

# Delta-Opioid Agonist SNC-121 Protects Retinal Ganglion Cell Function in a Chronic Ocular Hypertensive Rat Model

Yasir Abdul, Naseem Akhter, and Shahid Husain

**PURPOSE.** This study examined if the delta-opioid ( $\delta$ -opioid) receptor agonist, SNC-121, can improve retinal function and retinal ganglion cell (RGC) survival during glaucomatous injury in a chronic ocular hypertensive rat model.

**METHODS.** IOP was raised in brown Norway rats by injecting hypertonic saline into the limbal venous system. Rats were treated with 1 mg/kg SNC-121 (intraperitoneally [IP]) once daily for 7 days. Pattern-electroretinograms (PERGs) were obtained in response to contrast reversal of patterned visual stimuli. RGCs were visualized by fluorogold retrograde labeling. Expression of TNF- $\alpha$  and p38 mitogen-activated protein (MAP) kinase was measured by immunohistochemistry and Western blotting.

**RESULTS.** PERG amplitudes in ocular hypertensive eyes were significantly reduced ( $14.3 \pm 0.60$   $\mu$ volts) when compared with healthy eyes ( $18.0 \pm 0.62$   $\mu$ volts). PERG loss in hypertensive eyes was inhibited by SNC-121 treatment ( $17.20 \pm 0.1.3$   $\mu$ volts;  $P < 0.05$ ). There was a 29% loss of RGCs in the ocular hypertensive eye, which was inhibited in the presence of SNC-121. TNF- $\alpha$  production and activation of p38 MAP kinase in retinal sections and optic nerve samples were upregulated in ocular hypertensive eyes and inhibited in the presence of SNC-121. Furthermore, TNF- $\alpha$  induced increase in p38 MAP kinase activation in astrocytes was inhibited in the presence of SNC-121.

**CONCLUSIONS.** These data provide evidence that activation of  $\delta$ -opioid receptors inhibited the loss of PERG amplitudes and rate of RGC loss during glaucomatous injury. Mechanistic data provided clues that TNF- $\alpha$  is mainly produced from glial cells and activates p38 MAP kinase, which was significantly inhibited by SNC-121 treatment. Overall, data indicate that enhancement of  $\delta$ -opioid activity in the eye may provide retina neuroprotection against glaucoma. (*Invest Ophthalmol Vis Sci.* 2013;54:1816-1828) DOI:10.1167/iovs.12-10741

Glaucoma is a chronic optic neuropathy characterized by retinal ganglion cell (RGC) death, an excavated appearance of the optic nerve, and vision loss.<sup>1</sup> Studies have shown

that several factors including elevated IOP,<sup>2</sup> oxidative stress,<sup>3</sup> mitochondrial dysfunction,<sup>4</sup> glutamate neurotoxicity,<sup>5</sup> and pro-inflammatory cytokines<sup>6</sup> work concomitantly in the pathogenesis of the disease and in regulating RGC susceptibility to glaucomatous damage.<sup>7</sup> Current therapeutic management of glaucoma aims to halt or slow disease progression by reducing elevated IOP. Although IOP-lowering treatment can retard the disease progression in many glaucoma patients, it is not always sufficient to prevent disease progression. As a result, efforts in our laboratory are focused on developing neuroprotective treatment strategies for glaucoma.

TNF- $\alpha$  is a pro-inflammatory cytokine that is rapidly upregulated in several neurodegenerative disorders, such as multiple sclerosis, Parkinson's disease, Alzheimer disease, and glaucoma.<sup>6,8</sup> The levels of TNF- $\alpha$  and its receptor, TNF-R1, are also upregulated significantly in glaucoma.<sup>6,9</sup> Growing evidence supports that TNF- $\alpha$  through the binding of TNF-R1, a death receptor, is involved in mediating RGC death during glaucomatous neurodegeneration.<sup>6,9</sup> In addition, the expression of TNF- $\alpha$  receptors on astrocytes and axons of the glaucomatous optic nerve head (ONH) would suggest that TNF- $\alpha$  is stimulating cytodestructive processes in both the astrocytes and axons.<sup>10</sup> Considering a dire need to develop neuroprotective agents that can provide neuroprotection and counterbalance the actions of TNF- $\alpha$ -mediated cytodestructive signaling pathways, we set out to determine if a selective delta-opioid ( $\delta$ -opioid) receptor agonist, SNC-121, possessed these novel properties against glaucomatous injury.

The effects of opioids are mediated through activation of three opioid receptor subtypes:  $\delta$ -opioid,  $\kappa$ -opioid, and  $\mu$ -opioid.<sup>11</sup> Studies have shown that opioid receptor activation by exogenous agonists can facilitate a protective effect against hypoxia, ischemia, cold, or an acidic environment.<sup>12-14</sup> More recently, we published that morphine pretreatment can provide significant retinal neuroprotection against acute ischemic<sup>15</sup> and glaucomatous injury,<sup>16</sup> and this neuroprotection is mediated, in part, via inhibition of TNF- $\alpha$  production.<sup>16,17</sup> However, the neuroprotective roles of  $\delta$ -opioid receptors against glaucomatous injury have remained fully unexplored.

The data presented herein describes the potential participation of  $\delta$ -opioid receptors in a neuroprotective paradigm against glaucomatous injury in a chronic ocular hypertensive rat model. The data provide evidence that  $\delta$ -opioid receptor activation by the exogenous ligand, SNC-121, protects RGC function and integrity against glaucomatous injury. Mechanistic data provide clues that TNF- $\alpha$  is produced mainly from glial cells during the early phase of glaucoma development, which subsequently phosphorylate and activate p38 mitogen-activated protein (MAP). These studies provide clues that TNF- $\alpha$ -mediated signaling pathways could be a potential target for development of antiglaucoma therapies and enhancement of  $\delta$ -opioid activity by exogenous means may be a vital neuroprotective strategy for glaucoma therapy.

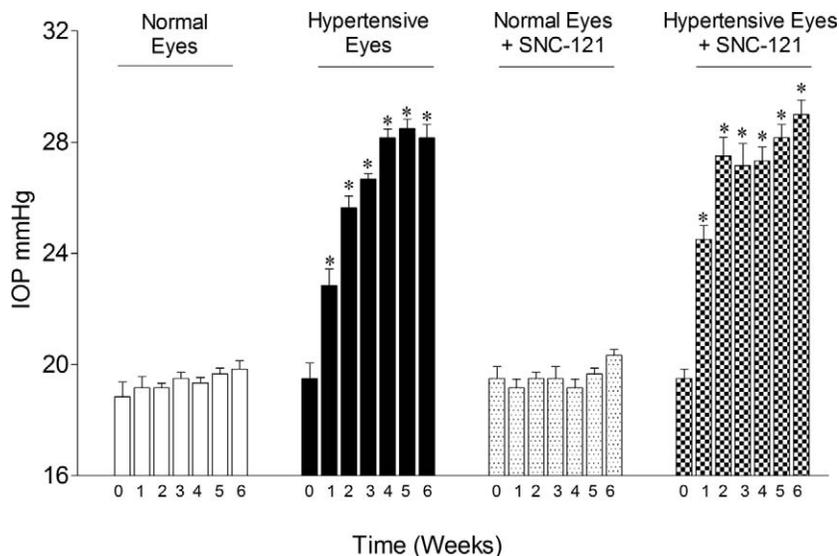
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**FIGURE 1.** IOP measurements of healthy eyes, and eyes from a chronic glaucoma model with and without SNC-121 (1 mg/kg) treatment for 7 days, once daily. Rats were divided into two groups: ocular hypertensive group ( $n = 8$ ) and SNC-121-treated ocular hypertensive group ( $n = 8$ ). IOP was elevated in one eye of brown Norway rats by injecting approximately 50  $\mu$ L of 2.0 M hypertonic saline, while the contralateral eye served as the control. Rats were maintained for up to 6 weeks post surgery. A total of 16 rats were used in this experiment. Data are mean  $\pm$  SE. \* $P < 0.05$ ;  $n = 8$  for each group.

## MATERIALS AND METHODS

### Animals

Adult male or female brown Norway rats (3–5 months of age; 150–200 g; Harlan Laboratories, Inc., Indianapolis, IN) were used in this study. Rats were kept under a cycle of 12 hours light and 12 hours dark for all the studies. Animal handling was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research; and the study protocol was approved by the Animal Care and Use Committee at the Medical University of South Carolina. Stock SNC-121 solutions (10 mg/mL) were made and diluted in normal saline (0.9%). SNC-121 (1 mg/kg) was injected intraperitoneally (IP) in brown Norway rats once daily for 7 days. Drug administration (100–150  $\mu$ L) was performed daily at the same time between 9 AM to 11 AM. The control group was handled in a similar fashion, except that normal saline was injected without SNC-121.

