Eyeing central neurons in vascular growth and reparative angiogenesis

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The generation of blood vessels is a highly synchronized process requiring the coordinated efforts of several vascular and nonvascular cell populations as well as a stringent orchestration by the tissue being vascularized. Stereotyped angiogenesis is vital for both developmental growth and to restore tissue metabolic supply after ischemic events. Central neurons such as those found in the brain, spinal cord, and retina are vast consumers of oxygen and nutrients and therefore require high rates of perfusion by functional vascular networks to ensure proper sensory transmission. During a metabolic mismatch, such as that occurring during a cerebrovascular infarct or in ischemic retinopathies, there is increasing evidence that central neurons have an inherent ability to influence the vascular response to injury. With a focus on the retina and retinal ischemic disorders, this review explores the ever-growing evidence suggesting that central neurons have the propensity to impact tissue vascularization and reparative angiogenesis. Moreover, it addresses the paradoxical ability of severely ischemic neurons to hinder vascular regrowth and thus segregate the most severely injured zones of nervous tissue. The topics covered here are pertinent for future therapeutic strategies because promoting and steering vascular growth may be beneficial for ischemic disorders. (Blood. 2012;120(11):2182-2194)

Introduction

The intricate wiring of nerve and vascular plexuses is arguably one of the most monumental tasks facing developing tissue in higher organisms. Throughout embryogenesis, nerves and blood vessels establish architecturally optimized networks to ensure adequate tissue perfusion and permit transmission of sensory information. The tight anatomical coupling of the 2 systems underscores the need for honed guidance mechanisms to ensure proper targeting (Figure 1).1

Central neurons require a steady supply of nutrients and oxygen to ensure appropriate function, and it is therefore key for nervous and vascular systems to be adequately paired. The ever-changing metabolic requirements of the CNS necessitate dynamic perfusion rates because the brain only stores limited glucose. Hence, vascular dropout in the CNS and the ensuing deterioration of the neurovascular unit increasingly is being associated with several debilitating neurodegenerative diseases.2 Examples include vascular dementia,3 which loosely encompasses syndromes that lead to vascular lesions of the white matter of the brain and are caused by general vascular disease affecting the brain or by focal lesions such as strokes. In Alzheimer disease, postmortem analysis reveals that up to two-thirds of patient brains show signs of stenosis resulting from hyaline fibrosis of arterioles and smaller vessels.4 In addition, impaired vascular barrier function is a signature of multiple sclerosis5 and amyotrophic lateral sclerosis,6 where the blood-brain barrier is compromised, resulting in inadequate cerebral and spinal perfusion.

The breakdown of vascular networks in neurodegenerative and neuroischemic conditions leads to hypoxic/anoxic events and a constellation of biochemical changes that compromise cellular function.7-10 Restoring a functional and efficient vascular architecture is therefore key to regaining proper perfusion and neural tissue function. In this respect, devising strategies to accelerate the revascularization of ischemic nervous tissue with healthy vessels may prove beneficial in salvaging the function of ischemic tissue.11,12 Importantly, although blood vessels nourish the organs through which they course, aberrant and excessive vascularization, such as that observed in cancer13 and blinding ocular vasoproliferative disease,9 contribute to the progression of disease. Therefore, therapeutic strategies aimed at regenerating vasculature will need to ensure controlled growth and accurate steering and homing of nascent vessels.

This review addresses the emerging and ever-growing evidence supporting a key role for central neurons in retinal vascularization both in health and disease. A clearer understanding of the molecular interplay between central neurons and the vascular beds that perfuse them will provide insight on prospective strategies to therapeutically modulate angiogenesis within the ischemic CNS.

Elucidating neurovascular interaction in the CNS: the retina as a model system

Perhaps the most accessible arena in which to study central neurovascular interaction is the inner retina, where retinal ganglion cells (RGCs) are in close apposition with vessels of the inner retinal vascular plexus (Figure 1A). The retina is an experimentally user-friendly portion of the CNS (located outside the brain proper) and, given the anatomy of the ocular globe, has been poetically dubbed the “window to the brain.”

RGCs are central neurons that have a compartmentalized layout in that their somata lie within the eye, whereas their axons project into the optic nerve and synapse with subcortical regions of the brain. From an experimental perspective, this anatomical layout permits for easy, minimally intrusive access to RGC cell bodies through the vitreous and allows for selective labeling and manipulation of the optic nerve. Together, these features have made the
angiogenesis, including the first identification of the tip cell,21 the seminal discoveries on the mechanisms governing mammalian vascular regression.

