Study Protocol

Optic nerve crush as a model of retinal ganglion cell degeneration

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Background: Axonal degeneration caused by damage to the optic nerve can result in a gradual death of retinal ganglion cells (RGC), leading to irreversible vision loss. An example of such diseases in humans includes optic nerve degeneration in glaucoma. Glaucoma is characterized by the progressive degeneration of the optic nerve and the loss of RGCs that can lead to loss of vision. The different animal models developed to mimic glaucomatous neurodegeneration, all result in RGC loss consequent optic nerve damage.

Methods: The present article summarizes experimental procedures and analytical methodologies related to one experimental model of glaucoma induced by optic nerve crush (ONC). Point-by-point protocol is reported with a particular focus on the critical point for the realization of the model. Moreover, information on the electroretinogram procedure and the immunohistochemical detection of RGCs are described to evaluate the morpho-functional consequences of ONC.

Discussion: Although the model of ONC is improperly assimilated to glaucoma, then the ONC model simulates most of the signaling responses consequent to RGC apoptosis as observed in models of experimental glaucoma. In this respect, the ONC model may be essential to elucidate the cellular and molecular mechanisms of glaucomatous diseases and may help to develop novel neuroprotective therapies.

Keywords: Axonal injury; retrograde degeneration; cell death

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Introduction

Neurodegenerative disorders of the retina involve optic nerve (ON) head injury and the progressive death of retinal ganglion cells (RGCs). The axons of the RGCs form the ON and transmit visual information to the visual cortex. The death of RGC is one of the main causes of irreversible blindness in the world (1).

Glaucoma is a collection of diseases that lead to an irreversible vision loss due to RGC damage. Although the causes leading to RGC loss are not fully understood, the onset and progression of glaucomatous damage and RGC death may share similar mechanisms.

Despite several models have been developed to mimic glaucomatous neurodegeneration, all of them result in RGC loss consequent to optic nerve damage, probably as the initial insult in glaucomatous diseases. In this respect, the optic nerve crush (ONC) model although not directly mimicking the pathophysiological mechanisms of the human glaucoma has allowed us to acquire information on RGC degeneration.

In particular, the ONC model has been used to investigate mechanisms involved in RGC degeneration in order to identify molecules with therapeutic potential to be eventually used as pharmacological treatments against glaucomatous neurodegeneration.

The ONC model is not novel, having been first described in detail in 1999 (2) and has been highly refined to demonstrate a clear association between RGC loss and nerve crush force-impulse and duration (3,4). In addition, both RGCs themselves and the mouse strain used for the ONC protocol may differ in their resilience to the mechanical injury (5,6). Nevertheless, its quick and easy procedure makes the ONC model attractive to replicate RGCs loss that characterizes optic neuropathies including glaucoma.

The purpose of the present manuscript is to review the protocol to induce ONC in mice. Although the protocol is well consolidated, here we emphasize those peculiar strategies that are often neglected in detail or not even

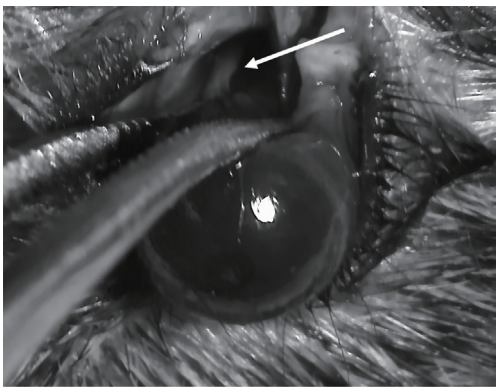

Figure 1 The optic nerve was exposed by blunt dissection of the conjunctiva and crushed at about 1 mm behind the globe. The white arrow points at the optic nerve.

discussed about the pros and cons of different strategies in order to provide readers with specific information, which may be of great help to determine the success of a given methodology. In the surgical procedure, for instance, the methodology used to access the ON, the force and the duration of the crush are critical to determine the extent of the ON damage. In addition, detailed information on the Pattern ERG procedure to evidentiate RGC function and the immunohistochemical detection of RGCs can make the difference when evaluating the morpho-functional consequences of ONC.

