Sphingosine-1-Phosphate Is a Key Regulator of Proliferation and Differentiation in Retina Photoreceptors

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PURPOSE. Identifying the cues required for the survival and development of photoreceptors is essential for treating retinal neurodegeneration. The authors previously established that glial-derived neurotrophic factor (GDNF) stimulates proliferation and that docosahexaenoic acid (DHA) promotes photoreceptor survival and differentiation. Later findings that ceramide triggers photoreceptor apoptosis suggested sphingolipids might also control photoreceptor development. The present study investigated whether sphingosine-1-phosphate (S1P), which promotes survival and differentiation in several cell types, regulates photoreceptor proliferation and differentiation and whether it is a mediator in GDNF and DHA effects.

METHODS. Rat retina neuronal cultures were supplemented at day 0 or 1 with S1P, GDNF, or DHA and were treated with DL-threo-dihydrosphingosine to inhibit S1P synthesis or with brefeldin A (BFA) to block intracellular trafficking. Proliferation was quantified to determine bromodeoxyuridine uptake and number of mitotic figures. Opsin, peripherin, and sphingosine kinase (SphK), the enzyme required for S1P synthesis, were quantified by immunocytochemistry and Western blot analysis.

RESULTS. S1P increased the proliferation of photoreceptor progenitors. It also stimulated the formation of apical processes, enhanced opsin and peripherin expression, and promoted their localization in these processes; DHA had similar effects. BFA prevented S1P and DHA enhancement of apical process formation without affecting opsin expression. GDNF and DHA enhanced SphK expression in photoreceptors, while inhibiting S1P synthesis blocked GDNF mitogenic effects and DHA effects on differentiation.

CONCLUSIONS. The authors propose S1P as a key regulator in photoreceptor development. GDNF and DHA might upregulate SphK levels to promote S1P synthesis, which would initially promote proliferation and then advance photoreceptor differentiation.

METHODS.

RESULTS.

CONCLUSIONS.
Little is known concerning S1P roles in the nervous system; it is involved in brain neurogenesis and promotes the proliferation of neuronal progenitors in embryonic rat brain. Information on S1P functions in the retina is even scarcer. It has been proposed to participate in rhodopsin trafficking to the OS, and lipid phosphatases involved in its hydrolysis have been described in rod OS.

In this study, we investigated whether S1P participated in the regulation of photoreceptor development in culture. Our results show for the first time that S1P enhanced the proliferation of photoreceptor progenitors and promoted their differentiation. These results also suggest that S1P might act as a second messenger for trophic factors, such as DHA and GDNF, which would enhance its synthesis to regulate the final number of photoreceptors and control their development.

**Materials and Methods**

**Materials**

Albin0 Wistar rats bred in our own colony were used in all the experiments. All proceedings concerning animal use were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Plastic culture 35- and 60-mm diameter dishes (CellStar) were from Greiner Bio-One (Frickenhausen, Germany). Dulbecco modified Eagle medium (DMEM), trypsin, insulin, gentamicin, 5-bromo-2'-deoxyuridine (BrdU), 5-bromo-2'-deoxyuridine-5'-triphosphate (BrdUTP), and terminal deoxynucleotidyl transferase (TdT) were from Invitrogen (Carlsbad, CA). Bovine serum albumin (Fraction V; fatty acid-free; low endotoxin, tissue culture tested), poly(dT) were from Invitrogen (Carlsbad, CA). Bovine serum albumin (Fraction V; fatty acid-free; low endotoxin, tissue culture tested), poly(dT) were from Invitrogen (Carlsbad, CA). Bovine serum albumin (Fraction V; fatty acid-free; low endotoxin, tissue culture tested), poly(dT) were from Invitrogen (Carlsbad, CA).

