·Basic Research·

β-Ⅲ-Tubulin: a reliable marker for retinal ganglion cell labeling in experimental models of glaucoma

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Absract

• AIM: To evaluate the reliability of β-III-Tubulin protein as a retinal ganglion cell (RGC) marker in the experimental glaucoma model.

• METHODS: Glaucoma mouse models were established by injecting polystyrene microbeads into the anterior chamber of C57BL/6J mice, then their retinas were obtained 14d and 28d after the intraocular pressure (IOP) was elevated. Retinal flat mounts and sections were double-labeled by fluorogold (FG) and β-III-Tubulin antibody or single-labeled by β-III-Tubulin antibody, then RGCs were counted and compared respectively.

• RESULTS: IOP of the injected eyes were elevated significantly and reached the peak at 22.8±0.7 mm Hg by day 14 after injection, then dropped to 11.3±0.7 mm Hg by day 28. RGC numbers counted by FG labeling and β-III-Tubulin antibody labeling were 64 807±4930 and 64 614±5054 respectively in the control group, with no significant difference. By day 14, RGCs in the experimental group decreased significantly compared to the control group, but there was no significant difference between the FG labeling counting and the β-III-Tubulin antibody labeling counting either in the experimental group or in the control group. The result was similar by day 28, with further RGC loss.

• CONCLUSION: Our result suggested that the β-III-Tubulin protein was not affected by IOP elevation and can be used as a reliable marker for RGC in experimental models of glaucoma.

• KEYWORDS: β-III-Tubulin; glaucoma; fluorogold; retinal ganglion cell

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INTRODUCTION

As the second leading cause of blindness worldwide, glaucoma is characterized by progressive and irreversible atrophy of the optic nerve or cupping of the optic disc, which is the result of loss of axon and soma of retinal ganglion cells (RGCs)[1,2]. The RGCs are the only neurons in retina that connect and relay visual signals to the central nervous system [3], while they are also the main target cells that are susceptible to glaucomatous damage [2,4]. Thus observation and quantification of RGCs has always been important for evaluation of glaucomatous damage or effect of neuroprotection in studies of glaucoma. RGCs are located in the ganglion cell layer (GCL) of the retina, where however the displaced amacrine cells also occupy part of the cell population [5-7], making it necessary to identify RGCs from other neurons.

One common way to identify RGCs is the retrograde labeling by fluorescent neuron tracers, among which fluorogold (FG) is the most popular [8]. Once applied to the superior colliculus (SC) where the axon of RGCs mainly project in the brain [9,10], FG would be collected by the axon terminal of RGCs, transported in a retrograde manner along RGC axons and finally, after a period of time (usually 7d)[11,12], accumulates in the RGC soma located in the GCL of retina. As RGCs are the only neurons in retina that reach the SC, theoretically FG would not be present in other neurons except RGCs, which makes the RGC labelling specific. However, retrograde transport of FG from SC to the RGC soma depends on the bioactivities of RGC axons [13,14], which have been demonstrated to progressively decrease as the intraocular pressure (IOP) elevates [15,16]. So the transport of FG might be affected in glaucoma models, leaving a proportion of RGCs unlabeled and the quantification of RGCs inaccurate. Also, a relatively long term of observation would not be achieved because more RGC axons would be dysfunctional as long as the high IOP persists[17].

Alternative approaches of identifying RGCs include immunodetection of proteins that are specifically expressed in RGCs rather than other neurons in the GCL. Thy-1 is a

glycoprotein in the cell membrane predominantly expressed in RGCs in the retina and has been used as a marker for RGCs [8]. However the mRNA level and expression of Thy-1 gene decreases before the actual loss of RGCs in pathological conditions including intraocular hypertension[18-20], which means surviving RGCs with decreased or no expression of Thy-1 would not be labeled nor counted in models of glaucoma, indicating that Thy-1 is not suitable for assessment of RGC loss in glaucoma. Other RGC markers such as the transcription factor family Brn3 [21-24] and neurofilament [25,26] have also been used to label RGC, but in models of glaucoma, none of them has been proven to be a better alternative to retrograde labeling, which is still considered to be the most reliable method of labeling RGC. Transgenic methods that integrate genes of fluorescent proteins and promote their expression in RGCs have been recently used to make the RGCs born with fluorescent signals and directly distinguishable [27]. However, the success rate of gene transduction and fully expression of the integrated gene still need demonstration when it comes to quantitative analysis of RGCs and establishment of the transgenic models takes rather long[28].

β-III-Tubulin, or class III β-Tubulin, is a neural specific type of the tubulin family comprising proteins that form the microtubule of the cytoskeleton. It has been used to identify and separate neurons in brain tissues from glial cells that do not express β-III-Tubulin [29]. High expression of β-III-Tubulin has also been found in RGCs due to their neuronal origin [30]. In fact, β-III-Tubulin has already been used to identify and quantify RGCs in various optic nerve injury models[31-34]. However, in models of glaucoma, whether the expression of the protein decreases in RGC or increases in other neurons as IOP elevates is not well studied. In this study we would like to assess the reliability of β-III-Tubulin as a marker for identification and quantification of RGCs in glaucoma models, by comparing numbers of RGCs in the whole retina counted by β-III-Tubulin immunolabeling to those obtained by FG retrograde labeling.

MATERIALS AND METHODS

Grouping of Animals The experimental procedures and use of animals were approved and monitored by the Animal Care Committee of the West China Hospital and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Healthy adult C57BL/6J mice with weight ranging from 35 g to 50 g were included in the experiment, and those with any eye disease such as keratitis, cataract or uveitis were excluded. The animals were housed in a room where light was turn on and off alternately every 12h with food and water provided without restriction and they were kept at least for 1wk in this environment before any procedures were performed. In total, 64 adult C57BL/6J mice (no gender discrimination) from Experimental Animal Center of West China Hospital were employed and divided into four groups, including two experimental groups (16 mice in each group), a control groups (16 mice) and a blank control group (16 mice). All mice in the experimental groups received injection of 10 μm fluorescent polystyrene microbeads (Invitrogen Trading Co., Ltd., Shanghai, China) in the anterior chamber while mice in the control group received injection of phosphate buffer solution (PBS). Mice in the blank control group received sham injection with nothing actually injected. For comparing β-III-Tubulin immunolabeling and FG retrograde labeling of RGCs, mice in one of the experimental groups and mice in the control group were treated with FG that was applied to their superior colliculi (SCi) one week earlier before microbeads or PBS was injected, and their retinas were finally analyzed by FG and β-III-Tubulin double labeling. Furthermore, in order to find out whether the presence of FG in the RGC soma would affect the immunoreactivity of β-III-Tubulin and decrease the number of β-III-Tubulin labeled RGCs, retinas of mice in the other experimental group and the blank control group were analyzed only by β-III-Tubulin immunolabeling. By the end of 2 and 4wk after PBS or microbeads injection, half of the mice in each group were sacrificed respectively.

