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Topical Administration of GLP-1 Receptor Agonists Prevents Retinal Neurodegeneration in Experimental Diabetes

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COMPLICATIONS

Retinal neurodegeneration is an early event in the pathogenesis of diabetic retinopathy (DR). Since glucagon-like peptide 1 (GLP-1) exerts neuroprotective effects in the central nervous system and the retina is ontogenically a brain-derived tissue, the aims of the current study were as follows: *1*) to examine the expression and content of GLP-1 receptor (GLP-1R) in human and *db/db* mice retinas; *2*) to determine the retinal neuroprotective effects of systemic and topical administration (eye drops) of GLP-1R agonists in *db/db* mice; and *3*) to examine the underlying neuroprotective mechanisms. We have found abundant expression of GLP-1R in the human retina and retinas from *db/db* mice. Moreover, we have demonstrated that systemic administration of a GLP-1R agonist (liraglutide) prevents retinal neurodegeneration (glial activation, neural apoptosis, and electroretinographical abnormalities). This effect can be attributed to a significant reduction of extracellular glutamate and an increase of prosurvival signaling pathways. We have found a similar neuroprotective effect using topical administration of native GLP-1 and several GLP-1R agonists (liraglutide, lixisenatide, and exenatide). Notably, this neuroprotective action was observed without any reduction in blood glucose levels. These results suggest that GLP-1R activation itself prevents retinal neurodegeneration. Our results should open up a new approach in the treatment of the early stages of DR.

Diabetic retinopathy (DR) has been classically considered to be a microcirculatory disease of the retina. However, before any microcirculatory abnormalities can be detected under ophthalmoscopic examination, retinal neurodegeneration is already present (1,2). In other words, retinal neurodegeneration is an early event in the pathogenesis of DR (3–5). Neuroretinal functional abnormalities can be detected by means of electrophysiological studies in patients with diabetes with <2 years of diabetes evolution, which is before microvascular lesions can be detected under ophthalmologic examination (6–8). In addition, there is emergent evidence that neurodegeneration and microvascular impairment are closely related and, consequently, that the term "neurovascular unit impairment" is currently used when describing the early events in the pathogenesis of DR (9,10). Therefore, therapeutic strategies based on neuroprotection could be effective not only in preventing or arresting retinal neurodegeneration but also in preventing the development and progression of the specific microvascular abnormalities that exist in the early stages of DR.

Recently, we have characterized the neurodegenerative process that occurs in the retinas of C57BL/KsJ-*db/db* mice (11). We found that the *db/db* mouse reproduces the features of the neurodegenerative process that occurs in the human diabetic eye. Thus, our results suggest that the C57BL/KsJ-*db/db* mouse is an appropriate experimental model for testing neuroprotective agents in DR.

Glucagon-like peptide 1 (GLP-1) exerts neuroprotective effects in both the central and peripheral nervous system (12–14). Given that the retina is ontogenetically a brain-derived tissue, it is reasonable to expect that GLP-1 could

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also be useful in preventing or arresting retinal neurodegeneration in the setting of DR. In fact, it has recently been shown that intravitreal injections of exendin-4 (a GLP-1 receptor [GLP-1R] agonist) prevent electroretinography (ERG) abnormalities and morphological features related to neurodegeneration in rats with streptozotocin-induced diabetes (15) and in Goto-Kakizaki rats (16). In addition, both GLP-1 and exendin-4 were able to completely protect cultured rat hippocampal neurons against glutamate-induced apoptosis (17). This is important because glutamate excitotoxicity is a major mediator of neurodegeneration in DR (10). Furthermore, GLP-1 also protects hippocampal neurons against advanced glycation end product–induced damage (18).

GLP-1R expression has been found in retinas from rats (19) and chickens (20), and in ARPE-19 cells (an immortalized line of human retinal pigment epithelium [RPE]) (21). However, the presence of GLP-1R has never been examined in the human retina. GLP-1R expression in the retina could be contemplated as a new target for treating neurodegeneration based on GLP-1 analogs. However, in the event that the systemic administration of GLP-1R agonists was effective in abrogating neurodegeneration, it would be very difficult to know whether the beneficial effect was directly due to GLP-1R activation or the result of their hypoglycemic action. Since the intraocular administration of GLP-1 analogs seems unlikely to lower blood glucose levels, this approach could be useful for answering this question. In addition, given that in the early stages of DR intravitreous injections are inappropriately invasive, a proof of concept on the effectiveness of GLP-1 topically administered (eye drops) seems necessary.

On this basis, the aim of the current study was to examine the expression and content of GLP-1R in human retinas from donors who did not have diabetes. In addition, the retinal neuroprotective effects of systemic and topical administration of GLP-1R agonists were evaluated. Finally, in order to shed light on the neuroprotective mechanisms, the apoptotic/survival signaling pathways and the levels of glutamate as well as glutamate/aspartate transporter (GLAST) have been assessed.

RESEARCH DESIGN AND METHODS

Human Studies

Human Retinas

Retinas were obtained from the tissue bank of our center. A total of eight donors with diabetes and eight donors without diabetes matched by age and sex were included in the study. One eye cup was harvested in order to separate neuroretina from RPE, and samples of both tissues were immediately frozen with liquid nitrogen and stored at −80°C. Tissue samples derived from this eye cup were used for the studies of gene and protein expression. The other eye cup was also harvested, and both RPE and neuroretina were soaked in paraffin and used for performing immunohistochemical studies. The time period from death to eye enucleation was <4 h. The general characteristics of patients with diabetes and control subjects and the cause of death are shown in Supplementary Table 1.

The procedure for eye cup donation and for the handling of this biologic material is rigorously regulated by the protocol of donations of the Blood and Tissue Bank of the Catalan Department of Health and was approved by the ethics committee.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Madrid, Spain). Then, RNA samples were treated with DNAse (Qiagen, Madrid, Spain) to remove genomic contamination and were purified on an RNeasy MinElute column (Qiagen). RNA quantity was measured on a Nanodrop spectrophotometer, and integrity was determined on an Agilent 2100 Bioanalyzer. Reverse transcription was performed with a High Capacity Kit (Applied Biosystems, Madrid, Spain) with random hexamer primers. The RT-PCR was performed using primers for GLP-1R (5′-TTGGGGTGAACTTCCTCATC-3′ and 5′-CTTGGCAAGTCTGCATTTGA-3′, forward and reverse, respectively) and GLP-1 (5′-CAGGAATAACATTGCCAAA-3′ and 5′-TCTGGGAAATCTCGCCTTC-3′, forward and reverse, respectively). β-Actin was used as a constitutive gene.

Protein Extraction and Western Blotting

Protein extracts from isolated RPE and neuroretina samples were prepared by homogenization with radioimmunoprecipitation assay lysis buffer containing 10 mmol/L EDTA acid and proteinase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L Na_3VO_4, and 20 mmol/L NaF) using the Brinkmann Polytron PT 10/35 (ALT, East Lyme, CT). Extracts were cleared by microcentrifugation at 10,000*g* for 10 min at 4°C. The supernatants were aliquoted and stored at −80°C. Protein concentrations were determined with a BCA kit (Thermo Scientific, Rockford, IL).

Total protein (5 μg) was separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ). The membranes were incubated with blocking 5% nonfat dried milk in 10 mmol/L Tris-HCl and 150 mmol/L NaCl, pH 7.5, over 1 h, and then were incubated in primary antibody against GLP-1R (1:4,000; Abcam, Cambridge, U.K.) in 0.05% Tween-20, 10 mmol/L Tris-HCl, and 150 mmol/L NaCl, pH 7.5. Immunoreactive bands were visualized using enhanced chemiluminescence (Millipore, Madrid, Spain). For densitometric analysis of Western blots we have used a GS-800 calibrated densitometer (Bio-Rad Laboratories, Madrid, Spain) and Quantity One software version 4.6.2 (Bio-Rad Laboratories).

Immunohistochemistry

Retinal sections (5 μm) of eyes from human donors (eight donors without diabetes and eight donors with diabetes) were deparaffinized in xylol and rehydrated in graded ethanol. To eliminate autofluorescence, slides were

washed in potassium permanganate. Then, sections were incubated for 1 h in 2% BSA and 0.05% Tween in PBS to block unspecificities. GLP-1R (ab39072) and GLP-1 (ab133329) primary antibodies (1:500; Abcam) were incubated overnight at 4°C in the same blocking buffer. Then, sections were washed and incubated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) at room temperature for 1 h. Slides were coverslipped with a drop of mounting medium containing DAPI for visualization of cell nuclei (Vector Laboratories, Burlingame, CA).

