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GDNF-Induced Osteopontin from Müller Glial Cells Promotes Photoreceptor Survival in the Pde6b^{rd1} Mouse Model of Retinal Degeneration

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KEY WORDS
Retinitis pigmentosa; neuroprotection; neuron–glia interaction; apoptosis; OPN; PI3K; illumina transcriptome arrays

ABSTRACT
Glial cell line-derived neurotrophic factor (GDNF) enhances the survival of a variety of neurons, including photoreceptors (PR) in the retina. In contrast to most other GDNF receptive neurons, GDNF does, however, not exert its neuroprotective activity directly on PR neurons but transmits it indirectly by inducing expression of yet unknown neurotrophic factors in retinal Müller glial (RMG) cells. Genome-wide differential transcriptome analyses of GDNF-treated mouse retinas revealed 30 GDNF-induced transcripts containing a total of six genes coding for secreted molecules. Among them was (OPN), a secreted glycoprotein which was expressed in mouse RMG and secreted from primary mouse RMG in culture. Furthermore, OPN secretion was significantly upregulated on GDNF treatment of primary RMG. To validate, whether OPN could qualify as a neuroprotective factor for PR, we evaluated its potential neurotrophic activity on isolated PR *in vitro* as well as on retinal explants from the *retinal degeneration 1* (Pde6b^{rd1}) mouse mutant. OPN exerted a significant, positive survival effect on primary porcine PR cells in a concentration-dependent manner and induced activation of PI3K/Akt pro-survival pathway. Moreover, in retinal explant cultures from Pde6b^{rd1} mice, OPN significantly reduced the percentage of apoptotic cells to levels comparable with that observed in explants from wild-type mice and led to survival of significantly more PR in long-term retinal explant cultures. Our findings suggest that RMG-derived OPN is a novel candidate protein that transmits part of the GDNF-induced neuroprotective activity of RMG to PR cells.

INTRODUCTION

The degeneration of photoreceptors (PR) associated with progressive retinal dystrophies is the inception to the major cause of blindness in the western world. Among these dystrophies, *retinitis pigmentosa* comprises a heterogeneous group of inherited disorders occurring in one of 4,000 people for a total of more than 1 million individuals worldwide (Hartong et al., 2006). Reports on rodent models of *retinitis pigmentosa* demonstrate that PR degeneration involves different cell death pathways with apoptosis as the final event (Adler, 1996; Chang et al., 1993; Lolley et al., 1994; Portera-Cailliau et al., 1994; Tso et al., 1994). The most extensively studied mouse model of *retinitis pigmentosa* is the *retinal degeneration 1* (Pde6b^{rd1}) mouse which carries a mutation in the gene for the rod PR-specific phosphodiesterase-6 beta subunit (Pde6b). This mutation renders Pde6b protein non-functional (Bowes et al., 1990) leading to cGMP accumulation and rapid apoptosis of rods (Farber and Lolley, 1974) and finally results in loss of cones as secondary effect (Hartong et al., 2006).

Irrespective of the death mechanisms, many investigations have followed the delay of PR apoptosis as a promising therapeutic strategy for the treatment of these disorders. The use of neurotrophic factors to prevent PR loss has been studied for more than a decade. Molecules like brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (FGF-2), and glial cell line-derived neurotrophic factor (GDNF) were shown to delay degeneration of PR in various *in vitro* and *in vivo* models (Faktorovich et al., 1990; Frasson et al., 1999; Gauthier et al., 2005; Li et al., 2010).

Additional Supporting Information may be found in the online version of this article.

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GDNF is one of the most effective neuroprotective factors for retinal cell survival (Frasson et al., 1999; Koeberle and Ball, 1998; Yan et al., 1999). Originally purified from a rat glioma cell line supernatant as a trophic factor for embryonic midbrain dopamine neurons (Lin et al., 1993), GDNF is widely distributed in diverse tissues (Nosrat et al., 1996) especially in the central and peripheral nervous systems. Its expression in the eye is restricted to the retina and many studies have shown its potential therapeutic value by providing neuroprotection in the retinal degeneration context. In the Pde6b^{rd1} model, subretinally injected GDNF was shown to delay PR death and to rescue PR function (Frasson et al., 1999). This result was subsequently confirmed in several models of PR degeneration *in vivo* (Lawrence et al., 2004; Wu et al., 2002).

To unravel the cellular mechanisms of the GDNF-induced PR rescue, we previously studied the expression of GDNF receptor components, namely transmembrane tyrosine kinase Ret and GPI-achored co-receptors GFRα-1, −2 and, −3 by means of immunohistochemistry in the porcine retina (Hauck et al., 2006). The findings supported expression of all receptor components on retinal Müller glial (RMG) cells among other cells, however, none of these receptor components was expressed in PR. These findings were complemented by other groups, studying receptor expressions in other species such as rodents and chickens (Brantley et al., 2008; Harada et al., 2002; Hauck et al., 2006; Jomary et al., 1999, 2004; Karlsson et al., 2002; Koeberle and Ball, 2002; Kretz et al., 2006; Rothermel et al., 2004) which show partly contradicting results but in summary all support that GDNF signaling components are not expressed in PR. This results in consent on the hypothesis that GDNF does not exert its neuroprotective function directly.

Indeed, GDNF modulates trophic factor release by RMG *in vitro*, suggesting that GDNF could exert its neuroprotective effect through both direct and indirect pathways (Harada et al., 2002, 2003; Hauck et al., 2006). Taken together, observations support that the neuroprotective effect of GDNF on PR survival in mouse models of *retinitis pigmentosa* is mediated by the interaction of GDNF with RMG, which release secondary neuroprotective factors acting directly on PR.

In this study we used a transcriptome approach to identify the molecules upregulated in RMG in response to GDNF stimulation. The secreted glycoprotein osteopontin (OPN) was found to exert a pro-survival effect on isolated PR *in vitro* as well as on PR in retinal explant cultures from the Pde6b^{rd1}. OPN prolonged the survival of isolated PR *in vitro*, and the number of apoptotic PR was reduced in Pde6b^{rd1} explants. These results provide evidence that GDNF induces differences in the gene expression profile of mouse retinas and suggest a role for RMG-derived OPN as a novel neuroprotective molecule for PR cells.

Abbreviations

BDNF	brain-derived neurotrophic factor
FGF-2	basic fibroblast growth factor
CNTF	ciliary neurotrophic factor
DMEM	Dulbecco's modified Eagle's medium
FCS	fetal calf serum
GDNF	glial cell line-derived neurotrophic factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUVEC	human umbilical vein endothelial cell
ONL	outer nuclear layer
OPN	osteopontin
PBS	phosphate buffered saline
PR	photoreceptors
RMG	retinal Müller glial cells
SD	standard deviations.

