

# Effect of GDNF on Neuroblast Proliferation and Photoreceptor Survival: Additive Protection with Docosahexaenoic Acid

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**PURPOSE.** In a previous study, it was reported that docosahexaenoic acid (DHA) is essential to postpone apoptosis and to promote differentiation of rat retina photoreceptors *in vitro*. In the current study, the protective effects of GDNF on photoreceptor cells during development *in vitro* and its action when combined with DHA were investigated.

**METHODS.** Rat retina neuronal cultures were incubated in a chemically defined medium, either without photoreceptor survival factors or supplemented with GDNF, DHA, or GDNF plus DHA. Evolution of survival, apoptosis, opsin expression, mitochondrial functioning, and cell proliferation were investigated at different times of development *in vitro*.

**RESULTS.** Incubation with GDNF selectively increased the number of surviving photoreceptors, reduced their apoptosis, and augmented opsin expression. Proliferative cell nuclei antigen (PCNA) determination and addition of [<sup>3</sup>H]-thymidine or bromodeoxyuridine showed that GDNF promoted neuroblast proliferation during the first hours of development *in vitro*. The combined addition of GDNF and DHA enhanced opsin expression and photoreceptor survival in an additive manner. The advance of photoreceptor apoptosis in cultures without trophic factors correlated with an increased impairment in mitochondrial functionality. Addition of GDNF and DHA significantly diminished the loss of mitochondrial activity.

**CONCLUSIONS.** These results show that GDNF stimulated the cell cycle progression, leading to neuroblast proliferation at early stages of development, and delayed the onset of apoptosis later on, improving differentiation and acting as a trophic factor for photoreceptors. The combination of GDNF with DHA had an additive effect both on photoreceptor survival and on opsin expression. Preservation of mitochondrial function may be involved in the antiapoptotic effect of both factors. (*Invest Ophthalmol Vis Sci.* 2001;42:3008-3015)

During the past few years, our knowledge of the trophic factors (TFs) required by retina photoreceptors has increased enormously. Several molecules, such as taurine, retinoic acid, basic fibroblast growth factor (FGF), brain-derived

neurotrophic factor, ciliary neurotrophic factor (CNTF), and interleukin-1 $\beta$  have been implicated in the survival and differentiation of these cells.<sup>1-3</sup> Previous work in our laboratory has shown that docosahexaenoic acid (DHA), the most abundant polyunsaturated fatty acid in photoreceptors,<sup>4</sup> is also essential for survival *in vitro* of these cells.<sup>5-7</sup> When cultured in chemically defined media in the absence of TFs, photoreceptors develop and differentiate normally for approximately 4 days; then, most of these cells selectively undergo a degeneration process that proceeds by apoptosis. DHA acts as a trophic molecule for photoreceptors. Its addition leads to a notorious delay in the onset of apoptosis in these cells and to an increase in their differentiation, enhancing opsin expression and promoting apical differentiation.<sup>6,7</sup>

In spite of the beneficial effects of added DHA, photoreceptor apoptosis still progresses, although at a slower rate, clearly implying that other trophic molecules are required for sustaining the development of these cells. A promising candidate is glial derived neurotrophic factor (GDNF), a distant member of the transforming growth factor (TGF)- $\beta$  family that has potent neurotrophic effects on several neuronal types,<sup>8,9</sup> including dopamine and motor neurons,<sup>10-12</sup> along with protective effects in *in vivo* models of Parkinson disease.<sup>13-15</sup> Retinal cells synthesize GDNF<sup>9</sup> and express the GDNF- $\alpha$  receptor for this TF, which in turn activates the Ret protein tyrosine kinase.<sup>16</sup> Moreover, GDNF has recently been shown to increase the number of viable mouse retinal photoreceptors in culture<sup>16</sup>; to protect rod photoreceptors in animal models of retinitis pigmentosa, a neurodegenerative disease affecting these cells<sup>17</sup>; and to improve rod outer segment maintenance.<sup>18</sup>

Besides their well-known effects on neuronal survival and differentiation, several TFs participate in the control of neuronal proliferation. In the retina, different growth factors, such as TGF- $\alpha$  and - $\beta$ 3, epidermal growth factor, and both basic and acidic FGFs promoted progenitor cell proliferation at different periods of development in rodents,<sup>19-21</sup> whereas insulin, its related growth factors, and neurotrophin (NT)-3 had a similar proliferative effect in fish and chick retinal cultures.<sup>22-24</sup> Because GDNF is a member of the TGF- $\beta$  family, its possible role as a promoter of cell proliferation was worth investigating.

Previous works have shown that some TFs, when added simultaneously, display synergistic protective effects on photoreceptors,<sup>25,26</sup> suggesting that these neurons may require a concert of different TFs during development. The identification of the main TFs required by photoreceptors, their possible interactions, and the molecular pathways activated during survival and death in photoreceptors remains to be determined. In this work, we investigated whether GDNF could stimulate photoreceptor survival, proliferation, and differentiation and whether its effects are modified when combined with DHA.

