Universitat Autònoma de Barcelona

NEURODEGENERATION AS AN EARLY EVENT IN THE PATHOGENESIS OF DIABETIC RETINOPATHY: THERAPEUTIC IMPLICATIONS

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DOCTORAL THESIS

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Laboratory of Diabetes and Metabolism
Vall d'Hebron Research Institute

Department of Biochemistry and Molecular Biology
Autonomous University of Barcelona

Vall d'Hebron
Institut de Recerca
VHIR

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Lidia Corraliza Márquez

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Dr. Rafael Simó Canonge
Thesis Director

Dra. Cristina Hernández Pascual
Thesis Director

Dra. Patricia Bogdanov Baruj
Thesis Director

Dr. Joan Xavier Comella Carnicé
Tutor

Lidia Corraliza Márquez

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ABBREVIATIONS

AC	Adenylate cyclase
ACCORD-Eye	Action to Control Cardiovascular Risk in Diabetes
AD	Alzheimer's Disease
AGE	Advanced Glycation End-products
ALE	Advanced Lipoxidation End-products
AMPA	amino-3-hydroxy-5-methyl-4-isoxazolepropionate
APP	Amyloid Precursor Protein
AR	Aldose reductase
ARE	Antioxidant Response Element
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
BRB	Blood Retinal Barrier
BREC	Bovine Retinal Endothelial Cells
BP	Blood pressure
BV	Blood vessels
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
DPN	Diabetic Peripheral Neuropathy
DPP-4	Dipeptidyl peptidase-4
DR	Diabetic Retinopathy
eNOS	endothelial Nitric Oxide Synthase
EPCs	Endothelial Progenitor Cells
Epo	Erythropoietin
Epo-R	Erythropoietin-Receptor
ER	Endoplasmic Reticulum
ERG	Electroretinogram
ETDRS	Early Treatment of Diabetic Retinopathy Study
FA	Fenofibric Acid

FasL	Fas Ligand
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
GABA	γ-Aminobutyric acid
GAD	Glutamic Acid Decarboxylase
GAPDH	Glyceraldehyde-3 Phosphate Dehydrogenase
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate Aspartate Transporter
GLP-1	Glucagon Like Peptide-1
GLP-1R	Glucagon Like Peptide-1 Receptor
GLT	Glutamate Transporters
GSH	Reduced Glutathione
HIF-1α	Hypoxia-Inducible Factor-α
IFL	Inner Fiber Layer
ILM	Inner Limiting Membrane
INL	Inner Nuclear Layer
iNOS	inducible Nitric Oxide Synthase
IPL	Inner Plexiform Layer
IRBP	Interphotoreceptor Retinoid-Binding Protein
IT	Implicit Time
LTP	Long Term Potentiation of synaptic transmission
MAPK	Mitogen-Activated Protein Kinase
MAs	Microaneurysms
MCAO	Middle Cerebral Artery Occlusion
mGluR	metabotropic Glutamate Receptors
$NADP^+$	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

NF-κB	Nuclear Factor-κB
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NOS	Nitric Oxide Syntase
Obrb	Obese Receptor B
OIR	Oxygen Induced Retinopathy
OLM	Outer Limiting Membrane
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
OPs	Oscillatory Potentials
p53RE	p53 Response Elements
PARP	poly(ADP-ribose) Polymerase
PC	Propeptide Convertase
PCs	Pericyte Cells
PD	Parkinson Disease
PDR	Proliferative Diabetic Retinopathy
PEDF	Pigment Epithelium-Derived Factor
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
POS	Photoreceptors Outer Segments
PPAR-ɣ	Peroxisome Proliferator-Activated Receptor ɣ
PPAR-α	Peroxisome Proliferator-Activated Receptor-α
PPG	Postprandrial Glucose
PPV	Pars Plana Vitrectomy
RAGE	Receptor for AGE
RBC	Rod Bipolar Cell

RPE	Retinal Pigment Epithelium
SDH	Sorbitol Dehydrogenase
SOD	Superoxide Dismutase
SST	Somatostatin
STZ	Streptozotocin
TZD	Thiazolidinedione
WT	Wild type

INTRODUCTION

1. DIABETIC RETINOPATHY

The term diabetic retinopathy (DR) refers to a diabetes-induced pathology of the retinal capillaries, arterioles and venules, and the subsequent effects of leakage from or occlusion of the small vessels[1]. It is a frequent and potentially blinding complication of diabetes. The main reasons accounting for the loss of vision are diabetic maculopathy and complications of proliferative diabetic retinopathy (PDR) such as vitreous hemorrhage, tractional retinal detachment, and neovascular glaucoma[2].

