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Caspase-3 inhibitor reduces apototic photoreceptor cell death during inherited retinal degeneration in *tubby* mice

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Purpose: The *tubby* mouse, previously suggested as an animal model for the human Usher Syndrome type I, was used in an analysis of pathophysiological processes leading to the inherited retinal degeneration, also shown in Usher syndrome patients. To evaluate pathogenic mechanisms causing retinal degeneration in *tubby* mice, we examined the time course of apoptotic photoreceptor cell death. Apoptotic pathways were determined by the inhibition of specific caspases in vivo.
Methods: Apoptotic cells were identified during retinal differentiation and degeneration by the TUNEL-method. Apoptotic events were confirmed by DNA-laddering. Intravitreal injection of apoptosis inhibitors was applied to reduce apoptotic photoreceptor cell death in *tubby* mice.
Results: During retinal differentiation there is no apparent difference between *tubby* and wild type mice in apoptotic events. Between post natal day 16 and 23, apoptosis was detected in the outer nuclear layer of *tubby* mice retinas, but was absent in control mice. The number of TUNEL-labeled photoreceptor cells peaked at post natal day 19. After this peak of apoptosis, the number of apoptotic photoreceptor cells gradually decreased. While a caspase-1 inhibitor did not reduce the number of apoptotic cells, a specific caspase-3 inhibitor caused a significant decrease of apoptotic photoreceptor cells in the *tubby* mouse retina.
Conclusions: Apoptosis is necessary for appropriate differentiation of the retina of *tubby* and wild type mice. In the fully developed tubby mouse retina, apoptotic photoreceptor cell death leads to retinal degeneration. Apoptosis in the *tubby* mouse retina is mediated by specific activation of members of the caspase-3 family. Caspase-3 inhibition drastically reduces photoreceptor cell death in the degenerating *tubby* mouse retina and may be a potential tool for therapeutic strategies of retinal degeneration in human Usher patients.

Inherited retinal degenerations are the major causes of hereditary blindness [1]. Retinal degeneration (rd) diseases are characterized by progressive reduction of the number of retinal photoreceptor cells, leading to vision loss. To date, more than 100 genes coding for proteins of retinal degeneration have been identified that can be divided into three groups: (1) proteins of the phototransduction cascade (e.g., rhodopsin, transducin, cyclic GMP-phosphodiesterase), (2) structure and metabolism proteins (e.g., peripherin, ROM1) and (3) proteins of the retinal pigment epithelium (e.g., RPE65). The defective genes causing retinal degeneration are listed in the OMIM (Online Medline Inheritance in Man) database. Nevertheless, the pathophysiological mechanisms leading to photoreceptor loss are not completely understood. In recent years new molecular techniques and test systems in the field of ophthalmologic research have identified programmed cell death, or apoptosis, as the final common pathway of the degradation of the retina [2,3]. To investigate inherited retinal degeneration, we used the rd5/rd5 or *tubby* mouse strain. The *tubby* mouse is a known animal model that exhibits retinal degeneration and was proposed by Heckenlively et al. as a model system for human Usher syndrome type 1 [4-6]. The Usher syndrome is the most common hereditary form of combined deafness and blindness in humans [7]. So far, three different Usher animal models (shaker-1, waltzer, ames waltzer) have been investigated, however their retinas do not degenerate [8-10]. In contrast, homozygous *tubby* mice exhibit progressive hearing loss through degeneration of their organ of Corti and also progressive retinal degeneration, characterized by a loss of photoreceptor cells [11]. Both types of sensoric degeneration result, at about 6 months, in complete deafness and blindness [5,6]. The mutation in the *tubby* gene is a G-to-T transversion that abolishes the donor splice site in exon 11, resulting in an aberrant transcript. Translation of the intron sequence results in the substitution of 44 amino acids at the C-terminal end of the tubby (TUB) protein with 24 different amino acids encoded by the intron [12]. Recent studies indicated that the TUB-protein is expressed in the ganglion cells and photorecptor cells of the mammalian retina [13]. Nevertheless, the cellular function of the *tubby* protein (TUB) remains elusive. It has been suggested that TUB functions as a membrane bound transcription regulator [14]. Other proposed molecular functions of the *tubby* protein are as a facilitator of rhodopsin transport in rod photoreceptors, or as a mediator of insulin signaling, respectively [15,16].

