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How can we study the glaucomatous pathology in an animal model?

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Abstract:	The DBA/2J mouse is a model of ocular hypertension and retinal ganglion cell (RGC) degeneration, due to the iris pigment dispersion (IPD) and iris stromal atrophy (ISA). These mice present increase in intraocular pressure (IOP) typical in many glaucoma types. This increase correlates with the death of the retinal ganglion cells. The RGCs can be investigated at different age by retrograde tracing with an analogue of fluorogold, hydroxystilbamidine methanesulfonate (OHSt), applied on the superior colliculi. Whole mount retinas were processed to quantify the population of RGCs identified by fluorogold tracing and were counted using image analysis software; an isodensity contour plot was generated for each retina. These progressive process could be also followed by means of electroretinographic recordings that clearly correlated with morphological changes (loss of RGCs). The DBA/2J mouse presented a rise in IOP which started at 9 months being maximal at 12 months with an increase of 70 % above the initial IOP. Quantification of the total number of RGCs identified by OHSt showed a clear increase in the cell death as long as the pathology advanced, this being also correlated by a decrease in the in pSTR epectroretinographic recordings. Our results indicate the existence of a correlation between the rise in IOP, retinal function impairment and RGC loss. This functional and morphological analysis allows a reliable assessment of the progression of the disease and permits the use of this animal as a preclinical model for investigation potential antiglaucomatous drugs.
Author Comments:	An important point is that this work is performed by three different labs. I have indicated my lab (in the Universidad Complutense de Madrid) as the reference one, but it would

	be necessary also to film in the Universidad de Murcia and in the Universidad de Alcala de Henares since the paper combines techniques developed and performed in three different labs as indicated.
Additional Information:	
Question	**Response**
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Dear Editor:

Please find enclosed the revised version of the manuscript entitled: “How can be studied the glaucomatous pathology in an animal model?” by Drs. Perez de Lara and co-workers to be published in JoVE.

We have done an extensive revision of the manuscript taking into account the comments raised by the three referees. Nonetheless, we wish to comment to the editor some aspects that make us feel quite uncomfortable.

1.- The reviewer 2, suggest the possible existence of better control strains that the C57. It is true that there are other genetically modified animals that apparently may be more suitable. Nevertheless electrophysiological studies demonstrate relevant functional changes indicating that they are not the best strains to be compared to the DBA/2J.

2.- Reviewer 3, is complaining about the fact of using STR for the recording of ganglion cell functionality. We agree with the referee 3 that PERG is an excellent technique but STR is also a good one to determine the electrical activity of ganglion cells.

That’s all we wanted to comment. Thank you very much for your attention and comprehension.

Looking forward to hear from you soon.

Yours,

Dr. Jesús Pintor.

TITLE:
Study of glaucomatous pathology in an animal model

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KEYWORDS:
Animal model, electroretinography, glaucoma, intraocular pressure, DBA/2J mouse, retinal ganglion cell

SHORT ABSTRACT:
In this paper, we present a mouse model of glaucoma that depicts the features of the glaucomatous pathology in humans and demonstrates how it might be used for preclinical studies.

LONG ABSTRACT:
Due to iris pigment dispersion (IPD) and iris stromal atrophy (ISA), the DBA/2J mouse is a model of ocular hypertension and retinal ganglion cell (RGC) degeneration. These mice present increases in intraocular pressure (IOP) typical in many glaucoma types. This increase correlates with the death of the RGCs. The RGCs can be investigated at different ages through retrograde tracing with an analogue of fluorogold, hydroxystilbamidine methanesulfonate (OHSt), applied on the superior colliculi. Whole-mount retinas were processed to quantify the population of RGCs identified by fluorogold tracing and were counted using image analysis software. An isodensity contour plot was generated for each retina. These progressive processes could also be followed by means of electroretinographic recordings that clearly correlated with morphological changes (loss of RGCs). The DBA/2J mouse presented a rise in IOP which started at 9 months and became maximal at 12 months, with an increase of 70% above the initial IOP. Quantification of the total number of RGCs identified by OHSt showed a clear increase in cell death as the pathology advanced; this was also correlated with a decrease in the in pSTR electroretinographic recordings. Our results indicate the existence of a correlation between rises in IOP, retinal function impairment, and RGC loss. This functional and morphological analysis allows for the reliable assessment of the progression of the disease and permits the use of this animal as a preclinical model for the investigation of potential antiglaucomatous drugs.

INTRODUCTION:
Glaucoma consists of a group of optic neuropathies that are characterized by the progressive loss of retinal ganglion cells (RGCs)[1-3], with a concomitant loss of visual function that may result in permanent blindness[2]. This pathology is often associated with an abnormal increase in the intraocular pressure (IOP), the only major risk factor that can be modified with medical or surgical treatment[4-7].

Patients with IOP values above 21 mmHg are further examined with ophthalmological tests for the presence of typical optic disk abnormalities and thinness of the retinal nerve fiber layer. Any loss of visual function is examined with visual field tests (perimetry) or electroretinography in order to fully confirm the existence of the pathology. When glaucoma is diagnosed, there are several therapeutic options, pharmacological or surgical, that can be implemented to avoid further progression of the disease.

Several glaucoma animal models have been developed to mimic some of the features of this pathology. A popular group of animal models is based on the elevation of the IOP. This may be achieved with the application of substances such as corticoids, alpha chemotrypsin, phenol[8-12], or hyaluronic acid, which increase the volume of the aqueous humour. Alternative strategies, such as cauterization of the episcleral or perilimbal veins or photocoagulation of the trabecular meshwork, diminish the aqueous humor outflow. Other, more dramatic models, such as optic nerve axotomy, may also contribute to the investigation of injuries associated with RGC loss.

Despite the existence of several animal models for experimental glaucoma[13,14], there was a lack of an animal model that spontaneously developed a glaucomatous pathology. Since 1927, the mouse strain DBA/2J has been used in laboratories for

many research purposes, including cardiovascular biology, neurobiology, and sensorineural research. It was only recently realized that this strain develops a spontaneous exfoliative glaucoma. Although this type of glaucoma is not the most representative of that in humans, its development in mice mimics the progression of human glaucoma.

DBA/2J presents a gradual increase in IOP, which is maximal between the 9th and 12th month of age. This rise in the IOP is mainly due to the iris pigment dispersion in the eye anterior chamber produced by an iris atrophy. The rise in IOP might be the key factor that produces retinal cell damage in this animal model. Retinal cell death concomitantly produces a visual dysfunction that can be recorded by electroretinography. In conclusion, the rise in IOP, the retinal damage, and the retinal loss-of-function are three hallmarks of the glaucomatous pathology in humans that are measurable in the DBA/2J mouse model.

In the present work, we introduce how to measure three aspects of glaucoma: IOP, retinal cell death, and changes in the retinal electrophysiology. Altogether, it will be possible to study how glaucoma progress in these animals. Moreover, this animal model will be helpful in the investigation of the major challenges of glaucoma treatment: the search for hypotensive compounds and the exploration of compounds with retinal neuroprotective properties.

PROTOCOL:

All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

NOTE: Experiments were performed on adult female C57BL/6J (n=8) and DBA/2J (n=8) mice. Mice were housed (1-4 mice per cage) in temperature- and light-controlled rooms maintained according to a 12-hr light/dark cycle. All animals were fed *ad libitum*. DBA/2J and C57BL/6J mice were studied at 3, 6, 9, 12, and 15 months of age. The experiments were performed on female DBA/2J and C57BL/6J mice since IOP increases earlier in females and remains elevated longer[15].

1. Assessment of intraocular pressure measurements

1.1 Ideally, perform the IOP measurements at the same time of day to avoid fluctuations due to circadian rhythms[16,17].

1.2 Anesthetize the mice in accordance with institutional guidelines. Inject mice with a mixture of ketamine (95 mg/kg) and xylazine (5 mg/kg) through an intraperitoneal (i.p.) injection.

1.2.1. Confirm anesthesia effectiveness with a gentle toe pinch, which should not break the skin or cause any deep tissue damage, or by corneal reflex (touching the edge of the cornea with a cotton tip).

