

Photoreceptor preservation induced by intravitreal controlled delivery of GDNF and GDNF/melatonin in rhodopsin knockout mice

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Purpose: To evaluate the potential of a poly(lactic-co-glycolic acid) (PLGA)-based slow release formulation of glial cell line–derived neurotrophic factor (GDNF) alone or in combination with melatonin to rescue photoreceptors in a mouse model of retinal degeneration.

Methods: GDNF and GDNF/melatonin-loaded PLGA microspheres (MSs) were prepared using a solid-in-oil-in-water emulsion solvent extraction-evaporation technique. A combination of PLGA and vitamin E (VitE) was used to create the microcarriers. The structure, particle size, encapsulation efficiency, and in vitro release profile of the microparticulate formulations were characterized. Microparticulate systems (non-loaded, GDNF, and GDNF/melatonin-loaded MSs) were administered intravitreally to 3-week-old rhodopsin knockout mice (*rho* $^{-/-}$; n=7). The functional neuroprotective effect was assessed with electroretinography at 6, 9, and 12 weeks old. The rescue of the structure was determined with photoreceptor quantification at 12 weeks (9 weeks after administration of MSs). Immunohistochemistry for photoreceptor, glial, and proliferative markers was also performed.

Results: The microspheres were able to deliver GDNF or to codeliver GDNF and melatonin in a sustained manner. Intravitreal injection of GDNF or GDNF/melatonin-loaded MSs led to partial functional and structural rescue of photoreceptors compared to blank microspheres or vehicle. No significant intraocular inflammatory reaction was observed after intravitreal injection of the microspheres.

Conclusions: A single intravitreal injection of GDNF or GDNF/melatonin-loaded microspheres in the PLGA/VitE combination promoted the rescue of the photoreceptors in *rho* $^{-/-}$ mice. These intraocular drug delivery systems enable the efficient codelivery of therapeutically active substances for the treatment of retinal diseases.

Retinal degenerative disorders are characterized by irreversible loss of retinal neurons. These disorders include retinal detachment (RD) [1], age-related macular degeneration (AMD) [2], diabetic retinopathy [3], glaucoma, and retinitis pigmentosa, among others [4]. Autophagy defects can cause apoptosis and necrosis of rods and cones [5]. The causes of photoreceptor degenerations include but are not limited to oxidative stress [6], ischemia [7], protein misfolding [8], physical damage [9], and lack of mechanical and trophic support from the neighboring photoreceptors, Müller glial cells, and RPE cells [10]. Two major causes of photoreceptor cell degeneration are considered to increase reactive oxygen

species (ROS) due to reduced oxygen utilization [11] and a decrease in neurotrophic factors from the rods [12].

Neuroprotection, a mutation-independent approach to protect central nervous system (CNS) cells, has demonstrated its therapeutic potential for retinal degenerations in various animal models. Neuroprotective effects can be achieved by several pathways. One is preserving neuronal cells from oxidation by harvesting ROS with antioxidants [13]. Another well-described strategy is to use neurotrophic factors that can improve retinal cell differentiation, survival, and function [14]. With these objectives, different active substances have been tested for the treatment of retinal degeneration. The usefulness of macromolecules, such as glial cell line–derived neurotrophic factor (GDNF) [15], brain-derived neurotrophic factor (BDNF) [16], nerve growth factor (NGF) [17], ciliary neurotrophic factor (CNTF) [18], cerebral dopamine neurotrophic factor (CDNF) [19], and basic fibroblast growth factor (bFGF) [20], has been previously reported. In addition, low molecular weight molecules, such as valproic acid [21],

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rasagiline [22], melatonin [23], and N-acetyl-L-cysteine [24], have been also studied.

GDNF has been demonstrated to enhance the survival of dopaminergic [25] and motor neurons [26] in the central, peripheral, and autonomic nervous systems. In the eye, GDNF is primarily expressed in the retina [27]. Several studies have shown the activity of GDNF to rescue retinal ganglion cells (RGCs) even at low concentrations [28-30]. Furthermore, the protective activity of GDNF has been also observed for photoreceptors and can be induced by the expression of certain yet unknown neurotrophic factors in retinal Müller glial cells [15,31].

The neurohormone melatonin is synthesized in the retina and the pineal gland of mammals through a biosynthetic pathway [32]. Melatonin plays an important role in retinal physiology and pathophysiology and acts as a local neuro-modulator within the eye [33,34]. In the human retina, two subtypes of melatonin receptors (MT1 and MT2) are present, with the MT1/MT2 heteromeric complex responsible serving as a specific pharmacological target, leading to improved photoreceptor function [35]. In addition, melatonin has antioxidant properties with free radical scavenger activity [36,37] that has been shown in vitro [37,38] and in vivo [39]. This active substance also has demonstrated a neuroprotective effect on RGCs in vitro [40] and a prosurvival effect on RPE cells and photoreceptors [23,38,41] in vitro and in vivo. In addition, this molecule is implicated in the modulation of intraocular pressure (IOP) [42,43] with potential usefulness in the treatment of elevated IOP-related glaucoma [44].

The therapeutic approaches using growth factors vary: They may be administered as recombinant proteins [15,30,45], overexpressed in host cells through administration of genetic vectors [46], induced in host cells by small molecules [47], or overexpressed in transplanted cells [48]. We explored the sustained release of the recombinant protein GDNF, because it provides better control of ocular levels of the substance and allows combination with other active molecules, such as antioxidants. Furthermore, taking into account the multifactorial character of the retinal diseases, a combination of neuroprotective substances acting through different pathways might offer a synergistic benefit. The main challenge in such combined neuroprotective therapies is to achieve a therapeutic concentration of the different compounds in the retina for an extended period. In the last decade, much effort has been made in developing intraocular drug delivery systems (IODDSs) based on biodegradable poly(lactic-co-glycolic acid) (PLGA) able to provide long-term delivery of active substances. Among the IODDSs, microparticulate systems are gaining more attention for personalized therapy

as different amounts of this therapeutic tool can be injected depending on the disease and patient needs [49].