**Neuronal Cultures**

Hill, NJ) and a kind gift from Ne´stor Carri (IMBICE, Buenos Aires, Argentina). Dulbecco modified Eagle medium (DMEM), trypsin, insulin, gentamicin, 5-bromo-2-deoxyuridine (BrdU), 5-bromo-2'-deoxyuridine-5'-triphosphate (BrdUTP), and terminal deoxynucleotidyl transferase (TdT) were from Invitrogen (Carlsbad, CA). Bovine serum albumin (Fraction V; fatty acid-free; low endotoxin, tissue culture tested), poly(dT) were from Invitrogen (Carlsbad, CA). Bovine serum albumin (Fraction V; fatty acid-free; low endotoxin, tissue culture tested), poly(dT) were from Invitrogen (Carlsbad, CA).

**Evaluation of Neuroblast Proliferation**

To investigate the S1P effect on the proliferation of photoreceptor progenitors, neuronal cultures were supplemented with S1P 1 hour after cells were seeded, and proliferation was evaluated 1 or 2 days later. BrdU uptake was determined by incubating 0- and 1-day cultures with 30 μM BrdU (final concentration in culture) for 16 to 18 hours. Cells were fixed for at least 30 minutes, treated with 2 N HCl for 30 minutes for DNA denaturation, and neutralized with 0.1 M boric acid. BrdU uptake was determined using a monoclonal antibody against BrdU. Mitotic figures were evaluated by fluorescence microscopy after cells were permeated with 0.1% Triton X-100 in PBS and incubated for 20 minutes with DAPI, a DNA marker.

**Addition of S1P and DHA**

Aliquots from a S1P stock solution (0.5 mg/mL, in methanol/water, 95:5) were evaporated under a stream of dry nitrogen, resuspended in a bovine serum albumin (BSA) solution in DMEM (4 mg/mL), and heated at 40°C to 50°C for 30 minutes with occasional vortexing and sonication to allow solubilization. S1P effect on opsin expression was evaluated at concentrations ranging from 0.5 to 5 μM. A final 1 μM S1P concentration (in the culture medium) was chosen for subsequent experiments. The same volume of the solution used as vehicle was added to controls.

DHA (6.7 μM) was added to day 1 cultures, complexed with BSA, in a 2:1 (DHA/BSA) molar ratio. The same volume of the BSA solution was added to controls.

**Evaluation of Photoreceptor Differentiation**

Opsin and peripherin expression and the amount of photoreceptors with either opsin or peripherin positive-apical processes were evaluated using Rho4D2 and anti-peripherin monoclonal antibodies, respectively. Cy2-conjugated goat anti–mouse antibody was used as the secondary antibody. Amplification with tyramine fluorescent compounds was occasionally used to improve visualization. Controls for immunocytochemistry were made by omitting the primary or the secondary antibody.

**Immunocytochemical Methods**

After different time periods, cultures were fixed for at least 1 hour with 2% paraformaldehyde in PBS at room temperature, followed by permeation with Triton X-100 (0.1% in PBS) for 15 minutes. Photoreceptors were identified by immunocytochemistry with the monoclonal antibody Rho4D2, by their morphology, and by other criteria as previously described.

Secondary antibodies biotin-conjugated goat anti–mouse and avidin-conjugated horseradish peroxidase (HRP), and goat anti–rabbit HRP, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Fluorophore-conjugated tyramine compounds and reaction buffers were synthesized according to previous reports. Secondary antibodies biotin-conjugated goat anti–rabbit and avidin-conjugated horseradish peroxidase were from Vector Laboratories (Burlingame, CA). GDNF was from PeproTech (Rocky Hill, NJ) and a kind gift from Néstor Carri (IMBICE, Buenos Aires, Argentina). Monoclonal antibodies anti–opsin (Rho4D2) and anti–peripherin (clone Per3B6) were generous gifts from Robert Molday (University of British Columbia, Canada). Polyclonal antibody anti–SphK1 was kindly provided by Linda Obeid (Medical University of South Carolina). Solvents were HPLC grade, and all other reagents were analytical grade.

