



Does GDNF exert its neuroprotective effects on photoreceptors in the *rd1* retina through the glial glutamate transporter GLAST?

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Purpose: We previously demonstrated that exogenous glial cell line-derived neurotrophic factor (GDNF) induces histological and functional protection of photoreceptors in the retinal degeneration (*rd1*) mouse model. The mechanisms underlying such neuroprotection remain elusive. In parallel to this work, we provided evidence for the occurrence of glutamate-mediated excitotoxic phenomena contributing to rod photoreceptor death in the *rd1* retina in the companion paper. In the present study, we investigated whether, as demonstrated in other models, GDNF could exert its neuroprotective effect on photoreceptors through Müller glial cells (MGC) by promoting the expression of the glial L-glutamate/L-aspartate transporter (GLAST), an endogenous neuroprotective mechanism against glutamate-mediated excitotoxicity.

Methods: Reverse transcription-polymerase chain reaction (RT-PCR) was used to compare the mRNA expression levels of GDNF receptors between *rd1* and wild-type mouse retinas as well as between MGC and mixed retinal cell cultures. Recombinant GDNF was applied to pure MGC cultures, to *rd1* retinal organ cultures and injected subretinally into *rd1* mouse eyes. GLAST expression following GDNF treatment was measured by RT-PCR, immunoblotting and immunohistochemistry. Free glutamate and glutamine levels were quantified in *rd1* retinas after GDNF or control treatment using an amino acid analyzer.

Results: mRNA expression studies of GDNF receptors, GFR α -1 and Ret, demonstrated that GDNF receptors were not exclusively expressed by the degenerating photoreceptor cells but mainly by MGC. Exogenous GDNF application to MGC cultures, *rd1* mouse retinal explants and in vivo *rd1* mouse retinas increased the expression of GLAST by 48% in retinal explants ($p < 0.005$) and by 25% in vivo ($p < 0.0005$). GLAST protein expression in MGC was particularly increased around degenerative photoreceptors. Free glutamate and glutamine levels in the *rd1* retina were not significantly modified by exogenous GDNF.

Conclusions: Our data suggest that, in the *rd1* mouse retina, GDNF neuroprotective effect on photoreceptors can be mediated indirectly through the activation of MGC. We demonstrate that injection of recombinant GDNF enhances the expression of GLAST and more particularly around the degenerating photoreceptors. Since we failed to demonstrate that GDNF decreases free glutamate levels, we could not ascertain whether GDNF promoted photoreceptor-survival via an increase of glutamate uptake and, therefore, a change in glutamate distribution.

Retinitis pigmentosa (RP) is a heterogeneous group of inherited retinal degenerative diseases, characterized by the progressive death of rod and cone photoreceptors leading to an irreversible loss of visual function. The retinal degeneration (*rd1*) mouse is an appropriate model for studying the cellular and molecular events leading to photoreceptor death and for evaluating new treatments as the pattern of photoreceptor loss is similar to that in affected humans and because the mutated gene encodes rod cGMP-phosphodiesterase (Pde6b) [1] as in some affected human families [2].

However, to this day, mechanisms leading to photoreceptor apoptosis are still unclear. Despite the lack of knowledge of these mechanisms, several therapeutic approaches have been

proposed and showed some efficacy in such a model. Besides therapies aimed at curing the genetic anomaly per se (corrective gene therapy), many works have demonstrated the benefit of using trophic factors to limit or to postpone the degeneration process [3-6]. This appears as a potential mutation-independent therapeutic approach addressing the challenges linked to the tremendous genetic heterogeneity associated with the RP phenotype [7]. We previously demonstrated that intraocular administration of glial cell line-derived neurotrophic factor (GDNF) not only significantly slows down photoreceptor degeneration, but also partially preserves visual function in the *rd1* mouse [8]. This result was further confirmed in several different models of photoreceptor degeneration in vivo: the transgenic S334ter rat expressing a mutated rhodopsin gene [9], the retinal detachment-induced photoreceptor degeneration model [10] and the RCS rat [11]. Yet, the mechanisms of such neuroprotection remain elusive. The work presented here stems from an attempt to elucidate one of the mechanisms of GDNF-induced neuroprotection.

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It is not formally known whether GDNF acts directly or indirectly on photoreceptors *in vivo* and what changes are induced by such a treatment in the dystrophic retina. Whereas other trophic factors are highly suspected to promote photoreceptor survival indirectly through an activation of Müller glial cells (MGC) [12-15], GDNF was first reported to promote newborn photoreceptor survival directly *in vitro* [16-18]. Harada et al. [19,20] demonstrated that GDNF modulates trophic factor release by MGC *in vitro* and thus proposed that GDNF could exert its neuroprotective effect through both direct and indirect pathways.

In the central nervous system (CNS), GDNF has been shown to protect from excitotoxic and ischemic damage [21,22], and to modulate the expression of the glutamate transporter GLT-1 [23,24] and/or of the glial L-glutamate/L-aspartate transporter (GLAST) [24,25]. In the retina, GLAST is expressed in MGC and is essential for glutamate clearance to avoid neurotoxicity and to allow normal signal transmission between photoreceptor and bipolar cells [26-29]. Since we demonstrated, in a parallel companion article, the occurrence of glutamate-mediated excitotoxic mechanisms contributing to rod cell death in the *rd1* retina [30], we here tried to learn whether GDNF could exert its neuroprotective effect on photoreceptors through MGC by promoting the expression of GLAST.

We first studied GDNF receptor distribution by analyzing the mRNA expression of its different components: GFR α -1 and Ret in wild-type and *rd1* mouse retinas, and in MGC cultures and mixed retinal cell cultures. Since our results demonstrated that GFR α -1 and Ret are expressed in MGC, we subsequently compared GLAST expression levels in MGC cultures in the presence and the absence of GDNF, and in GDNF-treated and PBS-treated *rd1* retinas. Finally, we measured free glutamate and glutamine levels in GDNF- and PBS-treated *rd1* retinas.

