Constitutive and AP20187-Induced Ret Activation in Photoreceptors Does Not Protect from Light-Induced Damage

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Purpose. Delivery of glial cell-derived neurotrophic factor (GDNF), either as a recombinant protein or by retinal gene transfer results in photoreceptor (PR) neuroprotection in genetic models of retinitis pigmentosa (RP). The mechanism of GDNF action and its direct targets in the retina remain unknown. The goal of the present study was to test the neuroprotective effect of GDNF from light-induced damage, a commonly used stimulus of PR degeneration, and to determine whether protection occurs directly on PRs.

Methods. Adeno-associated viral vectors (AAV) were developed that expressed either GDNF or a constitutively (RetMen2A) or pharmacologically activated chimeric GDNF receptor (Fv2Ret). Fv2Ret homodimerization and activation are induced by the administration of the small dimerizer drug AP20187. AAV2/2 vectors and the cytomegalovirus (CMV) promoter were used to transduce GDNF in the retina, whereas RetMen2A and Fv2Ret were transduced by AAV2/5 vectors and their expression restricted to PRs by the rhodopsin promoter. In vivo GDNF levels were measured by ELISA, RetMen2A and Fv2Ret expression and activation in vitro and/or in vivo were assessed by Western blot and immunofluorescence analyses. ERG measurements and histologic analyses were performed to assess morphologic and functional rescue, respectively.

Results. GDNF gene transfer resulted in sustained protein expression in the eye. In addition, the results confirmed in vivo that PR-restricted activation of Ret signaling occurred after either AAV-mediated expression of RetMen2A or AP20187-dependent Fv2Ret activation. However, this or AAV-mediated GDNF retinal gene transfer did not result in functional or morphologic PR protection from light-induced damage.

Conclusions. The results suggest that the apoptotic pathways responsible for light-induced PR degeneration are not inhibited by GDNF. However, GDNF signaling was shown to be regulated in time and levels in the retina by the AP20187/Fv2Ret system which is therefore available to be tested as gene-based therapeutic strategy in models of PR degeneration responsive to GDNF. (*Invest Ophthalmol Vis Sci.* 2007;48:5199–5206) DOI:10.1167/iovs.07-0140

Retinitis pigmentosa (RP) is a group of inherited (Mendelian in most of the cases) retinal diseases for which no treatment is available. It is characterized by progressive photoreceptor (PR) loss, generally due to apoptosis that is independent of the mutation underlying the disease.[1] A general antiapoptotic treatment therefore is highly desirable considering the high genetic heterogeneity of the condition. Molecules with neurotrophic activity have been shown to be effective in slowing PR cell death in various models of RP.[2–7] Among them, intraocular administration of glial cell-derived neurotrophic factor (GDNF) either as a recombinant protein or by retinal gene transfer results in retinal preservation in four different genetic models of RP: the *rd1*[8,9] and *rds*[10] mice and the RCS[10] and S334Ter rats.[11] The apoptotic pathways that mediate PR degeneration in these models have been partly elucidated.[12–18] GDNF activity is mediated by the interaction with a specific receptor, the GDNF family receptor α (GFRα1 or -2)[19] (Fig. 1A), which in turn interacts with either the transmembrane tyrosine kinase receptor Ret or the neural cell adhesion molecule N-CAM.[20,21] GFRα1 and -2 and Ret are expressed on PR outer segments as well as on other retinal cell types, whereas N-CAM expression in the adult eye is restricted to the inner retina.[22–24] GDNF-induced Ret dimerization juxtaposes the two intracellular catalytic domains, thereby allowing mutual transphosphorylation of its tyrosine residues, which propagate the signal to the nucleus by recruiting intracellular proteins.[25] Various Ret gain-of-function mutations responsible for autosomal dominant multiple endocrine neoplasia types 2A and 2B (RetMEN2A and -2B) result in the formation of Ret covalent dimers with constitutive kinase activity[25] (Fig. 1B). Among the intracellular proteins recruited in the Ret-mediated signal cascade, the SH2-containing protein (Shc) is a pivotal direct target of both activated Ret and RetMen2A.[26] This adaptor protein exists in three isoforms, namely p66, p52, and p46, which differ only in their N-terminal regions.[27] The phosphorylated Ret receptor binds and phosphorylates Shc on its tyrosine residues in a glycine/proline-rich region that is present in all three Shc isoforms.[27]

The light-damaged albino mouse is an animal model commonly used in quantitative tests of the PR survival-promoting activity of various potential neurotrophic agents.[2,3] This model involves the exposure of albino murine retinas to bright light.[28–30] Light-induced retinal degeneration can be induced at will, proceeds faster than in most RP animal models, and can be modulated by varying light intensity or exposure duration, all features highly desirable when testing an experimental

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Supported by Telethon Grant TIGEM P21; the Milton and Steinbach Fund; EC-FP6-projects LSHB-CT-2005-512146 DiMI and 018933 Clinigene; National Eye Institute Grant NIH1R01EY015136-01; and Grant DM589/7303/04 from the Italian Ministry of Agriculture (AA); and the FIRB Grant RBAUO15YHM from the Italian Ministry of University and Research (LD).

Submitted for publication February 5, 2007; revised May 8, 2007; accepted September 4, 2007.

