A Key Role for Calpains in Retinal Ganglion Cell Death

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PURPOSE. The purpose of this study was to examine the importance of calpains in retinal ganglion cell (RGC) apoptosis and the protection afforded by calpain inhibitors against cell death.

METHODS. Two different models of RGC apoptosis were used, namely the RGC-5 cell line after either intracellular calcium influx or serum withdrawal and retinal explant culture involving optic nerve axotomy. Flow cytometry analysis with Annexin V/PI staining was used to identify RGC-5 cells undergoing apoptosis after treatment. TdT-mediated dUTP nick end labeling (TUNEL) was used to identify cells undergoing apoptosis in retinal explant sections under various conditions. Serial sectioning was used to isolate the cell population of the ganglion cell layer (GCL). Western blotting was used to demonstrate calpain cleavage and activity by detecting cleaved substrates.

RESULTS. In the RGC-5 cell line, the authors reported the activation of μ-calpain and m-calpain after serum starvation and calcium ionophore treatment, with concurrent cleavage of known calpain substrates. They found that the inhibition of calpains leads to the protection of cells from apoptosis. In the second model, after a serial sectioning method to isolate the cells of the ganglion cell layer (GCL) on a retinal explant paradigm, protein analysis indicated the activation of calpains after axotomy, with concomitant cleavage of calpain substrates. The authors found that inhibition of calpains significantly protected cells in the GCL from cell death.

CONCLUSIONS. These results suggest that calpains are crucial for apoptosis in RGCs after calcium influx, serum starvation, and optic nerve injury. (Invest Ophthalmol Vis Sci. 2007;48: 5420–5430) DOI:10.1167/iovs.07-0287

Apoptosis, or programmed cell death, is an energy-dependent cell death process characterized by its morphologic features. Although apoptosis does not occur under normal conditions in the intact retina, under conditions of degeneration, such as glaucoma, it has been shown that retinal ganglion cells (RGCs) die by apoptosis. The biochemical pathways involved in apoptosis, namely the death receptor and mitochondrial/intrinsic pathway, both culminate in the activation of caspases. Recently, however, the involvement of a number of other proteolytic activities, such as calpains, cathepsins, and granzymes, have also been described in different experimental models of cell death.

Calpains are a family of cytoplasmic, calcium-activated, cysteine proteases consisting of at least 15 mammalian members. These structurally related enzymes include the ubiquitous calpains comprising two isoforms, μ-calpain (capn1) and m-calpain (capn2). They differ in their sensitivity to calcium because they are activated by low and high micromolar-free calcium, respectively. Each comprises a large 70- to 80-kDa catalytic subunit and a common 29-kDa regulatory subunit (encoded by capn1). In μ-calpain and m-calpain, at least four domains have been identified, and the small subunit has two regions. Synthesized as inactive proenzymes, these proteins require autophosphorylation to become active. μ-Calpain knockout mice were viable and fertile. When both ubiquitous calpains were inactivated by the knockout of their regulatory subunit, it was shown that they can promote death and survival, depending on the stimulus. Calpains have a number of known substrates, including their endogenous inhibitor calpastatin, plus caspase 12, 10, caspase 3 and PARP. Bid, Bax, Apaf 1 and Fodrin, p53 and p35. Cleavage of these substrates, including calpastatin, fodrin, and p35, correlates with increased cell death.

Models of RGC apoptosis include using the rat RGC line RGC-5 and using ex vivo retinal explants. RGC-5 is a transformed rat RGC line derived from P1 Sprague–Dawley rats. By expressing RGC markers, but not those of other retinal cells, this cell line has been shown to be susceptible to apoptosis after serum starvation, which mimics the deprivation of growth factors seen in glaucoma. This laboratory has previously described an ex vivo retinal explant model that mimics the in vivo ON transection model and results in death specifically in the ganglion cell layer (GCL). Previous studies reported calpain activation in models of retinal cell apoptosis involving ocular hypertension and hypoxia. The purpose of our study was to examine the role of active calpains in RGC apoptosis and to examine the effect of calpain inhibitors. It has been suggested that excitotoxic death plays a role in glaucoma and is caused by glutamate-mediated calcium influx and that this death could be mimicked using the calcium ionophore A23187. We used calcium ionophore-treated or serum-starved RGC-5 cells and retinal explant models. After treatment, calpain inhibitors were used in both paradigms to promote RGC survival. In our retinal explant model, we also describe a recently optimized retinal shaving procedure to isolate cells of the GCL. This allowed us to detect protein cleavage patterns in a layer-specific manner which is not possible using immunohistochemistry or Western blotting of whole retinal lysates.

