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Docosahexaenoic acid prevents apoptosis of retina photoreceptors by activating the ERK/MAPK pathway

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Abstract

Identifying the trophic factors for retina photoreceptors and the intracellular pathways activated to promote cell survival is crucial for treating retina neurodegenerative diseases. Docosahexaenoic acid (DHA), the major retinal polyunsaturated fatty acid, prevents photoreceptor apoptosis during early development *in vitro*, and upon oxidative stress. However, the signaling mechanisms activated by DHA are still unclear. We investigated whether the extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) or the phosphatidylinositol-3-kinase (PI3K) pathway participated in DHA protection. 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene (U0126), a specific MEK inhibitor, completely blocked the DHA anti-apoptotic effect. DHA rapidly increased ERK phosphorylation in photoreceptors, whereas U0126 blocked this increase. U0126 hindered DHA prevention of mitochondrial depolarization, and blocked the DHA-induced increase in opsin expression. On the contrary, PI3K inhibitors did not diminish the DHA protective effect. DHA promoted the early expression of Bcl-2, decreased Bax expression and reduced caspase-3 activation in photoreceptors. These results suggest that DHA exclusively activates the ERK/MAPK pathway to promote photoreceptor survival during early development *in vitro* and upon oxidative stress. This leads to the regulation of Bcl-2 and Bax expression, thus preserving mitochondrial membrane potential and inhibiting caspase activation. Hence, DHA, a lipid trophic factor, promotes photoreceptor survival and differentiation by activating the same signaling pathways triggered by peptidic trophic factors.

Keywords: Bcl-2, docosahexaenoic acid, extracellular signal-regulated kinase signalling pathway, mitochondrial depolarization, photoreceptor, survival.

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Identifying the trophic factors required for retina photoreceptor survival, and the intracellular pathways they activate in order to prevent photoreceptor apoptosis, is crucial for designing procedures to rescue photoreceptors from dying in retina neurodegenerative diseases. Several trophic factors, such as taurine, retinoic acid, basic fibroblast growth factor (FGF), brain-derived neurotrophic factor, ciliary neurotrophic factor (CNTF), pigment epithelium-derived factor, interleukin-1β and glial-derived neurotrophic factor (Altschuler *et al*. 1993; Fontaine *et al*. 1998; Lavail *et al*. 1998; Morrow *et al*. 1998; Frasson *et al*. 1999; Jablonski *et al*. 2000; Politi *et al*. 2001a) are known to promote photoreceptor survival and differentiation. Docosahexaenoic acid (DHA), the most abundant polyunsaturated fatty acid in the retina, is also essential for photoreceptor survival during early development in culture (Rotstein *et al*. 1996, 1997, 1998; Politi *et al*. 2001a,b). DHA has long been known to be critical for proper visual function; its deficiency impairs the electric response to illumination, decreases visual acuity and affects retinal development (Neuringer *et al*. 1984, 1985; Uauy *et al*. 1990). We demonstrated that, in addition to its structural relevance, DHA postpones the apoptosis of photoreceptors, which otherwise occurs in the absence of trophic factors

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Abbreviations used: BSA, bovine serum albumin; CNTF, ciliary neurotrophic factor; DAPI, 4,6-diamidino-2-phenylindole; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FGF, basic fibroblast growth factor; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MEK, ERK kinase; PBS, phosphate-buffered saline; PQ, paraquat; PI3K, phosphatidylinositol-3-kinase; RXR, retinoid X receptors; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene.

during their early development in culture (Rotstein *et al*. 1997; Politi *et al*. 2001b), and effectively prevents photoreceptor apoptosis induced by oxidative stress (Rotstein *et al*. 2003). DHA also induces photoreceptor progenitors to exit the cell cycle (Insua *et al*. 2003) and stimulates their differentiation (Rotstein *et al*. 1996, 1998). Hence, DHA acts as a lipid trophic molecule controlling several aspects of photoreceptor development and survival.

The protective effect of DHA has also been shown in a neuroblastoma cell line (Kim *et al*. 2000) and in retinal pigment epithelium cells subjected to oxidative stress (Mukherjee *et al*. 2004). Being a lipid molecule, the way in which DHA promotes survival is intriguing. Trophic factors usually promote cell survival by activating the phosphatidylinositol-3-kinase (PI3K) and/or the extracellular-signal regulated kinase (ERK) pathway. The PI3K pathway is actively involved in the regulation of survival and growth in the retina (Hernández-Sánchez *et al*. 1995), and is essential for the survival of amacrine neurons mediated by insulin growth factor 1 (Politi *et al*. 2001c) and in estrogen-mediated neuroprotection for retinal neurons (Yu *et al*. 2004). The signaling pathway of either the mitogen-activated protein kinases (MAPKs) or the ERKs, which participates in neuronal survival, proliferation and differentiation, is also functional in the retina. It comprises a family of serine/threonine kinases, named MAPKs, and MAPK kinases (either MAPKK or MEK), which activate ERK through phosphorylation (Chang and Karin 2001). Its activation is required for FGF2-stimulated survival in rat retinal neurons (Desire *et al*. 2000), and specifically in photoreceptors (Kinkl *et al*. 2001).

In this work we investigated whether these pathways were involved in the anti-apoptotic effect of DHA on photoreceptors, and whether DHA modulated Bcl-2 and Bax expression during the *in vitro* development of photoreceptors. Our results show that DHA prevented photoreceptor apoptosis exclusively through the activation of MEK and the subsequent phosphorylation of ERK, as blocking the PI3K pathway did not affect DHA protection. Activation of ERK then led to the regulation of the levels of anti- and pro-apoptotic proteins of the Bcl-2 family. This suggests that the ERK/MAPK pathway is the main pathway activated by DHA in photoreceptors to promote survival both early during development and upon oxidative stress; hence, although a lipid molecule, DHA activated the same pathways as well-known peptidic trophic factors to promote photoreceptor survival.

Materials and methods

Materials

Albino Wistar rats (1–2-days old) bred in our own colony were used in all the experiments. All proceedures concerning animal use were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Plastic 35-mm diameter culture dishes and multichambered slides were from Inter Medical (Nunc, Naperville, IL, USA). Fetal calf serum was from Centro de Virología Animal (CEVAN, Buenos Aires, Argentina) and Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies (Grand Island, NY, USA). Trypsin, trypsin inhibitor, transferrin, hydrocortisone, putrescine, insulin, polyornithine, selenium, gentamycin, 4,6-diamidino-2-phenylindole (DAPI), fluorescein-conjugated secondary antibodies, paraformaldehyde, TRITC-labeled phalloidin, paraquat dichloride (methyl viologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride; PQ) and monoclonal anti-syntaxin clone HPC-1 syntaxin were from Sigma (St Louis, MO, USA). Monoclonal antibodies for Bax, Bcl-2, phospho-ERK1/2 and secondary antibody, goat anti-mouse IgG-horseradish peroxidase (HRP) and goat anti-rabbit IgG-HRP, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and ERK1/2 was from Promega (Madison, WI, USA). Secondary antibody, Alexa 488-conjugated-goat anti-mouse, MitoTracker (CMXRos) was from Molecular Probes (Eugene, OR, USA). Tyramine was from NEN Life Science Products (DuPont, Wilmington, DE, USA) and ABC reagents from Vector Laboratories (Burlingame, CA, USA). 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene (U0126), wortmaninn, LY294002 and kainic acid were from Biomol (Plymouth Meeting, PA, USA). Monoclonal Rho4D2 was a generous gift from Dr R. Molday (University of South Columbia). DHA, from Sigma, was further purified following the method described by Rotstein *et al*. (1996, 1997). All other reagents used were analytical grade.

Neuronal cultures

Pure neuronal cultures were obtained following procedures previously established (Rotstein *et al*. 1996, 1997). Neuronal cell types were identified by their morphology using phase-contrast microscopy and by immunocytochemistry, using the monoclonal antibodies syntaxin (HPC-1) and Rho4D2, which selectively react with amacrine and photoreceptor neurons, respectively (Barnstable 1980; Hicks and Barnstable 1987; Kljavin *et al*. 1994).

Photoreceptors have a small round cell body (3–5 μm in diameter) with a single neurite, which usually end in a conspicuous synaptic 'spherule'; sometimes they display a connecting cilium at the opposite end, but they fail to develop their characteristic outer segments; opsin is diffusely distributed over their cell body, which is usually darker than that of amacrine neurons. To be identified as photoreceptors, the cells had to display at least three of the above described criteria. Amacrine neurons are larger than photoreceptors (7–20 μm in diameter) and have multiple neurites. Almost all of them show HPC-1 immunoreactivity starting at early stages of development, and this immunoreactivity is retained even after undergoing degenerative changes that alter their morphological appearance (Politi *et al*. 2001c).

Selective elimination of amacrine neurons

To specifically evaluate protein expression and activity in photoreceptors, amacrine neurons were selectively eliminated from the cultures with 60 μM kainic acid (Abrams *et al*. 1989).

DHA supplementation

DHA (6.7 μM), complexed with bovine serum albumin (BSA), was usually added at day 1 *in vitro* (Rotstein *et al*. 1996). However, in experiments using either U0126 or PI3K inhibitors, DHA was added

at different times *in vitro*, as indicated in each case. The same volume and concentration of BSA solution was added to control cultures.

