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# Caspase dependence of the death of neonatal retinal ganglion cells induced by axon damage and induction of autophagy as a survival mechanism

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## Abstract

We examined the degeneration of post-mitotic ganglion cells in *ex-vivo* neonatal retinal explants following axon damage. Ultrastructural features of both apoptosis and autophagy were detected. Degenerating cells reacted with antibodies specific for activated caspase-3 or -9, consistent with the presence of caspase activity. Furthermore, peptidic inhibitors of caspase-9, -6 or -3 prevented cell death (100 µM Ac-LEDH-CHO, 50 µM Ac-VEID-CHO and 10 µM Z-DEVD-fmk, respectively). Interestingly, inhibition of autophagy by 7-10 mM 3-methyl-adenine increased the rate of cell death. Immunohistochemistry data, caspase activation and caspase inhibition data suggest that axotomy of neonatal retinal ganglion cells triggers the intrinsic apoptotic pathway, which, in turn, is counteracted by a pro-survival autophagic response, demonstrated by electron microscopy profiles and pharmacological autophagy inhibitor.

Key words: Apoptosis; Autophagy; Caspases; Retina; Central nervous system

## Introduction

The mechanism by which central neurons undergo cell death after axonal injury is not clear (1-4). Several studies indicate that it may be due to blockade of retrograde axonal transport of neurotrophins (5,6), the triggering of damage signals (7), or other still unidentified mechanisms.

In adult rats, retinal ganglion cells (RGCs) undergo apoptosis throughout the course of several diseases, such as ocular hypertension, axotomy, ischemic insults, and stress-mediated degeneration of photoreceptors (4,8-13). In neonatal rats, axotomy also induces cell degeneration (4,14,15) and is prevented by inhibition of protein synthesis (4,16), growth factors (14,17), inhibition of caspases (4), reduced redox state (18), or overexpression of Bcl-2 (19).

A thorough study has shown the involvement of the apoptotic proteases, caspase-9 and -3, in axotomy-induced RGC death in retinal explants from postnatal day 6 (PD6) mice (20). Furthermore, ablation of the superior colliculus of PD2 rats induces a massive and rapid increase in RGC death that is not accompanied by activation of caspase-3 (21). Therefore, the involvement of caspases in axotomy-induced death of retinal ganglion cells in the neonatal retina needs to be established.

Programmed cell death can take several forms, such as autophagic cell death (for a review, see Ref. 22,23), and apoptosis is not the only program of execution of cell death that may be involved in the demise of the retina. Light stress induces ultrastructural changes in rat rod photoreceptors, and an early sign of abnormality is the proliferation of autophagic bodies in the inner segments (24), suggesting a role for autophagic cell death in retinal cell degeneration. Mutation of the OPA1 gene causes optic atrophy, in which loss of adult RGCs is accompanied by an increase in the number of autophagosomes in the RGC layer (25). Also, Kim et al. (26) reported that RGC death following optic nerve transection is accompanied by up-regulation of autophagy-related genes, such as beclin1 and several Atg genes, and that treatment of the cell line RGC5 with inhibitors of autophagy under serum-deprived

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conditions decreases cell viability by approximately 40% (26). Still, although the role of autophagy in adult RGCs is under scrutiny, there are no available data on the incidence of autophagy in RGCs in the neonatal retina during normal development or following axon damage.

Understanding the molecular pathways by which axonal injury induces neuronal death is important for the development of preventive strategies for rescuing these cells after pathological insults, and also to prevent secondary degeneration among other cell types within the retinal tissue (27). Thus, in the present study, we identified the mode of cell death induced by axotomy of immature RGCs using a model of retinal explants from neonatal rats.

## Material and Methods

### Material

Ac-Leu-Glu-His-Asp-H (aldehyde) (Ac-LEHD-CHO) and Ac-Val-Glu-Ile-Asp-H (aldehyde) (Ac-VEID-CHO) were obtained from the Peptide Institute (Japan) and were used at 100 µM from a stock solution in DMSO. Ac-Asp-Glu-Val-Asp-fluoro-methyl-ketone (Z-DEVD-fmk) (Calbiochem, USA) was used at 100 µM from a stock solution in DMSO. 3-Methyl-adenine (Sigma, USA) was used at 3 µM from a stock solution in culture medium. Ac-VEID-pNa (p-nitroanilide) and Z-DEVD-pNa (Calbiochem) were used at 200 µM from a stock solution in DMSO.

