Published in final edited form as:

Eur J Neurosci. 2009 July ; 30(2): 175–185. doi:10.1111/j.1460-9568.2009.06814.x.

Inactivation of astroglial NF- κ B promotes survival of retinal neurons following ischemic injury

Galina Dvorianchikova¹, David Barakat², Roberta Brambilla³, Christian Agudelo⁴, Eleut Hernandez¹, John R. Bethea³, Valery I. Shestopalov^{1,2}, and Dmitry Ivanov^{1,5}

¹Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami Miller School of Medicine, Miami, FL, USA

²Department of Cell Biology and Anatomy, University of Miami Miller School of Medicine, Miami, FL, USA

³The Miami Project to Cure Paralysis, University of Miami Miller School of Medicine, Miami, FL, USA

⁴Department of Physiology and Biophysics, University of Miami Miller School of Medicine, Miami, FL, USA

⁵Vavilov Institute of General Genetics RAS, Moscow, Russian Federation

Abstract

Reactive astrocytes have been implicated in neuronal loss following ischemic stroke. However, the molecular mechanisms associated with this process are yet to be fully elucidated. In this work, we tested the hypothesis that astroglial NF- κ B, a key regulator of inflammatory responses, is a contributor to neuronal death following ischemic injury. We compared neuronal survival in the ganglion cell layer after retinal ischemia-reperfusion in wild type and in GFAP-I κ B α -dn transgenic mice, where the NF- κ B classical pathway is suppressed specifically in astrocytes. The GFAP-I κ B α -dn mice showed significantly increased survival of neurons in the ganglion cell layer following ischemic injury as compared to WT littermates. Neuroprotection was associated with significantly reduced expression of pro-inflammatory genes, encoding *Tnf- α* , *Ccl2 (Mcp1)*, *Cxcl10 (IP10)*, *Icam1*, *Vcam1*, several subunits of NADPH oxidase and NO synthase in the retinas of GFAP-I κ B α -dn mice. These data suggest that certain NF- κ B-regulated pro-inflammatory and redox-active pathways are central to glial neurotoxicity induced by ischemic injury. The inhibition of these pathways in astrocytes may represent a feasible neuroprotective strategy for retinal ischemia and stroke.

Keywords

astrocytes; inflammation; ischemia; retinal pathology; transgenic mice

Introduction

Stroke is the third leading cause of death in the United States after heart disease and cancer, and is responsible for 10% of deaths world-wide. Ischemic stroke comprises about 80% of

Correspondence: Dr. D Ivanov and Dr. V Shestopalov, Bascom Palmer Eye Institute, Departments of Ophthalmology, University of Miami Miller School of Medicine, 1638 NW 10th Ave, Miami, FL 33136, USA, Tel 305-326-6344, Fax 305-547-3718. divanov@med.miami.edu, vshestopalov@med.miami.edu.
Editor: Dr. Jean-Marc Fritschy

all stroke cases (Feigin 2005). Similarly to other CNS diseases and insults, ischemic stroke triggers astrocytic activation, commonly referred to as astrogliosis, in and around the infarcted region of the brain (Stoll, Jander et al. 1998; Mabuchi, Kitagawa et al. 2000). Although astrogliosis appears to be beneficial during the initial stages of injury (Ridet, Malhotra et al. 1997; Sofroniew 2005), chronic astroglial activation sustains neuroinflammation, a major cause of neuronal dysfunction and death in neurodegenerative disorders (Pekny and Nilsson 2005). Indeed, when exposed to various stress stimuli, astrocytes synthesize and release a host of molecules ranging from cytokines, chemokines, immune mediators, adhesion molecules and nitric oxide, which establish a pro-inflammatory environment detrimental to CNS recovery (Falsig, Latta et al. 2004; Swanson, Ying et al. 2004; Pekny and Nilsson 2005). The transcription factor NF- κ B is a master regulator of the cellular responses to injury, inflammation and other stresses (Ridet, Malhotra et al. 1997; Nomoto, Yamamoto et al. 2001; Swanson, Ying et al. 2004; Sofroniew 2005). In the CNS, significant NF- κ B activation is detected in both neuronal and glial cells following injury or disease (Bethea, Castro et al. 1998; Brambilla, Bracchi-Ricard et al. 2005). The contribution of NF- κ B activation to CNS pathology has been clearly demonstrated in several models of CNS injury, including trauma (Brambilla, Bracchi-Ricard et al. 2005), cerebral ischemia (Herrmann, Baumann et al. 2005), experimental autoimmune encephalomyelitis (van Loo, De Lorenzi et al. 2006; Brambilla, Persaud et al. 2009), and Parkinson's disease (Dehmer, Heneka et al. 2004). It has been suggested that functional improvement from transgenic inhibition of NF- κ B in astrocytes is achieved via suppressing chronic CNS inflammation, highlighting the direct role of astrocytes and astroglial NF- κ B at the late phases of CNS pathologies (Brambilla, Bracchi-Ricard et al. 2005). In this work we sought to investigate the role of NF- κ B signaling at early stages following the ischemia-reperfusion (IR) injury (Selles-Navarro, Villegas-Perez et al. 1996; Kawai, Vora et al. 2001; Neufeld, Kawai et al. 2002) and utilized the same transgenic model with NF- κ B is inactivated in astrocytes by overexpressing a dominant negative form of the I κ B α super-repressor (I κ B α -dn). Our results demonstrate that selective inhibition of astroglial NF- κ B significantly reduces ischemic stroke severity in the retina, providing a direct demonstration of the crucial role of astrocytes in the pathophysiology of acute CNS injury.

Materials and Methods

Animals

All experiments were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and according to the IACUC approved protocols. GFAP-I κ B α -dn transgenic mice were generated on a C57BL/6 X SJL background and backcrossed at least 16 generations onto wild type C57BL/6 mice to establish transgenic lines (Brambilla, Bracchi-Ricard et al. 2005). All animals used in our experiments were 3 month old male mice (7 animals per group), obtained by breeding heterozygous GFAP-I κ B α -dn males with WT females. WT littermates from the same breeding were used as controls.

Transient retinal ischemia

After anesthesia with intraperitoneal ketamine (80 mg/kg) and xylazine (16 mg/kg), pupils were dilated with 1% tropicamide-2.5% phenylephrine hydrochloride (NutraMax Products, Inc., Gloucester, MA), and corneal analgesia was achieved with 1 drop of 0.5% proparacaine HCl (Bausch & Lomb Pharmaceuticals, USA). Retinal ischemia was induced for 60 minutes by introducing a 33-gauge needle attached to a normal (0.9% NaCl) saline-filled reservoir into the anterior chamber of the eye. The saline reservoir was elevated to increase intraocular pressure (IOP) above systolic blood pressure (IOP increased to 120 mm Hg). The contralateral eye was cannulated and maintained at normal IOP to serve as a normotensive control. Complete retinal ischemia, evidenced by a whitening of the anterior

segment of the eye and blanching of the retinal arteries, was verified by microscopic examination. After needle removal, 1% 5 atropine and 1% vetropolycin with hydrocortisone ointment (Fougera & Atlanta, Inc., Melville, NY) were applied to the conjunctival sac. Mice were sacrificed by CO₂ inhalation under anesthesia.

