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Increased expression and activation of poly(ADP-ribose) polymerase (PARP) contribute to retinal ganglion cell death following rat optic nerve transection

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Abstract

Excessive activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) by free-radical damaged DNA mediates necrotic cell death in injury models of cerebral ischemia-reperfusion and excitotoxicity. We recently reported that secondary retinal ganglion cell (RGC) death following rat optic nerve (ON) transection is mainly apoptotic and can significantly but not entirely be blocked by caspase inhibition. In the present study, we demonstrate transient, RGC-specific PARP activation and increased retinal PARP expression early after ON axotomy. In addition, intravitreal injections of 3-aminobenzamide blocked PARP activation in RGCs and resulted in an increased number of surviving RGCs when compared to control animals 14 days after ON transection. These data indicate that secondary degeneration of a subset of axotomized RGCs results from a necrotic-type cell death mediated by PARP activation and increased PARP expression. Furthermore, PARP inhibition may constitute a relevant strategy for clinical treatment of traumatic brain injury. *Cell Death and Differentiation* (2001) **8,** 801 – 807.

Keywords: PARP; retinal ganglion cell death; optic nerve transection; neuroprotection; 3-aminobenzamide; necrosis

Abbreviations: PARP, poly(ADP-ribose) polymerase; RGC, retinal ganglion cell; ON, optic nerve; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; 3-ABA, 3-aminobenzamide; FB, Fast Blue

Introduction

Poly(ADP-ribose) polymerase (PARP) is a tightly bound nuclear enzyme found in various organs, including the brain.[14] Under physiological conditions it is thought to play an important role in the repair of DNA strand breaks, thereby contributing to maintain the genomic integrity of cells.[34] PARP has also been implicated to participate in cellular differentiation, gene rearrangement and transpositions.[13] PARP is activated by DNA strand breaks which result from DNA damage mainly mediated by reactive oxygene species (ROS) and nitric oxide.[8,36] PARP activation results in the formation and transfer of negatively charged ADP-ribose polymers onto chromatin-associated proteins as well as ADP-ribosylation of PARP itself, utilizing nicotinamide-adenosine dinucleotide as its substrate. Poly-ADP-ribosylation of various nuclear proteins constitutes an important post-translational modification necessary for efficient repair of DNA strand breaks.[26] However, it has also been demonstrated that excessive PARP activation by various triggers resulting in depletion of NAD^+ and subsequent cellular ATP depletion ultimately leads to a necrotic-type cell death.[5,32,41,49] There is accumulating evidence that excessive PARP activation plays a key role in mediating ischemia-reperfusion-induced cerebral injury[10,11,38,42] and that infarct volume can be dramatically reduced by PARP inhibition and in PARP knock-out mice.[9,37,38] Cerebral ischemia results in massive activation of N-methyl-D-aspartat (NMDA) receptors *via* an increase of extracellular glutamate leading to elevated intracellular calcium concentrations, activation of neuronal NO synthase (nNOS), increased NO and ROS formation and ultimately to PARP-activating DNA damage.[4,7]

3-Aminobenzamide (3-ABA) has been most frequently used as a PARP inhibitor because it displays no toxicity *in vivo* and is highly selective for PARP compared with mono(ADP-ribose) transferase, another DNA repair enzyme.[36] PARP inhibition is achieved by binding of 3-ABA to the NAD^+-binding site of PARP due to its higher binding affinity compared with NAD^+. In contrast to nicotinamide, 3-ABA does not scavenge NO or peroxynitrite itself, thus not exerting relevant PARP-independent neuroprotective effects.[36]

Rat optic nerve (ON) transection consistently leads to secondary death of about 85% of the entire retinal ganglion cell (RGC) population within 14 days after injury.[20,43] In contrast to cell death occurring after ischemia-reperfusion injury, this secondary RGC death is mainly apoptotic.[1,15] It can partially but not entirely be blocked by intravitreal application of potent caspase inhibitors.[16] However, features of necrotic RGC death preceding the appearance of apoptotic RGCs have also been described after ON crush injury.[2] In addition, ON transection has been shown to induce upregulation of nNOS in RGCs and an increase of NOS activity in RGC axons.[21] To determine whether PARP plays a role in mediating secondary RGC death, we examined for the first time PARP expression and PARP activation following ON transection utilizing PARP and poly(ADP-ribose) polymer immunohistochemistry, and Western blot analysis. In addition, we tested the effect of

3-aminobenzamide, a widely used PARP inhibitor, on PARP activation and RGC survival 14 days after ON axotomy.