Addition of U0126

Cultures were treated with the MEK inhibitor U0126 at a final concentration of 10 μM. Three different protocols were assayed. In the first, U0126 was added at day 1 *in vitro* and cultures were supplemented with either BSA or DHA 30 min later. In the second, either BSA or DHA was added at day 1 and U0126 was added at day 4. In the third, U0126 was added at day 4 and 30 min later the cultures were supplemented with either BSA or DHA. As the inhibitor showed the same effect in all cases, the first protocol described was used for further experiments.

Addition of wortmaninn and LY294002

Cultures were also treated with two different inhibitors of PI3K, wortmaninn and LY294002, at 100 nM and 10 μM concentrations, respectively. Because of the short lifetime of wortmaninn, these inhibitors were added at day 4; DHA was added 1 h later and cells were fixed after 24 h.

Paraquat addition

Cultures treated with U0126 at day one *in vitro*, 30 min before DHA supplementation, were treated at day 3 with 48 μM PQ (Rotstein *et al*. 2003) for 24 h.

Measurement of caspase-3 activity

The activity of caspase-3 was determined in 6-day-old cultures by a fluorometric assay (EnzChek™ Caspase-3 Assay Kit #2; Molecular Probes); essentially following the method described by the manufacturer with slight modifications. Half of the culture media was removed from cultures supplemented with either BSA (control) or DHA, which were then treated with kainic acid. After 1 h, cells were washed twice with DMEM, and the original media was restored for another 1 h of incubation. Cells were harvested and washed in phosphate-buffered saline (PBS), lysed as reported in the manufacturer's instructions, and centrifuged at 13 000 rpm (14 400 *g*) for 15 min at 4°C. The protein content was determined by the method described by Bradford (1996); 70 μL of each sample was then mixed with the caspase-3 specific substrate Z-DEVD-R110 and incubated at room temperature (22°C) for 30 min. Substrate cleavage was monitored at room temperature (22°C) using an excitation wavelength of 485 nm and emission detection at 530 nm in a SLM model 4800 fluorimeter (SLM Instruments, Urbana, IL, USA). The temperature was set at 20°C with a thermostated circulating water bath (Haake, Darmstadt, Germany). A standard curve with the R110 reference standard was prepared to determine nmoles of product produced in the caspase-3 reactions, and results were shown as nmoles of product/μg protein. To confirm that substrate cleavage was the result of caspase activity, part of the lysed samples were incubated with the caspase-3-specific inhibitor Ac-DEVD-CHO, and caspase activity was then evaluated.

Immunocytochemical methods

Cultures were fixed for at least 1 h with 2% paraformaldehyde in PBS, followed by permeation with Triton X-100 (0.1%) for 15 min. Neuronal cell types were identified with specific monoclonal antibodies, as described above. Alexa 488-conjugated goat anti-mouse was used as the secondary antibody. Tyramide signal amplification was occasionally used to improve visualization, following the procedure described by the manufacturer. Controls for immunocytochemistry were performed by omitting either the primary or the secondary antibody.

The number of photoreceptors expressing either Bax or Bcl-2 with respect to the total number of photoreceptors was determined by immunocytochemistry using specific monoclonal antibodies.

Determination of apoptotic cells

Apoptotic cells were determined by evaluating the integrity of nuclei after staining cell nuclei with DAPI, a fluorescent dye that binds to DNA. Briefly, cells were permeated with 0.1% Triton X-100, washed with PBS and incubated with DAPI for 20 min. Cells were considered to be apoptotic when they showed either fragmented or condensed (pycnotic) nuclei.

Evaluation of mitochondrial membrane potential

To assess the number of cells preserving their mitochondrial membrane potential, cultures were incubated for 30 min before fixing with the fluorescent probe MitoTracker (0.1 μg/mL); the number of either photoreceptors or amacrine cells displaying fluorescent mitochondria with respect to the total number of each cell type was determined.

Expression of phospho-ERK in photoreceptors

Cultures were supplemented with either BSA or DHA after 24 h, and pERK expression was determined after 1 and 4 h by immunocytochemistry using a pERK monoclonal antibody.

To confirm the effects of DHA on pERK expression, the levels of ERK1/2 and phospho-ERK1/2 were determined by western blot. At day 3, cultures were treated overnight with kainic acid to eliminate amacrine neurons, which had a significant pERK expression, and were stimulated at day 4 with DHA. To evaluate whether U0126 blocked the DHA-mediated pERK increase, this inhibitor was added before DHA stimulation. At different time periods cells were rinsed with PBS and lysates were prepared. Proteins, dissolved in 6X Laemmli sample buffer, were separated on sodium dodecyl sulfate (SDS)-polyacrylamide (10%) gels (Laemmli 1970), and electro-transferred to polyvinylidene difluoride (PVDF) membranes, which were then blocked to avoid non-specific binding for either 2 h at room temperature or overnight at 4°C in PBS buffer containing 5% skimmed milk. Anti-phospho ERK1/2 (p42/44) or anti-ERK1/2 antibodies were then allowed to react with the membranes overnight at 4°C or for 2 h at room temperature, respectively. Membranes were washed three times in PBST (0.1% Tween 20), incubated with a 1 : 10 000 dilution of either peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody, respectively, for 1 h at room temperature, and washed three additional times with PBST. Visualization was performed by an enhanced chemiluminiscent technique (ECL) following the manufacturer's instructions. Images were obtained and bands were quantified as described by Gentili *et al*. (2001).

Statistical analysis

The results represent the average of at least three separate experiments (± SD), unless specifically indicated, and each experi-

ment was performed in triplicate. For cytochemical studies, 10 fields per sample were analyzed in each case. Statistical significance was determined by a Student's two-tailed *t*-test.

Results

The anti-apoptotic effect of DHA during photoreceptor development is closely related to the preservation of the mitochondrial membrane potential

In our culture conditions photoreceptors develop normally for 3–4 days and then degenerate through an apoptotic pathway (Rotstein *et al*. 1996, 1997). Consistent with previous results, most photoreceptors in control conditions showed pycnotic nuclei by day 6 (Figs 1c and g), whereas in DHA-supplemented cultures most photoreceptors presented intact nuclei at the same time of development (Fig. 1f). DHA reduced photoreceptor apoptosis at every time studied (Fig. 1g). Photoreceptor apoptosis during early development *in vitro* was parallel to a decrease in the number of photoreceptors maintaining their mitochondrial membrane potential (Figs 1b and h). Although about 50% of photoreceptors showed active mitochondria at early culture times, few had functional mitochondria after 11 days (Fig. 1h). DHA prevented mitochondrial depolarization (Figs 1e and h); by day 7, and still by day 11, the number of photoreceptors retaining their mitochondrial membrane potential in DHA-supplemented cultures was significantly higher than in controls (Fig. 1h).

The MEK inhibitor U0126 blocked DHA prevention of photoreceptor death during development

To establish whether the activation of the ERK/MAPK pathway was involved in the protective effect of DHA, we treated cultures with the MEK1/2 inhibitor U0126 at day 1, before DHA supplementation. At day 6, DHA-supplemented cultures showed less apoptotic photoreceptors than in controls, with more photoreceptors having active mitochondria (Figs 2b, e and h). The addition of U0126 blocked DHA

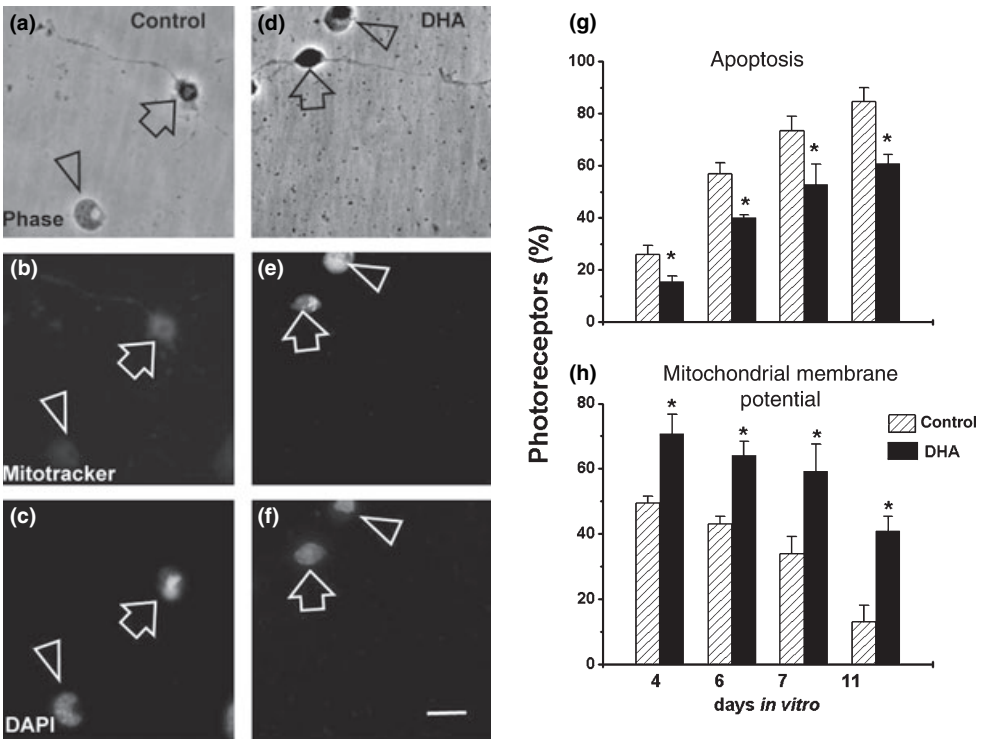

Fig. 1 Docosahexaenoic acid (DHA) prevented photoreceptor apoptosis and mitochondrial depolarization during development in culture. Phase contrast (a and d) and fluorescence (b, c, e and f), micrographs showing mitochondrial membrane potential with the fluorescent probe MitoTracker (b and e) and nuclei integrity observed with DAPI (c and f) in 6-day-old retinal neurons either with (d, e and f) or without (a, b and c) 6.7 μM DHA, added at day 1 in culture. Note that in DHA-supplemented cultures photoreceptors showed bright fluorescent mitochondria and intact nuclei (arrows in d, e and f), whereas in cultures without DHA, photoreceptors presented faintly fluorescent mitochondria and pycnotic nuclei (arrows in a, b and c). Amacrine neurons are indicated with arrowheads. The scalebar in panel (f) represents 10 μm. Retinal neurons were cultured for different times either without (control) or with DHA. (g) Bars represent the percentage of apoptotic photoreceptors, determined by analyzing nuclei integrity with 4,6-diamidino-2-phenylindole (DAPI), and the percentage of photoreceptors preserving their mitochondrial membrane potential (h). *Statistically significant differences, compared with control ($p < 0.01$).