Disclosure: **M. Allocca**, None; **U. Di Vicino**, None; **M. Petrillo**, None; **F. Carlomagno**, None; **L. Domenici**, None; **A. Auricchio**, None

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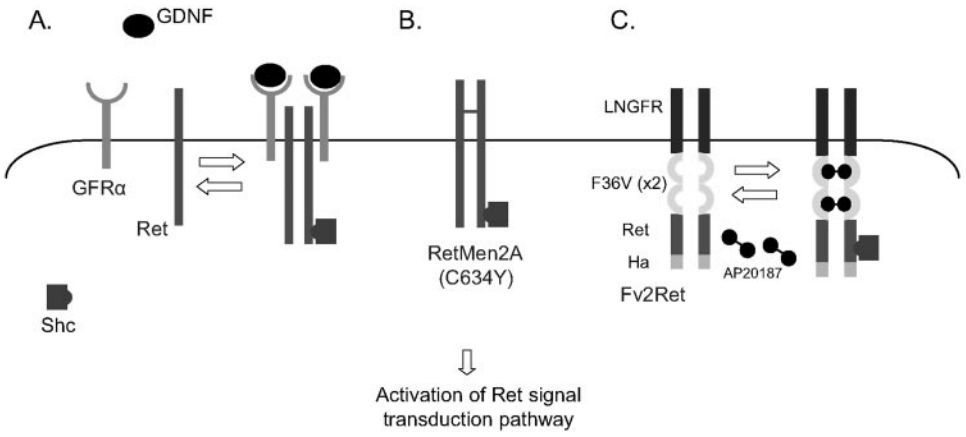

FIGURE 1. Schematic representation of the physiological, constitutive, and pharmacologically-activated Ret signaling. (**A**) Physiological GDNF-induced Ret dimerization allows mutual transphosphorylation of its tyrosine residues which propagate the signal to the nucleus by recruiting intracellular proteins. The SH2-containing protein (Shc) is an adaptor protein and one of the direct targets of activated Ret receptors. The direct binding of Shc to phosphorylated Ret receptor leads to its tyrosine phosphorylation. (**B**) The C634Y mutation responsible for autosomal dominant multiple endocrine neoplasia type 2A (RetMEN2A) results in constitutive Ret kinase activity. (**C**) In the chimeric Fv2Ret receptor the cytoplasmic domain of Ret has been fused to the human LNGFR extracellular and transmembrane domains, to two domains contained in the FKBP protein (F36V) and to a C-terminal hemoagglutinin (Ha) tag. When the AP20187 dimerizer drug binds to the F36V domain, the receptor dimerizes, and transphosphorylation of the receptor triggers an intracellular signaling cascade resulting in biological effects.

therapy.[30] In addition, retinal degeneration occurring in several genetic models of RP can be accelerated by exposure to light[31,32] and, conversely, light-induced retinal degeneration occurs by apoptosis similar to genetic RP,[30] thus suggesting that light represents a non-artificial damaging agent for the retina. Notably, GFRα-2 receptor is upregulated in Müller and PR cells during light-induced retinal degeneration.[33,34] This change in expression suggests that GDNF is an endogenous neuroprotective factor in the light-damaged model. Although GDNF stimulates survival of newborn PRs and directly delays PR outer segment collapse in vitro,[19,35] it is currently unclear whether this neuroprotective effect in vivo is exerted directly on PRs or through the Müller cell-PR network.[33,36]

The goal of our study was to test by gene transfer GDNF neuroprotection from light-induced damage and whether this occurs directly on PRs. We took advantage of the ability of vectors derived from the small adeno-associated virus (AAV) to transfer genes to the retina.[37] Exchanging the surface proteins (capsids) among various AAV serotypes allows the creation of hybrid AAV vectors (named AAV2/1, -2/2, and so on, where the first number defines the genome and the second the capsid of origin) that have retinal transduction characteristics dependent on the capsid.[38,39] We and others have demonstrated that vectors with AAV5 capsids (AAV2/5) transfer genes to retinal pigment epithelium (RPE) and PRs more efficiently than those with AAV2 capsids (AAV2/2) when injected subretinally.[37–40] The combination of AAV2/5 vectors with the PR-specific rhodopsin promoter results in transgene expression restricted to PRs.[41,42] Conversely, AAV2/2 vectors transduce retinal ganglion cells (RGCs) when injected intravitreally.[38] To address specifically whether GDNF neuroprotective effect is exerted directly on PRs, we used a system that pharmacologically regulates the homodimerization of tyrosine kinase (TK) receptors.[43–45] This system is based on the ability of a small dimerizer drug (AP20187) to bind and dimerize a specific modified domain (F36V) contained in the FK506-binding protein (FKBP12) thus resulting in reversible receptor dimerization and transphosphorylation (Fig. 1C).[46–52] We used the combination of AAV2/5 vectors and rhodopsin promoter to assess whether PR-restricted activation of a chimeric Ret receptor (Fv2Ret) responsive to the AP20187 drug protects from light-induced retinal degeneration. Along the same line, PR-restricted expression of the constitutively activated RetMen2A provides similar evidence. In addition, the AP20187/Fv2Ret system could be useful for RP gene therapy, avoiding the expression of the therapeutic growth factor while tightly regulating its activity in a drug dose-dependent and reversible manner.

MATERIALS AND METHODS

Generation of the Plasmid Constructs

The GDNF coding sequence was obtained from adult mouse brain cDNA with the primers *Not*I-forward (For) 5′-ATTTGCGGCCGCATGGGATTCGGGCCACTTGGAGTT-3′ and *Hin*dIII-reverse (Rev) 5′-CCCAAGCTTTCAGATACATCCACACCGTTTAGC-3′ to insert *Not*I and *Hin*dIII sites at the 5′ and 3′ ends, respectively. The PCR products were then digested with *Not*I and *Hin*dIII and cloned into pAAV2.1.CMV.EGFP[53] by removing the EGFP coding sequence (*Not*I-*Hin*dIII) to obtain the pAAV2.1.CMV.GDNF plasmid.

The pAAV2.1.RHO.EGFP was obtained exchanging the CMV promoter of pAAV2.1.CMV.EGFP with the rhodopsin (RHO) promoter sequence. The RHO promoter (U16824 PubMed sequence) was amplified from human genomic DNA. The PCR was performed to insert an *Nhe*I and an *Not*I site at the 5′ and 3′ ends, respectively, with the following primers: *Nhe*I-RHOFor 5′-AATTATGCTAGCAGATCTTCCCCACCTAGC-3′ and RHORev-*Not*I 5′-ATTAATGCGGCCGCGGATGACTCTGGGTTCTG-3′. The PCR products were then digested with *Nhe*I and *Not*I and cloned into pAAV2.1.CMV.EGFP after the CMV promoter was removed.