**Addition of Brefeldin A**

A stock solution of the fungal antibiotic brefeldin A (BFA; 4 mg/mL) was prepared in dimethylsulfoxide; dilutions (using Hanks balanced salt solution) were then prepared. Day 2 neuronal cultures were treated with 0.5 μg/mL BFA (final concentration); day 5 cultures were treated with 0.25 μg/mL BFA (final concentration) to avoid deleterious effects; DHA or S1P, or both were added 2 hours later, and cells were fixed at day 4 or 6, respectively. Photoreceptor differentiation was then evaluated.
Selective Elimination of Amacrine Neurons

To evaluate different parameters exclusively in photoreceptors, amacrine neurons were eliminated from the cultures by previously established methods. In brief, 3- or 4-day cultures were incubated overnight with 0.25 mM kainic acid, which selectively kills amacrine neurons while photoreceptors remain unaffected. Cells were then fixed or lysed.

Inhibition of S1P Synthesis

To investigate whether GDNF promoted S1P synthesis to stimulate proliferation, day 0 cultures were supplemented with 1 μM DHS, a potent, cell-permeable, competitive inhibitor of SphK1,34,35 and 30 minutes later without or with GDNF (4 ng/mL, final medium concentration). BrdU was added 4 hours later, and cells were fixed after 18-hour incubation. Proliferation was evaluated as described.

To evaluate whether the effect of DHA on photoreceptor differentiation required S1P synthesis, 1-day cultures were treated with DHS and were supplemented 1 hour later with or without DHA or S1P. Photoreceptor differentiation was evaluated at day 6.

Effect of DHA and GDNF on SphK Levels

To analyze the effect of GDNF on SphK expression, GDNF was added 1 hour after the cells were seeded. At this time in development, kainic acid is not toxic for amacrine neurons; hence, SphK expression in amacrine and photoreceptor cells was determined. To evaluate the effect of DHA on SphK expression in photoreceptors, amacrine neurons were eliminated, as described, and fresh media with or without DHA were added at day 4. Neurons were fixed or lysed 24 hours after the addition of DHA or GDNF. To improve the detection of SphK, its proteosomal degradation was inhibited by the treatment of cultures with the proteosome inhibitor MG-132 (0.1 μM)18 1 hour after fixation or lysis. SphK expression was evaluated by immunocytochemistry or Western blot analysis with the use of a specific polyclonal antibody, anti-SphK1.

Protein Extraction and Western Blot Analysis

Expression of opsin, peripherin, and SphK was evaluated by Western blot analysis. Media were removed. Cells were rinsed with PBS, collected in lysis buffer (3 mM KCl, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Tween-20, 1% NP-40) containing a protease inhibitor mixture, and lysed in ice for 20 minutes. Proteins were quantified with a protein assay (DC; Bio-Rad; Hercules, CA) based on the Lowry assay and were separated by one-dimensional SDS-PAGE. Briefly, samples were mixed with 6× Laemml sample buffer and were heated for 5 minutes at 95°C for SphK or not heated for opsin and peripherin. Proteins (either 10 or 20 μg/sample) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride (Immobilon P; Millipore, Billerica, MA) membranes. Membranes were then washed in buffer with 5% nonfat dry milk for 1 hour at room temperature to block nonspecific binding. Anti-opsin, anti-peripherin, anti-SphK1, and anti-actin antibody bodies were allowed to react with the membrane overnight at 4°C or for 2 hours at room temperature. Membranes were thoroughly washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies, in TBS-T for 1 hour at room temperature and were then visualized with enhanced chemiluminescence.

Bands were quantified with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Evaluation of Photoreceptor Apoptosis

Apoptosis was determined at day 6 by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and by evaluating nuclei integrity. For TUNEL staining, the cultures were fixed with 2% paraformaldehyde and stored in 70% ethanol for 48 hours at 20°C. Cells were then preincubated with 1× TdT buffer for 15 minutes and with the TdT reaction mixture (0.05 mM BrdUTP and 0.3 U/μL TdT in TdT buffer) at 37°C in a humidified atmosphere for 1 hour. The reaction was stopped with stop buffer (300 mM NaCl, 30 mM sodium citrate, pH 7.4) at room temperature. Negative controls were prepared by omitting TdT. The presence of BrdU was determined with an anti-BrdU monoclonal antibody.

Nuclei integrity was evaluated after staining cell nuclei with DAPI, as described. Cells were considered apoptotic when they showed fragmented or condensed (pyknotic) nuclei. The percentage of apoptotic photoreceptors was determined by double-labeling with DAPI and Rhod2 to unambiguously identify cells as photoreceptors and to establish the total number of these cells.

