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c-fos and retinal dystrophy

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The absence of *c-fos* prevents light-induced apoptotic cell death of photoreceptors in retinal degeneration *in vivo*

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Apoptotic cell death in the retina was recently demonstrated in animal models of the hereditary human retinal dystrophy known as retinitis pigmentosa[1,2]. Although recent evidence indicates that the proto-oncogene *c-fos* is a mediator of apoptosis[3–7], its precise role is unclear. In fact, under some conditions, *c-fos* may even protect against apoptotic cell death[8]. In the retina, *c-fos* is physiologically expressed in a diurnal manner and is inducible by light[9,10]. We previously observed a light-elicited, dose-dependent apoptotic response in rat photoreceptors[11]. To determine whether *c-fos* is involved in the light-induced apoptotic pathway we have used control mice and mice lacking *c-fos*. We found that following dark adaptation and two hours of light exposure both groups of animals exhibited only a few apoptotic cells. However, at 12 and 24 additional hours after light exposure, apoptosis increased dramatically in controls but was virtually absent in those mice lacking *c-fos*. Therefore, *c-fos* is essential for light-induced apoptosis of photoreceptors. Notably, *c-fos* is continuously upregulated concomitant with apoptotic photoreceptor death in our system and in animal models of retinitis pigmentosa (Agarwal, N. *et al., Invest. Ophthalmol. Vis. Sci. Suppl.* 36, S638 and Rich, K.A. *et al., Invest. Ophthalmol. Vis. Sci. Suppl.* 35, 1833). Inhibition of *c-fos* expression might therefore represent a novel therapeutic strategy to retard the time course of retinal dystrophies and light-induced retinal degeneration.

The cellular immediate early gene and proto-oncogene *c-fos* encodes a nuclear DNA binding phosphoprotein that forms heterodimeric complexes with members of the Jun-family of proteins to constitute the transcription factor AP-1 (activator protein 1) (ref. 12). In many systems, expression of *c-fos* precedes the initiation of apoptosis or is concomitant with apoptosis[5,6]. More recently, functional studies *in vitro* have demonstrated that neutralizing antibodies specific for the Fos-family can prevent apoptosis of nerve growth factor (NGF) deprived sympathetic neurons[3] and expression of the c-Fos protein can even actively induce apoptosis in susceptible cell lines[4]. However, *in vivo* studies have so far failed to demonstrate an essential role for *c-fos* in apoptosis[13].

In the retina, *c-fos* can be induced by light in the inner nuclear layer (INL) and ganglion cell layer (GCL) and by darkness in photoreceptors of the outer nuclear layer (ONL)[10]. Furthermore, it is expressed in a rhythmic fashion[9,10] suggesting an important function in the regulation of retinal physiology[14]. Previously we observed that exposure of rodents to light can induce apoptosis of retinal photoreceptors[11]. Therefore we tested the hypothesis that *c-fos* is involved in the initiation of apoptosis of photoreceptors *in vivo*.

c-fos$^{-/-}$ mice, as well as controls (*c-fos*$^{+/+}$ and *c-fos*$^{+/-}$ littermates)