METHODS

Animals: C3H/He mice (*rd1* mice) and C57BL/6 mice (wild-types or controls) were obtained from Iffa Credo (L'Arbresle, France). Animals were cared for and handled according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. They were maintained in clear plastic cages and subjected to light/dark cycles of 12 h. Day of birth was designated postnatal day 0 (PN0).

MGC cultures and GDNF application *in vitro*: Retinal MGC culture technique was adapted from Hicks and Courtois [31]. Since the mutated *Pde6b* gene has been shown to be specifically expressed in rod photoreceptors from PN8 in the *rd1* retina [32], we assumed that *rd1* and wild-type MGC are similar in both strains before the degeneration starts, and used C57BL/6 retinas to perform the cultures. Mice were killed at PN8. Eyes were immediately enucleated into Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) containing 10 U/ml penicillin and 10 μ g/ml streptomycin, and stored overnight at room temperature in the dark. Eyes were then incubated in DMEM containing 0.1% trypsin and 70 U/

ml collagenase (Worthington Biochemical, Freehold, NJ) at 37 °C for 30 min. Retinas were dissected free of the retinal pigment epithelium, cut into 1 mm² fragments, placed in a Petri dish containing DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and maintained at 37 °C in a humidified 5% CO₂ incubator. Two days later, retinal aggregates were removed. At confluence, MGC cultures were resuspended and seeded in two different plates. After two days (just before confluence) the medium was removed, and plates were rinsed twice with DMEM. After rinsing, cells were immediately placed in DMEM supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml sodium selenite, 1 mM sodium pyruvate, 10 U/ml penicillin and 10 μ g/ml streptomycin, with or without 50 ng/ml GDNF (rhGDNF, Promega, Madison, WI). The medium was removed 24 h later, cells were rinsed with phosphate buffered saline (PBS), and RNA was extracted.

Retinal cell cultures: Mouse retinal cell cultures were prepared from PN8 C57BL/6 mice as previously described [33].

Retinal organ cultures: *rd1* mice were killed at PN15 (n=6). Retinas were immediately dissected free of the retinal pigment epithelium and the sclera under a dissecting stereomicroscope. Retinal explants were then grown as organ cultures in a chemically defined medium (CDM, 1.5 ml/retina) in the presence (right eyes) or absence (left eyes) of GDNF (50 ng/ml). CDM was composed of DMEM, supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin, 63.6 nM progesterone, 0.1 mM putrescine, 5 ng/ml sodium selenite, 3 mM taurine, 2.7 μ M cytidine 5'-diphosphoethanolamine, 5.2 μ M cytidine 5'-diphosphocholine, 0.55 μ M hydrocortisone, 2.9 μ M 3-3'-5-triiodo-L-thyronine, 1 mM sodium pyruvate, 0.31 μ M prostaglandin D₂, 10 U/ml penicillin, and 10 μ g/ml streptomycin. Cultures were maintained at 37 °C in a humidified 5% CO₂ incubator. After 48 h of incubation, the medium was removed and proteins extracted from the retinas. The left and right eyes of each animal were compared for the protein expression study.

Subretinal injections of GDNF: GDNF was injected into *rd1* mice at PN15 (n=6). Mice were anesthetized intraperitoneally with a 1:1 (v/v) mixture of etomidate (0.5 mg/ml; Janssen-Cilag, Boulogne-Billancourt, France) and midazolam (0.5 mg/ml; Roche, Neuilly-sur-Seine, France) at a dose of 10 ml per kg body weight. GDNF (1 μ l) was injected at a dose of 330 ng/ μ l in sterile PBS with a 10 μ l microsyringe (Unimetrics, Folsom, CA), a microinjector (UltraMicroPump II-Micro 4, World Precision Instruments, Sarasota, FL) and a 30 gauge bevelled needle. With the aid of a stereomicroscope (Zeiss, Germany), the needle was inserted just posterior to the limbus, through the sclera and directed towards the posterior pole of the eye into the subretinal space. PBS (1 μ l) was injected into the contralateral eye as a control. The left and right retinas of each animal were compared, 48 h after injection, for the protein expression study.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis: Total RNAs were purified from cultures by use of a Qiagen RNeasy Kit (Qiagen, Valencia, CA) accord-

ing to the manufacturer's instructions, and from retinas by the cesium chloride centrifugation method [34]. cDNAs were synthesized by reverse transcription using random hexamers (pdN₆) according to standard protocols. The sequence of each primer, the annealing temperatures, the length of the amplified products and the GenBank accession numbers are given in Table 1.

Standard PCRs were performed at a final concentration of 1X PCR buffer, 260 μ M dNTPs, 2 μ M each primer, 2.5 U of Taq polymerase (Invitrogen, Carlsbad, CA) in a total volume of 25 μ l. The mixture was amplified with the RoboCycler-Gradient-96 (Stratagene, La Jolla, CA) for 35 cycles. Cycling conditions were as follows: initial denaturation at 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 s (see Table 1 for annealing temperatures) and elongation at 72 °C for 1 min, followed by a final elongation step at 72 °C for 3 min. PCR products (10 μ l) were separated by 1.2% agarose gel and quantified using the Phoretix 1D image analysis software (Phoretix International, Newcastle-upon-Tyne, UK).

Real-time PCRs were performed on a LightCycler instrument (Roche-Diagnostics, Indianapolis, IN) and with SYBR Green I, according to the manufacturer's recommendations. Cycling conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 0 s, annealing for 5 s (see Table 1 for annealing temperatures used) and elongation at 72 °C for 15 s. Melting curve analysis was performed as follows: denaturation at 95 °C for 2 min, annealing at 65 °C for 2 min, followed by a gradual increase (0.1 °C/s) in temperature to 95 °C. Real-time PCR efficiencies were evaluated by calculating the slope of a linear regression graph following recommended protocols. For each experiment, crossing points were calculated by the LightCycler Data Analysis Program (LightCycler-3.5 Software). Target gene expression levels were normalized with respect to β -actin expression. Mean crossing point deviations were calculated between samples and controls as specified in the figures. For comparative studies of in vivo gene expression between *rd1* and control retinas, 10 retinas were pooled