MATERIALS AND METHODS

Animals

Eyecups for GDNF treatment were dissected from 2-month-old human glial fibrilary acidic protein-enhanced green fluorescent protein (hGFAP-eGFP) transgenic mice (Nolte et al., 2001) on a strain of FVB genetic background. Cultures of RMG cells were obtained from hGFAP-eGFP transgenic mice on a C57Bl/6 background. Retinal explants were prepared from C3H/HeH (Pde6b^{rd1}) mice (Dalke and Graw, 2005). Wildtype (wt) mice used for explants were congenic-C3H*Pde6b*+ (Hart et al., 2005). PR survival assays were performed with PR from adult porcine eyes provided by a local slaughterhouse.

Isolation and *In Vitro* Culturing of Primary Mouse RMG and Mouse Eyecup Cultures

Mouse RMG (mRMG) were isolated employing a panning method (Hauck et al., 2003) and cultured in Dulbecco's modified Eagle's medium/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (DMEM/HEPES [GIBCO]) with 10% fetal calf serum (FCS) to confluence (7 days) resulting in a purity above 97% (Hauck et al., 2003). One day before stimulation, mRMG were washed, transferred into serum-free medium (DMEM/HEPES; GIBCO), and after 12 h of serum starvation stimulated with GDNF (100 ng/mL; Peprotech) for additional 24 h. Isolated supernatants were filtered (cut-off 0.2 μm) to eliminate debris. Mouse eyecup cultures with attached retinas from hGFAP-eGFP mice were incubated as previously described (Alonso-Gomez et al., 2000) with minor modifications. After dissection, eyecups were placed into 24-well cell culture plates (Falcon) with 1 mL of serum-free medium (DMEM/HEPES; GIBCO) per well and stimulated with GDNF (100 ng/mL; Peprotech) for 24 h (37°C, humidified atmosphere of 5% CO_2/95% air). After incubation, retinas were dissected from the eyecups and mRNA was isolated from retinas for microarray analysis.

RNA Isolation and Microarray Analysis

Mouse retinas were collected in two groups (using a total of 25 mice). The retinas were resuspended in lysis

buffer (QIAGEN) and dissociated with a 0.6-mm gauge needle for subsequent RNA purification (RNeasy kit, QIAGEN) including DNase treatment. RNA quality was assessed using the Agilent Bioanalyser and only high-quality RNA (RIN > 8) was used for array analysis. Five samples (two GDNF-treated, three controls) were processed. Total RNA of 120 ng was amplified in a single round with the Illumina TotalPrep RNA Amplification Kit (Ambion). Amplified RNA of 1.5 μg was hybridized on Illumina MouseWG-6 v1.1 arrays containing about 45k probes. Staining and scanning was done according to the Illumina expression protocol. Illumina Beadstudio software 3 was used for background correction. The Bioconductor software package implemented in CARMAweb (Rainer et al., 2006) was employed for normalization using the quantile algorithm. Upregulated probes were analyzed using the *t*-test implemented in CARMAweb and probes with *P*-values less than 0.01 were selected for further analysis. GO term enrichment analysis was performed with GePS (Genomatix) using standard settings ($P < 0.01$).

Photoreceptor Preparation and Survival Assay

Porcine PR were prepared and cultured as described (Hauck et al., 2008). Different concentrations of OPN (25 ng/mL, 50 ng/mL, 100 ng/mL of purified human recombinant OPN; R&D Systems) in DMEM/F-12 medium (GIBCO) or medium alone were applied to the PR cultures 20 h after preparation. PR survival was monitored by an esterase calcein-fluorophore assay (Molecular Probes), whereby living cells fluoresce bright green. Total fluorescence per well is linearly correlated to the number of living cells (Hauck et al., 2006) and was measured daily for 7 days, using a fluorescence reader (BioTek; Synergy HT) and compared with initial fluorescence. Every experimental condition was assayed at least in triplicate.

Retina Explant Culture

Retinas were isolated from 5 days old Pde6b^{rd1} and wild type (wt) mice with the retinal pigment epithelium attached essentially as described previously (Caffe et al., 2001). In brief, animals were killed and the eyes enucleated in aseptic environment. Right after the enucleation, the eyes were incubated at 37°C for 15 min in R16 serum-free medium (Invitrogen Life Technologies) containing 0.12% proteinase K (MP Biomedicals). The enzymatic digestion was stopped with 10% FCS (GIBCO) in R16 medium, and the eyes were dissected. The anterior segment, lens, vitreous, sclera, and choroids were carefully removed and the retina was cut perpendicular to its edges, resulting in a coverleaf-like shape. Subsequently, the retina was transferred to a 0.4-μm polycarbonate membrane (Costar) with the retinal pigment epithelium directly facing the membrane. The insert was placed into a six-well culture plate and incubated in R16 nutrient medium at 37°C. Every second day, 1.2 mL of the nutrient medium, was replaced. The retina was left in R16 culture medium without treatment for 2 days to adapt to culture conditions. Then explants were either exposed to 50 ng/mL OPN (R&D systems), 100 ng/mL GDNF (Peprotech) or kept as untreated control. Explants were cultured for 6 days (short-term cultures, PN5 + 6) or for 12 days (long-term cultures, PN5 + 12). Culturing was stopped by 1h fixation in 4% paraformaldehyde, washed in phosphate buffered saline (PBS), and then cryoprotected by incubation for 10, 20, and 30 min with 10%, 20%, and 30% sucrose, respectively. Explants were embedded in Tissue Tek (Sakura) for cryosectioning (14 μm) using a cryotome (Leica, CM 1850) and if necessary stored frozen for short periods.

Immunohistochemistry

For immunolabeling sections were rinsed in PBS and incubated for 10 min in 0.1% Triton X-100 in PBS, followed by blocking for 30 min in 5% normal donkey serum in PBS-T. Incubation with primary antibodies to mouse OPN (1:200; R&D Systems) or glutamine synthetase (GS; 1:200; BD Transduction Laboratories) diluted in 1% NDS-PBS-T was done overnight at 4°C, followed by incubation with Alexa 488/568-coupled secondary antibodies (1:200; Molecular Probes) for 2 h at room temperature. After a final washing, sections were mounted in FluorSave (Calbiochem) and microscopic images were obtained (Axio Imager Z1, Zeiss).

TUNEL Assay

Apoptotic cells in sections from retinal explant cultures were detected using a TUNEL assay kit (Roche Diagnostics) according to the manufacturer's instructions. Controls consisted of omitting the terminal deoxynucleotidyl transferase enzyme from the labeling solution (negative control) and in incubating sections for 30 min with DNase I (Roche Diagnostics, 3 U/mL) in 50 mM Tris-HCl pH 7.5, 1 mg/mL bovine serum albumin to induce DNA strand breaks (positive control). While no labeling was observed in the negative control sections, essentially all nuclei in all retinal layers were stained in the positive control sections (not shown). DAPI was applied at the end of the TUNEL assay to label nuclei.