Our goal is to provide simultaneous sustained release of two neuroprotective agents (GDNF and melatonin) from PLGA microspheres (MSs) able to protect retinal tissues long-term. To achieve this goal, the efficacy to rescue photoreceptors in a mouse model of retinal degeneration has been evaluated. Microspheres loaded with GDNF-PLGA/vitamin E (VitE) and GDNF/melatonin-PLGA/VitE were formulated and characterized. In addition to the potential neuroprotective effect, vitamin E was included in the polymeric PLGA matrix to improve encapsulation of the biotechnological product and to modulate the release of GDNF from the particles [50]. The GDNF-loaded PLGA/VitE MSs and GDNF/melatonin-loaded PLGA/VitE MSs were tested in a well-characterized model of inherited photoreceptor degeneration, the rhodopsin knockout mouse (*rho* (-/-)) [51,52]. This animal model is thought to resemble autosomal recessive rhodopsin null mutations in humans and has been proposed for rapid testing of therapies for retinal degenerations [53]. Initially, animals show normal retinal development followed by failure of the outer segment (OS) to form in rod photoreceptors. From postnatal week 4 to 6, the *rho* (-/-) mice appear to have normal cones and no detectable rod-derived electroretinogram (ERG). By the age of 3 months, most of the rod nuclei are lost (approximately 90%), leaving only a single, fragmented row of nuclei composed almost exclusively of cones.

METHODS

Elaboration of microspheres: PLGA MSs were prepared according to a solid-in-oil-in-water (S/O/W) emulsion-solvent evaporation technique previously described [30]. Briefly, 20 µg of GDNF (R&D, Systems, Minneapolis, MN) were first suspended in 20 µl of VitE (Sigma-Aldrich, Schnellendorf, Germany) via gentle sonication for 30 s at low temperature (0 °C; Sonicator XL, Heat System, Inc., Farmingdale, NY). Once formed, the suspension was added to 1 ml of PLGA (Resomer®503, Boehringer Ingelheim Pharma GmbH & Co, Ingelheim, Germany) solution in methylene chloride (20% w/v) to elaborate the GDNF-loaded PLGA/VitE MSs or a solution of 20 mg of melatonin (Sigma-Aldrich) and 200 mg of PLGA in 1 ml of methylene chloride to prepare the GDNF/melatonin-loaded PLGA/VitE MSs. The organic phase was then emulsified in both cases with 5 ml of polyvinyl alcohol (PVA) 72,000 g/mol (Merck KGaA, Darmstadt, Germany) MilliQ® water solution (2% w/v) in a homogenizer (Polytron® RECO, Kinematica GmbH PT 3000, Lucerna, Switzerland) at 5,000 rpm for 1 min and 6,000 rpm for 1 min. This emulsion was subsequently poured on 100 ml of an aqueous PVA

solution (0.1%) and kept under constant stirring for 3 h, to allow evaporation of the organic solvent. Once formed, microspheres were washed to eliminate PVA, filtered, freeze-dried, and kept at $-20\text{ }^{\circ}\text{C}$ under dry conditions until use.

Microsphere characterization:

Production yield percentage—The production yield percentage (PY%) of each formulation was calculated from the weight of the dried microspheres (W_1) recovered and the sum of the initial dry weight of the starting materials (W_2). The formula for calculating the % yield is as follows [54]:

$$\text{PY}\% = \frac{\text{Weight of MSs } (W_1)}{\text{Total weight of active substances and polymer } (W_2)} \times 100$$

Mean particle size and particle size distribution—The mean particle size and the particle size distribution were measured with light scattering in a Microtrac[®] S3500 Series Particle Size Analyzer (Montgomeryville, PA).

Morphological evaluation—The external morphology of freeze-dried microspheres was evaluated with scanning electron microscopy (Jeol, JSM-6335F, Tokyo, Japan). Samples were gold sputter-coated before observation.

Encapsulation efficiency—To determine the encapsulation efficiency (EE%) of GDNF, a liquid/liquid extraction method was used. Briefly, 5 mg of microspheres (GDNF-loaded PLGA/VitE as well as GDNF/melatonin-loaded PLGA/VitE MSs) were dissolved in 0.7 ml of methylene chloride. Then, the same volume of the reactive diluent composed of PBS (13.08 mM KH_2PO_4 , 11.13 mM $\text{Na}_2\text{HPO}_4(12\text{H}_2\text{O})$, 68.45 mM NaCl), pH 7.4, and 1% bovine serum albumin (BSA) provided in the enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems) was added. After vigorous mixing, the heterogeneous system was settled during 10 min. Finally, the aqueous phase was extracted after phase separation (centrifugation for 11,290 $\times g$, 15 min, $4\text{ }^{\circ}\text{C}$). The extraction procedure was repeated four times, to recover all of the encapsulated protein. The GDNF content in the extractive medium was quantified with the ELISA kit in triplicate. The amount of encapsulated melatonin in the microspheres was determined as follows: 10 mg of melatonin-loaded MSs were placed in 5 ml of methylene chloride to dissolve the polymer. Then, 12 ml of ethanol were added to promote polymer precipitation. After vortex mixing, the samples were centrifuged at 1,960 $\times g$ for 5 min, and the methylene chloride:ethanolic supernatant was recovered and filtered (0.45 μm). The melatonin content in the methylene

chloride:ethanol mixture was quantified with ultraviolet visual (UV-Vis) spectrophotometry at 278 nm.

For each active substance (GDNF and melatonin), encapsulation efficiency was calculated as follows [54]:

$$\text{EE}\% = \frac{\text{Actual active compound content}}{\text{Theoretical active compound content}} \times 100$$

In vitro release studies: To evaluate the rate of GDNF release from the microspheres, replicates of 5 mg of GDNF-loaded PLGA/VitE MSs and GDNF/melatonin-loaded PLGA/VitE MSs were suspended in a release medium composed of 1.5 ml of PBS (pH 7.4 isotonized with NaCl) containing 1% BSA as the protein carrier and 0.02% sodium azide. Eppendorf[®] LoBind vials (Hamburg, Germany) were used in all cases. Samples were placed in a water shaker bath and were maintained under constant agitation 100 rpm at $37\text{ }^{\circ}\text{C}$ (Clifton Shaking Bath NE5, Nickel-Electro Ltd, Avon, UK). At preset times (1 h, 24 h, and once a week until the end of the assay), the microsphere suspensions were gently centrifuged (1,960 $\times g$ for 5 min) and the supernatants were recovered and replaced with the same volume of fresh medium. If necessary, aliquots from supernatants were diluted with the diluent reactive provided in the ELISA kit for GDNF to be quantified with the ELISA technique.