MATERIALS AND METHODS

Reagents and Antibodies
Calpain inhibitors 4-fluorophenylsulfonylVal-Leu-CHO (SJA6017; Calpain Inhibitor VI), N-acetyl-Leu-Leu-Net-CHO (ALLN; Calpain Inhibitor I), carboxenoxyl-Val-Pheno-CHO (MDL28170); Calpain Inhibitor III), and benzoyloxy-carbonyl-Leu-lysyl-norleucinal (Calpeptin) were purchased from Calbiochem (Nottingham, UK). Calcium ionophore A23187 was purchased from Sigma (Dublin, Ireland). Antibodies were purchased from the following companies: Calbiochem (Calpain I, 208753; Calpain II,
Cell Culture

The RGC-5 cell line was a kind gift from Neeraj Agarwal (Fort Worth, TX) and were cultured in Dulbecco modified Eagle medium (DMEM; Sigma), supplemented with 10% FCS (Sigma) and 1% penicillin/streptomycin (Sigma). Cells were incubated at 37°C in humidified 5% CO₂. For induction of apoptosis, 100,000 cells/well were seeded in tissue culture six-well plates (Nalge Nunc International, Hereford, UK) and were allowed to attach for 16 hours. Cells were washed twice with phosphate-buffered saline (PBS), followed by addition of 2 mL serum-free medium or media containing 5 μM A23187. Calpain inhibitors were added at the indicated concentrations. After incubation for different periods of time, the cells were detached with trypsin-ethylene diamine tetraacetic acid (EDTA) solution (Sigma) and, together with their supernatants, were washed once with PBS.

Cell Death Measurements: Propidium Iodide Uptake and Annexin V Staining

Double staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI; Sigma) was performed for quantification of apoptosis. Cells were harvested, washed once with ice-cold PBS, and resuspended in 100 μL calcium-binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) containing 1:10 annexin V-FITC solution (IQ Products, Groningen, The Netherlands). After 10-minute incubation in the dark at room temperature, the cells were diluted with 300 μL binding buffer, and PI was added to a final concentration of 50 μg/μL immediately before flow cytometric analysis. Samples were analyzed (FACScan; Becton Dickinson, Franklin Lakes, NJ) using software (CellQuest; Becton Dickinson) for subsequent data treatment. Ten thousand events per sample were acquired.

Animal Treatment

C57BL/6 (wild-type) mice were obtained from Harlan UK (Bicester, UK). All experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Retinal Explant Culture

Retinal organ culture was performed according to a previously described protocol. Briefly, C57BL/6 (wild-type) mice were decapitated at P6 and P60, and the eyes were removed. The anterior segment, vitreous body, and sclera were removed, and the retina was mounted on nitrocellulose inserts (Millicell; Millipore, Billerica, MA) photoreceptor-side down. Explants (without RPE) were cultured in 1.2 mL R16 media (from Per Ekstrom, Wallenberg Retina Centre, Lund University, Sweden) without FCS. After indicated time points, retinal explants were fixed in formalin; this step was followed by cryoprotection in 25% sucrose overnight. Frozen sections (5–7 μm) were cut, and TUNEL was performed.
Terminal dUTP Nick End Labeling

