Involvement of c-Jun N-terminal kinase 2 (JNK2) in Endothelin-1 (ET-1) Mediated Neurodegeneration of Retinal Ganglion Cells

Bindu Kodati,[1,2] Dorota L. Stankowska,[1,2] Vignesh R. Krishnamoorthy,[3] and Raghu R. Krishnamoorthy[1,2]

[1]Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, Texas, United States
[2]North Texas Eye Research Institute, University of North Texas Health Science Center, Fort Worth, Texas, United States
[3]Department of Cellular and Molecular Physiology, Loyola University, Maywood, Illinois, United States

Correspondence: Raghu R. Krishnamoorthy, Department of Pharmacology and Neuroscience, North Texas Eye Research Institute, University of North Texas Health Science Center, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107, USA;
raghu.krishnamoorthy@unthsc.edu.

Received: November 3, 2020
Accepted: April 21, 2021
Published: May 12, 2021

Citation: Kodati B, Stankowska DL, Krishnamoorthy VR, Krishnamoorthy RR. Involvement of c-Jun N-terminal kinase 2 (JNK2) in endothelin-1 (ET-1) mediated neurodegeneration of retinal ganglion cells. *Invest Ophthalmol Vis Sci.* 2021;62(6):13.
https://doi.org/10.1167/iovs.62.6.13

Purpose. The goal of this study was to determine whether JNK2 played a causative role in endothelin-mediated loss of RGCs in mice.

Methods. JNK2$^{-/-}$ and wild type (C57BL/6) mice were intravitreally injected in one eye with 1 nmole of ET-1, whereas the contralateral eye was injected with the vehicle. At two time points (two hours and 24 hours) after the intravitreal injections, mice were euthanized, and phosphorylated c-Jun was assessed in retinal sections. In a separate set of experiments, JNK2$^{-/-}$ and wild type mice were intravitreally injected with either 1 nmole of ET-1 or its vehicle and euthanized seven days after injection. Retinal flat mounts were stained with antibodies to the RGC marker, Brn3a, and surviving RGCs were quantified. Axonal degeneration was assessed in paraphenylenediamine stained optic nerve sections.

Results. Intravitreal ET-1 administration produced a significant increase in immunostaining for phospho c-Jun in wild type mice, which was appreciably lower in the JNK2 $^{-/-}$ mice. A significant ($P < 0.05$) 26% loss of RGCs was found in wild type mice, seven days after injection with ET-1. JNK2$^{-/-}$ mice showed a significant protection from RGC loss following ET-1 administration, compared to wild type mice injected with ET-1. A significant decrease in axonal counts and an increase in the collapsed axons was found in ET-1 injected wild type mice eyes.

Conclusions. JNK2 appears to play a major role in ET-1 mediated loss of RGCs in mice. Neuroprotective effects in JNK2$^{-/-}$ mice following ET-1 administration occur mainly in the soma and not in the axons of RGCs.

Keywords: neurodegeneration, neuroprotection, glaucoma posterior segment

The vasoactive endothelin peptide, endothelin-1 (ET-1), has been shown to be elevated in the aqueous humor and circulation in animal models of glaucoma, as well as in glaucoma patients.[1–4] Several studies have shown the ability of ET-1 administration (intravitreal and retrobulbar) to produce optic nerve degeneration and apoptosis of retinal ganglion cells.[4–7] However, the detailed cellular and signaling mechanisms contributing to ET-1–mediated neurodegeneration in glaucoma are not completely understood. ET-1 act through two classes of G protein-coupled receptors called ET_A and ET_B receptors. The regulation of transcription of the preproET-1 gene involves activator protein-1 (complexes of combinations of c-Jun and c-Fos), nuclear factor-1 and GATA2.[8–10] Previously, we demonstrated that c-Jun and its phosphorylation was enhanced in primary RGCs treated with ET-1.[11] Moreover, ET-1 treatment also produced an increase in ET_A and ET_B receptor expression in primary RGCs,[11] possibly through elevation of c-Jun expression. For instance, c-Jun overexpression was found to produce an increase in expression of ET_A and ET_B receptors in human nonpigmented ciliary epithelial cells.[12] This suggests that various noxious stimuli including, TNF-α,[13] and hypoxia,[14] that activate c-Jun could trigger the expression of both ET-1 and its receptors. C-Jun is activated by phosphorylation by the stress activated protein kinase, c-Jun N-terminal kinase (JNK), which is activated in response to numerous stimuli relevant to glaucoma pathogenesis.

The stress induced MAPK, JNK, is involved in the various cellular activities from proliferation to apoptosis which is mainly dependent on the response of the specific cell type, external stimuli and its activation by specific upstream kinases.[15] There are three isoforms of JNK, JNK1, JNK2, which are ubiquitously expressed in most cells, and JNK3, which is expressed mainly in brain, heart, and testis.[16–18] JNK regulates the activity by phosphorylation of various downstream factors including the c-Jun (a component of transcription factor activator protein-1), ATF2 and ATF3. In addition, JNK regulates the activity/expression of other

transcription factors including, Elk1, p53, HSF-1, and c-Myc and the members of Bcl2 family, involved in both extrinsic and intrinsic mechanisms of apoptosis.[19–22] Studies on cerebellar granule neurons have shown that the JNK-mediated phosphorylation in the nucleus is more critical for the apoptosis than the JNK activity in the cytosol.[23] Studies have demonstrated apoptotic effects of JNK isoforms and their reversal by blocking JNK with various agents.[19,24–28] Our previous studies also showed increased phosphorylation of c-Jun (possibly through the involvement of JNK) in ET-1–mediated cell death in primary RGCs,[11] but the exact JNK isoforms contributing to phosphorylation of c-Jun and ET-1–mediated cell death have not been determined. The present study aimed to elucidate the role of JNK2 in ET-1 mediated cell death of RGCs in mice.

Methods

Animals

All animal procedures were carried out in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in ophthalmic and vision research and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee (animal protocol no. IACUC-2017-0024). Male and female, wild type (C57BL/6) and JNK2$^{-/-}$ mice (stock no. 004321) were purchased from Jackson Labs and were matched for age and gender. The animals were housed in rooms where the temperature, humidity and light were controlled. Food and water were provided as desired.

Intravitreal Injections of ET-1 or Vehicle

ET-1 obtained from Bachem (Torrance, CA, USA) was dissolved in the vehicle solution (0.25% glacial acetic acid neutralized to pH 7.0 with NaOH) and adjusted to a final concentration of 500 µM. Mice were anesthetized by injecting intraperitoneally using an anesthetic cocktail having xylazine (5.5 mg/kg)/ ketamine (55 mg/kg)/ acepromazine (1.1 mg/kg). One eye of the mice was injected with ET-1 and contralateral eye was injected with the vehicle using a Hamilton syringe. A single drop of 0.5% proparacaine hydrochloride (Alcon Laboratories, Inc., Fort Worth, TX, USA) and 1% tropicamide was applied to both eyes. An ultrafine 33-gauge disposable needle connected to a 10 microliters Hamilton syringe was be used to intravitreally inject 2 µL of either 500 µM ET-1 or vehicle into the vitreous chamber by injecting through the sclera, 1 mm behind the limbus region. The injection was carried out slowly, making sure to avoid the lens during insertion of the needle. After the injection, the needle was held in place for 1 minute and gradually withdrawn from the vitreal chamber. After the injection, triple antibiotic (bacitracin zinc, neomycin sulfate and polymyxin B sulfate) was applied at the injection site, and the animals were allowed to recover from the anesthesia. After various time points following the intravitreal injection, including two hours, 24 hours, and seven days, mice were humanely euthanized by an overdose of 120 mg/kg body weight of Fatal-Plus (pentobarbital) (Covetrus, Dublin, OH, USA) administered intraperitoneally.