Melatonin release was determined from 10 mg of GDNF/melatonin-loaded PLGA/VitE MSs dispersed in (pH 7.4 isotonized with NaCl) a water shaker bath and were maintained under constant agitation 100 rpm at $37\text{ }^{\circ}\text{C}$ (Clifton Shaking Bath NE5). At preset times (1 h, 24 h, 3 days, and once a week until the end of the assay), the supernatants were filtrated (0.45 μm), and the concentration of the melatonin released from the microspheres was measured with UV-Vis spectrophotometry at 278 nm.

Animal procedures: For all experimental procedures, animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, under a protocol approved by the Schepens Eye Research Institute Animal Care and Use Committee. Rhodopsin null mice, *rho* ($-/-$), from Peter Humphries (Trinity College, Dublin), were used as retinal degenerative experimental recipient animals. Intravitreal injections and electroretinography recordings were performed under general anesthesia induced by intraperitoneal injection of ketamine/xylazine (100 mg/kg and 20 mg/kg, respectively) and topical anesthesia with proparacaine drops (Alcon; Fort Worth, TX). Tropicamide (Alcon) was applied to dilate the pupils. After the intravitreal injection, the eye was treated with an antibiotic gel. The same

procedures were applied to the control eyes. At the end of the experiment, animals (12 weeks old) were euthanized with CO₂ inhalation followed by cervical dislocation. To study photoreceptor rescue, 3-week-old animals were randomized into three groups (n=7), which received intravitreal injection of GDNF-loaded PLGA/VitE microspheres, GDNF/melatonin-loaded PLGA/VitE microspheres, and non-loaded PLGA/VitE microspheres. Left eyes were used as untreated controls in all animals. For the microsphere injection procedure, 2.5% (w/v) suspensions of microspheres were prepared in Hanks Balanced Salt Solution (HBSS) and briefly vortexed immediately before each injection to ensure homogeneous dispersion of microspheres in the injected fluid. A volume of 2 μ l of the microsphere suspension was administered intravitreally to 3-week-old rhodopsin knockout mice using a beveled glass needle (50 μ m internal diameter), connected to a Hamilton syringe through polyethylene tubing. To reduce intraocular pressure, corneal paracentesis was performed simultaneously during the injection. The function of the retina was assessed with photopic electroretinography recordings (Espion Electroretinography System; Diagnosys LLC, Lowell, MA). ERG response from both eyes was recorded simultaneously using gold wire loop active electrodes on the cornea, a reference electrode on the forehead, and the ground electrode close to the tail bet-adapted for 10 min before recording.

The photopic ERG responses to a series of xenon-light flashes (25 cd \times c/m²) presented in a Ganzfeld bowl (Diagnosys LLC). The amplitude of the b-wave was defined as the absolute value from the trough of the a-wave to the peak of the first positive wave. If the a-wave was absent, the baseline to the peak of the first positive wave was measured. To compensate for the variability of degeneration between individual mice (*rho* (-/-)), the b-wave amplitude was normalized by the contralateral (the untreated eye). For normalization, the b-wave of a contralateral (the un-injected eye) was subtracted from the b-wave of the eye that received the treatment.

Histopathological examination: Nine weeks after the injection (12 weeks of age), the animals were euthanized, and the eyes were enucleated and fixed in Davidson solution, followed by processing and paraffin embedding. Six-micron-thick sections were cut in the area close to the optic nerve head (ONH) and processed for hematoxylin and eosin (H&E) staining and immunohistochemical analysis (IHC). We stained the tissue for photoreceptor (recoverin and S-opsin), glial (GFAP, glutamine synthetase, and CRAL-BP), and proliferative markers (PCNA and Ki67). The photoreceptor rescue was quantified by counting the total number of cells in the outer nuclear layer at each 100 μ m region, starting from the optic nerve head. Three total sections per animal, seven eyes per group were analyzed to build a spider plot.

Statistical analysis: In vitro experiments were repeated three times. In vivo experimental groups contained a minimum of seven retinas per condition. The data are expressed as the mean \pm standard error of the mean (SEM), unless otherwise noted. Statistical significance was defined at *p<0.05, **p<0.01, and ***p<0.001 by using one-way ANOVA with Bonferroni testing for post-hoc comparisons or the standard Student *t* test.

RESULTS

Elaboration and characterization of microspheres: The yield obtained using the microencapsulation method was 75.6 \pm 6.80% for the GDNF-loaded PLGA/VitE MSs and 71.4 \pm 2.10% for the GDNF/melatonin-loaded PLGA/VitE MSs. In both cases, the largest population of microspheres was found to be in the 20–40 μ m range. The mean particle size was 21.6 \pm 0.30 μ m for the GDNF-loaded PLGA/VitE MS formulation and slightly higher (24.6 \pm 0.40 μ m) for the GDNF/melatonin-loaded PLGA/VitE MSs (Figure 1 insets). Scanning electron microscopy studies showed spherical particles with several pores located on the surface (Figure 1).

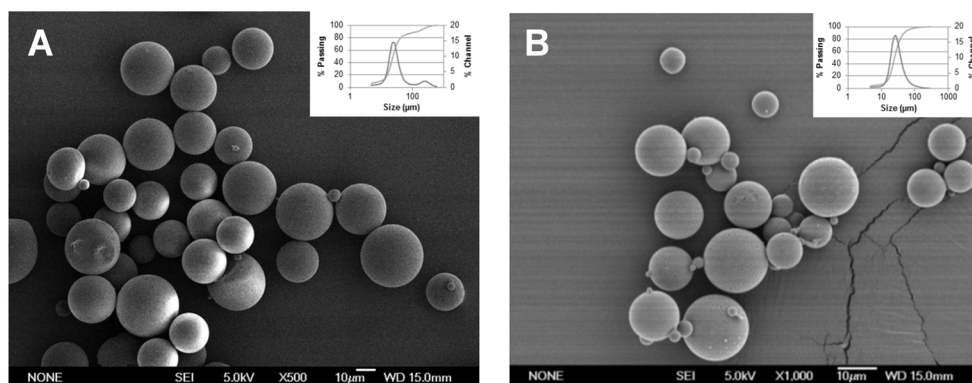


Figure 1. Morphological and size analysis. Scanning electron microscopy images of (A) glial cell line-derived neurotrophic factor/vitamin E (GDNF/VitE)-loaded microspheres and (B) GDNF/VitE/melatonin-loaded microspheres. Inset: Particle size distribution.

