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ARTICLE

Systemic but Not Intraocular *Epo* Gene Transfer Protects the Retina from Light- and Genetic-Induced Degeneration

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Molecules with neurotrophic activity are being evaluated for treatment of retinitis pigmentosa in animal models. In particular, great interest has been focused recently on erythropoietin (Epo). Evidence of its neurotrophic activity comes mainly from data demonstrating photoreceptor protection in a rodent light-damage model through systemic administration of a recombinant form of this hormone. Our goal was to test whether *Epo* retinal gene transfer can rescue or delay photoreceptor cell death. We delivered adeno-associated viral vectors encoding *Epo* intraocularly and, for comparison, intramuscularly to one light-induced and two genetic models of retinal degeneration. Intraocular *Epo* gene transfer resulted in sustained hormone expression in the eye, which was undetectable systemically. In contrast, *Epo* intramuscular gene transfer resulted in hormone secretion in the circulation, which was not detected in ocular fluids. The protein secreted from muscle and retina is of the same molecular weight as a commercial recombinant human Epo. Interestingly, following systemic but not intraocular *Epo* delivery, morphological photoreceptor protection was observed in the light-damage and *rds/peripherin* (*Prph2*) models of retinal degeneration. In the light-damage model, the morphological rescue was accompanied by a significant electrophysiological improvement of photoreceptor function. In contrast, no photoreceptor rescue was observed following *Epo* gene transfer in the *rd10* model. This suggests that different apoptotic mechanisms, with varying sensitivities to Epo, occur in different retinal degeneration models. In conclusion, our data support Epo as a neuroprotective agent in some, but not all, retinal degenerations. Further, rescue is observed in specific models after systemic but not intraocular *Epo* gene transfer.

Key Words: erythropoietin, retinal degeneration, AAV, apoptosis, animal models

INTRODUCTION

Retinitis pigmentosa (RP) is a group of inherited (Mendelian in most of the cases) retinal diseases for which no treatment is available. Molecules with neurotrophic actions have been shown effective in slowing photoreceptor cell death in various models of RP [1]. A general antiapoptotic treatment, independent of the specific genetic mutation, is highly desirable in a genetically heterogeneous condition such as RP. Delivery of a neuroprotective factor through a gene therapy approach can provide a persistent, theoretically regulatable supply of neurotrophic factor to the RP retina. Transgene delivery can be activated specifically in the eye by performing intravitreal or subretinal injections of the DNA-based drug [2]. Recently, erythropoietin (Epo) was discovered to be a neurotrophic molecule in the retina.

Epo is known as a hematopoietic cytokine produced by the fetal liver and adult kidney in response to hypoxia. It serves to maintain an erythrocyte mass appropriate to the oxygen supply. Recently, Epo has

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been shown to have neuroprotective and neurotrophic actions in the central and peripheral nervous systems [3–8]. As the retina is an extension of the central nervous system, it comes as no surprise that Epo is viewed as an important developmental, neuroprotective, and regenerative factor in this tissue. Epo and its receptor (Epo-R) are expressed in the human retina during early fetal development [9]. Epo can promote neural outgrowth from retinal ganglion cells in a dose-dependent manner and preserves them following axotomy [10,11]. In addition, hypoxia-induced retinal Epo expression protects retinal ganglion cells from transient global retinal ischemia and reperfusion injury through an antiapoptotic pathway [12]. This effect is inhibited by intraocular delivery of soluble Epo-R. Recently, the neurotrophic effect of Epo in the retina has been extended beyond damage from ischemia and axotomy. Systemic administration of recombinant human Epo protects retinal photoreceptors from light-induced damage in rats via a signaling cascade that inhibits the AP-1 apoptotic pathway [13]. The authors speculate that this occurs via the interaction of Epo with Epo-R in the photoreceptor inner segments [14]. A similar protection from light damage is seen in transgenic mice overexpressing Epo in several tissues, including the retina. When transgenic animals are crossed with models of genetic retinal degeneration, such as the *rd1* mouse, no rescue is observed [15]. Therefore, Epo represents an endogenous neuroprotective factor for the ischemic retina and a potential exogenous neurotrophic molecule for the treatment of some but not all retinal degenerations.