The molecular mechanism leading to apoptosis include as key events the activation of specific cellular proteinases, named caspases (**c**ysteinyl **asp**artate-specific protein**ases**) [17]. Caspases are synthesized as inactive proenzymes which are proteolyticly cleaved into active enzymes. This activation of

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caspases is mediated either through membrane bound receptors or through changes of the mitochondrial membrane integrity in a stimulus or tissue specific manner [18]. The hierarchy of caspases (initiator caspase-2, -8, -9) leads then to sequential proteolytic activation downstream, producing the caspases (caspase-3, -6, -7) [19]. In this group, caspase-3 is one of the main executioners of apoptosis, cleaving key apoptotic proteins such as poly(ADP-ribose) polymerase (PARP), amyloid precursor protein, lamin B, fodrin and gelsolin [17,20,21].

In this study, the apoptotic cell death in *tubby* mouse retinas, compared to control mouse retinas, were analyzed by the TUNEL-method (TUNEL: **T**dT-mediated X-d**U**TP **n**ick **e**nd **l**abeling) during retinal differentiation and degeneration. Our results show that regulatory apoptosis occurs during differentiation of *tubby* and wild type retinas which may indicate that the tubby mouse retina correctly develops. In contrast, in mature retinas, apoptosis was restricted to photoreceptor cells of *tubby* mice showing a peak of apoptosis at post natal day (PN) 19. Application of caspase inhibitors revealed that photoreceptor cell death is mediated by caspase-3. These results provide insight into therapeutic treatments by specific inhibition of apoptosis and thus for retinal degeneration in USH patients.

METHODS

Animals and Genotype Analysis: All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. For all eye preparations, animals were killed by CO_2 inhalation. The different mouse strains were all maintained on a 12-h light / dark schedule. Wild type mice (C57BL/6J) and *tubby* mice (C57BL/6J-tub Hbb^{S}/+Hbb^{P}) were purchased from the Jackson Laboratory (Bar Harbor, ME). For genotyping analysis of *tubby* mice,

