



# Intravitreal injection of PLGA microspheres encapsulating GDNF promotes the survival of photoreceptors in the *rd1/rd1* mouse

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**Purpose:** To evaluate the potential delay of the retinal degeneration in *rd1/rd1* mice using recombinant human glial cell line-derived neurotrophic factor (rhGDNF) encapsulated in poly(D,L-lactide-co-glycolide) (PLGA) microspheres.

**Methods:** rhGDNF-loaded PLGA microspheres were prepared using a water in oil in water (w/o/w) emulsion solvent extraction-evaporation process. In vitro, the rhGDNF release profile was assessed using radiolabeled factor. In vivo, rhGDNF microspheres, blank microspheres, or microspheres loaded with inactivated rhGDNF were injected into the vitreous of *rd1/rd1* mice at postnatal day 11 (PN11). The extent of retinal degeneration was examined at PN28 using rhodopsin immunohistochemistry on whole flat-mount retinas, outer nuclear layer (ONL) cell counting on histology sections, and electroretinogram tracings. Immunohistochemical reactions for glial fibrillary acidic protein (GFAP), F4/80, and rhodopsin were performed on cryosections.

**Results:** Significant delay of rod photoreceptors degeneration was observed in mice receiving the rhGDNF-loaded microspheres compared to either untreated mice or to mice receiving blank or inactivated rhGDNF microspheres. The degeneration delay in the eyes receiving the rhGDNF microspheres was illustrated by the increased rhodopsin positive signals, the preservation of significantly higher number of cell nuclei within the ONL, and significant b-wave increase. A reduction of the subretinal glial proliferation was also observed in these treated eyes. No significant intraocular inflammatory reaction was observed after the intravitreal injection of the various microspheres.

**Conclusions:** A single intravitreal injection of rhGDNF-loaded microspheres slows the retinal degeneration processes in *rd1/rd1* mice. The use of injectable, biodegradable polymeric systems in the vitreous enables the efficient delivery of therapeutic proteins for the treatment of retinal diseases.

The use of exogenous growth factors to delay photoreceptors death is gaining momentum as a neuroprotection strategy. LaVail and colleagues explored the potential of direct intravitreal injection of fibroblast growth factor 2 (FGF2), ciliary derived neurotrophic factor (CNTF), and brain derived neurotrophic factor (BDNF) to slow retinal degeneration in animal models [1-3]. Insights gained into the mechanisms of growth factor-induced neuroprotection [4-6] suggest that some neurotrophic factors may be used therapeutically to induce anatomic and functional photoreceptor rescue. Glial cell line-derived neurotrophic factor (GDNF) belongs to the transforming growth factor- $\beta$  super family. Its activity is mediated by the interaction with a specific receptor and a tyrosine kinase receptor, Ret [7]. GDNF and its receptor complex are produced by the retina and by the optic nerve head, suggesting possible endogenous neurotrophic physiological effects [8,9]. In vitro and in vivo studies have shown the potential of GDNF for rescuing retinal photoreceptors and ganglion cells functions [10-14].

Besides the selection of appropriate growth factors for different types of retinal diseases [15,16], another crucial aspect of neuroprotection as a therapy is the need for efficient

intraocular delivery of the therapeutic molecules. Direct injection of the protein in the subretinal space must be repeated in order to achieve photoreceptor survival in the *rd1/rd1* mouse model [12]. On the other hand, a single subretinal implantation of transfected cells or injection of recombinant adeno-associated virus allows for a sustained release of GDNF and higher protective effects [13,17]. Therefore, viral and nonviral delivery systems for the intraocular sustained release factors have been explored.

For the prospect of clinical application, sustained neurotrophic protein release systems that avoid the use of viral vectors and subretinal injections may have advantages. Adequate strategies for the encapsulation of GDNF in biodegradable PLGA microspheres has enabled the release of therapeutic levels for more than two months in vitro [18]. In vivo, the striatal implantation of these GDNF microspheres has promoted recovery of motor function in a model of Parkinson's disease [19].

The aim of this study was to evaluate the potential of a single intravitreal injection of GDNF-loaded PLGA microspheres to delay the retinal degeneration processes occurring in the *rd1/rd1* mouse.

## METHODS

**Microsphere formulation:** Microspheres were prepared using a water/organic phase/water (w/o/w) emulsion solvent extrac-

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tion-evaporation process as previously reported [20]. Briefly 75  $\mu\text{g}$  of recombinant human GDNF (rhGDNF, R&D Systems, Abingdon, UK) were suspended in an internal aqueous phase composed of 60  $\mu\text{l}$  of citrate buffer (pH 5 with 5% human serum albumin), and 90  $\mu\text{l}$  of polyethylene glycol 400 (Cooper, Melun, France). The mixture was emulsified in 2 ml of organic phase (3:1 methylene chloride: acetone) containing 50 mg of polymer (PLGA 37.5/25, Phusis, Saint Ismier, France). This water/organic phase emulsion was subsequently poured into an external aqueous solution of poly(vinyl alcohol), 30 ml, 5% w/v, Rhodoviol® 4/125 (Merck Eurolab, Paris, France) containing NaCl (10% w/v). The resulting w/o/w emulsion was added to deionized water (400 ml) containing NaCl (10% w/v) to extract the organic solvent. Finally, the formed microparticles were filtered and freeze-dried to obtain a free flowing powder. Blank microspheres or microspheres loaded with inactivated rhGDNF were prepared in the same conditions.

**Evaluation of rhGDNF release in vitro:** rhGDNF radiolabeling ( $^{125}\text{I}$ -labeled rhGDNF) was used to estimate an in vitro drug release profile. rhGDNF was labeled by a chemical method using Bolton Hunter reagent (Perkin Elmer, Paris, France). rhGDNF (10  $\mu\text{g}$ ) in 120  $\mu\text{l}$  of phosphate-buffered saline (pH 7.4) was placed in 250  $\mu\text{Ci}$  (9.25 MBq) of  $^{125}\text{I}$ -labeled Bolton-Hunter reagent. The reaction mixture was incubated at 4 °C under gently stirring for 1 h. A gel filtration system (PD10 column, Amersham Biosciences, Orsay, France), which had been equilibrated with phosphate buffer saline (PBS) containing 0.5% gelatine, was used to separate  $^{125}\text{I}$ -rhGDNF from the hydrolyzed products. The  $^{125}\text{I}$ -rhGDNF was eluted with PBS, and peak fractions were pooled. The radiolabeled protein was then purified in Nanosep® 10K centrifugal devices (Merck Eurolab, Paris, France).

**Animals:** C3H/HeN mice homozygous for the nonsense mutation (amino acid position 347) in the  $\beta$ -PDE gene (Janvier, Le Genest, France) were used. Mice were maintained in clear plastic cages and subjected to a standard 12 h:12 h light:dark cycle. Experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in ophthalmologic and vision research and the institutional guidelines regarding animal experimentation in Ophthalmic and Vision Research. The *rd1/rd1* mouse is a rapid retinal degeneration model where the photoreceptors degeneration begins early at postnatal day 8 (PN8) and is complete by day 28 (PN28) [21]. Therefore, the effects of rhGDNF-loaded microspheres were evaluated at PN28.