Statistical Analysis

For cytochemical studies, 10 fields per sample, randomly chosen, were analyzed in each case. Each value represents the average of at least three experiments, with three or four dishes for each condition ± SD. Statistical significance was determined by Student’s two-tailed t-test.

Results

Effect of S1P on the Proliferation of Photoreceptor Precursors

In our culture conditions, photoreceptor progenitors proliferated for 1 to 2 days before differentiating as photoreceptors; therefore, we investigated whether S1P promoted this proliferation. S1P addition at day 0 rapidly increased BrdU uptake in day 1 progenitors (Figs. 1A–D), from 23% in controls to 33% in S1P-supplemented cultures (Fig. 1E). S1P also augmented the amount of mitotic figures, which were more than 2.5-fold higher in S1P-supplemented cultures than in controls (Fig. 1F).

In normal conditions, proliferation decreased rapidly; in 2-day controls, few neuroblasts took up BrdU, and almost no mitotic cells were present. BrdU-labeled cells (Fig. 1E) and mitotic figures (Fig. 1F) were still present in S1P-supplemented cultures, suggesting S1P preserved some mitotic activity.

Effect of S1P on Photoreceptor Differentiation

Retinal photoreceptors in vitro, cultured in media lacking their trophic factors, develop as round cells with small bodies and short cilia, but their development seems to be hindered compared with their in vivo counterparts. They usually lack the high opsin levels and characteristic OS found in photoreceptors in vivo; few cultured photoreceptors express opsin or develop apical processes that resemble rudimentary OS. DHA supplementation enhances opsin expression and the development of apical processes and promotes opsin localization in these processes. Adding S1P at day 1 rapidly enhanced opsin expression; a slight but significantly higher percentage of photoreceptors expressing opsin, compared with controls, was already observed 4 hours after this addition (value corresponding to day 1 in Fig. 2A) and increased over time. S1P and DHA had similar effects on opsin levels, which followed the same time course of increase. Their combined addition at day 1 increased opsin expression compared with controls but had no additive effect. By day 6, cultures with S1P and DHA...
showed 11% ± 0.3% opsin-positive photoreceptors (not shown) compared with 10.8% ± 0.2% and 10.8% ± 0.03%, in S1P and DHA-supplemented cultures (Fig. 2A), respectively.

The increase in the percentage of opsin-positive photoreceptors was visible in a small range of S1P concentrations (Fig. 2B); 0.5 μM S1P did not increase opsin expression, whereas 1 and 2 μM S1P augmented it similarly, from nearly 4% to approximately 8% (Fig. 2B). At 2.5 μM, S1P concentration was already deleterious for the cells (not shown). Western blot analysis confirmed DHA, and S1P addition led to higher opsin levels compared with controls (Fig 2C).

We then explored the expression of peripherin, a structural protein found in disc rims in photoreceptor OS. S1P and DHA increased the percentage of peripherin-expressing photoreceptors approximately 30% compared with controls (Fig. 2A), respectively. The increase in peripherin levels after the addition of S1P and DHA was evidenced clearly by Western blot analysis (Fig. 2E).

We then investigated the effect of S1P on the development of apical processes. As previously reported, most photoreceptors in control cultures lacked apical processes (Figs. 3A, 3D, 3G, 3J, 3M, 3P, 3R, 4), and opsin labeling was distributed throughout the whole cytoplasm and neurite (Figs. 3A, 3P, 3R). A small number of them had short cilia, showing acetylated α-tubulin staining (Fig. 3G), and few were labeled with peripherin (Fig. 3D). S1P addition rapidly promoted the formation of apical processes and the localization in them of opsin (Fig. 3G) and peripherin (Figs. 3F, 3L, 3O). These peripherin-labeled processes (Figs. 3L, 3O, white arrows) were clearly observed protruding at the tip of cilia labeled with acetylated α-tubulin (Figs. 3I, 3O, open arrows). Opsin localization in apical processes was evident in confocal micrographs (Figs. 3Q, 3S, white arrows). S1P effects on photoreceptor differentiation were similar to those of DHA (Figs. 3B, 3E, 3K, 3N). The higher percentage of photoreceptors with apical processes was already visible 4 hours after S1P or DHA addition and augmented continually after 2 days in vitro (Fig. 4A), doubling that in controls at every time point studied. The combined addition of S1P and DHA showed the same effect as each molecule by itself.
on the development of apical processes at day 6, with 4.4% ± 0.1%, 4.6% ± 0.01%, and 4.6% ± 0.1% of photoreceptors having apical processes in S1P, DHA (Fig. 4A), and S1P plus DHA-supplemented cultures (not shown), respectively.