Results

Retinal PARP expression following ON transection

Immunohistochemistry employing a polyclonal anti-PARP antiserum showed only moderate PARP expression in the ganglion cell layer (GCL), the inner nuclear layer (INL), and the outer nuclear layer (ONL) of sham-operated control animals (Figure 1). No specific immunoreactivity was observed in the inner and outer plexiform layer (IPL, OPL). There were no significant changes in retinal PARP immunoreactivity 3 h following ON transection when compared to control animals (data not shown). In contrast, we observed an induction of nuclear PARP immunoreactivity in the GCL and INL beginning at 12 h and peaking at 1 day to 3 days after ON injury (Figure 1). Seven days after ON transection, however, PARP expression in the GCL did not differ from that of controls whereas PARP expression in the INL was still elevated compared to control animals (Figure 1). Only a slight increase in PARP immunoreactivity was detected in the ONL following ON axotomy (Figure 1). Negative controls did not show any specific staining.

Corresponding to these results, Western blot analysis using a polyclonal anti-PARP antiserum revealed an increase in retinal PARP expression after ON transection when compared to sham-operated control animals. Changes were detected at a single band of 113 kDa matching the band detected in lysates of the rat glioma cell line LN-18 which served as positive control (see Materials and Methods). Retinal PARP expression markedly increased 1 day and especially 3 days post-injury when compared to control animals (Figure 2). PARP protein levels had declined again at 7 days after ON transection but were still elevated when compared to control animals, corresponding to the sustained increase in PARP expression in the INL. Only a slight increase in retinal PARP expression was observed 3 h after axotomy (Figure 2).

Retinal PARP activation following ON transection

To examine whether ON transection results in retinal PARP activation, we performed immunohistochemistry employing a polyclonal antibody against poly(ADP-ribose) polymers, which are formed dependent on PARP activity. We detected increased GCL-specific, nuclear immunoreactivity at all time points following ON transection, indicating an RGC-specific PARP activation (Figure 3). Poly(ADP-ribose) polymer immunoreactivity in the GCL was prominent 12 h and 3 days after ON transection but peaked at 1 day after axotomy (Figure 3). In contrast, in sham-operated control animals, specific poly(ADP-ribose) polymer immunoreactivity was restricted to some scattered cells in the GCL, probably due to minor mechanical irritations at the surface of the ON during sham operation (Figure 3). Only a slight increase in poly(ADP-ribose) polymer immunoreactivity was observed 3 h post-lesion (data not shown). PARP activation appears to be transient since GCL-specific poly(ADP-ribose) polymer immunoreactivity had markedly declined at 7 days after ON transfection (Figure 4). Specific immunoreactivity was restricted to the GCL and not observed in any other retinal layer and in negative controls.

To quantify GCL-specific retinal PARP activation after ON transection we performed counts of poly(ADP-ribose) polymer positive cells in the GCL at different time points after axotomy (Figure 4). In sham-operated control animals we detected 24 ± 7 poly(ADP-ribose) polymer positive cells per retinal section. While there was only a slight, not significant increase to 34 ± 6 cells per section 3 h post-lesion, the number of poly(ADP-ribose) polymer positive RGCs increased to 86 ± 20 cells per section 1 day after