NOTE: Any observed movement (withdrawing the paw or movement of the eyelids) indicates that the animal is not sufficiently anesthetized for surgery.

1.3 Bring the tonometer close to the mouse's eye, fixing the tonometer with hands and/or to some solid object. The distance must be 1-4 mm from the tip of the probe to the cornea of the eye. Ensure that the tip of the probe hits perpendicularly to the central cornea. Measure both eyes of each animal at 3, 6, 9, 12, and 15 months of age.

NOTE: To perform intraocular pressure measurements, a non-invasive rebound tonometer[4,14,18,19] was used, and readings were taken just after the application of anesthesia[20].

1.4 Collect six consecutive measurements for each reading and repeat it three times to get a final IOP value[14,21].

2. Electroretinographic recordings

2.1 Dark-adapt the mice overnight. Carry out all manipulation procedures under dim red light (l > 600 nm).

2.2 Calibrate the light intensity with a dual-biosignal generator device specifically adapted for ERG responses in order to ensure consistent recording parameters across the different ages.

2.3 Anaesthetize the mice with an i.p. injection of a solution of ketamine (95 mg/kg) and xylazine (5 mg/kg).
NOTE: Confirm anesthesia effectiveness (see 1.2.1).

2.4 Dilate the pupils by applying a topical drop of 1% tropicamide in the right eye of the experimental animal five min before ERG testing.

2.5 Proceed to situating the electrodes:

2.5.1 Apply a topical non-allergenic ionic conductive drop of methylcellulose to each eye before situating the corneal electrode.

NOTE: This approach is necessary to minimize corneal desiccation and maximize conductivity of the generated response.

2.5.2 Keep the working electrode, a mouse Burian-Allen lens electrode, in contact on the corneal surface covered with methylcellulose. Place the reference electrode in the mouth. Place a needle subcutaneously at the base of the tail as the ground electrode.

2.6 Maintain the mice on a circulating warm water pad at 37 ºC. Place them in a Ganzfeld stimulator to ensure consistent experimental conditions.

2.7 Stimulate flash-induced ERG responses with a Ganzfeld dome light source, which ensures homogeneous illumination of the retina and emits a wide range of light intensities.

NOTE: Using a commercial amplifier, amplify the electrical signals generated in the retina and filter the bands between 0.3 and 1,000 Hz. Digitalize the electrical signals

at 20 kHz using a data acquisition board and display them on a PC computer monitor. Record the scotopic threshold response (STR) by stimulating the retina with light intensities ranging between 10^{-5} and 10^{-2} cd s m^{-2} in the right eye. Average a series of ERG responses (20 ERGs for each trace) at each light intensity after adjusting the time interval between flashes to ensure complete recovery of the response (10 sec for the dimmest stimulus intensities and 30 sec for the strongest stimuli).

2.8 Analyze the different recordings of the amplitudes of the different waves according to the criteria established by the International Society for Clinical Electrophysiology of Vision (ISCEV).

NOTE: This ERG protocol has been previously used by several authors as a functional test for the study of retinal ganglion cell population[5,6,22-27].

2.9 Maintain the mice on a heating pad to assure normal body temperature at 37 °C during recovery from the induced anesthesia.

2.10 Apply ocular ointment containing neomycin and prednisone to prevent corneal desiccation.

3. Retrograde labeling study

3.1 Anesthetize the mice with an i.p. injection of ketamine (70mg/kg) and xylazine (10mg/kg). Confirm anesthesia effectiveness (see 1.2.1). Apply sterile eye lubricant ointment to prevent drying of the corneas during surgery.

3.2. Shave the head with an electric clipper equipped with a surgical blade. Remove the clipped hair from the animal using a vacuum-system or tape. Disinfect the operation area with 10% povidone iodine solution.

NOTE: Retrograde labeling was performed as described elsewhere[4,6,26-30].

3.3. Cut the scalp along the midline with a #15 scalpel blade attach to a reusable scalpel blade handle. Expose the skull. Perform a bilateral parietal craniotomy. Use a dental drill to expose the dura mater, and cut it with fine forceps or spring scissors.

3.4. Carefully remove the brain cortex over the dorsal surface of the superior colliculi (SCi) using aspiration with a fine glass cannula attached to a vacuum pump. Operate under surgical microscope visual guidance.

NOTE: Aspirate the brain content until the SCi limits are observed under the microscope. Be careful not to touch the sinus

3.5. Place a small piece of gelatin sponge soaked in the tracer solution (10% hydroxystilbamidine methanesulfonate (OHSt) and 10% dimethyl sulfoxide (DMSO) in 0.9% saline), covering the entire surface of both SCi.

NOTE: Retinal ganglion cells (RGCs) are labeled retrogradely with the persistent marker OHSt**.** It is possible to identify RGCs due to the active retrograde axonal

transport from the superior colliculi (SCi) when this compounds is applied to the SCi one week before animal processing[4,6,28-30].

3.6 Close the skin with suture wound clips.
3.6.1. Leave the animals to recover in a breeding cage with flat paper bedding, and keep them warm with the aid of a heating pad. Monitor the animals and return them to the breeding house when they recover.

3.6.2. For five days, monitor the mice for general conditions and signs of infection after surgery.

4. Immunohistochemical study
4.1 Isolating the mouse retina
4.1.1 Anesthetize the mice and take the eye orientations. Place 6/0 suture silk through the ocular conjunctive on the superior pole of each eye to prepare retinal whole-mounts.
NOTE: The insertion point of the rectus muscle and the nasal caruncle were used as additional landmarks[31].

4.1.2 Perfuse the mice transcardially with saline and 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2-7.4) at 4 °C[41].

4.1.3 Enucleate and remove the cornea with curved tip forceps and sterile fine-angled dissecting scissors, following the protocol described by Mahajan and co-workers[42]. Cut around to the limbus. Move forceps and, with a slight back angle, pull up the cornea. Then, remove the ciliary body and lens with slight pressure on the eyecup.

4.1.4 Dissect out the mice retinas as flattened whole-mounts by making four radial cuts in the superior, inferior, nasal, and temporal retinal poles (the deepest in the superior pole). Carefully, remove the vitreous without damaging the retina by pulling it up with curved forceps located inside the posterior chamber.

4.1.5 Fix the retinas for an additional hour in the fixative solution (4.1.2), rinse them in 0.1 M PBS, mount them with the vitreal side up on subbed slides, and cover them with anti-fading mounting media.

4.2 Immunolabeling

4.2.1 Permeate the whole-mount retinas with PBS 0.5% Triton X-100 for 15 min at -70 °C, and then wash them in fresh PBS 0.5% Triton X-100 for 10 min.

4.2.2 Incubate the retinas in blocking buffer (PBS with 2% Triton X-100 and 2% normal donkey serum).

4.2.3 Incubate the whole-mount retinas, that were previously treated with OHSt[5,6,14,29,30,32-34], in the Brn3a primary antibody (diluted 1:100 in blocking buffer) overnight at 4 °C.

NOTE: In order to verify RGC survival in the retina when the mice were sacrificed, Brn3a goat antibody is used since it labels a transcription factor specifically expressed in adult RGCs[32,33,35,36].

4.2.4. Rinse the retinas three times in PBS.

4.2.5 Incubate for 2 hr at room temperature with the secondary antibody, diluted 1:500 in blocking buffer.

4.2.6 Finally, wash the retinas carefully in PBS and mount them with the vitreal side up on subbed slides. Cover them with antifading solution and place a cover glass onto the mounting medium. Seal with nail polish.

5. Retinal whole-mount reconstructions

5.1 Photograph the different groups of retinas under a fluorescence microscope equipped with an ultraviolet (BP 365/12, LP 397) and a rhodamine (BP 546/12, LP590) filter to observe the white-gold OHSt fluorescence and orange-fluorescent dye-conjugated antibodies, respectively. Analyze them.
NOTE: The microscope should be equipped with a digital high-resolution camera and a computer-driven motorized stage connected to an image analysis software through a microscope-controller module.

5.2. Photograph retinal multiframe acquisitions in a raster scan pattern, and capture the frames side-by-side with no gap or overlap between them with a 20x objective. Focus single frames manually before capturing each image, and then upload them into the image analysis program.

