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# Photoreceptor rescue and toxicity induced by different calpain inhibitors

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**Abstract**

Photoreceptor degeneration is the hallmark of a group of inherited blinding diseases collectively termed retinitis pigmentosa (RP); a major cause of blindness in humans. RP is at present untreatable and the underlying neurodegenerative mechanisms are largely unknown, even though the genetic causes are often established. The activation of calpain-type proteases may play an important role in cell death in various neuronal tissues, including the retina. We therefore tested the efficacy of two different calpain inhibitors in preventing cell death in the retinal degeneration (*rd1*) human homologous mouse model for RP. Pharmacological inhibition of calpain activity in *rd1* organotypic retinal explants had ambiguous effects on photoreceptor viability. Calpain inhibitor XI had protective effects when applied for short periods of time (16 h) but demonstrated substantial levels of toxicity in both wild-type and *rd1* retina when used over several days. In contrast, the highly specific calpain inhibitor calpastatin peptide reduced photoreceptor cell death *in vitro* after both short and prolonged exposure, an effect that was also evident after *in vivo* application via intravitreal injection. These findings highlight the importance of calpain activation for photoreceptor cell death but also for photoreceptor survival and propose the use of highly specific calpain inhibitors to prevent or delay RP.

**Keywords:** apoptosis, calcium, CAST, CNX295, retina.

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Retinitis pigmentosa (RP) designates a group of genetic diseases causing photoreceptor cell death and blindness in humans. RP usually follows a two-stage process in which first the rod-type photoreceptors and then the cone-type photoreceptors degenerate (Kennan *et al.* 2005). In the developed world, RP is the prevalent cause of hereditary blindness in the working age population (Farrar *et al.* 2002). Although RP causing mutations have been identified in 36 genes to date (Retnet database: http://www.sph.uth.tmc.edu/retnet, information retrieved in May 2010), the mechanisms responsible for photoreceptor cell death are still largely unknown and currently no treatment is available (Sancho-Pelluz *et al.* 2008).

The retinal degeneration (*rd1*) mouse is a human homologous animal model for RP that allows investigations into the pathological mechanisms of hereditary photoreceptor cell death (Keeler 1924; Farber and Lolley 1974; Paquet-Durand *et al.* 2009), and is characterized by a loss-of-function mutation in the gene encoding for the ß-subunit of rod photoreceptor cGMP phosphodiesterase 6 (*Pde6b*) (Bowes *et al.* 1990). This causes cGMP to accumulate in *rd1*

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*Abbreviations used*: CAST, calpastatin peptide; CNG, cyclic nucleotide gated ion channel; CPI XI, calpain inhibitor XI; CRB, calpain reaction buffer; DIV, days *in vitro*; HDAC, histone deacetylase; ONL, outer nuclear layer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PKG, protein kinase G; PN, postnatal day; PTX, phosphate buffered saline containing 0.2% Triton X100; rd, retinal degeneration; RP, retinitis pigmentosa; TUNEL, terminal dUTP nick end labelling; wt, wild-type.

photoreceptors and eventually triggers cell death (Farber and Lolley 1974; Paquet-Durand *et al.* 2009), via pathways that are now starting to be revealed (e.g., Sanges *et al.* 2006; Sancho-Pelluz *et al.* 2008).

An important mechanistic finding relates to calpain-type proteases, which have been shown to be expressed and hyperactive in degenerating *rd1* photoreceptors (Paquet-Durand *et al.* 2006, 2007a). The calpains are a group of calcium ($Ca^{2+}$)-activated proteases with 14 known isoforms (Croall and Ersfeld 2007), that have been implicated in neurodegeneration in a number of different tissues (Leist and Jaattela 2001; Blomgren *et al.* 2007) including retinal ganglion cells (Araujo *et al.* 2004; Das *et al.* 2006; Oka *et al.* 2006) and photoreceptors (Gomez-Vicente *et al.* 2005; Paquet-Durand *et al.* 2006; Sanges *et al.* 2006). Throughout cellular life certain calpains are regulated by the highly specific, endogenous calpain inhibitor, calpastatin (Goll *et al.* 2003; Hood *et al.* 2004). The dissociation of the calpain-calpastatin complex allows for $Ca^{2+}$-dependent activation of the protease (Hood *et al.* 2004; Li *et al.* 2004; Suzuki *et al.* 2004). Over-expression of calpastatin has been associated with an increased resistance to injury and reduced neuronal cell death in neurodegenerative disease models (Wingrave *et al.* 2004; Higuchi *et al.* 2005). Similarly, in *rd1* mouse photoreceptors but also in other neurodegenerative diseases such as Alzheimer's, excessive activation of calpains and cell death correlates with a strong down-regulation of calpastatin (Paquet-Durand *et al.* 2006; Rao *et al.* 2008).

Together these findings offered two important hypotheses for RP research: (i) that uncontrolled calpain activity strongly contributes to photoreceptor cell death and (ii) that specific calpain inhibitors may prove useful for neuroprotective retinal therapy. We therefore tested different calpain inhibitors in *rd1* and wild-type (*wt*) retinae to further investigate the importance of calpain for photoreceptor cell death. Our results show that the low molecular weight calpain inhibitor XI (CPI XI) can have both beneficial and detrimental effects on photoreceptor viability, depending on whether it is applied by an acute or chronic treatment, respectively. On the other hand, the highly specific inhibitor calpastatin peptide (CAST) consistently exerted strong neuroprotective effects across different *in vitro* paradigms. Furthermore, CAST reduced *rd1* photoreceptor cell death *in vivo*, suggesting a role for certain calpain inhibitors in the development of novel RP therapies.

