

Apoptotic Retinal Ganglion Cell Death After Optic Nerve Transection or Crush in Mice: Delayed RGC Loss With BDNF or a Caspase 3 Inhibitor

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PURPOSE. To investigate retinal ganglion cell (RGC) survival and activation of caspase 3 after optic nerve crush (ONC) or transection (ONT) and treatment with brain-derived neurotrophic factor (BDNF) or Z-DEVD_fmk.

METHODS. In albino Swiss mice, the left optic nerve was severed or crushed at 0.5 mm from the optic head and retinas were analyzed from 1 to 10 days. Additional groups were treated intravitreally with a single injection of BDNF (2.5 µg) or Z-DEVD_fmk (125 ng) right after injury, or with Z-DEVD_fmk at day 2, or with multiple injections of Z-DEVD_fmk. As controls intact or vehicle-treated retinas were used. In all retinas, Brn3a (RGCs) and cleaved-caspase 3 (c-casp3) were immunodetected and their numbers quantified. In an additional group, c-casp3 expression was assessed in RGCs retrogradely labeled before axotomy.

RESULTS. The temporal loss of RGCs was the same after ONC or ONT and occurred in two phases with 65% loss during the first 7 days and an additional 4% loss from day 7 to 10. The appearance of c-casp3⁺RGCs is Gaussian, peaking at 4 days and declining thereafter. Brn3a down-regulates when RGCs start expressing c-casp3. Retinal ganglion cell rescue rate for BDNF or Z-DEVD_fmk is similar and both delay RGC loss by 1 day. Delayed treatment with Z-DEVD_fmk does not rescue RGCs, and several injections are not better than a single one at the time of the injury.

CONCLUSIONS. Brn3a down-regulation marks the beginning of RGC death, which after axotomy occurs by caspase-dependent apoptosis in at least half of the RGCs. These data should be considered when designing neuroprotective strategies.

Keywords: Brn3a, active caspase 3, BDNF, Z-DEVD, neuroprotection

Retinal ganglion cells (RGCs) are the only neurons that relay information from the retina to the brain. Retinal ganglion cells die when their axons, that form the optic nerve, are injured, leading to irreversible loss of function. Because central nervous system neurons in mammals are not replaced by new ones, blindness ensues. Much effort is being devoted to find neuroprotective therapies to diminish or delay axotomy-induced RGC death. However, to date there are no successful treatments probably because neither the appropriate target(s) nor the right time window have been deciphered. Thus, the detailed knowledge of the temporal course of RGC death and of the mechanisms that lead to this loss is essential to finely tune the therapeutic time-window and the type of molecules to assay.

In rats it has been demonstrated that the rate of RGC loss depends on the distance at which injury is inflicted, the closer the injury to the cell soma the quicker the degeneration.¹ In this species, RGC loss also depends on the type of injury to the optic nerve, although the reasons behind are unknown. For instance, after intraorbital optic nerve transection (ONT) RGC death is quicker than after optic nerve crush (ONC),²⁻⁷ and after ocular hypertension, a pathologic situation that also harms the optic nerve, RGC death follows a slower course than after

simple axotomy.⁸⁻¹⁰ Genetic background also influences the course of RGC degeneration because RGC loss after axotomy varies between some mice strains¹¹ but not between others.¹²

Axotomy-induced RGC loss has been attributed, at least in part, to a withdrawal of trophic factors from the axonal terminals¹³ which, in turn, causes the apoptotic death of RGCs.¹⁴⁻²⁴ Apoptosis can be triggered by death receptor activation (extrinsic pathway) or mitochondrial release of cytochrome C (intrinsic pathway) reviewed in Ref. 25. Both pathways are up-regulated in axotomized rat RGCs.^{14,26} The extrinsic pathway starts by the activation of a cell surface receptor. Activated death receptors, such as Fas, recruit adaptor proteins that activate caspase 8 by proteolytic cleavage. Active caspase 8, in turn, activates downstream executioner caspases (caspases 3 and 7). The intrinsic pathway starts with the permeabilization of the mitochondrial outer membrane. This permeabilization causes the release of cytochrome C into the cytoplasm, which induces the formation of a complex called the apoptosome. This complex, then, binds and activates caspase 9. Active caspase 9 bound to the apoptosome recruits and activates the effector caspases 3 and/or 7. Thus, both the extrinsic and the intrinsic pathway converge at caspase 3. Caspase 3, together with the other effector caspases, dismantles



cellular structures by cleaving specific substrates causing the death of the cell.^{15,25,27} Recent studies show that besides its crucial role in neuronal death by apoptosis, caspase 3 performs other functions, death independent, such as dendrite pruning and synaptic plasticity roles.^{25,28}

Therefore, neuroprotective therapies to rescue axotomized RGCs are mostly directed to either administration of trophic factors or manipulation of the apoptotic pathway. Brain-derived neurotrophic factor (BDNF) is, to date, the best neuroprotectant,^{5,6,12,29–32} but its effect is only transitory even with long-lasting treatments.^{33,34} Likewise, caspase inhibition only results in transient neuroprotection.^{19,20,24,35,36,36–41}

In mice, there is not, to our knowledge, a comparative study of the loss of RGCs triggered by ONC or ONT, nor is there a detailed analysis of RGC loss versus expression of apoptotic signals with or without neuroprotection.

Thus, we have analyzed (1) the daily loss of Brn3a-positive RGCs (Brn3a⁺RGCs) in albino mice after ONT or ONC (from 1 to 10 days); to be comparable, both lesions were performed at the same distance from the optic nerve head (0.5 mm); (2) the appearance of RGCs undergoing caspase-dependent apoptosis immunodetecting active caspase 3 (cleaved caspase 3, c-casp3) in the same retinas; and (3) RGC survival and activation of caspase 3 after administration of BDNF, chosen as positive control,¹² or of an irreversible caspase 3 inhibitor (Z-DEVD_fmk).

MATERIALS AND METHODS

Animal Handling and Ethics Statement

Two-month-old male albino Swiss mice, Crl:CD1 (ICR), were purchased from Charles River (Barcelona, Spain). All experimental procedures were carried out in accordance with the Association for Research in Vision and Ophthalmology and the European Union Guidelines for the Use of Animals in Research and were approved by the Ethical and Animal Studies Committee of the University of Murcia (Spain).

Animals subjected to surgery: For anaesthesia, a mixture of xylazine (10 mg/kg body weight, Rompun; Bayer, Kiel, Germany) and ketamine (60 mg/kg body weight, Ketalar; Pfizer, Alcobendas, Madrid, Spain) was administered intraperitoneally (IP). After surgery, an ointment containing tobramycin (Tobrex; Alcon S.A., Barcelona, Spain) was applied to the cornea to prevent its desiccation. After surgery, mice were given oral analgesia (Buprex, Buprenorphine 0.3 mg/mL; Schering-Plough, Madrid, Spain) at 0.8 mg/kg (prepared in strawberry-flavored gelatine) the day of the surgery and up to a maximum of 3 days after the surgery.

All animals were killed with an IP overdose of pentobarbital (Dolethal, Vetoquinol; Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain).

Experimental Design

Experimental rationale and animal groups are detailed in Figure 1.

Surgery

Retinal Ganglion Cell Tracing. The retrogradely transported tracer hydroxystilbamidine methanesulfonate (OHSt, Molecular Probes, Leiden, The Netherlands) was diluted at 10% in 0.9% NaCl-10% DMSO and applied to both superior colliculi (SCi) 1 week before the axotomy as previously described.^{4,42–44} Briefly, after exposing the midbrain, a small pledget of gelatine sponge (Spongostan Film, Ferrosan A/S, Denmark) soaked in the tracer solution was applied over the

entire surface of both SCi. The craniotomy was covered with Spongostan and the skin sutured with 4/0 silk (Lorca Marín, Murcia, Spain).

Intraorbital Nerve Transection or Crush. An incision was made in the left superior orbital rim, the superoexternal orbital contents were dissected, the superior and lateral rectus muscles were removed, and the left optic nerve exposed. To spare the retinal artery and avoid retinal ischemia, the meninges were opened and then the left optic nerve was either transected (ONT) or crushed for 10 seconds (ONC) at 0.5 mm from the optic disc.^{12,42} After surgery, the eye fundus of each experimental eye was checked to verify that the retinal blood flow was intact.

