Video Article

Assessment of Vascular Regeneration in the CNS Using the Mouse Retina

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Abstract

The rodent retina is perhaps the most accessible mammalian system in which to investigate neurovascular interplay within the central nervous system (CNS). It is increasingly being recognized that several neurodegenerative diseases such as Alzheimer's, multiple sclerosis, and amyotrophic lateral sclerosis present elements of vascular compromise. In addition, the most prominent causes of blindness in pediatric and working age populations (retinopathy of prematurity and diabetic retinopathy, respectively) are characterized by vascular degeneration and failure of physiological vascular regrowth. The aim of this technical paper is to provide a detailed protocol to study CNS vascular regeneration in the retina. The method can be employed to elucidate molecular mechanisms that lead to failure of vascular growth after ischemic injury. In addition, potential therapeutic modalities to accelerate and restore healthy vascular plexuses can be explored. Findings obtained using the described approach may provide therapeutic avenues for ischemic retinopathies such as that of diabetes or prematurity and possibly benefit other vascular disorders of the CNS.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51351/>

Introduction

Throughout CNS development, nerves, immune cells and blood vessels establish remarkably coupled networks to ensure adequate tissue perfusion and allow transmission of sensory information¹⁻⁵. The breakdown of vascular systems results in insufficient tissue oxygenation and compromised metabolic supply and is increasingly recognized as an important contributor to the pathogenesis of neurodegenerative diseases⁶. Vascular dropout and the deterioration of the neurovascular unit within the brain, for example, is associated with vascular dementia, vascular lesions of the white matter of the brain⁷ and Alzheimer's disease with stenosis of arterioles and small vessels⁸. In addition, impaired vascular barrier function is thought to contribute multiple sclerosis⁹ and amyotrophic lateral sclerosis¹⁰.

Of direct relevance to the retinal model described in this protocol, blinding diseases such as diabetic retinopathy¹¹ and retinopathy of prematurity^{12,13} are characterized by a phase of early vascular degeneration. The ensuing ischemic stress on the neurovascular retina triggers a second phase of excessive and pathological neovascularization that likely originates as a compensatory response to re-instate oxygen and energy supply¹⁴⁻¹⁶. An attractive strategy to overcome the ischemic stress that is central to disease progression is to restore functional vascular networks specifically in the ischemic zones of the neuro-retina (**Figures 2 and 3**). Provoking a controlled angiogenic response may come across as counter-intuitive for a condition in which anti-angiogenic treatments such as anti-VEGFs are considered as adapted treatments. Yet, evidence for the validity of this approach is mounting. For example, enhancing "physiological-like" vascular regrowth in ischemic retinopathies has been elegantly demonstrated through introduction of endothelial precursor cells¹⁷, inhibition of Müller cell-expressed VEGF induced downregulation of other angiogenic factors¹⁸, injection of myeloid progenitors¹⁹, inhibition of NADPH oxidase induced apoptosis²⁰, increasing dietary ω -3 polyunsaturated fatty acid intake²¹, treatment with a carboxyl-terminal fragment of tryptophan tRNA synthetase²², and direct administration of VEGF or FGF-2 for protection of glial cells²³. Moreover, we have demonstrated that modulating classical neuronal guidance cues such as Semaphorins or Netrins in ischemic retinopathies accelerates vascular regeneration of healthy vessels within the retina and consequently reduces pathological angiogenesis^{24,25}. Of direct clinical relevance, several of the aforementioned animal studies provide evidence that promoting vascular regeneration during the early ischemic phase of retinopathies can significantly reduce sight-threatening pre-retinal neovascularization^{19,23,24,26}, likely through the reduction of ischemic burden.

Devising therapeutic strategies that stimulate regeneration of functional vessels remains a significant challenge for vascular biologists. Here we describe an experimental system that employs the mouse model of oxygen-induced retinopathy (OIR) to explore strategies to modulate vascular regrowth within the retina. Developed by Smith *et al.* in 1994²⁷, this model serves as a proxy for human proliferative retinopathies and consists of exposing P7 mouse pups to 75% O₂ until P12 and subsequently re-introducing the pups to ambient room O₂-tension (**Figure 1**). This paradigm loosely mimics a scenario where a premature infant is ventilated with O₂. The exposure of mouse pups to hyperoxia provokes degeneration of