The chimeric construct for Ret was produced at Ariad Pharmaceuticals (Cambridge, MA). In this chimeric construct the cytoplasmic domain of Ret (amino acids 658-1114) was fused to the human low-affinity nerve growth factor receptor (LNGFR) extracellular and transmembrane domains (LNGFR amino acids 1–274) and to two FKBP (F36V) domains in the LNGFR-FKBPx2-Ret configuration to obtain the pCLFv2Ret expression vector (Fig. 1C). The sequence encoding the chimeric Ret (Fv2Ret) was digested with *Hpa*I restriction enzyme and inserted into a pCDNA3 plasmid. Then, the fragment encoding Fv2Ret was cut with *Not*I and a *Hin*dIII and inserted into pAAV2.1.CMV.EGFP[53] and into pAAV2.1.RHO.EGFP to obtain the pAAV2.1.CMV.Fv2Ret and pAAV2.1.RHO.Fv2Ret plasmid, respectively. The RetMen2A (C634Y) coding sequence was amplified from the PCDNA3 plasmid[54] to insert a *Not*I and a *Hin*dIII site at the 5′ and 3′ ends, respectively, with the following primers: *Not*I-RetFor 5′-GCGGCCGCATGGCGAAGGCGACGTC-3′ and RetRev-*Hin*dIII 5′-AGGCTTCTAGAATCTAGTAAATGCATGGG-3′ and then cloned into pAAV2.1.RHO.EGFP digested with *Not*I and *Hin*dIII to obtain pAAV2.1.RHO.RetMen2A.

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AAV Vector Production

AAV vectors were produced by the TIGEM AAV vector core using pAAV2.1.CMV.GDNF, pAAV2.1.RHO.Fv2Ret, pAAV2.1.RHO.RetMen2A, pAAV2.1.RHO.EGFP, and pAAV2.1.CMV.EGFP.[53] Recombinant AAV2/5 and -2/2 viruses were produced by triple transfection of 293 cells followed by $CsCl_2$ purification of the AAV2/5 vectors and by affinity column purification of the AAV2/2 vectors, as previously described.[53] For each viral preparation, physical titers (genome copies [GCs]/mL) were determined by both PCR quantification (*Taq*Man; Perkin-Elmer, Life and Analytical Sciences, Inc., Wellesley, MA)[55] and dot blot analysis.

Transfection of 293 Cells with Chimeric Ret and Stimulation by AP20187

293 cells were plated in six-well plates to a concentration of 3×10^5 cells/well. Twenty-four hours later, the cells were transfected (Fugene 6; Roche, Basel, Switzerland) according to the manufacturer's instruction with 1 μg per well of pAAV2.1.CMV.EGFP or .CMV.Fv2Ret. Forty-three hours later, the cells were deprived of serum for 5 hours; stimulated with 0, 0.25, or 2.5 μM of AP20187 (Ariad Pharmaceuticals) for 5 minutes at 37°C; and harvested by scraping. The AP20187 drug was dissolved in ice-cold ethanol. To prevent detrimental effects of the solvent on the cells the final concentration of ethanol used in the medium was 0.25% for all samples. The unstimulated samples received only ethanol solution.

Vector Administration, AP20187 Treatment, and Tissue Collection

All procedures were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Four-week old male BALB/c mice (Harlan Italy, Udine, Italy) were used. Before administration of the vector, the mice were anesthetized with an intraperitoneal (IP) injection of Avertin at 2 mL/100 g body weight (1.25% [wt/vol] 2,2,2-tribromoethanol and 2.5% [vol/vol] 2-methyl-2-butanol; Sigma-Aldrich, St. Louis, MO).[56] Mice were injected subretinally with 2 μL (3×10^9 GC) of AAV2/2.CMV.GDNF ($n = 11$), AAV2/5.RHO.Fv2Ret ($n = 21$), or AAV2/5.RHO.RetMen2A ($n = 18$) in the right eye and with 2 μL of AAV2/2.CMV.EGFP or AAV2/5.RHO.EGFP in the left eye (3×10^9GC) as control. An additional group of animals ($n = 7$) was treated with combined subretinal (1 μL) and intravitreal (1 μL) injections of AAV2/2.CMV.GDNF in the right eye and AAV2/2.CMV.EGFP in the left eye. Subretinal and intravitreal vector administrations were performed as described.[57] Twenty-eight days after vector administration, the retinas of some animals ($n = 6$) injected with RetMen2A were harvested for Western blot analysis, and the remaining animals ($n = 12$) were exposed to light to induce retinal degeneration according to the protocol described later. The animals injected with AAV2/5.RHO.Fv2Ret ($n = 7$) received AP20187 treatment starting on day 28 after vector administration with two intravitreal injections of 4 μg (one at the beginning and one at the end of the light-exposure period). Both the AAV2/5.RHO.Fv2Ret- and the contralateral AAV2/5.RHO.EGFP-treated eyes were injected with AP20187. The treatment was supplemented by daily IP injections of AP20187 (10 mg/kg, the highest dose suggested for in vivo use by the manufacturer) during the light-exposure protocol and for an additional week after its end (totally 11 days). At this time the ERGs were performed, and eyes harvested for histologic section as described in the specific section. A group of five mice that received AAV2/5.RHO.Fv2Ret but not AP20187 was used as control of Fv2Ret basal activity during light-induced damage.