We have selected three models of retinal degeneration to study the effects of *Epo* gene delivery in preventing or ameliorating photoreceptor cell death. The models selected are the light-damaged albino Lewis rat and homozygous *rds* (*Prph2*^{Rd2}) and *rd10* (*Pde6b*^{Rd10}) mice. The first model involves the exposure of albino rodent retinas to bright light [16,17]. Exposure to high-intensity visible light is used as a model system to assess quantitatively the photoreceptor survival-promoting activity of various potential neurotrophic agents [18,19]. The *Prph2* gene encodes a photoreceptor-specific membrane glycoprotein, *peripherin/rds* [20], which is essential in maintaining the structure of photoreceptor outer segments. Homozygous *rds* mice show retinal degeneration of early onset and slow progression [21]. The *rds* mouse outer nuclear layer (ONL) is gone by 12 months of age at which time there is undetectable electroretinographic (ERG) activity [20]. Subretinal injection of recombinant adeno-associated viral vectors (AAV) encoding the *Prph2* transgene results in morphological and electrophysiological correction of the *rds* defect [22–24]. The *rd10* mouse is homozygous for a missense mutation in exon 13 of the β subunit of the rod phosphodiesterase gene [20]. This is the same gene affected in the *rd1* (*Pde6b*^{Rd1}) mouse treated with Epo by Grimm *et al.* [15]. The *rd10* mouse has a slightly slower time course of retinal degeneration than the *rd1* mouse, with histological evidence of retinal degeneration at 3 weeks of age [20]. ERG activity is never normal in the *rd10* mouse and is undetectable at 2 months of age [20]. To test whether *Epo* gene transfer results in a neurotrophic effect in the aforementioned models of retinal degeneration we delivered AAV vectors encoding Epo to the retinas and compared the rescue effects to those produced by intramuscular delivery.

RESULTS AND DISCUSSION

Assessment and Characterization of *Epo* Expression Following AAV-Mediated Gene Transfer in Albino Rats and *rds* and *rd10* Mice

We injected AAV2 vectors encoding either Epo or EGFP intraocularly and intramuscularly (im) into albino Lewis rats and *rds* and *rd10* mice. We injected the animals either intravitreally or subretinally with AAV2-CMV-*Epo* in the right eye and performed control injections in the left eye. AAV2 vectors injected intravitreally (iv) target mainly retinal ganglion cells, while subretinal (sr) injection targets photoreceptor and retinal pigment epithelium [25]. Epo is a secreted protein measurable with a commercial ELISA and physiological response is readily quantified by measuring the hematocrit levels. Intraocular transduction with vectors encoding a secreted protein results in diffusion of the protein through the vitreous in the anterior chamber (AC) fluid, which can then be sampled to assess the *in vivo* protein levels [26]. While AC fluid sampling is challenging in mice, the size of a rat eye allows this procedure to be performed repeatedly and reliably [26]. Therefore, to verify Epo expression and its ability to cross the blood–retina barrier we measured hematocrit and serum Epo levels in treated and control animals. In addition, we measured Epo levels in the AC fluid of rats (Table 1). Following intramuscular, but not intraocular, administration of the vector, circulating Epo and hematocrit were significantly increased. Epo was detected at high levels in the AC fluid of rats injected intraocularly but not in those injected im, suggesting that circulating Epo does not cross efficiently the blood–retina barrier and accumulate at detectable levels in the ocular fluids (Table 1).