Immunohistochemistry

Mice were euthanized after the two-hour (n = 4 mice/eyes per treatment group) and 24-hour (n = 4 mice/eyes per treatment group) time points, following which their eyes were enucleated and immediately fixed in 4% paraformaldehyde and placed on a shaker at room temperature for three hours. After the incubation the fixed retinas were washed with PBS and immersed overnight in 70% ethanol and thereafter embedded in paraffin and sectioned. The retinal sections obtained were deparaffinized using xylene, and rehydrated with a graded descending series of ethanol concentrations (100%, 95%, 90%, 80%, and 50% ethanol) and finally with PBS. Permeabilization with 0.1% sodium citrate and 0.1% Triton X-100 was carried out for eight minutes to facilitate the subsequent entry of antibodies. To prevent nonspecific binding of the secondary antibody, the sections were incubated with blocking buffer (5% normal donkey serum and 5% BSA in PBS) for approximately two to three hours. After blocking, the sections were incubated with the respective primary antibodies overnight at 4°C. The primary antibodies used for the experiment were mouse anti phospho-c-Jun (1:50, SC-822; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and goat anti-Brn3a (1:200, SC-31984, Santa Cruz Biotechnology, Inc.). After primary antibody incubation, the sections were washed three times with PBS and incubated with appropriate secondary antibodies for one to two hours at room temperature. Secondary antibodies used in the experiment are donkey anti-mouse Alexa 546 (1:1000 dilution, A10036; Life Technologies, Carlsbad, CA, USA), donkey anti-goat Alexa 488 (1:1000 dilution, A11055; Invitrogen, Carlsbad, CA, USA). To assess the nonspecific binding of secondary antibodies, "blank" sections (in which the primary antibody incubation was omitted) were used after incubation only with secondary antibody.

Retinal Flat Mount Immunostaining

Seven days after the intravitreal injections, mice (n = 6 mice/eyes per treatment group) were euthanized, their eyes were enucleated and immediately fixed overnight in 4% paraformaldehyde at 4°C. After three washes with 1x PBS, retinas were carefully separated from the globe and incubated overnight in blocking buffer (5% normal donkey serum and 5% BSA in PBS) at 4°C, cuts were made in the four quadrants (superior, inferior, nasal and temporal) and retinal flat mounts were prepared. The retinal flat mounts were then incubated with the primary antibody, goat anti-Brn3a (1:200, SC-31984, Santa Cruz Biotechnology, Inc.) for three days at 4°C. After three washes with PBS, the flat mounts were incubated overnight in the corresponding secondary antibodies: Alexa 488 conjugated donkey antigoat antibody (1:1000 dilution, A11055, Invitrogen) at 4°C. After washes, the flat mounts were mounted using Prolong Gold anti-fade (Life Technologies) and images were taken using the Keyence fluorescence microscope (Keyence, Osaka, Japan).

Quantification of Retinal Ganglion Cell Survival

Images of flat mounts were captured using magnification × 20 in a Keyence fluorescence microscope. Images were taken at two different eccentricities, located at one third (mid-peripheral) and two thirds (peripheral) of the distance between the optic nerve head and the periphery of the retina. Two images were captured at each eccentricity, in each of the four quadrants, including the superior, inferior, nasal, and temporal quadrants, for a total of 16 images per retina. RGC counts were determined by a semiautomatic cell counting procedure on ImageJ (Rasband, 1997–2018).

Investigative Ophthalmology & Visual Science

Briefly, all the images were first converted to an eight-bit images and run through FTT band pass filter. The images were then run through auto-threshold and converted to binary images. After removing outliers, binary functions like Fill holes and Watershed were applied. Cells were then counted by applying appropriate particle parameters, that is, size (0.01–infinity) and circularity (0.0–1). The counting was performed by a masked observer who was unaware of the genotypes and treatment groups of the animals.

PPD Staining of Optic Nerve for the Assessment of Axonal Damage

Degeneration of axons were examined using PPD staining which stains the myelin around the axons. Briefly, wild type (n = 8 mice/eyes per treatment group) and JNK2$^{-/-}$ (n = 6 mice/eyes per treatment group) mice were injected with vehicle and ET-1 and maintained for a week. The mice were then euthanized, and their eyes were enucleated, after which the optic nerves were excised posterior to the globe. The optic nerves were then immediately fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Before dehydration the optic nerves were transferred to 2% osmium tetroxide in PBS for one hour and embedded in Epon. Optic nerve cross sections were obtained using the ultramicrotome and stained with 1% PPD, and images of the stained sections were taken in a Zeiss LSM 510 META confocal microscope using an oil immersion magnification × 100. Images were taken at a few points in the center, as well as the peripheral region of each quadrant of every optic nerve section. The analysis was done in three different ways: (1) The images were graded in a masked manner by two individuals with a score ranging from 0-9 using the method described by Chauhan et al.[29] (2) Image J software was used for the axon counts. Briefly, after adjusting the brightness/contrast, the sharpest optic nerve section image was selected by using the stack option in the software and analyzed based on the particle size parameters like size, and circularity. The total axonal counts are the average of the axonal counts from the central and peripheral regions of each optic nerve. (3) The collapsed axons were manually counted on the basis of the PPD staining of the degenerative axons. Based on the statistical analysis the neuroprotective/neurodegenerative effects were analyzed further.

Statistical Analysis

The statistical analysis was performed using the GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). To compare the data between multiple groups, a two-way ANOVA followed by Tukey's multiple comparison test was used, and for comparison between two groups, an unpaired *t*-test was used. Values of $P < 0.05$ were considered to be statistically significant.

Results

Significant Upregulation of Phospho c-Jun in C57BL/6 Wild Type Compared to JNK2$^{-/-}$ Mice

ET-1 treatment has been shown to elevate JNK and its substrate: immediate early gene c-Jun in both primary RGCs and the human nonpigmented ciliary epithelial cells.[11,30] He et al.[11] also demonstrated a dramatic increase in immunostaining for phospho-c-Jun following treatment of primary RGCs with 100 nM ET-1 for 24 hours. In the present study, the involvement of JNK2 and its effects on ET-1 mediated RGC cell death was assessed in vivo. To assess the involvement of JNK2 signaling in the retina following intravitreal ET-1 administration (at two time points: 2h and 24h), immunohistochemical analysis of phospho c-Jun was performed in the retinal sections. The integrated densities from confocal z-stack images were analyzed separately for the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), and inner nuclear layer (INL) to determine the intensity of immunostaining of phospho-c-Jun and compared between the ET-1 and vehicle treated eyes.

At the two-hour time point after ET-1 injection, the wild type C57BL6/J mice showed a significant increase in immunostaining for phospho-c-Jun (Fig. 1A) mainly in ganglion cell layer ($P = 0.0098$, n = 4 mice , Fig. 1C), and inner plexiform layer ($P = 0.0278$, n = 4 mice, Fig. 1D) compared to the vehicle-treated eyes. There was a modest increase (not statistically significant) in immunostaining for phospho-c-Jun in the NFL (Fig. 1B) in the ET-1 treated eyes, compared to vehicle treated eyes in wild type mice. In the JNK2$^{-/-}$ mice in all the retinal layers, there was no appreciable increase in the phospho-c-Jun in the ET-1 treated eyes, compared to the vehicle treated eyes. There was a significant increase in phospho-c-Jun staining in ET-1 injected wild type eyes compared to that of JNK2$^{-/-}$ mice (n = 4 wild type mice and n = 4 JNK2$^{-/-}$ mice) eyes in the IPL ($P = 0.0006$, Fig. 1D) and INL ($P = 0.026$, Fig. 1E).