TABLE 1. OLIGONUCLEOTIDES USED FOR RT-PCR

Gene	Forward and reverse primer sequences (5' to 3')	Annealing temperature (°C)	Product length (bp)	GenBank accession number	Application
ABCR	F: ACTTCACCCTCTACCTACCACAC R: GAACAACCTTCACCACCAAGCC	57	355	AF000149	Standard RT-PCR (Figure 1F)
β -actin	F: AAAGACCTCTATGCCAACACAG R: AAAGAAAGGGTGTAACGCGAG	57	296	M12481	Standard and real time RT-PCR (Figure 1F)
GDNF	F: ACCAGATAAACAAGCGGCAG R: TCAGATACATCCACACCGTTTAG	60	402	XM122804	Real time RT-PCR (Figure 1B)
GFR α -1	F: GCAAGGAAACCAACTTCAGC R: TCGTATGGGGAATCTTCCAG	54	202	AF014117	Real time RT-PCR (Figure 1C)
GFR α -1	F: GAAGATTCCCATACGAGCC R: TGCCAATCAGTCCCGAGTAG	60	535	AF014117	Standard RT-PCR (Figure 1F)
GFR α -2	F: CCTGAACGACAACCTGCAAGA R: CAGTTGGGCTTCTCCTTGTC	50	246	AF002701	Real time RT-PCR (Figure 1E)
GLAST	F: GAAGGGGAGGCAACCTGAAATGAG R: AAGGGGGAGTGGGAAAATGAAAA	56	287	D63816	Standard RT-PCR (Figure 1F)
GLAST	F: GAAGTCTCCGACGTTCTAATCC R: GCTCTGAAACCGCCACTTACTATC	58	303	D63816	Real time RT-PCR (Figure 2)
Ret	F: ATCCACACCTTCGGACTCAC R: AACCCAGTGCTAGTGCCATC	54	201	AF209436	Real time RT-PCR (Figure 1D)
Ret	F: AGTATGCCAAGTATGGCTCTCTG R: ACTCCAAAGGACCACACATCAC	60	403	AF209436	Standard RT-PCR (Figure 1F)
Rod arrestin	F: CTATFACGTCAAGCCTGTAGCC R: CATCCTCATCTTCTTCCCTTC	56	434	M24086	Real time RT-PCR (Figure 1A)

This table lists all the oligonucleotides used for RT-PCR in this study with the corresponding annealing temperatures, PCR product lengths, and Genbank accession numbers.

for each age (PN1, PN8, PN15, PN35) and each strain. The amplification steps were performed in triplicate for each point and were repeated using three independent cDNA preparations. The levels of expression of rhodopsin, rod arrestin, and other markers of retinal degeneration were tested and found to be in accordance with the age and genotype analyzed, thus validating our RNA preparations (see Figure 1A for in vivo mRNA expression study of rod arrestin).

Immunohistochemistry: *rd1* mice were killed 48 h after being injected (n=10). GDNF- and PBS-treated eyes were immediately removed, fixed in 4% paraformaldehyde for 6 h at 4 °C. Eyes were then embedded in paraffin. Sections (6 μm) were cut in a transverse plane using a microtome (model HM340E; Microm International GmbH, Walldorf, Germany). Sections were saturated in PBS containing 3% BSA and 0.1% Tween 20 for 1 h and then incubated overnight at 4 °C with guinea pig anti-GLAST polyclonal antibody (1:4000; AB1782, Chemicon International, Temecula, CA). The primary antibody was localized using goat anti-guinea pig antibody coupled

with Alexa Fluor 594 (1:1000; Molecular Probes, Leiden, Netherlands). No immunoreactivity was observed when anti-GLAST antibody was preabsorbed with GLAST immunizing peptide (Sigma Genosys, The Woodlands, TX). Confocal microscopy was performed using a TCS-SP Leica microscope (Leica Microsystems Inc., Bannockburn, IL), equipped with a 40x objective (plan apo; NA=1.25). The same parameters and exposure times were used in all cases. For each optical section, the signal was treated by line averaging to integrate the signal collected over four lines in order to reduce noise. A focal series was collected for each specimen (11 optical sections per specimen).

Protein extraction and immunoblotting: *rd1* and control mice were killed at PN1, PN8, PN15, PN21, and PN35 (3 animals per age and strain). Eyes were immediately enucleated, and retinas dissected within 2 min in sterile PBS. Retinas were then homogenized in a lysis buffer containing 50 mM Tris-HCl pH 7.5, 1 mM PMSF, 1 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 1X mixture of protease in-