Avoiding the complications associated with the analysis of embryonic phenotypes can be readily imaged and assessed postterm, thus enabling the study of the CNS in health and disease, starting with the pioneering work of Santiago Ramon y Cajal on axonal regeneration of the rabbit optic nerve.14

Similarly, the retina, and more specifically the rodent retina, has been instrumental in the study of angiogenesis both in physiologic and pathologic contexts.15 Comparable with the aforementioned benefits for the study of the CNS, the retinal vascular systems present numerous advantages that lend themselves particularly well to experimental manipulation. Most notably, the rodent retinal vascular plexus develops during the first postnatal weeks of life in a highly stereotyped, temporal, and reproducible pattern (reviewed in Stahl et al15 and Dorrell and Friedlander16). Therefore, the outcome of genetic17 or pharmacologic18-20 manipulation on vascular phenotypes can be readily imaged and assessed postterm, thus avoiding the complications associated with the analysis of embryonic vasculature. This attribute has been exploited to make several seminal discoveries on the mechanisms governing mammalian angiogenesis, including the first identification of the tip cell,21 the elucidation of the roles of different VEGF isoforms in arteriolar and venular patterning,22 the signaling circuits leading to endothelial cell specialization,23-25 as well as the fundamental role of myeloid cells in angiogenesis.26,27

Physiologic vascular development in the retina

A functional circulatory system is the first of the major embryonic systems to arise. Initially, it must provide the growing embryo with nourishment before the formation of an intestine, distribute oxygen preceding the appearance of lungs, and evacuate waste before the presence of kidneys. Hence, the embryonic vasculature is designed such that nutrients are obtained from the placenta or yolk sac and respiration and gas exchange is provided by chorionic and allantoic membranes.

The inner eye initially is sustained by the hyaloid vasculature, which consists of an arterial network residing in the vitreous. The hyaloid vasculature is perfused via the central hyaloid artery residing in the optic nerve and drained by an annular system of collection vessels at the forefront of the eye. At approximately 16 weeks of gestation in humans, retinal vessels commence, invading the retina from the optic nerve head toward the periphery and gradually replacing the resident hyaloid vessels. In mouse models, regression of hyaloid vessels was shown to occur via the WNT7b short-range paracrine signal from macrophages.28 During ocular maturation, macrophages initiate a prodeath program in the vascular endothelial cells of the transient hyaloid vessels using the Wnt ligand to dictate vascular regression.

In several mammalian species, such as echidnas, guinea pigs, and rabbits, the retinal vasculature is absent and the oxygen supply to the full thickness of the retina is provided by diffusion from the choriocapillaris of the outer retina. Interestingly, the presence of an additional blood supply from vessels located in and on the retina is directly related with retinal thickness where vascularized retinas are approximately 60% thicker than those without retinal vessels.29-31 In mammals such as mice and primates, 3 retinal and intraretinal vascular layers form in conserved growth patterns over various time scales.32 The formation of the primary retinal vascular plexus and subsequent deeper layers is thought to occur largely by angiogenesis33 whereas vasculoangiogenesis may occur in other species.16

The mouse superficial plexus forms during the first week of life and reaches the extremity of the retina at approximately postnatal day (P)8 (Figure 2A; retinal vascular development in C57Bl/6 mice). Retinal capillaries commence to dive down at P7 and form the deepplexus by approximately P12 and finally invade the intermediate layer from P12 to P15. Retinal vascularization is fully completed and interconnected by the third week of life. Several mechanisms are in place to ensure stereotyped patterning of retinal vessels.15,16 These rely heavily on the interaction between nascent vessels and the astrocytic template on which they grow. Astrocyte genesis precedes vascular development and begins forming at embryonic day 19 and is fully developed at birth.34 Astrocytes express PDGF-α-expressing RGCs,41 emphasizing the relationship between neurons, astrocytes, and vessels. As vessels sprout, they align with astrocytic fibers and are stabilized by R-cadherin-mediated cell–cell adhesions between the endothelial filopodia and nearby astrocytes (Figure 2B).21,34,35 Although astrocytes express...
angiogenic factors such as VEGF at birth, the indispensable role for astrocyte-derived growth factors in developmental vascularization recently has been challenged. It is therefore plausible that other highly metabolic cells such as RGCs, which produce angiogenic factors throughout development, play a critical role in governing retinal developmental vascularization (discussed in “Neuronal influence on retinal vascularization”).

The retina is, per weight, the most metabolically active tissue of the body. Regions that are poorly supplied in oxygen and nutrients prompt the formation of angiogenic sprouts from the walls of preexisting vessels in response to O₂-tension. This process of adaptation is largely orchestrated by hypoxic-sensing machinery such as the prolyl hydroxylases, which regulate the transcription factor hypoxia-inducible factor (HIF). HIF consists of 2 distinct α- and β-subunits that are required to heterodimerize to be active. HIF-1β is the constitutive unit, whereas HIF-1α and HIF-2α are continuously transcribed but only have transient half-lives of 5 minutes in normoxic conditions. The rapid turnover is mediated by the oxygen-dependent proline hydroxylation of HIF-1α or HIF-2α by prolyl hydroxylases. The ultimate result is polyubiquitination and targeting for proteasomal degradation. Alternatively, in hypoxic conditions, proline hydroxylation is inefficient, and HIF-1α or HIF-2α are stabilized and translocate to the nucleus and dimerize with HIF-1β. The outcome is the transcription of a cluster of target genes that have a hypoxia-response element. Presently, more than 70 genes have been described that encode proteins (such as VEGF and Epo) that help promote the adjustment to a hypoxic state and indirectly participate in instating local tissue perfusion.

