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# Caspase-independent photoreceptor apoptosis *in vivo* and differential expression of apoptotic protease activating factor-1 and caspase-3 during retinal development

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## Abstract

**Apoptosis is the mode of photoreceptor cell death in many retinal dystrophies. Exposure of Balb/c mice to excessive levels of light induces photoreceptor apoptosis and represents an animal model for the study of retinal degenerations. Caspases have emerged as central regulators of apoptosis, executing this tightly controlled death pathway in many cells. Previously we have reported that light-induced photoreceptor apoptosis occurs independently of one the key executioners of apoptosis, caspase-3. This present study extends these results reporting on the lack of activation of other caspases in this model including caspases-8, -9, -7, and -1. Furthermore, photoreceptor apoptosis cannot be inhibited with the broad range caspase inhibitor zVAD-fmk indicating that light-induced retinal degeneration is caspase-independent. We demonstrate that cytochrome *c* does not translocate from mitochondria to the cytosol during photoreceptor apoptosis. We also show that during retinal development apoptotic protease activating factor (Apaf-1) protein levels are markedly decreased and this is associated with the inability to activate the mitochondrial caspase cascade in the mature retina. In addition, there is also a significant reduction in expression of caspases-3 and -9 during retinal maturation and these levels do not increase following light exposure. Finally, we show that the calcium-dependent proteases calpains are active during light-induced retinal degeneration and establish that the calcium channel blocker D-*cis*-diltiazem completely inhibits photoreceptor apoptosis.**
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**Keywords:** apoptosis; retinitis pigmentosa; caspase-independent; light; photoreceptor

**Abbreviations:** AcIETD-$\rho$NA, Ac-Ile-Glu-Thr-Asp-p-nitroanilide; AcYVAD-$\rho$NA, Ac-Tyr-Val-Ala-Asp-p-nitroanilide; Apaf-1, apoptotic protease activating factor; CFE, cell free extract; DEVD-fmk, Asp-Glu-Val-Asp-fluromethylketone; DMSO, dimethyl sulphoxide; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; INL, inner nuclear layer; ip, intraperitoneal; ONL, outer nuclear layer; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate buffered saline; RCS, Royal College of Surgeons; rd, retinal degeneration; RP, Retinitis Pigmentosa; TUNEL, Terminal dUTP nick end labelling; TdT, terminal deoxynucleotidyl transferase; UV, ultraviolet; zVAD-fmk, Z-Val-Ala-Asp.fluoromethylketone

## Introduction

Light-induced photoreceptor apoptosis represents an animal model for the study of retinal degenerations such as Retinitis Pigmentosa (RP), a genetically diverse group of disorders involving the progressive death of photoreceptor cells.[1] Apoptosis is the mode of photoreceptor cell death in both human cases and animal models of RP and is now recognised as the final common pathway in many other human retinal disorders.[2–5] Despite these observations the sequence of events that lead to photoreceptor apoptosis and the apoptotic pathways involved remain poorly characterised. As a genetically encoded programme of cell destruction, apoptosis is under the control of a network of highly ordered and conserved components. Regulatory components of the apoptotic pathways in photoreceptors therefore, represent possible therapeutic targets for the inhibition of apoptosis and the amelioration of blinding retinal disorders such as RP.

The caspase family of cysteine proteases have emerged as central regulators of apoptosis. The family is comprised of at least 14 members present in the cell as inactive precursors that undergo proteolytic processing and activation. Once activated, caspases can proteolyse additional caspases generating a cascade that cleaves key structural components as well as proteins critical for cell survival in a highly sequence-specific fashion, ultimately resulting in the systemic and controlled destruction of the cell.[6] Many of the typical morphological features associated with apoptosis such as internucleosomal DNA fragmentation, as well as membrane blebbing and apoptotic body formation can be orchestrated by caspase activation and consequently the caspase family are believed to play a key and central role in apoptosis.[7] Two major caspase pathways have been described. The receptor-mediated pathway entails ligand binding to cell surface receptors, receptor oligomerisation and recruitment and activation of caspase-8 while the mitochondrial pathway involves caspase activation from within the cell. This intrinsic pathway is initiated by release of cytochrome *c* from the mitochondrial intermembrane space. Cytochrome *c* associates with Apaf-1 in a dATP dependent manner to promote the activation of caspase-9.

Caspase-9 then cleaves and activates the executioner caspases-3 and -7. In turn, caspase-3, described as one of the key executioners of apoptosis has been demonstrated to activate at least four other caspases (-2, -6, -8 and 10).[8]

There is no doubt that caspases play a central role in many apoptotic systems. However, increasing evidence now indicates that apoptotic features can be found also in cells where caspase activation is not detected or where caspase inhibitors have been employed.[9–12] Indeed, we have previously provided evidence for a caspase-independent pathway of apoptosis in photoreceptors *in vitro*.[13] Furthermore, there is increasing evidence that other protease families including calpains are involved in apoptosis.[14,15] Calpain is a family of calcium-dependent cysteine proteases of which two major isoforms exist, $\mu$calpain (activated with $\mu$M $Ca^{2+}$) and mcalpain (activated with mM $Ca^{2+}$).[16] Like caspases, calpain substrates include a variety of cytoskeletal proteins such as the actin-binding protein fodrin[17] as well as proteins involved in apoptosis such as Bax,[18] p35,[19] p53,[20] procaspase-9,[15] procaspase-3 and PARP.[21] Increases in intracellular calcium levels occur in many apoptotic systems and may initiate activation of these calcium-dependent proteases. Rapid activation of calpains has been demonstrated during thymocyte and neuronal apoptosis where calcium influx precedes activation.[22,23] Moreover, in several studies calpain inhibitors have been demonstrated to block apoptosis.[24–26] We have previously reported absence of caspase-3 activation during light-induced photoreceptor apoptosis *in vivo*.[27] In this present study, we examine the activation status of several other members of the caspase family as well as the potential involvement of the calcium-activated proteases, calpains.

## Results

### Caspases-1, -7, -8 and -9 are not activated during light-induced photoreceptor apoptosis

Caspases-8, -9, and -3 are situated at pivotal junctions in apoptotic pathways. While caspase-8 is activated following ligand binding and receptor oligomerisation (TNF and TRAIL receptors), agents or insults that trigger release of cytochrome c from mitochondria result in caspase-9 activation. Caspase-8 then activates caspase-3 by proteolytic cleavage and caspase-9 activates both caspases-3 and -7. Caspase-7 has been identified as a contributor to the execution of apoptosis in several systems and can cleave many of the same substrates as caspase-3, including PARP.[28] We have previously reported the lack of caspase-3 activation during light-induced photoreceptor apoptosis *in vivo*. In this study we therefore initially examined the activation status of the effector caspase-7 as well as the initiator caspases-8 and -9.

Caspase-7 is synthesised as a 35 kD inactive proenzyme and is cleaved to generate active subunits of 20 and 12 kD while procaspase-9 (45 kD) is cleaved to generate active subunits of 37 and 39 kD. Analysis of caspases-7 and -9 activation by Western blot demonstrates the absence of active subunits in cell lysates taken from the retinas of light-induced Balb/c mice 3, 6, 14 and 24 h after light exposure (Figure 1a,b). The murine haematopoietic 32D cell line is included as a control to demonstrate the processing of procaspase-7 (35 kD) and procaspase-9 (46 kD) to the active 20, 37 and 39 kD fragments respectively as these cells undergo apoptosis following ultraviolet light (UV) irradiation. The differences in caspase-7 levels observed between these two cell types may be a reflection of the differences in the susceptibility of a dividing, short-lived, apoptosis-prone, haematopoietic cell and a mature, post-mitotic, irreplaceable neuronal cell to apoptosis.[29]

Caspase-8 is synthesized as a 53 kD inactive proenzyme and is cleaved initially to generate a 42 kD subunit. Analysis of the levels of procaspase-8 (53 kD) and the 42 kD subunit by Western blot demonstrates no detectable decrease in procaspase-8 and the absence of the 42 kD subunit in cell lysates taken from the retinas of light-induced Balb/c mice 3, 6, 14 and 24 h after light exposure (Figure 1c). The Jurkat cell line is included as a control to demonstrate the processing of procaspase-8 (53 kD) to the 42 kD fragment as these cells undergo apoptosis following incubation with anti-Fas IgM. To further confirm the absence of active caspase-8 in the retina of light-induced Balb/c mice, the activity of caspase-8 like proteases was assessed by measuring the cleavage of the colorimetric substrate AcIETD-$\rho$NA (Figure 1d). Again no evidence of AcIETD-$\rho$NA cleavage was obtained in light-induced Balb/c retina. Untreated and anti-Fas IgM treated Jurkat cells served as negative and positive controls, respectively.