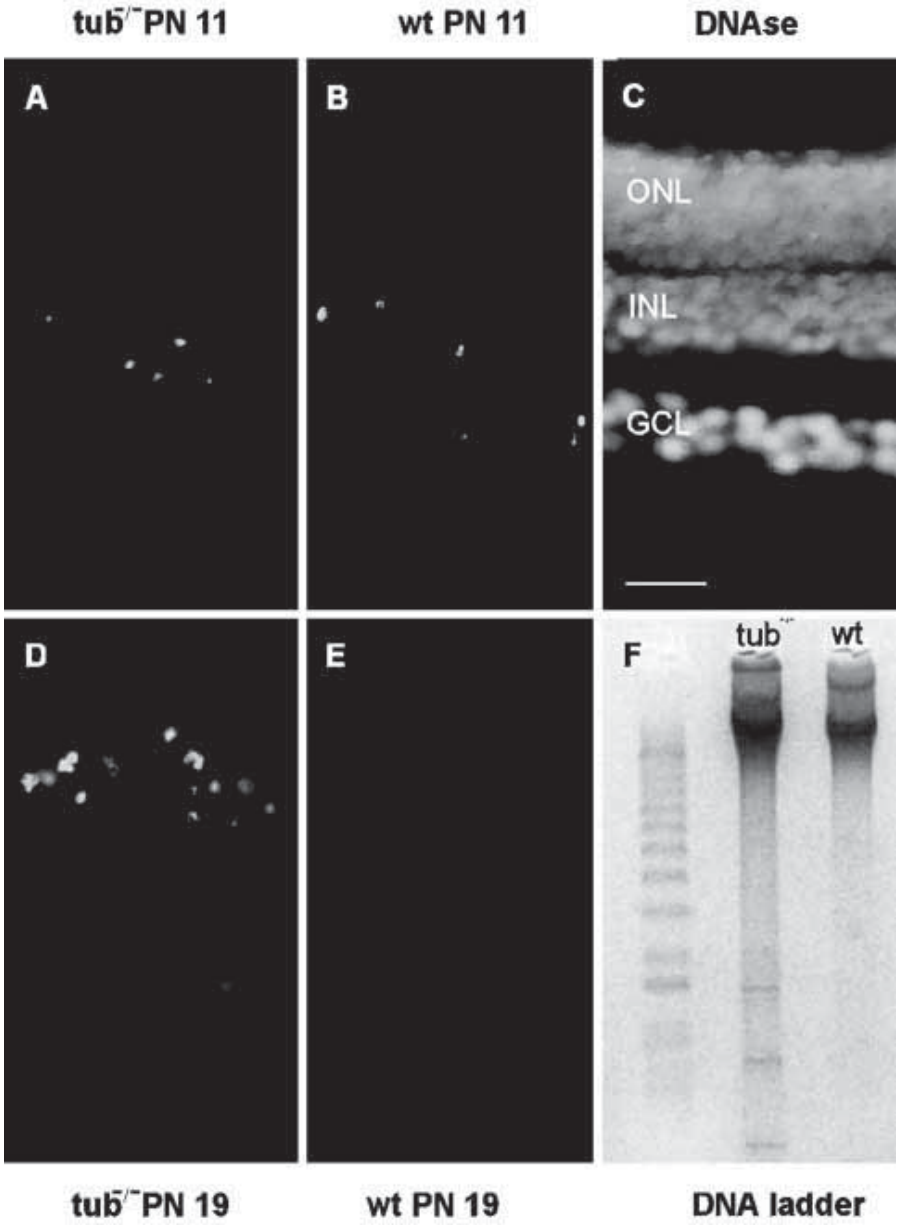

Figure 1. Apoptotic cells during the development of the retina in *tubby* and wild type mice. TUNEL-staining of longitudinal sections through retinas of PN 11 *tubby* mouse (**A**), PN 11 wild type mouse (**B**), control adult wild type retina treated with DNAse (**C**), PN 19 *tubby* mice (**D**) and PN 19 wild type mouse (**E**). In PN 11 TUNEL-positive apoptotic cells were present in the inner retina of both wild type and tubby mouse. In contrast, in PN 19 mice TUNEL-labeled apoptotic cells were restricted to the *tubby* retina where they localize to the outer nuclear layer (ONL). As expected, in the control DNAse-treated retina all nuclear layers were TUNEL-positive. INL, inner nuclear layer GCL, ganglion cell layer. Scale bar in **C** represents 20 μm. **F**: DNA ladder formation after agarose gel electrophoresis of genomic DNA confirmed apoptosis in PN 19 *tubby* mouse retinas (lane 1: DNA marker; lane 2: DNA ladder of PN 19 *tubby* mouse $tub^{-/-}$; lane 3: no DNA ladder of PN 19 wild type mouse (wt)).

genomic DNA from mouse tails was amplified by polymerase chain reaction (PCR) using the following primers: forward primer, ACA CGG AGA TCA TCA TTG AG and reverse primer, GAG ATG CAC ACA GAC AGC A. After 35 cycles, the PCR products were digested with the Sml I restriction enzyme (New England Bio Labs, Frankfurt/Main, Germany) and analyzed by agarose gel electrophoresis.

TUNEL-staining: For visualization of apoptotic cells, eyecups were fixed for 2 h with 2% paraformaldehyde in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), washed in PBS, dehydrated in ethanol and embedded in paraffin. Sections of 5-10 µm were cut on a JUNG microtom (JUNG AG Heidelberg, Germany). Collected paraffin sections were dewaxed for 20 min in xylene at 60°C and hydrated in graded ethanol to water. After washing the sections twice with PBS, they were incubated with proteinase K (Fluka, Germany) for 10 min at RT. Washed sections were permeabilized for 5 min with 0.1% Triton X 100 in 0.1% sodium citrate. For TUNEL staining, the "In Situ Cell Death Detection Kit" (Boehringer Mannheim, Germany) was used per instructions. The incubation with the "TUNEL-mixture" for 1 h at 37 °C in a humid chamber was terminated by multiple washes in PBS. Dried sections were mounted in Mowiol (HOECHST 4.88, Hoechst, Germany) and analyzed under a Axiovert M35 (Zeiss, Germany) or a Leitz DMRD (Leica, Germany) fluorescent microscope. Scanned images were processed with the computer graphic program (Corel Draw Photo-Paint 7, Ottawa, Canada).

DNA laddering: Internucleosomal DNA fragmentation analysis was performed according to Grimm et al. [22]. Genomic DNA was isolated from four *tubby* mice retinas as described and the DNA fragments were electrophoretically separated on a 1% agarose gel.