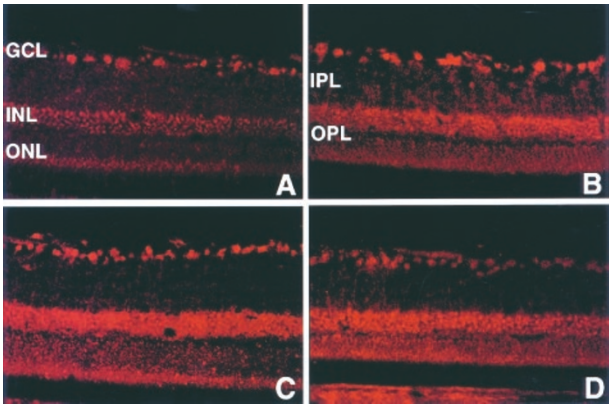

Figure 1 PARP immunohistochemistry on retinal sections. Note moderate PARP expression in the GCL, INL, and ONL of control animals (**A**). Increased nuclear PARP immunoreactivity in the GCL and INL 1 day (**B**) and 3 days (**C**) after ON transection. PARP expression returns to control levels in the GCL but is still elevated in the INL 7 days after ON injury (**D**). Only slight increase in PARP immunoreactivity in the ONL after ON axotomy (**A**–**D**). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer

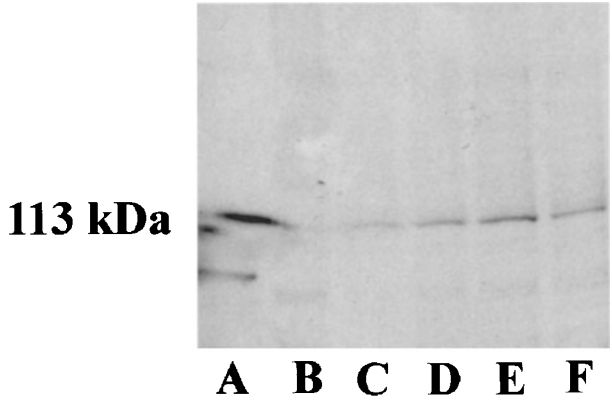

Figure 2 Western blot analysis of retinal PARP expression. (**A**) Positive control (see Materials and Methods). Note marked increase in retinal PARP protein 3 days (**E**) and less prominent, 1 day (**D**) and 7 days (**F**) after ON transection compared to control animals (**B**). Only a slight increase in retinal PARP expression was observed 3 h (**C**) after axotomy

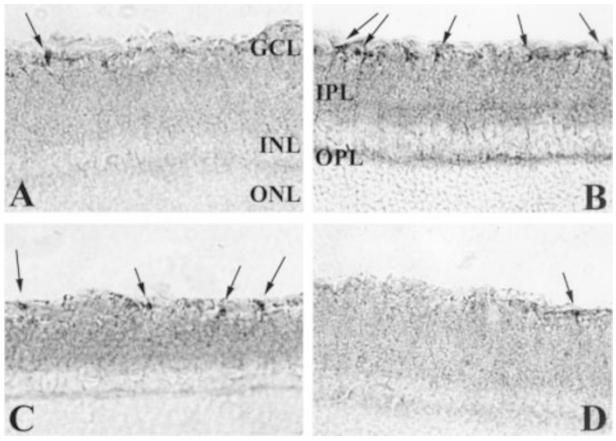

Figure 3 Poly(ADP-ribose) polymer immunohistochemistry on retinal sections to monitor PARP activation after ON transection with or without 3-ABA treatment. Note GCL-specific, nuclear immunoreactivity 1 day (**B**) and, less prominent, 3 days (**C**) after ON axotomy without a specific therapy, indicative for RGC-specific PARP activation. In contrast, only a small number of poly(ADP-ribose) polymer positive cells (see also Figure 4) was observed in retinae of sham control animals (**A**). Note that intravitreal 3-ABA application markedly reduced the number of poly(ADP-ribose) positive RGCs 1 day after ON transection (**D**). In these animals immunoreactivity is restricted to a small number of RGCs. Some immuno-reactive RGCs are marked with arrows. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer

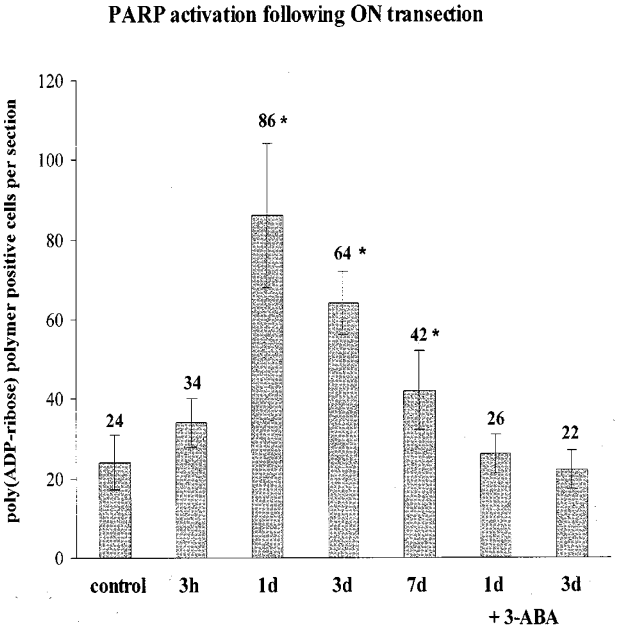

Figure 4 Counts of poly(ADP-ribose) polymer positive RGCs in retinal sections to assess PARP activation at different time points after ON transection with or without intravitreal 3-ABA application. Note marked increase in the number of immunopositive RGCs 1 day and 3 days post-lesion without therapy, which can be blocked by 3-ABA application. Data are given as mean $\pm$ standard error of the mean (S.E.M.). *, $P<0.05$ *vs* control (see Materials and Methods); control, sham-operated animals; 3-ABA, 3-aminobenzamide

axotomy. Three and 7 days following ON transection the number of immunpositive cells declined to 64 ± 8 and 42 ± 10, respectively, indicating a decrease in PARP activity (Figure 4).

Inhibition of PARP activation by intravitreal 3-ABA application

To examine whether intravitreal 3-ABA application can block retinal PARP activation induced by ON transection we performed poly(ADP-ribose) polymer immunohistochemistry on retinal sections of axotomized/3-ABA-treated animals. One day as well as 3 days after ON transection and 3-ABA treatment (injections either 2 and 20 h or 2 h, 20 h and 2 days post-lesion) we observed a marked decrease in the number of poly(ADP-ribose) polymer positive RGCs when compared to untreated animals 1 and 3 days after lesion. Immunoreactivity was only observed in some scattered cells in the GCL, indicating an effective inhibition of PARP activation by intravitreal 3-ABA application (Figure 3). Counts of poly(ADP-ribose) polymer positive cells in retinal sections of these animals revealed 26 ± 5 and 22 ± 5 cells per section 1 day and 3 days after ON transection and 3-ABA application (Figure 4). This indicates that intravitreal 3-ABA injections can efficiently block axotomy-induced PARP activation in RGCs.

Rescue of axotomized RGCs by PARP inhibition

The mean RGC density in unlesioned retinae was 2084 ± 59 Di-I-labeled cells per mm^2. Retrograde labeling of RGCs from the ON stump using FB revealed RGC densities in treated and control animals 2 weeks after ON transection. In untreated animals, mean RGC density declined to 348 ± 61, i.e. to 16.7% of control values within 14 days after ON transection (Figure 5). PARP-inhibition by intravitreal 3-ABA-injections at 2 h, 1, 2 and 4 days post-lesion significantly enhanced RGC survival 14 days after ON transection by 76% compared to vehicle-injected, axotomized control animals ($P<0.05$). The mean RGC density in retinae of 3-ABA-treated animals was 673 ± 36 FB-labeled cells per mm^2 compared to 383 ± 40 cells per mm^2 in vehicle-injected, axotomized control animals (Figure 5). This corresponds to an effective RGC rescue (ERR) of 17.0%, the percentage of RGCs which are protected from secondary cell death due to the treatment (see Materials and Methods). The difference in RGC density between vehicle-injected (PBS with 40% DMSO) and untreated animals (383 ± 40 *vs* 348 ± 61 cells per mm^2) was not statistically significant. In addition to labeled RGCs, we also observed labeling of endothelial cells of retinal vessels and microglial cells, which could easily be distinguished from RGCs by their different shape and diameter (see Materials and Methods).