Various endogenous and recombinant Epo isoforms exist with different biological activities [27–30]. The isoforms differ mainly in their glycosylation status [31].To evaluate the molecular weight of Epo produced from AAV-transduced retina and muscle, we analyzed AC fluids and serum samples from control and AAV-treated rats by Western blot and compared them to a sample of commercially available recombinant human Epo (Fig. 1). No differences in the molecular weights of the various recombinant Epo proteins were evident. Although iso-

TABLE 1: Hematocrit (Hct) and Epo levels in serum and/or anterior chamber (AC) fluid from (A) albino Lewis rats and (B) *rds* and (C) *rd10* mice after intramuscular, intravitreal, and subretinal (where indicated) administration of the AAV2-CMV-*Epo* vector

	Injection route	Hct (%)	Serum Epo (mU/ml)	AC Epo (mU/ml)
A	Control	44.28 ± 4.05 (n = 14)	0 (n = 14)	0 (n = 14)
	Intramuscular	77.8 ± 4.5 (n = 8)	26.4 ± 2.6 (n = 7)	0 (n = 10)
	Intravitreal	45.37 ± 2.30 (n = 6)	0 (n = 8)	1078 ± 372 (n = 5)
	Subretinal	47 ± 5.1 (n = 22)	0 (n = 23)	1257 ± 902 (n = 8)
B	Control	48.6 ± 0.36 (n = 23)	0 (n = 23)	—
	Intramuscular	75.7 ± 3 (n = 7)	21 ± 3.6 (n = 7)	—
	Intravitreal	56.9 ± 1.6 (n = 24)	—	—
	Subretinal	59.5 ± 3.23 (n = 6)	—	—
C	Control	49.3 ± 3.9 (n = 3)	0 (n = 3)	—
	Intramuscular	79.9 ± 3.6 (n = 6)	804 ± 76.4 (n = 2)	—
	Intravitreal	48.25 ± 2.8 (n = 5)	0 (n = 5)	—

Measurements were performed at P56 for rats and *rds* animals and at P28 for *rd10* mice. Uninjected albino Lewis rats and Balb/c and C57BL/6 mice were used as controls. Values shown are means ± SD. —, not measured.

electrofocusing or lectin-binding techniques are more sensitive than electrophoresis for differentiating various Epo isoforms [32,33], standard Western analysis has also been shown valid for this purpose [34,35]. However, our data do not completely exclude differences in Epo posttranslational modifications that may occur in AAV-transduced muscle versus retina.

Morphological and Functional Photoreceptor Rescue Following AAV-Mediated *Epo* Gene Transfer

We administered AAV vectors to Albino Lewis rats and *rds* and *rd10* mice, light-damaged in the case of the rats, and analyzed the animals as described under Materials and Methods. Histological analyses of retinas of treated and control light-damaged rats and *rds* mice showed a significantly higher number of rows of photoreceptor nuclei in the animals treated with AAV2-CMV-*Epo* intramuscularly compared to intraocularly ($P < 0.003$ for rats, $P < 0.0005$ for *rds;* Figs. 2A and B). We observed no histological rescue in *rd10* mice following AAV2-CMV-*Epo* administration, regardless of the route of delivery (systemic versus intraocular, Fig. 2A). The absence of morphological rescue following intraocular gene transfer is not due to the vector delivery procedure, as demonstrated by similar ONL nuclei counts in both uninjected and control-injected albino Lewis rats and *rds* mice, in agreement with previous reports (Fig. 2A and [19]).

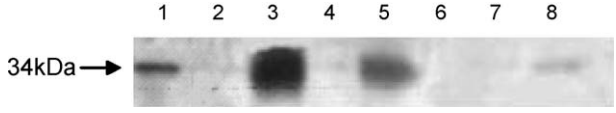

FIG. 1. Western blot analysis with anti-Epo antibodies of AC fluids and sera of albino Lewis rats injected with AAV2-CMV-*Epo*. Lane 1 contains 5 ng of commercial Epo. The remaining lanes contain AC fluids of animals treated intramuscularly (im; lane 2), subretinally (sr; lane 3), and intravitreally (iv; lane 5) with AAV2-CMV-*Epo* and sr (lane 4) and iv (lane 6) with AAV2-CMV-*EGFP*. Sera from animals before and a month after im injection with AAV2-CMV-*Epo* were loaded in lanes 7 and 8, respectively.