### Tissue culture

Lister hooded rats were used. Retinal explants were prepared as previously described (28). PD1 animals were killed instantaneously according to the policy of the National Institute of Health Guide for the Care and Use of Laboratory Animals, their eyes were removed and the retinas were dissected. Fragments of approximately 1 $mm^2$ were cut in culture medium and placed in 25-mL tight-lidded Erlenmeyer flasks with 5 mL Eagle's basal medium (Gibco BRL, USA) with 5% fetal calf serum (WL Immunochemicals, USA) and 20 mM HEPES. The flasks were kept in an orbital shaker at 80-90 revs/min and at 37°C for 24 h. Drugs were added at the beginning of the incubation period.

### Histology

The retinal explants were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 1 h and then infiltrated in a solution of 30% sucrose in 0.1 M sodium phosphate buffer, pH 7.2. The explants were embedded in optimal cutting temperature medium and 10-µm thick transverse sections were cut with a cryostat. The sections were either stained with neutral red or processed for immunohistochemistry.

### Immunohistochemistry

Immunohistochemistry for activated caspase-3 was performed using the CM1 polyclonal antiserum that recognizes activated caspase-3 without significant cross-reactivity with the 32-kDa zymogen form (CM1 was a kind gift from Dr. Anu Srinivasan, Idun Pharmaceuticals, USA). Paraformaldehyde-fixed tissue sections were processed for immunohistochemistry as described previously (29).

### Transmission electron microscopy

Tissue for transmission electron microscopy analysis was processed as described previously (29). Thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 1210 transmission electron microscope.

### Caspase activity assay

Protein samples were prepared as previously described (29) and Z-DEVD-pNa or VEID-pNA hydrolysis was measured by absorbance at 405 nm in a 3550-UV Microplate Reader (Bio-Rad, USA).

### Quantification of cell death and statistics

To estimate cell death rates, homogeneously neutral red-stained condensed profiles were counted within the RGC layer from each of three distinct explants in each experiment (minimum of 100 cells/explant). Data are reported as means ± SD of the replicates from several pooled experiments. The values were normalized with respect to the average rate of cell death within the ganglion cell layer (GCL) in each experiment, which was taken as 100%. Data were analyzed statistically by one- or two-way analysis of variance, using the SPSSPC statistical software.

## Results

### Ultrastructural features of axon-damaged neonatal RGCs

To identify the mode of cell death induced by axotomy in neonatal RGCs, we first examined retinal explants by transmission electron microscopy. Apoptotic hallmarks were observed in dying cells throughout the RGC layer, such as pyknotic nuclei and preserved organelles (Figure 1A,B). Morphologic features of autophagy were also clearly observed in RGCs, such as isolation membranes, i.e., the formation of double-membrane cisterns in the cytoplasm and autophagosomes (Figure 1B,C). The double-membrane cisterns surrounded cytoplasmic contents or whole organelles to form autophagosomes, such as the engulfed mitochondria seen in Figure 1C. Partially degraded cargo within autophagosomes was also observed (Figure 1A, arrows). The morphological pattern of dying RGCs suggested that components of both the apoptotic and autophagic cell death pathways were involved in neonatal RGCs axotomy-induced degeneration.

**Mitochondria are involved in cell death induced by axotomy**

Activation of caspase-9 depends on the assembly of the mitochondrial apoptosome, a macromolecular complex formed by procaspase-9, apoptotic protease activating factor 1, dATP, and cytochrome *c*. Release of cytochrome *c* is often associated with the opening of a mitochondrial permeability transition pore (mPTP). By using bongkrekic acid, an mPTP inhibitor, we partially blocked cell death induced by axotomy (Figure 2A), showing that cell death induced by RGC deafferentation depends on mitochondrial commitment.

**Axotomy induces caspase activation in neonatal RGCs**

To test if the apoptotic features observed at the ultrastructural level were a result of caspase activation, we cultured retinal explants in the presence of peptide inhibitors of caspase-9, -3 or -6. LEHD, an inhibitor of caspase-9, partially blocked cell death induced by axotomy (Figure 2B), reaching a maximum effect of nearly 40% inhibition of cell death. This is consistent with the immunoreactivity for activated caspase-9 found in the degenerating profiles in the GCL (Figure 2C). DEVD, an inhibitor of caspase-3, partially blocked cell death induced by axotomy (Figure 2D), also in agreement with immunohistochemical data for activated caspase-3 in the GCL (Figure 2E). VEID, an inhibitor of caspase-6, also partially blocked cell death induced by axotomy (Figure 2F). The involvement of caspase-3 and caspase-6 in RGC degeneration was further confirmed by the demonstration of its activity (Table 1).

