Topical administration of GLP-1 receptor agonists prevents retinal neurodegeneration in experimental diabetes

Short running title: GLP-1 and retinal neurodegeneration

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ABSTRACT

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 present study were: 1) To examine the expression and content of 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. 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.
INTRODUCTION

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 diabetic patients with less than two 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, 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 retina 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 C57BL/KsJ-db/db 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 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-1R agonist) prevent ERG abnormalities and
morphological features related to neurodegeneration in rats with streptozotozin—induced diabetes (STZ-DM) (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) chickens (20) and in ARPE-19 cells (an immortalized line of human retinal pigment epithelium) (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 analogues. 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 analogues seems unlikely to low 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 present study was to examine the expression and content of GLP-1R in human retinas from diabetic and non-diabetic donors. 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 signalling pathways and the levels of glutamate as well as glutamate/aspartate transporter (GLAST) have been assessed.
MATERIAL AND METHODS

Human Studies

Human retinas

Retinas were obtained from the Tissue Bank of our Centre. A total of 8 diabetic donors and 8 non-diabetic donors matched by age and gender were included in the study. One eye-cup was harvested in order to separate neuroretina from retinal pigment epithelium (RPE) and both tissues were immediately frozen with liquid nitrogen and stored at –80°C. Tissues 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 hours. The general characteristics of diabetic patients and controls and the cause of death are shown in table 1 of supplementary material.

The procedure for eye-cup donation and for the handling of this biologic material is rigorously regulated by the protocol of donations of the Tissue Bank of our Centre and was approved by the ethical 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 purified on an RNeasy MinElute column (Qiagen, Madrid, Spain). 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’-TTG GGG TGA ACT TCC TCA TC-3’ and 5’-CTT GGC AAG
TCT GCA TTT GA-3′ forward and reverse respectively) and GLP-1 (5´-CAGGAATAACATTGCCAAAA-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 were prepared by homogenization with RIPA lysis buffer containing 10 mM EDTA acid and proteinase inhibitors (1 mM PMSF, 2 mM Na3VO4 and 20 mM NaF) using the Brinkman PT 10/35 Polytron (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% non-fat dried milk in 10 mmol/l Tris-HCl and 150 mmol/l NaCl pH 7.5 during 1 h and then incubated in primary antibody against GLP-1R (1:4000, Abcam, Cambridge, UK) in 0.05% Tween-20, 10 mmol/l Tris-HCl, and 150 mmol/l NaCl pH 7.5. Immunoreactive bands were visualized using chemiluminescence (ECL, Millipore, Madrid, Spain). For densitometric analysis of western blots we have used a GS-800 calibrated densitometer (Bio-Rad Laboratories, Madrid, Spain) and the Quantity One software 4.6.2 (Bio-Rad Laboratories, Madrid, Spain).

**Immunohistochemistry**

Retinal sections (5 µm) of eye human donors (8 non-diabetic and 8 diabetic donors) 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 0.05% Tween in PBS to block unspecificities. GLP-1R (ab39072) and GLP-1
(ab133329) primary antibodies (1:500; Abcam, Cambridge, UK) were incubated overnight at
4ºC 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 40X using the 488 nm and 405 nm laser lines and each image was
saved at a resolution of 1024x1024 pixels.

Animal studies

Animals

C57BLKS/J db/db male mice (BKS.Cg-Dock7m+/+LeprdbJ) and db/+ (congenic non-diabetic
littermates) were obtained from Charles River Laboratories, Inc. A genotyping was performed
to confirm the absence of the rd8 mutation. Blood glucose concentrations were measured
from the tail vein (glucose assay kit; Abbott, Illinois, U.S.A.).

This study was approved by the Animal Care and Use Committee of 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 weeks-old diabetic mice (db/db) received 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 (phosphate-buffered saline, pH 7.3-7.5; n=12) or liraglutide (400 µg/kg/day; n=12) for 15 days. Twelve non-diabetic mice fed *ad libitum* and matched by age served as control group. At day 15 the animals were euthanized by cervical dislocation and the eyes 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 weeks-old mice. Twelve non-diabetic mice matched by age served as 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 approximately two hours prior to necropsy. Mice were euthanized 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 were evaluated. For this purpose, the animals were sacrificed at 2 hours after the single topical administration. GLP-1 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 three different doses of liraglutide: 80 mcg (n=4), 180 mcg (n=4) and 240 mcg (n=4). After 60 minutes of the topical administration the mice were euthanized 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 mcg/Kg/day (6 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.

**Electroretinogram**

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

**Tissue processing**

Mice were euthanized 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 approximately 6 hours after enucleation. Immunohistochemical studies were done on paraffin sections.