**Intravitreal injection:** Intravitreal injections were performed in the vitreous of PN11 mouse eyes using microfine (300  $\mu\text{l}$ ) syringes with 30 ga needles under topical anesthesia (tetracaine 1% drops, Novartis Ophthalmics SA, Rueil Malmaison, France). Microspheres were suspended in diluted (1/10) dispersing medium composed of distilled water, polysorbate 80 (0.5%), mannitol (4%), and carboxymethylcellulose sodium (1%). At PN11 the eyelids are fully differentiated and almost open. Thus, only a gentle manual maneuver is required for the separation of the eyelids before injection. One  $\mu\text{l}$  of

the suspension corresponding to 0.3 mg of microspheres was injected into the vitreous. Fourteen eyes received rhGDNF-loaded microspheres (corresponding to 0.38  $\mu\text{g}$  of rhGDNF), 18 eyes received unloaded (blank) microspheres and 12 eyes received microspheres encapsulating heat-inactivated rhGDNF. To limit loss of the injected solution and allow the intraocular pressure to equilibrate (as observed by the return of normal iris perfusion), the microfine needle was left in place for 10 s before withdrawal.

At the end of the experiment (PN28), mice were sacrificed by a lethal dose of pentobarbital (6 g/100 ml; Ceva Santé Animale, Libourne, France) injected intraperitoneally, and the eyes were enucleated and analyzed.

**Outer nuclear layer cell count:** At PN28, an average cell count of the outer nuclear layer (ONL) for each examined eye was obtained from five histology sections through the optic nerve stained with hematoxylin-eosin. Six independent eyes were evaluated for the untreated (control) mice (30 slides). Eleven independent eyes were evaluated for the group of mice receiving blank microspheres (55 slides). Seven independent eyes were evaluated for the group of mice receiving rhGDNF

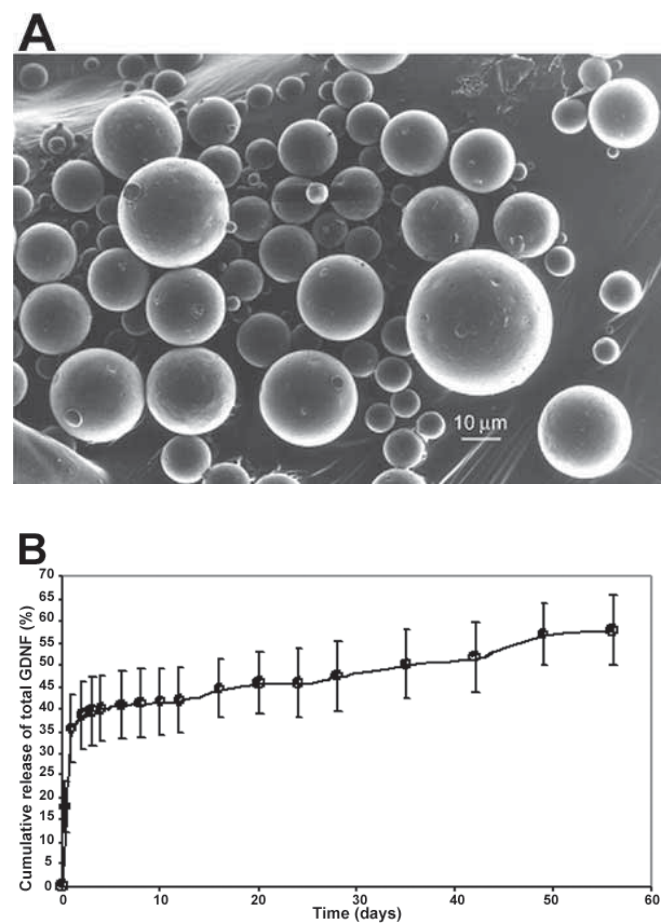


Figure 1. Characteristics of PLGA microspheres. **A:** Picture of rhGDNF-loaded microspheres analyzed by scanning electron microscopy. Scale bar in **A** represents 10  $\mu\text{m}$ . **B:** In vitro cumulative release of  $^{125}\text{I}$ -rhGDNF from PLGA microspheres (n=2); error bars represent the standard deviation.

microspheres (35 slides) and 6 independent eyes were evaluated for the group of mice receiving the inactivated rhGDNF microspheres (30 slides). For each section, the number of nuclei in the ONL was obtained in 400  $\mu\text{m}$  retinal length (400  $\mu\text{m}$  from the temporal and 400  $\mu\text{m}$  from the nasal side) within the same region (400  $\mu\text{m}$  away from the edge of the optic nerve). These counts were performed by an examiner unaware of the treatment. For group comparisons, the average count of all sections obtained from all independent eyes of the same treatment group were used.

*Rhodopsin immunohistochemistry on flat-mount retinas:*

Rhodopsin immunohistochemistry was performed on whole flat-mount retinas from mice injected with rhGDNF microspheres, microspheres with inactivated rhGDNF, blank microspheres, or untreated mice (6 retinas for each condition) as previously described [12]. Briefly, at PN28, ocular globes were fixed in 4% paraformaldehyde (Merck Eurolab, Paris, France) for 1 h. Retinas were isolated, placed in PBS in 1.5 ml microcentrifuge tubes, permeabilized in PBS, 0.1% Triton X-100 (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 5 min, and incubated in blocking buffer (PBS containing 0.1% bovine serum albumin, 0.1% Tween 20 and 0.1% sodium azide [Sigma-Aldrich, Saint-Quentin Fallavier, France]) for 15 min.

Retinas were incubated in rod photoreceptor-specific monoclonal mouse antibody rho-4D2 (1/100 dilution in blocking buffer; kindly provided by Dr. Robert Molday, University of British Columbia, Vancouver BC, Canada) [22]. As negative controls, normal mouse serum (Nordic Immunological Laboratories, Tebu-bio, Le Perray en Yvelines, France) or mouse monoclonal antibody Leu-M5 directed against macrophages and monocytes (BD Biosciences, Pont-de-Claix, France) were used instead of rho-4D2 antibody (1/100 dilution in blocking buffer, 1 h). Then, the retinas were washed 3 times for 5 min in blocking buffer and incubated with a secondary goat anti-IgG mouse antibody conjugated to Alexa Fluor 488 (1/250 dilution in blocking buffer; Molecular Probes, Leiden, Netherlands). Retinas were then washed and mounted in PBS-glycerol (1/1) and examined by fluorescence microscopy with a 2.5 objective and photographed using a digital SPOT camera (Optilas, Every, France). For each retina, three pictures (exposure time of 5 s) were taken to cover the whole retinal surface. Photographs of flat-mounts were merged in Photoshop 6.0 to reconstruct the whole retina.