Briefly, retinal explants or enucleated eyes were fixed in 10% neutral buffered formalin for a minimum of 4 hours, followed by cryoprotection in 25% sucrose overnight at 4°C. Frozen sections (7 μm) were permeabilized using 0.1% Triton X-100. After washes in PBS, sections were incubated with 50 μL reaction buffer containing terminal deoxynucleotidyl transferase (TdT; Promega, Southampton, UK) and fluorescein-labeled dUTP (Boehringer, Mannheim, Germany) for 1 hour at 37°C. Sections were then washed in PBS and incubated with fluorescein isothiocyanate-conjugated sheep anti-TdT (Boehringer, Mannheim, Germany) for 30 minutes at room temperature. Sections were washed again in PBS and counterstained with 1 μM 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, Taunton, UK) at room temperature for 5 minutes. Sections were washed in PBS and coverslipped with Vectashield mounting medium (Vector Laboratories, Peterborough, UK).

FIGURE 3. Changes in calpain cleavage products and calpain substrate cleavage products after calcium ionophore treatment of RGC-5 cells. Shown are densitometric plots of (a) the 78-kDa band of μ-calpain, (b) the 37-kDa band of m-calpain, (c) the 110-kDa band of calpastatin, and (d) the 145-kDa calpain-specific band of fodrin after Western blot analysis.

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cein-12-dUTP (Roche, Lewes, UK) according to the manufacturers’ instructions. Nuclei were counterstained (Hoechst 33342; Sigma). Sections were incubated for 1 hour at 37°C in a humidified chamber. After several washes in PBS, the sections were mounted (Mowiol; Calbiochem) and viewed under a fluorescence microscope (Eclipse E600; Nikon, Tokyo, Japan).

Serial Sectioning
To obtain an enriched population of the retinal GCL, a serial sectioning method was used and was carried out as previously described23 with several modifications. Briefly, four mouse eyes (P60) were explanted as described for each time point. The retina, still attached to the nitrocellulose insert, was placed on a glass coverslip, and the area around the retina was cut out and allowed to adhere to the surface of the coverslip. The vitreal side was facing up, and the RGCs were uppermost. Several sections were first cut from cryochromde and discarded to obtain a flat surface in line with the cryostat. The coverslip with the attached retina was then allowed to adhere to the surface of the cryochromde before sectioning. A 30-μm section was cut from the surface of each flat-mounted retina. The sections were pooled in frozen Eppendorf tubes and dissolved in 30 μL SDS-PAGE sample buffer and subjected to Western blotting, as described.

Western Blots
Cells were plated in tissue culture flasks and allowed to attach overnight, and apoptosis was induced by replacing the routine medium with serum-free medium or media containing 5 μM A23187 (Sigma). After the appropriate incubation times, whole cell extracts were obtained and resolved by denaturing SDS-PAGE. Briefly, cells were scraped and, together with the supernatant, washed once with ice-cold PBS followed by resuspension in cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM Na2VO4, 1 mM NaF, 1 mM ethylene glycol tetraacetic acid [EGTA], 1% Nonidet P-40 [NP40], 0.25% sodium deoxycholate, 0.2 mM AEBSF [Calbiochem], 1 μg/μL antipain, 1 μg/μL aprotinin, 1 μg/μL chymostatin, 1 μg/μL pepstatin, and 0.1 μg/μL leupeptin). After incubation on ice for 45 minutes, debris was pelleted by 15-minute centrifugation (14,000 rpm) at 4°C and protein concentration in the supernatants was normalized with the Bio-Rad (Hemel Hempstead, UK) assay, using bovine serum albumin as standard. In total, 20 μg protein was diluted in 2× sample buffer (10% SDS, 100 mM dithiothreitol, glycerol, bromophenol blue, Tris-HCl) and resolved on 8% to 12% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Membranes were blocked for 1 hour in 5% nonfat dried milk, followed by incubation overnight at 4°C with primary antibodies. After washes with TBs-Tween 20, membranes were incubated with the appropriate secondary antibodies for 1 hour at room temperature (Dako, Carpinteria, CA). After washes in TBs-Tween 20, membrane development was achieved using enhanced chemiluminescence (ECL, Pierce, Rockford, IL).

