Immediate Upregulation of Proteins Belonging to Different Branches of the Apoptotic Cascade in the Retina after Optic Nerve Transection and Optic Nerve Crush

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Purpose. To further investigate the molecular signals underlying optic nerve (ON) injury, the authors analyzed in adult control, ON-transected, and ON-crushed retinas the expression pattern and time-course regulation of the following proteins, all of which are linked to apoptosis through different pathways: Stat 1, caspase 11 (inflammation and death), cathepsins C and B (lysosomal death pathway), calpain 1 (endoplasmic reticulum stress), calreticulin (apoptosis marker), Jun (early response), and aryl hydrocarbon receptor (cell cycle arrest).

Methods. Adult female rats were subjected to intraorbital optic nerve transection (IONT) or intraorbital optic nerve crush (IONC). Protein from naive and ON-injured adult rat retinas was extracted at different times postlesion, and Western blotting experiments were performed. For immunohistofluorescence analyses, retinal ganglion cells (RGCs) were retrogradely identified with fluorogold applied to the superior colliculi 1 week before injury.

Results. Western blotting analyses revealed upregulation of all the analyzed proteins as early as 12 hours postlesion (hpl), peaking at 48 hpl, in agreement with our previous RNA study findings. Furthermore, immunohistofluorescence to radial sections showed that all but Stat 1 were expressed by the primarily injured neurons, the RGCs, as seen by colocalization with fluorogold.

Conclusions. All analyzed proteins were upregulated in the retina after IONT or IONC as early as 12 hpl, indicating that ON injury regulates several branches of the apoptotic cascade and suggesting that commitment to death might be an earlier event than previously anticipated. (*Invest Ophthalmol Vis Sci.* 2009; 50:424–431) DOI:10.1167/iovs.08-2404

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Supported by Instituto de Salud Carlos III Grants CP003/00119, PI070225, and RD07/0062/0001; Fundación Séneca Grant 04446/GERM/07; and MECSAF-2005-04812.

Submitted for publication June 9, 2008; revised July 24, 2008; accepted October 22, 2008.

Disclosure: **M. Agudo**, None; **M.C. Pérez-Marín**, None; **P. Sobrado-Calvo**, None; **U. Lönngren**, None; **M. Salinas-Navarro**, None; **I. Cánovas**, None; **F.M. Nadal-Nicolás**, None; **J. Miralles-Imperial,** None; **F. Hallböök**, None; **M. Vidal-Sanz**, None

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Axotomy to central nervous system (CNS) neurons results in a loss of function and leads eventually to the death of the wounded neurons. As part of the CNS, retinal ganglion cells (RGCs) die soon after optic nerve (ON) injury, irrespective of the type of lesion, transection, or crush (Parrilla-Reverter G, et al. *IOVS* 2004;45:ARVO E-Abstract 911).[1] Multiple efforts have been made to prevent or slow this neurodegenerative process but have resulted in little success,[2–7] highlighting the need for better understanding of the molecular mechanisms underlying RGC degeneration.

We recently carried out an extensive time-course analysis of the retinal transcriptome regulation comparing control and ON-injured retinas.[8] This work showed that, with time, numerous death-related genes were upregulated after intraorbital optic nerve transection (IONT) or intraorbital optic nerve crush (IONC). These death signals cover a wide range of molecular pathways, including the extrinsic apoptotic cascade, cell cycle deregulation, inflammatory responses linked to cell death, and, to a minor extent, the intrinsic pathway of apoptosis. Regulated death-related genes fall into several functional categories such as proteases, signaling proteins (kinases, phosphatases), transcription factors, and death receptors. Retinal transcriptome regulation in response to IONT or IONC shows some similarities but also some differences.[8] For instance, more death-related genes are regulated after transection than after crush, and those commonly regulated by both injuries show, as a general rule, longer lasting response after IONT than after IONC.[8]

We hypothesized that the major cause of RGC death associated with ON lesion might lie among those death-related genes that were commonly regulated by both types of injuries. Therefore, we further analyzed the time-course protein expression levels and patterns of eight of those genes. To date, with the exception of Jun[9] and calpain 1,[10] these proteins have not been described in these models of neuronal death. They were chosen because of their increased mRNA regulation after ON injury,[8] their function, and their role in different branches of the apoptotic cascade. Thus, we have studied caspase 11, a protease linked to the inflammatory response and to the extrinsic apoptotic pathway[11]; cathepsins C and B, proteases associated to the lysosomal death pathway[12]; calpain 1, an endoplasmic reticulum Ca^{2++}-dependent protease that, under pathologic circumstances, processes caspases among other apoptotic proteins[10,13,14]; calreticulin, a Ca^{2++}-buffering protein that is an early marker of apoptosis[15,16]; and three transcription factors, Jun, aryl hydrocarbon receptor (AhR), and the signal transducer and activator of transcription 1 (Stat 1). Increased expression of immediate early genes, such as *c-fos*[8] and *Jun*, is an early event observed in axotomized neurons.[17] AhR is a transcription factor activated by environmental toxins. Interestingly, it has been reported that when it is activated, it inhibits the cell cycle through the retinoblastoma protein, leading eventually to apoptosis.[18,19] Finally, Stat 1 is a latent cytoplasmic transcription factor regulated by cytokines that

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has been shown to be a key modulator of inflammatory cell death.[20]

We show here that these genes, which demonstrated upregulation in our previous array analysis study,[8] are also upregulated at the protein level in IONT- and IONC-injured retinas compared with control untouched retinas. Furthermore, all but Stat 1 are expressed by RGCs, as demonstrated by colocalization with fluorogold (FG). Finally, we discuss the results in conjunction with data from our previous array study.[8] All these data have been combined to generate custom signaling maps (MetaCore and MapEditor software; GeneGo Bioinformatics Software, Inc. [www.genego.com], San Diego, CA), which permit a hypothetical model recreation for easy understanding and visualization of the apoptotic signals triggered in adult retinas by optic nerve injury.

