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Lack of neuroprotection against experimental glaucoma in c-Jun N-terminal kinase 3 knockout mice

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Abstract

To determine if the absence of c-Jun N-terminal kinase 3 (JNK3) in the mouse retina would reduce retinal ganglion cell (RGC) loss in mice with experimental glaucoma. C57BL/6 mice underwent experimental intraocular pressure (IOP) elevation with a bead/viscoelastic injection into one eye. One-half of the mice were *Jnk3* homozygous knockouts (KO) and were compared to wild type (WT) mice. IOP was measured under anesthesia with the TonoLab, axial length was measured post-mortem with calipers after inflation to 15 mm Hg, and RGC layer counts were performed on retinal whole mount images stained with DAPI, imaged by confocal microscopy, and counted by masked observers in an image analysis system. Axon counts were performed in optic nerve cross-sections by semi-automated image analysis. Both WT and *Jnk3*^{-/-} mice had mean elevations of IOP of more than 50% after bead injection. Both groups underwent the expected axial globe elongation due to chronic IOP elevation. The absence of JNK3 in KO retina was demonstrated by Western blots. RGC layer neuron counts showed modest loss in both WT and *Jnk3*^{-/-} animals; local differences by retinal eccentricity were detected, in each case indicating greater loss in KO animals than in WT. The baseline number of RGC layer cells in KO animals was 10% higher than in WT, but the number of optic nerve axons was identical in KO and WT controls. A slightly greater loss of RGC in *Jnk3*^{-/-} mice compared to controls was detected in experimental mouse glaucoma by RGC layer counting and there was no protective effect shown in axon counts. Counts of RGC layer cells and optic nerve axons indicate that *Jnk3*^{-/-} mice have an increased number of amacrine cells compared to WT controls.

Keywords

glaucoma; c-Jun N-terminal kinase; retinal ganglion cell; pathogenesis; neuroprotection

Introduction

Injury to neuronal axons can cause their loss by either Wallerian degeneration or progressive dying back (Seif et al., 2001). Axon death can be a separate process from cell body injury

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and death. The effects on the retinal ganglion cell (RGC) body in glaucoma are probably initiated by signals generated by the injured axon. A comprehensive protective strategy for the RGC must include both avoidance of axonal injury, and inhibition of signals of axon injury to the RGC body. We formed the hypothesis that cJun N terminal kinase (JNK) may be one signal to the RGC body of axonal injury at the optic nerve head in experimental glaucoma, based on gene array data (Yang et al., 2007) that indicated rapid upregulation of transcription factors known to be responsive to JNK. There is evidence that phosphorylated JNK (p-JNK) moves in retrograde transport as part of the dynein complex after axon injuries that may share features with glaucoma, such as nerve ligation (Cavalli et al., 2005). In ligation models, p-JNK signals neurotrophin withdrawal (Lindwall et al., 2005) and its inhibition is neuroprotective against apoptotic death in experimental neuronal injury (Okuno et al., 2004; Eilers et al., 2001). Furthermore, pJNK is increased in human (Tezel et al, 2003) and rat glaucoma (Kwong et al., 2006) retinas, as well as in the rat retina after NMDA-induced excitotoxicity (Bessero et al., 2010).

