GDNF Stimulates Rod Photoreceptors and Dopaminergic Amacrine Cells in Chicken Retinal Reaggregates

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PURPOSE. To investigate the role(s) of glial cell line–derived neurotrophic factor (GDNF) on expression of rod photoreceptor and dopaminergic amacrine cell–specific genes in an in vitro reaggregate model of the chick retina.

METHODS. Retinal reaggregates derived from embryonic day (E)6 chicks (rossetted spheroids) were supplemented with 50 ng/mL GDNF, or, alternatively, endogenous GDNF expression was downregulated by transient transfection of spheroids with a pCMS-EGFP[GFN] antisense vector. Using mainly semiquantitative RT-PCR analyses, expression of rhodopsin, four separate opsins, and tyrosine hydroxylase (THase) was analyzed after either treatment.

RESULTS. Supplementation with GDNF accelerated rhodopsin mRNA expression and sustained it at an increased level, in contrast to untreated control subjects, where rhodopsin mRNA levels were lower and unmaintained. Expression of red, green, blue, and violet opsins were unaffected. Under these conditions, GDNF also massively increased the expression of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine. The expression of endogenous GDNF was blocked in spheroids by using antisense transfections, which resulted in both a significant decrease in rhodopsin mRNA expression and a complete suppression of THase expression, as determined by RT-PCR, Western blot analysis, and immunocytochemistry.

CONCLUSIONS. GDNF supports expression of both rhodopsin and THase in vitro, two critical molecules involved in the production of rod photoreceptors and dopaminergic amacrine cells, respectively; however, the presence of GDNF does not affect cone production and survival. (Invest Ophthalmol Vis Sci. 2007;48:5306–5314) DOI:10.1167/iovs.07-0313

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Supported by European Union SENSPESTI (Tissue Engineering of Living Biosensors to Evaluate Risks for Health by Pesticides Affecting the Cholinergic Neurotransmitter System) Consortium QLK4-CT-2002-02264 and Deutsche Forschungsgemeinschaft Grant DFG-La379/12-1.
Submitted for publication March 14, 2007; revised April 30 and June 26, 2007; accepted September 11, 2007.
Disclosure: K.N. Volpert, None; A. Rothermel, None; P.G. Layer, None.

Layer. None
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absence of GDNF. Moreover, knockdown of GDNF completely inhibited THase mRNA expression. Thus, GDNF appears to exert multiple influences that support expression and survival of both rod photoreceptors and dopaminergic ACs.

**Materials and Methods**

### Tissue Culture

Six-day-old chicken embryos (E6, white leghorn) were used to produce rosetted retinal spheroids. The central parts of the retina were isolated and collected in F12 medium on ice. The retinal tissue was dissociated by tryptic digestion in F12 medium containing 0.05% mg/mL trypsin (Worthington Biochemicals/Cell Systems, Remagen, Germany) for 8 minutes at 37°C. The remaining cell clusters were mechanically dissociated in Hanks’ balanced salt solution (HBSS) containing 0.5 mg/mL DNase I (Worthington Biochemicals/Cell Systems, Remagen, Germany) by 30 to 35 gentle strokes with a round-bored Pasteur pipette. For generation of retinal spheroids, 2 × 10^6 cells/mL were cultured in 35-mm dishes containing 2 mL aggregation medium (DMEM, 2% FCS, 1% t-glutamine, and 0.15% penicillin/streptomycin, all from Invitrogen-Gibco, Berlin, Germany) on a gyratory shaker in an incubator 37°C and 5% CO2 for at least 12 hours at 37°C and 5% CO2 on a gyratory shaker. After 12 hours, the spheroids were harvested, to isolate RNA and/or to produce established spheroids. The central parts of the retina were isolated by tryptic digestion in F12 medium containing 0.05 mg/mL trypsin (Worthington Biochemicals/Cell Systems, Remagen, Germany) for 1 minute for 29 cycles. Primers for PCR were 5'-TGCCAGAGATTACCCAGAT-3' and 5'-AGGTCATCTGATAGCTGTT-3' for GDNF, 5'-GGGAGTACGCTGTT-3' and 5'-CTTCTGTTGCGTATG-3' for GAPDH, 5'-GACCAGAGCTCCACGAGCA-3' and 5'-AGGTCATCTGATAGCTGTT-3' for red opsin; 5'-CATG-3' and 5'-TGTTGATCAGATAGCAGCA-3' for green opsin; 5'-GACCAGAGCTCCACGAGCA-3' and 5'-AGGTCATCTGATAGCTGTT-3' for THase. The relative expression of mRNA was quantified as a percentage of GAPDH band intensity at the appropriate stages.