To assess the functional rescue of photoreceptors following *Epo* gene transfer, we performed electrophysiological (ERG) analysis on all animals. For albino Lewis rats, retinal responses to flashes of different intensities, before phototoxicity, are reported in Fig. 3A. The peak *b*-wave amplitude under dark-adapted conditions was 0.5 cd/m^2 (mean amplitude 754 μV, SEM 106, N = 7) following *Epo* gene transfer. This suggests that (i) intraocular injections and (ii) elevated Epo intraocular concentrations do not impact on retinal function. After exposure for 48 h to constant illumination (1000 lux), the ERG was nearly abolished in control eyes injected with AAV2-CMV-*EGFP*. As shown in Fig. 3B (traces 3 and 5), ERG recorded at a luminance of 0.5 cd/m^2 were nearly flat in animals receiving AAV2-CMV-*EGFP* into the eye (intravitreal and subretinal injection, control rats). Neither sr nor iv administration of AAV2-CMV-*Epo* was able to prevent this dramatic alteration (Fig. 3B, traces 2 and 4). In contrast, in animals that received im injection of AAV2-CMV-*Epo,* the ERG was partially conserved (Fig. 3B, see trace 1). In Fig. 3C we plotted the transfer curve for *b*-wave mean amplitude as a function of luminance after photolesion. The mean values of *b*-waves recorded in control eyes treated with reporter genes were significantly reduced compared to those measured in animals before phototoxicity: subretinal injections with AAV2-CMV-*EGFP* (mean amplitude at 0.5 cd/m^2 42 μV, SEM 10, N = 7) and intravitreal injections with AAV2-CMV-*EGFP* (mean amplitude at 0.5 cd/m^2 56 μV, SEM 14, N = 5) gave similar results (data not shown). Neither intravitreal (mean amplitude at 0.5 cd/m^2 60 μV, SEM 6, N = 7) nor subretinal injections (mean amplitude 0.5 cd/m^2 100 μV, SEM 14, N = 5) of AAV2-CMV-*Epo* prevented ERG alteration,

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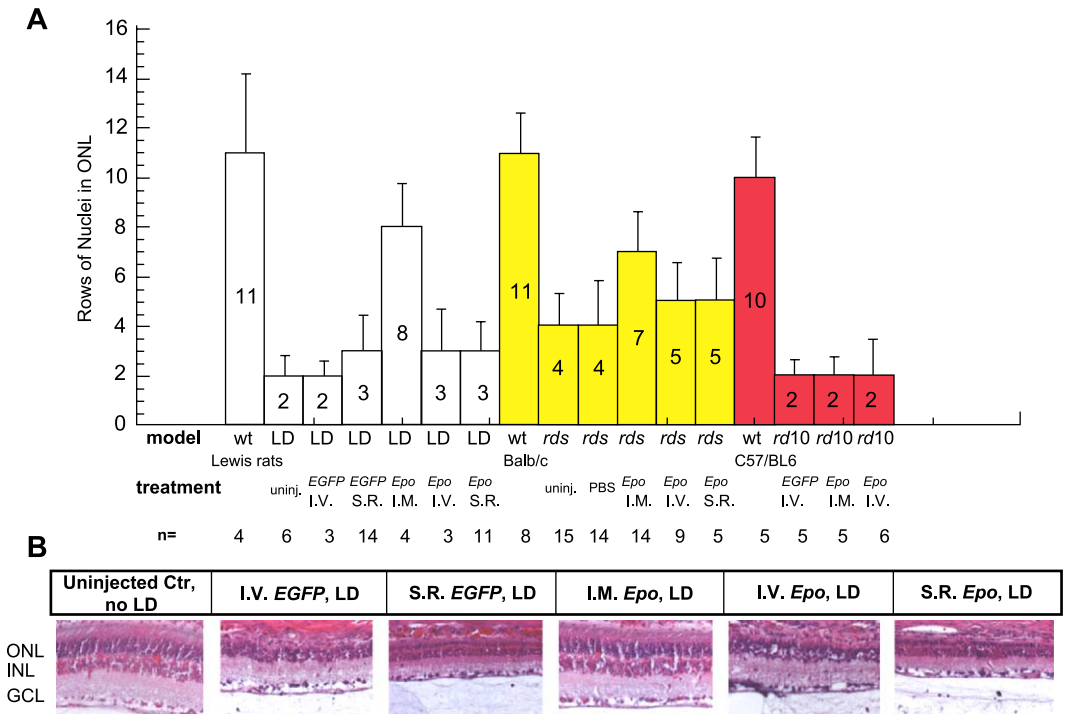