Caspase-1 activation has been detected during retinal degeneration in the Royal College of Surgeons (RCS) rat and inhibitors of caspase-1 partially delayed this process.[30] In addition, a recent study carried out by Grimm and colleagues on light-induced gene expression in the retina revealed caspase-1 to be the only gene differentially regulated by light exposure.[31] We therefore examined the activation status of caspase-1 by Western blot (Figure 1e) and by determination of YVAD-$\rho$NA cleavage (Figure 1f) using recombinant caspase-1 as a positive control. We could not detect caspase-1 activity by either method in light-induced Balb/c mice 3, 6, 14 or 24 h after light exposure.

### Caspase-inhibitor zVAD-fmk fails to prevent light-induced retinal injury *in vivo*

The major evidence for the apparent central role of caspases in apoptosis has come from studies which employed caspase inhibitors to block apoptosis. However other studies that have used the same inhibitors have demonstrated that apoptosis can occur in a caspase-independent manner.[10,32,33] We examined the effect of the irreversible, broad-spectrum caspase inhibitor, zVAD-fmk on photoreceptor apoptosis. Prior to light exposure age matched Balb/c mice, (12 mice in total) were injected with 80 $\mu$g of zVAD-fmk (in 2 $\mu$l of 2% DMSO) into the right eye and 2 $\mu$l of 2% DMSO into the left eye. As shown in Figure 2, zVAD-fmk failed to prevent apoptosis as assessed by TUNEL. In both the control retina (Figure 2a–d) and the retina treated with zVAD-fmk (Figure 2e–h) there is scattered TUNEL labelling at 6 h and abundant TUNEL positive cells at 14 and 24 h. The dose of caspase inhibitor (80 $\mu$g) employed in this study is significantly greater

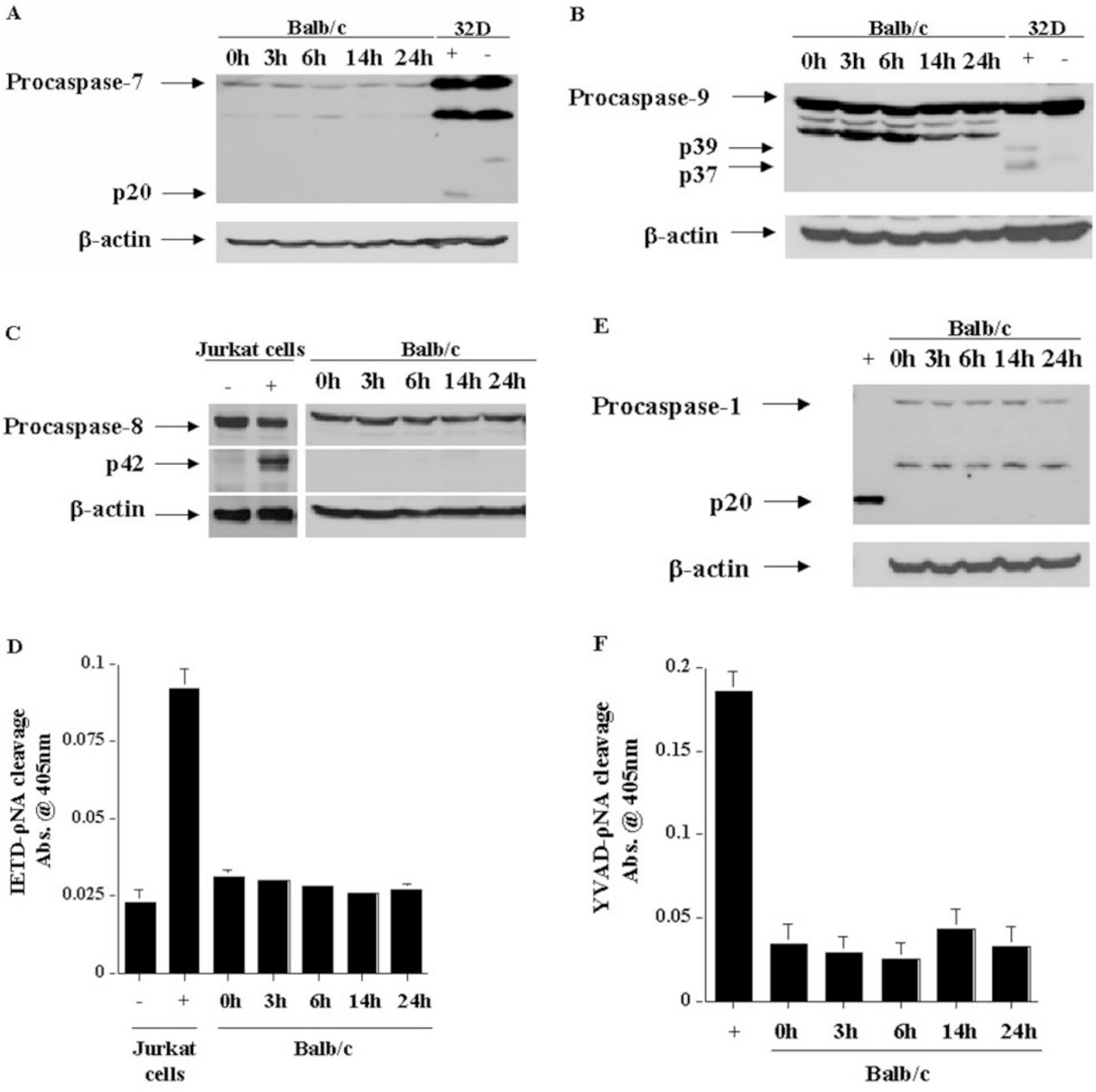


**Figure 1** (**A**) Analysis of caspase-7 activity by Western blot. Equivalent quantities of total protein from cell lysates taken prior to light exposure (0 h), and 3, 6, 14 and 24 h of darkness following light exposure were resolved using SDS–PAGE and transferred to a nitrocellulose membrane. The presence of procaspase-7 (35 kDa) and the proteolytically active 20 kDa fragment were determined using an anti-caspase-7 antibody. Untreated and UV treated 32D cells served as negative and positive controls respectively in order to determine the ability of this antibody to detect active murine caspase-7. The 32D protein lysates were extracted 16 h after a 10-min exposure to ultraviolet irradiation. These cells demonstrate the processing of procaspase-7 (35 kDa) to the active 20 kDa fragment as these cells undergo apoptosis. The 35 kDa procaspase species is present at all time points analysed in the Balb/c extracts, however, the 20 kDa fragment is absent up to 24 h. The blot was re-probed with an antibody to $\beta$-actin to demonstrate equal protein loading (bottom). A representative result of three independent experiments is shown. (**B**) Analysis of caspase-9 activity by Western blot. Equivalent quantities of total protein from cell lysates taken prior to light exposure (0 h), and 3, 6, 14 and 24 h of darkness following light exposure were resolved using SDS–PAGE and transferred to a nitrocellulose membrane. The presence of procaspase-9 (46 kDa) and the proteolytically active 39 and 37 kDa fragments were determined using an anti-caspase-9 antibody. Untreated and UV treated 32D cells served as negative and positive controls respectively. In contrast to the 32D cells, which show processing of procaspase-9 (46 kDa) to the active 39 and 37 kDa fragments as these cells undergo apoptosis following UV treatment, retinal cells retain capase-9 in its inactive form during light-induced apoptosis. The blot was re-probed with an antibody to $\beta$-actin to demonstrate equal protein loading (bottom). A representative result of three independent experiments is shown. (**C**) Analysis of caspase-8 activity by Western blot. Equivalent quantities of protein from cell lysates taken prior to light exposure (0 h), and 3, 6, 14 and 24 h of darkness following light exposure were resolved using SDS–PAGE and transferred to a nitrocellulose membrane. The presence of procaspase-8 (53 kDa) and the 42 kDa fragment were determined using an anti-caspase-8 antibody. Untreated and Fas IgM treated Jurkat cells served as negative and positive controls respectively in order to determine the ability of this antibody to detect the 42 kDa fragment. The Jurkat cell protein lysates were extracted immediately after incubation with apoptosis inducing anti-Fas IgM antibody (300 ng ml$^{-1}$) for 4 h. These cells demonstrate the processing of procaspase-8 (53 kDa) to the 42 kDa fragment as these cells undergo apoptosis. The 53 kDa procaspase species is present at all time points analysed in the Balb/c extracts, however, the 42 kDa fragment is absent up to 24 h. The blot was re-probed with an antibody to $\beta$-actin to demonstrate equal protein loading (bottom). A representative result of three independent experiments is shown. (**D**) Analysis of caspase-8 like activity by detection of IETD-$\rho$NA cleavage. The measurement of IETD-$\rho$NA cleavage was performed in a spectrophotometric assay by monitoring the liberation of $\rho$NA due to caspase activity prior to light exposure (0), and 3, 6, 14 and 24 h of darkness following light exposure. Untreated