At the 24 h time point, wild type mice showed a prominent and significant increase in immunostaining for phospho-c-Jun in the GCL ($p = 0.03$, n = 4 mice, Figs. 2A, 2C) in the ET-1 injected eyes compared to vehicle treated eyes. There was an appreciable increase (not statistically significant) in immunostaining for phospho-c-Jun in the NFL (Fig. 2B), IPL (Fig. 2D), and INL (Fig. 2E) in the ET-1 injected eyes, compared to the vehicle-injected eyes in wild type mice. In the JNK2$^{-/-}$ mice, there was no significant change in the phospho-c-Jun in all the retinal layers in the ET-1 treated eyes, compared to the vehicle treated eyes. On comparison of the ET-1 treated eyes between the wild type and the JNK2$^{-/-}$ mice, we found a significant increase in immunostaining for phospho-c-Jun in the NFL (0.0417, n = 4 mice, Fig. 2B), GCL ($P = 0.0009$, n = 4 mice, Fig. 2C), IPL ($P = 0.029$, n = 4 mice, Fig. 2D) and INL ($P = 0.0029$, n = 4 mice, Fig. 2E) in the wild type mice, compared to the JNK2$^{-/-}$ mice (Fig. 2A).

Protection of RGCs From Cell Loss in JNK2$^{-/-}$ Mice in Comparison With the Wild Type Mice

To determine the involvement of JNK2 signaling pathway in ET-1 mediated loss of RGCs, we intravitreally administered ET-1 in one eye and the vehicle in contralateral eye in both wild type and JNK2$^{-/-}$ mice and maintained for seven days. Retinal flat mounts were prepared and the survival of RGC were assessed by counting the viable RGC immunolabelled with the Brn3a antibody. The RGC counts between the ET-1 and vehicle administered eyes for each retinal eccentricity was compared between the wild type and JNK2$^{-/-}$ mice (Fig. 3A). In wild type mice, in the peripheral eccentricity, there was a significant decrease in RGC counts in the ET-1 injected eyes, compared to vehicle injected eyes ($P = 0.049$, Fig. 3B). However, although there was a decreasing trend, there was no statistical significance in RGC counts

Investigative Ophthalmology & Visual Science

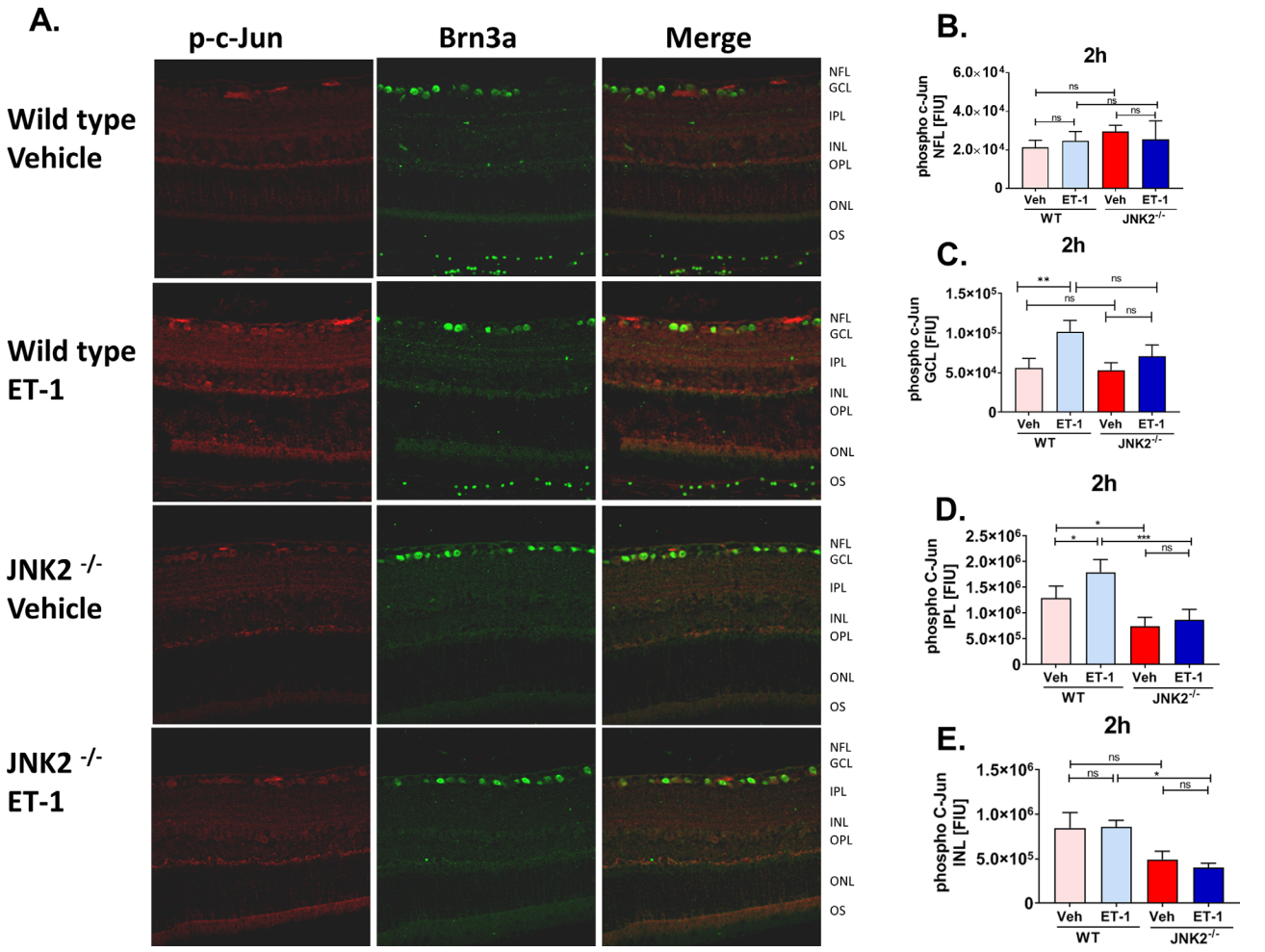

Figure 1. Phospho c-Jun levels in retinas of wild type and JNK2$^{-/-}$ mice, two hours after intravitreal administration of ET-1. Representative images from confocal microscopy using an objective lens ×40. Immunostaining of retina sections from wild type and JNK2$^{-/-}$ mice two hours after intravitreal ET-1 administration with 2 µL of 500 µM ET-1 using antibodies against phospho c-Jun (*red*) and Brn3a (*green*) (**A**). Relative fluorescent intensity quantification for NFL (**B**), GCL (**C**), IPL (**D**), and INL (**E**) layers, respectively. *Bars* represent mean ± SEM (n = 4 wild type mice/retinas and n = 4 JNK2$^{-/-}$ mice/retinas). ONL outer nuclear layer, OPL outer plexiform layer. *Asterisks* indicate statistical significance *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (two-way ANOVA and Tukey's multiple comparison tests).

in the midperipheral region between the vehicle-injected and ET-1 injected eyes in wild type mice (Fig. 3C). We also found no significant RGC loss in both eccentricities in ET-1 injected eyes, compared to vehicle injected eyes in JNK2$^{-/-}$ eyes. We observed a statistically significant difference in total RGC counts (peripheral and mid-peripheral eccentricities) between the wild type mice and JNK2$^{-/-}$ mice intravitreally injected with ET-1 ($P = 0.0104$, Fig. 3D). Taken together, the results demonstrate that the JNK2 isoform plays a critical role in signaling pathways contributing to RGC cell death after ET-1 treatment.