Statistical Analysis
Data are given as mean ± SD. In the case of two-sample comparisons, a Student’s t-test assuming unequal variances was used to determine whether there was a significant difference between the two sample means. Differences were considered significant if P < 0.05.

RESULTS
Time Course of RGC-5 Apoptosis
In Figure 1, we show the time course for cell death after treatment of the RGC-5 cell line with two stimuli thought to play a role in glaucomatous RGC apoptosis, elevation of intracellular calcium, and deprivation of survival factors. FACS analysis was used to measure the double staining of RGC-5 cells with Annexin V and PI. Annexin V measures the exposure of phosphatidyl serine on the external cell membrane, an early event in apoptosis, whereas PI indicates the permeability of the cell and nuclear membranes that are late apoptotic events. Our results show that 24 hours after insult, RGC apoptosis was barely above control levels. By 48 hours, however, there was approximately 25% apoptosis in cells treated with A23187 and 17% apoptosis in serum-starved cells. After 72 hours, almost 30% of cells underwent apoptosis in both treatment groups.

Calpain Activation after Ionophore Treatment and Serum Withdrawal in RGC-5 Cells
After a recent report describing a possible role for calpain proteases in the RGC-5 cell line using the calpain inhibitor benzoxycarbonyl-leucyl-norleucinal (Calpeptin; Calbiochem),22 we sought to examine whether calpains were activated in this cell line after treatment with calcium ionophore or serum starvation. Both stimuli have been used previously in this cell line to characterize RGC death as they mimic events in retinal abnormalities. In Figure 2, Western blotting indicated that μ-calpain and m-calpain were cleaved to their active fragments in a time- and concentration-dependent manner. At 24 hours, 5 μM A23187 was sufficient to cause cleavage of μ-calpain, m-calpain, and their substrates calpastatin and fodrin. Cleavage of calpastatin decreases its ability to inhibit calpain activity; the 110kDa calpastatin band is no longer detected after treatment with 5 μM A23187. The calpain-specific cleavage products (150/140 kDa) of fodrin were detected 24 hours after treatment. These bands were quantified using densitometry and are shown in Figure 3. After serum starvation, similar results were observed. In Figure 4, Western blot analysis of calpains and their substrates demonstrated that calpains are activated 24 hours after serum withdrawal. As shown in Figure 5, the proforms of μ- and m-calpain decrease after serum starvation, and their endogenous inhibitor calpastatin is cleaved. Fodrin is also cleaved to its calpain-specific fragments. However, the time course is longer when compared to the ionophore model.
Calpain Inhibition Protects RGC-5 Cells after Death Stimuli

Having shown that calpains were cleaved, we wanted to determine the effect of inhibiting calpains on cell death in the RGC-5 cell line after calcium insult or serum withdrawal at 48 hours. We used the calpain inhibitors SJA6017, benzylxycarbonylleucyl-norleucinal (Calpeptin; Calbiochem), ALLN, and MDL28170 at concentrations between 1 and 10 μM in both cell death treatments. In Figures 6a and 6b, we show that using...
SJA6017 at 1 μM significantly reduced apoptosis in both calcium ionophore (P = 0.003) and serum-starved (P = 0.02) cells. Increasing the concentration of inhibitor did not increase protection. In Figures 6c and 6d, we show that 1 μM SJA6017 significantly protected cells against ionophore-induced cell death (P = 0.003) but not against serum withdrawal. In Figures 6e and 6f, we show that ALLN used at 1 μM protected cells from both ionophore (P = 0.008) and serum withdrawal (P = 0.016) cell death. Cell death did increase at higher concentrations (5 and 10 μM) of ALLN after serum withdrawal, possibly because at higher concentrations ALLN may inhibit the proteasome. It has been shown that the proteasome plays a key role in degrading a number of proapoptotic proteins such as Bim. If this was inhibited, cell death might have been initiated earlier and through a different pathway than the one described. Using MDL at a number of different concentrations (1–10 μM) failed to protect cells from either stimulus. Figure 7 illustrates that after calpain inhibition using the indicated calpain inhibitors at 1 μM, the 145-kDa calpain-specific band of fodrin decreases.