5.2.1 Define a scan area to cover the entire retina consisting of a matrix of m frames in columns and n frames in rows. Indicate the total number of frames in the scan area by frames in columns times frames in rows (m x n).

5.3 Capture 140 consecutive frames at a resolution of 300 dpi. Perform retinal whole-mount reconstructions following previously-described procedures[5,6,14,28-31].

5.4 Measure the area of retinal whole-mounts on the high-resolution photomontage images of the complete retinas using a suitable software. Process reconstructed images with an image-editing computer software as needed to produce printouts.

6. Morphometric analysis of retinal whole-mounts

6.1 Process all images taken of the retinas with specific macros written in the image analysis program macro language that apply a sequence of filters and transformations in turn to each image of the stack.

6.2 Count the resulting cells and transfer the data to a spreadsheet for analysis. These subroutines have recently been described in detail[6,14,28-30].

6.3 Analyze the distribution of RGCs using isodensity maps for each retina. Calculate the cell densities and represent them as a filled contour plot graph, following previously-described methods[14].

NOTE: Every frame captured was divided into an equal number of 36 sampling areas of interest (AOI) for OHSt or Brn3a labeling. These AOI were automatically counted, and data were exported and saved to a spreadsheet software.

6.4 Represent these densities as filled contour plots using a graphic presentation software that constructs pseudo-colored isodensity maps on a scale with 38 different steps (each of 125) ranging from 0 to 4,750 cells/mm^2. The plots should demonstrate the topological distribution of RGC labeled FG$^+$ and Brn3a$^+$.

NOTE: This upper limit was chosen on the basis of earlier studies that showed mean upper densities around this value[14].

REPRESENTATIVE RESULTS:

DBA2/J MOUSE GLAUCOMATOUS PRESENTS FEATURES OF THE HUMAN DISEASE

Intraocular pressure measurements

The elevation of IOP is one of the hallmarks of most types of glaucoma. The measurements of mice IOP permit observation of the gradual increase in this physiological parameter, which starts to vary at 9 months and peaks at 12. Compared to the constant IOP of the control C57BL/6J strain, the glaucomatous mice demonstrate an increase. The maximal increase in IOP in the glaucoma mice (at 12 months) was 70%, compared either with the glaucoma strain before the onset of the pathology or with the control strain (Figure 1).

Retinal Ganglion Cell Death

Blindness is also another consequence of the glaucomatous pathology, and this is due to ganglion cell death. In this sense, the use of both OHSt retrograde tracing and Brn3a immunolabeling on the same retina allows for morphofunctional studies. While OHSt labels RGCs capable of retrograde axonal transport of the tracer from their target region in the brain towards their cell somata in the retina, Brn3a labels all surviving RGCs, regardless of their axonal transport capabilities[14]. Furthermore, the changes in the distribution of retrograde labeled (OHSt$^+$) or immunolabeled (Brn3a$^+$) RGCs can be examined and compared in detail by constructing contour plot isodensity maps for the retinas of each control or injured mouse (Figure 2).

Electroretinogram responses

To confirm the lack of functionality of mice retinas when glaucoma is developed, we performed STR recordings to assess the possible loss of function in the inner retina. ERG measurements are a useful tool to evaluate functional retinal changes in this animal model. At 12 months, compared with younger mice, glaucomatous mice showed significant reductions of electrical responses. However, there were no significant differences in ERG responses at any of the different ages studied in the control mice (Figure 3).

FIGURE LEGENDS:

Figure 1. Intraocular pressure (IOP) measurements in the C57BL/6J and DBA/2J animals as a function of age. IOP in C57BL/6J and DBA/2J animals was measured at 3, 9, and 15 months of age (white and black circles). Each circle corresponds to a mean ± SD of different animal measurements (n=8).

Figure 2. Electroretinogram responses from control C57BL/6J or DBA/2J glaucomatous mice as a function of age. Superposed ERG trace responses from C57BL/6J (A) and DBA/2J (B) mice recorded in response to light intensity (indicated to the left of the recorded traces in log $cd \cdot s \cdot m^{-2}$). Recordings obtained from animals of 3 (green traces), 6 (blue traces), 9 (purple traces), and 12 (red traces) months of age are shown. A significant reduction in the ERG amplitude response was observed for the DBA mice. Averaged amplitudes of the positive waves (pSTR, circles) and negative waves (nSTR, triangles) measured from the ERG flash response as a function of stimulus light intensity recorded from C57BL/6J (open symbols) and DBA/2J (closed symbols) mice at 6 (C) and 12 (D) months of age. Plot data correspond to mean values ± SD (n=8). A significant reduction of STR amplitudes in DBA/2J between 12 and 15 months is observed for pSTR amplitudes ($p < 0.0001$).

Figure 3. Retinal distribution of surviving RGCs identified by Brn3a immunohistochemistry on 15-month-old DBA mice. (A) Filled contour plots showing densities of $Brn3a^+$ RGCs in whole-mounts. Note the intensely-labeled RGCs distributed throughout the entire retina, with the typical high-density region along the naso-temporal streak in the superior retina of a representative animal without RGCs loss (6 months of age). (B) Gradual absence of labeled RGCs preferentially in the superior retina as the pathology develops, adopting the form of focal as well as diffuse loss, as evidenced by the cooler colors of the isodensity maps (12 months of age). (C) Representative animal with severe damage showing labeled RGCs restricted to a wedge located between the 3 and 4 o'clock positions (15 months of age). Maps are represented as filled contour plots generated by assigning to each of the 36 subdivisions of each individual frame a color code according to its RGC density value within a 38-step color scale range from 0 (purple) to 4,750 (red) or higher ($Brn3a^+$ RGCs/mm^2). For all retinas, the superior pole is directed to the 12 o'clock orientation. Scale bar = 1 mm.

DISCUSSION:

In the DBA/2J mouse, the glaucomatous pathology can be monitored with at least three parameters: IOP, retinal ganglion cell loss, and retinal dysfunction[37]. This is a clear advantage compared to several models in which it is possible to study either changes in IOP, modifications in the retina, or changes in the electrophysiology, but never simultaneously. This implies that, depending on the particular interest, one or the other glaucoma model must be chosen[38-40]. The DBA/2J might not be the best model, but it depicts changes that mimic in many ways human glaucoma pathology and therefore opens the possibility for the investigation of this pathology from different perspectives.

As an added value, the present animal model will be useful to test new compounds, either for the reduction of IOP or for neuroprotection. The DBA/2J model has the advantage of presenting the common symptom, abnormal IOP. In this sense, it is of interest to use this model to test potential anti-glaucomatous drugs designed to reduce IOP. The moment of maximal IOP (12 month of age) is an optimal time point to test these molecules. The application of classical hypotensive drugs significantly reduces IOP, indicating that this model is suitable for this type of study.

Since it is possible to follow retinal degeneration as a progressive event from nine months of age onwards, neuroprotective agents could also be explored. As controls, we may use animals treated with vehicle, while experimental groups may be treated with the desired compound. Moreover, ERG recordings from such animal groups will allow investigations of the putative neuroprotective effects of new substances. It will also be possible to establish optimal treatment timeframes in order to get the best final results. It is important to notice that the only problem of this approach is the time necessary for the animal to develop the pathology. It is necessary to wait a minimum of 9 months to start to see the changes in IOP and the retinal impairment.

These two treatment development strategies are possible due to the gradual progression of the pathology, which permits research into the establishment of the most suitable treatment start point. Although the DBA/2J mouse presents several limitations, it offers several advantages above the other existing animal models of glaucoma.

DISCLOSURES:
The authors have nothing to disclose.