### Development of Glaucoma Model by Hypertonic Saline Injection

Brown Norway rats (150–200 g body weight) were housed under a standard 12:12 (light:dark) cycle. A stable baseline IOP was documented prior to hypertonic saline injection. Rats were anesthetized with ketamine (75 mg/kg) and xylazine (8 mg/kg) and body temperature was maintained at 37°C with a heating pad. Topical proparacaine (0.5%) was applied to the cornea. A pulled-glass micropipette attached to a syringe by PE-50 tubing (Becton Dickinson & Co., Sparks, MD), was inserted into a circumferential limbal vein near the cornea and approximately 50  $\mu$ L of 2 M hypertonic saline was injected into the limbal venous system as described.<sup>16,18</sup> After surgery, an antibacterial ointment (neomycin) was applied at the injection site of each animal to prevent infections. IOP was recorded as the average of six to eight consecutive measurements prior to surgery (baseline IOP; 18–20 mm Hg) followed by IOP measurement on a weekly basis after treatment, using a calibrated Tonolab tonometer (Colonial Medical Supply Co., Inc., Franconia, NH), as described earlier.<sup>19</sup> IOP exposure for each animal was calculated by performing separate integration of the IOP over days of exposure for the treated and control eyes as described.<sup>16,20</sup> The control eye integral values were then subtracted

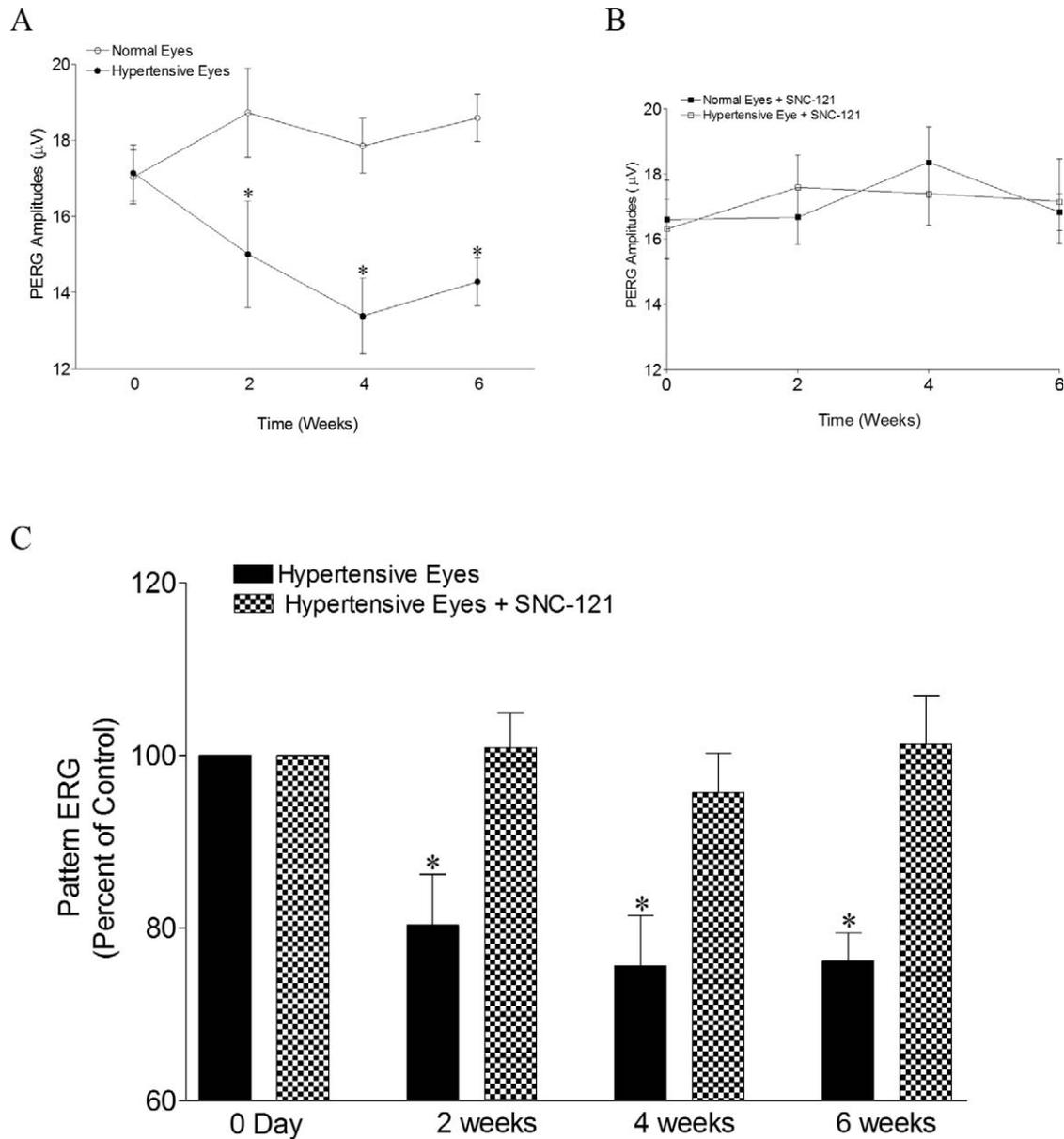
from the treated eye integral, yielding the “IOP-integral difference”; values are expressed as millimeters of mercury over the days. As inclusion criteria following saline injection, only animals with an elevated IOP that was at least 25% over baseline were included in the study.

### Pattern-Electroretinogram (PERG) Recordings

Rats were anesthetized with ketamine (75 mg/kg) and xylazine (8 mg/kg) and body temperature was maintained at 37°C with a heating pad. PERG recordings (without dark adaptation) were conducted in both eyes (sequentially) 3 days prior to IOP elevation by hypertonic saline injection, and then biweekly post surgery as described earlier.<sup>16</sup> Fifteen rats were used in this experiment. Rats were divided into two groups: an untreated hypertensive group and the SNC-121-treated hypertensive group. The PERG electrode was placed on the corneal surface by means of a micromanipulator and positioned in such a way as to encircle the undilated pupil without limiting the field of view. A small drop of saline was applied to keep the cornea and lens moist during each recording. A visual stimulus generated by black and white alternating contrast reversing bars (mean luminance, 50 cd/m<sup>2</sup>; spatial frequency, 0.033 cycle/deg; contrast, 99%; and temporal frequency, 1 Hz) was aligned with the projection of the undilated pupil at an 11-cm distance using the UTAS-2000 (LKC Technologies, Gaithersburg, MD). Each PERG was an average of 300 sweeps at an interval of 1 second. For the PERG amplitudes, measurements were made between a peak and adjacent trough of the waveform.

### Retrograde Labeling of Retinal Ganglion Cells

Rats were deeply anesthetized with ketamine (75 mg/kg), xylazine (8 mg/kg), and body temperature was maintained at 37°C with a heating pad. Retrograde labeling of RGCs was performed as described earlier.<sup>16</sup> Briefly, 3  $\mu$ L of a 5% solution of fluorogold (Fluorochrome, LLC; Denver, CO) in PBS was injected into the superior colliculus of twelve anesthetized ocular hypertensive animals immobilized in a stereotaxic apparatus. Using a small drill, a 1/8-inch hole was made in the skull 6 mm from bregma and 2 mm from lambda. After making this hole in the skull, a Hamilton syringe (Hamilton, Reno, NV) was filled with fluorogold and the syringe needle gently inserted at the hole and



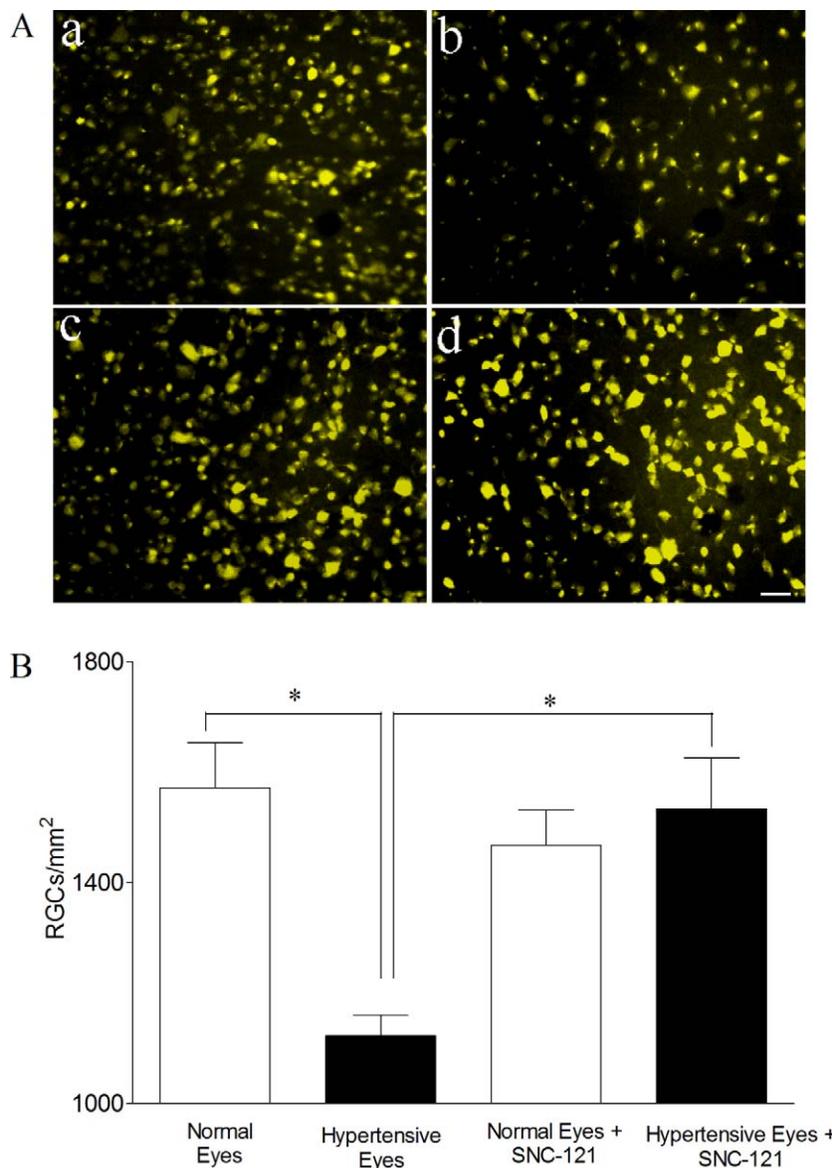
**FIGURE 2.** (A) PERG recording in normal and ocular hypertensive rat eyes. Each waveform is a mean of 300 individual waveforms taken at an interval of 1 second for each data point. (B) Example of PERG recorded in SNC-121-treated (1 mg/kg) healthy and ocular hypertensive rat eyes. Each waveform is also a mean of 300 individual waveforms. (C) Changes observed in PERG of untreated and SNC-121-treated ocular hypertensive rat eyes. In these experiments, brown Norway rats were treated with 1 mg/kg SNC-121 (IP) immediately after hypertonic saline injections, and subsequently once daily for 7 days. PERG data shown in this figure were collected at weeks 2, 4, and 6, post saline injection. Prior to the surgery, PERG values in both hypertensive and SNC-121-treated hypertensive eyes (e.g., ranging from 16–20  $\mu$ volts) were considered as 100% at day 0. The changes in PERG of hypertensive eyes or SNC-121-treated eyes at 2, 4, and 6 weeks were calculated using their respective values at day 0. A total of 15 rats (e.g., ocular hypertensive group,  $n = 8$  and SNC-121-treated ocular hypertensive group,  $n = 7$ ) were used in this experiment. IOP data for these rats is shown in Figure 1. Data are mean  $\pm$  SE; \* $P < 0.05$ ;  $n = 7$  to 8.

