Interactions between VEGFR and Notch signaling pathways in endothelial and neural cells

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Abstract

Notch cell interaction mechanism governs cell fate decisions in many different cell contexts throughout the lifetime of all Metazoan species. It links the fate of one cell to that of its neighbors through cell-to-cell contacts, and binding of Notch receptors expressed on one cell to their membrane bound ligands on an adjacent cell. Environmental cues, such as growth factors and extracellular matrix molecules, superimpose a dynamic regulation on this canonical Notch signaling pathway. In this review, we will focus on Notch signaling in the vertebrate vascular and nervous systems and examine its role in angiogenesis, neurogenesis, and neurovascular interactions. We will also highlight the molecular relationships of the Notch pathway with vascular endothelial growth factors (VEGFs) and their high-affinity tyrosine kinase VEGF receptors, key regulators of both angiogenesis and neurogenesis.

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Introduction

The vertebrate central nervous system (CNS) develops upon its colonization and irrigation by the vascular system. During embryonic development, the vessels provide trophic support and growth factors to proliferating and differentiating neural cells. In turn, neural cells provide cues to endothelial cells (ECs) for infiltration and expansion into the neural tissue [1, 2]. The close structural and functional relationship between the nervous and the vascular system persists in the adult brain. The importance of these interactions is apparent in maintenance of the remaining adult stem cell niches and in the dependence of injury responses triggered by trauma on cerebrovascular network properties [3].

The mutual interaction between neural and ECs is based on signaling molecules common to both cell types [4]. Notch is a major signaling pathway that regulates the development of many cell types, including endothelial and neural cells. It is one of the most well-conserved signaling pathways in multicellular organisms [5]. Notch has been shown to influence multiple cellular processes including: cell fate decisions, proliferation, apoptosis, migration, and plasticity. The canonical Notch pathway is initiated when Notch receptors expressed on one cell bind with their membrane-bound ligands on an adjacent cell, linking the fate of one cell to that of its neighbor [6–8]. In mammals, there are four Notch receptors (Notch1-4), which are single-pass transmembrane heterodimers, and five Notch ligands belonging to the Delta-Serrate-Lag (DSL) family, including Jagged1 and 2 (Jag1, Jag2) and Delta-like 1, 3, and 4 (Dll1, Dll3, Dll4) [9].

During angiogenesis, cell fate decisions among a group of ECs occur at the level of angiogenic sprouts, where ‘tip’ or leader cells segregate from the ‘stalks’ or trailing ECs forming the core of the vessel [10]. Tip cells localize at the very front of angiogenic sprouts and are characterized by extensive filopodia protrusions that sense guidance cues in the environment. Tip cells are also molecularly distinct from stalk cells, most notably by their high expression of Notch ligand Dll4 as well as VEGFR-2 [11, 12]. The role of Notch signaling in angiogenic sprouting has been demonstrated through genetic inactivation and/or pharmacological blocking of either Notch1 signaling or Dll4, which promotes tip cell formation and EC proliferation. Blocking the Notch pathway results in mispatterned angiogenic growth and the formation of a hyperplasic, immature, and nonfunctional vascular network [13–18]. Notch signaling is thus required for sprouting angiogenesis. Complex interactions between Notch and the VEGFRs, especially VEGFR-2 and VEGFR-3, have been shown to modulate Notch signaling and its effects on angiogenic activity [19–22].

Notch signaling also influences a wide array of cellular processes in the mammalian CNS and is essential during both embryonic and adult neurogenesis [6, 7]. Notch signaling mechanisms in neural cells are, however, far less understood than in ECs, most notably due to the difficulty in identifying ligand- and receptor-bearing cells within such a complexly structured tissue composed of such a wide diversity of cells. At early stages of CNS development, i.e., E11.5–E13.5 in the mouse, the embryonic neuroepithelium contains radial glia, neuronal progenitor cells, young neurons, as well as sprouting ECs, all of which establish Notch-mediated interactions. The neurogenic niches of the adult brain, the subventricular zone (SVZ) lining the lateral ventricles, and the subgranular zone (SGZ) of dentate gyrus (DG), are structured similarly, and are composed of the same types of cells, which also establish a highly complex network of Notch-mediated interactions. The first aim...
of this review will be to discuss the relationship between Notch and the VEGFRs during angiogenesis. We will then review the role and mechanisms of Notch signaling during neurogenesis, and explore the possibility of Notch–VEGFR signaling pathway interactions during regulation of neural stem cell.

The Notch signaling pathway

The canonical Notch pathway involves the cell-surface Notch molecule, which successively plays the role of a receptor and a transcription factor [23, 24]. Ligand binding initiates a series of proteolytic cleavages of the Notch receptor, which culminates in cleavage of the transmembrane domain by gamma-secretase and the subsequent release of the Notch intracellular domain (Notch ICD or NICD) into the cytosol. NICD translocates to the nucleus where it interacts with and converts the DNA-binding protein CBF1 (C promotor-binding factor, also known as RBPIk in mammals) complex into an activator complex. The NICD/CBF1 activator complex, which includes the coactivator Mastermind (MAML in mammals, [25]) among others [26], initiates the transcription of Notch target genes. Ubiquitous Notch target genes include the hairy and enhancer of split (HES) family genes and HES-related genes Hesr1 and Hesr2 (also known as Hey/Herp genes), which encode basic helix-loop-helix (bHLH) transcription factors that promote progenitor cell survival and suppress differentiation [27, 28].