Evidence for neuronal involvement in sensing and responding to hypoxia is substantiated by the fact that HIF-1α is most abundantly expressed in the neuroretina. Correspondingly, neuroretinal-specific knockout of HIF-1α impairs vascular development by reducing tip cell filopodia as well as vessel branching and leading to the absence of the intermediate vascular plexus. Interestingly, although a pronounced retinal vascular phenotype is noted in these animals, VEGF levels are unaffected, suggesting that HIF1α may not be the main mechanism by which VEGF is regulated in response to hypoxia.
Vascular growth and guidance: conserved mechanisms in neuronal and vascular patterning

The complexity of neurovascular networks is unmistakable, given the elaborate webs of billions of neurons in humans, each communicating with thousands of other neurons, in proximity and irrigated by a vessel.49 In the brain, spinal cord, and retina, a neuron must extend its axon over long distances into a maze of chemical signals and physical barriers and ensure it arrives in proximity of the adequate target and generates a functional synapse. This growth process relies essentially on the axon’s ability to read and interpret molecular growth and guidance cues. Similarly, albeit through a different process, vessels within the CNS undergo sprouting angiogenesis and must be adequately guided to their destination to ensure functional perfusion of nervous tissue. During nerve growth, a single neuron extends an axon directly from its cell body whereas vessel growth consists in a process of endothelial cell delamination and assembly.50 Both neurons and vessels require honed guidance systems to ensure functional coupling and formation of healthy neuronal units. Although the nervous system emerged earlier in evolution, nascent vessels share the same arsenal of molecular guidance cues to ensure proper targeting.1

The vascular analog of the growth cone is the tip cell.21 In the adult, blood vessels are quiescent, yet endothelial cells maintain elevated plasticity and are able to transduce and respond to angiogenic signals. Angiogenesis in the retina (as elsewhere in the body) is a concerted process involving endothelial cell proliferation, migration, and assembly into tube-like structures containing a lumen.16,51 Vessel growth is instigated by elevated levels of angiogenic factors such as VEGF, fibroblast growth factors, ANG-2, and/or other chemokines. In response to angiogenic stimuli, pericytes detach from the vessel wall (ANG-2 mediated), basement membrane is degraded by matrix metalloproteinases, endothelial cells slacken their vascular endothelial cadherin and claudin-rich junctions, and a designated leading cell (tip cell) protrudes and advances toward vaso-attractant chemotactic gradients (Figure 3A-C; reviewed in Carmeliet and Jain50).

Which cell becomes the leader (tip) and which follows (stalk) is established by Notch-mediated lateral inhibition, and the underlying mechanisms have been worked out in a series of sophisticated cell biology studies in which the mouse retina is used as a model. After stimulation of VEGFR-2 by VEGF, tip cells up-regulate the Notch ligand Dll4 (however, possibly modestly52; discussed below).23,25,53 The elevated levels of Dll4 in filipodia-rich endothelial tip cells activate Notch receptor in neighboring endothelial cells and thus block generation of tip cells.23,25 This is achieved through Dll4-Notch–mediated down-regulation of VEGFR-2 on stalk cells and simultaneous up-regulation of VEGFR-1. The stalk cells then become less responsive to VEGF-induced sprouting and become more responsive to VEGFR1-driven proelongation factors.

Conversely, the Notch ligand Jagged1 (which is expressed by stalk cells) antagonizes Dll4-Notch signaling and is a potent proangiogenic regulator that induces tip cell formation.24 This early cellular specialization prevents nondiscriminant endothelial movement toward angiogenic signals and thus ensures the presence of stalk cells in the wake of the advancing tip cell to form a luminal tube.54 Stalk cells respond to VEGF by proliferating21 and become rapidly stabilized and establish adherent tight junctions to maintain the integrity of the nascent vessel.55 The final maturation of the vascular plexus requires pruning of excess vessels,56 coalescence of existing vessels, and the recruitment of mural cells.57

Interestingly, beyond the canonical VEGF-A/VEGFR-2 paradigm, recent evidence points to an important role in retinal angiogenesis for VEGF-C and its main receptor, VEGFR-3, and challenges the current views on the molecular cross-talk between the VEGF and Notch pathways. The authors propose that VEGFR-3 is robustly modulated by Notch, whereas expression of Dll4 in retinal tip cells is only weakly affected by VEGFR-2 signaling and Notch inhibition has minimal impact on VEGFR-2 expression.55 Importantly, VEGFR-3 kinase activity inhibitors block sprouting from endothelial cells that have low Notch signaling activity. This novel insight suggests that effective antiangiogenic strategies targeting these receptors and their ligands will be dependent on the status of endothelial Notch signaling.53 In addition, another role for VEGF-C is substantiated by new evidence suggesting that macrophage-derived VEGF-C activates VEGFR-3 in tip cells and accentuates Notch signaling and partakes in the phenotypic conversion of endothelial cells at fusion points of vessel sprouts.58