Taken together, these results indicate that axotomy of neonatal RGCs induces activation of caspase-9, -3 and -6. Partial blockade of cell death by caspase inhibitors, immunohistochemistry and caspase activity data suggest the existence of alternative mechanisms of cell death induced

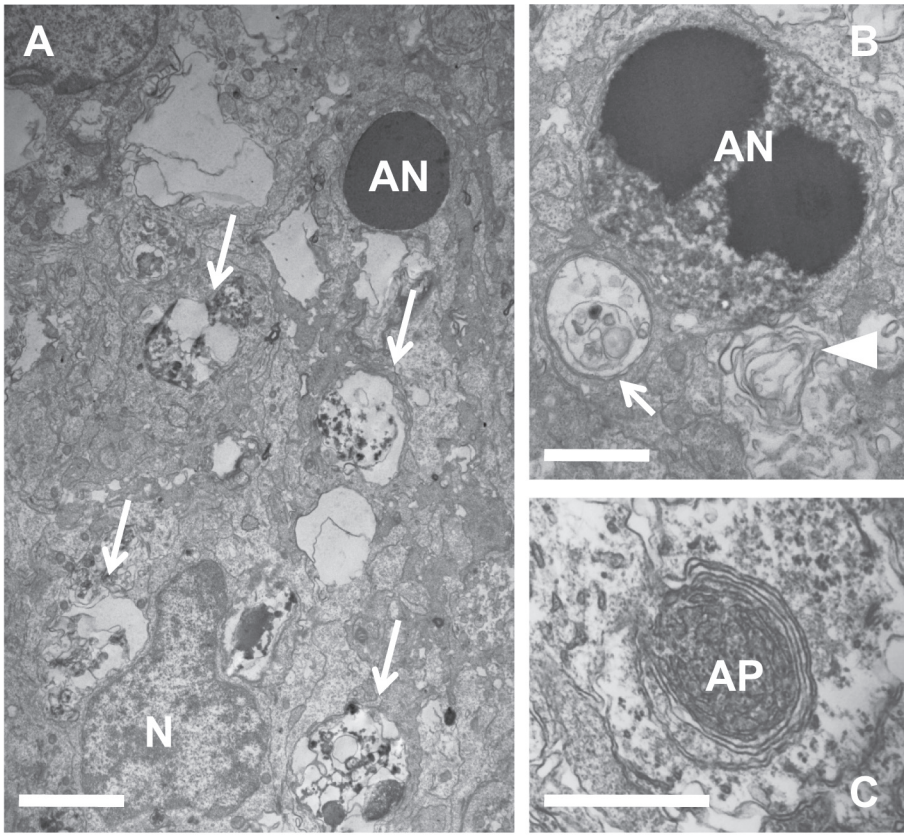


**Figure 1.** Electron micrographs of autophagy-related vesicular compartments 24 h after retinal ganglion cells axotomy. Retinal explant sections were obtained from axotomized retinae maintained *in vitro* for 24 h. Several types of autophagic vacuoles are shown: *A*, Autophagosome-filled profiles (arrows). Scale bar = 5 µm. *B*, Double-membraned cisterns (arrowhead) or autophagosome (arrows). Scale bar = 2.5 µm. *C*, An autophagosome containing recognizable mitochondria (AP). Scale bar = 2.5 µm. N = normal nucleus; AN = apoptotic nucleus.