In most biological situations, including in disease [29], the outcome of Notch signals depends on quantitative parameters [8]. The level of Notch target gene activation is intimately dependent on the ‘strength’ of the signal and Notch expressing cells display a dynamic response to temporal variations of Notch ligand expression on neighboring cells. Recent genetic and genomic approaches, moreover, showed that Notch signals can be attenuated by a large number of genes and that the above canonical pathway is integrated in a complex genetic circuitry with consequences on Notch signaling output [30–34]. Notch target genes can be regulated by other non-canonical Notch signaling pathways, which are independent of NICD, CSL, or even Notch receptor itself [9, 35], specifically, the VEGF-A/VEGFR-2 axis and its Notch independent-activation of Notch target genes in endothelial and neural cells, which we will discuss later. Consequently, despite the apparent simplicity of its canonical pathway, the Notch pathway is complexed with other pathways able to regulate and activate it. Therefore, a readout of Notch pathway target gene expression must be carefully interpreted and other steps in the pathway examined in order to properly identify Notch-dependent mechanisms.

VEGFs and VEGFRs

Vascular endothelial growth factor (VEGF or VEGF-A) strongly promotes angiogenesis and is required for vascular development [36, 37]. It binds the tyrosine kinase receptors VEGFR-1 (Flt1) and VEGFR-2 (Flk1), the latter being the primary receptor transmitting VEGF signals in ECs [38, 39].

VEGFR-1 binds VEGF-A with higher affinity than does VEGFR-2, but VEGFR-1 tyrosine kinase activity is only weakly activated by its ligands [40, 41], which makes that VEGFR-1, as well as its soluble form sVEGFR-1, acts as a VEGF decoy in ECs, regulating the spatial activation of VEGFR-2 and the formation of vascular sprouts [42]. VEGFR-2 is known to transduce the full range of VEGF-A responses in ECs, i.e., regulating EC survival, proliferation, migration, and formation of the vascular tube [41, 43].

VEGFR-3 is the third member of the VEGFR family and is expressed in the vascular system with a restriction to lymphatic ECs from stage E16.5 [44]. This receptor is activated by VEGF-C and VEGF-D. VEGF-C can also bind VEGFR-2 after proper proteolytic cleavage,
leading to the formation and activation of VEGFR-2/VEGFR-3 heterodimers [41, 45]. However, its highest binding affinity is for VEGFR-3 [46]. VEGFR-3 also regulates angiogenesis and Vegfr3 deletion causes severe defects in arterial-venous remodeling of the primary vascular plexus in mice, with a lethality at stage E10.5 [47], and defective segmental artery morphogenesis in zebrafish [48]. VEGF-C/VEGFR-3 is most well known for its role in development of the lymphatic vascular network. VEGF-C acts as an attractive cue for lymphatic progenitor cells. Bi-allelic deletion of Vegfc in the mouse leads to a complete failure of lymphatic vessel formation and embryonic lethality at stage E16.5. Mice heterozygous for Vegfc can survive as adults, with lymphatic vessel hypoplasia and lymphedema, but no marked defects of the blood vasculature [49].

Interestingly, double Vegfc−/−/Vegfd−/− mutants survive until E16.5 and do not recapitulate the phenotype of Vegfr3−/− mutants, suggesting VEGF-C/VEGF-D-independent activation of VEGFR-3. In support of this hypothesis, it was found that VEGFR-3 on cultured human dermal blood vascular ECs can be phosphorylated via stimulation by collagen-I in the absence of its ligands VEGF-C/VEGF-D, and even in the presence of blocking antibodies against VEGFR-3 [45]. This observation is in agreement with previous reports showing that a cooperative interaction between the β1-integrin and VEGFR-3 is required for EC endothelial cell migration [50] and that extracellular matrix induces integrin-mediated VEGFR-3 phosphorylation and downstream activation of CRKI/II—SHC (Src homology and collagen homology protein)—JNK cascade [51]. Therefore, VEGFR-3 mediates both ligand-dependent effects through an ‘active’ pathway and extra-cellular matrix-dependent effects though a ‘passive’ pathway which proved to be pro- and anti-angiogenic, respectively, as we will discuss later [19, 21, 52].