FIG. 2. (A) Retinal morphological rescue after AAV-mediated *Epo* gene transfer. Histological analyses at P65 in light-damaged albino Lewis rats (white bars), at P56 in *rds* mice (yellow bars), and at P28 in *rd10* (red bars) after intramuscular (I.M.), intravitreal (I.V.), and/or subretinal (S.R.) injection of AAV2-CMV-*Epo* are shown. *rds* PBS shows the mean value from animals injected with PBS, either iv or sr. LD, light damage; *n* = number of animals in each group. Values shown are means ± SD ($P < 0.003$ and $P < 0.0005$ for rats and *rds,* respectively). (B) Histological analysis of Lewis rat retinas either untreated or treated with AAV, before and after light damage and stained with hematoxylin and eosin. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

while im injections of the vector were able to prevent the effect of phototoxicity (mean *b*-wave amplitude at 0.5 cd/m^2 190 μV, SEM 22, $N = 6$), at least partially. The functional analysis of intramuscularly treated rats reflected the protection from cell death observed by morphological analysis.

In *rds* and *rd* mice scotopic and photopic ERG did not show any significant differences between PBS- or AAV2-CMV-*EGFP*-injected and uninjected mice (data not shown). Also, no ERG evidence of rescue was present in either *rds* or *rd10* mice injected intraocularly or intramuscularly with AAV2-CMV-*Epo* (data not shown). The absence of a significant functional rescue in *rds* animals despite higher numbers of photoreceptor nuclei in the vector- than in the PBS-treated controls is not surprising considering the functional role of *peripherin/rds*. It is possible that either the morphological rescue was not sufficient to preserve the function or that the preserved photoreceptors do not function properly, as observed with other neurotrophic molecules [36,37]. Therefore, functional photoreceptor rescue at the ERG level is evident only in the light-damaged rats following intramuscular delivery of vector and systemic production of Epo.

Our data show that *Epo* gene transfer results in preservation of photoreceptors in two different models of retinal degeneration (one induced and one genetic model) only when Epo is produced by muscle and secreted into the circulation. This effect is independent of the local concentration of the hormone, which is high in the AC fluid of animals receiving intraocular administration of the vector and undetectable in those that received the vector intramuscularly. In previous studies, photoreceptor protection from light damage was observed following systemic Epo administration or in transgenic animals overexpressing the hormone in the eye as well as in other tissues [13,15,38]. Taken together, these data support the hypothesis that both in the presence of light damage and in *Prph2* gene deficiency, Epo's neurotrophic effect might not be exerted directly on photoreceptors: for instance, it could be due to the increase in hematocrit levels. Nevertheless, a more detailed analysis (other than what was performed here) of the potential secretion of Epo isoforms from AAV-transduced retina and muscle would be required to rule out the possibility that a specific isoform secreted from transduced muscle but not in the retina can act directly on the Epo-R expressed in the eye [9]. In a previous study, no rescue was observed in an inherited light-independent transgenic *rd* mouse overexpressing Epo [15]. Our data support this finding. In fact, the absence of detectable

morphological or functional photoreceptor rescue by *Epo* gene transfer in *rd* mice suggests that in this model the mechanism leading to photoreceptor cell death is different than in *rds* or light-damaged albino animals, as shown in previous reports [39–42].

In conclusion, we show that *Epo* gene transfer protects against retinal degeneration induced by light or by a specific genetic cause in rodents both morphologically and functionally. We show that this effect is restricted to intramuscular *Epo* gene transfer, resulting in secretion of circulating Epo, and is independent of the intraocular concentration of the hormone. Further studies will be required to elucidate whether the Epo neuroprotective effect observed in retinal degeneration occurs via a direct or an indirect mechanism on photoreceptors.