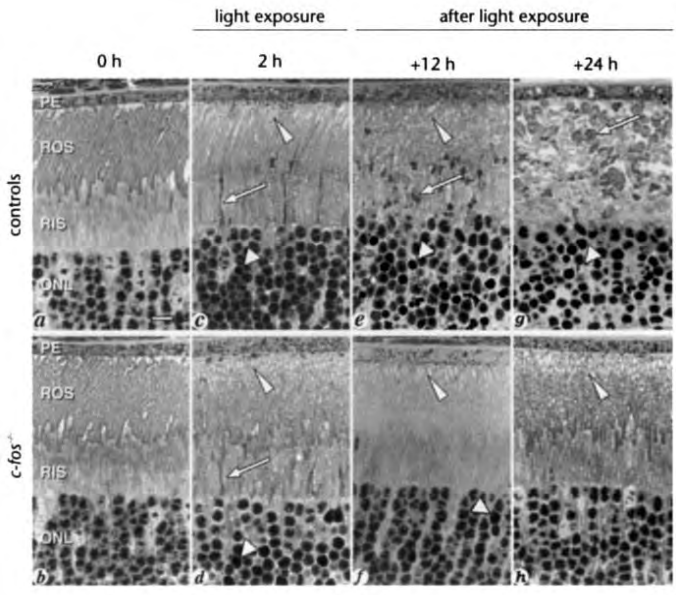


Fig. 1 *a–h*, Light microscopy of retinal apoptosis in control mice (*a*, *c*, *e* and *g*) and *c-fos*$^{-/-}$ mice (*b*, *d*, *f* and *h*). *a* and *b*, Dark adapted control mice (*a*) and mutant littermates (*b*) show normal retinal morphology. *c* and *d*, Immediate sacrifice after two hours of light exposure: both control (*c*) and *c-fos*$^{-/-}$ mice (*d*) reveal minimal lesions consisting of vesiculations of ROS (long arrowhead). Condensed cytoplasm of RIS (arrow) and scattered apoptotic nuclei (short arrowhead) in the ONL are seen in both groups. *e* and *f*, 12 hours after light exposure: retinas of control mice (*e*) display abundant apoptosis with condensed nuclei in the ONL (short arrowhead). ROS are distinctly disrupted (long arrowhead), RIS are disrupted and condensed (arrow). *c-fos*$^{-/-}$ mice (*f*) reveal an almost complete absence of apoptotic changes, still with only very few apoptotic nuclei present (short arrowhead). ROS and RIS are well preserved, ROS vesiculations are still present (long arrowhead). *g* and *h*, 24 hours after light exposure: In controls (*g*) ROS and RIS can not be differentiated (arrow), the ONL displays apoptosis of virtually all nuclei (small arrowhead). In *c-fos*$^{-/-}$ mice, vesiculations of ROS are still apparent (long arrowhead) and no apoptotic cells are seen (*h*). PE, pigment epithelium; ROS, rod outer segments; RIS, rod inner segments; ONL, outer nuclear layer. Scale bar, 10 μm.

Table 1 Counts of apoptotic photoreceptor nuclei in c-fos$^{-/-}$ and control mice immediately, 12 hours and 24 hours after light exposure

Analysis after exposure	c-fos$^{-/-}$	Controls
Immediate	3.3 ± 2.8	4.0 ± 1.8
After 12 hours	1.3 ± 1.0*	48.5 ± 11.4*
After 24 hours	0.65 ± 0.25*	63.15 ± 0.25*

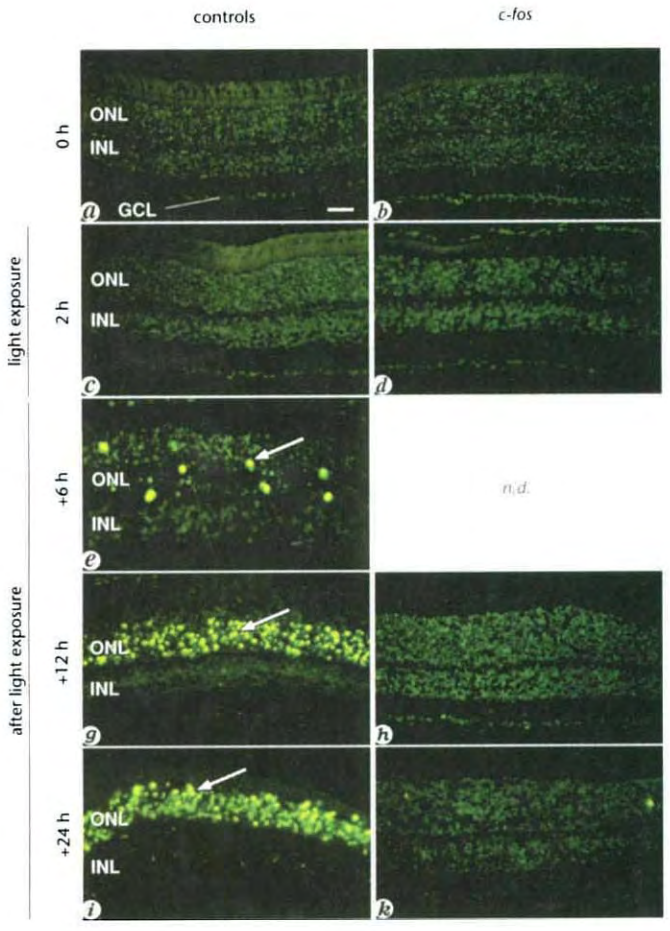
Student's unpaired *t*-test. Values are means ± s.d.
*$P < 0.001$.