Intensity of rhodopsin immunoreactivity was quantified by using the luminosity feature of Photoshop for each individual row picture. Tissue and background regions were manu-

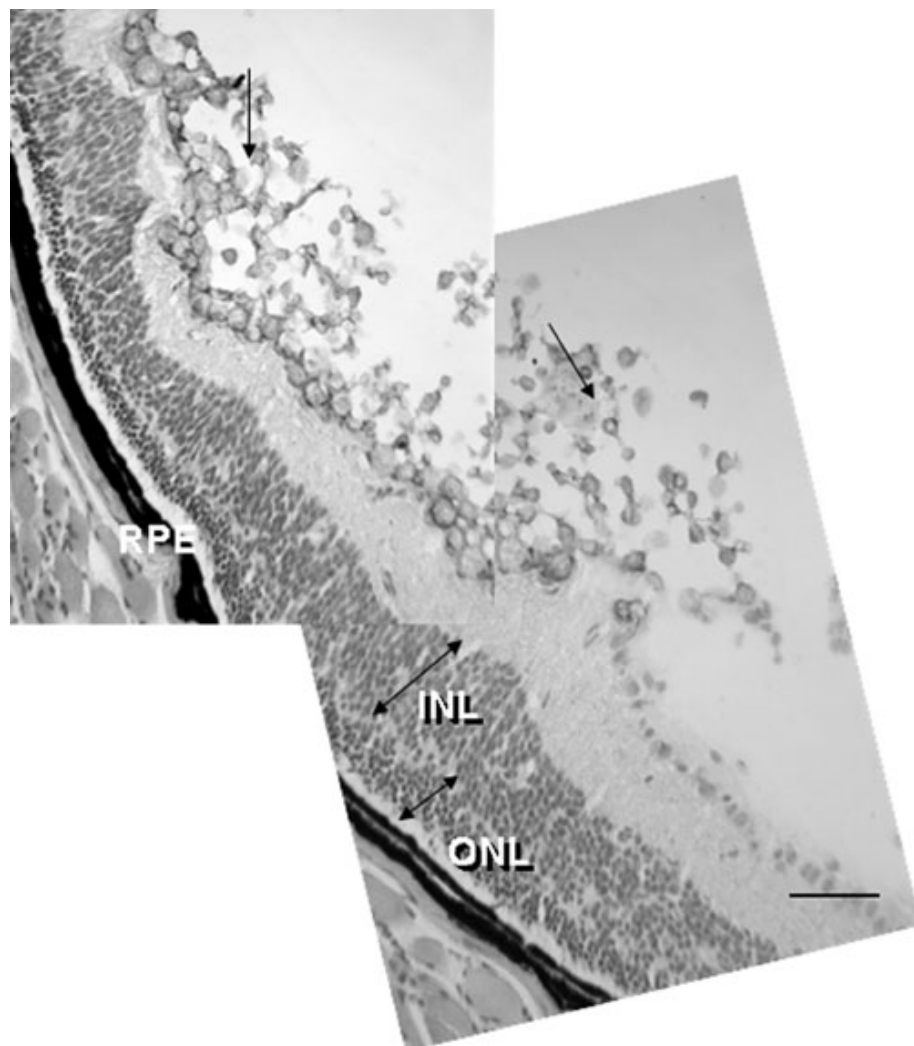


Figure 2. Aggregation of microspheres at the retinal surface as occurred in two of the injected eyes. Histology picture of retina section stained with hematoxylin-eosin shows that microspheres (arrows) are still present at 28 days and form aggregates at the retinal surface. Note that numerous photoreceptors have survived (double arrow showing ONL) at the site of microspheres accumulation. The outer nuclear layer (ONL), inner nuclear layer (INL), and retinal pigment epithelium (RPE) are identified. Scale bar represents 100  $\mu\text{m}$ .

ally selected. Any residual pigmented epithelium was excluded. Mean pixel brightness was determined for each region by using the "Histogram" imaging feature. To control for differences in background levels among images, the mean brightness level per pixel of the tissue region was divided by the background region from each flat-mount image.

**Rhodopsin, GFAP, and F4/80 immunohistochemistry:** Eyes of PN28 uninjected mice, mice injected with blank microspheres, rhGDNF microspheres, and microspheres with inactivated rhGDNF (6 eyes for each condition) were enucleated, snap frozen in Tissue-Tek OCT-compound (Bayer Diagnostics, Puteaux, France), and sectioned (10  $\mu$ m).

Sections were fixed in 4% paraformaldehyde (Merck Eurolab, Paris, France) for 5 min at room temperature and washed in PBS. For rhodopsin immunohistochemistry, sections were incubated 1 h in mouse rho-4D2 antibody (1/100 dilution in PBS). Slides were washed three times in PBS and incubated with mouse anti-IgG conjugated to Alexa Fluor 488 (1/250 dilution in PBS; Molecular Probes, Leiden, Netherlands).

The slides used for glial fibrillary acidic protein (GFAP) detection were incubated 1 h with monoclonal anti-GFAP antibody (Serotec, Varilhes, France) diluted 1/100 in PBS. After

washing, sections were incubated in a solution of 1/100 of secondary goat anti-rabbit antibody conjugated to Texas red (Molecular Probes, Interchim, Asnières, France) for 1 h.

For the detection of infiltrating inflammatory cells (mouse infiltrating macrophages and activated microglial cells), sections were incubated with rat F4/80 antibodies against mouse antigens diluted 1/100 in PBS (Pharmingen, B&D Biosciences, Le Pont de Claye, France). In these cases and after washing, the sections were incubated in a solution of rabbit anti-rat antibody conjugated to FITC.

Nuclei were stained 5 min with 4',6-diamino-2-phenylindole (DAPI) solution diluted 1/3000 (Sigma-Aldrich, Saint-Quentin Fallavier, France). Slides were washed, mounted in glycerol/PBS (1/1), examined under a fluorescence microscope Aristoplan (Leica, Rueil-Malmaison, France), and photographed with a digital SPOT camera (Optilas, Every, France). Experiments using nonimmune serum or omitting the primary antibody served as controls. Each experiment was performed on a minimum of three independent samples.

**Electroretinogram recordings:** At PN23, after 24 h dark adaptation, mice treated with rhGDNF microspheres (10 eyes), blank microsphere (n=14), or untreated mice (n=6) were anesthetized under dim red illumination with an intramuscular in-