Retrograde Labeling of Retinal Ganglion Cells Retrograde labeling of RGCs by FG was performed following the standard protocol [11]. In brief, after the mouse was deeply anesthetized with intraperitoneal injection of pentobarbital (1% saline solution, 60 mg/kg), its head fur was removed and skin incised in the midline to expose the skull, in which two holes were drilled at the site corresponding to bilateral SCi, exposing the cerebrum beneath. Then the cerebrum over SCi was carefully removed before a piece of sterile sponge pre-soaked in FG solution (Fluorochrome, Denver, CO, USA; 2% in PBS) was placed in each hole on the dorsal surface of SC, where FG would be picked up by axon terminals of RGCs and transported retrogradely to their somas in the GCL. After the surgery, mice were kept warm under an incandescent lamp and allowed to recover by their own. Noting that it takes time for thorough transport of FG to the RGC soma and elevated IOP induced by microbeads injection might stop the transport of FG by RGC axons and prevent the labeling of RGCs, FG was applied to the SCi one week earlier before microbeads or PBS injection.

Establishment of Models of Glaucoma The method of inducing elevated IOP by injecting polystyrene microbeads into the anterior chamber to obstruct outflow of aqueous humor were taken[31,35]. Mice in the experimental groups were anesthetized by intraperitoneal injection of pentobarbital solution, supplemented by topical anesthesia with Oxybuprocaine Hydrochloride (Santen Pharmaceutical Co.,

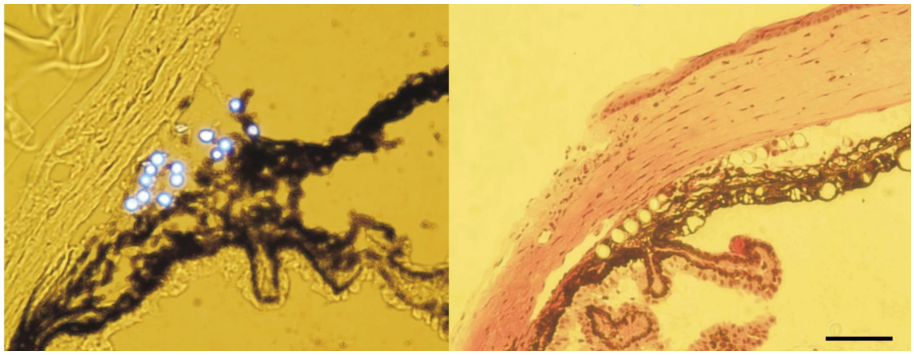

Figure 1 Microbeads accumulated at the anterior chamber angle or schlemm´s canal after injection of 2 μL polystyrene microbeads (HE-stained) Scale bar=50 μm.

Ltd., Japan). Then the polystyrene microbeads with a uniform diameter of 10 μm (Invitrogen Trading Co., Ltd., Shanghai, China) were injected unilaterally in their right eyes. The microbeads suspension whose original concentration was 3.6×10^6 beads per milliliter had been resuspended in PBS at a final concentration of 7.2×10^6 beads per milliliter to archive more effective obstruction [31]. More specifically, the right cornea of each mouse in the experimental group was gently punctured near the center using a 30-gauge needle to generate an easy entry for a glass micropipette connected with a Hamilton syringe, which was used to control precisely the volume (2 μL) of anterior chamber injection, then 2 μL microbeads suspension were injected into the right anterior chamber through the entry wound while the left eyes were left untreated. At the same time, 2 μL PBS was injected into the right eyes of mice in the control group following the same procedure and sham injection was performed to mice served as the blank control. Tobramycin dexamethasone eye ointment was administered to the injected eye of every mouse to prevent infection or inflammation.

Intraocular Pressure Measurement No sedative nor anesthetic drugs were applied to the mice in order to avoid effects on IOP due to anesthesia. All mice were subjected to IOP measurement every two days for 1wk before anterior chamber injection for their accommodation and the result was taken as the baseline. After microbeads or PBS injection, IOP of the injected eyes of the mice was measured every two days at the same time of the day with a tonometer (TonoLab; Colonial Medical Supply, Espoo, Finland) after applying topical anesthesia of the cornea. The tonometer would automatically take six measurements and eliminate the highest and lowest values to generate an average value as programmed. The average value was regarded as one reading and 3 readings obtained from each eye were averaged to figure out the final IOP value in each measurement.

Tissue Preparation and Immunofluorescence As described above, half of the mice in each group were sacrificed by cervical dislocation at the end of 2 and 4wk respectively after IOP elevation was induced. Then their eyes, with the cornea penetrated, were enucleated and fixed in 4% paraformaldehyde overnight after they received a transcardial perfusion with 4% paraformaldehyde in PBS. Retinal flat mounts and sections were then prepared for immunofluorescence analysis. Some of the flat mounts or sections that were treated following the same procedures except primary antibody incubation served as negative controls. To show the distribution of the polystyrene microbeads after injected into the anterior chamber, paraffin sections of the anterior segment of the eyeball were also prepared and stained with hematoxylin and eosin (Figure 1).

Retinal Flat Mounts Cornea and lens of the fixed eyes were removed and the whole retina was dissected from the eyecup, cut in 4 directions radially and flatly mounted on a glass slide with the GCL set upward. After permeabilized with 0.5% Triton X-100 diluted in PBS (PBS-T), the retinal flat mounts were immersed in 5% bovine serum albumin (BSA) for 60min to block nonspecific staining and then incubated with a primary antibody against β-III-Tubulin (Anti-Tubb3; Sigma-Aldrich Trading Co., Ltd., Shanghai, China) diluted 1:200 in blocking buffer consisting of 5% BSA and 0.05% triton in PBS at 4℃ overnight. The flat mounts were rinsed 3 times for 10min with PBS to remove excess primary antibody and incubated with the fluorescent secondary antibody (Alexa Fluor 568, Invitrogen Trading Co., Ltd., Shanghai, China) diluted 1:500 in blocking buffer for 2h at room temperature, protected from light. After thorough wash with PBS, the retinal flat mounts were prepared for RGC observation and quantification under a fluorescence microscope (Axio Imager 2, Carl Zeiss Microscopy GmbH, Germany). Retinal flat mounts prepared from eyes of mice previously treated with FG were finally double labeled by both FG and β-III-Tubulin antibody, and these flat mounts were protected from light throughout the entire process.