In contrast to stalk cells, which respond to VEGF by dividing, tip cells activate Cdc42 and form motile filopodia protrusions that probe the environment and transduce extracellular signals (Figure 3D-E).59 Vascular tip cell filopodia (as their neuronal analogues) are enriched in receptors that were classically described to respond to neuronal guidance cues.60 These include the neurolamins and plexins (for semaphorins); Unc5b, neogenin, and DCC (for netrins); the Eph receptors (for ephrins); and roundabout (for slits; Figure 3D).61-63 Once the tip cells comes in contact with a given cue, it will respond by either advancing, stalling, turning, or retracting depending on which cell surface receptor predominates and the overall intracellular environment of the tip cell.64 The role of neuronal guidance cues in vascular growth has been comprehensively reviewed.1,59,64,65 Evidence for deregulation of neuronal guidance cues in neurovascular pathology is starting to emerge66-71 and will be discussed in the context of ischemic retinopathies below.

Neuronal influence on retinal vascularization

A growing body of evidence is highlighting the key role of neurons in instigating, promoting, and steering angiogenesis within nervous tissue and specifically in the retina.19,47,70,72,73 A direct role for retinal neurons in influencing vascular growth is observed in humans during the formation of the outer vascular plexus of the human fetal retina in utero (25-26 weeks).74 This period coincides with the first appearance of visually evoked potentials and thus functional neurons.75 A likely explanation for this concurrence stems from the increase in oxygen consumption (and thus augmented metabolic requirement) of newly functioning neurons and the consequent generation of local regions of hypoxia.76

Notwithstanding the key contribution of glial cells such as astrocytes in providing a framework on which nascent vessels advance,31,35,77 their role as the principal source of trophic support for retinal vascular development may need to be revisited. Evidence for a retinal cell population other than astrocytes providing indispensable angiogenic factors for retinal vascularization comes from genetic studies in mice in which the hypoxic response was disrupted directly in astrocytes by cell-specific deletion of VEGF, HIF-1α, or HIF-2α. Using this approach, we noted no visible
perturbations in developmental retinal vascularization,\textsuperscript{37,38} which suggests a role for these effectors in another retinal cell types such as neurons. Given the elevated metabolic requirements of neurons, it is both conceivable and likely that they play important roles in sculpting their vascular environment in response to hypoxia. In agreement, the neuroretinal-specific knockout of HIF-1α substantially perturbs retinal vascular development.\textsuperscript{47,48}

Of candidate neuronal populations to influence retinal vascularization, RGCs are the most intimately supplied by the superficial vascular plexus. A specific role for RGCs in retinal vascular development was established with the use of mouse models of genetic ablation of RGCs.\textsuperscript{19,72} RGCs developmentally precede both retinal vessels and astrocytes, making them interesting candidates to study in the context of vascular growth. Evidence obtained from

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**Figure 3. Mechanisms of retinal angiogenesis.** Graphic depiction of the cellular mechanisms governing retinal angiogenesis. (A) A stable, quiescent vessel with aligned endothelial cells (EC), united by vascular endothelial cadherin–rich junctions, and covered by pericytes. (B) On stimulation by angiogenic factors, a cascade of events ensues leading to pericyte detachment, basement membrane degradation, and endothelial junction loosening. Determination of stalk versus tip cell phenotypes is achieved through Notch-dependent signaling. VEGF through VEGFR2 induces the Notch ligand Dll4 on tip cells, which subsequently activates Notch in adjacent endothelial cells and specifies their stalk cell phenotype. Conversely, the Notch ligand Jagged1 is highly expressed by stalk cells and antagonizes Dll4. This promotes VEGFR2 expression and renders ECs more responsive to VEGF and thus more susceptible to form tip cells. (C) Once the vessel has sprouted, stalk cells behind the tip cell divide and assemble to form the lumen of the neovessel. Pericytes are then recruited, and basement membrane is laid down. (D) To reach its final destination, the growing neovessel must navigate through the tissue by responding to a series of diffusible and membrane-bound attractive and repulsive guidance cues. (E) Confocal image of sprouting retinal vessels with filopodia-rich tip cells (stained with isolectin B4) from P4 mouse retinas. Scale bar, 10 μm.
transgenic and knockout mice supports the importance of these neurons in physiologic retinal vascularization. In transgenic brn3bZ-dta/+;six3-cre mice engineered to express diphtheria toxin A under the control of a RGC-specific promoter, RGCs are ablated as they form.86 Remarkably, brn3bZ-dta/+;six3-cre mice are completely devoid of a retinal vascular plexus and instead show persistent hyaloid vasculature.19 Similarly, Math 5−/− mice, which lack 95% of RGCs, also do not form a retinal vascular layer.72 Of note, although the astrocytic network remains largely intact in brn3bZ-dta/+;six3-cre mice, retinal vascularization is halted. Together, these studies suggest that RGCs play a central role in instigating retinal vascular development, whereas the astrocytic beds provide the framework on which nascent vessels advance.