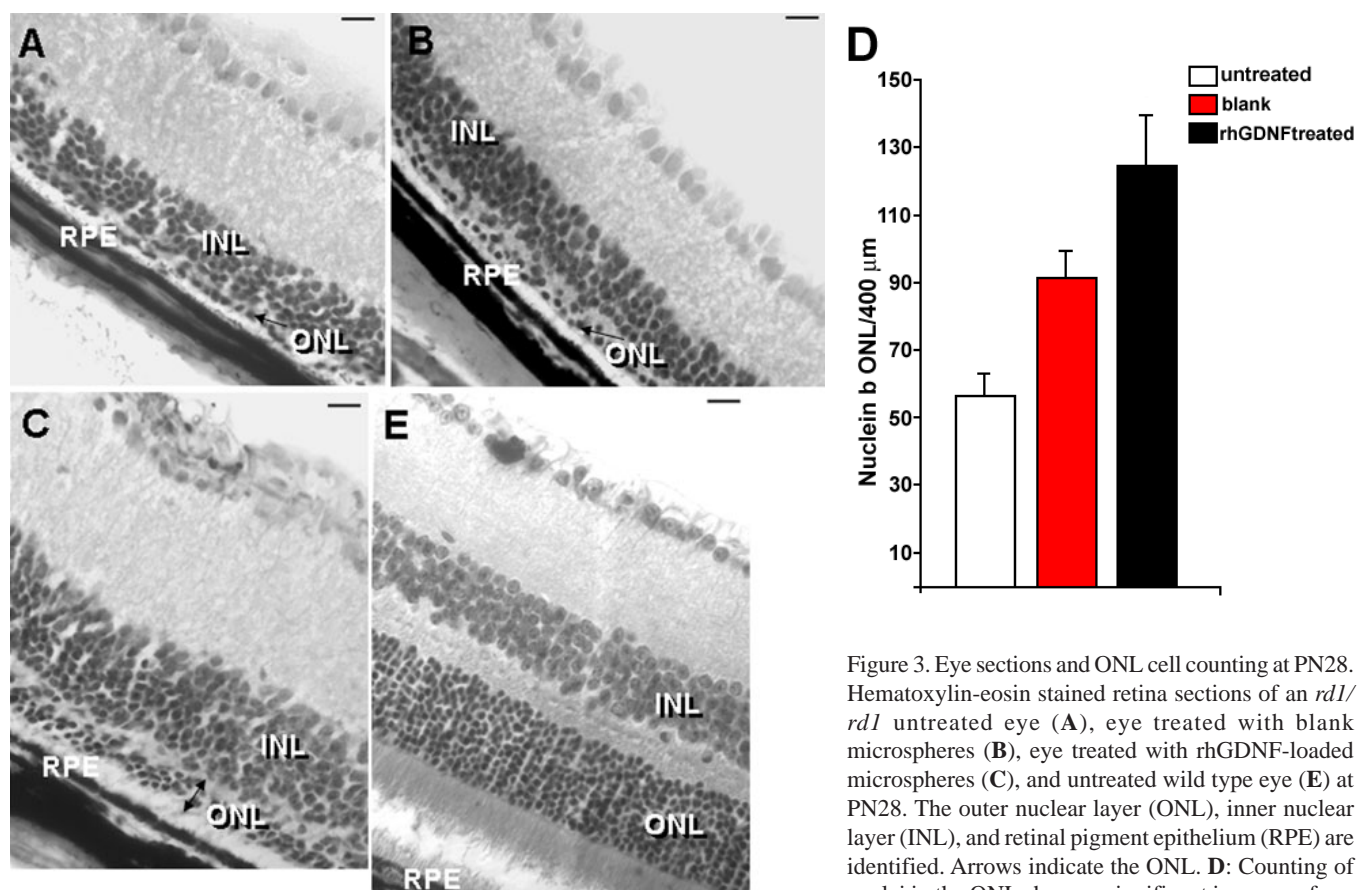


Figure 3. Eye sections and ONL cell counting at PN28. Hematoxylin-eosin stained retina sections of an *rd1/rd1* untreated eye (A), eye treated with blank microspheres (B), eye treated with rhGDNF-loaded microspheres (C), and untreated wild type eye (E) at PN28. The outer nuclear layer (ONL), inner nuclear layer (INL), and retinal pigment epithelium (RPE) are identified. Arrows indicate the ONL. D: Counting of nuclei in the ONL shows a significant increase of nuclei in eyes treated with rhGDNF-loaded microspheres compared to untreated eyes ( $p=0.001$ ) or eyes treated with blank microspheres ( $p=0.002$ ). Scale bars represent 20  $\mu$ m.

jection of ketamine 500 (100 mg/kg, Vibrac Laboratories, Carros, France) and xylazine (10 mg/kg, Rompun 2%, Bayer Diagnostics, Puteaux, France). Pupils were dilated with tropicamide application on the cornea (2 mg/0.4 ml, Novartis Ophthalmics SA, Rueil Malmaison, France). The cornea was locally anesthetized with topical application of tetracaine 1%. Upper and lower lids were retracted to proptose and maintain the eye open. The measuring electrode made with a gold wire and a plastic lens was placed on the cornea with a drop of methylcellulose whereas the stainless steel reference electrode was inserted subcutaneously on the head of the animal. Light stimulations were delivered every 20 s with a xenon lamp at 10 cds/m<sup>2</sup> in a Gantzfeld from Multiliner Vision (Toennies/Jaeger, Hoechberg, Germany). Fifty consecutive recordings were measured, high- and low-pass filtered at 0.1 Hz and 300 Hz and finally averaged. For the photopic ERG measurement, wild type mice were adapted for 10 min to a background light of 25 cd/m<sup>2</sup>. In wild type adult C57/Bl6 mice, photopic ERG responses were recorded to the same light intensity (10 cds/m<sup>2</sup>) after 10 min light adaptation to the background light (25 cd/m<sup>2</sup>). The b-wave amplitudes were measured from the minimum peak to the maximum peak following the light stimulation.

**Statistical analysis:** Results are expressed as means±standard error of the mean (SEM) and compared us-

ing the nonparametric Mann-Whitney test. An  $\alpha$  level of 0.05 was chosen for statistical significance.

## RESULTS

The use of microspheres loaded with inactivated rhGDNF yielded the same results as the blank microspheres. Therefore, for simplification, only the results of the blank microspheres are represented and compared to the other groups for all parameters.

**In vitro rhGDNF kinetics of release:** Microspheres containing rhGDNF were smooth and spherical with a mean±SD particle size of 27±10  $\mu$ m (Figure 1A). The encapsulation efficiency was 92.0±5.6% (n=4) as determined by radioactivity. The release profile was analyzed in cumulative conditions by taking into account the total rhGDNF radioactivity (Figure 1B). A burst (about 35% of the rhGDNF released) was observed within the first 24 h. Then, a low and continuous release occurred (10 ng/day) reaching about 60% of the total radioactivity at 56 days. In correlation with these findings, after 56 days, radioactivity counts indicated that 40% of the <sup>125</sup>I-rhGDNF remained within the microspheres. Previous studies have shown that this type of microsphere released bioactive rhGDNF [20].