Ocular symptoms, such as a slow and gradual decrease in visual acuity, metamorphopsia, and a sudden loss of vision in one eye, occur when the DR has reached a very advanced and irreversible stage[3].

1.1 EPIDEMIOLOGY

The world prevalence of diabetes among adults, aged 20–79 years, will be 7.7%, 439 million adults by 2030. Between 2010 and 2030, there will be a 69% increase in numbers of adults with diabetes in developing countries and a 20% increase in developed countries[4, 5].

Type 2 diabetes is the most common type, accounting for approximately 87% to 91% of cases in most Caucasian populations and western countries and up to 95% of diabetes in developing countries[5-8]. The main reason for this is thought to be because of these developing countries adopting a western life style. In virtually every developed society, diabetes is one of the leading causes of blindness, renal failure and lower limb amputation. It is also now one of the leading causes of death through its effects on cardiovascular disease (70-80% of people with diabetes die of cardiovascular disease). The main relevance of diabetes complications from a public health perspective is the relationship with human suffering and disability, and the huge socio-economic costs through premature morbidity and mortality[4, 9].

DR is the most common microvascular complication of diabetes mellitus and affects between 3%-4% of people in Europe[10, 11]. It is the main cause of blindness within the working-age population in industrialized countries[10].

In patients with type 1 diabetes retinal changes are rarely seen before adolescence while about one third of patients with type 2 diabetes have signs of DR at time of initial diagnosis of diabetes mellitus. Patients with type 1 diabetes have more risk of suffer from PDR than the ones with type 2 diabetes, while DME is more commonly found in type 2 diabetes[10].

DR prevalence in the diabetic population is around 35%, 7% of people with diabetes suffer from PDR, 7% has DME, and 10% is affected by vision-threatening stages (proliferative DR and clinically significant macular edema) [12].

The incidence of DR increases with the duration of diabetes, and during the first two decades of disease, nearly all patients with type 1 diabetes and more than 60% of those with type 2 diabetes will develop DR[13]. However, the early retinal changes are not noticed by patients[14].

1.2 CLASSIFICATION

Concepts:

The first ophthalmoscopic sign of DR are usually retinal **microaneurysms** (MAs). They are located predominantly within the inner nuclear layer of the retina and in the deep retinal capillary network[15]. On ophthalmoscopy, fresh MAs appear as small red dots. Microaneurysms may become later yellowish due to the increased thickening of the basement membrane. Finally they occlude[16] (Figure 1).

Intraretinal hemorrhages result from ruptured small vessels and MAs, and are mostly located deep in the retina. In the diabetic eye, retinal intraretinal hemorrhages are characteristically more numerous in the posterior pole.

Hard exudates are extracellular accumulations of lipids, proteins and lipoproteins derived from leakage from abnormal vessels.

Capillary closure is a major feature of vascular damage in diabetic patients. It has become recognized that areas of retinal capillary closure, or capillary non-perfusion, as a frequent feature of advanced DR. The areas of capillary non-perfusion enlarge as the disease progresses. Vasoregression may be the initial step in this process[17, 18].

Retinal edema is defined as thickening of the macula and is mainly due to accumulation of fluid in the central macular area resulting mainly from fluid leakage due to the alteration of the blood-retinal barrier.

The most commonly used grading system in clinical studies of DR is the ETDRS (Early Treatment of Diabetic Retinopathy Study) scale[19], which relies upon a number of photographically detectable microvascular lesions as indicators of disease progression[20].

There are five stages that are recognized in the classification of DR[21, 22]:

- No apparent retinopathy: as the name implies there are no diabetic fundus changes.