going down 4 mm, whereupon the fluorogold was injected. The needle was left in the brain for 30 to 60 seconds, then slowly removed. The skull hole was filled with bone wax 903 (Lukens Cat # 2007-05; World Precision Instruments, Inc., Sarasota, FL). Seven days post injection, animals were euthanized and their eyes were enucleated and fixed in 4% paraformaldehyde (PFA) for 24 hour at 4°C. After rinsing with PBS, each retina was detached from the eyecup and prepared as a flat mount by mounting the vitreous side up. Fluorescent RGCs were visualized under Zeiss microscopy (Jena, Germany). Each retina was divided into four quadrants, and each quadrant was further divided in two regions (inner and peripheral retina). RGCs in the inner and peripheral retina were counted at 1.5 to 2.00 mm and 3.5 to 4.00 mm from the optic

disc, respectively. RGCs were counted in exactly same fashion in normal, hypertensive, and SNC-121-treated hypertensive retinas. RGCs were counted and averaged per eight microscopic fields of identical size (150  $\mu$ m<sup>2</sup>;  $\times 20$  magnification) per retina by using ImageJ software (NIH, Bethesda, MD). The automated RGC numbers generated by ImageJ software were comparable when RGCs were counted manually by two operators in a masked fashion.

**Immunohistochemistry**

Eyes and optic nerve were removed at 3, 7, and 42 days, respectively, post hypertonic saline injections and immunohistochemistry was



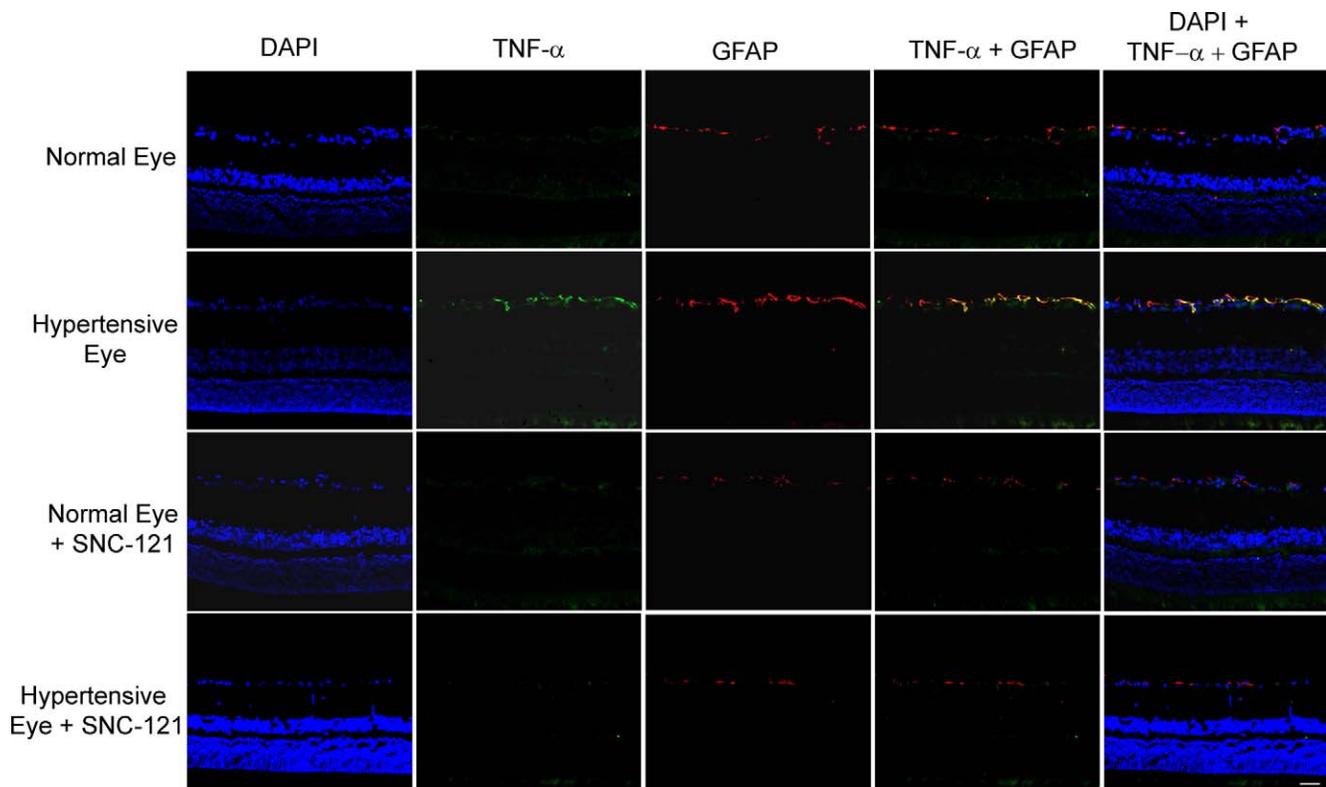
**FIGURE 3.** (A) Fluorescence micrographs of flat-mounted retinas depicting Fluorogold-labeled RGCs in normal (a), ocular hypertensive (b), SNC-121-treated normal (c), and SNC-121-treated ocular hypertensive (d) eyes. Briefly, 3  $\mu\text{L}$  of a 5% solution of fluorogold was injected into the superior colliculus of anesthetized animals. Seven days post injection, animals were euthanized and retinas were prepared as flat-mounts, vitreous side facing up. Fluorescent RGCs were visualized under Zeiss microscopy. Bar: 20  $\mu\text{m}$ . (B) Quantification of RGCs. Rats were divided into two groups: ocular hypertensive group ( $n = 6$ ) and SNC-121-treated ocular hypertensive group ( $n = 6$ ). A total of 12 rats were used in this experiment. IOP and PERG data for these rats are shown in Figures 1 and 2, respectively. RGCs were counted in eight microscopic fields of identical size (150  $\mu\text{m}^2$  area) for each retina using ImageJ software. \* $P < 0.05$ ;  $n = 6$  for each group.

performed as described previously.<sup>15</sup> After removing the anterior segment of the eye and the lens, eyes, and isolated optic nerves were fixed in 4% PFA for 4 hours, then cryoprotected in 25% sucrose solution overnight at 4°C. The eyecups and optic nerves were washed in ice cold PBS and frozen in optimal cutting temperature compound embedding medium over dry ice. Eyes and optic nerves were either stored at -20°C or cryosections were cut. Cryosections were cut at -20°C, fixed in cold methanol for 10 minutes and rinsed in 10 mM Tris-buffered saline (TBS), pH 7.5. Tissues were permeabilized with 0.2% Triton-X-100 in TBS and washed again with TBS. Tissues were then blocked with 5% BSA in TBS for 1 hour at room temperature, followed by incubation with primary antibodies (e.g., anti-TNF- $\alpha$  antibody, 1:100 dilution; anti-cellular retinaldehyde binding protein (CRALBP), 1:500 dilution; anti-glial fibrillary-acidic protein [GFAP], 1:150 dilution; anti-phospho-p38 MAP kinase, 1:500 dilution; or 0.5% BSA) for

overnight at 4°C. Cryosections were then washed with TBS and incubated with fluorescein-conjugated secondary antibody (antimouse Immunoglobulin [Ig] G, 1:400; DyLight 488 and anti-Rabbit IgG, 1:600 Rhodamine; Jackson Immuno Research Laboratories, Inc., West Grove, PA) at room temperature for 1 hour. Negative control slides were incubated with 0.5% BSA in place of the primary antibody. The sections were observed under a bright-field microscope equipped with epifluorescence, and digitized images were captured by a digital camera (Zeiss).

