss recent findings on the vascular and nonvascular roles of VEGF-A in the nervous system to complement and update several excellent previous reviews (e.g. Greenberg and Jin, 2005; Rosenstein and Krum, 2004; Ruiz de Almodovar et al., 2009).

VEGF isoform expression and function

Early in vertebrate development, endothelial cells condense into blood vessels in a process termed vasculogenesis (see Glossary, Box 1) (Risau and Flamme, 1995). This process occurs first extra-embryonically to create the yolk sac vasculature and subsequently in the embryo proper to give rise to the dorsal aorta, cardinal vein and pharyngeal arch arteries. Endothelial cell differentiation

depends on VEGF, and vasculogenesis therefore fails when VEGF-A expression is abolished by gene targeting in the mouse, causing early embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996).

Throughout most embryonic tissues, VEGF-A is synthesized as a collection of three major isoforms as a consequence of alternative splicing; in humans, they consist of 121, 165 or 189 amino acids and are therefore termed VEGF121, VEGF165 and VEGF189. The corresponding mouse isoforms are termed VEGF120, VEGF164 and VEGF188 (Fig. 1A), as they are all one amino acid shorter (reviewed by Ruhrberg, 2003). Expression of any one of the VEGF-A isoforms rescues the vascular defects and lethality of the full *Vegfa* knockout in mice (Carmeliet et al., 1999; Ruhrberg et al., 2002; Stalmans et al., 2002). This is explained by the ability of each isoform to induce endothelial cell differentiation and proliferation (e.g. Ruhrberg et al., 2002). However, the relative ratio of the different VEGF-A isoforms varies in different tissues (Ng et al., 2001) because they have specific roles during the later stages of vascular development, which relate to their differential affinity for the extracellular matrix and various VEGF-A receptors. Accordingly, biochemical assays revealed that the human VEGF189 isoform with its two heparin-binding domains is retained in the extracellular matrix after secretion, whereas VEGF165, which contains only one heparin-binding domain, is partly matrix-bound and partly diffusible; VEGF121 has no heparin-binding domains and is the most diffusible isoform (Park et al., 1993). The diffusibility of the isoforms is thought to reflect their differential affinity for heparan sulphate proteoglycans (HSPGs) in the extracellular matrix, although genetic or other physiological evidence that HSPGs are essential for VEGF-A isoform-induced signalling events is still lacking.

All VEGF-A isoforms bind to the transmembrane tyrosine kinase receptors VEGFR1 (FLT1) and VEGFR2 (FLK1 or KDR), whereas the two non-tyrosine kinase receptors of the neuropilin family, NRP1 and NRP2, preferentially bind VEGF164 (Fig. 1B) (reviewed by Ruhrberg, 2003). One recent study reported that VEGF121 also binds NRP1 in endothelial cells (Pan et al., 2007). However, the significance of this observation for vessel growth has not yet been established in vivo and it does not agree with the similarity of neuronal patterning defects in mice lacking NRP1 or VEGF164 but expressing VEGF120 (see below).

Below we discuss the roles of VEGF-A signalling in modulating vascular and neuronal behaviour in the nervous system. For a discussion of specific intracellular signalling pathways activated by the various VEGF-A isoforms and their receptors, we refer the reader to an excellent recent review on this topic (Koch et al., 2011).

VEGF-A signalling during CNS angiogenesis The CNS vasculature is essential for brain development and homeostasis

Like all organs, the central nervous system (CNS) requires a good vascular supply, both during development and in the adult. The requirement for blood vessels in the developing CNS is likely to be

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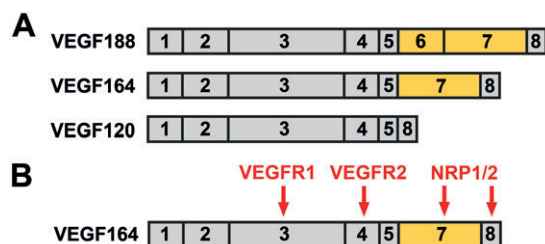


Fig. 1. VEGF-A isoforms and their receptors. (A) The mouse *Vegfa* gene encodes three major splice forms: VEGF120, VEGF164 and VEGF188. The yellow boxes represent domains that can each promote binding to heparin sulphate proteoglycans (HSPGs) in the extracellular matrix and to the neuropilin cell surface receptors. VEGF120, which does not possess HSPG-binding domains, is thus able to diffuse over the greatest distance. (B) The tyrosine kinase receptors VEGFR1 (FLT1), VEGFR2 (KDR) and the non-tyrosine kinase neuropilin receptors NRP1/2 bind to different VEGF-A domains (red arrows). Because it lacks domain 7, VEGF120 is thought not to bind to the neuropilins effectively.

due to the need for delivering oxygen and nutrients to the rapidly growing and therefore metabolically active CNS tissue. Accordingly, ablating VEGF-A expression from neural progenitors and their progeny impairs brain vascularisation and leads to widespread neuronal apoptosis in the mouse embryo (Breier et al., 1992; Haigh et al., 2003; Raab et al., 2004). In the adult, reduced VEGF-A levels in *Vegfa^{delta/delta}* mice due to mutation of the hypoxia response element in the *Vegfa* promoter impairs neuroprotection; for example, these mutant mice do not recover motor function after transient spinal cord ischemia, whereas wild-type littermates show only a transient clinical deficit (Lambrechts et al., 2003). Moreover, these mutants suffer late-onset motoneuron degeneration and paralysis, leading to a disease akin to amyotrophic lateral sclerosis (ALS; see Glossary, Box 1) (Oosthuysen et al., 2001). Surprisingly, however, adult mice with the *Vegfa^{delta/delta}* mutation have a normal capillary density in the CNS (Oosthuysen et al., 2001). This finding raises the possibility that hypoxia regulation of *Vegfa* expression is not essential for vascular development.