Methods

ONC procedure

(I) Use the C57BL/6J mouse strain because the eye anatomy is better evident in pigmented mice than in albino mice.

(II) Anesthetize the mouse by intraperitoneal injection of avertin (1.2% tribromoethanol and 2.4% amylene hydrate in distilled water, 0.02 mL/g body weight; Sigma-Aldrich, St. Louis, MO) or an approved cocktail in agreement with laboratory protocols or local regulations. Confirm the depth of anesthesia by pinching the hind limbs.

(III) Transfer the mouse to the surgical microscope and instill 1 drop of topical anesthetic in each eye.

(IV) Perform lateral canthotomy to increase access to the optic nerve.

(V) Insert the tips of Dumont n. 5 forceps between the eyelids so that the eye protrudes from the socket.

(VI) Scrape the scleral surface with fine forceps after the dissection of the overlying conjunctiva to reduce the risk of damaging the vascular plexus leading to excessive bleeding.

(VII) Pinch the conjunctiva along the upper temporal boundary of the globe with Dumont No. 7 curved forceps that makes a small fold in the tissue to avoid damaging the sclera. With the Vannas spring scissors, make a hole of about 1 mm in the superior temporal conjunctiva and then dissect the conjunctiva to widen the hole.

(VIII) Gently separate the tissue with forceps until you have a clear view, through the newly formed opening, of the superior muscle and remove the fat blocking the optic nerve. Be careful not to disrupt any blood vessels otherwise the cavity will be filled by blood thus obstructing the vision of the optic nerve.

(IX) Once exposed, use the curved Dumont #N7 self-closing forceps in the open position around the optic nerve 1–2 mm from the eyeball, loosen grip and hold steady for 5 s to crush the ON (*Figure 1*). Depending on the forceps, try different force and duration of the crush to find the optimal parameters to induce adequate degeneration of ON axons and significant RGC cell death.

(X) Following crush, check for adequate blood flow in the retina. Apply an antibiotic ointment over the incision and cornea. Place mice on a heating pad to fully recover before transferring back to their home cages and check for infection every 12 h.

Survival time

The survival time between ONC and electrophysiological detection of RGC function has a dramatic bearing on monitoring RGC pathology and has been established in several studies. After mechanical optic nerve damage, RGC degeneration begins rather quickly. RGCs die in a proportion of approximately 50% and 70% one or two weeks after injury, respectively. However, different outcomes are likely to result from substantial differences in the extent of crush thus confirming the necessity to check for the severity (strength and duration) of the crush. In fact, injury to RGCs after ONC can vary between studies and depends on how much force is applied at the injury site, the distance from the globe where the injury is applied and the species or strain of the experimental animal (7-9).

Let the mice to survive for 7 days until to proceed

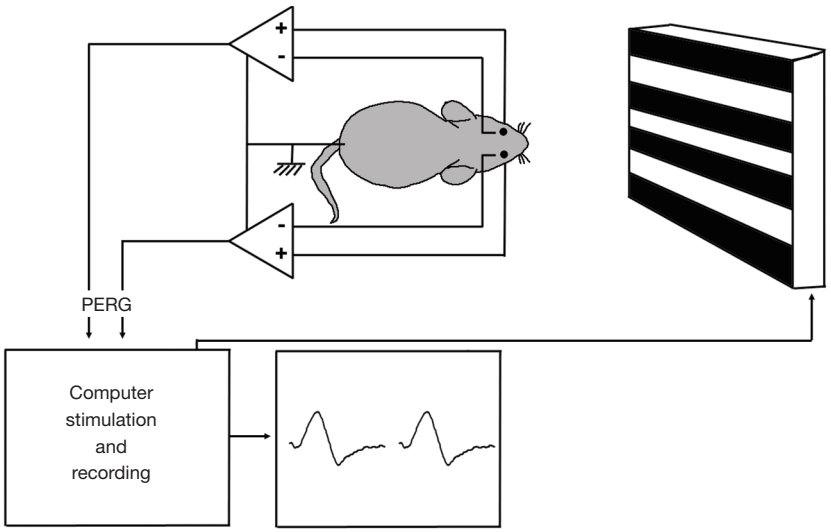

Figure 2 Schematic diagram of the recording setup. PERG, pattern-induced electroretinogram.

with further investigations including recording of the pattern-induced electroretinogram (PERG) that is an accurate measure of RGC function and the RGC immunohistochemical detection to determine RGC loss in comparison to control retinas.