Axonal Degeneration

One week after injection with either ET-1 or vehicle, wild type and the JNK2$^{-/-}$ mice were sacrificed and enucleated. The integrity of the optic nerve axons was assessed by PPD staining, which darkly stains the myelin of damaged axons (Fig. 4A). In wild type mice, the optic nerve of ET-1 injected eye showed intense staining of myelin, disruption of axonal bundles, and glial scar formation. Wild type C57BL6/J mice showed significant disruption of the axonal bundles, based on the axon grading performed in a masked manner by two individuals ($P = 0.0056$, n = 8 wild type mice) (Fig. 4B), and counts obtained using image J in the ET-1 injected eye ($P = 0.029$, n = 8 wild type mice) (Fig. 4C), compared the vehicle treated eye respectively. When ET-1 injected eyes were compared between the wild type and JNK2$^{-/-}$ mice, we did not observe any significant difference between them in the axon counts, as well as their grades. Further, we manually counted the degenerated axons which stained darkly in the axoplasm of PPD stained optic nerve cross-sections. Following ET-1 injection, analysis of optic nerve sections from wild type and the JNK2$^{-/-}$ mice did not show any significant difference in the number of degenerated axons. We observed a significant decline in axon counts ($P = 0.029$, n = 8 wild type mice) and increase in collapsed axons ($P = 0.0018$, n = 8 wild type mice) in the ET-1 injected eyes, compared to the vehicle-injected eyes in wild type mice (Figs. 4C and 4D). However, there was no significant difference in the number of axon counts (Fig. 4C), as well as collapsed axons (Fig. 4D), between wild type (n = 8) and JNK2$^{-/-}$ mice (n = 6) after

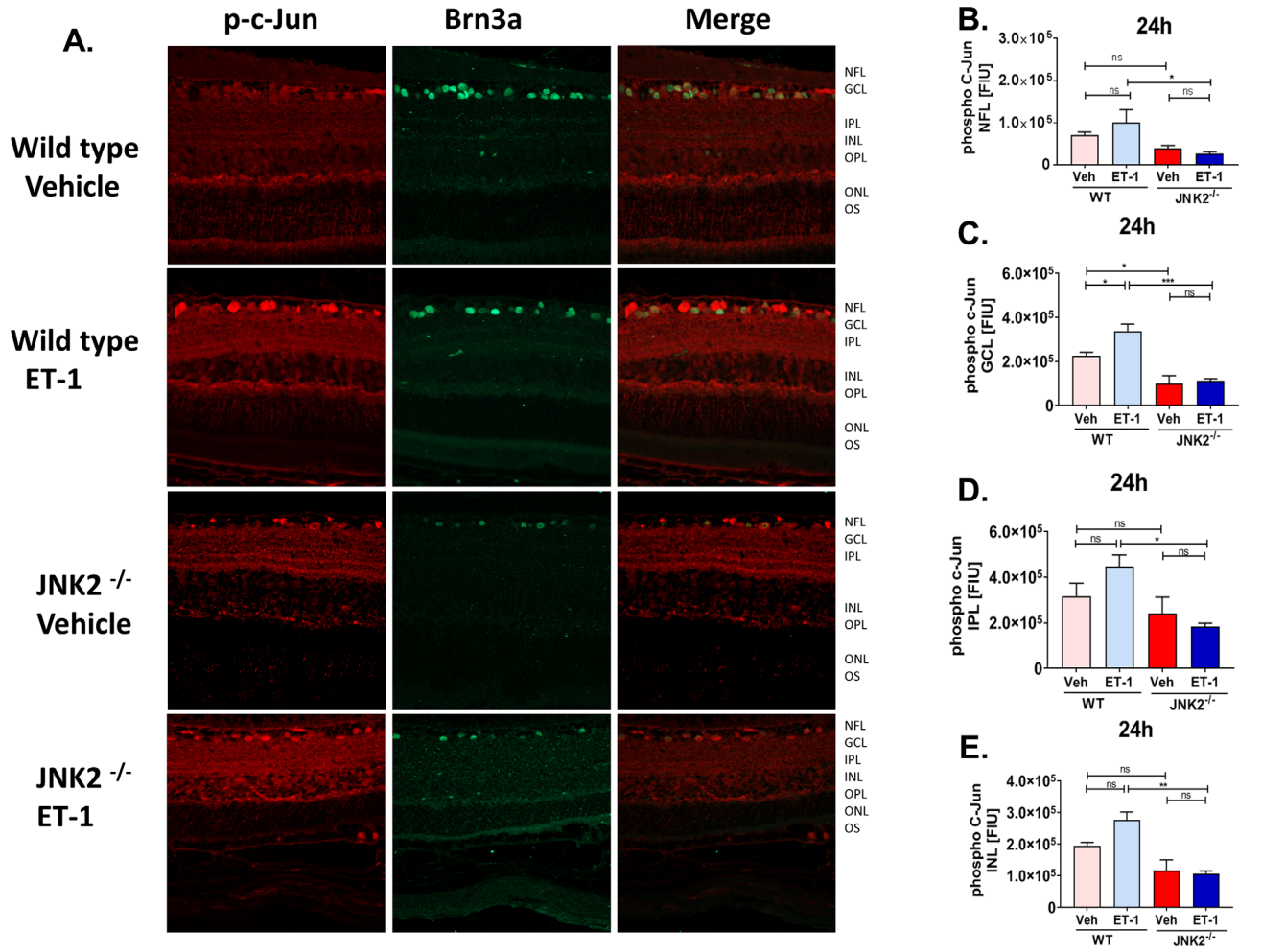

Figure 2. Phospho c-Jun expression in retinas of wild type and JNK2$^{-/-}$ mice, 24 hours after intravitreal administration of ET-1. Representative images from confocal microscopy using an objective lens ×40. Immunostaining of retina sections from mice after 24 hours of intravitreal injection with 2 µL of 500 µM ET-1, using antibodies to phospho c-Jun (*red*) and Brn3a (*green*) (**A**). Relative fluorescent intensity quantification for NFL (**B**), GCL (**C**), IPL (**D**), and INL (**E**), respectively. *Bars* represent mean ± SEM (n = 4 wild type mice/retinas and n = 4 JNK2$^{-/-}$ mice/retinas). ONL, outer nuclear layer; OPL, outer plexiform layer. *Asterisks* indicate statistical significance *P < 0.05; **P < 0.01; ***P < 0.001 (two-way ANOVA and Tukey's multiple comparison tests).

ET-1 injection, suggesting that blocking JNK2 signaling does not protect axons from ET-1-mediated degeneration.

Discussion

The currently available treatments for glaucoma, including, pharmacological and surgical approaches of lowering IOP greatly slow down the progression of the disease.[31] However, in some patients, there is continued neurodegenerative effects which produce visual deficits.[32,33] Besides the primary insult of elevated IOP, additional factors including, ischemia,[34] excitotoxicity,[35] TNF-α[36] or a combination of these are thought to be contributors to glaucomatous neurodegeneration. One of the endogenous vasoconstrictors that produces ischemic insult in the ONH, with a potential role in glaucoma and other neurodegenerative diseases is the vasoactive peptide endothelin-1 (ET-1).[37–39] In vivo studies in rabbits and rhesus monkeys showed that administration of low doses of ET-1 generated ischemic effects leading to the optic nerve damage as seen in glaucoma.[40,41] Previous studies have reported the upregulation of ET_B receptors occurs after IOP elevation in rats.[42] ET-1 may activate optic nerve astrocytes, leading to ECM changes in the lamina cribrosa and ONH, which in turn could cause disruption in axoplasmic transport and eventual RGC loss via loss of trophic support and reactive gliosis.[38,43–45] However, the signaling events contributing to ET-1 mediated neurodegeneration are not completely understood.