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REFERENCES:
1 Howell, G. R. *et al.* Axons of retinal ganglion cells are insulted in the optic nerve early in DBA/2J glaucoma. *J Cell Biol.* **179** (7), 1523-1537, doi:10.1083/jcb.200706181, (2007).
2 Quigley, H. A. & Broman, A. T. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol.* **90** (3), 262-267, doi:10.1136/bjo.2005.081224, (2006).
3 Schlamp, C. L., Li, Y., Dietz, J. A., Janssen, K. T. & Nickells, R. W. Progressive ganglion cell loss and optic nerve degeneration in DBA/2J mice is variable and asymmetric. *BMC Neurosci.* **7** 66, doi:10.1186/1471-2202-7-66, (2006).
4 Cuenca, N. *et al.* Changes in the inner and outer retinal layers after acute increase of the intraocular pressure in adult albino Swiss mice. *Exp Eye Res.* **91** (2), 273-285, doi:10.1016/j.exer.2010.05.020, (2010).
5 Salinas-Navarro, M. *et al.* Ocular hypertension impairs optic nerve axonal transport leading to progressive retinal ganglion cell degeneration. *Exp Eye Res.* **90** (1), 168-183, doi:10.1016/j.exer.2009.10.003, (2010).
6 Salinas-Navarro, M. *et al.* Functional and morphological effects of laser-induced ocular hypertension in retinas of adult albino Swiss mice. *Mol Vis.* **15** 2578-2598 (2009).
7 Schuettauf, F. *et al.* Retinal neurodegeneration in the DBA/2J mouse-a model for ocular hypertension. *Acta Neuropathol.* **107** (4), 352-358, doi:10.1007/s00401-003-0816-9, (2004).

8 El-Hossary, G. G., El-Gohary, A.A., A.H., E.S. Topical Instillation of aminoguanidine reducing intraocular pressure and improving visual evoked potential in rabbits with experimental glaucoma. *Research Journal of Medicine and Medical Sciences.* **5** 18-24 (2010).
9 Iskender Ince, I., Karasulu E., Ates H., Yavasoglu A, and L., K. A Novel Pilocarpine Microemulsion as an Ocular Delivery System: In Vitro and in Vivo Studies. *J Clin Exp Ophthalmol.* **6** 1-6 (2015).
10 Luntz, M. H. Experimental glaucoma in the rabbit. *Am J Ophthalmol.* **61** (4), 665-680 (1966).
11 Salwa, A., Mervat, A. Impact of elevated intraocular pressure and 0,15% brimonidine tartrate on aqueous humor and retina of experimental animal. . *Int J Pharm Pharm Sci.* **4** 464-470 (2012).
12 Shafaa, M. W., Sabra, N. M. & Fouad, R. A. The extended ocular hypotensive effect of positive liposomal cholesterol bound timolol maleate in glaucomatous rabbits. *Biopharm Drug Dispos.* **32** (9), 507-517, doi:10.1002/bdd.778, (2011).
13 McKinnon, S. J., Schlamp, C. L. & Nickells, R. W. Mouse models of retinal ganglion cell death and glaucoma. *Exp Eye Res.* **88** (4), 816-824, doi:10.1016/j.exer.2008.12.002, (2009).
14 Vidal-Sanz, M. *et al.* Understanding glaucomatous damage: anatomical and functional data from ocular hypertensive rodent retinas. *Prog Retin Eye Res.* **31** (1), 1-27, doi:10.1016/j.preteyeres.2011.08.001, (2012).
15 Libby, R. T. *et al.* Inherited glaucoma in DBA/2J mice: pertinent disease features for studying the neurodegeneration. *Vis Neurosci.* **22** (5), 637-648, doi:10.1017/S0952523805225130, (2005).
16 Aihara, M., Lindsey, J. D. & Weinreb, R. N. Twenty-four-hour pattern of mouse intraocular pressure. *Exp Eye Res.* **77** (6), 681-686 (2003).
17 Drouyer, E. *et al.* Glaucoma alters the circadian timing system. *PLoS One.* **3** (12), e3931, doi:10.1371/journal.pone.0003931, (2008).
18 Danias, J. *et al.* Quantitative analysis of retinal ganglion cell (RGC) loss in aging DBA/2NNia glaucomatous mice: comparison with RGC loss in aging C57/BL6 mice. *Invest Ophthalmol Vis Sci.* **44** (12), 5151-5162 (2003).
19 Wang, W. H., Millar, J. C., Pang, I. H., Wax, M. B. & Clark, A. F. Noninvasive measurement of rodent intraocular pressure with a rebound tonometer. *Invest Ophthalmol Vis Sci.* **46** (12), 4617-4621, doi:10.1167/iovs.05-0781, (2005).
20 Ding, C., Wang, P. & Tian, N. Effect of general anesthetics on IOP in elevated IOP mouse model. *Exp Eye Res.* **92** (6), 512-520, doi:10.1016/j.exer.2011.03.016, (2011).
21 Valiente-Soriano, F. J. *et al.* Effects of ocular hypertension in the visual system of pigmented mice. *PLoS One.* **10** (3), e0121134, doi:10.1371/journal.pone.0121134, (2015).
22 Bui, B. V. & Fortune, B. Ganglion cell contributions to the rat full-field electroretinogram. *J Physiol.* **555** (Pt 1), 153-173, doi:10.1113/jphysiol.2003.052738, (2004).
23 Frishman, L. J., Reddy, M. G. & Robson, J. G. Effects of background light on the human dark-adapted electroretinogram and psychophysical threshold. *J Opt Soc Am A Opt Image Sci Vis.* **13** (3), 601-612 (1996).
24 Frishman, L. J. & Steinberg, R. H. Intraretinal analysis of the threshold dark-adapted ERG of cat retina. *J Neurophysiol.* **61** (6), 1221-1232 (1989).

25 Saszik, S. M., Robson, J. G. & Frishman, L. J. The scotopic threshold response of the dark-adapted electroretinogram of the mouse. *J Physiol.* **543** (Pt 3), 899-916 (2002).
26 Alarcon-Martinez, L. *et al.* Short and long term axotomy-induced ERG changes in albino and pigmented rats. *Mol Vis.* **15** 2373-2383 (2009).
27 Alarcon-Martinez, L. *et al.* ERG changes in albino and pigmented mice after optic nerve transection. *Vision Res.* **50** (21), 2176-2187, doi:10.1016/j.visres.2010.08.014, (2010).
28 Salinas-Navarro, M. *et al.* Retinal ganglion cell population in adult albino and pigmented mice: a computerized analysis of the entire population and its spatial distribution. *Vision Res.* **49** (6), 637-647, doi:10.1016/j.visres.2009.01.010, (2009).
29 Galindo-Romero, C. *et al.* Axotomy-induced retinal ganglion cell death in adult mice: quantitative and topographic time course analyses. *Exp Eye Res.* **92** (5), 377-387, doi:10.1016/j.exer.2011.02.008, (2011).
30 Galindo-Romero, C. *et al.* Effect of brain-derived neurotrophic factor on mouse axotomized retinal ganglion cells and phagocytic microglia. *Invest Ophthalmol Vis Sci.* **54** (2), 974-985, doi:10.1167/iovs.12-11207, (2013).
31 Salinas-Navarro, M. *et al.* A computerized analysis of the entire retinal ganglion cell population and its spatial distribution in adult rats. *Vision Res.* **49** (1), 115-126, doi:10.1016/j.visres.2008.09.029, (2009).
32 Nadal-Nicolas, F. M. *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.* **50** (8), 3860-3868, doi:10.1167/iovs.08-3267, (2009).
33 Nadal-Nicolas, F. M. *et al.* Whole number, distribution and co-expression of brn3 transcription factors in retinal ganglion cells of adult albino and pigmented rats. *PLoS One.* **7** (11), e49830, doi:10.1371/journal.pone.0049830, (2012).
34 Nadal-Nicolas, F. M. *et al.* Displaced retinal ganglion cells in albino and pigmented rats. *Front Neuroanat.* **8** 99, doi:10.3389/fnana.2014.00099, (2014).
35 Quina, L. A. *et al.* Brn3a-expressing retinal ganglion cells project specifically to thalamocortical and collicular visual pathways. *J Neurosci.* **25** (50), 11595-11604, doi:10.1523/JNEUROSCI.2837-05.2005, (2005).
36 Weishaupt, J. H., Klocker, N. & Bahr, M. Axotomy-induced early down-regulation of POU-IV class transcription factors Brn-3a and Brn-3b in retinal ganglion cells. *J Mol Neurosci.* **26** (1), 17-25, doi:10.1385/JMN:26:1:017, (2005).
37 Perez de Lara, M. J. *et al.* Assessment of inner retina dysfunction and progressive ganglion cell loss in a mouse model of glaucoma. *Exp Eye Res.* **122** 40-49, doi:10.1016/j.exer.2014.02.022, (2014).
38 Morrison, J. C. *et al.* A rat model of chronic pressure-induced optic nerve damage. *Exp Eye Res.* **64** (1), 85-96, doi:10.1006/exer.1996.0184, (1997).
39 Mabuchi, F., Aihara, M., Mackey, M. R., Lindsey, J. D. & Weinreb, R. N. Optic nerve damage in experimental mouse ocular hypertension. *Invest Ophthalmol Vis Sci.* **44** (10), 4321-4330 (2003).
40 Moreno, M. C. *et al.* A new experimental model of glaucoma in rats through intracameral injections of hyaluronic acid. *Exp Eye Res.* **81** (1), 71-80, doi:10.1016/j.exer.2005.01.008, (2005).