## MATERIALS AND METHODS

### Materials

Albino Wistar rats (1-2 days old) bred in our own colony were used in all the experiments. All proceedings concerning animal use were in

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accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Plastic 35-mm diameter culture dishes and multichambered slides (Nunc) were purchased from Inter Med (Naperville, IL). Fetal calf serum (FCS) was from Centro de Virología Animal (CEVAN, Buenos Aires, Argentina). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco-Life Technologies (Grand Island, NY). Trypsin, trypsin inhibitor, transferrin, hydrocortisone, putrescine, insulin, polyornithine, selenium, gentamicin, 4,6-diamidino-2-phenylindole (DAPI), fluorescein-conjugated secondary antibodies, propidium iodide, bromodeoxyuridine (BrdU), and paraformaldehyde were from Sigma (St. Louis, MO). Secondary antibody (Alexa 488 conjugated-goat anti-mouse) and red mitochondrial stain (MitoTracker; CMXRos) were from Molecular Probes (Eugene, OR). GDNF was from Peptotech, Inc. (Rocky Hill, NJ). Mouse monoclonal antibody against the proliferating cell nuclear antigen (PCNA, p36 antigen) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies HPC-1 and Rho4D2 were generous gifts from Colin Barnstable (Yale University, New Haven, CT) and Robert Molday (University of British Columbia, Vancouver, Canada), respectively. DHA was isolated from bovine retinas by a combination of chromatographic procedures.<sup>5</sup> [<sup>3</sup>H]-thymidine, (specific activity, 17.9 Ci/mmol) was from New England Nuclear (Boston, MA). NTB2 autoradiographic emulsion (NTB2) and developer (Dektol) were from Eastman Kodak (Rochester, NY). All other reagents used were of analytical grade.

### Retinal Cultures

Pure retinal cultures were obtained according to procedures previously established.<sup>27</sup> In brief, rat retinas were dissected and dissociated under mechanical and trypsin digestion. After dissociation, the cells were resuspended in a chemically defined medium, without the specific TFs required for photoreceptor cells, as previously described.<sup>5,27</sup> The resultant cell suspension was seeded on 35-mm diameter dishes, sequentially pretreated with polyornithine (0.1 mg/ml) and schwannoma-conditioned medium.<sup>28</sup> Cultures were incubated at 36°C in a humidified atmosphere of 5% CO<sub>2</sub>. At different times, neurons were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS), and the number of amacrine and photoreceptor neurons, the two major cell types in the cultures, was determined. Neuronal cell types were identified by their morphology using phase-contrast microscopy and by immunocytochemistry, using the monoclonal antibodies HPC-1 and Rho4D2, which selectively react with amacrine and photoreceptor neurons, respectively.<sup>29-31</sup> Photoreceptors have a small, round cell body (3–5 μm) with a single neurite at one end that usually ends in a conspicuous synaptic spherule; sometimes they display a connecting cilium at the opposite end, but the characteristic outer segments fail to develop. Opsin is diffusely distributed over the 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 these features. Amacrine neurons are larger than photoreceptors (7–20 μm) and have multiple neurites.

### GDNF and DHA Supplementation

GDNF in DMEM was added to the cultures immediately after seeding the cells at a final concentration of 4 ng/ml, and the same volume of DMEM was added to control cultures. DHA (6.7 μM), complexed with bovine serum albumin in a 2:1 molar ratio, in DMEM, was added at day 1 *in vitro*.<sup>5</sup> The same volume and concentration of a bovine serum albumin solution were added to control cultures.

### Determination of Apoptotic Cells

Apoptotic cells were quantified by first permeating the cells with 0.1% Triton X-100 in PBS and then analyzing nuclei fragmentation by labeling nuclei for 20 minutes with DAPI, a DNA marker.

### Determination of Surviving and Dead Cells

Dead cells were determined by incubating the cultures with propidium iodide (PI), at a final concentration of 0.5 μg/ml in culture, for 30

minutes before fixing the cells.<sup>32</sup> Surviving cells were quantified, taking into account the simultaneous absence of PI-labeling plus a healthy morphologic appearance, such as that described earlier. To establish the identity of dead cells, neuronal cultures were first labeled with PI and then with the monoclonal antibodies Rho4D2 or HPC-1, and the simultaneous labeling with PI and each monoclonal antibody was determined by fluorescence microscopy.

### Identification of Proliferating Neuroblasts by Studying DNA Synthesis and G1-Cyclin Expression

Most photoreceptor progenitors complete their last cell division cycle during the first postnatal days,<sup>2,33</sup> the highest number of photoreceptors being generated at postnatal day 0. To estimate cell division of photoreceptor precursors *in vitro*, [<sup>3</sup>H]-thymidine or 5-BrdU, at a final concentration of 1 μCi/ml and 50 μM, respectively, were added to the cultures immediately after the cells were seeded and left for 24 hours at 36°C. Longer incubations with BrdU (i.e., 48 hours) were performed to assess the fate of proliferating precursors as either photoreceptors or amacrine neurons. The cultures were finally fixed with either paraformaldehyde or 2% glutaraldehyde in PBS. To determine cell labeling with [<sup>3</sup>H]-thymidine, cultures fixed with 2% glutaraldehyde were dehydrated with increasing concentrations of ethanol, exposed to autoradiographic emulsion (NTB2) for 15 days in the dark, developed for 3 minutes (Dektol), washed with water, and fixed as previously described.<sup>27,34</sup> BrdU-labeled cells were estimated by immunocytochemical analysis. The number of cells expressing PCNA p36 antigen, a G1-S cyclin used as a marker for proliferating cells were detected by immunocytochemical methods using a mouse monoclonal anti-PCNA antibody.

### Mitochondrial Functionality

To assess mitochondrial functionality, cultures were incubated for 30 minutes with mitochondrial stain (0.1 μg/ml; MitoTracker), fixed with 2% paraformaldehyde and examined by fluorescence microscopy. Functional mitochondria took up the fluorescent marker and retained it, emitting a bright fluorescent signal.

