Université de Montréal

Neuron-Derived Semaphorin 3A is an Early Inducer of Vascular Permeability in Diabetic Retinopathy

par

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Résumé

La détérioration de la barrière hémato rétinienne et l'œdème maculaire consécutif est une manifestation cardinale de la rétinopathie diabétique (RD) et la caractéristique clinique la plus étroitement associée à la perte de la vue. Alors que l'œdème maculaire affecte plus de 25% des patients souffrant de diabète, les modalités de traitement actuellement disponibles tels que les corticostéroïdes administrés localement et les thérapies anti-VEGF récemment approuvés présentent plusieurs inconvénients. Bien que le lien entre une rupture de l'unité neuro-vasculaire et la pathogénèse de la RD ait récemment été établi, l'influence de la signalisation neuro-vasculaire sur la vasculopathie oculaire diabetique a jusqu'à présent reçu peu d'attention. Ici, à l'aide d'ètudes humaines et animales, nous fournissons la première preuve du rôle essentiel de la molécule de guidage neuronale classique Sémaphorine 3A dans l'instigation de la perméabilité vasculaire maculaire pathologique dans le diabète de type 1. L'étude de la dynamique d'expression de Sémaphorine 3A révèle que cette dernière est induite dans les phases précoces hyperglycèmiques du diabète dans la rétine neuronale et participe à la rupture initiale de la fonction de barrière endothéliale. En utilisant le modèle de souris streptozotocine pour simuler la rétinopathie diabétique humaine, nous avons démontré par une série d'approches analogue que la neutralisation de Sémaphorine 3A empêche de façon efficace une fuite vasculaire rétinienne. Nos résultats identifient une nouvelle cible thérapeutique pour l'œdème maculaire diabétique en plus de fournir d'autres preuves de communication neuro-vasculaire dans la pathogènese de la RD.

Mots-clés

Sémaphorine 3A,

œdème,

diabète,

rétinopathie diabétique,

cellules ganglionnaires de la rétine

Abstract

The deterioration of the blood retinal barrier and consequent macular edema is a cardinal manifestation of diabetic retinopathy (DR) and the clinical feature most closely associated with loss of sight. While macular edema affects over 25% of patients suffering from diabetes, currently available treatment modalities such as locally administered corticosteroids and recently approved anti-VEGF therapies, present several drawbacks. Although recent insight on the pathogenesis of DR points to a breakdown in the neurovascular unit, neurovascular cross-talk and its influence on diabetic ocular vasculopathy has thus far received limited attention. Here we provide the first evidence from both human and animal studies for the critical role of the classical neuronal guidance cue Semaphorin3A in instigating pathological macular vascular permeability in type I diabetes. Investigation of the dynamics of expression reveal that Semaphorin3A is induced in the early hyperglycemic phases of diabetes within the neuronal retina and precipitates initial breakdown of endothelial barrier function. Using the streptozotocin mouse model as a proxy for human diabetic retinopathy, we demonstrate by a series of orthogonal approaches (gene silencing or treatment with soluble Neuropilin-1 employed as a Semaphorin3A trap), that neutralization of Semaphorin3A efficiently prevents retinal vascular leakage. Our findings identify a new therapeutic target for DME and provide further evidence for neurovascular cross-talk in pathogenesis of DR.

Key Words

Semaphorin3A,

Edema,

Diabetes,

Diabetic Retinopathy,

Retinal Ganglion Cells

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List of Abbreviations

Adherens junction (AJ); age-related macular degeneration (AMD) basement membrane (BM); basic fibroblast growth factor (bFGF); blood-brain barrier (BBB); blood-nerve barrier (BNB); blood- retinal barrier (BRB); central nervous system (CNS); diabetic macular edema (DME); diabetic retinopathy (DR); diabetic nephropathy (DNeph); diabetic neuropathy (DN); Evans blue (EB); epiretinal membrane (ERM); focal adhesion kinase (FAK); glomerular filtration barrier (GFB); ganglion cell layer (GCL); Hôpital Maisonneuve-Rosemont (HMR); human retinal microvascular endothelial cells (HRMEC); human umbilical vein vascular endothelial cells (HUVEC); inner nuclear layer (INL); macular hole (MH); neuropilin 1 (Nrp-1); NG2 proteoglycan (*NG2*); lentivirus (Lv); outer nuclear layer (ONL); pericyte (PC); placental growth factor (PlGF); plasmalemma vesicle associated protein (Plvap); platelet derived growth factor receptor (PDGFR); peripheral nervous system (PNS); proliferative diabetic retinopathy (PDR); Public Health Agency of Canada (PHA); retinal ganglion cells (RGC); semaphorin 3A (Sema3A); small hairpin RNA (shRNA); smooth muscle actin (SMA); spectral-domain optical coherence tomography (SD-OCT); stomatal diaphragms (SD); streptozotocin (STZ); whole-animal tamoxifen-inducible Cre mouse ($Tam^{Cre\text{-}Esr1}$); tight junction (TJ); Type 1 diabetes mellitus (T1DM); Type 2 diabetes mellitus (T2DM); vascular endothelial cadherin (VE-cadherin); vascular endothelial growth factor (VEGF); VEGF Receptor-2 (VEGFR-2); vascular permeability (VP); vesiculo-vacuolar organelles (VVOs).

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Chapter1:

Background

1.1 Diabetes

1.1.1 A major public health issue

Diabetes encompasses a group of diseases characterized by hyperglycemia and glucose intolerance as a result of insulin deficiency and/or impaired sensitivity to insulin. This condition is classified into two groups: Type 1 diabetes mellitus (T1DM), which is characterized by an autoimmune destruction of pancreatic beta cells, and Type 2 diabetes mellitus (T2DM), which occurs in 90% of cases, has a more diverse etiology and manifests itself later in life (*1*).

Diabetes worldwide has reached epidemic proportions, affecting both developed and developing countries. Globally, the prevalence of diabetes in the age group from of 20 to 79—number of diabetics over total population—was estimated at 6.4% in 2010 (285 million adults) and is expected to reach 7.7% by 2030 (439 million adults) (*2*). Specifically in North America, the prevalence of diabetes was reported to be 10.2% in 2010 and represented the highest in the world.

As of 2009 there were approximately 2.4 million Canadians (6.8%) living with diabetes. The Public Health Agency of Canada (PHA) has estimated that for the 11-year period between 1998/99 to 2008/09 the age-standardize prevalence rate of diabetes increased by 70% (*3*). Additionally, assuming incidence rates continue to rise in the context of the 2008/09 mortality rates, the PHA projects that by 2018/19 there will be 3.7 million Canadians with diabetes. It is therefore clear that diabetes represents a major public health issue that will continue to significantly burden healthcare systems given the annual per capita health care cost for diabetics is approximately four times that of non-diabetics.

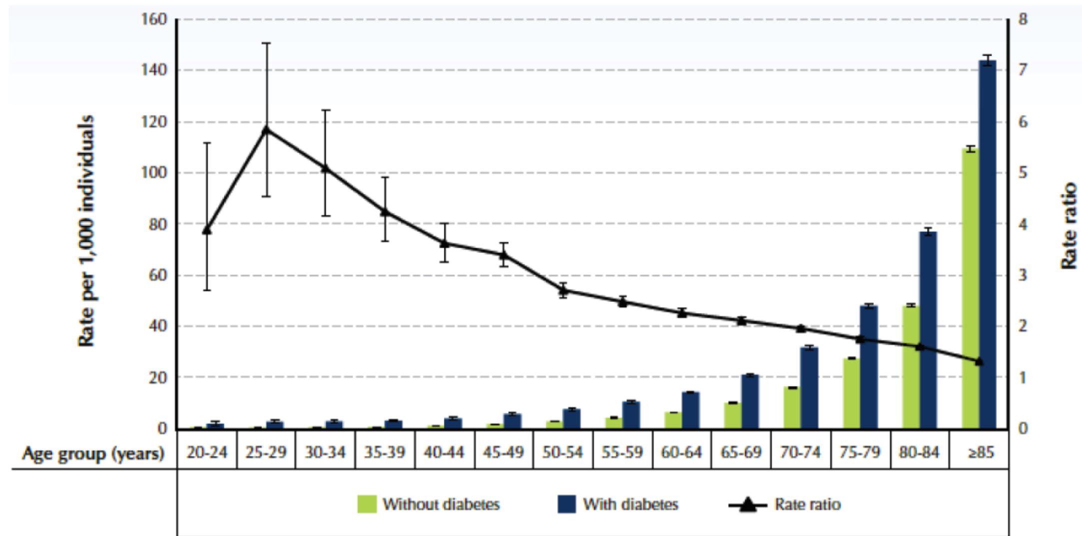
It is therefore crucial to gain further pathophysiological insight to this condition and consequently develop more cost-effective and efficient treatments for this condition (*3-5*).

In terms of the impact of diabetes on mortality rates, Vital Statistics data in Canada underestimate the association between diabetes and mortality because diabetes is rarely recorded as cause of death on death certificates (*3, 6*). For instance, in 2007 3.1% of all deaths were attributed to diabetes as a primary cause, although 29.9% of the total diseased had been diagnosed with diabetes (*6*). In addition, in 2008/09, Canadians aged 20 to 39 and 40 to 74 years showed all-cause mortality rates of 4.2 to 5.8 and 3.6 to 2 times higher, respectively, in diabetics versus non-diabetics (Figure 1a). It is therefore clear that people affected by diabetes are more likely to die prematurely in every age group, and that it is a life-threatening disease.

1.1.2 Complications Related to Diabetes

The underestimated relation between diabetes and death comes from the fact that numerous diabetes- related deaths are reported to arise from the complications associated with diabetes and not from the disease itself (Figure 1b). Diabetes-related comorbidities also contribute significantly to the total burden of the disease on the healthcare system since the medical- and mortality-associated costs of diabetes increase by 3.6 fold (1.024 to 3.701 billion US dollars) when the cost of complications are included (*5*). Therefore, it is necessary to further understand the genesis of diabetes-related comorbidities in order to prolong the life expectancy of patients suffering from this condition.

a



b

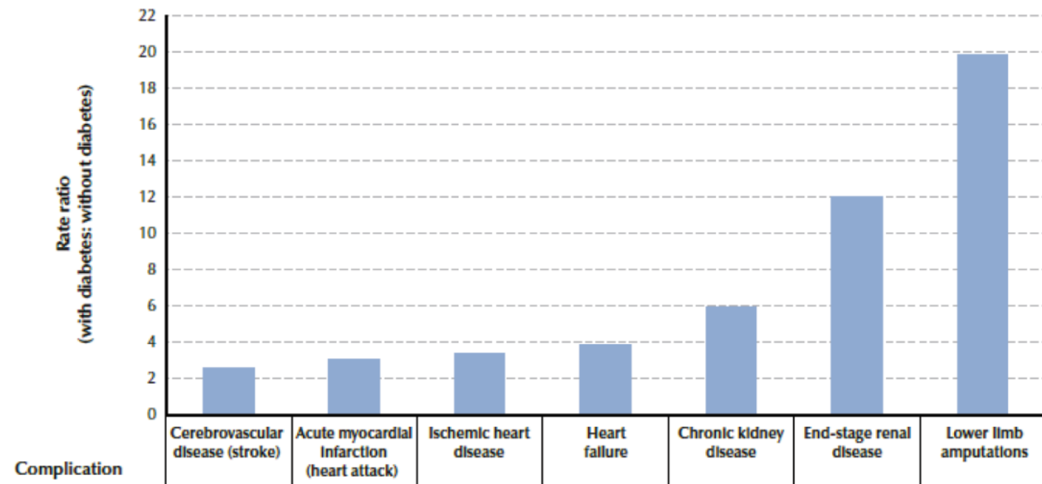


Figure 1. Diabetes: mortality and complications. (a) All-cause mortality rates and rate ratios among individuals aged 20 years and older, by diabetes status, Canada, 2008/09 **(b)** Prevalence rate ratios, standardized to 1991 population, of complications among hospitalized individuals aged 20 years and older, Canada, 2008/09. Source: Public Health Agency of Canada (August 2011); using 2008/09 data from the Canadian Chronic Disease Surveillance System (Public Health Agency of Canada). Modified from (*3*).

Complications secondary to diabetes can be divided in short- and long-term. Short-term complications are associated with: 1) poor glycemic control and hyperglycemia that can lead to infection/slow wound healing, as well as diabetic ketoacidosis (DKA) and hyperglycemic hyperosmolar nonketotic syndrome (HHNS); and 2) inadequate utilization of glycemic control treatments, e.g. insulin, which can trigger hypoglycemic episodes. Nevertheless, the most health-threatening complications that have a more direct impact on quality of life and life-expectancy are those that arise due to long-term exposure to hyperglycemia. These include, but are not limited to cardiovascular, ophthalmic (e.g. retinopathy, glaucoma, cataracts) and renal disease (nephropathy), as well as nerve damage (central and peripheral neuropathy), lower extremity lesions (peripheral vascular pathology, which can lead to amputation), gingivitis/periodontis and depression (*3, 7-11*).

Interestingly, however, the effects of hyperglycemia on the vasculature are the main source of morbidity and mortality in Type 1 and 2 diabetes (*12*). At the macrovascular level, complications arise in the form of coronary artery disease, peripheral arterial disease and stroke, whereas microvascular complications originate mainly in the retina, brain and kidneys (see Discussion for more information on diabetic microvascular pathology on brain and kidney).

1.1.3 Diabetic microvascular pathology in the retina

Diabetic retinopathy (DR) is the most common complication associated with diabetes (*12*). DR also represents the leading cause of blindness in working age adults, affecting approximately 50 % of diabetics (*13*). This form of microvascular diabetic pathology is characterized by a decrease in the barrier function of the endothelium that leads to increased extravasation of plasma components into the underlying interstitium and

tissue. In the retina, as well as in other tissues, diabetes-linked vascular deterioration of the retinal capillaries and endothelial barrier breakdown— blood- retinal barrier (BRB) in this case—have been associated with an overall state of hypoxia, which represents the first stage of diabetic retinopathy. DR generally manifests itself through an initial microvascular degeneration that can induce a destructive and excessive vascularization, during its proliferative stage (Proliferative DR, PDR), in an attempt to reinstate metabolic equilibrium in the hypoxic retina. This shortage in oxygen is believed to be triggered by hemoglobin glycation (i.e. non-enzyme mediated glycosylation), which increases the pigment's oxygen affinity thus hindering efficient delivery of O_2 (*14*). Hypoxia is further enhanced in the diabetic retina as a result of increased resistance to blood flow that reduces capillary circulation to the already stressed neural retina due to: a thickening of the basement membrane (BM; thicker BM reduces the rate O_2 diffusion into the irrigated tissue and decreases capillary elasticity); and an abnormally high blood viscosity, which is most salient in diabetics with retinopathy (*15*).

In addition, PDR is characterized by two prominent pathological features: 1) a decrease in the barrier function of retinal vessels that leads to a detrimental increase in vascular permeability, which leads to vasogenic oedema, retinal thickening and a subsequent loss of central vision; 2) an abnormal and misdirected growth of these leaky vessels towards the vitreous, which can ultimately cause retinal detachment.

However, increased retinal vascular permeability is not only seen during the proliferative stage of DR, which is the most common cause of vision loss in Type 1 diabetics. Vascular leakage can also arise independently, leading to diabetic macular edema (DME), which represents the main cause of loss of visual acuity in Type 2 diabetics (*16*). In DME, the retina secretes vascular endothelial growth factor (VEGF) as a result of

hypoxic conditions, which decreases inter endothelial cells (EC) adherens junction interactions, increases endothelial fenestrations, thus promoting vascular permeability, which leads to retinal thickening and visual complications. As a result, there is a new approach in the treatment of DME that involves the use of anti-VEGF agents that appear to be more efficient and less invasive in mitigating vascular leakage, and therefore edema, than previous treatments such as corticosteroids and photocoagulation (*16*).

Notably, the effects of hyperglycemia on the macro- and microvasculature are the main source of illness and death in diabetes (*12*). As the term indicates, diabetic microvascular pathology involves the microvessels of the organism, which include the arterioles, metarterioles, capillaries and venules. Unlike the macrovessels, e.g. arteries, that are composed of endothelium, internal elastic lamina, basement membrane, external elastic lamina and adventitia, the microscopic vessels of the vasculature are structurally characterized by a much simpler tissue make up. As such, capillaries are primarily composed of endothelium, basement membrane and pericytes. However, the capillary endothelium exhibits a high degree of structural variation depending of the nature and requirements of the tissue it irrigates.

1.2 The vasculature

1.2.1 *The endothelium*

In vertebrates, blood circulates in a closed system from the heart to every tissue in the organism through the arteries, arterioles, capillary beds. Once the tissue is perfused, venules and veins return the blood to the heart, thus completing the circuit. The collective of these structures form the circulatory system, which is in charge of delivering oxygen

and nutrients to the various tissues, and collecting wastes and carbon dioxide from them for their disposal. The common denominator to the different components of the vasculature is the endothelium, formed by a layer of cells (endothelial cells) that lines the lumen through which the blood circulates.

The endothelium exhibits a wealth of phenotypes as determined by the relative expression of numerous junctional and adherence proteins, specialized structures for transport (e.g. channels, transporters, etc.), as well as the coverage of the basement membrane. Different combinations of these components allow the endothelium to adapt to the numerous physiological requirements imposed by the several tissues that the vasculature irrigates. Such heterogeneity of the endothelium can be already observed in hagfish, whose ancestor is the last common ancestor of all modern vertebrates, thus suggesting that heterogeneity evolve as an early and necessary characteristic of ECs (*17*).

During development, ECs arise from mesoderm through the differentiation of hemangioblast/angioblast, yet other cell lineages, such as adipose and neural stem cells, have the ability to transdifferentiate into ECs as well (*18-20*). In addition, ECs have in common very few specific protein or mRNA markers, of which the most uniformly expressed, though not unique to them, are vascular endothelial cadherin (VE-cadherin), platelet/endothelial cell adhesion molecule 1 (PECAM-1) and thrombomodulin (*20*). The varied ontogenical origins of ECs and the difficulty to find characteristic molecular markers reflect, once again, the phenotypic variability that characterizes the endothelium.