Pattern electroretinogram

(I) After overnight dark adaptation, anesthetize and prepare the mice for recording under dim red light. Use a heating pad to keep the body temperature at 37 ℃.

(II) Record the PERG responses through a corneal electrode. Place two stainless-steel needles as reference electrode on the forehead and a ground electrode on the tail root. PERG responses can be evoked in anesthetized mice using an alternating pattern of black and white horizontal bars delivered on a stimulus display unit from a commercially available PERG system (SB700 Advanced; Nikon-Europe, Amsterdam, Netherlands). Stimuli consist in 98% contrast-reversing bars with 0.05 cycles/deg spatial frequency and 1 Hz temporal frequency. The pattern stimuli are administered through a light emitting diode display with a mean luminance of 50 cd/m^2 aligned at about 20 cm from the corneal surface (*Figure 2*).

(III) A total of 200 signals needs to be averaged. PERG responses can be analyzed by measuring the amplitude and the implicit time of the N35-P50 and P50-N95 waves.

Immunohistochemical detection of RGCs

(I) After PERG recording, leave the animal to recover for almost for 1 day. Then proceed to RGC detection by sacrificing the mice with an overdose of avertin. After the mouse is euthanized, enucleate its eyes.

(II) Use small scissors to remove the cornea by cutting along the *ora serrata* and pull out the lens with forceps.

(III) To remove the net from the cup, slowly move the forceps along the circumference line. Cut the optic nerve to ease the separation of the retina from the eyecup.

(IV) Make incisions from the periphery towards the optic disk to make 4 quadrants. The incisions will allow to make a flat preparation and to refer the RGCs to a specific retinal quadrant (*Figure 3*).

(V) Fix the whole mounts in 4% paraformaldehyde [dissolved in 0.1 M phosphate buffer saline (PBS)] for 2 h at 4 ℃ and store at 4 ℃ in 25% sucrose dissolved in 0.1 M PBS.

(VI) Then incubate the retina with the guinea pig polyclonal antibody directed to RNA-binding protein with multiple splicing (RBPMS, ABN1376, dilution 1:100; Merck, Darmstadt, Germany), a RGC marker (10) in PBS containing 5% bovine serum albumin (BSA) and 2% TritonX-100 for 24 h at 4 ℃.

(VII) Aspirate the primary antibody and wash the retina 3 times with 1× PBB for 15 min each, then incubate the retinas overnight with FITC-conjugated anti-guinea pig secondary antibody (F6261; Merck) at 4 ℃.

(VIII) Aspirate the secondary antibody and wash the retina 3 times with 1× PBS for 15 min each.
(IX) Mount the retinas on slides with the ganglion cell side facing up. Place 1–2 drops of mounting medium (ab104139; Abcam, Cambridge, United Kingdom) on the surface of each retina and cover with a 22 mm × 22 mm micro coverslip.
(X) Acquire 10× retinal images with a fluorescence microscope (Ni-E; Nikon-Europe, Amsterdam, The Netherlands) in order to obtain four radial tiles (500 × 500 µm) in the central and peripheral retina (center: 500 µm from the optic nerve head; periphery: 500 µm from the peripheral edge).
(XI) Count the RGC density in retinas as the average of the number of RBPMS immunopositive somata per mm^2.

Results

Pattern electroretinogram

To evaluate RGCs function, analyze the amplitude and implied time of the components of the PERG responses. In particular measure the amplitude of the N35-P50 wave from the depression of the negative peak, N35, to the peak of the positive peak, P50, and the amplitude of the P50-N95 wave from the peak of the positive peak, P50, to the depression. respectively of the negative peak, N95. In addition, measure the implicit time from the onset of the stimulus to the P50 and N95 peaks. After 7 days from ONC, both components N35-P50 and P50-N95 are significantly reduced compared to controls, while implicit times are increased (*Figure 4*).