## Experimental procedures

### Animals

Animals were housed under standard white cyclic lighting, had free access to food and water, and were used irrespective of gender. C3H *rd1*/*rd1* (*rd1*) and control C3H *wt* mice (Sanyal and Bal 1973; Sanges *et al.* 2006) were used for *in vivo* and *in vitro* experiments. All procedures were performed in accordance with the Swedish National Animal Care and Ethics Committee (permits # M213/03, # M225/04, # M242/07), with the Italian Animal Care Ethics Committee (PROT. No. 6916/07), and with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and visual research. Because of the critical changes at postnatal day (PN) 11 (Hauck *et al.* 2006; Sancho-Pelluz *et al.* 2008), comparisons between *rd1* and *wt* were carried out at this age.

### Organotypic retinal explant culture

For *in vitro* experiments, PN5 animals were killed by decapitation and retinal explants were generated in principle as described previously (Caffe *et al.* 2001). In the present study, explants were allowed to adjust to culture conditions for 2 days *in vitro* (DIV) before being treated for another 4 days for what will be referred to here as chronic treatment, or for the last 16 h of the 6 day total incubation, which is hereafter addressed as acute treatment. The end point for the experiments thus corresponds to PN11 *in vivo* (5 + 2 + 4 days or 5 + 5 days + 16 h). In addition to these two different short-term treatments, a long-term treatment was used in which cultures where explanted at PN5, allowed to adjust to *in vitro* culture for 2 DIV, and then treated for another 12 DIV until PN19. Explants were maintained under serum-free conditions in R16 nutrient medium (Invitrogen, Paisley, Scotland), and the culture medium with or without treatment was changed every 2 days.

Two calpain inhibitors were used: CPI XI [Z-l-Abu-CONH $(CH2)_3$-morpholine; sometimes referred to as CX295; Calbiochem, San Diego, CA, USA] and CAST (Sigma, Stockholm, Sweden). Calpain inhibitor XI concentration ranged from 0.5 μM to 100 μM, whereas 20 μM was used for CAST treatment (Movsesyan *et al.* 2004). Controls for CPI XI treated retinae were exposed to equivalent amounts of vehicle (dimethylsulfoxide), whereas controls for water soluble CAST received regular R16 medium. Upon completion of each experiment, the preparations were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 h, followed by rinsing with PBS and cryoprotecting with 25% sucrose in PBS. Some preparations were left unfixed to enable the calpain activity assay (below).

### *In vivo* experiments

Because of the rapid progression and early onset of the *rd1* degeneration, intravitreal injections were performed at PN10, approximately 2 days before eye opening. Animals were anesthetized with an intraperitoneal injection of 2 mL/100 g body weight of avertin [1.25% (w/v) 2,2,2-tribromoethanol and 2.5% (v/v) 2-methyl-2-butanol; Sigma]. The eye lid was opened carefully, a small incision made below the *ora serrata* and 1 μL of a solution containing 100 μM CAST in PBS was injected into the eye. Sham-treated animals received only PBS. The capillary was maintained in the eye for approximately 3 s to avoid reflux. As the free intraocular volume of the mouse eye at this age is approximately 5 μL, the effective inhibitor concentration was estimated to be at 20 μM. Animals were killed between 4 h and 16 h (approx. PN11) after injection. The eyes were enucleated and immediately snap frozen on dry ice without fixation. The whole eyes were then embedded in Tissue Tek® (Sakura Finetek, Zoeterwoude, The Netherlands) and sectioned (12 μm) in a cryotome (HM560 Microm, Walldorf, Germany). The fresh, unfixed sections were then used for enzyme activity assays. A total of 12 animals were used for intravitreal injection.

### Calpain activity assay

Tissue sections from unfixed retinae were incubated for 15 min in calpain reaction buffer (CRB: 25 mM HEPES, 65 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 2 mM dithiothreitol, pH 7.2). The fluorescent calpain substrate CMAC, t-BOC-Leu-Met (A6520, Invitrogen, Carlsbad, CA, USA) was then added to CRB at a final concentration of 2 μM and incubated in the dark for 2 h at 37°C. The sections were washed twice for 10 min each in CRB and then mounted with Vectashield (Vector, Burlingame, CA, USA). The activity assay generally labeled the cell membranes, while calpain-activity-positive cells additionally showed a bright labeling of the nucleus and perinuclear cytoplasm.

### Terminal dUTP nick-end labeling

Sections were fixed in 4% PFA, and washed four times in PBS, then incubated for 1 h in 20% goat serum. Terminal dUTP nick-end labeling (TUNEL) staining was done using an *in situ* cell death detection kit (Roche, Mannheim, Germany) conjugated with tetramethylrhodamine red. Controls were performed by omitting the terminal deoxynucleotidyl transferase enzyme from the labelling solution (negative control) and in pre-treating the sections for 30 min with DNAse I (Roche, 3 U/mL) in 50 mM Tris–HCl, pH 7.5, 1 mg/mL bovine serum albumin to induce DNA strand breaks (positive control). The negative control gave no staining at all, while the positive control resulted in general staining of all nuclei in all layers of the retina (Paquet-Durand *et al.* 2007b).

### Staining protocol

Frozen tissue sections or PFA fixed retinae were stained for general histological light microscopic analysis with hematoxylin-eosin according to standard protocols.

Immunostaining was performed on fixed tissue sections. These were washed four times for 10 min each in PBS containing 0.2% Triton X100 (PTX). Blocking solution containing PTX and 5% normal serum from the host animal, from which the secondary antibody was obtained, was applied for 1 h. Primary mouse anti-rhodopsin antibody (MAB5316, Millipore, Solna, Sweden) was diluted (1 : 800) in PTX containing 2% normal serum and applied overnight at 4°C. Sections were then washed four times for 10 min each in PTX and incubated with secondary anti-mouse antibody labelled with Alexafluor 488 fluorescent dye (1 : 250, Invitrogen). After three more washing steps in PBS, the sections were mounted with Vectashield (Vector) or Glycergel (DAKO, Carpinteria, CA, USA). Controls consisted of sections processed in parallel without primary antibody and application of the fluorescence detection system.