JNKs are stress activated protein kinases that were originally identified in cells subjected to various cellular stresses including DNA damage, inflammatory cytokines (e.g. interleukin-1), TNF-α, heat shock, and hypoxia.[46] The isoforms of JNK respond to these various external stimuli in different ways, producing changes in gene expression resulting in different phenotypic changes depending on specific cell types. For instance, Dvoriantchikova and Ivanov[47] showed that treatment of RGCs with TNF-α produced sustained activation of JNK leading to RGC death, while astrocytes treated with TNF-α showed transient activation of JNK detected by its phosphorylation. Various studies have shown the importance of the JNK pathway mediated neurodegenerative effects in animal models of glaucoma

Investigative Ophthalmology & Visual Science

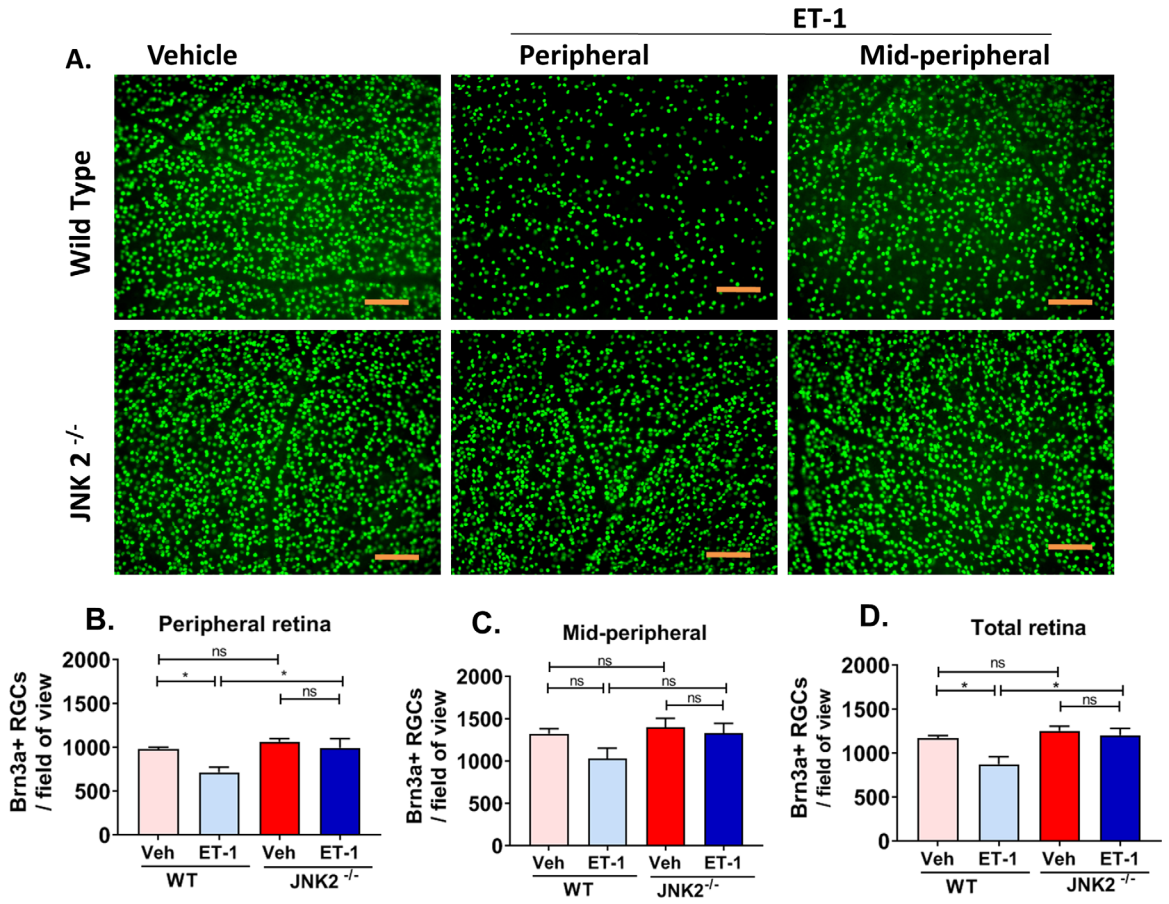

Figure 3. Survival of RGC in JNK2$^{-/-}$ mice compared to C57BL/6 mice after intravitreal administration of ET-1: Mice were intravitreally injected either with vehicle or ET-1 and maintained for seven days. The mice were euthanized, and RGC loss was analyzed by the counts obtained from the retinal flat-mounts in two eccentricities (peripheral and mid-peripheral). Representative images of the retinal flat mounts obtained from vehicle and ET-1 injected mice eyes, stained with Brn3a marker are shown for the wild-type and JNK2$^{-/-}$ mice for the peripheral and mid-peripheral eccentricities (**A**). Graphs are shown for peripheral (**B**) mid-peripheral (**C**), and total counts (average counts of peripheral and mid-peripheral) (*$P = 0.0211$, *$P = 0.0104$) (**D**). Significant loss of RGCs (26% loss) is observed in wild type mice. Data are shown as mean ± SD (n = 6 wild type mice/retinas and n = 6 JNK2$^{-/-}$ mice/retinas, (two-way ANOVA and Tukey's multiple comparison tests). *Scale bar*: 100 µm.

involving ischemic injury, and optic nerve crush.[27,48–50] In the laser photocoagulation method of IOP elevation, RGC loss was accompanied by JNK activation, suggesting the role of JNK in RGC apoptosis.[51,52] JNK acts by phosphorylating several key proteins including c-Jun, c-Fos, Elk-1, ATF-2, and p53, which could form homodimers and heterodimers and act as transcription factors to influence gene expression and cell fate. An increase in phospho-c-Jun was found as early as two days after IOP elevation, as well as after optic nerve transection in Wistar rats.[52] In an optic nerve crush model, the combined deletion of JNK2 and JNK3 and the conditional deletion of JUN inhibited the loss of RGC and showed long-term protection[27] and the upstream blockade of the JNK signaling also enhanced the survival of RGCs.[48] In an ischemia/reperfusion model, administration of the JNK inhibitor SP600125 demonstrated a protective role in RGCs, as well as in non-RGC cells in various inner retinal layers.[49]

In our present study we focused on the involvement of JNK2 in ET-1 mediated RGC loss in mice. We used intravitreal administration of 1 nmole of ET-1 which was similar to the dose that produced a significant decline in axonal transport of mitochondrial sub-components following intravitreal administration in prior experiments in rats.[53] To assess the increased phosphorylation of c-Jun we used the antibodies which can recognize the phosphorylation at Ser 63/73 residue, the specific site targeted by the kinase activity of JNK.[54] Though the phosphorylation of c-Jun could occur through the actions of any of the three JNK isoforms, JNK2$^{-/-}$ mice did not show significant increase in phospho-c-Jun in the RGC layer, when compared to the wild type mice in both the 2 h and 24 h time points. Interestingly, we observed significant elevation of phospho c-Jun in GCL for the 2 h and 24 h time points. Statistical comparison of the ET-1 treated eyes between the JNK2$^{-/-}$ mice and wild type revealed a significant elevation of phospho-c-Jun in the wild type eyes in both RGC and non RGC layers (Fig. 1 and Fig. 2). Interestingly, JNK2$^{-/-}$ mice displayed significant phosphorylation of c-Jun, compared to the wild type mice regardless of treatment (Fig. 1 and Fig. 2). It is possible that there is a compensatory upregulation of JNK1 and /or JNK3 in the JNK2 knockout mice. Despite this, JNK2 knockout mice displayed a neuroprotective effect, indicating a key role of JNK2 in ET-1 mediated neurodegeneration.

To further determine whether the increase of phospho c-Jun could lead to the RGC death we assessed RGC survival by counting Brn3a-positive cells in retinal flat mounts. We