### Optic Nerve Head Astrocyte Cultures

Ten human eyes from five donors were obtained from National Disease Research Interchange (NDRI, Philadelphia, PA) to isolate ONH astrocytes as described previously.<sup>17</sup> Briefly, primary ONH astrocytes



**FIGURE 4.** Eyes of brown Norway rats were enucleated 7 days post hypertonic saline injections. Contralateral eyes were used as the normal control. Cryosections were immunostained by anti-TNF- $\alpha$  and anti-GFAP antibodies as indicated horizontally. Ocular treatments are indicated vertically. *Green color* indicates staining for TNF- $\alpha$ , *red* for GFAP, and *blue* nuclei for DAPI. *Far right panels* represent double-labeling of TNF- $\alpha$  and GFAP. There was no positive staining when primary antibodies were omitted (not shown). Fluorescence microscopy; *bar* is 20  $\mu$ m. Data shown in this figure are a representation of at least four independent experiments. A total of 10 animals were used in this experiment. Comparable staining for TNF- $\alpha$  and GFAP was seen in at least four animals in each treatment group.

were isolated from ONH tissue of human eyes. Optic nerve freed of sclera tissue and central retinal vessels was cut into four pieces. These pieces were then plated onto a collagen-I-coated cell culture plate and allowed to grow 2 to 3 weeks in Dulbecco's minimum essential medium F12 (DMEM F12; Cellgro, Pittsburg, PA) containing 10% fetal bovine serum (FBS) and antibiotics. ONH astrocytes from the mixed cell culture were purified by immunopanning as described earlier.<sup>17</sup> Briefly, culture plates were coated with the C5-neuroepithelial monoclonal antibody, which was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). The mixed population of cells derived from tissue explants was trypsinized and resuspended in DMEM and placed on the C5-antibody-coated plates for 40 minutes. Nonadherent cells were removed and adherent cells were washed gently and cultured in astrocyte growth medium (Cambrex; Lonza, Walkersville, MD) containing 3% FBS. The purity of the astrocyte culture was determined by positive immunostaining for the astrocyte markers GFAP and neural cell adhesion molecule (a cell surface adhesion-molecule) in each batch. In this study, ONH astrocytes of passages two to six were used. ONH astrocytes were pretreated with the  $\delta$ -opioid agonist, SNC-121 (1  $\mu$ mol/L), for 15 minutes followed by treatment with 25 ng/mL TNF- $\alpha$  for 6 hours for the measurement of phospho- and total-p38 MAP kinase expression.

### Western Blotting

Equivalent amounts of optic nerve extracts or cell lysates of human ONH astrocytes (15  $\mu$ g protein/lane) were loaded onto 10% SDS-PAGE, proteins separated, and proteins transferred to nitrocellulose membranes as described earlier.<sup>17</sup> The membranes were blocked with 5% nonfat dry milk followed by incubation for 12 hours at 4°C with appropriate primary antibodies (e.g., TNF- $\alpha$ , total-p38 MAP kinase,

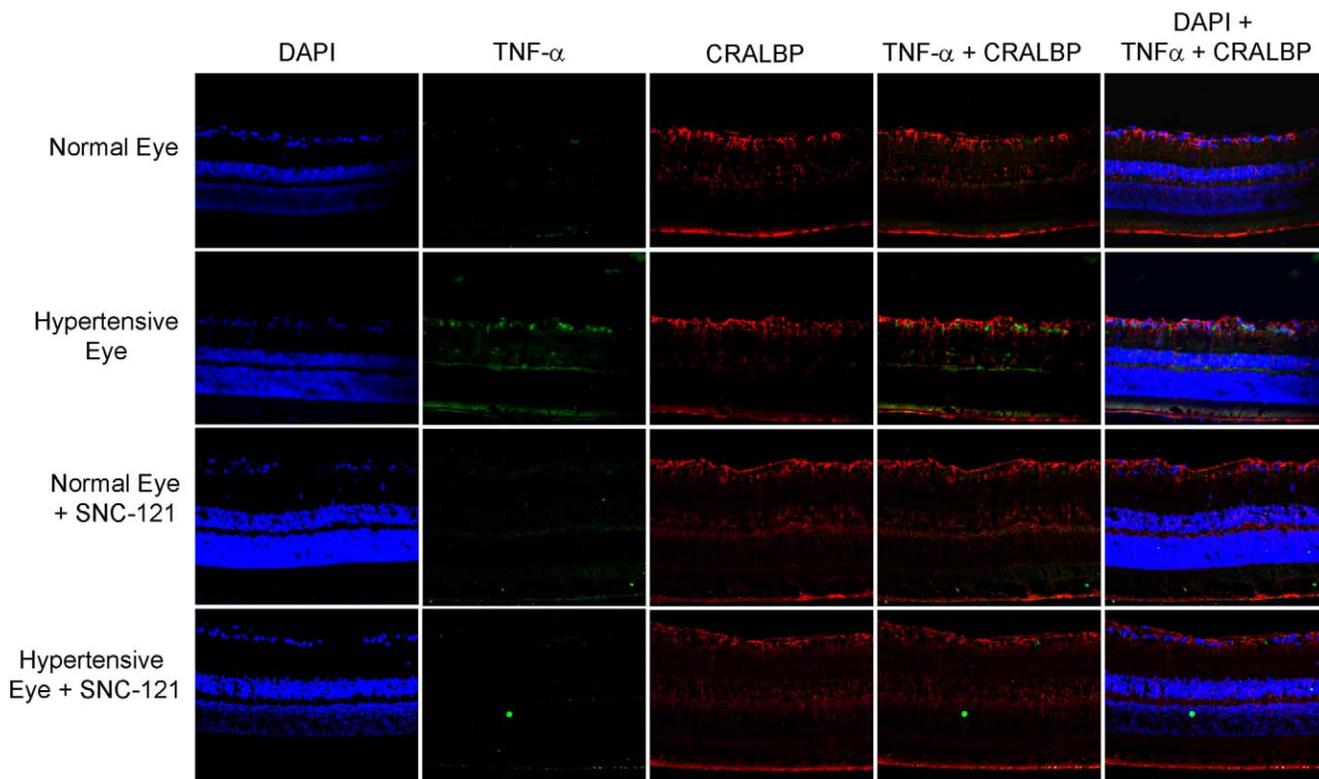
phospho-p38 MAP kinase, at 1:1000 dilutions or  $\beta$ -actin at 1:3000 dilutions). After washing, membranes were incubated for 1 hour at 20°C with appropriate secondary antibodies (horseradish peroxidase [HRP]-conjugated; dilution 1:3000). Prestained molecular weight markers were run in parallel to identify the molecular weight of proteins of interest. For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent reagent, and the signal was monitored using a Biorad Versadoc imaging system (Biorad, Hercules, CA).

### Statistical Analysis

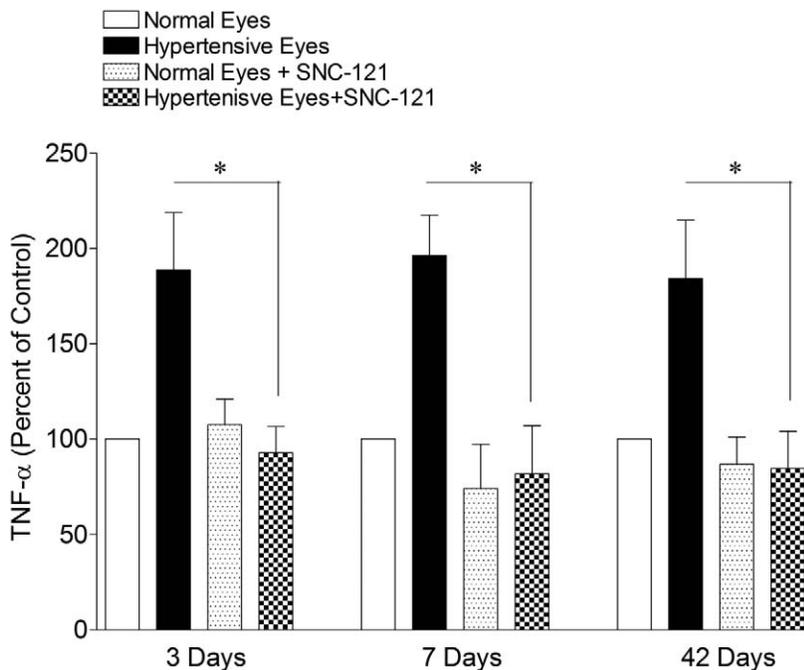
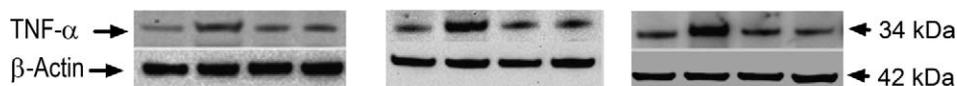
Statistical comparisons were made using the Student's *t*-test for paired data or ANOVA using the Bonferroni posttest for multiple comparisons (GraphPad Software, Inc., San Diego, CA). *P* less than or equal to 0.05 was considered significant.

### RESULTS

A significant elevation in IOP was seen as early as 7 days, which was maintained for up to 6 weeks post surgery in an ocular hypertensive rat model of glaucoma. To determine the effects of a selective  $\delta$ -opioid receptor agonist, SNC-121, animals were treated with 1 mg/Kg SNC-121 for 7 days, once daily. We have not seen any significant difference in retina neuroprotection between the groups that were either treated 7 or 28 days with 1 mg/kg SNC-121 (data not shown). Since there was no added benefit for a prolonged SNC-121 treatment, we chose to treat animals with SNC-121 (1 mg/kg) for 7 days in all subsequent studies. As shown in Figure 1, treatment of SNC-121 (1 mg/kg;



**FIGURE 5.** Eyes of brown Norway rats were enucleated 7 days post hypertonic saline injection. Contralateral eyes were used as the healthy control. Cryosections were immunostained by anti-TNF- $\alpha$  antibodies and anti-CRALBP, as indicated horizontally. Ocular treatments are indicated vertically. *Green color* indicates staining for TNF- $\alpha$ , *red* for CRALBP, and *blue* nuclei for DAPI. *Far right panels* represent double-labeling of TNF- $\alpha$  and CRALBP. There was no positive staining when primary antibodies were omitted (not shown). Fluorescence microscopy; *bar* is 20  $\mu$ m. Data shown in this figure are representative of at least four independent experiments. A total of 10 animals were used in this experiment. Comparable staining for TNF- $\alpha$  and CRALBP was seen in at least four animals in each treatment group.