The specific method by which p-JNK may initiate changes in the RGC body are hypothesized to be similar to events in other neuronal systems. For example, our glaucoma array data showed early upregulation of c-Jun and activating transcription factor-3 (ATF3). pJNK phosphorylates c-Jun, which has been shown to be increased in experimental glaucoma (Levkovitch-Verbin et al., 2005), and it in turn combines with ATF3 in injured RGC (Takeda et al., 2000) to act as a transcriptional activator of other genes, including *Hsp27*, known to be upregulated in human glaucoma RGC (Tezel et al., 2000). pJNK activates a component of the mitogen-activated protein (MAP) kinase pathway, potentiating RGC death, and this is known to occur in rat glaucoma (Levkovitch-Verbin et al, 2007). pJNK arrival at the neuronal cell body is also associated with activation of CCAAT/enhancer binding protein (Cebp δ) (Fischer et al., 2004; Chen et al., 2004), a member of the phosphatidylinositol-3 (PI-3) kinase/Akt pathway, which typically supports neuronal survival (Sekine et al., 2002). Cebp δ is known to stimulate expression of signal transducer and activator of transcription 3 (Stat3) (Hanz et al., 2006), which was consistently elevated in our glaucoma model. Another upregulator of Stat3 is arrival at the cell body of vimentin/JNK complexes. In the central nervous system, JNK plays a role in neuronal death after axotomy by retrograde signaling (Herdegen et al, 1998; Cavalli et al., 2005). For example, injured axons exhibit enhanced pJNK retrograde transport as a signaling program in the CNS (Hanz et al., 2006). Nitric oxide acts to block anterograde transport through a pJNK-mediated mechanism (Stagi et al, 2005), in which pJNK interacts with JNK-interacting protein (JIP) (Yasuda et al., 1999), a key component of the motor proteins responsible for axonal transport.

We therefore tested the hypothesis that *Jnk3* knockout (KO) mice would be relatively protected from chronic experimental glaucoma. Of the isoforms of JNK expressed throughout the body, JNK3 is the form most highly expressed in the central nervous system. It is expressed in the retina, although JNK1 and JNK 2 are also expressed in the retina. *Jnk3* (*Mapk10*) KO mice had decreased neuronal death after kainate exposure or ischemia (Yang et al., 1997). If pJNK3 is a necessary positive signal for RGC death in glaucoma, its elimination should decrease RGC death. In a chronic IOP elevation model, we compared *Jnk3* KO to control mice, to measure relative RGC survival. We additionally studied potential changes in retina structure that might result from elimination of JNK3 in control animals.

Methods

Generation of *Jnk3* KO mice

A total of 125 mice at 4–5 months of age were utilized in this study. *Jnk3* KO animals were obtained from Jackson Laboratories (Bar Harbor, ME) as heterozygotic breeding pairs generated from frozen embryos, on a C57BL/6 background. Animals produced from the original breeding pairs with two normal JNK alleles were used as controls. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the EC Directive 86/609/EEC for animal experiments, using protocols approved and monitored by the Johns Hopkins University School of Medicine Animal Care and Use Committee.

Methods for experimental glaucoma by bead injection

We produced chronic IOP elevation by a published method (Cone et al., 2010) using injections of Polybead Microspheres® (Polysciences, Inc., Warrington, PA, USA) of 6 µm diameter and viscoelastic solution (10 mg/ml sodium hyaluronate: Healon, Advanced Medical Optics Inc., Santa Ana, CA, USA). Briefly, we first sterilized the beads in 100% ethanol, centrifuged them after washing in sterile phosphate buffered saline, and aspirated the centrifuged pellet directly into a glass micropipette at approximately 3×10^6 beads per µL. Mice were anesthetized with intraperitoneal injection of ketamine, xylazine, and acepromazine (50, 10 and 2 mg/kg, respectively). One eye was proptosed and a 50 µm glass cannula was inserted into the anterior chamber, with injection of 2 µL of beads followed by 3 µL of viscoelastic solution using a Hamilton syringe (Hamilton Company Reno, NV, USA). The needle was left in place for two minutes to minimize efflux of injected material.

IOP measurement and tonometer calibration

IOP measurements were made in both eyes with the TonoLab tonometer (TioLat, Helsinki, Finland) under combined topical anesthesia with 0.5% proparacaine hydrochloride eyedrops (Akorn Inc, Buffalo Grove, IL, USA) and general anesthesia (as above). IOP was measured prior to baseline, immediately after injection, 3 days after injection, and weekly thereafter until sacrifice at 6 weeks after injection. We have confirmed the accuracy of the TonoLab as a measure of true IOP in glaucoma and normal mice in a calibration study (Pease et al., 2010).