Investigative Ophthalmology & Visual Science

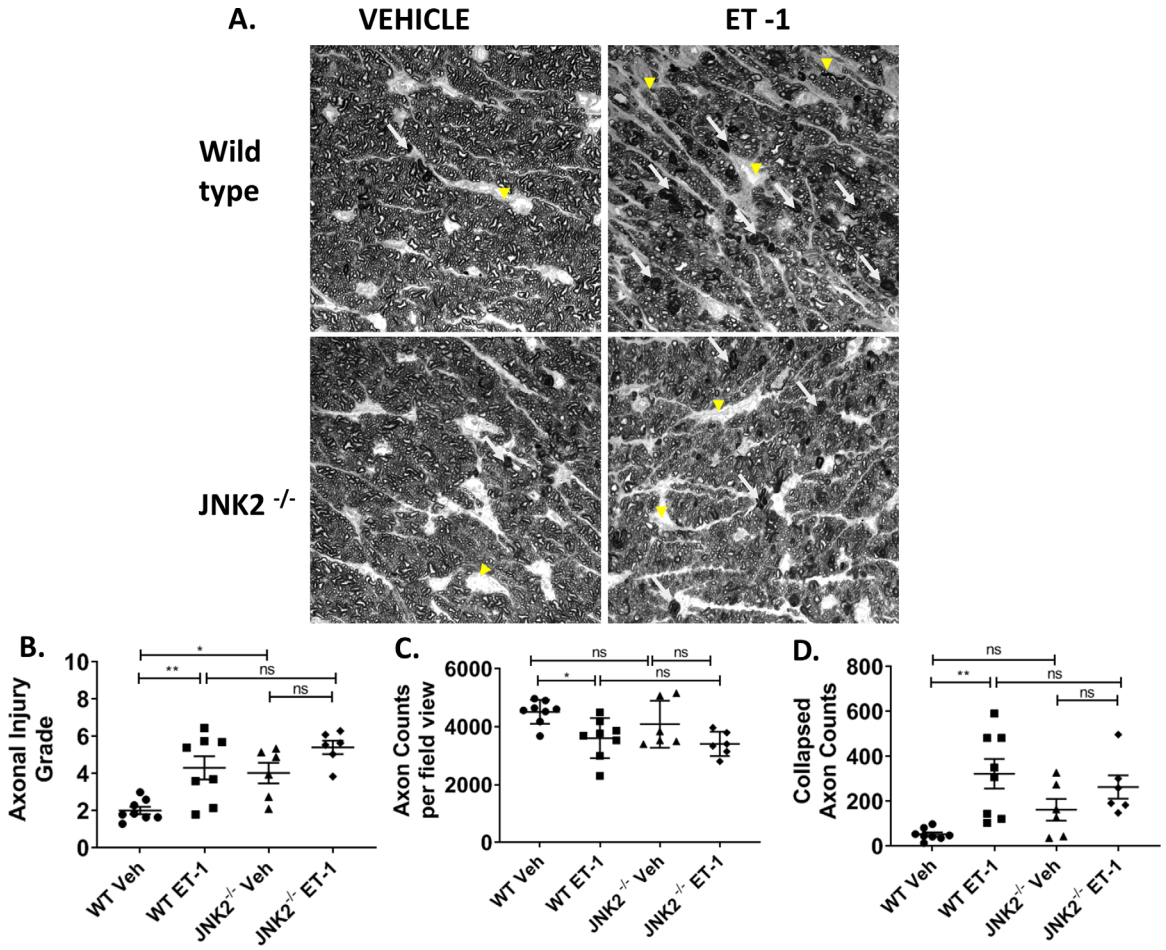

Figure 4. Optic nerve axonal degeneration in wild type and JNK2$^{-/-}$ mice. Both wild type and JNK2$^{-/-}$ mice were intravitreally injected in one eye with vehicle and contralateral eye with ET-1 and maintained for a week. Seven days after the intravitreal injections, animals were euthanized, and optic nerve sections obtained were subjected to PPD staining to assess the optic nerve degeneration. Severe axonal degeneration accompanied by gliosis and glial scar were observed in ET-1 injected eyes compared to vehicle injected eye in both wild type and JNK2$^{-/-}$ mice. Dark spots indicate the degenerating axons (**A**). Axon grading for wild type and JNK2$^{-/-}$ mice in a scale of 0 to 9 (healthy to degenerating axons) showed a significant difference between ET-1 and vehicle injected eyes in wild type mice (**$P = 0.0056$, *$P = 0.0289$, n = 8 wild type mice/optic nerves and n = 6 JNK2$^{-/-}$ mice/optic nerves) (**B**). The mean axon counts in ET-1 injected wild type mice showed a significant reduction in number of healthy axons compared to vehicle injected eyes (*$P = 0.029$, n = 8 wild type) (**C**). There was a significant increase in counts of collapsed axons in the ET-1 injected eyes, compared to the vehicle injected eyes in wild type mice (**$P = 0.0018$, n = 8 wild type mice) (**D**). Mean ± SD (n = 8 in Wild type and n = 6 in JNK2$^{-/-}$ mice (one-way ANOVA followed by Tukey's multiple comparisons test). *Arrows* indicate collapsed axons and arrow head indicates the glial scarring. *Scale bar*: 20 µm.

observed a significant, 26% loss of RGCs in the retina in wild type mice, 7 days post injection with ET-1, compared to that in vehicle injected wild type mice. JNK2$^{-/-}$ mice showed no significant loss of RGCs after ET-1 administration, compared to JNK2$^{-/-}$ mice treated with the vehicle (Fig. 3). The peripheral retina was found more susceptible to the apoptotic effects of ET-1, similar to that seen in glaucoma. The precise mechanisms underlying these effects are not clear. It is possible that JNK2 plays an important role in signal transduction mechanisms following ET-1 mediated neurodegeneration, since phosphorylation of c-Jun is significantly attenuated in JNK2 knockout mice. A recent publication by Marola et al.[55] demonstrated increased immunolabeling for phospho-c-Jun in RBPMS-positive RGCs following intravitreal ET-1 administration in C57BL/6J mice. The authors found that 60% of the Jun-positive cells were immunoreactive for caspase-3 indicative of apoptotic changes and also observed that ET-1–mediated RGC loss was significantly decreased in c-Jun conditional knockout mice.[55]

In glaucoma and many neurodegenerative diseases, the axonal degeneration often leads to the apoptotic cell death of the soma. Studies in a genetic mouse model by over expression of Bcl2, showed the prevention of somal loss but not the degeneration of axons.[56,57] On similar lines, Libby et al.[58] found that Bax deficiency protects against degeneration of the soma but not the axons following optic nerve crush in mice. Similarly, in an optic nerve crush model, Syc-Mazurek et al.[59] found that combined deletion of Jun and Ddit3 (CHOP) conferred somal protection; however, it did not attenuate axonal degeneration. These findings suggest that there is a compartmentalization of signals that contribute different neurodegenerative effects in the RGC soma versus the axons.

There was appreciable axonal damage observed in vehicle injected JNK2 knockout, compared to vehicle injected wild type mice. Perhaps, JNK2 signaling has a constitutive role in axon development; hence, knockout of JNK2 exhibited some degenerative effects in the axons. The role

of JNK in axon development been reported in other CNS neurons.[60,61] It is possible that the effects of ET-1 were not evident in the JNK2-deficient mice injected with ET-1, since the JNK2 knockout mice already had unhealthy axons; hence, the further protective/detrimental effects of ET-1 could not be assessed in this genetic background. It would be important to quell noxious signals contributing to neurodegenerative effects in the soma as well as the axons to produce robust neuroprotection.[58] Most of the studies in rodent models with induced ocular hypertension have shown that axonal degeneration and dysfunction precedes RGC death early in course of disease progression, which ultimately leads to the axonal transport obstruction, thereby leading to the RGC loss.[62–68] In C57BL/6 background mice, the combined null mutation of JNK2 and JNK3 protected dopamine neurons, however, was not able to protect against axon degeneration, after intrastriatal administration of 6-hydroxydopamine.[69] On similar grounds, DBA/2J mouse model of ocular hypertension, the knockout of JNK2 and JNK3 protected the somas of RGCs but not the axons from the degeneration.[70] However, Libby's group has shown that JNK2/JNK3 double knockout mice show significant protection from axonal degeneration after optic nerve crush in mice.[27] Presently in our studies we also observed that the JNK2 deletion delayed the apoptotic RGC loss after the intravitreal injection administration of ET-1 but did not block axonal degeneration in mice. JNK2 deletion was also not protective in the DBA/2J model of ocular hypertension and produced increased axonal degeneration.[70] Species differences in the role of the different JNK isoforms in neurodegeneration could possibly account for these findings.

In summary, ET-1 binding to its receptor could activate the JNK signaling mechanism, thereby phosphorylating its downstream transcription factors. The extrinsic pathway of apoptosis that can cause RGC loss occurs mainly by increasing the phosphorylation of c-JUN, which is the immediate early gene response activated by JNK. Our data suggest that JNK2 plays an important role in ET-1–mediated phosphorylation of c-Jun, which can lead to the loss of RGCs, a key event in glaucoma pathology.

Acknowledgments

Supported by NEI (EY028179).