For Western blot analysis of Fv2Ret phosphorylation, we used the injection protocol just described, but the animals were not exposed to light, and the retinas were harvested 5 hours after the second intravitreal injection of AP20187 ($n = 3$) and treated as described in the next section. An additional group of animals ($n = 3$) received daily IP injection of AP20187 for 4 days, and their retinas were harvested 5 hours after the last AP20187 administration. A group ($n = 3$) that did not received the AP20187 was used as control. The AP20187 drug was dissolved in a solution of ethanol, PEG-400, and Tween 20 according to the manufacturer's instructions (Ariad Pharmaceuticals).

GDNF Measurements

GDNF levels were measured in the animal's anterior chamber (AC) fluid by GDNF immunoassay (Emax; Promega, Madison, WI). AC fluid was tapped from harvested eye balls at the time of euthanatization (P56) after ERG measurements and before histologic analysis.[58]

Protein Extraction from 293 Cells and Retinas and Western Blot Analysis

Harvesting of retinas was performed as described elsewhere.[58] Retinas and transfected 293 cells were harvested in lysis buffer (Tris [pH7.4] 40 mM, EDTA 4 mM, $MgCl_2$ 5 mM, Triton X-100 1%, Na_3VO_4 100 μM, phenylmethylsulfonyl fluoride [PMSF] 1 mM, NaCl 150 mM, and protease inhibitors [Roche]) and lysed on ice for 30 minutes. Retina samples were passed through pipettes with progressively smaller tips (p1000–p200) to disperse cells. Samples were spun at 14,000 rpm for 15 minutes and the supernatants collected. Total proteins were measured with the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co, Boston, MA). Proteins were denatured by heating to 98°C for 3 minutes and separated by SDS-PAGE (7% gel to detect chimeric Ret or RetMen2A and 10% to detect Shc proteins) under reducing conditions with 4% stacking gel in 1× Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS [pH 8.3]) in a miniprotean cell (Bio-Rad; Munich, Germany). The separated proteins were electrotransferred to a nitrocellulose filter (Schleicher & Schuell GmbH, Dassel, Germany) in a transfer buffer (0.004 M Tris, 2 g/L glycine, 0.05% SDS, and 20% methanol [pH 8.3]) in a minitransfer cell (Bio-Rad) at 100 V in a cold room for 1 hour. Membranes were incubated overnight in blocking buffer and subsequently probed with the specific antibodies: anti-Ret,[54] anti-PyRet,[54] anti-Shc, and anti-PyShc (Upstate Biotechnology, Waltham, MA), anti-Ha antibody (Sigma-Aldrich, Milano, Italy) and anti-Py (Santa Cruz Biotechnology, Santa Cruz, CA) The blots were developed by chemiluminescence (Enhanced Chemiluminescence Kit; Pierce Chemical Co.). The bands detected by Western blot were quantified on computer (Image Processing Tool Kit ver. 5 plus, Fovea Pro 3.0; Reindeer Graphics Inc., Asheville, NC).

Exposure to Light of the Albino Mice

The protocol derives from that described by LaVail et al.,[2] with modification of the duration of exposure to light. Briefly, the animals were reared in a 12-hour light–dark cycle until day 28 after vector injection. At that time, the animals were housed separately in clear Plexiglas cages and exposed to continuous light produced by eight 36-W white fluorescent bulbs (Osram Sylvania; Munich, Germany). Light sources were suspended 30 cm above (four bulbs) and below (four bulbs) the cages to produce an illumination of ~2000 lux. The animals had free access to food and water. After continuous illumination for 96 hours, the animals were housed in a room in a 12-hour light–dark cycle for 1 week before the electroretinogram (ERG) recording.

Electroretinogram Measurements

ERGs were performed before and 1 week after exposure to constant light for all injected and sham-injected animals. Briefly, the animals were dark adapted for 3 hours and then anesthetized and placed in a stereotaxic apparatus under dim red light. ERGs were evoked by flashes of different light intensities ranging from 10^{-4} to 20 cd $\cdot$ m^{-2} $\cdot$ s^{-1} generated through a Ganzfeld stimulator (Lace Elettronica, Pisa, Italy). To minimize the noise, three different responses evoked by light were averaged for each luminance step (the time interval between light stimuli was 4 to 5 minutes). The electrophysiological signals were recorded through gold plate electrodes inserted under the lower eyelids. Electrodes in each eye were referred to a needle electrode in-

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serted subcutaneously at the level of the corresponding frontal region. The different electrodes were connected to a two-channel amplifier. Amplitudes of a- and b-waves were plotted as a function of increasing light intensities. After completion of responses obtained in dark-adapted conditions, the recording session continued with the purpose of dissecting the cone pathway mediating the response to light. For this purpose, the ERG in response to light of 20 cd · m^{-2} was recorded in the presence of a continuous background light set at 15 cd · m^{-2}. The amplitude of the b-wave for each eye was plotted as a function of luminance (transfer curve) under scotopic and photopic conditions. For each group, the mean b-wave amplitude was plotted.

Histologic Analysis

After ERG measurements, mice were killed, their eye cups were harvested and fixed by immersion in 4% paraformaldehyde. Then, the mouse eye cups were infiltrated with 30% sucrose for cryopreservation and embedded in tissue-freezing medium (OCT Matrix; Kaltek, Padova, Italy). For each eye, 150 to 200 serial sections (12-μm-thick) were cut along the horizontal meridian, and the sections were progressively distributed on 10 slides so that each slide contained 15 to 20 sections representative of the whole eye at different levels. The sections were stained with 4′,6′-diamino-2-phenylindole (DAPI; Vectashield; Vector Laboratories, Inc. Burlingame, CA) and retinal histology was analyzed by light microscopy. To quantify PR rescue, the nuclei in the ONL of each eye were counted. A minimum of three sections per slide, representative of the entire eye cup, were analyzed. For each section, the number of nuclei in the ONL was separately counted on the nasal, central, and temporal sides. The nasal, temporal, and central counts of each section were independently averaged, therefore obtaining a number that was the average of the three sides for each eye. The counts from each group were then averaged, and standard errors were calculated. Untreated BALB/c ($n = 8$) albino mice were used as the control.