### Microscopy, cell counting and statistics

Histological work was performed on 12 μm frozen sections. Morphological observations and routine light microscopy were performed on a Zeiss Axiophot (Zeiss, Jena, Germany) microscope equipped with a Zeiss Axiocam digital camera. Fluorescence excitation was provided by a HBO 100W halogen lamp. Images were captured using Zeiss Axiovision 4.2 software; image overlays and contrast enhancement were done using Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA, USA).

Percentages of both calpain activity and TUNEL-positive cells were assessed and calculated in a blinded fashion as reported previously (Paquet-Durand *et al.* 2006, 2009). For each animal the central areas (in proximity to the optic nerve) of at least three sections were quantified to yield an average value, and at least three different animals were analyzed for each time-point and genotype. The number of photoreceptor rows remaining after long-term *in vitro* culture (PN5–19) was counted manually on hematoxylin-eosin stained specimens. At least nine values from different sectioning levels in central areas of the retina were taken down and averaged for each explant, and these values were then used for further calculations and comparisons. Values are given as mean ± standard error of the mean (SEM), except for long-term *in vitro* treatment where lower *n*-values required the use of median instead of mean. In cases where single sample groups were compared to a control group, statistical significance was tested using unpaired, two-tailed, Student's *t*-test and Microsoft Excel Software. For multiple comparisons, statistical significance was tested using GraphPad Prism 4.01 Software (GraphPad Software, La Jolla, CA, USA) and employing one-way ANOVA followed by Bonferroni's multiple comparison post-test. Significance levels were: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

## Results

### Effect of calpain inhibitor XI on photoreceptor viability varies by treatment

We had previously established that, when included in the calpain assay system, CPI XI potently reduced the calpain activity that could be visualized in *rd1* photoreceptors *ex vivo* (Paquet-Durand *et al.* 2006). We therefore tested the efficacy of this inhibitor to rescue *rd1* photoreceptors in an *in vitro* organotypic retinal explant system. Two different treatment paradigms were utilized: chronic and acute, as described in Experimental Procedures (PN5 + 2 + 4 DIV and PN5 + 5 DIV + 16 h *in vitro*, respectively). The TUNEL assay for dying cells identified a significant, and concentration dependent, difference between the outcomes of the chronic and acute treatments (Fig. 1). As CPI XI concentration increased, the chronically-treated retinae showed more TUNEL-positive cells, particularly in the outer nuclear layer (ONL), but opposite to this, in acutely-treated retinae, their number decreased (Fig. 1a, e; b, f). Thus, at the highest CPI XI concentration (100 μM), chronic treatment resulted in a 195% increase in TUNEL-positivity in the ONL (8.3 ± 0.8% SEM, $n = 4$, $p < 0.001$) compared to vehicle treated (dimethylsulfoxide) *rd1* retinae (4.3 ± 0.2%, $n = 14$), while after acute treatment the ONL of retinae exhibited 43% less TUNEL-positive cells (CPI XI treated: 2.5 ± 0.4%, $n = 4$; control: 4.4 ± 0.3%, $n = 12$, $p < 0.01$; Fig. 1i). The discrepancy between the effects of chronic and acute treatment was even more obvious when the percentage of TUNEL-positive cells was plotted against CPI XI concentration, and a linear regression fitted through the data points (Fig. 1j).

Wild-type retinae responded to CPI XI treatment in similar ways (Fig. 1c, g; d, h). A comparatively low number of TUNEL-positive cells were seen in the vehicle treated

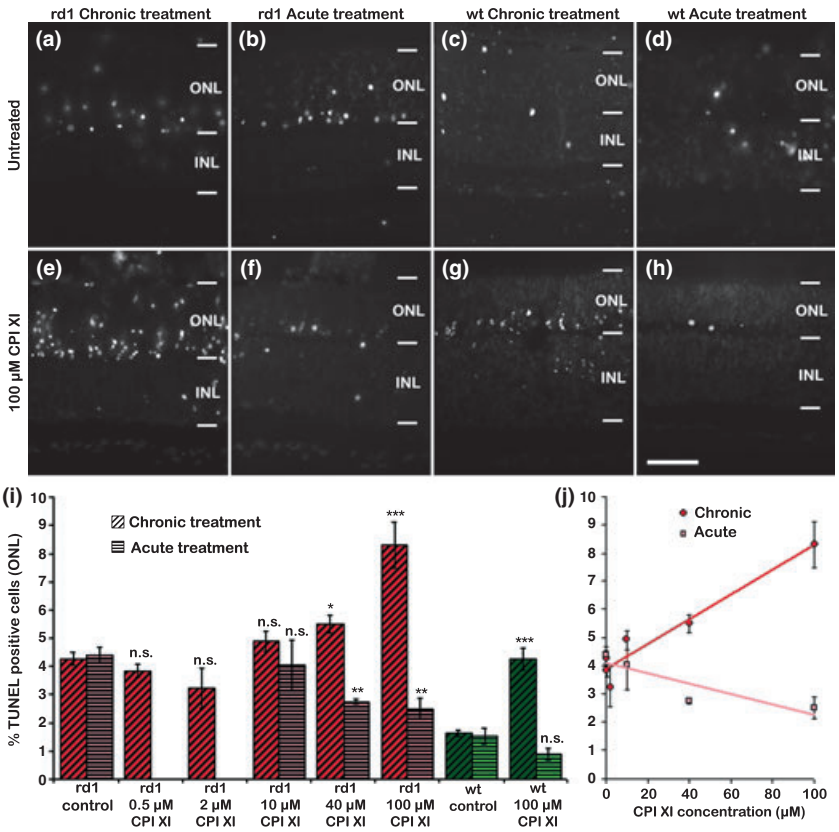


**Fig. 1** Acute CPI XI treatment preserves photoreceptors while chronic treatment induces cell death. The percentages of TUNEL-positive cells in the ONL of *rd1* and *wt* retinae illustrate the drastic differences induced by 96 h chronic (a, e; c, g) and 16 h acute (b, f; d, h) inhibition. The quantification of this effect for different concentrations of CPI XI in *rd1 and wt* retinae (i) showed that as inhibitor concentration increased, chronic treatment caused more cell death, whereas photoreceptors were dose-dependently rescued after acute treatment. This strong discrepancy was also exemplified by linear regression analysis for *rd1* photoreceptor cell death after acute and chronic treatment (j). Images shown are representative for the central area of retinal explant cultures. For each time-point retinae from at least four different *wt* or *rd1* animals were analyzed. Error bars represent SEM, scale bar in (h) = 100 µm. Significance levels were: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