Disclosure: **B. Kodati,** None; **D.L. Stankowska,** None; **V.R. Krishnamoorthy,** None; **R.R. Krishnamoorthy,** None

References

1. Lepple-Wienhues A, Becker M, Stahl F, Berweck S, Hensen J, Noske W, Eichhorn M, Wiederholt M. Endothelin-like immunoreactivity in the aqueous humour and in conditioned medium from cultured ciliary epithelial cells. *Curr Eye Res*. 1992;11:1041–1046.
2. Kallberg ME, Brooks DE, Garcia-Sanchez GA, Komaromy AM, Szabo NJ, Tian L. Endothelin 1 levels in the aqueous humor of dogs with glaucoma. *J Glaucoma*. 2002;11:105–109.
3. Choritz L, Machert M, Thieme H. Correlation of endothelin-1 concentration in aqueous humor with intraocular pressure in primary open angle and pseudoexfoliation glaucoma. *Invest Ophthalmol Vis Sci*. 2012;53:7336–7342.
4. Lau J, Dang M, Hockmann K, Ball AK. Effects of acute delivery of endothelin-1 on retinal ganglion cell loss in the rat. *Exp Eye Res*. 2006;82:132–145.
5. Chauhan BC, LeVatte TL, Jollimore CA, Yu PK, Reitsamer HA, Kelly ME, Yu DY, Tremblay F, Archibald ML. Model of endothelin-1-induced chronic optic neuropathy in rat. *Invest Ophthalmol Vis Sci*. 2004;45:144–152.
6. Howell GR, Macalinao DG, Sousa GL, Walden M, Soto I, Kneeland SC, Barbay JM, King BL, Marchant JK, Hibbs M, et al. Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. *J Clin Invest*. 2011;121:1429–1444.
7. Krishnamoorthy RR, Rao VR, Dauphin R, Prasanna G, Johnson C, Yorio T. Role of the ETB receptor in retinal ganglion cell death in glaucoma. *Can J Physiol Pharmacol*. 2008;86:380–393.
8. Stow LR, Jacobs ME, Wingo CS, Cain BD. Endothelin-1 gene regulation. *FASEB J*. 2011;25:16–28.
9. Inoue A, Yanagisawa M, Takuwa Y, Mitsui Y, Kobayashi M, Masaki T. The human preproendothelin-1 gene. Complete nucleotide sequence and regulation of expression. *J Biol Chem*. 1989;264:14954–14959.
10. Hynynen MM, Khalil RA. The vascular endothelin system in hypertension–recent patents and discoveries. *Recent Pat Cardiovasc Drug Discov*. 2006;1:95–108.
11. He S, Park YH, Yorio T, Krishnamoorthy RR. Endothelin-mediated changes in gene expression in isolated purified rat retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2015;56:6144–6161.
12. He S, Minton AZ, Ma HY, Stankowska DL, Sun X, Krishnamoorthy RR. Involvement of AP-1 and C/EBPbeta in upregulation of endothelin B (ETB) receptor expression in a rodent model of glaucoma. *PLoS One*. 2013;8:e79183.
13. Prasanna G, Dibas A, Tao W, White K, Yorio T. Regulation of endothelin-1 in human non-pigmented ciliary epithelial cells by tumor necrosis factor-alpha. *Exp Eye Res*. 1998;66:9–18.
14. Desai D, He S, Yorio T, Krishnamoorthy RR, Prasanna G. Hypoxia augments TNF-alpha-mediated endothelin-1 release and cell proliferation in human optic nerve head astrocytes. *Biochem Biophys Res Commun*. 2004;318:642–648.
15. Lin A. Activation of the JNK signaling pathway: breaking the brake on apoptosis. *Bioessays*. 2003;25:17–24.
16. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell*. 2000;103:239–252.
17. Bogoyevitch MA, Kobe B. Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. *Microbiol Mol Biol Rev*. 2006;70:1061–1095.
18. Bode AM, Dong Z. The functional contrariety of JNK. *Mol Carcinog*. 2007;46:591–598.
19. Johnson GL, Nakamura K. The c-jun kinase/stress-activated pathway: regulation, function and role in human disease. *Biochim Biophys Acta*. 2007;1773:1341–1348.
20. Dhanasekaran DN, Reddy EP. JNK-signaling: A multiplexing hub in programmed cell death. *Genes Cancer*. 2017;8(9-10):682–694.
21. Thomas CN, Berry M, Logan A, Blanch RJ, Ahmed Z. Caspases in retinal ganglion cell death and axon regeneration. *Cell Death Discov*. 2017;3:17032.
22. Dhanasekaran DN, Reddy EP. JNK signaling in apoptosis. *Oncogene*. 2008;27:6245–6251.
23. Bjorkblom B, Vainio JC, Hongisto V, Herdegen T, Courtney MJ, Coffey ET. All JNKs can kill, but nuclear localization is critical for neuronal death. *J Biol Chem*. 2008;283:19704–19713.
24. Liu J, Minemoto Y, Lin A. c-Jun N-terminal protein kinase 1 (JNK1), but not JNK2, is essential for tumor necrosis factor