Injections of apoptotic inhibitors: For the inhibition of apoptotic photoreceptor cells in *tubby* mice retinas, the following two specific caspase inhibitors were used: caspase-1

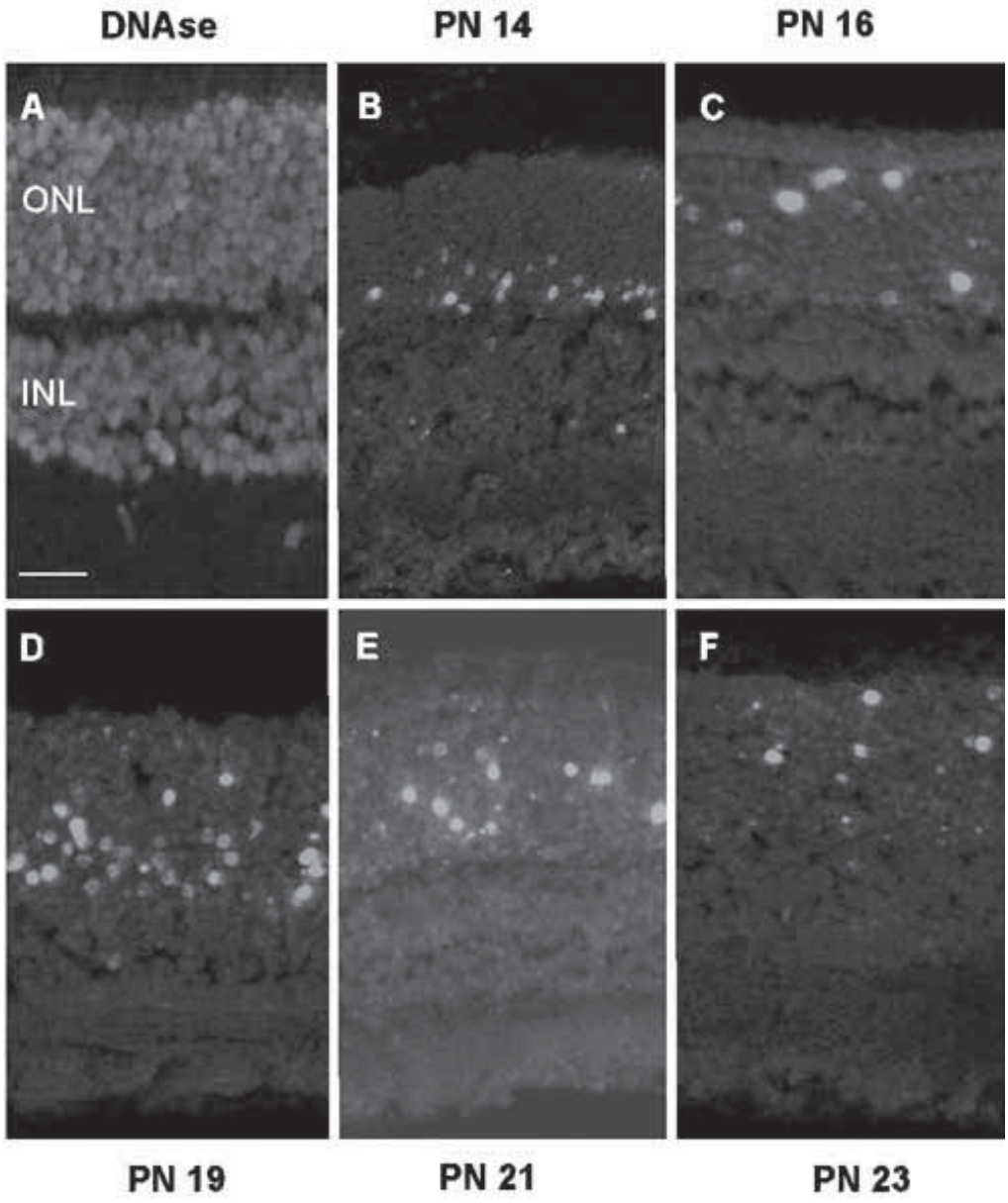

Figure 2. Apoptotic photoreceptor nuclei during retinal degeneration in *tubby* mice. TUNEL-labeled longitudinal sections through retinas of an adult wild type mouse (**A**) and *tubby* mice at the age of PN 14 (**B**), PN 16 (**C**), PN 19 (**D**), PN 21 (**E**), and PN 23 (**F**). The number of TUNEL-positive apoptotic photoreceptor cells in the outer nuclear layer (ONL) gradually increased from PN 14 to the peak at PN 19, followed by a decrease to a basic level at PN 23. Note: While a few TUNEL-positive nuclei were found in the inner nuclear layer (INL) in the PN 14 *tubby* retina, apoptotic cells are absent in older *tubby* mice in the inner retina. Scale bar represents 15 µm.