From a functional perspective, the endothelium serves several roles including the regulation of vasomotor tone, angiogenesis, innate and acquired immunity, leukocyte trafficking and hemostasis (*20*). Throughout the range of different physiological functions

that the endothelium must perform, it is key that vascular integrity is not compromised for prolonged periods of time. Controlled vascular permeability is crucial for proper transport and delivery of water and solutes between the blood and the underlying interstitium. Conversely, breakdown of vascular barrier function is the hallmark of several pathologies.

1.2.2 Across the endothelium: paracellular and transcellular routes

The molecular exchange across the vasculature occurs mostly at the level of capillaries either via the paracellular route, i.e. in between ECs, or via the transcellular route, through the cell. The former is determined and regulated by two major types of intercellular junctions: a) tight junctions (TJ) composed of occludins and claudins; and b) adherens junctions (AJ) formed by cadherins. Both TJs and AJs not only connect ECs and limit the movement of macromolecules across the endothelium while allowing smaller solutes and water to diffuse, but also contribute to setting up their polarity by establishing the border between luminal and abluminal sides. Some examples of EC-specific junctional proteins include VE-cadherin and claudin-5 (*21, 22*) (Figure 2).

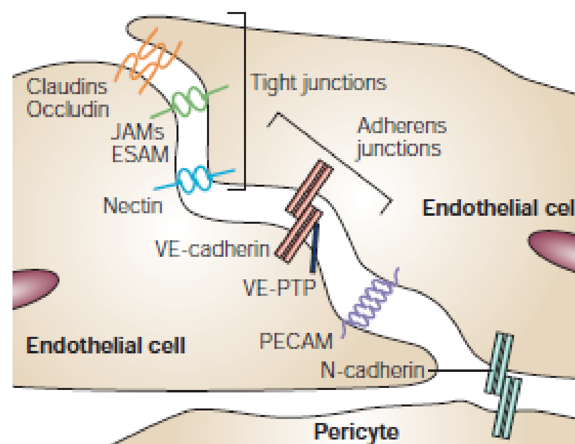


Figure 2. Examples of tight and adherens junctions found in the endothelium. The movement of blood components via the paracellular route is determined and regulated by two major types of intercellular junctions: tight junctions composed of occludins and

claudins; and adherens junctions formed by cadherins. Both TJs and AJs determine the border between luminal and abluminal aspects, thus helping establish proper endothelial cell polarity. Some examples of EC-specific junctional proteins are VE-cadherin and claudin-5. Modified from (*23*).

In addition to the paracellular transport that occurs through the intercellular cleft, molecules can permeate the endothelium directly through ECs via the transcellular route following three different paths. First, transmembrane transporters differentially positioned and distributed along the opposite ends of the ECs, carry water and small molecules vectorially. Second, macromolecules move across the epithelium in membrane-bound carriers in a process denominated transcytosis. This system operates through several components: 1) caveolae (or plasmalemmal vesicles) are plasma membrane spherical invaginations of regular size (70 nm) that occur individually or in grape-like clusters on the luminal and abluminal aspects of ECs (*24, 25*), and whose openings or stomata can contain selectively permeable stomatal diaphragms (SDs) (*26*); 2) vesiculo-vacuolar organelles (VVOs) are conglomerates of interconnected vesicles that span from luminal to abluminal ends across the cytoplasm of ECs and are separated by SDs (*27*). Even though, caveolae and VVOs share a high morphological resemblance, EM data from caveolin 1-knockout mice lacking caveolae (but still containing VVO-like organelles) suggest that they are functionally distinct structures (*28*).

Third, molecules can also diffuse transcellularly through pores or channels that may be selective or not. Examples of such structures include: 1) fenestrae (Latin for “window”), which are regular circular openings that cover the entire length of the EC and run individually or in groups to form a “sieve plate”. Most fenestrae carry fenestral diaphragms (FDs) on the luminal side and, unlike SDs, contain protein tufts that limit their permeability; 2) transendothelial channels (TEC) are pores that span across ECs, arise from

the interconnection of two to four luminal and abluminal caveolae and contain two SDs on each aspect of the EC (*29*). The relative abundance and localization of transporters, caveolae, VVOs, fenestrae, TECs and their related structures determine the phenotypic and, thus, functional heterogeneity of the endothelium (Figure 3).

Another important structure associated with the endothelium is the basement membrane (BM), which is a layer on which the EC monolayer lies. It is made of secreted extracellular proteins that include elastin, collagen IV, enactin/nidogen, heparan sulfate proteoglycans and laminin (*30*). Based on the degree of coverage (i.e. continuous/discontinuous) and organization of the BM, as well as by the presence or absence of fenestrae, the endothelium can be classified into three types: continuous fenestrated, continuous non-fenestrated, and discontinuous/sinusoidal (Figure 3). Continuous non-fenestrated endothelium is found in the capillaries of the brain, retina, lung and skin, as well as in veins and arteries and is characterized by low and highly selective permeability (*20*). In turn, fenestrated continuous is present in tissues where transendothelial transport and filtration are crucial, such as endocrine glands, choroid capillaries, glomeruli and gastric and enteric mucosae. Finally, discontinuous or sinusoidal epithelium is highly permeable and is characteristic of liver vascular beds. Fenestrations in the liver are larger (100-200 vs. 70 nm in diameter), contain gaps within individual cells and lack diaphragms when compared to continuous fenestrated tissue. The different BM arrangements and degrees of fenestrations allow the endothelium to regulate its permeability accordingly and, thus, adapt to the numerous and specific physiological requirements of the various tissues that are irrigated the given vasculature.

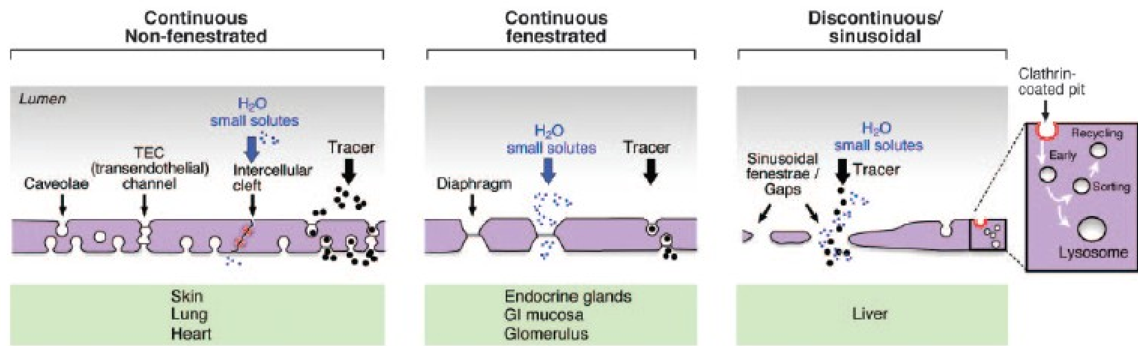


Figure 3. Types of endothelia and their associated components. Continuous non-fenestrated endothelium is found in the capillaries of the brain, retina, lung and skin, as well as in veins and arteries and is characterized by a low and highly selective permeability. Fenestrated continuous endothelium can be encountered in tissues where transendothelial transport and filtration are crucial, such as endocrine glands, choroid capillaries, glomeruli and gastric and enteric mucosa. Discontinuous or sinusoidal endothelium is highly permeable and is characteristic of liver vascular beds. Modified from (*20*) .

1.2.3 The Pericytes

Embedded in the BM, resides another important component of the vasculature: the pericytes (PC). These cells play a unique role as evidenced in their association with the BM and special contacts shared with the ECs (*31*). PCs are found in precapillary arterioles, capillary beds, postcapillary venules and collecting venules (*32*), and have been shown to develop from various embryonic tissues including trunk vessels in the axial and the lateral plate mesenchyme (*33*), neural crest cells in the brain (*34*) and epicardial cells in the splanchnic mesoderm (*35*).

In most vascular beds, pericytes are found embedded in the BM, which separates them from the ECs, and contribute to vascular structure. PCs thus interact with ECs indirectly via fibronectin-rich adhesion plaques (*36*). However, in areas of the endothelium

where the BM is not present or is very thin, pericytes interconnect with ECs through membrane invaginations called *peg-socket contacts* that possess an array of junctional complexes that include tight-, gap- and adherens junctions, such as N-cadherin (Figure 4) (*31, 37*). Interestingly, one pericyte is usually associated with several ECs, thus, suggesting a role in the coordination and integration of cellular communication among neighboring ECs.

PCs provide a plethora of physiological functions. Pericytes show the highest rate of microvascular coverage within the central nervous system (CNS) where they actively partake in the formation of the blood-brain barrier (BBB) during embryogenesis and provide neuroprotection (*38, 39*). During this process, increased pericyte coverage strengthens barrier function and decreases vascular permeability of the developing brain vasculature by: inducing tight junction formation, hindering EC expression of molecules that enhance transcytosis such as Plvap, and limiting immune cell infiltration (*38*).

Similarly, in the retina, pericytes interact with the underlying continuous non-fenestrated endothelium and regulate regional blood flow by contraction through the action of contractile proteins such as actin, myosin and tropomyosin (*37, 40*). They contract in hyperoxic conditions (*41*), as well as in the presence of ATP, and relax when exposed to nitric oxide and CO_2 (*42, 43*). Pericytes have also been involved in the control of capillary structure, inhibition of endothelial cell proliferation and angiogenesis (*14*). Moreover, PCs play an important role in maintaining the integrity of the internal blood-retinal barrier (iBRB), thus controlling the vascular barrier function of the retinal capillaries. This is achieved through EC-pericyte-induced of the tight junctional proteins occludin and zona occludens 1 (ZO-1) during normoxia (*44*).

The maintenance of blood-barriers, BBB and BRB, in the CNS is crucial for the proper functioning of the brain and retina, respectively, and their breakdown is associated with the etiology of several pathologies, including diabetic neuropathy and retinopathy, amyotrophic lateral sclerosis and stroke (*14, 45-47*).

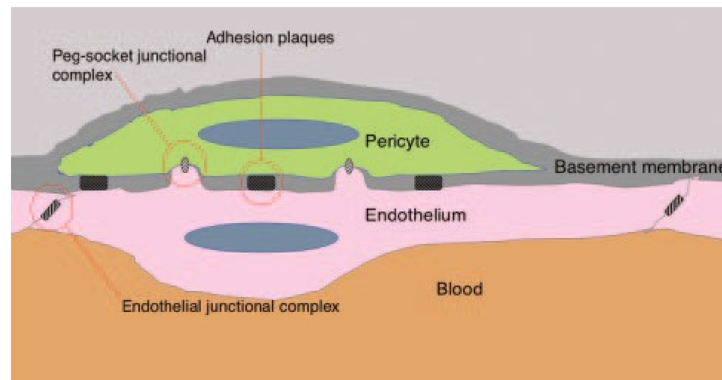


Figure 4. Endothelial-pericyte interaction in the microvasculature. The pericytes are found embedded in the BM in most vascular beds. This layout separates the PC from the ECs .PCs interact with ECs indirectly via fibronectin-containing adhesion plaques. However, in ~~those~~ areas where the BM is not present, pericytes contact ECs through membrane invaginations called peg-socket contacts that posses an array of junctional complexes that include tight-, gap- and adherens junctions such as N-cadherin. One pericyte is typically associated with several ECs, suggesting a role in the coordination and integration of cellular communication among neighboring ECs. Modified from (*31*).

1.3 Nerves and vessels

1.3.1 Common guidance

Since the appearance of metazoans, there has been evolutionary pressure to develop sensory and motor capabilities to allow a more efficient interaction between the organism and its environment. The advent of nervous tissue and subsequent development of a central

nervous system allowed for such a sensory-motor processing. As organisms became larger with more complex body plans, they required a more elaborate nutrient and oxygen delivery system. This was achieved with the development of a convection system such as the circulatory system.

In order for the nervous and circulatory systems to innervate and irrigate a given tissue, vessels and the axons of neurons have to successfully extend and navigate through the developing organism. Interestingly, vessels and nerves follow a common stereotyped path through the body, even though macroscopically they seem to develop distinctly (*48*). Microscopically, nerves and vessels share an analogous structure at their growing fronts: the growth cone of the neuron's axon and the tip cell of the developing/branching endothelium (Figure 5). These specialized structures are highly chemosensitive, malleable and dynamic, constantly projecting and retracting filopodia as a result of attractive and repulsive cues found in the surrounding interstitium. These cues help nerves and vessels find their targets and are determined by the relative concentration of guidance molecules present in their microenvironment, as well as by the differential expression and distribution of the corresponding receptors for these guidance cues.

Beyond anatomical similarities, the molecules and receptors involved in nerve pathfinding have also been found to guide vessels. Examples include netrins and their Unc5 and DCC receptor families (*49-58*); slits and their robo receptors (*59-66*); ephrins and their eph receptors (*67-73*); and semaphorins and their neuropilin and plexin receptors (*49-51, 74-79*).

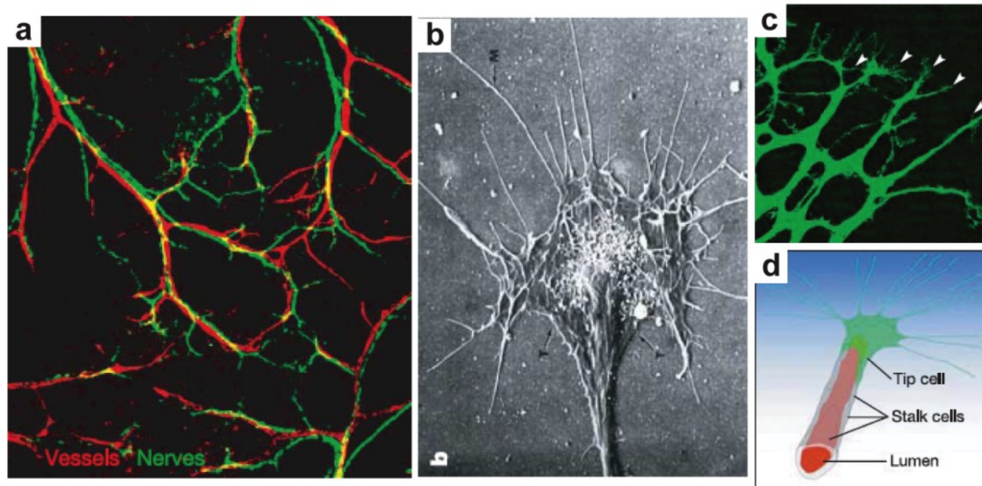


Figure 5. Nerves and vessels follow stereotyped trajectories and share similar cellular structural features. (a) Nerves and vessels follow the same path; murine skin sensory nerves assist in proper arteriogenesis (*80*). **(b)** Axon and growth cone containing several filipodia (scanning electron image). **(c)** Proliferating vascular network, showing a growing front containing tip cells that project several filipodia (white arrowheads). **(d)** Schematic of extending stalk cell attached to the leading filipodia-rich tip cell. Figured modified from (*48, 80*).

Interestingly, however, semaphorins serve other roles beyond axon and vessel guidance. They are expressed in endocrine, gastrointestinal, hepatic, immune, musculoskeletal, renal, reproductive and respiratory systems, where they serve distinct functions (*79, 81-94*). The numerous biological functions of semaphorins arise from their shared ability to affect cytoskeleton dynamics. By altering actin filaments and the microtubule network, semaphorins are able to influence cellular morphology, attachment, migration, polarization and growth; this is a characteristic that makes them an attractive target for study (*92*).

1.3.2 The Semaphorins

While the biological functions of semaphorins have been studied for the past 20 years, novel physiological roles continue to emerge (*74*). There are eight main classes of semaphorins: class 1 and 2 are found in invertebrates, 3 to 7 are found in vertebrates, in addition to the V class that is only found in viruses (*95*). Semaphorins can be secreted, diffusible and act long-distance, or be membrane-bound and act short-distance , but all of them have in common a 500 amino acid sequence, the *semaphorin* domain (*49-51, 74, 92, 96*). This is the most highly conserved domain across all the classes and is responsible for mediating their effects (*97-99*). Semaphorins are ubiquitously expressed in the organism, but they were originally characterized as axonal guidance molecules in the development of nervous system (*100*).

The first dicovered semaphorin of the secreted family was Semaphorin3A (Sema3A), which was initially discovered to promote axonal collapse (*74*). Sema3A signals to target cells through a direct interaction with neuropilin 1 (Nrp-1) receptor, which is also a co-receptor for vascular endothelial growth factor (VEGF) (*101, 102*). Sema3A also acts indirectly via the coreceptor plexinD1 (*92*). In the nervous system, Sema3A generally affects growth cone morphology by destabilizing the peripheral cytoskeleton; it promotes the depolymerization and hinders the repolymerization of F-actin, also trumping microtubule dynamics, thus promoting partial or total cellular structural collapse (*51*). Moreover, Sema3A can induce apoptosis as evidenced: *in vitro*, by exposing primary neurons to this protein; and *in vivo* by the protective effect of its neutralization on retinal ganglion cells (RGCs) following optic nerve axotomy, which is a phenomenon known to lead to RGCs apoptosis (*85, 103-105*).

More recently, Sema3A and its receptors were found to be involved in cardiovascular development (*106*). Endothelial cells not only express Nrp1 and plexinD1, but in the presence of Sema3A, EC proliferation is impaired thus hindering vessel branching (*78, 101*). Interestingly, one study found Sema3A to affect mature vessels by destabilizing inter endothelial cell junctions and thereby promoting increased vascular permeability, in addition to inhibiting VEGF-induced proliferation (*93*).

From a pathological point of view, Sema3A has been found to participate in nervous system pathologies, such as schizophrenia and Alzheimer's disease (*107, 108*). In addition, Sema3A has been shown to block tumor growth and normalize tumor vasculature, as well as blocking and misdirecting physiological revascularization in a model of retinopathy of prematurity (*86, 89*).

It is not surprising that Sema3A serves an important role during development, homeostasis and even pathology, considering its effects on cell survival, proliferation and cytoskeletal dynamics. Even though Sema3A has been mostly characterized in the nervous and circulatory systems, the evidence indicating its involvement in other tissues continues grow.