Images were acquired with a confocal laser-scanning microscope (FV1000; Olympus, Hamburg, Germany) at ×40 using the 488- and 405-nm laser lines, and each image was saved at a resolution of 1,024 × 1,024 pixels.

Animal Studies

C57BL/KsJ-*db*/*db* male mice (BKS.Cg-*Dock7*^{m}+/+*Lepr*^{db}/J) and *db*/+ (congenic nondiabetic littermates) were obtained from Charles River Laboratories. Genotyping was performed to confirm the absence of the rd8 mutation. Blood glucose concentrations were measured from the tail vein (glucose assay kit; Abbott Laboratories, Abbott Park, IL).

This study was approved by the Animal Care and Use Committee of the Vall d'Hebron Research Institute. All the experiments were performed in accordance with the tenets of the European Community (86/609/CEE) and ARVO (Association for Research in Vision and Ophthalmology).

Interventional Study

Systemic Treatment

Eight-week-old *db*/*db* mice received a restrictive diet (normal chow diet [Teklad Global 18% protein rodent diet; Harlan Laboratories] restricted to 60% of total daily calories; 13 kcal/day; n = 10) or normal chow diet with subcutaneous injections of either vehicle (PBS, pH 7.3–7.5; n = 12) or liraglutide (400 μg/kg/day; n = 12) for 15 days. Twelve nondiabetic mice that were fed ad libitum and were matched by age served as the control group. At day 15, the animals were killed by cervical dislocation and the eyes were enucleated.

Topical Ocular Treatment

Liraglutide (400 μg/kg/day; 5 μL) (n = 10) or vehicle (0.9% sodium chloride; 5 μL) (n = 10) eye drops were administered directly onto the superior corneal surface of each eye using a micropipette in 8-week-old mice. Twelve nondiabetic mice matched by age served as the control group. The treatment (liraglutide or vehicle) was administered twice daily for 15 days. On day 15, the drop of liraglutide or vehicle was administered ~2 h prior to necropsy. Mice were killed by cervical dislocation.

To assess whether liraglutide topically administered reaches the retina, GLP-1 concentration after the last topical dose of liraglutide (n = 4 *db*/*db* mice; 8 eyes) or vehicle (n = 4 *db*/*db* mice; 8 eyes) administered on day 15 was evaluated. For this purpose, the animals were killed at 2 h after the single topical administration. GLP-1 level was measured by immunohistochemistry following the methodology described below. In addition, in order to estimate the ocular dose-dependent absorption of liraglutide administered by eye drops, 12 mice were treated with the following three different doses of liraglutide: 80 μg (n = 4), 180 μg (n = 4), and 240 μg (n = 4). Sixty minutes after the topical administration, the mice were killed and the retinas were processed. Finally, a dose efficacy study was performed. For this purpose, we used eye drops of liraglutide at 40, 200, and 400 μg/kg/day (six mice for each dose).

Apart from liraglutide, native GLP-1 (400 μg/kg/day; 5 μL/eye twice) (n = 6), lixisenatide (20 μg/kg/day; 5 μL/eye twice) (n = 6), and exenatide (40 μg/kg/day; 5 μL/eye twice) (n = 6) were also tested in order to assess their effectiveness in preventing retinal neurodegeneration.

ERG

Full-field ERG recordings were measured using the Ganzfeld ERG platform (Phoenix Research Laboratories, Pleasanton, CA), as reported elsewhere (11) and following the recommendations of the International Society for Clinical Electrophysiology of Vision (22).

Tissue Processing

Mice were killed by cervical dislocation. The eyes were immediately enucleated, and the neuroretina was separated. The neuroretina from one of the eyes was frozen in liquid nitrogen and stored at −80°C for protein assessments. The other eye was fixed in 4% paraformaldehyde within ~6 h after enucleation. Immunohistochemical studies were performed on paraffinized sections.

mRNA Expression of GLP-1R

GLP-1R expression was analyzed by RT-PCR (GGGTCTC TGGCTACATAAGGACAAC and AAGGATGGCTGAAGCG ATGAC were the primers used [forward and reverse, respectively]).

Immunohistochemical Analysis for Glial Activation Assessment

Glial activation was evaluated by fluorescence microscopy using specific antibodies against glial fibrillar acidic protein (GFAP) following the methodology described elsewhere (11). To evaluate the degree of glial activation, we used a scoring system based on the extent of GFAP staining (23) that had been used previously by our group (11).

Immunohistochemical Analysis for Apoptosis Assessment

Apoptosis was evaluated using the TUNEL method, as previously described (11). Sections of retina were permeabilized by incubation at room temperature for 5 min with 20 μg/mL freshly prepared proteinase K solution. Apoptotic cells were identified using green fluorescence (1:200 dilution prepared in PBS; Alexa Fluor 594 goat-anti-rabbit; Invitrogen). For evaluation by fluorescence microscopy, an excitation wavelength in the range of 450–500 nm (e.g., 488 nm)

and detection in the range of 515–565 nm (green) was used. The results are presented as the percentage of TUNEL-positive cells with respect to the Hoechst-stained cells obtained by ImageJ software.

Other Immunohistochemistry Analyses

GLAST was evaluated by fluorescence microscopy using specific antibodies, as previously reported (11). cAMP immunofluorescence was quantitated using a mouse monoclonal antibody (1:200; ab24851; Abcam). The disruption of the blood-retinal barrier (BRB) was assessed by measuring albumin leakage (sheep polyclonal; 1:500; ab8940; Abcam). In addition, immunofluorescence for vascular endothelial growth factor (VEGF; rabbit polyclonal; 1:150; ab46154; Abcam) and interleukin-1β (IL-1β; rabbit polyclonal; 1:100; ab9722; Abcam) was measured.

Glutamate Quantification

Quantification of glutamate was performed by reverse-phase ultra-performance liquid chromatography (Acquity UPLC; Waters, Milford, MA) as aminoquinoline derivatives (AccQTag Chemistry, MassTrak AAA method and instruments; Waters), following the methodology previously described by Narayan et al. (24).

Western Blot Analyses

Neuroretinas were extracted in 50 μL of lysis buffer. A total of 20 μg protein was resolved by 10% (v/v) SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA). The blots were probed with rabbit anti–GLP-1R (1:1,000; ab39072; Abcam). The same blots were stripped and reblotted with a rabbit primary antibody specific to CypA (1:5,000; Enzo Life Sciences Inc., Farmingdale, NY) to normalize protein levels. Densitometric analysis of the autoradiographs was performed with ImageJ software. Results are presented as densitometry arbitrary units.

Several representative mediators of apoptotic (Fas/FasL, caspase 8, Bax, p53), antiapoptotic (Bcl-xL), neuroinflammatory (inducible nitric oxide synthase [iNOS]), and insulin signaling (phosphorylated AKT [pAKT]/AKT) pathways were also analyzed by Western blot. The list of antibodies used in Western blotting are described in Supplementary Table 2.

Statistical Analysis

The results are expressed as the mean ± SD. Statistical comparisons were performed with Student unpaired and paired *t* tests. When multiple comparisons were performed, one-way ANOVA followed by the Bonferroni test was used. The Fisher exact test was used to analyze categorical variables. Levels of statistical significance were set at $P < 0.05$.

RESULTS

GLP-1 and GLP-1R Are Expressed in Human Retinas

GLP-1 was expressed in human retinas, mainly in the ganglion cell layer (GCL). We found significantly lower levels of both mRNA and protein content in the retinas from patients with diabetes compared with control subjects without diabetes matched by age (Fig. 1*A*, *C*, and *E*).

GLP-1R was expressed in human retina at levels even higher than those observed in human bowel and liver (Fig. 1*B*). No significant differences in GLP-1R mRNA were detected in either the RPE or neuroretina between donors with and without diabetes.

No differences in GLP-1R protein concentration were observed between retinas from donors with and without diabetes assessed by Western blot in the neuroretina (Fig. 1*F*) and the RPE. Furthermore, no differences in GLP-1R immunofluorescence (arbitrary units) were detected between donors with and without diabetes (Fig. 1*D*). As occurred with GLP-1, GLP-1R was mainly expressed in the GCL. However, sparse staining was also observed in the inner nuclear layer (INL) and the outer nuclear layer (ONL) (Fig. 1*D*).

GLP-1R Is Expressed in *db/db* Retinas

mRNA expression of GLP-1R was detected in retinas of *db/db* mice as well as in *db/+* mice, and, as occurred in humans, GLP-1R protein abundance was independent of the presence of diabetes (Supplementary Fig. 1).