inhibitor (Z-YVAD-CMK, 50 mM, Alexis, Switzerland) and caspase-3 inhibitor (Z-DEVD-FMK, 50 mM, R& D, Germany). Mice were first anesthetized with an intraperitoneal injection of AVERTIN (2-2-2 tribrom-ethanol, 15 µl/g, Fluka, Germany) 10 min before treatment. Injections were performed with a Hamilton syringe according to previous reports [23]. 2 ml of the caspase inhibitors (right eye) or physiological saline (left eye) as a control were injected into the vitreous space, puncturing the mouse eye at the cornea-sclera junction. 24 h after injection, the treated eyes were processed for TUNEL-labeling as described above. For quantification, all values expressed as the mean and standard error of the mean were analyzed using the Student's t-test (α=0.05) after assuring the homogeneity of variances. For each experiment at least three anesthetized animals were injected and analyzed as described above.

RESULTS

Apoptotic cell death in the tubby *mouse retina:*

Apoptotic cell death in the *tubby* mouse retina was investigated in comparison to wild type retina during retinal differentiation and in the mature retina. For this purpose, apoptotic cells were stained by the TUNEL-method in longitudinal sections through the retina. In all states of post-natal retinal differentiation, TUNEL-positive cells were predominantly found in the inner nuclear layer of the developing retina in both *tubby* mice and wild type mice (Figure 1A,B). In contrast, in differentiated mature retina, TUNEL-positive cells were not commonly found in the wild type retina, but were frequently detected in the outer nuclear layer of the *tubby* mouse retina (Figs. 1D, E). The apoptotic cell death of photoreceptor cells in the outer nuclear layer determined by TUNEL-labeling was confirmed by internucleosomal DNA fragmentation (DNA ladder) of genomic DNA (Figure 1F).

To examine the pathologic cell death underlying the degeneration of the *tubby* mouse retina, the time course of apoptotic cell death was analyzed by the TUNEL-stained sections of *tubby* mouse retinas of different post natal ages. As shown in Figure 2, TUNEL-positive photoreceptor cells were detected in all the mature stages of the *tubby* mouse retina investigated. The quantification of TUNEL-positive cells in *tubby* mouse retina sections revealed that the number of apoptotic photoreceptor cells increased from PN 14 until PN 19 and drops back to a constant low number of dying cells after passing an apoptotic peak at PN 19 (Figure 3).

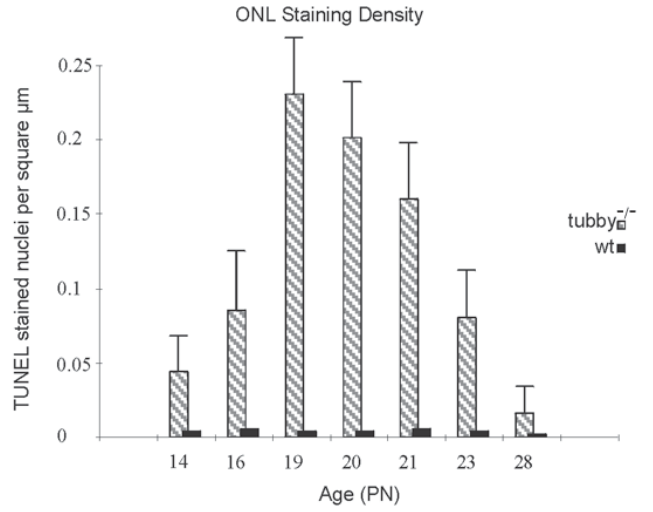

Figure 3. Time course of apoptotic photoreceptor cells of *tubby* and wild type mice. Retinas were collected at PN 14-28 from wild type and *tubby* mice. The number of TUNEL-positive apopototic photoreceptor cells in *tubby* mouse retinas increased after retinal differentation and passed an peak ("apoptotic peak"), before dropping to a small basic number. For each time point, the left, stripped column represents the number of TUNEL-positive nuclei per square µm of the outer nuclear layer in *tubby* mice. The right, black column represents that of wild type mice. The data of each time point represent the mean of counts of three representative areas of two retina samples; the error bars represent the standard error of the mean.