Materials and Methods

Animal Handling and Surgery

Sprague-Dawley female rats (weight range, 180–220 g) were obtained from the university breeding colony. For anesthesia, a mixture of xylazine (10 mg/kg body weight; Rompun; Bayer, Kiel, Germany) and ketamine (60 mg/kg body weight; Ketolar; Pfizer, Alcobendas, Madrid, Spain) was used. All experimental procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animals were divided into a control group that did not undergo any experimental manipulation and two experimental groups, one undergoing intraorbital nerve transection (IONT) and the other undergoing intraorbital nerve crush (IONC). Sterile precautions were maintained for all surgical procedures. The left ON was intraorbitally injured according to procedures that are standard in our laboratory.[1,8,21–23] When performing IONT, the dura mater was opened longitudinally to spare the blood supply, and the ON was sectioned 0.5 mm from the optic disc. To perform IONC, the ON was crushed for 10 seconds 3 mm from the optic disc, with the use of watchmaker forceps (Parrilla-Reverter G, et al. *IOVS* 2004;45:ARVO E-Abstract 911). Before and after the procedure, the eye fundus was observed through the operating microscope to assess the integrity of the retinal blood flow.

To identify RGCs in cross-sections processed for immunohistofluorescence analyses, we applied the retrogradely transported tracer fluorogold (FG) (3% diluted in 10% dimethyl sulfoxide-saline; Fluorochrome, Denver, CO) to the superior colliculi according to a previously described technique that is standard in our laboratory.[5,6,22,24,25] For this part of the study, three animal groups were prepared: control untouched rats in which FG was applied 1 week before processing ($n = 5$), and two experimental groups (IONT, $n = 5$; IONC, $n = 5$) in which FG was applied 1 week before surgery. Optic nerve injuries were carried out as mentioned, and animals were processed at 48 hours postlesion (hpl) because, according to our Western blotting results, most of the analyzed proteins were upregulated at this time.

Immunohistofluorescence

Immunohistofluorescence analyses were carried out as previously described.[8] Briefly, animals with FG-traced RGCs were deeply anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer after a saline rinse. Dissected retinas were cryoprotected in 30% sucrose (Sigma, Alcobendas, Madrid, Spain) before they were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) for cryostat sectioning. Sections (15 μm) were blocked in 2% donkey serum in phosphate-buffered saline (PBS) with 0.1% Triton-100 and were incubated overnight a 4°C with the appropriate primary antibody. Immunoreactivity was detected using fluorescence-conjugated secondary antibodies. Images were taken with a charge-coupled device camera using imaging software (Image-Pro Plus 5.1 for Windows; Media Cybernetics, Silver Spring, MD) and were further processed (Adobe Photoshop 7.0; Adobe Systems, San Jose, CA).

Western Blot Analysis

Western blot analyses were carried out as previously described.[8] Briefly, freshly dissected retinas from control, IONT-, and IONC-injured animals (12 hpl, 48 hpl, and 3 or 7 days postlesion [dpl]; $n = 4$ per group and time point) were homogenized in 300 μL lysis buffer (1% Nonidet-p40, 20 mM Hepes, pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA with 1× protease inhibitor cocktail; Roche Diagnostics, Barcelona, Spain). The amount of protein was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, ThermoFisher Scientific, Cultek SL, Madrid, Spain). As a loading control, β-actin detection was carried out for each Western blot. Protein samples were run on 16% or 12% SDS-PAGE and were transferred to a polyvinylidene fluoride (PVDF; Amersham, GE Healthcare Europe GmbH, Barcelona, Spain) membrane and were incubated with the appropriate antibody overnight at 4°C. Secondary detection was carried out with horseradish peroxidase (HRP)-conjugated secondary antibodies that were visualized by enhanced chemiluminescence (ECL; Amersham, GE Healthcare Europe GmbH) and exposure to x-ray film. Exposed films were analyzed with a bioinformatics software package (Gene Tool, Syngene; Synoptics, Cambridge, UK).

The signal intensity of treated retinas was referred to the signal of the control retinas, which was arbitrarily considered as 100%. To avoid biological variability, extracts from four animals were loaded in parallel. To avoid technical variability, each Western blot was performed three times. Data shown are the averaged values of these replicas with their SEM.

Antibodies and Working Dilutions

Antibodies were diluted in PBS with 0.1% Triton-100 for immunohistofluorescence (IHF) or 0.1% Tween-20 for Western blotting (W). Rabbit anti–β-actin (1:1000 W) was from Sigma. The remaining primary antibodies—rabbit anti–caspase 11 (1:200 W and IHF), rabbit anti–cathepsin C (1:100 W, 1:50 IHF), rabbit anti–cathepsin B (1:100 W, 1:50 IHF), rabbit anti-Jun (1:200 W, 1:50 IHF), goat anti–calreticulin (1:200 W and IHF), goat anti-AhR (1:200 W and IHF), rabbit anti–Stat 1 (1:200 W, 1:5000 IHF), rabbit anti–calpain 1 (1:200 W and IHF)—were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Secondary Antibodies

Western Blotting. Secondary antibodies were peroxidase-conjugated goat anti–mouse IgG and goat anti–rabbit IgG 1:5000 (AffiniPure H+L; Jackson ImmunoResearch, Suffolk, UK), and peroxidase-conjugated donkey anti–goat IgG 1:5000 (Santa Cruz Biotechnology).