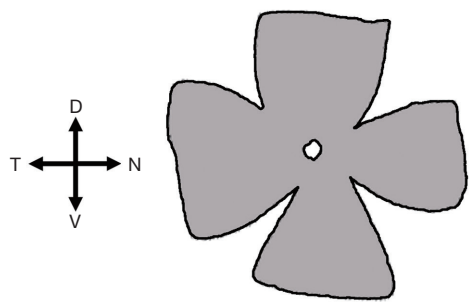

Figure 3 Schematic drawing of retina whole mount cut in to 4 quadrants. D, dorsal; T, temporal; N, nasal; V, ventral.

Quantification of RGC loss

To demonstrate the efficacy of ONC, count the RBPMS immunopositive cells/mm^2 in a masked manner. As shown in *Figure 5*, in the ONC model the number of RBPMS-positive cells is significantly reduced compared to the control retina, indicating a consistent loss of RGCs as successfully induced by ONC.

Discussion

While the ONC is used as a model for glaucoma in the research setting, the causes of glaucoma cannot be attributed to axonal injury alone. Intraocular pressure (IOP) may also play an important role and there are rodent models that simulate glaucoma by increasing the IOP. In fact, IOP elevation, caused by an imbalance in the production and drainage of aqueous humor in the anterior chamber of the eye, is considered one of the main risk factors in glaucoma (11). Although mouse models of ocular hypertension can be considered as good models of glaucoma, minimum acceptable standards need to be evaluated to validate these models. Each model has its strengths and weaknesses, for example, models using injection of viscous agent or laser photocoagulation are useful to study the effect of IOP elevation on RGC degeneration. In addition,

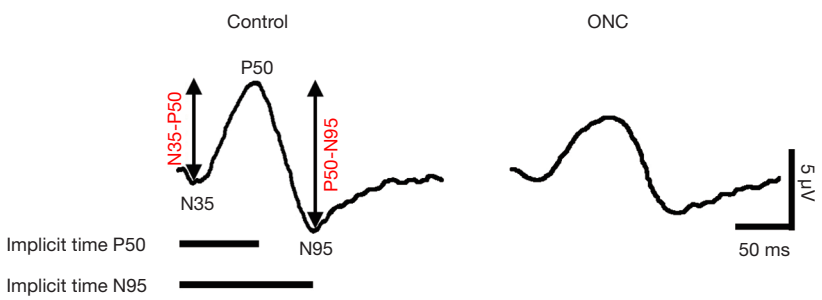

Figure 4 Representative example of PERG waveform and measurements of N35-P50 and P50-N95 amplitudes and their implicit time in control and ONC mice. PERG, pattern-induced electroretinogram; ONC, optic nerve crush.

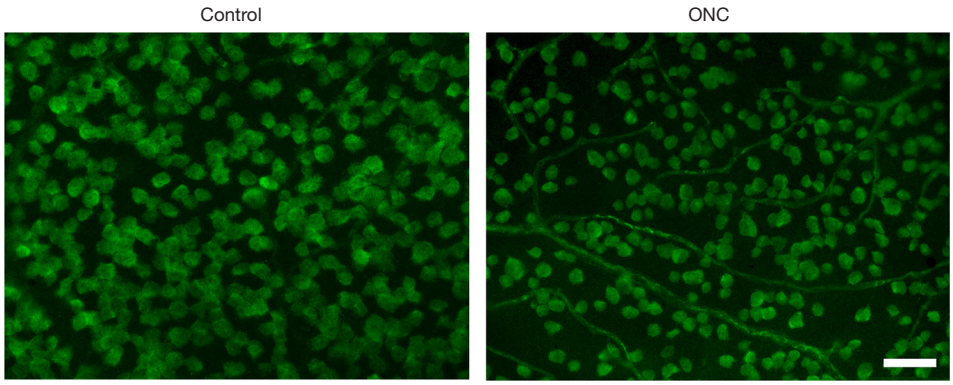

Figure 5 Representative images of RBPMS-immunostained RGCs in retinal whole mounts from control and ONC mice. Scale bar: 50 µm. RBPMS, RNA-binding protein with multiple splicing; RGCs, retinal ganglion cells; ONC, optic nerve crush.

the DBA/2J mouse is used as a model for congenital experimental glaucoma. However, when using this model, it can lead to difficulties in assessing disease progression due to its variability (12,13). In the ONC model discussed here, the contribution of axonal injury to the glaucomatous pathology is independent on the effects of ocular hypertension on RGC dysfunction thus mimicking more precisely traumatic optic neuropathies and representing an excellent model for studying to which extent axonal injury plays a role in glaucomatous pathology.