Sacrifice and axial length measurements

Animals were anesthetized with intraperitoneal injection of ketamine, xylazine, and acepromazine (50, 10 and 2 mg/kg, respectively) prior to perfusion fixation with 4% paraformaldehyde in 0.1M Na₃PO₄ at 6 weeks after bead injection. Both eyes were enucleated and immersion fixed for an additional hour, then cleaned of extraocular tissues. The eyes were manually inflated to 15mm Hg with a needle inserted into the anterior chamber connected to a reservoir of saline that determined the IOP prior to measurement of axial length and width with a digital caliper (Instant Read Out Digital Caliper, Electron Microscopy Sciences, Hatfield, PA, USA). The length was measured from the center of the cornea to a position just temporal to the optic nerve, while width was measured at the largest dimension at the equator, midway between cornea and optic nerve.

RGC Counts

The outcome measure for RGC loss was the number of RGC layer cells in flat mounts of both glaucoma and fellow control eyes. After axial length measurements, both retinas were removed from perfusion-fixed eyes, incised for flat mounting, placed on SuperFrost Plus slides for 24 hours, washed in PBS for 10 minutes, and treated with 0.1% Triton/PBS for 5

minutes before being exposed to 1:250 dilution of DAPI stain for 5 minutes (Invitrogen, Carlsbad, CA, USA). After buffer washes, retinas were cover-slipped with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA, USA) and imaged using the Zeiss LSM 510 Meta Confocal Microscope (Zeiss MicroImaging, Thornwood, NY, USA). Twenty 40x images were taken per retina, 5 fields from each of 4 quadrants (superior, nasal, inferior, temporal), equaling a 7% sample of total retinal area. Each image was taken at the level of the RGC layer and analyzed with Metamorph Image Analysis software (Molecular Devices, Downingtown, PA, USA). Because the confocal imaging was performed only at the level of the RGC layer, astrocytes of the nerve fiber layer are not included. All cells with a round or slightly oval nucleus and morphology compatible with that of neurons were identified manually by trained technical staff masked to the protocol details for each retina. Interobserver reproducibility of the method comparing 3 technicians showed a mean difference of only 0.5% between observer A and B, and a mean difference of 1.5% between observer A and observer C, and 1.9% between observer B and observer C, counting a total of nearly 6,000 cells from 20 images in each test. Cells that were not counted included: 1) those with elongated nuclei more typical for endothelial cells or astrocytes and 2) polymorphonuclear cells assumed to be white blood cells. Experimental retinas were compared to mean RGC layer counts from the data of pooled, fellow eyes.

Optic nerve axon counts

After perfusion fixation, optic nerves were placed in 1% osmium, dehydrated in ascending alcohol concentration, and placed in 1% uranyl acetate in 100% ethanol for 1 hour. Tissues were embedded in epoxy resin mixture at 60°C for 48 hours. One micron thick cross-sections of the optic nerve were stained with 1% toluidine blue in 1% sodium borate. Digital images of the nerves were taken at low power to measure the total optic nerve area in each nerve and then at 100x using a Cool Snap camera and Metamorph Image Analysis software. For each nerve, five 40 × 40 μm fields were acquired, equaling a 9% sample of the total nerve area. A masked observer edited non-axonal elements from each image, generating an axon density measure from the software. Average axon density/mm² was multiplied by the individual nerve area to estimate axon number. Nerve area and density were calculated for each nerve. To estimate axon loss in statistical analysis, the value for each glaucoma nerve was compared to the pooled average axon count for matching animals of the same strain.

Retinal histology and immunolabeling for synuclein

We quantitatively measured mid-retinal and outer retinal layer thicknesses to evaluate possible damage to zones other than to the RGC layer and nerve fiber layer. An ideal glaucoma model should duplicate the fact that only RGC layer cells atrophy and that there is no change in the middle or outer retina. Should the latter occur, it might mean that gross vascular compromise was responsible for the effects noted rather than experimental IOP elevation. Samples of the optic nerve head and adjacent retina from both injected and fellow control eyes were epoxy embedded following the same protocol as for optic nerve cross-sections above. The thickness of the inner nuclear and outer nuclear layers of the retina was measured at 6 locations on each section by a masked observer in 15 mice in paired injected and control eyes.