Growth factor gradients comprising VEGF-A isoforms regulate CNS angiogenesis

It was first hypothesized more than 20 years ago that VEGF-A is released by rapidly growing neuronal precursors to recruit filopodia-extending blood vessels from perineural vessels into the brain (Fig. 2A) (Risau, 1997). This idea was corroborated by genetic studies, which showed that VEGF-A isoforms through their differential matrix affinities establish chemoattractive gradients, and that these gradients induce filopodia extension from endothelial cells at the leading edge of growing brain vessels (Ruhrberg et al., 2002). In this fashion, VEGF-A isoforms regulate the morphology and connectivity of capillary networks in the brain (Ruhrberg et al., 2002). The analysis of endothelial cell behaviour in retinal VEGF-A gradients (Gerhardt et al., 2003; Ruhrberg et al., 2002) led to the concept that VEGF-A promotes the migration of filopodia-studded endothelial tip cells and the proliferation of lumen-forming stalk cells (reviewed by Geudens and Gerhardt, 2011). Subsequent to VEGF-induced vessel sprouting, CNS microglia promote fusion of sprouts into vascular loops (Fig. 2A) in a mechanism that does not rely on VEGF-A (Fantin et al., 2010).

Box 1. Glossary

Age-related macular degeneration. A late-onset eye disorder characterised by progressive degeneration of the central macula, leading to loss of central vision.

Amyotrophic lateral sclerosis (ALS). A progressive and fatal disease characterised by degeneration of upper and lower motoneurons.

Angiogenesis. The formation of new blood vessels from pre-existing vessels by sprouting and/or remodelling.

Angiogenic niche of neurogenesis. The existence of new blood vessels and new neurons in the brain at the same time, in close proximity and requiring each other.

Commissural axon. An axon that crosses the midline of the neural tube or its derivatives.

Diabetic retinopathy. An eye condition characterised by detrimental changes in the retinal vasculature as a complication of diabetes mellitus.

Neurogenesis. The generation of new neurons from embryonic neural progenitors or adult neural stem cells.

Subventricular zone (SVZ). A region in the lateral ventricles of the adult brain containing neural stem cells that supplies new neurons to the olfactory bulb.

Vasculogenesis. The formation of new blood vessels from single endothelial or endothelial precursor cells.

Distinct roles for different VEGF-A receptors during CNS angiogenesis

VEGF-induced autophosphorylation of VEGFR2 induces endothelial cell differentiation, proliferation, migration and survival (reviewed by Koch et al., 2011; Ruhrberg, 2003). Accordingly, VEGFR2 is essential for vasculogenesis and angiogenesis in both the mouse yolk sac and embryo proper (Shalaby et al., 1995). Function-blocking antibodies specific for VEGFR2 inhibit blood vessel sprouting in the perinatal retina, demonstrating that VEGFR2 is also required for CNS vascularisation (e.g. Gerhardt et al., 2003). Mice lacking the alternative VEGF-A tyrosine kinase receptor VEGFR1 also have severe vascular defects, but they include endothelial cell overcrowding and vascular disorganisation (Fong et al., 1995). This phenotype is not explained by an ability of VEGFR1 to convey VEGF-A signals in endothelial cells; instead, VEGFR1 has a higher affinity than VEGFR2 for VEGF-A and is therefore able to control VEGF-A availability to VEGFR2 (e.g. Hiratsuka et al., 1998; Kearney et al., 2004).

Unlike mice lacking VEGFR1 or VEGFR2, mice lacking NRP1 survive embryogenesis long enough to form a multilayered brain and spinal cord; it was therefore possible to show that this receptor is important for vessel sprouting within the CNS (Gerhardt et al., 2004; Kawasaki et al., 1999). The most popular hypothesis of NRP1 function in vessel growth postulates that VEGF164 binds to NRP1 on endothelial cells to potentiate VEGFR2 signalling (Soker et al., 1998). Consistent with this idea, loss of NRP1 from endothelial cells in mice results in vascular phenotypes that are similar to those of full *Nrp1* knockout mice (Gu et al., 2003). Nevertheless, a demonstration that VEGF-A signalling through NRP1 is essential for angiogenesis would require an NRP1 mutant specifically deficient in VEGF-A binding, analogous to the mutant that was previously created to selectively abolish binding of semaphorins to NRP1 and that phenocopied the axonal defects of full *Nrp1* knockouts (Gu et al., 2003). Although abolishing NRP1 in the C57BL/6 genetic background causes severe yolk sac vascular defects and embryonic lethality prior to brain vascularisation at