Immunohistofluorescence. Antibodies were previously checked for unspecific labeling (4-hour incubation onto retinal sections without primary antibody incubation): Alexa Fluor-568 goat anti–mouse IgG (H+L) 1:500, Alexa Fluor-568 donkey anti–goat IgG (H+L) 1:500 (Molecular Probes, Invitrogen, Barcelona, Spain), and Cy3 goat anti–rabbit IgG (H+L) 1:500 (Jackson ImmunoResearch).

Results

Proteases: Caspase 11, Cathepsin C, Cathepsin B, and Calpain 1

Caspase 11. Western blot analysis shows that caspase 11 is weakly expressed in control retinas and that after IONT and IONC, its expression increases as early as 12 hpl, peaks at 48 hpl, and lasts up to 7 dpl, the longest time point analyzed in the present experiments (Fig. 1A). Immunohistofluorescence analysis illustrated that caspase 11 was not detected in control retinas (Fig. 1D). However, after IONT (Fig. 1E) and IONC (Fig. 1F), a strong signal was evident in the nerve fiber layer. In addition, it was observed that some RGCs express caspase 11,

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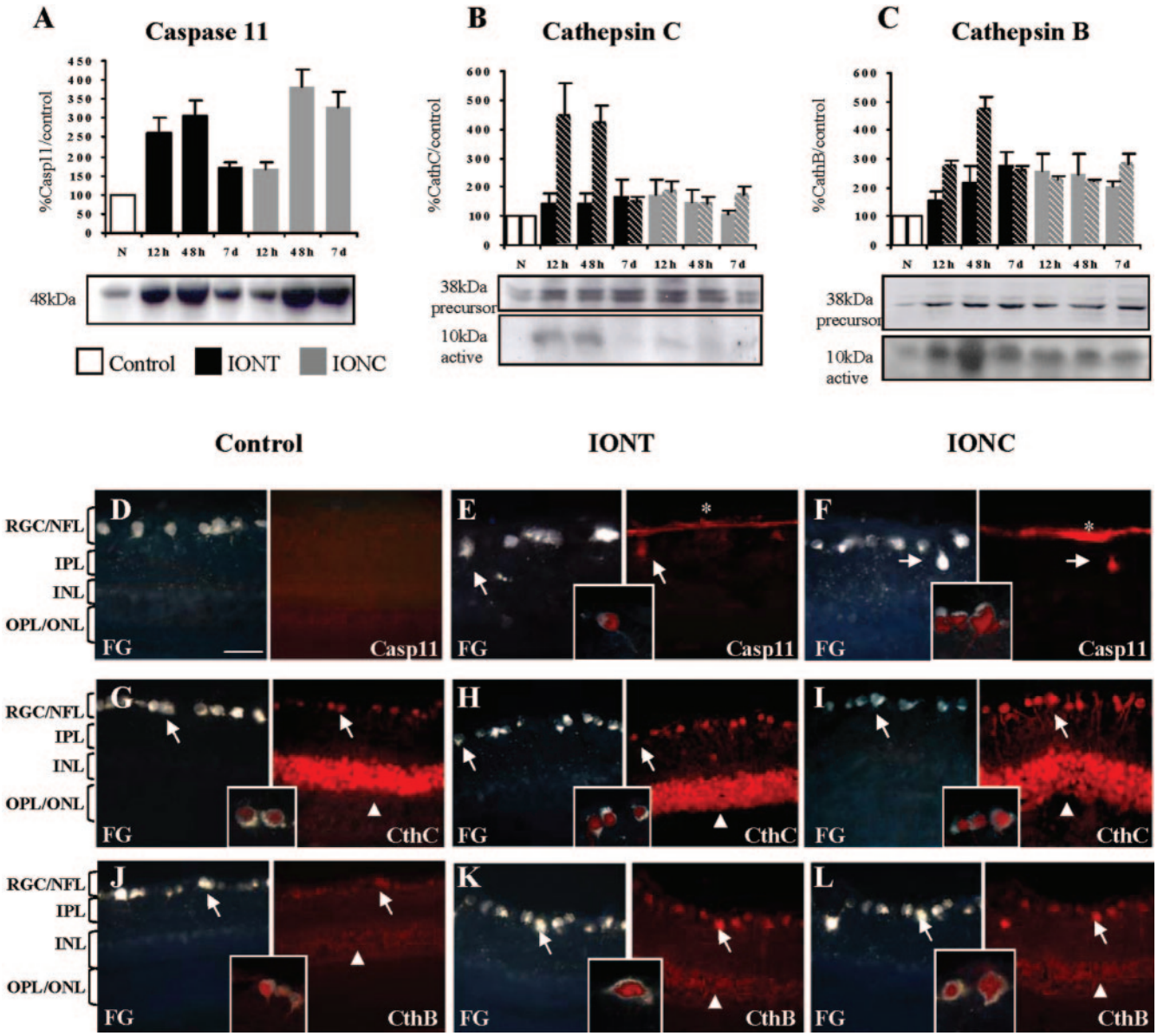