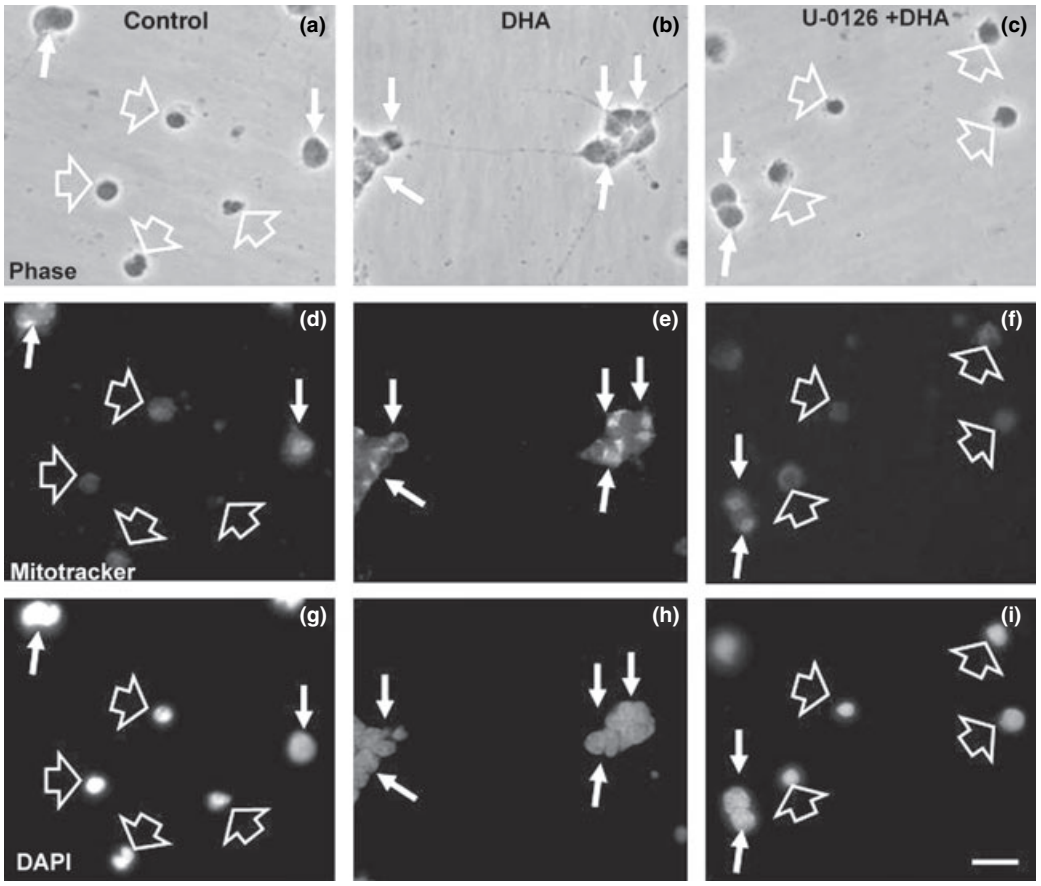

Fig. 2 Effect of 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene (U0126) on docosahexaenoic acid (DHA) prevention of photoreceptor death during development. Phase contrast (a–c) and fluorescence (d–i) micrographs showing mitochondrial membrane potential (d–f) and nuclei labeled with DAPI (g–i), in 6-day-old retinal neurons cultured with DHA (b, e and h), without DHA (a, d and g), or with DHA plus U0126 (c, f and i). DHA prevented both the loss of mitochondrial membrane potential and nuclear fragmentation in photoreceptors (thin white arrows in b, e and h). This protective effect was blocked by U0126; note mitochondrial depolarization and the presence of fragmented nuclei (open arrows in f and i). The scalebar in (i) represents 10 μm.

protection: photoreceptor cell bodies shrunk, they lost their neurites and their characteristic morphology, most of their nuclei were either fragmented or pycnotic (arrows in Figs 2c and i), and they failed to preserve their mitochondrial membrane potential (Fig. 2f). The percentage of apoptotic photoreceptors increased to about the same level as that found in controls (Fig. 3a). U0126 did not affect the apoptosis of photoreceptors in 6-day-old control cultures, which remained at 60% of the total photoreceptors, as in cultures lacking U0126 (Fig. 3a).

By day 6, the percentage of photoreceptors still preserving mitochondrial membrane potential in control conditions was about 40%, and this percentage was unaffected by U0126. In contrast, the addition of U0126 prior to DHA supplementation decreased the percentage of photoreceptors maintaining mitochondrial membrane potential (Fig. 3b) from 60%, in DHA-supplemented U0126-lacking cultures, to about 40% when U0126 was added before DHA.

U0126 blocked DHA protection of photoreceptor apoptosis induced by oxidative stress

To find out whether the activation of the MEK/ERK pathway was also involved in the DHA protection of photoreceptors from oxidative stress, DHA-supplemented cultures, pretreated either with or without U0126, were treated with the oxidant PQ at day 3. In the absence of U0126, DHA effectively prevented photoreceptor apoptosis occuring after PQ treatment (Figs 4a, c and e). The addition of U0126 inhibited this protection, and PQ increased photoreceptor death with a concomitant loss of mitochondrial membrane potential in these cells, in spite of DHA supplementation (Figs 4b, d and 5a). As previously demonstrated (Rotstein *et al*. 2003), PQ increased photoreceptor apoptosis from around 25% in controls to about 60% of total photoreceptors in PQ-treated cultures (Fig. 5(a)), whereas DHA supplementation reduced PQ-induced apoptosis to about 40% of photoreceptors. However, when U0126 was added before

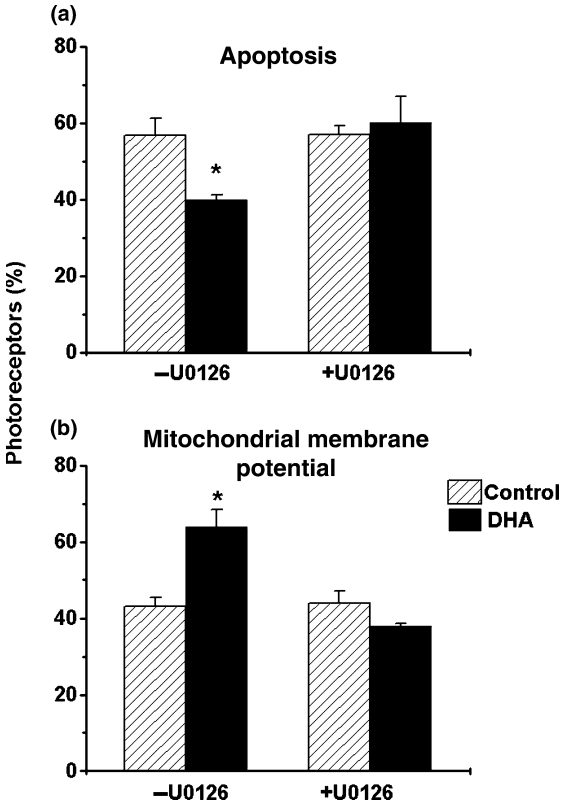

Fig. 3 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene (U0126) inhibited docosahexaenoic acid (DHA) prevention of photoreceptor death during development. Retinal neurons were cultured for 6 days either without (control) or with DHA in cultures incubated either with (+U0126) or without U0126 (–U0126). The percentage of apoptotic photoreceptors (a) was determined with DAPI and the percentage of photoreceptors preserving mitochondrial membrane potential (b) was determined by using MitoTracker. *Statistically significant differences, compared with control ($p < 0.01$).

DHA, the number of apoptotic photoreceptors was the same as that found in PQ-treated cultures lacking DHA (Fig. 5a), suggesting MEK1/2 activation was essential for DHA protection.

Although DHA prevented PQ-induced mitochondrial depolarization in photoreceptors (Figs 4c and 5b), U0126 hampered this protection; in its presence, PQ treatment of DHA-supplemented cultures decreased the number of photoreceptors preserving mitochondrial membrane potential to the same values observed in samples without DHA (Figs 4d and 5b).