than the effective dose of caspase inhibitors shown to have protective effects in photoreceptors in previous studies.[34,35]

## Cytochrome-*c* is not released from the mitochondria following light-exposure

Release of cytochrome *c* from the mitochondrial intermembrane space is a fundamental event in apoptosis as it sets in motion the assembly of the apoptosome, resulting in activation of caspase-9, the downstream effector caspases-7 and -3 and ultimately cell death.[8] We therefore investigated the cellular distribution of cytochrome *c* during light-induced photoreceptor apoptosis using subcellular fractionation studies. Cytochrome *c* was not detected in cytosolic fractions by Western blot during light-induction (1 h), immediately following light exposure (2 h) or following a further incubation of 3, 6, 14 and 24 h in darkness (Figure 3a). These results demonstrate that cytochrome *c* does not translocate from mitochondria to the cytosol during light-induced retinal degeneration.

## Addition of cytochrome *c* to cell-free extracts does not initiate activation of caspases-9 or -3

Several studies have demonstrated that cytochrome *c* in association with dATP is capable of inducing proteolytic processing of procaspases-9 and -3 in cell free systems derived from a variety of cell types.[8,36–38] We therefore investigated the ability of cytochrome *c* to initiate the activation of caspases-9 and -3 in cell free extracts derived from adult Balb/c retina used in this study (Figure 4). The murine haematopoietic 32D cell line is included as a control to demonstrate the rapid processing of procaspase-9 (45 kD) to the active 37 and 39 kD fragments and procaspase-3 to its active p17 kD subunit following incubation with cytochrome *c* for up to three hours (Figure 4c). In direct contrast, analysis of the conversion of procaspases-9 and -3 to their active forms in cytosolic extracts prepared from untreated retina (Figure 4a) or from light-induced retina (14 h) (Figure 4b), following incubation with cytochrome *c* demonstrates no detectable decrease in the procaspases-9 or -3 and the absence of the

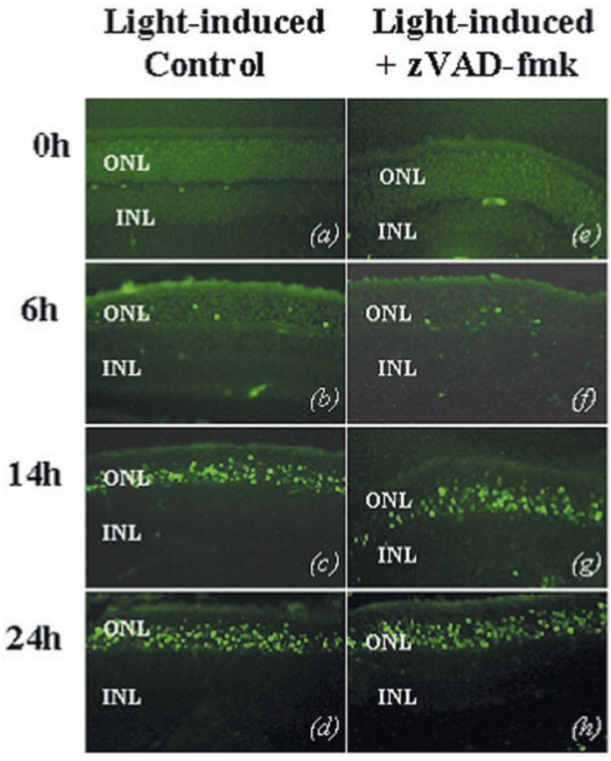


**Figure 2** Effect of zVAD-fmk, a broad-spectrum caspase inhibitor on light induced retinal degeneration. ZVAD-fmk was administered sub-retinally to anaesthetised mice prior to light exposure. The right eye of each animal was injected with 2 $\mu$l of DMSO, 0.9% W/V NaCl buffer containing 40 ug/$\mu$l zVAD-fmk (**e**–**h**) and the left eye was treated with 2 $\mu$l of DMSO, 0.9% W/V NaCl alone as a control (**a**–**d**). In control mice and in mice treated with the caspase inhibitor zVAD-fmk, the retina of dark-adapted control mice does not show labelling (**a** and **e**). Photoreceptors of mice from both experimental groups that were sacrificed at 6 h following light exposure have scattered labelling in the ONL (**b** and **f**) and mice sacrificed 12 h (**c** and **g**) and 24 h (**d** and **h**) after light exposure show significant labelling of photoreceptors. ONL: outer nuclear layer; INL: inner nuclear layer

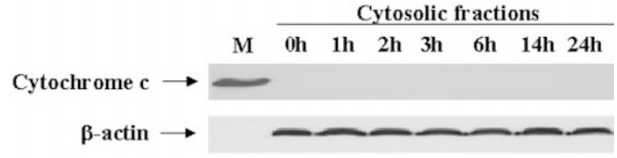


**Figure 3** Analysis of cellular distribution of cytochrome *c* during photoreceptor apoptosis. Cells were fractionated at each time point (prior to light exposure (0 h), during light-induction (1 h), immediately following light exposure (2 h) or following a further incubation of 3, 6, 14 and 24 h in darkness) and equal amounts of cytosolic protein loaded into each lane. As a positive control for cytochrome *c*, 20 $\mu$g of mitochondrial fraction (M) was loaded in the first lane. The blot was initially probed with an antibody to cytochrome *c* (top) and then re-probed with an antibody to $\beta$-actin (bottom) to demonstrate equal protein loading. Cytochrome *c* was readily detected in the mitochondrial fraction (M) but is absent from the cytosolic fractions up to 24 h after light exposure. A representative result of three independent experiments is shown

and anti-Fas IgM treated Jurkat cells served as negative and positive controls respectively. Jurkat cells were suspended at $5 \times 10^5$ cells/ml and treated with apoptosis inducing anti-Fas IgM antibody (300 ng ml$^{-1}$) for 4 h. Data is expressed as the mean $\pm$ S.E.M. of three independent experiments. (**E**) Analysis of caspase-1 activity by Western blot. Equivalent quantities of protein from cell lysates taken prior to light exposure (0 h), and 3, 6, 14 and 24 h of darkness following light exposure were resolved using SDS–PAGE and transferred to a nitrocellulose membrane. The presence of procaspase-1 (44 kDa) and the 20 kDa fragment were determined using an anti-caspase-1 antibody. Recombinant caspase-1 served as a positive control. The 44 kDa procaspase species is present at all time points analyzed in the Balb/c extracts, however, the 20 kDa fragment is absent up to 24 h. The blot was re-probed with an antibody to $\beta$-actin to demonstrate equal protein loading (bottom). A representative result of three independent experiments is shown. (**F**) Analysis of caspase-1 like activity by detection of YVAD-$\rho$NA cleavage. The measurement of YVAD-$\rho$NA cleavage was performed in a spectrophotometric assay by monitoring the liberation of $\rho$NA due to caspase activity prior to light exposure (0 h), and 3, 6, 14 and 24 h of darkness following light exposure. Recombinant caspase-1 served as a positive control. Data is expressed as the mean $\pm$ S.E.M. of three independent experiments