Electrophoretic mobility shift assay (EMSA)

The preparation of retinal nuclear extracts and determination of the NF- κ B DNA-binding activity were performed with a nuclear and cytoplasmic reagent kit (NE-PER; Pierce) and an electrophoretic mobility shift assay (EMSA) chemiluminescence kit (LightShift; Pierce), respectively, according to the manufacturer's protocols. A double-stranded oligonucleotide containing an NF- κ B DNA-binding consensus sequence, 5'-AGT TGA GGG GAC TTT CCC AGG C-3' was used to study NF- κ B DNA-binding activity, as previously described (Zeng, Tso et al. 2008). Briefly, 2 μ g of nuclear extracts from the whole retina was preincubated in a reaction mixture for 20 min, and a biotin end-labeled, double-stranded oligonucleotide containing the κ B consensus sequence was added. Next, 5 μ l of loading buffer was added to each sample. A 20 μ l aliquot of each sample was electrophoresed through a 6% nondenaturing polyacrylamide gel. The hybridization signal was quantified by intensitometry using Quantity One Image Software (Bio-Rad Laboratories)

Immunohistochemistry for NeuN in flat mounted retinas

Seven days after IR, we analyzed the loss of retinal ganglion cell layer neurons. Eyes were enucleated upon euthanasia, incised at the ora serrata and immersion-fixed in a 4% paraformaldehyde solution (in PBS, pH 7.4) for 1 hour, and the retinas were removed. The retinas were cryoprotected overnight in 30% sucrose followed by 3 freeze-thaw cycles, were rinsed 3 $\times$ 10 minutes in 0.1 M PBS, blocking by 5% donkey serum, 0.1% Triton X-100 in 0.1M tris buffer (TB) for 1 hour, and incubated overnight with monoclonal FITC-conjugated NeuN antibody (Chemicon, USA; dilution 1:300). After 3 $\times$ 10 minutes rinse in 0.1M TB retinas were flatmounted, coverslipped and imaged using a Leica TSL AOBS SP5 confocal microscope (Leica Microsystems, Exton, PA).

Counting of NeuN positive GCL neurons

NeuN-positive neurons in the ganglion cell layer (GCL), including retinal ganglion cells (RGCs) and displaced amacrine cells, were imaged by confocal microscopy in flatmounted retinas. To avoid topological irregularities, stacks of 5 serial images were collapsed to generate "maximum projections" (standard feature of the Leica LAS AF software), where all imaged cells appear in sharp focus. Individual retinas were sampled randomly to collect a total of 20 images located at the same eccentricity (1–1.5 mm from the optic disk) in the four retinal quadrants using 20 $\times$ objective lens. NeuN-positive neurons with the size range of 6–30 μ m were counted semi-automatically using MetaMorph (Universal Imaging Co., USA) software, after image thresholding and manual exclusion of artifacts. Cell loss in the ischemic retinas was calculated as percentile of the mean cell density in normotensive fellow control eyes. We observed IR-induced loss of retinal neurons 7 days after reperfusion.

Real-time PCR analysis

Real-time PCR analysis was performed as described previously (Ivanov, Dvorianchikova et al. 2006) using gene-specific primers (Table 1). The GFAP-I κ B α -dn transgene was amplified using of forward I κ B α (5'-TTC ATA AAG CCC TCG CAT CC-3') and reverse I κ B α (5'-ACA GCC AGC TCC CAG AAG TG-3') primers. Total RNA was extracted from retinas using Absolutely RNA Nanoprep kit (Stratagene, USA), reverse transcribed with Superscript III (Invitrogen, USA) polymerase to synthesize cDNA. Real time PCR was performed in the Rotor-Gene 6000 Cyclor (Corbett Research, Australia) using the SYBR

GREEN PCR MasterMix (Qiagen, USA). For each gene, relative expression was calculated by comparison with a standard curve, following normalization to the housekeeping genes β -actin (*Actb*), *Sdha*, *Thp* expression chosen as controls. We assessed gene expression 24 hours post-reperfusion.

Immunohistochemistry

Fixed retinas were sectioned to a thickness of 100 μ m with a vibratome (Vibratome, St. Louis, MO) and immunostained using the protocol described previously (Ivanov, Dvorianchikova et al. 2006; Ivanov, Dvorianchikova et al. 2008). Sections were incubated with various primary antibodies (Table 1), followed by species-specific secondary fluorescent antibodies (AlexaFluor, Invitrogen, USA). Control sections were incubated without primary antibodies. Imaging was performed with a Leica TSL AOBS SP5 confocal microscope (Leica Microsystems, PA).

Protein oxidation assay

We evaluated protein oxidation by detecting the carbonylated derivatives of Pro, Arg, Lys and Thr aminoacids, the most common products of protein oxidation by ROS and secondary by-products of oxidative stress. We utilized the OxiSelect™ Protein Carbonyl ELISA Kit (Cell Biolabs, CA) to quantify protein carbonyls determined by comparing the absorbance with that of a known reduced/oxidized BSA standard curve, according to manufacturer's instructions. Protein carbonylation was assayed 1 and 3 days post-reperfusion.

Statistical analysis

Statistical analysis of real time PCR and cell density data was performed with one-way ANOVA followed by the Tukey test for multiple comparisons. In the case of single comparisons, the Student's T test was applied. P values equal to or less than 0.05 were considered statistically significant.

Results

Inhibition of astroglial NF- κ B reduced severity of ischemic damage to the retina

To evaluate the effect of astroglial NF- κ B inhibition on the severity of acute ischemia, we took advantage of the transgenic mouse model previously characterized by Brambilla and colleagues (Brambilla, Bracchi-Ricard et al. 2005; Bracchi-Ricard, Brambilla et al. 2008; Brambilla, Persaud et al. 2009). Briefly, the NF- κ B inhibition in GFAP-I κ B α -dn transgenic mice was induced by overexpressing a dominant negative form of the inhibitor of NF- κ B-alpha (I κ B α) under the control of the GFAP promoter that is active in astrocytes. An extensive testing has demonstrated that GFAP-I κ B α -dn mice are indistinguishable from the WT littermates in every aspect, showing no phenotypic abnormalities (Brambilla, Bracchi-Ricard et al. 2005; Bracchi-Ricard, Brambilla et al. 2008; Brambilla, Persaud et al. 2009). The expression of the transgene is limited to astrocytes and GFAP-expressing non myelinating Schwann cells. No ectopic expression of the transgene was found in neurons or in any other tissue outside of the nervous system (Brambilla, Bracchi-Ricard et al. 2005; Bracchi-Ricard, Brambilla et al. 2008; Brambilla, Persaud et al. 2009). We measured the transgene expression by qRT-PCR, which was detected only in the retinas of the GFAP-I κ B α -dn but not the WT mice. However, in the ischemic retinas of the GFAP-I κ B α -dn mice, the expression of the transgene was up-regulated 2.3 ± 0.2 fold change ($P=0.04$, $n=6$) relative to sham operated eyes 24 hours after reperfusion. To functionally confirm that the truncated I κ B α activates in the retina leading to the suppression of NF- κ B, a series of electrophoretic mobility shift assays (EMSAs) were performed using the nuclear extracts from the ischemic and sham operated eyes of WT and GFAP-I κ B α -dn mice (Fig. 1, A and B). WT ischemic