41. Gage, G. J., Kipke, D. R., Shain, W. Whole Animal Perfusion Fixation for Rodents. *J. Vis. Exp.* (65), e3564, doi:10.3791/3564 (2012).
42. Mahajan, V. B., Skeie, J. M., Assefnia, A. H., Mahajan, M., Tsang, S. H. Mouse Eye Enucleation for Remote High-throughput Phenotyping. J. Vis. Exp. (57), e3184, doi:10.3791/3184 (2011).

Figure

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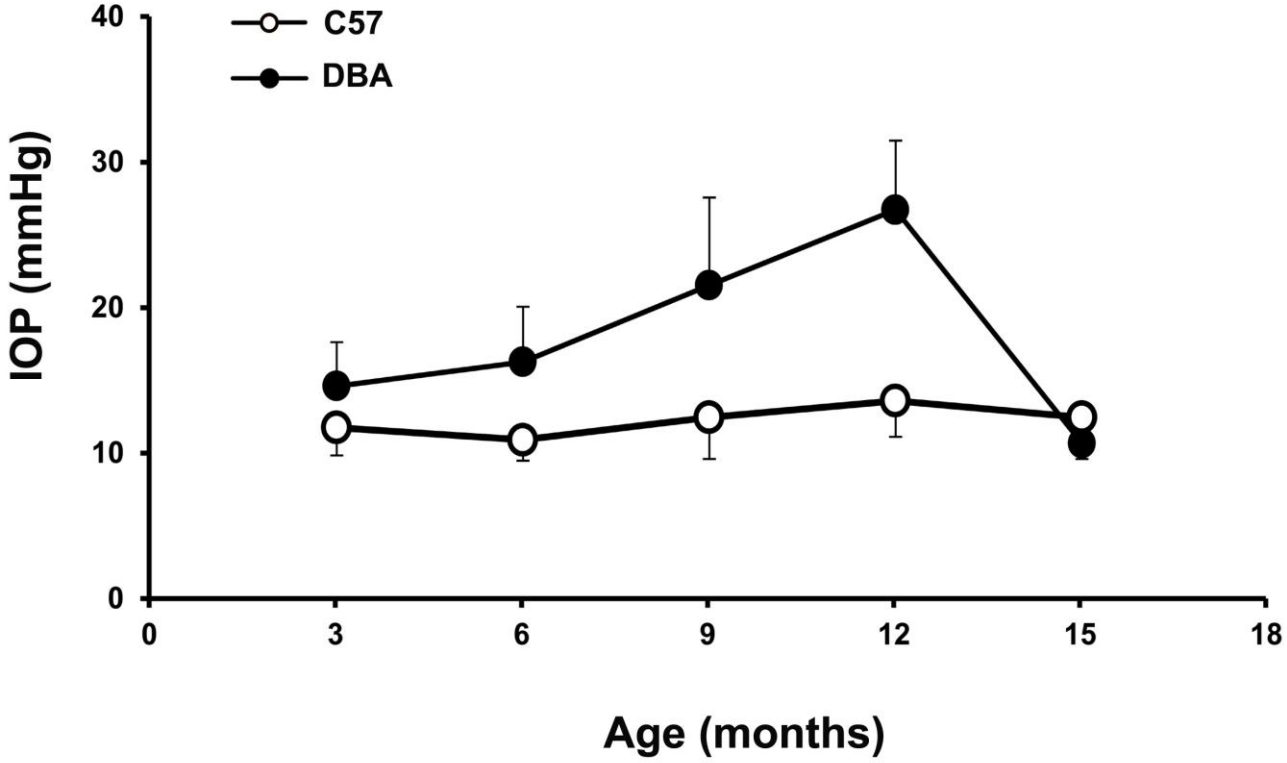

FIGURE 1

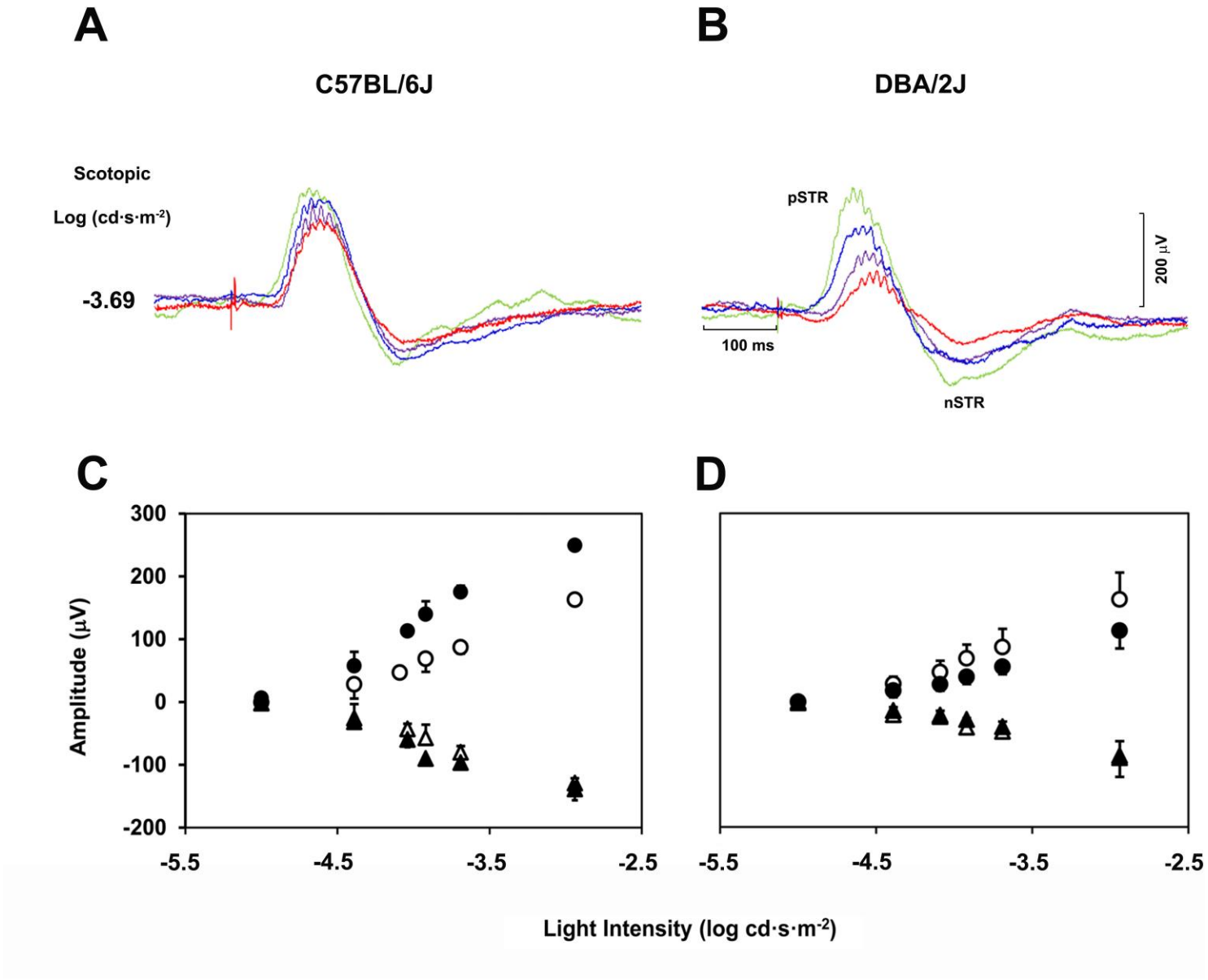
A
B
C57BL/6J
DBA/2J
Scotopic
Log (cd·s·m-2)
-3.69
pSTR
nSTR
100 ms
200 µV
C
D
Amplitude (µV)
300
200
100
0
-100
-200
-5.5
-4.5
-3.5
-2.5
Light Intensity (log cd·s·m-2)