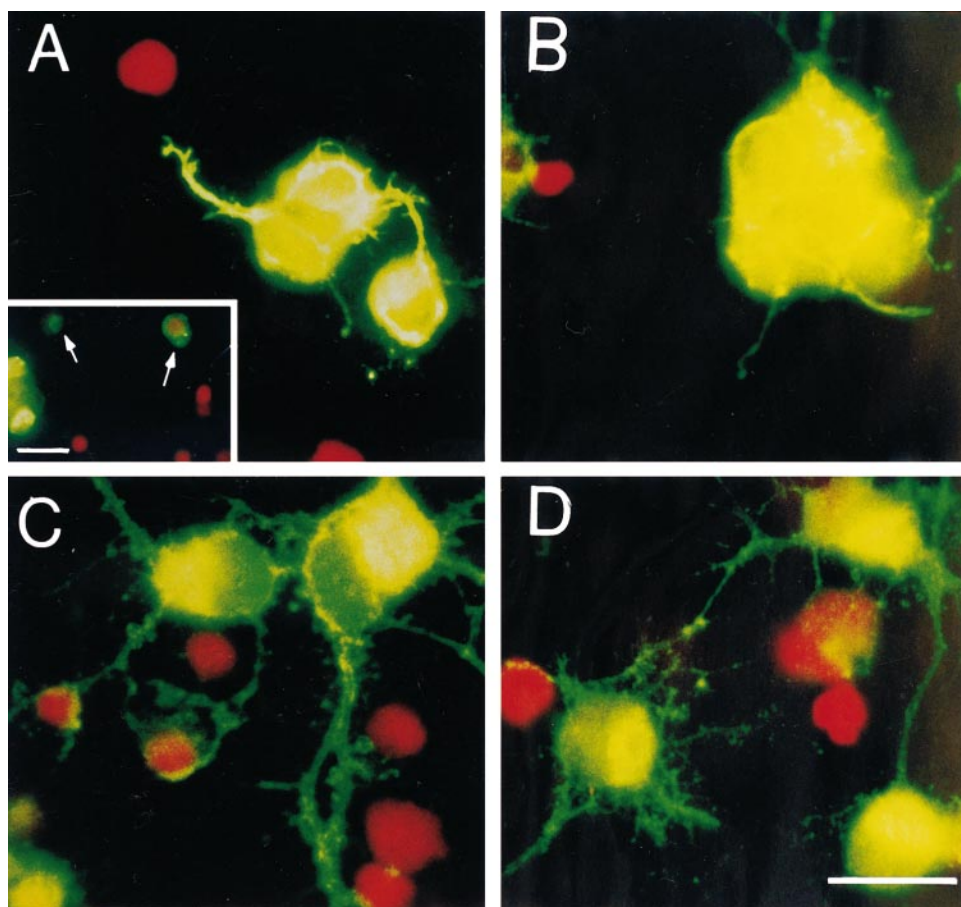
### Statistical Analysis

The results represent the average of three experiments (±SD). Unless specifically indicated, each experiment was performed in triplicate. For cytochemical studies, 10 fields per sample were analyzed in each case. Statistical significance was determined by Student's two-tailed *t*-test.

## RESULTS

### Effect of GDNF on Survival of Photoreceptor Cells

Dissociated retinal cells grown in the chemically defined medium used in these studies give rise to a pure neuronal culture, mainly comprising photoreceptors and amacrine cells, together adding up to approximately 95% of the total cells, as previously described.<sup>5-7</sup> Two other populations of presumptive bipolar and ganglion cells, jointly representing less than 5% of the cells, can also be observed. After 4 days *in vitro*, photoreceptors start an apoptotic pathway leading to the death of most of them by day 14.<sup>6</sup> After 7 days *in vitro*, control cultures showed a high number of dead PI-labeled cells (red-labeled cells in Figs. 1A, 1C). Double labeling of the cultures with PI and monoclonal antibodies Rho4D2, for photoreceptor cells, or HPC-1, for amacrine cells, showed that many PI-positive cells were also labeled with Rho4D2 (Fig. 1A, inset; Table 1), but not with HPC-1—evidence that most PI-positive cells were photoreceptors. Addition of GDNF significantly decreased the amount of PI-labeled cells and of PI- and Rho4D2 double-labeled photoreceptors (Figs. 1B, 1D; Table 1). GDNF also



**FIGURE 1.** GDNF selectively stimulates opsin expression and protects photoreceptors against cell death. Fluorescence photomicrographs of 7-day cultures of retinal cells treated without (A, C), or with (B, D) GDNF and double-stained with propidium iodide (red cells) and with the monoclonal antibodies Rho4D2 (A, B) or HPC-1 (C, D), which selectively recognize photoreceptor and amacrine neurons, respectively. The increase in opsin expression in GDNF-treated cultures is clearly visible. *Inset:* 7-day culture double-stained with Rho4D2 and propidium iodide. *Arrows:* dead photoreceptors expressing both Rho4D2 and propidium iodide. Scale bar, 20  $\mu$ m.

promoted expression of the visual protein opsin; more opsin-expressing photoreceptors were observed in GDNF-treated than in control cultures (Fig. 1B). Amacrine neurons, which depend on insulin-like growth factor (IGF)-1 or insulin for their survival,<sup>35</sup> continued their growth and development for up to 12 to 14 days in culture, unaffected by addition of GDNF (Figs. 1C, 1D; Table 1). Therefore, GDNF effects were selective for photoreceptor cells.