retinas 24 hours after reperfusion exhibited a strong induction of the NF- κ B DNA-binding activity, whereas in ischemic retinas of the GFAP-I κ B α -dn mice such DNA-binding activity was significantly reduced by 1.40 ± 0.02 fold change ($P=0.01$, $n=5$, Fig. 1, A and B). This implies that activation of NF- κ B induced by IR injury is attenuated in the retinas of GFAP-I κ B α -dn mice, this finding is similar to the effect observed in the spinal cord (Brambilla, Bracchi-Ricard et al. 2005; Bracchi-Ricard, Brambilla et al. 2008; Brambilla, Persaud et al. 2009).

We induced unilateral retinal ischemia in the WT and GFAP-I κ B α -dn mice by raising the intra ocular pressure (IOP) above normal systolic levels and evaluated neuronal survival. This model of retinal IR injures cells located predominantly in the ganglion cell layer (Kawai, Vora et al. 2001; Goto, Ota et al. 2002; Levin and Gordon 2002; Neufeld, Kawai et al. 2002; Wang, Niwa et al. 2002; Kim, Ju et al. 2004; Osborne, Casson et al. 2004). Similar to ischemic stroke, IR-induced degeneration of RGCs is biphasic with a primary degeneration occurring within 24 hours after reperfusion and a secondary degeneration progressing over several days (Selles-Navarro, Villegas-Perez et al. 1996), we decided to evaluate neuronal survival in the ganglion cell layer (GCL) one week after reperfusion to be able to detect both waves of degeneration. We observed IR-induced loss of retinal neurons 7 days after reperfusion in both WT and GFAP-I κ B α -dn retinas (Fig. 2). However, when neuronal loss in the GCL was compared between WT and GFAP-I κ B α -dn mice, significant differences in neuronal viability became apparent. Retinas from experimental eyes of WT mice had significantly ($P=0.0016$, $n=7$) lower numbers of surviving NeuN-positive neurons ($63 \pm 7\%$) in the GCL as compared to GFAP-I κ B α -dn mice ($91 \pm 3\%$). GFAP-I κ B α -dn mice were protected from IR injury as no significant difference was found between ischemic and naïve retinas in the number of viable GCL neurons (Fig. 2). NeuN immunohistochemistry in flat-mounted retinas demonstrated that affected neurons were homogeneously distributed across ischemic retinas, without any geographic pattern of neuronal degeneration in the GCL (Fig. 2, A and B).

Inhibition of astroglial NF- κ B resulted in reduced expression of pro-inflammatory genes following retinal ischemia

To study the molecular changes associated with the elevated resistance to ischemia we compared the expression of several anti-inflammatory and pro-inflammatory genes, the latter known to be involved in IR-induced cytotoxicity, between the two genotypes following IR-injury. We chose to assess gene expression at the early time point of 24 h post-reperfusion, since most changes in gene expression for these molecules typically occur shortly after ischemic injury. Because most of the events proceeding IR retinal injury occur in the GCL, our analysis of expression in the whole retina reflects events occurring mainly in this layer (Kawai, Vora et al. 2001; Goto, Ota et al. 2002; Levin and Gordon 2002; Neufeld, Kawai et al. 2002; Wang, Niwa et al. 2002; Kim, Ju et al. 2004; Osborne, Casson et al. 2004). Transcriptional upregulation of the cytokines *Il1 β* , *Il6*, *Lif*, *Tnf- α* , the chemokines *Ccl2* (*Mcp1*), *Ccl5* (*Rantes*), *Cxcl10* (*IP10*) and the cell adhesion molecules *Icam1* and *Vcam1* was evident in all experimental eyes 24 h after reperfusion (Fig. 3). In GFAP-I κ B α -dn mice, however, the expression of *Tnf- α* , *Ccl2*, *Cxcl10*, *Icam1* and *Vcam1* was significantly reduced compared to WT. In contrast, we did not detect statistically significant differences in IR-induced upregulation of *Il1 β* , and *Ccl5* genes between the two genotypes. Furthermore, *Il6* was significantly upregulated only in WT retinas (Fig. 3) and *Ncam1* expression remained largely unchanged in both genotypes (Fig. 3).

The gene expression profiles for *Tnf- α* and *Vcam1* were consistent with the levels of protein accumulation detected by immunohistochemistry 24 h after reperfusion, which were higher in WT mice compared to GFAP-I κ B α -dn (Fig. 4). Additionally, the expression of GFAP appeared to be increased in the WT mice compared to the transgenics (Fig. 4), suggesting a

higher level of astrocytic activation in this genotype following ischemic injury. Combined, these data indicate that, as a result of astrocytic NF- κ B inactivation, the inflammatory response observed in the retina 24 h after reperfusion was significantly suppressed in GFAP-I κ B α -dn eyes.

Ischemic astrocytes were shown to release multiple anti-inflammatory immunoregulatory molecules such as *Tgfb1*, *Il10*, *Cx3cl1* (fractalkine) (Hughes, Botham et al. 2002; Dhandapani, Hadman et al. 2003; Tichauer, Saud et al. 2007) and neurotrophins (*Ngf* and *Ntf3*) (Widenfalk, Lundstromer et al. 2001), with protective effects following CNS injury. Our gene expression data indicated a statistically significant elevation of *Tgfb1* gene expression in WT mice compared to transgenic after IR-injury, while *Ngf* and *Ntf3* remained largely unchanged in GFAP-I κ B α -dn vs WT littermates (Fig. 3).

Inhibition of astroglial NF- κ B resulted in reduced expression of genes associated with oxidative stress following IR

IR injury is typically associated with overproduction of damaging ROS and NO⁻ by NAD(P)H oxidase and NO synthase (Facchinetti, Dawson et al. 1998). The NAD(P)H oxidase complex includes two membrane-bound elements: Cybb (gp91^{PHOX}) and Cyba (p22^{PHOX}), three cytosolic components: Ncf2 (p67^{PHOX}), Ncf1 (p47^{PHOX}) and Ncf4 (p40^{PHOX}), and a low-molecular-weight G protein (either Rac2 or Rac1) (Babior 1999). We, therefore, investigated the expression of genes encoding subunits of NO synthase, NAD(P)H oxidase and other components of these pathways in the retinas of GFAP-I κ B α -dn and WT mice 24 h after IR injury. Upregulation of *Nos2* (*iNos*) gene, as well as genes encoding subunits of the NAD(P)H oxidase protein complex: *Cybb*, *Ncf1* and *Ncf2*, was evident in the retinas of both genotypes (Fig. 5). However, such IR-induced upregulation was significantly reduced in GFAP-I κ B α -dn mice compared to WT (Fig. 5). In parallel, we detected by immunohistochemistry a reduction in the levels of protein accumulation for *Cybb* and *Ncf1* in GFAP-I κ B α -dn mice compared to WT after IR-injury, consistently with the gene expression data (Fig. 5B). Furthermore, as additional assessment of oxidative damage, we measured protein oxidation by quantifying IR-induced accumulation of carbonylated proteins. In agreement with the gene expression data, we found a time-dependent increase in protein carbonylation only in WT mice. No IR-induced increases in protein carbonyls was detected in GFAP-I κ B α -dn retinas (Fig. 4D). Taken together these data suggest that suppression of oxidative stress in GFAP-I κ B α -dn mice might contribute to the improved survival of GCL neurons.