### Western Blot Analysis

For Western blot analyses, retinal spheroids from the appropriate stages were stored in homogenization buffer (1 mM NaHCO3, 0.2 mM MgCl2 × 6 H2O, 0.2 mM CaCl2 × H2O, 1 mM spermidine; pH 8.0; all from Merck) and homogenized. Appropriate amounts of homogenates of spheroids were boiled in Laemmli buffer (Bio-Rad Laboratories, Munich, Germany) with 5% β-mercaptoethanol (Merck), fractionated by SDS polyacrylamide gel electrophoresis and then blotted to nitrocellulose membranes (GE Healthcare, Braunschweig, Germany). Blots were probed with specific primary monoclonal antibody against THase (Chemicon, Hampshire, Great Britain) at a dilution of 1:1000 for 12 hours and then incubated with peroxidase-conjugated anti-IgG (Dianova, Hamburg, Germany) at a dilution of 1:5000 for 90 minutes. Then, blots were developed with chemiluminescence (ECF Western detection system; GE Healthcare) and visualized using exposure to x-ray film for 4 hours.

### Cell Counting and Statistical Analysis

To determine the number of immunostained positive cells, we stained frozen sections with DAPI, rhod2, and CERN906 antibody, the percentage of immunolabeled cells per section of a single spheroid were calculated in relation to DAPI-positive cells of the same spheroid. At least six cryosections of different spheroids derived from two individuals were performed for each spheroid. To determine the relative expression of mRNA, the percentage was calculated in relation to the GAPDH band intensity for each culture stage. Each experiment was performed at least three times.

### Microscopy and Photography

Photomicrographs of sections were taken with a microscope (Axio-phot; Carl Zeiss, Jena, Germany) combined with a charge-coupled device, three-color (CCD-3) digital camera (Intas, Göttingen, Germany). Photomicrographs were processed on a computer with documentation and analysis software (Diskus Histologie MAN F70B; Hilgers, Königswinter, Germany; Multianalysts 2.0, Bio-Rad; Adobe Photoshop CS, Adobe Systems, Inc., San Jose, CA; and Excel, Microsoft, Redmond, WA).
RESULTS

Effect of GDNF Supplementation on rho4D2+ and Rhodopsin mRNA–Expressing Cells

When 50 ng/mL GDNF was added to the rosetted spheroids from E6 chick retinas under serum-reduced culture conditions, the number of rho4D2+ cells (immature rods) increased significantly in a time-dependent manner (Fig. 1; and see the introduction). At the same time, the expression of endogenous rhodopsin mRNA showed a parallel increase (Fig. 2). Specifically, the initial expression of rhodopsin mRNA in GDNF-treated cultures was low at 6% (relative to GAPDH controls), but increased rapidly to 21% at day in culture (dic)4 and remained high until dic10 (Figs. 2B, 2C). By contrast, control cultures maintained in serum-reduced medium demonstrated a relative rhodopsin mRNA expression level of 7% at dic2, increasing to 17% at dic6 before declining to 4% by dic10 (Figs. 2A, 2C).

As opposed to its affects on rod photoreceptors, the number of CERN906+ cells (immature red and green cones) were not affected by treatment with 50 ng/mL GDNF, since immunostaining revealed no significant differences between treated and control cultures (Figs. 1B, 1D, 1H). Semiquantitative RT-PCR for red, green, blue, and violet cone opsins confirmed this finding (Fig. 3).

Downregulation of GDNF Expression Using GDNF Antisense Oligonucleotides

By transfecting rosetted spheroids with a pCMS-EGFP(GDNF) antisense vector, we then tested the consequences of downregulation of endogenous GDNF expression. We tested four different GDNF antisense constructs and then used the sequence that achieved the highest transfection efficiency (approximately 30%). The temporal expression of endogenous GDNF was analyzed by semiquantitative RT-PCR (Fig. 4). Control cultures showed a somewhat variable pattern of GDNF expression, with the highest signals of mRNA transcripts found at dic9 and 11 (Fig. 4A). After treatment with the GDNF antisense vector, the intensity of GDNF mRNA expression indeed was very low, with expression of GDNF mRNA trans-
scripts detectable only during the first two culture days (Fig. 4B). Quantitatively (Fig. 4C), the highest relative GDNF mRNA expression occurred at dic5 (5%), thereafter diminishing gradually to 1% at dic13. By contrast, control cultures maintained expression levels of 8 to 22% between dic5 and 13.