Discussion

Excessive activation of poly(ADP-ribose) polymerase has been implicated to mediate cell death in different models of ischemia-reperfusion injury[10,40,42] as well as streptozotocin-induced diabetes,[32] glutamate-induced neurotoxicity[5,49] and Alzheimer's disease.[27] The common feature in the pathogen-

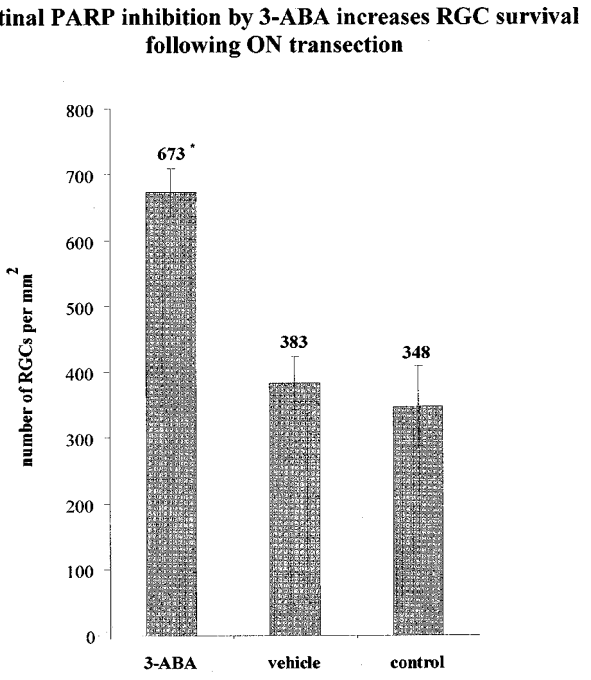

Figure 5 Survival-promoting effect of retinal PARP inhibition on axotomized RGCs 14 days after ON transection by intravitreal 3-ABA injections. Data are given as mean ± standard error of the mean (S.E.M.). Intravitreal injections of 3-aminobenzamide in vehicle (3-ABA), 40% DMSO in PBS (vehicle) or ON transection only (control). *, $P<0.05$ *vs* vehicle and control (see Materials and Methods); RGCs, retinal ganglion cells

esis of these clinically relevant conditions leading to PARP activation is oxidative DNA damage *via* increased production of nitric oxide and the formation of reactive oxygen species. Downstream events of massive PARP activation are NAD^+ depletion resulting in depletion of cellular ATP and subsequent necrotic-type cell death.[41] Inhibition of PARP activity using benzamide analogs, nicotinamide, benzopyrones or isoquinoline derivatives[36] has been demonstrated to attenuate ischemia-reperfusion injury in various models of transient cerebral,[10,37,38] heart,[40] renal,[3] and retinal ischemia.[24] In addition, PARP inhibition has been shown to protect from glutamate- and MPTP-induced neurotoxicity.[5,6] It has also been suggested, however, that the neuroprotective effect of PARP inhibition may require conditions of cellular NAD^+ depletion and that PARP inhibition may be neurotoxic after mild, sublethal ischemia without NAD^+ depletion.[29]

In multiple studies, transection of the rat ON has been used as a model to characterize secondary cell death following diffuse axonal injury.[1,15,18,43] Within 2 weeks after axotomy about 85% of all RGCs undergo secondary cell death which has been demonstrated to display hallmarks of apoptosis like caspase activation[18] and upregulation of Bax protein expression.[15] However, caspase inhibition employing different caspase inhibitors only partially blocks secondary death of axotomized RGCs and neuroprotective effects observed are transient.[16,17] In addition, neuroprotective effects of several neurotrophic factors on axotomized RGCs seem to be partially due to mechanisms other than inhibition of intracellular caspase activity.[20,47] In the present study, we therefore examined the role of PARP, which has been identified as an important mediator of a necrotic-type cell death, in axotomy-induced RGC death. Following ON transection, we observed an RGC-specific, transient increase in PARP activity as shown by GCL-specific enhancement of poly(ADP-ribose) polymer formation. In addition, we demonstrate a transient increase in retinal PARP expression localized in the GCL and the INL. Furthermore, intravitreal application of the PARP inhibitor 3-aminobenzamide significantly enhanced RGC survival 14 days after ON transection, indicating that PARP activation in axotomized RGCs is accompanied with NAD^+ depletion.[29]