We also performed immunolabeling for synuclein gamma (Sneg) in a subset of retinal wholemounts of WT and KO control retinas to estimate directly the number of RGC (as compared to other neurons, such as amacrine cells, that are included in DAPI counts). Following overnight fixation at 4°C, these retinas were washed in buffer and stored in methanol at -20°C until ready for immunolabeling. After serial rehydration using methanol/PBST (PBS, 1% TritonX-100), retinas were incubated for 3 days in primary antibody, mouse anti-human synuclein [2C3] antibody (GTX91423) 1:500 (GeneTex, Irvine, CA)

diluted in PBST/10% normal goat serum at 4°C. Following primary antibody incubation, retinas were washed with PBST and incubated for two hours at room temperature with goat anti-mouse Alexa 488 1:200 secondary antibody (Invitrogen, Carlsbad, CA), diluted in PBST. After secondary antibody incubation, retinas were washed in PBST, then PBS, mounted and coverslipped in Fluorescent Mounting Medium.

Western blot for JNK

To confirm that JNK3 was not expressed in the KO animals, we isolated unfixed retina from WT and KO eyes and lysed the tissue by sonication in RIPA buffer containing a cocktail of protease inhibitors (Roche, Indianapolis IN). Cell debris was removed by centrifugation and protein concentration in the supernatant was determined by Bradford method. Ten µg protein was loaded on 10% SDS-PAGE, and separated by electrophoresis. Protein was transferred to nitrocellulose membranes, which were then blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Triton (TBST) and probed with anti-Jnk3 and anti-total JNK antibodies (Cell Signaling Technology, Danvers, MA) at 4°C overnight. The protein loading control was alpha Tubulin. Membranes were then washed with TBST 6 times, and probed with HRP-linked anti-rabbit IgG. The signals were developed with enhanced chemiluminescent substrate before film exposure.

Statistical methods

The following data were tabulated and compared between treated and control eyes in each animal of both KO and WT mice: axial length increase in glaucoma eye compared to pooled fellow eyes, IOP average level, IOP average difference between glaucoma and control eye, and RGC layer cell count. Axon counts were conducted in control nerves of KO and WT mice. Mean values were compared with parametric statistical tests (t tests) for data that were normally distributed and median values with non-parametric testing (Mann Whitney) for those whose distributions failed normality testing. RGC layer loss and optic nerve axon loss were each the dependent variable in separate multivariable linear regression analyses with the independent variables: diagnosis (glaucoma or control), axial length increase, and average IOP increase.

Results

1. Western blot and immunolabeling

Western blots performed with two antibodies, one specific for JNK3 and one pan-JNK antibody, showed that there was no detectable JNK3 protein in the KO retina (Figure 1). The antibody that recognizes all JNK isoforms showed a normal level of the other forms of JNK. The finding that the signal intensity with the pan-JNK antibody was similar in the KO and WT retinas suggests that there is significant expression of Jnk1 and/or Jnk2 in the KO retinas, and that there may be compensatory increases in isoforms 1 and 2 in the KO retina.

2. IOP data

The IOP under anesthesia in C57BL/6 mice was approximately 10 mm Hg. After bead/viscoelastic injection, IOP in both WT and KO mice rose significantly ($p < 0.001$ at 3 days, 1 week, and 6 week time points, t test; Figure 2). There was no significant difference in the IOP increase in the WT compared to KO mice at any of the measured time points to 6 weeks (p all greater than 0.20, t test), nor was the average IOP increase at any time point different between WT and KO (Mann Whitney, $p = 0.46$). The first data point on the graph in Figure 2 is immediately after bead injection, showing a very slight elevation.