displayed normal ocular and retinal morphology when not exposed to acute light pulses (Fig. 1, *a* and *b*). The transparency of the ocular optical media, the pupillary light reflex and the light transmission of homogenized crystalline lenses measured by spectrophotometry were all similar in control and in *c-fos*$^{-/-}$ mice (data not shown).

In a pilot study, a dose-response relationship of retinal light damage was established in control mice (F1-hybrids derived from C57BL/6xSV129, data not shown.) The threshold light dose to induce apoptosis of photoreceptors was 5'000 lux for two hours. Pure background strains (C57BL/6 and SV129) were also examined and they both exhibited light-induced apoptosis under these conditions. The kinetics of apoptosis was dependent on the pigmentation state. SV129 mice, which are less pigmented than C57BL/6 mice showed an earlier onset of light-induced apoptosis. However, analysis at 12 and 24 hours after light exposure revealed massive apoptosis of photoreceptors in both strains. In this study we were careful to use only equally pigmented C57BL/6xSV129 hybrids in order to avoid variability in our results. In all our experiments, no differences in the extent of light damage were observed between *c-fos*$^{+/+}$ and *c-fos*$^{+/-}$ control mice.

Unanaesthetized, free-moving experimental *c-fos*$^{-/-}$ mice[15] and littermate controls were either analyzed prior to light exposure (Fig. 1, *a* and *b*) or following exposure to the above light regimen. In analogy to our light damage model in the rat retina, acute lesions, consisting of vesiculations of rod outer segment (ROS) disks[16] were seen immediately after the two hour light exposure (Fig. 1, *c* and *d*). Only few apoptotic rod cells were observed in both groups of animals (Fig. 1, *c* and *d*). At 12 hours (Fig. 1, *g* and *h*) and 24 hours (Fig. 1, *i* and *k*) in darkness following light exposure, however, striking differences emerged. In controls, abundant apoptosis occurred in the photoreceptor layer with deterioration of ROS (Fig. 1, *g* and *i* and Table 1). In contrast, there was no progression of apoptosis in *c-fos*$^{-/-}$ mice, the majority of photoreceptors exhibiting complete structural integrity except the vesiculations of ROS (Fig. 1, *h* and *k* and Table 1). Analysis of total retinal DNA by agarose gel electrophoresis confirmed the occurrence of apoptosis in the retinas of controls but not *c-fos*$^{-/-}$ mice at 12 hours following illumination (Fig. 2*l*).

Analysis of DNA strand breaks by *in situ* nick end-labeling (TUNEL) in the retinas of control animals showed practically no signals in photoreceptor nuclei immediately after light exposure. At six hours after light exposure, positive labeling increased and at 12 hours and 24 hours, there were abundant TUNEL positive cells in the ONL (Fig. 2, *a*, *c*, *e*, *g* and *i*). In *c-fos*$^{-/-}$