Retinal Sections Fixed eyes were transferred in 30% sucrose solution overnight for dehydration and cut into sections as thin as 10 μm vertically through the optic nerve

papillary after embedded in the optimal cutting temperature compound. Immunofluorescence analyses were performed as previously described. The sections were immersed in 0.5% Triton X-100 for permeablization and blocked in 5% BSA for 30min. After blocking, the retinal sections were incubated with the mouse anti-β-III-Tubulin antibody (Anti-Tubb3; Sigma-Aldrich Trading Co., Ltd., Shanghai, China) at 4℃ overnight. Then the sections were rinsed with PBS 3 times for 5min before incubated with the donkey anti-mouse secondary antibody (Alexa Fluor 488, Invitrogen Trading Co., Ltd., Shanghai, China) for 2h at room temperature. Anti-fading mounting medium containing DAPI (BeiJingZhongShanJingqiao biotechnology Co., Ltd) were mounted on the sections to stain the cell nucleus, providing better recognition and observation of cells under the fluorescence microscope. To find out whether the displaced amacrine cells in the GCL would also be labeled by the β-III-Tubulin antibody and miscounted as RGCs, part of the retinal sections without FG labeling were double immunostained with antibodies against β-III-Tubulin and Syntaxin (Santa Cruz Biotechnology Co., Ltd., Shanghai, China) which is a specific marker for amacrine cells. The secondary antibodies were donkey anti-mouse (Alexa Fluor 488, Invitrogen Trading Co., Ltd., Shanghai, China) and goat anti-rabbit (Alexa Fluor 568, Invitrogen Trading Co., Ltd., Shanghai, China) fluorescent antibodies respectively.

Quantification of Retinal Ganglion Cells Quantification of RGCs were accomplished in retina flat mounts. The retinal flat mounts were divided into 4 quadrants (superior, temporal, nasal, and inferior) as we cut the whole retina in 4 directions during preparation. Six standard regions (0.3×0.3 mm^2 each) distributed at a 1 mm interval from the center of the optic nerve head (ONH) along the radius were selected in each quadrant, among which three were from the peripheral region (3 mm from the ONH), two were from the intermediate region (2 mm from the ONH), and one was from the central region (1 mm from the ONH). In total 24 regions were selected in each retina flat mount, and in each region, a photograph of the retina was taken by the fluorescence microscopy (Axio Imager 2, Carl Zeiss Microscopy GmbH, Germany) at 400 times magnification. The 24 images taken from one flat mount were then analyzed with the AxioVision software (Carl Zeiss MicroImaging GmbH, Germany). RGCs were identified among FG, β-III-Tubulin or both positive cells by morphological characteristics in the 24 images and were manually counted in a masked manner. The RGC density (cells/mm^2) of the whole retina was calculated by averaging the cell counts of every image and divide the result by the area of the standard region (0.3×0.3-mm^2). The total area of the retinal flat mount was measured using Image J software (available at http://imagej.nih.gov/ij/download.html), and then the number of RGCs of the whole retina was estimated by multiplying the RGC density and the retina area.

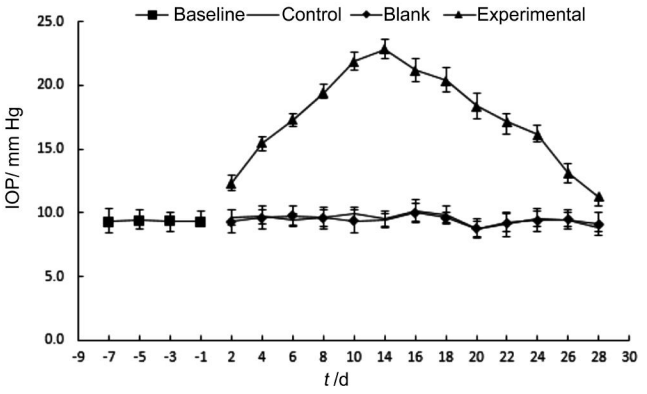

Figure 2 IOP elevation after microbeads injection IOP measurement for 1wk before anterior chamber injection revealed the baseline of IOP to be 9.3±0.9 mm Hg. Mice in the two experimental groups experienced IOP elevation after injection with an average peak level at 22.8±0.7 mm Hg by day 14 while IOP of mice in the control group remained at 9.5±0.7 mm Hg throughout the experiment. There was no significant difference between the IOP of the control group and the blank control group (P>0.2).

Statistical Analysis The statistical analysis was performed using SPSS 16.0 software (IBMM SPSS, Inc.). The data were expressed as mean±standard error of the mean (SEM). Statistical difference among groups was analyzed by Student's t-test or one-way analysis of variance (ANOVA). P< 0.05 was considered statistically significant.

RESULTS

Intraocular Pressure Elevation after Microbeads Injection After microbeads injection, none of the eyes showed any sign of inflamation which would be indicated by conjunctiva congestion or cloudiness in the anterior chamber, slight opacity of the cornea were found in some of the eyes during IOP measuerment and was considered to be a result of elevated IOP and injuryof the endothelia during microbeads injection. The measurement of IOP was performed every two days for 1wk before anterior chamber injection and every two days after the injection until the mice were executed. Before the injection, the avarage IOP of all the mice turned out to be 9.3±0.9 mm Hg, and this result was regarded as the baseline. After injection of PBS, the average IOP of mice in the control group stayed at 9.5±0.7 mm Hg, while that of mice in the blank control group was 9.5±0.9 mm Hg throughout the experiment. IOP of either the control group (t=1.22, P>0.2) or the blank control group (t=1.14, P>0.2) was not significantly different from the baseline and there was no significant difference between the control group and the blank control group (P>0.5), indicating that PBS injection would not induce IOP elevation. On the contrary, mice in the experimental groups experienced a significantly elevated IOP in eyes that received microbeads injection (Figure 2). The elevated IOP

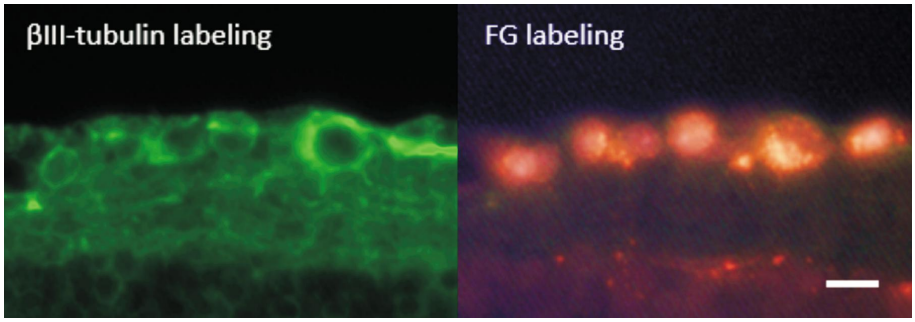

Figure 3 β-III-Tubulin and FG double labeling of RGCs from mice in the control group Both β-III-Tubulin and FG positive cells located in the GCL and could be identified as RGCs. With almost the same cell arrangement, the β-III-Tubulin positive RGCs corresponded well with the FG positive RGCs, indicating that RGCs were labeled by both markers. Scale bar=10 μm.