Systemic Administration of a GLP-1R Agonist Prevents Retinal Neurodegeneration in *db/db* Mice

As expected, *db/db* mice that were fed a restrictive diet for 15 days presented with lower weight and blood glucose levels than the placebo group that was fed ad libitum (Supplementary Fig. 2). Compared with the placebo group, the group of *db/db* mice treated with subcutaneous injections of liraglutide also presented with a reduction of blood glucose levels, but it was significantly less than that observed in *db/db* mice treated with a restrictive diet.

Glial Activation

In the retinas of diabetic mice treated with placebo, GFAP expression was prominent along the inner limiting membrane (in Müller cell endfeet) and in Müller cell radial fibers extending through both the inner and outer retina (Fig. 2*A*). Diabetic mice treated with liraglutide presented with a significantly lower GFAP immunofluorescence score than diabetic mice treated with vehicle ($P < 0.001$), and with a score similar to that of nondiabetic mice (P = NS) (Fig. 2*B*). Notably, the prevention of glial activation with liraglutide tended to be higher than that obtained with a restrictive diet ($P = 0.07$), even though the reduction in blood glucose levels was significantly lower in the former group.

Retinal Apoptosis

The total percentage of retinal apoptotic cells as well as the percentage of apoptotic cells in retinal layers (ONL, INL, and GCL) in diabetic mice was significantly higher compared with that observed in retinas from age-matched nondiabetic controls ($P < 0.001$) (Fig. 2*C* and *D*). Diabetic mice treated with the GLP-1R agonist presented with a significantly lower

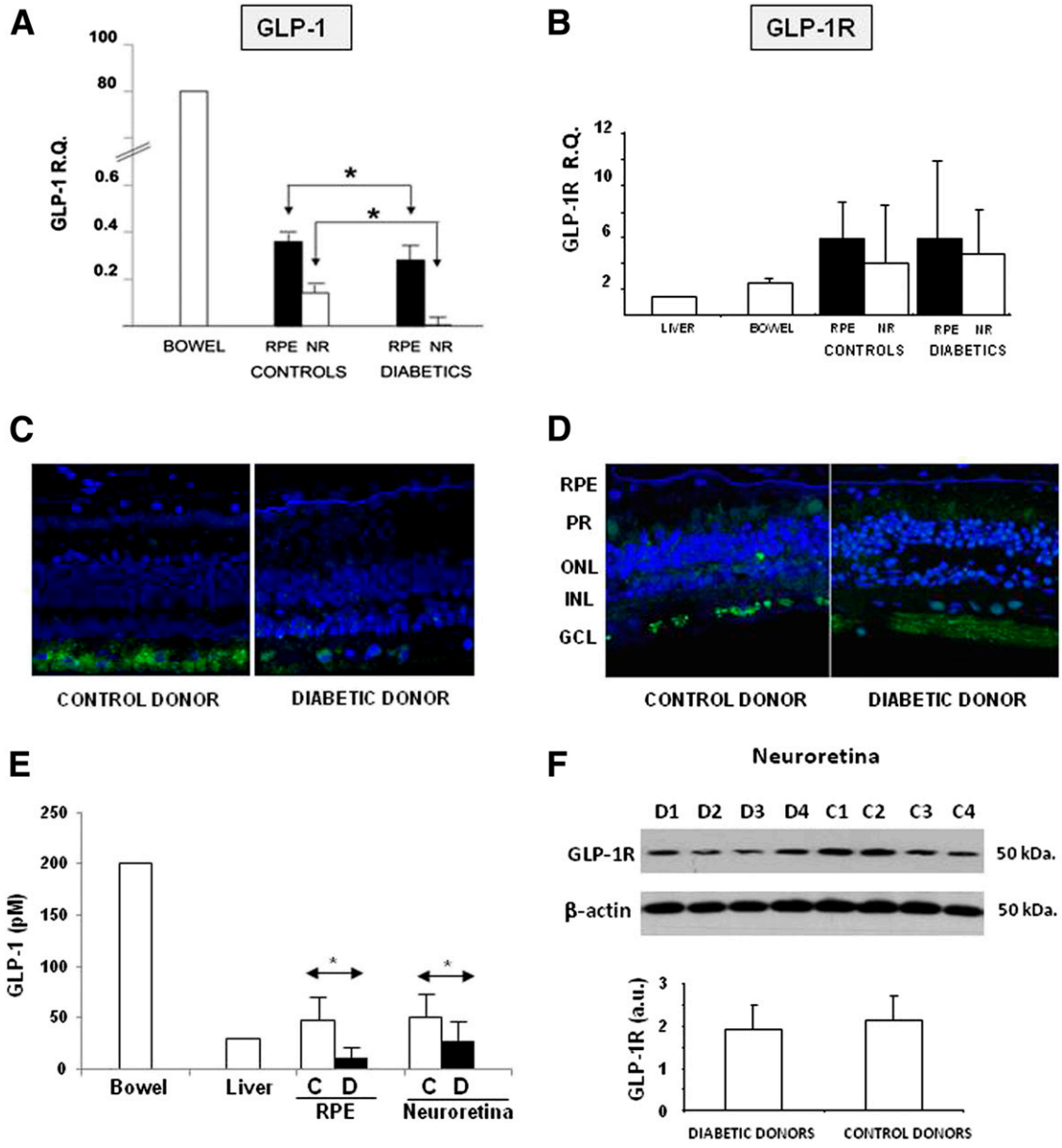


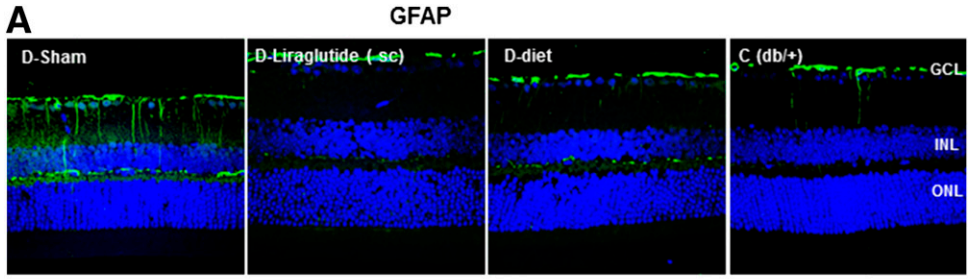
Figure 1—*A*: Real-time quantitative RT-PCR analysis of GLP-1 mRNA in human retinas. *B*: Real-time quantitative RT-PCR analysis of GLP-1R mRNA in human retinas. NR, neuroretina. *C*: Comparison of GLP-1 immunofluorescence (green) in the human retina between representative samples from a donor without diabetes and a donor with diabetes. *D*: Comparison of GLP-1R immunofluorescence (green) in the human retina between representative samples from a donor without diabetes and a donor with diabetes. Nuclei were labeled with DAPI (blue). PR, photoreceptors. *E*: GLP-1 assessment by ELISA (Millipore, Madrid, Spain). *F*: GLP-1R assessment by Western blot in the neuroretina. D1–D4, donors with diabetes 1–4; C1–C4, control donors 1–4; R.Q., relative quantification. The study was performed in eight donors with diabetes and eight donors without diabetes. The Student *t* test was used for comparisons. $^*P < 0.05$.

rate of apoptosis than diabetic mice treated with vehicle ($P < 0.001$). No differences in the percentage of apoptotic cells were observed between diabetic mice treated with liraglutide compared with those treated with a restrictive diet.

Neurodegeneration Was Prevented in Diabetic Mice Treated With GLP-1R Agonists Topically Administered Without Changes in Blood Glucose Levels

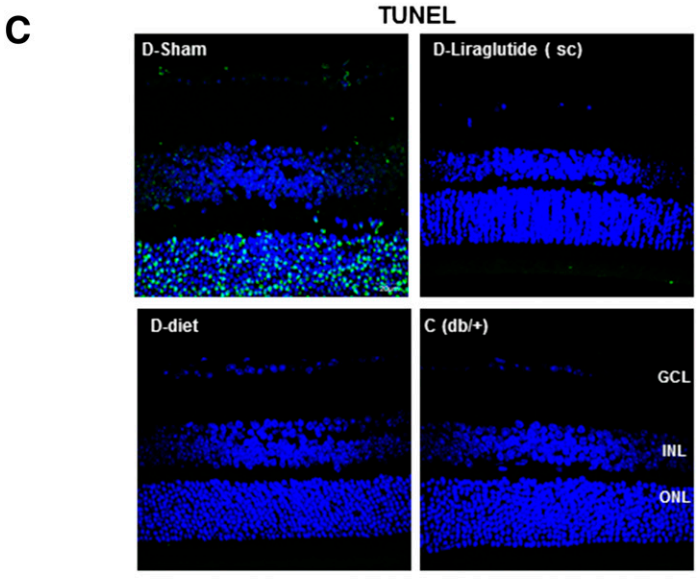
We observed that liraglutide administered by eye drops was able to reach the retina. This was demonstrated by the significant increase of GLP-1 immunofluorescence in the retina 2 h after topical administration (Fig. 3*A*).