1.4 The retina

The retina contains the photosensitive tissue of the eye. It is part of the central nervous system (CNS) and is highly organized in three layers of neurally derived cells (the neural retina) that are closely associated with the retinal vasculature (vascular retina), which feeds this highly metabolic tissue. The neural retina, from the outside to the inside of the eye, is composed of: the retinal pigmented epithelium (RPE), the photoreceptor layer (rods and

cones), the outer nuclear layer (ONL, cell bodies of rods and cones), the outer plexiform layer (synapses between photoreceptors and bipolar cells), the inner nuclear layer (INL, nuclei and cell bodies of the bipolar cells), inner plexiform layer (synapses between bipolar cells), the ganglion cell layer (ganglion neuron bodies and nuclei) and the nerve fiber layer (axons from the ganglion neurons) (Figure 6). When light enters the eye, it stimulates the photoreceptors resulting in a chemoelectrical impulse that travels via the afore-mentioned cellular path. From the ganglion neurons' nerve fibers the transduction signal is relayed to the primary visual cortex where it is further processed to produce the phenomenon of vision. Because of the high energy demand required for phototransduction and its related metabolic pathways, the retina consumes oxygen at a higher rate than in any other tissue (*14, 109*). The highest oxidative enzymatic activity of the retina has been localized to the RPE and inner segment of photoreceptors, where there is a constant, arduous synthesis and daily replacement, respectively, of phototransduction-specific cell components (i.e. pigments) (*14, 110-112*). High metabolic activity in these tissues can also be evidenced by the large density of mitochondria present in them, which indicates a strong reliance on aerobic metabolism (*14, 113-115*).

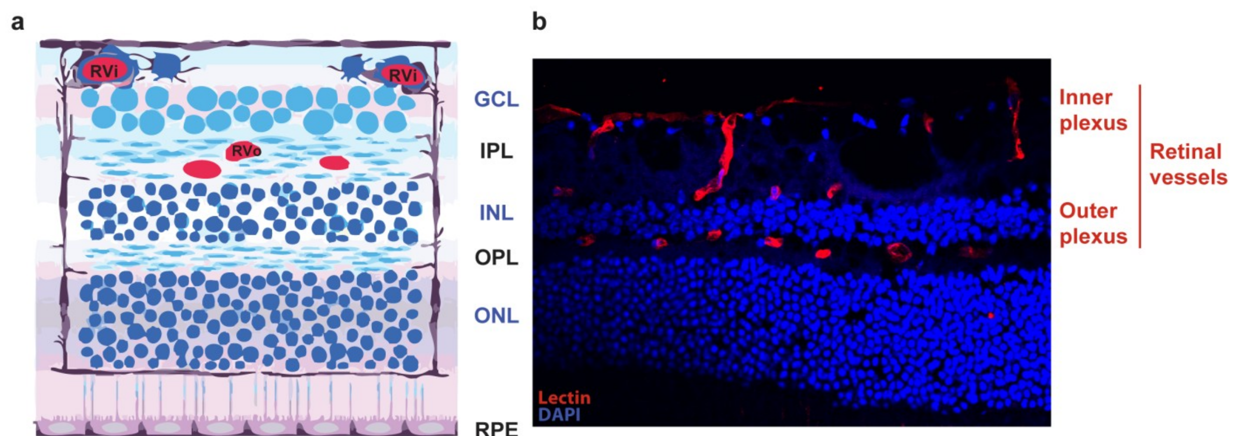


Figure 6. The neural and vascular retina: cross-section. (a) Schematic of the retina and **(b)** immunofluorescent staining of a retina cryosection dyed for nuclei (blue, DAPI) and retinal vessels (red, Lectin) illustrate the close association between the neural and vascular retinae. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigmented epithelium. (a) Figure modified from (*14*).

In order to maintain a high supply of oxygen, the adult mammalian retina is fed by two vascular systems: 1) the choroidal vessels that lie outside the retina, are highly fenestrated and, thus, permeable and provide support to the RPE and photoreceptors; 2) the retinal vessels irrigate the remaining neural components of the retina via an outer plexus that lies between the INL and outer plexiform layer, and a second plexus located in the proximity of the GCL and nerve fibers (*116*). The retinal vasculature posses a similar barrier to that found in the rest of the CNS vasculature termed blood-retina barrier, which makes the retinal endothelium very selectively permeable. In some clinical conditions such as central retinal vein occlusion, retinopathy of prematurity and diabetic retinopathy, the resulting hypoxia that affects the retina leads to a degradations of the BRB, yielding increased and pathological vascular permeability (*14*).

1.5 Hypothesis and Objectives

The pathological barrier-breakdown observed in DR has received considerably less attention than the pathological pre-retinal vascularisation (i.e. *neovascularization*) that is characteristic of the advanced stages of DR (*117-119*). As a result, the current standards of care present side-effects that cannot be ignored. These include increased cataract formation and a detrimental rise in intraocular pressure with intravitreal use of corticosteroid (*117*). Comparably, anti-VEGF therapies, which are generally effective, may be associated with

increased thromboembolic events, possible neuronal toxicity and geographic atrophy when used frequently as long term regimens (*120-122*). In addition, even though panretinal photocoagulation and grid/focal laser are the most widely used forms of treatment for PDR and DME, respectively, they destroy hypoxic retinal tissue, which inadvertently leads to reduced visual field and promotes secretion of pro-angiogenic factors. As a result, these therapeutic limitations emphasize the need for novel pharmacological approaches.

In addition, from a physiological perspective, the presence of VEGF in the diabetic retina does not explain other prominent pathological features of DR, such as the initial vascular decay or the misdirected nature of the pathological neovascularization observed in PDR. While several other factors such as hyperglycemia and oxidative stress have been linked to vascular decay in DR, the mechanisms that precipitate vessel breakdown remain largely ill-defined.

Here we focused on the neurovascular guidance cue semaphorin 3A (Sema3A) and its potential role in mediating barrier function compromise in diabetic retinopathy. Our lab has previously shown that Sema3A participates in vascular degeneration and later blocks physiological vascularization in a different model of proliferative retinopathy that shares common features with PDR and is used to study retinal pathology in the premature newborn retina (*86*). At the same time, Sema3A has been shown to be a strong inducer of vascular permeability (*93*). In this context, we hypothesize that Semaphorin 3A provokes vascular permeability in Diabetic Retinopathy. Thus, we set out:

1) To elucidate the dynamics of semaphorin 3A induction in diabetic retinopathy

2) To evaluate the role of semaphorin 3A in the barrier function of the retinal vasculature

3) To elucidate the mechanism through which semaphorin 3A acts on barrier function

Chapter 2: Article

(Accepted for revision in a peer-reviewed journal)

Neuronal-Derived Semaphorin 3A is an Early Inducer of Vascular Permeability in Diabetic Retinopathy

<u>Contributions by Figure:</u>

Candidate's name: Agustin Cerani (AC)

1. **Figure 1:** ***Sema3A is elevated in the vitreous of human diabetic patients suffering from diabetic retinopathy and in retinal neurons in the early phases of STZ-induced diabetes.***
   Preparation and set up of the entire Figure 1 was performed by AC.
   - **A-D:** OCT and 3D retinal map data were obtained from Dr. Rezende (ophthalmologist). Images were selected by AC.
   - **E:** Human vitreous samples were obtained and provided by Dr. Rezende; western blot was perfomed by Catherine Menard (CM); picture was prepared by AC.
   - **F:** Patient data was organized by AC
   - **G:** drawing modified by AC from Wei Li's design.
   - **H-J:** STZ diabetes induction protocol was carried out by AC. Tissue extraction and preparation was performed by AC. Data analysis, statistics and graph preparation were performed by AC.
   - **K-N:** Retinas were extracted and prepared by AC (except for some stainings and imaging).
   - **O:** Retinas were extracted and prepared by AC; laser capture microscopy (i.e. collection of micro-cuts) was perfomed by AC.

2. **Figure 2:** ***Retinal barrier function is compromised by Sema3A.***
   - **A:** microsurgery/intravitreal injections and Evans Blue Permeation assay (technique adapted and optimized by AC) were carried out by AC. Data analysis, statistics and graph preparation done by AC.
   - **B:** microsurgery/intravitreal injections of Evans Blue performed by AC. Extractions of eyes and sample preparation done by AC.
   - **C:** Part of cell culture of HUVECs, part of statistics and graph preparation by AC. ECIS performed by Chintan Patel.
   - **D:** Drawing by Dr. Sapieha.
   - **E:** Drawing done by AC.
   - **F-H:** Part of HRMEC culture and cell treatment by AC. Blots and quantification by CM. Picture and graph preparation for figure by AC.
   - **I:** HRMEC culture by AC. The rest performed by Dr. Nicolas Tetreault (NT).
   - **J:** HRMEC culture and plan by AC. Treatment and Blot by Dr. Agnieszka Dejda (AD). Preparation for figure by AC.

3. **Figure 3:** ***Targeted silencing of neuron-derived Sema3A or intravitreal neutralization of Sema3A efficiently reduces diabetes-induced retinal vascular permeability.***
    - **A:** STZ diabetes induction protocol was carried out by AC. Tissue extraction and preparation was performed by AC. Immunofluorescence, imaging and panel preparation by NT.
    - **B, E, G:** microsurgery/intravitreal injections and Evans Blue Permeation assay (technique adapted and optimized by AC) were carried out by AC. Data analysis, statistics and graph preparation done by AC.
    - **C:** Perfomed by Nicholas Sitaras (NS).
    - **D:** Microsurgery/intravitreal injections of Lv.shGFP and Lv.shSema3A by AC; STZ diabetes induction protocol was carried out by AC. Tissue extraction and preparation was performed by AC. RT-qPCR by NT. Data analysis, statistics and graph by AC.
    - **F:** Drawing by Wei Li

4. **Figure 4:** ***Conditional knockout of Nrp-1 prevents Sema3A-induced retinal barrier function breakdown.***
    - **A:** Drawing by Ac.
    - **B-D:** Planning, Tamoxifen protocol adaptation and optimization by AC. Samples were extracted and prepared by AC; blot and RT-qPCR by CM. Data analysis, statistics and panel preparation for figure by AC. Immunofluorescence by AD.
    - **E-F:** Planning, Tamoxifen protocol adaptation and optimization by AC. microsurgery/intravitreal injections and Evans Blue Permeation assay (technique adapted and optimized by AC) were carried out by AC. Data analysis, statistics and graph preparation done by AC.
    - **G-I:** Part of HRMEC culture of AC. Blot, quantification and statistics by CM. Panel preparation by CM and AC.
    - **J:** Drawing by Dr. Sapieha.

Note: For Evans Blue (EB) Permeation, Dominique Leboeuf assisted with intravenous injections of EB.

Title: Neuron-Derived Semaphorin 3A is an Early Inducer of Vascular Permeability in Diabetic Retinopathy

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Key words: Semaphorin3A, Edema, Diabetes, Diabetic Macular Edema, Diabetic Retinopathy, Retinal Ganglion Cells, Vascular Permeability.

One Sentence Summary: The neuro-vascular guidance cue Semaphorin3A is produced by retinal neurons early in diabetes and provokes vascular leakage.
Running title: Semaphorin 3A Induces Vascular Permeability in Diabetic Retinopathy

SUMMARY:

The deterioration of the inner blood retinal barrier and consequent macular edema is a cardinal manifestation of diabetic retinopathy and the clinical feature most closely associated with loss of sight. Currently available treatments such as locally administered corticosteroids and anti-VEGF therapies, present several drawbacks. Here we provide the first evidence from both human and animal studies for the critical role of the classical neuronal guidance cue, Semaphorin3A, in instigating pathological vascular permeability in diabetes. We reveal that Semaphorin3A is induced in the early hyperglycemic phases of diabetes within the neuronal retina and precipitates initial breakdown of endothelial barrier function. We demonstrate by a series of orthogonal approaches that neutralization of Semaphorin3A or conditional knockout of its receptor Neuropilin-1 in Tam$^{Cre\text{-}Esr1}$/*Nrp1*$^{flox/flox}$ mice efficiently prevents diabetes-induced retinal vascular leakage. Our findings identify a new therapeutic target for macular edema and provide further evidence for neurovascular cross-talk in the pathogenesis of DR.

HIGHLIGHTS

-The classical neuronal guidance cue Semaphorin3A is induced in the early hyperglycemic phases of diabetes within the neuronal retina.

- Semaphorin3A instigates pathological retinal vascular permeability in type I diabetes.

- Neutralization of Semaphorin3A or its receptor Neuropilin-1 efficiently prevents retinal vascular leakage secondary to diabetes.

INTRODUCTION

Diabetic retinopathy (DR) is the most prominent complication of diabetes and the leading cause of blindness in working age individuals (Kempen et al., 2004). It is characterized by an initial microvascular degeneration followed by a compensatory but pathological hyper-vascularization mounted by the hypoxic retina in an attempt to reinstate metabolic equilibrium (Cheung, 2008; Sapieha, 2012). Although often initially asymptomatic, loss of sight is provoked primarily by diabetic macular edema (DME), vitreous hemorrhages and in advanced cases, pre-retinal neovascularization and tractional retinal detachment (Antonetti et al., 2012; Wang et al., 2012). Of these, DME is the most common cause of central vision loss in diabetics affecting over 25% of patients suffering from diabetes (Moss et al., 1998). It is triggered secondary to the deterioration of the blood-retinal barrier (BRB) and the consequent increase in extravasation of fluids and plasma components into the vitreous cavity. Ultimately, the decrease in retinal vascular barrier function leads to vasogenic edema and pathological retinal thickening.

Although significant effort has been invested in elucidating the mechanisms that govern destructive pre-retinal neovascularization in DR (Silva et al., 2010; Stahl et al., 2010; Wang et al., 2012), considerably less is known about the cellular processes that lead to increased retinal vascular permeability. Consequently, the current standards of care present non-negligible side-effects. These include increased cataract formation and a harmful rise in intraocular pressure with intravitreal use of corticosteroid (Silva et al., 2010). Similarly, anti-VEGF therapies, which in general exhibit respectable safety profiles, may be associated with increased thromboembolic events (Stewart, 2012), possible neuronal toxicity (Robinson et al., 2001) and geographic atrophy (Comparison of Age-related Macular Degeneration Treatments Trials Research et al., 2012; Group et al., 2011) when used as frequent long term regiments. Moreover, the first and most widely used form of treatment is panretinal photocoagulation, either for proliferative diabetic retinopathy PDR or grid/focal laser for DME. Laser-based photocoagulation approaches destroy hypoxic retinal tissue secreting pro-angiogenic factors and inadvertently lead to reduced visual field or central or paracentral scotomas. These therapeutic limitations highlight the need for novel pharmacological interventions.

Current investigations into the molecular mechanisms that cause DME have largely focused on VEGF. This may in part be attributed to the fact that the prominent clinical features of DR have led to the general inference that it is entirely of a microvascular nature. Yet evidence points to early changes in the neural retina (Kern and Engerman, 1996) (Barber et al., 2005; Barber et al., 1998; Gastinger et al., 2008). While there is irrefutable evidence for a neurovascular link in the progression of DR (Antonetti et al., 2012), neurovascular cross-talk has received limited attention in the context of DR pathogenesis. Consistent with a breakdown in neurovascular cross-talk in ischemic retinopathies, we have recently shown that Semaphorin 3A (Sema3A), a classic neuronal guidance cue that also affects endothelial cell behaviour is produced by stressed retinal ganglion neurons (RGCs) and partakes in deviating neo-vessels towards physiologically avascular regions of the eye (Joyal et al., 2011).

In neurons, binding of Sema3A to its cognate receptor Neuropilin-1 (Nrp-1) provokes cytoskeletal collapse (Takahashi et al., 1999); the transduction mechanism in endothelial cells remains ill-defined (Gelfand et al., 2009). Neuropilin-1 has the particular ability to bind two structurally dissimilar ligands via distinct sites on its extracellular domain (Gluzman-Poltorak et al., 2001; Lee et al., 2002; Mamluk et al., 2002). It binds Sema3A (Klagsbrun and Eichmann, 2005; Miao et al., 1999) provoking cytoskeletal collapse and $VEGF_{165}$ (Gluzman-Poltorak et al., 2001; Klagsbrun and Eichmann, 2005; Klagsbrun et al., 2002; Mamluk et al., 2002) enhancing binding to VEGFR2 and thus increasing its angiogenic potential (Soker et al., 2002). Crystallographic evidence revealed that $VEGF_{165}$ and Sema3A do not directly compete for Nrp-1 but rather can simultaneously bind to Nrp-1 at distinct, non-overlapping sites (Appleton et al., 2007). Moreover, genetic studies show that Nrp-1 distinctly regulates the effects of VEGF and Sema3A on neuronal and vascular development (Vieira et al., 2007). Of note, it was proposed that similar to VEGF, Sema3A may itself promote vascular permeability (Acevedo et al., 2008); a counter-intuitive observation given the divergent biological roles of VEGF and Sema3A. However, the role of Sema3A in mediating the breakdown of barrier function such as that observed in diabetic retinopathy had to date not been explored.

Here we provide the first evidence for the role of Sema3A in disrupting retinal barrier function in diabetic retinopathy. We demonstrate in both human patients and animal

models that ocular Sema3A is robustly induced in the early stages of diabetes and mediates via Nrp-1, the breakdown of the inner blood retinal barrier. Neutralizing Sema3A may represent an attractive alternative therapeutic strategy to counter pathologic vascular permeability in DR.

RESULTS

Sema3A is elevated in the vitreous of human patients suffering from diabetic retinopathy.

In order to evaluate the potential role of Sema3A in mediating the edematous phenotype observed in DR, we first sought to determine the presence of this guidance cue in the vitreous of patients suffering from DME. Vitreous was recovered during standard vitroretinal surgery from 7 patients. Four samples were obtained from patients suffering from DME and 3 from control patients (non-vascular pathology) undergoing surgery for macular hole (MH) or epiretinal membrane (ERM). Spectral-domain optical coherence tomography (SD-OCT) was performed and 3D retinal maps were generated to evaluate the extent of retinal damage and edema. In contrast to controls, sampled DME patients showed significant retinal swelling, specifically in the macular and peri-macular zones (**Fig 1a-d**).