### Effect of GDNF and DHA on Apoptosis and Opsin Expression

To investigate the combined effect of GDNF and DHA, photoreceptor survival, opsin expression, and evolution of apoptosis were analyzed at day 11 in control cultures and in cultures

**TABLE 1.** GDNF's Effect on the Amount of Propidium Iodide-Labeled Photoreceptors

	Photoreceptors (%)	Amacrine Neurons (%)
Control	60.4 $\pm$ 7.0	3.84 $\pm$ 1.6
GDNF added	35.7 $\pm$ 6.7*	4.09 $\pm$ 0.6

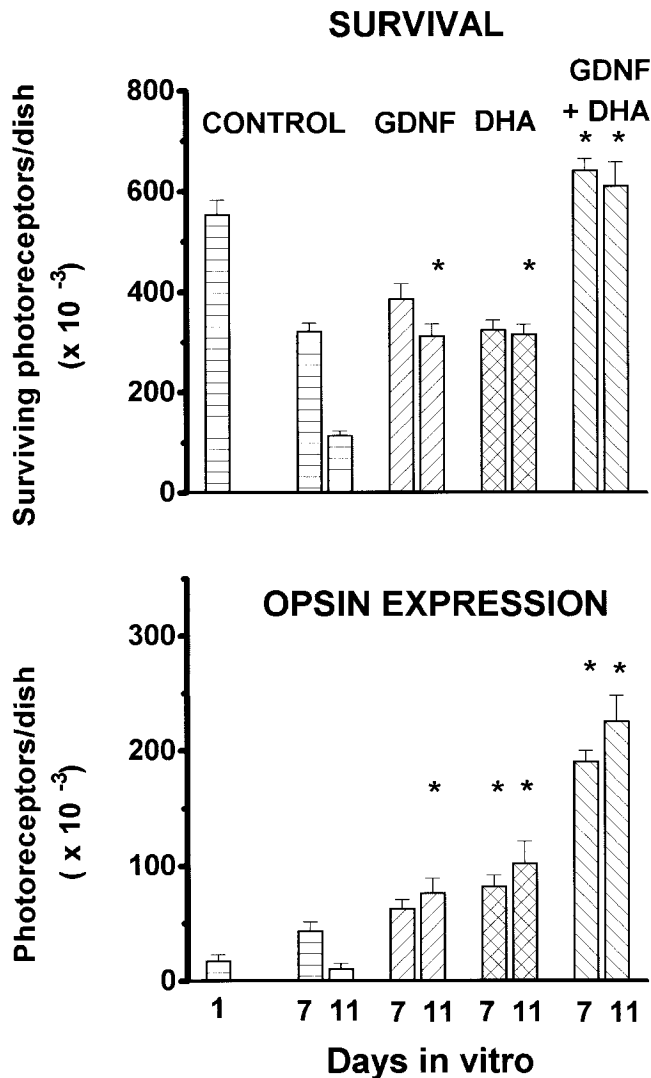
Retinal cultures, treated without (control) or with GDNF added at day 0 in vitro were analyzed after 7 days. The table depicts the percentage of photoreceptors simultaneously labeled with PI and Rho4D2 compared with the total number of Rho4D2-labeled photoreceptors, and the percentage of amacrine cells simultaneously labeled with HPC-1 and PI compared with the total number of HPC-1-labeled amacrine cells. The decrease in the percentage of dead PI-labeled photoreceptor cells after GDNF treatment is significant.

\* Significant difference compared with control conditions ( $P < 0.05$ ).

supplemented with GDNF, DHA, or both factors together. Analysis of photoreceptor survival showed that approximately 550,000 cells per dish differentiated as photoreceptors at day 0 (Fig. 2, top), and this number decreased to more than 300,000 cells per dish by day 7, in both control and GDNF- or DHA-treated cultures. After this time, photoreceptor degeneration progressed steadily in control cultures, but degeneration was almost completely stopped in GDNF-supplemented cultures. Thus, only 110,000 photoreceptors per dish survived by day 11 in control cultures (Fig. 2, top) and just approximately 10% of them expressed opsin at this time (Figs. 2, bottom; 3A). Addition of GDNF protected photoreceptor cells, increasing their survival and the amount of these cells that expressed opsin (Figs. 2, 3C, 3D). At day 11, the number of photoreceptors still present in the cultures was only slightly lower than at day 7 (Fig. 2, top), and approximately 25% of them expressed opsin (Fig. 2, bottom). The effects of DHA on photoreceptor survival and opsin expression were quantitatively similar to those of GDNF (Fig. 2).

The combined addition of GDNF plus DHA dramatically increased both the number of photoreceptors and their opsin expression (Fig. 2); approximately 640,000 photoreceptors per dish were present in the cultures at day 7, slightly more than those determined at day 0, and almost all of them still survived after 11 days in vitro. In addition, the amount of photoreceptors expressing opsin was strikingly higher than in control cultures (Figs. 2, 3G, 3H): nearly 40% of photoreceptors showed opsin expression. Therefore, both GDNF and DHA by themselves promoted photoreceptor survival and opsin expression, and these effects were additively enhanced when both factors were added together. On the other hand, the





**FIGURE 2.** GDNF and DHA promote photoreceptor survival and opsin expression. Retinal cultures were treated without (control) or with GDNF, DHA, or GDNF plus DHA, and the number of surviving (*top*) or opsin-expressing (*bottom*) photoreceptors was determined at 0, 7, and 11 days in culture. The effect of GDNF and DHA on survival and opsin expression was additive. \*Significant differences from the corresponding day in control conditions ( $P < 0.05$ ).

number of amacrine neurons was almost the same at the different times and in every condition studied.

We have previously demonstrated that under control conditions, photoreceptor degeneration follows an apoptotic pathway.<sup>6</sup> This is clearly evident in Figure 3B, where most of these cells showed either pyknotic or fragmented nuclei. The percentage of apoptotic photoreceptors rose steadily with time in vitro, increasing from 26% to 85% between days 4 and 11, respectively (Fig. 4, top). Addition of GDNF reduced the percentage of photoreceptors progressing to apoptosis to approximately 70% at day 11. This protective effect of GDNF was similar to the effect of DHA on apoptosis (Figs. 3E, 3F, 4, top<sup>6</sup>). The combined addition of GDNF and DHA led to a decrease in the number of apoptotic photoreceptors, resembling that promoted by each factor by itself (Fig. 4, top). Hence, these TFs, either individually or combined, partially blocked the advance of apoptosis in culture, showing no additive effect on this blockade.