Liraglutide topically administered (eye drops) did not reduce blood glucose levels (Supplementary Fig. 2*B*). However, it was able to prevent the morphological and functional neurodegenerative abnormalities caused by diabetes at the same level as after subcutaneous administration. The effect of eye drops containing liraglutide on preventing glial activation and apoptosis is shown in Fig. 4.



B

SCORE	D-Sham	D-Liraglutide (sc)	D-diet	Control
1	0.00	65.00	50.00	100.00
2	0.00	35.50	20.83	0.00
3	20.00	0.00	16.67	0.00
4	40.00	0.00	12.50	0.00
5	40.00	0.00	0.00	0.00



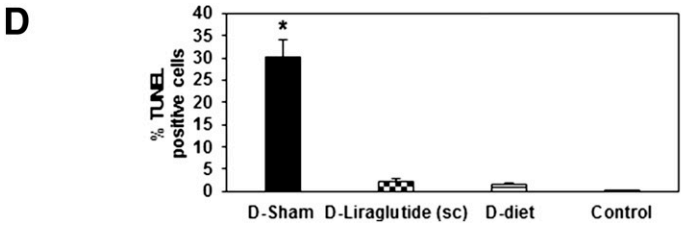


Figure 2—Systemic liraglutide administration. *A*: Glial activation; comparison of GFAP immunoreactivity (green) in the retina among representative samples from diabetic mice treated with vehicle, liraglutide, and restrictive diet and from a nondiabetic mouse. Nuclei were labeled with Hoechst stain (blue). *B*: Quantification of glial activation based on the extent of GFAP staining. The scoring system was as follows: Müller cell endfeet region/GCL only (score 1); Müller cell endfeet region/GCL plus a few proximal processes (score 2); Müller cell endfeet plus many processes, but not extending to ONL (score 3); Müller cell endfeet plus processes throughout with some in the ONL (score 4); and Müller cell endfeet plus lots of dark processes from GCL to outer margin of ONL (score 5). *C*: Apoptosis; TUNEL-positive immunofluorescence (green) in a representative mouse from each group. *D*: Percentage of TUNEL-positive cells in the neuroretina. Results are the mean ± SD. $n = 10$ mice per group. One-way ANOVA and Bonferroni multiple comparison test were used. $^*P < 0.05$ compared with the other groups. C, control mice; D, diabetic mice.

Eye drops of native GLP-1, lixisenatide, and exenatide were also effective in preventing glial activation (Fig. 4*A* and *B*) and apoptosis (Fig. 4*C* and *D*) without differences among them and were very similar to that observed with liraglutide. As expected, no reduction in blood glucose levels was observed after topical treatment with of all these GLP-1R agonists (data not shown).

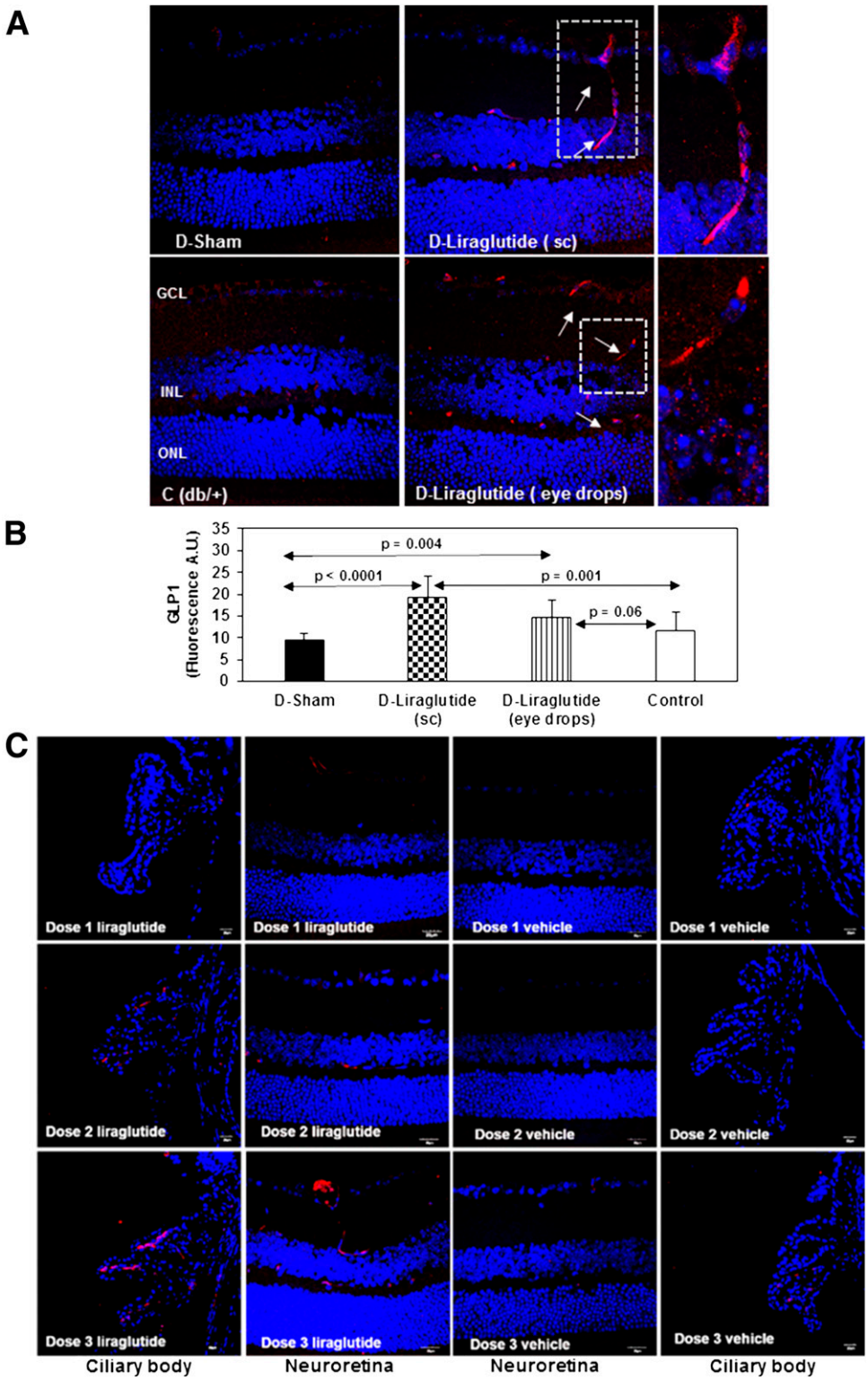
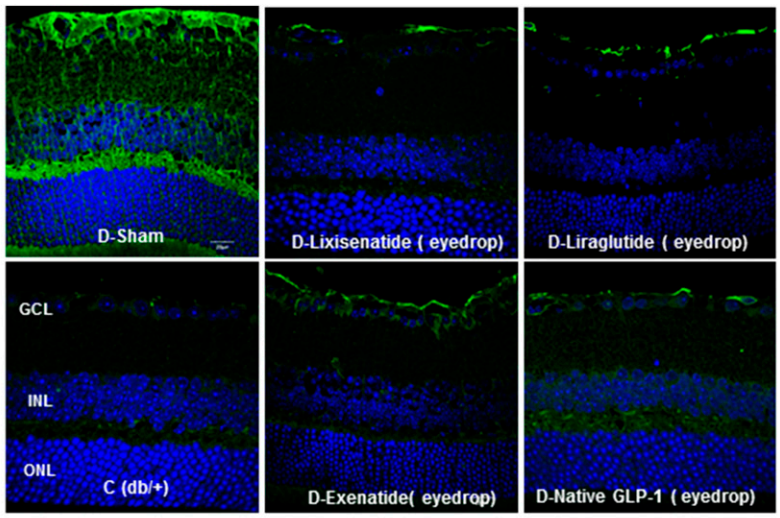


Figure 3—*A*: Immunofluorescence showing the increase of GLP-1 (magenta) in the retina after liraglutide administration in representative samples from a diabetic mouse treated with subcutaneous liraglutide and a diabetic mouse treated with eye drops containing liraglutide. A representative sample from a diabetic mouse treated with vehicle (sham) and a nondiabetic mouse are also shown. Nuclei were labeled with Hoechst stain (blue). Arrows show how the liraglutide reached the retina. *B*: Quantification of GLP-1 immunofluorescence. $n = 4$ mice (8 eyes) per group. Results are the mean ± SD. One-way ANOVA and the Bonferroni multiple comparison test were used. *C*: Immunofluorescence staining for GLP-1 (magenta) in sections of neuroretina (middle panels) and ciliary body from a diabetic mouse showing a dose-dependent liraglutide accumulation. Mice were treated with a single dose of liraglutide at different concentrations (Dose 1 80 μg [$n = 4$], Dose 2 180 μg [$n = 4$], Dose 3 240 μg [$n = 4$]). After 60 min of topical administration, the mice were killed and the retinas were processed. C, control mice; D, diabetic mice.