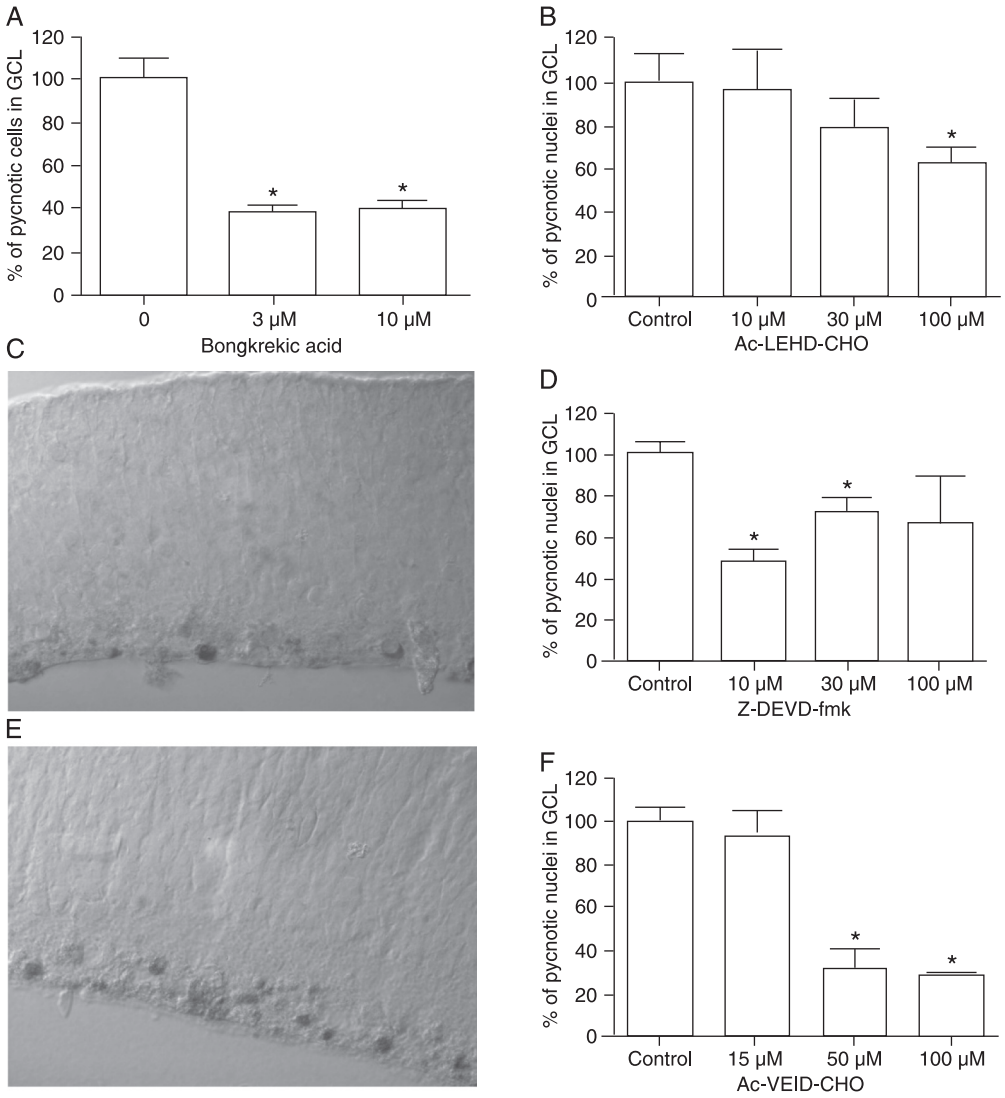


**Figure 2.** Induction of apoptosis in neonatal retinal ganglion cells. The graph shows the effect of increasing concentrations of: *A*, Bongkrekic acid; *B*, caspase-9 inhibitor; *D*, caspase-3 inhibitor, and *F*, caspase-6 inhibitor on cell death in the ganglion cell layer (GCL) of retinal explants. Cell death was estimated from the density of pyknotic profiles after staining with neutral red. Data were pooled from three independent experiments (N = 3-9 for each data), and are reported as mean percent ± SD density of dead cells in the control preparation (100%). *P < 0.05 *vs* respective control (Mann-Whitney test). *C*, Immunostaining for activated caspase-3; *E*, immunostaining for activated caspase-9.

by optic nerve transection in RGCs.

### Induction of autophagy is an anti-apoptotic event in neonatal RGCs

The ultrastructural analysis unveiled autophagic profiles within the GCL. To test the role of autophagy in the RGCs activated by axon damage, we cultured retinal explants in the presence of 3-methyladenine (3MA), an inhibitor of autophagy (Figure 3). 3MA increased the rate of cell death in GCL (Figure 3). This effect of 3MA is consistent with the view that autophagy has a protective role in immature RGCs in this experimental paradigm.

**Table 1.** Caspase activation induced by axotomy in neonatal retinal ganglion cells.

| | Enzyme activity (nmol/min) |
|---|---|
| Caspase-3 | 26.74 ± 9.25 |
| Caspase-6 | 6.28 ± 1.06 |
| Control | 3.13 ± 0.36 |

Caspase activity is reported as means ± SD in nmol/min. Cleavage of the caspase-3 substrate DEVD-p-nitroanilide or caspase-6 substrate VEID-p-nitroanilide pooled from two independent experiments (N = 2-4 for each data).

## Discussion

The major findings of the present study were: 1) axotomy induces ultrastructural signs of both apoptosis and autophagy in neonatal RGCs, 2) neonatal RGC death induced by axotomy partially depends on activation of caspase-9, -3 and -6, and 3) inhibition of autophagy potentiated the cell death induced in the GCL by axon damage.