Notch and VEGFRs interactions during angiogenesis

Notch signaling is essential for vascular development. Notch-1 and Notch-4 receptors as well as JAG-1, Dll-1, and Dll-4 ligands are expressed in ECs where they play a key role in the induction of arterial cell fate and in sprouting angiogenesis [53]. The necessity of Dll-4/Notch-1 signaling in ECs was made apparent when deletion of Notch-1, or a single copy of Dll-4 resulted in severe vascular defects and embryonic lethality [54, 55]. In vascular sprouts, the tip cells express high levels of Notch ligand Dll4 and are Notch-1-deficient, while the trailing ECs, or stalk cells, as a result of their contact with the Dll4-expressing tip cells [14–18, 56]. Heterozygous deletion of Notch ligand Dll4 increases the number of filopodia and sprouting tips [17]. Similarly, blockade of Notch increases the number of filopodia and sprouting tips, with an associated expansion of VEGFR-3 (Flt4) expression [16, 57]. Tip cells with low Notch activity show high VEGFR-2 and low VEGFR-1 expression, indicative of an increase in VEGF-A signaling activity, as well as higher levels of Dll4 expression in tip cells [13, 15–17, 22, 58, 59]. In contrast, stalk cells have high levels of Notch signaling due to activation by the adjacent tip cells. Stalk cells, however, strongly express another Notch ligand, Jagged-1, which has a lower binding affinity for Notch compared to Dll4, but nevertheless can antagonize Dll4 activity by competing for Notch receptors. Modification of Notch by FRINGE glycosyltransferase favors activation by Dll4, but and reduces Notch activation upon Jagged-1 [60]. Consequently, the forward induction of Notch signaling is reduced in the vascular front which maintains EC responsiveness to VEGF stimulation, sustaining tip cell sprouting activity and also allowing new tip cells to form along the front to form branching vessels [61]. Vessel branching within the developing vascular network is also the consequence of another down-stream Notch target, Notch-regulated ankyrin-repeat protein (Narp), which counteracts Notch signaling and is expressed in stalk cells at the branch points [11].
Silencing of Nrarp results in a reduction in vessel density, due to lowered endothelial cell proliferation, a consequence of an up-regulation of Notch and VEGFR-1. The resulting vessels are also poorly lumened and vessel regression can be seen in the network, a consequence of increased Wnt signaling, which Nrarp also balances in the stalk cells [11]. In this context, a balance is created by anti-angiogenic Dll4 and proangiogenic Jagged-1 to regulate Notch activation, the level of which determines its interactions with VEGF/VEGFR and other signaling pathways. All of this pathway cross-talk is essential for proper development and patterning of the vasculature as well as its stabilization [62].

A group of studies determined that VEGFR-3 also interacts with Notch to regulate sprouting angiogenesis. High levels of VEGFR-3 expression were detected in angiogenic sprouts and when VEGF-C/VEGFR-3 interactions were inhibited with function blocking anti-VEGFR-3 antibodies the authors saw decreased sprouting, vascular density, vessel branching, and endothelial cell proliferation in different mouse angiogenic models, both in vivo and in vitro. Conversely, they showed that VEGF-C-mediated stimulation of VEGFR-3 promoted angiogenesis, even in the presence of antibodies against VEGFR-2, and that a combination of blocking antibodies against VEGFR-2 and VEGFR-3 resulted in additive inhibition of angiogenesis and tumor growth [22]. Further investigations focused on VEGF-C-mediated VEGFR-3 revealed that Vegfc homozygous mutants displayed reduced vascular branching and that macrophages served as a source of VEGF-C ligand for the VEGFR-3+ tip cells localized at branching points [21]. In conjunction, the authors showed that the cell-type-specific deletion of VEGFR-3 in ECs led to excessive angiogenic sprouting and branching, which was associated with a decreased level of Notch. FoxC2+/−; VEGFR-3−/− compound heterozygote mice were also examined and found to recapitulate homzygous deletion of VEGFR-3, implicating that VEGF-C binding to VEGFR-3 activates PI(3) K and its downstream target FoxC2, a known regulator of Notch ligand, Dll4, and target gene expression [21, 63]. This phenotype, contrasted with the suppressed angiogenesis induced by anti-VEGFR-3 blocking antibodies, revealing that the VEGFR-3 intracellular domain is endowed with signaling properties, independently of VEGF-C-activation of VEGFR-3 extracellular domain. The action of this domain moreover antagonizes the effect of VEGF-C stimulation. Using cultured human blood vascular ECs, the authors showed that VEGFR-3 could be phosphorylated, in absence of its ligands, through contact with extracellular matrix collagen I, and that VEGFR-3 activation was then Src-dependent. Together, these findings demonstrate the dual action of VEGFR-3 in angiogenesis, i.e., on the one hand, passive, ubiquitous and anti-angiogenic through interactions with the extracellular matrix and, on the other hand, active, local and pro-angiogenic following VEGFR-3 stimulation by its ligands VEGF-C and VEGF-D.

Subsequent studies focused on Notch-VEGFR-2 interactions during sprouting angiogenesis and showed that Dll4 can be highly expressed by ECs in the absence of VEGF-A/VEGFR-2 signaling, provided that ECs are without Notch signaling [19]. Although surprising with regards to the convergent observations reported above that VEGF-A induces the Notch ligand Dll4 in tip cells which leads to suppression of excess sprouts in adjacent ECs, this result indicates that Notch signaling regulates and determines the level of response of ECs to VEGF-A, as well as the prevalent expression of VEGFR-2 versus VEGFR-3 in ECs. The authors carried out cell-type-specific deletions of Notch and RBPJk in ECs (Notch1; RBPJkiΔEC), which strongly upregulated VEGFR-3 protein, without altering VEGFR-2 expression, in ECs. This observation confirmed the potent inhibitory control of Notch signaling on VEGFR-3 expression, previously reported by Tammela et al. [22]. The Notch1 RBPJkiΔEC mice showed a misoriented vascular growth and excessive sprouting which were not rescued by blocking antibodies against VEGFR-3, but instead by MAZ51, an inhibitor of VEGF-3 tyrosine kinase activity. These results confirm that VEGFR-3 receptor acts independently of VEGF-C in ECs, as reported by Tammela et al. [21] and

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suggests that ‘passive’ VEGFR-3 signaling can also promote angiogenic sprouting, provided that the ECs have little to no Notch activity. This led Benedito and Hellstrom [52] to propose that, while VEGFR-3 is normally an antagonist of sprouting, in this situation of minimal Notch activity, it may become an agonist. These elegant and complementary studies, which are summarized in Fig. 1a, demonstrate the highly dynamic balance of Notch and VEGFR-2/VEGFR-3 expression in ECs, as well as the highly versatile role of VEGFR-3 in angiogenesis.