ONL (1.6 ± 0.1%, $n = 8$), representing a low level of photoreceptor degeneration caused by the culture procedure. However, the levels of cell death were significantly increased by chronic CPI XI treatment (4.3 ± 0.4%, $n = 10$, $p < 0.001$), while acute treatment led to a numerical reduction of TUNEL-positive cells in the ONL (CPI XI treated: 0.9 ± 0.2%, $n = 6$; control: 1.5 ± 0.3%, $n = 4$, $p = 0.08$; Fig. 1i).

## Calpastatin peptide protects *rd1* photoreceptors *in vitro*

The results of the chronic CPI XI treatment prompted the need to look for a different, less toxic calpain inhibitor. When calpastatin peptide (CAST), at the concentration of 20 µM, replaced CPI XI under the identical chronic treatment, the percentage of TUNEL-positive cells in the *rd1* ONL drastically diminished from 3.9% (± 0.4 SEM, $n = 6$) in untreated preparations to 1.8% (± 0.2, $n = 12$, $p < 0.001$) in CAST treated specimens. This was statistically equivalent to *wt* control (1.6 ± 0.1%, $n = 8$) and correlated with a statistically significant ($p < 0.001$) decrease of calpain activity-positive cells in the ONL (Fig. 2). Beyond the ONL, cell death labeling in the inner nuclear layer (INL) and ganglion cell layer of CAST-treated *rd1* and *wt* retinae did not identify detrimental effects of CAST treatment on cultured tissue.

As judged by TUNEL analyses, CAST had a neuroprotective effect and CPI XI a photoreceptor toxic effect in the chronic treatment, in cultures ending at a time-point corresponding to PN11. However, at this time-point the *rd1* degeneration had not yet led to an obvious decrease in the number of ONL cell rows, and by an experiment of this kind it was thus not possible to conclude that such treatments affect the long-term survival of photoreceptors. To study this, we prolonged the *in vitro* treatment to 14 DIV until a time-point corresponding to PN19 (PN5 + 2 + 12). Under these conditions, control *wt* retinae displayed 9.3 rows of photoreceptors in the ONL (± 0.3 SEM, $n = 3$; Fig. 3a) which was significantly decreased by CPI XI (40 µM) treatment to 6.8 (± 0.7, $n = 3$, $p < 0.05$; Fig. 3b). Vehicle treated degenerating *rd1* retinae exhibited 3.3 (± 0.3, $n = 9$; Fig. 3c) rows of photoreceptors at PN19. This was further reduced by CPI XI treatment (2.8 ± 0.2, $n = 6$; Fig. 3d), although this difference did not attain statistical significance. In clear opposition to this, CAST (20 µM) treatment significantly increased the number of surviving *rd1* photoreceptor rows by almost 70% (CAST: 5.5 ± 0.9, $n = 3$, $p < 0.05$; Fig. 3e, quantification in 3f).

To obtain a further index on the status of the photoreceptors, the expression of rhodopsin was analyzed (Fig. 3g–k). Healthy, *wt* retinae *in vivo* show rhodopsin expression restricted to the outer segments of rod photoreceptors (Fig. 3g), while *rd1* retinae, where outer segments do not develop properly, present with a partial mislocalization in the cytoplasm of photoreceptors, that is, staining is here readily