Intravitreal administration was done in the left eye. Brain-derived neurotrophic factor (Peprotech EC Ltd., London, UK) was diluted to 1 µg/µL in 1% bovine serum albumin-PBS, and Z-DEVD_fmk (ApexBio Tech, LLC, Houston, TX, USA) to 50 ng/µL in 0.5% dimethylsulfoxide/saline. The injection volume was 2.5 µL.

Retinal Dissection and Immunodetection

Unless otherwise stated, all reagents were from Sigma-Aldrich Química S.A. (Madrid, Spain).

After euthanasia, all animals were perfused transcardially first with saline to remove the blood and then with 4% paraformaldehyde in 0.1 M phosphate to fix the tissue.

All the retinas were dissected as flattened whole-mounts and double-immunostained for Brn3a and c-casp3. Immunodetection was carried out as previously described.^{12,42} Briefly, retinas were permeated in PBS 0.5% Triton X-100 (Tx) by freezing them during 15 minutes at –70°C. Then, they were thawed at room temperature, rinsed in new PBS-0.5% Tx and incubated overnight at 4°C with the primary antibodies diluted in blocking buffer (PBS, 2% normal donkey serum, 2% Tx). Retinas were washed three times in PBS and incubated 2 hours at room temperature with the secondary antibodies diluted in PBS-2% Tx. Finally, retinas were thoroughly washed in PBS-0.5% Tx and, after a last rinse in PBS, retinas were mounted vitreal side up on subbed (cleaned and gelatine-coated) slides and covered with antifading media (Vectashield mounting medium; Vector Laboratories, Palex Medical, Barcelona, Spain).

Antibodies

In all retinas, Brn3a (diluted 1:500, goat anti-Brn3a C-20; Santa-Cruz Biotechnology, Heidelberg, Germany) and active-caspase 3 (diluted 1:500, rabbit anti-cleaved caspase 3 [Asp175]; Cell Signaling, Werfen/Izasa, Barcelona, Spain) were double immunostained. Secondary detection was carried out with donkey anti-goat Alexa 488 and donkey anti-rabbit Alexa 594 (1:500; Molecular Probes, Life Technologies, Madrid, Spain).

Image Acquisition, Automated Quantification, and Topographic Maps

OHSt, Brn3a, and c-casp3 signals were acquired under an epifluorescence microscope (Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany) equipped with a computer-driven motorized stage (ProScan H128 Series; Prior Scientific Instruments, Cambridge, UK) controlled by Image-Pro Plus (IPP 5.1 for Windows; Media Cybernetics, Silver Spring, MD, USA) as described.^{42–45} Briefly, retinal multiframe acquisitions were photographed in a raster-scan pattern in which frames were captured side-by-side with no gap or overlap between them with a 20× objective. Single frames were focused manually before acquisition of the image. All frames from each retina (154 acquisitions/retina) were then fed into the IPP image

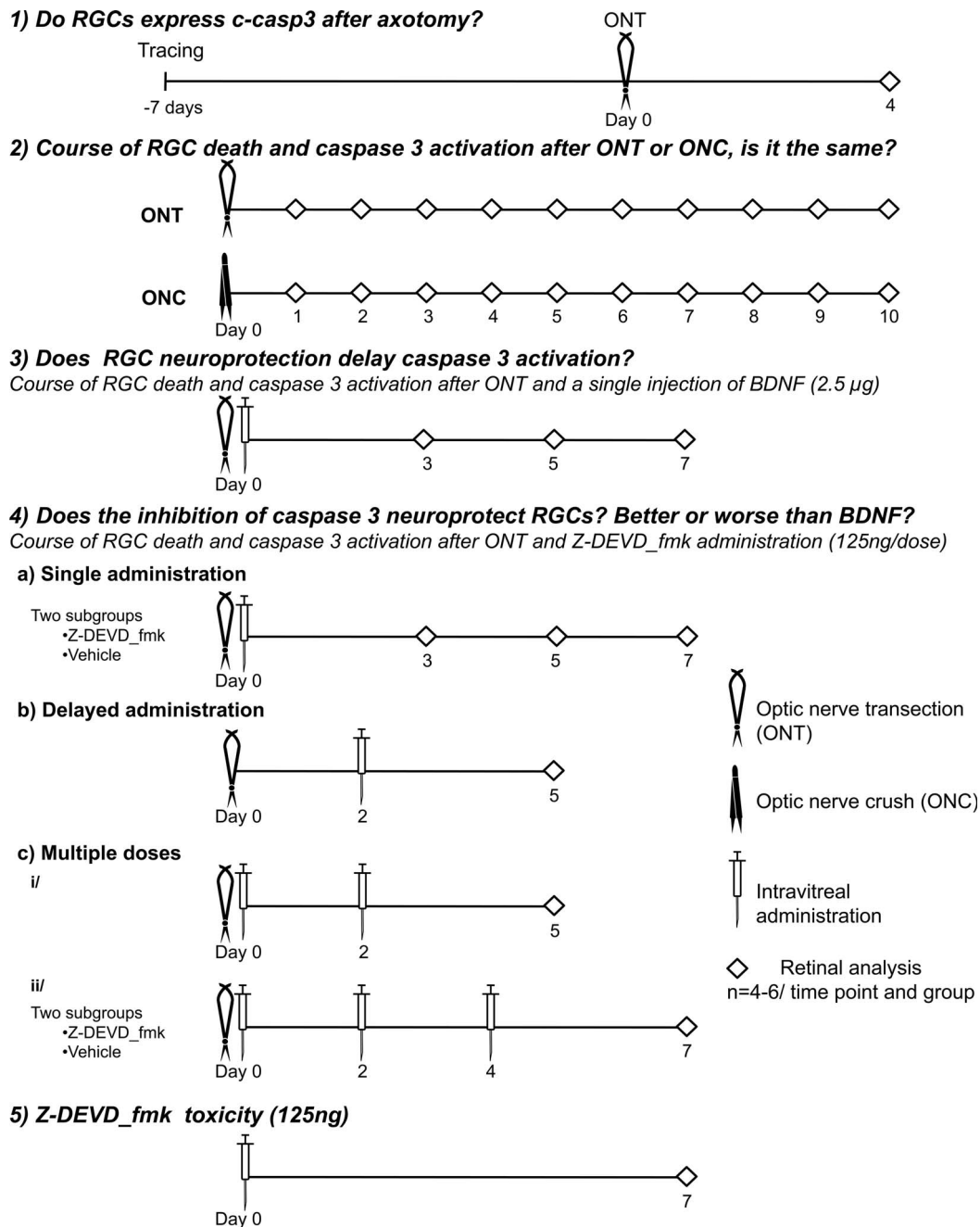


FIGURE 1. Experimental design. All retinas were dissected as flat-mounts and subjected to double immunodetection of Brn3a and c-casp3. Intact retinas ($n = 6$) were used as control of the RGC population. Z-DEVD_fmk vehicle (0.5% dimethylsulfoxide in saline) control groups are detailed in the figure. Brain-derived neurotrophic factor-vehicle (1% bovine serum albumin in PBS) control groups were not done since recently we have published that this vehicle formulation does not have any effect on RGC survival after ONT in adult mice.¹²

analysis program, to tile them and reconstruct the retinal photomontages.

Brn3a⁺RGCs were automatically quantified as reported.^{12,42} Briefly, the IPP macro language was used to apply a sequence of filters and transformations to each image in order to clarify cell limits and separate individual cells for automatic cell counting. Cleaved-caspase 3 RGCs were dotted on the retinal photomontage, and their number automatically quantified using the IPP software.⁴⁵ Finally, each count was exported to a spreadsheet (Microsoft Office Excel 2003; Microsoft Corporation, Redmond, WA, USA) for statistical analysis.