The advantages of this model are the ease of carrying out the procedure and the rapid onset of pathology. Furthermore, this model involves some features similar to human glaucoma. In fact, a significant portion of RGC injury in glaucoma is believed to occur through mechanical stress at the optic nerve head. Therefore, the ONC model represents a good model for studying the role of axonal injury in glaucomatous pathology (14).

Overall, each of the glaucomatous models has inherent advantages and disadvantages. In general, experimental designs attempt to maximize the information available for each research task. An optimal experiment allows the inferred models with the highest degree of confidence expected. However, in the situation where some models are incomplete, the role of the experimental design must be carefully examined to avoid a complex interpretation of the results.

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Footnote

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Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

1. Alqawlaq S, Flanagan JG, Sivak JM. All roads lead to glaucoma: Induced retinal injury cascades contribute to a common neurodegenerative outcome. Exp Eye Res 2019;183:88-97.
2. Li Y, Schlamp CL, Nickells RW. Experimental induction of retinal ganglion cell death in adult mice. Invest Ophthalmol Vis Sci 1999;40:1004-8.
3. Liu X, Feng L, Shinde I, et al. Correlation between retinal ganglion cell loss and nerve crush force-impulse established with instrumented tweezers in mice. Neurol Res 2020;42:379-86.
4. Norat P, Gao J, Soldozy S, et al. A standardized crush tool to produce consistent retinal ganglion cell damage in mice. Neural Regen Res 2021;16:1442-3.
5. Tran NM, Shekhar K, Whitney IE, et al. Single-Cell Profiles of Retinal Ganglion Cells Differing in Resilience to Injury Reveal Neuroprotective Genes. Neuron 2019;104:1039-1055.e12.
6. Li Y, Schlamp CL, Nickells RW. Variable susceptibility of ganglion cells to optic nerve crush in inbred mice. Invest Ophthalmol Vis Sci 2003;44:E-abstract 1131.
7. Galindo-Romero C, Avilés-Trigueros M, Jiménez-López M, et al. Axotomy-induced retinal ganglion cell death in adult mice: quantitative and topographic time course analyses. Exp Eye Res 2011;92:377-87.
8. Parrilla-Reverter G, Agudo M, Nadal-Nicolás F, et al. Time-course of the retinal nerve fibre layer degeneration after complete intra-orbital optic nerve transection or crush: a comparative study. Vision Res 2009;49:2808-25.
9. Nadal-Nicolás FM, Jiménez-López M, Sobrado-Calvo P, et al. Brn3a as a marker of retinal ganglion cells: qualitative and quantitative time course studies in naive and optic nerve-injured retinas. Invest Ophthalmol Vis Sci 2009;50:3860-8.
10. Rodriguez AR, de Sevilla Müller LP, Brecha NC. The RNA binding protein RBPMS is a selective marker of ganglion cells in the mammalian retina. J Comp Neurol 2014;522:1411-43.
11. Kastner A, King AJ. Advanced glaucoma at diagnosis: current perspectives. Eye (Lond) 2020;34:116-28.
12. McDowell CM, Kizhatil K, Elliott MH, et al. Consensus Recommendation for Mouse Models of Ocular Hypertension to Study Aqueous Humor Outflow and Its Mechanisms. Invest Ophthalmol Vis Sci 2022;63:12.
13. Turner AJ, Vander Wall R, Gupta V, et al. DBA/2J mouse model for experimental glaucoma: pitfalls and problems. Clin Exp Ophthalmol 2017;45:911-22.
14. Johnson TV, Tomarev SI. Rodent models of glaucoma. Brain Res Bull 2010;81:349-58.

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