The encapsulation efficiency of GDNF was $39.5 \pm 3.60\%$ (34.7 ± 5.30 ng GDNF/mg MSs) and $26.4 \pm 0.90\%$ (22.1 ± 0.80 ng GDNF/mg MSs) for the GDNF-loaded PLGA/VitE MSs and the GDNF/melatonin-loaded PLGA/VitE MSs, respectively. However, melatonin entrapment for the GDNF/melatonin-loaded PLGA/VitE MSs formulation was $39.2 \pm 0.30\%$ (32.8 ± 0.20 μ g melatonin/mg MSs).

The GDNF release observed within 24 h (burst) represented 75% (26.12 ± 1.2 ng GDNF/mg MSs) of the encapsulated protein for the GDNF-loaded PLGA/VitE MSs. Subsequently, a slow and continuous release occurred from day 7 to day 42 at 65 pg GDNF/mg MSs/day. After that, a moderate release step occurred from day 42 to day 56, in which the protein was released at 48 pg GDNF/mg MSs/day followed by a rapid release step at 140 pg GDNF/mg MSs/day during the next week and until the end of the assay.

In the case of the GDNF/melatonin-loaded PLGA/VitE MSs an initial burst was observed for GDNF, releasing 44% of the loaded protein (9.7 ± 0.2 ng GDNF/mg MSs) in the first 24 h of in vitro testing. Next, a slow sustained release occurred from day 7 to day 35 at 36 pg GDNF/mg MSs/day. Contrary to the MSs prepared without melatonin, GDNF did not show any increased release at that point but was continuously released at 19 pg GDNF/mg MSs/day from day 35 to the end of the assay (day 63; Figure 2).

Melatonin released from the GDNF/melatonin-loaded PLGA/VitE MSs also showed a different sequence of fast and slow release rates. After rapid delivery during the first 7 days (burst effect at 24 h of 1.23 μ g melatonin/mg MSs), a sustained release was observed from day 7 to day 14 (97 ng melatonin/mg MSs/day). After that, a very slow melatonin release was observed until day 56 at a rate of 14 ng melatonin/mg MSs/day. Finally, an increase in the melatonin release rate (1 μ g melatonin/mg MSs/day) was observed in the last week of the release study (Figure 2).

Rescue of retinal structure and function in rhodopsin knockout mice: Photoreceptor quantification showed significant rescue by GDNF-PLGA/VitE MSs and GDNF/melatonin-PLGA/VitE MSs, with a 1.7- and 1.6-fold increase in cell number compared to vehicle or non-treated. The architecture of the retina was not affected by the treatment (Figure 3). We observed similar expression of photoreceptor and glial markers throughout the retina in all treatment groups. At 12 weeks of age (9 weeks after administration of the MSs), we did not observe any significant inflammation or infiltration in any of the treatment groups (Figure 4A,B). The glia remained slightly reactive in all groups as detected with GFAP and glutamine synthetase staining, an expected result for this retinal degeneration model.

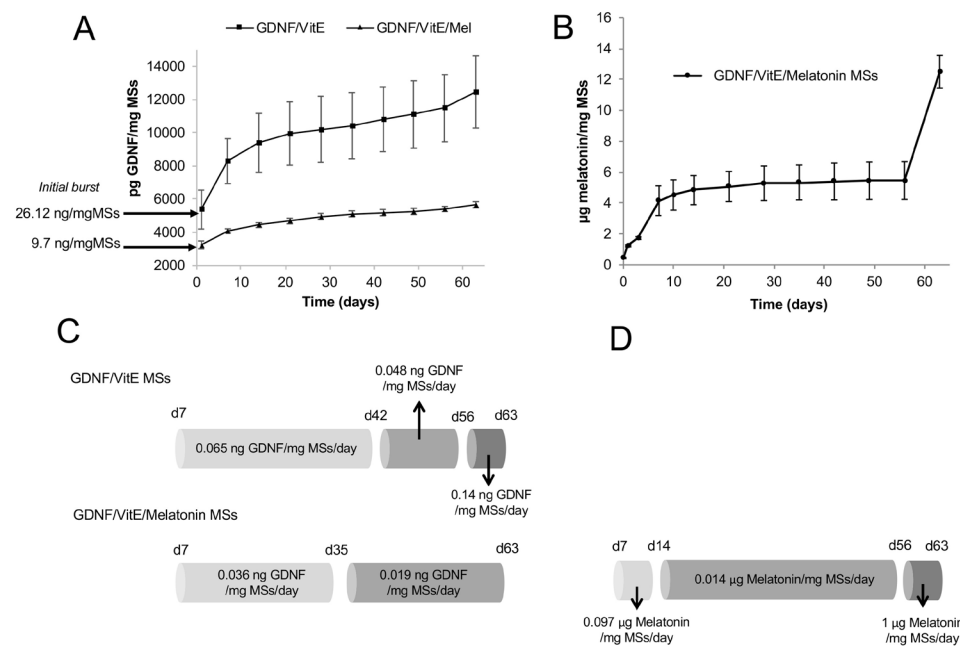


Figure 2. In vitro release studies. A: Cumulative release of glial cell line-derived neurotrophic factor (GDNF; ng/mg microspheres) over 63 days from the GDNF/vitamin E (VitE)-loaded formulation (■) and from the GDNF/VitE/melatonin-loaded formulation (▲). Release media: PBS (pH 7.4) with 1% bovine serum albumin (BSA) and 0.02% Na Azide. B: Cumulative release of melatonin (μ g/mg microspheres) over 63 days from the GDNF/VitE/melatonin-loaded formulation (●). Release media: PBS (pH 7.4). C: Schematic representation of the GDNF release rate (ng GDNF/mg microspheres/day) from the GDNF/VitE-loaded microsphere formulation and the GDNF/VitE/melatonin-loaded microsphere formulation during the 63 days. D: Schematic representation of the melatonin release rate (μ g melatonin/mg microspheres/day) from the formulation of GDNF/VitE/melatonin-loaded MSs during the 63 days. Panel A, B, C sampling sizes (n=3); error bars are SEM.

formulation during the 63 days. D: Schematic representation of the melatonin release rate (μ g melatonin/mg microspheres/day) from the formulation of GDNF/VitE/melatonin-loaded MSs during the 63 days. Panel A, B, C sampling sizes (n=3); error bars are SEM.