**Knockdown of GDNF Decreases Rhodopsin Expression**

The consequence of transfection with GDNF antisense oligonucleotides on the expression of endogenous rhodopsin mRNA was analyzed by semiquantitative RT-PCR (Fig. 5). Control cultures showed a strong expression of rhodopsin mRNA transcripts at dic5 to 9–48 hours after transfection (17%–20% relative expression), and then a significant decrease at dic11 and 13 (6%; Figs. 5A, 5C). During the first culture days, rhodopsin mRNA expression after GDNF downregulation was high at levels of 8% to 12%. This level subsequently declined to approximately 2% by dic13 (Figs. 5B, 5C).

These findings were independently confirmed by immunostaining cryosections using the rod-specific antibody rho4D2 (Fig. 6). Compared with a nontransfected (Figs. 6A, 6C) and a transfected control (Figs. 6D, 6F), the GDNF antisense-treated cultures revealed a lower number of rho4D2 cells (Figs. 6G, 6I). For quantification, we determined the ratios of rho4D2 cells in control and GDNF-treated cultures based on the total number of DAPI-positive cells. In transfected control cultures at dic7 and 9 (48 hours after transfection), the number of rho4D2 cells reached 6% to 7%, while in nontransfected cultures, the percentage was approximately 1% higher. In contrast, cultures after knockdown of GDNF possessed less than or equal to half this number of rho4D2 cells. In fact, at the first and last time points tested after GDNF downregulation.

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**FIGURE 3.** Temporal expression of endogenous opsin mRNA in rosetted spheroids in serum-reduced culture conditions (A) or when treated with 50 ng/mL GDNF (B). Opsin mRNA for red, green, blue, and violet cones was analyzed by semiquantitative RT-PCR at different days in culture. For quantification, the percentage of opsin mRNA was calculated in relation to expression of GAPDH mRNA (C, D). Densitometric quantification of PCR products revealed no significant difference between control and GDNF-treated cultures.

**FIGURE 4.** Expression of GDNF mRNA in anti-GDNF-transfected rosetted spheroids. After the indicated culture periods, spheroids were transfected under control conditions (A) or with an antisense-probe specific for GDNF (B). Transfection continued for 12 hours, and harvest was 48 hours after transfection. Agarose gel electrophoresis of RT-PCR products revealed that GDNF mRNA expression was effectively downregulated (B, cf. A). Densitometric measurements document a successful GDNF knockdown (C). *P < 0.01; **P < 0.001; ***P < 0.0001.
The rod-specific effect mediated by GDNF was verified by immunostaining for red and green cones using the specific antibody CERN906 (Figs. 6B, 6E, 6H). Only at dic7, 9, and 11 (48 hours after transfection) was a weak decrease in the number of red and green cones detected in GDNF antisense transfected cultures (e.g., 0.5% fewer CERN906+ cells; Fig. 6K) compared to control cultures (Fig. 6J). Therefore, while rod photoreceptors were clearly diminished after downregulation of endogenous GDNF, cone photoreceptors were not significantly affected.

Supplementation with GDNF Enhanced THase Expression

Since GDNF has been reported to support the expression of THase, we analyzed the effect of GDNF supplementation on THase expression in rosetted spheroids using semiquantitative RT-PCR at different culture stages (Fig. 7). Under serum-reduced control conditions, initial expression occurred at dic8 (Fig. 7A), and remained constant until dic10. In contrast, addition of exogenous 50 ng/mL GDNF caused a significant temporal shift of THase expression (Fig. 7B), appearing at least 2 days earlier than in control cultures. Moreover, the amount of THase expression was significantly increased in response to GDNF supplementation. Quantitatively (Fig. 7C), the degree of THase mRNA expression increased gradually both under control and GDNF-supplemented culture conditions. However, while in control cultures relative THase expression increased from 5% to 6.5% between dic8 and 10, application of GDNF accelerated this expression. Specifically, at dic6, relative expression was 4%, increasing to 6.5% at dic8 and 9% at dic10.