- Mild non-proliferative diabetic retinopathy (NPDR): this stage is characterized by the presence of a few microaneurysms.
- Moderate NPDR: is characterized by the presence of microaneurysms, intraretinal hemorrhages or venous beading that do not reach severity.
- Severe NPDR: this stage is the key level that ophthalmologist should identify. If hemorrhages are present in all 4 quadrants, then by definition, severe NPDR is present. Also, if 2 quadrants or more have venous beading and if one or more quadrant has intraretinal microvascular abnormalities of great magnitude.
- Proliferative diabetic retinopathy (PDR): is the final stage, it is characterized by definite neovascularization of the disc, the retina, the iris and/or neovascularization of the angle, or vitreous hemorrhage or tractional retinal detachment.

When DR affects the macula and threatens central visual acuity, it is named diabetic maculopathy. In this area of the retina, excessive vasopermeability and edematous damage is referred to as Diabetic Macular Edema (DME) and is the commonest cause of blindness in diabetes[20, 23]. DME can occur at virtually any stage of retinopathy development, however, it is most prevalent during the later phases of the disease[24]. With regards to macular edema severity classification, it should be noted if it is present or absent. If it is present it can be further classified as mild, moderate and severe depending on the distance of the exudates and thickening from the center of the fovea[21].

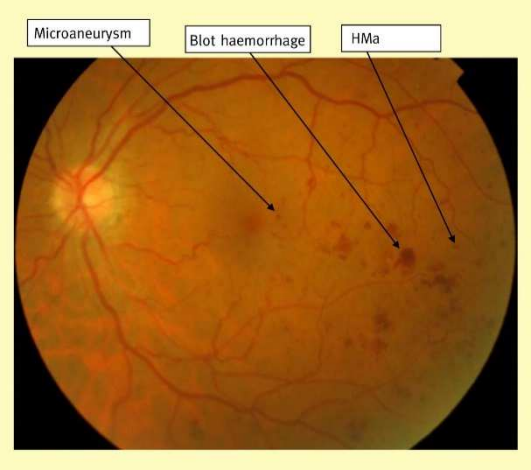

Figure 1. Eye fundus showing microaneurysms, small hemorrhage/microaneurysms (HMa) and blot hemorrhages.

1.3 PATHOPHYSIOLOGY

The retinal changes in patients with diabetes result from five fundamental processes[25]:

1- Formation of retinal capillary microaneurysms
2- Development of excessive vascular permeability
3- Vascular occlusion
4- Proliferation of new blood vessels and accompanying fibrous tissue on the surface of the retina and optic disk
5- Contraction of these fibrovascular proliferations and the vitreous

The above mentioned retinal changes are due to microcirculatory disease. Microangiopathy threatens sight when it leads to macular edema and/or retinal ischemia with attendant unregulated angiogenesis (Figure 2).

Macular edema develops when abnormal permeability of retinal capillaries causes the passive influx of plasma or blood into the retina which overwhelms the active reabsorbing transport.

Retinal ischemia develops when a critical number of capillaries become nonperfused and obliterated. The simplest paradigm to explain capillary permeability and closure centers on vascular endothelium. In the retina, as in the brain, endothelial cells are the site of the blood-tissue barrier, and as in all vessels, provide a non-thrombogenic surface for blood flow. Both of these properties are compromised by diabetes[26].

There is plenty of evidence that the visible manifestations of the disease are accompanied by insidious degenerative changes. Both retinal glia and neurons are compromised early in the disease progression, and thus play causative or contributory role in the microangiopathy[26-41].

The main features of retinal neurodegeneration, which are apoptosis and glial activation, have been found in the retinas of donors with diabetes without any microcirculatory abnormalities appearing in ophthalmoscopic examinations performed during the year before death[42-44].

Diabetes increases apoptosis in neurons, especially in the retinal ganglion cell layer[39]. This loss of neural cells results in a reduction in the thickness of retinal nerve fiber layer[45-47]. It should be noted that his thinning of the ganglion cell layer has been found in patients with diabetes with no or only minimal DR[46-48]. Moreover, a study conducted to measure the loss of photoreceptors in the early stages of DR showed that the parafoveal cone density was on average 10% lower in the diabetic group than in the control group but the results were not clinically significant[49].