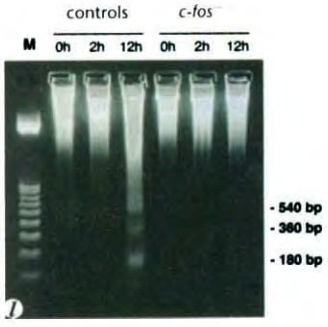


Fig. 2 *a–k*, Detection of DNA strand breaks in photoreceptor nuclei in light microscopic sections by *in situ* nick end-labeling (TUNEL). *a* and *b*, Retinas of dark adapted control mice (*a*) and *c-fos*$^{-/-}$ littermates (*b*) do not show labeling. *c* and *d*, Photoreceptors of control (*c*) and *c-fos*$^{-/-}$ mice (*d*) remain negative immediately after light exposure. *e*, Sacrifice at six hours in darkness reveals scattered labeling in the ONL in control mice (arrow). *g* and *i*, In control mice, sacrifice 12 hours (*g*) and 24 hours (*i*) after light exposure shows massive labeling of photoreceptors. *h* and *k*, *c-fos*$^{-/-}$ mice show no staining after 12 hours (*h*) and 24 hours (*k*). Apoptosis at six hours in darkness was not determined in *c-fos*$^{-/-}$ mice (n.d.). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 100 µm. *l*, DNA fragmentation analysis. Control mice do not show internucleosomal DNA fragmentation prior to (0 hours) and immediately after (two hours) light exposure. Internucleosomal DNA fragmentation is found after additional 12 hours in darkness (12 hours). No DNA fragmentation is observed in *c-fos*$^{-/-}$ mice at any time point investigated. M, 100 bp marker ladder.

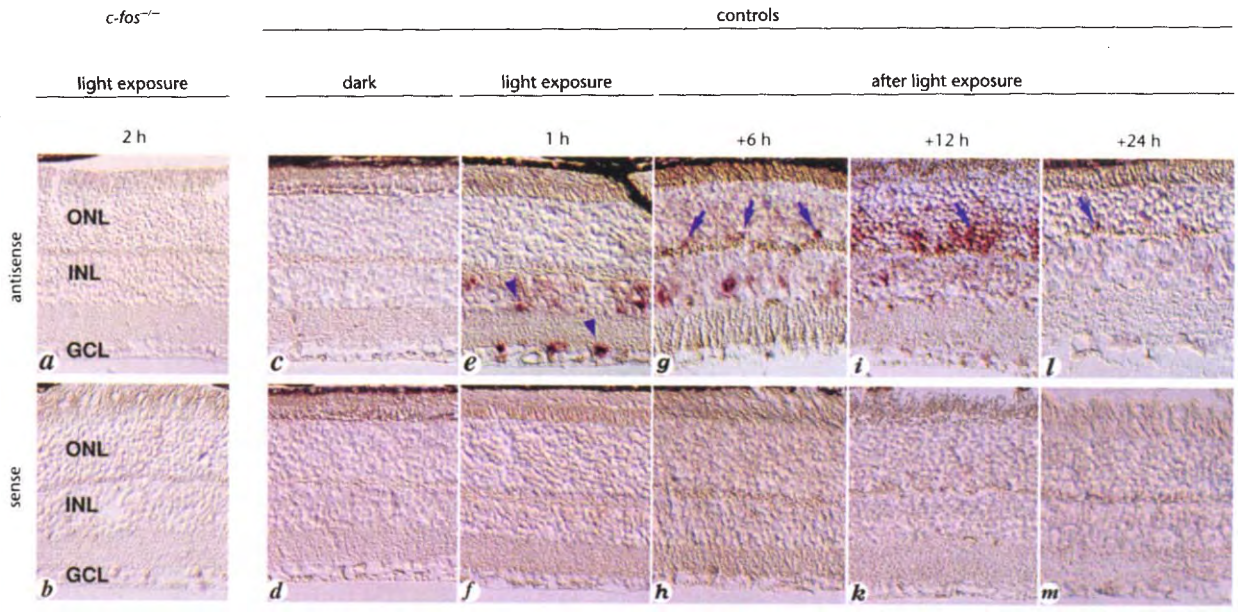


Fig. 3 *a–m*, *In situ* hybridization detection of *c-fos* mRNA. *a* and *c*, No signal detected in the retinas of *c-fos*$^{-/-}$ mice or dark-adapted controls. *e*, After one hour of light exposure, strong expression of *c-fos* is induced in the GCL and the INL (arrowheads). *g*, After two hours of illumination and six hours in darkness the expression is mainly observed in the INL, with some positive cells present in the ONL (arrows). *i*, After 12 hours in darkness, strong expression is observed in the degenerating ONL (arrow). *l*, After 24 hours in darkness, expression returns to a low level with single cells in the ONL still positive (arrow). Lower panels (*b, d, f, h, k* and *m*), hybridization sense controls. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