**Safety of PLGA microspheres:** Hematoxylin-eosin staining of cryosections showed that, at PN28, few injected

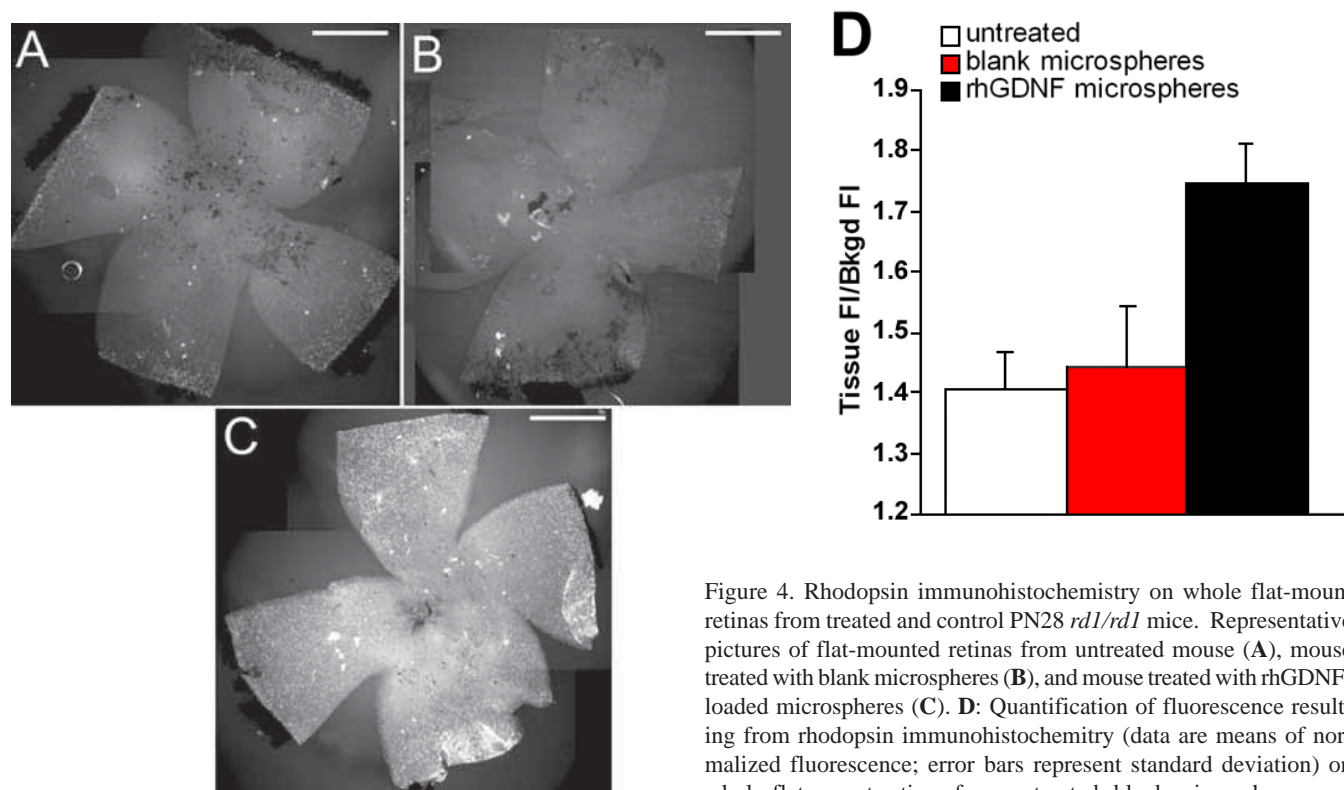


Figure 4. Rhodopsin immunohistochemistry on whole flat-mount retinas from treated and control PN28 *rd1/rd1* mice. Representative pictures of flat-mounted retinas from untreated mouse (A), mouse treated with blank microspheres (B), and mouse treated with rhGDNF-loaded microspheres (C). D: Quantification of fluorescence resulting from rhodopsin immunohistochemistry (data are means of normalized fluorescence; error bars represent standard deviation) on whole flat-mount retinas from untreated, blank microspheres, and rhGDNF-loaded microspheres treated groups. A significant increase of immunofluorescence is observed in eyes treated with rhGDNF-loaded microspheres when compared to the eyes treated with blank microspheres ( $p=0.04$ ) or to the untreated eyes ( $p=0.003$ ). Scale bars represent 1 mm.

microspheres were still present within the vitreous cavity of treated eyes, dispersed into the vitreous. The injection of microspheres into the vitreous cavity was well tolerated and no vitreous tractions or retinal folds were seen. However, in two of the eyes, microspheres formed aggregates adherent to the retinal surface, localized vitreous tractions with formation of retinal folds but no retinal detachment (Figure 2). Interestingly, in these eyes, the “trophic” effect was most evident in front of these aggregates.

*Number of photoreceptor nuclei at PN28:* At PN28, a single row of cells was observed in the retinal ONL of untreated eyes (Figure 3A), while three to five rows of cells were observed in the ONL homogeneously across retinas of mice

treated with rhGDNF releasing microspheres (Figure 3C). Counting the average number of nuclei demonstrated that retinas of mice treated with rhGDNF releasing microspheres had a significant increase in the number of cells within the ONL ( $123 \pm 13$  nuclei/400  $\mu\text{m}$ ) as compared to the retinas from untreated mice ( $56 \pm 3$  nuclei/400  $\mu\text{m}$ ;  $p=0.001$ ; Figure 3D). Interestingly, eyes treated with blank microspheres ( $91 \pm 14$  nuclei/400  $\mu\text{m}$ ) also had an increased number of cells in the ONL when compared to the untreated control eyes ( $p=0.001$ ; Figure 3A,B,D). This increased number was however, significantly lower than that observed in eyes treated with rhGDNF-loaded microspheres ( $p=0.002$ ; Figure 3D). For comparison and illustration of the degenerative processes occurring in the