**mRNA expression of GLP-1R**

GLP-1R expression was analyzed by RT-PCR (GGGTCTCTGGCTACATAAGGACAAC and AAGGATGGCTGAAGCGATGAC 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 GFAP (Gial fibrillar acidic protein) following the methodology described elsewhere (11). To
evaluate the degree of glial activation, we used a scoring system based on extent of GFAP staining (23) and previously used by our group (11).

**Immunohistochemical analysis for apoptosis assessment**

Apoptosis was evaluated using the TUNEL (Terminal Transferase dUTP Nick-End Labeling) method as previously described (11). Sections of retina were permeabilized by incubation at room temperature for 5 min with 20 µg/ml Proteinase K solution, freshly prepared. Apoptotic cells were identified using green fluorescence [Alexa Fluor 594 goat-anti-rabbit (Invitrogen) (1:200 dilution prepared in PBS)]. 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 staining cells obtained by Image J software.

**Other immunohistochemistry analyses**

GLAST was evaluated by fluorescence microscopy using specific antibodies as previously reported (11). cAMP immunofluorescence were quantitated using a mouse monoclonal antibody (1:200, ab24851, Abcam, Cambridge, UK). The disruption of the blood-retinal barrier (BRB) was assessed by measuring albumin leakage (sheep polyclonal, 1:500, Ab8940, Abcam). In addition, immunofluorescence for VEGF (rabbit polyclonal, 1:150, ab46154, Abcam) and IL-1β (rabbit polyclonal, 1:100, ab9722, Abcam) were measured.

**Glutamate quantification**

Quantification of glutamate was performed by reverse phase ultra-performance liquid chromatography (UPLC) (Acquity-UPLC, Waters) as aminoquinoline derivatives (AccQ-Tag chemistry, MassTrak AAA method and instruments, Waters, Milford, MA), following the methodology previously described by Narayan et al. (24).
**Western blots analyses**

Neuroretinas were extracted in 50 µL of lysis buffer. A total of 20 µg protein was resolved by 10 % (vol. /vol.) SDS–PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The blots were probed with rabbit anti-GLP-1R (1:1000, ab39072, Abcam Ltd, Cambridge, UK). The same blots were stripped and rebalotted with a rabbit primary antibody specific to CypA (1:5000, Enzo Life Sciences Inc., Framingdale, 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), anti-apoptotic (BclxL), neuroinflammatory (iNOS), and insulin signaling (pAKT/AKT) pathways were also analyzed by Western blot. The list of antibodies used in Western blotting are described in Table 2 of supplementary material.

**Statistical Analysis**

The results are expressed as means ± SD. Statistical comparisons were performed with Student’s unpaired and paired t tests. When multiple comparisons were performed, one-way ANOVA followed by the Bonferroni test was used. The Fisher’s exact test was used to analyze categorical variables. Levels of statistical significance were set at p<0.05.
RESULTS

GLP1 and GLP-1R is expressed in human retinas

GLP-1 was expressed in human retinas, mainly in ganglion cell layer (GCL). We found a significantly lower levels of both mRNA and protein content in the retinas from diabetic patients in comparison with non-diabetic controls matched by age (Figure 1A,C,E).

GLP-1R was expressed in human retina at levels even higher than those observed in human bowel and liver (Figure 1B). No significant differences in GLP-1R mRNA were detected in either the RPE or neuroretina between diabetic and non-diabetic donors.

No differences in GLP-1R protein concentration were observed between retinas from diabetic and non-diabetic donors assessed by Western-blot in the neuroretina (Figure 1F) and the RPE. Furthermore, no differences in GLP-1R immunofluorescence (arbitrary units) were detected between diabetic and non-diabetic donors (Figure 1D). As occurred with GLP-1, GLP-1R was mainly expressed in the ganglion cell layer (GCL). However, sparse staining was also observed in the inner nuclear layer (INL) and in the outer nuclear layer (ONL) (Figure 1D).

GLP-1R is expressed in db/db retinas

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

Systemic administration of a GLP-1R agonist prevents retinal neurodegeneration in db/db mice

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

**Glial activation**

In the retina 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 (Figure 2A). Diabetic mice treated with liraglutide presented significantly a lower GFAP immunofluorescence score than diabetic mice treated with vehicle (p<0.001), and similar to non-diabetic mice (p=n.s) (Figure 2B). Notably, the prevention of glial activation with liraglutide tended to be higher than the obtained with restrictive diet (p=0.07) even though the blood glucose reduction 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 in comparison to that observed in retinas from age-matched non-diabetic controls (p<0.001) (Figure 2C-D). Diabetic mice treated with the GLP-1R agonist presented a significantly lower 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 in comparison with those treated with 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 hours after topical administration (Figure 3A).