U0126 blocked the increase in opsin expression promoted by DHA addition

DHA improves the early steps of photoreceptor differentiation, increasing opsin expression in these cells (Rotstein *et al.* 1998). We now investigated if this effect also depended on the activation of the ERK/MAPK pathway. DHA addition doubled the number of photoreceptors expressing opsin, compared with control cultures (Fig. 6). However, the addition of U0126 before DHA supplementation barred this increase, and the number of opsin-expressing photoreceptors in cultures with DHA and U0126 was the same as that found in controls, suggesting that the increase in opsin expression required the activation of the ERK/MAPK pathway.

DHA promoted phosphorylation of ERK

Next, we investigated whether the addition of DHA promoted ERK phosphorylation. In 1-day-old cultures supplemented with either DHA or BSA, no increase in pERK labeling was observed 1 h after the addition of DHA. However, after 4 h, pERK labeling in photoreceptors in DHA-supplemented cultures markedly increased, compared with controls (Fig. 7).

We then analyzed pERK levels by western blot. To eliminate the amacrine neuron contribution to ERK phosphorylation, 3-day-old cultures were treated with kainic acid overnight, and the time-course of ERK phosphorylation after DHA stimulation was then analyzed. Both ERK isoforms, ERK1 (44 kDa) and ERK2 (42 kDa), were present in photoreceptors, with ERK2 being the major isoform (Fig. 8a). DHA activation of MEK was very rapid; although the levels of pERK1/2 were the same in DHA-treated and untreated cultures 5 min after the addition of DHA (not shown), an increase in ERK phosphorylation was already observed after 10 min (Fig. 8a). Although total ERK levels were constant after DHA supplementation, ERK presented two peaks of phosphorylation, one at 10 min and the other one after 4 h of DHA addition, to finally decrease after 9 h (Figs 8a, b and c).

The addition of U0126 at day 4, 30 min before the addition of DHA, blocked the DHA-induced increase in pERK (Fig. 9); 10 min after the addition of DHA, cultures treated with U0126 showed no increase in pERK levels (Fig. 9b). These results suggest that DHA activated MEK, which in turn phosphorylated ERK. The inhibition of MEK activation blocked the increase in pERK levels prompted by DHA, consequently impeding DHA effects on photoreceptors.

Interestingly, under basal conditions, amacrine neurons, the other major cell type in the cultures, also showed an intense pERK labeling; however, ERK phosphorylation in these cells was not enhanced by DHA (Fig. 7, arrowheads). Moreover, the addition of U0126 did not increase amacrine cell apoptosis (not shown). This indicates that although the ERK/MAPK pathway was operative in these neurons, it was not regulated by DHA, and ERK activation was not involved in promoting amacrine cell survival.

Wortmaninn and LY294002 did not inhibit the protective effect of DHA

To establish if the PI3K pathway also participated in DHA protection of photoreceptors, cultures were treated with either

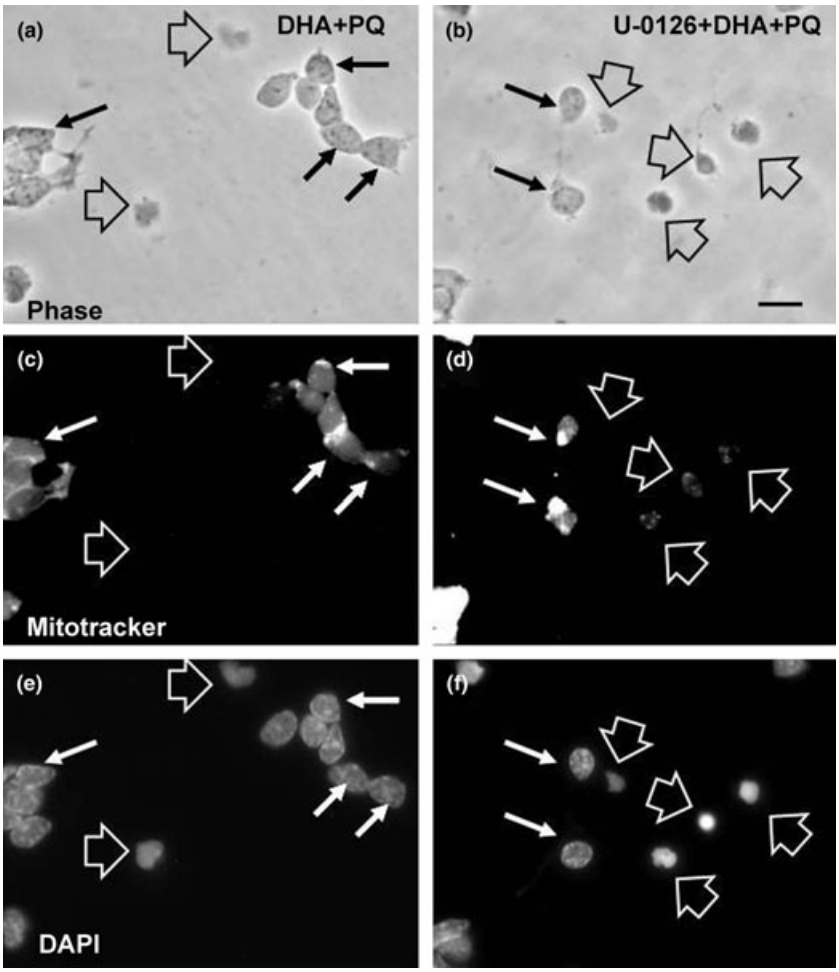

Fig. 4 Effect of 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene (U0126) on docosahexaenoic acid (DHA) prevention of photoreceptor apoptosis upon oxidative stress. Cultured retinal neurons were incubated either with DHA (a, c and e) or with DHA plus U0126 (b, d and f) and treated at day 3 with 48 μM paraquat (PQ) for 24 h. Phase contrast (a and b) and fluorescence (c–f) micrographs showing mitochondria labeled with MitoTracker (c and d) and nuclei labeled with 4,6-diamidino-2-phenylindole (DAPI) (e and f). DHA prevented the loss of mitochondrial membrane potential and nuclear fragmentation of photoreceptors (thin white arrows in c and e) induced by oxidative stress, and U0126 blocked this protection, as shown by the pale fluorescence indicating mitochondrial depolarization and the noticeable nuclei fragmentation (open arrows in d and f). The scalebar in (b) represents 10 μm.

wortmaninn or LY294002 at day 4, and apoptosis in cultures with and without DHA was evaluated 24 h later. None of these inhibitors diminished the anti-apoptotic effect of DHA (not shown). At day 5 in culture, already 46% of photoreceptors were apoptotic in the control BSA-supplemented conditions, either with or without wortmaninn and LY294002. DHA reduced photoreceptor apoptosis to 30%, and this reduction was unaffected by treatment with either wortmaninn or LY294002 before the addition of DHA.

On the contrary, amacrine neurons, which depend on the PI3K pathway for their survival (Politi *et al*. 2001c), markedly increased their apoptosis in cultures treated with wortmaninn and LY294002 (not shown). Altogether, these results strongly suggest that DHA did not activate the PI3K pathway to achieve photoreceptor protection.

DHA increased Bcl-2 expression and decreased Bax expression in photoreceptors

We then investigated whether DHA affected the expression of Bcl-2 and Bax, anti- and pro-apoptotic proteins of the Bcl-2 family, respectively, which are crucial regulators of mitochondrial outer membrane permeability. In cultures lacking trophic factors, about 37% of the total photoreceptors expressed Bcl-2 at day 4, and this expression decreased during development reaching 20% by day 9 (Fig. 10a). DHA promoted an early and significant increase in Bcl-2 expression: at day 4, about 60% of photoreceptors expressed this protein, almost doubling the value in control cultures. Although Bcl-2 expression then decreased with time, it was still significantly higher than in controls by day 6 (30% and 40% of photoreceptors, in control and DHA-supplemented cultures, respectively) and only matched control values by day 9.

The percentage of photoreceptors expressing Bax, almost negligible during the first days in culture, markedly increased during development *in vitro*, consistent with the increase in photoreceptor apoptosis (Fig. 10b), from 2% at day 4 to 10% and almost 30% of these cells at days 6 and 9, respectively. DHA did not affect the percentage of Bax-expressing photoreceptors at early culture times, but reduced it significantly by day 9.