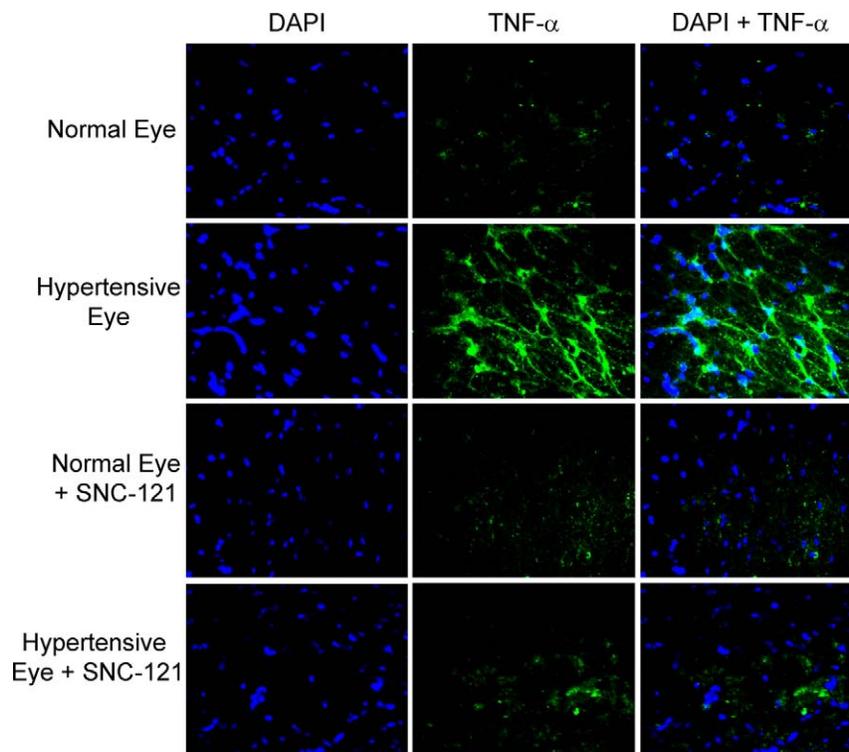
**FIGURE 6.** TNF- $\alpha$  production in response to glaucomatous injury and its suppression by SNC-121 treatment in the optic nerve extracts. Optic nerves of untreated and SNC-121-treated healthy and hypertensive eyes were collected at 3, 7, or 42 days post injury and analyzed for TNF- $\alpha$  expression by Western blotting using anti-TNF- $\alpha$  antibodies. Rats were divided into two groups: ocular hypertensive group ( $n = 4$ ) and SNC-121-treated ocular hypertensive group ( $n = 4$ ). Rats were treated with SNC-121 (1 mg/kg) once daily for 7 days. IOP was elevated in one eye of brown Norway rats by injecting approximately 50  $\mu$ L of 2.0 M hypertonic saline into limbal veins, while the contralateral eye served as the control. Band intensities of Western blots were quantitated by densitometry. A total of 24 animals were used in this experiment. Data are expressed as mean  $\pm$  SE. \* $P < 0.05$ ;  $n = 4$  for each group.

IP) had no significant effect on IOP when measured once a week. Additionally, IOP was measured at 2, 4, 6, 24, and 72 hours, post SNC-121 treatment and we have not seen any significant changes in the IOP (data not shown). Furthermore, the IOP-integral difference of IOP elevation over the days of exposure for both the control and hypertensive eyes was calculated using the area under the curve (e.g., elevated IOP minus baseline IOP), as described earlier.<sup>16</sup> IOP-integral differences were averaged for each group and compared. The cumulative average group IOP-integral differences rose steadily over the 6 week duration. At week 6, cumulative IOP of the untreated ocular hypertensive group ( $319 \pm 5$  mm Hg;  $n = 8$ ) was not significantly different from the SNC-121-treated ocular hypertensive group ( $331 \pm 8$  mm Hg;  $n = 8$ ). However, the IOP remained significantly elevated in both the untreated and SNC-121-treated ocular hypertensive groups, when compared with healthy eyes (Fig. 1).

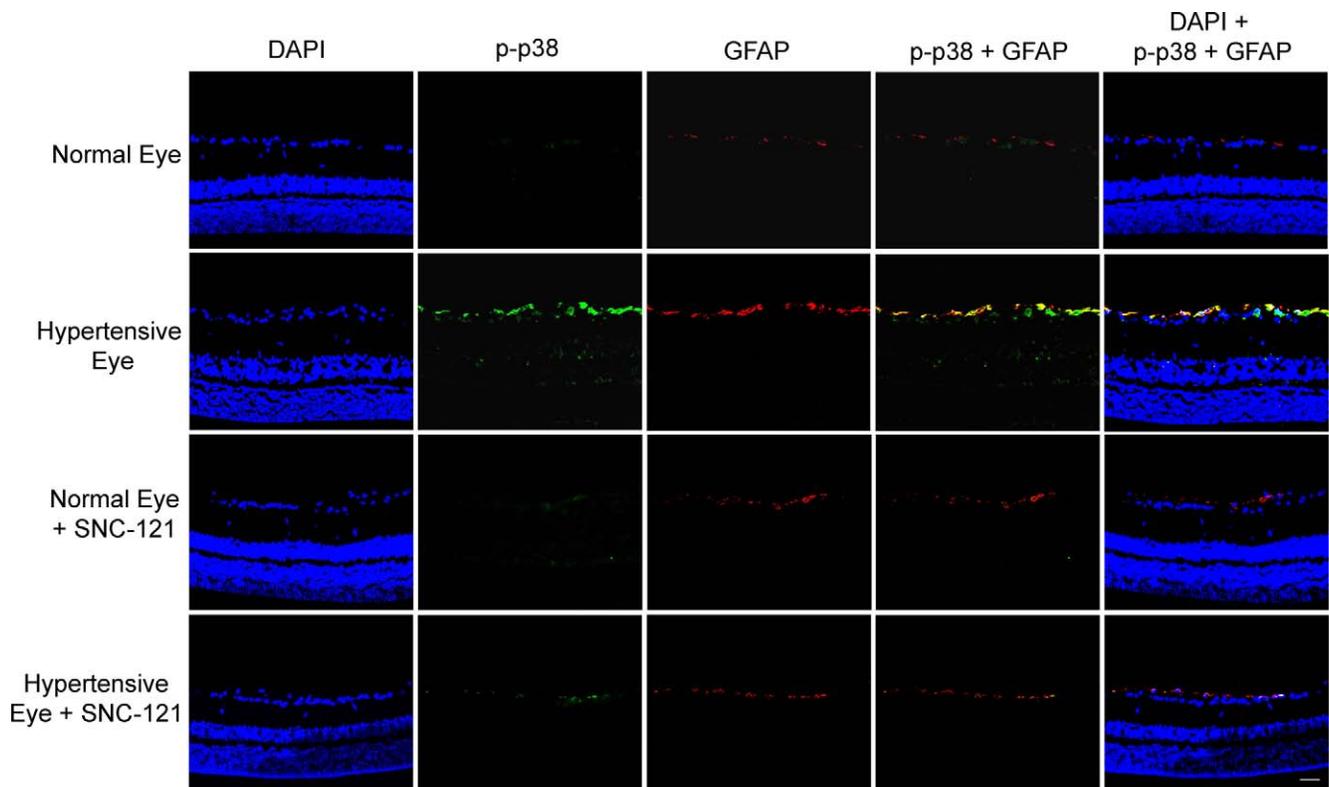
To determine the functional response of RGCs in healthy and ocular hypertensive rats, PERGs were performed. Figure 2A presents changes in PERG amplitudes in normal and ocular hypertensive eyes, whereas Figure 2B shows changes in PERG amplitudes of SNC-121-treated healthy and hypertensive eyes. Figure 2C presents the change in the PERG of ocular hypertensive eyes and SNC-121-treated ocular hypertensive

eyes at different time points, post injury. PERG amplitudes were significantly reduced in ocular hypertensive eyes when compared with healthy eyes ( $P < 0.05$ ; Fig. 2A). To determine if  $\delta$ -opioid receptor activation during glaucomatous injury preserved the function of RGCs, animals were treated with SNC-121 (1 mg/kg, IP) each day for 7 days. SNC-121 treatment preserved RGC function significantly ( $P < 0.05$ ; Fig. 2B), as determined by PERG. Overall, PERGs were reduced to 80%, 76%, and 76% in hypertensive eyes at 2, 4, and 6 weeks post injury, respectively, which were increased to 101%, 96%, and 101% when animals were treated with SNC-121 (Fig. 2C).

To confirm that declines in PERG amplitudes are due to the loss of RGCs, they were visualized by retrograde labeling with bilateral injections of fluorogold into the superior colliculus. Representative micrographs of fluorogold-labeled cells indicate a clear loss of RGCs in the ocular hypertensive eye as compared with the contralateral healthy eye (i.e., Fig. 3A-b compared with Fig. 3A-a, respectively). The loss of RGCs was reduced when the ocular hypertensive animals were treated with SNC-121 (1 mg/kg; IP) each day for 7 days (Fig. 3A-d; Fig. 3A-c serving as the treatment control). Quantification of RGCs in healthy eyes, ocular hypertensive eyes, SNC-121-treated healthy eyes, and SNC-121-treated ocular hypertensive eyes are shown in Figure 3B. The mean number ( $\pm$  SE) of



**FIGURE 7.** The ONH of brown Norway rats was removed 42 days post hypertonic saline injection. Contralateral optic nerve was used as the normal control. Cryosections were immunostained by anti-TNF- $\alpha$  antibodies as indicated horizontally. Ocular treatments are indicated vertically. *Green color* indicates staining for TNF- $\alpha$  and *blue* nuclei for DAPI. There was no positive staining when primary antibodies were omitted (not shown). Data shown in this figure are a representation of at least four independent experiments. A total of eight animals were used in this experiment. Comparable staining for TNF- $\alpha$  was seen in at least four animals in each treatment group.