Consistent with a prospective role in DME, Western blot analysis of patient vitreous revealed that Sema3A (125kDa) was robustly induced in most patients affected by DME (**Fig 1e**). In addition, a second heavier Sema3A band (>200 kDa) was detected in DME patients and corresponds to a reported functional Sema3A dimmer (Koppel and Raper, 1998). Detailed patient characteristics are presented in **Figure 1f**. These data on human subjects provide the rational to explore the role of Sema3A in the context of diabetes-induced retinal vasculopathy.

Neuronal Sema3A is upregulated in the early phases of streptozotocin-induced diabetes.

Given the elevated levels of Sema3A in the vitreous of DME and PDR patients, we sought to elucidate the dynamics and pattern of Sema3A expression in a mouse model of T1DM. Streptozotocin (STZ) was administered to ~6 week-old C57BL/6J mice and

glycemia monitored according to the scheme depicted in **Figure 1g**. Mice were considered diabetic if their non-fasted glycemia was higher than 17 mM (300 mg/dL).

As early as 4 weeks after induction of diabetes, retinal levels of Sema3A where over 2-fold higher in STZ treated mice when compared to vehicle injected controls (*P*=0.0045, n=5) (**Fig. 1h**). Significantly higher retinal levels of Sema3A persisted at 8 weeks (*P*=0.0011, n=8). Importantly, throughout these early time points of diabetes, VEGF levels in STZ-treated mice remained at similar levels to that observed in vehicle treated congener mice as has been previously described (Mima et al., 2012). As expected, at all analyzed time-points, STZ-treated mice showed pathologically elevated blood glucose levels of ~30mM (p<0.0001 for both 4 and 8 weeks of diabetes) (**Fig 1i)**.

Importantly, the rise in Sema3A expression was an early event in pathogenesis as it preceded pericyte loss as both STZ and vehicle-treated mice showed no significant difference in transcript levels for pericyte markers platelet derived growth factor-receptorβ (*PDGFR-β)* (*P*=0.219, n=11), NG2 proteoglycan (*NG2*) (*P*=0.316, n=4), and smooth muscle actin (*SMA*) (*P*=0.494, n=4) (**Fig 1j).** Similarly, immunohistochemistry on retinal flatmounts from control and STZ animals confirmed similar vascular coverage by NG2 and SMA-expressing pericytes (**Fig 1k**).

We next investigated the cellular source of Sema3A in the diabetic retina. Immunohistochemistry on retinal cryosections revealed that Sema3A was strongly expressed by retinal neurons of the ganglion cell layer (GCL) (**Fig 1l&m**). Co-localization with the RGC marker *β*III-tubulin confirmed that retinal ganglion cells (RGCs) were an important source of Sema3A within the diabetic retina (**Fig 1m; inset**). Consistent with this retinal immuno-localization, laser-capture micro-dissection of the retinal ganglion cell layer from normal and diabetic mice followed by quantitative RT-PCR revealed a 5-fold increase of Sema3A transcript in RGCs from STZ retinas (*P*=0.014, n=3) (**Fig 1n&o**). These data provide evidence for the local production of Sema3A by diabetic neurons in close proximity to the retinal vascular plexus and agree with a role for Sema3A in mediating the vascular phenotype associated with DME and PDR.

Retinal barrier function is compromised by Sema3A

Given the increase in retinal Sema3A levels observed in the vitreous samples of patients with DME and mouse retinas in the early stages of diabetes (**Fig 1**), we proceeded to investigate the propensity of Sema3A to disrupt vascular barrier function. A single intravitreal injection of Sema3A (100µg/ml) into adult mouse retinas resulted in a significant ~2-fold increase (**Fig 2a**; $P<0.01$, n=3 distinct experiments with a total of 9 mice) in retinal vascular permeability as determined by Evans Blue (EB) permeation. This increase was similar to that observed with intravtireal administration of VEGF (50µg/ml) (**Fig 2a;** $P<0.05$, n=3 distinct experiments with a total of 9 mice) or a combination of both Sema3A and VEGF (**Fig 2a**; $P<0.001$, n=3 distinct experiments with a total of 9 mice). The propensity of Sema3A to induce vascular leakage was corroborated by confocal imaging of retinal sagittal sections where increased EB permeation throughout the retina (red) signifies elevated plasma albumin extravasation and translates into increased retinal edema (**Fig 2b)**.

Further evidence for the ability of Sema3A to compromise endothelial barrier function, was obtained from real-time analysis of trans-endothelial electric resistance (**Fig 2d**). Treatment of an intact monolayer of endothelial cells with Sema3A reduced endothelial monolayer impedance (interval from 3.26h to 6h = $0.048>P>0.009$; n=3) and hence a drop in barrier function in the first 6 hours by a magnitude similar to, yet lower than VEGF (interval from 1.12h to 6h = $0.045>P>0.001$; n=4)(**Fig 2c**).

We next proceeded to determine if Sema3A activated classical signaling pathways that have reported roles in promoting vascular permeability. In this respect we investigated, by Western blot analysis, the activation profiles of Src and focal adhesion kinase (FAK) that are known to transduce extracellular signals that provoke the loosening of endothelial cell tight junctions(Acevedo et al., 2008; Eliceiri et al., 1999; Scheppke et al., 2008) (**Fig 2e**). Stimulation of Human Retinal Microvascular Endothelial Cells (HRMECs) by either Sema3A (100ng/ml) or VEGF (50ng/ml) lead to robust phosphorylation of Src at Tyr416 in the activation loop of the kinase domain which is reported to enhance enzyme activity (Hunter, 1987) (**Fig 2f**). In turn, FAK was phosphorylated on Tyr576 and 577 (sites for

Src-kinases) (**Fig 2g**). Ultimately, the tight junction protein VE-cadherin became phosphorylated on tyrosine-731, which is a posttranslational modification associated with increased vascular permeability (Potter et al., 2005; Schlaepfer et al., 1994) (**Fig 2h**). Consistent with the above data on retinal permeability (**Fig 2a,b**), we did not observe an additive or synergistic effect when simulation of HRMECs was performed with a combination of Sema3A and VEGF suggesting a potential eventual convergence of signaling pathways for both factors.

The ability of an endothelial cell to maintain intact intra-cellular junctions dictates the quality of barrier function. Consistent with a role in inducing vascular permeability, confocal microscopy of Sema3A-treated HRMECs revealed pronounced formation of vascular retraction fibers as determined by VE-cadherin and phalloidin staining (white arrows; **Fig 2i**). The retraction was similar to that observed with VEGF alone or with a combination of VEGF and Sema3A. Importantly, at the doses employed in our study (100-200ng/ml), Sema3A did not induce cell death or apoptosis as determined by assessment of activation (cleavage) of caspase-3 (**Fig 2j**). These data support role of Sema3A in mediating the breakdown of endothelial cell barrier function and further substantiate the involvement of Sema3A in diabetes-induced retinal vascular permeability.

Inhibition of neuron-derived Sema3A efficiently reduces pathological vascular permeability in T1DM.

To investigate the therapeutic potential of blocking Sema3A in diabetic retinopathy, we proceeded to inhibit Sema3A using 2 distinct approaches, namely virally delivered interference RNA or a Sema3A trap. The magnitude of retinal vascular leakage was assessed 8 weeks after administration of STZ in adult mice. At this time-point, flat-mount retinas from STZ mice show elevated expression of phosphorylated VE-cadherin in lectin-stained retinal endothelial cells (**Fig 3a**) and animals have a significant ~57% increase in retinal vascular leakage (**Fig 3b)** (*P*=0.027; n=4 distinct experiments with a total of 12 mice).

Recent evidence suggests that retinal neurons exert an important influence on the blood vessels that perfuse them (Antonetti et al., 2012; Binet et al., 2013; Fukushima et al.,

2011; Joyal et al., 2011; Kim et al., 2011; Sapieha et al., 2008). In light of the robust expression of Sema3A in diabetic RGCs (**Fig 1**), we sought to inhibit production of this guidance cue directly in RGCs using a lentiviral (Lv) vector carrying small hairpin RNAs (shRNAs) against Sema3A (Joyal et al., 2011). We generated Lv vectors with a VSVG capsid which exhibits high tropism for RGCs when delivered intravitreally (Binet et al., 2013; Joyal et al., 2011; Sapieha et al., 2008) (**Fig 3c**). Efficiency of this approach was confirmed as a single intravitreal injection of Lv.shSema3A at 5 weeks of life, (1 week prior to STZ administration) lead to a significant ~63% reduction in retinal Sema3A expression at the 8 week time point after STZ administration when all analysis was carried out (*P*=0.0014, n=3 distinct experiments with a total of 9 mice) (**Fig 3d)**. Lv.shSema3A-mediated reduction in retinal Sema3A expression provoked a ~50% decrease in vascular leakage when compared to control Lv.shGFP (*P*=0.022, n=3 distinct experiments with a total of 9 mice) (**Fig 3e)** thus validating the strategy of targeting Sema3A in neurons of the GCL to counter pathological vascular leakage in diabetes.

In order to therapeutically neutralize vitreal Sema3A, we employed recombinant mouse(rm) soluble Nrp-1 as a bivalent trap for both Sema3A and VEGF. Neuropilin-1 is a single-pass receptor with its extracellular domain subdivided into distinct sub-domains of which a1a2 bind Sema3A and b1b2 bind VEGF (Geretti et al., 2008) (**Fig 3f**). Intravitreal injections of rmNrp-1 at 6 and 7 weeks after STZ administration lead to a ~50% reduction in retinal permeability when compared to vehicle injected controls, as measured at 8 weeks post STZ (*P*=0.012, n=6 distinct experiments with a total of 18 mice) (**Fig 3g**). This reduction was of similar magnitude to that observed with gene silencing of Sema3A (**Fig 3e**). Given that VEGF is not increased in diabetic retinas at this early time point while Sema3A is robustly induced (**Fig 1**), the observed reduction of Evans Blue permeation in rmNrp-1 injected retinas is likely attributed to neutralization of Sema3A. Together, these data suggest that neutralization of Sema3A in the diabetic retina is an effective strategy to reduce vasogenic edema.

Conditional knockout of Nrp-1, prevents Sema3A-induced retinal barrier function breakdown

In light of Nrp-1 being the cognate receptor for Sema3A, we sought to determine whether deletion of *Nrp-1* protects against Sema3A-induced vascular permeability. Because systemic germline deletion of *Nrp-1* is embryonic lethal (Jones et al., 2008; Kawasaki et al., 1999; Kitsukawa et al., 1997), we generated a whole-animal tamoxifen-inducible (Tam-inducible) Cre mouse ($Tam^{Cre\text{-}Esr1}$) to induce the conditional deletion of exon 2 of *Nrp-1* (**Fig 4a**). To validate Cre recombination at the *Nrp-1* locus and confirm disruption of *Nrp-1 in vivo*, $Tam^{Cre\text{-}Esr1}$ mice were crossed with $Nrp1^{fl/fl}$ mice. Progeny were systemically administered tamoxifen (400µg/mouse) or vehicle over a period of 5 consecutive days at 6-9 weeks of age. This dosing regimen lead to an efficient knockout of Nrp-1 in the vascular system as determined by Western blot (**Fig 4b**) and qPCR (*P*=0.0012) (**Fig 4c**) and resulted in an near complete absence of Nrp-1 in retinal vessels (**Fig 4d**). Tam-treated $Tam^{Cre\text{-}Esr1}/Nrp1^{fl/fl}$ mice did not show any difference in body weight, size or open-field activity when compared with littermates from 4 through 20 weeks of age (data not shown). Importantly, Tam-treated $Tam^{Cre\text{-}Esr1}/Nrp1^{fl/fl}$ mice with disrupted retinal vascular *Nrp-1* were protected against Sema3A-induced retinal vascular permeability (*P*=0.36; n=7 distinct experiments with 21 mice) (**Fig 4e;** while control $Tam^{Cre\text{-}ESR1}/Nrp1^{+/+}$ controls showed 3-fold higher vascular leakage in response to Sema3A (*P*=0.00065; n=3 distinct experiments with a total of 9 mice). Conversely, disruption of *Nrp-1* did not influence VEGF-induced vascular retinal permeability ($Tam^{Cre\text{-}Esr1}/Nrp1^{fl/fl}$ - Vehicle vs VEGF: Ttest *P*=0.0024, n=3; $Tam^{Cre\text{-}Esr1}/Nrp1^{+/+}$ - Vehicle vs VEGF: Ttest *P*=0.032, n=3) (**Fig 4f**) suggesting that VEGF-induced retinal vascular permeability is independent of Nrp-1 as previously suggested (Pan et al., 2007). In line with a role for Nrp-1 in mediating Sema3A-induced vascular permeability, knockdown of Nrp-1 in HRMECs by Lv.shNrp1 prevents phosphorylation of Src, FAK and VE-Cadherin (**Fig 4 g-i**). Collectively, these data confirm that Sema3A-mediated inner-blood retinal barrier function breakdown is Nrp-1 dependent.

DISCUSSION

Therapeutic strategies to treat complications associated with diabetic retinopathy until recently consisted predominantly in controlling systemic metabolic deregulation (Silva et al., 2010). While laser photocoagulation and targeted treatments such as locally

administered corticosteroids and recently approved anti-VEGF therapies are currently available, their off-target effects underscore the need to explore novel therapeutic avenues. In the present study, we provide the first evidence that Sema3A provokes vascular barrier breakdown in the early phases of diabetic retinopathy and ultimately precipitates DME when vascular pericyte coverage is still unperturbed. While the biological functions of semaphorins have been studied for the past 20 years (Luo et al., 1993), novel physiological roles continue to emerge (Acevedo et al., 2008; Bernard et al., 2012; Bouvree et al., 2012; Fukushima et al., 2011; Gu et al., 2002; Gu et al., 2003; Guttmann-Raviv et al., 2007; Joyal et al., 2011; Kim et al., 2011; Le Guelte et al., 2012; Maione et al., 2009; Matsuoka et al., 2011; Serini et al., 2003; Suto et al., 2007). We demonstrate that in a healthy mature retina, Sema3A is modestly expressed, whereas in diabetes, retinal ganglion neurons which are in intimate proximity of retinal vessels (Sapieha, 2012) significantly increase production of this classic guidance cue. Through its cognate receptor Nrp-1, Sema3A provokes loosening of endothelial cell junctions and leads to vasogenic edema (**Fig 4j**).

Sema3A presents itself as an attractive candidate for therapeutic neutralization in adult ocular vasculopathies given that its physiological roles are largely limited to embryogenesis. In addition, further properties that make Sema3A a noteworthy drug target are its ability to induce apoptosis and promote cytoskeleton remodeling (Guttmann-Raviv et al., 2007; Klagsbrun and Eichmann, 2005; Miao et al., 1999; Neufeld et al., 2012), which are both salient features of ischemic and proliferative retinopathies such as that of diabetes (Duh, 2011; Sapieha et al., 2010; Wang et al., 2012). Importantly, at the early time points in disease where levels of Sema3A are elevated, VEGF levels remain low and relatively unchanged compared to non-diabetic controls. Given these expression kinetics, inhibition of Sema3A may be warranted in the early phases of disease when low VEGF levels are not yet reflective of level of pathological vascular permeability.

While the introduction of anti-VEGF therapy to attenuate neovascular age-related macular degeneration (AMD) and more recently DME has resulted in a profound change in clinical treatment paradigms, inhibition of a molecule that plays key roles in vascular homeostasis warrants contemplation in a condition such as diabetes where vascular

stability is already compromised. Hence neutralizing Sema3A instead of currently targeted factors such vaso-protective and neuroprotective VEGF and placental growth factor (PlGF) may provide a valid therapeutic alternative for diabetic retinopathy. Exclusive neutralization of Sema3A in the early phase of diabetes would thus permit the baseline levels of VEGF present to play out their protective ocular and systemic roles (Robinson et al., 2001; Stewart, 2012). Alternatively, in later phases of disease, neutralization of Sema3A may also be sought as an adjunct to currently employed anti-VEGF therapies such as bevacizumab (Avastin), ranibizumab (Lucentis) or aflibercept (Eylea) given that both proteins seem to have disparate expression kinetic during disease progression yet have similar effects on retinal vascular barrier function. In this regard soluble Nrp-1 could be employed as a bivalent Semaphorin3A and VEGF trap, due to its intrinsic ability to bind both molecules (20-25).

Using $Tam^{Cre\text{-}Esr1}/Nrp1^{fl/fl}$ to conditionally delete *Nrp-1* in mature animals, we provide *in vivo* evidence for the role of this receptor in mediating the effects of Sema3A on vascular barrier function breakdown. Nrp-1 also acts as a co-receptor for $VEGF_{165}$ increasing its affinity towards VEGF Receptor-2 (VEGFR-2) thus enhancing VEGFR-2 mediated chemotaxis, growth of endothelial cells and angiogenesis (Miao et al., 2000; Soker et al., 2002; Soker et al., 1998). However, the effects of VEGF on vascular permeability do not seem to require Nrp-1 since $Tam^{Cre\text{-}Esr1}/Nrp1^{fl/fl}$ mice showed an identical magnitude of retinal edema as control $Tam^{Cre\text{-}Esr1}/Nrp1^{+/+}$ mice secondary to elevated VEGF administration. This is consistent with previous studies demonstrating that blocking NRP-1 with distinct monoclonal antibodies had no effect on VEGF-induced vascular permeability(Pan et al., 2007). It is likely that the point of signaling convergence lies downstream of Nrp-1 given that co-stimulation of ECs with both VEGF and Sema3A does not enhance permeability beyond levels noted when each is applied independently,

Because there is a certain mechanistic overlap in the etiologies of several ocular vasculopathies such as neovascular AMD, diabetic retinopathy, retinal vein occlusions and retinopathy of prematurity, treatment paradigms such as anti-VEGFs are being used or explored for more than one ocular vasculopathy. Similarly, Sema3A neutralization may be useful for treating vessel leakage and edema associated with neovascular AMD(Yancopoulos, 2010). Moreover, we have previously demonstrated in a model of

oxygen induced retinopathy(Smith et al., 1994) that in late stages of pathological retinal neovascularization, neuronal-derived Sema3A forms a repulsive barrier that hinders normal revascularization by misdirecting vessels away from the ischemic retina(Joyal et al., 2011). Hence inhibition of Sema3A could potentially simultaneously benefit the two main pathognomonic features of DR, i.e. barrier function deterioration and pathological pre-retinal neovascularization which separately can lead to loss of vision in diabetes.