3. Axial length increase

The axial length at baseline was 3.57 ± 0.30 mm. As seen in previous bead/viscoelastic glaucoma experiments, the eyes elongated in response to IOP elevation. In WT mice, the increase at 6 weeks was 0.15 ± 0.20 mm (4.5% elongation), while in KO mice it was 0.32 ± 0.34 mm (9.7% elongation). The difference between WT and KO mice achieved statistical significance (Mann Whitney, $p = 0.04$). The increase in width was similar magnitude to that of the length increase, but was not significantly different between WT and KO ($p > 0.05$, data not shown)

4. Retinal layer thickness

Examination of cross-sections of the retina in separate bead-injected eyes from those in which whole mounts were used for counting showed only loss of cells from the RGC layer and thinning of the nerve fiber layer. As expected in glaucomatous optic neuropathy, there was no thinning in the inner nuclear, outer nuclear, or photoreceptor layers. The mean thickness of the outer nuclear layer in bead-injected compared to fellow, control eyes differed by only 3% (larger), while the inner nuclear layer differed by -1% (smaller in bead-injected retina), but both were statistically insignificant differences ($p = 0.4$ and 0.9 , t test).

5. RGC layer counts

The number of RGC layer cells in JNK KO control (non-glaucoma) retinas was significantly higher in all quadrants than in WT controls (Table 1). The mean increase across all quadrants was 8.4 ± 0.22 % in the KO RGC layer. While the difference was slightly greater in KO males (compared to WT males) than in KO females (compared to WT females), the gender difference was not statistically significant (Table 2). In the Tables, field 1 is nearest to the optic disc and field 5 is the most peripheral retina.

In order to determine if the increase in RGC layer neurons among KO mice was an increase in RGC themselves, in amacrine cells, or in both, we carried out labeling with antibodies to Sncg, a specific marker for RGC, in 4 eyes of 3 mice from both WT and KO animals (Figure 3). As a proportion of total neurons labeled with DAPI in the RGC layer, Sncg positive cells presented 40.3 ± 5.9 % of total neurons in the WT eyes and 36.1 ± 5.2 % of neurons in the KO ($p = 0.003$, t test). Since we found that the number of RGC axons in the optic nerves of WT and KO animals were identical (see below), these data suggest that the 10% increase in DAPI-counted RGC layer cells was due to an increase in amacrine cells. For example, if WT retina has 100 neurons, 40 of which are RGCs (40%), and if the number of total neurons is increased by 10% to 110, all comprised of amacrine cells, then the RGC proportion (Sncg positive) should be $40/110 = 36.3$ % -- almost exactly the figure we found for Sncg positive cells in the KO retinas.

The loss of RGC layer cells at 6 weeks in the bead-treated eyes of WT animals was under 4%, while in KO animals it was nearly 7% (Table 3). By eccentricity (field) and by quadrant, there was significantly greater loss in KO than in WT animals in 2 of 5 fields and in the superior quadrant, but the differences were not significant in the other zones. In no region was damage proportionately greater in WT compared to KO animals.

In regression models with RGC layer loss as dependent variable, we adjusted the RGC layer counts by using the estimated number of RGCs among total neurons in the RGC layer. For example, since 40% of RGC layer cells in WT animals are RGCs, a 5% loss of DAPI-counted RGC layer cells would be corrected to 5% times $100/40 = 12.5$ %, making the assumption that all neurons lost were RGCs, as we and others have demonstrated in rodent glaucoma models (Kielczewski et al., 2005). Likewise, the KO data were corrected by multiplying RGC layer loss by $100/36$ (to account for the lower proportion of RGC). In

univariable models with RGC loss as dependent variable, diagnosis was significantly related to loss (worse loss in KO animals, $p = 0.003$), as was axial length increase ($p = 0.03$), but average IOP increase was not ($p = 0.8$). Use of peak IOP or exposure to IOP over time (IOP integral) gave similar results as average IOP, so we used the latter in multivariable models. In a multivariable model with dependent variable RGC loss (corrected), diagnosis (WT vs KO) was significantly related to loss ($p = 0.045$) when adjusted for axial length increase ($p = 0.012$). Thus, when taking into account the greater axial length increase of the KO animals, there remained a slightly greater loss of RGC in the KO animals.

6. Optic nerve axon counts

To corroborate the possible change in RGC number in control KO compared to control WT animals, we counted optic nerve axon number in 10 WT and 10 KO control nerves. The WT were $46,807 \pm 4785$, while the KO were $47,056 \pm 4672$ (mean $\pm$ standard deviation, $p = 0.22$, t test). Thus, the apparent increase in DAPI counts of neurons in the RGC layer is most likely due to an increase in non-RGC, namely amacrine cells in the KO mice.