**FIGURE 8.** Eyes of brown Norway rats were enucleated 7 days post hypertonic saline injection. Contralateral eyes were used as the healthy control. Cryosections were immunostained by anti-phospho-p38 antibodies and anti-GFAP as indicated horizontally. Ocular treatments are indicated vertically. *Green color* indicates staining for phospho-p38, *red* for GFAP, and *blue* nuclei for DAPI. *Far right panels* show double labeling of phospho-p38 and GFAP. There was no positive staining when primary antibodies were omitted (not shown). Fluorescence microscopy; *bar* is 20  $\mu$ m. A total of 10 animals were used in this experiment. Data shown in this figure are a representation of at least five independent experiments.

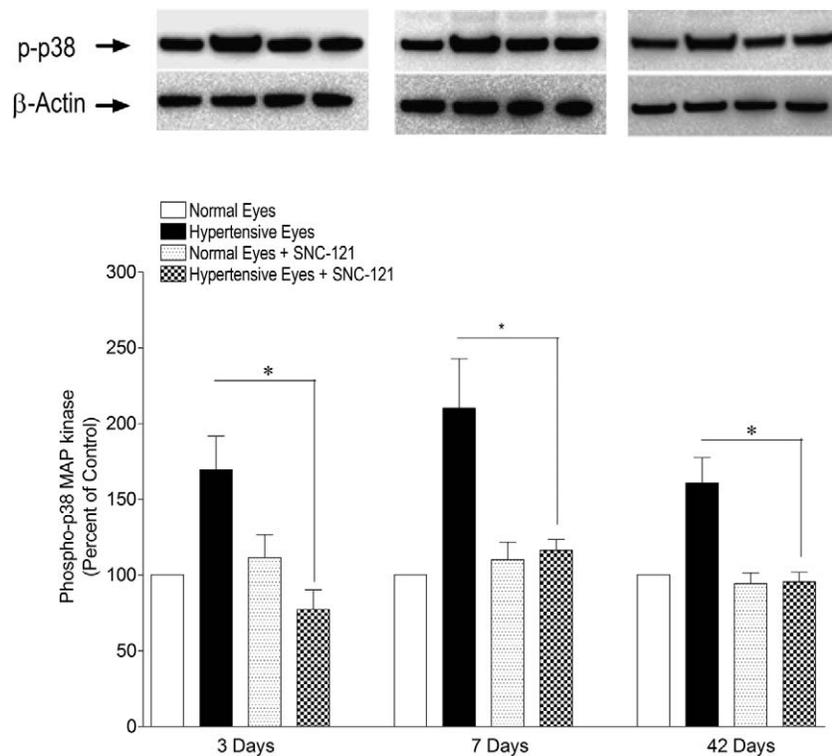
fluorogold-positive RGCs were: healthy eyes,  $1572 \pm 81$ ; ocular hypertensive eyes,  $1122 \pm 38$  (28.6% less than healthy eyes); and SNC-121-treated ocular hypertensive eyes,  $1533 \pm 92$  (36.6% greater than the ocular hypertensive eyes).

To determine if  $\text{TNF-}\alpha$  plays a detrimental role in the early stages of glaucoma development and  $\delta$ -opioid receptor agonist, SNC-121, counterbalances  $\text{TNF-}\alpha$  production, retinal sections of chronic ocular hypertensive rat eyes were analyzed for  $\text{TNF-}\alpha$  expression by immunohistochemistry. As shown in Figure 4, no staining for  $\text{TNF-}\alpha$  was seen in normal eyes (contralateral eye); however, the staining for  $\text{TNF-}\alpha$  was increased in ocular hypertensive eyes, which was inhibited in SNC-121-treated animals. Strong immunostaining was observed for  $\text{TNF-}\alpha$  in the nerve fiber layer and RGC layer. In addition, an occasional punctate staining was observed in the inner plexiform and inner nuclear layers of ocular hypertensive eyes (Fig. 5). To identify the cell type responsible for  $\text{TNF-}\alpha$  production, a dual immunolabeling using GFAP (a glial cell marker) and CRALBP (an RPE and Müller cell marker) was performed. As shown in Figure 4,  $\text{TNF-}\alpha$  and GFAP immunostaining were colocalized (far right panels). Additionally, we have noticed a mild staining for GFAP in healthy eyes and it was increased in the nerve fiber layer of ocular hypertensive eyes, which was also inhibited in SNC-121-treated ocular hypertensive eyes.

As shown in Figure 5, strong CRALBP staining was observed in the RPE and glial Müller cells. However, CRALBP staining was not colocalized with  $\text{TNF-}\alpha$  staining. To further confirm that  $\text{TNF-}\alpha$  seen in immunohistochemistry data are mainly produced from glial cells, we have analyzed the ONH (non-myelinated) samples of normal and ocular hypertensive animals at 3, 7, and 42 days, post hypertonic saline injections.

We chose 3 days as the earliest time point for  $\text{TNF-}\alpha$  detection in optic nerve samples because we have not seen an appreciable increase in the IOP prior to 3 days, in the hypertonic saline injected eyes. As shown in Figure 6,  $\text{TNF-}\alpha$  production was significantly increased in the optic nerve ( $189 \pm 30\%$  over the control) as early as 3 days post injury, and remained significantly elevated up to 42 days ( $184 \pm 31\%$  over the control). The increase in  $\text{TNF-}\alpha$  production was completely inhibited when animals were treated with SNC-121 at each time point. To further confirm our Western blot data, we analyzed optic nerve sections by immunohistochemistry for  $\text{TNF-}\alpha$  production at 42 days, post glaucomatous injury. In this experiment, IOP was measured at 3, 7, 14, 28, and 42 days, and pattern ERG was measured at 14, 28, and 42 days (data not shown). Changes seen in IOP and PERG were comparable to the data shown in Figures 1 and 2. As shown in Figure 7, we saw mild  $\text{TNF-}\alpha$  staining in normal optic nerve, which was further increased in the hypertensive optic nerve. The increased  $\text{TNF-}\alpha$  staining in the ocular hypertensive optic nerve was inhibited in SNC-121-treated animals.

To determine the downstream signaling events, we analyzed retinal sections for the activation of stress-induced protein kinases such as p38 MAP kinase. As shown in Figure 8, phospho-p38 staining was upregulated in ocular hypertensive eyes and colocalized with GFAP staining. The increase in p38 MAP kinase phosphorylation was partially inhibited in the presence of SNC-121. Furthermore, we have analyzed ONH (nonmyelinated) samples of ocular hypertensive eyes for p38 MAP kinase activation. As shown in Figure 9, phosphorylation of p38 MAP kinase was significantly increased at 3 days ( $169 \pm 22\%$  over the control), 7 days ( $210 \pm 33\%$  over the control),



**FIGURE 9.** Activation of p38 MAP kinase in response to glaucomatous injury and its suppression by SNC-121 treatment in the optic nerve extracts. Optic nerves of untreated and SNC-121 treated healthy and ocular hypertensive eyes were collected at 3, 7, or 42 days post injury. Changes in phosphorylation of p38 MAP kinase were analyzed by Western blotting using selective anti-phospho-p38 MAP kinase antibodies. Rats were divided into two groups: ocular hypertensive group ( $n = 6$ ) and SNC-121-treated ocular hypertensive group ( $n = 6$ ). IOP was elevated in one eye of brown Norway rats by injecting approximately 50  $\mu$ L of 2.0 M hypertonic saline, while the contralateral eye served as the control. A total of 36 animals were used in this experiment. Band intensities of Western blots were quantitated by densitometry. Data are expressed as mean  $\pm$  SE. \* $P < 0.05$ ;  $n = 6$  for each group at each time point.

and 42 days ( $161 \pm 17\%$  over the control), post injury. In optic nerve, the increase in p38 MAP kinase phosphorylation was completely inhibited when animals were treated with SNC-121 at all time points. In contrast, we have not seen any changes in the expression pattern of total p38 MAP kinase in the untreated and SNC-121-treated ocular hypertensive eyes (data not shown).

To establish a causal link between TNF- $\alpha$  and p38 MAP kinase activation in glial cells, we used primary cultures of optic nerve head (ONH) astrocytes. Cells were pretreated with SNC-121 followed by TNF- $\alpha$  (25 ng/mL) treatment for 6 hours and phosphorylation of p38 MAP kinase was determined by Western blotting using selective anti-phospho-p38 MAP kinase antibodies. A concentration response study was performed in ONH astrocytes using TNF- $\alpha$  for p38 MAP kinase activation, where we found that 25 ng/mL TNF- $\alpha$  provided maximum phosphorylation and activation of p38 MAP kinase (data not shown). As shown in Figure 10, TNF- $\alpha$  increased the phosphorylation of p38 MAP kinase over 2-fold at 6 hours (control  $100 \pm 00$  versus TNF- $\alpha$   $231 \pm 31$ ;  $n = 4$ ;  $P = 0.024$ ). TNF- $\alpha$ -induced increase in p38 MAP kinase phosphorylation in ONH astrocytes was significantly inhibited in the presence of SNC-121 (TNF- $\alpha$   $231 \pm 31$  versus SNC-121 + TNF- $\alpha$   $128 \pm 25$ ;  $n = 4$ ;  $P = 0.041$ ).