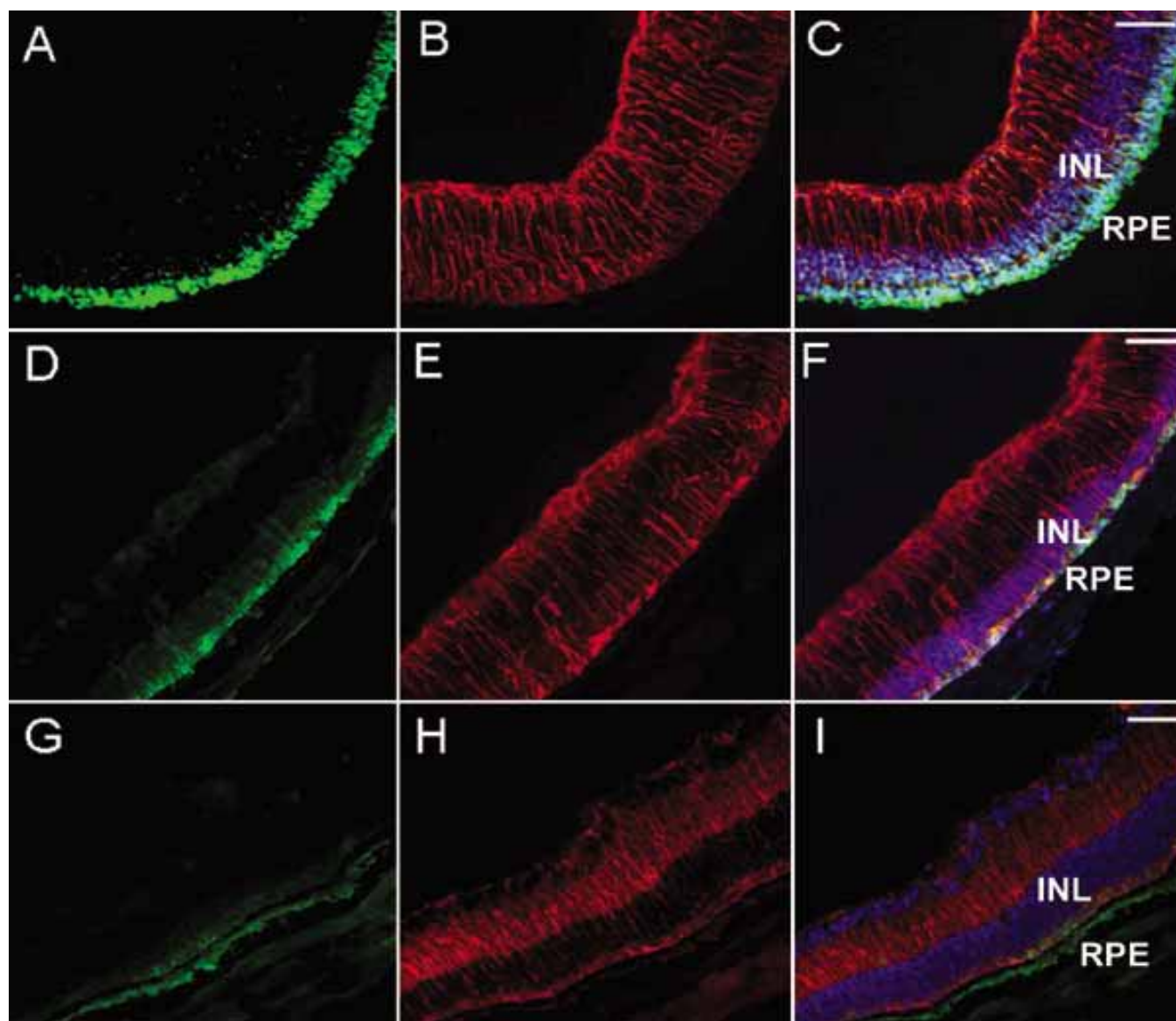


Figure 5. Rhodopsin and GFAP immunohistochemistry in eye sections from treated and control PN28 *rd1/rd1* mice. Rods were identified by rho-4D2 immunostaining (in green) on sections from PN28 mice eyes treated with rhGDNF-loaded microspheres (A) or blank microspheres (D), or untreated (G). Glial cells were identified by GFAP immunostaining (in red) on sections from PN28 mice eyes treated with rhGDNF-loaded microspheres (B) or blank microspheres (E), or untreated (H). Rho-4D2, GFAP and nuclei (DAPI in blue) staining were combined on sections from PN28 mice eyes treated with rhGDNF-loaded microspheres (C) or blank microspheres (F), or untreated (I). The inner nuclear layer (INL) and retinal pigment epithelium (RPE) are identified. Scale bars represent 100  $\mu\text{m}$ .

*rd1/rd1* mice, a picture of the normal wild type retina is illustrated (Figure 3E).

**Rhodopsin immunostaining on whole flat-mount retinas:** At PN28, the rhodopsin immunoreactivity in untreated (control) *rd1/rd1* flat-mount retinas was nearly extinguished (Figure 4A), reflecting the advanced total degeneration of rods in these mice retinas [21,23,24]. The rhodopsin signal in eyes treated with blank microspheres or with inactivated rhGDNF-loaded microspheres was not statistically different from the one observed in the untreated control group of mice (Figure 4A,B). At the same postnatal stage (PN28), the retinas of eyes treated with rhGDNF-loaded microspheres had a high rhodopsin signal (Figure 4C). The mean calculated ratio of tissue to background fluorescence (Tissue FI/Bkgd FI) was significantly increased in the group of eyes treated with rhGDNF-loaded microspheres when compared to the untreated eyes ( $p=0.003$ ) or to the eyes treated with blank microspheres ( $p=0.04$ ; Figure 4D). No significant difference regarding the mean calculated ratios of tissue to background fluorescence was found between the untreated eyes and eyes treated with blank microspheres or with inactivated rhGDNF-loaded microspheres ( $p=0.1$ ; Figure 4D).

**Rhodopsin and GFAP immunostaining:** In eyes treated with rhGDNF releasing microspheres, multiple rows of rhodopsin positive cells were observed throughout the retina (Figure 5A). GFAP-positive retinal Müller glial cells (RMG) prolongations appeared thicker and denser than in retinas of eyes treated with blank microspheres (Figure 5B,E). The com-

bination of rhodopsin and GFAP staining is shown in Figure 5C. In eyes injected with blank microspheres, a few rhodopsin positive cells were detected (Figure 5D). GFAP-positive RMG prolongations were less dense than in rhGDNF-treated eyes with a strong signal detected at the external limiting membrane level (Figure 5E). The combination of these signals is shown in Figure 5F. In *rd1/rd1* untreated (control) eyes, a weak fluorescent rhodopsin signal was observed at the ONL layer level (Figure 5G). Similar pattern of GFAP staining as in blank microspheres was observed (Figure 5H,I).

Higher magnification shows that in rhGDNF-loaded microspheres treated eyes, GFAP positive outer prolongations surround and envelop the surviving photoreceptor nuclei (Figure 6A,D). On the other hand, in blank microspheres treated eyes and in untreated control *rd1/rd1* eyes, RMG prolongations were less dense at the inner nuclear layer level (INL) contrasting with their denser subretinal GFAP positive extensions (Figure 6B,C,E,F).

**F4/80 immunostaining:** Sparse infiltrating F4/80-positive cells were observed at the level of the inner limiting membrane in most eyes injected with rhGDNF-loaded microspheres (Figure 7A) or in eyes injected with blank microspheres (not shown). In the two eyes where particle aggregates were observed on the inner limiting membrane, infiltrating F4/80-positive cells were also observed in the subretinal space (Figure 7B).