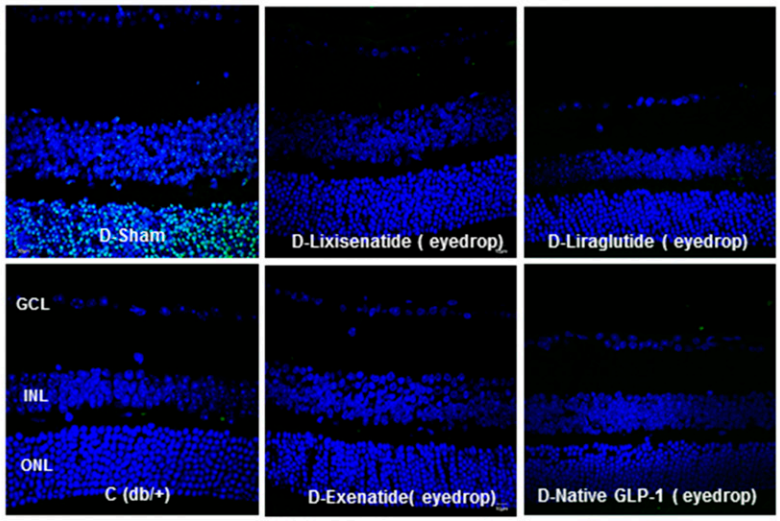
A



B

% positive GFAP labeling						
score	D-Sham (eyedrop)	D- Lixisenatide (eyedrop)	D-Liraglutide (eyedrop)	D-Native GLP-1 (eyedrop)	D-Exenatide (eyedrop)	control (db/+)
1	0.00	100	100	65.00	70.00	75.00
2	0.00	0.00	0.00	20.00	30.00	25.00
3	15.00	0.00	0.00	15.00	0.00	0.00
4	42.50	0.00	0.00	0.00	0.00	0.00
5	42.50	0.00	0.00	0.00	0.00	0.00

C



D

% TUNEL positive cells					
D-Sham (eyedrop)	D- Lixisenatide (eyedrop)	D-Liraglutide (eyedrop)	D-Native GLP-1 (eyedrop)	D-Exenatide (eyedrop)	control (db/+)
23.83 ± 1.82*	1.23 ± 0.36	0.18 ± 0.10	1.28 ± 0.53	1.50 ± 0.17	0.10 ± 0

Figure 4—Topical administration of GLP-1R agonists. *A*: Comparison of GFAP immunoreactivity (green) in the retina between representative samples from diabetic mice treated with vehicle and GLP-1R agonists (native GLP-1, lixisenatide, liraglutide, and exenatide) and a nondiabetic mouse. Nuclei were labeled with Hoechst stain (blue). *B*: Quantification of glial activation based on the extent of GFAP staining. *C*: Apoptosis; TUNEL-positive immunofluorescence (green) in a representative mouse from each group. *D*: Percentage of TUNEL-positive cells in the neuroretina. $n = 6$ mice per group. Results are the mean ± SD. One-way ANOVA and Bonferroni multiple comparison test were used. *$P < 0.05$ compared with the other groups. C, control mice; D, diabetic mice.

GLP-1R Agonist Treatment Prevents ERG Abnormalities

Treatment with liraglutide, systemically or topically administered, prevented the decreased amplitude of a-wave, b-wave, and oscillatory potentials (OPs). In addition, liraglutide abrogated the increase of implicit time of OPs observed in the diabetic mice treated with vehicle (Fig. 5).

Dose-Dependent Absorption of Topical GLP-1R Agonist and Dose-Efficacy Study

We observed that liraglutide accumulation in the retina was dependent on the dose administered (Fig. 3*B*). In addition, a dose response in terms of efficacy was observed. In this regard, the effectiveness of topical

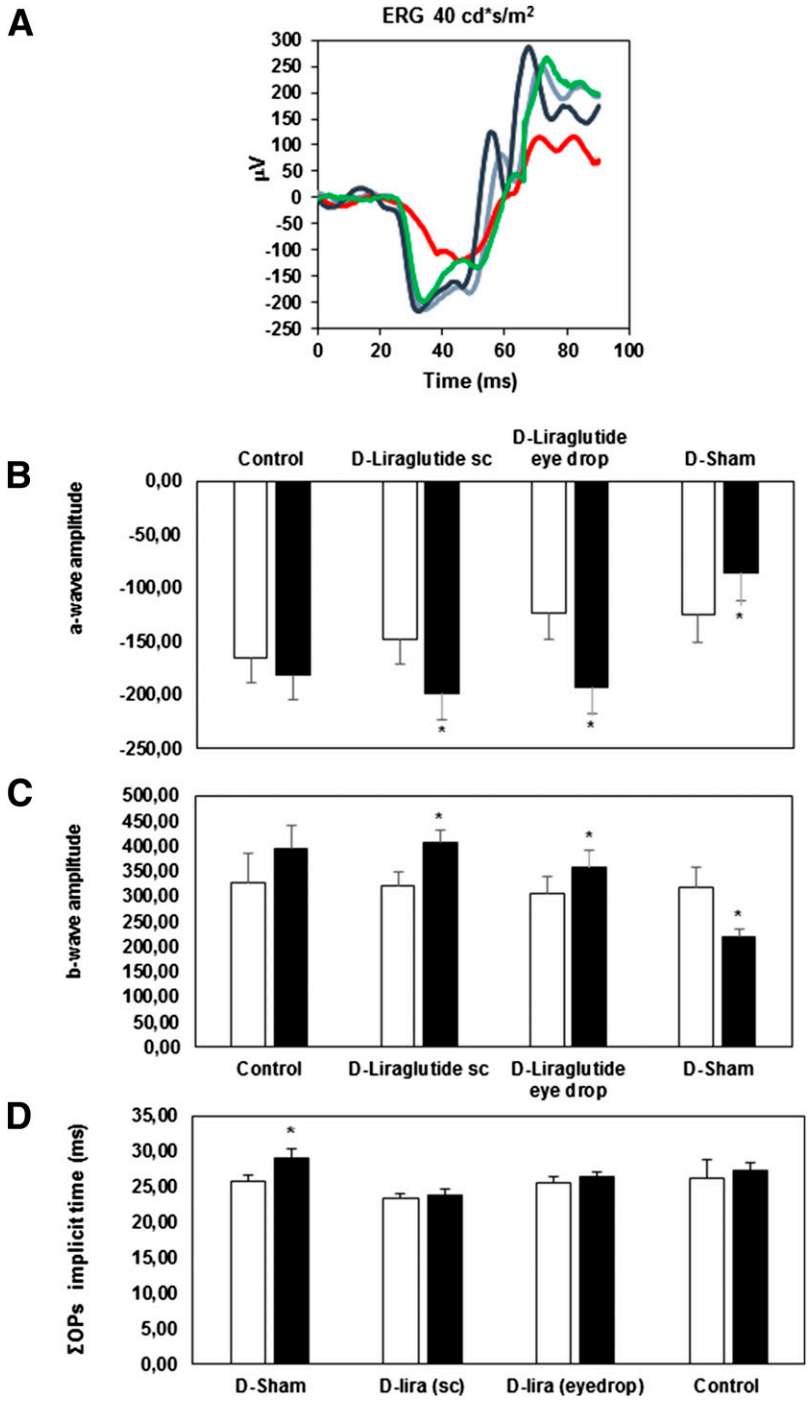


Figure 5—*A*: ERG traces in response to stimulus intensity of 40 cd/s/m^2 in a representative nondiabetic mouse (dark blue), a *db*/*db* mouse treated with vehicle (red), a *db*/*db* mouse treated with liraglutide (lira) by the subcutaneous route (gray), and a *db*/*db* mouse treated by the topical ocular route (green). cd, candela. *B*: Prevention of the decrease in ERG a-wave amplitude by liraglutide (subcutaneously and topically administered). *C*: Prevention of the decrease in ERG b-wave amplitude by liraglutide (subcutaneously and topically administered). *D*: Liraglutide (subcutaneously and topically administered) prevented the increase of OPs implicit time. We added up OP implicit time (ΣOP implicit time) for the first five OPs. Black columns, at day 14; white columns, baseline. n = 10 mice/group. Results are the mean ± SD. Differences between final and baseline ERG parameters in each group were assessed by paired Student t test. *$P < 0.05$. D, diabetic mice.

administration of liraglutide with eye drops at concentrations of 200 μg/kg/day was slightly lower than with eye drops at 400 μg/kg/day in preventing glial activation and apoptosis, and the dose of 40 μg/day was significantly less effective than the dose of 200 μg/kg/day (data not shown).