Topographical distribution of Brn3a⁺RGCs was obtained through quadrant analysis and visualized on isodensity maps as reported,^{12,42,43} while the distribution of c-casp3⁺RGCs was carried out using the next neighbor algorithm.⁴⁵ Isodensity maps depict the density (number of cells/mm²) with a color scale that goes from 0 RGCs/mm² (purple) to ≥ 5700 RGCs/mm² (red). In the neighbor maps, each dot represents a c-casp3⁺RGC and its color indicates the number of c-casp3⁺RGCs (neighbors) around it in a radius of 0.088 mm from 0 to 3 (purple) to a maximum of ≥ 12 to 14 neighbors (yellow).

Measurement of Fluorescence Intensity

Magnifications acquiring Brn3a signal from Brn3a⁺:c-casp3⁻ and Brn3a⁺:c-casp3⁺ cells, or acquiring c-casp3 signal from c-casp3⁺:Brn3a⁻ and c-casp3⁺:Brn3a⁺ cells were taken from retinas analyzed at 4 days after ONT using the same acquisition settings for all of them. The intensity of Brn3a or c-casp3 signal was measured individually in each cell using ImageJ software (Rasband WS, ImageJ, <http://imagej.nih.gov/ij/>; provided in the public domain by the US National Institutes of Health, Bethesda, MD, USA). The signal intensity of each cell was measured in fluorescence arbitrary units, assigning to the maximum signal within marker the value of 100. Data were plotted using the GraphPad Prism version 5 software (GraphPad, San Diego, CA, USA).

Statistics

Regression analysis and comparison of two groups (*t*-test) or more than two groups (pairwise multiple comparison procedures, Kruskal-Wallis followed by Dunn's post hoc test) were done with GraphPad Prism. Comparisons were as follows: (1) ONT versus intact retinas; (2) ONC versus intact retinas; (3) ONT versus ONC; (4) ONT+BDNF versus intact retinas; (5) ONT+Z-DEVD_fmk versus intact retinas; (6) ONT versus ONT+BDNF; (7) ONT versus ONT+vehicle; (8) ONT+vehicle or ONT versus ONT+Z-DEVD_fmk; and (9) ONT+BDNF versus ONT+Z-DEVD_fmk. Differences were considered significant when $P < 0.05$.

Regression analyses were done using as variables time after lesion (independent) and RGC number (dependent).

Retinal ganglion cell rescue rate (RRR) was calculated using the criteria by Straten et al.⁴⁶ as follows: $RRR = (RGCs_{Tbe} - RGCs_{noTbe}) / (RGCs_{intact} - RGCs_{noTbe}) * 100$, where $RGCs_{Tbe}$ is the total number of RGCs after a given treatment, $RGCs_{noTbe}$ is the number of RGCs that survive without treatment (ONT or vehicle), and $RGCs_{intact}$ is the number of RGCs in intact retinas.

RESULTS

Active-Caspase 3 Is Expressed by RGCs After Axotomy

In a pilot experiment where Brn3a⁺RGCs and c-casp3 were immunodetected in retinas processed at 2, 4, or 6 days after ONT, we observed a peak of c-casp3⁺ cells at 4 days, but these cells did not always express Brn3a. This could imply that after axotomy, c-casp3 was expressed by other cells in the ganglion cell layer, and/or c-casp3 was only expressed by RGCs but those that had entered apoptosis ceased to express Brn3a.

Taking advantage of the fact that dead RGCs do not disappear from the tissue until microglial clearance,¹² we performed ONT on traced retinas. Four days after the axotomy, retinas were dissected and Brn3a and c-casp3 were double immunostained. Traced RGCs without Brn3a expression but that had not yet been engulfed by microglial cells would still be present in the retina. Figure 2A illustrates that all c-casp3⁺ cells were traced-RGCs. We then measured Brn3a expression level in c-casp3 expressing and nonexpressing RGCs (Fig. 2B, left graph) and vice versa: We measured the level of c-casp3 expression in Brn3a⁺ or Brn3a⁻ RGCs (Fig. 2B, right graph). These graphs show that while Brn3a expression level is highly variable, as reported for rat,⁷ those RGCs that also express c-casp3 have, on average, a significantly lower expression of Brn3a (*t*-test: $P < 0.001$). Conversely, there is on average a significantly higher expression of c-casp3 in those RGCs that

TABLE 1. Temporal Course of RGC Loss and Activation of Caspase 3 After Optic Nerve Transection or Crush

	Brn3a ⁺ RGCs	c-Casp3 ⁺ RGCs
Intact ($n = 6$)	49,677 ± 4,013	0.5 ± 0.5
Contralateral ($n = 50$)	49,631 ± 4,633	0.6 ± 0.7
ONT ($n = 4$ -6/time point)		
1 d	48,933 ± 3,545	19 ± 5†
2 d	47,416 ± 4,699	24 ± 9
3 d	39,821 ± 3,579*	636 ± 34
4 d	37,559 ± 2,338	1,633 ± 446
5 d	30,228 ± 4,156	1,124 ± 115
6 d	20,849 ± 1,979	856 ± 56
7 d	17,672 ± 1,143	296 ± 84
8 d	16,424 ± 393	284 ± 77
9 d	15,860 ± 756	192 ± 47
10 d	15,406 ± 500	92 ± 4
ONC ($n = 4$ -6/time point)		
1 d	49,569 ± 3,900	20 ± 3†
2 d	44,415 ± 4,315	30 ± 4
3 d	42,122 ± 2,670*	659 ± 138
4 d	35,370 ± 1,235	1,450 ± 234
5 d	29,991 ± 4,620	1,114 ± 185
6 d	20,579 ± 1,418	768 ± 153
7 d	19,366 ± 2,450	246 ± 39
8 d	17,066 ± 1,589	222 ± 48
9 d	15,546 ± 1,187	177 ± 31
10 d	15,161 ± 1,187	98 ± 6

Mean ± standard deviation of the total number of Brn3a⁺RGCs and c-casp3 RGCs in the same retinas. In the uninjured retinas, contralateral to the injured ones, the number of RGCs did not differ from intact retinas. There was no difference between ONC or ONT at any time point ($P > 0.05$ Kruskal-Wallis test, followed by Dunn's multiple comparison test).

* After both lesions the loss of RGCs is first significant at day 3 ($P < 0.01$). There is a significant loss of RGCs between days 1 and 3 ($P < 0.01$), days 3 and 5 ($P < 0.001$), and days 5 and 6 ($P < 0.001$, see Fig. 4). From 6 to 10 days, even though there is a decrease of RGCs, this is not significant.

† The number of c-casp3⁺RGCs increases significantly already at day 1 (*t*-test, $P < 0.0001$), but when comparing all time points together, the increase of c-casp3⁺RGCs is significant at day 3 ($P < 0.01$), peaks at days 4 and 5, declines until day 7, and stabilizes from then until day 10. Statistical analysis: Kruskal-Wallis test, and Dunn's multiple comparison test.

have already lost Brn3a than in those that still express it (*t*-test: $P < 0.01$).

Our results are consistent with previous reports⁷ showing that Brn3a expression after axotomy decreases in the retina for two reasons: first, the death of RGCs and second, a decreased expression of Brn3a in the injured ones. Moreover, these results also indicate that down-regulation of Brn3a coincides with the beginning of RGC death.

Temporal Loss of RGCs and Activation of Caspase 3

In intact retinas, the number of Brn3a⁺RGCs was 49,677 ± 4013, in accordance with previous reports.^{12,42} In these retinas, we found on average 0.5 ± 0.5 c-casp3⁺ cells/retina. The number of RGCs and c-casp3⁺ cells in the unlesioned right contralateral retinas did not differ from intact ones (Table 1). There were no significant differences in the number of Brn3a⁺RGCs or c-casp3⁺RGCs between ONC or ONT at any time point (Table 1) and both, the loss of RGCs and the appearance of c-casp3⁺RGCs is diffuse and occurs across the retina (Figs. 3, 4).