Investigative Ophthalmology & Visual Science

alpha-induced c-Jun kinase activation and apoptosis. *Mol Cell Biol*. 2004;24:10844–10856.
25. Sun H, Wang Y, Pang IH, Shen J, Tang X, Li Y, Liu C, Li B. Protective effect of a JNK inhibitor against retinal ganglion cell loss induced by acute moderate ocular hypertension. *Mol Vis*. 2011;17:864–875.
26. Syc-Mazurek SB, Fernandes KA, Libby RT. JUN is important for ocular hypertension-induced retinal ganglion cell degeneration. *Cell Death Dis*. 2017;8:e2945.
27. Fernandes KA, Harder JM, Fornarola LB, Freeman RS, Clark AF, Pang IH, John SW, Libby RT. JNK2 and JNK3 are major regulators of axonal injury-induced retinal ganglion cell death. *Neurobiol Dis*. 2012;46:393–401.
28. Yoshida K, Behrens A, Le-Niculescu H, Wagner EF, Harada T, Imaki J, Ohno S, Karin M. Amino-terminal phosphorylation of c-Jun regulates apoptosis in the retinal ganglion cells by optic nerve transection. *Invest Ophthalmol Vis Sci*. 2002;43:1631–1635.
29. Chauhan BC, Levatte TL, Garnier KL, Tremblay F, Pang IH, Clark AF, Archibald ML. Semiquantitative optic nerve grading scheme for determining axonal loss in experimental optic neuropathy. *Invest Ophthalmol Vis Sci*. 2006;47:634–640.
30. Wang J, Ma HY, Krishnamoorthy RR, Yorio T, He S. A feed-forward regulation of endothelin receptors by c-Jun in human non-pigmented ciliary epithelial cells and retinal ganglion cells. *PLoS One*. 2017;12:e0185390.
31. Adams CM, Stacy R, Rangaswamy N, Bigelow C, Grosskreutz CL, Prasanna G. Glaucoma—next generation therapeutics: impossible to possible. *Pharm Res*. 2018;36(2):25.
32. Leske MC, Heijl A, Hussein M, Bengtsson B, Hyman L, Komaroff E, Early Manifest Glaucoma Trial G: factors for glaucoma progression and the effect of treatment: the early manifest glaucoma trial. *Arch Ophthalmol*. 2003;121:48–56.
33. Johnson LN. Glaucoma as a neurodegenerative disease: why we must 'look for the protein'. *R I Med J (2013)*. 2016;99(6):18–21.
34. Flammer J, Orgul S, Costa VP, Orzalesi N, Krieglstein GK, Serra LM, Renard JP, Stefansson E. The impact of ocular blood flow in glaucoma. *Prog Retin Eye Res*. 2002;21:359–393.
35. Casson RJ. Possible role of excitotoxicity in the pathogenesis of glaucoma. *Clin Exp Ophthalmol*. 2006;34:54–63.
36. Tezel G. TNF-alpha signaling in glaucomatous neurodegeneration. *Prog Brain Res*. 2008;173:409–421.
37. Yorio T, Krishnamoorthy R, Prasanna G. Endothelin: is it a contributor to glaucoma pathophysiology? *J Glaucoma*. 2002;11:259–270.
38. Chauhan BC. Endothelin and its potential role in glaucoma. *Can J Ophthalmol*. 2008;43:356–360.
39. Shoshani YZ, Harris A, Shoja MM, Rusia D, Siesky B, Arieli Y, Wirostko B. Endothelin and its suspected role in the pathogenesis and possible treatment of glaucoma. *Curr Eye Res*. 2012;37:1–11.
40. Orgul S, Cioffi GA, Wilson DJ, Bacon DR, Van Buskirk EM. An endothelin-1 induced model of optic nerve ischemia in the rabbit. *Invest Ophthalmol Vis Sci* 1996;37:1860–1869.
41. Orgul S, Cioffi GA, Bacon DR, Van Buskirk EM. An endothelin-1-induced model of chronic optic nerve ischemia in rhesus monkeys. *J Glaucoma* 1996;5:135–138.
42. Minton AZ, Phatak NR, Stankowska DL, He S, Ma HY, Mueller BH, Jiang M, Luedtke R, Yang S, Brownlee C, et al. Endothelin B receptors contribute to retinal ganglion cell loss in a rat model of glaucoma. *PLoS One*. 2012;7(8):e43199.
43. Prasanna G, Krishnamoorthy R, Clark AF, Wordinger RJ, Yorio T. Human optic nerve head astrocytes as a target for endothelin-1. *Invest Ophthalmol Vis Sci*. 2002;43:2704–2713.
44. Rao VR, Krishnamoorthy RR, Yorio T. Endothelin-1 mediated regulation of extracellular matrix collagens in cells of human lamina cribrosa. *Exp Eye Res*. 2008;86:886–894.
45. Trivli A, Koliarakis I, Terzidou C, Goulielmos GN, Siganos CS, Spandidos DA, Dalianis G, Detorakis ET. Normal-tension glaucoma: Pathogenesis and genetics. *Exp Ther Med*. 2019;17:563–574.
46. Paul A, Wilson S, Belham CM, Robinson CJ, Scott PH, Gould GW, Plevin R. Stress-activated protein kinases: activation, regulation and function. *Cell Signal*. 1997;9:403–410.
47. Dvoriantchikova G, Ivanov D. Tumor necrosis factor-alpha mediates activation of NF-kappaB and JNK signaling cascades in retinal ganglion cells and astrocytes in opposite ways. *Eur J Neurosci*. 2014;40:3171–3178.
48. Welsbie DS, Yang Z, Ge Y, Mitchell KL, Zhou X, Martin SE, Berlinicke CA, Hackler L, Jr., Fuller J, Fu J, et al. Functional genomic screening identifies dual leucine zipper kinase as a key mediator of retinal ganglion cell death. *Proc Natl Acad Sci USA*. 2013;110:4045–4050.
49. Kim BJ, Silverman SM, Liu Y, Wordinger RJ, Pang IH, Clark AF. In vitro and in vivo neuroprotective effects of cJun N-terminal kinase inhibitors on retinal ganglion cells. *Mol Neurodegener*. 2016;11:30.
50. Roth S, Shaikh AR, Hennelly MM, Li Q, Bindokas V, Graham CE. Mitogen-activated protein kinases and retinal ischemia. *Invest Ophthalmol Vis Sci*. 2003;44:5383–5395.
51. Kwong JM, Caprioli J. Expression of phosphorylated c-Jun N-terminal protein kinase (JNK) in experimental glaucoma in rats. *Exp Eye Res*. 2006;82:576–582.
52. Levkovitch-Verbin H, Quigley HA, Martin KR, et al. The transcription factor c-jun is activated in retinal ganglion cells in experimental rat glaucoma. *Exp Eye Res*. 2005;80(5):663–670.
53. Stokely ME, Brady ST, Yorio T. Effects of endothelin-1 on components of anterograde axonal transport in optic nerve. *Invest Ophthalmol Vis Sci*. 2002;43:3223–3230.
54. Coffey ET, Smiciene G, Hongisto V, et al. c-Jun N-terminal protein kinase (JNK) 2/3 is specifically activated by stress, mediating c-Jun activation, in the presence of constitutive JNK1 activity in cerebellar neurons. *J Neurosci*. 2002;22:4335–4345.
55. Marola OJ, Syc-Mazurek SB, Howell GR, Libby RT. Endothelin 1-induced retinal ganglion cell death is largely mediated by JUN activation. *Cell Death Dis*. 2020;11:811.
56. Sagot Y, Dubois-Dauphin M, Tan SA, et al. Bcl-2 overexpression prevents motoneuron cell body loss but not axonal degeneration in a mouse model of a neurodegenerative disease. *J Neurosci* 1995;15:7727–7733.
57. Kitaoka Y, Kitaoka Y, Kwong JM, et al. TNF-alpha-induced optic nerve degeneration and nuclear factor-kappaB p65. *Invest Ophthalmol Vis Sci*. 2006;47:1448–1457.
58. Libby RT, Li Y, Savinova OV, et al. Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage. *PLoS Genet*. 2005;1:17–26.
59. Syc-Mazurek SB, Fernandes KA, Wilson MP, Shrager P, Libby RT. Together JUN and DDIT3 (CHOP) control retinal ganglion cell death after axonal injury. *Mol Neurodegener*. 2017;12(1):71.
60. Hirai S, Cui DF, Miyata T, et al. The c-Jun N-terminal kinase activator dual leucine zipper kinase regulates axon growth and neuronal migration in the developing cerebral cortex. *J Neurosci*. 2006;26:11992–12002.
61. Eminel S, Roemer L, Waetzig V, Herdegen T. c-Jun N-terminal kinases trigger both degeneration and neurite outgrowth in primary hippocampal and cortical neurons. *J Neurochem*. 2008;104:957–969.
62. Maddineni P, Kasetti RB, Patel PD, et al. CNS axonal degeneration and transport deficits at the optic nerve head

Investigative Ophthalmology & Visual Science

precede structural and functional loss of retinal ganglion cells in a mouse model of glaucoma. *Mol Neurodegener*. 2020;15:48.

63. Howell GR, Libby RT, Jakobs TC, et al. Axons of retinal ganglion cells are insulted in the optic nerve early in DBA/2J glaucoma. *J Cell Biol*. 2007;179:1523–1537.
64. Buckingham BP, Inman DM, Lambert W, et al. Progressive ganglion cell degeneration precedes neuronal loss in a mouse model of glaucoma. *J Neurosci*. 2008;28:2735–2744.
65. Soto I, Oglesby E, Buckingham BP, et al. 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.
66. Quigley HA, McKinnon SJ, Zack DJ, et al. Retrograde axonal transport of BDNF in retinal ganglion cells is blocked by acute IOP elevation in rats. *Invest Ophthalmol Vis Sci*. 2000;41:3460–3466.
67. Johnson EC, Jia L, Cepurna WO, Doser TA, Morrison JC. Global changes in optic nerve head gene expression after exposure to elevated intraocular pressure in a rat glaucoma model. *Invest Ophthalmol Vis Sci*. 2007;48:3161–3177.
68. Pease ME, McKinnon SJ, Quigley HA, Kerrigan-Baumrind LA, Zack DJ. Obstructed axonal transport of BDNF and its receptor TrkB in experimental glaucoma. *Invest Ophthalmol Vis Sci*. 2000;41:764–774.
69. Ries V, Silva RM, Oo TF, et al. JNK2 and JNK3 combined are essential for apoptosis in dopamine neurons of the substantia nigra, but are not required for axon degeneration. *J Neurochem*. 2008;107:1578–1588.
70. Harder JM, Williams PA, Soto I, et al. Jnk2 deficiency increases the rate of glaucomatous neurodegeneration in ocular hypertensive DBA/2J mice. *Cell Death Dis*. 2018;9:705.

Investigative Ophthalmology & Visual Science