retinal capillaries and microvasculature, and yields a reproducible area of vaso-oblivation (VO) typically assessed upon exit from O₂ at P12, although maximal VO area is reached at 48 hr (P9) after exposure to O₂²⁸. In the mouse, the avascular VO zones spontaneously regenerate over the course of the week following re-introduction to room air and eventually VO zones are completely re-vascularized (**Figure 2**). Re-introduction to room air of mice subjected to OIR also provokes pre-retinal neovascularization (NV) (maximal at P17) that is typically assessed to determine the efficacy of anti-angiogenic treatment paradigms. In its purest form, the OIR model provides a highly reproducible and quantifiable tool to assess oxygen-induced vascular degeneration and determine the extent of destructive pre-retinal neovascularization²⁹⁻³¹.

Various explorative treatment paradigms that modulate CNS vascular regeneration can be investigated using the OIR model including use of pharmacological compounds, gene therapy, gene deletion and more. The propensity of a given approach to influence vascular regrowth is assessed step-wise in the window between P12 (maximal VO after exit from hyperoxia) and P17 (maximal NV). Evaluation of treatment outcome on pathological NV can be rapidly and easily determined in parallel and has been thoroughly described by Stahl and colleagues^{30,31}. Here we provide a simple step-by-step procedure to investigate the modulation of physiological revascularization within the neural retina by pharmacological compounds, prospective therapeutics, viral vectors or to study the influence of candidate genes in transgenic or knockout mice.

Protocol

Ethics statement: All animal experimentation adheres the animal care guidelines established by the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the Canadian Council of Animal Care.

1. Oxygen Induced Retinopathy (OIR)

1. Record date of birth of mouse pups as P0.
2. Record all weights of animals upon entry into O₂ to ensure an adequate weight range. Note: For C57BL/6 mice at P17, body weight should range between 5 and 7.5 g for maximal NV³². In order to maintain environmental consistency, it is recommended to use littermates as control (for genetically modified mice as well as mice receiving experimental treatments). When assessing effects of a viral vector, one must consider tropism of the virus and allow sufficient time for full expression of virally-delivered transgenes. Rapidly expressing viral vectors such as 3rd generation lentiviruses^{24,25,33} are recommended.
3. Place mouse pups at P7 (C57BL/6 or desired strain) and a CD1 fostering mother into an oxygen chamber set at 75% O₂ for 5 days²⁷. Environmental humidity and temperature were held constant throughout O₂ exposure. Note: Research facilities equipped with a central source of O₂ are ideal and limit the cumbersome replacement of empty O₂ tanks. If working with transgenic or knockout mice, it is important to ensure that control and experimental mice are acquired from the same vendor to limit genetic drifts within the same strains^{30,29}.
4. At P12, remove mice from the oxygen chamber and return animals to ambient O₂.

2. Intravitreal Injection for Delivery of Compounds to the Inner Retina (When Assessing Effects of a Pharmacological Compound or Recombinant Protein)

1. At P14, anesthetize mice with 2% isoflurane in oxygen 2 L/min (or animal protection committee-approved anesthetic of choice). In order to verify the effectiveness of the anesthesia, sequentially pinch the tail, rear foot and ear with forceps.
2. Place the mouse on its belly.
3. Using a sterile 10 µl syringe fitted with a beveled pulled-glass needle, perform an injection of a maximal volume of 1 µl of solution containing the compound being investigated or vehicle (physiological saline) at the posterior limbus of the eye, with a 45° angle avoiding the lens. Note: The pulled glass-needle is attached to the syringe using a drop of epoxy-resin.
4. Apply a drop of lubricant ophthalmic ointment (ideally with antibiotic) with a swab to the mouse's eye.
5. Return the mouse back to the cage with fostering mother. Mice are then carefully monitored until recovered and fully ambulatory.