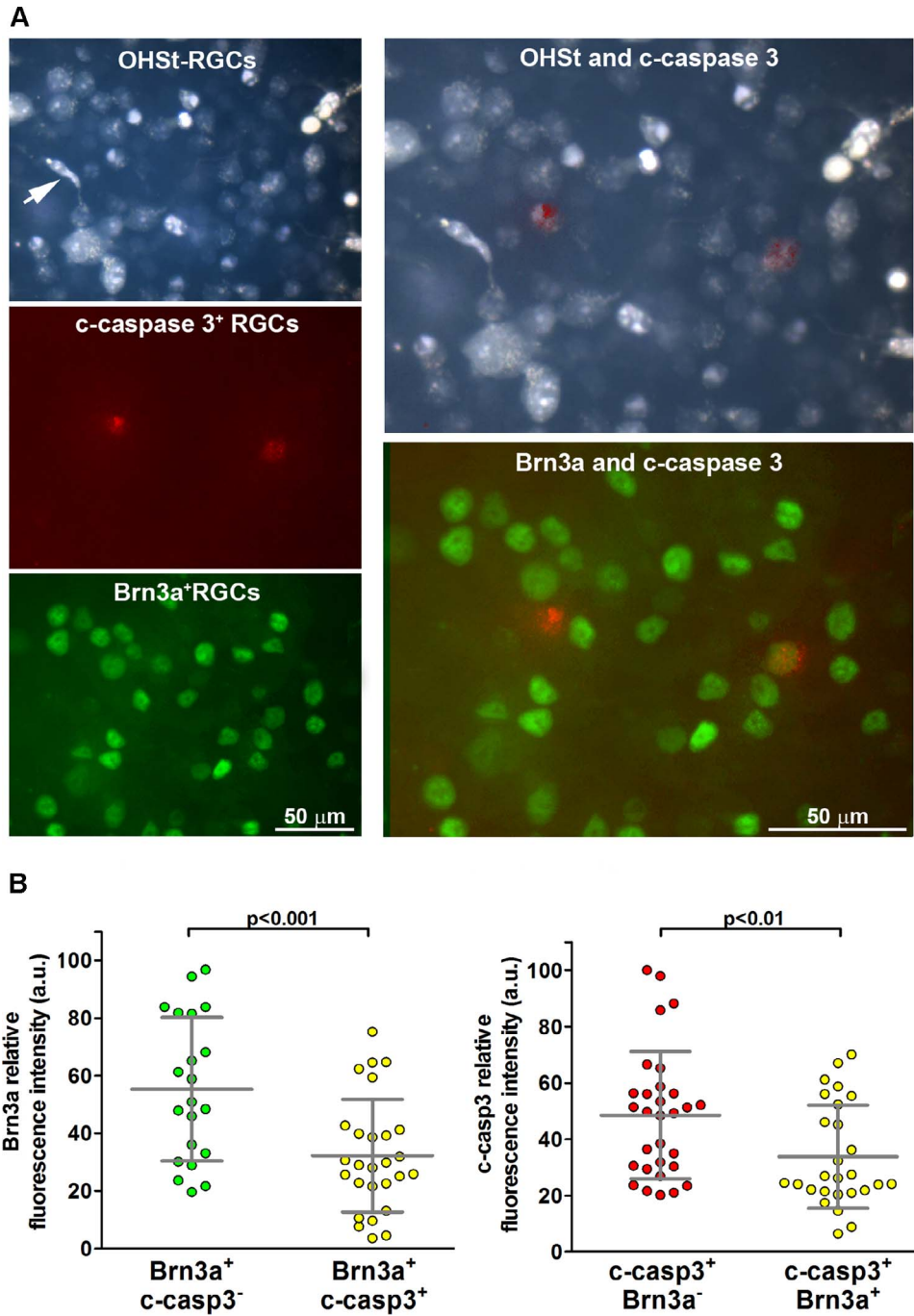


FIGURE 2. After axotomy, only RGCs express active caspase 3. (A) Magnifications from a traced (OHSt, blue) flat mounted retina analyzed 4 days after ONT and immunodetected for Brn3a (green) and c-casp3 (red). The arrow points to a phagocytic microglial cell transcellularly labeled with the tracer after engulfing a traced-RGC. While not all c-casp3⁺ cells expressed Brn3a, all c-casp3⁺ cells were always traced, indicating that all c-casp3⁺ cells were RGCs. (B) Left: Scatterplot showing the intensity of Brn3a signal (left graph) in RGCs that did not express c-casp3 (green circles) and in RGCs that express it (yellow circles). Right: Scatterplot showing the intensity of c-caspase3 signal in RGCs that did not express-Brn3a (red circles) and in RGCs that express it (yellow circles). Gray lines indicate the mean ± standard deviation. a.u., arbitrary units.

Our daily assessment of RGC death after ONC and ONT (Fig. 1) shows that RGC loss is first significant at day 3 and progresses significantly until day 7 (Kruskal-Wallis test, post hoc Dunn's test, $P < 0.001$, Table 1). From day 7 to day 10, RGC loss amounts for a further 4% of the original population, but this decrease is not significant (Kruskal-Wallis test, post hoc Dunn's test, $P > 0.05$, Table 1; Fig. 5A).

Retinal ganglion cell loss fits to a linear regression with two phases, one quicker than the other (Fig. 5A). The initial rapid phase occurs from day 0 to day 7 (7.4 according to the regression analysis, X0 point). Approximately 30,000 RGCs die during these initial 7 days, whereas only ~2000 RGCs die during the course of the subsequent 3 days (8–10). Thus, on average, ~5000 RGCs are lost every day until day 7 (m1: slope