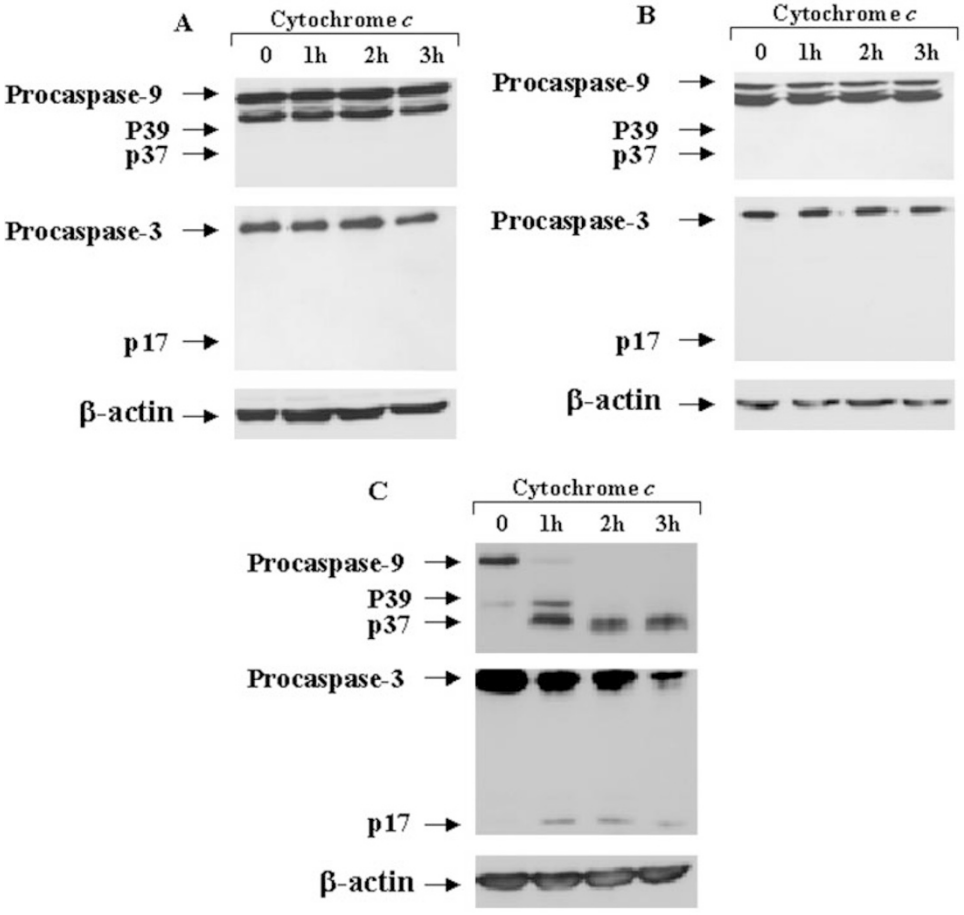


**Figure 4** Addition of cytochrome *c* and dATP to cell-free extracts and analysis of caspases-9 and -3 activation by western blot. Equivalent quantities of protein from untreated Balb/c cell-free extracts (**A**), cell free extracts prepared from light-induced retinas (14 h) (**B**) and 32D cells (c), incubated in cytochrome *c* and dATP for 1, 2 and 3 h were resolved using SDS–PAGE and transferred to a nitrocellulose membrane. The presence of procaspase-9 (46 kDa) and the proteolytically active 39 and 37 kDa fragments were determined using an anti-caspase-9 antibody (top). The presence of procaspase-3 (32 kDa) and the proteolytically active 17 kDa fragment was determined using an anti-caspase-3 antibody (middle). In contrast to the 32D cells which clearly show processing of procaspases-9 and -3 to their active fragments (p39, p37 and p17 respectively) following incubation with cytochrome *c* (Figure 3c) and dATP, retinal cells (0 h and 14 h) retain caspases-9 and -3 in their inactive form (46 and 32 kDa respectively) (Figure 3**A**,**B**). The blots were re-probed with an antibody to $\beta$-actin to demonstrate equal protein loading (bottom). A representative result of three independent experiments is shown

active subunits. Due to the low level of procaspase-3 in the retinal cell free extracts and the relative intensity of the p17 kD product, we further examined the activation status of caspase-3 by determination of DEVD-$\rho$NA cleavage and did not detect caspase activity (data not shown). These cell free studies demonstrate that the caspase cascade downstream of cytochrome *c* release cannot be activated in adult photoreceptor cells.

## Apaf-1, caspases-3 and -9 protein levels are markedly reduced in the mature retina and do not increase following light exposure

A recent study by Yakovlev and colleagues demonstrated that the potential of the mitochondrial caspase cascade is reduced during brain development and this repression is associated with down regulation of Apaf-1 and caspase-3.[39] We therefore examined the expression levels of each component of the apoptosome at the protein level to determine the potential mechanism by which cytochrome *c* fails to activate the mitochondrial caspase cascade in the retina. Expression levels of Apaf-1, caspases-9 and -3 in the mature retina ($P_{60}$) were compared to levels expressed at postnatal day 10 ($P_{10}$), a time when developmental photoreceptor cell death in the retina is just complete. Western blot analysis revealed a marked reduction of both Apaf-1 and caspase-3 expression at $P_{60}$. Caspase-9 protein levels were also observed to decrease albeit to a lesser extent (Figure 5a). The blots were re-probed with GAPDH to ensure equal protein loading. We also assessed protein expression levels of apoptosome components in the pigmented C57 retina. A similar age-dependent reduction in protein expression was observed indicating that this observation is not restricted to the albino Balb/c retina. Furthermore, we assessed the expression levels of Apaf-1, caspases-9 and -3 following light exposure in the Balb/c retina at $P_{60}$ by Western blot and found no significant increase in these proteins at 3, 6, 14 and 24 h after light induction (Figure 5b).

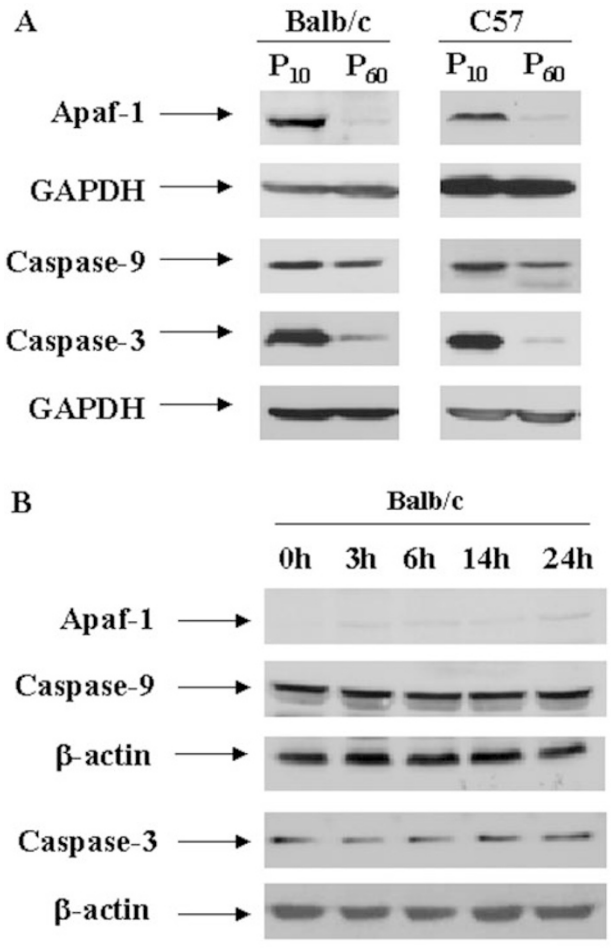


**Figure 5** Western blot analysis of Apaf-1, caspases-9 and -3 levels at $P_{10}$ and $P_{60}$ in Balb/c and C57 retina and during light-induced photoreceptor apoptosis. Sixty micrograms of protein from Balb/c and C57 retina at $P_{10}$ (postnatal day 10) and $P_{60}$ (postnatal day 60) (**A**) and from cell lysates taken prior to light exposure (0 h), and 3, 6, 14 and 24 h of darkness following light exposure at $P_{60}$ (**B**) were resolved by 8% (Apaf-1) and 12% (caspase-9 and -3) SDS–PAGE and transferred to nitrocellulose membranes. Expression levels of Apaf-1 were determined using an anti Apaf-1 antibody (top). Expression levels of caspases-9 and -3 were determined using anti-caspases-9 and -3 antibodies respectively (middle). Both blots were re-probed for GAPDH to demonstrate equal protein loading (middle and bottom). These blots were repeated three times with similar results

These results suggest that cytochrome *c* fails to activate caspases-9 or -3 in the mature retina (even in extracts prepared 14 h after light induction) due to diminished levels of Apaf-1 in the adult retina, which do not significantly increase after exposure to light.