By studying neurovascular interplay in diabetes, we obtained insight into a novel fundamental neuro-vascular mechanism that mediates pathological barrier function breakdown in diabetic retinopathy. In doing so, we identify a potential novel therapeutic target, Sema3A, that may be involved beyond DME in diseases where elevated vascular permeability is a contributing factor such as neovascular AMD, retinopathy of prematurity, cancer and stroke.

Materials and Methods:

Human samples

Approval of human clinical protocol and informed consent form by Maisonneuve-Rosemont Hospital (HMR) ethics committee (*Ref. CER: 10059*) and rectuitment of patients for local core vitreal biopsy sampling from patients afflicted with T1DM or T2DM. The entire procedure was performed as an outpatient procedure in the minor procedure room within the ambulatory clinic from the Department of Ophthalmology at Maisonneuve-Rosemont Hospital. All instruments were opened and handled in a sterile manner. The study conforms to the tenets of the declaration Helsinki and was approved by the Institutional Review Board of the Maisonneuve-Rosemont Hospital affiliated with the University of Montreal.

Vitrectomy

All patients previously diagnosed with DME or PDR were followed and operated by a single vitreoretinal surgeon (FAR). Control patients were undergoing surgical treatment for non-vascular pathology (ERM or MH) by the same surgeon. A 5% povidone-iodine solution was used to clean the periocular skin and topical instillation into the eye and

within the cul-de-sac was left in place for 5 minutes. For sampling of DME vitreous, patients were then draped in a standard sterile manner with placement of a lid speculum. A 27-ga self-retaining line (Insight Instruments, Stuart, FL) for balanced salt solution (BSS) infusion was first placed, followed by a 29-ga chandelier placement connected to a mercury vapor light source (Synergetics, O'Fallon, Mo). The surgical view during the procedure was provided through a surgical operative microscope and a Volk contact lens (Volk direct image 1.5x magnifying disposable vitrectomy lens, OH, USA). The vitrectomy was performed using a 25-ga sutureless Retrector® system (Insight Instruments, Stuart, FL) in all patients. The model used in the study is a portable, battery-powered system with a maximum cut rate of 600 cpm and features a single-use retractable sheathed guillotine cutter (25-ga) with an in-built needle (23-ga). The needle was introduced bevel down through displaced conjunctiva in an oblique one-plane tunnel into the vitreous cavity 3-4mm from the limbus. With the exception of the portable vitrector motor handpiece, which was placed within a sterile plastic cover when in use, all other instruments used were sterile and disposable. For PDR and control patients a standard 25-gauge 3-port *pars plana* vitrectomy was performed in the operating room.

Animals

All studies were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of the University of Montreal in agreement with the guidelines established by the Canadian Council on Animal Care. C57Bl/6 wild-type were purchased from The Jackson Laboratory. Tamoxifen-inducible (Tam-inducible) Cre mice ($Tg^{Cre\text{-}Esr1}$; no. 004682) and Neuropilin 1 floxed mice ($Nrp1^{tm2Ddg}$/J; no. 005247) were purchased from The Jackson Laboratory.

Streptozotocin (STZ) mouse model

C57BL/6J mice of 6- to 7-week were weighted and their baseline glycemia was measured (Accu-Chek, Roche). Mice were injected intraperitoneally with streptozotocin (Sigma-Alderich, St. Lois, MO) for 5 consecutive days at 55 mg/Kg. Age-matched controls were injected with buffer only. Glycemia was measured again a week after the last STZ

injection and mice were considered diabetic if their non-fasted glycemia was higher than 17 mM (300 mg/dL).

Real-time PCR analysis

RNA was isolated using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and digested with DNase I to prevent amplification of genomic DNA. Reversed transcription was performed using M-MLV reverse transcriptase and gene expression analyzed using SybrGreen in an ABI Biosystems Real-Time PCR machine. β-actin was used as a reference gene.

Laser-capture microdissection

Eyes were enucleated from 14-week old adult C57BL/6J that had been diabetic for 8 weeks and flash-frozen in OCT. We then cut 12µm sections using a Leica cryostat at -20˚C and air-dried for 10min. We dissected retinal layers using a Zeiss Observer microscope equipped with a Palm MicroBeam device for laser-capture microdissection. We isolated mRNA from these sections and performed qPCRs as described above.

Western-blotting

Equal volumes of vitreous fluid from PDR and controls (20uL), DME and controls (40uL) or lysates (30ug of protein as determined by BCA assay, Sigma) from HRMECs cultured in endothelial growth media 2 for microvascular cells (EGM-2 MV, Lonza) treated after 2-hours of starvation in endothelial basal media (EBM-2, Lonza) with 50 ng/mL $VEGF_{165}$, 100 ng/mL Sema3A or vehicle (EBM-2). For assessment of retinal protein levels, we enucleated eyes at varying time points and rapidly dissected and homogenized retinas. Protein concentrations were assessed by BCA assay (Sigma), and then 30ug of protein analyzed for each condition by standard SDS-PAGE technique. Antibodies used for Western-blotting are: Nrp-1 (R&D Systems, #AF566), pVE-Cadherin (Invitrogen, #441145G), Src (Cell Signaling, #2108), pSRC (Cell Signaling, #2101), FAK (Cell Signaling, #3285), pFAK (Cell Signaling, #3281), b-Actin (Sigma, #A2228), Sema3A (Santa Cruz, #sc-1148 OR ABCAM #ab23393).

Immunohistochemistry

To localize protein expression, eyes were enucleated from mice and fixed in 4% paraformaldehyde for 4h at RT and incubated in 30% sucrose overnight and then frozen in OCT compound. We then embedded the whole eye in optimal cutting temperature compound at -20˚C and performed 12um serial sections. We carried out immunohistochemistry experiments and visualized the sections with an epifluorescent microscope (Zeiss AxioImager) or or confocal microscope (Olympus confocal FV1000). Antibodies used for immunohistochemistry are: Sema3A (ABCAM #ab23393), Smooth Muscle Actin (SMA) (ABMCA, #ab7817), NG2 proteoglycan (ABCAM #ab50009) and βIII Tubulin (ECM). Secondary antibodies are Alexa 594 (Invitrogen, #A11005) and Alexa 488 (Invitrogen, #A11008).

For visualization of pan-retinal vasculature, flat-mounted retinas were stained with stained with fluoresceinated Isolectin B4 (Alexa Fluor 594 – I21413, Molecular Probes) in 1 mM $CaCl_2$ in PBS for retinal vasculature. For assessment of vascular permeability (see Evans Blue -EB- permeation), we injected mice in the vitreous chamber with vehicle, Sema3A and VEGF. EB was injected intravenously and 2 hours later, eyes were harvested and retinas were dissected for flatmount or prepared for cryosections and visualization under a confocal microscope (Olympus confocal FV1000).

Evans Blue Permeation Assay

Retinal EB permeation was performed with modifications as described in *(Xu et al., 2001)*. EB was injected at 45 mg/kg intravenously and it was allowed to circulate for 2 hours prior to retinal extraction. Evans Blue permeation was expressed relative to controls.

Electric Cell-substrate Impedance Sensing (ECIS) Assay

Real-time analysis of trans-endothelial electric resistance was performed by plating human umbilical vein endothelial cells (HUVECs) onto 8W10E+ standard 8-well arrays (Applied BioPhysics, NY) at a density of 10^5 cells per well. Cells were allowed to grow

to establish a monolayer leading to a capacitance of less than 10nF. Once confluent, they were starved for 8 hours with endothelial basal media (EBM-2, Lonza) and treated with 50 ng/mL $VEGF_{165}$, 200 ng/mL Sema3A or vehicle (EBM-2) and impedance was measured using a ECIS Zθ impedance instrument (Applied BioPhysics, NY). Measurements were taken for 6 hours post treatment.

Preparation of lentivirus

We produced infectious lentiviral vectors by transfecting lentivector and packaging vectors into HEK293T cells (Invitrogen) as previously described(Dull et al., 1998). Viral supernatants were concentrated by ultra-centrifugation (>500-fold) as previously described by us (Binet et al., 2013; Joyal et al., 2011).

Soluble Recombinant Nrp-1

STZ treated diabetic C57BL/6J mice were intravitreally injected with rmNrp-1 from plasmid(Mamluk et al., 2002) or R&D Systems at 6 and 7 weeks after STZ administration. Retinal Evans blue permeation assay was performed at 8 weeks after STZ treatment as described above.

Statistical analyses

Data are presented as mean ± s.e.m. We used Student's T-test and ANOVA, where appropriate, to compare the different groups; a $P < 0.05$ was considered statistically different.

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Competing interests: The University of Montreal, Hopital Maisonneuve-Rosemont and PS have filed a patent pertaining to the results presented in the paper.

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Author contributions: P.S. and A.C. conceived and designed the experiments. A.C., N.T., C.M., F.A.R., A.D., C.P., E.L., N.S., D.L., V.D., F.B., performed the experiments. A.C., N.T., C.M., C.P., E.L., A.D, F.A.R and P.S. analyzed the data. A.C., N.T. and C.M. assembled figures. P.S. wrote the manuscript.

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Figure Legends

Figure 1. Sema3A is elevated in the vitreous of human diabetic patients suffering from diabetic retinopathy and in retinal neurons in the early phases of STZ-induced diabetes. (A) Spectral-domain optical coherence tomography (SD-OCT) and **(B)** 3D retinal maps from healthy eyes. **(C-D)** In patients suffering from DME, significant retinal swelling mostly in the macular and peri-macular zones was noted. **(E)** Western blot analysis of equal volumes of vitreous humor revealed a pronounced induction of Sema3A (~125 kDa), including the presence of a heavy weight isoform (>200k Da). **(F)** Detailed patient characteristics. **(G)** Streptozotocin (STZ) was administered to ~6 week-old C57BL/6J mice and glycemia monitored according to scheme; a mouse with non-fasted glycemia higher than 17 mM (300 mg/dL) was considered diabetic. **(H)** 4 weeks after

induction of diabetes, retinal Sema3A mRNA levels rose more than 2-fold higher in STZ treated mice when compared to vehicle-injected controls (*P*=0.0045, n=5); elevated levels of Sema3A persisted at 8 weeks (*P*=0.0011, n=8). At both time points, VEGF levels remained unchanged. **(I)** At both 4 and 8 weeks of diabetes, STZ-treated mice showed pathologically elevated blood glucose ~30mM (*P*<0.0001, for both time points). The rise in Sema3A expression preceded pericyte loss as evidenced by **(J)** similar levels of transcripts for pericyte-related markers platelet derived growth factor-receptorβ (*PDGFR-β)*, neuron-glial antigen 2 (NG2) proteoglycan, and smooth muscle actin (*SMA*) in STZ- and vehicle-treated mice. **(K)** Equivalent pericyte vascular coverage as depicted by pericyte-specific dyes for NG2 and SMA (representative of at least three separate experiments). Scale bar: 100um **(L, M)** Immunohistochemistry on retinal cryosections revealed that Sema3A protein was robustly expressed by retinal ganglion cells (RGCs) in the ganglion cell layer (GCL), as confirmed by co-localization with the RGC marker *β*III-tubulin. Representative images of three independent experiments. Scale bar: 20um, inset: 10um **(N, O)** Laser-capture micro-dissection of the GCL from normal or diabetic mice followed by quantitative RT-PCR confirmed a ~5-fold induction of Sema3A in neurons in close proximity to the inner retinal vascular bed (*P*=0.014, n=3). Scale bar: 20um.

Figure 2. Retinal barrier function is compromised by Sema3A. (A) Intravitreal injection of Sema3A resulted in a ~2-fold increase (*P*<0.01, n=3 (9 animals)) in retinal vascular permeability (VP) as determined by Evans Blue (EB) permeation; a similar increase was observed with intravitreal administration of VEGF (*P*<0.05, n=3 (9

animals)) and with a combination of both Sema3A and VEGF (*P*<0.001, n=3 (9 animals)). Values expressed relative to vehicle injected retinas ± s.e.m. **(B)** Confocal images of retinal sections injected with vehicle, VEGF orSema3A; red signal depicts leakage of Evans Blue/Albumin into the vitreous/retina. Representative images of three independent experiments. Scale bar: 30um. (**C**) Trans-endothelial resistance measured in real time by ECIS demonstrates that Sema3A effectively reduces endothelial barrier function (3.26h to 6h; 0.048>*P*>0.009; n=3) to a similar level as that observed with VEGF (1.12h to 6h; 0.045>*P*>0.001; n=4). (**D**) ECIS measures true barrier function by assessing the resistance of the paracellular pathway between the cells. (**E**) Working hypothesis for Sema3A mediated vascular permeability. Treatment of HRMECs with either Sema3A or VEGF leads to robust phosphorylation of **(F)** Src at Tyr416; **(G)** FAK on Tyr576 and 577 and **(H)** the adherence junction protein VE-cadherin on tyrosine-731. An additive or enhanced effect was not observed when simulation was performed with a combination of Sema3A and VEGF. *P<0,05, **P<0,01,***P<0,001 relative to vehicle ± s.e.m. **(I)** Confocal microscopy of Sema3A-treated HRMECs revealed formation of vascular retraction fibers as determined by VE-cadherin and phalloidin staining (white arrows); retraction was similar to that seen with VEGF alone or with a combination of VEGF and Sema3A. Scale bar: 20um. **(J)** Sema3A doses employed in our study, (100-200 ng/ml), did not induce cell death or apoptosis as determined by assessment of activation of caspase-3. Representative of three independent experiments.

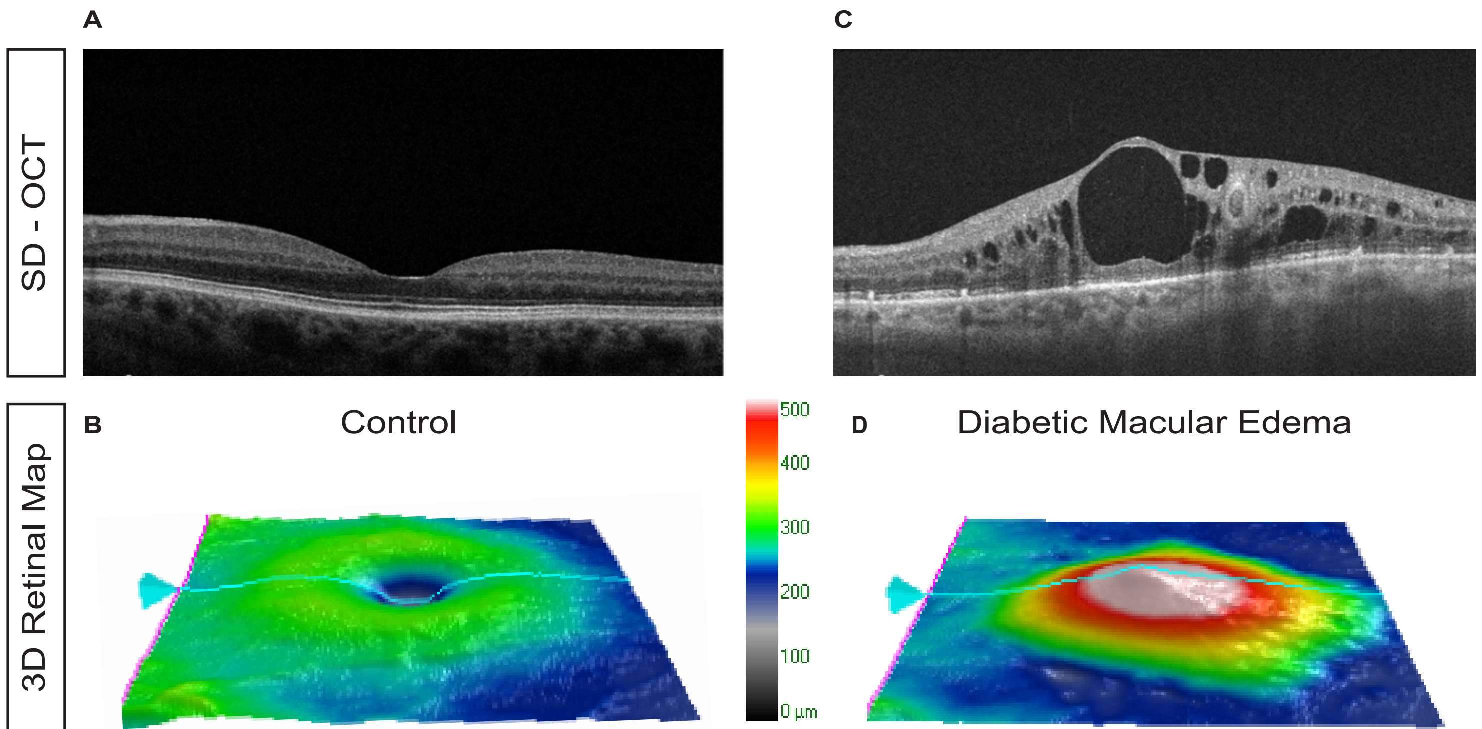
Figure 3. Targeted silencing of neuron-derived Sema3A or intravitreal neutralization of Sema3A efficiently reduces diabetes-induced retinal vascular

permeability. (A) Retinal flatmounts 8 weeks following STZ-injection show elevated VE-cadherin phosphorylation (linked to higher permeability) on lectin-positive retinal vessels. Representative images of three independent experiments. Scale bar: 20μm **(B)** STZ-treated mice show a 56.8% increase in permeability (*P*= 0.027; n=4 (12 mice)). **(C)** Lentiviral vectors with a VSVG capsid exhibit high tropism for RGCs when delivered intravitreally, as depicted by a reporter Lv vector carrying GFP. Scale bar: 20μm. **(D)** A single intravitreal injection of Lv.shSema3A at 5 weeks of diabetes lead to a significant 62.3% reduction in retinal Sema3A expression (*P*=0.0014, n=3) and **(E)** provoked a proportional 49.5% decrease in vascular leakage (*P*=0.022, n=3 (9 mice)). **(F)** Vitreal Sema3A was neutralized with a recombinant mouse (rm) soluble Nrp-1 protein employed as a bivalent trap for both Sema3A and VEGF. **(G)** Intravitreal injection of rmNrp-1 in STZ mice at weeks 6 and 7 after induction of diabetes lead to a 48.1% reduction in retinal permeability at week 8 of diabetes (*P*=0.012, n=6 (18 mice)). Values expressed relative to vehicle injected retinas ± s.e.m.