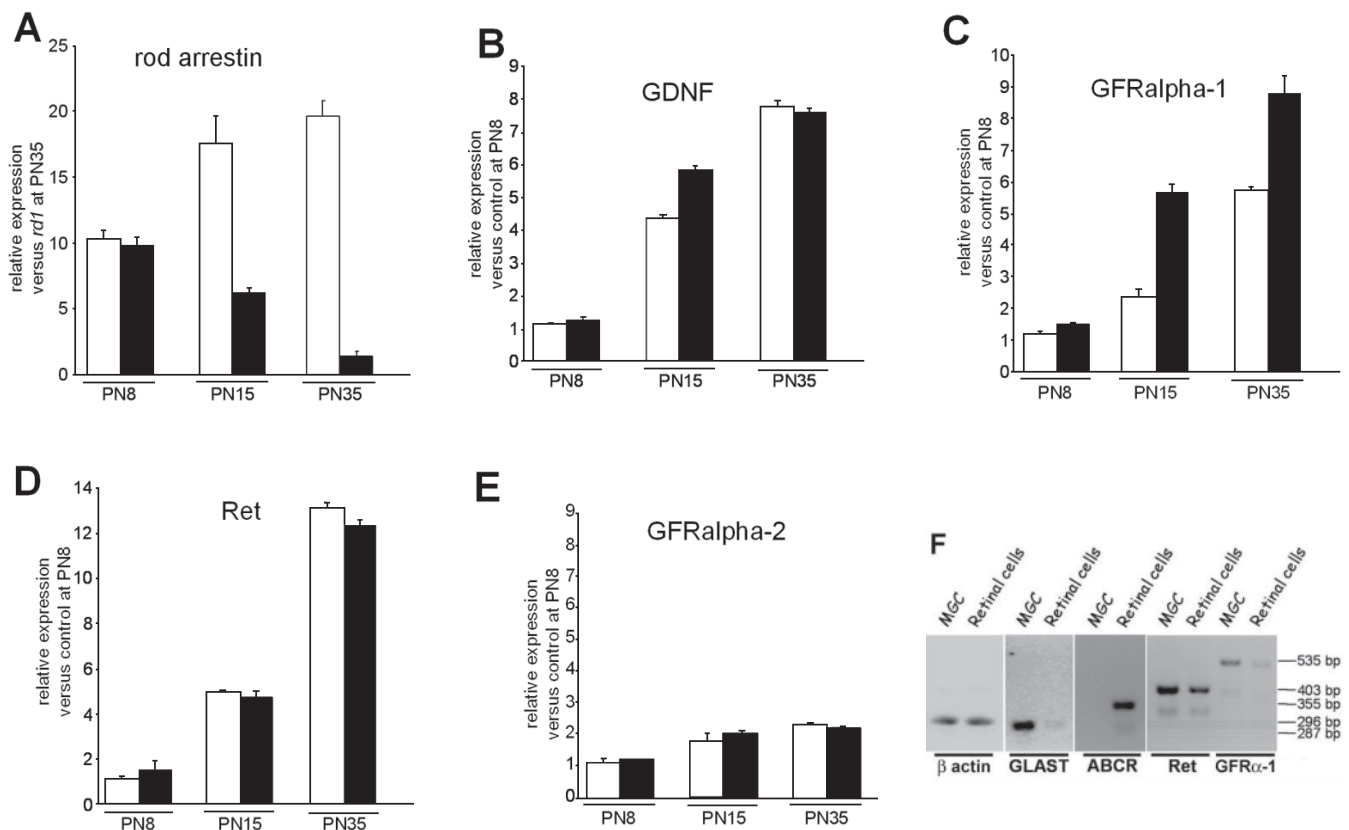


Figure 1. Rod arrestin, GDNF, GFR α -1, Ret, and GFR α -2 mRNA expression in the *rd1* retina. In vivo levels of rod arrestin (A), GDNF (B), GFR α -1 (C), Ret (D), and GFR α -2 (E) mRNA expression were measured in *rd1* and wild-type retinas during rod degeneration (PN8, PN15, and PN35) using semi-quantitative real-time RT-PCR. Bars represent the mean of triplicate determinations; the error bars represent the standard error of the mean. The open bars represent wild-type mice, and the solid black bars represent *rd1* mice. These graphs show data relative to the lowest value of PN8 control and not relative to the mean value of PN8 control. In the case of rod arrestin (A), expression data was plotted relative to the lowest value obtained, regardless of the age or strain. Concerning markers of rod photoreceptors, the lowest value was observed in 35-day-old *rd1* mouse retinas because there are no more surviving rods then. Therefore, rod arrestin data were expressed in comparison to PN35 *rd1* retinas. F: Gel electrophoresis of PCR products after amplification of first-strand cDNA from pure Müller glial cell (MGC) cultures and mixed retinal cell cultures. PCR products for β -actin (296 bp), GLAST (287 bp), ABCR (355 bp), Ret (403 bp), and GFR α -1 (535 bp) of the correct and indicated sizes were obtained. Results are representative of three experiments.

hibitors, 45 $\mu\text{g}/\text{ml}$ TLCK, 1 mM sodium fluoride, and 1 mM sodium orthovanadate. Protein concentrations were estimated by Bradford's technique [35]. Proteins were shock-frozen and maintained at -80°C until analysis. Proteins were further diluted 1:1 in sample buffer (final concentrations: 10 mM Tris-HCl pH 8, 1 mM EDTA, 20 mM dithiothreitol, 3% SDS, 10% glycerol, 0.1% bromophenol blue, 4 M urea) and heated at 50°C for 45 min. Proteins (10 $\mu\text{g}/\text{lane}$) were separated by 10% SDS-PAGE gel electrophoresis containing 4 M urea, and transferred onto nitrocellulose membranes. Membranes were blocked with PBS, 0.1% Tween 20, 3% nonfat dry milk, and 5% horse serum overnight at 4°C and then incubated with anti-GLAST antibody (1:15000) for 2 h at room temperature. Membranes were washed and incubated with the appropriate horseradish peroxidase-labeled secondary antibody (1:15000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Antibody binding was visualized by a chemiluminescence detection system (ECL+, Amersham, Arlington Heights, IL) as recommended by the manufacturer. To ensure that equal quantities were loaded in each lane, membranes were stripped and subsequently reprobed with mono-

clonal anti- α -tubulin antibody (1:500; T5168, Sigma, Saint Louis, MO). Alternatively, an identical gel was run in parallel and subjected to Coomassie staining to control equal loading. Band intensities were quantified by scanning densitometry using Phoretix 1D software.

GDNF quantification: GDNF protein levels in untreated *rd1* and control retinas at PN15 were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (GDNF-Emax ImmunoAssay System, Promega, Madison, WI).

Quantification of free amino acids: Retinas were rapidly dissected on ice and deproteinized with 10% (w/v) trichloroacetic acid containing 0.5 mM EDTA. After sonication and centrifugation, supernatants were collected and stored at -80°C until amino acid analysis. Amino acids were separated and quantified by ion exchange chromatography using an amino acid analyzer (AminoTac JLC-500/V; JEOL, Tokyo, Japan). The concentrations of each amino acid are expressed, as a percentage, with respect to the sum of all the concentrations of the amino acids quantified by the analyzer (which include taurine, threonine, serine, asparagine, glutamate, glutamine, glycine, alanine, citrulline, valine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, lysine, and arginine).