To evaluate functional rescue of retinal neurons, ERGs were performed in the 6-, 9-, and 12-week-old retinal degeneration mouse model (Figure 5). The amplitude of the b-waves was measured and compared between GDNF-loaded PLGA/VitE MSs, GDNF/melatonin-loaded PLGA/VitE MSs, non-loaded PLGA/VitE MSs, and untreated mice. An averaged ERG from a wild-type (*wt*) mouse is included for comparison. There was no statistically significant difference between GDNF-loaded PLGA/VitE MSs, GDNF/melatonin-loaded PLGA/VitE MSs, and non-loaded PLGA/VitE MSs in the amplitude of the b-wave ($p>0.05$) at 3 and 6 weeks after injection. We also observed a trend toward slight depression of ERG in all treatment groups, which is likely related to the intravitreal injection procedure. However, when the mice were 12 weeks old, the ERG b-wave amplitudes were statistically significantly higher in the GDNF-loaded MS-treated eyes (39 μ V) and the GDNF/melatonin-loaded MSs (47 μ V) compared to the non-loaded-microsphere-treated eyes (31 μ V; $p=0.03$) or the untreated eyes (32 μ V). No statistically significant difference in the b-wave amplitude was observed between the blank microsphere-treated eyes and the untreated control eyes. These results suggest that the GDNF-loaded PLGA/VitE MSs and the GDNF/melatonin-loaded PLGA/VitE MSs provide functional rescue of photoreceptors after 9 weeks of treatment. The statistically significant difference in

photopic ERG recordings was observed only at the terminal time point (12 weeks of age) and only after normalization by the contralateral eye. The lack of more significant functional rescue may be related to the variability in the degeneration rate between animals.

DISCUSSION

Direct delivery of GDNF into the eye enhances the survival of a variety of neurons in the retina, including retinal ganglion cells and photoreceptors [15,30,45]. This neurotrophic factor, which belongs to the transforming growth factor β superfamily, was first described as a stimulant of survival of dopaminergic neurons in vitro with an EC_{50} of 40 pg/ml [25] and has been found to stimulate the survival of rods in P5 mouse retinal cultures with a maximal effect at 200 pg/ml (ED_{50} of about 30 pg/ml) after 6 days in culture [55,56]. In vivo, direct subretinal injection of 330 ng/ μ l of rhGDNF in the *rd1/rd1* mouse at P13 and P17 has been shown to exert histologic and functional neuroprotective effects on rod photoreceptors, reducing the degeneration at P23 [57]. However, the real utility of neurotrophic factors is linked to the development of effective formulations able to deliver the active substance to the target tissue as adverse reactions and complications have been shown with repeated intravitreal administration [58]. To reduce the frequency of injections and lengthen the

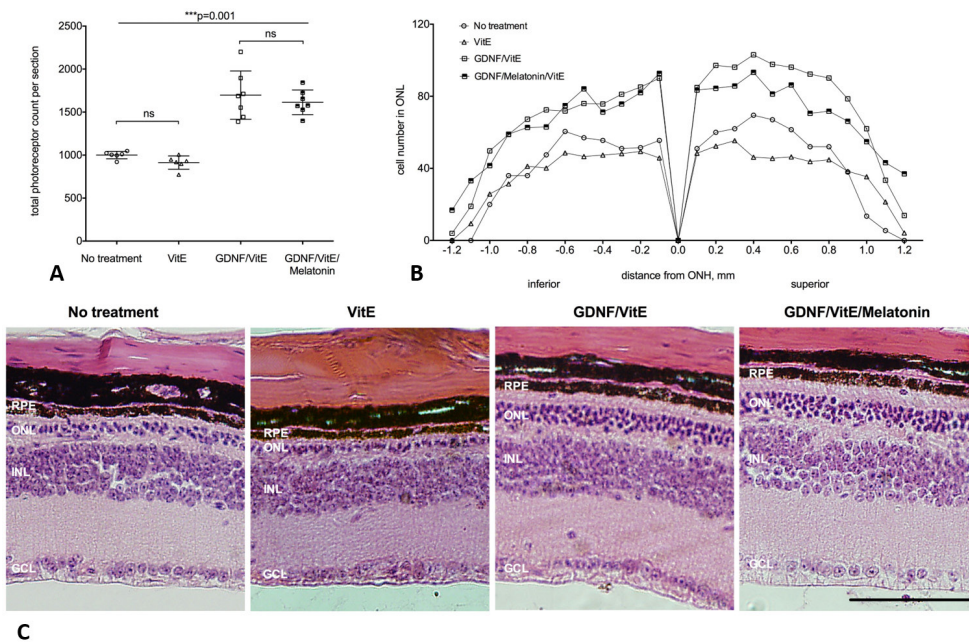


Figure 3. The rescue of photoreceptors with microspheres. **A:** Total photoreceptor count per section of glial cell line-derived neurotrophic factor/vitamin E (GDNF/VitE)-loaded microspheres (MSs), GDNF/VitE/melatonin-loaded MSs, VitE-loaded MSs, and untreated *rho* (*-/-*) mice. We observed a more than 1.5-fold increase in the photoreceptor number in both treatment groups compared to the control groups. No difference between the control groups was observed. Error bars indicate standard error of the mean (SEM); levels of significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Sampling size for each group: 7 animals/group. **B:** The spider plot demonstrates the pan-retinal rescue of photoreceptors in both treatment

groups 12 weeks after the intravitreal injection of the microspheres. **C:** Hematoxylin and eosin (H&E) staining of paraffin sections of the retinas for GDNF/VitE-loaded MSs, GDNF/VitE/melatonin-loaded MSs, VitE-loaded MSs, and untreated *rho* (*-/-*) mice. We did not observe any signs of inflammation in the treated eyes at the 12-week time point.