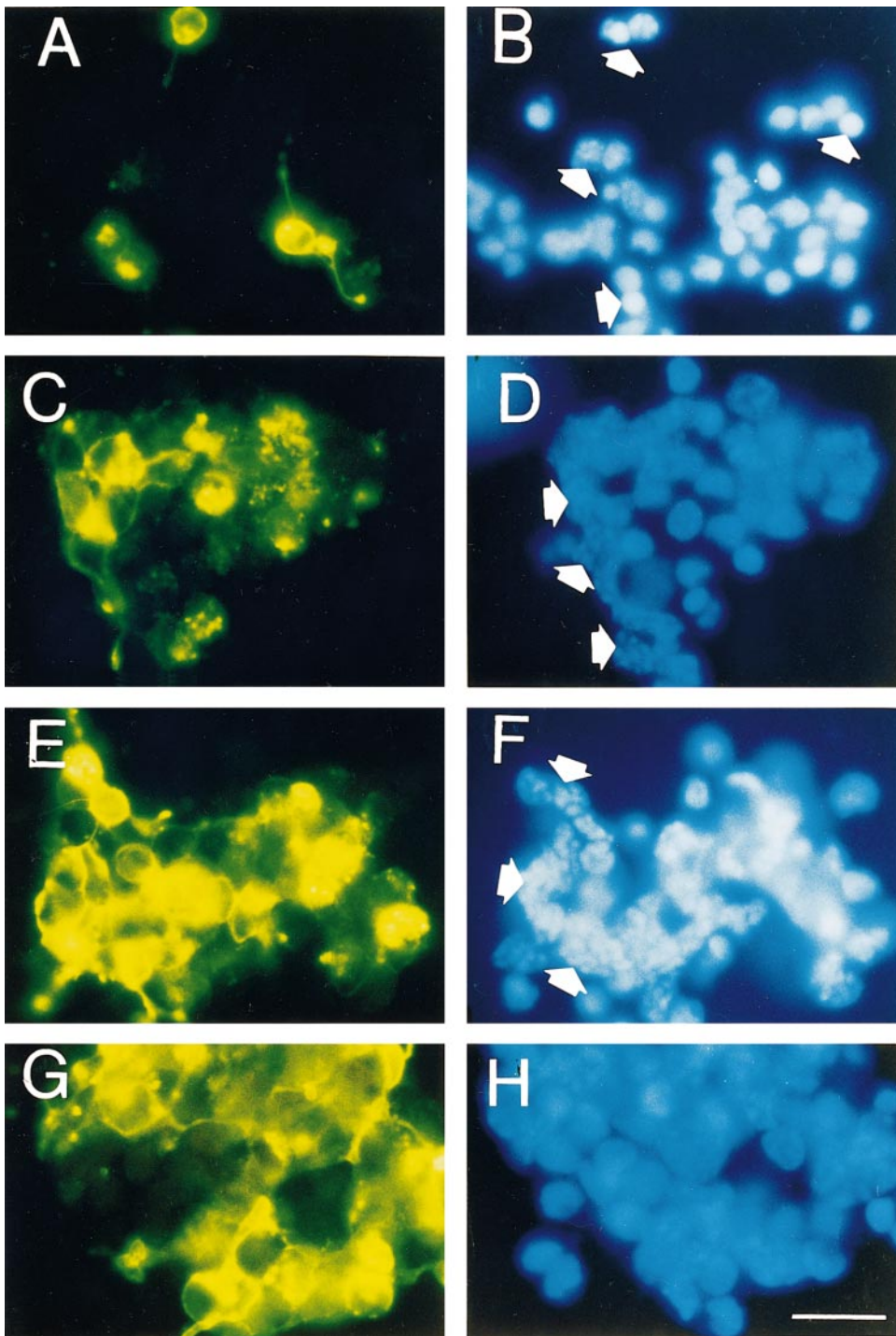
### Protection of Mitochondrial Functionality in Photoreceptors

Mitochondria are actively implicated in the process of apoptosis, and major changes in mitochondrial membrane integrity and permeability have been proposed as one of the hallmarks of apoptosis.<sup>36,37</sup> Searching for the intracellular pathways leading to photoreceptor apoptosis, we investigated whether mitochondrial function was affected during development in vitro of these cells. In cultures devoid of their specific TFs, apoptosis of photoreceptors ran parallel to a progressive decrease in the percentage of these cells bearing active mitochondria. Nearly 55% of photoreceptors showed normal mitochondria at day 4, and this percentage decreased steadily during development in vitro; by day 11, only approximately 10% of these cells retained functional mitochondria (Fig. 4, bottom). On the contrary, amacrine neurons, which showed no signs of apoptosis, preserved their mitochondrial activity throughout their development in culture. Their healthy appearance corresponded with the presence of brightly fluorescent mitochondria and with the absence of nuclei fragmentation (not shown). Addition of either GDNF or DHA partially prevented the loss of mitochondrial permeability in photoreceptor cells, simultaneously reducing their apoptotic progression; whereas at day 4 the amount of photoreceptors with functional mitochondria was similar in all experimental conditions and remained constant up to day 7, by day 11 this amount was three- to four times higher in GDNF- and DHA-supplemented than in control cultures (Fig. 4, bottom). When added together, GDNF and DHA preserved mitochondrial activity in about the same manner as each factor by itself (Fig. 4). Thus, the combined addition of these molecules showed no additive effect on either apoptosis advance or mitochondrial protection.