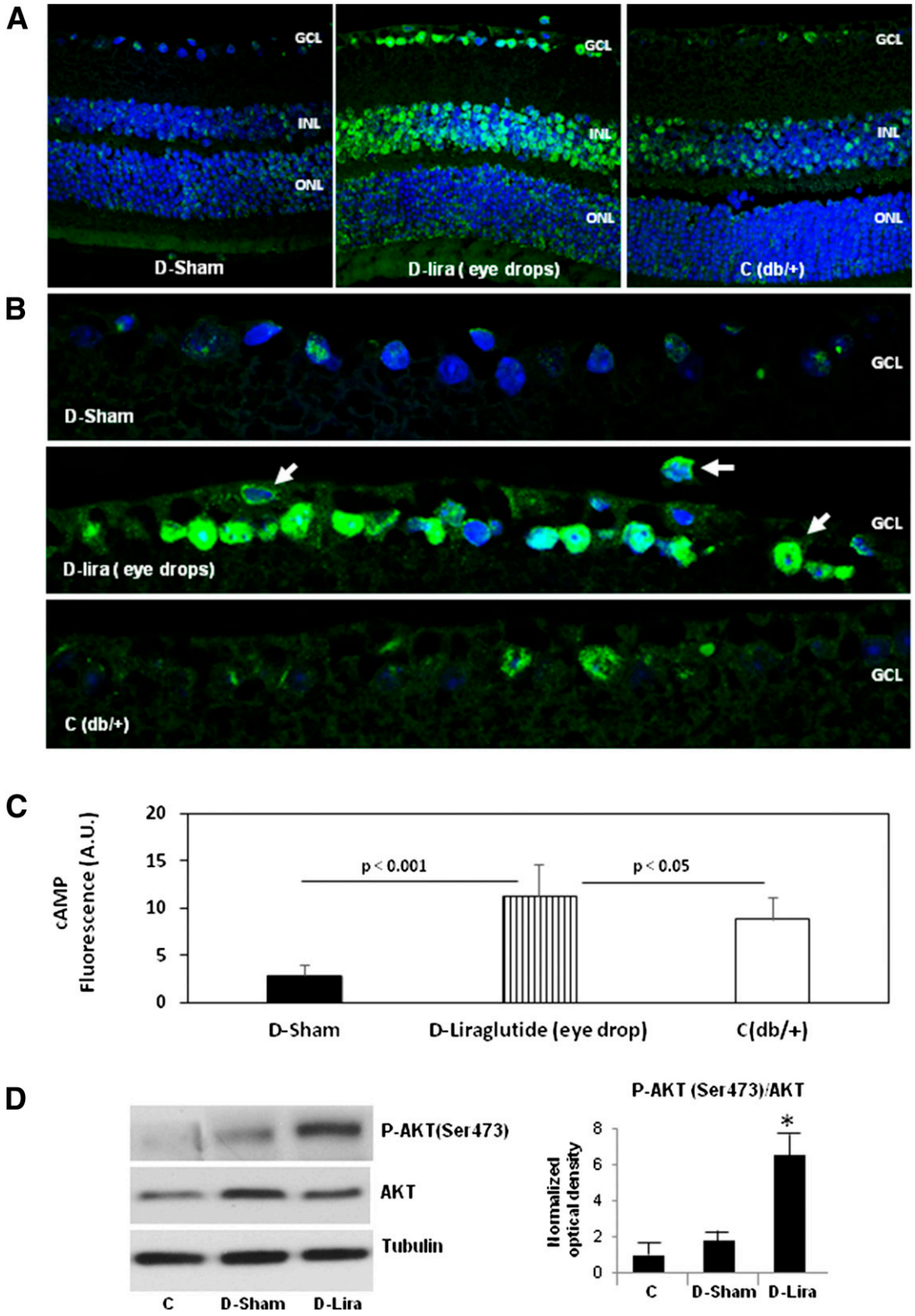


Figure 6—*A*: Comparison of cAMP immunofluorescence (green) among representative retinal samples from a *db/db* mouse treated with sham, a *db/db* mouse treated with liraglutide (lira) eye drops, and a nondiabetic mouse. Nuclei were labeled with Hoechst stain (blue). *B*: Detailed images of GCL showing the expression of cAMP (white arrows). *C*: Quantification of cAMP immunofluorescence in arbitrary units (A.U.). n = 10 mice per group. Results are the mean ± SD. One-way ANOVA and Bonferroni multiple comparison test were used. *D*: Representative Western blot analysis and quantification of the expression of pAKT and AKT in the neuroretina. Tubulin was used as a loading control. Immunoreactive pAKT was normalized to the total AKT, and the quotient of controls was set to unity. n = 6 mice per group. $^*P < 0.001$ compared with the diabetic group treated with vehicle (D-Sham). C, control mice; D, diabetic mice.