**Time Course of Cell Death in the GCL after Optic Nerve Axotomy**

In Figure 8, we show the time course for cell death in retinal explant cultures. This paradigm, involving optic nerve axotomy, is a widely used ex vivo model of RGC death. TUNEL was used to measure DNA fragmentation, a marker of apoptosis, in cells of the GCL. In Figure 8a, we show representative pictures of Hoechst-stained nuclei of the GCL. TUNEL staining indicates cells undergoing cell death in the corresponding sections. We quantified cell death by counting the number of TUNEL-positive cells as a percentage of the Hoechst-stained cells per field. We counted cells in three fields per explant and three explants per treatment. As in agreement with our previous results, we show in Figure 8b that, 48 hours after insult, cell death was barely above control levels. By 72 hours, however, more than 15% of cell death had occurred. After 96 hours, there was more than 25% cell death in cells of the GCL.

**Calpain Is Activated in the GCL of Retinal Explants after Optic Nerve Axotomy**

Recent reports describe a possible role for calpain proteases in retinal cell death after hypoxia and N-methyl-D-aspartate
were detected, and p35 was cleaved to p25 after retinal ex- 

plant culture, indicating the calpain was active in the GCL of retinas after axotomy.

**Calpain Inhibition Protects Cells in the GCL from Cell Death after Optic Nerve Axotomy**

In Figure 12, we show the inhibition of cell death in the GCL of retinal explants using calpain inhibitors after 96 hours in culture. TUNEL was used to measure DNA fragmentation, a marker of apoptosis, in cells of the GCL. In Figure 12a, we show representative pictures of Hoechst-stained nuclei of the GCL and TUNEL staining of the corresponding fields after treatment with the indicated calpain inhibitors. We quantified cell death by counting the number of TUNEL-positive cells per field. We counted three fields per explant and three explants per treatment. In Figure 12b, we show that in untreated retinal explants there was more than 25% cell death. Whereas benzylxoycarbonylleucyl-norleucinal (Calpeptin; Calbiochem) or MDL28170 do not significantly protect against the axotomy-induced cell death, we show that there is nearly a 50% protection with SJA6017 (P = 0.01) and also a significant protection with ALLN (P = 0.024). Figure 13 illustrates that after inhibition of calpain with the indicated calpain inhibitors at 5 μM, there was a decrease in the 145-kDa calpain-specific band of fodrin.

**Figure 7.** Inhibition of calpain prevents cleavage of fodrin to its 145-kDa fragment. Shown is a Western blot of fodrin cleavage products after calcium ionophore treatment of RGC-5 cells for 72 hours with or without the indicated calpain inhibitors. Shown also are densitometric plots of the 145-kDa calpain-specific band after Western blot analysis.

(NMDA) excitotoxicity. We wanted to localize our analysis of calpain activation to the GCL. Because immunohistochemistry does not allow observation of the cleavage pattern and Western blotting of whole retinal lysates is not cell layer specific, we explored the possibility of isolating the GCL. A method described in recent work on protein translocation within photoreceptors persuaded us to attempt “retinal shaving” of the retinal GCL as a possible method of observing protein expression changes in RGC. Optimization of this procedure for the RGC allowed us to use Western blotting to examine the purity of the shavings. Shown in Figure 9a is a Western blot of GCL shavings (30-μm sections from four retinas), whole retinal lysate, untreated RGC-5 lysate, and HL60 lysate. Shaving of the GCL from four retinas we pooled enhances the expression of Thy1, a RGC marker, compared with a whole retinal lysate. The expression of Chx10 (rod bipolar cell marker) is absent in the shavings but present in whole retinal lysate, confirming that the shavings contain only cells of the GCL. With retinal shavings of the GCL after explant culture, we used Western blotting to detect calpain cleavage and activity by substrate cleavage. Figure 9b (see also Fig. 11) shows a decrease in the proforms of μ-calpain and m-calpain; we also detected the cleavage products of m-calpain after 24 hours in culture. With retinal shavings of the GCL of explanted retinas, we used Western blotting of calpain substrates to determine whether calpain was indeed active after cleavage. As can be seen in Figure 10 and Figure 11, calpastatin cleavage first appeared after 24 hours. The calpain-specific fodrin products were detected, and p35 was cleaved to p25 after retinal ex-
After our laboratory’s findings on photoreceptor death, we sought to determine whether activated calpains were involved in RGC apoptosis. Using the RGC-5 cell line, we have shown for the first time that ubiquitous calpains are cleaved after both calcium ionophore treatment and serum withdrawal. Calpain cleavage of known substrates was shown in both models and is in agreement with a previous report that detected the calpain-specific cleavage fragment of fodrin after calcium influx. Cleavage of calpain substrates was dependent on the concentration of calcium ionophore, with cleavage of calpains, calpastatin, and fodrin obvious at 48 hours after treatment with 5 μM A23187. The serum-starved cells took 72 hours to reach a similar level of death.