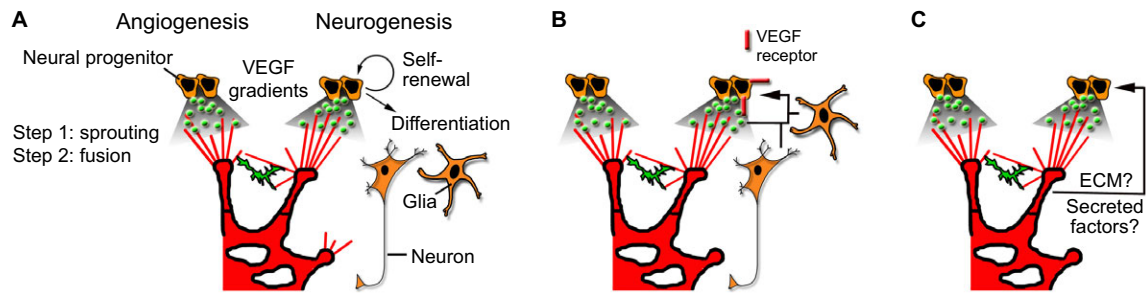


Fig. 2. Spatiotemporal relationship of angiogenesis and neurogenesis in the embryonic brain. (A) VEGF-A (green circles) is secreted from neural stem/progenitor cells (small orange cells) as three isoforms that form chemoattractive VEGF-A concentration gradients (grey) as a result of their differential matrix affinities. These gradients are sensed by endothelial tip cell filopodia at the tips of blood vessels (red) during vessel sprouting (step 1: sprouting). Microglia (green cell) help neighbouring tip cells to fuse into new vessel loops (step 2: fusion). At the same time, neural progenitors self-renew or give rise to progeny that differentiate into neurons (arborised orange cell with axon) or glia (stellate orange cell). (B,C) Alternative hypotheses to explain the coordinated growth of blood vessels and neural tissue in the angiogenic niche during neurogenesis. (B) VEGF-A from neural progenitors or differentiated neural progeny signals directly back to neural progenitors expressing VEGF-A receptors (red). (C) Blood vessels growing in response to VEGF-A gradients secrete neurogenic/trophic factors or lay down an extracellular matrix (ECM) that promotes self-renewal or differentiation of neural stem/progenitor cells.

approximately embryonic day (E) 10.5 (Jones et al., 2008), losing NRP1 in the JF1 genetic background allows embryos to survive until ~E14.5 and has enabled the study of VEGF-A isoform signalling in developing neurons (Schwarz et al., 2004) (see below). The genetic modifiers of NRP1 signalling in the different mouse strains have not been identified.

In summary, there is now good experimental evidence to suggest that the different VEGF-A receptors have essential and complementary roles in vascular development. However, the mechanisms that ensure signal integration from these receptors to promote normal brain vascularisation remain to be identified. Finally, the physiological significance of NRP2 binding to specific VEGF-A isoforms has not yet been determined.

Direct roles of VEGF-A in neurons

Increasing evidence shows that VEGF-A can support neural cells independently of its roles in vessels. Accordingly, VEGF-A is mitogenic for astroglia and Schwann cells *in vitro* (e.g. Krum et al., 2002; Mani et al., 2005; Schratzberger et al., 2000; Silverman et al., 1999; Sondell et al., 1999a; Sondell et al., 1999b). In addition, both glial cell types produce a number of different growth factors, including VEGF-A, to support neuronal growth in explant cultures (Eddleston and Mucke, 1993; Krum and Rosenstein, 1998). The observation that VEGF-A treatment increases neurite number, length and size as well as soma size in primary CNS neuron cultures lacking glia provided initial evidence that VEGF-A can directly affect neurons, independently of glia (e.g. Khaibullina et al., 2004; Rosenstein et al., 2003). Recently, several lines of *in vivo* experimentation demonstrated direct and essential roles for VEGF-A in regulating neural progenitor proliferation, survival, migration, axon/dendrite patterning and synaptic function.

VEGF-A receptors in neurons

VEGFR2 has been implicated as a VEGF-A receptor for survival signalling in mature neurons *in vitro* (e.g. Ogunshola et al., 2002; Oosthuyse et al., 2001; Sondell et al., 2000). In agreement, the forced expression of VEGFR2 in motoneurons enhances their survival in a mouse model of ALS-like motoneuron degeneration (Storkebaum et al., 2005). However, ablating VEGFR2 in neuronal progenitors or in their progeny does not obviously impair brain morphogenesis or viability in the mouse, suggesting

that VEGFR2 does not have a general role in neuronal survival (Haigh et al., 2003). Instead, VEGFR2 might convey VEGF-A signals in select subsets of neurons, for example to control axon pathfinding across the spinal cord midline (see below). Additionally, VEGFR2 can act in a complex with NRP1 and the semaphorin receptor plexin D1 to convey semaphorin signals in axon guidance (Bellon et al., 2010).

The alternative VEGF-A receptor tyrosine kinase VEGFR1 is expressed in several types of developing and adult neurons (e.g. Islamov et al., 2004; Yang et al., 2003). The genetic requirement for VEGFR1 as a VEGF-A receptor specifically in the neural lineage has not been examined so far, but we know that mice lacking the tyrosine kinase activity of VEGFR1, similar to mice lacking neural VEGFR2, undergo at least grossly normal brain morphogenesis (Hiratsuka et al., 1998). However, a recent study suggested that VEGFR1 might negatively regulate neurogenesis (see Glossary, Box 1) by regulating VEGF-A levels (see below) (Wittko et al., 2009).