In controls, nearly 75% of peripherin-labeled photoreceptors had intensely labeled cilia, but few (< 29%) had peripherin-labeled-apical processes (Fig. 4B). In contrast, in S1P- and DHA-supplemented cultures, most photoreceptors (approximately 70%) showed peripherin-positive apical processes, and those with peripherin-labeled cilia decreased to 30% (Fig. 4B). These results are evidence that S1P and DHA further advanced photoreceptor differentiation.

**Brefeldin A Inhibition of DHA- and S1P-Induced Formation of Apical Processes**

To gain insight into the processes leading to OS formation, cultures were treated with BFA, which inhibits intracellular trafficking pathways, before DHA or S1P was added. Confocal micrographs confirmed that S1P and DHA promoted the development of opsin-labeled apical processes (white arrows in Figs. 5A, 5D, 5G, 5J), virtually absent in controls, in which opsin labeled photoreceptor neurites and cell bodies (thin arrows in Figs. 5A, 5D, 5G, 5J). BFA hindered DHA and S1P stimulatory effect, blocking this development and simultaneously increasing opsin delocalization (thin arrows in Figs. 5C, 5F, 5I, 5L). At day 4, the percentage of opsin-labeled photoreceptors with apical processes in DHA- and S1P-supplemented cultures was twice that in controls (Fig. 5M), and the addition of BFA reduced it almost to control levels (Fig. 5M), concurrently augmenting the percentage of photoreceptors having cilia (not shown). DHA and S1P decreases in opsin expression were unaffected by BFA (Fig. 5N); the same percentage of opsin-expressing photoreceptors was found in DHA- and S1P-supplemented cultures with or without BFA. At these concentrations and lengths of incubation with BFA, no increase in photoreceptor apoptosis was observed (not shown).

**S1P Effect on Photoreceptor Apoptosis**

Photoreceptors cultured in media lacking their specific trophic factors developed normally for 3 to 4 days and then started to degenerate through an apoptotic pathway. S1P addition significantly reduced photoreceptor apoptosis; after 6 days in vitro, approximately 50% of photoreceptors...
**FIGURE 3.** Effect of S1P on the formation of apical processes. Cultures were treated at day 1 with BSA (Ctl, left), DHA (middle), or S1P (right) and were fixed at day 6. Fluorescence micrographs show coimmunostaining of photoreceptors with opsin (A–C) and peripherin (D–F) antibodies and with acetylated α-tubulin (G–I) and peripherin (J–L) antibodies and their merge (M–O). Intense staining with opsin and peripherin (thick arrows in B, C, E, F, K, L) was observed in apical processes in DHA and S1P-supplemented cultures but was present only in cilia in controls (wide arrows in A, D). Acetylated α-tubulin-labeled photoreceptor cell bodies and cilia (open arrows in G–I, M–O) but did not label apical processes (H, I, N, O). Confocal micrographs (P–S) show opsin expression (P, Q) and the merge of this expression with Nomarsky images (R, S) in control (P, R) and S1P-supplemented (Q, S) cultures. Opsin was distributed throughout the cytoplasm and neurite in controls (open arrows in P, R), and S1P promoted its localization in photoreceptor apical processes (thick arrow in Q, S). Scale bars, 5 μm.
were apoptotic in controls, and S1P decreased their apoptosis to less than 20% (Fig. 6). This decrease was confirmed by the reductions in the amounts of TUNEL-labeled photoreceptors in S1P-supplemented cultures compared with controls (not shown).