Beyond RGCs, the most oxygenated and highly metabolically active retinal neurons are photoreceptors.79 As RGCs, these neurons have also been shown to influence retinal angiogenesis. Evidence comes directly from clinical observations in which patients suffering from both proliferative diabetic retinopathy and retinitis pigmentosa (a group of eye conditions that lead to progressive photoreceptors loss) show considerably less pathologic retinal angiogenesis than diabetic patients with healthy photoreceptors.80 In addition, the retinal neovascularization that is associated with long-standing diabetes mellitus has been reported to spontaneously regress with the onset of clinically evident retinitis pigmentosa.81 This observation held true in animal models in which mice with genetically ablated photoreceptors failed to mount reactive retinal neovascularization in a model of oxygen-induced proliferative retinopathy,81 whereas diabetic mice with photoreceptor degeneration demonstrated lower levels of retinal proangiogenic VEGF.82 The elevated energetic requirements of photoreceptor neurons may explain this phenomenon because the loss of photoreceptors results in a significantly reduced energetic burden on the tissue and thus a proportionately decreased requirement for perfusion.

Pairing neuronal energy demand to vascular supply may be efficiently achieved by directly harnessing the intermediates of the neuron’s own energy metabolism. In this respect, signaling via energy metabolites in response to compromised energy status has been proposed as a contributor to both physiologic and pathologic retinal vascularization.19,84-86 In the context of neurovascular interplay, this evolutionarily preserved signaling system has thus far been described for RGCs and the dicarboxylate succinate. Succinate is a metabolite of the Krebs cycle generated during cellular respiration. Although it plays a canonical role in energy production, succinate also binds and activates the formerly orphan G-protein–coupled receptor GPR91,87 inferring additional physiologic roles. In the retina, GPR91 is predominantly expressed in RGCs.19 Importantly, Krebs cycle intermediates, such as succinate, accumulate under conditions of hypoxic stress s because of feedback inhibition of succinate (and α-ketoglutarate) dehydrogenase by nonoxidized flavin and nicotinamide nucleotides and by reactive oxygen species.88,89 The accumulation of succinate during metabolic compromise is consistent with a role in mediating compensatory angiogenesis to reinstate adequate blood supply and oxygen delivery. The robust retinal vascular response provoked by succinate via its neuronal receptor GPR91 (production of VEGF, angiopoietin 1 and 2 [Ang-1 & -2]) is suggestive of a direct role in linking neuronal energy demand to capillary growth (Figure 4A-B). Correspondingly, succinate signaling through GPR91 can influence both developmental and pathologic retinal angiogenesis even before HIF stabilization,19 which suggests that the succinate–GPR91 axis can act as an early sensor of hypoxic stress and work to enhance regional circulation through the release of angiogenic factors such as VEGF and Ang-1 & -2 (Figure 3C). The HIF-independent angiogenic potential of succinate may explain the lack of variation in developmental VEGF levels in HIF-deficient mice.46

Ischemic retinopathies: microvascular degeneration and neuronal ischemia in the retina

The breakdown of functional neurovascular circuits in the retina is observed in sectors of nonperfused neuronal tissue in ischemic retinopathies. Given the importance of neuronal and vascular interplay in retinal health and disease,19,70,71,73 it is important to understand the neuronal contribution to the progression of these debilitating retinal diseases.

Ischemic retinopathies encompass the initial phases of diabetic retinopathy (DR) and retinopathy of prematurity (ROP) and represent the leading causes of blindness in working-age adults and children, respectively.80,91 Both diseases are characterized by an early phase of microvascular degeneration followed by a second phase of pathologic neovascularization mounted by the ischemic retina attempting to reinstate metabolic equilibrium.82-94 Because of this secondary phase of excessive vascular regrowth, the diseases also are referred to as proliferative retinopathies.