The effect of GDNF on THase expression was confirmed by Western blot analysis of GDNF-treated and control cultures at dic6, 8, and 10. Figure 8 shows immunoblots probed with an antibody specific for THase. The GDNF-treated samples show initial THase protein expression at dic8, whereas in control cultures THase protein expression was first detected at dic10. By this stage, the immunoblots of GDNF-treated rosetted spheroids revealed a much higher intensity of THase expression than nontreated samples.

Effect of Knockdown of GDNF on Expression of THase

Final confirmation of the dependence of THase on GDNF was illustrated by downregulating GDNF by using antisense transfection (Fig. 9). As expected, this treatment suppressed expression of THase mRNA transcripts completely (Figs. 9B, 9C), while in control cultures, THase transcripts were detected from dic7 onward (Fig. 9A), consistent with previous data.

DISCUSSION

There is tremendous interest in the possibility of using GDNF therapeutically for maintenance of photoreceptors in blinding diseases. Therefore, a precise knowledge of its actions on development and maintenance of retinal tissue and its various cell types is essential. Using GDNF supplementation or knockdown techniques and RT-PCR analyses, we offer further support for a specific effect of GDNF on rhodopsin expression, and therefore on rod photoreceptors, during in vitro development of chick retinal tissue (3-D retinal spheroids). This result had been elucidated by other, mainly histologic, techniques in an earlier report. In addition, we applied antisense techniques to downregulate endogenous expression of GDNF to establish firmly the role of GDNF in rod development. Moreover, we detected a pronounced effect of GDNF on THase expression and thus on development of dopaminergic ACs of the retina. Two topics will be discussed in relation to these findings: first, the individual specificity and mode of GDNF action on rods and dopaminergic ACs, and second, the possibility of a common link between the actions of GDNF on these two different cell types.

Rod Photoreceptors as a Major Target for GDNF

The loss of photoreceptors caused by cell degeneration leads to retinitis pigmentosa (RP), a primary cause of blindness worldwide. Trophic factors capable of protecting neurons from cell death provide a promising strategy for neuroprotective intervention, as indicated by retinal degeneration studies in various animal models. A survival-promoting effect of GDNF has been reported for ganglion cells and photoreceptors and more specifically for rod survival. GDNF was shown to delay degeneration of rod photoreceptors in mouse and in a transgenic rat model of human retinitis pigmentosa. Moreover, the survival time and functional maintenance of rod outer segments were significantly improved by GDNF.

In an earlier study, we demonstrated multiple effects of GDNF on rod photoreceptors during in vitro development of the chicken retina, such as the ability of GDNF to affect proliferation, differentiation, and survival of rod photoreceptors.

![Figure 5. Temporal expression of rhodopsin mRNA in anti-GDNF-transfected rosetted spheroids. Spheroids were transfected at different culture days in control conditions (A) or with a GDNF antisense probe (B), analyzed by semiquantitative RT-PCR, and quantified by comparison to the expression of GAPDH mRNA (C). Electrophoresis of RT-PCR products revealed a similar expression pattern under both conditions (A, B), but note the different expression intensities (C). *P < 0.01; **P < 0.001.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933442/)
In that study, the role of GDNF as a mitogenic factor was established by demonstrating dose-dependent, cell-specific increases in rod proliferation. We also applied exogenous GDNF to chicken retinal spheroids and documented its effects on photoreceptors by immunohistochemistry. In the present study, we extended the analysis to examine the mRNA levels of rhodopsin using rod-specific RT-PCR probes. Adding 50 ng/mL GDNF to retinal spheroids enhanced the expression of rhodopsin mRNA significantly. In contrast to control cultures, a very high expression level of rhodopsin mRNA was sustained over the whole culture period; therefore, GDNF appears to promote the survival of rod photoreceptors.

Using a loss-of-function approach, we achieved an almost complete gene knockdown and concurrent reduction in rhodopsin mRNA levels of rhodopsin using rod-specific RT-PCR probes. Adding 50 ng/mL GDNF to retinal spheroids enhanced the expression of rhodopsin mRNA significantly. In contrast to control cultures, a very high expression level of rhodopsin mRNA was sustained over the whole culture period; therefore, GDNF appears to promote the survival of rod photoreceptors.