**Functional assessment:** In the *rd1/rd1* mouse, the rod phosphodiesterase is not functional, they do not respond to

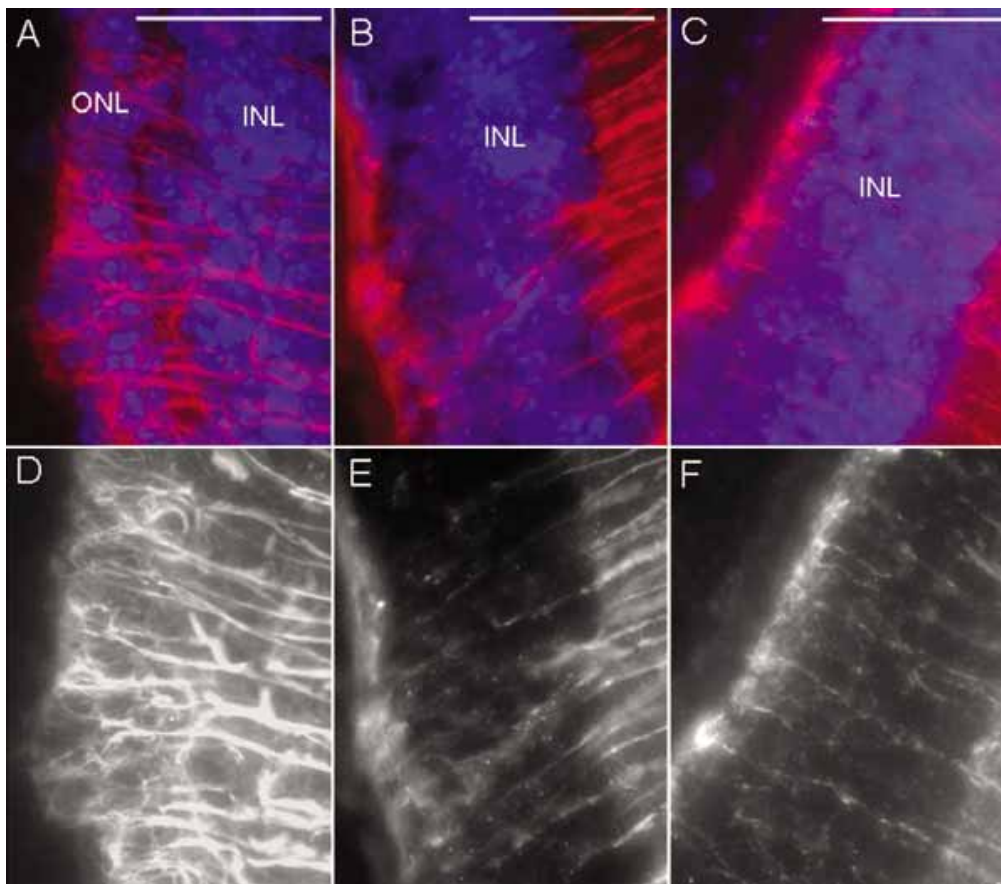


Figure 6. Glial reaction in the outer retina of rhGDNF treated eyes. GFAP immunostaining in red and DAPI staining in blue on sections from PN28 *rd1/rd1* mouse: treated with rhGDNF-loaded microspheres (A) showing thick prolongation closely surrounding photoreceptors, treated with blank microspheres (B) or untreated (C) showing subretinal glial reaction and thin RMG prolongations. Corresponding black and white images of GFAP immunostaining are also shown in this figure (D-F). The outer nuclear layer (ONL) and inner nuclear layer (INL) are identified. Scale bars represent 100  $\mu\text{m}$ .

light stimulation and do not generate a scotopic ERG. As a consequence, ERG recordings were limited to the photopic ERG produced by the cone circuit. Because these photopic ERGs are very small in *rd1/rd1* mice, 50 flashes were delivered and the recordings averaged in order to increase the signal to noise ratio. Figure 8A illustrates representative averaged recordings from the different animal groups at PN23. An averaged photopic ERG from a wild type (WT) mouse is also included for comparison. ERG b-wave amplitudes were statistically significantly higher in the rhGDNF-loaded microspheres treated eyes ( $34.4 \pm 5.2 \mu\text{V}$ ;  $n=10$ ) than in the blank-microsphere treated eyes ( $26.7 \pm 2.4 \mu\text{V}$ ;  $n=13$ ;  $p=0.01$ ) or the untreated eyes ( $19.2 \pm 4.3 \mu\text{V}$ ;  $n=6$ ;  $p=0.01$ ; Figure 8B). No significant difference in the b-wave amplitude was observed between the blank-microsphere treated eyes and the untreated control eyes ( $p=0.2$ ). These results suggest that the rhGDNF-loaded microspheres provided a functional rescue of photoreceptors.

### DISCUSSION

It has been reported that subretinal injection of a recombinant adeno-associated virus driving the human GDNF transgene has a significant rescue effect on retinal degeneration in TgN S334ter-4 rhodopsin rats [13]. Direct subretinal injection of rhGDNF in *rd1/rd1* mice at PN13 and PN17 reduced the extent of photoreceptor degeneration at PN23 in these treated eyes [12], demonstrating that GDNF has the potential for both anatomical and functional rescue of photore-

ceptors in rodent models of inherited retinal degenerations. Due to their complexity and potential danger, intraocular injections in the subretinal space may not be optimal for clinical use. Therefore, we evaluated the potential of single intravitreal injections of polymeric microspheres slowly releasing rhGDNF for the rescue photoreceptors in the *rd1/rd1* mouse.

Our results show that rhGDNF-loaded microspheres injected in the vitreous cavity are well tolerated and allow for a sustained release of bioactive rhGDNF. This slow release of the trophic factor induces anatomical and functional rescue of photoreceptor degeneration in *rd1/rd1* mice. Despite the limited time effect obtained with this nonviral strategy, its advantages include the possible modulation of trophic factor delivery according to the initial clinical response. It also can enable the sequential administration of different factors, since the release can be programmed for a limited period of time. In these respects, the present strategy differs for the viral gene therapy strategy that aims at the constant production of high levels of a specific protein for a very long period of time.

Biodegradable microspheres have been widely used for vitreoretinal drug delivery in many animal models [25]. When injected in the vitreous cavity, most of microparticles remain in the vitreous space, while in aphakic eyes, some of them may migrate to the anterior chamber [26]. The more commonly used polymers for microparticles have been polylactide (PLA) and poly(D,L-lactic acid-co-glycolic acid) (PLGA). More recently, a solid PLGA implant has been developed for the delivery of corticosteroids into the vitreous cavity of eyes with