As Bcl-2 and Bax both form heterodimers, which of these proteins predominates seems to be critical in determining cell fate (Bernardi *et al*. 2001). In control cultures, the ratios

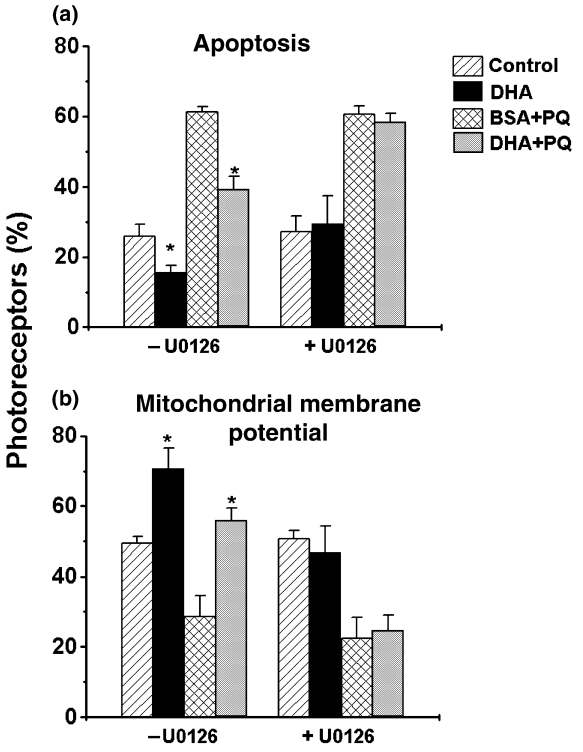

Fig. 5 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene U0126 blocked docosahexaenoic acid (DHA) protective effects on photoreceptors subjected to oxidative stress. Retinal neurons (3-days old) were pre-incubated with either the MEK inhibitor U0126 (+U0126) for 30 min or with vehicle (–U0126), and then supplemented either without (control) or with DHA at day 1. The cultures were finally either treated or not with paraquat (PQ) for another 24 h. The percentage of apoptotic photoreceptors (a) was determined with 4,6-diamidino-2-phenylindole (DAPI) and the percentage of photoreceptors preserving mitochondrial membrane potential (b) was quantified using Mito-Tracker. *Statistically significant differences, compared with control ($p < 0.01$).

between the percentages of photoreceptors expressing Bcl-2 vs. those expressing Bax were the highest, about 13%, by day 4 and markedly decreased during development, to less than 1% by day 9 (Fig. 10c), consistent with the increase in photoreceptor apoptosis. DHA significantly increased the Bcl-2/Bax ratio, compared with controls; it was almost 25 after 4 days *in vitro,* and although it decreased during development, it was still double that of controls by day 6, and only dropped to the same value as found in controls by day 9.

DHA decreased caspase-3 activity

Caspase activation leads to much of the orchestrated cellular destruction that occurs in apoptosis. However, involvement of caspases in photoreceptor apoptosis is still controversial. Because mitochondrial depolarization leads to the subsequent activation of caspase-9 and -3, we investigated whether

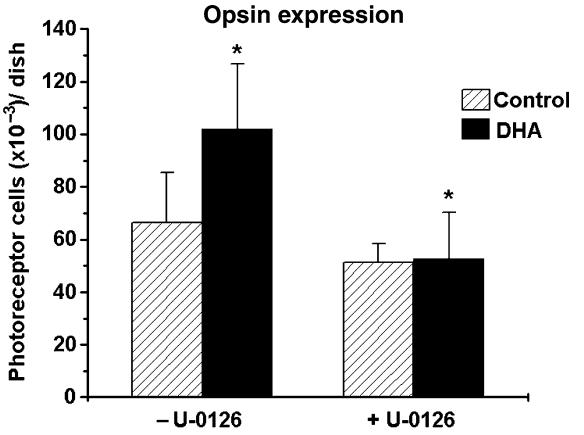

Fig. 6 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene (U0126) inhibited docosahexaenoic acid (DHA) up-regulation of opsin expression in photoreceptors. The bars represent the number of photoreceptor cells that expressed opsin in 6-day-old cultures either supplemented or not with DHA, and in either the presence (+U0126) or absence (–U0126) of U0126. *Statistically significant differences, compared with control ($p < 0.05$; $n = 6$).

the activation of the latter participated in photoreceptor apoptosis during development *in vitro*, and whether the addition of DHA prevented this activation. Caspase-3 activity was measured in photoreceptors treated overnight with kainic acid. In 6-day-old control cultures, a time of development at which about 60% of photoreceptors were apoptotic, caspase-3 activity was four times higher than in cultures treated with a caspase-3 inhibitor, indicating that the enzyme was markedly stimulated (Fig. 11). DHA reduced caspase-3 activity by 25%, suggesting that it partially prevented caspase activation, but did not completely block it.

Discussion

DHA has been shown to be an effective survival factor for retinal photoreceptors both during development *in vitro* (Rotstein *et al*. 1997; Politi *et al*. 2001a,b) and during oxidative damage (Rotstein *et al*. 2003). Our present results demonstrate that DHA enhanced ERK phosphorylation in photoreceptors, whereas the addition of U0126, a specific MEK inhibitor, completely blocked the anti-apoptotic effect of DHA. On the contrary, inhibitors of the PI3K signaling pathway did not decrease DHA protection of photoreceptors. This suggests that DHA promoted photoreceptor survival during early development in culture and upon oxidative stress exclusively through the activation of the ERK/MAPK pathway.

ERK expression is distributed throughout the whole mouse retina at P0, and decreases in a time-dependent manner in the outer nuclear layer, although it is still detectable in adult retina (Rhee and Yang 2003). In the neonatal retina, activation of ERK by cytokines is develop-

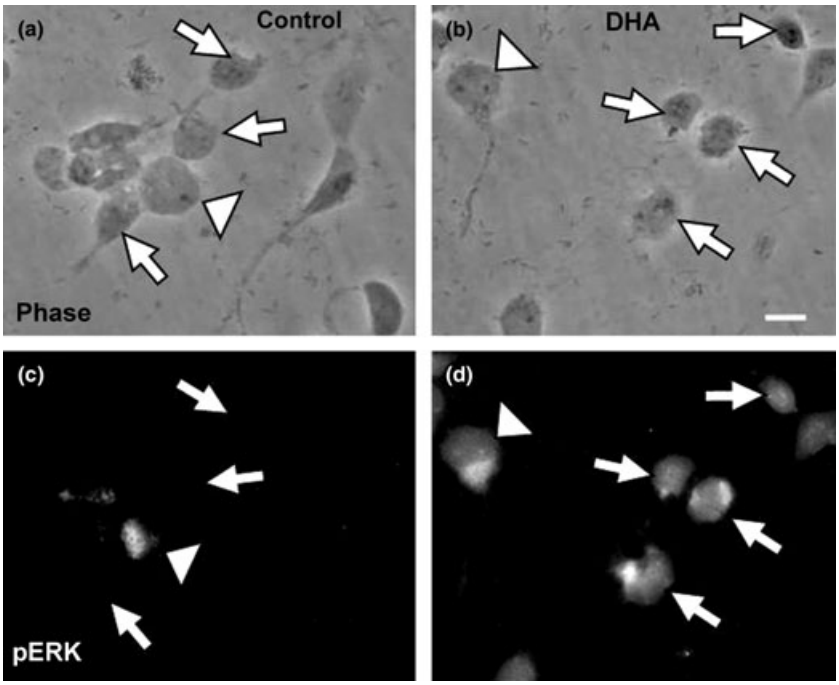

Fig. 7 Docosahexaenoic acid (DHA) induced extracellular signal-regulated kinase 1/2 (ERK1/2) activation *in vitro*. Phase contrast (a and b) and fluorescence (c and d) photomicrographs of 1-day-old retinal neurons cultured either without (a and c) or with (b and d) DHA for 4 h. pERK expression was then analyzed using a specific monoclonal antibody. The increase in the number of photoreceptors (arrows) expressing pERK was clearly visible in DHA-supplemented cultures (arrows in b and d), whereas amacrine neurons showed an intense pERK labeling in both conditions (arrowheads in a–d). The scalebar in (b) represents 10 μm.

mentally regulated, and is mediated by its upstream kinase, MEK1/2; increased pERK levels in response to cytokine signals are found both in retinal proliferating progenitors and in postmitotic photoreceptor precursors, in which it might influence cell fate specification and promote differentiation and survival, respectively (Rhee *et al*. 2004). The ERK/MAPK pathway has been shown to participate in the protective response to bright light damage in the retina (Liu *et al*. 1998). Trophic factors such as FGF2 and CNTF activate this pathway to promote photoreceptor survival (Kinkl *et al*. 2001; Rhee *et al*. 2004) and prevent light-induced damage after optic nerve section (Valter *et al*. 2005). Our results show that DHA stimulated this kinase cascade, activating MEK1/2 to enhance ERK1/2 phosphorylation. Activation of these enzymes was essential for the anti-apoptotic effect of DHA; MEK inhibition, which prevented ERK phosphorylation, completely abolished the DHA protection of photoreceptors, both during development and during oxidative damage. In addition, DHA improvement of the early steps of photoreceptor differentiation, i.e. the up-regulation of opsin expression and the increased development of apical processes (Rotstein *et al*. 1998) was completely blocked by U0126, indicating that this effect depended on ERK activation. Hence, DHA triggered the ERK/MAPK pathway to promote both survival and early differentiation of photoreceptors. Interestingly, although these are long-term effects and despite the continuous presence of DHA in the incubation medium, DHA-induced phosphorylation of ERK was transient *in vitro*. Cytokine signal transduction has been shown to have feedback regulatory mechanisms that lead to a rapid decrease of signaling events (O'Shea *et al*. 2002); similar mechanisms might be operative in DHA-activated signaling pathways, the elucidation of which demands further investigation. Another intriguing question is how DHA activates this pathway, which is usually activated by the binding of ligands to tyrosine kinase-like membrane receptors (Trk), then leading to receptor dimerization and autophosphorylation. DHA might: (i) directly bind as a ligand to these receptors; (ii) activate one of the proteins involved in the signaling cascade, downstream of the Trk receptor; or (iii) given its lipidic characteristics, modify the biophysical properties of neuronal membranes to induce the dimerization and consequent activation of these receptors. An alternative pathway might be the activation of nuclear receptors prior to the activation of the ERK/MAPK pathway, as in mouse brain DHA has been identified as a ligand for nuclear retinoid X receptors (RXR) (Mata de Urquiza *et al*. 2000), which act as ligand-activated transcription factors. Given the pleiotropic effects of these nuclear receptors, it is plausible that the DHA-induced RXR activation might activate different survival/differentiation pathways, including the regulation of the ERK/MAPK pathway. Additional research is required to establish which of the listed events is triggered by DHA to activate the ERK/MAPK pathway.