Immunolabeling on Retinal Sections

Sections were rinsed in PBS and incubated for 20 minutes in 0.2% Triton 100 and 1% normal goat serum (NGS) in PBS, followed by blocking for 1 hour in 10% NGS in PBS. The incubation with anti-Ha (1:100 in PBS; Sigma-Aldrich) was performed for 2 hours at RT followed by the incubation with AlexaFluor 568–coupled secondary antibody (1:500 in PBS; Invitrogen-Molecular Probes, Milano, Italy) for 1 hour at RT. After a final wash, the sections where mounted with DAPI (Vectashield; Vector Laboratories, Inc.), and images were obtained (Axiocam; Carl Zeiss Meditec, Inc., Berlin, Germany).

Results and Discussion

Assessment of GDNF Expression and of Fv2Ret and RetMen2A Localization and Phosphorylation

AAV2/2 vectors encoding either GDNF or EGFP under the control of the CMV promoter were injected subretinally in BALB/c mice. GDNF is a secreted protein measurable with a commercial ELISA. Retinal transduction with vectors encoding secreted proteins results in diffusion of the protein through the vitreous in the anterior chamber (AC) fluid which can then be sampled to assess in vivo protein levels.[58] Therefore, to verify GDNF expression, its levels were measured in the AC fluid of treated and control animals. As expected, GDNF was detected at high levels in the AC fluid of eyes injected with AAV2/2.CMV.GDNF (4479 ± 1638 pg/mL, $n = 7$), but not in those injected with AAV2/2.CMV.EGFP, where it was undetectable.

Fv2Ret is a chimeric Ret receptor fusion protein responsive to the soluble AP20187 (Fig. 1C). To test in vitro the AP20187-dependent Fv2Ret receptor transphosphorylation, 293 cells were transiently transfected with pAAV.CMV.Fv2Ret and stimulated 48 hours later with 0, 0.25, and 2.5 μM AP20187. Cells

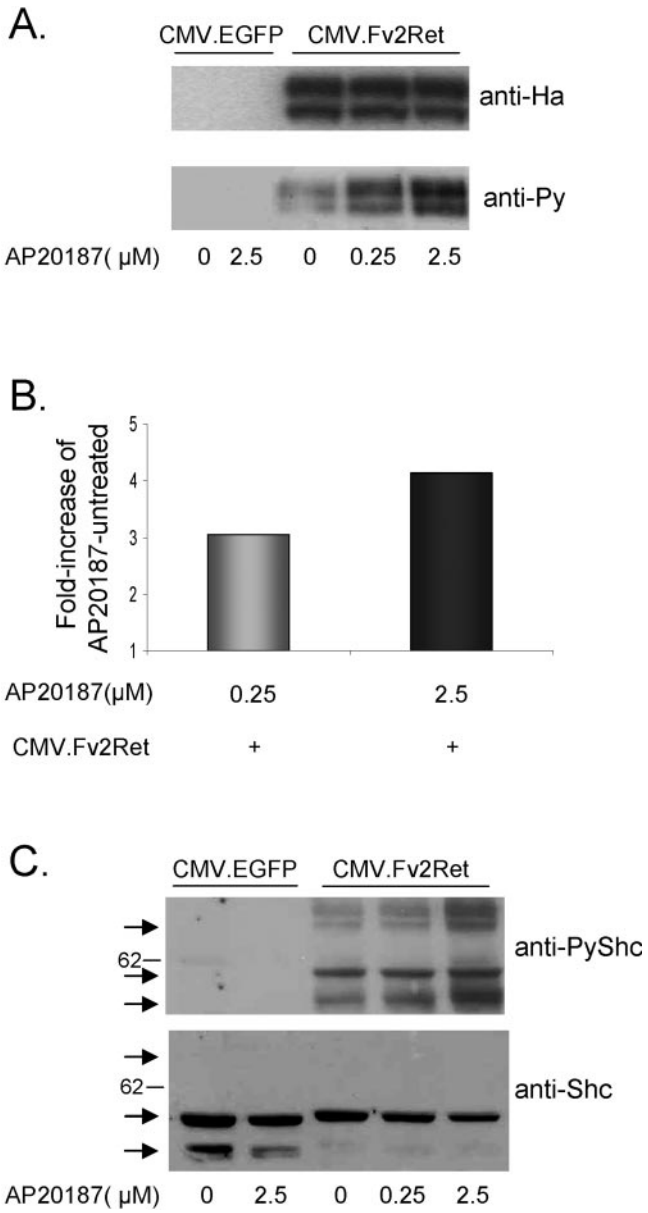

Figure 2. AP20187-dependent Fv2Ret tyrosine phosphorylation in vitro. (**A**) Western blot analysis with anti-hemoagglutinin (anti-Ha, *top*) and anti-phosphotyrosine (anti-Py, *bottom*) antibodies of total cellular lysates (0.5 μg) from 293 cells transfected with pAAV.CMV.Fv2Ret or -EGFP and stimulated for 5 minutes with various doses of AP20187, as indicated. (**B**) Increase in Fv2Ret phosphorylation upon AP20187 stimulation compared with unstimulated control. The histogram shows the average of two independent experiments. (**C**) Phosphorylation of SH2-containing protein (Shc) upon AP20187 administration in 293 cells expressing Fv2Ret. Western blot analysis with anti-PyShc (*top*) and anti-Shc (*bottom*) antibodies of total cellular lysates from 293 cells transfected with pAAV.CMV.Fv2Ret or .EGFP and stimulated for 5 minutes with various doses of AP20187 as indicated. *Left*: bands corresponding to three Shc isoforms (*arrows*) and the marker's molecular masses (kDa).

transfected with pAAV.CMV.EGFP and stimulated with 0 and 2.5 μM AP20187 were used as the control. A double-phosphorylated band corresponding to Fv2Ret is evident in the samples transfected with pAAV.CMV.Fv2Ret but not in those transfected with pAAV.CMV.EGFP (Fig. 2A). The presence of a double Fv2Ret band may be due to the presence of a degradation product of lower molecular weight. Basal Fv2Ret phos-