Image Analysis and Statistics

Fluorescent and TUNEL stainings were quantified and averaged (minimum four animals) at 200-fold magnification or 400-fold magnification (Axio Imager Z1, Zeiss).

Images of the entire section using the mosaic picture mode were acquired for the TUNEL sections. For each section, subsets (regions of interest) were defined and

analyzed using a software (Definiens Enterprise Image Intelligence™ Suite, Fa. Definiens AG, Munich) (Baatz et al., 2009). A specific ruleset was developed to detect and quantify semantic classes. In a first step, the algorithm segments pictures iteratively, recognizing groups of pixels as objects. The objects were classified based on fluorescence-layer, intensity, shape, and neighborhood grading two semantic classes "TUNEL signal" and "outer nuclear layer." For each image, the number of detected TUNEL-signals and the area of DAPI stained outer nuclear layer were calculated. The total number of cells was determined by measuring the area of the outer nuclear layer (ONL) and dividing it through the average cell size. The number of positive cells was then divided by the total number of cells in the ONL to calculate the percentage of TUNEL-positive cells. Statistical comparisons among different experimental groups were made assuming a normal data distribution using a two-tailed Student's *t*-test and Microsoft Excel software. Error bars indicate standard deviations (SD), levels of significance are $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Western Blots

Supernatants obtained from primary mRMG 7 days in culture (untreated or treated with 100 ng/mL GDNF) were resolved by 10% dodecyl sulphate-polyacrylamide gel electrophoresis and blotted semidry onto polyvinylidene difluoride membranes. Blots were incubated with primary antibodies in 5% BSA TBS-T overnight at 4°C (anti-OPN, 1:1,000; R&D systems) or anti-phosphoAkt (ser473; 1:1,000; Cell Signaling Technology), washed, and incubated with appropriate horseradish peroxidise-coupled secondary antibodies (1:15,000; Jackson Laboratories). Signal was developed with the ECL+ enhanced chemiluminescence kit (GE Healthcare). Blots were stripped and reprobed with anti-Akt (1:1,000; Cell Signaling Technologies) antibodies to verify equivalent protein loading.

Enzyme-Linked Immunosorbent Assay (ELISA)

OPN levels in filtered mRMG supernatants (untreated or treated with 100 ng/mL GDNF) were determined after a culture period of 7 days using ELISA (Quantikine® mouse OPN immunoassay; R&D systems). Absorbances were measured in a Synergy HT Multi Detection Microplate Reader (Synergy) at 450 nm and all experimental conditions were performed in triplicates.

RESULTS

GDNF Induces Expression of OPN in Mouse Retina

We performed a microarray study using total mouse eyecup cultures from hGFAP-eGFP transgenic mice treated with GDNF to identify genes that are upregulated under GDNF stimulation at the transcript level. After treatment, the retinas were isolated and directly used for RNA extraction. Amplified aRNA was hybridized to Illumina Mouse WG-6 v1.1 expression beadchips which provide almost complete coverage of the mouse genome (about 45k probes). The statistical analysis of the obtained dataset resulted in the identification of 30 genes ($P < 0.01$), upregulated after 24 h of GDNF stimulation in comparison with untreated controls (Table 1 and Supp. Info. Fig. 1). Six of these genes coded for secreted proteins. OPN, previously reported with anti-apoptotic functions (Geissinger et al., 2002) was selected for further analysis.

GDNF Increases the Expression of Intracellular OPN in Mouse Retinal Explants Exclusively in RMG cells

OPN is expressed in neurons of the developing and adult central nervous system (CNS), (Iczkiewicz et al., 2004; Lee et al., 2001; Shin et al., 1999) and in microglia of the rat retina after excitotoxic injury (Chidlow et al., 2008). OPN is also expressed in the retina of Pde6b^{rd1} and wild type (wt) mice, and similar levels of OPN were detected by Western blots of PN11 tissues from both genotypes (data not shown). Since we found that OPN transcript expression increased after GDNF treatment and since we had demonstrated before that GDNF induces activation of RMG cells in the retina (Hauck et al., 2006), we aimed to determine the *in situ* localization of induced OPN protein expression. For this purpose, we treated explants from wt C3H mice with GDNF (2 and 4 days after explantation) and after fixation (6 days after explantation) labeled for OPN as well as for GS, a RMG marker. OPN expression in untreated controls was restricted to the retinal ganglion cell layer (Fig. 1A). Since OPN precisely co-localized with GS, we conclude that in untreated control retinas, OPN expression is confined to the endfeet of RMG cells. In contrast, GDNF-treated explants (treated with 100 ng/mL GDNF) showed a marked increase of OPN expression (Fig. 1B). OPN staining was now also evident in the inner nuclear layer (INL), and again co-localized with GS (Fig. 1C). Fluorescence intensities and immunoreactive section areas revealed a significant increase in OPN staining intensity ($P < 0.01$, Fig. 1D) and OPN-positive area ($P < 0.05$, Fig. 1E). Moreover, we observed a significant increase in GS staining intensity as well as GS-immunoreactive section area after treatment with GDNF.

These findings confirmed the upregulation of OPN and GS in RMG cells in response to GDNF treatment and additionally revealed upregulation of GS expression in mouse retinal explant cultures.

GDNF Stimulation Increases the Secretion of OPN from Isolated mRMG cells

Even though we have shown that OPN is a molecule expressed in RMG cells, it was necessary to confirm that

TABLE 1. Upregulated Genes in hGFAP-eGFP Mouse Retinas in Response to GDNF Stimulation. mRNA from hGFAP-eGFP Explanted Retinas Treated with GDNF (100 ng/mL) Was Used for Microarray Analysis. Shown are 30 Genes with $p < 0.01$