The non-tyrosine kinase VEGF-A receptor NRP1 is upregulated on neurons and endothelium after CNS ischemia (Zhang et al., 2001), but mouse knockout studies or equivalent physiological experiments have not yet determined whether neuropilins primarily act as VEGF-A receptors in these adult situations, or whether they instead function as receptors for axon guidance molecules of the class 3 semaphorin family (Raper, 2000). By contrast, several embryonic studies have provided conclusive evidence that NRP1 does convey VEGF-A signals in subpopulations of developing neurons, including facial branchiomotor (FBM) neurons, retinal ganglion cells (RGCs) and gonadotropin-releasing hormone (GnRH) neurons (see below).

Taken together, it appears that VEGFR1, VEGFR2 and NRP1 are all expressed in distinct subtypes of developing and adult neurons, but only VEGFR2 and NRP1 have so far been shown to cell-autonomously affect the behaviour of differentiated neurons during embryogenesis. Potential roles for the VEGF receptors in the generation of neurons are discussed in the following section.

VEGF-A and the angiogenic niche of neurogenesis

Two regions in the mammalian brain undergo neurogenesis in normal adults: the subventricular zone (SVZ; see Glossary, Box 1) of the lateral ventricles, which supplies the olfactory bulb with new

neurons that are destined to become interneurons; and the dentate gyrus, which supplies the hippocampus with new neurons that will participate in memory formation. In both regions, neurogenesis occurs in close proximity to growing blood vessels (Fig. 2A). This observation led to the concept of the 'angiogenic niche of neurogenesis' (see Glossary, Box 1) (Palmer et al., 2000). Two possibilities might explain the association of new vessel growth with neurogenesis: VEGF-A might stimulate neurogenesis directly by acting on neural progenitors expressing appropriate receptors, in analogy to its role in blood vessel growth (Fig. 2B); alternatively, VEGF-A-induced blood vessels might promote neurogenesis by providing structural and matrix support or by secreting other neurogenic factors, in addition to their general role in supplying oxygen and nutrients (Fig. 2C). These alternative hypotheses need not be mutually exclusive.

One of the first studies that experimentally validated the existence of an angiogenic niche for neurogenesis concerned seasonal neuron production in the higher vocal cord centre (HVC) of adult male songbirds (Louissaint et al., 2002). In this organ, testosterone stimulates the secretion of VEGF-A and causes upregulation of VEGFR2 on HVC blood vessels to increase angiogenesis; the VEGF-A-stimulated blood vessels in turn secrete brain-derived neurotrophic factor (BDNF) to support neuronal progenitor recruitment into the neural networks of the HVC (Hartog et al., 2009; Louissaint et al., 2002; Rasika et al., 1994). Mechanistically, vessel-derived BDNF is thought to promote neuronal progenitor migration and, possibly, survival (Louissaint et al., 2002). Blood vessels may also play a more structural role, as neural progenitor cells in the adult mouse SVZ directly contact blood vessels (Mirzadeh et al., 2008) and maintain close proximity to blood vessels when proliferating (Tavazoie et al., 2008). Moreover, disrupting this interaction alters the position and proliferation of these neural progenitors (Shen et al., 2008).

Several other lines of *in vitro* experimentation support a role for VEGF-A in adult neurogenesis. Firstly, VEGF-A expression is upregulated in CNS regions that undergo neurogenesis in rodents after transplantation of bone marrow-derived mesenchymal stem cells (Bao et al., 2011), after exercise (Wong-Goodrich et al., 2010), following traumatic brain injury (Lu et al., 2011) or in response to environmental enrichment (Cao et al., 2004). Secondly, manipulating VEGF-A expression alters the level of neurogenesis in these experimental settings. For example, sequestering circulating VEGF-A with a soluble VEGFR1 receptor disrupts exercise-induced neurogenesis (Fabel et al., 2003). Moreover, delivery of exogenous VEGF-A to the brain enhances neurogenesis in adult rats, whereas inhibiting VEGF-A expression by siRNA disrupts neurogenesis, learning and memory formation in healthy animals (Cao et al., 2004; Sun et al., 2010a) and in rodents with traumatic brain injury or ischemia (Li et al., 2009; Thau-Zuchman et al., 2010; Wang et al., 2009; Wang, Y. Q. et al., 2007).

VEGF-A also plays a role in antidepressant therapies that stimulate neurogenesis. Treatment with several different classes of antidepressants induces VEGF-A expression, and the pharmacological inhibition of VEGF-A signalling by intraventricular infusion of a VEGFR2 inhibitor reduces the neural stem/progenitor proliferation induced by electroconvulsive treatment or antidepressant therapy in rodents (Segi-Nishida et al., 2008; Warner-Schmidt and Duman, 2007). Inhibition of VEGFR2 also blocks stress-related behavioural changes in rats (Greene et al., 2009). Although it is not known whether VEGF-A levels are decreased in depression, polymorphic variation in *VEGFA* might explain the variable success of antidepressant treatment in humans (Viikki et al., 2010).

The studies described above imply that VEGF-A is important for adult neurogenesis. However, VEGF-A and VEGF-A-induced blood vessel growth may also be important for developmental neurogenesis. For example, endothelial cells enhance the ability of embryonic neural progenitors to self-renew in co-culture models and thereby inhibit their differentiation (Shen et al., 2004). Neonatal SVZ-derived neural stem cells also proliferate in a VEGF-A-dependent fashion when co-cultured with endothelial cells (Sun et al., 2010b).