Inhibition of astroglial NF- κ B modulated astrocyte-dependent microglial responses

Astrocytes play an active role in attracting and modulating the activation of both resident microglia and infiltrating inflammatory cells, which can exacerbate damage to nervous tissue (Hughes, Botham et al. 2002; Tichauer, Saud et al. 2007). To evaluate whether inactivation of NF- κ B in astrocytes could affect these processes, we measured the expression of cell-specific markers in the whole retina of WT and GFAP-I κ B α -dn mice 24 h after IR-injury. We found an increased expression of the astrocyte-specific marker *Gfap* and the microglia/macrophage marker *Cd11b* in both genotypes, with significantly higher upregulation of *Gfap* in WT compared to GFAP-I κ B α -dn retinas. Even though the levels of *Cd11b* transcript did not differ between the two genotypes, we observed a higher presence of hypertrophic Cd11b-positive cells with less ramified processes in WT versus GFAP-I κ B α -dn retinas (Fig. 6). We then examined changes in genes involved in astrocyte-microglia crosstalk, such as colony-stimulating factors (M-CSF/Csf1, GM-CSF/Csf2 and G-CSF/Csf3) and *Cx3cl1*/fractalkine. Expression of *Csf1* and *Csf2* was upregulated in the IR-challenged retinas of both genotypes, however, GFAP-I κ B α -dn retinas showed significantly reduced

expression as compared to the WT ones (Fig. 6). In contrast, we did not detect any expression of *G-CSF/Csf3* in either normal or ischemic retinas.

Discussion

In this study, we demonstrated that astrocytes play an essential role in the pathophysiology of acute CNS injury and identified the transcription factor NF- κ B as a major regulator of ischemic injury severity in a model of retinal IR. Inhibition of astroglial NF- κ B *in vivo* reduced the severity of ischemic damage to the retina and was associated with suppressed inflammation and oxidative stress, thus providing a mechanism of neuroprotection.

Reactive astrocytes are known to facilitate both protective and toxic effects in the CNS following IR injury. While glutamate uptake by astrocytes is critically important in restraining excitotoxic elevations of this neurotransmitter in brain extracellular space (Chen and Swanson 2003; Hertz 2008; Malarkey and Parpura 2008), glutamate efflux from reactive astrocytes by reversal of glutamate uptake machinery contribute to extracellular glutamate elevations (Chen and Swanson 2003; Hertz 2008; Malarkey and Parpura 2008). Moreover, astrocytic glutathione, which is known to serve as a sink for nitric oxide and ROS-induced stress during ischemia (Dringen, Gutterer et al. 2000; Chen and Swanson 2003), is rapidly depleted upon reactivation and NO exposure, thus exacerbating injury (Garcia-Nogales, Almeida et al. 1999; Bishop, Dringen et al. 2007). The reactive astrocytes were also shown to influence neuronal survival in the post-ischemic period via secretion of nitric oxide, TNF- α , and other factors contributing to delayed neuronal death, such as overexpression of aquaporin-4 channels facilitating brain edema (Ridet, Malhotra et al. 1997; Swanson, Ying et al. 2004). Many stress-response and pro-inflammatory processes in astrocytes are regulated by NF- κ B (O'Neill and Kaltschmidt 1997; Swanson, Ying et al. 2004). In brain ischemia, NF- κ B is involved in excitotoxic, oxidative and inflammatory events associated with neurodegeneration, and plays a dual role in the modulation of neuronal survival. It has been proposed that the contrasting effects of NF- κ B activation in the CNS may depend on the type of stimulus or target cell (Mattson and Meffert 2006), suggesting a model where NF- κ B is neuroprotective when induced in neurons and is neurotoxic when chronically activated in astro- and microglial cells (Bethea, Castro et al. 1998; Domanska-Janik, Bronisz-Kowalczyk et al. 2001; Mattson and Camandola 2001; Fridmacher, Kaltschmidt et al. 2003; Brambilla, Bracchi-Ricard et al. 2005; Mattson and Meffert 2006). To test whether this model is true in acute IR injury, we focused on the role of astroglial NF- κ B in facilitating degeneration of neurons in the GCL of the inner retina.

Our *in vivo* data demonstrated that the inhibition of astroglial NF- κ B in the ischemic retinas significantly increased survival of the GCL neurons one week after reperfusion. RGCs, the major neuronal subpopulation of the GCL, are highly energy-dependent neurons and were shown to be particularly susceptible to IR injury (Kawai, Vora et al. 2001; Neufeld, Kawai et al. 2002). Upon reactivation, retinal astrocytes, which maintain active crosstalk with RGC axons, resident microglia and infiltrating leucocytes (Hall and Berry 1989; Meyer-Franke, Shen et al. 1999), activate a transcriptional pro-inflammatory program through both NF- κ B-dependent and independent pathways (Widenfalk, Lundstromer et al. 2001; Swanson, Ying et al. 2004). Over-stimulation of this program exacerbates the injury-induced stress by challenging neurons with over-exposure to cytokines such as IL1 β and TNF- α , which can promote neurotoxicity and oxidative damage to neurons (Saud, Herrera-Molina et al. 2005; Thomas, Zhang et al. 2005). As expected, our data showed an upregulation of both *Tnf- α* and *Il1 β* 24 hours after reperfusion. In contrast to *Il1 β* levels that remained unchanged, the expression of *Tnf- α* was significantly reduced, in GFAP-I κ B α -dn vs. WT retinas, suggesting that the neuroprotective effect is due to the NF- κ B-dependent suppression of *Tnf- α* . This is in agreement with previous reports on blocking astroglial NF- κ B in other CNS injury

models (Brambilla, Bracchi-Ricard et al. 2005; Maragakis and Rothstein 2006; Brambilla, Persaud et al. 2009). Other NF- κ B-regulated transcripts differentially expressed in TG vs. WT retinas included *Ccl2*, *Cxcl10*, *Icam1* and *Vcam1*. The chemokines CCL2 (MCP1) and CXCL10 (IP10) are essential for immune cell activation and trafficking of peripheral immune cells across the blood-brain barrier (BBB) (Hofmann, Lachnit et al. 2002; Ubogu, Cossoy et al. 2006). Interactions with adhesion molecules ICAM1 and VCAM1 on endothelium and astrocyte end-feet is another key event in T cell migration into the CNS, and functional inactivation of ICAM1 either in knockout mice or by injections of anti-ICAM1 antibodies resulted in significant reduction of infarct volume and general neurologic deficits following focal cerebral ischemia (Zhang, Chopp et al. 1994; Connolly, Winfree et al. 1996). Reduced levels of these transcripts in GFAP-I κ B α -dn exposed to IR suggest that some beneficial effects of NF- κ B downregulation in astrocytes could also be attributed to a vascular effect, via the inhibition of astrocytic NF- κ B, might also be associated with a reduced infiltration of inflammatory cells into the retina.