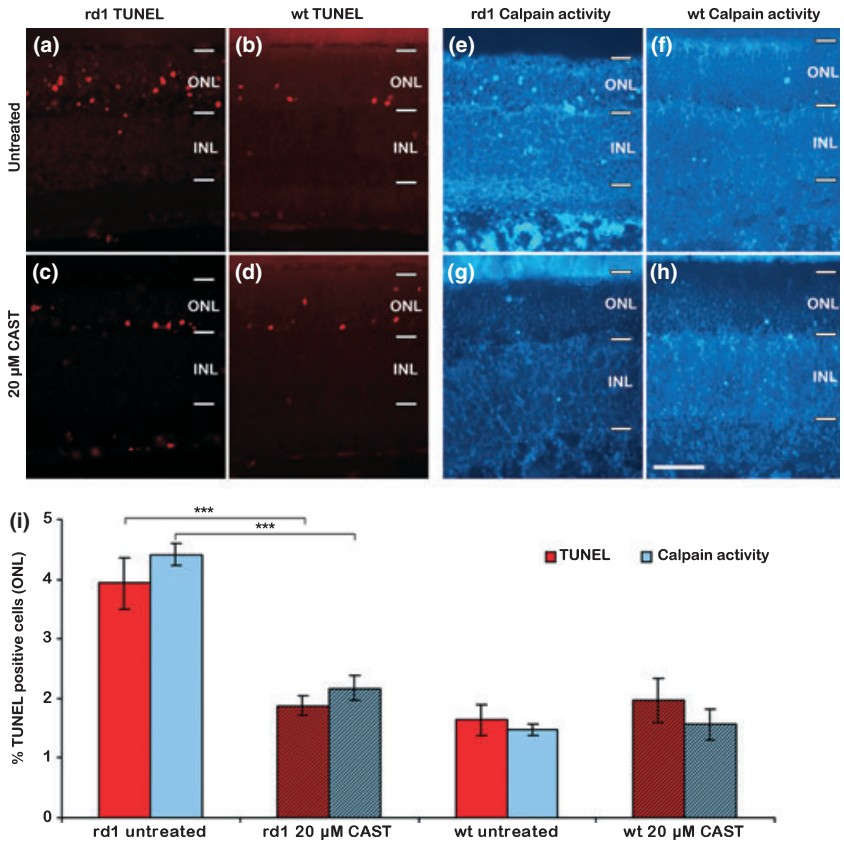


**Fig. 2** Calpastatin peptide reduces calpain activity and photoreceptor cell death *in vitro*: treatment of cultured retinal explants from PN7 to PN11 with 20 μM CAST resulted in a significant decrease in the percentage of TUNEL-positive cells (red bars in i) in the *rd1* ONL (a, c), but not in *wt* (b, d). Similarly, the number of ONL cells showing high calpain activity (blue bars in i) was decreased by CAST (e, g). *wt* ONL was not affected by the treatment (f, h). The quantification (i) is based on cell counts obtained from at least three different *wt* or *rd1* retinae. Error bars represent SEM. Images shown are representative for the central area of retinal explant cultures, bright staining sometimes visible in the upper part of an image relates to the culture membrane. Scale bar in (h) = 100 μm. Significance level was: ***$p < 0.001$.

seen in the ONL (Fig. 3i) (Bowes *et al.* 1988; Sancho-Pelluz *et al.* 2010). In degenerating *rd1* retinae cultured until PN19 the size of rod outer segments and rhodopsin immunofluorescence was strongly reduced when compared to *wt* (Fig. 3g, i). In *wt* retinae, CPI XI appeared to increase rhodopsin immunoreactivity in the ONL (Fig. 3g, h). In *rd1* explants after long-term CAST treatment, survival of photoreceptors was increased as judged by the number of ONL cell rows (Fig. 3f) with most ONL cells expressing rhodopsin (Fig. 3i, k) thus confirming rod photoreceptor identity. In the untreated *rd1* control, however, many of the remaining ONL cells were rhodopsin-negative and hence most likely cone photoreceptors. Still, we cannot exclude the possibility that among the rhodopsin-negative cells, in the untreated *rd1* situation, there might have been occasional rods which had lost rhodopsin expression.

The use of the rhodopsin staining also illustrated a remarkable photoreceptor neurite outgrowth into the inner nuclear layer of the untreated *rd1* specimens (20.9 ± 2.4 neurites/mm) when compared to *wt* retinae (5.0 ± 0.6 neurites/mm), which resembled what has been reported previously for degenerating human rod photoreceptors (Li *et al.* 1995). However, such rod neurite sprouting was strongly reduced in CPI XI treated *rd1* retinae (11.2 ± 3.0 neurites/mm) but not affected by CAST treatment (24.3 ± 2.9 neurites/mm), suggesting a potential dependency of this process on the activity of only certain types of calpains (Fig. 3i–l).

Taken together, CAST treatment in both short- and long-term retinal culture demonstrated a strong pro-survival effect, suggesting a major contribution of calpastatin targets to mutation induced *rd1* photoreceptor death. At the same time, chronic application of CPI XI clearly had detrimental effects on photoreceptors, while the inner retina appeared to be less, or not at all, affected.

### Inhibition of calpain reduces calpain activity and cell death *in vivo*

To explore the outcome of calpain inhibition *in vivo* in *rd1* mice, CAST was injected into the vitreous body of the eye. In the first series of experiments, CAST was injected at PN10 and the percentage of dying photoreceptors was assessed using the TUNEL assay at different time-points post-injection. When compared to untreated specimens (Fig. 4a), at 4 h post-injection there was no significant effect, but at 8 h and 16 h (Fig. 4b) post-injection a strong and significant decrease in the number of TUNEL-positive cells was observed (Fig. 4c). At 48 h an obvious difference between treated and untreated specimens could no longer be noted, presumably because of drug clearance and/or degradation. As the damage to the early post-natal mouse eye caused by intravitreal injections is substantial, repeated