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phorylation, probably due to its overexpression, is present in the absence of AP20187 but increases with AP20187 dosage. The levels of Fv2Ret phosphorylation in samples stimulated with 0.25 and 2.5 μM AP20187 were 3.0 and 4.1 times those of unstimulated samples (Fig. 2B), respectively. Western blot analysis with anti-Ha antibodies shows that similar amounts of Fv2Ret were present in lysates of cells transfected with pAAV.CMV.Fv2Ret. Higher doses of AP20187 (25 μM) did not result in a further increase of Fv2Ret phosphorylation levels (data not shown). To demonstrate that the Fv2Ret receptor is able to transduce intracellular signals, we performed Western blot analysis with anti-Shc and anti-PyShc antibodies on the same cellular lysates from 293 cells, as described earlier (Fig. 2C). Shc phosphorylation is evident in the samples transfected with pAAV.CMV.Fv2Ret but not in those transfected with pAAV.CMV.EGFP, as expected (Fig. 2C). In addition, Fig. 2C shows that the levels of Shc phosphorylation increase with the AP20187 dose in agreement with the increase of Fv2Ret phosphorylation. Shc phosphorylation in the samples transfected with pAAV.CMV.Fv2Ret but not stimulated with AP20187 reflects the basal Fv2Ret dimerization and tyrosine phosphorylation observed. In Figure 2A, Western blot analysis with anti-Shc antibodies showing higher levels of Shc protein in the AP20187-unstimulated sample than in those stimulated with 0.25 and 2.5 μM drug strongly supports AP20187-induced Shc phosphorylation.

To test the AP20187-dependent Fv2Ret and constitutive RetMen2A phosphorylation in vivo in PR cells, we generated recombinant AAV2/5.RHO.Fv2Ret, -.RetMen2A, and -.EGFP vectors. Four-week-old BALB/c mice received subretinal injections of AAV2/5.RHO.Fv2Ret or -.RetMen2A vectors in the right eye and of AAV2/5.RHO.EGFP vector in the left eye as control. To confirm that the combination of AAV5 capsid and rhodopsin promoter results in transgene expression restricted to PRs, 28 days after vector administration we analyzed some retinas injected with AAV2/5.RHO.Fv2Ret and .EGFP by immunofluorescence with anti-Ha antibody and by direct fluorescence, respectively. Figure 3A shows that EGFP expression was restricted to PR cell bodies and outer segments (left); Fv2Ret was localized to PR outer segments similarly to endogenous Ret[23] (right). Twenty-eight days after vector administration (a time point selected to allow sustained gene expression from AAV2/5 in the retina), the animals injected with AAV2/5.RHO.Fv2Ret were divided into three groups (n = 3) as described in the Materials and Methods section. One group received IP administration of AP20187 (10 mg/kg) for 5 days, one group received combined AP20187 IP and intravitreal (IV, 4 μg) administration on days 1 and 5 of treatment, and the last group did not receive AP20187. The IV injections localize AP20187 directly to the vitreous, whereas IP injections require the drug to cross the blood–retina barrier. All retinas were harvested 5 hours after the last AP20187 injection (32 days after vector administration) and analyzed by Western blot with anti-Py and anti-Ha antibodies. The bands obtained by Western blot with anti-Py antibodies were quantified and the results are shown in Fig. 3B as the increase in Fv2Ret phosphorylation on AP20187 administration compared with AP20187-untreated, Fv2Ret-transduced control retinas. Fv2Ret tyrosine phosphorylation occurred in the absence of AP20187 increases of 3.7 ± 1.0- and 6.7 ± 2.2-fold after IP and combined IP and IV AP20187 injections, respectively (Fig. 3B). There was no increase in the Fv2Ret phosphorylation levels when higher doses (40 μg) of AP20187 were injected IV (data not shown). Our data indicate that AP20187 given systemically can cross the blood–retina barrier, although the levels of AP20187-dependent Fv2Ret phosphorylation are higher when the drug is given IV. Twenty-eight days after the vector was administered, the animals with AAV2/5.RHO.RetMen2A subretinally administered in one eye and AAV2/5.RHO.EGFP in the contralateral eye were killed and their retinas harvested and analyzed by Western blot (Fig. 3C). We detected a phosphorylated band with a molecular weight that corresponded to RetMen2A in the retinas injected with AAV2/5.RHO.RetMen2A, but not in those injected with AAV2/5.RHO.EGFP (Fig. 3C). In addition, all Shc isoforms are strongly phosphorylated on tyrosine residues in