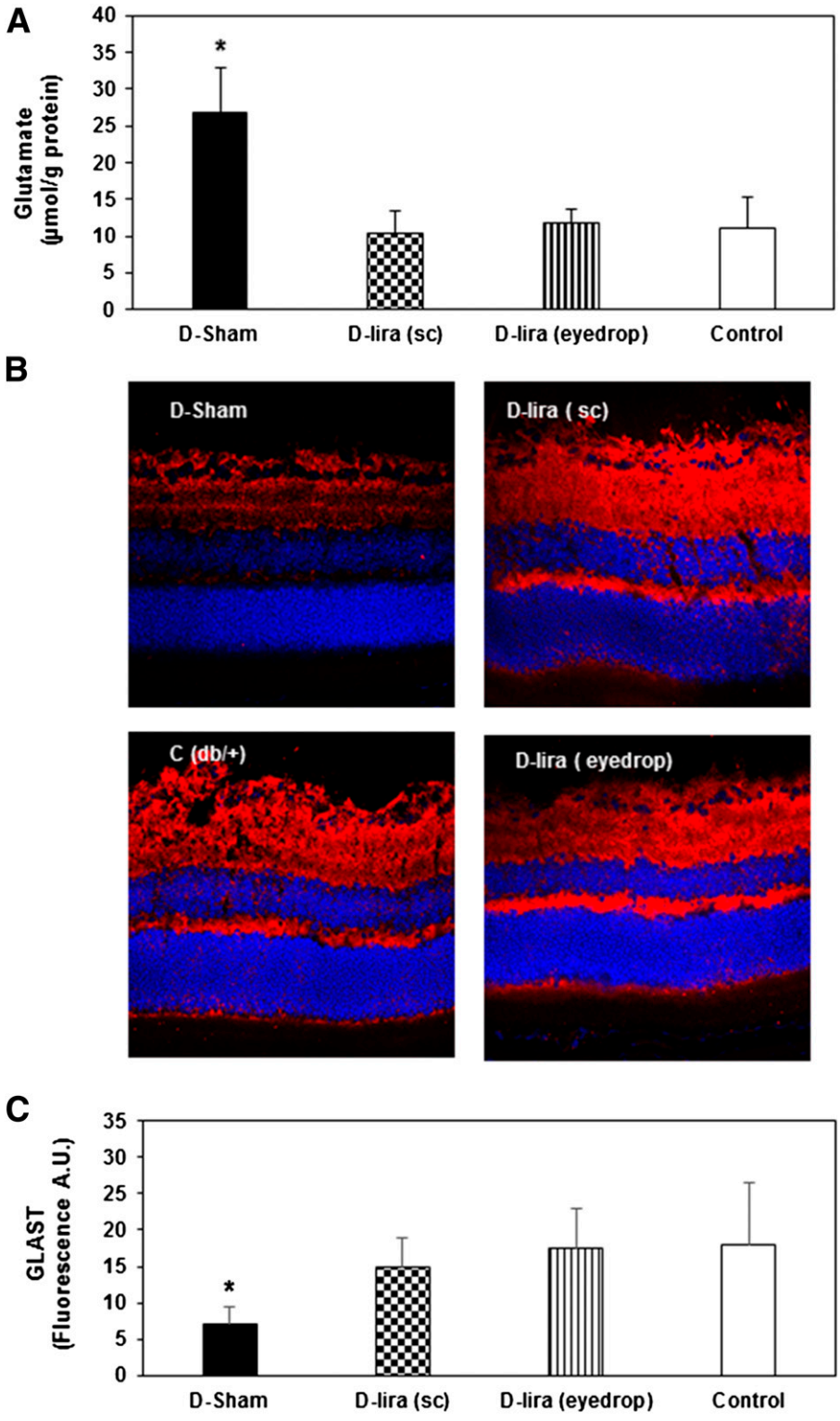


Figure 7—*A*: Retinal concentration of glutamate measured by high-performance liquid chromatography in the following groups: *db*/*db* mice treated with vehicle (black bars), *db*/*db* mice treated subcutaneously with liraglutide (lira) (chessboard bars), *db*/*db* mice treated with liraglutide eye drops (vertical stripes bars), and nondiabetic mice (white bars). *B*: Comparison of GLAST immunofluorescence (red) among representative samples from a *db*/*db* mouse treated with vehicle, a *db*/*db* mouse treated with liraglutide subcutaneously, a *db*/*db* mouse treated with liraglutide eye drops, and a nondiabetic mouse. Nuclei were labeled with Hoechst stain (blue). *C*: Quantification of GLAST immunofluorescence in arbitrary units (A.U.). $n = 10$ mice per group. Results are the mean ± SD. One-way ANOVA and Bonferroni multiple comparison tests were used. *$P < 0.001$ compared with the other groups. C, control mice; D, diabetic mice.

Mechanisms of Neuroprotective Action of GLP-1R Agonists

Since the neuroprotective effect was similar for all the GLP-1R agonists, we decided to select liraglutide to explore the underlying mechanisms of action. This was decided not only because liraglutide was the agonist used for both systemic and topical treatments but also to reduce the number of animals used in order to fulfill the ethical regulations for animal experimentation.

Demonstration of Specific Response of GLP-1R Activation

GLP-1, acting via the GLP-1R, leads to the activation of a downstream specific signaling pathways such as adenylyl cyclase and phosphatidylinositol 3-kinase/Akt. The activation of adenylyl cyclase results in an increase of cAMP, the primary second messenger of the GLP-1R (25–27). We found a significant increase in cAMP and pAKT in the retinas of diabetic mice treated with GLP-1R agonists, and, therefore, it can be assumed that the activation of the GLP-1R was produced (Fig. 6).

Effect of GLP-1R Agonist on Retinal Glutamate and GLAST Content

Liraglutide was able to prevent the increase in glutamate levels induced by diabetes (Fig. 7*A*). We did not find any difference in glutamate concentration between subcutaneous and ocular liraglutide administration.

GLAST content was downregulated in retinas from diabetic mice treated with vehicle (Fig. 7*B* and *C*). In diabetic mice treated with liraglutide, administered subcutaneously or by eye drops, GLAST downregulation was prevented (Fig. 7). We did not find any differences in GLAST immunofluorescence between subcutaneous and topical liraglutide administration.

Effect of GLP-1R Agonist on Proapoptotic/Survival Signaling

Topical liraglutide treatment prevented the upregulation of proapoptotic/proinflammatory markers (iNOS, FasL, caspase 8, P53/p-P53, Bax) and the downregulation of survival pathways (Bcl-xL) induced by diabetes (Supplementary Fig. 3). In addition, a significant increase in insulin signaling assessed by the ratio pAKT/AKT was observed (Fig. 6). Similar results were observed in retinas of diabetic mice after subcutaneous administration of liraglutide (data not shown).

Topical Administration of GLP-1R Agonists Prevents the Disruption of the BRB

In order to assess the effect of GLP-1R agonists on early microvascular impairment, we examined the albumin leakage, as well as two of the most important players in the pathogenesis of the breakdown of the BRB: VEGF and IL-1β. As expected, an overexpression of both VEGF and IL-1β was observed in diabetic mice, and it was associated with albumin leakage, thus revealing the disruption of the BRB. All these abnormalities were prevented by topical administration using eye drops containing native GLP-1 or liraglutide (the other GLP-1R agonists were not tested) (Fig. 8).

DISCUSSION

In the current study, we found for the first time the expression of both GLP-1 and GLP-1R in the human retina. Notably, the expression of GLP-1R was even higher than that detected in the liver or the bowel. However, we did not find any difference in GLP-1R (both mRNA and protein) between donors with and without diabetes. By contrast, GLP-1 was downregulated in retinas from donors with diabetes. Furthermore, we provide evidence that GLP-1R agonists prevent retinal neurodegeneration independently of their hypoglycemic action.

It has been reported that GLP-1 and longer-lasting protease-resistant analogs cross the blood-brain barrier and exert a neuroprotective action in the brains of mouse models of several neurodegenerative diseases, such as Alzheimer disease (28,29). In this regard, several clinical trials (30,31) aimed at exploring the effects of GLP-1R agonists on preventing the development of Alzheimer disease are in progress.

In this study, we demonstrated that systemic treatment with the GLP-1R agonist liraglutide prevents retinal neurodegeneration in diabetic mice. Liraglutide crosses the blood-brain barrier (14), and, therefore, it can be assumed that circulating liraglutide reaches the retina. However, given that liraglutide exerts a hypoglycemic action, it is difficult to separate the effects observed in preventing neurodegeneration because of its reduction of blood glucose levels from those induced directly by GLP-1/GLP-1R activation in the retina. Our finding that the neuroprotection obtained with subcutaneous administration of liraglutide was even higher than that observed with a restrictive diet, besides achieving less blood glucose reduction, suggests an insulin-mediated effect or a direct effect on GLP-1Rs expressed in the retina. In order to shed light on this issue, we tested the effect of the topical administration (eye drops) of liraglutide, as well as of native GLP-1, lixisenatide, and exenatide. We found that the topical administration of all these GLP-1R agonists prevented retinal neurodegenerative features induced by diabetes without any effect on blood glucose levels. These findings strongly support a direct neuroprotective effect of GLP-1R agonists that is independent of their capacity for lowering blood glucose levels or increasing insulin secretion. In this regard, native GLP-1 prevented glial activation and apoptosis in porcine retinal explants cultured under diabetic conditions (high glucose plus IL-1β) (data not shown).

It should be noted that we found a high rate of apoptosis in the retina of *db/db* mice compared with other experimental models of DR. However, apoptosis was not observed in nondiabetic mice, and a significant reduction in the most important features of neurodegeneration had been found in a previous study (11) after lowering blood glucose levels by using a restrictive diet. Therefore, the