Anti-apoptotic strategies using multiple injections of different caspase inhibitors resulted in survival of 20 to 34% of the RGCs which, without a specific therapy, would have undergone secondary cell death within 2 weeks after lesion (ERR), indicating that apoptosis constitutes the predominant mode of RGC death after ON transection.[16] However, retinal PARP inhibition by intravitreal 3-ABA injections after ON transection also led to significantly enhanced RGC survival (ERR of 17%), demonstrating that at least for a subset of axotomized RGCs secondary degeneration seems to be mediated by a necrotic-type cell death. Multiple injections of neurotrophic factors like BDNF, CNTF and GDNF, which are thought to interfere with multiple, intracellular pathways, resulted in ERRs of 27, 22 and 21%, respectively.[19,20,22,28]

Our results indicate that early secondary RGC death following ON transection involves non-apoptotic mechanisms like necrosis, which is mediated by post lesional PARP activation and increased PARP expression in RGCs. This hypothesis is further supported by a study demonstrating an upregulation of nNOS in RGCs as well as an increase of NOS activity in RGC axons after ON transection,[21] since PARP activation is strongly related to NO and peroxynitrite formation by nNOS.[11,36] It has been shown that nNOS can be regulated post-translationally by increased intracellular calcium concentrations.[30] Elevation of intracellular calcium, however, can result from activation of ionotropoic glutamate receptors, and a member of this family, NMDAR 1b, has been demonstrated to be upregulated early after ON crush.[23] Furthermore, intravitreal glutamate levels were found to be elevated as early as 3 days after ON crush injury[48] and intravitreal application of the NMDA receptor antagonist MK-801 increased RGC survival following crush-axotomy.[33] Moreover, appearance of necrotic cell death preceding apoptotic cell death has been reported as early as 2 days after ON crush injury.[2]

PARP has been identified as a substrate for activated caspase-3, which is a crucial mediator of apoptotic cell death.[31,39] However, both the precise role of PARP in apoptosis and the purpose of PARP cleavage by apoptotic proteases remain unclear. It has been demonstrated that caspase-3-mediated cleavage of PARP blocks DNA repair and prevents depletion of intracellular NAD^+ stores due to increased PARP activity suggesting that PARP cleavage secures intracellular energy levels necessary for the apoptotic process.[31,41,45] In addition, several studies

indicate that under certain experimental conditions apoptotic cell death may be PARP independent.[25,46] However, transient ribosylation of nuclear proteins by PARP has been reported necessary for Fas-induced apoptosis[35] and another study noted abnormal apoptosis in PARP knockout mice.[8] In our model of ON transection, caspase-3 activity was shown to peak at day 4 to 6 post-lesion.[18] Therefore, it is plausible that the level of activated caspase-3 during days 1 to 3 post-injury is not sufficient for relevant PARP cleavage, especially since the increase of caspase-3 in axotomized RGCs does not seem to occur simultaneously in the entire RGC population, but rather constitutes a process lasting several days.[15,18,43] In turn, this may provide a time window for activated PARP to induce secondary cell death following ON transection.

Taken together, these data indicate that secondary death of axotomized RGCs, even though mainly induced by caspase activation, may in part be mediated by PARP activation and increased PARP expression. PARP activation and increased PARP expression in RGCs occur at day 1 to 3 after ON transection and are almost back to control levels 7 days post-injury, thus preceding caspase-3 activation in our model of ON injury. In accordance with these results, intravitreal application of the PARP inhibitor 3-ABA increases survival of axotomized RGCs *in vivo* by blocking post lesional PARP activation suggesting that PARP inhibition may also constitute a relevant therapeutic strategy for traumatic brain injury. Future studies will have to elucidate the therapeutic potential of combining caspase and PARP inhibition for limiting axotomy-induced RGC death.