3. Assessment of Vessel Perfusion and Barrier Function (Integrity) by Fluorescein Angiography

1. Anesthetize mice with 2% isoflurane in oxygen 2 L/min (or animal protection committee-approved anesthetic of choice). In order to verify the effectiveness of the anesthesia, sequentially pinch the tail, rear foot and ear with forceps. Note: This is typically performed at P17 when regeneration is assessed. Also carry-out the analysis at P19 and P21 to determine if vascular integrity is preserved over time.
2. Once anesthetized, weigh the mouse.
3. Make a midline abdomen incision with dissecting scissors. Note: Dissecting instruments should be regularly checked and sharpened.
4. Cut ribs laterally and raise the ribcage with the aid of forceps. Note: It is necessary to cut as laterally as possible to avoid damage to the heart.
5. After removing peripheral tissue from the heart, clamp the descending aorta with hemostatic forceps.
6. Slowly inject fluorescein-dextran to the left ventricle using a 25 G needle. Note: If vascular barrier function is investigated, 70 kDa fluorescein-dextran is employed as it will leak out of vessels when vessel integrity is compromised. If the investigator wants to cast blood vessels, 2 MDa fluorescein-dextran is used. Critical steps: 1) To ensure a homogenous repartition, centrifuge fluorescein-dextran and inject the supernatant, 2) In order to prevent vessel constriction, inject warmed fluorescein-dextran solution, 3) Its circulation time shouldn't exceed 4 min.
7. Decapitate mice 2 min after injection with operating scissors.

4. Enucleation and Eye Fixation

Note: When assessing rates of vascular regeneration, first collect retinas at P12 and additionally at P14 and P17. Increase the number of sampled time points for more accurate determination of rates of revascularization²⁴.

1. Tilt the mouse head and place it on its side.
2. Remove skin and eyelids covering the eye using dissecting scissors.
3. Place curved forceps below the eye and gently pull it up until the optic nerve is severed.
4. Turn the mouse's head onto its other side and perform the same steps (steps 4.2 and 4.3).
5. To ensure better penetration of fixative, puncture a hole in the anterior chamber of the eye using 30 G needle.
6. Transfer eyes to a tube containing 4% paraformaldehyde (PFA) and fix for 1 hr at room temperature.
7. Remove PFA and wash eyes 4 times with a solution of ice-cold PBS.

5. Retinal Dissection

1. Place mouse eyes in a Petri dish containing cold PBS and perform dissection of the retinas under a stereomicroscope.
2. Remove extra fat/tissue surrounding the eye with micro-dissection scissors.
3. Cut off the cornea with micro-dissection scissors.
4. Using two pairs of forceps, minutely peel the sclera away from the periphery towards the optic nerve and discard.
5. Pinch the lens (whitish ball beneath the cornea) with forceps and extract it from the eye cup. Use one pair of forceps as a support, and the other to grip and carefully raise and remove the lens.
6. Detach the hyaloid vessels from the inner side of the retina using small brushes (size 0) and forceps.
7. Remove bundles of hyaloid vessels connected to the optic disc using forceps.
8. Transfer dissected retinas to 2 ml microcentrifuge tubes containing PBS and place on ice prior to starting the staining procedure.

6. Retinal Vascular Staining

1. Incubate dissected retinas overnight with gentle shaking at 4 °C in a solution of fluorescently coupled-isolectin B4 (rhodamine-lectin or other) in PBS containing 1 mM CaCl₂ (a 1:100 dilution of a 2 mg/ml isolectin B4 solution is recommended). During the entire staining procedure, cover tubes with aluminum foil or an opaque foil to protect from light.
2. On the following day, remove staining solution and wash retinas 3x in PBS for 10 min at room temperature.

7. Preparation of Retinal Flatmounts

1. Transfer retinas, photoreceptor-side down, onto a microscope slide and make four deep equidistant radial incisions using a surgical scalpel to divide the retina into four equal-sized quadrants. During the incisions, brace the retina with a brush so that it does not move.
2. Using two brushes soaked in PBS, carefully flatten the quadrants photoreceptor side-down and immerse the retina in mounting medium to prevent photo-bleaching. Then carefully place a coverslip on the surface of the mounted retina without applying pressure and making sure that air bubbles do not accumulate under the cover slip.

8. Imaging and Quantification of Vasoobliteration (VO) and Neovascularization (NV) as Previously Described³¹

1. Take images of whole-mounted retinas with an epi-fluorescence microscope at a magnification of 10X.
2. Open the retinal image in photo editing software, stitch together and measure the total retinal area, and avascular area. Area can be expressed in pixels.
3. Determine extent of VO by dividing the number of pixels in the avascular area by the number of pixels in the total retinal area.
4. Determine extent of NV by dividing the number of pixels of NV by the number of pixels in the total retinal area as described³¹.

Representative Results

The OIR model is widely used to study oxygen-induced vascular degeneration and ischemia-induced pathological neovascularization in the retina and has been instrumental in the development of currently employed anti-angiogenic treatments for ocular diseases^{27,29,30}. Findings obtained using this model can be loosely extrapolated to ischemic retinopathies such as proliferative diabetic retinopathy and retinopathy of prematurity³⁰.