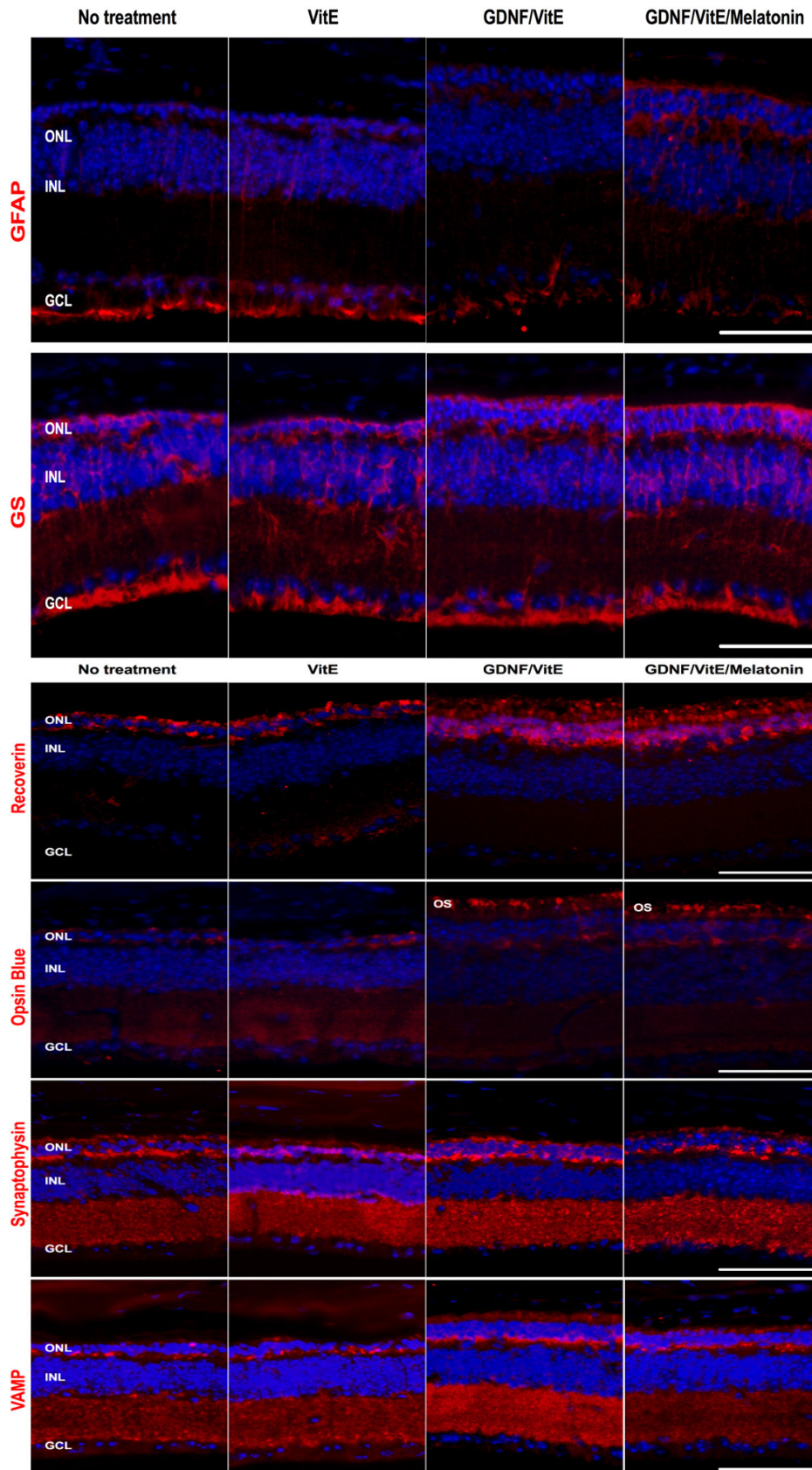


Figure 4. Immunohistochemical analysis. **A:** Glial marker expression. The expression of glial markers glial fibrillary acidic protein (GFAP), LIM/homeobox 2 protein (Lhx2), and glutamine synthetase (GS) was similar across the control and treatment groups. The Müller glia processes are visible spanning the entire retinal thickness. Scale bar, 50 μ m. 4',6-diamidino-2-phenylindole (DAPI; blue) is used for nuclei counterstaining. **B:** Expression of the photoreceptor and synaptic markers. The immunohistochemical analysis of the treated and control eyes at 12 weeks following injection of the microspheres demonstrated partial preservation of the photoreceptors. We observed increased expression of the pan-photoreceptor marker recoverin in both treatment groups, which correlates with increased cell counts and outer nuclear layer (ONL) thickness. We also confirmed the preservation of outer segments in short-wavelength cones as detected with S-opsin. The increased expression of synaptic markers vesicle-associated membrane protein (VAMP) and synaptophysin correlated with the partial photoreceptor rescue in the treatment groups. Scale bar, 50 μ m. DAPI (blue) is used for nuclei counterstaining.

active substance release time, neurotrophic factors have been encapsulated in biodegradable microspheres [30] or nanoparticles [59].

Several studies have shown that the use of microcarriers for drug delivery in the eye results in effective targeting, high availability in targeted tissues, and few adverse effects [49]. In this line, drug delivery systems based on PLGA microspheres loaded with neurotrophic factors such as GDNF offer great promise for long-term rescue of retinal cells in many neurodegenerative pathologies [30,45,60].

Recently, there has been increased interest in the simultaneous use of active substances targeting different pathophysiological pathways for the treatment of multifactorial diseases.