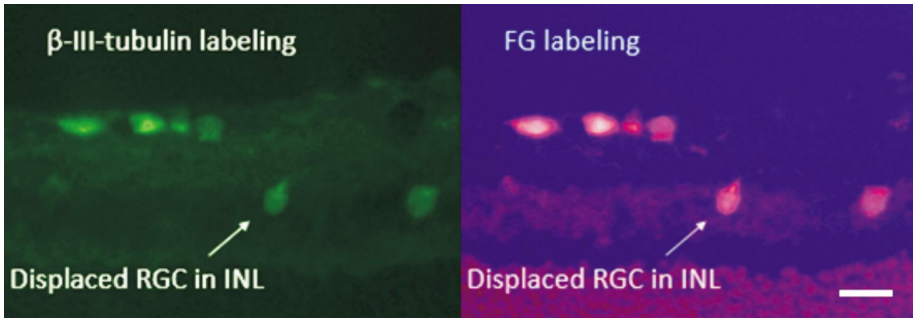

Figure 4 Fluorescent signals were detected in the inner nuclear layer (INL) These cells have the same fluorescent intensity and morphologic characteristics as those in the GCL and were considered to be displaced RGCs. Double labeling of displaced RGCs by both β-III-Tubulin antibody and FG further confirmed the specificity of β-III-Tubulin expression in RGC. Scale bar=15 μm.

kept rising and reached an average peak level at 22.8± 0.7 mm Hg by day 14, and started to go downafterward. By day 28, the IOP of eyes with microbeads injection had dropped to 11.3±0.7 mm Hg, which was close to the control group. The difference between the IOP of microbead-injected eyes and PBS-injected eyes was significant at each timepoint of measurement (day 14: P<0.002; day 28: P<0.002).

Quantification of Retinal Ganglion Cells Labeled by Fluorogold and β -III -Tubulin under Normal Intraocular Pressure We counted RGCs in the selected regions of the retinal whole mounts obtained from mice in the control group and the blank control group, which did not go through IOP elevation and calculated the average RGC density of each retina. After measuring the area of each retina, RGC counts of the whole retina were then estimated by multiplying the RGC density and the retina area. The retina flat mounts were labeled by both FG and β-III-Tubulin antibody (control group) or β-III-Tubulin antibody alone (blank control group), then numbers of RGCs were counted respectively by FG and/or β-III-Tubulin antibody. Among the FG and/or β-III-Tubulin positive cells, the ones with large size and neurites were identified as RGCs. As expected, in double labeled flat mounts, cells immunostained by β-III-Tubulin antibody highly corresponded to the FG positive cells identified as RGCs. To make sure that those labeled cells were RGCs, retinal sections were prepared to reveal layers of the retina and the exact position of the labeled cells (Figure 3). Both FG and β-III-Tubulin positive cells were in the GCL in retinal sections and corresponded well, indicating that RGCs were labeled by both markers, though in some cases fluorescent signals were also detected in the inner nuclear layer (INL; Figure 4), which was considered to be from displaced RGCs in the INL. More importantly, there was no significant difference between the average count of β-III-Tubulin labeled cells and that of FG labeled cells (64 614±5054 and 64 807±4930, respectively; t=0.11, P>0.5). Also, the number of β-III-Tubulin labeled cells in double labeled flat mounts did not differ significantly from the average RGC count of retinal flat mounts that were only labeled by β-III-Tubulin antibody (63 830±3894; t=0.49, P>0.5; Figure 5).

Comparison of Retinal Ganglion Cells Labeled by Fluorogold and β -III -Tubulin Antibody after Intraocular Pressure Elevation Half of the mice in each group were sacrificed at days 14 and 28 respectively after they received microbeads or PBS injection. Retinas of mice in each group were prepared as retinal flat mounts or sections. The retina flat mounts of mice in the FG-treated experimental group showed an obviously sparser distribution of FG positive or β-III-Tubulin positive RGCs than retina flat mounts of mice in the control group by day 14 (Figure 6). In the FG-treated experimental group, the average count of FG positive RGCs was 48 065 ±3556 and that of β-III-Tubulin positive RGCs was 48 051±2957 while in the control group, the corresponding numbers were 64 161±3415 and 63 732 ±3372 respectively. While there was no significant

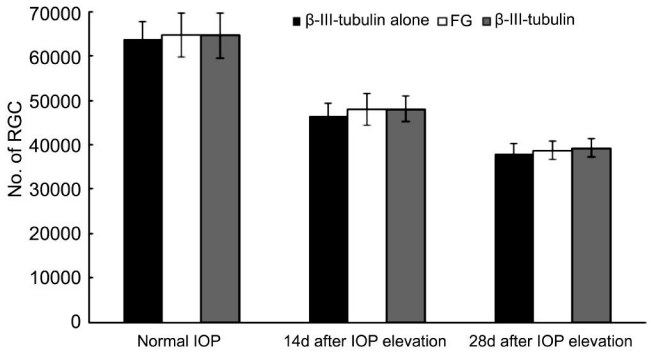

Figure 5 Comparison of the number of RGCs counted by FG and β -III -Tubulin double labeling and β -III -Tubulin labeling alone at different time points There was no significant difference between the number of RGCs counted by β-III-Tubulin labeling alone and that counted by β-III-Tubulin labeling in double labeled flat mounts either under normal (P >0.5) or elevated IOP (P >0.2), indicating that the immunoreaction of β-III-Tubulin antibody and the RGCs was not affected by FG accumulation in the RGC soma and the results of the double labeling was reliable.