VEGF-A can also affect neural progenitor migration and survival. For example, VEGF-A stimulates the migration of fibroblast growth factor (FGF)-stimulated progenitors from explanted neonatal rat SVZ (Zhang et al., 2003) and the migration of a neuroectodermal progenitor cell line derived from a human cerebellar tumour (Bagnard et al., 2001). *In vivo*, overexpression of VEGF-A, either from a recombinant adeno-associated virus vector in adult rats or from a neuronal *vegfa* transgene in mice, correlates with increased neuronal progenitor migration from the SVZ after ischemia (Li et al., 2009; Wang, Y. et al., 2007). Whether this is secondary to VEGF-A enhancing neuronal progenitor proliferation and/or new progenitor survival is not known. Furthermore, VEGF-A infusion after traumatic brain injury in rats decreases the number of apoptotic neurons in the hippocampus without an appreciable effect on progenitor proliferation (Lee and Agoston, 2010).

Even though the *in vivo* studies described above collectively support the concept of an angiogenic niche of neurogenesis, they did not unequivocally establish whether VEGF-A acts directly on neurons that express VEGF-A receptors or instead increases neurogenesis indirectly by stimulating pro-neurogenic angiogenesis (Fig. 2B,C). By contrast, it has been convincingly shown that the closely related factor VEGF-C signals directly to neural stem cells and niche astrocytes through its receptor VEGFR3 (FLT4) independently of its alternative roles in lymphatic or blood vessels (Calvo et al., 2011).

Evidence for direct effects of VEGF-A in neurogenesis

Several *in vitro* studies have raised the possibility that VEGF-A, similar to VEGF-C, regulates neurogenesis by acting directly on neural progenitors, independently of blood vessels. For example, VEGF-A stimulates the proliferation of primary embryonic cortical neuronal progenitors in a VEGFR2-dependent manner in the absence of endothelial cells (Jin et al., 2002). Yet, few studies have attempted to dissociate VEGF-A-induced angiogenesis and neurogenesis in a whole-animal context. One study showed that a low dose of exogenous VEGF-A increased neurogenesis in the absence of angiogenesis in the SVZ and dentate gyrus, but this was due to reduced neuronal progenitor apoptosis rather than increased proliferation (Schanzer et al., 2004). Perhaps the most convincing evidence that VEGF-A directly increases neuronal progenitor proliferation *in vivo* comes from studies in the developing chick retina, which is devoid of blood vessels throughout much of its development and can therefore be exploited to analyse vascular-independent effects of VEGF-A on neurons. The implantation of VEGF-A-expressing cells into the vitreous humour of E5 chick eyes mildly increased proliferation, whereas the implantation of cells expressing a soluble VEGFR2 to sequester VEGF-A or the infection of chick E1.5 optic vesicles with a virus expressing dominant-negative VEGFR2 decreased the proliferation of retinal cells (Hashimoto et al., 2006). Taken together, it appears likely that VEGF-A can be neurogenic by increasing the proliferation or decreasing the apoptosis of neural progenitors.

In the developing murine neural retina, VEGFR2 is expressed in areas of ganglion cell differentiation and blood vessel growth (Yang and Cepko, 1996), and treatment of the central retina of perinatal mice with the VEGFR2/VEGFR1 inhibitor SU5416 reduces the retinal vasculature and the thickness of the nonvascularised peripheral retina (Robinson et al., 2001). Mice lacking VEGFR1 signalling (*Flt1^{tk-/-}*) have increased VEGF-A levels in the brain, which correlates with enhanced neural progenitor cell proliferation in the SVZ and rostral migratory stream and leads to an enlarged olfactory bulb; this effect is likely due to a direct effect of VEGF-A on VEGFR2-expressing neural progenitors rather than to altered angiogenesis (Wittko et al., 2009). By contrast, hippocampal neurogenesis is not affected in *Flt1^{tk-/-}* mutant mice (Wittko et al., 2009) or by intracerebral infusion of SU5416 after traumatic brain injury in rats (Lee and Agoston, 2010). Ultimately, a detailed analysis of cell-specific VEGF-A receptor knockouts will be required to fully understand whether VEGF-A is directly neurogenic in the developing or adult CNS of mammals.

VEGF-A signalling in neuronal patterning

NRP1 serves as a receptor for the VEGF165 isoform on endothelial cells (Soker et al., 1998) and, together with plexins, acts as a receptor for semaphorins on neurons (e.g. Kolodkin et al., 1997; reviewed by Schwarz and Ruhrberg, 2010). Several studies have tested the idea that VEGF-A also controls neuronal development, both by binding to NRP1 on neurons and through other VEGF-A receptors.

VEGF-A regulates neuronal migration

Two independent studies demonstrated that VEGF-A directly regulates the migration of differentiated neurons, independently of its roles in neurogenesis or blood vessel growth. The first study showed that isoform-specific VEGF-A signalling through NRP1 guides the cell body (soma) of FBM neurons in the developing mouse brainstem (Fig. 3A) (Schwarz et al., 2004). In this system, FBM neurons differentiated normally, as demonstrated by the analysis of transcription factor expression; moreover, the VEGF164/NRP1-mediated guidance occurred independently of blood vessels, as an endothelial-specific *Nrp1* knockout mouse did not have FBM migration defects (Schwarz et al., 2004). Intriguingly, VEGF164-NRP1 signalling was selectively important for FBM cell bodies, but not axons, the patterning of which was instead controlled by SEMA3A signalling through NRP1 and plexin A4 complexes (Schwarz et al., 2004; Schwarz et al., 2008). This observation suggests that NRP1 preferentially acts as either a VEGF-A or SEMA3A receptor according to the cellular compartment that it localises to in these neurons.