Liraglutide topically administered (eye drops) did not reduce blood glucose levels (Supplementary Figure 2B). 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 of liraglutide in preventing glial activation and apoptosis is shown in figure 4.

Eye drops of native GLP-1, lixisenatide and exenatide were also effective in preventing glial activation (Figure 4A-B) and apoptosis (Figure 4C-D) without differences among them and 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).

**GLP-1R agonist treatment prevents ERG abnormalities**

Treatment with liraglutide, systemic 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 (Figure 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 (Figure 3B). In addition, a dose-response in terms of efficacy was observed. In this regard, the effectiveness of topical administration of eye drops of liraglutide at concentrations of 200 mcg/Kg/day was slightly lower than eye drops at 400 mcg/kg/day in
preventing glial activation and apoptosis, and the dose of 40 mcg/day was significantly less effective than 200 mcg/Kg/day (data not shown).

**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 not only because liraglutide was the agonist used both for systemic and topically 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 downstream specific signaling pathways such as adenylyl cyclase and phosphatidylinositol 3-kinase (PI3-kinase)/Akt. The activation of adenylyl cyclase results in an increase of AMPc, the primary second messenger of the GLP-1R (25-27). We found a significant increase in AMPc and pAKT in the retina of diabetic mice treated with GLP-1R agonists and, therefore, it can be assumed that the activation of the GLP-1R was produced (Figure 6).

*Effect of GLP-1R agonist on retinal glutamate and GLAST content*

Liraglutide was able to prevent the increase of glutamate levels induced by diabetes (Figure 7A). 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 (Figure 7B-C). In diabetic mice treated with liraglutide, administered subcutaneously or by eye drops, GLAST downregulation was prevented (Figure 7). We did not find any differences in GLAST immunofluorescence between subcutaneous vs. 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 Figure 3). In addition, a significantly increase in insulin signaling assessed by the ratio p-AKT/AKT was observed (Figure 6). Similar results were observed in diabetic retinas after subcutaneous administration of liraglutide (data not shown).

Topical administration of GLP-1R agonists prevents the disruption of the blood-retinal barrier (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 of eye drops containing native GLP-1 or liraglutide (the other GLP-1R agonists were not tested) (Figure 8).
DISCUSSION

In the present 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 diabetic donors and non-diabetic donors. By contrast, GLP-1 was downregulated in the diabetic retinas. 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 analogues 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 aimed at exploring the effects of GLP-1R agonists in preventing the development of Alzheimer disease are in progress (30, 31).

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 due to its reduction of blood glucose levels from those induced directly by GLP-1/GLP-1R activation in the retina. Our finding that neuroprotection obtained with the subcutaneous administration of liraglutide was even higher than that observed with 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 to this issue we tested the effect of topical administration (eye drops) of liraglutide, as well as native GLP-1, lixisenatide and exenatide. We found that 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 which 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 interleukin 1β) (data not shown).

It should be noted that we found a high rate of apoptosis in the retina of db/db mice in comparison with other experimental models of DR. However, apoptosis was not observed in non-diabetic mice and a significant reduction in the most important features of neurodegeneration had been found in a previous study after lowering blood glucose levels by using restrictive diet (11). Therefore, 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+ 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, on the contrary, that it is 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 present 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, it was able to prevent the diabetes-induced increase of caspase-8 and the BAX (pro-apoptotic)/Bcl-xL (anti-apoptotic) 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 elevated in the extracellular space in experimental models of diabetes, as well as in the vitreous fluid of diabetic patients with PDR (10). This extracellular and synaptic excess of glutamate leads to 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 (GDNF) and fenofibrate) in diabetic murine models were related to 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 topical therapy (eye drops) in the early stages of DR. In the present 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 of diabetic patients including those patients in whom the systemic administration of GLP-1R agonists is not
recommended (i.e. pancreatitis, gastrointestinal adverse effects). In addition, given that topical GLP-1R agonists action is not insulin-mediated, they could also be useful in the subset of patients for whom these systemic treatments are not currently indicated (i.e. type 1 diabetic patients or type 2 diabetic patients with insulinopenia). However, topical drug administration might not work in humans despite its effects in rodents and, therefore, clinical trials addressed to answering these relevant questions, as well as to exploring the effectiveness of eye drops of GLP-1R agonists in other neurodegenerative retinal diseases seem warranted.