Figure 4. Conditional knockout of Nrp-1 prevents Sema3A-induced retinal barrier function breakdown. Tamoxifen (Tam) was administered systemically during a 5 day period to tamoxifen-inducible Nrp-1 *floxed* B6 mice ($\mathrm{Tam}^{Cre\text{-}Esr1}/Nrp1^{fl/fl}$). **(A)** Inducible Cre-loxP system: estrogen receptor 1 (Esr1) forms a complex with Cre recombinase; Tam, when available, binds the Cre-Esr1 complex, allowing its nuclear translocation and the subsequent recombination/excision of the *floxed* target gene *Nrp-1*. Efficiency of *Nrp-1* deletion by CRE-Esr1 recombinase is evidenced by decreased **(B)** protein and (**C**) mRNA transcript in vascular tissue (*P*=0.0012; n=2). **(D)** Immunohistochemistry in

retinal cryosections reveals the efficiency of tamoxifen-induced knockdown of Nrp-1 in lectin-stained retinal vessels. **(E)** In absence of Nrp-1, intravitreally administered Sema3A did not increase vascular leakage (*P*=0.36; n=7 (21 mice)), while $Tam^{Cre-ESR1}/Nrp1^{+/+}$ controls show 3-fold higher vascular leakage (*P*=0.00065; n=3 (9 mice)). **(F)** Conversely, disruption of *Nrp-1* did not influence VEGF-induced vascular retinal permeability (p=0.0024; n=3 (9 mice)), suggesting that VEGF-induced retinal vascular leakage is independent of Nrp-1 as previously reported. **(G)** *In vitro* knockdown of Nrp-1 in HRMECs by Lv.shNrp1 prevents phosphorylation of Src (*P*= 0.004; n=3), **(H)** FAK (*P*= 0.0002; n=3) and (**I**) VE-Cadherin (*P*=0.0081; n=3). (**J**) Graphic depiction of the main findings of the study. In a healthy mature retina, levels of Sema3A are low whereas in diabetes, retinal ganglion neurons in intimate proximity of retinal vessels significantly increase their production. Through Nrp-1, Sema3A provokes loosening of endothelial cell junctions and leads to vasogenic edema.

Figure 1 - Part 1



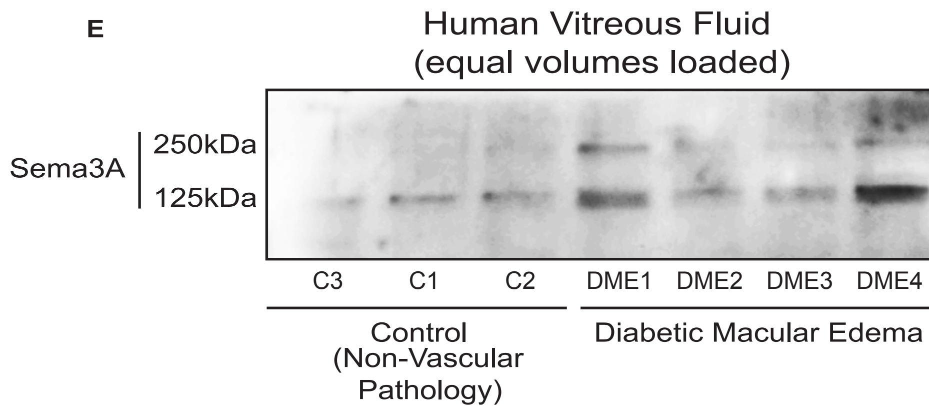


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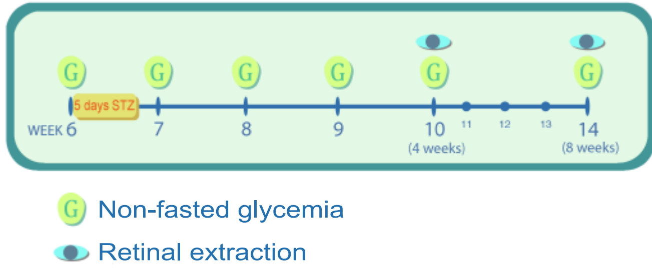
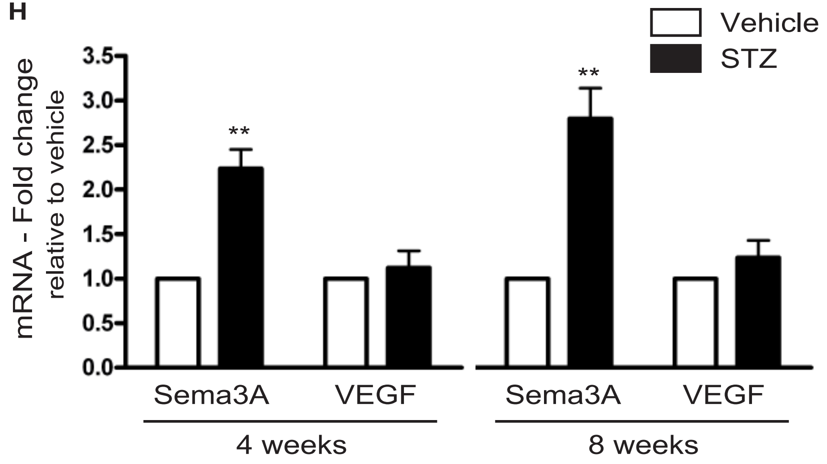
Table 1: Patient Information

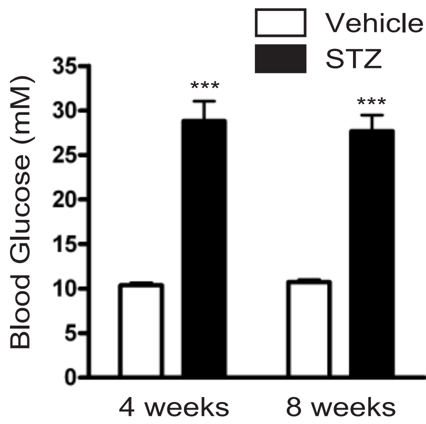
Sample	Age	Type Db	Duration (years)	Retinopathy
C1	63	n.a.	n.a.	MH
C2	71	n.a.	n.a.	ERM
C3	62	n.a.	n.a.	ERM
DME1	70	2	10	DME
DME2	61	2	25	DME
DME3	75	2	10	DME
DME4	74	2	15	DME

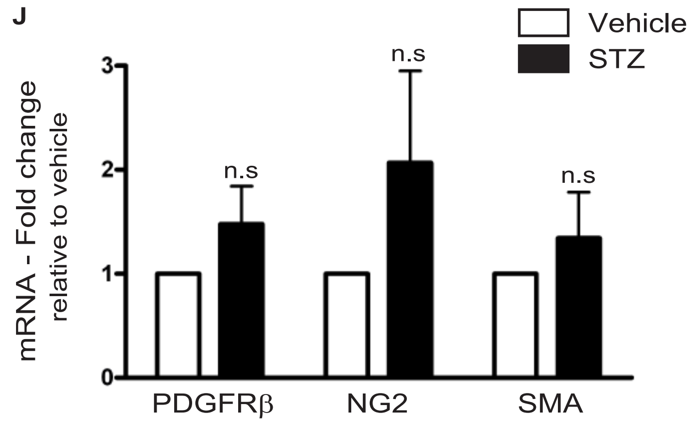
Notes: C Control (Non-vascular Pathology); Db Diabetes; DME Diabetic Macular Edema; ERM Epiretinal Membrane; MH Macular Hole

Figure 1 - Part 2









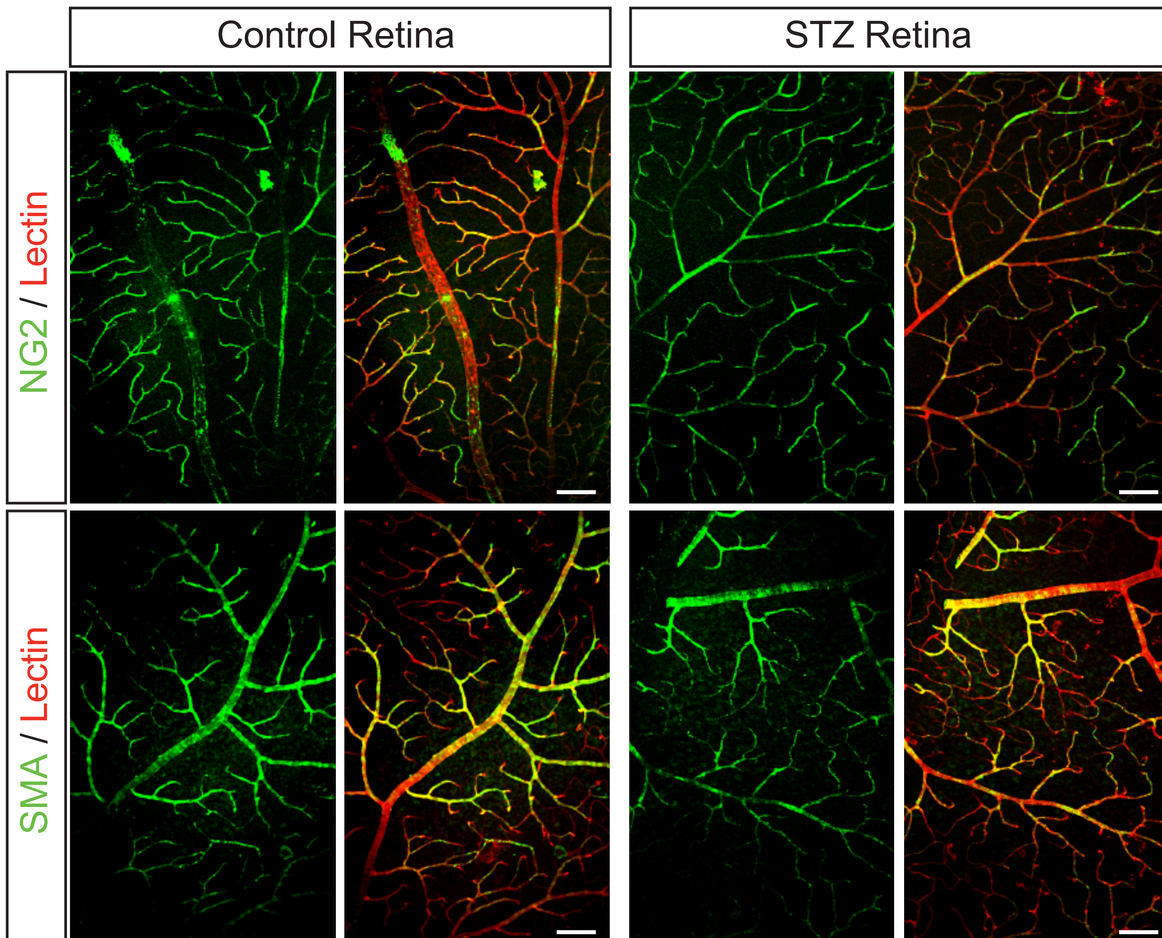


Figure 1 - Part 3

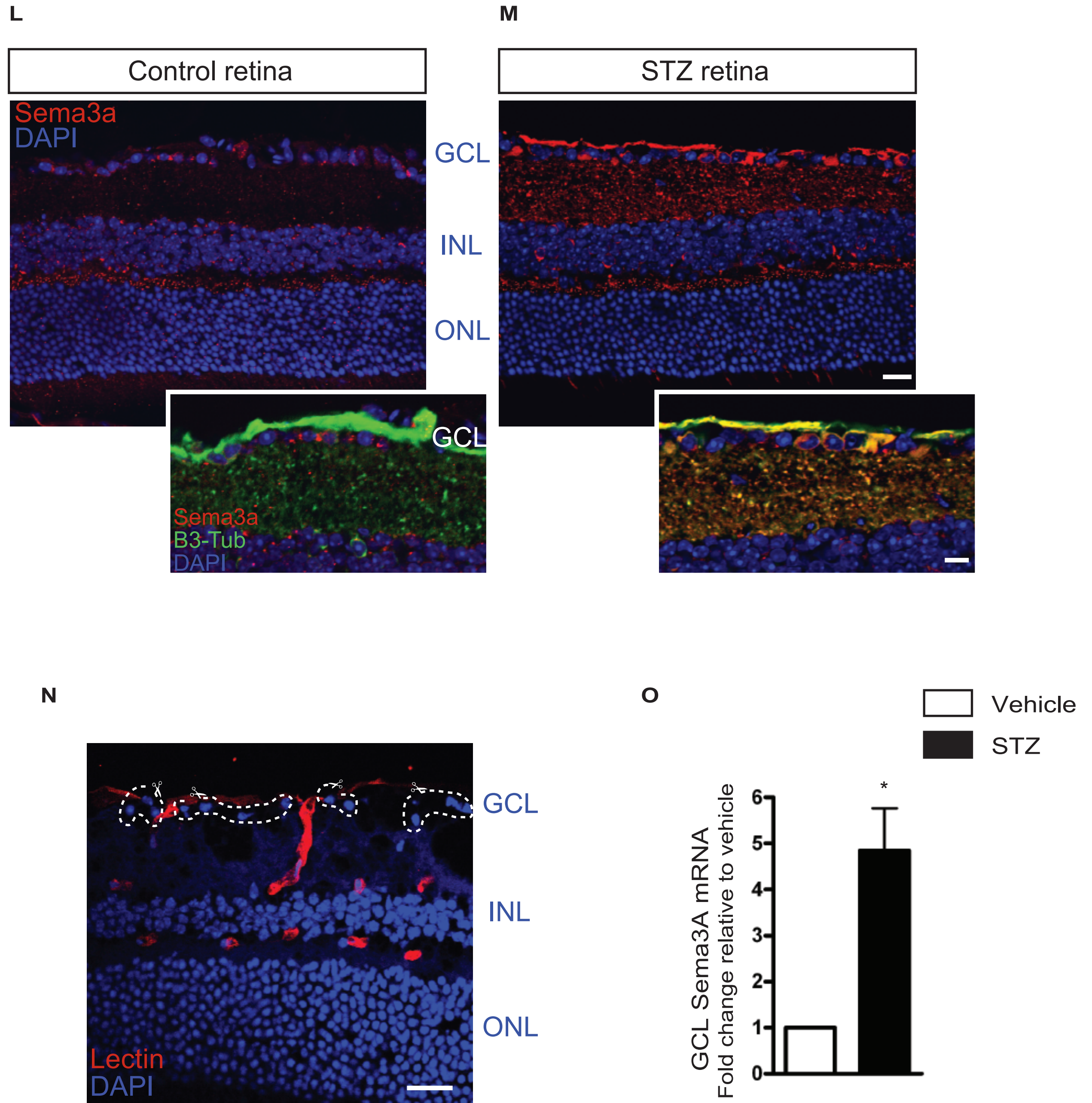
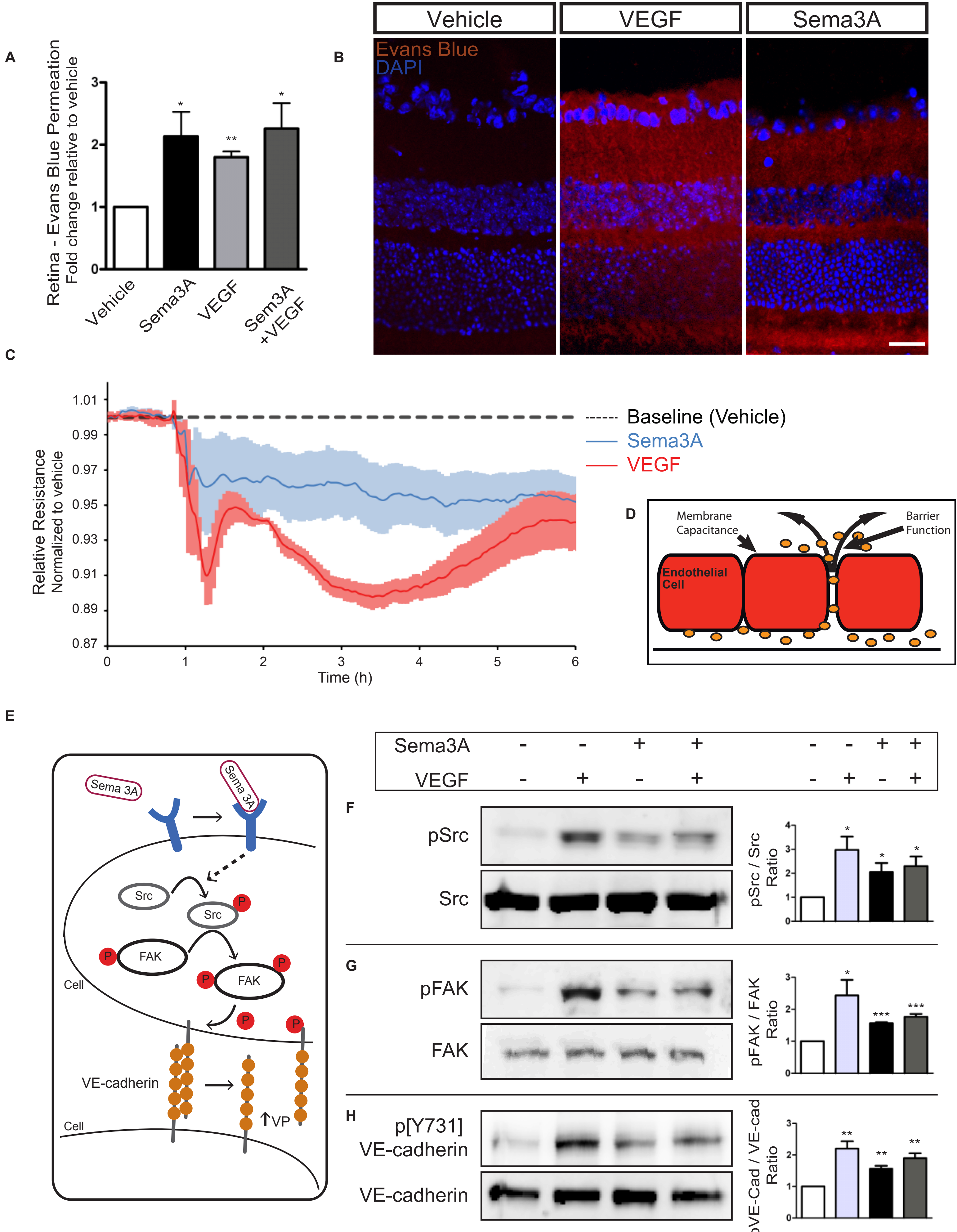