## Age-dependent susceptibility of retinal CFE to cytochrome *c* dependent activation of caspases-9 and -3

$P_{10}$ and $P_{60}$ retinal extracts were incubated in the presence and absence of cytochrome *c* and dATP for 2 h. Western blot analysis of caspases-9 and -3 revealed processing of procaspase-9 (45 kD) to the active 37 and 39 kD fragments and procaspase-3 to its active p17 kD subunit in $P_{10}$ extracts following incubation with cytochrome *c*. In contrast, analysis of the conversion of procaspases-9 and -3 to their active forms in cytosolic extracts prepared from $P_{60}$ retinal extracts following incubation with cytochrome *c* demonstrates no detectable decrease in the procaspases-9 or -3 and the absence of the active subunits (Figure 6a). Similar results were obtained for $P_{10}$ and $P_{60}$ C57 retinal extracts (Figure 6b). These results demonstrate that susceptibility of retinal CFE to cytochrome *c* dependent activation of caspases is age-dependent and that defective cytochrome *c*-dependent caspase activation in the mature retina correlates with diminished Apaf-1 and caspase-3 levels. (It is interesting to note that a single immuno-reactive band can be seen at $P_{60}$ that is absent at $P_{10}$. This band does not appear to be an active product as it is detected above the p39 kD active band. However, several splice variants of caspase-9 that act as endogenous inhibitors of apoptosis have been identified in several species, including mouse.[40–43] It is possible that this band is a potential novel isoform of caspase-9 that may act to inhibit apoptosis by a similar mechanism. It would not be surprising then to find this band present in post-mitotic fully differentiated cells at p60 and absent from dividing, apoptosis-prone, haematopoietic cells and retinal cells at p10.

## Calpains are activated during light-induced photoreceptor apoptosis

The lack of activation of caspases in this model prompted us to explore the possible involvement of other proteases. We previously reported elevated intracellular calcium levels during photoreceptor apoptosis and therefore examined the activation status of calpains, calcium activated proteases. The activity of calpains was assessed by measuring the cleavage of the fluorogenic substrate Suc-Leu-Tyr-AFC (Figure 7). This substrate is a membrane permeable, calpain-specific substrate. Increased calpain activation was detected in light-induced Balb/c mice as early as 30 min after light induction and activity continued to increase up to 24 h after the light insult. To ensure that fluorescence activity was calpain specific we employed the calpain inhibitor, calpeptin. At 24 h calpeptin completely inhibited calpain activity in retinal cell lysates. These results demonstrate the early and sustained activation of calpains during light-induced retinal degeneration.

## D-*cis*-diltiazem, a calcium channel blocker inhibits early elevation of intracellular calcium, and photoreceptor apoptosis

Calcium elevation is an early and rapid event in light-induced retinal degeneration.[27] In this present study we show that the previously observed increase in intracellular calcium results in the activation of calcium-dependent proteases. To determine if elevated calcium levels play a significant role in light-induced photoreceptor apoptosis, animals were treated with the calcium channel blocker D-*cis*-diltiazem, 1 h prior to light exposure. Intracellular calcium levels were analyzed using the fluorescent probe fluo-3 AM (Figure 8a). Increased levels of calcium were detected as before immediately after light exposure and the number of cells with elevated calcium continued to increase up to 3 h. This transient elevation of

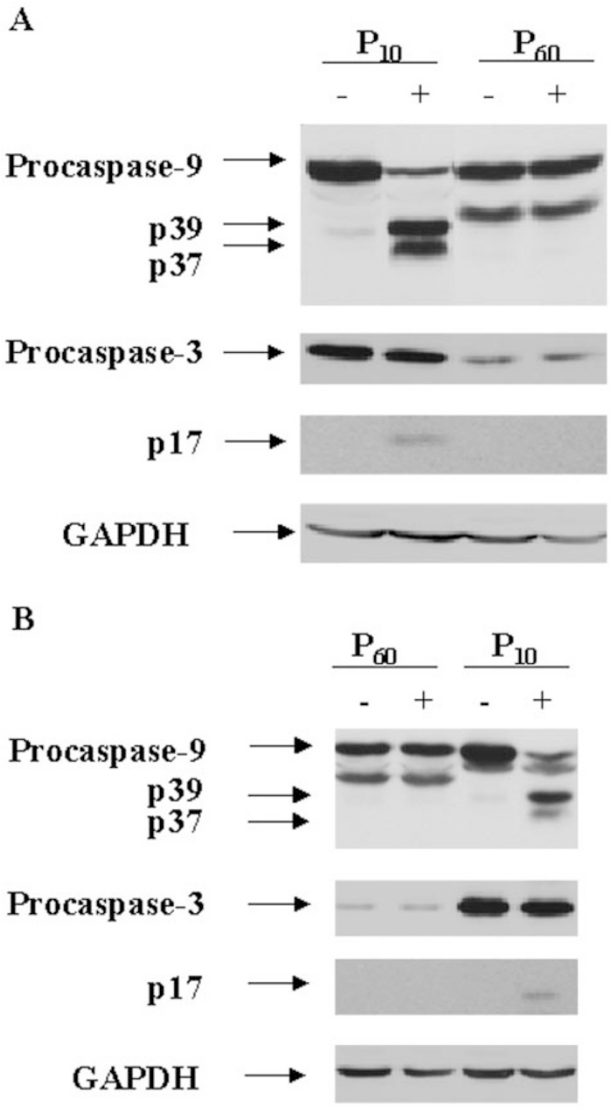


**Figure 6** Analysis of age-dependent susceptibility of retinal CFE to cytochrome *c* dependent activation of caspases-9 and -3. Equivalent quantities of protein from $P_{10}$ and $P_{60}$ Balb/c cell-free extracts (**A**), and from $P_{10}$ and $P_{60}$ C57 cell-free extracts (**B**), incubated with (+) or without (−) cytochrome *c* and dATP for 2 h were resolved using SDS–PAGE and transferred to a nitrocellulose membrane. The presence of procaspase-9 (46 kDa) and the proteolytically active 39 and 37 kDa fragments were determined using an anti-caspase-9 antibody (top). The presence of procaspase-3 (32 kDa) and the proteolytically active 17 kDa fragment was determined using an anti-caspase-3 antibody (middle). As before (Figure 3B), the adult Balb/c retina ($P_{60}$) retains caspases-9 and -3 in the inactive form even after incubation with cytochrome *c* and dATP. This is also the case for the adult C57 retina. In contrast, the $P_{10}$ Balb/c and C57 retina show processing of caspases-9 and -3 to their active fragments (p39, p37 and p17 respectively) following incubation with cytochrome *c* and dATP. The blots were re-probed with an antibody to $\beta$-actin to demonstrate equal protein loading (bottom). A representative result of three independent experiments is shown