Symbol[a]	Protein name[b]	Probe ID[c]	Accession number[d]	Ratio[e]	Average expression %[f]	Signal peptide[g]
Stfa	Stefin-1	ILMN_2774410	CYT1_MOUSE	231.0	124	
Sox10	Transcription factor SOX-10	ILMN_1228105	SOX10_MOUSE	61.0	16	
Tns4	Tensin-4	ILMN_1236029	TENS4_MOUSE	43.7	12	Yes
Mlana	Melan-A	ILMN_1233568	Q2TA50_MOUSE	21.3	73	
Tyrp2	L-dopachrome tautomerase	ILMN_1251894	TYRP2_MOUSE	17.6	118	Yes
Plac8	Placenta-specific gene 8 protein	ILMN_2766415	PLAC8_MOUSE	16.6	14	
Spp1	Osteopontin	ILMN_2690603	OSTP_MOUSE	5.1	580	Yes
Col17a1	Collagen alpha-1(XVII) chain	ILMN_1240481	COHA1_MOUSE	2.4	45	Yes
Fbxo32	F-box only protein 32	ILMN_2752994	FBX32_MOUSE	2.4	15	
3110021N24Rik	3110021N24Rik	ILMN_1252656		1.8	19	
D930020B18Rik	Uncharacterized protein C12orf56 homolog	ILMN_1241856	CL056_MOUSE	1.7	23	
Glra	Glycine receptor subunit alpha-1	ILMN_2451750	GLRA1_MOUSE	1.7	43	Yes
Bgn	Bone/cartilage proteoglycan I	ILMN_2637249	PGS1_MOUSE	1.6	18	Yes
Kcnj8	ATP-sensitive inward rectifier potassium channel 8	ILMN_1253409	IRK8_MOUSE	1.6	18	
Ly6c1	Lymphocyte antigen 6 complex, locus C1	ILMN_1254927	Q91XG0_MOUSE	1.6	47	
Kcnk6	Potassium channel subfamily K member 6	ILMN_1258376	KCNK7_MOUSE	1.6	21	
Txnip	Thioredoxin-interacting protein	ILMN_1213609	TXNIP_MOUSE	1.6	317	
D230044L08Rik	D230044L08Rik	ILMN_1257426		1.6	87	
Btbd14a	Nucleus accumbens-associated protein 2	ILMN_2597978	NACC2_MOUSE	1.5	30	
Arhgef16	Rho guanine nucleotide exchange factor 16	ILMN_1239332	ARHGG_MOUSE	1.5	25	
LOC236598	LOC236598	ILMN_1248092		1.5	19	
2210410D02Rik	2210410D02Rik	ILMN_1239137		1.5	19	
Fam82b	Regulator of microtubule dynamics protein 1	ILMN_2482897	RMD1_MOUSE	1.5	21	
Fes	Tyrosine-protein kinase Fes/Fps	ILMN_2695793	FES_MOUSE	1.4	20	
2310046K01Rik	Uncharacterized protein C20orf54 homolog	ILMN_2675760	CT054_MOUSE	1.4	25	
Mmrn2	Multimerin-2	ILMN_2494159	MMRN2_MOUSE	1.4	52	Yes
5730416F02Rik	5730416F02Rik	ILMN_2737046		1.4	39	
E230026L19Rik	E230026L19Rik	ILMN_1240942		1.4	27	
Grrp1	Glycine/arginine-rich protein 1	ILMN_2705434	GRPP1_MOUSE	1.3	44	
Usp7	Ubiquitin carboxyl-terminal hydrolase 7	ILMN_1221908	UBP7_MOUSE	1.3	39	

[a]Protein recognition short symbol.
[b]Name of identified proteins according to Swiss-Prot database(number of unique identifications).
[c]Probe identification Illumina ID.
[d]Swiss-Prot identifier.
[e]Linear ratio treated/untreated.
[f]Average expression of the upregulated proteins in the retina.
[g]Extracellular localization information given by Swiss-Prot database and SignalP software.

OPN is secreted under GDNF stimulation to qualify OPN as a potential RMG-derived neurotrophic factor. Primary mouse RMG (mRMG) were isolated by the panning method (day 1 *in vitro*, Fig. 2A) and after 7 days in culture (Fig. 2B) treated for 24 h with GDNF (100 ng/mL). Supernatants were collected and monitored for OPN expression by Western blots and ELISA. Secreted OPN was detected with around 66 kDa in Western blots (Fig. 2C) and was induced under the influence of GDNF. The previously reported sizes of secreted OPN vary from 44 to 75 kDa, due to post-translational modifications (Sodek et al., 2000). An OPN-specific ELISA confirmed a significant increase of OPN in the mRMG supernatants (**$P < 0.01$) (Fig. 2D).

OPN Promotes Survival in Primary PR Through the Activation of PI3K/Akt Pathway

GDNF-induced upregulation of OPN secretion could directly influence PR survival. To test this hypothesis, OPN was directly applied to isolated primary procine PR cultures and the survival of PR was monitored by a calcein-estearase assay as described before (Hauck et al., 2008). The survival supporting effect of OPN was already detectable after 3 days *in vitro* (data not shown) and was significantly enhanced after 5 days *in vitro* (Fig. 3A). Concentrations below 25 ng/mL OPN did not increase survival compared with the negative control (data not shown), but OPN at 25, 50, and 100 ng/mL robustly enhanced survival of PR.

Cell survival can be promoted by several distinct or interacting intracellular pathways. One of the best known pathways related to cell survival is the PI3-K/Akt pathway. We investigated whether OPN could induce activation of PI3-K/Akt pathway in isolated PR cultures. PR were stimulated with OPN for different time periods and western blots of the lysates were probed with an antibody specific for phospho-Akt (Ser473). Akt showed a time-dependent increase of phosphorylation as compared with non-phosphorylated Akt (Fig. 3B). Already after 5 min, a slight increase in Akt phosphorylation was observed which increased continuously during the time course of the experiment (30 min).

OPN Reduces Cell Death in Pde6b^{rd1} Retina Explant Cultures

To test whether OPN promotes PR cell survival in the context of a mutation-induced retinal degeneration, we analyzed retinal explants from Pde6b^{rd1} which carries a mutation in the PR-specific PDE6beta resulting in complete PR loss at 4 weeks of age. Explants were prepared