Materials and Methods

Animal surgery and intravitreal injections

Adult female Sprague Dawley rats (200–250 g) were anesthetized by intraperitoneal injection of chloral hydrate (7% in PBS; 420 mg/kg body weight). The optic nerve (ON) was exposed and transected as described previously leaving the retinal blood supply intact.[20,47] To determine RGC densities in lesioned animals, cells were retrogradely labeled with Fast Blue (FB; Dr Illing Chemie, Gross-Umstadt, Germany). For FB staining, a small piece of gel foam soaked in a 2% aqueous FB solution was placed at the ocular nerve stump after ON axotomy. To determine RGC numbers in unoperated control animals using retrograd labeling, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Di-I; 5% in dimethyl-formamide; Molecular Probes Inc., Oregon, USA) was applied to both superior colliculi on postnatal day 5 employing a micropipette. Double-staining protocols with Di-I applied to the superior colliculi and FB applied to the transected ON revealed similar label efficiencies of the two tracers, thus allowing comparison of Di-I and FB data.[12]

3-aminobenzamide (3-ABA; Sigma, Munich, Germany) was dissolved in 10 mM PBS containing 40% dimethyl-sulfoxide (DMSO). Animals were anesthetized by inhalation of diethylether and intravitreally injected with either 0.6 μmol of 3-ABA (n=6) or vehicle only (n=4). Injection volume was 2 μl. Intravitreal injections were performed 2 h, 1, 2 and 4 days following ON transection employing a glass micro-electrode according to a protocol described in more detail elsewhere.[20]

Assessment of RGC survival: cell densities and statistical analysis

Rats were sacrificed by an overdose of chloral hydrate 14 days after ON transection. Operated eyes were removed, retinae dissected, fixed in 4% paraformaldehyde (PFA) in PBS for 20 min and flat-mounted onto glass slides. Retinae were examined by fluorescent microscopy (Axiophot 2; Zeiss, Göttingen, Germany) using a rhodamine (546/590 nm) or a DAPI filter (365/397 nm) for Di-I or FB fluorescence, respectively. RGC densities were determined by counting tracer-labeled cells in 12 distinct areas (three areas per retinal quadrant) of 62 500 μm^2 each.[12,20] Labeled endothelial cells of retinal vessels (fusiform shape) and microglial cells (rod-shaped, ramifications, smaller diameter than RGCs) were excluded from cell counts. Cell counts were performed in duplicate by two independent and blinded investigators.

Data are given as mean $\pm$ standard error of the mean (S.E.M.). Statistical significance was determined using the Mann–Whitney U-test. For exact evaluation of survival-promoting effects due to the specific treatment, we defined an effective RGC rescue (ERR): $ERR=(N_{ther}-N_{con})/(N_{tot}-N_{con}) \times 100$ (N_{tot}: RGC number in unlesioned retinae, N_{con}: RGC number surviving axotomy without therapy, N_{ther}: RGC number surviving after a given therapy.[47])