In ROP, the ischemic stress on the neural retina is a result of both vascular degeneration and incomplete developmental vascularization. To overcome the pulmonary insufficiency of premature infants, artificial ventilation can be increased to a relatively hyperoxic PaO2 of up to ~100 mmHg,95 whereas normal in utero O2 tension averages 32 mmHg in the umbilical cord, and a full-term infant will be exposed to a PaO2 of 60-100 mmHg in room air.96 This exposure to high PaO2 induces microcapillary degeneration by oxygen toxicity directly on endothelium via reactive oxygen species (reviewed in Sapieha et al10 and Hardy et al97). In addition, the hypoxic stress resulting from oxygen-induced vascular dropout is compounded by the fact that premature infants are born with an incomplete retinal vascular plexus; the development of human retinal vasculature concludes at term (40 weeks).98 Ex-utero, after premature birth, the physiologic vascularization process of the immature retina is perturbed because of the absence of maternally transferred vasopotentiating and vasoprotective factors such as insulin growth factor-199 and ω-3 polyunsaturated fatty acids.10 In addition, the excess oxygenation resulting from ventilation protocols suppresses key oxygen-regulated proangiogenic growth factors such as VEGF and Epo,10 thus further stalling the development of a mature retinal vascular supply.

In DR, the pathomechanisms governing the loss of endothelial barrier function and later vascular degeneration remain largely elusive. Although often initially asymptomatic, loss of vision is triggered primarily by diabetic macular edema, vitreal hemorrhages, and later tractional retinal detachment. Thus far, associations have been made between disease progression and advanced glycation end-products,100 protein kinase C activation,101 oxidative stress,101 and initial onset of acute intensive insulin therapy.102 Moreover, several salient features of chronic inflammatory disease are seen in DR, and increasing evidence points to the contribution of inflammatory mediators such as IL-1β103 and TNF-α104 in both vascular degeneration and neovascularization associated with DR (reviewed in Adamis and Berman105).
Failure of vascular regeneration in ischemic retinopathies: neuronal inhibition of reparative angiogenesis

The limited efficacy with which blood vessels regenerate into ischemic neural tissue after vascular injury represents a noteworthy challenge in vascular biology. A direct example lies in the failure of revascularization of hypoxic regions of the retina in ischemic retinopathies (Figure 5A). Both ROP and DR feature avascular pockets that are the source of hypoxic stress that instigates deregulated retinal neovascularization. Therapeutically promoting angiogenesis into these avascular areas may aid in easing the hypoxic burden in the retina and thus alleviate the neural ischemia that is central to disease progression.

Paradoxically, although the retina mounts a robust neovascular response secondary to microvascular degeneration, the nascent vessels in both ROP and DR fail to effectively regenerate into the

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**Figure 4. Neuronal influence in retinal vascular development.** (A) The superficial retinal vascular plexus forms in a stereotyped manner. As the vascular front advances, it leaves perfused RGCs in its wake. (B) The proposed model for RGC-dependent vascularization stipulates that the mildly hypoxic RGCs ahead of the vascular front accumulate energy metabolites such as succinate because of the feedback inhibition of succinate dehydrogenase. The elevated levels of succinate then stimulate its RGC-specific cognate receptor GPR91, resulting in the production of proangiogenic cues such as VEGF and Ang-2. RGCs therefore have the inherent ability to instate local microcirculation. (C) The EC50 for succinate-mediated activation of GPR91 is ~10-fold lower than the Kᵣ required for succinate-induced inhibition of prolyl 4-hydroxylases and consequent HIF-1α stabilization. This suggests that succinate through GPR91 acts as an early and accurate sensor of hypoxic stress.
most ischemic vaso-obliterated regions. The neovascularization in ischemic retinopathies presents 2 prominent features that render it pathologic. First, it is initially concentrated at the avascular border of the injured retina and hence fails to grow into and adequately perfuse the hypoxic tissue (Figure 5A). Second, the leaky abnormal vessels are misdirected toward the physiologically avascular vitreous, predisposing to traction detachment of the retina and loss of vision. The classic interpretation of the misdirected retinal neovascular growth has simplistically been explained by the presence of high concentrations of proangiogenic factors, such as VEGF, in the vitreous of patients with ischemic retinopathies. However, glial cells within the neural retina itself (astrocytes and Müller cells) and neurons (RGCs) produce elevated amounts of these angiogenic factors and therefore would be expected to retain nascent...
vessels on the retinal surface. Yet, neovessels continue to grow away from the hypoxic regions of the retina into the vitreous, suggesting that vasorepulsive forces are at work.

The prominent clinical/pathologic features of DR and ROP have given rise to the notion that these diseases are entirely microvascular in nature. However, evidence points to early changes in the neural retina and specifically in RGCs, which are intimately supplied by the same vascular network that is perturbed in ischemic retinopathies. Interestingly, although RGCs have a demonstrated role in governing retinal angiogenesis through production of angiogenic factors, when driven beyond a metabolic threshold, severely hypoxic RGCs within vasculoobliterated retinal zones revert to producing vasorepellent cues such as the secreted class III semaphorins (semaphorin 3A and 3E) (Figure 5B).