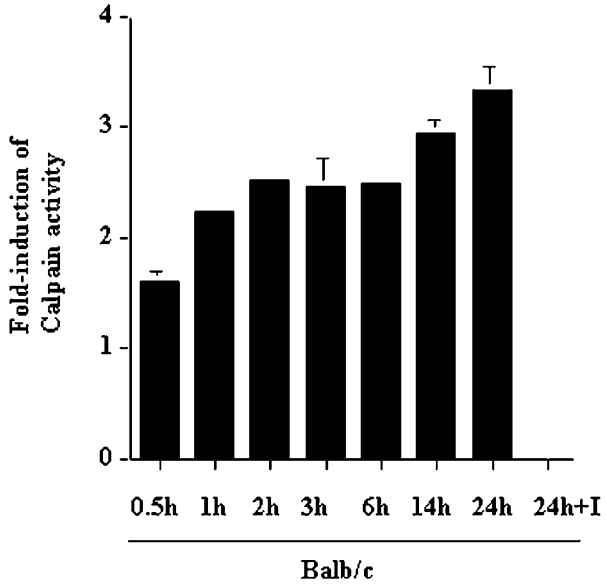


**Figure 7** Analysis of calpain activity by detection of Suc-Leu-Tyr-AFC cleavage. The measurement of Suc-Leu-Tyr-AFC cleavage was performed in a fluorogenic assay by monitoring the liberation of AFC due to calpain activity prior to light exposure (0 h), during light-induction (0.5 and 1 h), immediately following light exposure (2 h) or following a further incubation of 3, 6, 14 and 24 h in darkness. As an additional control to ensure that fluorescence activity was calpain specific the last lane (24 h+I) represents calpain activity after incubation of the 24 h lysate in calpeptin (100 $\mu$M). Calpain activity is completely inhibited in this sample. Data is expressed as fold induction of calpain activity and as the mean ± S.E.M. of three independent experiments

intracellular calcium is blocked by D-*cis*-diltiazem. Finally, the effect of D-*cis*-diltiazem on photoreceptor apoptosis was examined. In treated animals no TUNEL positive photoreceptor cells were detected immediately following light exposure or following a further incubation of 6, 14 and 24 h in darkness (Figure 8c, e–g). Animals that were injected with PBS alone as a control, showed similar TUNEL labelling to untreated light-induced animals (Figure 8a–d). These results demonstrate that D-*cis*-diltiazem completely inhibits photoreceptor apoptosis and establish a key role for calcium in this model of light-induced retinal degeneration.

## Discussion

Analysis of caspase activity in this study reveals that light-induced photoreceptor apoptosis *in vivo* occurs independently of caspase activation. Caspase-independent apoptosis has been described in several other models of apoptosis including cell death induced by vitamin D compounds[44] nitric oxide,[9] depletion of heat shock protein 70,[45] Bax,[46] Bak,[33] and the c-Myc interacting adaptor protein Bin 1.[47] Furthermore, this laboratory has previously described a retinal cell apoptosis pathway *in vitro* which does not involve caspase activity and retains key features of apoptotic cell death including DNA strand nicking, phosphatidylserine externalisation and cell shrinkage.[13] So despite the central role once attributed to caspases in apoptosis, emerging evidence now supports the existence of caspase-independent pathways in a number of cells under certain cellular stresses.[12]

To determine the molecular basis by which caspases fail to be activated in this model we further explored the intrinsic mitochondrial caspase pathway in photoreceptors. Analysis of the cellular distribution of cytochrome *c* revealed the absence of cytochrome *c* from retinal cytosolic

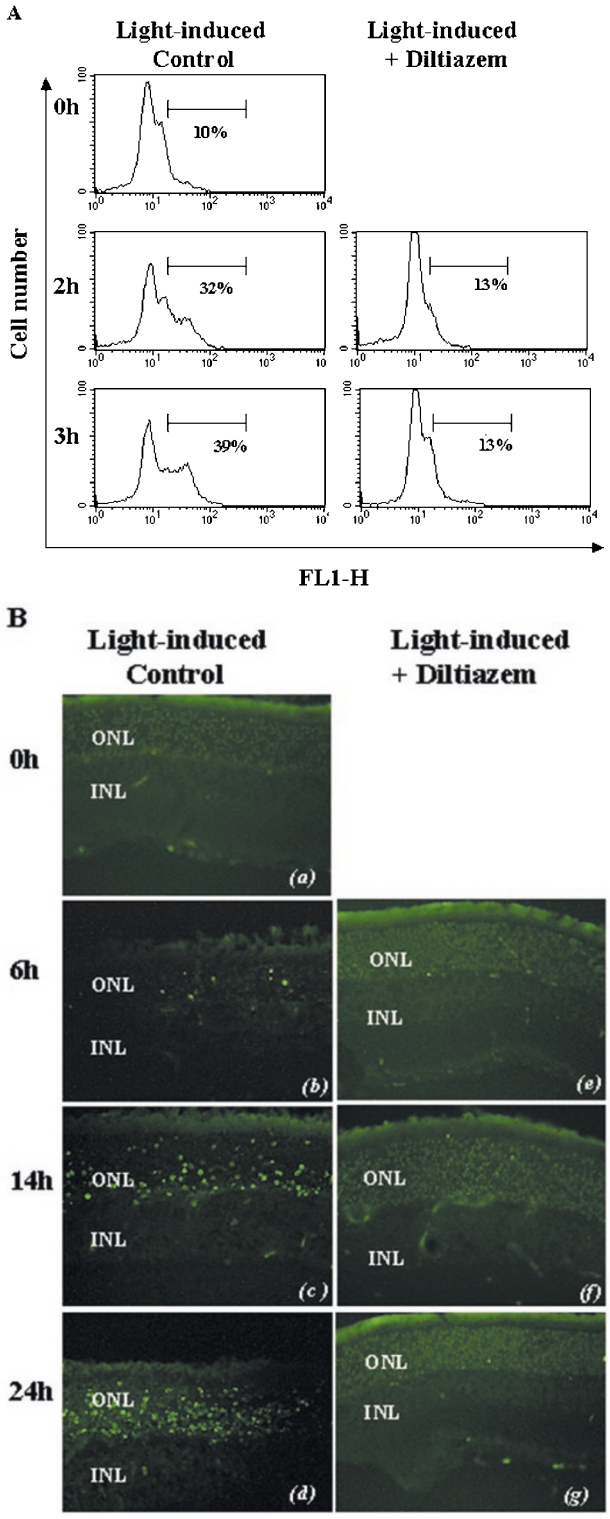


**Figure 8** (**A**) Analysis of intracellular calcium levels in the retinas of untreated light-induced Balb/c mice and mice treated with 50 mg kg$^{-1}$ of the calcium channel blocker D-*cis*-diltiazem. Intracellular calcium levels were monitored using the fluorescent probe Fluo-3 in photoreceptors from mice treated with D-*cis*-diltiazem (i.p.50 mg/kg) in PBS or PBS alone as a control at the following time points: prior to light exposure (0 h), immediately after light exposure (2 h) and 3 h of darkness following light exposure. Increased fluorescence in the FL-1 channel indicates increased levels of calcium. The percentage of cells displaying increased levels of calcium is shown at each time point. Results are representative of three independent experiments. (**B**) Effect of D-*cis*-diltiazem, a calcium channel blocker on photoreceptor apoptosis. Mice were treated with D-*cis*-diltiazem (i.p.50 mg/kg) in PBS or PBS alone as a control, 1 h prior to light exposure. Retinas of mice treated with PBS alone (**A**–**D**). Retina of dark-adapted control mouse (**A**) does not show labelling. Photoreceptors of mice sacrificed 6 h in darkness following light exposure reveals scattered labelling in the ONL (**B**). Mice sacrificed 14 h (**C**) and 24 h (**D**) after light exposure show significant labelling of photoreceptors. Retinas of mice treated with 50 mg kg$^{-1}$ of the calcium channel blocker D-*cis*-diltiazem (**E**–**G**). Photoreceptors of mice sacrificed 6 (**E**), 14 (**F**) and 24 (**G**) h of darkness following the light exposure show no labelling. ONL: outer nuclear layer; INL: inner nuclear layer