Angiogenesis is not a continuous process and recent reports showed that in the developing zebrafish circadian oscillations control developmental angiogenesis. It was found that the circadian regulator Bmal1 directly targets the promoter region of the Vegf gene leading to an elevated expression of VEGF-A [64, 65]. These data confirm previous reports in mice of the circadian expression of VEGF-A in murine tumor cells [66]. Interestingly, Notch signaling is also regulated in an oscillatory fashion and the gene Hes7, which is regulated by the Notch and Fgf/Mapk pathways is a crucial component of the segmentation clock [67, 68]. The Notch-VEGF-A interactions in the oscillatory regulation of angiogenesis have, however, not yet been explored.

Notch signaling in neurogenesis

The embryonic phenotype associated with the haploinsufficiency of the Notch locus was one of the first genetic mutation characterized in the fly (Drosophila melanogaster) [69]. It was described as a ‘neurogenic phenotype’ resulting from the failure of the neurogenic ectoderm to segregate neural and epidermal cell lineages. All cells become neuroblasts, which causes hypertrophy of neural tissue at the expense of epidermal structures. In the mouse, targeted mutation of the Notch pathway genes Notch1 and RBPJk also implicated a role for these genes in neurogenesis implicating that the Notch pathway and its regulatory mechanisms were conserved from fly to mouse [70]. Notch1 and RBPJk mutations led to decreased expression of the Notch target gene Hes-5 and upregulation of proneural gene Mash-1 and Notch ligand Dll1, resulting in enhanced neurogenesis. Interestingly, no change in Hes-1 expression were detected in these mutants, suggesting that Hes-1 expression during early embryonic neurogenesis is not, or not mainly, controlled by the canonical Notch pathway. The RBPJk mutation showed stronger effects on proneural gene expression than the Notch1 mutation, consistent with functional redundancy of Notch genes in neurogenesis. These findings supported a role for Notch signaling in the regulation of neural stem cell differentiation, but the early lethality of Notch1 mutants (E11.5) did not allow assessment of the long term consequences of deletion on the neural stem cell pool. To circumvent this difficulty, several studies have used Cre/loxP-mediated recombination to remove Notch1 in subsets of neural cells [71–73]. Interestingly, the telencephalic deletion of Notch led to a reduced neuron number in vivo later in development, confirming expectations of the effects of progressive depletion of neural progenitor pool [72]. Neurosphere assays from single embryonic neural stem cells derived from mice deficient for Notch1, RBPJk or Presenilin1, another key regulator in Notch signaling, were used to support in vivo results in RBPJk−/− or Notch1−/− embryonic mice and in the adult PS1+/− mutants all of which revealed a neural stem cell depletion [74]. Interestingly, these authors also found that RBPJk and Notch signaling were, however, dispensable for generation of neural stem cells from embryonic stem cells.

In the adult brain, Notch signaling pathway molecules are expressed in both the SVZ and SGZ [75–79]. Converging pharmacological and genetic evidence indicate that the canonical Notch pathway regulates maintenance of adult NSCs through promotion of their self-renewal and inhibition of their exit from the cell cycle, as well as regulating generation of neuronal and glial precursor cells within the regions. The SGZ of adult inducible Notch-
deficient mice displays an increased cell cycle exit of early precursor types [75], an impaired expansion of the progenitor pool [80] and a depleted stem cell population [77]. Conditional inactivation of RBPJk resulted in an initial increase in DG neurogenesis, by inducing premature neuronal differentiation of the pool of Sox2-positive neural stem cells depleting them, eventually leading to repression of adult hippocampal neurogenesis [81]. Conditional deletion of RBPJk in the Nestin-expressing stem cells had a similar effect in the adult SVZ. The type B stem cells within the region differentiated into transit-amplifying cells and neurons, depleting the neural stem cell pool resulting in premature cessation of neurogenesis [77]. Treatment of neural stem cells in vitro with the Notch inhibitor DAPT inhibited the cell cycle and extended the G1-S transition, allowing the cell to exit the cell cycle when levels of Notch signaling remain low [82]. Conditional genetics combined with mosaic analysis of Notch mutants showed that, in the adult SVZ, active neural stem cells display a selective reliance on Notch signaling, while Notch1 function is compensated in quiescent neural stem cells [83].