difference between FG-positive and β-III-Tubulin positive cells in both the experimental (t =0.14, P>0.5) and the control group (t =0.25, P>0.5), the average RGC number of mice in the experimental group was significantly less than that in the control group, counting by either β-III-Tubulin labeling (t =9.89, P<0.01) or FG labeling (t =9.23, P<0.01). By day 28, the number of survived RGCs labeled by FG and β-III-Tubulin antibody in the experimental group was respectively 38 761±2045 and 39 266±1976 while there were 65 453 ±6010 FG positive RGCs and 65 497 ±6178 β-III-Tubulin positive RGCs of the controlled eyes left. However, what's really inspiring was, in the experimental group, when analyzing with β-III-Tubulin and FG double labeling, the β-III-Tubulin positive cells turned out to highly overlap with the FG-positive cells in both retina flat mounts and sections by day 14 (Figure 6) and day 28 (Figure 7), which means the β-III-Tubulin antibody still labeled FG-positive cells even when IOP kept high for a period of time. Moreover, by β-III-Tubulin immunolabeling alone, the average RGC count of mice in the experimental group without FG treatment was 46 458±2769 by day 14 and 37 841±2314 by day 28, with no significant difference from the result obtained by β-III-Tubulin labeling in the FG-treated experimental group at the same time points (day 14: t =1.01, P>0.2; day 28: t =1.32, P>0.2; Figure 5).

DISSCUSION

In many studies of glaucoma, despite some immunoreactive approaches, the identification of RGCs is done by retrograde labeling with FG[36-41], which is still considered to be the most reliable and accurate method. As FG is not directly applied to the retina but to SCi in the CNS and could only be transported to the RGC soma by RGC axons, there would be less possibility for any other cells in the retina to be labeled. The result of our study shows that the average FG positive RGC count of mice in the control group without IOP elevation was 64 807±4930, within the normal range of RGC count of the mouse retina [7], which confirmed the reliability of FG retrograde labeling in RGC quantification. However, realizing that the transport function of RGC axons would be affected under high IOP, preventing FG from reaching the RGC soma, we applied FG to the mice 1wk earlier before inducing IOP elevation because the axonal transport of FG usually takes 7d [12]. This pretreatment definitely prolonged the period of our experiments. And also, the requirement of an intracranial surgery increased the complexity and difficulty of the experimental procedures and led to early death of some animals, which was excluded from our experiment. So we would regard FG labeling as a relatively accurate method with less efficiency and convenience.

β-III-Tubulin is one of the isotypes of the tubulin protein family. While the tubulins are constituents of the microtubule that constructs the cytoskeleton in mammalian cells, β-III-Tubulin is expressed specifically in neurons [42-44]. In the retina, β-III-Tubulin has been found in neurons of the early born cell lineage including RGCs [30] and could be used to identify RGCs *in vitro* [33], showing the potential to be a RGC marker. Actually, in the past few years, this neuronal specific cytoskeleton component has already been used as a marker for RGC in various models of optic nerve injury such optic nerve crush or optic nerve transection [31-34]. Our result that the RGC count of retina flat mounts in the blank control group that was immunolabeled by β-III-Tubulin alone showed no significant difference with the number of RGC of the FG prelabeled flat mounts in the control group has confirmed that β -III-Tubulin could be used as an alternative of FG for labeling RGC under normal condition. However, in glaucoma models, as RGSs get lost during the pathological process, the reliability of β-III-Tubulin to identify and quantify RGCs has not been seriously assessed yet. As with the glaucoma model establishment, we chose the polystyrene microbeads with a relatively small diameter. In our experiment, IOP of mice in the experimental groups did not initially reach the peak level right after the anterior chamber injection, but encountered a gradual increase of about 14d. That might be due to that the injected microbeads only accumulated and partially blocked the anterior chamber angle at first, while they gradually got into the Schlemm's canal and further blocked the outflow of aqueous humour, inducing a higher IOP elevation. The microbeads were finally removed from the Schlemm's canal by episcleral veins, which explains the dropdown of IOP afterward.

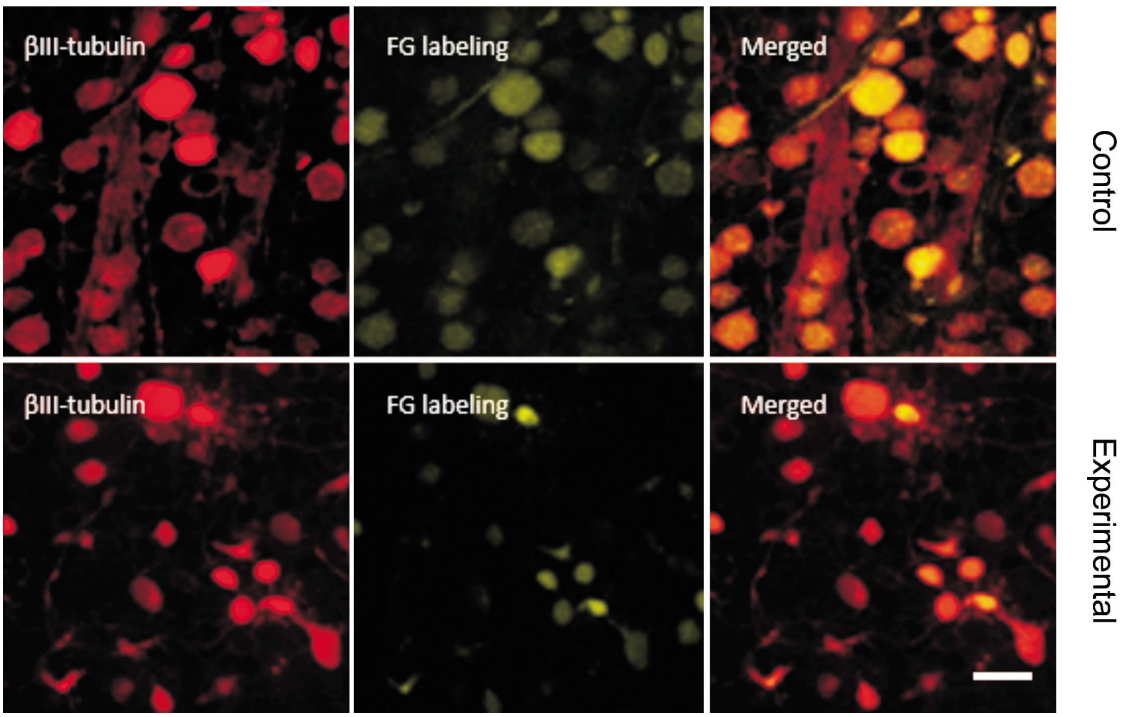

Figure 6 Retinas obtained from mice executed at day 14 in the experimental group and the control group that were pretreated with FG A sparser distribution of RGCs were observed by both FG and β-III-Tubulin labeling in the experimental group while there was still no significant difference between numbers of RGCs counted by FG and β-III-Tubulin labeling within the same group and the β-III-Tubulin positive cells highly overlapped with the FG positive cells. Scale bar=10 μm.