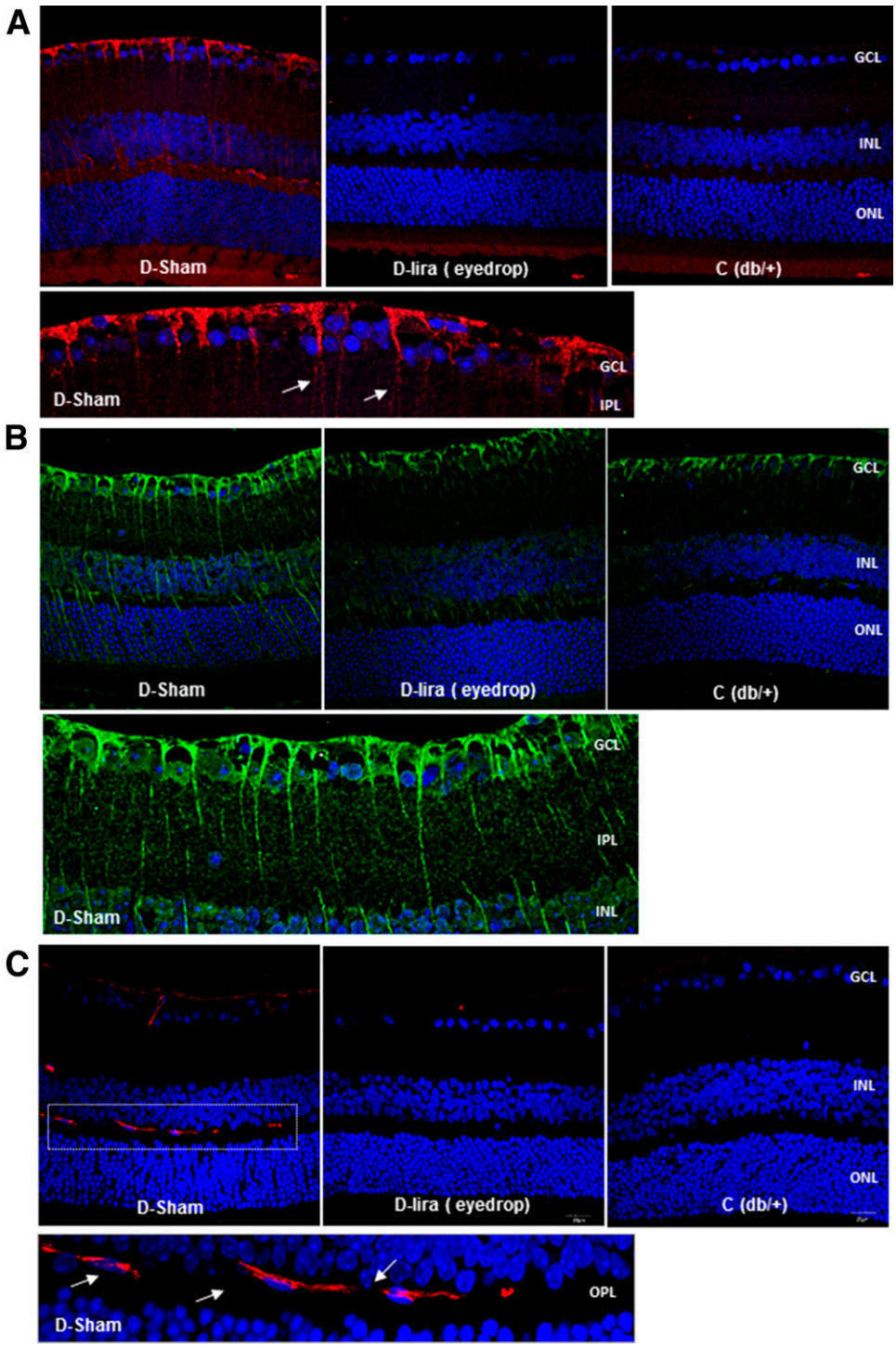


Figure 8—VEGF (red) (*A*), IL-1β (green) (*B*), and albumin (red) (*C*) immunofluorescence from a representative case of a diabetic mouse treated with vehicle (D-Sham; left image), a diabetic mouse treated with liraglutide (D-lira eye drop; middle image), and a nondiabetic mouse (control *db*/+; right image). C, control mice; D, diabetic mice; IPL, inner plexiform layer; OPL, outer plexiform layer.

neurodegeneration detected in homozygous *db*/*db* animals is related to hyperglycemia and cannot be attributed to a genetic nature. In addition, it should be emphasized that a similar high rate of TUNEL-positive cells have been reported previously, not only by our group (11,32) but also by other authors (33,34). We believe that this is not an impediment but is, on the contrary, a good model for testing the effectiveness of neuroprotective drugs such as GLP-1 agonists.

The mechanisms by which GLP-1R agonists mediate neuroprotection are still not fully understood, but there is evidence suggesting that the activation of common

pathways to insulin signaling is a relevant mechanism (35). In the current study, we found that liraglutide (systemic and topically administered) activates the AKT pathway, which is essential for the survival of retinal neurons (36,37). In addition, liraglutide was able to prevent the diabetes-induced increase of caspase-8 and the Bax (proapoptotic)/Bcl-xL (antiapoptotic) ratio. In addition, we found that liraglutide (systemically and topically administered) was able to prevent the diabetes-induced retinal activation of p53, a factor that participates in hypoxic and oxidative stress–mediated retinal cell death (38), as well as several key molecules involved in retinal apoptosis and inflammation such as FasL and iNOS (39–41).

Glutamate is the major excitatory neurotransmitter in the retina, and it has been found to be elevated in the extracellular space in experimental models of diabetes, as well as in the vitreous fluid of patients with proliferative DR (10). This extracellular and synaptic excess of glutamate leads to the overactivation of ionotropic glutamate receptors (excitotoxicity), which results in an uncontrolled intracellular calcium response in postsynaptic neurons and cell death (10). Our results suggest that liraglutide could prevent glutamate accumulation by abrogating the downregulation of GLAST induced by diabetes. GLAST, the main glutamate transporter expressed by Müller cells (42), is the most dominant glutamate transporter, accounting for at least 50% of glutamate uptake in the mammalian retina (43). In this regard, it has been reported that the retinal neuroprotective effect of other drugs (somatostatin, glial cell line–derived neurotrophic factor, and fenofibrate) in diabetic murine models was related to the upregulation of GLAST (32,44,45).

From the clinical point of view, the early identification of neurodegeneration will be crucial for implementing an early treatment based on drugs with a neuroprotective effect. However, at these stages patients are practically asymptomatic, and, therefore, aggressive treatments such as intravitreal injections are not appropriate. This opens up the possibility of developing a topical therapy (eye drops) for use in the early stages of DR. In the current study, we provide evidence that GLP-1R agonists topically administered prevent retinal neurodegeneration to the same degree as systemic administration. This finding strongly supports the concept that these drugs have a direct neuroprotective effect in the retina independent of their ability to reduce blood glucose levels. Apart from the ease of application and the possibility of their being self-administered, GLP-1R agonists administered by eye drops limit their action to the eye and minimize the associated systemic effects. Therefore, it seems reasonable to postulate that eye drops of GLP-1R agonists could be used for DR treatment in most patients with diabetes, including those patients in whom the systemic administration of GLP-1R agonists is not recommended (i.e., those with pancreatitis and those experiencing adverse gastrointestinal effects). In addition, given that the action of topically applied GLP-1R agonists is not insulin mediated, these agonists could also be useful in the subset of patients for whom these systemic treatments are not currently indicated (i.e., patients with type 1 diabetes or patients with type 2 diabetes with insulinopenia). However, topical drug administration might not work in humans despite its effects in rodents, and, therefore, clinical trials addressing these relevant questions as well as exploring the effectiveness of eye drops of GLP-1R agonists in treating other neurodegenerative retinal diseases seem warranted.

In the current study, we have demonstrated that the retinal neuroprotective effect of subcutaneous liraglutide administration was even higher than that observed with restrictive diet and achieved less of a reduction in blood glucose levels. Since liraglutide is able to cross the blood-brain barrier, it is very likely that the same could occur with the BRB, thus activating downstream survival signaling through GLP-1R expressed in the retina. Therefore, systemic administration of the GLP-1R agonists currently used in clinical practice could have an extra value for preventing DR. Head-to-head clinical trials comparing GLP-1R agonists with other antidiabetic drugs are needed to confirm this hypothesis.

It should be emphasized that we found local production of GLP-1 by the retina. This is an important finding because the local production of GLP-1 is colocalized with GLP-1R mainly in the GCL and, therefore, could exert significant autocrine/paracrine actions. In this regard, the administration of GLP-1R agonists can be contemplated as a replacement treatment of a natural neurotrophic factor that is downregulated in the diabetic retina.

Finally, we found that diabetic mice presented albumin leakage associated with an overexpression of IL-1β and VEGF. These abnormalities were prevented by eye drops containing both native GLP-1 and liraglutide. These findings give us another mechanistic reason why GLP-1R agonists can be useful for preventing not only neurodegeneration but also early microvascular impairment.

In conclusion, GLP-1R agonists, by means of a significant reduction of extracellular glutamate and an increase of prosurvival signaling, are useful in preventing retinal neurodegeneration. This could be envisaged as a useful tool for the treatment of the early stages of DR. However, specific clinical trials aimed at testing their advantages for the treatment of DR compared with other antidiabetic agents are needed. In addition, their topical administration could open up a new approach in the treatment of the early stages of DR.

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Duality of Interest. Vall d'Hebron Research Institute holds intellectual property related to the use of ocular glucagon-like peptide 1 receptor agonists to treat diabetic retinopathy. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. C.H. designed the project, obtained funds, led the analysis, wrote the manuscript, reviewed and edited the manuscript, and approved the final version of the manuscript. P.B. led the analysis, wrote the first draft of the manuscript, and approved the final version of the manuscript. L.C., M.G.-R., C.S.-A., J.A.A., and A.I.A. led the analysis, reviewed the manuscript, and approved the final version of the manuscript. A.M.V. contributed to discussion, reviewed the manuscript, and approved the final version of the manuscript. R.S. designed and coordinated the project, obtained funds, wrote the manuscript, reviewed and edited the manuscript, and approved the final version of the manuscript. C.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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