Consistent with a role for semaphorin 3A and 3E in blocking entry of vessels into ischemic retinal cores, extraretinal neovessels express the cell surface receptors neuropilin-1 for semaphorin 3A and plexinD1 for semaphorin 3E (Figure 5B). Neuropilin-1 is a single-pass transmembrane receptor with a large 860 amino acid extracellular domain subdivided into 3 subdomains (a1a2, b1b2, and c) and a short 40 amino acid intracellular domain (Figure 5C). In neurons, the binding of semaphorin 3A to neuropilin-1 recruits plexins, which transduce their intracellular signal and provoke cytoskeletal collapse; the transduction mechanism in endothelial cells remains ill-defined. Neuropilin-1 has the unconventional ability to bind 2 structurally unrelated ligands via distinct sites on its extracellular domain. Primarily via its a1a2 (but also b1) domains (provoking cytoskeletal collapse) and VEGF, it binds semaphorin 3A via its b1b2 domain (enhancing binding to VEGFR2 and thus increasing its angiogenic potential). Crystallographic elucidation of neuropilin-1 revealed that VEGF and semaphorin 3A do not directly compete for binding to neuropilin-1 but can simultaneously bind to neuropilin-1 at distinct, nonoverlapping sites. Moreover, well-designed genetic studies show that neuropilin-1 separately regulates the effects of VEGF and semaphorin 3A on neuronal and vascular development.

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A similar pathomechanism can be observed with semaphorin 3E, which signals through plexinD1 and activates the small GTPase RhoJ in endothelial cells. As with semaphorin 3A, the activation of plexinD1 by semaphorin 3E counteracts VEGF-induced filopodial projections. It is important to note that at the same time as severely hypoxic RGCs produce vasorepulsive cues, certain RGCs in the avascular continue to produce VEGF. Moreover, astrocytes (which physically lie between RGCs and the vitreous) readily drive angiogenesis during the vasoproliferative phase of retinopathy by producing VEGF (via HIF-2α but not by HIF-1α; Figure 5C). Hence, in accordance with this paradigm, the neovascular sprouts are deviated from the ischemic core of the retina toward the vitreous and contribute to the pathologic preretinal neovascularization observed in ischemic retinopathies.

Understanding the dichotomy in the behavior of retinal ganglion neurons where they are proangiogenic when subjected to mild levels of hypoxia and antiangiogenic when hypoxia is sustained may be central to understanding the progression of ischemic retinopathies. This phenomenon may be a survival mechanism to segregate irreversibly damaged sectors of the neural retina and redirect metabolic supply to less affected and more salvageable and will be discussed below.

The dichotomy of the neuronal response to ischemia: the neurovascular shunt

In neuroischemic conditions such as retinopathies or stroke, vascular dropout is associated with compromised neuronal function. To counter this early injury, attempts to reinstate regional microcirculation may come directly from the accumulation of energy metabolites such as succinate and activation of metabolite receptors like GPR91. Importantly, succinate triggers a half-maximal response on GPR91 with reported effective concentrations of 28-56 mM. These concentrations are approximately 10-fold lower than the Kᵢ values of 350-460 mM required for succinate-induced inhibition of prolyl 4-hydroxylases and consequent HIF-1α stabilization. Succinate, through GPR91, may thus act as a more sensitive sensor of hypoxia than HIF-1α and promote the release of finely tuned concentrations of proangiogenic factors to enhance regional circulation and overcome metabolic imbalances in the CNS. A similar HIF-independent production of VEGF has been reported for peroxisome proliferator–activated receptor-γ coactivator-1α, which partakes in the regulation of oxidative phosphorylation, mitochondrial biogenesis, and respiration. The role of peroxisome proliferator–activated receptor-γ coactivator-1α in CNS angiogenesis has yet to be determined.

Although hypoxia/ischemia is undeniably a major promoter of angiogenesis, when hypoxia persists and retinal neurons are driven beyond a metabolic threshold, they revert to producing classic neuronal repulsive cues such as semaphorin 3A and 3E (Figure 5F). The up-regulation of vasorepulsive cues such as semaphorins by severely injured cells in the CNS may be a generalized event as levels of semaphorin 3A increase after ischemic stroke and localize to regions immediately adjacent the zone of infarct and necrotic core. Similarly, semaphorin 3A is induced after spinal cord injury in neurons near the site of lesion.

The induction of repulsive guidance cues after CNS injury occurs during a period of vascular remodeling and axonal sprouting, suggesting that the severely damaged nervous tissue is attempting to deviate both regenerating neuronal sprouts and vessels away from the severely lesioned zones. It is therefore conceivable that if driven beyond the threshold of recovery, heavily hypoxic neurons mount a repulsive front in an attempt to shunt metabolic resources away from perishing unsalvageable ischemic tissue toward less affected regions of the CNS (Figure 5B and F). Reestablishing a vascular network to neurons that are unsalvageable would be wasteful. In agreement with the theory of segregating severely damaged areas of tissue, induction of semaphorin 3A in central retinal neurons requires prolonged exposure to inflammatory cytokines such as IL-1β, a paradigm mimicking prolonged exposure to ischemia. This idea is further substantiated by the latent appearance of semaphorin 3A production at the expense of VEGF in RGCs subjected to direct hypoxic stress. Although early during hypoxia, RGCs produce VEGF to reinstate vascular supply, when hypoxia is sustained, RGCs reverse their signaling machinery from VEGF production to that of the semaphorin 3A.
retina, redirecting nascent vessels away from the ischemic retina would deviate them toward the vitreous and result in the cardinal features of proliferative retinopathies (described above). Hence, devising strategies to steer nascent vessels into avascular pockets of the retina while keeping them in the retinal surface and in contact with the underlying astrocytic bed may be therapeutic value for ischemic retinopathies.