Functional abnormalities

It has been shown that patients with diabetes and mild non-proliferative DR (NPDR) exhibit a wide variety of **visual function abnormalities**, including loss of contrast sensitivity and impaired visual fields, even in the absence of clinically evident vascular lesions or macular edema[50-57]. These observations suggest that diabetes may directly insult the neural retina, given that ganglion cell function is thought to be primarily responsible for contrast sensitivity[58]. The alterations can be detected by means of electrophysiological studies in patients with diabetes with less than 2 years of diabetes duration, which is before microvascular lesions can be detected under ophthalmological examination[40]. The characteristic abnormalities found in the electroretinogram of diabetic patients are the reduction in the amplitude and delay in the b-wave and in the latency of the oscillatory potentials, even without any evidence of microvascular abnormalities[59-65]. Interestingly, there are functional abnormalities related to glucose control that are partly reversible and, therefore, do not necessarily implicate cellular loss or major structural damage[66].

Several studies have shown that, in diabetic subjects without signs of retinopathy, there is a reduction of the vasodilator capacity of retinal vessels in response to flicker stimulation[3, 67, 68].

All these findings suggest that neuronal apoptosis precedes overt vascular dysfunction.

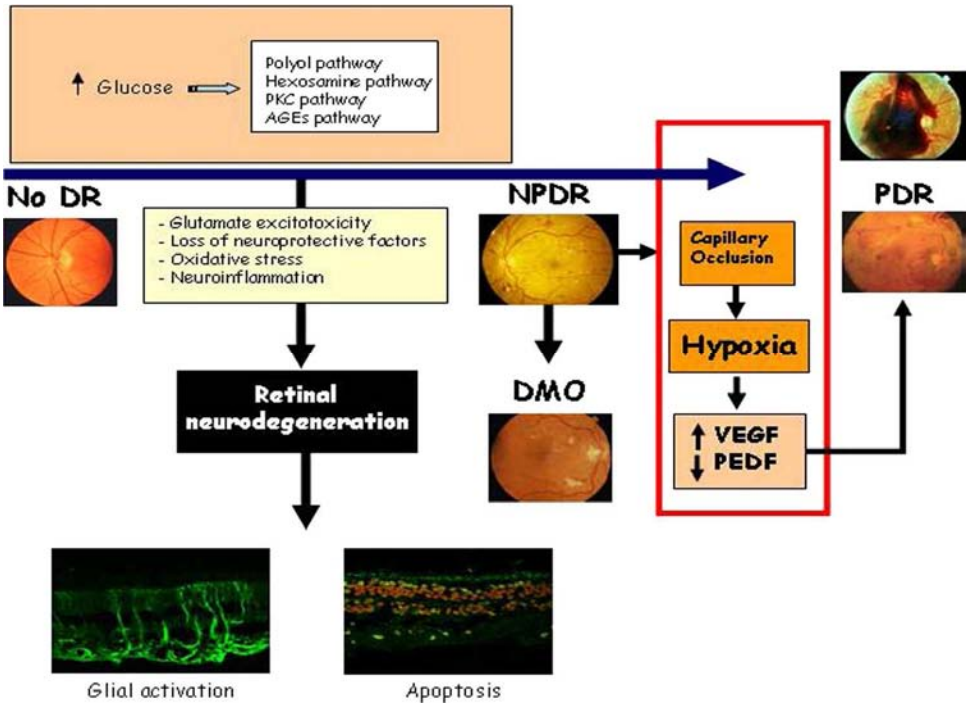

Figure 2. Schematic representation of DR development. Extracted from Simó and Hernández, 2012.

1.4 TREATMENT

Current treatments for DR target late stages of DR when vision has already been significantly affected.

Treatment of the classic risk factors such as hyperglycemia and hypertension is currently recommended for preventing or arresting the development of DR. But, by now, nothing specific can be done until advanced stages of the disease, then, laser photocoagulation, intravitreal injections of corticosteroids of anti-VEGF agents and vitreoretinal surgery are performed. All these treatments are expensive and have to be done by a trained-expert and have a significant number of secondary adverse effects. For all these reasons, new treatments targeting the early stages of the disease are needed[69].