The group of Kageyama [84, 85] made an interesting observation that the level of Notch signaling in neural stem cells regulates the balance between quiescence and proliferation, the high Notch activation inducing growth arrest while low Notch signaling causes cell proliferation. This balance results from a constitutive oscillation of expression of the downstream Notch effector Hes1, with a period of 2–3 h by negative feedback. Consequently, Hes1 oscillations induce the oscillatory expression of the proneural gene Neurogenin2 (Ngn2) and the Notch ligand gene Delta-like1 (Dll1). Dll1 oscillation maintains a group of cells in the undifferentiated state while the different dynamics of Hes1 and Ngn2 lead to different outcomes. Hes1 allows cell proliferation and differentiation when its expression oscillates but induces dormancy when its expression is sustained. Ngn2 leads to the maintenance of neural stem/progenitor cells by inducing Dll1 oscillation when its expression oscillates but to neuronal differentiation when its expression is sustained. It is worth noting that, in contrast with neural stem cells, neuroblasts are less dependent on Notch and more responsive to environmental cues for regulation of their proliferation [80], which confirms the cell-type dependent effect of Notch signaling in neurogenesis. In addition to HES genes, EphB2 has been reported as a downstream mediator of Notch signaling which prevents differentiation of ependymal cells into niche astrocytes in the adult SVZ [86].

Regarding the upstream regulators of Notch signaling, several growth factors have been implicated. Interaction between EGF receptor signaling and Notch is required for maintenance of neural stem and progenitor cells in the adult SVZ. EGF receptor signaling in transit-amplifying type C cells non-cell-autonomously suppresses Notch signaling in type B cells and consequently inhibits their proliferation and self-renewal. This action results from Notch 1 ubiquitination and degradation via induction of Numb [87]. FGF signaling is also regulating Notch-dependent neurogenesis, with consequences on the expansion of embryonic cortical surface area, as shown by changes in the expression of Notch pathway genes in FgrR mutant embryos [88]. Several evidence have also been provided that Wnt signaling regulated embryonic neurogenesis upstream of Notch, in the hindbrain, cortex and retina [89–91]. SFRPs, which are Wnt signaling antagonists, also play an important role for Notch activation in the retina where Notch signaling was transiently upregulated in Sfrp1−/−; Sfrp2−/− embryos, because SFRPs bind ADAM10 metalloprotease and downregulate Notch activity [92]. Sonic Hedgehog (SHH) morphogen is another upstream regulator of Notch signaling as shown for embryonic retinal progenitors ([93]; see next paragraph) and cortical progenitors. Conditional inactivation of Patched1, a negative regulator of the SHH pathway, in Nestin-positive neural progenitors of the neocortex led to an increase in the number of symmetric proliferative divisions of radial glial with a concomitant upregulation of Hes1 and Blbp, downstream targets of Notch signaling [94]. Finally, a role for the Slit1/2-Robo1/2 axis, which is known as a regulator of axonal and EC guidance, has also been
shown in embryonic cortical progenitor cells where it activates transcription of the Notch effector Hes1, independently of Notch. Proliferation of radial glial stem cells is inhibited while production of intermediate progenitors is enhanced in Robo1/2 and Slit1/2 mutants, suggesting that Slit/Robo signaling modulates the transition between primary and intermediate progenitors [95]. Together, these regulators may modulate ‘strength’ of Notch signaling directly through interactions with the pathway components or indirectly by targeting the same genes Notch does.

One key question that still remains is which cell types act as the main Notch ligand-presenting cell for these NSCs in the developing neuroepithelium and in the neurogenic niches of the adult brain. During development, ECs are in intimate contact with radial glia which have been shown to regulate vessel stabilization via modulation of canonical Wnt signaling in the late embryonic cortex [96]. In the adult subventricular zone, neural stem cells are also closely apposed to local blood vessels, and able to directly contact ECs due to decreased pericyte coverage [97, 98]. Since the two Notch ligands Dll4 and Jag1 are highly expressed by ECs [99], this opens possibility that ECs may also be Notch-ligand-presenting cells for embryonic and adult neural stem cells (Fig. 1b).

**VEGFs and VEGFRs in neurogenesis**

An increasing body of evidence shows that VEGF-A and its tyrosine kinase receptors also regulate development of the nervous system. In small invertebrates such as nematodes, which lack a vascular system and distribute oxygen by diffusion, orthologues of tyrosine kinase VEGF receptors are expressed by the nervous system and regulate neuronal and glial cell development [100, 101]. In vertebrates, all of the VEGFs and VEGF receptors are also expressed in the CNS throughout development [102]. Their instructive role for neural cell development and their implication in brain diseases and repair have been discussed elsewhere [103–106].

VEGF transcript levels are abundant in the ventricular neuroepithelium of embryonic brain when neural stem/progenitors cells and ECs proliferate rapidly [107]. This temporal and spatial expression of VEGF suggests that VEGF is synthesized and released by the ventricular neuroectoderm and that it may simultaneously induce the ingrowth of capillaries from the perineural vascular plexus and regulate neurogenic activity [108]. This is supported by findings that VEGF can act as a neurotrophic factor and as guidance cue for neural progenitors [109, 110]. Recently, in vitro studies using co-cultures of EC and neonatal rat brain slices suggested that VEGF-A is the main neurogenic factor delivered by ECs since RNA interference against VEGF-A strongly inhibited the proliferation of Nestin+ neural progenitors in the presence of ECs [111]. Interestingly, VEGF-A depletion also altered expression of *Pten, Akt1*, and *PI3P* genes encoding key regulators of VEGFR signaling pathway [41].