A more recent study showed that altering VEGF-A isoform gradients or overall VEGF-A levels also regulates neuronal migration (Ruiz de Almodovar et al., 2010). Specifically, altering the ratio of matrix-binding to diffusible VEGF-A isoforms affects the migration of newborn granule cell neurons in the mouse embryo cerebellum (Fig. 3B). Granule cell migration was also impaired in *Vegfa^{delta/delta}* mouse mutants with low VEGF-A levels, although these mice showed normal generation, survival and differentiation of these neurons and lacked obvious blood vessel defects in the cerebellum (Ruiz de Almodovar et al., 2010). A granule cell-specific mouse knockout of *Vegfr2* further demonstrated that the signal-transducing neural VEGF-A receptor is VEGFR2 (Ruiz de Almodovar et al., 2010).

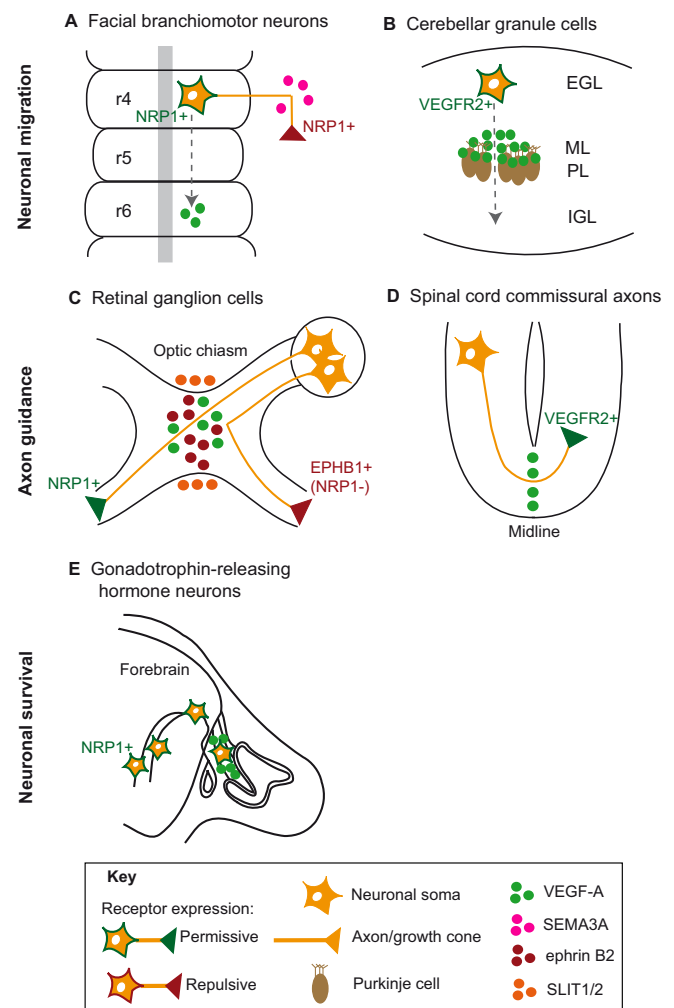


Fig. 3. Roles for VEGF-A in neuronal development. (A) The somata of facial branchiomotor neurons (orange) in rhombomere (r) 4 express NRP1 (dark green outline) and are guided by VEGF-A to r6, where they form the 7th cranial motor nuclei. Their axons also express NRP1 on the growth cone (dark red triangle); they extend from the hindbrain into the second branchial arch, where they are patterned by SEMA3A-mediated repulsion. (B) Newborn cerebellar granule cells (orange) in the external granule cell layer (EGL) express VEGFR2 (dark green outline); they are attracted by VEGF-A gradients emanating from the molecular and Purkinje layers (ML and PL) to guide them to the internal granule cell layer (IGL). (C) Retinal ganglion cells (orange) express NRP1 on their growth cones (dark green triangle) and are guided by VEGF-A across the optic chiasm to innervate the contralateral brain. Retinal ganglion cells that do not express NRP1 but express the ephrin receptor EPHB1 on their growth cones (dark red triangle) are repelled by ephrin B2 at the chiasm and thus project ipsilaterally. The position of the optic chiasm is determined by SLIT1/2 expression. (D) Spinal cord commissural axons (orange) express VEGFR2 on their growth cone (dark green triangle) and are guided by VEGF-A across the midline. (E) A subpopulation of newly born gonadotrophin-releasing hormone neurons (orange) expresses NRP1 (dark green outline) and requires VEGF-A survival signals, which are present along the migratory path, to survive the journey from the vomeronasal organ through the nose into the forebrain.

Together, these studies demonstrated that VEGF-A is a physiological guidance cue for subpopulations of neurons that acts independently of its vascular effects or role in neuronal progenitor proliferation to promote neuronal migration. However, the precise

molecular mechanism involved differs between neurons. Further studies will be necessary to determine whether VEGF-A-mediated neuronal guidance is of widespread significance for the CNS or affects only a few subsets of neurons.