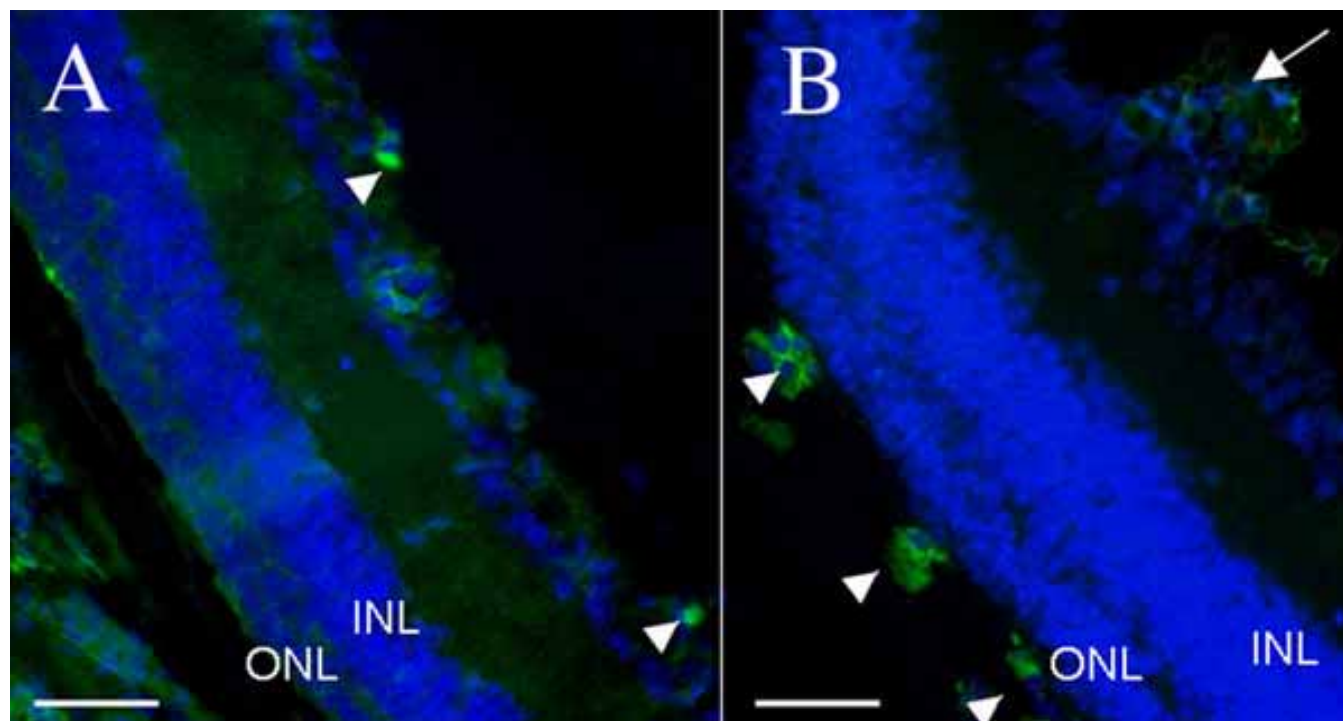


Figure 7. F4/80 immunohistochemistry on eye sections from treated and control PN28 *rd1/rd1* mice. In order to evaluate cell infiltration, macrophages and infiltrating cells were labeled with F4/80. F4/80 immunostaining in green and DAPI staining in blue. Infiltrating macrophages (arrowheads) in rhGDNF-loaded microspheres treated eyes without microspheres aggregation (A) or with microspheres aggregates at the retina surface (B; white arrow). The outer nuclear layer (ONL) and inner nuclear layer (INL) are identified. Scale bars represent 100  $\mu\text{m}$ .



intraocular inflammation [27], demonstrating the potential clinical application of these polymers. When compared to solid implants, PLGA microspheres have the advantage of being easily injected into the vitreous cavity through a small gauge needle.

The PLGA microspheres are well tolerated and induce only a mild inflammatory reaction [28]. This inflammatory reaction may partly contribute to the observed “survival” effect observed after the intravitreal injection of blank microspheres. This inflammatory reaction may amplify the effect of endogenous growth factors release detected after sham intravitreal needle penetration or saline injection [29].

In vivo, the implantation of rhGDNF-releasing microspheres in the cerebral striatum, promoted the recovery of motor function [19]. The total dose of rhGDNF released by these PLGA microspheres was low but the clinical response and biological effects were optimized by the use of microspheres. Similarly, in the retina the total dose of rhGDNF contained in the injected microspheres was 0.38  $\mu\text{g}$ . This dose

is lower than that injected twice in the subretinal space by Frasson et al. [12] in the same animal model. However, in the present study, we have observed that a single intravitreal injection of these PLGA microspheres preserved partial rhodopsin expression and photoreceptor survival at PN28 slowing down the retinal degenerative processes occurring in the *rd1/rd1* mice. The optimal dose of trophic factor needed for maximal photoreceptor rescue effect is not known. Nonetheless, it is conceivable that a cumulative and long standing stimulus delivered by the slow releasing microspheres could have a greater therapeutic potential. The elucidation of these parameters is an attractive and important goal for further research.

Glial cells and particularly the retinal Müller glial cells (RMG) play an important but incompletely elucidated role in the pathogenesis of retinal degeneration [30,31]. In eyes treated with rhGDNF loaded microspheres, RMG cell prolongations surrounded the rescued photoreceptors within the ONL but did not progress to the subretinal space. On the other hand, in the untreated eyes or in eyes treated with blank microspheres, an evident invasion of the subretinal space by the RMG cells is observed. A similar process was observed when adenovirus was used to produce GDNF in the subretinal space of rats with retinal detachments [32]. In these eyes, as in our rhGDNF treated mice eyes, an inhibition of subretinal fibrosis was observed.

In the prospect of clinical application, biodegradable sustained delivery systems may allow for a slow and gradual release of factors within the vitreous cavity circumventing the need for repeated injections or the use of viral vectors. We are not aware of previous reports demonstrating that encapsulation of neurotrophic factor in polymeric biodegradable microspheres can be used as a slow release system in the vitreous cavity efficiently delaying ongoing retinal degeneration processes. Before this technology can be used in the clinic however, data regarding sterilization process of PLGA microspheres, optimal doses and the kinetics of factors release in larger animal eyes are needed. These necessary aspects are now under study in our laboratory.

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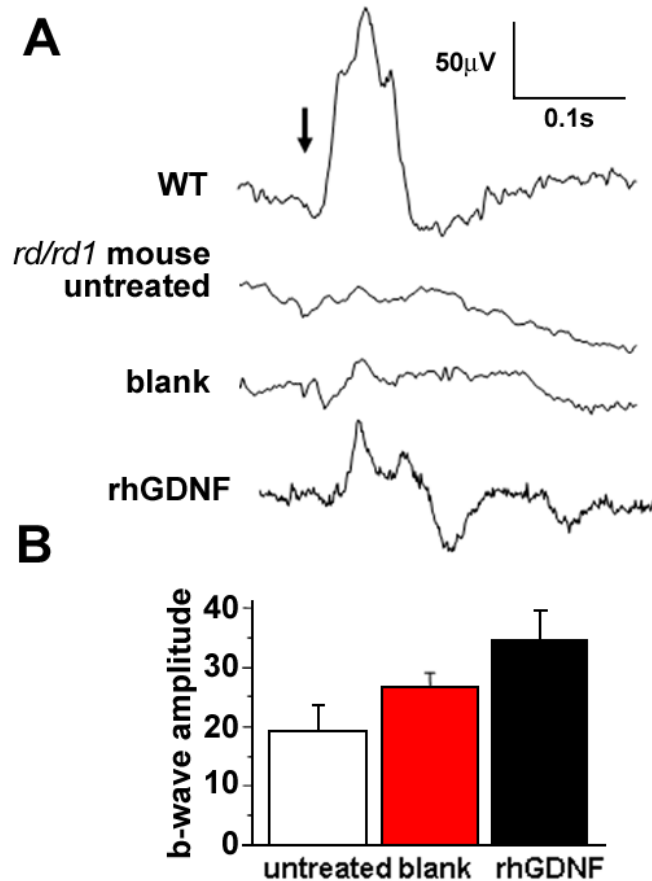


Figure 8. Electrophysiological recordings of treated and control PN23 *rd1/rd1* mice. **A:** Representative averaged recordings from the different animal groups at PN23. An averaged photopic ERG from a wild type adult animal is also shown (WT) to illustrate the difference in amplitude. **B:** ERG b-wave amplitudes were significantly higher ( $p=0.01$ ) in the rhGDNF-loaded microspheres ( $34.4 \pm 5.2 \mu\text{V}$ ;  $n=10$ ) compared to the blank-microsphere treated eyes ( $26.7 \pm 2.4 \mu\text{V}$ ;  $n=13$ ) and the untreated eyes ( $19.2 \pm 4.3 \mu\text{V}$ ;  $n=6$ ).

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