Statistical analysis: All data are presented as the mean and the standard error of the mean (SEM). Unpaired Student's t-test was used to compare amino acid levels in *rd1* and control mice and to compare GLAST mRNA expression in GDNF- and PBS-treated MGC. Paired Student's t-test was used to compare GLAST protein expression in GDNF- and PBS-treated eyes both in retinal organ cultures and in vivo. Values of $p < 0.05$ were considered to be significant.

RESULTS

GDNF expression during photoreceptor degeneration in the *rd1* mouse retina: A review of the literature enabled us to determine that (1) PN8 most likely represents the first time point when the degenerative process becomes detectable in the *rd1* mouse retina [32,36-40], (2) PN35 can be considered as the terminal stage of rod degeneration [41], and (3) PN15 represents an intermediate stage between the two [42].

We confirmed these data by studying rod arrestin mRNA relative expression between *rd1* and control retinas using quantitative RT-PCR (Figure 1A). At PN8 (corresponding to the onset of photoreceptor degeneration), no difference was noticed between *rd1* and control retinas. At PN15 (corresponding to an intermediate stage of degeneration), *rd1* mouse retinas expressed 2.8 fold less rod arrestin mRNA than did controls and at PN35 (representing the terminal stage of rod degeneration), over 14 fold less.

As GDNF has recently been shown to be upregulated endogenously in a model of light-induced retinal degeneration [20], we examined GDNF mRNA expression in *rd1* and control mouse retinas during rod photoreceptor degeneration using real-time RT-PCR selecting times corresponding to early, intermediate, and terminal phases of degeneration as above (Figure 1B). At PN8, no difference was noticed between *rd1* and control retinas. At PN15, GDNF mRNA expression was

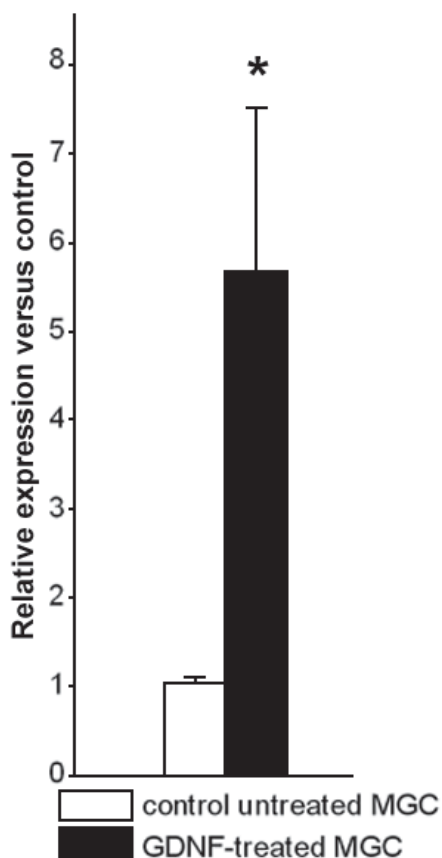


Figure 2. GLAST mRNA expression in Müller glial cell (MGC) cultures treated with GDNF. GLAST mRNA levels were studied in MGC cultures in the presence and absence of GDNF using real-time RT-PCR. Bars represent the mean of three independent experiments; the error bars represent the standard error of the mean (asterisk indicates $p < 0.05$, $n=3$).

1.35 fold higher in *rd1* mice than in controls. At PN35, no more difference was observed.

We further investigated whether there could be a difference in GDNF protein expression between the two strains at PN15, but the level of GDNF protein expression was undetectable at PN15 when measured by ELISA. Our results thus suggest that GDNF mRNA is moderately and transiently upregulated at PN15 in the *rd1* mouse.

GDNF receptor mRNA expression in the *rd1* mouse retina: GDNF acts through a receptor complex composed of the binding receptor GFR α -1 and the Ret protein tyrosine kinase [16,43-45]. We used real-time RT-PCR to study GFR α -1 and

Ret mRNA expression in the retinas of *rd1* and control mice, selecting times corresponding to early, intermediate and terminal phases of degeneration as above. In both control and *rd1* mouse retinas, GFR α -1 mRNA expression increased with age (Figure 1C). However, at each age, the mRNA expression was significantly higher in *rd1* retinas than in controls. At PN8, GFR α -1 mRNA expression was 1.3 fold higher in *rd1* retinas than in controls. At PN15, the mRNA expression difference reached a maximum of 2.4 fold. At PN35, GFR α -1 mRNA increase returned to a 1.5 fold difference. In both strains, Ret mRNA expression increased with age (Figure 1D). But in contrast to GFR α -1, no difference in Ret mRNA expression was