FIGURE 1. Caspase 11, cathepsin C, and cathepsin B are overexpressed in ON-injured retinas. (**A–C**) Western blot time-course analysis showing the regulation of total caspase 11 (**A**) and of cathepsin C (**B**) and cathepsin B (**C**) precursor and active forms in control, IONT-, and IONC-injured retinas. In cathepsin C and B, *solid columns* quantify the precursor forms and *striped columns* quantify the active ones. Graphs show quantification of protein signal ($n = 4$ animals per lesion and time point; Western blots were replicated three times). Signal from injured retinas was referred to as the control signal, which was arbitrarily considered 100%. Error bars show the SEM for each experiment. (**D–L**) Immunohistofluorescence analysis for caspase 11 (**D–F**, *red signal*), cathepsin C (**G–I**, *red signal*), and cathepsin B (**J–L**, *red signal*) to FG-traced RGCs (corresponding *left* images, *blue signal*) on control, IONT-, and IONC-injured retinas at 48 hpl ($n = 5$ per group). *Boxed areas*: magnified positive RGCs for a given protein. *Arrows*: RGC. *Arrowhead*: inner nuclear layer. *Asterisk*: nerve layer. h, hours postlesion; d, days postlesion; Casp11, caspase 11; CthC, cathepsin C; CthB, cathepsin B; RGC/NFL, retinal ganglion cell/nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL/ONL, outer plexiform layer/outer nuclear layer. Scale bar, 100 μm.

as shown by colocalization to FG-traced RGCs. Interestingly, Western blotting and immunohistofluorescence showed that IONC induced stronger caspase 11 upregulation than IONT.

Cathepsin C and Cathepsin B. Western blot analysis (Fig. 1B) showed that cathepsin C precursor (molecular weight [MWt], 38 kDa) was upregulated after both lesions as early as 12 hours, lasting up to 7 days after IONT, but that it decreased to control levels at this time after IONC. The cathepsin C active form (MWt, 10 kDa) was upregulated from 12 hours until 3 dpl after IONT and peaked at 12 hpl, when its signal was fourfold the control level of expression. IONC also triggered upregulation of the active form of cathepsin C at these time points, but to a lesser extent than IONT did. Cathepsin B precursor was weakly detected in control extracts, and its expression doubled from 12 hours until 7 days after transection and crush (Fig. 1A). The level of the active form of Cathepsin B increases after both injuries, being at 48 hours after IONT when a higher expression is observed.

Immunohistolocalization of cathepsin C and B in sectioned retinas is shown in Figures 1G to 1L. In control retinas, cathepsin C expression was observed in the ganglion cell layer (GCL), where colocalization of FG and CY3-signal (red) indicated that cathepsin C was expressed by RGCs and by other non–FG-traced cells located in this layer. In addition, the cathepsin C

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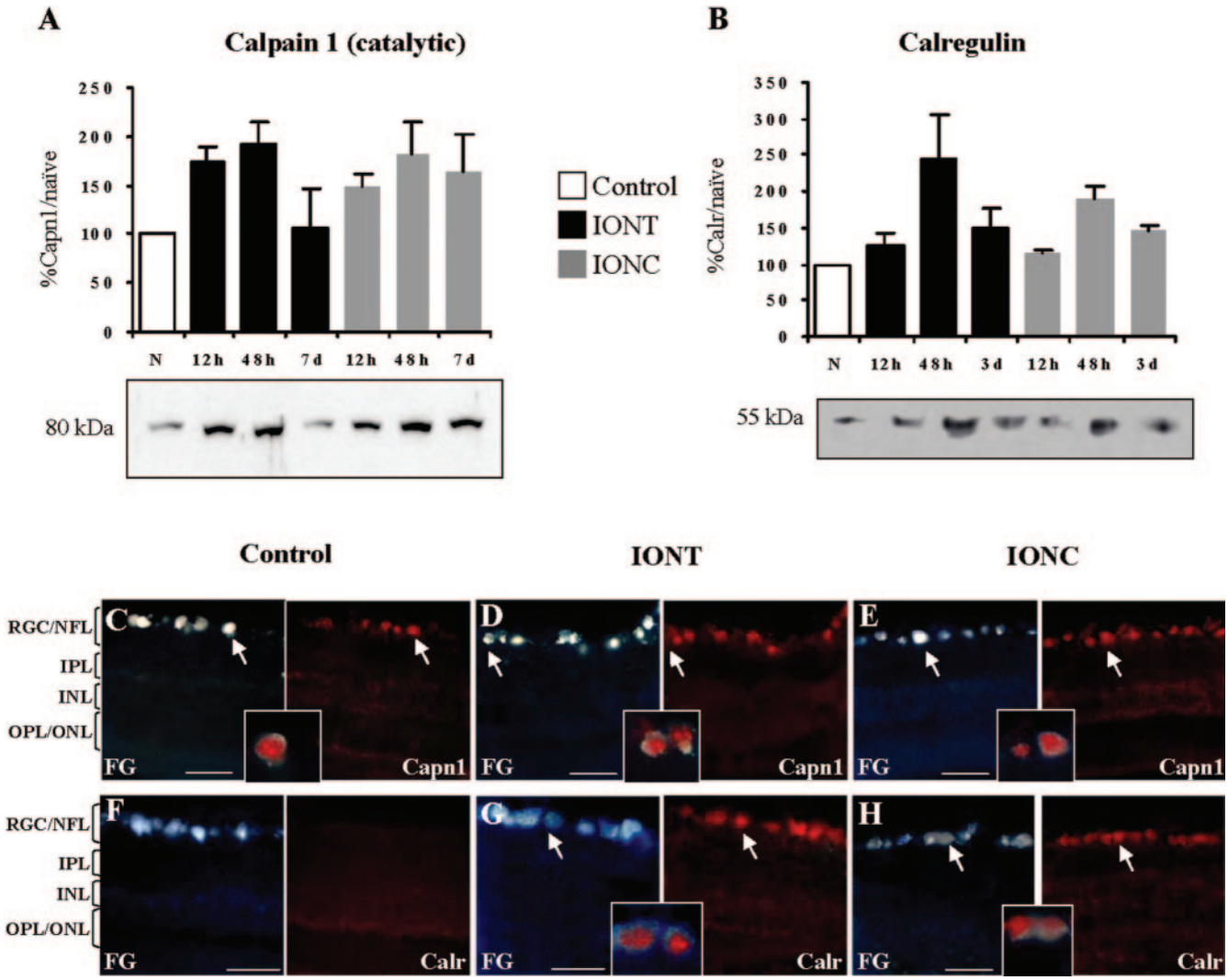