The axon loss in WT and KO groups did not differ significantly, with 2.7% loss in WT and 2.1% in KO animals ($p = 0.8$, t test, $n = 18$ and 24 animals, respectively) (Figure 4). The difference between WT and KO animals was also not significant in multivariable regression in which axon loss was dependent variable and either average IOP or axial length (or width) increase were included in the models to adjust for exposure to the effects of the bead injection among animals.

Discussion

Jnk3 gene KO has been shown to be neuroprotective in models such as kainate excitotoxicity in hippocampus (De Lemos et al., 2010) and rotenone toxicity in dopaminergic neuron culture (Choi et al., 2010). In fact, JNK3 is specifically implicated in the fast axonal transport blockade by huntingtin protein (Morfini et al., 2009); a process analogous to the transport obstruction known to occur in glaucoma. It was unexpected, therefore, that lack of JNK3 not only did not protect RGC layer cells, and even slightly increased RGC layer loss compared to WT mice in our glaucoma model. However, axon loss was not significantly different between KO and WT animals, though at these low levels of axon loss damage was apparently more sensitively detected in the whole mounts compared to nerve cross-sections. The IOP was significantly elevated in both WT and KO animals to the same degree as in our prior series of over 150 mice (Cone et al., 2010). There was no significant difference between KO and WT animals in mean IOP. Both WT and KO animals underwent axial elongation (and widening) of the globe in response to chronic IOP elevation, again as seen in our prior experiments. Thus, the greater RGC loss between KO and WT was not likely due to unequal exposure to IOP and remained after adjustment for axial length increase of KO animals.

There is some precedent for detrimental effects of JNK3 KO on neuronal survival. In mice deficient for the retinoblastoma gene, extensive central neuron loss occurs during embryogenesis and this loss of neurons is actually accentuated in *Jnk3*^{-/-} animals (Kerameris et al., 2008). In that model, KO of either JNK2 alone or JNK2 and 3 together actually blocks the increased cell loss. In similar fashion, cultured cerebellar granule cells subjected to neurotrophin withdrawal continue to show c-Jun phosphorylation in *Jnk3*^{-/-} cells, and are only inhibited in this response when both JNK2 and 3 are knocked out (Bjorkblom et al., 2008). In a review of research on inhibition or KO of JNK, it was concluded: "constitutive knockout techniques are handicapped by compensation of the lacking protein by functionally and/or structurally related molecules." (Haeusgen et al., 2009) In experimental vascular brain occlusions, mitochondrial JNK expression changes

rapidly from JNK1 to JNK3, illustrating the possibility of changes in expression among JNK isoforms (Zhao and Herdegen, 2009). Likewise, in a model of 6-hydroxydopamine toxicity in substantia nigra, KO of either JNK2 or JNK3 alone had some protective effect, but only double KOs had complete protection from neuronal loss, illustrating a cross-compensation effect (Ries et al., 2008). These findings indicate that the effects of Jnk3 KO can be tissue-specific, compartment-specific, and even opposite in direction within the central nervous system.

It is possible that if the baseline glaucoma damage were greater, we might have a better opportunity to identify neuroprotective effects. Six weeks after bead injection, C57BL/6 animals that have been injected at 2 months of age show 10–20% loss of RGCs and about 5% their axons with the methods used here. We have recently increased the cell loss at 6 weeks by altering the composition of the beads to cause greater obstruction of outflow. We have also determined that RGC loss in experimental mouse glaucoma is strain specific and age-related (Cone et al., 2010). Relevant to the present experiment, C57BL/6 mice are significantly less susceptible to RGC loss than CD1 albino mice. At this time, most KO mice that are available commercially come on a C57BL/6 background, leading to lower RGC death rates in the setting of our transgenic animals.