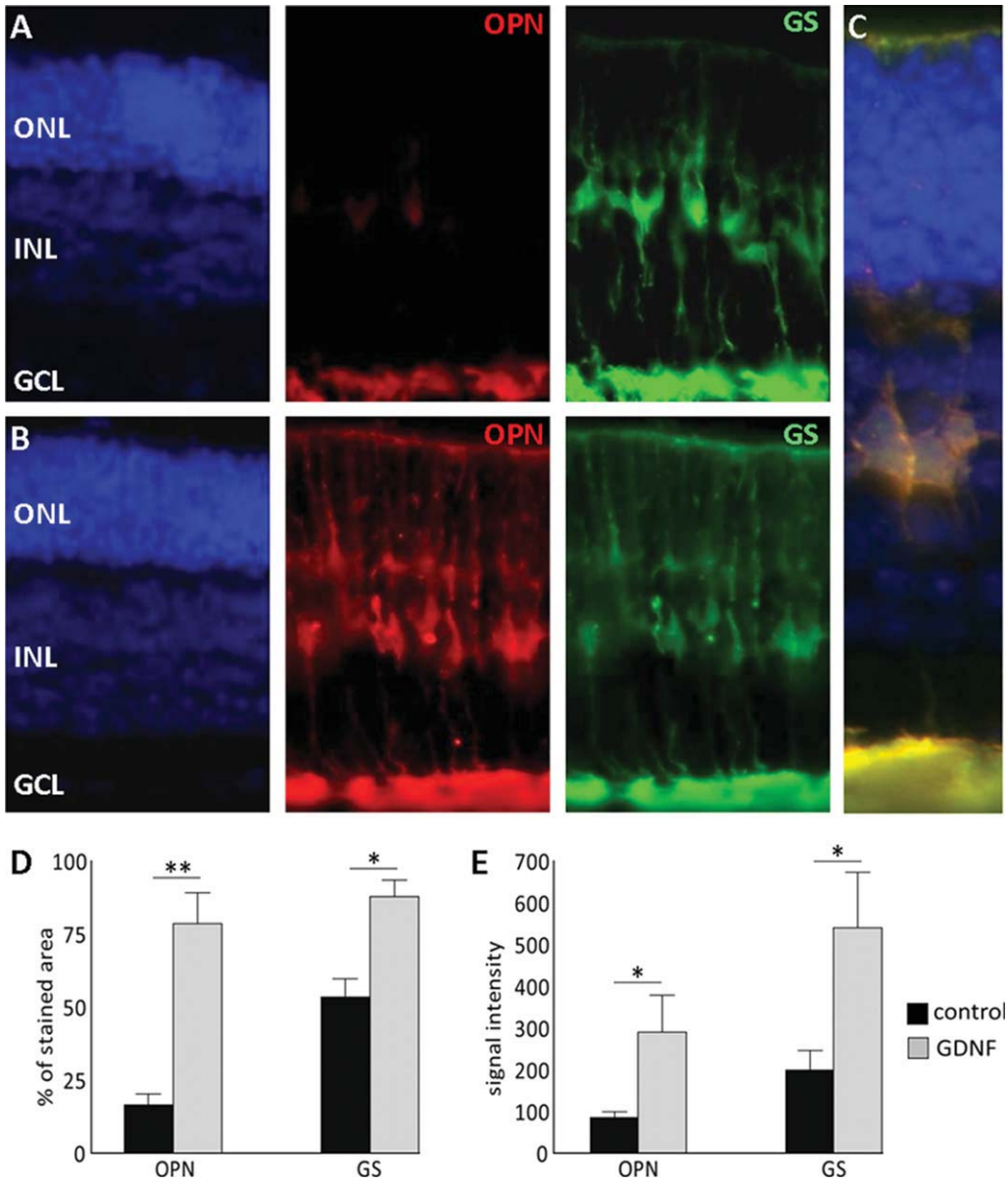

Fig. 1. OPN expression in mouse retinal explants after GDNF stimulation. Immunohistochemical analysis of wt mouse retinal explants shows expression of OPN and GS specifically in RMG cells. Non-treated sections of wt retinal explants (**A**), and wt explants treated with GDNF (100 ng/mL) (**B**) were stained with anti-OPN and anti-GS antibodies ($n = 3$). The percentage of OPN stained area was significantly increased (**$P < 0.01$) and also slightly increased for GS (*$P < 0.05$) under GDNF stimulation (**D**).The intensity of the fluorescence signal was also significantly increased under GDNF stimulation for both OPN and GS signals (*$P < 0.05$) (**E**). The specificity of OPN staining in RMG end feet (GCL layer) and cell bodies (INL) was confirmed under high magnification (**C**). Images are representative for at least three independent experiments.

from PN5 Pde6b^{rd1} and wt mice, cultivated for 6 days (short-term) in a medium lacking OPN or in a medium supplemented with OPN (50 ng/mL) and analyzed at a time point when PR degeneration is at a peak in the Pde6b^{rd1} mutant *in vivo* (i.e., at PN11). Apoptotic cells were detected by TUNEL staining and quantified in relation to the total number of cells in the ONL. Treatment with OPN resulted in a significant decrease in the percentage of TUNEL positive cells (see Fig. 4) in Pde6b^{rd1} retina explants to similar values as observed in untreated wt explants (untreated Pde6b^{rd1} explants: 6.11% ± 2.08, $n = 4$, Fig. 4A; untreated wt explants: 2.02% ± 0.57, $n = 4$, $P = 0.014$, Fig. 4B; OPN-treated Pde6b^{rd1} explants: 1.62% TUNEL-positive cells ±0.80, $n = 4$, $P = 0.0096$, Fig. 4C).

To ensure that the effect of OPN translates into long-term survival of PR, explants were cultivated for 12 days (long-term cultures) and the PR number was measured. The quantification of PR nuclei in the ONL revealed a significant increase of the PR numbers in Pde6b^{rd1} long-term explants treated with OPN (50 ng/mL) (see Fig. 5) as compared with the untreated controls

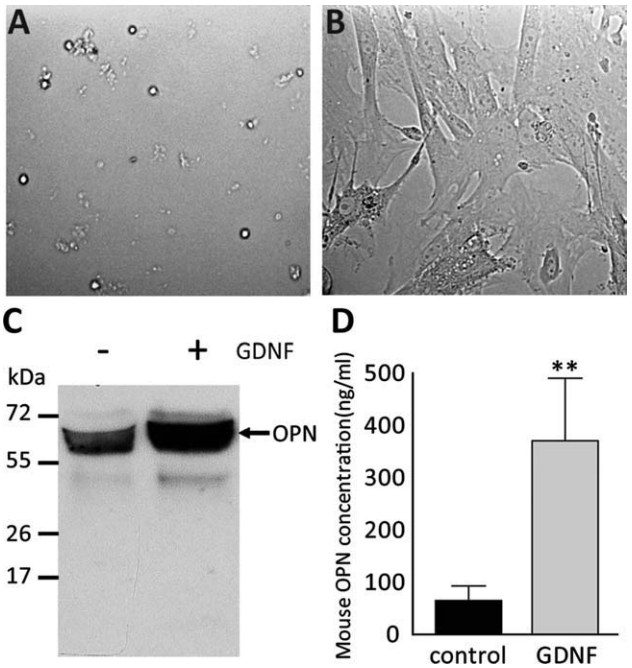

Fig. 2. Increase of OPN secretion by mRMG cells after GDNF stimulation. mRMG cells were isolated from 2-month-old wt mouse retinas and cultured in non-coated 6-well plates. After 24 h of incubation the culture medium was replaced to discard non-attached cells from the culture (panning). Only RMG cells remain attached and their morphology transformed to a rounded cell shape (**A**). After 7 days in culture, RMG developed a fibroblast-like morphology (**B**). RMG cells (after 7 days in culture) were treated with GDNF (100 ng/mL) for 24 h. (**C**) Western blot analysis of RMG supernatants induced with GDNF and controls shows higher amounts of secreted OPN. (**D**) Quantification by OPN-specific ELISA assay confirmed the significantly upregulated expression of secreted OPN (**$P < 0.01$) (concentration measured in ng/mL) in RMG supernatants treated with GDNF. (**$P < 0.01$, Student's t-test).