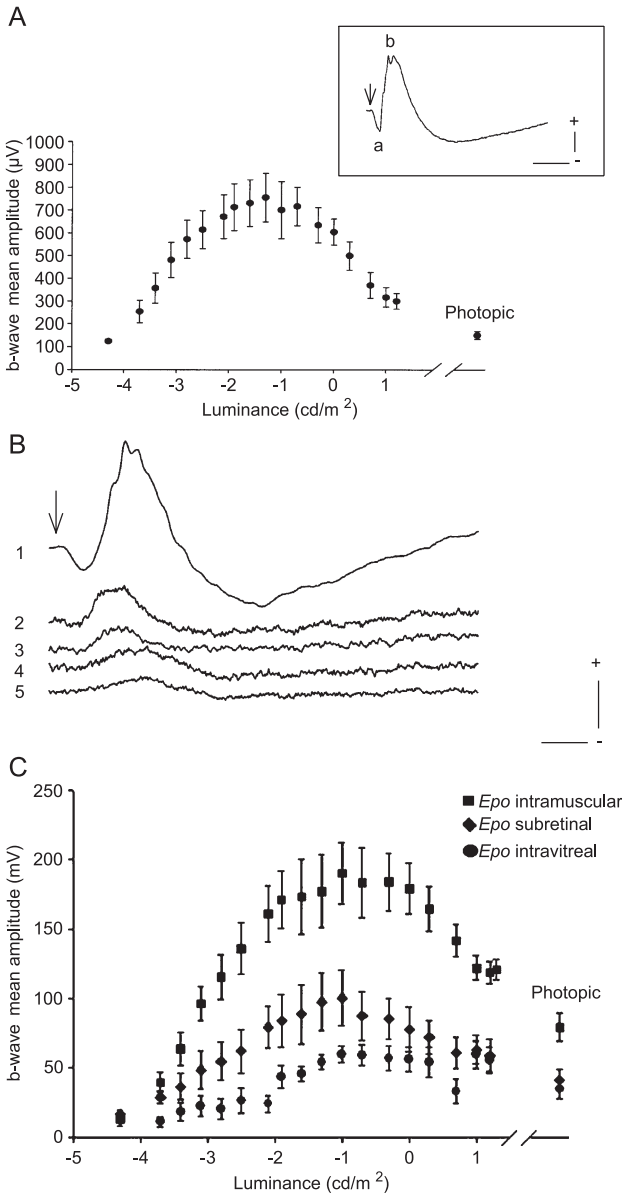

FIG. 3. ERG measurements in albino Lewis rats. (A) Transfer curve of mean *b*-wave amplitude as a function of luminance before photolesion. ERG were recorded for increasing luminances under scotopic conditions. Photopic ERG were those recorded under light conditions, with a luminance of flash set at 20 cd/m^2 and in the presence of a background whose luminance was 25 cd/m^2. The luminance is plotted on a log scale. In the inset, we report a typical ERG recorded at a luminance of 0.5 cd/m^2 under scotopic conditions: *a*- and *b*-wave are indicated; horizontal calibration bar, 100 ms, and vertical calibration bar, 200 μV. The arrow indicates the time when the flash was delivered. (B) ERG in animals treated with AAV2-CMV-*Epo* or AAV2-CMV-*EGFP* (control) and recorded after photolesion. Trace 1 shows a representative ERG recorded in an animal injected intramuscularly with AAV2-CMV-*Epo*. Traces 2 and 3 show ERG recorded from eyes treated with subretinal injections of AAV2-CMV-*Epo* and AAV2-CMV-*EGFP,* respectively. Traces 4 and 5 show ERG recorded from eyes treated with intravitreal injections of AAV2-CMV-*Epo* and AAV2-CMV-*EGFP*, respectively. ERG were recorded at a luminance of 0.5 cd/m^2 under dark-adapted conditions. Horizontal calibration bar, 100 ms, and vertical bar, 100 μV. (C) Transfer curve of mean *b*-wave amplitude as a function of luminance in animals treated with AAV2-CMV-*Epo* and recorded after photolesion. *Epo* intramuscular, eyes of animals treated with intramuscular injections of AAV2-CMV-*Epo (N* = 6). *Epo* intravitreal, right eyes treated with intravitreal injections of AAV2-CMV-*Epo (N* = 7). *Epo* subretinal, right eyes treated with subretinal injections of AAV2-CMV-*Epo (N* = 5). Other conventions as in (A).