### Effect of GDNF on Neuroblast Proliferation

Noteworthy, the number of surviving photoreceptors at days 7 and 11 in cultures supplemented with both GDNF and DHA tended to be higher than those present at day 0. Because only GDNF had been added at day 0, it seemed reasonable for this factor to be responsible for an upregulation of the cell cycle progression. To find out whether GDNF could stimulate the photoreceptor cell cycle, PCNA-cyclin reactivity, which has been shown to be a useful marker for proliferating retinal cells,<sup>38</sup> was assessed in control and GDNF-treated cultures at day 1. This revealed that addition of GDNF doubled the number of proliferating neuroblasts. Nearly 10% of progenitor cells expressed PCNA antigens in control cultures at day 1, whereas this percentage increased to almost 23% after the addition of GDNF (Table 2).

To further investigate the effect of GDNF on cell proliferation, cultures were incubated for 24 hours with either [<sup>3</sup>H]-thymidine or its analogue, BrdU, added immediately after seeding the cells to assess the number of proliferating neuroblasts. Autoradiographic analysis of [<sup>3</sup>H]-thymidine-labeled cultures revealed that progenitor cells underwent their last mitotic division in culture in control conditions (Figs. 5A, 5B), and addition of GDNF significantly increased the number of proliferating cells (Figs. 5C, 5D). In the absence of TFs, the percentage of [<sup>3</sup>H]-thymidine-labeled cells was approximately 8%, and this percentage increased to more than 22% in GDNF-supplemented cultures (Table 2). A similar result was observed in cultures incubated with BrdU. The percentages of BrdU-positive cells in control and GDNF-treated cultures were 10% and 20.6%, respectively. Some BrdU-labeled cells also expressed photoreceptor antigens, and the percentage of these labeled cells simultaneously expressing opsin increased with time in vitro (not shown). After 48 hours in vitro, the proportion of BrdU-positive cells remained constant in control cultures,



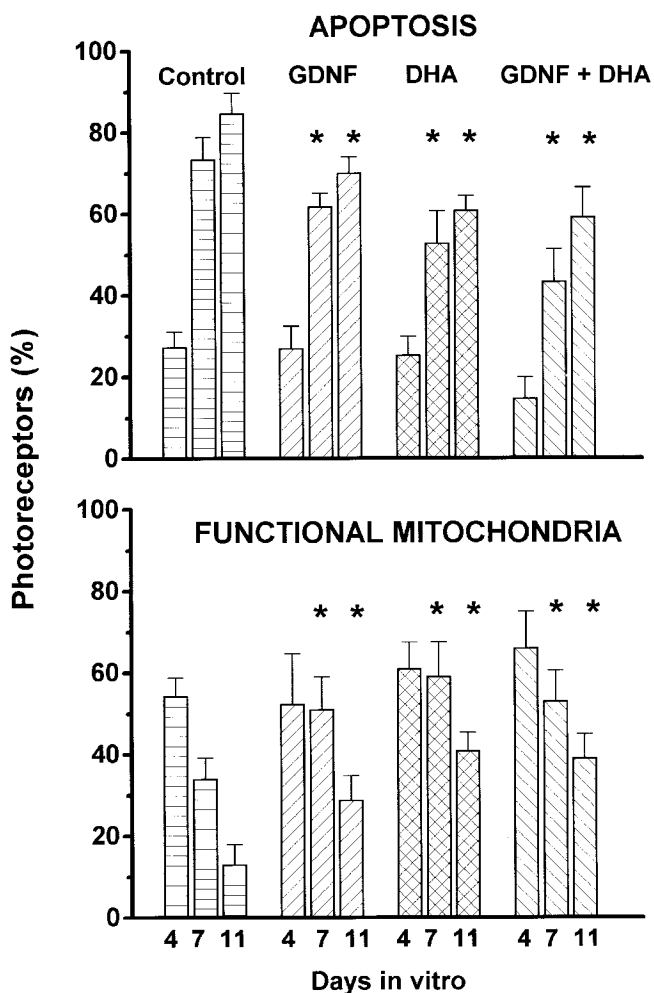
**FIGURE 3.** GDNF and DHA protect photoreceptors from apoptosis and additively promote opsin expression. Fluorescence photomicrographs of 11-day cultures incubated without (A, B) or with GDNF (C, D), DHA (E, F), or GDNF plus DHA (G, H) and processed to detect opsin expression (A, C, E, G) and to evaluate nuclei integrity with DAPI (B, D, F, H). *Arrows:* fragmented or pyknotic nuclei. Opsin expression increased after individual supplementation with either GDNF or DHA and increased further after their combined addition. Scale bar, 15  $\mu$ m.

whereas in GDNF-treated cultures this value increased approximately 50% with respect to that determined at day 1. Addition of DHA, alone or in combination with GDNF did not increase the percentages of BrdU-positive cells (not shown). Altogether, these results suggest that GDNF has a proliferative effect, promoting progenitor division mainly in neuroblasts committed to differentiate as photoreceptors.