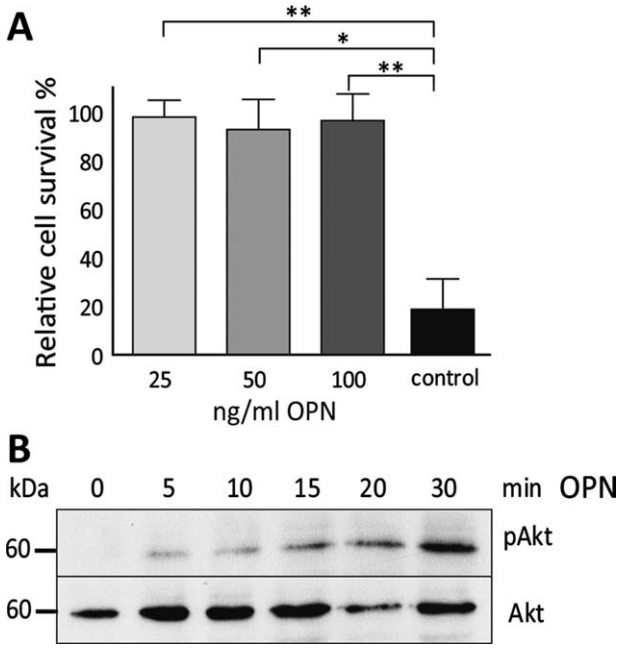

Fig. 3. OPN directly promotes PR survival *in vitro*. Porcine PR were isolated and cultured until they attached to the culture plate (24 h). After incubation in FCS-free medium, PR were treated with OPN (25, 50, and 100 ng/mL) and maintained in culture for additional 5 days. For all concentrations tested, a significant maintenance of the PR survival was detected (*$P < 0.05$, **$P < 0.01$) compared with untreated control PR (**A**). Western blots of lysates from primary porcine cultured PR treated with OPN (50 ng/mL) for different time points revealed a time-dependent increase of the phosphorylated form of Akt (Ser473) as compared with total amount of Akt protein (**B**).

(untreated wt explants: 1094 ± 70, $n = 4$, Fig. 5A; OPN-treated Pde6b^{rd1} explants: 626 PR in ONL ±43, $n = 3$, $P = 0.00024$, Fig. 5B; untreated Pde6b^{rd1} explants: 288 ± 20, $n = 3$, Fig. 5C).(1,089)

DISCUSSION

GDNF has previously been identified as one of the most potent neurotrophic factors for PR. In fact, GDNF has been shown to delay PR degeneration and to preserve retinal function in an animal model of human RP, the Pde6b^{rd1} mouse (Frasson et al., 1999).

However, the GDNF-mediated neuroprotection is likely transmitted to PR cells via an indirect mechanism involving RMG. Indeed, direct application of GDNF to PR *in vitro* does not enhance their survival (Hauck et al., 2006). During retinal degenerations RMG play an active role in the regulation of PR survival by releasing neurotrophic factors providing protection from deleterious mutations (Bringmann et al., 2006). In line with this, we have demonstrated that GDNF application to isolated RMG induced the expression and secretion of bFGF (FGF-2) (Hauck et al., 2006). FGF-2 has been thoroughly studied for its potential neuroprotective properties in various animal models of retinal degeneration (Faktorovich et al., 1990; LaVail et al., 1992; Sakai et al., 2007; Uteza et al., 1999). Unfortunately, the effect of this molecule improving PR survival has not been completely successful to date (Lau et al., 2000; Yamada et al., 2001). Of note, FGF-2 does not exert any functional rescue in rodents (LaVail et al., 1998), in contrast to GDNF (Frasson et al., 1999). These findings suggest that other neuroprotective factors, secreted by RMG in response to GDNF stimulation, might mediate the GDNF-related functional rescue effect.

In this work we identified several transcripts induced in mouse retina under the influence of GDNF. Among these transcripts was OPN which is expressed as a secreted and an intracellular variant and which has previously been demonstrated to exert neuroprotective functions (Iczkiewicz et al., 2007). We confirmed localization of OPN to RMG and could also demonstrate that isolated RMG respond with elevated secreted OPN to exogenously applied GDNF. This confirms the hypothesis of RMG cells as target cells for GDNF-induced signaling (Hauck et al., 2006). Given the wide range of functions attributed to OPN, it is surprising that it has not been more extensively investigated in relation to neurodegenerative diseases. In this study, we identified for the first time OPN as a secreted factor from RMG cells under GDNF stimulation and established a novel

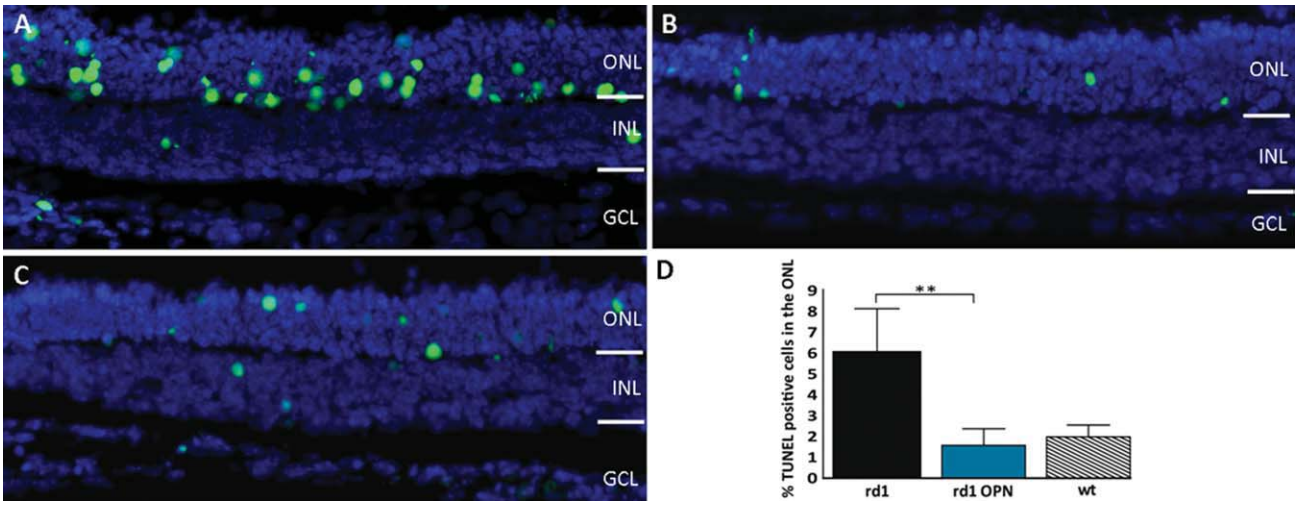

Fig. 4. Effect of OPN on PR cell survival in Pde6b^{rd1} short-term cultured retinal explants. Mouse retinal explants were explanted on PN5 and cultured for additional 6 days *in vitro*. TUNEL stainings on day PN5+div6 in Pde6b^{rd1} retinal explants (**A**) showed considerably more TUNEL-positive cells than their corresponding wt counterparts (**B**). Treatment with 50 ng/mL of OPN significantly decreased the number of TUNEL-positive cells in the ONL of Pde6b^{rd1} retinal explants (**C**). Percentage of TUNEL-positive cells in the ONL compared with total number of ONL cell nuclei for each condition (**$P > 0.01$) (**D**). Images were taken at 200 magnification and are representative for at least three independent experiments.

neuroprotective activity of this molecule for PR in an animal model for retinitis pigmentosa, the Pde6b^{rd1} mouse mutant.