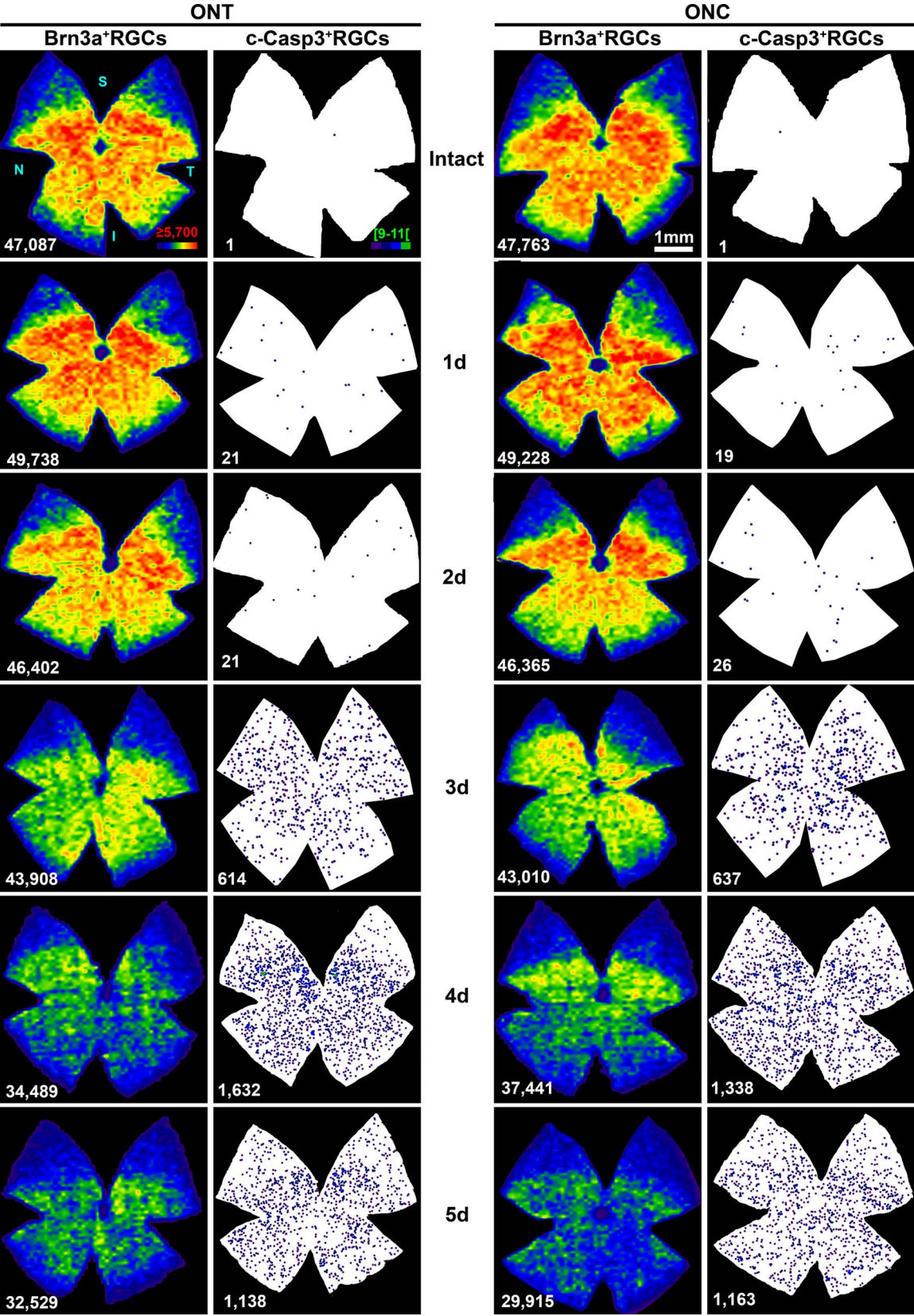


FIGURE 3. Topographical loss of RGCs and appearance of c-casp3⁺RGCs from 1 to 5 days after ONT or ONC. Isodensity and neighbor maps showing the distribution of Brn3a⁺RGCs and c-casp3⁺RGCs in intact retinas and in injured retinas from 1 to 5 days after ONT or ONC. Note that each pair of isodensity and neighbor maps is from the same retina. Below each map is shown the number of Brn3a⁺ or c-casp3⁺ RGCs quantified in that retina. Isodensity and neighbor maps color scales: first row. S, superior; N, nasal; T, temporal; I, inferior.

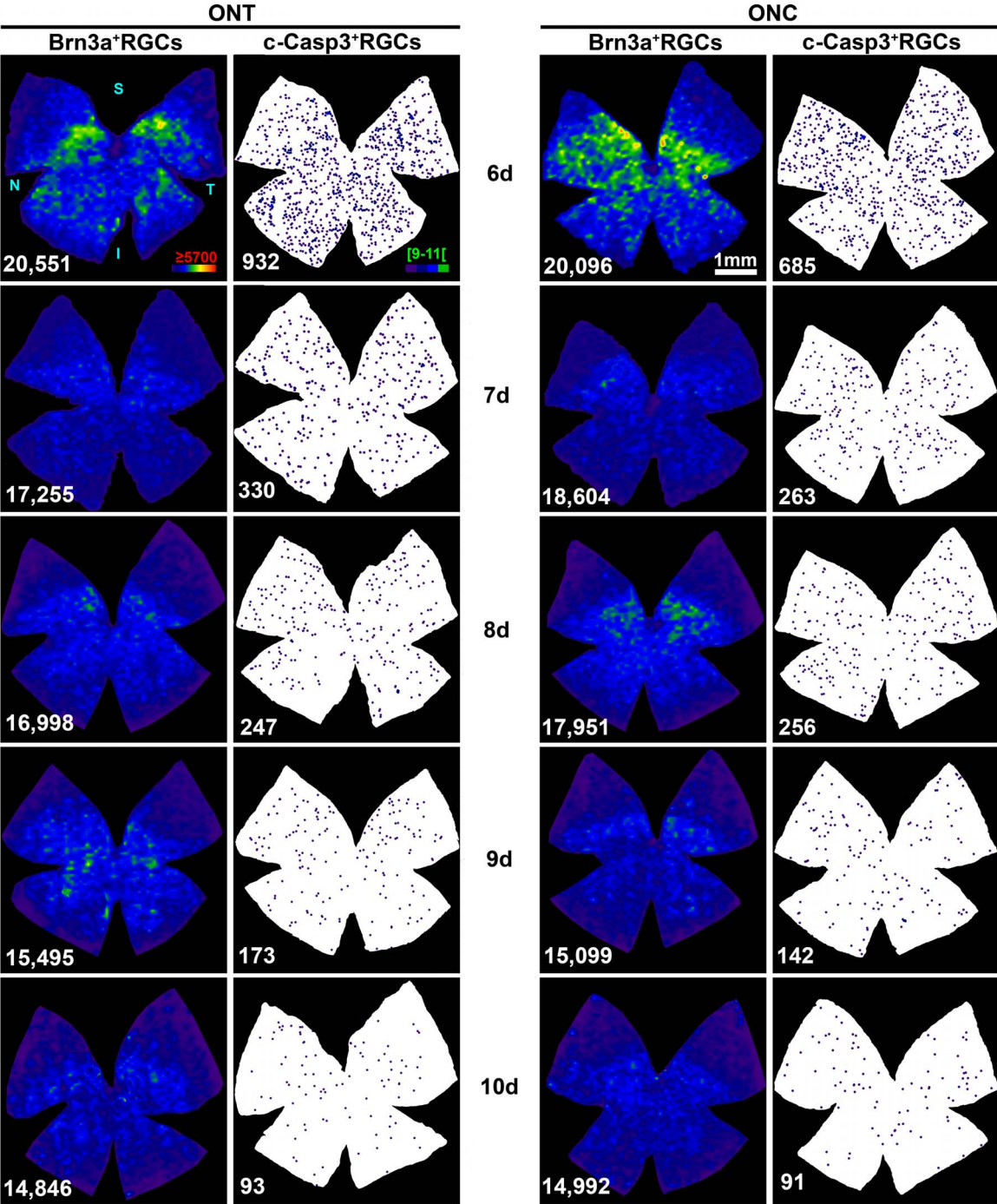


FIGURE 4. Topographical loss of RGCs and appearance of c-casp3+RGCs from 6 to 10 days after ONT or ONC. Isodensity and neighbor maps showing the distribution of Brn3a+RGCs and c-casp3+RGCs in injured retinas from 6 to 10 days after ONT or ONC. Note that each pair of isodensity and neighbor maps is from the same retina. Below each map is shown the number of Brn3a+ or c-casp3+ RGCs quantified in that retina. Isodensity and neighbor maps color scales: first row. S, superior; N, nasal; T, temporal; I, inferior.

value) and then, this rate decreases to ~900 after ONT and ~600 after ONC (m2: slope value).

The activation of caspase 3 follows a Gaussian distribution (Table 1; Fig. 5A). At day 1 after either lesion, the number of c-casp3+RGCs has already increased compared to control retinas. This change is significant when using the *t*-test ($P < 0.0001$), but when comparing all times together (Kruskal-Wallis) the significant increase occurs at day 3. The peak of c-casp3 occurs at day 4, the time point when half of the RGCs dying during the quick exponential phase are lost.

If we calculate the actual RGC loss between individual days (e.g., after ONT a mean of 2262 RGCs are lost between days 3 and 4 and 7331 between days 4 and 5), and consider 100% the number of c-casp3+RGCs quantified in that interval (e.g., 2269 c-casp3+RGCs appear between day 3 and 4, and 2757 between day 4 and 5), we obtain the percent of RGCs dying by caspase-dependent apoptosis. This percent ranges between 9% and 100% depending on the time interval considered, and on average is $50\% \pm 31$ for ONT and $42\% \pm 24$ for ONC. Thus,

TABLE 2. Delay of RGC Death After ONT by BDNF or a Caspase 3 Inhibitor

Treatment	Injection at	Analysis at	Brn3a ⁺ RGCs	c-casp3 ⁺ RGCs	RRR
BDNF 2.5 μ g	0 d	3 d	49,038 $\pm$ 1,274*	226 $\pm$ 160†	93
		5 d	37,628 $\pm$ 2,055*	1,511 $\pm$ 118	38
		7 d	23,154 $\pm$ 3,916	786 $\pm$ 219	17
Vehicle (0.5% DMSO)	0 d	3 d	39,612 $\pm$ 3,591	607 $\pm$ 71	
		5 d	30,619 $\pm$ 1,654	1,147 $\pm$ 157	
		7 d	17,103 $\pm$ 646	268 $\pm$ 107	
Z-DEVD_fmk 125 ng/injection	0, 2, and 4 d	7 d	16,754 $\pm$ 289	306 $\pm$ 133	
	Intact retinas	7 d	49,806 $\pm$ 1,523		
		0 d	49,216 $\pm$ 1,478*	368 $\pm$ 193†	95
	0 d	3 d	37,604 $\pm$ 3,437*	2,122 $\pm$ 162‡	38
		5 d	23,619 $\pm$ 1,867	566 $\pm$ 212	18
		7 d	31,477 $\pm$ 4,002	1,971 $\pm$ 137	6
	0 and 2 d	5 d	37,758 $\pm$ 1,655*	1,487 $\pm$ 221	39
	0, 2, and 4 d	7 d	20,508 $\pm$ 2,872	617 $\pm$ 71	9