MATERIALS AND METHODS

Vector Production

For AAV2 vector production, pAAV2-CMV-rh*Epo*3 and pAAV2-CMV-*eGFP*3 [43] were used. pAAV2-CMV-rh*Epo*3 contains the rhesus cDNA driven by the CMV promoter. Recombinant AAV2 viruses were produced by triple transfection of 293 cells followed by affinity column purification of the vectors as described elsewhere [43]. For each viral preparation, physical titers (genome copies (GC)/ml) were determined by PCR quantification using TaqMan (Perkin–Elmer, Foster City, CA,USA) [44]. Vectors were produced by the TIGEM AAV vector core. The AAV vectors were produced by the TIGEM AAV Vector Core.

Vector Administration to the Animals

Light damage. All procedures were performed in accordance with institutional guidelines for animal research. Four-week-old male albino Lewis rats (Charles River Italia, Lecco, Italy) were used. Before vector administration, rats were anesthetized with an intraperitoneal injection of avertin at 2 ml/100 g body weight (1.25% (w/v) 2,2,2-tribromoethanol and 2.5% (v/v) 2-methyl-2-butanol; Aldrich, St. Louis, MO, USA) [45]. Albino Lewis rats were injected in the right gastrocnemius (im, n = 10) with 100 μl of the AAV2-CMV-*Epo* vector (5.6 × 10^{12} GC) or iv (n = 8) or sr (n = 23) with 2 μl of AAV2-CMV-*Epo* (1 × 10^{11} GC) in the right eye and the same dose of AAV2-CMV-*EGFP* in the left eye, as control. Subretinal and intravitreal vector administrations were performed as described [46].

rds and rd10 mice. Three- to five-day-old *rds*/*rds* and *rd10* mice were treated with AAV2-CMV-*Epo*. Early postnatal administration was chosen to provide the optimal therapeutic effect with respect to disease progression. Before vector administration, pups were anesthetized by hypothermia. Mouse pups were injected in the right gastrocnemius (im, n = 14 *rds*, n = 6 *rd10*) with 10 μl of the AAV2-CMV-*Epo* vector (5.6 × 10^{11} GC) or iv (n = 9 *rds*, n = 6 *rd10*) or sr (n = 5 *rds*) with 2 μl of AAV2-CMV-*Epo* (1 × 10^{11}GC) in the right eye. For the left eyes, PBS and AAV2-CMV-*EGFP* were injected as controls in *rds* and *rd10* mice, respectively. All analyses were performed at P56 for *rds* mice and P28 for *rd10* mice.

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Light Exposure of Albino Rats

The protocol derives from that described by LaVail *et al.* [19], with modification of the duration of light exposure. Briefly, animals were reared on a 12-h light/dark cycle until day 56. At that time animals were housed separately in clear Plexiglas cages and exposed to continuous light produced by four 36-W white fluorescent bulbs (Osram Sylvania, Munich, Germany). Light sources were suspended 30 cm above (two bulbs) and under (two bulbs) cages to produce an illumination of 1000 lux. Animals had free access to food and water. After continuous illumination for 48 h animals were housed in a room with a 12-h light/dark cycle for 1 week before the ERG recording.