Although VEGF-A proved to be a strongly neurogenic factor, its direct action has only been demonstrated for subsets of neural progenitor cells, as indicated above. It has not been established if this local effect was liked to a region-specific pattern of expression and if other members of VEGF family had eventually a complementary expression pattern and may substitute to VEGF-A for regulating neurogenesis. VEGFR-2 expression is also restricted to subpopulations of neural cells, mostly neurons [112, 113], suggesting that VEGF-A may not preferentially target neural stem cells but rather neuronal progenitors or neuroblasts. We have recently provided evidence that the lymphangiogenic ligand VEGF-C and its high-affinity receptor VEGFR-3 also regulated neurogenesis by acting on neural stem cells and niche astrocytes, thus with a distinct cell-type specificity than the VEGF-A/VEGFR-2 axis.
VEGF-C, VEGF-D, and VEGFR-3 are expressed at low level by blood ECs endothelial cells and by other cell types, including neural cells [114]. In contrast to VEGF-A and VEGFR-2, which show a restricted spatio-temporal expression pattern in the CNS [4, 115, 116], VEGFR-3- and VEGF-C-expressing cells have a broad and complex distribution in the developing and adult brain. As illustrated in Fig. 2, VEGF-A and VEGF-C show different patterns of expression in the developing brain. VEGF-A is highly expressed in ECs while VEGF-C expression is rather restricted to radial glia, in specific domains of the ventricular zone. Using Vegfc<sup>lacZ/+</sup> and Vegfr3<sup>lacZ/+</sup> mice to detect β-galactosidase reporter expression of Vegfc and Vegfr3 respectively, as well as antibodies to stain VEGF-C and VEGFR-3, we have described overlapping or adjacent expressing patterns of VEGF-C and VEGFR-3 in different territories of the embryonic forebrain, midbrain and hindbrain, suggesting functional ligand/receptor interactions between VEGF-C and VEGFR-3 [117]. VEGF-C/VEGFR-3 are especially coexpressed in proliferative neuroepithelial domains of the ventral forebrain, such as the preoptic region and the olfactory bulb anlage, where proliferation of neural progenitors and migration of oligodendrocyte precursors was altered in both Vegfc<sup>lacZ/+</sup> and Vegfc<sup>lacZ/lacZ</sup>-deficient embryos. Additional loss-of function mutations in *Xenopus* confirmed the specific requirement of VEGF-C for the proliferation of neural progenitor cells expressing VEGFR-3. Therefore, VEGF-C provides a trophic support to neural progenitor cells during vertebrate brain development.

**VEGF-C and Vegfr3 expression in the embryonic and postnatal brain was characterized in detail in rats, using a combination of in situ hybridization, RT-PCR and immunolabeling analyses which confirmed overlapping cerebral expression of Vegfr3 and Vegfc, notably in the hippocampus of control and ischemic animals [118–121]. These studies showed a specific VEGF-C expression in radial glial cells of the embryonic forebrain as well as in adult SVZ cells, suggesting that VEGF-C may contribute to forebrain neurogenesis during the lifespan. Interestingly, VEGF-C expression persisted in adult radial glia and in tanyocytes of third ventricle, retinal Muller cells and cerebellar Bergmann glia, which display neural stem cell properties in vitro [118]. We have recently addressed the role of VEGF-C in adult neurogenesis, first focusing on the SVZ [122]. Using transgenic BAC VEGFR-3::YFP mice to visualize and isolate living Vegfr3-expressing cells, we found that adult SVZ VEGFR-3::YFP<sup>+</sup> cells were predominantly (around 90 %) quiescent astrocytes and neural stem cells (Fig. 3a). Using the Cre/loxP system and a loxP-flanked (floxed) Vegfr3 allele (Vegfr3<sup>floxed</sup>) [123] to generate inducible cell-type-specific deletions of Vegfr3 in neural cells, with a Bm4Cre driver line, or in astroglial cells, with a GlastCreERT2 inducible driver line, we also showed that VEGF-C expression by SVZ astrocytes was required for proper adult neurogenesis and that VEGF-C mediated the promoting effect of VEGF-C on adult neurogenesis.

The dentate gyrus (DG) of the hippocampal formation is the second neurogenic niche of the adult brain, playing an important role in learning, memory, and mood regulation [124–126]. We have investigated recently Vegfr3 expression and role in DG neurogenesis and found that SGZ VEGFR-3<sup>+</sup> cells were GFAP<sup>+</sup> astrocytes and radial glia/stem cells as well amplifying progenitors (Fig. 3b). Vegfr3 expression was not detectable in immature neurons and granule cells. Additional neurosphere assays performed on FACS-sorted Vegfr3-expressing cells from the DG of Vegfr3::YFP mice showed self-renewal and multipotency of these cells (data not shown; Calvo and Han, unpublished results). VEGF-C expression is therefore a hallmark of adult neural stem cells in the mouse. These findings suggest VEGF-C action in DG neurogenesis and raise the question of the nature of VEGF-C ligand, which may be VEGF-C or/and VEGF-D, because both are expressed in the DG [117, 121, 127]. They also lead to explore whether VEGFR-3 signaling in SGZ stem cells may mediate the effects of environmental changes, such as physical activity or stress, on DG neurogenesis and eventually modulate hippocampus-dependent behaviors.
Notch and VEGFRs interactions during neurogenesis