Previous investigations have confirmed that the total RGC layer counts correspond well to the optic nerve grading of cell loss in mouse glaucoma (Jakobs et al., 2005). Many neurons in the RGC layer are amacrine cells and are included in our counts of DAPI-stained neurons. We chose not to backfill RGC with dye from the superior colliculus, since doing so at the beginning of a 6 week period leads to non-specific dye uptake by non-neurons, which must be manually identified to avoid inclusion as RGC (Blair et al., 2005). Backfilling later, during high IOP, leads to inconsistent filling and underestimation of remaining RGC (Salinas-Navarro et al., 2010). The specific RGC markers for normal RGC (such as Thy-1, Brn3, and Snrg) are rapidly down-regulated with injury, leading to possible underestimation of RGC numbers (Yang et al., 2007). The number of amacrine cells is unaffected by RGC loss in experimental rat glaucoma (Kielczewski et al., 2005). In addition, Jakobs et al. (2005) concluded that total neuron counts from the RGC layer by DAPI staining combined with specific labeling of other retinal neurons revealed no cell loss (including amacrine cells) other than RGC in the DBA/2J mouse.

This suggests that most dying RGC layer cells in mouse glaucoma models would be RGC. Thus, the estimated loss of RGC would be derivable from the proportions of RGC layer cells that are amacrine and RGC in the mouse, estimated to be 59% amacrine and 41% RGC (Jeon et al., 1998). To confirm that the proportion of RGC layer cells that are RGC in the mouse strains utilized, we carried out immunolabeling for Snrg in normal C57BL/6 retinas (protocol from (Soto et al., 2008)). Forty percent of RGC layer cells labeled by specific antibodies for Snrg. Counts in retinas from 12 month DBA/2J mice with prolonged, spontaneous glaucoma damage found only 2.3% and 0.7% of RGC layer cells labeled for Snrg RNA (data not shown), and the number of remaining neurons (negative for Snrg) was 59% of the number in normal whole mounts, as would be expected from the data of Jeon et al., 1998). Taken together, these data suggest that 40% of RGC layer cells in the WT mice studied are RGC. In our initial experimental bead glaucoma paper, we calculated the ratio of RGC layer cell loss to axon count loss in 75 mice with both retinal and optic nerve data for the same eye. The median ratio of RGC layer to axon loss was 0.34 (as would be expected if ~40% of RGC layer cells were RGC as estimated by Snrg labeling).

Taken together, our retinal and optic nerve axon data suggest that one major effect on the RGC in control KO animals is a relative increase in amacrine cells in the RGC layer, but no change in the number of RGC. As a result, to estimate the number of RGCs lost in KO

animals, we took account of the fact that the proportion of neurons that are RGC is not 40% as in WT but 36%. Thus, when we measure 7.2% RGC layer cell loss in KO animals, and taking this proportion into account, the estimated loss of RGC in KO mice is 19.8% (7.2/0.36), while in controls (with 40% RGC in the RGC layer) the estimated loss of RGC is 8.3% (3.3/0.40).

We found a modestly greater death of RGC layer cells in *Jnk3* KO mice exposed to experimental glaucoma compared to WT mice, at least in some zones of the retina. It is possible that the removal of this gene during development leads to compensatory upregulation of other pathways in *Jnk3*^{-/-} null animals, including JNK1 and 2. The potential alterations caused by JNK3 removal may render RGC more susceptible to glaucoma injury. Despite these findings, it is still quite possible that pharmacological inhibition of JNK3 or of all JNK isoforms would be effective in RGC protection in glaucoma. Conditional KO of the *Jnk* genes in the adult mouse is a reasonable approach to test these hypotheses further, as would be the study of kinase inhibitors that are specific to the various JNK isoforms.

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Figure 1.

Western blot of whole retina tissue from WT and JNK3 KO animals using specific antibody to JNK3 (top) and pan-JNK antibody (middle). KO retina has no detectable JNK3, but does have two bands corresponding to locations of the other two JNK isoforms. Alpha Tubulin (bottom) was included as loading control and shows similar band density in both.

Figure 2.