DHA has been shown to promote neuroblastoma cell survival upon serum deprivation by facilitating the translocation and activation of Akt, a downstream effector of the PI3K pathway, without stimulating PI3K activity (Akbar *et al*. 2005). Activation of this pathway was nevertheless required for DHA protection, as either wortmaninn or LY294002 blocked the effect of DHA on neuroblastoma

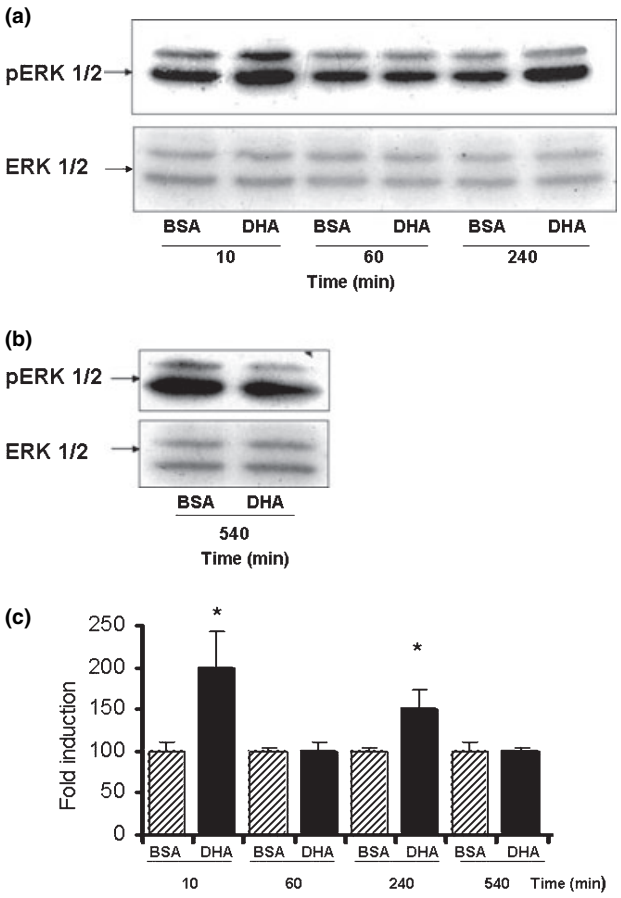

Fig. 8 Docosahexaenoic acid (DHA) stimulated the rapid phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). Retinal neurons (3-days old) were treated with kainic acid for 24 h to eliminate amacrine neurons, and then photoreceptors were stimulated with either DHA or bovine serum albumin (BSA) and finally lysed at the indicated timepoints. Total protein extracts (10 μg/lane) were analyzed by western blot using an anti-pERK1/2 antibody. After stripping, loading of equal quantities of ERK1/2 expression was verified by re-probing the same blots with anti-ERK1/2 antibody (lower panels). (a and b) Time course of pERK activation stimulated by DHA on photoreceptors. (c) Densitometric scanning of the p42ERK2 phosphorylation shown in (a and b). Western blots are representative of three independent experiments with similar results. *Statistically significant differences, compared with control ($p < 0.05$).

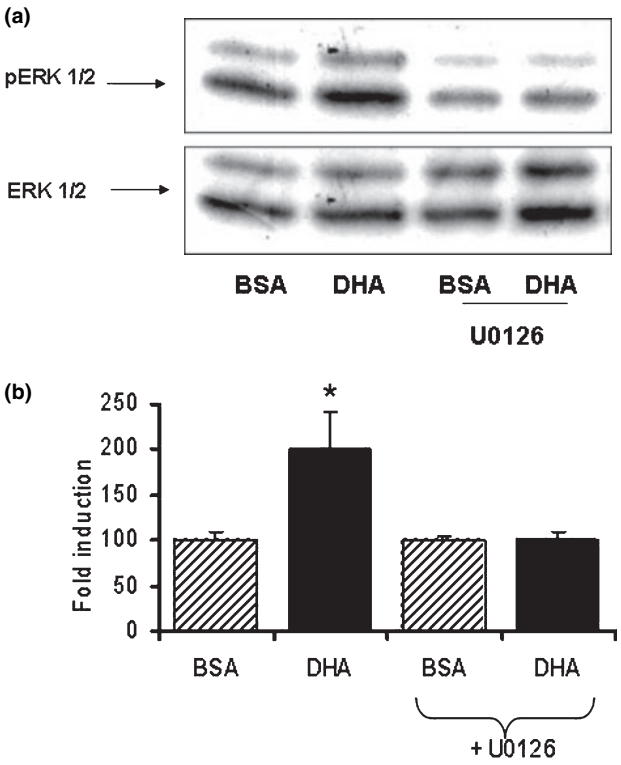

Fig. 9 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene (U0126) blocked extracellular signal-regulated kinase (ERK) activation induced by docosahexaenoic acid (DHA). Three-day cultures were treated with kainic acid for 24 h; photoreceptors were then pre-incubated with the ERK kinase (MEK) inhibitor U0126 (10 μM) for 30 min and then stimulated with either DHA or bovine serum albumin (BSA) for 10 min. Total protein extracts (10 μg/lane) were analyzed by western blot: (a) upper panels show immunodetection using an anti-pERK1/2 antibody and lower panels show immunodetection using anti-total-ERK1/2 (western blots were stripped and re-probed with an anti-ERK1/2 antibody). ERK activation was partially blocked by U0126 leading to a 50% reduction in the intensity of pERK immunolabeling, compared with cultures stimulated with DHA alone. (b) Densitometric scanning of the p42ERK2 phosphorylation shown in (a). Western blots are representative of three independent experiments with similar results. *Statistically significant differences, compared with control ($p < 0.05$).

cell survival. On the contrary, our results show that these inhibitors did not reduce the DHA protection of photoreceptors. Concurrently, LY294002 and wortmannin increased the apoptosis of amacrine neurons, which do depend on this pathway for their survival (Politi *et al*. 2001c), demonstrating that they effectively inhibited PI3K. This indicates that DHA did not stimulate the PI3K pathway to prevent photoreceptor death, and suggests that DHA might activate distinct signaling pathways in different cell types. Altogether, these results lead us to propose that the ERK/MEK pathway was the only signaling pathway involved in promoting retinal photoreceptor survival after DHA stimulation.

Increasing evidence has demonstrated the role of mitochondria in the regulation of apoptotic cell death (reviewed in Budihardjo *et al*. 1999; Hengartner 2000; Green and Kroemer 2004). Preservation of the mitochondrial membrane potential was crucial for the prevention of photoreceptor apoptosis; its loss was parallel to the increase in photoreceptor apoptosis during early development *in vitro* and upon oxidative damage, and DHA protection was closely related to the avoidance of mitochondrial depolarization. In parallel to the inhibition of DHA protection, U0126 markedly reduced

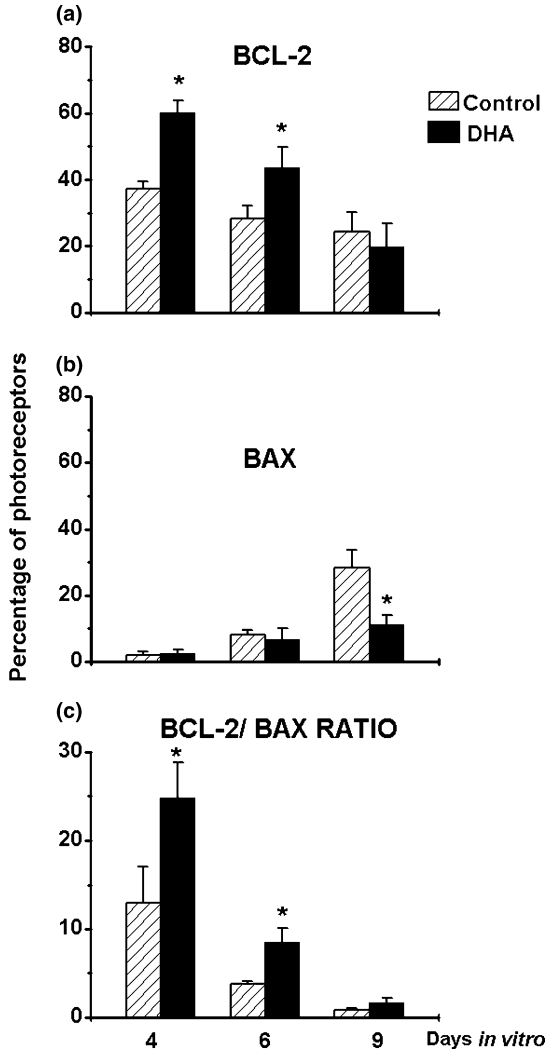

Fig. 10 Docosahexaenoic acid (DHA) regulated Bcl-2 and Bax expression in photoreceptors in culture. The bars show the percentage of photoreceptors expressing Bcl-2 (a), Bax (b), quantified by using specific monoclonal antibodies, and the Bcl-2/Bax ratio (c), in cultures either without (control) or with DHA at different times *in vitro*. *Statistically significant differences, compared with control ($p < 0.01$; $n = 5$).