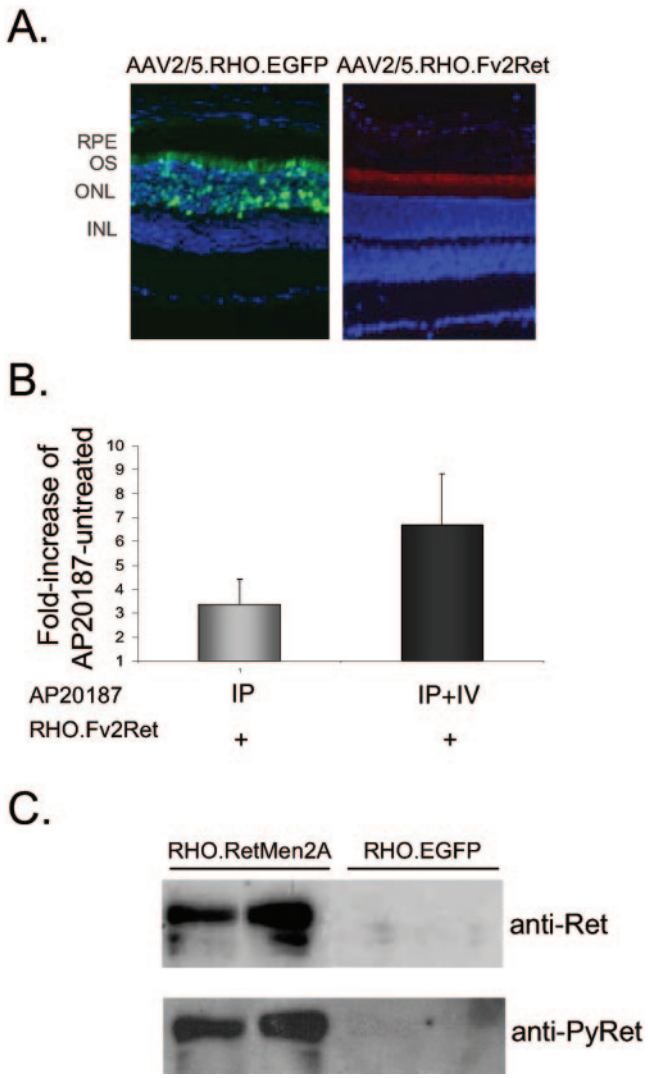

FIGURE 3. Assessment of Fv2Ret and RetMen2A localization and phosphorylation in the retina transduced with AAV2/5. (**A**) Photoreceptor-restricted Fv2Ret and EGFP expression after subretinal injections of AAV2/5. Fluorescence microscopy evaluation of EGFP expression from retinas injected subretinally with AAV2/5.RHO.EGFP (*left*). RPE: retinal pigment epithelium; OS: outer segments; ONL: outer nuclear layer; INL: inner nuclear layer. *Right*: immunofluorescence with anti-Ha antibodies on cryosections from retinas injected with AAV2/5.RHO.Fv2Ret (*red*). Magnification, ×20. (**B**) Increase of Fv2Ret phosphorylation in lysates from retinas subretinally injected with AAV2/5.RHO.Fv2Ret on intraperitoneal or combined intraperitoneal and intravitreal AP20187 administration compared with the controls, which did not receive the drug. The histogram shows the average ± SE of results obtained from three eyes/group injected and analyzed independently by Western blot. IP, intraperitoneal AP20187 administration (10 mg/kg); IP+IV = combined intraperitoneal (10 mg/kg) and intravitreal (4 μg) AP20187 administration. (**C**) RetMen2A expression and phosphorylation in the retina transduced with AAV2/5 vectors. Western blot analysis with anti-Ret (*top*) or anti-PyRet (*bottom*) antibodies of whole lysates from retinas subretinally injected with AAV2/5.RHO.RetMen2A or AAV2/5.RHO.EGFP.

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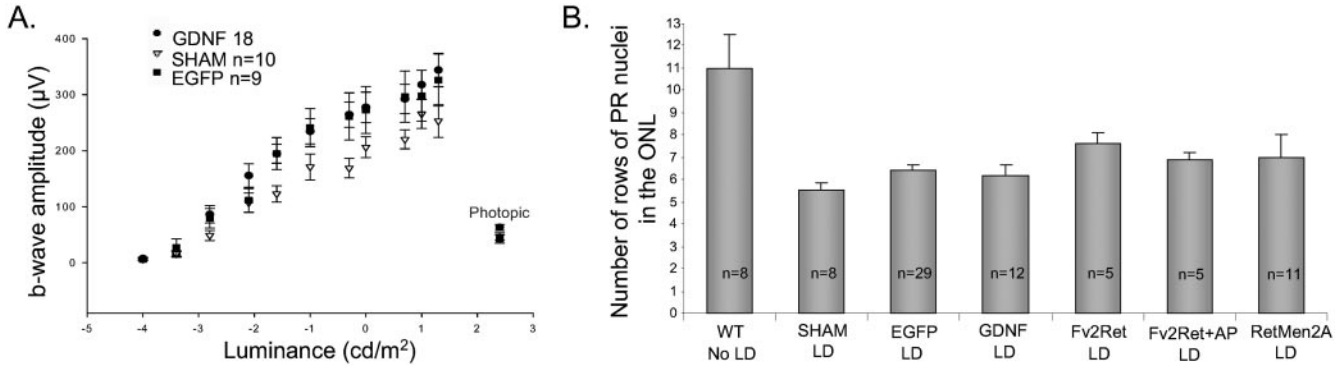

Figure 4. Assessment of photoreceptor function (ERG) and morphology after AAV-mediated gene transfer. (**A**) Transfer curve of mean b-wave amplitude (± SE) as a function of luminance recorded after photolesion (96 hours of exposure to light) in animals treated with AAV2/2.CMV.EGFP or.GDNF. The luminance is plotted on a log scale. GDNF, animals injected with AAV2/2.CMV.GDNF subretinally or subretinally/intravitreally; SHAM, sham-injected animals; EGFP, animals injected with AAV2/2.CMV.EGFP subretinally or subretinally/intravitreally; photopic, recordings in light adapted conditions. (**B**) Histologic analyses in light-damaged albino mice after AAV mediated expression of EGFP, GDNF, RetMen2A, and Fv2Ret with or without AP20187 treatment. LD, light-induced damage (96 hours of exposure to light). n = number of eyes in each group; WT, uninjected wild-type mice; No LD, no light-induced damage; ONL, outer nuclear layer; EGFP, animals injected with AAV2/2.CMV.EGFP subretinally or subretinally/intravitreally or with AAV2/5.RHO.EGFP, treated or not with AP20187; GDNF, animals injected with AAV2/2.CMV.GDNF subretinally or subretinally/intravitreally; Fv2Ret, animals injected with AAV2/5.RHO.Fv2Ret; Fv2Ret+AP, animals injected with AAV2/5.RHO.Fv2Ret and treated with AP20187; RetMen2A, animals injected with AAV2/5.RHO.RetMen2A.

the retinas injected with AAV2/5.RHO.RetMen2A but not with AAV2/5.RHO.EGFP (data not shown). After subretinal administration of AAV2/5 vectors, recombinant Ret expression and tyrosine phosphorylation in PRs were constitutive and robust in the case of RetMen2A and dependent on AP20187 administration in the case of Fv2Ret. We therefore tested whether AAV-mediated overexpression of GDNF, Fv2Ret, or RetMen2A protects PRs from light-induced damage, a model widely used to test neurotrophic molecules.