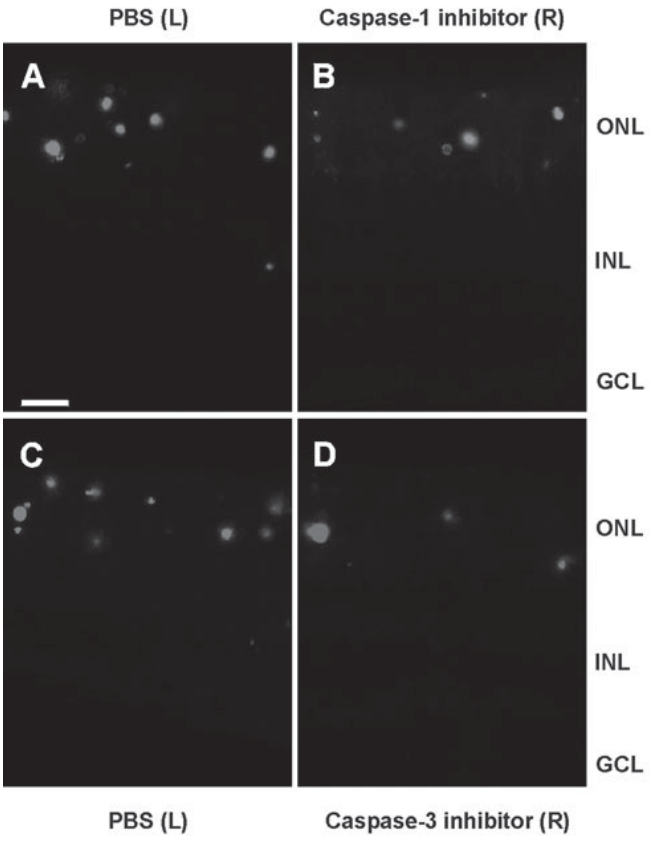

Figure 4. Apoptotic photoreceptor cells in retinas of *tubby* mice after injection of caspase inhibitors. TUNEL-stained longitudinal section through retinas of a PN 19 *tubby* mouse, previously injected with physiological saline (left eye control; **A**) and caspase-1 inhibitor (right eye; **B**). Between the control and the caspase-1 treated retina no difference in the number of apoptotic nuclei were visible. TUNEL-stained longitudinal section through the retinas of a PN 19 *tubby* mouse, previously injected with physiological saline (left eye control; **C**) and caspase-3 inhibitor (right eye). Comparison of both retinas revealed an obvious reduction of TUNEL-positive (**D**). INL, inner nuclear layer; GCL, ganglion cell layer; L, left eye; R, right eye. Scale bar represents 10 µm.

Intravitreal injections of caspase inhibitors: To evaluate the execution pathway of apoptotic photoreceptor cell death in the *tubby* mouse retina the specific inhibitors to caspase-1 and caspase-3 were applied in vivo. For this purpose, the caspase-1 inhibitor Z-YVAD-CMK and the caspase-3 inhibitor Z-DEVD-FMK were injected into the vitreous (or "crystalline lens") of the *tubby* mice eye at PN 18, the day before the determined "apoptotic peak" of photoreceptor cells. 24 h after the intravitreal injection, the effects of the caspase inhibitors on photoreceptor cell death were determined in retinal sections by the TUNEL-method. The number of TUNEL-positive cells in the outer nuclear layer of the caspase injected *tubby* mouse eyes were counted in areas next to the injection site and compared with the counts obtained from the control eye of the same test animal injected with physiological saline.