Electroretinogram Measurements

ERG measurements were performed according to previously published methods [47–49]. ERG analysis was performed at P56 and at P28 in *rds* and *rd* mice, respectively. In albino Lewis rats, ERG were recorded 1 week (P65) after exposure to constant light from eyes of rats treated with AAV2-CMV-*Epo* and AAV2-CMV-*EGFP*. In a separate group of control rats ERG were recorded before and after exposure to constant light. Briefly, animals were dark adapted (overnight for *rds,* 180 min for *rd10* and albino Lewis rats) and then anesthetized and loosely mounted in a stereotaxic apparatus with the body temperature maintained at 37.5°C and the heart rate monitored. ERG were evoked by 10-ms flashes of light generated through a Ganzfeld stimulator (Lace, Pisa, Italy). For the Lewis rats, and *rd10* mice, the electrophysiological signals were recorded through gold plate electrodes inserted under the lower eyelids in contact with the cornea previously anesthetized with ossibuprocaine (Novesine, Novartis Pharma, Switzerland). Electrodes in each eye were referred to a needle electrode inserted subcutaneously at the level of the corresponding frontal region. In the case of the *rds* mice, the mice bit down onto the reference electrode. The different electrodes were connected to a two-channel amplifier. To minimize the noise, 10 different responses evoked by light were averaged for each luminance step. For recording under scotopic conditions we adopted the following protocol. After dark adaptation, animals were anesthetized and mounted in a stereotaxic apparatus under dim red light. ERG were recorded in response to flashes of different light intensities ranging from 10^{-4} to 20 cd m^{-2} s^{-1}. Amplitudes of *a*- and *b*-waves were plotted as functions of increasing light intensities. After completion of responses obtained in dark-adapted conditions the recording session continued with the aim to dissect the cone pathway mediating the light response. To this aim the ERG in response to light of 20 cd m^{-2} was recorded in the presence of a continuous background light (background light set at 25 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.

Epo and Hematocrit Measurements, and Western Blot Analysis

Epo levels were measured in mice and rats using the Quantikine Human Epo Immunoassay (R&D Systems, Minneapolis, MN, USA). Hematocrit levels were measured by microcapillary centrifugation.

In light-damaged rats, hematocrit and Epo levels in the serum were measured before and a month after (at P56) vector administration. In addition, animals' AC fluid was tapped at P56 [26]. In *rds* and *rd10* animals, hematocrit and Epo levels were measured at P56 and at P28, respectively. AC fluid was not tapped due to technical limitations in the small mouse eye. In addition to immunoassay measurements, Epo levels in rat AC fluid were assessed by Western analysis a month after intraocular or intramuscular injections and in serum samples before and a month after injections. AC fluid and serum samples were denatured by heating to 98°C for 3 min and electrophoresed on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis with 4% stacking gel in 1× Tris–glycine buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) in a miniprotean cell (Bio-Rad, Munich, Germany) at 130 mA for 2 h. The separated proteins were electrotransferred to a nitrocellulose filter (Schleicher and Schuell GmbH, Dassel, Germany) using 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 160 V in a cold room for 1.3 h. The filters were incubated at room temperature for 1.3 h in blocking buffer containing 5% dried nonfat milk and 0.02% sodium azide in PBS and then probed with anti-Epo (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h. Anti-goat HRP (Amersham Pharmacia, Milan, Italy) and Pico chemiluminescence substrate (Pierce, Rockford, IL, USA) were used to detect the protein on the filter. Prestained molecular weight markers (Bio-Rad) were used to determine protein size. A recombinant human Epo (Santa Cruz Biotechnology) was used as positive control for the Western blot.

Histological Analysis

After ERG measurements, rats and mice were sacrificed and their eyecups were harvested and fixed by immersion in 4% paraformaldehyde. Then, rat and *rd10* mouse eyecups were embedded in paraffin and sectioned, while *rds* or wild-type (Balb/c, $n = 8$) eyecups were infiltrated with 30% sucrose for cryopreservation and embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA). For each eye 150 to 200 serial sections (11 μm thick) were cut along the horizontal meridian; 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 hematoxylin and eosin (Richard-Allen Scientific, Kalamazoo, MI, USA; Aldrich, Milan, Italy) and retinal histology was analyzed by light microscopy. To quantify photoreceptor rescue, the number of nuclei in the ONL of each eye was counted. For rats and *rd10* eyes a minimum of 3 sections/slide, representative of the entire eyecup, was 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. For *rds* eyes, retinal areas adjacent to the optic nerve were used. The counts from each group were then averaged and standard deviations were calculated. For *rds* eyes injected iv or sr with PBS there was no difference in ONL thickness between them; therefore all the data from these groups were compiled into one category. *P* values were calculated using Student's *t* test. Untreated albino Lewis rats, Balb/c mice, and C57BL6 mice were used as wild-type controls, respectively, for rats, *rds* mice, and *rd10* mice.

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