FIGURE 2

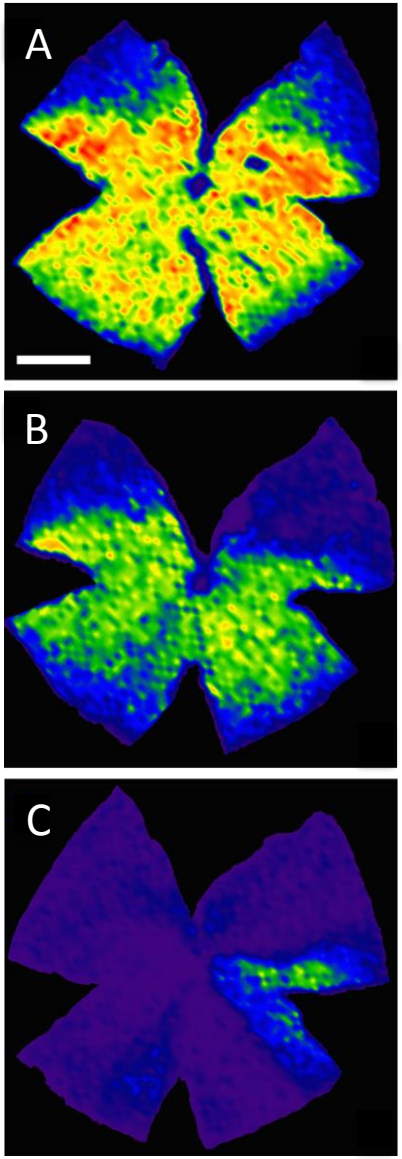

FIGURE 3

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number
Tonolab	Tiolat, Helsinki, Finland	TV02
Burian-Allen lens electrodes	Hansen Labs, Coralville, Iowa, USA	1000-540-305-D
CP511ACamplifier	Grass Instruments, Quincy, MA, USA	CP511AC
Power Lab data acquisition board	AD Instruments, Chalgrove,UK	16/35
Photometer	Mavo Monitor USB,Gossen, Nürenberg, Germany	5032 B
GraphPad Instat	GraphPad Software, San Diego California USA	v 3.0
Hydroxystilbamidine methanesulfonate	OHSt; Molecular ProbesInc, Eugene, OR, USA	H22845
Fluorescence microscope	Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany	000000-1116-576
Digital high-resolution camera	ProgRes C10plus; Jenoptik, Jena, Germany	456140-0000-000
Computer driven motorized stage	ProScan H128 Series; Prior Scientific Instruments, Cambridge, UK	00-H101AFN1
Image analysis software	Image-Pro Plus 5.1 for Windows (IPP); Media Cybernetics, Silver Spring, MD, USA	MAN SU 41N51000 20040830
DBA/2J mouse	Charles River Spain, Cerdanyola del Vallès, Spain	Stain code 026
Ketamine	Imalgene 1000, Merial, Barcelona, Spain	2529 ESP
Xylazine	Rompún, Bayer, S.A., Barcelona, Spain	*750 DB*
Colircusí Tropicamida 1%	Alcon-Cusí, S.A., El Masnou, Barcelona, Spain	653486 ESP
Methocel 2%	Novartis Laboratories CIBA Vision, Annonay, France	336503
Prednisone	Oftalmolosa Cusí Prednisona-Neomicina; Alcon S.A., Barcelona, Spain	NDC 61314-637-05
Gelatin sponge	Espongostan Film, Ferrosan A/S, Denmark	150865
Image-editing computer software	Adobe Photoshop CS ver. 8.0.1; Adobe Systems Inc., San Jose, CA, USA	20150722. r168 x64

Analysis software	Microsoft Office Excel 2003; Microsoft Corporation, Redmond,WA	160329
Sigma Plot	SigmaPlot 9.0 for Windows; Systat Software, Inc., Richmond, CA	90075
Immunolabeling	Subbed slides Anti-Brn3a, Santa Cruz	sc-31984

Comments/Description
Device to measure IOP
Electrodes for ERG
Amplifier
Data adquisition system
Photometer
Plotting and data analysis
Retrograde axonal transport
Retinas visualization
Picture capture
Analysis of the images
Glaucomatous mice
Anesthesia
Anesthesia
ERG measurements
Animal Care
ERG measurements
Retrograde labeling

Retinas whole mount
Immunohystochemistry

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Author(s): PEREZ DE CORO, UMIENTE-SORIANO, QUILES-TRIGUEROS, VIDAL-SAINZ, DE LA VILLA and PINTOR

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Editorial comments:

•Grammar:
-Please copyedit the entire manuscript for numerous grammatical errors. This editing should be performed by a native English speaker and is essential for clarity of the protocol. A small subset of errors is included below (these are only examples).

-Please rephrase the title to remove the personal pronoun “we.”

It has been changed.

-Please correct the grammar in the long abstract. There are comma splices and sentences that need commas for clarity. In addition, please make sure the verb tense is consistent throughout the abstract.
-2.2 – “recordings” should be “recording.”

It has been changed.

-2.5.2 – “Locate” should be “Place.”

It has been changed.

-2.7 – “Provide” should be “Stimulate”

It has been changed.

-2.7 note – Please use complete sentences. Also, this should be multiple steps rather than a note.
-3.3 – “duramater” should be “dura mater”

It has been changed.

-3.5 – Please correct “a small piece of gelatin sponge thin layer of gelatin sponge”

It has been changed.

-3.6.1 – “on a breeding cage” should be “in a breeding cage.”

It has been changed.

-4.1.3 – “tip curved” should be “curved tip.”

It has been changed.

-4.2.5 – “Incubated” should be “Incubate.”

It has been changed.

-5.2.1 – Please correct the grammar in the last sentence.

-Discussion – Please correct the following sentence: “In this sense, it results quite interesting to test potential anti-glaucomatous drugs designed to reduce IOP.”

We have changed it and now it appears as follows:

“... In this sense, it is of interest to use this model to test potential anti-glaucomatous drugs designed to reduce IOP…”

•Formatting:
-There should be a space between steps 5.2 and 5.2.1.

It has been change.

-The ethics statement should be separate from the information regarding housing of animals and type of mice used. It should appear as a note or a step.

The required change has been done and now appears as:

"…6.5 All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research…"

-4.1.5 – The "subbed slides" should appear in the materials table.

It has been included.

-Triton X-100 should always be written out as Triton X-100, and not abbreviated as "Triton."

It has been changed.

-Please include catalog numbers for the materials in the materials table. Also please include the antibodies in this table.

The table has been completed

•Additional detail is required:
-3.3 – Is the dental drill used to cut the dura mater? If not, how is it cut?

It has been changed.

-4.1.1 – How are sutures placed?

It has been changed.

-4.1.2 – Substantially more stepwise detail is required. How is the heart accessed for this step? Since this is not to be filmed, a citation would be sufficient.

It has been changed.

-4.1.3 – Please describe how enucleation is performed.

We consider this detail is totally unnecessary

-4.1.4 – Please clarify "Dissect out mice retinas and by means of four radial cuts in the superior, inferior, nasal, and temporal retinal poles"

Done
-4.1.5 – What same fixative solution? Are coverslips used? Is mounting media used prior to immunolabeling?

Done

The details are in 4.2.6

-4.2.1 – What is frozen for 15 min?