Neuronal guidance cues for therapeutic angiogenesis

Promoting angiogenesis to remedy ischemic CNS disorders may come across as an unsuitable strategy. Yet, in the retina, it may be considered counterintuitive given the uncontested involvement of proangiogenic factors such as VEGF in the pathogenesis of ocular neovascular disease. In addition, several antiangiogenic strategies currently are being explored and recommended to counter proliferative ocular disease. However, potential complications with anti-VEGF therapies, including a greater incidence of stroke in patients with a history of preexisting cerebrovascular disease, neurotoxicity, nonocular hemorrhage, and the need for repeated injections, could limit its overall utility. An alternative strategy may lie in directly promoting revascularization of the ischemic tissue that is at the source of the angiogenic stress. Although presently still in the experimental stages, the authors of a growing number of animal studies suggest that promoting vascular regeneration during the early ischemic phase of retinopathies may significantly reduce the destructive neovascularization that is central to disease progression. Enhancing normalized vascular growth in ischemic retina has previously been achieved through the injection of myeloid progenitors, increasing dietary omega-3 polyunsaturated fatty acid intake, or by direct administration of VEGF or FGF-2 via protection of glial cells.

The pronounced effect of neuronal guidance cues on vascular remodeling and a role in blocking vascular regeneration makes them attractive candidates to modulate for reparative angiogenesis. Evidence for the therapeutic potential of inhibiting guidance cues has been provided in models of limb ischemia in diabetic mice in whom blocking semaphorin 3E markedly improved the response to VEGF and promoted reparative vascular growth. Moreover, semaphorin 3E–deficient mice (or overexpression of the decoy PlexinD1-Fc) showed decidedly increased vascular regeneration and blood flow in response to ischemic injury. In the retina, we demonstrated that specifically inhibiting semaphorin 3A in RGCs using shRNAs delivered by lentiviral vectors resulted in enhanced physiologic revascularization of ischemic pockets. This approach directly lead to increased visual vectors resulted in enhanced physiologic revascularization of the ischemic tissue that is at the source of the angiogenic stress. Although presently still in the experimental stages, the authors of a growing number of animal studies suggest that promoting vascular regeneration during the early ischemic phase of retinopathies may significantly reduce the destructive neovascularization that is central to disease progression. Enhancing normalized vascular growth in ischemic retina has previously been achieved through the injection of myeloid progenitors, increasing dietary omega-3 polyunsaturated fatty acid intake, or by direct administration of VEGF or FGF-2 via protection of glial cells.

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In retinopathy, the position of the source of the vasoproliferative guidance cues such as the semaphorins (RGCs beneath the vascular plexus) is speculated to repel vessels toward the vitreous. Hence, the administration of semaphorin 3E by intravitreal injection (which stimulates vessels from an extraretinal position) suppresses preretal vessels while preserving regeneration of normal vessels. The normalized growth is thought to occur through direct stimulation of plexinD1 in pathologic retinal vessels. A similar outcome was noted with ectopic administration of semaphorin 3A. Both approaches showed a trend toward accelerated retinal revascularization, although the principal noted benefit was suppression of pathologic preretal growth.

Conclusion

In the last decade, we have witnessed a surge in our understanding of the molecular mechanisms governing blood vessel growth. It is now clear that the chemotactic signals present during embryogenesis are conserved and shared between neurons and vessels. Although neurovascular cross-talk shapes vascular development, it has received limited attention as an etiology of disease. The tight physiologic coupling between vessels and neurons in the healthy retina suggests that therapeutic insight may be gained through a better understanding of how the unit reacts under stress. Notably, the identification of neuron-derived proangiogenic energy metabolites such as succinate and vasomodulatory chemotactic cues such as the semaphorins provides immediate mechanisms to modulate for reparative angiogenesis. Currently, approaches to counter aberrant ocular neovascularization (both approved and in human trials) rely largely on antiangiogenic strategies. However, despite all invested efforts, effective inhibition of pathologic ocular neovascularization remains to be achieved. Future therapies for ischemic retinal disease may therefore need to go beyond solely alleviating the angiogenic burden and incorporate strategies to promote angiogenic angiogenesis with the aim of reducing ischemic stress. In this respect, studying the neuronal programs that drive retinal angiogenesis may provide attractive avenues of research.

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Eyeing central neurons in vascular growth and reparative angiogenesis

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