fractions up to 24 h after light exposure indicating that cytochrome *c* is not released from the mitochondria in this model (Figure 3). Surprisingly, addition of cytochrome *c* to retinal cell free extracts did not result in activation of caspases-9 or -3 (Figure 4). To address this issue we examined expression levels of apoptosome components, Apaf-1 and caspase-9 as well as caspase-3 during retinal development. A marked decrease in expression of Apaf-1 and caspase-3 in both the Balb/c and C57 adult retina was observed compared to levels at $P_{10}$ (postnatal day 10), while caspase-9 expression also decreased albeit to a lesser extent (Figure 5a). These results are consistent with reports demonstrating that caspase-3 mRNA and protein expression strongly decrease during brain development.[48,49] In addition, differential expression of Apaf-1 and caspase-3 during brain maturation has recently been demonstrated and shown to correlate with susceptibility to cytochrome *c*-dependent caspase activation.[39] Furthermore, defective cytochrome *c*-dependent caspase activation due to diminished or absent Apaf-1 has been reported in cancer cell lines.[50,51] Consistent with these results, we demonstrate in this study that loss of Apaf-1 correlates with the inability of cytochrome *c* to activate caspases. At $P_{10}$ when Apaf-1 and caspase-3 proteins are highly expressed, the intrinsic mitochondrial caspase pathway can be activated on addition of cytochrome *c* and dATP. In contrast, when Apaf-1 and caspase-3 levels are markedly reduced as in the adult retina, cytochrome *c* can no longer activate caspases-9 or -3 (Figure 6). It is crucial that apoptosis in the adult brain is tightly controlled. Rapid caspase activation and cell death, which occur normally during neuronal development, has serious implications in the mature brain. This is exemplified by the many neurological diseases including retinal disorders in which excessive apoptosis is observed. The photoreceptor cells of the retina, 'an accessible part of the brain' are mature, fully differentiated, post-mitotic cells and like other neuronal cells do not undergo apoptosis under normal physiological conditions. Down regulation of Apaf-1 to such an extent in the adult retina as observed in this study may be a mechanism by which mature photoreceptors retain tight control of apoptosis. Further down regulation of caspase-3 would protect the cell from apoptosis initiated by granzyme B or other caspases[8,11,12] and may act as a complimentary mechanism for regulation of apoptosis in the adult retina.

The results of this study are in contrast to recent reports describing caspase activation in several rat models of

photoreceptor apoptosis. These are the RCS rat,[30] rhodopsin S334ter rats[34] and in apoptosis induced by N-methyl-N-nitrosourea in Sprague–Dawley rats.[35] The apparent differences between these studies and this present study may be explained by the use of two different animal models i.e. rats and mice. Mice may not activate a caspase-dependent apoptotic pathway in the retina due to diminished levels of Apaf-1 and caspase-3, as reported in this study. Alternatively, stimuli other than light may induce caspase and Apaf-1 expression, thereby making the mitochondrial caspase pathway available. A recent study which demonstrates an increase in Apaf-1 mRNA and protein levels during traumatic brain injury supports this hypothesis.[39] This study suggests that in order for caspase-dependent apoptosis to occur in the mature brain, Apaf-1 must be reactivated. Furthermore, p53 transcriptional activation of Apaf-1 has been shown to play a pivotal role in the regulation of apoptosis after neuronal injury.[52] The possibility that Apaf-1 and caspase-3 may be reactivated in the rat models of photoreceptor apoptosis mentioned above, remains to be elucidated, as investigation of Apaf-1 and procaspase gene/protein expression during retinal degeneration in these models has not been carried out, to our knowledge. In contrast, we examined Apaf-1, procaspases-9 and -3 expression during light-induced photoreceptor apoptosis and did not detect any significant increase in expression of these proteins (Figure 5b). This correlates with the repression of cytochrome *c*-dependent activation of caspases-9 or -3 in lysates prepared from light-induced retina (Figure 4b).

There is increasing evidence to support the role of other proteases such as calpains, cathepsins and the proteosome complex in promoting apoptosis-like events.[15,53,54] The lack of caspase activation in this model prompted us to investigate the possible activation of other proteases. Analysis of the calcium-activated proteases, calpains, in this study revealed their activation during light-induced photoreceptor apoptosis (Figure 7). Calpain activation has been implicated in several neurodegenerative diseases including alzheimer's disease,[55] cerebral ischaemia[56] and cataract formation.[57] Moreover, irregular calpain activity has been detected in the RCS rat, a model for inherited retinal degeneration.[58] Calpains have an absolute requirement for calcium for activation.[59] We have previously described an increase in intracellular calcium levels during photoreceptor apoptosis.[27] In this present study, intraperitoneal injection of the calcium channel blocker D-*cis*-diltiazem completely inhibits photoreceptor apoptosis demonstrating a key role for calcium in retinal degeneration (Figure 8b). D-*cis*-diltiazem has previously been reported to enhance photoreceptor survival in the *rd* mouse model of human RP.[60] However, the mechanism of protection of this drug in photoreceptors is not yet established although it is postulated to modulate calcium levels by blocking L-type voltage gated channels. Here we show that administration of D-*cis*-diltiazem completely inhibits the increase in intracellular calcium (Figure 8a), thereby providing a potential mechanism for the inhibition of photoreceptor apoptosis by D-*cis*-diltiazem in this model.

In conclusion, this study describes apoptosis of photoreceptor cells *in vivo* via a pathway that does not involve activation of several key caspases and cannot be prevented by caspase inhibitors. The preclusion of caspase activation during light-induced photoreceptor apoptosis in the retina appears to be regulated at two levels: by the prevention of cytochrome *c* release from the mitochondria and due to diminished levels of Apaf-1, a critical component of the apoptosome, and caspase-3, a key executioner of apoptosis. This study may have implications for therapeutic strategies aimed at the prevention of photoreceptor apoptosis and blinding retinal disorders such as RP. These results taken together with previous reports of caspase activation during retinal apoptosis imply that photoreceptors are capable of caspase-dependent and independent cell death with the initial insult shaping cellular demise. Therapeutic strategies based on caspase inhibition, therefore, may not be as effective as strategies that target initiating signals of apoptosis, such as calcium channel blockers, as our findings demonstrate.

## Materials and Methods

### Retinal light-damage

Adult male Balb-c mice were maintained in the dark for 18 h before being exposed to constant light. Immediately prior to light exposure their pupils were dilated with 5% cyclopentolate. The mice were then exposed to 2 h of cool white fluorescent light at a luminescence level of 5000 lux. The mice were sacrificed after treatment by cervical dislocation at the following time points: 1 h after light onset, immediately after light exposure (2 h) and after 3, 6, 14 and 24 h of darkness that followed the 2 h light exposure.

### Cell lines

Jurkat T-cells were maintained in RPMI containing 10% FCS. 32D cells were cultured in RPMI containing 10% FCS and 10% WEHI conditioned media. Agents used to induce apoptosis were anti-human Fas (300 ng/ml) (Upstate Biotech New York, USA) and exposure to ultraviolet (UV) irradiation (10 min).

### Sub-retinal and intraperitoneal injections

For sub-retinal injections aged-matched (12 weeks) Balb/c mice were firstly anaesthetised with 750 $\mu$l of avertin administered intraperitoneally. The right eye of each animal was injected sub retinally under a surgical microscope with 2 $\mu$l of DMSO, 0.9% W/V NaCl buffer containing 40 ug/$\mu$l zVAD-fmk (Bachem Chemicals). The left eye was treated with 2 $\mu$l of DMSO, 0.9% W/V NaCl alone as a control. Following injection animals were allowed to recover completely prior to dark adaptation overnight followed by light insult. For intraperitoneal injections, mice were administered with 50 mg $kg^{-1}$ of Diltiazem (Calbiochem) in PBS or with PBS alone as a control, 1 h prior to light exposure.