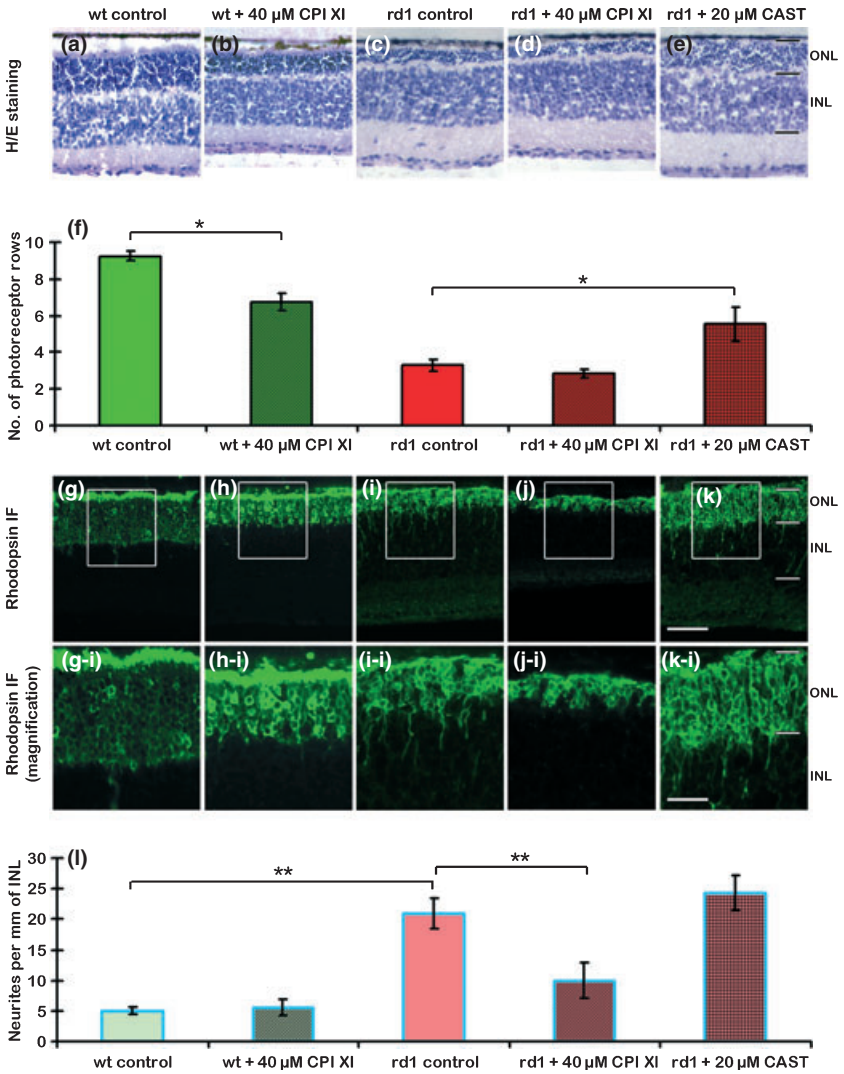


**Fig. 3** Effects of long-term calpain inhibitor treatment on photoreceptor survival: retinal explants from *wt* and *rd1* were cultured for 14 days *in vitro* (PN5 + 2 + 12) and exposed to either CPI XI or CAST. H/E staining revealed a significant decrease in the number of surviving photoreceptor rows when *wt* retina was treated with 40 μM CPI XI (a, b), suggesting photoreceptor toxicity (quantification of cell rows given in f). Degenerating *rd1* retina (c) was not significantly affected by CPI XI (d) but showed a considerable and significant increase in photoreceptor survival when treated with CAST (e). In cultured *wt* retina, immunofluorescence showed typical distribution of rhodopsin in rod outer segments with ONL cells occasionally showing accumulation of rhodopsin in the cytoplasm (g). A magnification of the area surrounded by a white rectangle is shown in (g-i). CPI XI treated *wt* retina showed a marked increase in rhodopsin immunoreactivity in the ONL (h, h-i). In cultured *rd1* retina, rod outer segments and rhodopsin immunofluorescence were strongly decreased, while there was unusual photoreceptor neurite outgrowth into the INL (i, i-i). The latter phenomenon was not observed in CPI XI treated *rd1* retina (j, j-i). CAST treatment did not alter the neurite outgrowth phenotype but increased photoreceptor survival, without apparent preservation of rod outer segments (k, k-i). A quantification of the calpain inhibition effect on photoreceptor neurite outgrowth is given (l). Images shown are representative for the central area of retinal explant cultures. For each time-point at least three different *wt* or *rd1* retinae were analysed. Error bars represent SEM, scale bars in k = 100 μm, k-i = 50 μm. Significance levels were: $^{*}p < 0.05$, $^{**}p < 0.01$.

injection traumas would result in severely increased retinal cell death. The considerable delay in the neuroprotective effects of CAST treatment *in vivo* could be because of the fact that at the time of treatment a certain number of cells were already compromised to an extent that did not allow rescue anymore.

As the beneficial effect of the treatment was most pronounced at 16 h post-injection, in a second set of experiments the effects of CAST treatment on calpain activity and TUNEL assay were assessed using this treatment-analysis interval, which hence ends the test at an age roughly corresponding to PN11. When compared to untreated or

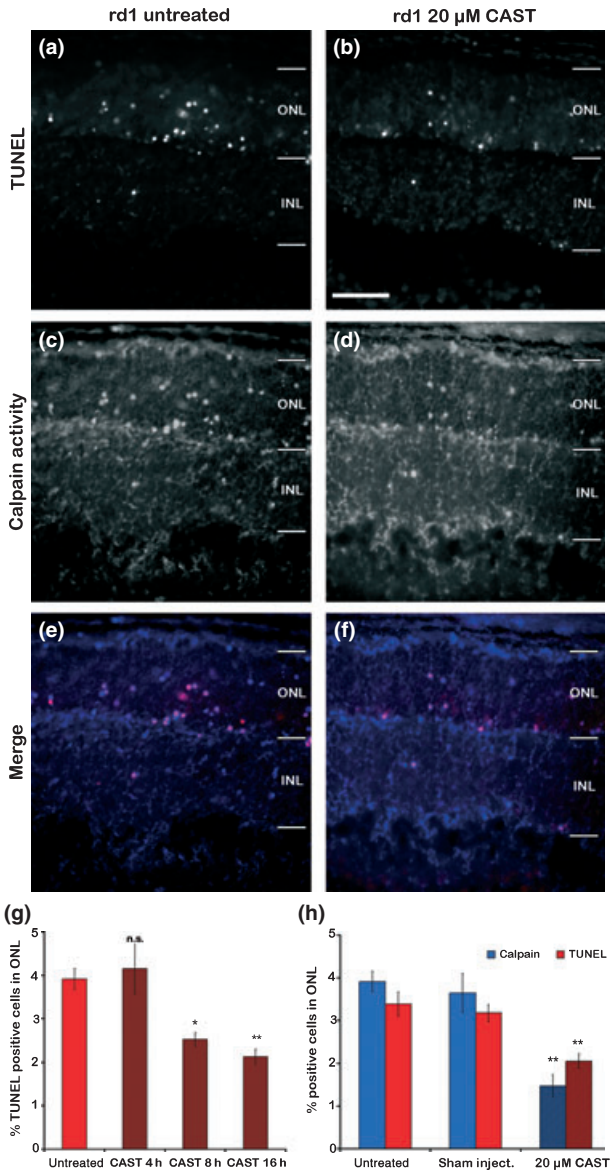


**Fig. 4** CAST treatment reduces calpain activity and cell death *in vivo*: CAST (20 µM) was injected into the vitreous body at PN10. When compared to untreated *rd1* retina, 16 h following CAST treatment the number of cells positive for cell death (TUNEL assay; a, b) and calpain activity (c, d) was strongly reduced. The merged image (e, f) illustrates the high degree of colocalization between TUNEL (red) and calpain activity (blue) positive cells. The protective effect of CAST treatment was maximal at 16 h post-injection (g). At this time-point, CAST decreased the number of cells positive for calpain (blue bars) and cell death (red bars) significantly (h). For each time-point at least three different *rd1* animals were analyzed, error bars represent SEM, scale bar in (b) = 100 µm. Significance levels were: $^{*}p < 0.05$, $^{**}p < 0.01$.