mice, no TUNEL positive photoreceptor cells were detected (Fig. 2, *b, d, h* and k).

Induction of *c-fos* was observed in the GCL and INL after one hour of light exposure (Fig. 3*e*). In the ONL, we observed *c-fos* expression starting from six hours after light exposure (Fig. 3*g*), and at 12 hours the major proportion of *c-fos* mRNA was located in the ONL (Fig. 3*i*). While expression in the ONL decreased almost to baseline levels within 24 hours, some single cells remained positive for *c-fos* mRNA expression (Fig. 3*i*). Thus, *c-fos* was expressed in the ONL concomitant with the induction of apoptosis.

The early appearance of *c-fos* mRNA during light exposure in the INL and GCL may reflect a classical immediate early gene response, perhaps mediated by the release of excitatory amino acids. Glutamate receptors were shown to mediate *c-fos* expression in the rat retina after light pulses or intraocular agonist injection[17,18]. After light exposure, when the animals were kept in darkness, *c-fos* mRNA levels were elevated in the ONL. This expression pattern is reminiscent of what has been observed for the activity of *c-fos* mRNA in the retina during the day and night periods as well as after acute light exposure[9,10].

In control mice we observed apoptosis directly at the end of the two hours light exposure at a much lower frequency than at 12 hours and 24 hours after light exposure. Notably, the same quantity of immediate apoptosis was registered in both groups of animals, at a time when *c-fos* mRNA was not detected in photoreceptors of control mice. Therefore, this immediate apoptotic response may not be mediated by *c-fos*. We have previously demonstrated a light-induced release of polyunsaturated fatty acids and their metabolites in the retina[19] which can mediate a rapid apoptotic response *in vitro* (Reinboth, J.-J. *et al.*, *Invest. Ophthalmol. Vis. Sci. Suppl.* **37**, S1048). Similar effects of hydroperoxides are observed in other systems[20].

The observed alterations of ROS have been described in previous studies and represent early and reversible signs of moderate light damage[16,21]. They may be due to ionic changes within ROS and/or photochemically induced free radical formation with lesions of phospholipid membranes or alterations of cytoskeletal elements. The similar extent of ROS vesiculations in both control and in *c-fos*$^{-/-}$ mice is confirmation for equal retinal irradiance levels.

The AP-1 constituents Fos and Jun both represent small families of proteins[12]. Depending on their composition and biological context, different AP-1 complexes may or may not show functional redundancy for one another[22]. Our data suggest that in the adult retina the lack of c-fos cannot be compensated for by other AP-1 members during light-induced apoptosis. In contrast, redundancy may exist in the developing retina where apoptosis occurs during normal histogenesis[23]. In *c-fos*$^{-/-}$ mice, developmental apoptosis occurs normally in other tissues[13]. In accordance, we found a regular morphology of the retina of *c-fos*$^{-/-}$ mice in our study.

In conclusion, in this study we report that *c-fos* is an essential mediator of delayed light-induced apoptosis of photoreceptor cells in the adult mouse retina. However, it remains to be shown whether the absence of light-induced apoptosis in *c-fos*$^{-/-}$ mice is due to the acute lack of Fos during light exposure or rather to its chronic deficit throughout development and in retinal rhythmicity. Therefore, the exact role of *c-fos* in retinal physiology and light-induced apoptosis needs to be clarified.