**Suppression of GDNF and DHA Effects by Inhibition of S1P Synthesis**

Previous work showed that GDNF stimulated the proliferation of photoreceptor progenitors. Our present data confirmed those results: approximately 26% of the cells took up BrdU in controls, and GDNF increased BrdU uptake to 40% of the cells (Fig. 7A). GDNF also increased the amount of mitotic figures from 3325 ± 707 to nearly 9013 ± 1573 per dish (Fig. 7B). To investigate whether S1P might be a mediator of the GDNF effect on photoreceptor proliferation, we inhibited S1P synthesis adding DHS, an SphK inhibitor, immediately after seeding the cells. Although the addition of DHS did not affect proliferation in day 1 control cultures (Figs. 7A, 7B), it completely inhibited GDNF-induced increases in proliferation (Figs. 7A, 7B), reducing BrdU uptake and the amount of mitotic figures to control values. These results strongly suggest that basal neuroblast proliferation was independent of S1P, but GDNF effect on proliferation required S1P synthesis.

We then investigated whether DHA required S1P synthesis to advance photoreceptor differentiation. In the absence of DHS, DHA promoted the development of opsin-labeled apical processes (Figs. 8A, 8D), doubling the percentage of photoreceptors expressing opsin and having apical processes by day 6 (Figs. 8G, 8H). DHA addition had no effect on opsin expression and did not induce photoreceptor apoptosis in controls (not shown). DHS blocked the DHA effect on the formation of apical processes and the increase in opsin levels (Figs. 8B, 8E, 8G, 8H) but did not affect those of S1P (Figs. 8G, 8H). S1P addition to cultures treated with DHS and DHA restored the increases in opsin expression and apical process development (Figs. 8C, 8F, 8G, 8H).

**DHA- and GDNF-Upregulated Sphingosine Kinase Expression**

Next, we investigated whether DHA and GDNF upregulated SphK levels in photoreceptors. Photoreceptors and amacrine neurons had a low and diffuse basal expression of SphK (Figs. 9A, 9F). GDNF addition to day 1 cultures increased the amount of photoreceptors expressing SphK 24 hours later, particularly in cultures treated with the proteosome inhibitor MG-132 (Figs. 9B, 9D). The higher SphK levels were confirmed by Western blot analysis (Fig. 9E).

To evaluate the effect of DHA on SphK expression in photoreceptors, 5-day cultures were treated with kainic acid to eliminate amacrine neurons. Almost no increase in SphK expression was visible after DHA addition in 4-day cultures because of its short lifetime (Fig. 9G). When cultures were treated with MG-132 to prevent SphK degradation, DHA enhancement of SphK expression was clearly evident (Fig. 9I). DHA upregulation of SphK levels was also evidenced by Western blot analysis (Fig. 9E).

In addition to the increased expression of SphK in DHA and GDNF-supplemented cultures, many photoreceptors localized this expression in their plasma membranes, which showed intense SphK labeling (thin arrows in Figs. 9D, 9I). This suggested that DHA and GDNF might promote the translocation of this enzyme from the cytosol to the plasma membrane, a mechanism activated by other trophic factors to induce SphK activation.

**DISCUSSION**

Uncovering cues for controlling the proliferation and development of retinal photoreceptors is essential for advancing the development of new tools for generating functional photoreceptors in neurodegenerative diseases of the retina. Our results demonstrate for the first time that S1P has a crucial role in controlling the proliferation and differentiation of photoreceptors. S1P stimulated the proliferation of photoreceptor progenitors at early culture times and advanced their differentiation as photoreceptors. These results also provide strong evidence that GDNF and DHA enhance the synthesis of S1P by enhancing the expression of SphK. Increased levels of S1P are required for GDNF and DHA stimulation of photoreceptor proliferation and differentiation, respectively, suggesting S1P might be an essential intracellular signal for controlling these processes.