In addition to their pro-inflammatory activity, reactive astrocytes at the site of ischemic injury have been shown to secrete numerous growth factors and cytokines with anti-inflammatory activity, including Ngf, Ntf3, Tgf β 1, Il10 and Cx3cl1 (Ridet, Malhotra et al. 1997; Hughes, Botham et al. 2002). However, with the exception of a marginal increase in *Tgf β 1*, we did not find significant differences in the IR-induced expression of these genes between GFAP-I κ B α -dn and WT retinas. These results imply that, in agreement with previous observations (Swanson, Ying et al. 2004), the protection provided by astroglial NF- κ B suppression in the IR model was not facilitated by activation of trophic and anti-inflammatory pathways.

An overproduction of ROS and RNS following IR injury is damaging to cell structures, including lipids, membranes, proteins, and DNA, due to uncoupling of the mitochondrial electron-transport chain and excessive production of O $_2^-$ and NO $^-$ by NAD(P)H oxidases and NO synthases (NOS), respectively. Precipitous activation of these enzymes under oxidative stress conditions such as ischemia occurs in activated resident cells, microglia and astrocytes, and in infiltrating cells, neutrophils and macrophages (Neufeld, Kawai et al. 2002; Swanson, Ying et al. 2004), and can inflict damage to cell structural proteins, lipids and DNA. In the context of brain ischemia, excessive activity of inducible NO-synthase, encoded by the *Nos2* gene, is broadly deleterious and its inhibition or inactivation is neuroprotective (Neufeld, Sawada et al. 1999). In addition to causing the synthesis of nitric oxide, brain ischemia leads to the generation of superoxide, through the action of NAD(P)H oxidase isoforms (Sun, Horrocks et al. 2007). In the resting cell, Ncf2 (p67^{PHOX}), Ncf1 (p47^{PHOX}) and Ncf4 (p40^{PHOX}) form a large molecular weight complex which can be recovered from the cytosolic fraction (Babior 1999). Activation of the NADPH oxidase is initiated by the assembly of Ncf2 (p67^{PHOX}), Ncf1 (p47^{PHOX}), and Rac1 (or Rac2) with Cybb (gp91^{PHOX}) (or Cyba (p22^{PHOX})) in a 1:1:1:1 complex in the plasma membrane (Babior 1999). Our analysis of IR-challenged retinas revealed that expression of *Nos2*, *Cybb*, *Ncf1* and *Ncf2* was significantly suppressed in the GCL of GFAP-I κ B α -dn eyes relative to WT controls. Furthermore, protein carbonylation, an indicator of an oxidative environment, was detected in WT but not in transgenic ischemic retinas. This suggests a key role for astroglial NF- κ B in facilitating oxidative conditions following retinal IR injury, and provides an explanation for improved neuronal survival in GFAP-I κ B α -dn retinas.

Astrocyte-mediated production of cytokines, such as CSFs, IL1 β , TNF- α , and, IL-6, can attract and activate microglia/macrophages thus creating paracrine and autocrine feedback loops where astroglial-derived factors amplify microglial/macrophage responses (Mallat, Calvo et al. 1996; Zawadzka and Kaminska 2005). Our hypothesis suggests that suppressing pro-inflammatory astroglial NF- κ B signaling could block such feedback loops and suppress

the inflammatory response. Indeed, we observed suppressed expression of the genes for *Tnf- α* , *Il6*, *M-CSF/Csf1* and *GM-CSF/Csf2* in the IR-challenged GFAP-I κ B α -dn vs. WT retinas, paralleled by increased expression of anti-inflammatory *Tgfb1*, which has been shown to antagonize the activity of *Il1 β* and *Tnf- α* (Boche, Cunningham et al. 2006). We also found that GFAP expression was higher in WT retinas compared to transgenic following IR injury, and the morphology of microglial cells clearly hypertrophic in WT mice compared to GFAP-I κ B α -dn. Taken together, these data suggest that NF- κ B-regulated activation of cytokine signaling in WT reactive astrocytes can exacerbate neurotoxicity in the CNS exposed to IR by triggering secretion of pro-inflammatory and toxic molecules in neighboring microglia and/or infiltrating macrophages.

In conclusion, our findings demonstrate that suppression of astroglial NF- κ B in GFAP-I κ B α -dn mice protects retinal neurons, RGCs in particular, against acute IR injury. Our results provide the first direct evidence that the NF- κ B-dependent responses to the IR injury in astrocytes directly facilitate post-ischemic neurotoxicity, and that inhibition of astroglial NF- κ B leads to neuroprotection. Our analysis indicates that the coordinated inhibition of pro-inflammatory mediators, ROS and RNS synthesis, and other toxicity pathways can contribute to the mechanisms of neuroprotection observed in the ischemic retinas of GFAP-I κ B α -dn mice.

Acknowledgments

This study was supported by: AHA Scientist Development Award 0735014B (D.I.); NIH grant EY017991 and Research to Prevent Blindness (RPB) Career Development Award (V.S.); NIH grant NS051709 and The Miami Project To Cure Paralysis (JRB); NIH grant P30 EY014801 and unrestricted RPB grant to the University of Miami Department of Ophthalmology. We thank Julia Shestopalov for expert assistance with illustrations and Dr. Miguel A Perez-Pinzon for critical reading of the manuscript.

Abbreviations

GCL	ganglion cell layer
RGCs	retinal ganglion cells
IOP	intra ocular pressure
IR	ischemia/reperfusion
WT	wild type
ROS	reactive oxygen species

References

- Babior BM. NADPH oxidase: an update. *Blood*. 1999; 93(5):1464–1476. [PubMed: 10029572]
- Bethea JR, Castro M, et al. Traumatic spinal cord injury induces nuclear factor-kappaB activation. *J Neurosci*. 1998; 18(9):3251–3260. [PubMed: 9547234]
- Bishop GM, Dringen R, et al. Zinc stimulates the production of toxic reactive oxygen species (ROS) and inhibits glutathione reductase in astrocytes. *Free Radic Biol Med*. 2007; 42(8):1222–1230. [PubMed: 17382203]
- Boche D, Cunningham C, et al. TGFbeta1 regulates the inflammatory response during chronic neurodegeneration. *Neurobiol Dis*. 2006; 22(3):638–650. [PubMed: 16510291]
- Bracchi-Ricard V, Brambilla R, et al. Astroglial nuclear factor-kappaB regulates learning and memory and synaptic plasticity in female mice. *J Neurochem*. 2008; 104(3):611–623. [PubMed: 17953667]
- Brambilla R, Bracchi-Ricard V, et al. Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury. *J Exp Med*. 2005; 202(1): 145–156. [PubMed: 15998793]