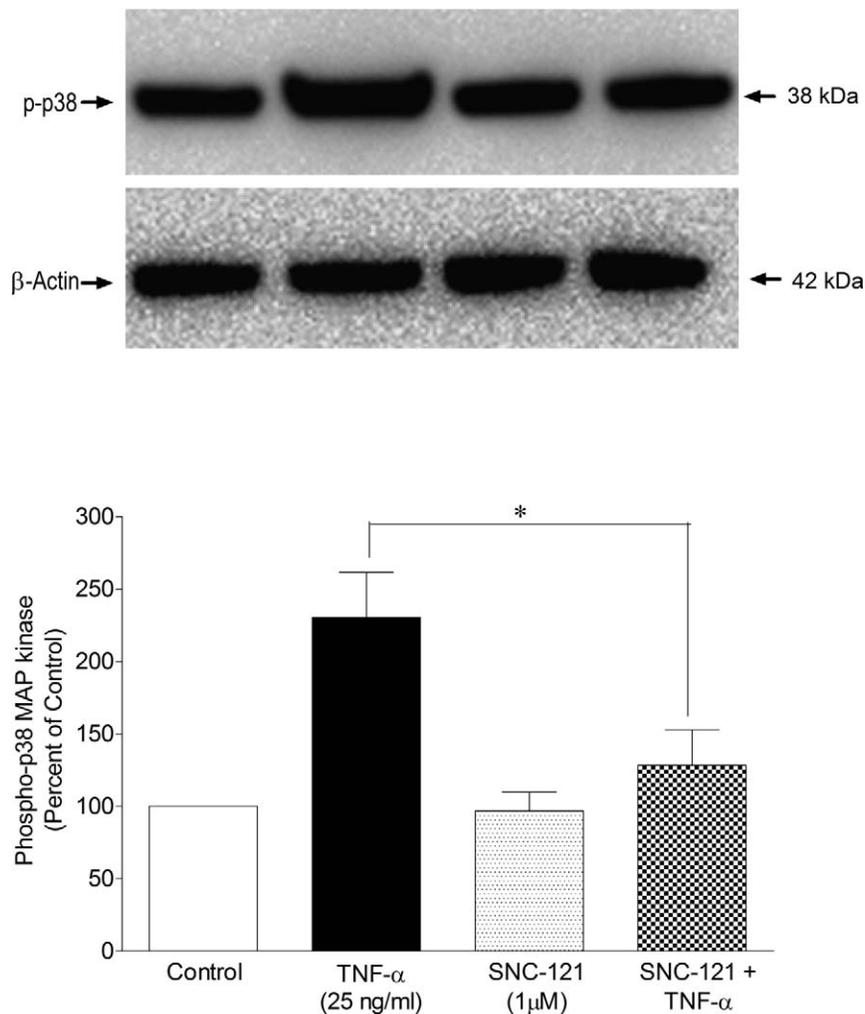
To determine if  $\delta$ -opioid receptors were downregulated or upregulated in response to glaucomatous injury, expression of  $\delta$ -opioid receptors was measured in ocular hypertensive eyes at 3, 7, and 42 days post injury. IOP was increased when measured at 3 and 7 days. As shown in Figures 11A and 11B,  $\delta$ -opioid receptors were moderately upregulated in ocular

hypertensive eyes at 3 days (44.6% over the healthy eye;  $n = 7$ ;  $P = 0.0078$ ) and 7 days (49% over the healthy eye;  $n = 7$ ;  $P = 0.0028$ ).

## DISCUSSION

Opioids have been used clinically for centuries as analgesics. However, other biological effects induced by opioids include cytoprotection, immunomodulation, neuroendocrine regulation, and behavioral modification. Most of these biological responses are presumed to be manifested through the activation of G-protein-coupled  $\delta$ -,  $\kappa$ -, and  $\mu$ -opioid receptors. In the eye, activation of opioid receptors has been implicated in the regulation of iris function, accommodative power, regulation of aqueous humor dynamics (e.g., IOP), corneal wound healing, retinal development, and retina neuroprotection.<sup>15,17,21-23</sup>

$\delta$ -opioid receptors (also known as DOR or DOP receptors in the International Union of Basic and Clinical Pharmacology [IUPHAR] nomenclature) are increasingly attractive due to their therapeutic potential. Along with the development of highly selective  $\delta$ -opioid receptor agonists and rapid progress in mouse mutagenesis approaches targeting the  $\delta$ -opioid receptor gene (*Oprd1*), novel functions of  $\delta$ -opioid receptors have emerged. For example, activation of  $\delta$ -opioid receptors reduced infarct size in stroke and myocardial ischemia models.<sup>24,25</sup> Activation of  $\delta$ -opioid receptors is protective against hypoxia/excitotoxic injury in cortical neurons.<sup>26</sup> DADLE, a  $\delta$ -opioid receptor agonist, has been shown to improve hippocampal neuronal survival against ischemia in a

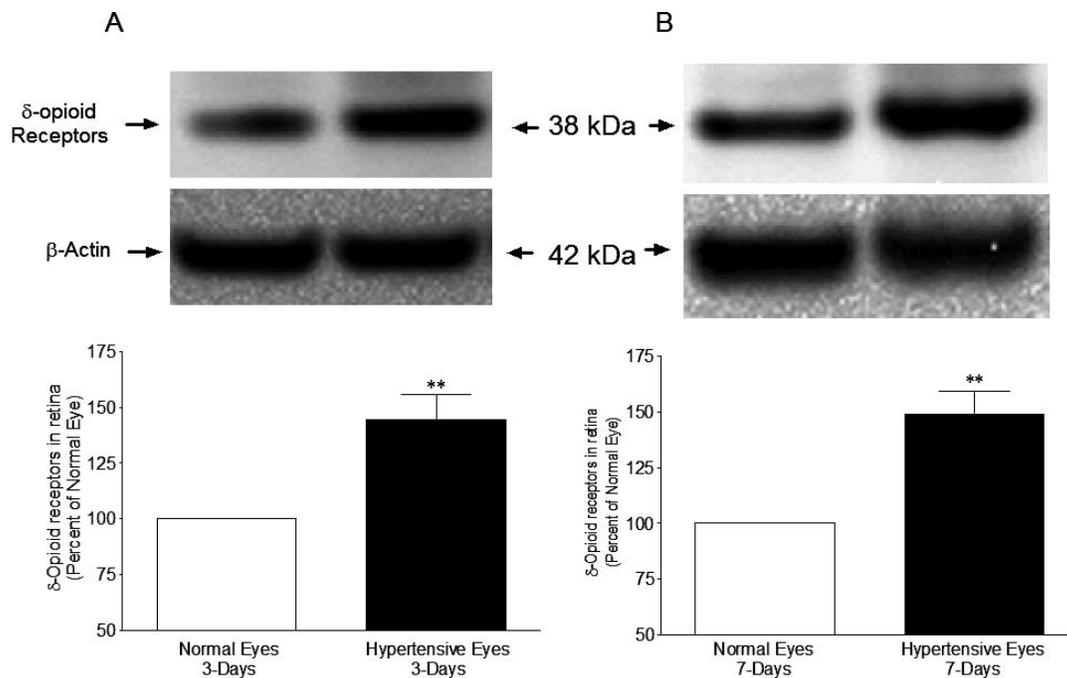


**FIGURE 10.** TNF- $\alpha$ -induced activation of p38 MAP kinase in ONH astrocytes. ONH astrocytes were starved in serum-free medium for overnight. Cells were then pretreated with SNC-121 (1  $\mu$ mol/L) for 15 minutes followed by TNF- $\alpha$  (25 ng/mL) treatment for 6 hours. Cell lysate (15  $\mu$ g protein) was analyzed by Western blotting using selective anti-phospho-p38 MAP kinase antibodies followed by incubation with appropriate secondary antibodies (HRP-conjugated; dilution 1:3000). The signal was captured using enhanced chemiluminescent reagent and the Biorad Versadoc imaging system. Data shown is a representative of four independent experiments. Data are expressed as mean  $\pm$  SE. \* $P$  < 0.05;  $n$  = 4.

rat model.<sup>27</sup> Studies have also shown that  $\delta$ -opioid receptor activation attenuates oxidative injuries in the brain exposed to ischemia/reperfusion by enhancing antioxidant ability and inhibition of caspase activity.<sup>28</sup> Moreover,  $\delta$ -opioid receptors have been involved in neuroprotective mechanisms of Alzheimer<sup>29</sup> and Parkinson's<sup>30</sup> diseases. In this manuscript we have shown an upregulation of  $\delta$ -opioid receptors in response to the glaucomatous injury, which may have been involved in the endogenous neuroprotection. Although  $\delta$ -opioid receptors are upregulated in response to glaucomatous injury, there may not be enough endogenous ligand to initiate downstream neuroprotective signaling cascades to protect the retina against glaucomatous injury. When animals were treated with an exogenous ligand, such as SNC-121,  $\delta$ -opioid receptors were activated and initiated a neuroprotective downstream signaling cascade to protect the retina.  $\delta$ -opioid receptor activation curtailed the detrimental cellular events at an early stage of the injury. Once the initial cellular events were counteracted by SNC-121 in the initial phase of glaucomatous injury, RGCs can be preserved, as seen here in Figures 2 and 3. Although beneficial effects of  $\delta$ -opioid receptor agonists in nonocular systems have been clearly established in the areas of

chronic pain, emotional disorders, and neuroprotection against various forms of injuries, the usefulness of  $\delta$ -opioid receptor agonists against glaucomatous injury remains to be clarified.