### Terminal dUTP nick end-labelling of fragmented DNA

DNA strand breaks in photoreceptor nuclei were detected by Terminal dUTP Nick End-Labelling (TUNEL). Briefly, enucleated eyes were fixed in 10% buffered formalin for 24 h, and cryo-protected in 30% sucrose overnight. The eye was then bisected along the vertical

meridian through the optic nerve and the hemi-cups embedded in tissue freezing medium (Shandon, PA, USA). Cryosections (5 $\mu$m) were permeablized with 0.1% Triton X-100 (Sigma, UK) in PBS for 2 min on ice, followed by three 5 min washes in PBS. The sections were then incubated in 50 $\mu$l of reaction buffer containing 2.5 mM $CoCl_2$, 0.1 U/ml terminal deoxynucleotidyl transferase (TdT) in a 0.1 M Na cacodylate (pH 7.0) buffer and 0.75 nM fluorescein-12-dUTP (Boehringer Mannheim, Germany). These sections were incubated at 37°C for 1 h in a humidified chamber. Following several washes in PBS, the sections were mounted in mowiol (Calbiochem) and viewed under a fluorescence microscope (Nikon Eclipse E600) using a fluorescein isothiocyanate (FITC) filter. Three animals were used for each of the time points; 0, 6, 14 and 24 h after light exposure.

## YVAD-$\rho$NA and IETD-$\rho$NA cleavage assays

Enucleated eyes were placed in PBS and retinal dissection was carried out using a watchmaker's forceps. The choroid, sclera and pigmented epithelium were removed and the retina was then separated from the vitreous and lens and washed with cold PBS. Total protein was obtained by homogenising retinas in 50 $\mu$l of chilled lysis buffer containing 10 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 5 mM EGTA, 50 mM NaCl, 1 mM PMSF, 2 $\mu$g/ml aprotinin and 2 $\mu$g/ml leupeptin. The cells were incubated on ice for 20 min and then lysed by 3–4 cycles of freezing and thawing. Insoluble material was pelleted by centrifuging at 20 000 $\times g$ for 15 min at 4°C. The protein content of each sample was determined by the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK) using bovine serum albumin as a standard and 80 $\mu$g of protein in 50 $\mu$l of lysis buffer was dispensed into each well of a microtiter plate. An equal volume of 2x reaction buffer (50 mM HEPES pH 7.4, 0.2% 3-[(3-cholomidopropyl) dimethylammonio] propane-1- sulphonic acid [CHAPS], 20% glycerol, 2 mM EDTA and 10 mM dithiothreitol [DTT]) was added to each sample with 200 $\mu$M caspase-1 substrate-AcYVAD-$\rho$NA (Calbiochem) (10 mM stock in DMSO), or 200 $\mu$M caspase-8 substrate—AcIETD-$\rho$NA (Calbiochem) (10 mM stock in DMSO). Reactions were incubated at 37°C for 1 h and then cleavage of each substrate was monitored by liberation of the chromogenic $\rho$NA in a SpectraMax-340 plate reader (Molecular Devices, CA, USA) by measuring absorption at 405 nm.

## Western blot analysis

The retina was dissected and total protein was obtained by lysing in RIPA buffer (50 mM Tris-HCL pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovandate, 1 mM sodium fluoride) containing antipain (1 $\mu$g/ml), aprotinin (1 $\mu$g/ml), chymostatin (1 $\mu$g/ml), leupeptin (0.1 $\mu$g/ml), pepstatin (1 $\mu$g/ml) and PMSF (0.1 mM). The amount of total protein of each sample was determined by the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK) using bovine serum albumin as a standard. Sixty $\mu$g of total protein from each sample was electrophoresed on polyacrylamide gels followed by transfer to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and incubated overnight with the appropriate antibodies. Antibodies reactive to caspase-7, caspase-9, caspase-3, (Cell Signaling Technology, MA, USA), caspase-8 (Bioquote Ltd. North Yorkshire, UK), caspase-1 (Upstate Biotechnology), cytochrome *c* (PharMingen International, San Diego, CA, USA), Apaf-1 (Santa Cruz), and GAPDH (Advanced Immuno-Chemical, CA, USA) were used in this study. Membrane development was achieved using Enhanced Chemiluminescence (ECL) (Amersham, Buckinghamshire, UK).

## Subcellular fractionation

Cytosolic and mitochondrial fractions were prepared for detecting cytochrome *c* as follows: Briefly, for each sample two retina were dissected and resuspended in 50 $\mu$l of buffer (100 mM HEPES, 500 mM mannitol, 400 mM sucrose, 50 mM EGTA, 1% BSA, 1 mM DTT, 0.1 mM PMSF, 0.1 $\mu$g/ml leupeptin and 1 $\mu$g/ml aprotinin). The retina was then disrupted in a 2 ml glass dounce homogeniser with 25–30 strokes of a B-type pestle (Kontes Glass company, New Jersey). The resultant homogenate was centrifuged at 1000 $\times g$ for 5 min at 4°C to remove nuclei. Supernatants were centrifuged at 10 000 $\times g$ for 15 min at 4°C. The resulting supernatants were used as the cytosolic fraction for detecting cytochrome *c*. The pellet was washed three times in ice-cold PBS and used as a positive control for mitochondrial cytochrome *c*.

## Preparation of cell-free extracts and cell-free reactions

Cell-free extracts were prepared from Balb/c retina as follows: A total of six retinas for each sample were dissected and washed in PBS. The retinas were resuspended in 150 $\mu$l of cell extraction buffer (CEB) and transferred to a 2 ml dounce homogeniser (CEB; 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM Dithiothreitol, 100 $\mu$M PMSF, 10 $\mu$g/ml leupeptin, 2 $\mu$g/ml aprotinin). The retinas were then placed on ice for 20 min and flicked regularly prior to disruption with 25–30 strokes of a B-type pestle. Lysates were then transferred to Eppendorf tubes and were centrifuged at 14 000 r.p.m. for 15 min at 4°C. The supernatant was removed and the amount of total protein was determined by the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK) using bovine serum albumin as a standard. For cell-free reactions 50 $\mu$g of protein was brought to a final volume of 20 $\mu$l in CEB and apoptosis induced by addition of bovine heart cytochrome c (Sigma, UK) and dATP at final concentrations of 50 $\mu$g/ml and 1 mM respectively. Extracts were then incubated at 37°C for 1, 2 and 3 h to initiate apoptosis. At these time points extracts were removed and stored at $-70$°C for subsequent Western blot analysis.

## Measurement of calpain activity by a fluorogenicsubstrate assay

A fluorogenic-substrate assay was preformed with Suc-Leu-Tyr-AFC (Enzyme System Products, CA, USA), a membrane permeable, calpain-specific substrate. Briefly, retinas were dissected and washed with cold PBS. Total protein was obtained by homogenising retinas in 50 $\mu$l of chilled lysis buffer containing 10 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl, 1 mM PMSF, 2 $\mu$g/ml aprotinin and 2 $\mu$g/ml leupeptin. The cells were incubated on ice for 20 min and then lysed by 3–4 cycles of freezing and thawing. Insoluble material was pelleted by centrifuging at 20 000 $\times g$ for 15 min at 4°C. The protein content of each sample was determined by the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK) using bovine serum albumin as a standard and 100 $\mu$g of protein in 200 $\mu$l of reaction buffer (100 mM imidazole, pH 7.3, 5 mM L-cysteine, 1 mM mercaptoethanol, 10 mM $CaCl_2$ and 4% DMSO) was dispensed into each well of a microtiter plate. Suc-Leu-Tyr-AFC substrate (62.5 $\mu$M) was added to each well and reactions were incubated at 37°C for 30 min. As an additional control to ensure that fluorescence activity was calpain specific, the calpain inhibitor, calpeptin (100 $\mu$M) was added to one of the wells (24 h), 15 min prior to addition of the substrate. Calpain activity was detected by measuring the proteolytic cleavage of the fluorogenic substrate using a SpectraMax Gemini fluorometer (Molecular Devices, CA, USA)

with excitation and emission wavelengths of 400 nm and 505 nm respectively.

### Intracellular free calcium measurement

Intracellular calcium levels were determined using the intracellular calcium probe, fluo-3 AM (acetoxymethyl ester) (Molecular Probes, Leiden, The Netherlands). Cells were incubated in darkness with fluo-3 (250 nM), prepared from a 500 $\mu$M stock, for 15 min at 37°C and fluorescence measured in FL-1 (530 nm) on a Becton-Dickenson FACScan flow cytometer with excitation at 488 nm.

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