It has been changed.

-4.2.6 – Is a coverslip used?

It has been changed.

-Please define the error in Figure 1 in the figure legend. Is it SD or SEM?

It is SD and it has been added to the figure legend.

•There are instances of unnecessary branding which should be removed:

-Jackson Laboratories should be mentioned in the materials table rather than the description of mice in the protocol.

Done.

-2.7 note – Power Lab

It has been changed.

-6.1 – IPP

It has been changed. DONE

-6.4 – Sigma Plot

It has been changed. DONE

•Discussion: Please discuss the critical steps, potential modifications/troubleshooting, and limitations of the protocol. It is mentioned that there are limitations, but these are not specified.

We have included the following sentence:

"…It is important to notice that the only problem of this approach is the time necessary for the animal to develop the pathology. It is necessary to wait a minimal of 9 months to start to see the changes in IOP and retinal impairment…"

•Length warning: Protocol is at the limit for highlighted material. Any additional detail added may require adjusting the portion highlighted.

We have tried to do our best regarding this but the referees suggested lots of changes that have been included.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such

as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

Reviewers' comments:

Reviewer #1:

The authors provide many practical details on how to study the course of the eye disease in the DBA2J mouse. It is a good model of experimental glaucoma mimicking the pigmentary glaucoma in humans. The authots have a functional approach with the ERG and a strutural approach with the RGC count. This allows an evaluation of the structure function relation ship. Most of the studies evaluating RGC density have been flawed because the investigators did not explore the whole surface of the retina. It has been shown than the degenerescence of RGCs due to ocular hypertension is not homogenous.Therefore it is mandatory to use the system mentioned in this paper using on average about 140 frames to cover the full field of the flat mount retina. Unfortunately many papers did not use this system.Consequently the technique used in this revised mansucript is really the strenght of the study.

All the work of this team has been acknowledeged as a seminal article published in the highest ranked journal in ophthalmology (Progress in retinal and eye research).

We are very thankful to the reviewer's comments. And we greatly appreciate your comments on the manuscript.

Reviewer #2:

Manuscript Summary:

The authors present a whole variety of methods for glaucoma analysis in the well-known DBA/2J mice. Especially the analysis of the immunostaining is very interesting and useful for readers/viewers of JoVE.

I am personally sorry to say this -as I appreciate the work form this group a lot- but I have severe problems in accepting this manuscript in the state it is now (see major comments).

I regard JoVE as an extremely useful tool to explain methods to other scientist or interested people. Many people including myself get to know and learn new methods using these videos. With this in mind, I reviewed the manuscript.

The methods as they are presented here are given without alternatives, limitations and discussion. And there are limitations (by animal protection law or the be precise they way it is enforced in different countries), which require alternatives and the ERG part requires discussion and alternatives as this is not the best functional test for glaucoma.

Major Concerns:

The methods the authors describe here are -without any doubts- the classic scientific gold standard to perform these experiments. I also learned these methods that way.

However nowadays they are in conflict with the animal protection law. Form my personal and the experience from others, section 1, 2, 3 and 4 would not be granted as they are described in the text. (This might differ from country to country)
To be clear, I used to perform the methods very similar as they are described here but in several animal applications, I wrote over the last years I needed to modify and change them. The authors therefore at least need to address this issue in the manuscript and should present alternatives.

This is a controversial point. The referee is right, things may change among countries, but originally this contribution was suggested by JOVE being the journal particularly interested in the way we proceed methodologically speaking, which is highly reproducible. If we modify this methodological aspect we would move from the original perspective and moreover we would come into other protocols we do not perform.

The issues in detail are:

- Ketamin / Xylazin anesthesia is generally regarded as outdated. Alternatives should be given.

We are very sorry, but we disagree with the reviewer on the use of ketamine/xylazine. Ketamine / Xylazine anesthesia cocktail is widely used in rodent research or in combination with others drugs (**Gargiulo et al., 2012**). When it is browsed through scientific databases it can be found that its use has been growing since 2000 till 2006, together with isofluorane , in all the research areas with rodent (**Stokes EL, Flecknell PA, Richardson CA. Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. Lab Anim. 2009 Apr;43(2):149-54.**). We think ketamine/Xylazine anesthetic mix cannot be regarded as outdated. It just one more option to use, like ketamine/xylazine/ acepromazine, ketamine/xylazine/ buprenorphine, ketamine/dexmedetomidine, ketamine/medetomidine, isofluorane, or others. Induction of general anesthesia in mice can be achieved by a variety of drugs and techniques (**Flecknell, 1989**). Each researcher has to choose the best option to his experimental procedure according to the local and international regulations that applied in his institution.

The most commonly used anesthetics in mice include the injectable agents avertin, pentobarbital, and ketamine, which are often combined with other agents such as acepromazine, xylazine, diazepam, several narcotic analgesics, and the inhalation agents halothane, isoflurane, and sevoflurane. Compared with injectable techniques, inhalation anesthesia provides greater safety, particularly for prolonged procedures, due to a lesser cardiovascular depression, a reduced impact on liver and kidney functions, and because it promotes rapid recovery and allows quick adjustments and easy maintenance of a steady anesthetic depth. However, inhalant agents foster respiratory depression (particularly in the presence of respiratory diseases), myocardial depression, vasodilation, and hypotension (Paddleford, 2000), exhibiting weak analgesic effects.

Finally, compared with injectable drugs, the modern inhalation anesthetics require complex and expensive equipment such as precision vaporizers and flowmeters, specific breathing systems, and efficient scavenging systems to prevent pollution (**Gargiulo S, Greco A, Gramanzini M, Esposito S, Affuso A, Brunetti A, Vesce G. Mice anesthesia, analgesia, and care, Part I: anesthetic considerations in preclinical research. ILAR J. 2012;53(1):E55-69).**

We will give a note with references for anesthesia alternatives (**Gargiulo S, Greco A, Gramanzini M, Esposito S, Affuso A, Brunetti A, Vesce G. Mice anesthesia, analgesia, and care, Part I: anesthetic considerations in preclinical research. ILAR J. 2012;53(1):E55-69).**

- However, in special cases (ERG measurements) there is a chance to get allowance for that. The authors should provide the user with useful information to get the allowance.

To perform an ERG is mandatory to induce an anesthesia degree to provide good and adequate immobilization to carry out the ERG measurements without any kind of interference between the stimulus and the eye. And ketamine /xylazine mix provide it. Inhalation anesthesia is administered by anesthesia machines and delivered by breathing system that can interfere with the ERG measurements. The reference electrode localization in the mouth could be a serious inconvenience to the inhalation anesthesia administration too.

- There is no need to anesthetize mice for IOP measurement. A sedation is sufficient.

We agree with the reviewer. However, IOP is measured under general anesthesia in most studies using mouse models (**Ding et al., 2011**), maybe because accurately and reliably measuring IOP is critical in glaucoma investigation. Others alternatives to general anesthesia to measure the IOP are sedation or anesthesia at low dose, immobilized conscious mice by restrainer and, even, mice with behavioral training to perform awake IOP measurement without immobilization (**Ding et al., 2011**). But in the case of DBA/2J the development of intraocular hypertension is associated with the age of the animal, and these older animals (more than 12 months old) could be more susceptible to stress by manipulation and usually the time course of a study with DBA/2J mice is spread almost a year.

- Neither craniotomy nor the removal of the brain cortex is needed to label retrograde. No allowances are therefore given any more to perform retrograde labelling with craniotomy and removal of the brain cortex in this country. Please also give alternatives (injections).

As suggested by the reviewer, there is other alternative method to RGC retrograde tracing from both superior colliculi and it can be achieved by multiple stereotactic injections (Barnstable and Drager, 1984; Siddiqui et al., 2014; Soto et al., 2008) but, if it is not done properly may lead to regions of the retina left untraced. Other alternatives methods are from the intact optic nerve, that is without severing the optic nerve (Nadal-Nicolas et al., 2014, 2015), or by a single stereotactic injection in the optic tract (Nadal-Nicolas et al., 2015).