Poly(ADP-ribose) polymer and PARP immunohistochemistry

Rats were anesthetized and perfused transcardially with saline, followed by 4% paraformaldehyde (PFA) in PBS at different time points after ON transection (3, 12, 24 and 72 h, 7 days; n=3 for each time point). Sham-operated animals served as controls. For poly(ADP-ribose) polymer immunohistochemistry additional animals were sacrificed 1 and 3 days following ON transection (n=3 for each time point), which had been intravitreally injected with 3-ABA at either 2 h and 20 h or 2 h, 20 h and 2 days after axotomy (for injection procedure see above). Retinal cryostat sections (16 μm, cryoprotected by sucrose incubation, 4% PFA-fixed) were prepared as described earlier.[20] For poly(ADP-ribose) polymer immunohistochemistry, sections were incubated with 2% Triton-X in PBS for 15 min at room temperature (RT) followed by treatment with 2% H_2O_2 (30 min at RT), incubation with blocking solution (2% BSA, 0.1% Triton-X, 10% NGS in PBS; 1 h at RT) and incubation with a polyclonal guinea-pig anti-poly(ADP-ribose) antibody (1 : 60 in PBS with 2% BSA and 0.1% Triton-X; 48 h at 4°C; Trevigen, MD, USA), which is specific for poly(ADP-ribose) polymers 2–50 units long. A biotin-conjugated goat anti-guinea-pig IgG was utilized as secondary antibody (1 : 100, 1 h at RT; Vector Laboratories, CA, USA) followed by signal amplification using an immunoperoxidase avidin-biotin complex kit according to the manufacturer's instructions (Vectastain elite; Vector Laboratories, MD, USA) and incubation with diminobenzidine as a chromogen (0.05% in PBS; Sigma, Munich, Germany).

For PARP immunohistochemistry, sections were incubated with blocking solution (2% BSA, 0.1% Triton-X, 10% NGS in PBS; 1.5 h at RT) followed by incubation with a polyclonal rabbit anti-PARP antibody (1 : 400 in PBS with 2% BSA and 0.1% Triton-X; overnight at 4°C; Santa Cruz Biotechnology, Inc., CA, USA). After application of a secondary, Cy-3-coupled, goat anti-rabbit antibody (1 : 600; 1 h at RT; Dianova, Hamburg, Germany), sections were examined under fluorescence microscopy using a rhodamine filter. For both poly(ADP-ribose) polymer and PARP immunohistochemistry negative controls were performed by omission of the primary antibody. All sections were counterstained with DAPI.

Cell counts of poly(ADP-ribose) polymer positive cells

To assess the number of poly(ADP-ribose) polymer positive RGCs at different time points after ON transection (control, 3 h, 1, 3 and 7 days) as a marker for PARP activation cell counts were carried out in the GCL of 12 corresponding retinal sections ($\pm$1.5 mm from the ON head) of three animals per time point stained for poly(ADP-ribose) polymer formation. To assess the efficacy of retinal PARP inhibition by 3-ABA identical cell counts were done in additional animals 1 and 3 days following ON transection (n=3 for each time point), which had been intravitreally injected at either 2 h and 20 h or 2 h, 20 h and 2 days after axotomy. Cell counts were performed by two independent and blinded investigators. Data are given as mean of immunopositive RGCs per section $\pm$ standard error of the mean (S.E.M.). Statistical significance was determined using the Mann–Whitney U-test.

Western blot analysis of retinal PARP expression

For Western blot analysis, retinae were dissected at different time points after ON transection (3, 12, 24, 72 h, 7 days; n=4 for each time point). Retinae of sham-operated animals served as controls. Retinae were complemented with lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% desoxycholate, 0.1% SDS, 5 mM EDTA, protease inhibitors), homogenized, centrifuged and supernatants used for SDS–PAGE. Equal amounts of protein were diluted in 6 $\times$ sample buffer, boiled for 5 min and loaded onto 8% polyacrylamide gels. To test for antibody specificity, 2 μl of a rat glioma cell line (LN-18) lysate, in which PARP protein had successfully been detected using Western blot analysis,[44] were loaded onto a separate lane as positive control. Proteins were transferred onto nitrocellulose membranes, immersed in blocking solution (5% milk, 0.1% Tween 20 in PBS; 12–15 h at 4°C) and incubated with a polyclonal rabbit anti-PARP antibody (1 : 2500 in PBS with 1% milk, 0.1% Tween 20; 2 h at RT; Boehringer-Mannheim, Germany). Subsequently, membranes were incubated with a peroxidase-coupled, goat anti-rabbit secondary antibody (1 : 200.000; Dianova, Hamburg, Germany), washed several times, immersed in ECL solution and exposed to ECL-Hyperfilm (Amersham, Braunschweig, Germany).

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