GDNF-Induced Gene Expression

Given that GDNF has been shown to rescue PR in the Pde6b^{rd1} mouse (Frasson et al., 1999) and that RMG comprise the major targets cells for GDNF in the retina (Hauck et al., 2006), we screened for GDNF-induced expression changes of secreted molecules with putative neuroprotective activities. We found that mouse retinas responded to GDNF application with an upregulation of 30 genes (Table 1). Six of them encode for secreted proteins: Tensin-4 (Tns4), L-dopacrome tautomerase (Tyrp2), Placenta-specific gene 8 protein (Plac8/Onzin), Osteopontin (Spp1/OPN), Collagen α-1(XVII) chain (Col17a1), Bone/cartilage proteoglycan I (Bgn) and Multimerin-2 (Mmrn2). Not all of these genes are yet known to be expressed in the retina. Tyrp2 (also known as Dct) is also involved in melanoma generating resistance to cis-diamminedichloroplatinum (CDDP), a treatment which promotes DNA damage and subsequent induction of apoptosis (Chu et al., 2000). Among the upregulated transcripts was also Sox10, which is known as a melanocytes transcription regulator in transcriptional pathways involved in development, differentiation and cell survival having implications for disease states of the pigment cell lineage such as melanoma (Loftus et al., 2009). We speculate that GDNF may induce the transcription of Sox10 influencing the activation of Tyrp2 transcription and subsequent secretion. Another interesting factor, Plac8 (also know as Onzin) promotes survival and transformation modulating the Akt-Mdm2-p53 pathway (Rogulski et al., 2005). The expression of this molecule has not yet been detected in the retina but could be an interesting therapeutic candidate for PR survival.

In our microarray-based screening for new secreted neurotrophic candidates, OPN was the only secreted molecule with known anti-apoptotic functions (associated with the GO term GO:0006916, "anti-apoptosis") that was upregulated in response to GDNF treatment (Geissinger et al., 2002). OPN-mediated cell survival was first demonstrated for kidney proximal tubule epithelial cells exposed to hypoxia and re-oxygenation (Denhardt et al., 1995). OPN also promotes cancer cell metastasis preventing programmed cell death and allowing uncontrolled proliferation of tumor cells (Hsieh et al., 2006). In case of harmful stimuli, OPN blocks the activation-induced cell death of macrophages, T lymphocytes as well as fibroblasts and endothelial cells (Denhardt et al., 1995; Standal et al., 2004). In addition, OPN has been characterized as a survival factor for adherent endothelial cells deprived from growth factors (Khan et al., 2002). The pro-survival activity of OPN possibly triggers different signaling pathways dependent on the cell type.

OPN Expression in the Retina

RMG cells in mouse retinas account for 16%–22% of the cell bodies in the INL (Jeon et al., 1998). Thus, any selected potential neurotrophic candidate from the total retina transcriptome study had to be validated with respect to its specific localization. We found OPN expression restricted to the GCL in untreated retinas, which is in line with previous studies (Chidlow et al., 2008; Hikita et al., 2006; Ju et al., 2000). We also found a complete co-localization with GS, a marker for RMG, and

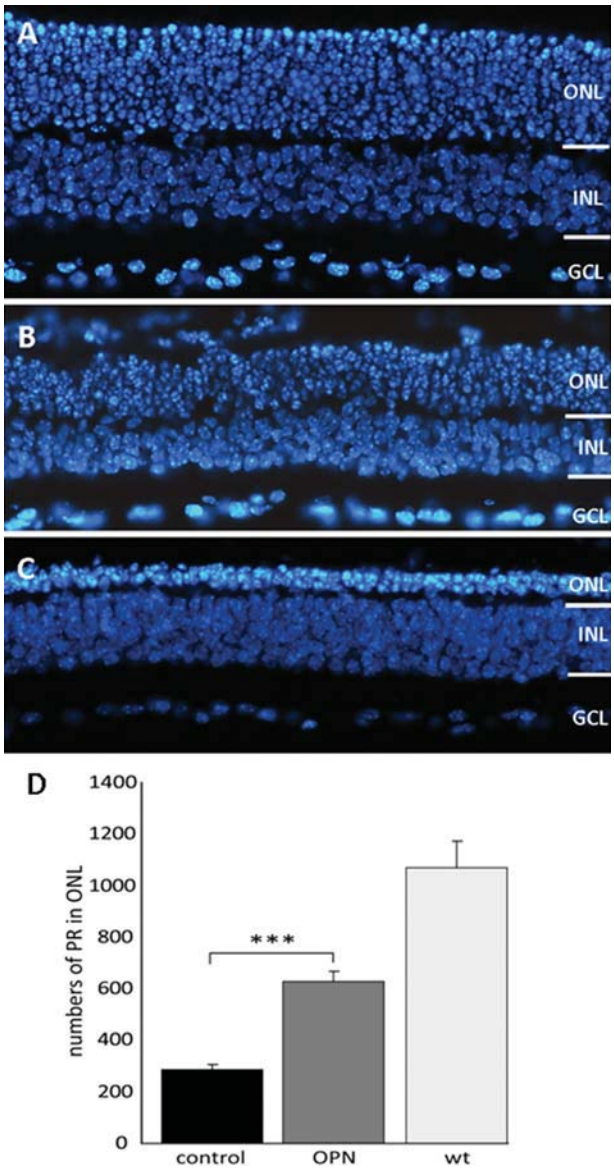

Fig. 5. Effect of OPN on PR cell survival in Pde6b^{rd1} long-term cultured retinal explants. Mouse retinal explants from wt (**A**) and Pde6b^{rd1} (**B**, **C**) mice were explanted on PN5 and cultured for additional 12 days *in vitro*. DAPI staining on day PN5+div12 in Pde6b^{rd1} OPN treated retinal explants showed a significant increase in the numbers of PR in ONL (B) compared with the untreated controls (C). Images were taken at 200 fold magnification and are representative for three independent experiments. Total number of ONL cell nuclei for each condition (**D**) (***$P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

thus conclude that expression of OPN is largely confined to RMG rather than to retinal ganglion cells. The expression of OPN in RMG is further corroborated by the observation that after GDNF stimulation not only OPN expression increases but also expression of the RMG marker GS was induced. Expression of GS can be regulated by steroid hormones in chicken retina (Linser and Moscona, 1979; Linser and Moscona, 1983). Furthermore, this upregulation could be explained via the induction of glutamate receptors by GDNF. It has been prompted that GLAST, a key protein in glutamate clearance and GS may operate in concert to terminate the neurotransmitter action of glutamate (Derouiche and Rauen, 1995). In line with this, a GDNF-elicited increase in GLAST protein in RMG cells has been observed (Delyfer et al., 2005), raising the possibility that GDNF could exert part of its neuroprotective effect by regulating intracellular glutamate levels, thereby preventing its exitotoxic effects.