- Brambilla R, Persaud T, et al. Transgenic Inhibition of Astroglial NF- κ B Improves Functional Outcome in Experimental Autoimmune Encephalomyelitis by Suppressing Chronic Central Nervous System Inflammation. *J Immunol.* 2009; 182(5) in press.
- Chen Y, Swanson RA. Astrocytes and brain injury. *J Cereb Blood Flow Metab.* 2003; 23(2):137–149. [PubMed: 12571445]
- Connolly ES Jr, Winfree CJ, et al. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *J Clin Invest.* 1996; 97(1):209–216. [PubMed: 8550836]
- Dehmer T, Heneka MT, et al. Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with I κ B α induction and block of NF κ B and iNOS activation. *J Neurochem.* 2004; 88(2):494–501. [PubMed: 14690537]
- Dhandapani KM, Hadman M, et al. Astrocyte protection of neurons: role of transforming growth factor-beta signaling via a c-Jun-AP-1 protective pathway. *J Biol Chem.* 2003; 278(44):43329–43339. [PubMed: 12888549]
- Domanska-Janik K, Bronisz-Kowalczyk A, et al. Interrelations between nuclear-factor κ B activation, glial response and neuronal apoptosis in gerbil hippocampus after ischemia. *Acta Neurobiol Exp (Wars).* 2001; 61(1):45–51. [PubMed: 11315321]
- Dringen R, Gutterer JM, et al. Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur J Biochem.* 2000; 267(16):4912–4916. [PubMed: 10931173]
- Facchinetti F, Dawson VL, et al. Free radicals as mediators of neuronal injury. *Cell Mol Neurobiol.* 1998; 18(6):667–682. [PubMed: 9876873]
- Falsig J, Latta M, et al. Defined inflammatory states in astrocyte cultures: correlation with susceptibility towards CD95-driven apoptosis. *J Neurochem.* 2004; 88(1):181–193. [PubMed: 14675162]
- Feigin VL. Stroke epidemiology in the developing world. *Lancet.* 2005; 365(9478):2160–2161. [PubMed: 15978910]
- Fridmacher V, Kaltschmidt B, et al. Forebrain-specific neuronal inhibition of nuclear factor κ B activity leads to loss of neuroprotection. *J Neurosci.* 2003; 23(28):9403–9408. [PubMed: 14561868]
- Garcia-Nogales P, Almeida A, et al. Induction of glucose-6-phosphate dehydrogenase by lipopolysaccharide contributes to preventing nitric oxide-mediated glutathione depletion in cultured rat astrocytes. *J Neurochem.* 1999; 72(4):1750–1758. [PubMed: 10098886]
- Goto W, Ota T, et al. Protective effects of timolol against the neuronal damage induced by glutamate and ischemia in the rat retina. *Brain Res.* 2002; 958(1):10–19. [PubMed: 12468025]
- Hall S, Berry M. Electron microscopic study of the interaction of axons and glia at the site of anastomosis between the optic nerve and cellular or acellular sciatic nerve grafts. *J Neurocytol.* 1989; 18(2):171–184. [PubMed: 2732757]
- Herrmann O, Baumann B, et al. IKK mediates ischemia-induced neuronal death. *Nat Med.* 2005; 11(12):1322–1329. [PubMed: 16286924]
- Hertz L. Bioenergetics of cerebral ischemia: a cellular perspective. *Neuropharmacology.* 2008; 55(3):289–309. [PubMed: 18639906]
- Hofmann N, Lachnit N, et al. Increased expression of ICAM-1, VCAM-1, MCP-1, and MIP-1 α by spinal perivascular macrophages during experimental allergic encephalomyelitis in rats. *BMC Immunol.* 2002; 3:11. [PubMed: 12196270]
- Hughes PM, Botham MS, et al. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, during acute and chronic inflammation in the rodent CNS. *Glia.* 2002; 37(4):314–327. [PubMed: 11870871]
- Ivanov D, Dvorianchikova G, et al. Differential gene expression profiling of large and small retinal ganglion cells. *J Neurosci Methods.* 2008; 174(1):10–17. [PubMed: 18640154]
- Ivanov D, Dvorianchikova G, et al. Microarray analysis of gene expression in adult retinal ganglion cells. *FEBS Lett.* 2006; 580(1):331–335. [PubMed: 16376886]

- Kawai SI, Vora S, et al. Modeling of risk factors for the degeneration of retinal ganglion cells after ischemia/reperfusion in rats: effects of age, caloric restriction, diabetes, pigmentation, and glaucoma. *Faseb J*. 2001; 15(7):1285–1287. [PubMed: 11344115]
- Kim KY, Ju WK, et al. Neuronal susceptibility to damage: comparison of the retinas of young, old and old/caloric restricted rats before and after transient ischemia. *Neurobiol Aging*. 2004; 25(4):491–500. [PubMed: 15013570]
- Levin LA, Gordon LK. Retinal ganglion cell disorders: types and treatments. *Prog Retin Eye Res*. 2002; 21(5):465–484. [PubMed: 12207946]
- Mabuchi T, Kitagawa K, et al. Contribution of microglia/macrophages to expansion of infarction and response of oligodendrocytes after focal cerebral ischemia in rats. *Stroke*. 2000; 31(7):1735–1743. [PubMed: 10884481]
- Malarkey EB, Parpura V. Mechanisms of glutamate release from astrocytes. *Neurochem Int*. 2008; 52(1–2):142–154. [PubMed: 17669556]
- Mallat M, Calvo CF, et al. Recruitment of brain macrophages: roles of cytokines and extracellular matrix proteins produced by glial or neuronal cells. *Braz J Med Biol Res*. 1996; 29(9):1173–1177. [PubMed: 9181060]
- Maragakis NJ, Rothstein JD. Mechanisms of Disease: astrocytes in neurodegenerative disease. *Nat Clin Pract Neurol*. 2006; 2(12):679–689. [PubMed: 17117171]
- Mattson MP, Camandola S. NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J Clin Invest*. 2001; 107(3):247–254. [PubMed: 11160145]
- Mattson MP, Meffert MK. Roles for NF-kappaB in nerve cell survival, plasticity, and disease. *Cell Death Differ*. 2006; 13(5):852–860. [PubMed: 16397579]
- Meyer-Franke A, Shen S, et al. Astrocytes induce oligodendrocyte processes to align with and adhere to axons. *Mol Cell Neurosci*. 1999; 14(4–5):385–397. [PubMed: 10588392]
- Neufeld AH, Kawai S, et al. Loss of retinal ganglion cells following retinal ischemia: the role of inducible nitric oxide synthase. *Exp Eye Res*. 2002; 75(5):521–528. [PubMed: 12457864]
- Neufeld AH, Sawada A, et al. Inhibition of nitric-oxide synthase 2 by aminoguanidine provides neuroprotection of retinal ganglion cells in a rat model of chronic glaucoma. *Proc Natl Acad Sci U S A*. 1999; 96(17):9944–9948. [PubMed: 10449799]
- Nomoto Y, Yamamoto M, et al. Expression of nuclear factor kappaB and tumor necrosis factor alpha in the mouse brain after experimental thermal ablation injury. *Neurosurgery*. 2001; 48(1):158–166. [PubMed: 11152341]
- O'Neill LA, Kaltschmidt C. NF-kappa B: a crucial transcription factor for glial and neuronal cell function. *Trends Neurosci*. 1997; 20(6):252–258. [PubMed: 9185306]
- Osborne NN, Casson RJ, et al. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retin Eye Res*. 2004; 23(1):91–147. [PubMed: 14766318]
- Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. *Glia*. 2005; 50(4):427–434. [PubMed: 15846805]
- Ridet JL, Malhotra SK, et al. Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci*. 1997; 20(12):570–577. [PubMed: 9416670]
- Saud K, Herrera-Molina R, et al. Pro- and anti-inflammatory cytokines regulate the ERK pathway: implication of the timing for the activation of microglial cells. *Neurotox Res*. 2005; 8(3–4):277–287. [PubMed: 16371322]
- Selles-Navarro I, Villegas-Perez MP, et al. A quantitative in vivo study. Retinal ganglion cell death after different transient periods of pressure-induced ischemia and survival intervals. *Invest Ophthalmol Vis Sci*. 1996; 37(10):2002–2014. [PubMed: 8814140]
- Sofroniew MV. Reactive astrocytes in neural repair and protection. *Neuroscientist*. 2005; 11(5):400–407. [PubMed: 16151042]
- Stoll G, Jander S, et al. Inflammation and glial responses in ischemic brain lesions. *Prog Neurobiol*. 1998; 56(2):149–171. [PubMed: 9760699]
- Sun GY, Horrocks LA, et al. The roles of NADPH oxidase and phospholipases A2 in oxidative and inflammatory responses in neurodegenerative diseases. *J Neurochem*. 2007; 103(1):1–16. [PubMed: 17561938]