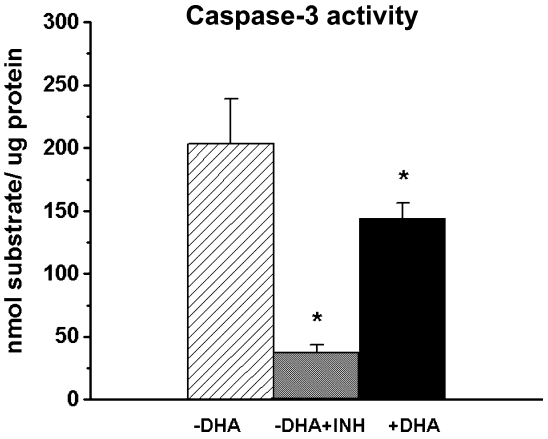

Fig. 11 Docosahexaenoic acid (DHA) decreased caspase-3 activity. Neuronal cultures were supplemented at day 1 either with (+DHA) or without (–DHA) DHA, and then treated at day 6 with kainic acid for 1 h. Cell lysates were incubated with a caspase-3 substrate in either the absence or presence of Ac-DEVD-CHO, a caspase-3 inhibitor (–DHA + INH) and then caspase activity was analyzed by a fluorimetric assay. The bars depict the activity of caspase-3, shown as nmoles of product/μg photoreceptor protein. Note that although DHA partially blocked caspase-3 activity, Ac-DEVD-CHO almost completely blocked this activity in cultures lacking DHA.

the percentage of photoreceptors with intact mitochondria. This is consistent with DHA activating a signaling pathway able to block an early event in apoptosis induction, upstream of mitochondria depolarization.

Increasing evidence supports the relevant role of the Bcl-2 family in the regulation of apoptosis during retina development. This apoptosis is almost completely absent in *Bax–/–* retina (Hahn *et al*. 2003), and is effectively prevented by the overexpression of Bcl-2 (Strettoi and Volpini 2002). The role of Bcl-2 family members appears to be different in mature and degenerating retinas (Donovan *et al*. 2006). No Bcl-2 expression was found in the photoreceptors of mature rat retinas (Chen *et al*. 1999), and conflicting data exists on the role of Bcl-2 in retinal degeneration. Although Bcl-2 overexpression has been shown to enhance photoreceptor survival in several animal models of retinal degeneration (Chen *et al*. 1996; Nir *et al*. 2000), other studies show either little or no protection (Joseph and Li 1996; Tsang *et al*. 1997). We here show that the variations in the levels of Bcl-2 and Bax observed in photoreceptors during their early development *in vitro* were consistent with the changes in apoptosis and in mitochondrial potential. Bax levels noticeably increased during development and also upon oxidative damage (Rotstein *et al*. 2003); on the contrary, the Bcl-2/Bax ratio decreased, accompanying the increase in apoptosis. DHA protection correlated with an increase in Bcl-2 expression in photoreceptors, and a concomitant prevention of the increase in Bax expression. This led to an increase in the Bcl-2/Bax ratio, which was probably related to DHA preservation of mitochondrial functionality at this early developmental time. As development proceeded, DHA failed to keep a high Bcl-2/Bax ratio and this decrease accompanied mitochondrial depolarization and photoreceptor apoptosis. DHA protection upon oxidative stress at an early developmental time similarly involved the up-regulation of Bcl-2 levels and down-regulation of those of Bax (Rotstein *et al*. 2003) in photoreceptors, and an enhancement of Bcl-2 and Bcl-x_L expression in pigment epithelium cells (Mukherjee *et al*. 2004). Our results suggest that the effectiveness of DHA as a survival molecule is closely related to its capacity to maintain high Bcl-2 levels in photoreceptors, and emphasize the relevance of the relative levels of Bcl-2 and Bax in

preserving mitochondrial polarization and, hence, photoreceptor survival during early development.

Caspase involvement in retinal cell death is still a matter of considerable controversy. Caspase-3, one of the main effector caspases, responsible for many of the proteolytic events of apoptosis, is activated in rat retinas subjected to intense light exposure (Chang *et al*. 2005). However, conflicting results exist regarding its involvement in photoreceptor death during development and in animal models of retinal degeneration (Liu *et al*. 1999; Carmody and Cotter 2000; Bode and Wolfrum 2003; Sanvicens *et al*. 2004; Zeiss *et al*. 2004; Donovan *et al*. 2006). We have determined that caspase-3 was active in 6-day-old photoreceptors, a time of development at which about 60% of photoreceptors are already apoptotic. The addition of DHA partially decreased caspase-3 activity, without blocking it completely. Similar results, showing the down-regulation of caspase-3 activity by DHA, were described in neuroblastoma cells (Kim *et al*. 2000). This partial reduction was consistent with the inability of DHA to completely prevent photoreceptor cell death, but rather to postpone it during early development *in vitro*. As caspase activation in the mitochondrial pathway is closely dependent on mitochondrial outer membrane permeabilization (reviewed in Hengartner 2000; Green and Kroemer 2004), the preservation of mitochondrial membrane barrier properties was probably the cause of this reduction in caspase-3 activity. This protection prevented the exit of death factors from mitochondria, thus precluding caspase-3 activation.

As a whole, our results suggest that DHA promotes photoreceptor survival and differentiation by the same mechanisms used by well-known peptidic trophic factors. Interestingly, this lipid survival molecule coincides with other photoreceptor trophic factors, such as CNTF and FGF2, in the activation of the ERK/MAPK survival pathway to prevent photoreceptor death. Sensory neurons have been shown to switch their trophic requirements, but preserve the signaling pathways mediating their survival (Salvarezza *et al*. 2003). As photoreceptors depend on several trophic factors for their survival, it can be proposed that these trophic molecules might act during development in either a sequential or overlapping manner, through the activation of different receptors, and, in spite of this trophic switch, the same signaling pathway is conserved to stimulate the cellular machinery leading to survival.

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References

Abrams L., Politi L. and Adler R. (1989) Differential susceptibility of isolated mouse retinal neurons and photoreceptors to kainic acid toxicity. *In vitro* studies. *Invest. Ophthalmol. Vis. Sci.* **30**, 2300–2308.

Akbar M., Calderon F., Wen Z. and Kim H.-Y. (2005) Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival. *Proc. Natl Acad. Sci. USA* **102**, 10858–10863.

Altschuler D., Lo Turco J. J. and Cepko C. (1993) Taurine promotes the differentiation of a vertebrate retinal cell type *in vitro*. *Development* **119**, 1317–1328.

Barnstable C. J. (1980) Monoclonal antibodies which recognize different cell types in the rat retina. *Nature* **286**, 231–235.

Bernardi P., Petronilli V., Di Lisa F. and Forte M. (2001) A mitochondrial perspective on cell death. *Trends Biochem. Sci.* **26**, 112–117.

Bode C. and Wolfrum U. (2003) Caspase-3 inhibitor reduces apototic photoreceptor cell death during inherited retinal degeneration in tubby mice. *Mol. Vis.* **9**, 144–150.

Bradford M. (1996) A rapid and sencitive method for quantification of microgram quantities of proteins utilizing the principe of protein binding. *Analit. Biochem.* **72**, 248–254.

Budihardjo I., Oliver H., Lutter M., Luo W. and Wang X. (1999) Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell Dev. Biol.* **15**, 269–290.

Carmody R. J. and Cotter T. G. (2000) Oxidative stress induces caspase-independent retinal apoptosis *in vitro*. *Cell Death Differ.* **7**, 282–291.

Chang L. and Karin M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**, 37–40.

Chang C. J., Cherng C. H., Liou W. S. and Liao C. L. (2005) Minocycline partially inhibits caspase-3 activation and photoreceptor degeneration after photic injury. *Ophthalmic. Res.* **37**, 202–213.

Chen J., Flannery J. G., La Vail M. M., Steinberg R. H., Xu J. and Simon M. I. (1996) bcl-2 overexpression reduces apoptotic photoreceptor cell death in three different retinal degenerations. *Proc. Natl Acad. Sci. USA* **93**, 7042–7047.

Chen S.-T., Wang J. P., Garey L. J. and Jen L. S. (1999) Expression of β-amyloid precursor and Bcl-2 proto-oncogene proteins in rat retinas after intravitreal injection of aminoadipic acid. *Neurochem. Int.* **35**, 371–382.

Desire L., Courtois Y. and Jeanny J. C. (2000) Endogenous and exogenous fibroblast growth factor 2 support survival of chick retinal neurons by control of neuronal bcl-x (L) and bcl-2 expression through a fibroblast growth factor receptor 1- and ERK-dependent pathway. *J. Neurochem.* **75**, 151–163.

Donovan M., Doonan F. and Cotter T. G. (2006) Decreased expression of pro-apoptotic Bcl-2 family members during retinal development and differential sensitivity to cell death. *Dev. Biol.* **291**, 154–169.

Fontaine V., Kinkl N., Sahel J., Dreyfus H. and Hicks D. (1998) Survival of purified rat photoreceptors *in vitro* is stimulated directly by fibroblast growth factor-2. *J. Neurosci.* **18**, 9662–9672.