Evidence that VEGF and Notch signaling regulates neurovascular interactions resulted from pioneer works of Sally Temple’s group [128]. In this study, the authors showed that endothelial coculture stimulated embryonic and adult neural stem cells by promoting their self-renewal, inhibiting their differentiation, and enhancing their neuron production. This effect resulted from the delivery of soluble factors from EC, and not vascular smooth muscle cells. Moreover, neuroepithelial and ECs established cell-to-cell contacts activating Notch and Hes1 in neural stem cells to promote their self-renewal. Additionally, gene-chip analysis of EC-stimulated rat brain slices indicated that VEGF-A siRNA strongly altered expression of Notch2 and Hes1 transcripts, suggesting that VEGF-A modulates Notch signaling pathway induced by contact between neural cells or between neural cells and ECs [111]. Associative evidence of a link between VEGF-A, Notch activation and neurogenic activity was also provided for neonatal SVZ neural stem cells, where an enriched expression of both VEGF-A and cleaved notch-1 was found in the CD-1 mouse strain, which displays a robust neurogenic response to neonatal hypoxic insult, compared to C57BL/6 mice, which are less responsive and recover poorly from hypoxia [62].

Proof of a relationship between VEGF-A/VEGFR-2 and the Notch signaling pathway during neurogenesis was directly demonstrated early neuronal progenitors of the developing retina [129]. Using the model of the chicken retina, which is completely devoid of blood vessels throughout development [130], the authors showed that perturbing VEGF signals, as well as VEGFR-2 receptor function, during early stages of retinal neurogenesis influenced retinal progenitor cell proliferation and commitment. Augmenting VEGF signals increased progenitor cell proliferation and decreased retinal ganglion cell genesis. Conversely, absorbing endogenous VEGF ligand or disrupting VEGFR-2 activity attenuated cell proliferation and enhanced retinal ganglion cell production. VEGF signals transmitted by VEGFR-2 appeared to activate divergent intracellular signaling components, which regulate different responses of progenitor cells. VEGF-induced proliferation is influenced by the MEK-ERK pathway, as well as by the basic helix-loop-helix factor HES1. By contrast, VEGF-dependent ganglion cell suppression does not require MEK-ERK activation, but instead relies on VEGF-stimulated Hes1 activity, which is notably independent of Notch signaling. Based on their findings, the authors proposed that Hes1 is a signaling hub in early retinal progenitor cells to integrate various cell-extrinsic cues, including VEGF and also SHH, in order to control cell proliferation and neuronal specification (Fig. 1b).

To explore whether VEGFR-3 and Notch pathways interact to regulate neural stem cell maintenance in adult neurovascular niches, we performed microarray analysis of Vegfr3::YFP/CD31− FACS-sorted cells (NSC and not EC) from the adult SVZ and showed that Vegfr3 transcripts were coexpressed with high level of Notch1 and Notch target gene (Hes5, Hes1) RNAs, compared to other niche cell types (Table 1). VEGF-C stimulation of SVZ niche cells moreover strongly increased expression of Notch1, Hes5 and Hes1 transcripts (data not shown). Further experiments testing in vivo the effects of Notch activation or blocking on VEGFR-3 activation, and of VEGF-C on Notch activity will determine whether VEGF-C and VEGFR-3 regulate and reinforce Notch signaling in adult SVZ NSCs, alike VEGF-A/VEGFR-2 in embryonic retinal progenitor cells.

Concluding remarks

The findings reported above indicate that proper growth of blood vessels requires a dynamic interaction between VEGFR-2, VEGFR-3 and Notch signaling pathways. Notch appears to regulate the relative importance of VEGFR-2 and VEGFR-3 for angiogenesis. Moreover, VEGFR-2 and Notch both modulate VEGFR-3 expression, independently and in opposite
directions. Consequently, while VEGFR-2 signaling is active and key regulator in ECs with high Notch activity and low VEGFR-3 expression, VEGFR-3 drives growth of ECs with low Notch signaling activity, even in the absence of VEGF ligands and VEGFR-2 signaling. In addition, VEGFR-3 can promote the stabilization of tip cells at vessel fusion sites by reinforcing Notch signaling through macrophage-derived VEGF-C activation. VEGFR-3 seems thus to have a dual pro-(active) and anti-(passive) angiogenic function and to complement the angiogenic action of VEGFR-2 in environmental context inappropriate for the VEGF-A/VEGFR-2 axis.