Combinatory therapeutic strategies are currently being evaluated by other groups for the treatment of ocular diseases. For example, Wyględowska-Promieńska et al. successfully tested the combination of an anti-VEGF agent (aflibercept) solution intravitreally injected in patients suffering exudative age-related macular degeneration with topical administration of bromfenac [61]. In addition, ongoing studies are exploring the efficacy of combining anti-VEGF treatment with sustained-release corticosteroids to treat diabetic macular edema [62].

In this work, we developed GDNF-loaded PLGA/VitE MSs and GDNF/melatonin-loaded PLGA/VitE MSs according to a previous elaboration protocol that allows for the release of the neurotrophic factor in its bioactive form, based on its encapsulation in a solid state and to the presence

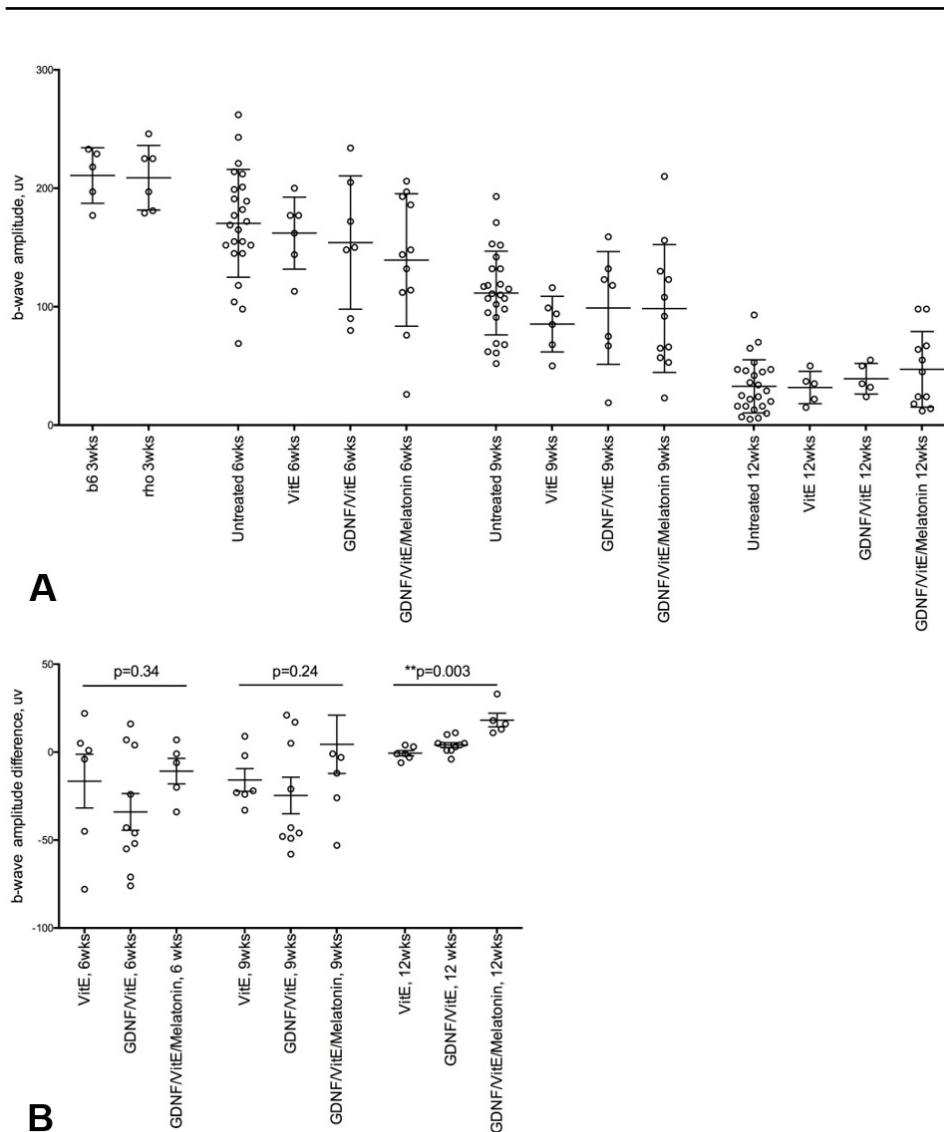


Figure 5. The rescue of retinal function in rhodopsin knockout mice with microspheres **A**: Photopic electroretinography recordings from *rho* (-/-) mice at 6, 9, and 12 weeks old. The amplitude of the b-waves was measured in glial cell line-derived neurotrophic factor/vitamin E (GDNF/VitE)-loaded microspheres (MSs), GDNF/VitE/melatonin-loaded MSs, VitE-loaded MSs, and untreated *rho* (-/-) mice. An averaged electroretinogram (ERG) from a 3-week-old wild-type (wt) mouse and a 3-week-old *rho* (-/-) mouse is also included. Error bars indicate the standard error of the mean (SEM). **B**: Comparison of photopic electroretinography recordings from *rho* (-/-) mice at 6, 9, and 12 weeks after intravitreal injection of microspheres. The amplitude of the b-waves was compared between the GDNF/VitE-loaded MSs, GDNF/VitE/melatonin-loaded MSs, VitE-loaded MSs, and untreated *rho* (-/-) mice. Error bars indicate standard error of the mean (SEM); levels of significance: *p<0.05, **p<0.01, ***p<0.001. Sampling size for each group: 11 animals/group. (graph shows only animals that were included in the analysis).

of vitamin E [50]. The two formulations compared in the present work show a particle size and morphology (spherical) suitable for injection as a suspension through needles typically used for intravitreal administration (25G to 32G) [63]. From the technological point of view, the presence of pores on the MS surface was previously explained as a consequence of the rapid removal of the organic solvent during the elaboration process and to the presence of an oily additive (vitamin E) in the formulation [30]. In this work, microspheres were prepared with a biodegradable polymer (PLGA). This family of copolymers has been approved for clinical use with a biodegradable intraocular implant for sustained release of dexamethasone (Ozurdex®, Allergan, Inc., Irvine, CA) indicated in the treatment of macula edema following branch retinal vein occlusion or central retinal vein occlusion, non infectious posterior segment uveitis, and diabetic macular edema [64,65].