sham-treated specimens, CAST injection *in vivo* led to a clear decrease in the number of cells positive for either calpain activity (*rd1* CAST: 1.5 ± 0.3%, $n = 3$; *rd1* sham: 3.6 ± 0.5%, $n = 4$, $p < 0.01$) or for TUNEL (*rd1* CAST: 2.1 ± 0.2%, $n = 3$; *rd1* sham: 3.2 ± 0.2%, $n = 4$, $p < 0.01$).

The reduction in the percentage of dying, TUNEL-positive cells in the *rd1* ONL *in vivo* corresponds to the previous *in vitro* observations and thus confirms the neuroprotective effects of CAST treatment.

## Discussion

Calpains have been widely addressed as key agents in processes of degeneration, especially in the CNS (Movsesyan *et al.* 2004; Paquet-Durand *et al.* 2007a; Vosler *et al.* 2008). The present study supports these notions and exhibits the therapeutic potential of calpain inhibition in photoreceptor degeneration. Our work further underlines the complexity of the calpain system, as the data also demonstrate that certain calpain isoforms or related proteases may be necessary components for photoreceptor survival.

### Mechanisms of photoreceptor cell death and activation of calpains

The mutation in the murine homologue of the phosphodiesterase-6, *PDE6B* gene causes excessive accumulation of cGMP in the *rd1* mouse model for RP (Farber and Lolley 1974; Paquet-Durand *et al.* 2009). The cGMP targets include cGMP-dependent protein kinase G (PKG), which is activated in *rd1* photoreceptors, and cGMP activated cyclic nucleotide gated (CNG) ion channels (Pilz and Broderick 2005). While CNG channels may allow for increased $Ca^{2+}$-influx from extracellular sources (Frasson *et al.* 1999), PKG dependent phosphorylation of inositol 1,4,5-trisphosphate receptors could lead to $Ca^{2+}$ release from intracellular stores (Wagner *et al.* 2003). In either case, elevated cytosolic $Ca^{2+}$ levels would permit activation of calpains. As PKG is 100-fold more sensitive to cGMP than are CNG channels (Lincoln and Cornwell 1993), rising cGMP levels might induce a biphasic increase in cytosolic $Ca^{2+}$, first by PKG-dependent mobilization of $Ca^{2+}$ from intracellular stores and then by CNG channel mediated extracellular $Ca^{2+}$ influx. Interestingly, a cGMP-PKG-dependent activation of calpains was demonstrated in osteoclasts (Yaroslavskiy *et al.* 2007), and this may happen also in *rd1* photoreceptors, as the increase in PKG activity in these cells (Paquet-Durand *et al.* 2009) is concomitant with calpain activation (Paquet-Durand *et al.* 2006).

In addition, we have recently shown that *rd1* photoreceptor degeneration involves high activity of poly-ADP-ribose-polymerase (Paquet-Durand *et al.* 2007b), which may have a bearing on $Ca^{2+}$ homeostasis and cause activation of calpains (Vosler *et al.* 2009). Rising intracellular $Ca^{2+}$ levels may cause activation of histone deacetylases (HDACs) (Qiu and Ghosh 2008), which was indeed observed in *rd1* photoreceptors (Sancho-Pelluz *et al.* 2010). HDAC activation strongly impacts gene transcription (Gallinari *et al.* 2007), and correlates with down-regulation of neuroprotective genes

such as cyclic AMP response element binding protein and calpastatin (Kitagawa 2007) in the degenerating *rd1* retina (Azadi *et al.* 2006; Paquet-Durand *et al.* 2006). Down-regulation of calpastatin in turn facilitates activation of calpains. Together, cGMP-dependent activation of PKG, CNG channels, poly-ADP-ribose-polymerase and HDAC might therefore constitute an auto-feedback loop that, once triggered, culminates in excessive activation of calpains and eventual cell death.

### Calpastatin peptide and calpain inhibitor XI

The effect of calpain inhibitors on photoreceptor cell death in the *rd1* mouse was approached using both *in vitro* and *in vivo* techniques. For the *in vitro* studies, an organotypic retinal explant culture system (Caffe *et al.* 2001) was used with both acute and chronic treatment schedules, and revealed that the outcome on photoreceptor viability depended on both the inhibitor and the treatment paradigm used.

At PN11, under acute (16 h) application used here, CPI XI promoted photoreceptor survival in a concentration dependent manner. However, chronic inhibition (4 days), which was six times longer in treatment duration, resulted in the complete opposite effect, causing more cells to undergo cell death than in untreated explants, again in a concentration dependent fashion. The detrimental effect by CPI XI was further corroborated in long-term, PN19 cultures of *wt* retinae. The opposing results from acute and chronic inhibition suggest that, apart from being able to induce photoreceptor degeneration, calpain activity may simultaneously be required for normal functioning of photoreceptors in both *rd1* and *wt* retinae.