## DISCUSSION

In cultures without TFs for photoreceptors, after 4 days these neurons begin a degeneration process that proceeds by apo-

ptosis.<sup>5,6</sup> This degeneration, at a time roughly coincident with that of synaptogenesis of photoreceptor cells *in vivo*,<sup>2</sup> shows many of the hallmarks of programmed cell death triggered by the absence of suitable TFs. The present report shows that GDNF acted as a TF for photoreceptors, promoting their survival and differentiation *in vitro*. Its addition had a significant protective effect on these cells, increasing their survival, enhancing opsin expression, and reducing apoptosis. These effects were similar to those induced by DHA supplementation. GDNF's effects were selective for photoreceptors. Amacrine neurons grew and differentiated, regardless of the presence of this TF. GDNF is a potent neurotrophic factor, able to improve



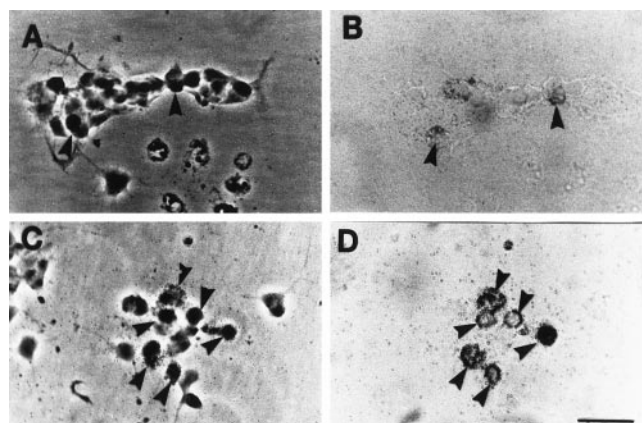
**FIGURE 4.** GDNF and DHA reduced photoreceptor apoptosis, simultaneously preventing the loss of mitochondrial function. The percentages of apoptotic photoreceptors (*top*) or photoreceptors showing functional mitochondria (*bottom*) were determined in 4-, 7-, and 11-day retinal cultures, treated without (control) or with GDNF, DHA, or GDNF plus DHA. The increase in apoptosis corresponds with a decrease in the amount of functional mitochondria. \*Significant differences from the corresponding day in control conditions ( $P < 0.05$ ).

survival and differentiation *in vivo* and *in vitro* in a wide variety of brain neuronal types,<sup>10,39,40</sup> and its expression has been detected in the rat retina since embryonic day 15.<sup>9</sup> GDNF has been shown to slow down degeneration and preserve the functionality of photoreceptor cells in *rd* mouse,<sup>17,26</sup> to improve mouse photoreceptor survival,<sup>16</sup> and to enhance rod outer segment maintenance.<sup>18</sup> Taken together, this evidence strongly supports GDNF as one of the TFs required for retina photoreceptor cells.

**TABLE 2.** Effect of GDNF Supplementation on Cell Cycle and Mitosis of Retinal Neuroblasts

Condition	Control	GDNF Added
PCNA	9.8 ± 2.1	22.7 ± 4.7
[ <sup>3</sup> H]-thymidine	7.8 ± 2.9	22.2 ± 5.4
BrdU	10.0 ± 3.0	20.6 ± 3.9

Retinal neurons supplemented without (control) or with GDNF were cultured for 24 hours, and the percentages positive for PCNA, BrdU, or [<sup>3</sup>H]-thymidine were determined. The values represent the percentages of labeled cells, and are shown as means ± S.D.



**FIGURE 5.** GDNF stimulates the proliferation of retinal neuroblasts. Phase (A, C) and bright-field (B, D) micrographs of 1-day control (A, B) and GDNF-treated (C, D) cultures, incubated with [<sup>3</sup>H]-thymidine for 24 hours and processed for autoradiographic analysis to detect mitosis (arrowheads). Scale bar, 15  $\mu$ m.

Our results show that GDNF also enhanced the number of photoreceptors expressing opsin. Even when considering the reduction in the total number of photoreceptors in control versus GDNF-treated cultures at day 11, GDNF promoted a notable increase in the amount of opsin-expressing photoreceptors at this culture time. DHA had a similar effect on opsin expression (Fig. 3).<sup>7</sup> Previous studies using several well-known TFs had shown that this was not a usual effect for TFs. Among several factors tested, only basic FGF gives rise to an increase in opsin expression.<sup>41</sup> Because opsin is the major protein in photoreceptor outer segments, the increase in the number of opsin-expressing photoreceptors implies that GDNF has an important role not only in promoting photoreceptor cell survival but in inducing the further differentiation of these cells as well.

To further investigate the mechanisms by which GDNF and DHA protect photoreceptor cells, we analyzed the changes in mitochondrial function, because mitochondria play a critical role in the onset and development of apoptosis.<sup>36</sup> In photoreceptor cells, a strong correlation was observed between apoptosis development and mitochondrial failure. As a rule, the increase in photoreceptor apoptosis during development *in vitro* was consistent with a decrease in mitochondrial functionality. In contrast, amacrine neurons, which develop normally in the insulin-supplemented medium used for these experiments, preserved their mitochondrial activity. Both GDNF and DHA reduced the apoptotic death of photoreceptors, simultaneously protecting mitochondrial functionality and membrane integrity. The combined addition of both factors induced a similar effect, diminishing the alterations in mitochondrial permeability. These results suggest that the loss of mitochondrial functionality is closely linked to the evolution of apoptosis in photoreceptors and, conversely, that prevention of mitochondrial impairment may be at least one of the protective mechanisms triggered by GDNF and DHA to slow down the progression of apoptosis in photoreceptors.