**S1P as a Cue for the Proliferation of Photoreceptor Progenitors**

Most rod photoreceptors are born between postnatal day (P) 0 and P2 in rodent retinas; they are the last neuronal type to be born in the retina in vivo. We have previously demonstrated that in neuronal cultures from day 0 rat retinas, photoreceptor progenitors accomplish their last rounds of mitosis in

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**FIGURE 4.** Effect of S1P and DHA on the development of apical processes. Neuronal cultures were supplemented at day 1 with BSA solution (Ctl), DHA, or S1P. Cells were fixed at day 1, 4 hours after the addition of S1P or DHA (indicated as day 1), after 2 to 6 days of development in vitro to evaluate the percentages of photoreceptors with apical processes (A), or at day 6 to evaluate the percentages of peripherin-expressing photoreceptors developing either only cilia or cilia plus apical processes (B). Note that in DHA- and S1P-treated cultures, more photoreceptors developed apical processes than only cilia, and the opposite was observed in control cultures. *P < 0.05; statistically significant differences compared with controls.
vitro. DHA and GDNF have opposing roles in controlling this proliferation. DHA induces the exit of photoreceptor progenitors from the cell cycle, while GDNF has a mitogenic effect, promoting proliferation.\(^7\) Identifying the molecular cues that induce undifferentiated cells to proliferate and eventually differentiate as photoreceptors has become even more relevant since the recent finding of stem cells in the retina because they can provide a therapeutic alternative to replace lost neurons. S1P is involved in the regulation of mammalian cell proliferation and growth in many tissues.\(^12\) In the nervous system, S1P induces the proliferation of embryonic neural progenitor cells,\(^22\) whereas SphK1/2 null mice show decreased mitogenesis and increased neuronal apoptosis in almost all brain regions.\(^43\) We show here for the first time that S1P stimulated the proliferation of photoreceptor progenitors at early times of development, prolonging their permanence in the cell cycle. Interestingly, S1P effects on proliferation were similar to those of GDNF,\(^7\) suggesting that S1P might be a key signal in controlling photoreceptor proliferation.

**S1P in the Advancement of Photoreceptor Differentiation**

Once photoreceptor progenitors exit the cell cycle, their differentiation proceeds in vivo through several steps. They initially develop a distal connecting cilium and then start expressing opsin and accumulating membranes at the cilium tip, which will finally form the rhodopsin-containing disks and the OS characteristic of mature photoreceptors. This differentiation appeared to be arrested in vitro. Cultures lacking S1P and DHA had poor opsin expression, as diffusely distributed over the entire plasmalemma as that found in immature photoreceptors, which show opsin labeling in inner and outer segment membranes and in synaptic terminal. It has been proposed that this distribution results from an inefficient, immature machinery for opsin transport.\(^44\) Photoreceptors in control cultures failed to develop apical processes; some of them concentrated peripherin, the disc rim protein essential for the morphogenesis and maintenance of the OS, in their cilia. This also mimics...
and DHA. These molecules stimulate the synthesis of proteins essential for OS in a BFA-independent process. They also promote the morphogenesis of OS, targeting these proteins to them, a process that requires the intracellular trafficking of opsin-containing vesicles to the tip of photoreceptor cilia and that is inhibited by BFA. DHA represents approximately 50% of the acyl chains esterified in rod OS phospholipids. The increase in DHA content that accompanies its effects on photoreceptor differentiation in vitro suggested the requirement for DHA-containing phospholipids for the formation of apical processes. These phospholipids are closely associated with rhodopsin in the newly formed disc membranes and have been proposed to have a relevant role in rhodopsin trafficking. S1P has also been proposed to participate in lipid and rhodopsin trafficking to OS in the frog retina, stimulating the uptake and esterification of DHA into phospholipids, followed by their increased delivery, together with rhodopsin, to the OS. Our results show that blocking S1P synthesis completely inhibited DHA-induced enhancement of opsin synthesis and apical process morphogenesis, maintaining photoreceptor differentiation in control levels. This suggests that S1P synthesis is essential for DHA effects and points to an indispensable role for S1P in the assembly of the OS.