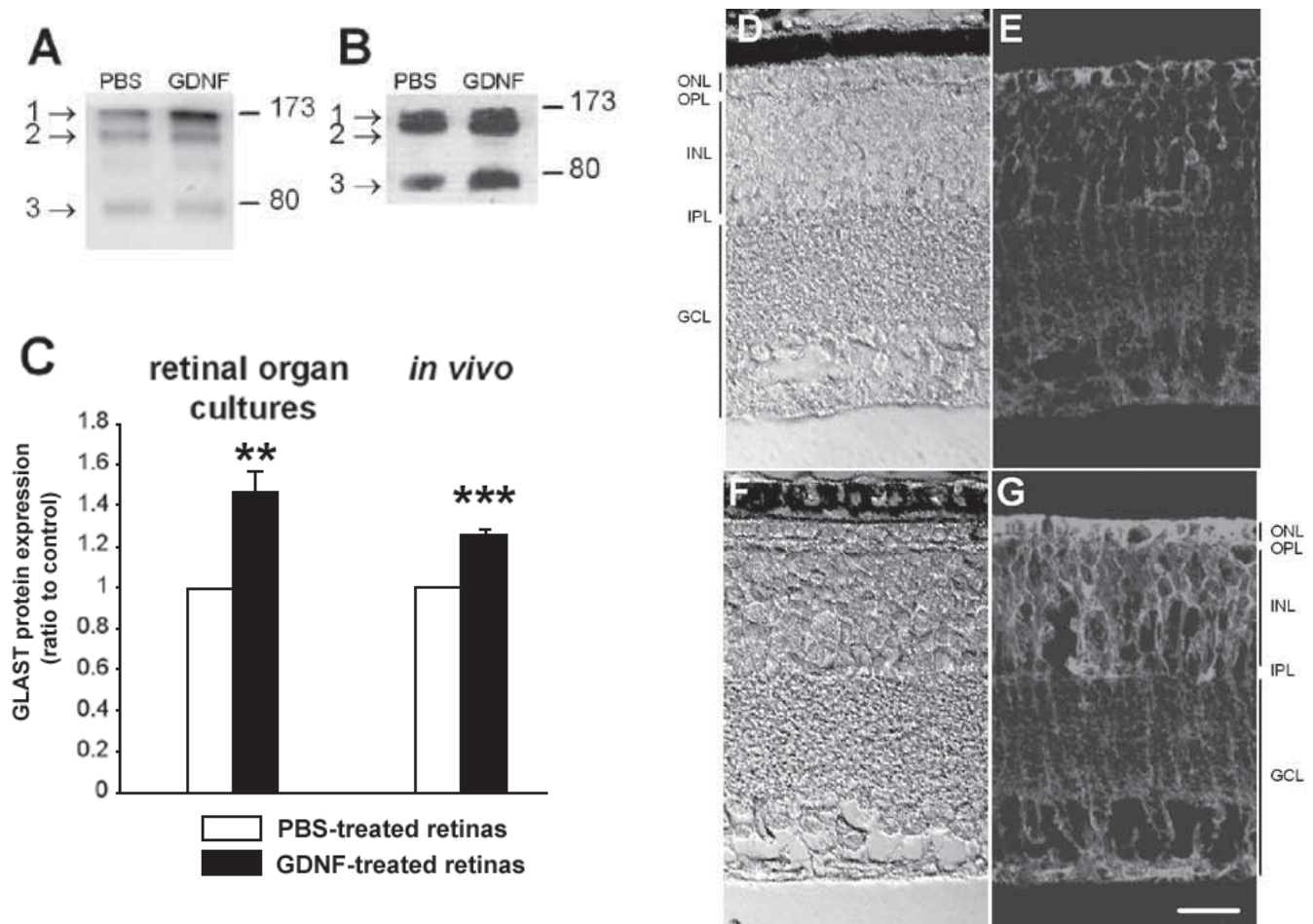


Figure 3. GLAST protein expression in *rd1* mouse retinas in the presence and absence of GDNF. **A,B:** GLAST protein levels were studied in retinal organ cultures (**A**) and in vivo (**B**) in the presence and absence of GDNF. Western blots of total proteins (10 μ g/lane) prepared from retinas 48 h after treatment with either GDNF or PBS, were probed with anti-GLAST. The blots demonstrated one band at about 76 kDa (GLAST monomeric form, labeled 3) and two bands at about 160 kDa (GLAST multimeric forms, labeled 1 and 2). **C:** GLAST protein was 1.48 times more abundant in GDNF-treated retinal organ cultures than in controls (6 samples per group). In vivo, GLAST protein was 1.25 times more abundant in GDNF-injected retinas than in controls (6 samples per group). Data are presented as the mean and SEM of the values obtained in three independent experiments (two asterisks indicate $p < 0.005$, three asterisks indicate $p < 0.0005$). **D-G:** Transverse sections of a PBS-injected eye and a GDNF-injected eye, from the same *rd1* mouse, were examined with a confocal microscope. Sections were analyzed using the same parameters and exposure times. Pictures were always taken at the same distance from the optic nerve. **D:** PBS-injected eye, Nomarski optics. **E:** PBS-injected eye, immunostaining with anti-GLAST antibody. **F:** GDNF-injected eye, Nomarski optics. **G:** GDNF-injected eye, immunostaining with anti-GLAST antibody. Due to photoreceptor degeneration, the outer nuclear layer (ONL) is no longer composed of one or two layers of photoreceptors. Pictures are representative of four independent experiments with similar results. The outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) are also labeled. The scale bar represents 20 μ m.

observed between *rd1* and control retinas from PN8 to PN35. As GDNF signaling through GFR α -2 has also been reported [46-48], we measured GFR α -2 mRNA expression from PN8 to PN35, but observed no difference between *rd1* and control retinas (Figure 1E). Hence, our results indicated that Ret and GFR α -2 mRNA expression levels remained stable during rod degeneration whereas GFR α -1 mRNA is upregulated.