FIGURE 2. Calpain 1 and calreticulin are overexpressed in ON-injured retinas. (**A**, **B**) Western blot time course analysis showing the regulation of (**A**) calpain 1 (**B**) and calreticulin in control, IONT-, and IONC-injured retinas. Graphs show quantification of protein signal ($n = 4$ animals per lesion and time point; Western blots were replicated three times). Signal from injured retinas is referred to as the control signal, which was arbitrarily considered 100%. Error bars show the SEM for each experiment. (**C–H**) Immunohistofluorescence analysis for calpain 1 (**C–E**, *red signal*) and calreticulin (**F–H**, *red signal*) to FG-traced RGCs (corresponding *left* images, *blue signal*) on control, IONT-, and IONC-injured retinas at 48 hpl ($n = 5$ per group). *Boxed areas*: magnified positive RGCs for a given protein. *Arrows*: RGC. Capn1, calpain 1; Calr, calreticulin. Scale bar, 100 μm.

signal was strongly observed in the inner nuclear layer, where bipolar and amacrine cell bodies lay (Fig. 1G). After ON injury, cathepsin C immunolabeling was similar to that of control retinas, but it stained some fibers that crossed the inner plexiform layer (Figs. 1H, 1I). The cathepsin B (Figs. 1J–L) expression pattern resembled that of cathepsin C, but its signal was weaker, in agreement with the Western blot findings.

Calpain 1. Figure 2A shows that after IONT, the calpain 1 protein level increased from 12 hpl until 48 hpl and decreased at 7 dpl to control levels. After IONC, calpain 1 was overexpressed from 12 hpl to 7 dpl (Fig. 2B). Immunohistofluorescence studies revealed that calpain 1 signal was circumscribed to the GCL in control and injured retinas. Colocalization with FG-traced RGCs indicated that it was expressed by these neurons (Figs. 2C–E).

Eat-Me Signal: Calreticulin

Figure 2B shows that calreticulin was upregulated at 48 hpl after IONT and IONC, suggesting that at this time there was a wave of apoptosis. This was strengthened by the transitory upregulation of active caspase 3 at the same postlesion time.[8]

Calreticulin was not detected in control retinas (Fig. 2F), but its expression was evident in the GCL of IONT-injured (Fig. 2G) and IONC-injured (Fig. 2H) retinas, where its signal colocalized with FG-labeled RGCs.

Transcription Factors: Stat 1, Jun, and AhR

Signal Transducer and Activator of Transcription 1. Stat 1 was highly expressed in control retinas, and its level increased after ON injuries from 48 hpl until 7 dpl (Fig. 3A). Stat 1 was expressed in the GCL layer, but not by RGCs, because there was no colocalization to FG. After optic nerve lesion, either crush or transection, it was observed that the Stat 1 signal broadened and occupied a wider area of the GCL (Figs. 3D–F).

Jun. Western blot analysis for Jun is shown in Figure 3B. Jun protein was upregulated from 12 hours to 48 hours after IONT and was transitorily upregulated at 48 hours after IONC.

Jun expression pattern within the retina is shown in Figures 3G to 3I. In control and injured retinas, the Jun signal was circumscribed to the GCL, where it was expressed by most FG-labeled RGCs.