Mitochondrial membrane permeability increases at an early stage in the apoptotic process, giving rise to the collapse of the inner mitochondrial transmembrane potential.<sup>36,37</sup> The involvement of altered mitochondrial permeability in rod apoptosis has been shown recently in the rat retina, where lead and calcium produce rod-selective apoptosis by opening the permeability transition pore, thus leading to mitochondrial depolarization.<sup>42</sup> The question remains as to which are the molecular pathways involved in GDNF- and DHA-induced protection of mitochondrial functionality. The recent finding that



DHA is a ligand for retinoid X receptors provides a new clue to understanding how this fatty acid may influence neural function.<sup>43</sup> The neuroprotective mechanisms triggered by DHA and GDNF to prevent apoptosis in retinal neurons are still to be established, and may involve the regulation of Bcl-2 and Bax expression.<sup>44</sup>

A striking finding was the significant increase in the number of surviving photoreceptors present in GDNF-treated cultures at day 11. In vivo, genesis of mouse rod photoreceptors starts at embryonic day 13, but their generation peaks at birth and goes on until as late as postnatal days 3 to 5, because many progenitor cells remain mitotically active after birth.<sup>2,33</sup> Injection of mouse eyes with [<sup>3</sup>H]-thymidine at day 0, before dissecting the retina, gives rise to labeled progenitor cells that develop and differentiate as photoreceptors in culture.<sup>34</sup> Because in our experiments retinal cultures were obtained from 1-day-old rats, a significant number of proliferating multipotent progenitors were still present. The higher number of [<sup>3</sup>H]-thymidine and BrdU-labeled neurons and the increase in PCNA-cyclin reactivity in GDNF-supplemented cultures compared with control cultures clearly demonstrate that GDNF increased the mitotic activity of proliferative neuroblasts, which would then differentiate as photoreceptors, augmenting the number of these cells at early stages of development in vitro.

To our knowledge, this is the first report demonstrating that GDNF may contribute to the control of cell proliferation. Because neurogenesis persists in the adult central nervous system, this finding may have therapeutic relevance for several neurodegenerative diseases affecting photoreceptor cells. In addition, these results provide further evidence regarding a role for TFs in the regulation of cell cycle progression in the retina. Some TFs, such as NT-3, seem to be important in early retinal development, establishing the size of the pool from which all future differentiated cells are to derive.<sup>24</sup> Our results show that GDNF has, at least in vitro, a mitogenic effect promoting neuroblast division mainly in photoreceptor precursors at a time equivalent to postnatal days 1 and 2, when most photoreceptors are generated. It has been recently proposed that retinal progenitor cells pass through a series of stages at which they become progressively competent to differentiate as different cell types.<sup>45</sup> If so, this would be the reason that addition of GDNF at a time in vitro at which neuroblasts are competent to differentiate mainly as photoreceptors would contribute to increasing the number of photoreceptor cells. Hence, GDNF's proliferative effect may partially counterbalance cell losses due to the apoptotic death of photoreceptors during development in vitro.

GDNF's effects on survival and opsin expression were similar to those promoted by DHA supplementation.<sup>5-7</sup> When both molecules were added together, their combined action additively stimulated photoreceptor survival and differentiation during development. As suggested in Figure 6, GDNF could play a dual role: early in development it may stimulate mitosis of photoreceptor neuroblasts, thus increasing the number of these cells and promoting their differentiation. The larger size of the initial photoreceptor pool may be preserved later by the combined protective and differentiation enhancing effects of GDNF and DHA, acting in a coordinated or complementary manner. It has recently been proposed that photoreceptor survival in the developing retina may require several TFs, probably interacting with each other and with other molecular and cellular components.<sup>1,26</sup> Thus, the combination of CNTF with either GDNF or basic FGF has been shown to partially prevent photoreceptor degeneration in *rd* mouse retina, whereas addition of the individual molecules had no protective effect.<sup>26</sup>

Previous studies from several laboratories, including ours, have shown that CNTF, basic FGF, DHA, and GDNF improve

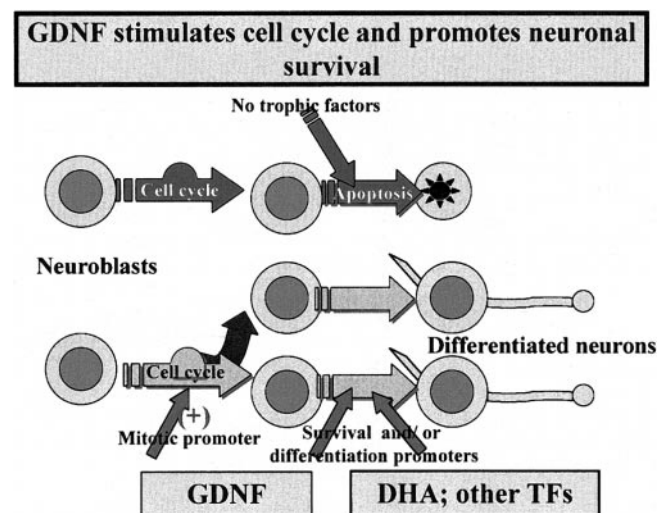


FIGURE 6. Proposed model for the combined action of GDNF, DHA, and other TFs during photoreceptor development. In the absence of suitable TFs, photoreceptors undergo apoptosis. GDNF may increase the number of photoreceptors during early development, by stimulating the cell cycle in neuroblasts that later on will differentiate as photoreceptors. Once the cells acquire the photoreceptor phenotype, GDNF, along with DHA, FGF, and/or other molecules, would act as survival and differentiation promoters.

photoreceptor survival and differentiation.<sup>1,3,5-7,16,17,41</sup> These TFs either ameliorate the apoptotic death of these cells in models of retinal degeneration or postpone the onset of apoptosis in vitro. However, none of them individually could effectively halt apoptotic death and lead to fully functional and differentiated photoreceptors. Altogether, these results open the interesting possibility that different TFs may act coordinately at distinct stages of retinal histogenesis, contributing to regulation of the final number of neuronal types in the retina by controlling not only their survival but also their proliferation rate. The search for a combination of several trophic molecules may eventually provide the tools for a better understanding of photoreceptor development and degeneration.

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