During the course of the injection experiments, all treated mice were in good health and none of the test animals died. Furthermore, the intravitreal injections did affect neither the retinal organization nor the distribution of apoptotic cells in the retina. Analysis of the treatments with caspase inhibitors revealed that the caspase-3 inhibitor Z-DEVD-FMK specifically reduced the number of apoptotic photoreceptor cells in the *tubby* mouse retina, while the caspase-1 inhibitor (Z-YVAD-CMK) had no effect (Figure 4). TUNEL-stained retinas of caspase-1-inhibitor injected eyes do not differ from physiological saline injected eyes (Figure 4A,B). The number of apoptotic cells determined in the outer nuclear layer of retina of the caspase-1 inhibitor treated and saline injected eyes is equivalent to that obtained in untreated *tubby* mouse retinas. In contrast, after application of the caspase-3 inhibitor, the number of TUNEL-positive photoreceptor cells in the *tubby* mouse retina was drastically reduced (Figure 4C,D). Further quantification was based on the analysis of 10 sections of 3 *tubby* mice eyes for each caspase inhibitor. The caspase-3 inhibitor application reduced the number of apoptotic cells in the outer nuclear layer by 40% compared with the controls (Figure 5). Our results indicate that photoreceptor cell death in the *tubby* mouse retinas is mediated by a specific activation of caspase-3.

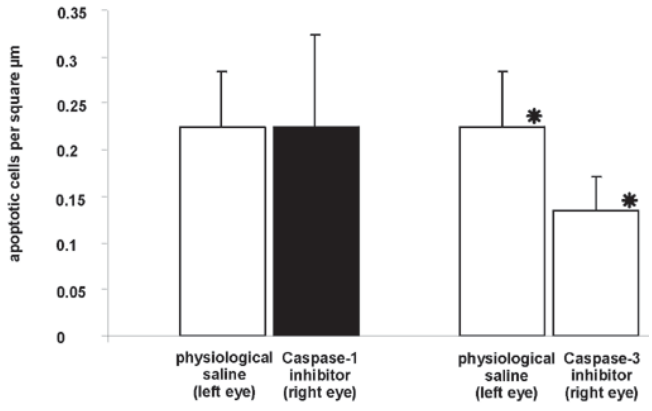

Figure 5. Quantification of TUNEL-stained photoreceptor cells of *tubby* mice retinas after injection of caspase inhibitors. No reduction of apoptotic nuclei was found after injection of caspase-1 inhibitor. Inhibition of caspase-3 activity alleviated apoptotic cell death in the outer nuclear layer. There was a statistical difference between physiological saline treated and caspase-3 inhibitor treated *tubby* mice ($p<0.05$; groups marked with asterisks). Each group consisted of three mice.

DISCUSSION

We have demonstrated in this study that caspase-3 is activated during the apoptotic retinal degeneration of *tubby* mice, thus playing a major role in photoreceptor cell death. First we observed that apoptotic cell death in *tubby* mice during retinal differentiation is not effected by the mutated *tub* gene. In both *tubby* and wild type mouse retinas, TUNEL-positive cells were localized predominantly in the inner nuclear layer. Our results are consistent with data previously obtained in wild type rodents and in other mouse models for retinal degeneration [2,24,25]. These observations confirm that cell death via apoptosis is a common and necessary process for the correct differentiation of the mammalian retina [3].

For several other animal models, apoptotic photoreceptor cell death has been identified as the molecular patho-mechanism leading to retinal degeneration [2,25,26]. Indeed, previous observations strongly suggest that photoreceptor cells in the *tubby* mouse retina also die due to apoptosis [4,13,27]. By TUNEL-staining analysis, Ikeda et al. [13] have detected a low number of apoptotic cells in the in *tubby* mouse retina at PN 28, which is consistent with our data obtained from similarly aged mice (Table 1). However, our TUNEL-labeling analysis revealed a greater number of apoptotic photoreceptor cells in the outer nuclear layer of mature *tubby* mice retinas in the age between PN 16 and PN 22 (Figure 3). The shape of the histogram of apoptotic photoreceptor cells in *tubby* mouse retinas is similar to those previously described for the retinal degeneration in other animal models (Figure 3). Time course comparison of apoptotic retinal degeneration between different mouse models reveals that the peak of apoptotic photoreceptor cells is dependent on the rate of retinal degeneration (Table 1). In *tubby* mice retinas, the apoptotic peak is later than in the faster degenerating retina of rd/rd mice, but earlier than in the slower degenerating retina of rds/rds mice [2,25]. Interestingly, this apoptotic peak occurs in the retina much earlier than the morphological indication for retinal degeneration previously reported [4]. Furthermore, these morphological studies showed that degenerating retinas also lose photoreceptor cells in later stages of retinal degeneration when only a few apoptotic cells are detected by the TUNEL method. To explain the discrepancy between the reduction of cell number in the photoreceptor layer and the number of observed apoptotic cells, it has been suggested that apoptotic retinal cells may be quickly removed from the degeneration retina by phagocytic microglial cells [28-32]. In the early stage of retinal degeneration (approximately during the increase of the apoptotic peak, PN 14 to PN 19), ramified macrophages might be transactivated into phagocytic active microglial cells, probably via signals from dying photoreceptor cells. In later degeneration stages (approximately during the reduction of the apoptotic peak and later steady state of apoptosis, after PN