We are very pleasant to give all these alternatives to the reader. But we have to keep in mind that we are going to study the functional integrity of RGC retrograde transport. And we need to place the tracer on the retino-topic projection nuclei available to the RGC axon terminals. One characteristic of DBA mice, as others mouse glaucoma models, is that when the animal expresses the pathology there are retina areas where the retrograde transport is compromised, showed by untraced retina areas or regions, and you need to be secure to have a good, complete and homogeneous tracer available to the axonal terminal and that the areas devoid of tracer are not artifact.

- There is no need to transcardially perfuse a living mouse to enucleate and fixate an eye and perform a whole-mount. Mice can be killed and then the eyes can be fixed and whole-mounts can be prepared or the other way around. There is multiple publications performing whole-mount preparation these ways. Please mention these alternatives and discuss them.

We are very sorry but we are disagreeing with the reviewer comments on this point. The animals are transcardially perfuse after a deep anesthesia with an overdose of 20% sodium pentobarbital and when all the nociceptive reflexes are abolished the perfusion is done. On the other hand, the main reason to carry out a transcardial perfusion is to remove the blood cells from the retina vascular system to avoid as much as possible the presence of

autofluorescence elements or background that can interfere the RGC quantification by automated methods.

- I also do not regard STR as a hallmark for glaucoma. Yes, there are differences. However, the authors also should address, this in the introduction and discussion, that there are better (although harder to perform) hallmarks: VEP!

The authors mix the terms RGC death and retinal cell death often in the text. Especially when it comes down to the ERG part. For the unexperienced reader this might be misleading as there is definite difference. Please straighten this out. This is especially crucial in Line 438. An ERG does definitely not "fully confirm the lack of functionality"

We agree and we have changed retinal by ganglion. We have also removed the word fully as indicated in the comment.

The authors should also mention that there are other control strain than the C57/BL6J mouse. Especially as the C57/BL6J is not regarded as the best strain for comparisons.

We agree with the reviewer with respect to the existence of different controls. DBA/2J-Gpnmb+/SjJ provides a genetically matched control for DBA/2J mice, this coisogenic strain has a functional allele of Gpnmb which does not develop glaucoma. In spite of this comments, Porciatti et al, 2010 reported a comparison between different strains including C57BL/6J, DBA/2J and DBA/2J-Gpnmb^{+}/SjJ and their results showed different characteristics in these mouse strains. Furthermore, retinal ganglion cell population is reported to be larger in DBA/2J than in C57BL/6J mice,(Williams RW , genetic and enviromental…)

16 out of 31 citations are self-citations. There is so much literature about the model and these methods available. Please cite and mention also the work of others in this field.

There are no so many researchers working on ERG and this animal model. Indeed the number of papers included in the text by the three different groups participating in this study are additive and therefore it is not strange that 50% of the reference belong to any of the groups. What is important is that all the quoted references (ours or not) are necessary for a better understanding of the manuscript, particularly when this type of paper is read by not specialist in the topic.

Minor Concerns:

Instead of "mouse" I would use DBA/2J mouse a key word.

It has been changed.

Introduction:

Regrettably, there are almost no citations! See also comment above. We all depend on being citied, so please respect and appreciate the work of the people you mention and cite them. Personally, I was really wondering who uses the application of phenol to induce IOP. I never heard of this and could also not find this in the literature.

We have included several references regarding this.

The first paper describing the DBA/2J mouse as a glaucoma model is from 1998. This is not "recently" (lines 152 and 156).

Done.

Methods:

The order of methods should be changed according to the results and figures. ERG is always last there.

The referee is right and we have changed the figures according to the text.

Line 203. No surgery was performed afterwards.

Line 209/210. Should be transferred to Line 217

Line 265-267: A link where to find these guidelines would be nice.

We have included a new reference

Line 312: define "normal"

It has been changed.

Line344: should be "fresh PBS" and "for 10 minutes"

Done.

Line384: This is a method article: The procedures should be in the article somewhere (or supplements)

The method has been already described elsewhere and moreover the lack of space obliged us to put the mentioned reference.

Line391: Please give more details about the macros

The macro designed to count RGC has been previously published (Salinas-Navarro et al., 2009). In brief, we used macro language to apply a sequence of filters and transformations to each image in order to clarify cell limits and separate individual cells for automatic cell counting. In a first step, the images are converted to 8-bit gray scale images. Illumination aberrations caused by the microscope optics are removed by the software flatten enhancement filter which evens out the background variations. This was followed by enhancement of the edges of the cells using the large spectral filter edge+ command, which extracts positive edges (in this case fluorescent stained bright cells) from the dark background. A setting of 8% (kernel size 20×20) was sufficient to enhance the cell edges making detection simpler. Large spectral filters are used where large kernels are required and cut down on the processing overheads. Small artifacts and noise are removed by running three passes of the median enhancement filter (kernel size 3×3). Cell clusters are then separated by two passes of the watershed split morphological filter which erodes objects until they split and then dilates them until they do not touch. The cells in each image are counted using predetermined parameters to exclude objects that are larger than 300 μm^2 or smaller than 7 μm^2. These parameters correspond to the largest or smallest individual OHSt-labeled object detected as RGC. Finally, each count was exported by dynamic data exchange to a spreadsheet (~~Microsoft® Office Excel 2003, Microsoft Corporation, Redmond, WA, USA).~~

Line395: see Line 384

??

Line 426: Should be: Retinal Ganglion Cell Death

It has been changed.

Line 427: It is a bit cynical to call "blindness another feature of glaucoma pathology".

We have changed it to "…consequence…"

Figure Legend:

Line 449/450: I also see measurement points of 6 and 12 months. Please clarify.

We commented the most representative changes when they occured nevertheless we performed a time course along 15 months.

Line454: According to the following text the mice are aged 6, 12 and 15 months and not only 15 months.

See previous comment.

Figure 3: Would be helpful if the A and B would also have a legend in the Figure, which color belongs to which line. C and D also here a bit text in the figure would help: 6 month above C 12 months above D. Explain circles and triangles.

The explanation has been done in the figure legend.

What are the last two pages "comments / Description" meant for?

We do not understand what the referee is talking about.

Please proofread this article once more. There are still major unmentioned language flaws.

Done

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
N/A

Major Concerns:
RGC counting based on OHSt retrograde tracing is fundamentally flawed, as it assumes that axon transport is intact in glaucoma models. Instead, axon transport is well known to be impaired early in the disease, including DBA/2J glaucoma. Thus, loss of OHSt-RGC staining cannot distinguish between RGC loss and loss of axon transport.

We think that there is a misunderstanding.

Of course, it has been showed important alterations in the retrograde and orthograde axonal transport in RGCs associated with OHT (for a review, see Vidal-Sanz et al., 2012).

In the DBA/2J mouse the fact that RGCs are identified with OHSt applied to their target regions is that this technique does not reflect RGC survival but rather an alteration of the retrograde axonal transport. Indeed, Brn3a immunodetection is used to determine RGC survival.

The presence of the tracer within the RGC bodies implies an active retrograde transport from the axon terminals in the SCi all the way back to the retina. It is possible that the absence of retrograde-labeled RGCs observed in DBA/2J mice retinas is related to a functional impairment of the axoplasmic flow, as has been observed following other types of experimental glaucoma models (Vidal-Sanz et al., 2012).

With this experimental approach it is possible to identify RGCs maintaining a functional retrograde axoplasmic transport, while Brn3a immunohistofluorescence is used to identify surviving RGCs. This observation further supports the concept that not all surviving RGCs retain normal physiological properties.

The pSTR does not seem an adequate method to assess RGC dysfunction. Several studies show that another functional measure –the PERG– is extinguished before 12 months of age in DBA/2J mice. Figure 3 of this study instead shows only a moderate reduction of pSTR between 12 and 15 months. According to current literature, at this age a large population of RGCs is already lost. So, what is the purpose of recording the pSTR? RGC function would be expected to deteriorate before RGC loss. This should be discussed

The referee is right about the importance of this approach (PERG). We are now fixing the conditions to perform PERG although by now pSTR is well established in our group and others to determine retinal ganglion cell degeneration.

Minor Concerns:
N/A

Additional Comments to Authors:
N/A