To confirm the crucial role played by calpains after both treatments, we treated the RGC-5 cells with a number of calpain inhibitors at different concentrations. We found that 3 of 4 inhibitors afforded protection from cell death. Three inhibitors exhibited varying degrees of efficacy with both treatments, whereas MDL28170 did not protect at any tested concentration after either treatment. ALLN gave the best protection, but ALLN also inhibits the proteasome, and this may enhance the protection that is actually attributed to calpain inhibition. A previous study has reported a greater percentage of protection from cell death by benzyloxycarbonylleucyl-nor-leucinal (Calpeptin; Calbiochem) after calcium influx. However, there were differences between that study and the one presented here; a lower concentration of ionophore was used and a higher percentage of apoptosis was detected with a different assay.

Optic nerve axotomy in vivo has been shown to result in cell death of RGCs. This death seems to be specific for RGCs because it has recently been reported that it may take 1 to 3 months after axotomy before statistically significant differences in the number of amacrine cells are detected. Our group has shown that retinal explant culture can mimic the time course of cell death seen in the in vivo model. Recently, we optimized a serial sectioning method that had originally been used to isolate photoreceptors to obtain the GCL cell population. RGCs comprise a small per-

**FIGURE 9.** Calpains are activated in the GCL after optic nerve axotomy. (a) Shown are Western blots of retinal markers after retinal shaving of the GCL. Thy1, a marker of RGCs, is enriched in retinal shavings and is present in a whole retinal lysate and RGC-5 lysate but not in HL60 lysate. Chx10, a marker of rod bipolar cells, is not present in retinal shavings of the GCL but is present in the whole retinal lysate, indicating the purity of the shavings. (b) Shown are Western blots of calpains and their substrates after optic nerve axotomy for 24, 48, and 72 hours. Calpain cleavage increases with time in culture. β-Actin was used as a loading control.

**FIGURE 10.** Calpain substrates are cleaved in the GCL after optic nerve axotomy. Shown are Western blots of calpain substrates after optic nerve axotomy for 24, 48, and 72 hours. Calpain activity, indicated by substrate cleavage, increases with time in culture. β-Actin was used as a loading control.

**DISCUSSION**

Apoptosis of RGCs is central to glaucomatous progression. It is therefore important to develop models representing the biochemical events that take place during the progression of this disease. A number of models of RGC-specific death have emerged in the recent past. In this study, we have chosen to use the RGC-5 cell line and cultured retinal explants to mimic certain events seen in RGC death.