- Swanson RA, Ying W, et al. Astrocyte influences on ischemic neuronal death. *Curr Mol Med*. 2004; 4(2):193–205. [PubMed: 15032713]
- Thomas MS, Zhang W, et al. Signaling pathways mediating a selective induction of nitric oxide synthase II by tumor necrosis factor alpha in nerve growth factor-responsive cells. *J Neuroinflammation*. 2005; 2:19. [PubMed: 16144552]
- Tichauer J, Saud K, et al. Modulation by astrocytes of microglial cell-mediated neuroinflammation: effect on the activation of microglial signaling pathways. *Neuroimmunomodulation*. 2007; 14(3–4):168–174. [PubMed: 18073510]
- Ubogu EE, Cossoy MB, et al. The expression and function of chemokines involved in CNS inflammation. *Trends Pharmacol Sci*. 2006; 27(1):48–55. [PubMed: 16310865]
- van Loo G, De Lorenzi R, et al. Inhibition of transcription factor NF-kappaB in the central nervous system ameliorates autoimmune encephalomyelitis in mice. *Nat Immunol*. 2006; 7(9):954–961. [PubMed: 16892069]
- Wang X, Niwa M, et al. Neuronal degradation in mouse retina after a transient ischemia and protective effect of hypothermia. *Neurol Res*. 2002; 24(7):730–735. [PubMed: 12392214]
- Widenfalk J, Lundstromer K, et al. Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. *J Neurosci*. 2001; 21(10):3457–3475. [PubMed: 11331375]
- Zawadzka M, Kaminska B. A novel mechanism of FK506-mediated neuroprotection: downregulation of cytokine expression in glial cells. *Glia*. 2005; 49(1):36–51. [PubMed: 15390105]
- Zeng HY, Tso MO, et al. Activation of nuclear factor-kappaB during retinal degeneration in rd mice. *Mol Vis*. 2008; 14:1075–1080. [PubMed: 18552981]
- Zhang RL, Chopp M, et al. Anti-ICAM-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in the rat. *Neurology*. 1994; 44(9):1747–1751. [PubMed: 7936308]

Figure 1.

Inhibition of NF- κ B DNA binding activity in retinas from GFAP-I κ B α -dn mice 24 hours after IR injury **A**. Representative gel shift analysis of NF- κ B DNA and nuclear protein combination in retinas of ischemic and sham operated eyes of WT and GFAP-I κ B α -dn mice. NF- κ B DNA-binding activity in the nuclei of retinal cells of sham operated WT eyes (Lane 1), sham operated GFAP-I κ B α -dn eyes (Lane 2), WT eyes 24 hours after IR injury (Lane 3), GFAP-I κ B α -dn eyes 24 hours after IR injury (Lane 4), NF- κ B DNA-binding activity by competition electrophoretic mobility shift assay (EMSA) with a hundredfold excess of cold NF- κ B oligonucleotides (Lane 5). **B**. Quantification of NF- κ B DNA-binding activity in

retinas shown in (A). (* $p < 0.05$, compared with the control retina, $n=5$, ■- wild type, □- GFAP-I κ B α -dn mice)

Figure 2.

Inhibition of astroglial NF- κ B results in neuroprotective effects in the GCL after IR. **A.** Numbers of NeuN-labeled neurons in regions of central, middle and peripheral retina were compared between sham operated and ischemic eyes of wild type and GFAP-I κ B α -dn animals. Values are means $\pm$ SEM of each microscopic field. * p <0.05 ($\square$ - wild type sham operated, $\blacksquare$ - wild type 7 days after IR injury, $\square$ - GFAP-I κ B α -dn sham operated, $\blacksquare$ - GFAP-I κ B α -dn 7 days after IR injury) **B.** Percentage of GCL neurons lost at one week following IR of wild type and GFAP-I κ B α -dn mice. Central, middle and peripheral regions were compared bilaterally for individual animals. ($\blacksquare$ - wild type, $\square$ - GFAP-I κ B α -dn mice) **C.** In contrast to protected GFAP-I κ B α -dn post-ischemic retinas that have lost an average of 9.6% of cells, neuronal density decreased significantly (37.5%, p <0.002) in WT ischemic retinas 7 days after reperfusion. **D.** Representative confocal images of NeuN-labeled GCLs (green) in flat-mounted retinas acquired at centre, middle and periphery from optic nerve in sham-operated controls and ischemic retinas 7 days after reperfusion.

Figure 3.

Differential expression of the cytokines, the chemokines, the cell adhesion molecules and neurotrophins in WT and GFAP-IκBα-dn retinal tissues after IR. Gene expression was assessed in sham operated controls and experimental retinas following IR (24 hours after reperfusion). For each gene, results are expressed as folds of corresponding WT sham-operated eye $\pm$ SEM after normalization to β -actin. *P<0.05 with respect to corresponding WT. (■ - WT, □ - GFAP-IκBα-dn)

Figure 4.