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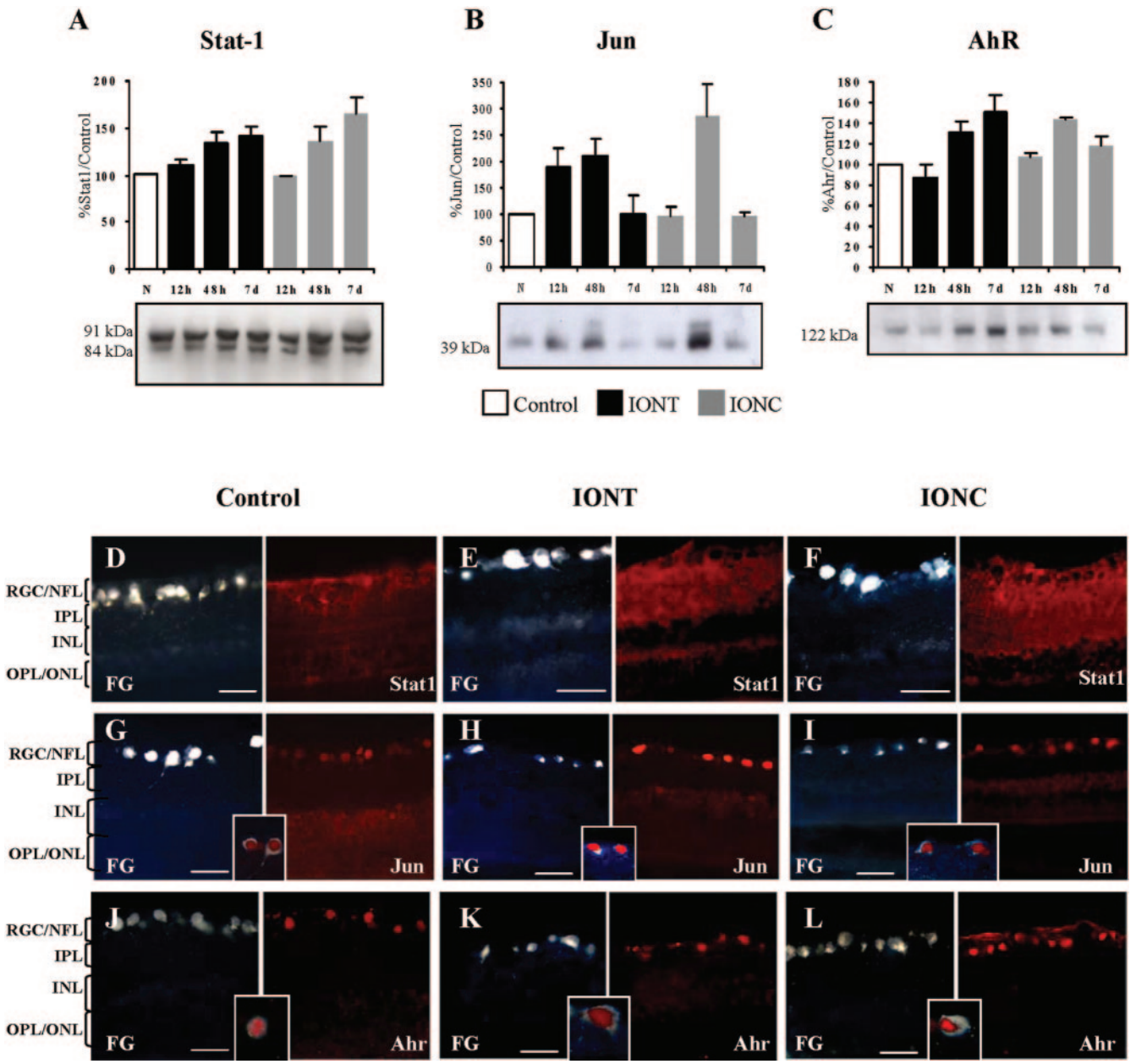

Figure 3. Jun, AhR, and Stat 1 are overexpressed in ON-injured retinas. (**A–C**) Western blot time-course analysis showing the regulation of Stat 1 (**A**), Jun (**B**), and AhR (**C**) in control, IONT-, and IONC-injured retinas. Graphs show quantification of protein signal ($n = 4$ animals per lesion and time point; Western blots were replicated three times). Signal from injured retinas is referred to as the control signal, which was arbitrarily considered 100%. Error bars show the SEM for each experiment. (**D–L**) Immunohistofluorescence analysis for Stat 1 (**D–F**, *red*), Jun (**G–I**, *red*), and AhR (**J–L**, *red*) to FG-traced RGCs (corresponding *left* images, *blue signal*) on control, IONT-, and IONC-injured retinas at 48 hpl ($n = 5$ per group). *Boxed areas*: magnified positive RGCs for a given protein. Scale bar, 100 μm.

Aryl Hydrocarbon Receptor. The AhR protein was barely detected in control extracts. However, after ON injury, AhR levels were upregulated from 48 hpl to 7 dpl (Fig. 3C). The AhR signal was detected in the GCL of control and injured retinas, where it colocalized with FG-traced RGCs. In addition, AhR stained the nerve layer of IONC-injured retinas (Figs. 3J–L).

Discussion

In mammals, optic nerve injury induces the death of most RGCs, impairing any chance of functional repair. This massive degeneration does not occur to such an extent in the visual system of lower vertebrates, such as fish[26] and frogs.[27] In these species, the surviving RGCs are able to regenerate and heal the wounded system. In fact, in the goldfish retina, it has been shown that optic nerve injury triggers the upregulation of antiapoptotic proteins and surviving signaling pathways, together with the downregulation of proapoptotic proteins such as caspase 3,[28] in a manner opposite to what occurs in the rat retina.[8] One of the main goals, then, is to decipher the death-molecular signals triggered by ON injury in adult mammal retinas. We describe here, for the first time, the temporal regulation of eight proteins related to cell death in the rat retina after crushing or transecting the optic nerve.