Number $\pm$ standard deviation of Brn3a⁺RGCs and c-casp3⁺RGCs in mice treated either with BDNF or Z-DEVD_fmk, and RRR for each treatment; $n = 4$ retinas per group and time point. A single intravitreal injection of Z-DEVD_fmk in intact retinas is not toxic for RGCs. There were no differences in the number of surviving RGCs or c-casp3⁺RGCs between the vehicle groups and ONT at the same time point ($P > 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparison test).

* Compared to vehicle groups or ONT alone, the number of surviving RGCs is significantly higher at 3 ($P < 0.01$) and 5 ($P < 0.001$) days after BDNF or Z-DEVD_fmk treatment. At 7 days, there are more RGCs in the treated retinas, but they do not reach statistical significance. Administration of Z-DEVD_fmk at day 2 after the lesion does not have a protective effect on RGCs, and several injections do not elicit a higher neuroprotection than one alone.

† At day 3 after either treatment, the number of c-casp3⁺RGCs is significantly smaller than in ONT or vehicle retinas ($P < 0.05$). At days 5 and 7, their number is significantly higher ($P < 0.01$) than at the same time points after ONT, but does not differ from days 4 and 6, respectively (Table 1).

‡ At day 5, the number of c-casp3⁺RGCs in the group treated at day 0 with a single injection of Z-DEVD_fmk is significantly higher ($P < 0.001$) than in the BDNF group at the same time point. Statistical analysis: Kruskal-Wallis, followed by Dunn's multiple comparison test.

after axotomy at least one half of the RGC population dies by apoptosis.

Effect of Treatment With BDNF or a Nonreversible Caspase 3 Inhibitor on RGC Survival and Activation of Caspase 3

Next we analyzed RGC survival and activation of caspase 3 after neuroprotection. As positive control of neuroprotection, retinas were treated with a single injection of BDNF following ONT and analyzed 3, 5, and 7 days later. These time points were chosen because at 3 days RGC death is first significant, at 5 days 50% of RGC have died, and at 7 days RGC death slows down. To determine if caspase 3 inhibition had the same effect as BDNF treatment, in a parallel group retinas were treated with the irreversible caspase 3 inhibitor Z-DEVD_fmk (Fig. 1; Table 2; Figs. 5B, 6).

Our data show that BDNF treatment delays RGC death after ONT by 1 day (Kruskal-Wallis test, post hoc Dunn's $P < 0.001$), in agreement with previous reports.¹² In fact, in the BDNF-treated retinas, all RGCs are alive at day 3. At day 5 there is a significant loss, similar to the loss observed at 4 days after ONT alone. At 7 days there is a further decrease, surviving as many RGCs as those observed at day 6 after ONT. The same shift was observed for the activation of caspase 3, indicating that BDNF slows down the progression of apoptosis. Topographically, the neuroprotective effects of BDNF were observed throughout the retina and not limited to the area around the injection site (within the superotemporal quadrant), showing that BDNF neuroprotection reaches the whole retina (Fig. 6).

The same rate of RGC survival and caspase 3 activation was observed after a single injection of Z-DEVD_fmk (Table 2; Figs. 5B, 6), with the exception that at 5 days there are significantly more c-casp3⁺RGCs in the Z-DEVD_fmk-treated group. Nevertheless, as observed for BDNF, both the number of surviving RGCs and of c-casp3⁺RGCs at 5 and 7 days are similar to those

found at 4 and 6 days without treatment. Importantly, a single injection of this inhibitor is not toxic for RGCs (Table 2).

Next we purposed to determine whether Z-DEVD_fmk injected after the lesion was as efficient as when injected at the time of the injury (Fig. 1). To this end, we administered the inhibitor 2 days after the axotomy, when neither RGC death nor caspase 3 activation were significant, and analyzed the retinas 3 days later (5 days after the ONT). Our results (Table 2) show that the delayed treatment with Z-DEVD_fmk does not increase RGC survival nor decreases caspase 3 activation.

Finally, we wanted to assess whether multiple doses of Z-DEVD_fmk elicited a better neuroprotection than a single one (Table 2). Because the delayed treatment with Z-DEVD_fmk did not neuroprotect, in this experiments all animals were treated at the time of the injury, and received a second dose 2 days after the lesion. Half of the animals were killed 3 days later, while the other half received a third dose at day 5 after the lesion (Fig. 1; Table 2) and retinas were analyzed at day 7 (3 days after the last injection). Our data show that multiple injections, being one of them at the time of the axotomy, are not better than a single one delivered at day 0. Thus, the effects Z-DEVD_fmk on early RGC survival are transitory, so this inhibitor must be administered at the time of ONT injury.

DISCUSSION

In this work we present several new findings regarding the response of RGCs to axotomy in mice. Firstly, we show that Brn3a expression in axotomized RGCs wanes as the expression of c-caspase 3 increases, thus marking the beginning of RGC death. Secondly, we have assessed that ONT and ONC induce the same temporal loss of RGCs and of activation of caspase 3. Thirdly, our data show that RGC death by axotomy has two phases: one quick and very steeped from day 3 to day 7, and