To improve our understanding of these mechanisms, we inhibited intracellular trafficking with BFA. BFA did not affect the increase in opsin expression induced by S1P and DHA, but it almost completely blocked the formation of apical processes and opsin localization in them, keeping photoreceptors at the same differentiation stage as in controls. Opsiin is synthesized in the endoplasmic reticulum, then modified in the Golgi, and is then vectorially transported in intracellular vesicles to the base of the connecting cilia for OS assembly. In frog photoreceptors, BFA does not affect opsin synthesis but reversibly inhibits its transport and that of DHA-containing phospholipids. Our results are consistent with a dual action of S1P and DHA: first, they potentiate their effects because the combined addition of both lipid molecules did not further enhance their effects. Second, our results are consistent with a dual action of S1P and DHA: they potentiate their effects because the combined addition of both lipid molecules did not further enhance their effects.
S1P as a Promoter of Photoreceptor Survival

Photoreceptors in our culture conditions start degenerating after 4 days in vitro, and trophic factors, such as DHA, prevent this degeneration. S1P remarkably enhanced photoreceptor survival, thereby preventing apoptosis. S1P has been shown to suppress apoptosis in many cell types. Although few data exist on the nervous system, S1P has been reported to protect mesencephalic neurons against excitotoxicity. S1P antiapoptotic effects have been associated with activation of the ERK/MAPK pathway, the same pathway activated by DHA to prevent photoreceptor death. Further work is required to establish which intracellular pathways are involved in S1P protection. However, our results demonstrate that S1P is involved in photoreceptor survival and suggest that DHA and S1P have similar effects not only on photoreceptor differentiation but on their survival as well.

S1P as a Second Messenger for DHA and GDNF Signaling

S1P is unique as a signaling molecule because it has two distinct mechanisms to mediate its biological effects. It acts as an extracellular ligand, binding to and activating G-protein-related membrane receptors, to regulate processes such as cytoskeletal rearrangements, cell migration, angiogenesis, and embryonic development of the heart. In mammals, S1P acts also as an intracellular second messenger in a receptor-independent manner. Activation of SpHK leads to the accumulation of S1P, which then activates diverse downstream effectors, such as signaling pathways regulating calcium mobilization, DNA synthesis, cell growth, tumorigenesis, and suppression of apoptosis. Increasing evidence shows that there is mutual cross-talk between S1P and growth factor–activated signaling cascades. The overlap in the biological functions of S1P and those of DHA or GDNF prompted us to investigate whether these trophic factors might require the synthesis of S1P to exert their effects on photoreceptor development. Nothing is known concerning the regulation of S1P synthesis in the retina. Our results show that blocking S1P synthesis by inhibiting SpHK1 activity completely blocked the GDNF mitogenic effect. Similarly, inhibiting SpHK1 abolished DHA enhancement of opsin expression and apical process formation. The addition of S1P to DHA and DHS-supplemented cultures restored the increase in opsin levels and the development of apical processes. This strongly supports the proposal that GDNF and DHA might promote S1P synthesis and then use S1P as a second messenger to mediate their biological effects.
activated by DHA and GDNF to augment S1P synthesis. They also suggest that these factors might induce the translocation, and consequent activation, of SphK to the plasma membrane, implying that a combination of enhanced transcription and higher activity might contribute to augment S1P levels. SphK1 synthesizes S1P through phosphorylation of the sphingosine produced from ceramide hydrolysis. Given that S1P and its precursors, ceramide and sphingosine, have opposing roles in the control of cell survival and growth, with S1P implicated in cell survival and growth and ceramide involved in proapoptotic and growth-inhibitory effects, favoring the synthesis of either sphingolipid might have crucial effects on cell fate. Hence, SphK might have a key role in defining this balance; the upregulation of its expression by trophic factors such as GDNF and DHA would increase S1P levels, which would promote the proliferation of photoreceptor progenitors at early developmental times and then advance their differentiation, ensuring the morphogenesis of OS and the development of mature photoreceptors.

In conclusion, we propose that S1P is a key mediator for regulating the final amount of photoreceptors in the retina, by initially controlling neuroblast proliferation and later promoting photoreceptor survival and differentiation. Photoreceptor trophic factors, such as GDNF and DHA, might elicit their biological effects by enhancing the synthesis of S1P, which would then act as a crucial second messenger for photoreceptor development.

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