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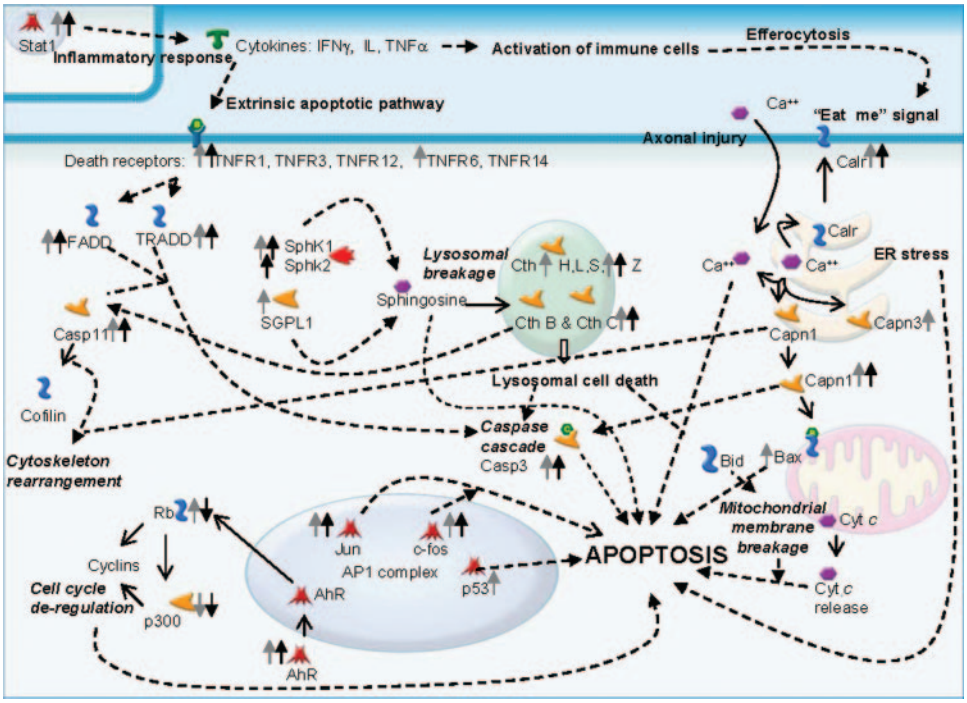

FIGURE 4. Apoptotic signals regulated in RGCs after ON injury. This map has been made based on our previous study[8] and on the results shown in this article. Software packages (MetaCore and MapEditor; GeneGo, Ltd.; www.genego.com) were used. All shown genes were regulated after one or both injuries at their respective RNA level, with the exception of TRADD and FADD, for which only protein regulation was observed (TRADD[8] and FADD [Agudo et al., unpublished data, 2008]). For TNFR1, Casp3, c-fos, Casp11, CathC, CathB, Capn1, Calr, AhR, Jun, and Stat 1, protein upregulation has also been shown (Agudo et al.[8] and this study). Note that all these proteins except Stat 1 are expressed by RGCs. *Yellow arrows*, *pink arrows*: regulation (RNA, protein, or both) after IONT and IONC, respectively.

Proteases: Caspase 11, Cathepsin C, Cathepsin B, and Calpain 1

Inflammatory Response: Caspase 11. Caspase 11 is an inflammatory caspase induced by interferon and Stat 1[29] whose role in apoptosis is mediated by activating caspases 3 and 8.[11,20] Furthermore, a role for caspase 11 in actin cytoskeleton depolymerization through its interaction with cofilin has been recently reported.[30,31] In our system we observed that after IONT and IONC, caspase 11 expression in the retina increased from 12 hpl to 7 dpl. Because both optic nerve injuries trigger RGC death, the role of caspase 11 in the injured retina may be related to the activation of the inflammatory response, which results in central nervous system neuronal apoptosis.[32–34] In addition, because the caspase 11 signal in the nerve fiber layer is very strong after ON injury, this may result in an imbalance toward actin depolymerization, causing axonal cytoskeleton destabilization. This, in conjunction with calpain 1 overexpression, may be part of the molecular events leading to the axonal degeneration observed after optic nerve injury.

Lysosomal Death Pathway: Cathepsin C and Cathepsin B. Some cathepsins have been detected in different regions of the eye. The optic nerve and the cornea express cathepsins A, B, D, and L, and the choroid expresses cathepsin S.[35]In the retina, the presence of cathepsin D[36–38] and cathepsin B has been reported.[35,36] Cathepsins are proteases located in the lysosomes. For years their biological functions have been circumscribed to unspecific intracellular protein degradation. Current evidence points to a role of lysosomes and, more specifically, of cathepsins in apoptosis. In fact the lysosomal pathway of apoptosis is a new phenomenon that is widely recognized.[12,39] To promote cell death, these proteases must be released from the lysosomes. Lysosomal permeabilization is facilitated by sphingosine, a lipid whose overexpression is linked to apoptosis.[40] Our array study[8] revealed that the mRNAs of some enzymes of the sphingosine metabolism were soon upregulated after IONT (sphingosine kinase 1 and sphingosine-1-phosphate lyase) and IONC (sphingosine kinases 1 and 2). The upregulation of these enzymes increases the amount of cytosolic sphingosine, which may eventually cause lysosomal permeabilization.[40,41] The role of cathepsins in apoptosis is twofold. First, they act as proteases, cleaving and consequently activating caspases. Second, they act on the mitochondria to induce mitochondrial dysfunction.[12,39,42] Cathepsin B is essential in different models of apoptosis through the generation of reactive oxygen species and the release of cytochrome *c* from the mitochondria.[10,32,33] It has been related as well to the activation of caspase 11.[34] Our array data showed that the mRNA of several cathepsins were upregulated in the retina after IONT (cathepsins B, C, H, L, S, and Z) and after IONC (cathepsins C and Z). The present studies show that the precursor and active forms of cathepsin B and C proteins are upregulated after both ON injuries and that both are expressed by RGCs. Interestingly, the upregulation of the active form is stronger after IONT than IONC, and this may be responsible in part for the quicker RGC degeneration observed anatomically after transection than after crush (Parrilla-Reverter G, et al. *IOVS* 2006;47:ARVO E-Abstract 1248). Because both cathepsins are upregulated and are active earlier (12 hours) than occurs with the upregulation of caspase 3 (48 hours) and concomitantly caspase 11 (12 hours) and because both caspases are activated by cathepsins, it is tempting to speculate that the upregulation of cathepsins B and C in the axotomized retinas is an early event of apoptosis caused by optic nerve injury.