Here we present an alternative use of this model to study vascular regeneration. We will describe an example of a strategy to regenerate the ischemic retina that was recently published by our lab. In the presented study, we demonstrate that sustained neuronal ischemia activates endoplasmic reticulum (ER) stress and via one of its effector endoribonucleases IRE1 α , cleaves the mRNA of the classical neuronal guidance cue netrin-1. We show that intra-ocular delivery of the Netrin-1, stimulates a program of reparative angiogenesis in retinal myeloid cells and thus accelerates neural tissue revascularization after OIR²⁵. Moreover, we provide an example of accelerated vascular regeneration using lentiviral mediated silencing of IRE1 α .

The described experimental paradigms can be modified to investigate the explorative treatment of choice. Importantly, the time point of intravitreal injection is determined based on the nature of the explored compound and must take into consideration the mechanism by which the investigated treatment acts. For example, to study a pharmacological compound (receptor agonist, antagonist, etc.), P14 may be selected

as it corresponds to a time point where retinal revascularization is accelerating yet a rapid-acting intervention may help speed up the rate of revascularization (**Figures 2 and 3a**). When viral vectors are employed, enough time must be allotted to allow for full expression of the passenger gene and an earlier time point may be selected to ensure full expression of the transgene or full silencing of the target gene (**Figure 4**). Lentiviral based vectors are particularly well suited in this regard due to their rapid expression, low inflammatory response and ease of production^{24,25}. It is also crucial to make certain that the target gene is delivered to the appropriate retinal cell population (RGC, Endothelial cell, Müller Cell, etc.), hence the tropism of the selected viral vector must be considered. Alternatively, studying the role of a gene in vascular regeneration using transgenic or knockout animals offers the advantage of not needing to perform intra-ocular injections.

Figure 1. Schematic depiction of the mouse OIR model. Mouse pups and nursing mothers are exposed to 75% O₂ from P7 to P12. A ventilated oxygen chamber with steady O₂ delivery and oximeter is required. During this initial period, retinal vaso-obliteration occurs. At P12, mice are returned to room air (21% O₂) until P17 when maximal pathological pre-retinal tufting occurs. Neovascularization recedes over the following days. The ideal period to investigate vascular regeneration is the window between P12 and P17. Sampling and assessing several time points for extent of vaso-obliteration is key to elucidating accurate rates of revascularization.

Figure 2. Time course of retinal revascularization following oxygen-induced vaso-obliteration. A) Graphic depiction of a retinal flatmounting procedure. An incision is made through the top of the cornea (black-grey dome) and the sclera (dotted red lines) and the retina is teased out. The lens can be extracted either after opening the cornea/sclera or once the sclera is removed as shown in the diagram. Hyaloid vessels are then removed and staining of retinal vessels performed. Once the staining protocol is finished, the retina is then cut into 4 equally spaced sections and mounted on a microscope slide vessel-side-up. **B)** Lectin-stained flatmount retinas from mice subjected to OIR. As vessels regenerate, the area of vaso-obliteration (here maximal at P12) fills in. Pathological neovascularization is maximal at P17 and appears as saturated spots in lectin stained OIR retinas. By P19-P21, retinas have fully regenerated their vascular networks. (This figure has been modified from Binet *et al. Cell Metabolism* 2013²⁵)

Figure 3. Example of an injectable treatment that accelerates retinal vascular regeneration: Netrin-1. **A)** Netrin-1 is injected at P14 and extent of vascular regrowth assessed at P17. A dose-dependent increase in vascular regeneration is observed. **B)** Vascular integrity can be assessed by perfusion with a low molecular weight fluorescent Dextran. If vessels show compromised barrier function, the fluorescent Dextran will leak outside the vessel (arrows). (This figure has been modified from Binet *et al. Cell Metabolism* 2013²⁵)