**Involvement of caspases in neonatal RGC degeneration**

Caspase activation in adult RGC death has been extensively documented. Caspase-2, -3, -8, and -9 were shown to be involved in *in vivo* degeneration of RGCs caused by optic nerve section in adult rats (8,9), transient global retinal ischemia degeneration of RGCs and amacrine cells (11) and chronic ocular hypertension-induced cell death, which may contribute to the pathology of glaucoma (10).

Our results demonstrate the activation of at least one initiator and two executor caspases following the axotomy of neonatal RGCs. Activation of caspase-3 in RGC death induced by axotomy was demonstrated in a similar explant model of retinas kept *in vitro* for 10 days (4), but our results implicate not only caspase-3, but also caspase-6 and -9 in cell death induced by axotomy of neonatal RGCs. Taken together, these results contribute to the mapping of the mechanisms of the execution of cell death in the developing retina.

**Autophagy and neonatal RGC death induced by axon damage**

The ultrastructural analysis of axotomized ganglion cells demonstrated the presence of autophagic profiles within the GCL. Although morphological signs of autophagy are observed in physiological processes in the nervous system (30), several studies implicate autophagy as an important mechanism of programmed cell death both in retina (28,29,31) and other structures of the nervous system (31-35).

Inhibition of autophagy in RGCs potentiated the damaging effect of axotomy, suggesting that autophagy is a tissue response to an insult and a mechanism to delay or block cell degeneration. In fact, such an event was already observed in colon cancer cells, in which mutants with a low rate of autophagy were more sensitive to apoptosis than parental cells, suggesting that autophagy may delay apoptosis by sequestering mitochondrial death-promoting factors such as cytochrome *c* (36). Our data also agree with studies of the adult retina, in which autophagy-related genes were up-regulated upon induction of cell death by optic nerve transection, and the use of inhibitors of autophagy potentiated cell death induced by serum deprivation in a retinal cell line (26). Thus, autophagy may represent a reaction of the RGCs towards recovery from axon damage, rather than a necessary mechanism of execution of cell death.

Similar to apoptosis, autophagy is also thought to be mediated by the opening of the mPTP (37). Since the opening of the mPTP can cause the release of harmful proteins,

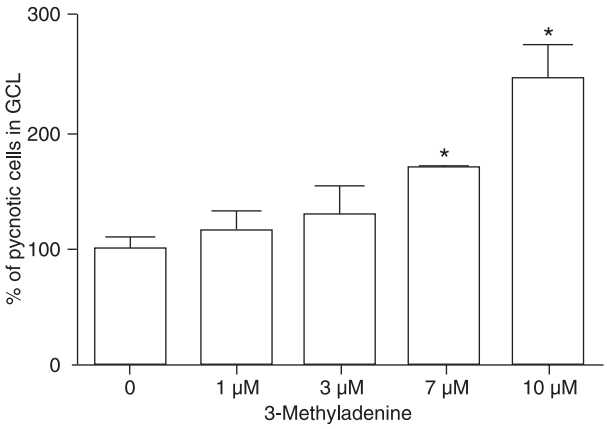


**Figure 3.** Induction of autophagy in neonatal retinal ganglion cells. The graph shows the effect of increasing concentrations of 3-methyladenine on cell death in the ganglion cell layer (GCL) of retinal explants. Cell death was estimated from the density of pyknotic profiles following staining with neutral red. Data were pooled from two independent experiments (N = 3-6 for each data), and are reported as mean percent ± SD density of dead cells in the control preparation (100%). *P < 0.05 *vs* control (Mann-Whitney test).

the authors suggested that autophagy is a protective device used by cells to rid themselves of damaged mitochondria. Mitochondrial disappearance is also noticed in pathological conditions (38). However, the exact role of mitochondrial disappearance in such conditions is debatable and remains to be elucidated (39).

Our data suggest that axotomy of neonatal RGCs triggers the intrinsic apoptotic pathway. However, it appears that retrograde stress also induces a pro-survival autophagic response, corroborating the growing body of evidence that autophagy can either synergize or counteract the apoptotic pathway. We have shown previously that autophagy constitutes an early stage of the apoptotic process induced by protein synthesis inhibition (29) and its inhibition partially blocks cell degeneration. Both the present and the previous study were conducted on retinal explants at the same postnatal age, but in each case cell death was induced by distinct insults on distinct cell layers, which contain cells at differing stages of differentiation. Taken together, the data from both studies imply that the interaction of apoptosis and autophagy depends on the nature of the insult, the cell type and the stage of differentiation. These observations underscore the need to identify specific pathways and the critical components of cell death programs, since autophagy-inducing drugs are readily available and undergoing clinical trials.

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