In contrast to CPI XI, CAST was found to counteract photoreceptor death in all *in vitro* and *in vivo* paradigms tested and thus served a neuroprotective role regardless of treatment design. The difference between the outcomes from the two inhibitors could originate in the manner of inhibition. The dipeptidyl-alpha-ketoimide CPI XI (CX295) inhibits the active site of calpains (Donkor 2000) and is hence predicted to act on all known calpain isoforms. In addition, it may also weakly inhibit cathepsin B (Blomgren *et al.* 2001). On the other hand, the peptidic calpain inhibitor CAST is based on the sequence of the endogenous calpain inhibitor calpastatin (Maki *et al.* 1989) and has thus a different operation mode, as it prevents the autolytic cleavage that leads to calpain activation (Goll *et al.* 2003). CAST is generally regarded to be the most specific inhibitor of calpains, and displays high selectivity for calpain isoforms associated with the small calpain subunit (calpain 4, CAPN4), which include the two main isoforms calpain 1 and 2 (μ- and m-calpain, respectively) as well as calpain 9 (Suzuki *et al.* 2004). This distinction in selectivity and mechanisms may be at the root of our differing results in the sense that the photoreceptors may, for their survival, rely on the continuous activity of calpain isoforms that can be inhibited by CPI XI, but not by CAST. With prolonged time of inhibition by CPI XI, a pro-survival effect would then be outbalanced by the detrimental loss of the activities of some calpains. Alternatively, CPI XI may have unwanted side-effects such as inhibition of other proteases like cathepsins or the proteasome, which again with time might render photoreceptor survival impossible. While both inhibitors may score similarly well in inhibiting calpains in cell-free or other test systems, our results from a complex neuronal tissue thus strongly indicate vital differences in their respective mode of action and/or specificity. Another aspect worth considering is that we previously reported a reduction in calpastatin mRNA in the retina of the *rd1* mouse (Paquet-Durand *et al.* 2006). As CAST in essence mimics calpastatin, its addition to the *rd1* retina may be seen as a way of counteracting the calpastatin discrepancy, which again fits with a better protective effect than when inhibiting calpains in general. The fact that the CAST effect was lost relatively soon after *in vivo* injection, could be because of metabolism or to increased demand after the time of eye opening (PN12–13). Additional *in vivo* studies with improved delivery methods will be needed to further establish neuroprotection mediated by calpastatin or structurally related inhibitors. Nevertheless, the results obtained with CAST treatment underline the benefits of calpain inhibition during photoreceptor degeneration, provided the right conditions are applied.

### Calpain activity in photoreceptor physiology

High intracellular $Ca^{2+}$-concentrations are known to occur in many degenerative processes and may lead to an excessive activation of calpains, which in turn may cause cell death (Suzuki *et al.* 2004; Vosler *et al.* 2008). By comparison, the functions of calpains under physiological $Ca^{2+}$-concentrations are less understood, although reports from a range of studies suggest involvement in several and diverse processes (Croall and Ersfeld 2007). An extended absence of CPI XI sensitive calpain activities – even if this is only partial, as with lower inhibitor concentrations here – could thus lead to a cumulative disruption of calpain-dependent cellular activities. As photoreceptors have one of the highest protein turn-over rates of any cell type (Young 1976), they may be particularly vulnerable to inhibition of proteolysis and improper disposal of surplus proteins. Interestingly, calpains of the types Lp82/85, which appear to be insensitive to inhibition by calpastatin (Goll *et al.* 2003), are found to play a role in rodent eye lens maturation (Ma *et al.* 2000). We have reported the presence of Lp82/85 calpain in the mouse retina (Paquet-Durand *et al.* 2006), and these might therefore be amongst the calpains inhibited by CPI XI but not by calpastatin. Furthermore, calpains have been implicated in proteolysis of arrestin in photoreceptors (Azarian *et al.* 1995) and

prolonged deactivation of the phototransduction cascade. CPI XI seemingly induced an increase in rhodopsin immunofluorescence in the *wt* ONL. Although this may simply reflect general protein accumulation in photoreceptors, it would also be compatible with an involvement of calpains in the hydrolysis and/or trafficking of rhodopsin. Dysregulated arrestin breakdown, alone or in combination with effects on rhodopsin, could thus promote degeneration of photoreceptors in ways similar to those causing retinal degeneration via abnormal rhodopsin/arrestin complexes (Chuang *et al.* 2004; Chen *et al.* 2006). Independent of the exact cause for the observed effects, the non-photoreceptor cells of the retina appeared to be much more tolerant to calpain inhibition.

Neurite sprouting in retinal degeneration has been found also in the human retina (Li *et al.* 1995), and may be linked to phenomena associated to degeneration in general, such as the presence of activated Müller cells, which are known to produce factors that could act stimulatory on various neuronal processes (Bringmann *et al.* 2006). In addition to such a mechanism, the excessive neurite sprouting in *rd1* photoreceptors may be boosted by their high levels of cGMP (Paquet-Durand *et al.* 2009), as a role of cGMP-PKG signalling in neurite outgrowth has been described (Ditlevsen *et al.* 2007). Interestingly, the sprouting effect was blocked by CPI XI, while it was unaffected after CAST treatment. Apart from the possibility that this result could be secondary to effects on, for instance, calpain sensitive Müller glia events, calpains are also known to regulate filopodial motility, neurite outgrowth (Robles *et al.* 2003), cell migration and adhesion (Glading *et al.* 2004; Franco and Huttenlocher 2005), which may all be relevant in this context. Although the mechanisms of neurite outgrowth are outside of the scope of this report, the results again demonstrate the different properties of the two inhibitors and emphasize the importance of studying them in parallel.

## Conclusion

While numerous studies have suggested calpain inhibition as a potential approach to prevent neurodegeneration in many different experimental systems, including the retina, our study for the first time provides data that calpain inhibition may have not only beneficial but also detrimental effects. The results bring about important considerations for the implementation of calpain inhibitors as therapeutic agents for RP and highlight the need to more precisely define calpain substrates. Nevertheless, the strong protective effect of CAST both *in vitro* and *in vivo* suggests that restriction of calpain inhibition to specific calpains is a feasible approach for the treatment of inherited retinal degeneration. In the future, carefully targeted and highly selective calpain inhibitors might further improve this.

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