VEGF-A in axon guidance

Although it has been reported that VEGF-A promotes neurite outgrowth from perinatal dorsal root ganglion explants containing VEGFR2-positive sensory neurons (Sondell et al., 1999a), a subsequent study showed that VEGFR2 expression in these ganglia is restricted to endothelial cells, and that VEGF-A sequestration with soluble VEGFR2 or VEGFR2 inhibition with a small molecular inhibitor only impaired endothelial, and not axon, growth (Kutcher et al., 2004). Consistent with the latter study, cranial sensory and limb axons grow normally in the absence of the NRP1-binding isoform VEGF164 (Schwarz et al., 2004; Vieira et al., 2007). Taken together, the *in vivo* data suggest that VEGF-A signalling through NRP1 is not important for axon guidance in the peripheral nervous system (PNS), but studies in mice lacking neuronal VEGFR2 are required to unequivocally exclude a role for VEGF-A signalling through this receptor in the PNS.

Several studies, however, agree that VEGF-A plays a role in the autonomous nervous system. Thus, exogenous VEGF-A increases neurite outgrowth from sympathetic ganglion explants (Long et al., 2009) and promotes sympathetic axon growth for re-innervation of surgically denervated femoral arteries in adult rats (Marko and Damon, 2008). VEGF-A also promotes the sympathetic regulation of resistance arteries, as their constriction is reduced in response to cold stress in *Vegfa^{delta/delta}* mice with low levels of endogenous VEGF-A (Storkebaum et al., 2010). However, this defect is not due to abnormal sympathetic axon guidance but rather to defective neuromuscular effector junctions (Storkebaum et al., 2010). It is not yet known whether defective sympathetic regulation of blood flow in the CNS is also the underlying cause of the reduced brain and spinal cord perfusion in *Vegfa^{delta/delta}* mice.

The first physiological evidence that VEGF-A directly guides CNS axons *in vivo* was provided by two recent studies on commissural axon (see Glossary, Box 1) guidance in the mouse (Erskine et al., 2011; Ruiz de Almodovar et al., 2011). Thus, VEGF-A helps RGC axons destined for the opposite brain hemisphere to migrate through the optic chiasm, which is the diencephalic midline structure where the optic nerves from both eyes partially cross (Fig. 3C) (Erskine et al., 2011). VEGF-A also promotes midline crossing of axons in the spinal cord (Fig. 3D) (Ruiz de Almodovar et al., 2011). Both studies combined genetic and explant experiments to demonstrate that VEGF-A promotes axon guidance independently of its vascular roles. Even though VEGF-A acts through VEGFR2 on spinal commissural axons (Ruiz de Almodovar et al., 2011), it requires NRP1 to guide contralateral RGC axons (Erskine et al., 2011). The observation that embryonic RGC axons use NRP1 was surprising, as *in vitro* studies suggested that VEGFR2 is important for the regeneration of postnatal RGC neurons after axotomy (Bocker-Meffert et al., 2002). The neuronal VEGF-A receptor used to mediate VEGF-A signals might therefore vary according to neuron type and developmental stage.

VEGF-A as a survival factor for developing and adult neurons

VEGF-A is a potent survival factor for many neuronal populations *in vitro*. For example, VEGF-A promotes neuronal survival in explanted CNS (Rosenstein et al., 2003; Silverman et al., 1999) and

PNS (Sondell et al., 1999a; Sondell et al., 2000) tissue. VEGF-A also protects stressed neurons in culture, for example after serum withdrawal (Jin et al., 2000a), hypoxia (e.g. Jin et al., 2000b; Svensson et al., 2002), mechanical trauma (Ma et al., 2011), chemical toxicity (Cui et al., 2011), or excitotoxicity caused by glutamate or the glutamate mimetic AMPA (Bogaert et al., 2010; Matsuzaki et al., 2001; Svensson et al., 2002). As these culture models did not contain blood vessels, it was concluded that the neuronal survival effects of VEGF-A were vessel independent.

Consistent with a role for VEGF-A in neuroprotection, *Vegfa^{delta/delta}* mice with reduced levels of VEGF-A suffer late-onset motoneuron degeneration (Oosthuysen et al., 2001). Additionally, VEGF-A treatment protects against AMPA-induced or ischemia-induced motoneuron degeneration *in vivo* (Lambrechts et al., 2003; Tovar-y-Romo et al., 2007). VEGF-A also protects against the effect of the G93A-SOD1 mutation that causes motoneuron degeneration in a mouse model of ALS (Azzouz et al., 2004). However, none of these *in vivo* studies directly distinguished whether the protective effects of VEGF-A are due to direct effects of VEGF-A on motoneurons, as observed *in vitro*, or whether neuroprotection was due to improved vascular performance. By contrast, VEGF-A does appear to play a direct role in neuroprotection of newborn dentate gyrus neurons, as VEGF-A infusion into the hypoxic adult rat brain enhances the survival of these neurons without inducing angiogenesis in this region (Sun et al., 2003).