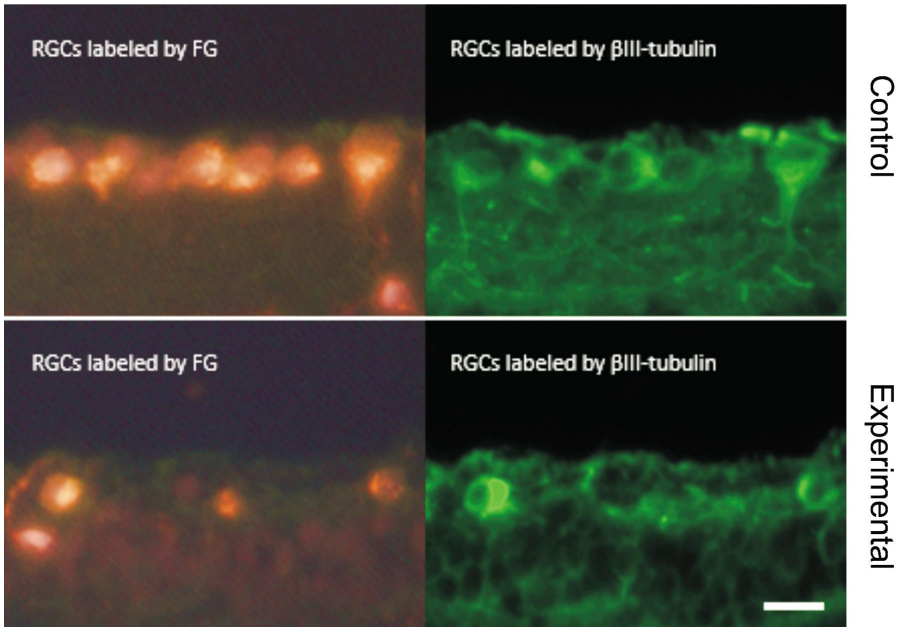

Figure 7 Retinal sections from the FG-treated experimental group showing RGCs labeled by β-III-Tubulin and FG by day 28 The β-III-Tubulin positive cells and FG positive cells still kept good correspondence while their distribution got sparser as time proceeded. Scale bar=10 μm.

Microbeads with smaller diameter had a lager quantity, which delayed the process of totally being removed, thus leading to a relatively long period of IOL elevation [31], making the glaucomatous damage more severe and the difference between the experimental group and the control group more obvious. After 14d of IOP elevation, RGCs of mice in the experimental group showed an obvious loss, compared to the control group, counting by either FG labeling or β-III-Tubulin antibody labeling, but no difference was noted between RGC counts obtained by FG labeling and β-III-Tubulin antibody labeling while the FG positive cells and β-III-Tubulin positive cells overlapped in retinal flat mounts and sections. The result was quite similar by day 28 after elevated IOP was induced with further loss of RGCs. It is already known that the expression of various genes would alter under high IOP [41]. The limitation of Thy-1 as a RGC

marker in glaucoma models is that its expression would be down regulated as the IOP elevates, even in surviving RGCs [18,20], which definitely would leave part of the RGCs uncounted in glaucoma models. Brn3a, as a transcript factor in the nucleus, has also been used as a RGC marker, but the loss of Brn3a expression or labeling happens much earlier than FG labeling [22]. Although the early loss of expression might indicate a higher sensitivity of Brn3a for detecting RGC death, the possibility that it is the cell function disturbance but not actual cell death that leads to the loss of Brn3a expression can still not be excluded, as Brn3a is after all a transcript factor, which reflects active function of cells. What's more, when it comes to time dependent analyzing, the possible discordance of Brn3a labeling and the actual surviving RGCs might lead to inaccurate results. FG labeling of RGC is reliable in glaucoma models because FG is a kind of foreign matter that accumulates in the soma of RGCs instead of something that would be affected by gene expression, so it would not disappear until the RGC is really dead and cleared by phagocytes such as microglia in the retina. In our study, we have demonstrated that under high IOP, the β-III-Tubulin antibody still labeled all the survived RGCs, which would certainly be FG positive, and the distribution of β-III-Tubulin positive RGCs corresponded well to that of FG-positive RGCs. The consistency of intensity of fluorescent signal of β-III-Tubulin labeling in the experimental group and the control group was also observed as time proceeded, which means that the β-III-Tubulin protein in each RGC did not decrease after IOP had been elevated. Interestingly, the expression of β-III-Tubulin increases when there is axonal regeneration after optic nerve injury [45,46], of which the reason might be the skeleton of the RGC axon needs reconstruction during regeneration and more β-III-Tubulin proteins need to be synthesized to provide materials. The glaucomatous damage also starts from the RGC axon [16,46], so there might also be reconstruction of the cell skeleton at the early stage of the damage when the RGC is still alive, resulting in an upregulation of β-III-Tubulin, which kept its immunoreactivity and fluorescent intensity. Another possible explanation might be that β-III-Tubulin is a kind of structural protein that contributes to the formation of cytoskeleton, it would less likely be affected by the cell function disturbance than functional proteins and would exist as long as the RGC is alive or cleared together with the RGC by phagocytes, which is more like the characteristics of FG. Furthermore, the number of β-III-Tubulin positive cells did not differ significantly between FG treated experimental group and the other experimental group that was finally analyzed by β-III-Tubulin alone, which means the presence of FG in the RGC soma did not affect the immunoreaction of β-III-Tubulin antibody or its labeling of the RGCs, making the correspondence of FG-positive cells and β-III-Tubulin positive cells trustworthy.