These findings now lead us to investigate the distinct signaling cascades activated by varying Notch activity in ECs. They also raise questions about the mechanisms that allow ECs to change frequently from a tip to a stalk position and to regulate Notch-VEGFRs interactions in a highly dynamic manner, likely by involving post-transcriptional regulations that have not yet been characterized. The highly dynamic Notch activity in ECs correlates with their ability to rapidly switch between tip and stalk cell fates during sprouting angiogenesis. Although neurogenesis does not always happen quite so rapidly, these signaling studies in angiogenesis offer a unique model for interpreting Notch-VEGFRs interaction in other cell types, including NSCs and their dynamic plasticity through development. Both VEGFR-2 and VEGFR-3 are expressed by different neural cell types and have already been implicated in various parts of neurogenic regulation. Interplay between Notch-VEGFR-2-VEGFR-3, as in ECs and angiogenic sprouts, may thus occur between different cell types within the neurogenic niches. These interactions may vary according to CNS region, developmental stages, and, or the cell types interacting to elicit regulation over a wider variety of processes and cell types in these complex tissues. For example, VEGF-A-VEGFR-2-Hes1 regulation may be prevalent in neuronal progenitors of the embryonic retina, whereas VEGF-C-VEGFR-3-Notch signaling governs astroglial/stem cell activity in the adult niches. Pharmacological approaches and genetic models are now available, allowing us to manipulate VEGFRs or Notch signaling molecules in these cell-type and spatio-temporal-specific patterns. Their combination will provide a better understanding of the neurogenic niche cells and will help us determine the diversity and the extent of regulation these pathways have on brain development and adult neurogenesis. These findings will set the groundwork for developing new therapies and improving current treatments for neural diseases, traumatic injuries, and age-related decline in humans.

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References


Fig. 1.
Proposed models of Notch-VEGFRs interactions during angiogenesis and neurogenesis. a In ECs, VEGF-A-activated VEGFR-2 signaling up-regulates VEGFR-3, but only weakly induces Dll4 expression in tip cells, in vivo. Dll4 expression is regulated by either Notch signaling, extracellular matrix signals or other unknown regulators (blue arrow). Activation of Notch strongly down-regulates the level of VEGFR-3, but not VEGFR-2, in adjacent ECs. VEGFR-3 expression in ECs is thus regulated both by VEGFR-2 and Notch signaling, independently. Little to no Notch signaling in ECs leads to ligand-independent activation of VEGFR-3, which induces mispatterned angiogenic growth, even in the absence of VEGF-2 signaling (modified from Benedito et al. [12]). b In neural progenitor/stem cells, Delta-Notch cell membrane interactions induces cleavage and intra-nuclear translocation of NICD which activates HES1 transcription. It is not yet known whether Delta-presenting cells that stimulate Notch-presenting progenitor/stem cells are exclusively neural cells or if they also include ECs, both in the early embryonic neuroepithelium or in adult niches where ECs and some neural progenitor/stem cells establish direct cell membrane contacts. In embryonic retinal progenitors, VEGF-A-mediated activation of VEGFR-2 induces the MEK-ERK cascade and enhances HES1 activity, independently of Notch. Cell proliferation requires independent inputs from both MEK-ERK and HES1, whereas blocking of cell differentiation mainly involves HES1 activity. SHH signaling also activates HES1 (from [129]). In adult neural stem cells, VEGFR-3, rather than VEGFR-2, is expressed predominantly and may mediate Notch target activation following VEGF-C stimulation.

ECs EC stalk cell, EC T EC tip cell

ECs EC stalk cell, EC T EC tip cell
Fig. 2.
VEGF-A/C expression in the embryonic mouse brain cortex. 

a β-galactosidase reporter activity (X-gal staining) in the brain of a Vegfc<sup>lacZ</sup>+/embryo at stage E13.5 reveals a high dorsal to low lateral gradient of Vegfc expression in the cortex of a whole-mount brain. C caudal, R rostral. 

b Immunolabeling of VEGF-C (green) in E13.5 brain shows expression in the dorsal cortex (large arrows) and the medial wall of telencephalon (thin arrows), especially at the ventricular surface, which correlates with the expression pattern of the Vegfc<sup>lacZ</sup>+/brain (white arrow). While VEGF-A (red), on the same sections, is expressed by ECs (arrowhead) throughout the dorsal and ventral parts of the forebrain (arrowhead).

Pictures at higher magnification display the vesicular pattern of expression for VEGF-C in neuroepithelial cells contrasting with the endothelial expression of VEGF-A. dCX dorsal cortex, H hem, LGE lateral ganglionic eminence, LV lateral ventricle. Scale bar 100 μm
Fig. 3.
Synthetic scheme of VEGFR-3 expression by neural stem cells in the SVZ and SGZ of adult Vegfr3::YFP mice. a In the SVZ, VEGFR-3 is expressed by a subpopulation of astrocytes (b) and almost all NSCs (B1), but not in the majority of amplifying progenitors (c), neuroblasts (a). VEGF-C is produced by SVZ astrocytes among other cell types, promoting activation of VEGFR-3 expressing cells, including the stem cells, as evidenced by an increase in amplifying progenitor and neuroblast cell numbers following VEGF-C overexpression [122]. b In the SGZ, VEGFR-3 expression can be seen on immature cells including radial glia and horizontal cells, the region’s stem cells, as well as the Mash1+ intermediate progenitors. It is not expressed by the less plastic neuronal precursors or the differentiated granule cells (Han, unpublished data).
Table 1

Microarray data showing upregulated transcripts of Notch and Notch signaling pathway genes in Vegfr3::YFP\(^+\) compared to Vegfr3::YFP\(^-\) cells

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Ratio</th>
<th>p value</th>
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<td>Notch signaling pathway genes</td>
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