The first unequivocal evidence that VEGF-A conveys physiological survival signals in neurons expressing VEGF-A receptors was provided by the finding that mice lacking VEGF164 or NRP1 in the neural lineage contain reduced numbers of GnRH neurons, which are essential for gonad development and fertility (Fig. 3E) (Cariboni et al., 2011). Treatment of immortalised GnRH neurons showed that VEGF164 enhanced survival, but not proliferation, and that the survival pathways involved co-activating phosphoinositide 3-kinase and mitogen-activated protein kinase signalling in an NRP1-dependent fashion (Cariboni et al., 2011). In agreement with the *in vitro* findings, apoptotic bodies were abundant along the VEGF-A-positive path that is taken by these neurons during embryogenesis as they travel from their birthplace in the nasal cavity to their destination in the hypothalamus (Cariboni et al., 2011). Importantly, this study also showed that mice lacking VEGFR2 in the neural lineage or NRP1 in blood vessels contained normal numbers of these neurons, establishing that VEGF164 signals through NRP1 in a cell-autonomous fashion, independently of vascular VEGF-A signalling or blood vessels.

New roles for VEGF-A in dendritogenesis and neuronal function

The observation that VEGF-A promotes neurite extension and upregulates expression of the dendrite-enriched microtubule-associated protein MAP2 (MTAP2) *in vitro* (Rosenstein et al., 2003) raised the possibility that VEGF-A affects dendrite and synapse development. Several studies have since begun to investigate the role of VEGF-A signalling in dendritogenesis and synaptic function. *In vitro*, administration of VEGF-A modulates calcium channels in hippocampal neurons (Kim et al., 2008; Ma et al., 2011) and upregulates the AMPA receptor subunit GluR2 (GRIA2) (Bogaert et al., 2010). VEGF-A also reduces stimulus-evoked depolarisation of hypoglossal motoneurons in organotypic slice culture (McCloskey et al., 2008). Interestingly, upregulation of VEGF-A through hypoxia-induced factor HIF1A enhances synaptic transmission in hippocampal neurons *in vitro* (Huang et al., 2010), suggesting that hypoxia regulates synaptic transmission

via VEGF-A. Consistent with this idea, VEGF-A administration increases phrenic nerve output in adult rats to regulate spinal respiratory functions (Dale-Nagle et al., 2011). In vivo, blocking VEGF-A activity by antibody administration also increases pain thresholds after chronic nerve constriction injury in rats (Lin et al., 2010).

The first in vivo evidence that VEGF-A promotes dendrite patterning came from a recent study in mice which showed that the forced expression of soluble VEGFR1 impairs dendrite development of newly born neurons migrating from the SVZ to the olfactory bulb, independently of vascular changes (Licht et al., 2010). A similar approach was subsequently used to show that VEGF-A increases synaptic strength independently of neurogenesis and angiogenesis to promote hippocampus-dependent memory formation (Licht et al., 2011). Together, these studies point to a crucial role for VEGF-A in dendrite development and synaptic plasticity, but further work will be required to elucidate the VEGF-A receptors and downstream signalling pathways involved.

VEGF-A in neurovascular co-patterning

Studies in genetically modified mouse embryos revealed that nerve-secreted VEGF-A acts on vascular NRP1 to promote arterial differentiation adjacent to sensory nerves in the limb skin (Mukouyama et al., 2005; Mukouyama et al., 2002). However, the factors that control the nerve-vessel co-alignment prior to arterial differentiation have remained elusive. Unpublished evidence presented in a recent review on neurovascular cross-talk suggests that the Schwann cell-derived chemokine CXCL12 activates its receptor CXCR4 on a subset of blood vessels to promote nerve-vessel co-alignment (James and Mukouyama, 2011). Whether VEGF-A also controls the co-patterning of arteries and nerves in the CNS is not known.

Conclusions

The studies described in this review demonstrate that VEGF-A plays multiple roles in the developing and adult nervous system by acting both on blood vessels and neurons. Interestingly, VEGF-like orthologues function in the nervous system of invertebrates, which do not have a vascular system other than a heart tube, e.g. *Caenorhabditis elegans* (Popovici et al., 2002; Procko et al., 2011). It is therefore possible that the VEGF family was used to shape the nervous system before it was adapted to induce and regulate the vasculature. If this hypothesis is correct, it seems likely that further roles for VEGF-A at different stages in the life of different neuronal subtypes remain to be discovered. Importantly, the dual effect of VEGF-A on neurons and blood vessels could be both beneficial and detrimental in a therapeutic context. Using anti-VEGF-A therapy to treat unwanted angiogenesis and vascular leakage in cancer and eye diseases might inadvertently inhibit adult neurogenesis and/or neuroprotection. On the other hand, VEGF-A could be used to treat neurodegenerative and neuropathic conditions by simultaneously enhancing blood vessel growth, neurogenesis and neuroprotection, provided that we learn to control its potential negative effects. Most importantly, VEGF-A can increase vascular permeability and thereby disrupt the blood-brain barrier and promote inflammatory cell infiltration (Kalaria et al., 1998; Krum and Khaibullina, 2003; Krum et al., 2002; Proescholdt et al., 1999; Zhang et al., 2000). Defining the role of VEGF-A and its receptors in neurodevelopment and in adult neural homeostasis and pathology is therefore a priority for the future. Specifically, delineating the components of downstream signalling pathways activated by VEGF-A binding to its receptors in neurons versus endothelial cells, and in angiogenesis versus vascular

permeability, will help to pioneer novel therapeutic strategies that selectively affect subsets of VEGF-A responses to promote effective and safe nervous system repair.

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Competing interests statement

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