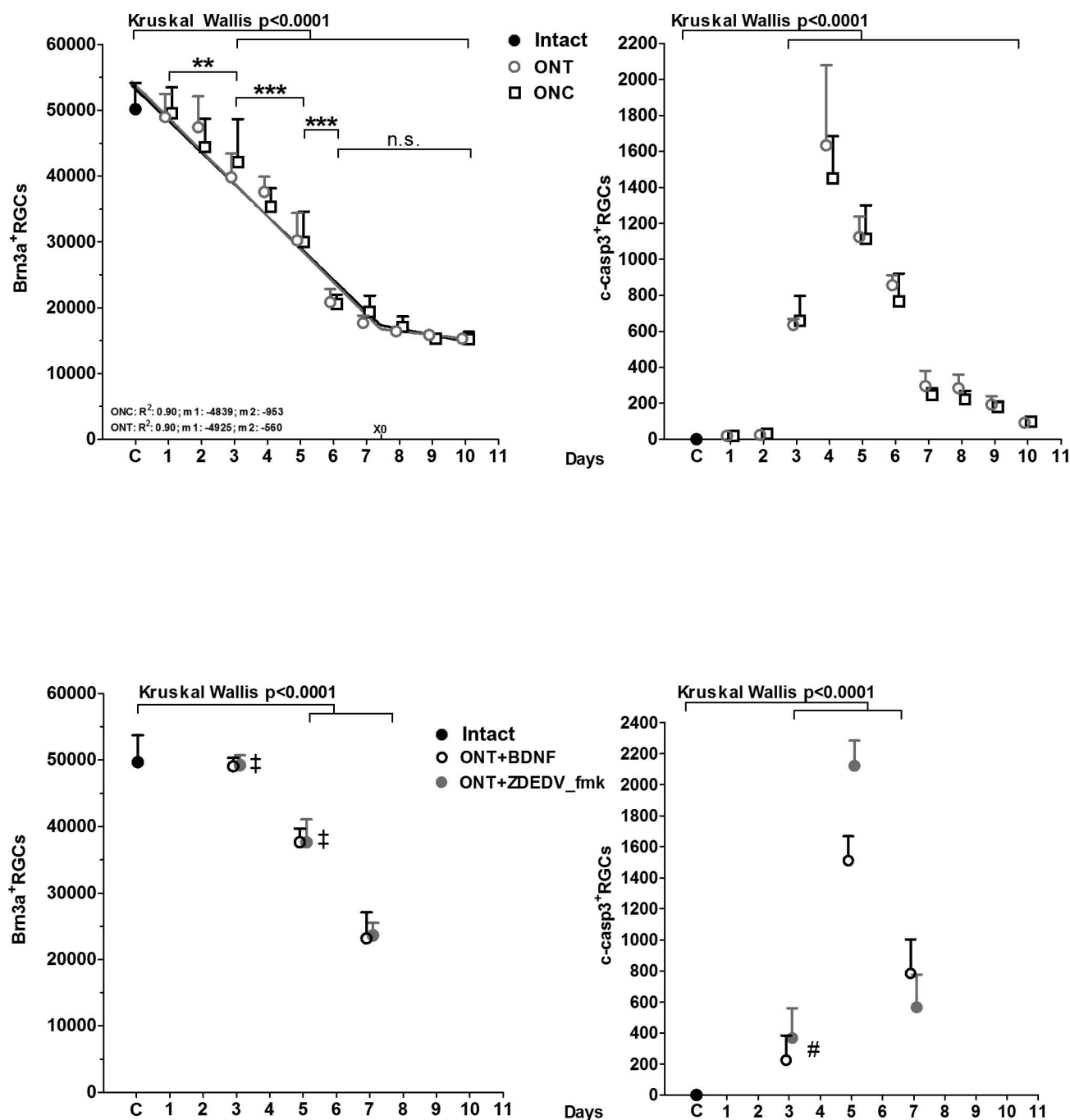


FIGURE 5. Retinal ganglion cell loss after ONT or ONC has two phases and it is delayed by BDNF or Z-DEVD_fmk administration. (A) X,Y graphs (time versus number of cells) showing the mean $\pm$ standard deviation of Brn3a⁺RGCs (left) and c-casp3⁺RGCs (right) after ONT or ONC. †At 3 days, the loss of Brn3a⁺RGCs is first significant compared to control. There is a significant loss of RGCs between 1 and 3 days, 3 and 5 days, and 5 and 6 days (** P < 0.01, *** P < 0.001). From 6 to 10 days the loss of RGCs progresses but is not significant (Kruskal-Wallis test, and Dunn's multiple comparison test). Retinal ganglion cell death occurs in two exponential phases, one quick from 0 to 7.4 days (X0) and a second one, slower, from this time point up to 10 days. The number of c-casp3⁺RGCs peaks at 4 days coinciding with the loss of half of the RGCs during the first exponential death, and declines thereafter. (B) X,Y graphs (time versus number of cells) showing the mean $\pm$ standard deviation of Brn3a⁺RGCs (left) and c-casp3⁺RGCs (right) after ONT+BDNF or ONT+Z-DEVD_fmk. In these groups, RGC loss is significant at day 5 († P < 0.001). The number of c-casp3⁺RGCs at 3 days is significantly lower than after ONT alone, and at days 5 and 7 reaches the values observed at days 4 and 6 when no treatment is delivered, respectively. At day 5 after ONT+Z-DEVD_fmk, there are significantly more c-casp3⁺RGCs than after BDNF treatment (‡ P < 0.001). Statistical analysis: Kruskal-Wallis test, and Dunn's multiple comparison test.

one slower from day 7 to day 10. Fourthly, our data indicate that at least half of the RGCs dying during the first phase die through caspase-dependent apoptosis. And, finally, we show that administration of BDNF or caspase 3 inhibitors at the time of the injury elicit the same rescue rate of RGCs.

Is Brn3a a Good Marker to Evaluate RGC Viability?

In albino mice, Brn3a is expressed by 92.6% of the RGCs traced from the superior colliculi.¹² In previous works it has been shown that down-regulation of Brn3a precedes the loss of

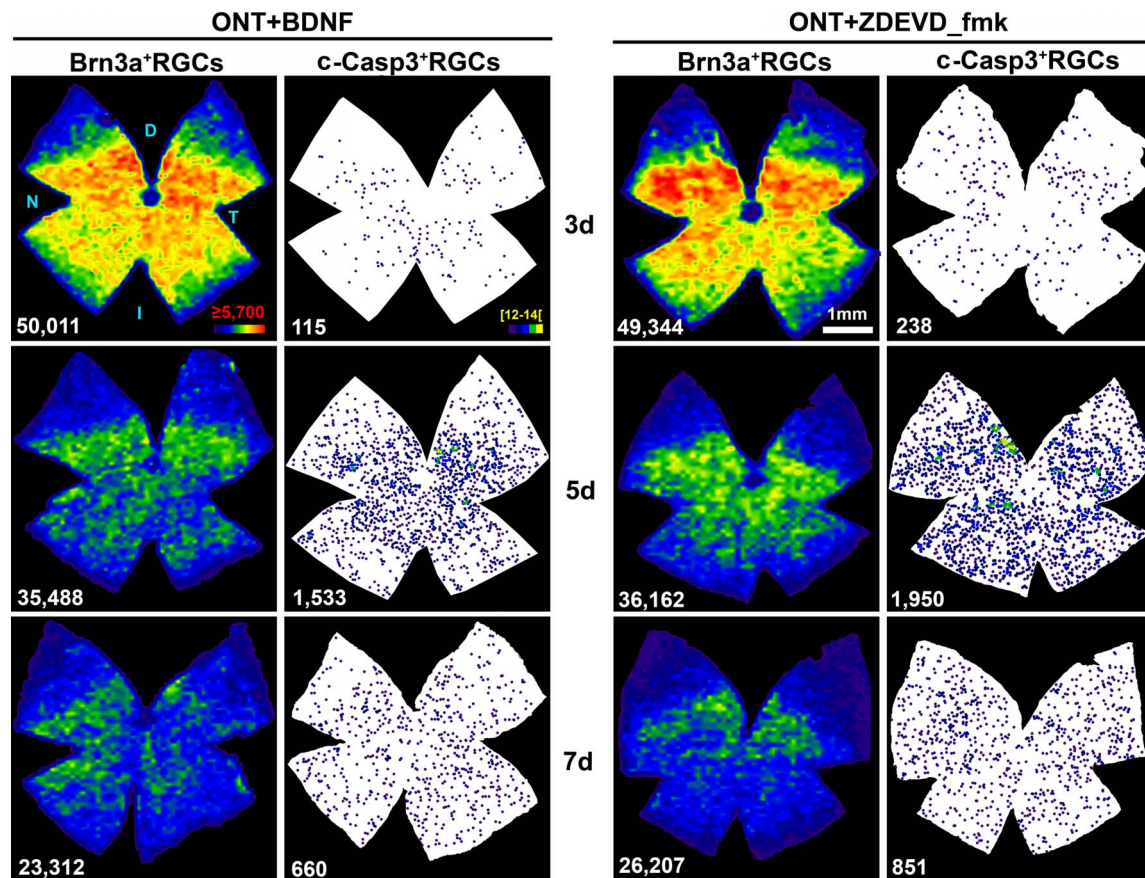


FIGURE 6. Brain-derived neurotrophic factor or Z-DEVD_fmk treatment delays ONT-induced RGC degeneration across the retina. Isodensity and neighbor maps showing the distribution of Brn3a⁺RGCs and c-casp3⁺RGCs in retinas treated just after the injury with BDNF or Z-DEVD_fmk and analyzed at 3, 5, or 7 days after the lesion. Note that each pair of isodensity and neighbor maps is from the same retina. Below each map is shown the number of Brn3a⁺ or c-casp3⁺ RGCs quantified in that retina. Isodensity and neighbor maps color scales: first row, S, superior; N, nasal; T, temporal; I, inferior.