To obtain information on the retinal localization of these

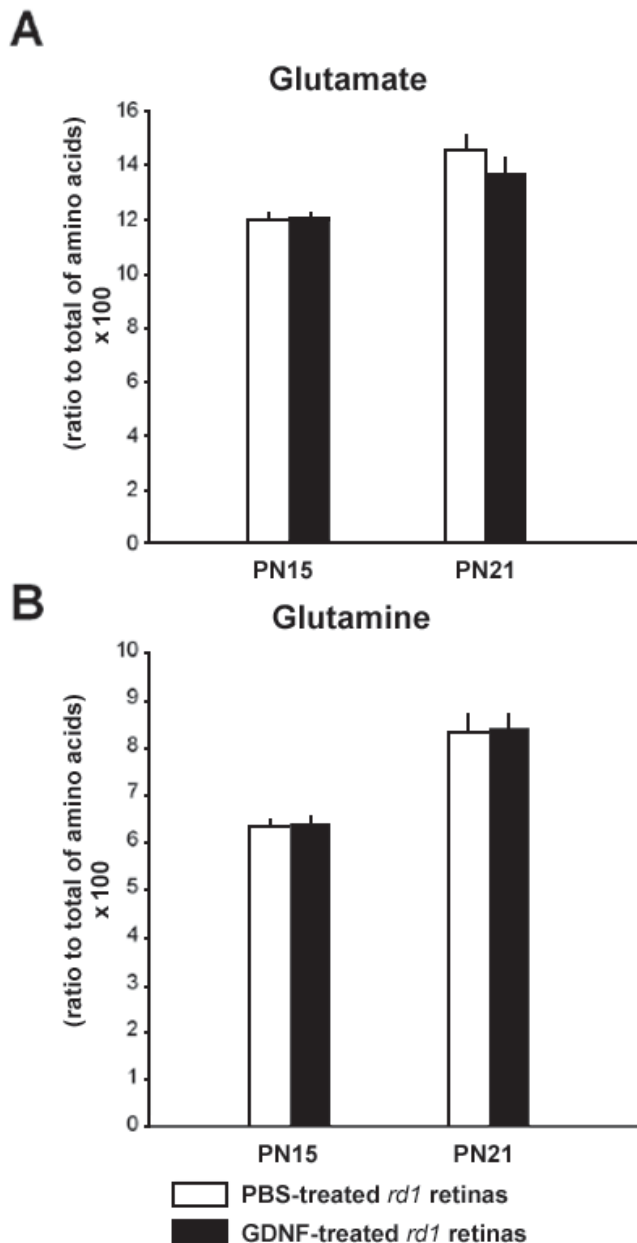


Figure 4. Free glutamate and glutamine levels in *rd1* and control retinas after subretinal injection of GDNF or PBS. Free glutamate (A) and glutamine (B) levels were measured in *rd1* and control retinas at PN15 and PN21, 48 h after subretinal injection of either GDNF or PBS. The bars represent the mean proportion (as a percentage) of amino acids that are glutamine and glutamate. Bars represent the mean (n=11); the error bars represent the standard error of the mean.

receptors, Ret and GFR α -1 mRNA expression levels were then compared in pure MGC cultures and in mixed retinal cell cultures (including all types of retinal cells, glial and neuronal) by using standard RT-PCR (Figure 1F). Figure 1F illustrates that both Ret and GFR α -1 mRNAs were expressed at higher levels in pure MGC than in retinal cell cultures. Quantitative measurements of RT-PCR products demonstrated that in vitro, in our RT-PCR conditions, Ret and GFR α -1 mRNAs were 1.7 and 5.3 times more abundant in pure MGC than in retinal cells, respectively, whereas there was no difference in the amount of housekeeping gene beta-actin mRNA between the two types of cultures. We quantified the glial glutamate transporter GLAST and the photoreceptor-specific gene ABCR to assess the quality of our cultures. GLAST mRNA was 8.7 times more abundant in MGC cultures than in retinal cell cultures. By contrast, ABCR mRNA expression was only detected in retinal cell cultures, thus confirming the purity of our MGC cultures. The comparison of GFR α -1 and GLAST mRNA expression ratios between MGC and mixed retinal cells suggested that GFR α -1 was not exclusively, like GLAST, but predominantly expressed in MGC whereas Ret was more widely distributed in retinal cells including MGC.

Exogenous administration of GDNF enhances GLAST expression: Since retinal MGC express GDNF receptors (Figure 1) and GDNF upregulates the expression of the glial glutamate transporter GLAST in the brain [24] and in a model of glaucoma [25], we next investigated whether exogenous GDNF could modulate GLAST expression by MGC in the *rd1* retina. The effect of GDNF on GLAST mRNA expression was first studied in vitro in pure MGC cultures. MGC cultures were treated with or without GDNF for 24 h. RNAs were then extracted and GLAST mRNA levels were analyzed by real-time RT-PCR. Figure 2 shows that GDNF-treated MGC expressed 5.5 fold more GLAST mRNA than did untreated MGC (SEM 1.8, n=3; p<0.05).

To further confirm that GDNF upregulates GLAST expression, we studied GLAST protein levels in retinal organ cultures and in vivo, in the presence and absence of GDNF. As previously reported [49-51], GLAST immunoblots showed different immunoreactive bands corresponding to monomeric (about 76 kDa) and multimeric forms (about 160 kDa) of GLAST. Figure 3A illustrates that GDNF treatment increased GLAST protein expression in *rd1* retinal organ cultures. Densitometric analysis of band intensities (monomeric and multimeric forms) revealed that GLAST protein levels were 1.48 fold higher in GDNF-treated explants than in paired controls (SEM 0.1, n=6, p<0.005; Figure 3C). GLAST expression levels were measured in vivo after subretinal injection of either GDNF or PBS. Immunoblot analyses confirmed the GDNF-induced increase in GLAST (Figure 3B). Figure 3C illustrates that, on average, 1.25 times more GLAST was present in *rd1* GDNF-injected eyes than in controls (SEM 0.02, n=6, p<0.0005).

Immunoreactivity experiments have shown that GLAST is present on MGC membranes and processes throughout the whole retina, with more intense labeling in both the outer limiting membrane and the outer plexiform layer [26,27]. Immu-

nohistochemical analyses of *rd1* mice after in vivo subretinal injection of either GDNF or PBS showed a similar immunoreactivity across the whole retina corresponding to MGC (Figures 3E and 3G) [26,27]. However, both in GDNF- and PBS-treated eyes, the staining appeared more intense in the outer nuclear layer around the degenerating photoreceptors (Figure 3E,G). In addition, GDNF injection increased GLAST labeling throughout the whole tissue, with more conspicuous labeling around the degenerating photoreceptors. Because of the resolution limit in confocal microscopy, we could not determine whether the observed increase in GLAST labeling was located at the membrane or in the cytoplasm (reticulum).

Our data show that GDNF upregulated GLAST expression in MGC cultures, in retinal organ cultures and in vivo, particularly around photoreceptors.

GDNF-induced upregulation of GLAST does not significantly modify free glutamate and glutamine levels in the degenerating rd1 retina: We demonstrated, in a parallel work, that free glutamate level increases during rod degeneration in the *rd1* retina and is toxic to rods [30]. In the present study, we showed that neuroprotective GDNF enhanced GLAST expression particularly around photoreceptors (Figure 2 and Figure 3). Hence we next investigated whether GDNF treatment could decrease glutamate level in *rd1* retinas and, at the same time, increase the level of one of its metabolite: glutamine. Subretinal injections of GDNF or PBS were performed on 15-day-old and 21-day-old *rd1* mice. Retinal amino acid levels were measured 48 h after injection. GDNF treatment did not alter free glutamate and glutamine levels at PN15 or PN21 (Figure 4).