TABLE 1. COMPARISON OF APOPTOTIC TIME COURSES OF MOUSE MODELS FOR RETINAL DEGENERATION

Mouse mutations	Ref	Apoptotic peak (PN)	Apoptotic cells per square micron (PN) 14	16	19	20	21	22	28
rd / rd	25	16	0.32	0.44	-	-	0.08	-	-
rds / rds	25	21	-	-	-	-	0.18	-	0.03
tub-/-	13	?	-	-	-	-	-	-	0.05
tub-/-	*	19	0.04	0.08	0.23	0.21	0.16	0.08	0.02

All three mouse models compared show an apoptotic peak during retinal degeneration. Nevertheless, the time points of the apoptotic peak differ between the mouse models. The asterisk ("*") in the "Reference" column indicates that this entry is based on the data reported herein.

19), apoptotic cells may be quickly removed from the degenerating retina by migratory phagocytes, as apoptotic photoreceptor cells were found to remain as TUNEL-positive cells only for a short time period approximately a few hours in the retina. Therefore, the number of TUNEL-stained cells after the apoptotic peak found in *tubby* mice and other mouse models for retinal degeneration [2,25] probably does not reflect the absolute number of dying photoreceptor cells. Our preliminary data from a current study on the role of the microglia during the retinal degeneration in *tubby* mice confirm this hypothesis (Bode and Wolfrum, unpublished observations).

Caspase-3 inhibitor reduces photoreceptor cell death in the tubby *mouse retina.:*

We show in the present study that neuronal cell loss via apoptosis is the final pathway of photoreceptor cell death during retinal degeneration in the *tubby* mouse, as found in other mouse models for retinal degeneration and in human retinal diseases [33,34]. In the absence of final knowledge on the linkage between the *tub* gene mutation and degeneration through apoptosis, there is increasing evidence that members of the caspase family of proteases play a critical role as mediators of neuronal and retinal cell death through apoptosis [23,35].

Our results demonstrated that the irreversible inhibition of caspase-3 protease activity by Z-DEVD-FMK, but not by the caspase-1 inhibitor Z-YVAD-CMK, significantly reduced photoreceptor cell death in the degenerating *tubby* mouse retina. This result provides strong evidence that caspase-3 proteases act as inducible cell death effectors in the photoreceptors of the degenerating retina of *tubby* mice. An upregulation of caspase-3 protease expression and a substantial reduction of apoptotic cell death induced by caspase-3 proteases inhibitors has been also demonstrated in cells of the inner retina [23,36,37]. However, our results indicate that caspase-3 also plays a key role as a mediator of apoptotic photoreceptor cell death in the outer retina during the degeneration of the *tubby* mouse retina. Our findings confirm the data previously obtained on rd/rd-mouse and on chemically induced retinal degeneration in rats as well as transgenic rats with a mutation in rhodopsin [38-41]. Nevertheless, the incomplete inhibition of photoreceptor cell death by the caspase-3 inhibitor does not exclude participation of other apoptotic pathways in the inherited retinal degeneration of *tubby* mice. Previous studies suggested that the downstream caspase-3 proteases can either be auto-activated or be activated by other upstream caspases [17]. The fact that the caspase-1 inhibitor Z-YVAD-CMK did not protect photoreceptor cells of the *tubby* mouse retina from apoptotic cell death argues against a major involvement of caspase-1 proteases in the sequentially activation of caspase-3 in *tubby* mouse photoreceptor cells during retinal degeneration.

In conclusion, our experiments demonstrate that photoreceptor cell death in the *tubby* mouse occurs through caspase-3 mediated apoptosis. Substantial alleviation of apoptotic photoreceptor cell death by inhibition of caspase-3 activity provides a strong rationale for anti-apoptotic strategies in the treatment of retinal degenerative diseases. In particular, anti-apoptotic therapies may be useful in halting the progression of retinitis pigmentosa in Usher patients.

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