In the present study we have demonstrated that the retinal neuroprotective effect of subcutaneous liraglutide was even higher than that observed with restrictive diet besides achieving less blood glucose reduction. 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 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 anti-diabetic 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 co-localized with GLP-1R mainly in 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-1b 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 for preventing retinal neurodegeneration. This could be envisaged as a useful tool for the treatment of early stages of DR. However, specific clinical trials aimed at testing their advantages for the treatment of DR in comparison 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.

**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, C.S, J.A. and A.A. led the analysis, reviewed the manuscript, and approved the final version of the manuscript. A.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.

**Acknowledgments**

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Conflicts of interest

Vall d'Hebron Research Institute holds intellectual property related to the use of ocular GLP-1R agonists to treat diabetic retinopathy.
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FIGURE LEGENDS

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, RPE: retinal pigment epithelium. C) Comparison of GLP-1 immunofluorescence (green) in the human retina between representative samples from a non-diabetic donor and a diabetic donor. D) Comparison of GLP-1R immunofluorescence (green) in the human retina between representative samples from a non-diabetic donor and a diabetic donor. Nuclei were labeled with DAPI (blue). PR: photoreceptors; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. E) GLP-1 assessment by ELISA (Millipore, Madrid, Spain). F) GLP-1R assessment by Western blot in the neuroretina. D1-D4: diabetic donors; C1-C4: control donors. The study was performed in 8 diabetic donors and 8 non-diabetic donors. Student t test was used for comparisons. * p<0.05.

Figure 2. Systemic liraglutide administration. Glial activation: A) Comparison of GFAP immunoreactivity (green) in the retina between representative samples from diabetic mice treated with vehicle, liraglutide and restrictive diet, and from a non-diabetic mouse. Nuclei were labeled with Hoechst (blue). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. B) Quantification Quantification of glial activation based on 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); Müller cell endfeet plus lots of dark processes from GCL to outer margin of ONL (score 5). Apoptosis: C) TUNEL positive immunofluorescence (green) in a representative mouse from each group. D) Percentage of TUNEL positive cells in the neuroretina. Results are mean ± SD. n = 10 mice per group. One-
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**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 non-diabetic mouse are also shown. Nuclei were labeled with Hoechst (blue). B) Quantification of GLP-1 immunofluorescence. n = 4 mice (8 eyes) per group. Results are mean ± SD. One-way ANOVA and the Bonferroni multiple comparison test were used. C) Immunofluorescence staining for GLP-1 (magenta) in sections of neuroretina (central 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 mcg (n=4), Dose 2: 180 mcg (n=4), Dose 3: 240 mcg (n=4)]. After 60 minutes of topical administration, the mice were euthanized and the retinas were processed.

**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, GLP-1R agonists (native GLP-1, lixisenatide, liraglutide, exenatide) and a non-diabetic mouse. Nuclei were labeled with Hoechst (blue). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. B) Quantification of glial activation based on extent of GFAP staining. Apoptosis: C) 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 mean ± SD. One-way ANOVA and Bonferroni multiple comparison test were used. * p<0.05 in comparison with the other groups.
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Figure 6. A) Comparison of cAMP immunofluorescence (green) between representative retinal samples from a db/db mouse treated with sham, a db/db mouse treated with liraglutide eye drops, and a non-diabetic mouse. Nuclei were labeled with Hoechst (blue). B) Detailed images of GCL showing the expression of cAMP (white arrows). C) Quantification of AMPc immunofluorescence in arbitrary units (A.U). n = 10 mice per group. Results are mean ± SD. One-way ANOVA and Bonferroni multiple comparison test were used. D) Representative western-blot analysis and quantification of expression of pAKT and AKT in the neuroretina. Tubulin was used as a loading control. Immunoreactive of pAKT was normalized to the total AKT and the quotient of controls was set to unity. n = 6 mice per group. * p<0.001 in comparison with the diabetic group treated with vehicle (D-Sham).

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**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) (central image) and a non-diabetic mouse (control db/+)(right image).
FIGURE LEGENDS SUPPLEMENTAL MATERIAL

Figure 1. A) Comparison of GLP-1R levels between 3 diabetic (db/db) and 3 non-diabetic mice (db/+). Autoradiograms were quantified by scanning densitometry. Results (mean ± SD) are expressed as arbitrary units of protein expression. n = 6 mice per group.

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Table 1. Clinical characteristics of diabetic and non diabetic donors

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<th>Non diabetic donors N= 8</th>
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<td>Age (years)</td>
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<td>Gender (M/F)</td>
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<td>Diabetes duration (years)</td>
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<tr>
<td>A1c (%)*</td>
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<td>Cause of death</td>
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<tr>
<td>- other</td>
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*: last A1c before death.
Supplementary table 2. Description of antibodies used in western blotting

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<th>Host</th>
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<td>Abcam, Cambridge, UK</td>
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