DISCUSSION

Our results can be summarized as follows: (1) In the *rd1* retina, GDNF receptor mRNAs did not decrease during photoreceptor degeneration and were expressed by MGC. (2) GDNF application to MGC cultures, *rd1* mouse retinal explants, and *rd1* mouse retinas in vivo enhanced the expression of GLAST. GLAST protein expression in MGC was particularly increased around degenerative photoreceptors. (3) Free glutamate and glutamine levels in the *rd1* retina were not significantly modified by exogenous GDNF.

A potential indirect neuroprotective pathway for GDNF: In the retina, we demonstrated that exogenous GDNF induces histological and, more importantly, functional protection of photoreceptors in the *rd1* mouse [8], as documented since then in other models of photoreceptor degeneration [9-11]. The mechanisms underlying GDNF neuroprotection remain unknown. In vitro, a direct survival-promoting effect on newborn rodent photoreceptors was observed [16,17]. Now, several arguments support the hypothesis that GDNF exerts its trophic effect on photoreceptors in the *rd1* mouse also indirectly through activation of intermediate MGC.

First, our results indicate that GDNF receptors, GFR α -1 and Ret, were not exclusively expressed by the degenerating photoreceptor cells in the *rd1* retina since both GFR α -1 and Ret mRNA levels in vivo are not reduced by photoreceptor degeneration (Figure 1C,D). Ret mRNA expression, on the one hand, is not altered by the degeneration process in accor-

dance with its immunohistochemical localization in the whole retina [52]. GFR α -1 localization in the retina, on the other hand, is still a matter of debate. Conflicting data have been reported, GFR α -1 has been localized in MGC, in the inner plexiform layer and retinal ganglion layer, with a weak staining also being observed in photoreceptor outer segments by some authors [53] or restricted to the outer nuclear layer by others [19,20]. Such discrepancies in immunohistochemical observations might come from the use of different antibodies. Here our results demonstrate that GFR α -1 mRNA expression did not decrease as one could have expected if its expression had been restricted to photoreceptors, but, on the contrary, increases in the *rd1* mouse retina during and after complete rod degeneration (Figure 1C). This implies that GFR α -1 mRNA is not exclusively expressed by photoreceptor cells, at least in the degenerative retina.

Second, our comparative in vitro experiments further demonstrate that GFR α -1 mRNA was predominantly expressed by MGC, whereas Ret mRNA was more widely distributed among cultured retinal cells (Figure 1F).

Therefore, all our observations suggest that GDNF could exert its trophic effect on photoreceptors in the *rd1* mouse retina through an indirect pathway via MGC.

The presence of GDNF receptors in MGC is further supported by the GDNF-induced changes in these cells. For instance, we have previously demonstrated that subretinal injection of GDNF increases glial fibrillar acidic protein (GFAP) immunoreactivity, a MGC marker, in the *rd1* mouse, thus implying that MGC are activated after GDNF treatment [8]. GDNF also stimulates the expression of endogenous trophic factors in MGC cultures [20]. Here we further confirmed the glial activation by demonstrating a GDNF-elicited increase in GLAST protein surrounding degenerating photoreceptors in the *rd1* retina (Figure 2 and Figure 3).

Does neuroprotective GDNF promote endogenous anti-excitotoxic mechanisms?: We demonstrated, in a parallel article, that glutamate-mediated excitotoxic mechanisms occur in the degenerating *rd1* retina and contribute to rod cell death [30]. These results are consistent with the data reported here.

First, Marco et al. [54] observed that, in the striatum, injection of excitatory amino acids: (1) induced an early and transient increase of GDNF mRNA expression, (2) clearly upregulated intrastriatal GFR α -1 mRNA, (3) and did not modify GFR α -2 mRNA levels.

As shown in Figure 1B,C,E we obtained similar profiles of expression during the degenerative process. Hence, although endogenous GDNF was only moderately and transiently increased, our observations suggest that GDNF-activated pathways are upregulated during photoreceptor degeneration in the *rd1* retina, possibly in relation to the alterations in glutamate metabolism we described [30].

Second, the observed upregulation of GLAST (which represents a key element in glutamate clearance) following GDNF neuroprotective treatment (Figure 2 and Figure 3) strengthened the hypothesis of a toxic glutamate increase during photoreceptor degeneration in the *rd1* mouse.

In the brain, GDNF is neuroprotective in both acute and

chronic neurodegenerative disorders [55,56] including an excitotoxic model of Huntington's disease [21]. Therefore, it was worth investigating whether GDNF could exert its neuroprotective effect on photoreceptors in the *rdl* retina, at least partially, by promoting an endogenous anti-glutamate-mediated excitotoxic mechanism, that is, an upregulation of GLAST.

In order to determine whether GDNF promoted photoreceptor survival through such a mechanism, we next measured free amino acid levels in *rdl* retinas following GDNF or PBS treatment. As shown in Figure 4, we failed to demonstrate a significant modification of glutamate and glutamine levels in GDNF-treated retinas when compared to controls. Two main explanations can be put forward for such results. First, the upregulation of GLAST may not be sufficient to prevent glutamate levels from rising. Second, our free amino acid measurements reflected both extra- and intracellular contents of whole *rdl* retinas, hence displacement of glutamate from one cellular compartment to another (from the extracellular environment of photoreceptors to the cytoplasm of MGC through GLAST) without metabolism of glutamate into glutamine by glutamine synthetase could not be demonstrated. Thus, we could not formally establish nor rule out that glutamate levels were effectively modified around photoreceptors.

Our results altogether shed some light on the central role of MGC in the neuroprotective response linked to GDNF in the *rdl* retina. Numerous published studies have dealt with the identification of trophic factors promoting photoreceptor survival, but only few of them focused on the mechanisms underlying trophic factor neuroprotective effects. Undoubtedly, understanding of these mechanisms would lead to new pharmacological approaches in RP. Further work is certainly needed to better understand the mode of action of GDNF on glial and rod photoreceptor cells and to design refined pharmacological intervention in the rod degenerative process.

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