Assessment of Morphologic and Functional PR Protection from Light-Induced Damage after AAV-Mediated GDNF, Fv2Ret, and RetMen2A Retinal Gene Delivery

Albino BALB/c mice were injected either subretinally (to transduce PR and RPE) or by a combined intravitreal/subretinal injection (to transduce the GCL in addition to PRs and the RPE) with AAV2/2.CMV.GDNF in the right eye and AAV2/2.CMV.EGFP in the left eye. An additional group was injected subretinally with AAV2/5.RHO.RetMen2A or .Fv2Ret in the right eye with the contralateral receiving AAV2/5.RHO.EGFP. Animals' eyes were light damaged 28 days after gene transfer, administered with AP20187 where indicated, and analyzed. To assess PR functional rescue after AAV-mediated gene delivery, we analyzed the retinal electrical activity after light-induced damage by means of Ganzfeld flash electroretinograms (ERG). Retinal responses before phototoxicity to flash of different intensities were recorded 20 days after AAV-mediated GDNF, Fv2Ret, RetMen2A, EGFP, or sham delivery and showed normal retinal function (the average maximal a-wave amplitude before the photolesion is 240 ± 12 μV, the average maximum b-wave is 526 ± 19 μV, n = 76). After animals were exposed to constant illumination for 96 hours, the retinal response to light decreased in AAV2/2.CMV.EGFP- and sham-injected eyes (Fig. 4A; average a-wave amplitudes: 128 ± 31 μV [n = 9] and 76 ± 16 μV [n = 10], respectively). No functional improvement was observed in the eyes injected either subretinally or subretinally/intravitreally with AAV2/2 encoding GDNF (Fig. 4A; average a-wave amplitude: 107 ± 19 [n = 19]). Given their similar results, the subretinal and combined subretinal/intravitreal injections were grouped in Figures 4A (GDNF group) and 4B (GDNF LD group). Similarly, administration of AAV2/5.RHO.RetMen2A or .Fv2Ret, with and without AP20187 treatment, did not protect PR electrical activity from light-induced damage (data not shown). We additionally applied a protocol resulting in more severe damage by exposing the animals to constant illumination for 168 hours. This prolonged exposure abolishes the generation of a- and b-waves in AAV2/5.RHO.RetMen2A-, .Fv2Ret-, and .EGFP-treated eyes. No significant differences were observed in the a- and b-wave intensities among animals injected with AAV encoding RetMen2A or Fv2Ret+AP20187 and control animals (Table 1).

The absence of PR functional rescue after either GDNF or RetMen2A and Fv2Ret gene transfer was confirmed at the

Table 1. Mean a- and b-Wave Amplitudes of AAV2/5-Treated Retinas after Light-Induced Damage

Treatment	AP20187	a-Wave Amplitude (μV)	b-Wave Amplitude (μV)	n
AAV2/5.RHO.Fv2Ret	+	26.3 ± 8.8	7.9 ± 1.8	5
AAV2/5.RHO.Fv2Ret	−	5.8 ± 2.2	6.1 ± 2.3	6
AAV2/5.RHO.RetMen2A	−	5.6 ± 1.7	12.3 ± 6.7	9
AAV2/5.RHO.EGFP	−	7.7 ± 2.0	4.7 ± 1.0	15
AAV2/5.RHO.EGFP	+	26.0 ± 10	14.2 ± 2.2	4

Average maximum a- and b-wave amplitudes after light-induced damage (168 hours of exposure to light) in mice subretinally injected with AAV2/5.RHO.Fv2Ret, .RetMen2A, or .EGFP and treated with AP20187, as indicated. Flash intensity, 1.8 log cd/m^2. Data are expressed as the mean ± SE. n, number of eyes in each group.

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histologic level. A significantly lower number of rows of PRs nuclei was present in the light-damaged retinas injected with AAV when compared with retinas that were not light damaged, independent of the treatment (Fig. 4B). This finding suggests that no functional or morphologic protection from light-damage was observed in PR after AAV-mediated delivery of GDNF or of constitutively or pharmacologically activated Ret. The absence of GDNF-mediated neurotrophic activity in the light-damage model may be due to differences in the apoptotic pathways responsible for PR death between this and the genetic models of RP where GDNF has been proven neuroprotective.[12–18] For instance, two caspase-independent cascades starting from calpain, one involving the mitochondrion and one the endoplasmic reticulum, mediate PR apoptosis in the *rd1* model.[14,16–18] Caspase-dependent cascades mediate apoptosis in other models responsive to GDNF, such as caspase-3 in S334Ter rats[12] and caspase-1 and -2 in RCS rats.[13,15] After light-induced damage, AP-1-dependent activation of caspase-1 has been documented.[30,59]

Independent of the impact on PR survival and function in light-induced retinal degeneration, we have shown that GDNF signaling can be triggered in the retina by the AP20187/Fv2Ret system after PR transduction with AAV2/5 vectors containing the rhodopsin promoter. AAV-mediated PR-restricted expression of this system in RP animal models in which GDNF neuroprotective effect has been described can be used to understand GDNF neuroprotective mechanism and targets as well as a potential gene-based therapeutic strategy for RP.

Acknowledgments

The authors thank Victor M. Rivera (ARIAD Pharmaceuticals) for providing the pCLFv2Ret construct and the AP20187 drug and for useful discussions and Carolina Iodice (Tigem, Naples, Italy) for help with the histology.

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