Endoplasmic Reticulum Stress: Calpain 1. High intracellular Ca^{2++} concentration is an early event in apoptosis and may occur through endoplasmic reticulum stress or by axonal injury.[41,43] Calpain 1 is a Ca^{2++}-activated protease normally located in the endoplasmic reticulum. Its upregulation concurs with apoptosis activation and myelin breakdown.[13,14,43] In the visual system, calpain 1 has been extensively analyzed in photoreceptor degeneration models[13] and in hypoxia-induced retinal death, the latter through the proteolysis of Tau and α-spectrin, two cytoskeletal proteins.[44] Calpain 1 activates apoptosis by cleaving and activating proapoptotic silent molecules, such as caspase 3, Bax, Bid, and p53, among others.[14] In our injury models, the catalytic form of calpain 1 was upregulated 12 hours after both injuries, again before caspase 3 activation was

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observed.[8] This is consistent with a recent report in which calpain 1 implication in RGC death after axotomy has been demonstrated in an in vitro model.[10]

Eat-Me Signal: Calreticulin. Calreticulin is another Ca^{2++}-activated protein, located in the endoplasmic reticulum, that modulates calcium homeostasis, protein folding, and cellular development in healthy cells. Recently, a new role for calreticulin as an eat-me signal has emerged.[15,16] Apoptotic cells must be cleared from the tissue before their membrane disintegrates.[16] Eat-me signals are markers for phagocytes to identify and remove apoptotic cells, a process known as efferocytosis. The better-characterized eat-me signals are phosphatidylserine and Annexin. Both are located inside the cell, and on apoptosis activation they relocate to the cell surface, where they are recognized by phagocytes. Calreticulin undergoes a similar process, so its upregulation and expression on the cell surface mark cells entering in apoptosis.[15,16] Our results show that calreticulin is transitorily overexpressed in the retina 48 hours after IONT and IONC and, more interestingly, that it is detected in injured but not in healthy RGCs. These data are consistent with the temporal expression of cathepsin B, cathepsin C, calpain 1, caspase 11, and caspase 3[8] and indicate that the induction of apoptosis by ON injury occurs early after the system is wounded.

Transcription Factors: Stat 1, Jun, and AhR

Inflammation and Cell Death: Signal Transducer and Activator of Transcription 1. Stat 1 is a transcription factor that plays an important role in promoting apoptotic cell death by mediating proapoptotic activities of cytokines.[20] Our study shows that Stat 1 is upregulated in the retina 48 hours after both ON injuries. Immunohistofluorescence experiments revealed that this transcription factor is located in the GCL layer but that its signal leaves a void where RGCs appear. Thus, it is localized in cells or at cell terminals located in the vicinity of RGCs. Stat 1 regulates the expression of many cytokines, which, in turn, sensitize cells to TNFα-mediated cell death that is dependent on death receptors and TRADD signaling.[20] TNFR1 and TRADD are upregulated after IONT and IONC, and they are expressed by RGCs.[8] It is possible, then, that if Stat 1 has a role in RGC death induced by ON injury, the role is mediated by its effect on cytokine production and not by its direct role in apoptosis.[20]

Immediate Early Response: Jun. Jun is another transcription factor whose role in cell death is controversial because it has been involved in cell survival and in cell death.[45,46] However, Jun downregulation by means of siRNA significantly increased the number of surviving RGCs 14 days after axotomy.[9] In addition, activation of the AP-1 complex, formed by Jun, c-fos, and ATF-family proteins, is an essential trigger of apoptosis in retinal degeneration.[47] In agreement with these reports, we have shown that c-fos[8] and Jun are upregulated in axotomized retinas and are expressed by RGCs.

Detoxification and Cell Cycle Deregulation: Aryl Hydrocarbon Receptor. AhR is a ligand-activated transcription factor that regulates the transcription of detoxification enzymes.[19] In addition to its classical role in the xenobiotic signaling pathway, AhR is implicated in cell cycle arrest, cell proliferation, differentiation, and apoptosis.[48] Its role in cell cycle arrest is mediated through the retinoblastoma protein and p300, whose RNA levels are regulated after IONT and IONC in the retina.[8,18,48] This, together with the retinal upregulation of AhR after both injuries when its expression was restricted to RGCs, suggests that cell-cycle deregulation may be part of the molecular events that trigger axotomy-induced RGC death

All these data are graphically shown in Figure 4, in which the molecular relationship among all these signals is summarized.

Conclusions

This is the first time that caspase 11, cathepsin C, cathepsin B, calpain 1, calreticulin, AhR, Stat 1, and Jun temporal protein expression have been analyzed in control, IONT, and IONC adult rat retinas. All were overexpressed in the retina at 12 hpl, and most peaked at 48 hpl, far earlier than the initial anatomic RGC loss was observed (Parrilla-Reverter G, et al. *IOVS* 2004; 45:ARVO E-Abstract 911),[1,5,49] suggesting that the commitment to death is an earlier event than thought. Furthermore, with the exception of Stat 1, which locates to the GCL, these death-related proteins are expressed primarily by the injured cells, RGCs. Given that they are all implicated in apoptosis, this extensive analysis highlights the complexity of the molecular events triggered in the retina by ON injury and points to new targets for designing future neuroprotective strategies.

Acknowledgment

The authors thank Maria Paz Villegas for critical reading of the manuscript.

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