traced-RGC bodies.^{2,30,42} This mismatch is more evident in rats than in mice and occurs during the first days after ONT. In fact, from 7 or 9 days onwards in mice or rats, respectively, the number of traced- and Brn3a⁺ RGCs does not differ. The reason of this mismatch may lie in the fact that Brn3a is an endogenous protein and a tracer is an exogenous compound. Thus, while a protein may be down-regulated as soon as the neuron enters in apoptosis, the traced-RGC body will disappear after being phagocytosed by a microglial cell that, in turn, will become transcellularly labeled.^{12,47–49} Our data here support this explanation, as we show that as soon as an RGC is expressing c-caspase 3, its Brn3a expression declines. This decline in Brn3a expression concurs with a previous study from our group.⁷ In that work, we demonstrated that Brn3a down-regulation after axotomy in rat occurs for two reasons: first the death of RGCs and, second, a down-regulation of Brn3a in the surviving ones. Importantly, we also observed that the decrease of Brn3a expression per RGC was more marked after ONT than after ONC in agreement with the quicker RGC death observed in rat after ONT than after ONC.^{2,4,7}

It is worth noting that in this study we observed traced RGCs that were c-casp3 positive and Brn3a negative. Because Brn3a is expressed by the majority of RGCs, these are dead RGCs that no longer have detectable Brn3 expression. However, part of them are also those RGCs that do not express Brn3a in the first place, such as melanopsin positive RGCs, which amount for 2% to 3% of the total RGC population in rats and mice.^{7,45,50}

Brn3a expression is maintained in neuroprotected retinas, as shown for rat and mouse,^{12,30} and here using two different approaches: administration of BDNF or inactivation of caspase 3. Brain-derived neurotrophic factor is a trophic factor that induces survival activating the MAPK/PI3K pathway^{51,52} that in turn blocks the activation of caspases through inhibition of the mitochondrial membrane permeabilization and release of cytochrome C.¹⁵ Z-DEVD-fmk is a synthetic peptide that mimics the cleavage sequence of caspase 3, so when the c-caspase 3 binds the peptide, it is inactivated. Thus, BDNF prevents the activation of caspase 3 while Z-DEVD blocks its activity. With both treatments, RGC survival was delayed in the same fashion. This indicates that Brn3a expression declines not when caspase 3 is active, but when it is acting (i.e., when it is cleaving its physiological substrates to execute apoptosis).

This loss of Brn3a signal when caspase 3 is active may indicate that Brn3a is a substrate of caspase 3. To test this possibility, we fed the mouse Brn3a protein sequence (FASTA accession number >gi|51921650|gb|AAU13951.1| Brn3a [*Mus musculus*]) into ExPASy PeptideCutter⁵³ and PROSPER⁵⁴ Web-based tools to predict in silico the cleavage sites of caspase 3 within a given protein. Peptide Cutter takes into account, for Caspase 3, the preferred sites DMQD|XDEVD|X, where X is any amino acid but Pro, Glu, Asp, Gln, Lys, or Arg. PROSPER computes as well structure characteristics. Using these specificities, Brn3a protein does not have a cleavage site for caspase 3.

Thus, there may be another explanation for Brn3a down-regulation. It is possible that the gene expression of Brn3a is

silenced by deacetylation of its promoter, as it has been shown for Brn3b and other RGC genes in axotomized mice retinas.⁵⁵ In line with this, nuclear atrophy (heterochromatic formation and nuclear shrinkage) is an early signal of RGC degeneration.^{56,57} In fact, Janssen et al.⁵⁷ observed that the nuclear area of axotomized RGCs in mice decreased already 3 days after the lesion, when RGC loss and caspase 3 activation is significant. Importantly, nuclear atrophy and gene silencing also occurs in *Bax*-deficient mice, which are blocked to complete apoptosis and therefore their RGCs survive after axotomy.^{17,56–59}

Brn3a has antiapoptotic functions^{60–65} and *Brn3a*-deficient mice show a higher expression of Bax during developmental apoptosis.⁶¹ This indicates that Brn3a is up-stream Bax. Thus the question arises, is Brn3a down-regulation cause or consequence of RGC death? Does Brn3a down-regulation trigger caspase 3 activation or is it the other way round? This should be further investigated.

Brn3a expression declines when RGCs express active caspase 3, a very early event after axotomy,^{14,26} but its expression is maintained in neuroprotected retinas and in the few RGCs that survive to long-term axotomy.³ Therefore, Brn3a immunodetection is a very sensitive tool to study the fate of injured RGCs.

ONT and ONC

The course of RGC death and activation of caspase 3 in these mice after ONC or ONT is indistinguishable. This is surprising since in rat^{2–4,7} ONC causes a slower RGC loss than ONT. This apparent contradiction may be explained by the distance from the neuronal soma where the injuries were inflicted.¹ In this work both lesions were done at 0.5 mm from the optic head, while in rats ONC was performed at 2 to 3 mm and ONT at 0.5.^{2–4,7}

Retinal ganglion cell death is not instantaneous, but rather a process lasting several days. It occurs across the retina in a diffuse pattern, and has two exponential phases. The loss of RGCs is first statistically significant 3 days after either lesion, and there are two further significant decreases from days 3 to 5 and between days 5 and 6, so that by day 7, 35% of RGCs survive. Then the process slows down and there is not significant RGC loss until day 10 but, nevertheless, during these days a further 4% of the original population is lost. These data are in agreement with previous papers showing that between 9 and 21 days after ONT RGC death stabilizes.⁴²

In the same retinas, c-casp3⁺RGCs appear across the retina already at day 1, although they are few (~19–20/retina). At day 3, when RGC loss is first significant, the number of c-casp3⁺RGCs is 25 times higher than at day two, peaks at day 4 and thereafter declines up to day 10. The peak of c-casp3⁺RGCs occurs at the time when half of the RGCs that are lost between 0 and 7 days have died.

Our data also show that approximately 50% of the dying RGCs express c-casp3, indicating that at least half of them die by an apoptosis caspase dependent mechanism. This percentage could be higher because the expression of c-casp3 is transitory. Alternatively, part of the RGCs may not die by this mechanism, but through the caspase independent apoptosis, as reported in an in vitro study using primary cultures of RGCs.²²

Delay of RGC Loss by BDNF and Z-DEVD_fmk

A single administration of BDNF on the day of the injury delays RGC death and caspase 3 activation by 1 day, indicating that there is a correlation between activation of caspase 3 and RGC death after optic nerve axotomy. The same RRR was observed in retinas treated the day of the injury with a single dose of the

irreversible caspase 3 compound, Z-DEVD_fmk. Z-DEVD_fmk is also, although with lower affinity, an inhibitor of caspases 7, 8, and 10, and it has been reported that inhibits as well the activity of calpain 1.⁶⁶ In rat, it has been shown that calpain 1 is overexpressed in RGCs after axotomy^{14,67} and inhibition of caspase 8²⁴ increases RGC survival after axotomy. Thus, the neuroprotection afforded with this compound may not only be ascribed to the inhibition of caspase 3, although the signaling of those proteases converges on it.

A very interesting result was that the delayed treatment with Z-DEVD_fmk did not rescue RGCs. At the time of the injection, 2 days after ONT, there is some caspase 3 activation but RGC death is not yet significant. This may mean that RGCs are committed to death before day 2 and/or that the dose of antagonist injected is not enough to block the increased activity of caspase 3. But because administration of multiple doses were not better than a single one, it is plausible that caspase 3 inactivation only works before the death of RGCs is quantifiable. In addition, these data suggest that alternative pathways, caspase 3 independent, play a role in RGC death by axotomy.

Finally, in mice these models of optic nerve axotomy, performed at 0.5 mm from the optic disk, are very drastic and cause a very quick and steep RGC death. In fact the therapeutic window is very narrow since RGC loss is first significant at 3 days after either lesion and progresses exponentially until day 7. This is a drawback because even the best neuroprotectant, BDNF, only delays RGC death by 1 day. Thus, these axotomies are not suitable to perform long-lasting experiments to test neuroprotection. To this end, gentler injuries are needed, injuries that cause a slower course of RGC death. On the other hand, both axotomy models may be used in proof of concept assays: they serve to test in vivo and in a short time frame the potential neuroprotective properties of a given molecule against a traumatic axonal injury. For instance, once we know that RGC loss starts at day 3 and that 50% of them have died by day 5, RGC survival after treatment should be assessed during this time frame.

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