Figure 2 - Part 1



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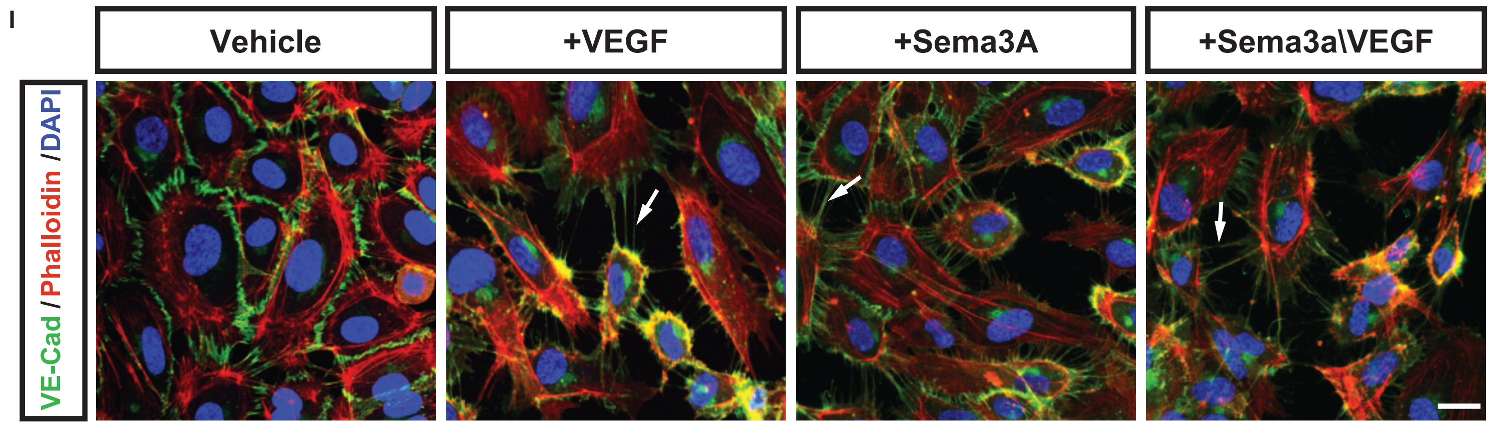


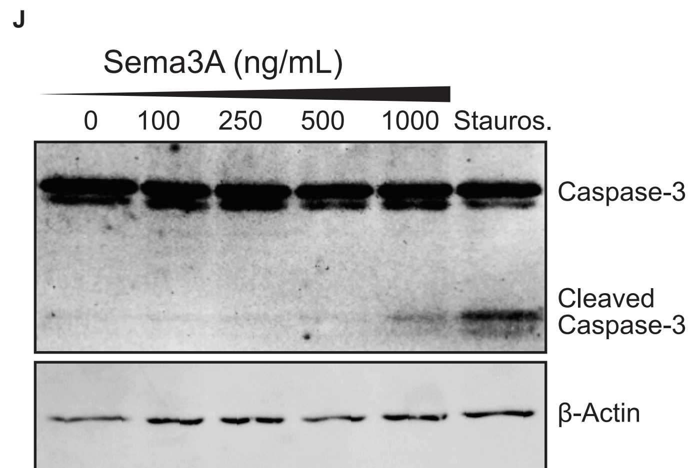
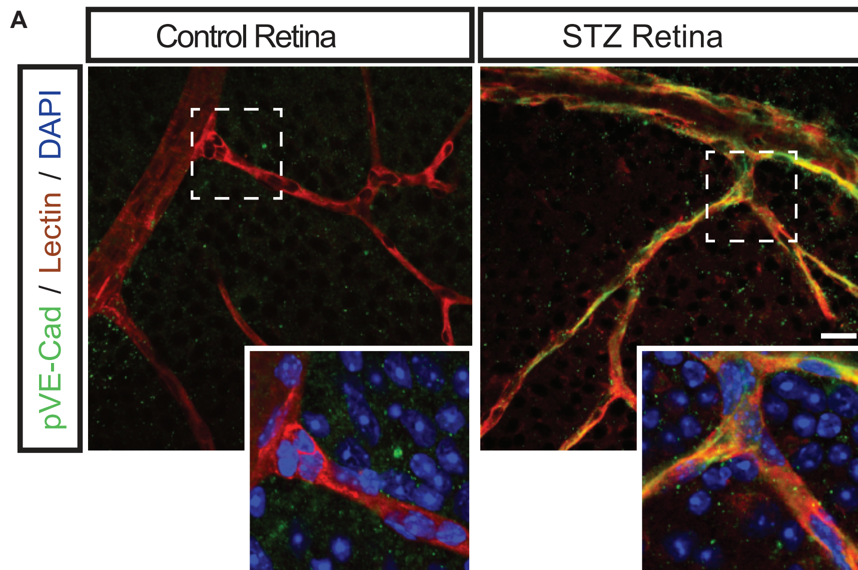
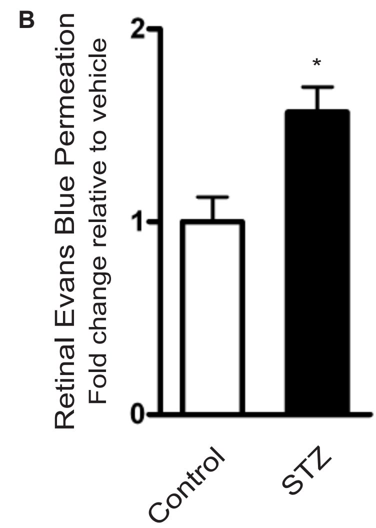
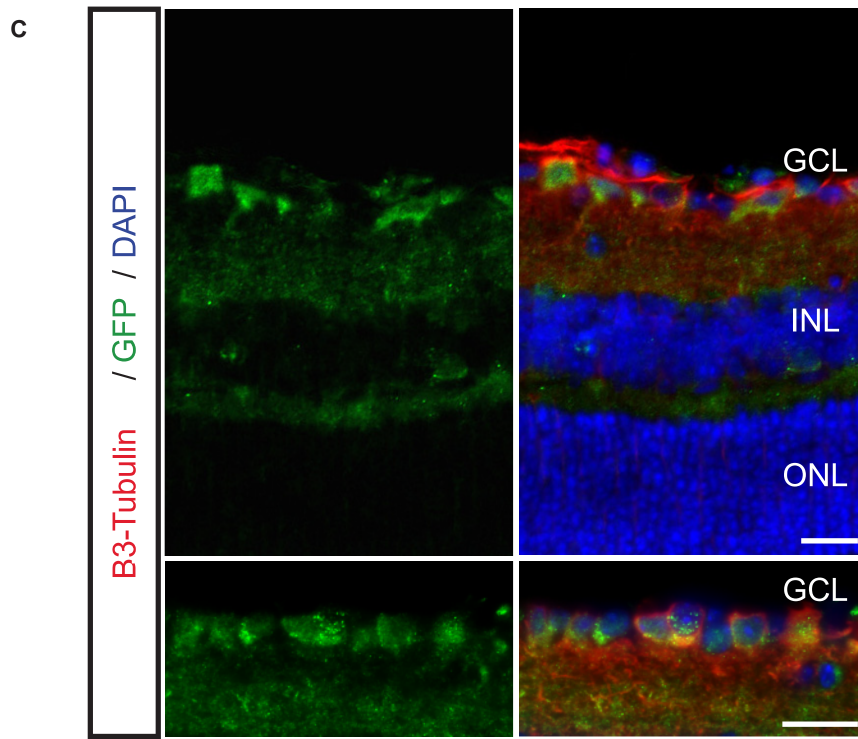
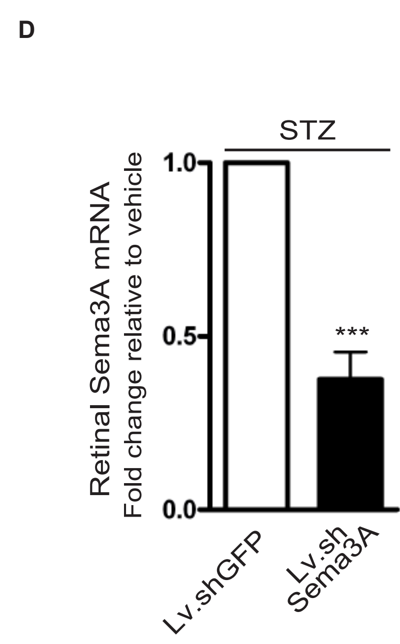


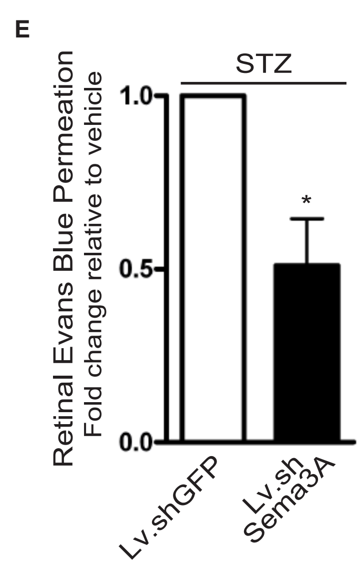
Figure 3

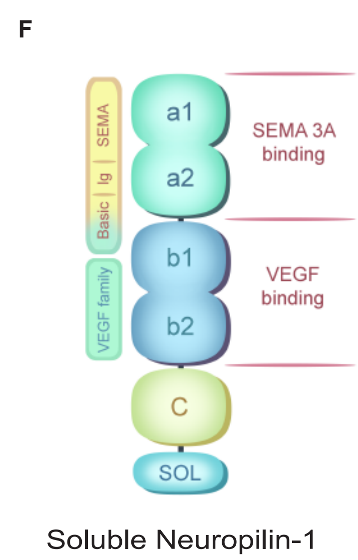












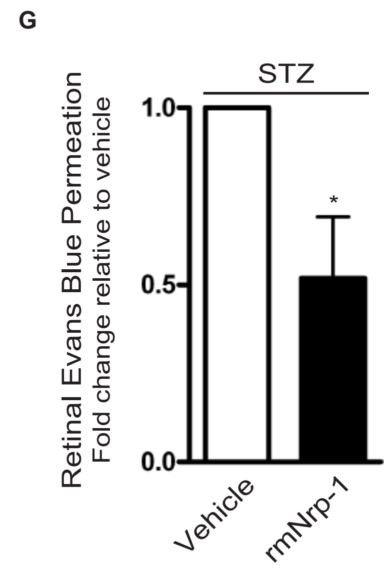


Figure 4 - part 1

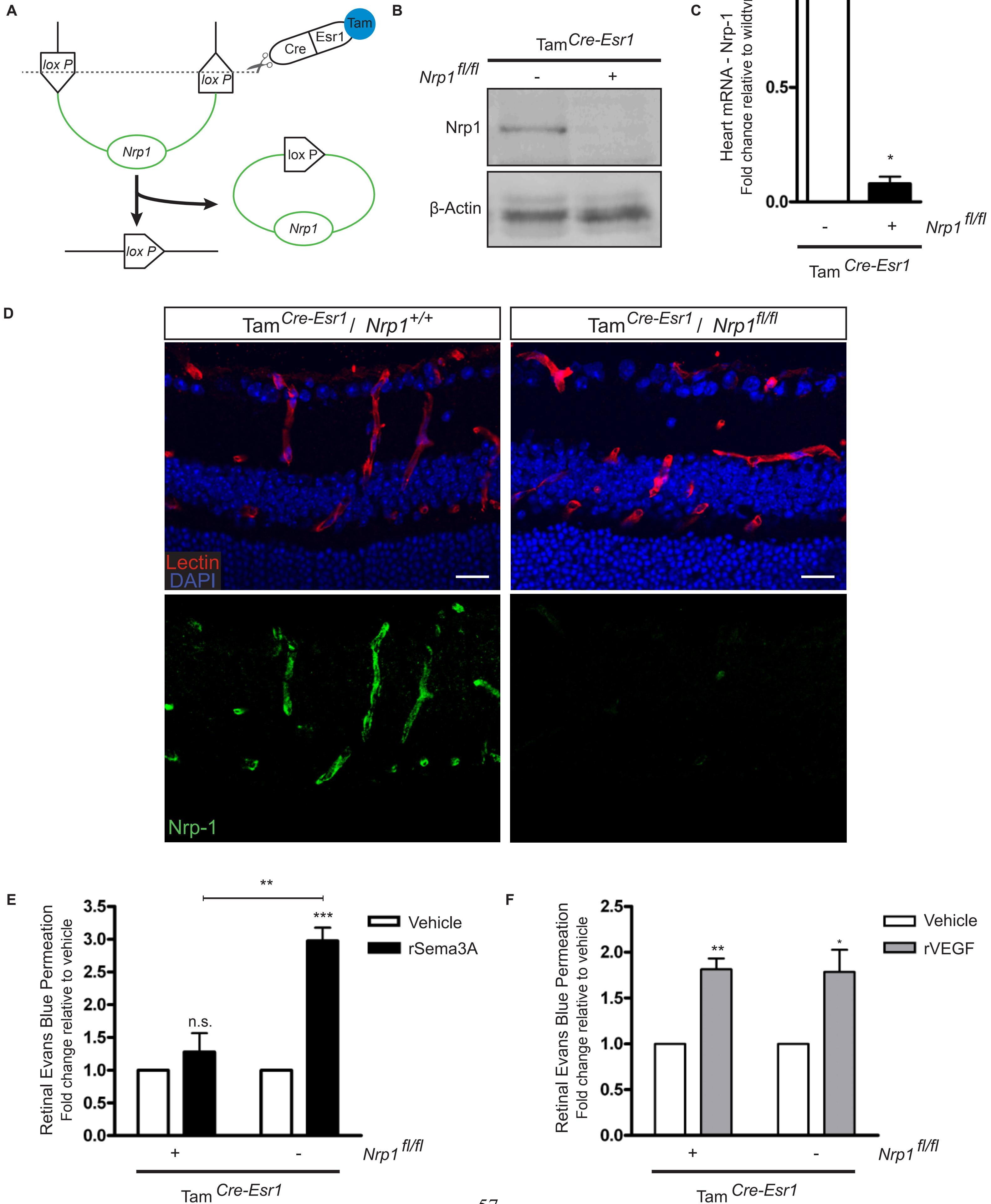
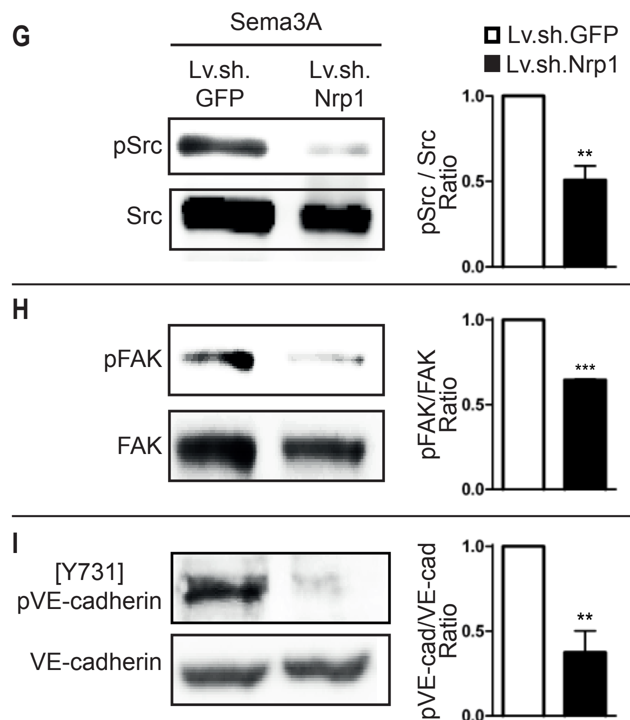


Figure 4 - Part 2



J

Healthy Retina

Diabetic Retina

Plasma extravasation

Endothelial Cell

RGC

RGC

RGC

Diabetic RGC

Diabetic RGC

P

Semaphorin3A

Neuropilin-1

VE-Cadherin

Chapter 3: Discussion

Discussion

In the present study, we demonstrate that neuronally derived semaphorin 3A induces vascular permeability in diabetic retinopathy. We initially found a significant increase of Sema3A protein in the vitreous of diabetic DME patients with advanced macular (central retina) oedema, which is a clinical manifestation associated with increased vascular leakage (Figure 1A-E) (*12*). In order to further investigate the dynamics of Sema3A induction and to study its potential involvement in the pathogenesis of DR, we used a "physiological" model of T1DM, where hyperglycemia is induced in mice by injecting STZ, a compound that induces the deterioration of the insulin-producing β-islets of the pancreas (*123, 124*). By using this model we saw that Sema3A is induced early on in the diabetic murine retina, i.e. 4 weeks after induction of hyperglycemia, and its expression remains elevated at 8 weeks of diabetes (Figure 1H). Interestingly, the principal site of expression of Sema3A was located to the RGC layer of neurons that lies adjacent to the inner retinal vascular plexuses (Figure 1L-O). Even though the link between hyperglycemia and Sema3A induction remains to be elucidated, Sema3A expression could have an inflammatory origin. Hyperglycemia is pro-inflammatory and is counteracted by the anti-inflammatory properties of insulin, which is secreted in response to increased glycemia (*125, 126*). When the hyperglycemia/insulin secretion feedback loop is interrupted as a result of decreased insulin availability (insulitis) or decreased insulin sensitivity, increased inflammation can occur in the retina. This in turn contributes to the pathogenesis of DR (*127-129*). Specifically, the pro-inflammatory factor IL-1β was recently reported to be induced in retinal vessels in a rodent model of STZ-induced diabetes (*130*). In line, our lab has previously reported that Sema3A is induced via IL-1β in the retina in the context of a hypoxia-induced inflammatory state (*86*). Therefore, Sema3A

in the diabetic retina could potentially be induced via pro-inflammatory cues such as IL-1β, yet this remains to be studied.