Immunolabeling is much easier to perform and it would leave the animals less suffering. However, the reliability of identification and quantification of RGCs by immunolabeling relies on the specificity of the cell marker. As mentioned previously, β-III-Tubulin is found in neurons of the early born cell lineage, which also included amacrine cells and some photoreceptors [30]. It is easy to tell RGCs apart from neurons that might be β-III-Tubulin positive in cell layers other than GCL by the position and morphological characteristics. However, both located in the GCL and being morphologically similar, it would be difficult to tell whether displaced amacrine cells (DACs) were also labeled by β-III-Tubulin, leading to over count of RGCs. So double labeling of RGCs and DACs by β-III-Tubulin and syntaxin (a specific marker of amacrine cells) respectively in radial retinal sections were performed. As syntaxinis a neuronal membrane protein involved in exocytosis [47], the fluorescent signal of syntaxin positive DACs was found surrounding the cell body, leaving the plasma unstained, whilethe β-III-Tubulin positive cells showed intensive fluorescent signal in the cell body,without membrane staining. The β-III-Tubulin positive RGCs also characterized with axonslabeled by β-III-Tubulin that converged to form the nerve fiber layer, which were found absent in syntaxin positive cells. These differences proved that those β-III-Tubulin positive cells in the GCL were RGCs other than DACs.

In conclusion, RGCs express β-III-Tubulin due to their neuronal origin and can be identified and quantified by β-III-Tubulin antibody in both normal mice and mice with IOP elevation. The expression of β-III-Tubulin in the RGC does not decrease as IOP elevates and time proceeds, making it a reliable marker of the RGC in experimental models of glaucoma while its advantages of convenience and efficiency also make it a good alternative of FG.

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REFERENCES

1 Diekmann H, Fischer D. Glaucoma and optic nerve repair. *Cell Tissue Res* 2013;353(2):327-337

2 Vidal-Sanz M, Salinas-Navarro M, Nadal-Nicolás FM, Alarcón-Martínez L, Valiente-Soriano FJ, de Imperial JM, Avilés-Trigueros M, Agudo-Barriuso M, Villegas-Pérez MP. Understanding glaucomatous damage: anatomical and functional data from ocular hypertensive rodent retinas. *Prog Retin Eye Res* 2012;31(1):1-27

3 Kaneda M. Signal processing in the mammalian retina. *J Nippon Med Sch* 2013;80(1):16-24

4 Agudo-Barriuso M, Villegas-Pérez MP, de Imperial JM, Vidal-Sanz M. Anatomical and functional damage in experimental glaucoma. *Curr Opin Pharmacol* 2013;13(1):5-11

5 Galindo-Romero C, Jiménez-López M, García-Ayuso D, Salinas-Navarro M, Nadal-Nicolás FM, Agudo-Barriuso M, Villegas-Pérez MP, Avilés-Trigueros M, Vidal-Sanz M. Number and spatial distribution of intrinsically photosensitive retinal ganglion cells in the adult albino rat. *Exp Eye Res* 2013;108:84-93

6 Samuel MA, Zhang Y, Meister M, Sanes JR. Age-related alterations in neurons of the mouse retina. *J Neurosci* 2011;31(44):16033-16044

7 Schlamp CL, Montgomery AD, Mac Nair CE, Schuart C, Willmer DJ, Nickells RW. Evaluation of the percentage of ganglion cells in the ganglion cell layer of the rodent retina. *Mol Vis* 2013;19:1387-1396

8 Leung CK, Weinreb RN. Experimental detection of retinal ganglion cell damage in vivo. *Exp Eye Res* 2009;88(4):831-836

9 Gaillard F, Karten HJ, Sauvé Y. Retinorecipient areas in the diurnal murine rodent Arvicanthis niloticus: a disproportionally large superior colliculus. *J Comp Neurol* 2013;521(8):Spc1

10 Cao J, Naito J, Chen Y. Retrograde tracing with fluorescent microspheres reveals bifurcating projections from central retina to tectum and thalamus in chicks. *Anat Histol Embryol* 2012;41(4):306-310

11 Chiu K, Lau WM, Yeung SC, Chang RC, So KF. Retrograde labeling of retinal ganglion cells by application of fluoro-gold on the surface of superior colliculus. *J Vis Exp* 2008;(16). pii:819

12 Huang TL, Huang SP, Chang CH, Lin KH, Sheu MM, Tsai RK. Factors influencing the retrograde labeling of retinal ganglion cells with fluorogold in an animal optic nerve crush model. *Ophthalmic Res* 2014;51 (4): 173-178

13 Takihara Y, Inatani M, Hayashi H, Adachi N, Iwao K, Inoue T, Iwao M, Tanihara H. Dynamic imaging of axonal transport in living retinal ganglion cells in vitro. *Invest Ophthalmol Vis Sci* 2011;52(6):3039-3045

14 Yu DY, Cringle SJ, Balaratnasingam C, Morgan WH, Yu PK, Su EN. Retinal ganglion cells: Energetics, compartmentation, axonal transport, cytoskeletons and vulnerability. *Prog Retin Eye Res* 2013;36:217-246

15 Chidlow G, Ebneter A, Wood JPM, Casson RJ. The optic nerve head is the site of axonal transport disruption, axonal cytoskeleton damage and putative axonal regeneration failure in a rat model of glaucoma. *Acta Neuropathol* 2011;121(6):737-751

16 Salinas-Navarro M, Alarcón-Martínez L, Valiente-Soriano FJ, Jiménez-López M, Mayor-Torroglosa S, Avilés-Trigueros M, Villegas-Pérez MP, Vidal-Sanz M. Ocular hypertension impairs optic nerve axonal transport leading to progressive retinal ganglion cell degeneration. *Exp Eye Res* 2010;90(1):168-183

17 Abbott CJ, Choe TE, Lusardi TA, Burgoyne CF, Wang L, Fortune B. Evaluation of retinal nerve fiber layer thickness and axonal transport 1 and 2 weeks after 8 hours of acute intraocular pressure elevation in rats. *Invest Ophthalmol Vis Sci* 2014;55(2):674-687

18 Huang W, Fileta J, Guo Y, Grosskreutz CL. Downregulation of Thy1 in retinal ganglion cells in experimental glaucoma. *Curr Eye Res* 2006;31(3): 265-271

19 Levkovitch-Verbin H, Makarovsky D, Vander S. Comparison between axonal and retinal ganglion cell gene expression in various optic nerve injuries including glaucoma. *Mol Vis* 2013;19:2526-2541

20 Schlamp CL, Johnson EC, Li Y, Morrison JC, Nickells RW. Changes in Thy1 gene expression associated with damaged retinal ganglion cells. *Mol Vis* 2001;7:192-201

21 Nadal-Nicolás FM, Jiménez-López M, Salinas-Navarro M, Sobrado-Calvo P, Alburquerque-Béjar JJ, Vidal-Sanz M, Agudo-Barriuso M. Whole number, distribution and co-expression of brn3 transcription factors in retinal ganglion cells of adult albino and pigmented rats. *PLoS One* 2012;7(11):e49830