Figure 4. Example of gene silencing with lentiviral-delivered shRNAs for retinal vascular regeneration: Lv.shIRE1α. To assess the ability of gene silencing of IRE1α in retinal ganglion neurons to aid vascular regeneration, an intravitreal injection of a lentivirus coding for an shIRE1α was injected at P3 to allow sufficient time for gene silencing when vascular growth and vaso-obliteration is assessed. The 3rd generation lentivirus employed in this example contains a modified vesicular stomatitis virus glycoprotein (VSV-G), engineered to target plasma membranes. Although these vectors effectively infect retinal ganglion cells, expression can also be noted in other local cell types. **(A)** This treatment did not lead to noticeable variations in oxygen-induced vaso-obliteration as determined at P12, signifying that all observed benefits on vaso-obliteration measured at later time points are due to increased vascular regeneration. **(B)** Inhibition of IRE1α in this paradigm dramatically enhanced vascular regeneration in the regrowth phase of OIR at P17. (This figure has been modified from Binet *et al. Cell Metabolism* 2013²⁵) **(C)** Assessing a third time point of vaso-obliteration such as P14, is required to determine accurate rates of revascularization. (This figure has been modified from Joyal *et al. Blood* 2011²⁴) [Please click here to view a larger version of this figure.](#)

Discussion

What is the most effective way to stimulate growth of new healthy vessels in ischemic nervous tissue? Is it therapeutically valid to interfere with and accelerate naturally occurring vascular regrowth? In neuro-ischemic pathologies such as ischemic retinopathies or stroke, vascular degeneration is associated with reduced neuronal function³⁵⁻³⁸. Hence to counter early injury, reinstating regional micro-circulation during the immediate/early segment of disease may prove beneficial. In an ocular context, experimental paradigms that accelerate revascularization of the ischemic retina reduce pathological neovascularization^{21-25,34} and thus this approach merits further investigation.

Since its introduction 20 years ago, the OIR model²⁷ has revolutionized retinal angiogenesis research³⁰. Here we provide an additional application for this model to study prospective strategies to modulate physiological vascular regeneration in the ischemic retina. An ideal animal model should encompass several criteria such as proximity to human patho-physiology, high reproducibility, rapid execution and ideally, low cost. The mouse model of OIR meets all these criteria and can be carried-out in less than 3 weeks.

Despite the numerous listed benefits, drawbacks include the current need to sacrifice the mouse prior to analyses and hence accurate longitudinal monitoring of an animal is not yet possible with current imaging tools. Currently, *in vivo* imaging techniques such as OCT or fluorescein angiography are largely unsuccessful due to the inherent optical limitation of the mouse eye (curvature radius and small size) which reduces imaging coverage³⁹ to the most central regions of the retina which are not relevant to the early stages of the model.

When employed to study vascular regeneration as proposed in this protocol paper, all considerations that apply to studying neovascularization should also be monitored. These include recording animal weight to estimate the metabolic health of the pup, which heavily influences angiogenesis³². Accurate control of oxygen tension in the hyperoxic chamber is also critical. Variation in oxygen concentration has a direct impact on vasoobliteration, which can inevitably lead to critical data misinterpretation. It is therefore recommended to either implement continuous electronic monitoring of chamber oxygen concentration or in the least, monitor daily changes in oxygen levels and limit oxy-cycler door opening to maintain constant oxygen levels. Optimal processing of retinas also takes practice. Extraction, mounting and staining of the retina must be executed with care as the relatively fragile retina must be delicately handled. In addition, as for all experimentation with genetically modified animals, the genetic drift of a colony can confound results. In absence of a selective force (in a mouse colony for example), the allelic or genetic drift is a random process that can lead to large changes in population over a short period of time. Allele frequencies can change from generation to generation and result in the formation of a sub-colony^{40,41}. These mutations are largely undetectable and it is thus important to limit the number of generations produced by the same breeder pairs in the same colony. The most efficient way to attain the highest genetic stability is to refresh "stocks" every five generations or proceed to backcrossing with a purchased mouse of identical background.

It is also recommended to test for retinal degeneration mutations (rd) 1 and 8⁴², at least once before starting a new line in order to avoid confounding phenotypes that can be attributed to premature photoreceptor degeneration. For transgenic animals, it is vital to ensure that both control and mutant mice are obtained from the same vendor and are necessarily on the same genetic background.

As we continue to elucidate the molecular mechanisms of blood vessel formation and growth, novel approaches to accelerate, slow, or steer nascent blood vessels are arising. A model system in which modulation of vascularization can be explored *in vivo* in a pathological context may provide a valuable tool to explore prospective therapeutic paradigms to counter neuronal ischemia within the CNS. Adapting the mouse OIR model to study vascular regeneration provides such a venue and will continue to be a valuable tool to further our understanding of the molecular basis for physiological and pathological angiogenesis.

Disclosures

The authors have nothing to disclose.

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