The biodegradation of PLGA microspheres occurs through a homogeneous hydrolytic chain cleavage mechanism where the rates of polymer degradation are similar for the surface and the bulk of the microspheres [66]. This phenomenon typically produces in vitro release profiles characterized by a combination of slow and fast release rates [67,68]. It has been demonstrated that the inclusion of hydrophobic substances in the formulation such as vitamin E promotes a decrease in the protein release rate [30]. In the present work, we demonstrate that the encapsulation of another hydrophobic compound (melatonin), solid in this case, promotes changes in the GDNF release profile, not only reducing the initial burst but also increasing the duration and lowering the delivery rate values of the neurotrophic factor observed in the slow release steps. The typical release profile from PLGA MSs was also observed in the melatonin release profile, also combining fast and slow release rates.

The formulation of more than one active compound in the same microparticulate system remains a challenge from a technological point of view. Several strategies have been evaluated in the last few years. For example, Feng et al. included a hydrophilic drug (doxorubicin) in the inner aqueous phase (W1) and a hydrophobic drug (paclitaxel) joined with the polymer in the organic phase (O) of a double W1/O/W2 emulsion to obtain porous PLGA microspheres useful for inhalation therapy in lung cancer [69]. Zhang et al. included two growth factors (bone morphogenetic protein and vascular endothelial growth factor) dissolved in the inner aqueous phase of a double W/O/W emulsion to prepare PLGA microspheres able to control the release of the two proteins. Subsequently, they were included in inorganic scaffolds and evaluated in vitro and in vivo for the treatment of bone

necrosis [70]. To our knowledge, this is the first time that the S/O/W emulsion method has been employed to microencapsulate more than one active compound in the same MS.

Approximately 0.05 mg of microspheres was injected intravitreally in each treated eye. According to that, the administered GDNF dose in each formulation was 1.735 ng and 1.105 ng for the GDNF-PLGA/VitE MSs and the GDNF/melatonin-PLGA/VitE MSs, respectively. Although the GDNF burst observed for the GDNF/VitE MSs (1.30 ng) and the GDNF/VitE/Mel MSs (0.48 ng) would mimic bolus injections, the half-life of the neurotrophic factor would assure effective concentrations only for several days. In pharmacokinetic studies performed after a single injection of GDNF (bolus) in porcine eyes, the levels of the neuroprotective factor in the vitreous were quantified for 7 days [71]. According to these data, frequent injections would be necessary to assure effective concentrations in the target site over the long term. Recently, it has been postulated that low concentrations of GDNF would remain active if they were maintained for a long time and that low amounts of GDNF (0.8 pg/day) delivered from PLGA microspheres resulted in RGC and axonal survival 11 weeks after injection in rats with induced glaucoma supporting the fact that controlled delivery of GDNF can be useful in preserving RGCs from degeneration [72].

The neuroprotective effect of GDNF is not limited to RGCs. The utility of GDNF delivered through a microparticulate system in rescuing photoreceptors in retinal degeneration animal models has been previously demonstrated by other authors. For example, Andrieu-Soler et al. showed the efficacy of released rhGDNF after single intravitreal injections of polymeric microspheres in the rescue of photoreceptors in the rd1/rd1 mouse [73]. In this study, a statistically significant delay of rod degeneration was observed at P28 in mice receiving rhGDNF-loaded MSs compared to either untreated mice or to mice receiving blank MSs with a protective effect lasting 17 days with a total dose of 380 ng of GDNF. In the present work, lower initial doses of the neurotrophic factor delivered from PLGA microspheres led to photoreceptor rescue, and was observed after 63 days of intravitreal injection of GDNF MSs in the *rho* (-/-) mice. Furthermore, considering that the GDNF-loaded MSs prepared in this study were able to release the neurotrophic factor for at least 6 months, according to recently published pharmacokinetic studies in rabbits [74], it can be assumed that the neuroprotective effect of the systems presented might be longer lasting. In this work, intravitreal injections of GDNF-PLGA/VitE MSs and GDNF/melatonin-PLGA/VitE MSs showed a prosurvival

effect on photoreceptors in the *rho* ($-/-$) mouse. Effects were demonstrated through measurements.

Interestingly, a similar neuroprotective effect was observed when GDNF was codelivered with melatonin, even with a less than twofold amount of GDNF released from GDNF/melatonin-loaded PLGA/VitE MSs according to in vitro studies. In this study, the amount of GDNF released from the particles resulted in a lower combination of GDNF and melatonin reinforcing the hypothesis that low amounts of neuroprotective active substances released for a long duration showed useful results in chronic neurodegenerative diseases affecting the back of the eye. However, further studies are necessary to confirm this hypothesis. An interesting result observed in the present study is that photoreceptor rescue appeared uniform across the entire retina when both formulations, GDNF-loaded PLGA/VitE MSs and GDNF/melatonin-loaded PLGA/VitE MSs, were injected, which might be indicative of the continuous delivery of the active compounds in the retina, an additional benefit of the prolonged sustained release of the two active compounds delivered to the vitreous. Morphological studies were further supported by the ERG experiments, confirming that visual function was extended in *rho* ($-/-$) retinas treated with GDNF-PLGA/VitE MSs and GDNF/melatonin-PLGA/VitE MSs.

Attempts have been made to explore the use of novel drug delivery systems for codelivery of bioactive factors in the eye. Recently, Nagai et al. prepared a microreservoir system intended for transcleral implantation composed of a non-biodegradable polymer combination capable of coreleasing small active molecules for 4 weeks [75]. The system was successfully employed for coadministration of a prostaglandin analog and a free radical scavenger for neuroprotection purposes against light-induced retinal damage in rats [76]. However, to our knowledge, the present study is the first study evaluating the long-term codelivery of two active substances (of large and small molecular weights) included in the same biodegradable drug delivery system for intravitreal administration.

Conclusions: A single intravitreal injection of GDNF-loaded PLGA/VitE MSs and GDNF/melatonin-loaded PLGA/VitE MSs promoted the anatomic and functional rescue of photoreceptors in *rho* ($-/-$) mice. The use of these novel intraocular drug delivery systems enables the efficient codelivery of therapeutic active substances for the treatment of retinal diseases and may be useful in protection against photoreceptor degeneration in retinal disease. We suggest that the combinatorial approach presented here should be useful in other retinal degenerative models.

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