GDNF in Dopaminergic Cells in the Retina

Dopaminergic neurons in the retina possess a typical morphology that is consistent across different vertebrate classes. Be-
cause GDNF had been reported to prevent dopaminergic neurons from cell death,\textsuperscript{7,8,51} and since an effect of knockdown of GFR\alpha4 on ACs was demonstrated,\textsuperscript{27} we analyzed its role on dopaminergic cells in retinal spheroids. This approach was technically difficult because of the very small subpopulation of dopaminergic cells in retinal spheroids. This approach was technically difficult because of the very small subpopulation of dopaminergic ACs in the inner nuclear layer of the normal chicken retina.

The influence of GDNF on dopaminergic ACs of the retina has not been extensively studied, whereas the effectiveness of brain-derived neurotrophic factor (BDNF) has been firmly established. In the nervous system, BDNF can regulate the release of several neurotransmitters, including dopamine. The dopamine synthesis and release is modulated by light,\textsuperscript{52} as is the synthesis of BDNF,\textsuperscript{53,54} establishing a link between photoreceptors and dopaminergic cells. Dopaminergic ACs present a primary GDNF target. Indeed, a rescue of photoreceptors from cell death,\textsuperscript{7–9,51} and since an effect of knockdown of GFR\alpha4 on ACs was demonstrated,\textsuperscript{27} we analyzed its role on dopaminergic cells in reaggregated chicken retinal spheroids by immunohistochemistry, since less than 1% of ACs are dopaminergic in vivo and in vitro. However, by using RT-PCR to analyze THase mRNA levels in developing spheroids, we showed a very strong effect of GDNF on THase expression. Although this does not prove that differentiation and survival of these cells is sustained by GDNF, the results support such a notion. Amplification of the rate-limiting enzyme THase for dopamine synthesis revealed an earlier and much increased expression in GDNF-treated cultures. These findings were further confirmed by Western blot analysis, revealing a similar protein expression pattern as that shown for mRNA expression. Even more impressively, the downregulation of GDNF in chicken retinal spheroids suppressed expression of THase mRNA completely. Therefore, it is very likely that the differentiation of dopaminergic ACs is affected by GDNF.

Why does GDNF affect both rods and dopaminergic ACs? These latest results, together with our earlier findings, strongly suggest that GDNF supports differentiation and survival of both dopaminergic ACs and rod photoreceptors. This notion is in line with other reports that show that GDNF enhances differentiation of various cell types in the nervous system.\textsuperscript{61–63} In the retina, not much is known about the effect of GDNF on other cell types, such as ganglion, bipolar, amacrine, horizontal, or Müller glia cells (MGCs). Given the present results, we have to ask how GDNF achieves similar effects on two separate cell types in the developing retina. GDNF has been suggested to act on photoreceptor precursors in a direct manner,\textsuperscript{43} whereas some other trophic factors mediate their protective effects indirectly by activation of MGCs.\textsuperscript{64,65} In porcine retina, GFR\alpha1, -2, and -3 and cRET were expressed on MGCs but not on photoreceptors, strongly suggesting that MGCs could represent a primary GDNF target. Indeed, a rescue of photoreceptors by GDNF was mediated by retinal MGCs, possibly due to upregulation and release of FGF-2, which in turn could act on photoreceptors.\textsuperscript{65} This appealing mechanistic scheme puts MGCs at the center of GDNF action in the retina and certainly deserves further attention. With its processes extending across the entire retinal width, MRCs could well be cellular mediators...
for the effects of GDNF on both photoreceptors and on dopaminergic ACs.

Altogether, we have shown that GDNF possesses different functions during in vitro development of the chicken retina. GDNF strongly affected both rhodopsin and THase expression; thus, GDNF and its receptor(s) are likely to function in the differentiation and protection of rod photoreceptors and dopaminergic ACs. For a further understanding of the function of GDNF and its possible interaction with MGCs and the rest of the retina, additional investigations are needed. However, results from these studies offer more evidence that GDNF and its receptors could become therapeutic tools and/or targets for extending the lifespan of degenerating photoreceptors.

Acknowledgments

The authors thank Colin Barnstable, David Gamm, David Hicks, and Joyce Tombran-Tink for helpful discussions and Jutta Huhn and Meike Stotz-Reimers for expert technical assistance.

References