Given the pronounced upregulation of Sema3A in the diabetic retina and its proximity to retinal vessels, we next studied the effect of Sema3A on the retinal vasculature. First, intravitreal injections of rSema3A in non-diabetic mice yielded an increased retinal vascular leakage (Figure 2A-B). Similarly, STZ-injected mice showed increased VP as previously reported (Figure 3B) (*131-133*). With the purpose of investigating the potential involvement of Sema3A in barrier function deterioration of the retinal vessels in the diabetic eye, we sought to decrease levels of Sema3A in the retina by implementing two different approaches: first, we targeted RGCs via a Lentivirus (Lv) carrying a shSema3A that successfully infects these cells and silences Sema3A expression (Figure 3C-D)(*86*); separately, we injected intravitreally a recombinant and soluble form of Sema3A's cognate receptor Neuropilin-1 (Nrp-1) as a "trap" method (Figure 3F). By silencing RGC-specific Sema3A expression or "trapping" and thus decreasing its availability in the retina, we saw a similar significant decrease in retinal vascular permeability (Figure 3E-G) thereby illustrating that Sema3A triggers vascular leakage in DR. Based on the ability of soluble Nrp-1 to reduce abnormal VP *in vivo*, soluble Nrp-1 could represent a potential new therapeutic approach for the treatment of diabetic macular edema.

After observing that Sema3A induces vascular permeability in DR, we next assessed if the vascular effects that are mediated by Sema3A in the retina are directed and transduced by Sema3A binding to its only cognate receptor Nrp-1. For this end, we investigated if conditional knockdown of *Nrp-1* protects against Sema3A-induced vascular permeability. We used *Nrp-1* floxed (*Nrp-1*$^{fl/fl}$) whole-animal tamoxifen-induced (Tam^{Cre-}

^{Esr1}) conditional knockout mice because systemic germline deletion of *Nrp-1* is embryonic lethal (*134-136*) (Figure 4A). As observed in Figure 4B-D, tamoxifen administration provided an efficient knockout of *Nrp-1* in the retinal vasculature of Tam$^{Cre\text{-}Esr1}$/*Nrp1*$^{fl/fl}$, yet *Nrp-1* expression was not affected in Tam$^{Cre\text{-}Esr1}$/*Nrp1*$^{+/+}$. Notably, mouse retinas were protected against Sema3A-induced vascular permeability, while Tam$^{Cre\text{-}Esr1}$/*Nrp1*$^{+/+}$ displayed a 3-fold increase in VP under the same conditions (Figure 4E). In addition, we observed that *Nrp1* deletion did not affect VEGF-induced permeability since intravitreally injected VEGF led to similar increases in retinal vascular permeation in Tam$^{Cre\text{-}Esr1}$/*Nrp1*$^{fl/fl}$ and Tam$^{Cre\text{-}Esr1}$/*Nrp1*$^{+/+}$ mice (Figure 4F).

From a mechanistic perspective, via Nrp-1 activation, Sema3A triggered signaling pathways involved in vascular permeability (Figure 2E). Western blot analysis of retinal endothelial cell lysates showed activation profiles of Src and focal adhesion kinase (FAK) that transduce extracellular signals, provoking the loosening of endothelial cell tight junction (Figure 3F-H) (*137-139*). The *in vitro* stimulation of HRMECs by either Sema3A or VEGF led to robust phosphorylation of Src at Tyr416 in the activation loop of the kinase domain, which is reported to enhance enzyme activity (*140*). In turn, FAK was phosphorylated on Tyr576 and 577, sites know to be phosphorylated by Src and required for upregulation of FAK activity (*141, 142*). Ultimately, the tight junction protein VE-cadherin, involved in calcium-dependent homophilic binding between endothelial cells , became phosphorylated on tyrosine-731, which is a posttranslational modification that has been shown to be sufficient to promote detachment of VE-cadherin adherens junctions, allowing higher paracellular diffusion and, thus, increased VP (Figure 3A,E) (*143, 144*). Consistent with the non-additive effect of combined VEGF and Sema3A observed on retinal permeability (Figure 3A), an additive or enhanced effect was not observed when

stimulation *in vitro* was performed with a combination of Sema3A and VEGF suggesting that both factors signal via redundant pathways. Importantly, infection of HRMECs with lentivirus (Lv) carrying a shNrp-1 (Lv.shGFP used as control) that successfully infects these cells and silences *Nrp-1* expression, significantly decreased phosphorylation of Src, FAK and VE-cadherin, thus further demonstrating that Sema3A-induced endothelial barrier breakdown is triggered via Nrp-1 activation (Figure 4G-I).

In addition, we observed that in diabetic mouse eyes where we had observed Sema3A-induced VP (Figure 3B, D, G), retinal vessels displayed VE-cadherin phosphorylation at tyrosine-731 (Figure 3A). Notably, when VE-cadherin is phosphorylated, its intracellular domain dissociates from the complex formed by the armadillo family of proteins β-catenin and p-120, which connects VE-cadherin with the actin cytoskeleton and is crucial for stable inter-EC binding (*144-146*). At the same time, p120 hinders the endocytosis of VE-cadherins, a process that leads to the intracellular degradation of this junctional protein (*147-149*). Sema3A-induced phosphorylation of VE-cadherin at tyrosin-731, especially in the context of normal VEGF levels (Figure 1B), could in part explain the degradation of VE-cadherin previously reported early in diabetes, further contributing to BRB breakdown in DR (*143*).

Another important junctional protein involved in barrier function stabilization that is also affected during diabetes is occludin (*150-152*). Interestingly, phosphorylation of occludin at serine-490 is a posttranslational modification that has recently been linked to VEFG-induced vascular permeability (*153*). As a result, we are currently investigating if Sema3A could induce phosphorylation [S-490] of occludin (thanks to Dr. Antonetti's generous donation of phoshoserine-490 antibody).

Thus far, we observed that Sema3A is induced early on in the diabetic retina and is responsible for a decrease in retinal barrier function (BRB breakdown) and a subsequent increased VP. Most interestingly, the main source of Sema3A mRNA and protein expression was localized to the neuronal retina, specifically to the retinal ganglion neurons (Figure 1L-O). This suggests a novel direct neuronal role in the etiology of DR and highlights the importance of neurovascular interplay in the pathogenesis of DR (*12*). In addition, we obtained insight on a prospective therapeutic target, i.e. Sema3A, which could address simultaneously the two main pathological features of DR, i.e. vascular decay and barrier function deterioration, which separately through pathological neovascularization and edema, respectively, can lead to loss of vision in diabetic patients.

During the onset of DR, however, blood-retinal barrier breakdown is not only associated to VP-inducing posttranslational modifications and decreased expression of adherens junctional proteins like VE-cadherin, but also to an initial degradation and subsequent loss of the endothelium-regulating mural cells known as pericytes (*12, 154*). As a result, we assessed pericyte physiology and coverage in the diabetic retina. Notably, we observed that the rise in Sema3A expression was an early event in pathogenesis since it preceded pericyte loss as both STZ and vehicle-treated mice did not show a significant difference in transcript levels for pericyte markers platelet derived growth factor receptor (*PDGFRb*), NG2 proteoglycan (*NG2*) and smooth muscle actin (*SMA*) (Figure 1K). Moreover, NG2 and SMA immunofluorescence confirmed similar pericyte coverage of the retinal endothelium in control and STZ-treated mice. Thus, these data suggest that Sema3A-induced VP predates pericyte dropout in DR (*155, 156*).

The two main pathological features of DR, namely the abnormal and misdirected growth of leaky vessels towards the vitreous and the decline of endothelial barrier function

of these vessels that leads to abnormal leakage and edema, have been linked to a loss of pericytes. Endothelium-specific knockout of platelet-derived growth factor β (PDGFβ) that trumps PDGFβ/PDGFRb signaling required for PC survival yields a variable degree of retinal pericyte loss with DR-like lesions located in the areas of greatest loss (*155, 157, 158*). It has therefore been suggested that pericyte dropout in the retina due to hyperglycemia-induced apoptosis is a sufficient cause for capillary occlusion, regression and, ultimately, retinal ischemia which leads to the onset of PDR complications (*45, 159*).

The decreased microvascular barrier function that is observed in the diabetic retina can also be found in other parts of the nervous system and represents an important pathological feature of diabetic neuropathy (DN) (*160*). This condition can affect the central and peripheral nervous system (PNS) and involves BBB and blood-nerve barrier (BNB) deregulation, respectively. Notably, as seen in DR, there is evidence of pericyte involvement in the onset of DN. In the brain, BBB breakdown and the associated increase in vascular permeability has been linked to a decrease in the pericyte population (*38, 161*). Pericyte involvement has also been suggested in the development of peripheral neuropathy, since these cells contribute to the strengthening of the BNB through the secretion of growth factors such as basic fibroblast growth factor (bFGF), which up-regulate tight junction claudin-5 expression in peripheral nerve microvascular ECs (*162*). Overall, there is a common role played by pericytes in the development of diabetic microvascular pathology in the nervous system that involves, as seen in the retina, brain (CNS) and PNS, a deregulation of endothelial barrier function and endothelial permeability. Even though the effects of Sema3A on the retinal vasculature are based on a direct effect on microvascular endothelial cells (Figure 2C-H), Sema3A could parallely be impacting pericyte regulation of the endothelium's homeostasis, such as junctional and

adherent complex expression, especially since pericytes express Nrp-1, yet this remains to be studied (*163*).

Microvascular complications secondary to diabetes, such as those observed in the brain and retina, are also found to affect the glomeruli in the kidney. Diabetic nephropathy (DNeph), which affects 30 % of diabetics (*164*), is initially characterized by microalbuminuria—increased albumin levels in the urine due to decreased barrier function of the glomeruli, i.e. glomerular filtration barrier, GFB—, often derives in nephrotic-range proteinuria, and represents the main cause of end-stage renal disease (ESRD) (*165*). From a hemodynamic perspective, hyperperfusion and hyperfiltration are also early features of this disease, which arise from deregulated afferent arteriole tone and are associated with high intraglomerular pressure (*166*). Such an increase in pressure has been associated with excessive production of mesangial cell matrix, podocyte injury and thickening of the glomerular basement membrane (*167*). Most interestingly, podocytes and mesangial cells are pericyte-like cells and play an important role in the etiology and progression of DNeph, both in terms of glomerular atrophy and permeability. As a result of hyperglycemia, mesangial cells produce high levels of bioactive transforming growth factor β (TGFβ) after a short period of self-regulated proliferation (*168*). Activation of TGFβ pathway plays an important role in mesangial cell hypertrophy and extracellular matrix overproduction, but it has no impact on the glomerular barrier function and the associated microalbuminuria as evidenced in *db/db* leptin receptor knockout mice—model of Type 2 diabetes—where treatment with neutralizing anti-TGFβ antibody yields decreased mesangial hypertrophy, but no significant effect on albumin permeability (*169-171*). This observation suggests the involvement of other cues, histological and biochemical, in the genesis of glomerular barrier decay in diabetes.

In the kidney, based on histology, decreased podocyte coverage and number in glomeruli demonstrates a close correlation to the level of GFB decay and proteinuria, similar to how decreased pericyte coverage of the retinal vasculature is associated with BRB breakdown and edema (*172*). Steffes *et al.*, while studying Type 1 diabetics, found a decrease in podocyte number per glomerulus for all ages, even in diabetes with short duration. Similarly, renal biopsies from Type 2 diabetics showed broaden podocyte foot processes and a reduction in number of these cells per glomerulus (*173*). Notably, a subsequent study of the same population showed that, among different glomerular morphological markers, number of podocytes per glomerulus represented the strongest predictor of diabetic renal disease progression, with lower cell count indicating faster progression (*174*). Furthermore, a study of Type 2 European diabetics found a significant reduction in podocyte density per glomerulus in patients with normal urine albumin levels, with a stronger decrease in proteinuric patients (*175*). In addition, Nakamura *et al.* found podocytes in the urine of 53 % of microalbuminuric Type 2 diabetic patients and in 80 % macroalbuminuric patients (*176*). These studies show that during diabetes, either Type 1 or 2, there is a loss of the pericyte-like podocyte cells in the glomeruli of the kidney analogous to that seen in the diabetic retina with similar endothelium-compromising results. Podocyte loss in diabetics leads to diabetic nephropathy, continues during its progression and causes the disruption of the glomerular filtration barrier, thus increasing glomerular vascular permeability and protein content in urine.

Moreover, from a biochemical perspective, in the adult mouse kidney, Sema3A is endogenously expressed in collecting tubules and podocytes, and controls the GFB, a structure analogous to the BRB (*177, 178*). Sema3A is also involved and required in normal nephron development (*177*) (*179*). Interestingly and in analogy to our reported

results, exogenous administration of Sema3A induced acute nephrotic range proteinuria, i.e. increased leakage of the glomerular vessels, via podocyte foot process effacement from the endothelium ("pericyte detachment") and concomitant EC damage (*180*). Similarly, Sema3A mRNA and protein were induced in a murine model of diabetic glomerular disease triggering increased proteinuria, as well as other pathognomonic aspects of diabetic nephropathy (*164, 181*). As a result, the effects of Sema3A upregulation on the microvascular endothelium could represent a common feature of diabetic microvascular pathology and not an isolated retina-specific mechanism. However, some aspects such as the Sema3A effect on retinal pericytes or targeted silencing of Sema3A in the diabetic glomerulus—since Sema3A-induced proteinuria is only correlational at this point—remain to be elucidated in order to understand more clearly the involvement of this guidance cue in diabetes vasculopathy.

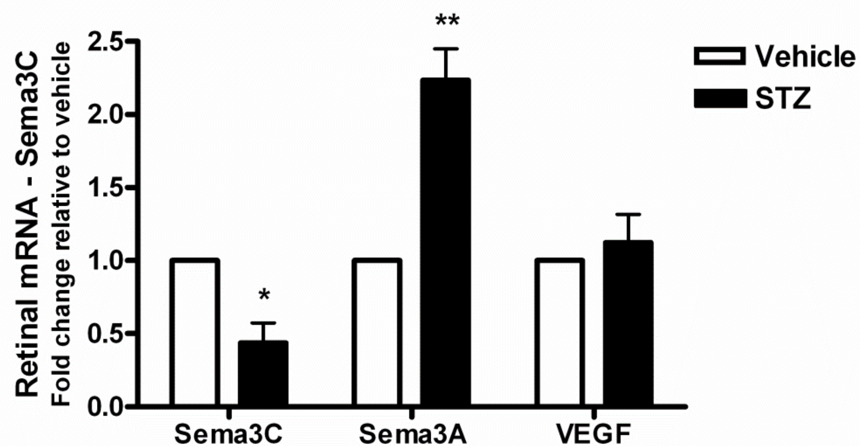


Figure 7. Retinal Semaphorin 3C expression decreases early on in STZ-induced diabetes. Total retinal Sema3C mRNA expression levels decline significantly ($p=0.015$, $n=3$) in the early stages of streptozotocin (STZ)-induced diabetes concurrently with a robust increase in Sema3A ($p<0.01$, $n=5$) and normal levels of VEGF.

Another interesting semaphorin that is less characterized with respect to vascular physiology is Sema3C. This axonal guidance cue has been described to promote glomerular endothelial cell survival, proliferation, migration, *in vitro* EC-tube formation and $VEGF_{120}$ secretion, opposite effects from those seen triggered by Sema3A (*92, 182*). Such observation prompted us to assess Sema3C's mRNA expression in the diabetic retina, by utilizing the same diabetic model from this study, at the same time points where Sema3A was increased. Notably, preliminary results show that retinal Sema3C was significantly decreased by 56.4% in STZ-injected mice relative to vehicle-injected mice (Figure 7; $p=0.015$, $n=3$). Hence, if Sema3C acts in the retina as an EC-protective compound, a decline in its expression in the context of a vascular pathology such as DR makes Sema3C an interesting target of study. Moreover, because Sema3C signals through an Nrp-1/Nrp-2 heterodimer and Sema3A, via Nrp-1, these two molecules may compete for receptor binding. Moreover, given that these two semaphorins have been described to have generally opposite effects on endothelial cell proliferation, migration, survival and adhesion, it would be interesting to further characterize the relative abundance and kinetics of expression of these cues in the normal and diabetic retinas, as well as their joint impact on vascular permeability and barrier function (*182, 183*).

While newer targeted treatments such as locally administered corticosteroids and recently approved anti-VEGF therapies for complications associated with diabetic retinopathy are currently available, their off-target effects highlight the need for novel therapeutic approaches. In the present study we disclose the previously undescribed role of

Sema3A as a potent mediator of vascular permeability in diabetic retinopathy. Using a combination of investigative approaches in both human diabetic patients and animal models, we demonstrate that Sema3A is induced in the early stages of DR and precipitates the breakdown of the retinal barrier function. In addition, using a series of orthogonal approaches, including Sema3A gene silencing and a Sema3A trap, we provide evidence that neutralization of Sema3A reduces pathological vascular permeability associated with the early pathogenesis of DR.

Although recent insight on the etiology of DR points to a breakdown in the neurovascular unit, neurovascular cross-talk and its influence on diabetic ocular vasculopathy has thus far received limited attention. Here we provide the first evidence from both human and animal studies for the critical role of the classical neuronal guidance cue Semaphorin3A in instigating pathological macular vascular permeability in type I diabetes.

Interestingly, however, as evidenced in models of diabetic retinopathy and nephropathy, Sema3A induction in diabetes and its related effect on vascular barrier function—via pericyte-like cells and/or directly on ECs—could represent a common etiologic mechanism for diabetic microvascular pathology throughout the body and not an isolated tissue-specific phenomenon. Together, our findings provide insight that may lead to the development of therapeutics that could have applications beyond DR to diseases where VP is a contributing factor such as retinopathy of prematurity, age-related macular degeneration, cancer and stroke.

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