Mean (SE) IOP for experimental glaucoma eyes after bead/viscoelastic injection (red for KO, blue for WT) and fellow control eyes (purple for KO and green for WT). The number of observations at each time point was: 124 immediately after injection, 69 at 3 days post injection, and 121 at both 1 week and 6 weeks post injection. Significant elevation is seen at all measured time points, but differences between WT and KO were not significant at any time point.

Figure 3.

Representative confocal image of mid-peripheral superior retina stained with an antibody against human synuclein (blue) and DAPI (yellow) for nuclei at 40x magnification. Note the DAPI and synuclein labeled RGC (white arrow) for comparison to DAPI only labeled presumed amacrine nuclei (pink arrow). The figure shows how easily the oval, elongated endothelial cell nuclei are to differentiate from neuronal nuclei (white arrow head, bar = 20 microns). Data for this study were collected by enumerating all neuronal nuclei in the RGC layer.

Figure 4.

Representative image of bead glaucoma induced axon loss in a wild type eye exposed to experimental glaucoma. Dying axons (black arrow heads) are scattered among normal appearing ones (white arrow) and the space between bundles has increased. This mouse had an overall estimated 13% loss compared to pooled controls (bar = 10 micron).

Table 1

Comparison of RGC layer cells in WT compared to KO mice

Field counts		Quadrant counts								
		Field 1	Field 2	Field 3	Field 4	Field 5	Inferior	Nasal	Superior	Temporal
WT	Mean	1381.1	1426.6	1387.3	1260.6	1096.1	1703.4	1753.7	1559.8	1579.5
	St Dev	95.4	95.5	100.0	113.9	74.4	91.5	135.0	133.2	124.6
	N =	35	36	33	35	17	31	27	33	28
KO	Mean	1497.6	1562.2	1517.2	1369.6	1171.7	1847.2	1844.2	1715.9	1738.0
	StDev	108.2	129.8	134.8	128.6	138.7	162.5	162.7	192.8	150.4
	N =	39	39	37	38	27	39	36	42	32
p value KO vs WT		<0.0001	<0.0001	<0.0001	0.0003	0.045	<0.0001	0.022	0.0002	<0.0001

Table 2

Comparison of RGC layer cells in KO males and females

KO	Field counts			Quadrant counts						
	Field 1	Field 2	Field 3	Field 4	Field 5	Inferior	Nasal	Superior	Temporal	
Male	Mean	1508.8	1574.0	1541.5	1393.4	1168.9	1870.3	1837.7	1717.0	1745.0
	St Dev	107.3	125.1	136.6	113.9	151.8	153.7	154.8	181.3	152.0
	N =	24	24	23	23	18	23	23	27	22
Female	Mean	1479.7	1543.3	1477.4	1332.9	1177.2	1814.1	1855.8	1713.9	1722.8
	St Dev	110.8	139.4	126.5	144.8	116.3	173.9	181.8	218.7	153.7
	N =	15	15	14	15	9	16	13	15	10
p value male vs female		0.42	0.48	0.16	0.16	0.89	0.29	0.75	0.96	0.71

Table 3

Comparison of RGC layer cell loss in KO and WT mice

Loss by Field												Loss by Quadrant				
		Field 1	Field 2	Field 3	Field 4	Field 5	Mean	Inferior	Nasal	Superior	Temporal	Mean				
WT	Mean	2.60%	1.19%	0.97%	3.42%	8.45%	3.3%	3.20%	8.41%	3.26%	0.74%	3.9%				
	St Dev	7.66%	7.37%	7.65%	8.23%	10.40%		8.43%	7.80%	12.84%	8.05%					
	N =	34	35	35	30	21		28	36	35	37					
KO	Mean	5.45%	8.58%	7.57%	7.00%	6.37%	7.0%	5.68%	11.42%	9.16%	2.92%	7.3%				
	St Dev	7.98%	7.64%	7.76%	6.62%	9.71%		8.86%	10.27%	8.90%	6.84%					
	N =	32	36	35	32	24		34	27	35	26					
p value WT vs KO		0.14	<0.0001	0.0006	0.06	0.49		0.27	0.19	0.03	0.27					