A. Tnf- α protein (green) accumulation in GCL co-localized with astrocyte marker Gfap (red) and was significantly increased in WT relative to GFAP-I κ B α -dn post-ischemic retinas (24 hours after reperfusion). Schematic on the right shows localization of the field of view (framed) relative to the rest of the retina. Arrows point to RGCs, arrowheads – to astrocytes. Retinal layers: INL, inner nuclear, ONL, outer nuclear, PhRL, photoreceptors. **B.** Immunofluorescent labeling for Icam1 in the GCL after IR significantly co-localized with astrocyte vascular endfeet labeled with Gfap (red), but not with microglia (labeled with Cd11b, blue/cyan) in both WT and GFAP-I κ B α -dn flat-mounted retinas (24 hours after reperfusion). Icam1 labeling was significantly increased in WT vs. GFAP-I κ B α -dn retinas. Microglial Cd11b-positive cells in GFAP-I κ B α -dn retinas appear ramified, in contrast to severely pruned and hypertrophic cells in WT post-ischemic retinas. Scale bar: 100 μ m.

Figure 5.

A. Differential expression of genes encoding *Nos2* and NADPH-oxidase in GCL of WT and GFAP-IκBα-dn retinas following IR. Gene expression was assessed in sham-operated controls and experimental eyes following IR (24 hours after reperfusion). For each gene, results are expressed as folds of corresponding WT sham-operated eye $\pm$ SEM after normalization to β -actin. * $P < 0.05$ with respect to corresponding WT. **B.**

Immunohistochemistry for the *Cybb* and *Ncf1* proteins accumulation in GCL of WT and GFAP-IκBα-dn post-ischemic retinas (green) validated increased levels of the transcripts at the level of corresponding proteins 24 hours after reperfusion; GFAP, red. Scale bar: 25 μm. **C.** Protein carbonylation assayed 1 and 3 days post-reperfusion, revealed statistically significant ($P < 0.05$) increase in oxidative damage to proteins in WT, but not in GFAP-IκBα-dn retinas.

Figure 6.

A. Differential expression of genes encoding glial cell markers in sham-operated controls and experimental eyes following IR (24 hours after reperfusion). For each gene, results are expressed as folds of corresponding WT sham-operated eye $\pm$ SEM after normalization to β -actin. * $P < 0.05$ with respect to corresponding WT. (■ - WT, □ - GFAP-I κ B α -dn) **B.** Microglial cell (Cd11b-labeled, crimson) morphology assessed in flat-mounted retinas was similar in sham-operated WT and GFAP-I κ B α -dn eyes, but changed dramatically in WT post-ischemic eyes 24 hours after reperfusion, showing both hypertrophy and presence of significant amounts of undifferentiated cells. In contrast, GFAP-I κ B α -dn microglial cell morphology changed insignificantly following IR. Scale bar: 25 μ m.

Table 1

List of PCR primers and the primary antibodies

Gene	Oligonucleotides		Primary antibodies
<i>Il1b</i>	Forward	GACCTTCCAGGATGAGGACA	
	Reverse	AGGCCACAGGTATTTGTCTG	
<i>Il6</i>	Forward	ATGGATGCTACCAAAGTGGAT	
	Reverse	TGAAGGACTCTGGCTTTGTCT	
<i>Il10</i>	Forward	GGTTGCCAAGCCTTATCGGA	
	Reverse	ACCTGCTCCACTGCCTTGCT	
<i>Tnf</i>	Forward	CAAAATTCGAGTGACAAGCCTG	Sc-1348 (Santa Cruz)
	Reverse	GAGATCCATGCCGTTGGC	
<i>Tgfb1</i>	Forward	TGAGTGGCTGTCTTTTGACG	
	Reverse	TCTCTGTGGAGCTGAAGCAA	
<i>Ccl2</i>	Forward	AGGTCCCTGTCATGCTTCTG	
	Reverse	ATTTGGTTCCGATCCAGGTT	
<i>Ccl5</i>	Forward	AGCAGCAAGTGCTCCAATCT	
	Reverse	ATTTCTTGGGTTTGCTGTGC	
<i>Cxcl10</i>	Forward	GCTGCAACTGCATCCATATC	
	Reverse	CACTGGGTAAAGGGGAGTGA	
<i>Icam1</i>	Forward	TGGTGATGCTCAGGTATCCA	Sc-53553 (Santa Cruz)
	Reverse	CACACTCTCCGGAACGAAT	
<i>Vcam1</i>	Forward	GTGGTGCTGTGACAATGACC	
	Reverse	ACGTCAGAAACAACCGAATCC	
<i>Ncam1</i>	Forward	ACGTCCGTTTCATAGTCCTG	
	Reverse	CTATGGGTTCCCCATCCTTT	
<i>Nos2</i>	Forward	CAGAGGACCCAGAGACAAGC	
	Reverse	TGCTGAAACATTTCTGTGC	
<i>Cybb</i>	Forward	GACTGCGGAGAGTTTGAAG	Sc-5827 (Santa Cruz)
	Reverse	ACTGTCCACCTCCATCTTG	
<i>Ncf1</i>	Forward	CGAGAAGAGTTCGGGAACAG	
	Reverse	AGCCATCCAGGAGCTTATGA	
<i>Ncf2</i>	Forward	CTACCTGGAGCCAGTTGAGC	Sc-7663 (Santa Cruz)
	Reverse	AGCGCCAGCTTCTTAGACAC	
<i>Ngf</i>	Forward	GTGCCTCAAGCCAGTGAAAT	
	Reverse	TCTCCTTCTGGGACATTGCT	
<i>Ntf3</i>	Forward	CGAACTCGAGTCCACCTTTC	
	Reverse	GCAGGGTGCTCTGGTAATTT	
<i>Csf1</i>	Forward	ATGCCAGATTGCCTTTGAATTT	
	Reverse	CTCATGGAAAGTTCGGACACAG	

Gene	Oligonucleotides		Primary antibodies
<i>Csf2</i>	Forward	GCCATCAAAGAAGCCCTGAA	
	Reverse	GCGGGTCTGCACACATGTTA	
<i>Pkcb</i>	Forward	CTCAGAGCGGAAGGGTACAG	
	Reverse	AACAGACCGATGGCAATCTC	
<i>Pcna</i>	Forward	TTGGAATCCCAGAACAGGAG	
	Reverse	CAGTGGAGTGGCTTTTGTGA	
<i>Gfap</i>	Forward	AGAAAGGTGAATCGCTGGA	C9205 (Sigma)
	Reverse	CGGCGATAGTCGTTAGCTTC	
<i>Cd11b</i>	Forward	ACAATGTGACCGTATGGGATC	RM2805 (Invitrogen)
	Reverse	GCAAACGCAGAGTCATTAAAC	
<i>Actb</i>	Forward	CACCCTGTGCTGCTCACC	
	Reverse	GCACGATTCCCTCTCAG	
<i>Sdha</i>	Forward	ACACAGACCTGGTGGAGACC	
	Reverse	GCACAGTCAGCCTCATTCAA	
<i>Tbp</i>	Forward	ACATCTCAGCAACCCACACA	
	Reverse	ATGATGACTGCAGCAAATCG	