Glaucoma is a long term optic neuropathy characterized by optic disc cupping, RGC death, and vision loss. POAG is the most common type of glaucoma. Glaucoma pathogenesis is multifactorial; however, a major risk factor for the development of glaucoma is elevated IOP. The pathophysiologic mechanisms by which elevated IOP leads to optic nerve atrophy and retinal degeneration are unknown. This manuscript provides novel findings that  $\delta$ -opioid receptor agonist treatment, such as with SNC-121, provides significant retinal neuroprotection against glaucomatous injury, thus, suggesting that  $\delta$ -opioid receptor agonists have a potential to be used as therapeutic agents against glaucomatous injury. The possible involvement of other opioid receptors (e.g.,  $\kappa$  and  $\mu$ ) in retina neuroprotection has not been ruled and it will be tested in our future studies. Recently, we have demonstrated that opioid receptor activation is required for the development of ischemic preconditioning within the retina, and that the administration of morphine can reduce retinal ischemic and glaucomatous injury.<sup>15,16</sup> Although the cellular mechanisms that are involved



**FIGURE 11.** Determination of  $\delta$ -opioid receptors in healthy and ocular hypertensive rat retinas at 3 (A) and 7 days (B) post injury. Retinal extract (15–20  $\mu$ g proteins) were analyzed by Western blotting using selective anti- $\delta$  antibodies followed by incubation with appropriate secondary antibodies (HRP-conjugated; dilution 1:3000). The signal was captured using enhanced chemiluminescent reagent and the Biorad Versadoc imaging system. A total of 14 rats were used in this experiment. Data shown are representative of seven independent experiments. Data are expressed as mean  $\pm$  SE. \* $P < 0.05$ ;  $n = 7$  for each time point.

in opioid-mediated retina neuroprotection are poorly understood, we have shown that morphine-induced retina neuroprotection against ischemia- and glaucoma-induced retina injury was mediated via, in part, inhibition of TNF- $\alpha$  production, caspase-8, and caspase-3 activation.<sup>16,17</sup>

Data presented in this manuscript provide evidence that  $\delta$ -opioid receptor activation acts to mitigate injurious events that lead to RGC death against glaucomatous injuries, as determined by PERG (Fig. 2). Studies in humans, primates, and rodents have shown that PERG is an indicator of RGC function.<sup>31,32</sup> Studies have shown that overall PERG response is reduced in glaucomatous conditions.<sup>33</sup> In the current study, a significant reduction (24%) in PERG was seen in ocular hypertensive eyes 6 weeks post injury. The loss in PERG amplitudes was significantly blocked in the presence of SNC-121 (Fig. 2). Furthermore, the rate of RGC loss was reduced in SNC-121-treated ocular hypertensive animals, as determined by fluorogold retrograde labeling (Fig. 3). In this study, we have not determined axonal loss in ocular hypertensive eyes. However, the reduction in fluorogold labeling of RGCs in ocular hypertensive eyes could be due to axonal loss, which might have also been improved in SNC-121-treated animals. Furthermore, data presented in this manuscript demonstrated that SNC-121-induced retina neuroprotection is IOP-independent, because IOP was not reduced in SNC-121-treated ocular hypertensive animals, while both PERG and RGC counts were significantly improved. Although studies have shown that opioid receptor agonists (e.g., morphine and bremazocine; 100  $\mu$ g/2–4 kg body weight) do lower IOP in rabbits when applied topically,<sup>34–36</sup> it has remained unknown if  $\delta$ -opioid agonists lower IOP in other rodents. In contrast, we have not seen any decline in IOP when rats were treated with SNC-121 (1 mg/kg) daily intraperitoneally for 7 days. This discrepancy could be due to the dosage of SNC-121, route of drug administration (e.g., topically versus IP), or species differences.

In glaucoma, numerous retina proteins have been demonstrated to be upregulated during the progression of disease, including TNF- $\alpha$ , TNF-R1, various protein kinases, and proteolytic caspases.<sup>6</sup> To dissect out downstream signaling targets in  $\delta$ -opioid receptor-mediated retina neuroprotection, first we determined the effects of SNC-121-treatment on TNF- $\alpha$  production within the retina. Interestingly, TNF- $\alpha$  production was completely inhibited in SNC-121-treated ocular hypertensive eyes (Fig. 4). These data support the hypothesis that  $\delta$ -opioid receptor activation opposes the production of TNF- $\alpha$  during early phases of glaucomatous injury. The identification of the main source(s) of TNF- $\alpha$  production under the glaucomatous condition had remained in question. A variety of cell types including activated macrophages, astrocytes, microglia, Müller cells, and/or neuronal cells under stress/glaucomatous conditions have been proposed for the enhanced production of TNF- $\alpha$ . To identify the cell type(s) involved in TNF- $\alpha$  production in glaucomatous conditions, we dually immunostained retinal sections with TNF- $\alpha$  along with activated glial cell markers (GFAP) and a glial Müller cell marker (CRALBP). As shown in this manuscript, TNF- $\alpha$  staining was colocalized with GFAP (Fig. 4) and not with CRALBP (Fig. 5), suggesting that glial cells, but not the Müller cells, are the primary source for TNF- $\alpha$  production in glaucomatous conditions. Earlier we have shown that resident ONH astrocytes and microglia cells are the major sources of TNF- $\alpha$  production and the site of opioid agonist actions for TNF- $\alpha$  suppression during acute ischemic injury.<sup>17</sup> New data presented in this manuscript and other published reports provide insight that glial cells are one of the major sources for TNF- $\alpha$  production under glaucomatous injury. Studies have shown that opioid receptor activation regulates multiple cellular processes including activation of MAP kinases.<sup>37</sup> Considering the detrimental roles of p38 MAP kinase in various injury models, we have determined the changes in the phosphory-

lation and activation of p38 MAP kinase in response to TNF- $\alpha$ -and/or glaucoma-induced injury. Immunostaining data shown in this manuscript clearly demonstrated a sustained increase in the phosphorylation of p38 MAP kinase in ocular-hypertensive eyes, which was inhibited in SNC-121-treated hypertensive eyes. Dual immunostaining data in retinal sections and Western blot data in optic nerve samples further support the idea that activation of p38 MAP kinase is taking place within the optic nerve at an early stage of glaucomatous injury. Studies have shown that p38 MAP kinase and c-Jun N-terminal kinases (JNK) are activated by stress and contribute in the neurodegenerative signaling pathways, whereas ERK1/2 have been shown to play neuroprotective roles against various forms of injuries.<sup>38-41</sup> Studies also have shown that increased activity of p38 MAP kinase plays a critical role in cell death in neurons under stress conditions.<sup>41</sup> Inhibition of p38 MAP kinase confers neuroprotection in vitro against excitotoxic exposure<sup>42</sup> and reduces acute ischemic injury in vivo.<sup>43</sup> Studies also have shown that  $\delta$ -opioid receptor agonist, DADLE, provides neuroprotection against oxygen-glucose deprivation-induced neuronal injury via inhibition of p38 MAP kinase phosphorylation and activation.<sup>40</sup> Additionally, p38 MAP kinase plays a key role in the glutamate-induced apoptosis of RGCs,<sup>44</sup> and the increased phosphorylation of p38 and JNK have been noted in an experimental glaucoma model.<sup>45</sup>

To establish a direct link between TNF- $\alpha$  and p38 MAP kinase, we determined the effect of TNF- $\alpha$  on p38 MAP kinase activation in isolated ONH astrocytes. TNF- $\alpha$ -induced increase in p38 MAP kinase phosphorylation was significantly inhibited in the presence of SNC-121. These data provide concrete support that p38 MAP kinase is a downstream target of TNF- $\alpha$  within the optic nerve.

Based on the data shown in this manuscript, and previously published work from our laboratory in acute ischemia and chronic glaucoma models,<sup>15-17</sup> it is evident that TNF- $\alpha$  production in response to retinal injuries is an early event. The current manuscript provides novel information that sustained IOP elevation and glial cell activation caused a sustained TNF- $\alpha$  production within the optic nerve, and activated downstream signaling targets such as p38 MAP kinase. Activation of these signaling pathways may subsequently enhance the production of extracellular matrix degrading enzymes, matrix metalloproteinases (MMPs), which will further destabilize the optic nerve by excessive remodeling. These detrimental signaling molecules directly and/or indirectly weaken axons and lead to the RGC death.  $\delta$ -opioid receptor agonists represent a novel class of drugs/agents that counteract detrimental events (e.g., TNF- $\alpha$  production and p38 MAP kinase activation) within the optic nerve at an early stage of disease development, which delays and/or prevents the loss of RGCs. Thus, therapeutic approaches that primarily inhibit excessive astrocyte reactivity, proinflammatory cytokine activity, and downstream activation and/or expression of signaling targets (e.g., stress-activated protein kinase; p38 kinase), should impede deleterious changes in the optic nerve and RGCs in eyes predisposed to glaucoma.

In summary, our study provides evidence that  $\delta$ -opioid receptors play key roles in retina neuroprotection against glaucomatous injury because: (1) RGC function was preserved by exogenous SNC-121 treatment in chronic ocular hypertensive rat eyes, as determined by PERG; (2) loss of RGCs was reduced in SNC-121-treated hypertensive eyes, as determined by retrograde labeling of RGCs; (3) TNF- $\alpha$  is mainly produced from glial cells in chronic ocular hypertensive eyes, as determined by immunohistochemistry; (4) TNF- $\alpha$  production in response to glaucomatous injury is inhibited in the presence of SNC-121; and (5) p38 MAP kinase, a downstream target of TNF- $\alpha$ , activation is suppressed by SNC-121 treatment. Overall,

TNF- $\alpha$  and p38 MAP kinase appear to be potential therapeutic targets to achieve neuroprotection against glaucomatous injury. Additionally, enhancement of  $\delta$ -opioidergic activity in the eye may present a viable neuroprotective strategy for the treatment of glaucoma.

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