Much attention has been paid to the importance of caspases in RGC apoptosis, and inhibiting caspases has been shown to afford some protection from certain stimuli. Only very recently have other proteases, including calpains, been suggested to play a role in such death. A growing body of evidence indicates an important role for calpains in neuronal cell death. Studies involving calpastatin knockout mice and mice overexpressing the calpain inhibitor, calpastatin, indicate that calpain activity is critical for excitotoxic cell death in the hippocampus. The importance of calpain in retinal degeneration has become apparent in recent years. This laboratory has revealed the importance of calpains in a number of models of photoreceptor death, including light-induced death and methyl nitrosourea-induced death in the rd1 mouse and in the photoreceptor cell line 661W after serum withdrawal.
FIGURE 11. Changes in calpain cleavage products and calpain substrate cleavage products after optic nerve axotomy. Shown are densitometric plots of (a) the 78-kDa band of \( \mu \)-calpain, (b) the 37-kDa band of m-calpain, (c) the 110-kDa band of calpastatin, (d) the 145-kDa calpain-specific band of fodrin and, (e) the 35-kDa band of p35 after Western blot analysis.
centage of the total retinal population; hence, using whole retinal lysates might not detect changes in this cell population. Immunohistochemistry can be used to detect some proteins, but it is not useful to detect cleavage patterns, phosphorylation shifts, or small changes in expression. Immunopanning has also been used to study RGCs; however, it is not possible to purify most cells with a 25% to 50% yield reported.38 By sequential sectioning, it is possible to use Western blotting to detect protein changes in the GCL. We show that Thy1, a retinal-specific marker, is present and enhanced in retinal shavings compared with whole retinal lysate. The rod bipolar cell marker Chx10 was not present in the shavings, indicating the absence of bipolar cells.

Using retinal shavings, we show for the first time that calpains are cleaved after ON axotomy. Both latent forms of \( \mu \)-calpain and m-calpain decrease over time; the cleaved product of m-calpain is detected at 24 hours. After ON from calpain cleavage, we show that calpain substrates, including calpastatin, fodrin, and p35, are cleaved at 24 hours. p35 Cleavage has been shown to be a significant event in neurodegeneration.17 Our group has previously shown that caspases are active after retinal explant culture,18 and it has been shown that caspase inhibition gave up to 34% protection from cell death after axotomy.39 We wanted to determine the extent of calpain involvement in axotomy-induced cell death and so used a number of different calpain inhibitors at an optimized concentration of 5 \( \mu \)M for retinal explants. We found that ALLN and SJA6017 gave protection against cell death. SJA6017 gave nearly 50% protection 96 hours after axotomy. MDL28170 and benzyloxycarbonyl-leucyl-norleucinal (Calpeptin; Calbiochem) failed to show statistically significant protection.

Calpain activity has been shown in models of ocular hypertension,20 NMDA excitotoxicity,27 hypoxia,21,26 and serum withdrawal,25 and these reports demonstrate a degree of protection from various calpain inhibitors. Our study shows that after calcium influx and serum withdrawal in vitro, calpains are active and calpain inhibition affords protection. We also show for the first time that calpain is active after optic nerve axotomy and that calpain inhibition protects RGCs. Although RGC death is a shared event in the models described in this article and in glaucoma models, it would be sensible to stress that conclusions drawn from axotomy models can be misleading with respect to glaucoma.

**Acknowledgments**

The authors thank Neeraj Agarwal for providing the RGC-5 cell line and Per Ekstrom for providing R16 explant culture medium. The authors thank members of the laboratory for helpful discussions. The authors also thank the Science Foundation Ireland for funding the study.

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**Figure 12.** Inhibition of calpain activity attenuates cell death in the GCL after optic nerve axotomy. (a) Shown are Hoechst 33342-stained nuclei of retinal explant sections from C57/Bl6 wild-type mice left in culture for 96 hours and treated with the indicated calpain inhibitors. DNA fragmentation was measured using TUNEL staining of the same sections and photographed using a fluorescence microscope. Representative sections are shown. (b) Percentages of TUNEL-positive cells in the GCL of explants were quantified. TUNEL-positive cells were counted for three sections per time point in three independent experiments. Mean ± SD were calculated and plotted on a histogram. Statistical significance was evaluated using the Student’s t-test.

**Figure 13.** Inhibition of calpain prevents cleavage of fodrin to the 145-kDa fragment. Shown is a Western blot of fodrin cleavage products after optic nerve axotomy for 72 hours, with or without the indicated calpain inhibitors. Shown also is a densitometric plot of the 145-kDa calpain-specific band after Western blot analysis.