Frasson M., Picaud S., Leveillard T., Simonutti M., Mohand-Said S., Dreyfus H., Hicks D. and Sahel J. (1999) Glial cell line-derived neurotrophic factor induces histologic and functional protection of rod photoreceptors in the rd/rd mouse. *Invest. Ophthalmol. Vis. Sci.* **40**, 2724–2734.

Gentili C., Morelli S., Boland R. and Russo de Boland A. (2001) Parathyroid hormone activation of map kinase in rat duodenal cells

is mediated by 3P,5P-cyclic AMP and Ca2. *Biochimica Biophysica Acta* **1540**, 201–212.

Green D. R. and Kroemer G. (2004) The patophysiology of mitochondrial cell death. *Science* **305**, 626–629.

Hahn P., Lindsten T., Ying G. S., Bennett J., Milam A. H., Thompson C. B. and Dunaief J. L. (2003) Proapoptotic bcl-2 family members, Bax and Bak, are essential for developmental photoreceptor apoptosis. *Invest. Ophthalmol. Vis. Sci.* **44**, 3598–3605.

Hengartner M. O. (2000) The biochemistry of apoptosis. *Nature* **407**, 770–776.

Hernández-Sánchez C., Lopez-Carranza A., Alarcón C., de La Rosa E. J. and de Pablo F. (1995) Autocrine/paracrine role of insulin-related growth factors in neurogenesis: local expression and effects on cell proliferation and differentiation in retina. *Proc. Natl Acad. Sci. USA* **92**, 9834–9838.

Hicks D. and Barnstable C. J. (1987) Different rhodopsin monoclonal antibodies reveal different binding patterns on developing and adult rat retina. *J. Hystochem. Cytochem.* **35**, 1317–1328.

Insua M. F., Garelli A., Rotstein N. P., German O. L., Arias A. and Politi L. E. (2003) Cell cycle regulation in retinal progenitors by Glia-Derived Neurotrophic Factor and Docosahexaenoic Acid. *Invest. Ophthalmol. Vis. Sci.* **44**, 2235–2244.

Jablonski M. M., Tombran-Tink J., Mrazek D. A. and Iannaccone A. (2000) Pigment epithelium-derived factorsupports normal developmentof photoreceptor neurons and opsin expression after retinal pigment epithelium removal. *J. Neurosci.* **20**, 7149–7157.

Joseph R. M. and Li T. (1996) Overexpression of Bcl-2 or Bcl-XL transgenes and photoreceptor degeneration. *Invest. Ophthalmol. Vis. Sci.* **37**, 2434–2446.

Kim H. Y., Akbar M., Lau A. and Edsall L. (2000) Inhibition of neuronal apoptosis by docosahexaenoic acid (22: 6n−3). Role of phosphatidylserine in antiapoptotic effect. *J. Biol. Chem.* **275**, 35 215–35 223.

Kinkl N., Sahel J. and Hicks D. (2001) Alternate FGF2-ERK1/2 signalling pathways in retinal photoreceptors and glial cells *in vitro*. *J. Biol. Chem.* **276**, 43871–43878.

Kljavin I. J., Lagenaur C., Bixby J. L. and Reh T. A. (1994) Cell adhesion molecules regulating neurite growth from amacrine and rod photoreceptor cells. *J. Neurosci.* **14**, 5035–5049.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lavail M. M., Yasumura D., Matthes M. T., Lau-Villacorta C., Unoki K., Sung C. H. and Steinberg R. H. (1998) Protection of mouse photoreceptors by survival factors in retinal degeneration. *Invest. Ophthalmol. Vis. Sci.* **39**, 592–602.

Liu C., Peng M., Laties A. M. and Wen R. (1998) Preconditioning with bright light evokes a protective response against light damage in the rat retina. *J. Neurosci.* **18**, 1337–1344.

Liu C., Li Y., Peng M., Laties A. M. and Wen R. (1999) Activation of Caspase-3 in the Retina of Transgenic Rats with the Rhodopsin Mutation S334ter during Photoreceptor Degeneration. *J. Neurosci.* **19**, 4778–4785.

Mata de Urquiza A., Liu S., Sjoberg M., Zetterstrom R. H., Griffiths W., Sjovall J. and Perlmann T. (2000) Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* **290**, 2140–2144.

Morrow E. M., Furukawa T. and Cepko C. L. (1998) Vertebrate photoreceptor cell development and disease. *Trends Cell Biol.* **8**, 353–358.

Mukherjee P. K., Marcheselli V. L., Serhan C. N. and Bazán N. G. (2004) Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc. Natl Acad. Sci. USA* **101**, 8491–8496.

Neuringer M., Connor W. E., Van Petten C. and Barstad L. (1984) Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. *J. Clin. Invest.* **73**, 272–276.

Neuringer M., Sturman J. A., Wen G. Y. and Wisniewski H. M. (1985) Dietary taurine is necessary for normal retinal development in monkeys. *Prog. Clin. Biol. Res.* **179**, 53–62.

Nir I., Kedzierski W., Chen J. and Travis G. M. (2000) Expression of Bcl-2 protects against photoreceptor degeneration in retinal degeneration slow (rds) mice. *J. Neurosci.* **20**, 2150–2154.

O'Shea J. J., Gadina M. and Schreiber R. D. (2002) Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* **109**, S121–S131.

Politi L. E., Rotstein N. P. and Carri N. G. (2001a) Effects of GDNF on neuroblast proliferation and photoreceptor survival: additive protection with docosahexaenoic acid. *Invest. Ophtahlmol. Vis. Sci.* **42**, 3008–3015.

Politi L. E., Rotstein N. P. and Carri N. (2001b) Effects of docosahexaenoic acid on retinal development: cellular and molecular aspects. *Lipids* **36**, 927–935.

Politi L. E., Rotstein N. P., Salvador G., Giusto N. M. and Insua M. F. (2001c) Insulin-like growth factor-I is a potential trophic factor for amacrine cells. *J. Neurochem.* **76**, 1199–1211.

Rhee K. D. and Yang X. J. (2003) Expression of cytokine signal transduction components in the postnatal mouse retina. *Mol. Vis.* **9**, 715–722.

Rhee K. D., Goureau O., Chen S. and Yang X. J. (2004) Cytokine-induced activation of signal transducer and activator of transcription in photoreceptor precursors regulates rod differentiation in the developing mouse retina. *J. Neurosci.* **24**, 9779–9788.

Rotstein N. P., Aveldaño M. I., Barrantes F. J. and Politi L. E. (1996) Docosahexaenoic acid is required for the survival of rat retinal photoreceptors *in vitro*. *J. Neurochem.* **66**, 1851–1859.

Rotstein N. P., Aveldaño M. I., Barrantes F. J., Roccamo A. M. and Politi L. E. (1997) Apoptosis of retinal photoreceptors during development *in vitro*: protective effect of docosahexaenoic acid. *J. Neurochem.* **69**, 504–513.

Rotstein N. P., Politi L. E. and Aveldaño M. I. (1998) Docosahexaenoic acid promotes differentiation of developing photoreceptors in culture. *Invest. Ophthalmol. Vis. Sci.* **39**, 2750–2758.

Rotstein N. P., Politi L. E., German O. L. and Girotti R. (2003) Docosahexaenoic acid protects retina photoreceptors from oxidative stress-induced apoptosis. *Invest. Ophthalmol. Vis. Sci.* **44**, 2252–2259.

Salvarezza S. B., Lopez H. S. and Masco D. H. (2003) The same cellular signaling pathways mediate survival in sensory neurons that switch their trophic requirements during development. *J. Neurochem.* **85**, 1347–1458.

Sanvicens N., Gomez-Vicente V., Masip I., Messeguer A. and Cotter T. G. (2004) Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6. *J. Biol. Chem.* **279**, 39 268–39 278.

Strettoi E. and Volpini M. (2002) Retinal organization in the bcl-2-overexpressing transgenic mouse. *J. Comp. Neurol.* **446**, 1–10.

Tsang S. H., Chen J., Kjeldbye H., Li W. S., Simon M. I., Gouras P. and Goff S. P. (1997) Retarding photoreceptor degeneration in Pdegtm1/Pdegtml mice by an apoptosis suppressor gene. *Invest. Ophthalmol. Vis. Sci.* **38**, 943–950.

Uauy R. D., Birch D. G., Birch E. E., Tyson J. E. and Hoffman D. R. (1990) Effect of dietary omega-3 fatty acids on retinal function of very-low-birth-weight neonates. *Pediatr. Res.* **28**, 485–492.

Valter K., Bisti S., Gargini C., Di Loreto S., Maccarone R., Cervetto L. and Stone J. (2005) Time course of neurotrophic factor upregulation and retinal protection against light-induced damage after optic nerve section. *Invest. Ophthalmol. Vis. Sci.* **46**, 1748–1754.

Yu X., Rajala R. V., Mcginnis J. F., Li F., Anderson R. E., Yan X., Lis S., Elias R. V., Knapp R. R., Zhou X. and Cao W. (2004) Involvement of insulin/phosphoinositide 3-kinase/Akt signal pathway in 17 β-estradiol-mediated neuroprotection. *J. Biol. Chem.* **279**, 13 086–13 094.

Zeiss C. J., Neal J. and Johnson E. A. (2004) Caspase-3 in postnatal retinal development and degeneration. *Invest. Opthalmol. Vis. Sci.* **45**, 964–970.