Secretion of OPN by RMG

The two different OPN isoforms represent alternative translational products of a single full-length OPN mRNA (Shinohara et al., 2008). The difference in post-translational modifications allows the intrinsic OPN to localize in the cytoplasm but not in the secretory vesicles. In contrast, secreted OPN maintains its signal peptide and colocalizes to perinuclear secretory vesicles (Shinohara et al., 2008). The secretory form of OPN has been reported as an apoptotic suppressor for human umbilical vein endothelial cells (HUVECs) deprived of growth factors (Khan et al., 2002). In RMG supernatants, OPN levels increase in response to GDNF. However, we also observed low levels of OPN in the supernatants of untreated RMG. This suggests that OPN is constitutively expressed in mouse RMG but is upregulated on mRNA and protein levels by GDNF as supported by the staining for OPN in sections from retinal explants (see Fig. 1).

sOPN Induces PR Survival by Activation of PI3K/Akt Pathway

The activation of phosphoinositide-3-kinase (PI3K) mediates diverse cellular functions including proliferation, chemotaxis, and more importantly, inhibition of apoptosis (Katso et al., 2001). The anti-apoptotic effects of the PI3K cascade are mediated by phosphorylation of the key downstream effector substrate Akt (Protein kinase B) a serine/threonine kinase (Dudek et al., 1997; Kauffmann-Zeh et al., 1997). PI3K controls the activity of Akt by regulating its location and activation. Active PI3K enzymes catalyze the formation of the lipid 3′-phophorylated phosphoinositides, which regulate the localization and activity of Akt that is a key molecule in neuronal survival (Philpott et al., 1997). OPN induces sustained activation of the PI3K downstream mediator Akt (Meller et al., 2005; Robertson and Chellaiah, 2010). Akt targets several proteins for cell survival, including apoptosis regulators and transcription factors. OPN has been shown to activate survival pathways via integrin receptors (Meller et al., 2005) especially through the integrin $\alpha_v\beta_3$ (Scatena et al., 1998). Since we have demonstrated that the application of OPN directly prolongs survival of PR in culture, and this correlates with an intrinsic activation of the PI3K/Akt pathway already detectable after 5 min of treatment, we assume a direct

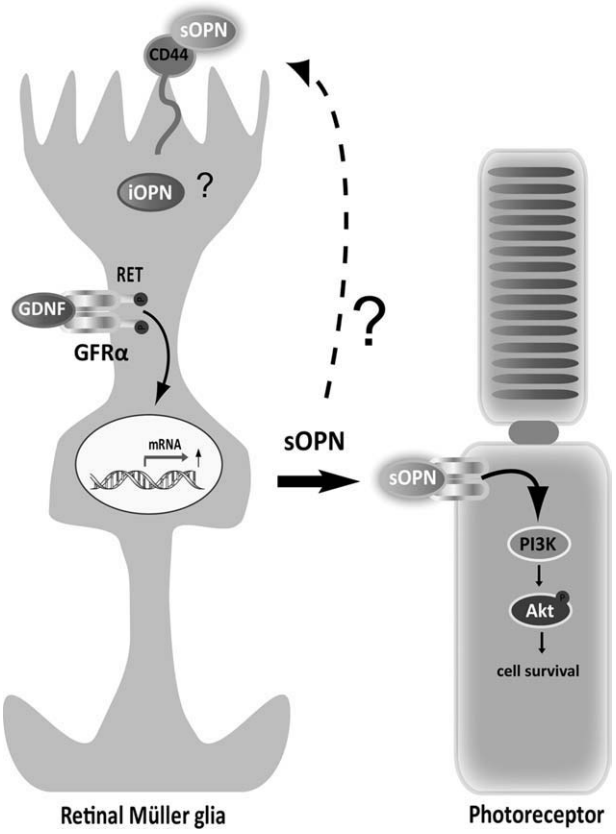

Fig. 6. Model of RMG–PR interaction system. As a result of GDNF stimulation, RMG induce secretion of OPN. OPN activates the PI3-K prosurvival pathway in isolated PR resulting in increased phosphorylation of its downstream effector Akt and resulting in prolonged PR survival. OPN could additionally induce a feedback process in RMG through CD44 receptors increasing the RMG-derived OPN and thus the transcription of additional growth factors and cytokines.

activation of the PI3K pathway by OPN in PR cells, where this pathway has been found to be crucial for survival of PR *in vitro* (Hauck et al., 2008).

OPN Exerts Neuroprotection in Retinal Explants

The use of retinal explants to study effects of neurotrophic factors on PR cells has many advantages when compared with cultures of isolated PR. In the explants, PR are maintained in their physiological context with regard to both, other retinal cell types and extracellular matrix. We applied this method to study the Pde6b^{rd1} model, which is a well known mouse model of retinal degeneration. With this culture paradigm, we could evaluate whether application of OPN would translate into protecting PR survival under the influence of mutation-induced degeneration. OPN application to Pde6b^{rd1} retinal explants significantly enhanced PR survival, maintaining the retinal morphology and the PR numbers even for long culture periods. Accordingly, these data suggest that a direct sustained treatment with OPN is sufficient to translate into a long-term survival of PR. This survival effect correlated with the results obtained with isolated PR cells. However, the observed neuroprotective activity of OPN may not only result from a direct effect of OPN on PR but also additionally from a OPN-mediated activation of RMG through CD44 receptor variants. Since OPN is an extracellular ligand of CD44 variants (Weber et al., 1996) permitting the activation of cellular responses (Sodek et al., 2000) and CD44 receptors are localized in RMG (Chaitin et al., 1994, 1996), we hypothesize that RMG could be an additional target cells for OPN action. We propose a model of GDNF-induced intrinsic OPN expression and OPN secretion from RMG, where secreted OPN exerts a direct survival effect on PR and additionally might stimulate RMG to overexpress other cytokines with a neuroprotective activity on PR (see Fig. 6). Recently, it was published that OPN was significantly upregulated in a animal model for glaucoma and found to protect RGCs in *ex vivo* cultures of D2Rj mouse retinas in a concentration dependent manner (Birke et al., 2010). At very high levels, however, OPN can also induce apoptosis selectively in the ganglion cell layer (Birke et al., 2010). These findings might indicate an ambivalent function of OPN, speculating that there is an initial increase of OPN to counteract deleterious processes where OPN can be neuroprotective, but also might have opposite effects if the insult persists. In relation to our study, we can confirm that the neuroprotective OPN effect depends on the application of the molecule in an adequate concentration range. Whether OPN has a survival effect on other retinal neurons or can be applied to other retinal degenerative diseases or injuries has to be further investigated.

Therefore, with this study we contribute to discern the action of GDNF and identify OPN as a novel neuroprotective factor for PR, opening a new avenue for future clinical applications.

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