With the absence of apoptotic cell death in mice lacking *c-fos*, an almost complete suppression of light-induced lesions is achieved. Notably, *c-fos* is also continuously upregulated concomitant with photoreceptor apoptosis in animal models of retinitis pigmentosa, a hereditary retinal disease characterized by progressive loss of visual cells. Inhibition of *c-fos* expression could therefore represent a novel strategy for therapeutic interventions of retinal dystrophies and light-induced retinal degen-

eration. This might extend the survival of retinal photoreceptors prolonging the period of visual function in diseases that otherwise lead to blindness or severe visual impairment.

Methods

Animals. All animal procedures adhered to the ARVO resolution for the care and use of animals in Vision Research. *c-fos* mutants[15] were bred from heterozygous matings on a mixed C57BL/6x129Sv background and maintained in a 12:12 light-dark cycle (lights on at 6:00 a.m.) with 10–20 lux within the cages. All animals were equally pigmented, verified by transillumination of the eye and observation of iris transparency.

Light exposure. Four to six week old male *c-fos*$^{-/-}$ mice and heterozygous and wild-type littermate controls were dark adapted for 36 hours and the pupils were dilated with cyclopentolate 0.5% (Alcon Pharmaceuticals, Cham, Switzerland). Animals were either immediately killed or exposed to 5'000 lux of diffuse, cool, white fluorescent light for up to 2 h (8–10 p.m.). During illumination, particular care was taken that the eyes were evenly illuminated. Mice were anaesthetized with CO_2 and killed by cervical dislocation at the following times: 1 h after light onset (middle of light exposure); immediately after a 2 h light exposure; after 6 h, 12 h and 24 h of darkness which followed the 2 h light exposure. Eyes were rapidly enucleated, fixed in 2.5% glutaraldehyde for 12 h, dehydrated and processed for light microscopic analysis or fixed in 2% paraformaldehyde for 2 h followed by dehydration and paraffin embedding.

Quantification of apoptotic nuclei and statistical analysis. Apoptotic photoreceptor nuclei were counted in one 0.5 µm semithin section per animal over a length of 10 µm per section. Data are shown as mean ± s.d. of 8 control mice and 8 *c-fos*$^{-/-}$ littermates used per experiment. Two animals from each group were killed either prior to light exposure (dark control), immediately after the 2 h light exposure or following an additional 12 h and 24 h in darkness. All experiments were performed twice (total number of controls and *c-fos*$^{-/-}$ mice was 16). The unpaired Student's *t*-test was used in this study.

TUNEL staining. TdT-mediated dUTP nick-end labeling (TUNEL)[24] was performed with modifications using the *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Germany) on 5 µm thick paraffin sections. DNA strand breaks were labeled with fluorescein and visualized with a FITC filter.

DNA fragmentation analysis. Retinas were rapidly removed through a slit in the cornea and frozen in liquid nitrogen. Retinas from two animals were pooled (total controls, $n = 6$; total *c-fos*$^{-/-}$ mice, $n = 6$). Total retinal DNA was extracted as described[25]. Five µg of total DNA were analyzed on a 1.8% agarose gel. DNA was visualized at 254 nm by staining with SYBR GREEN (Molecular Probes, Leiden, The Netherlands) and compared with a 100 bp ladder molecular weight marker (Pharmacia Biotech, Uppsala, Sweden).

***In situ* hybridization.** Sense and antisense RNA probes were transcribed *in vitro* with T3 and T7 RNA polymerase from linearized plasmid pBFos (A. Grigoriadis and E.F.W., unpublished) in the presence of digoxigenin-11-dUTP (Boehringer Mannheim, Germany). 50–200 ng of labeled transcripts were hybridized to paraformaldehyde fixed tissue sections at 65 °C as described[26]. Digoxigenin was detected with alkaline phosphatase-labeled anti-digoxigenin Fab fragments and 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Germany). Paraffin sections of the retinas of c-fos$^{-/-}$, dark adapted controls and control mice illuminated for 1 h and 2 h; and of controls illuminated for 2 h and kept in the dark for additional 6 h, 9 h, 12 h and 24 h were analyzed.

Acknowledgments

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