22 Nadal-Nicolás FM, Jiménez-López M, Sobrado-Calvo P, Nieto-López L, Cánovas-Martínez I, Salinas-Navarro M, Vidal-Sanz M, Agudo M. 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(8):3860-3868

23 Piri N, Kwong JMK, Song M, Caprioli J. Expression of hermes gene is restricted to the ganglion cells in the retina. *Neurosci Lett* 2006;405(1-2): 40-45

24 Sánchez-Migallón MC, Nadal-Nicolás FM, Jiménez-López M, Sobrado-Calvo P, Vidal-Sanz M, Agudo-Barriuso M. Brain derived neurotrophic factor maintains Brn3a expression in axotomized rat retinal ganglion cells. *Exp Eye Res* 2011;92(4):260-267

25 Gutierrez C, McNally M, Canto-Soler MV. Cytoskeleton proteins previously considered exclusive to ganglion cells are transiently expressed by all retinal neuronal precursors. *BMC Dev Biol* 2011;11:46

26 Chidlow G, Osborne NN. Rat retinal ganglion cell loss caused by kainate, NMDA and ischemia correlates with a reduction in mRNA and protein of Thy-1 and neurofilament light. *Brain Res* 2003;963 (1-2): 298-306

27 Raymond ID, Pool AL, Vila A, Brecha NC. A Thy1-CFP DBA/2J mouse line with cyan fluorescent protein expression in retinal ganglion cells. *Vis Neurosci* 2009;26(5-6):453-465

28 Raymond ID, Vila A, Huynh UC, Brecha NC. Cyan fluorescent protein expression in ganglion and amacrine cells in a thy1-CFP transgenic mouse retina. *Mol Vis* 2008;14:1559-1574

29 Xu SY, Wu YM, Ji Z, Gao XY, Pan SY. A modified technique for culturing primary fetal rat cortical neurons. *J Biomed Biotechnol* 2012; 2012:803930

30 Sharma RK, Netland PA. Early born lineage of retinal neurons express class III beta-tubulin isotype. *Brain Res* 2007; 1176:11-17

31 Chen H, Wei X, Cho KS, Chen G, Sappington R, Calkins DJ, Chen DF. Optic neuropathy due to microbead-induced elevated intraocular pressure in the mouse. *Invest Ophthalmol Vis Sci* 2011;52(1):36-44

32 Hu Y, Cho S, Goldberg JL. Neurotrophic effect of a novel TrkB agonist on retinal ganglion cells. *Invest Ophthalmol Vis Sci* 2010;51 (3): 1747-1754

33 Hu Y, Cui Q, Harvey AR. Interactive effects of C3, cyclic AMP and ciliary neurotrophic factor on adult retinal ganglion cell survival and axonal regeneration. *Mol Cell Neurosci* 2007;34(1):88-98

34 Oono S, Kurimoto T, Nakazawa T, Miyoshi T, Okamoto N, Kashimoto R, Tagami Y, Ito Y, Mimura O. Pyroglutamic acid promotes survival of retinal ganglion cells after optic nerve injury. *Curr Eye Res* 2009;34(7):598-605

35 Matsumoto Y, Kanamori A, Nakamura M, Negi A. Rat chronic glaucoma model induced by intracameral injection of microbeads suspended in sodium sulfate-sodium hyaluronate. *Jpn J Ophthalmol* 2014;58 (3): 290-297

36 Pérez de Lara MJ, Santano C, Guzmán-Aránguez A, Valiente-Soriano FJ, Avilès-Trigueros M, Vidal-Sanz M, de la Villa P, Pintor J. Assessment of inner retina dysfunction and progressive ganglion cell loss in a mouse model of glaucoma. *Exp Eye Res* 2014;122:40-49

37 Biermann J, van Oterendorp C, Stoykow C, Volz C, Jehle T, Boehringer D, Lagrèze WA. Evaluation of intraocular pressure elevation in a modified laser-induced glaucoma rat model. *Exp Eye Res* 2012;104:7-14

38 Husain S, Abdul Y, Crosson CE. Preservation of retina ganglion cell function by morphine in a chronic ocular-hypertensive rat model. *Invest Ophthalmol Vis Sci* 2012;53(7):4289-4298

39 Sandalon S, Könnecke B, Levkovitch-Verbin H, Simons M, Hein K, Sättler MB, Bähr M, Ofri R. Functional and structural evaluation of lamotrigine treatment in rat models of acute and chronic ocular hypertension. *Exp Eye Res* 2013;115:47-56

40 Wang DY, Ray A, Rodgers K, Ergorul C, Hyman BT, Huang W, *et al*. Global gene expression changes in rat retinal ganglion cells in experimental glaucoma. *Invest Ophthalmol Vis Sci* 2010;51(8):4084-4095

41 Karki R, Mariani M, Andreoli M, He S, Scambia G, Shahabi S, Ferlini C. β III-Tubulin: biomarker of taxane resistance or drug target? *Expert Opin Ther Targets* 2013;17(4):461-472

42 Van Oterendorp C, Lagrèze WA, Gutekunst E-M, Sgouris S, Maier P, Biermann J. Enrichment of retinal ganglion cells in rat retinal lysate by excimer laser ablation of the outer retina. *Invest Ophthalmol Vis Sci* 2013; 54(3):2061-2067

43 Romero-Alemán MM, Monzón-Mayor M, Santos E, Yanes C. Expression of neuronal markers, synaptic proteins, and glutamine synthetase in the control and regenerating lizard visual system. *J Comp Neurol* 2010;518(19):4067-4087

44 Romero-Alemán MM, Monzón-Mayor M, Santos E, Lang DM, Yanes C. Neuronal and glial differentiation during lizard (Gallotia galloti) visual system ontogeny. *J Comp Neurol* 2012;520(10):2163-2184

45 Fournier AE, McKerracher L. Expression of specific tubulin isotypes increases during regeneration of injured CNS neurons, but not after the application of brain-derived neurotrophic factor (BDNF). *J Neurosci* 1997; 17(12):4623-4632

46 Soto I, Oglesby E, Buckingham BP, Son JL, Roberson ED, Steele MR, Inman DM, Vetter ML, Horner PJ, Marsh-Armstrong N. Retinal ganglion cells downregulate gene expression and lose their axons within the optic nerve head in a mouse glaucoma model. *J Neurosci* 2008;28(2):548-561

47 Wang Y, Tang BL. SNAREs in neurons--beyond synaptic vesicle exocytosis (Review). *Mol Membr Biol* 2006;23(5):377-384