Risk factors control

The management of DR begins with improved glycemic control. The diabetes control and complications trial (DCCT) reported up to a 76% decrease in the progression of DR in type 1 diabetic patients receiving intensive insulin therapy during a 9 year period[70] and a persistent reduction in risk for four additional years, despite hyperglycemia[71]. The United Kingdom Prospective Diabetes Study was performed in type 2 diabetics and reported similar findings to DCCT, as well as decreased diabetes-related complications in patients undergoing aggressive blood pressure management[72, 73].

Nordwall and collegues[74] have recently reported that keeping HbA1c below 7.6% (60 mmol/ mol)
as a treatment seems to prevent PDR proliferative retinopathy and persistent macroalbuminuria
for up to 20 years in type 1 diabetic patients. On the other hand, it seems to be little effect of serum lipids or statins on the incidence of PDR and macular edema[75, 76].

Multiple randomized controlled trials have demonstrated the benefits of tight blood pressure (BP) control as a major modifiable factor for DR incidence and progression. The UKPDS was the first randomized controlled trial that showed the importance of tight BP control in reducing retinopathy[77]. It has been shown that every 10 mmHg increase in systolic blood pressure was associated with 10% increased risk of early DR and 15% risk of PDR or DME[78-80].

Laser photocoagulation

This intervention prevents further deterioration of vision if applied sufficiently early in the progression of the disease, but does not usually restore lost vision.

Laser photocoagulation is a proven effective treatment for preserving vision and reducing the risk of vision loss from DR by 60%[81, 82]. ETDRS states that focal laser surgery diminishes by 50% the probability of suffering loss of vision acuity in patients with DME[19, 83].

However, laser photocoagulation has adverse side effects. It can reduce a patient's night vision or his/her ability to function well when going from a lighted environment to a darkened one. It can cause a loss of peripheral vision or blind spots in a person's central vision, depending upon where the laser burns are placed. The laser burns themselves can become a site for the development of abnormal blood vessels beneath the retina, leading to vision loss. Finally, and most importantly, sometimes laser therapy does not work, and patients lose vision despite receiving timely treatment[84].

Vitreo-retinal surgery

Pars plana vitrectomy (PPV) is a surgical procedure that involves removal of vitreous gel from the eye. PPV allows the removal of opacities from the media, such as non-clearing vitreous hemorrhage, and offers relief from vitreoretinal traction[85]. Usually, it is reserved for the ultimately blinding complications of DR[86] because it is a complex and expensive treatment. The improvement in visual outcome achieved during the past 25 years has therefore resulted from advances in vitreoretinal surgical instrumentation and technique enabling more effective dissection of complex fibrovascular membranes. However, the visual outcome after vitrectomy continues to remain unpredictable[87].

The adverse effects associated with this technique are related to post-operative complications such as, cataract formation, recurrent vitreous cavity hemorrhage (early or delayed), rhegmatogenous retinal detachment, and neovascular glaucoma[69, 86].

Intravitreal drugs

Recently, new pharmacologic therapies targeting to block angiogenesis have been developed. Systemic administration of these drugs has several drawbacks, for instance, the high dose that has to be administered in order to overcome BRB and ensure the arrival of the drug to the vitreous and retina, additionally to the systemic adverse effects that could generate. For these reasons administration is local, by eye drops or intravitreal injections.

In recent years two groups of drugs for intravitreal use have become available[84]:

- Anti-vascular endothelial growth factor (VEGF) drugs, bevacizumab, pegatanib, ranibizumab and aflibercept. These inhibit the action of VEGF or bind it. Aflibercept also

blocks plancental growth factor. VEGF increases vascular permability and promote the growth of abnormal new vessels (neovascularisation).

- Steroids, including triamcinolone, the long-acting dexamethasone implant and the longer-acting fluocinolone implant.

The rational for using the anti-VEGF drugs in PDR and NPDR lies on the fact that it has been shown that raised VEGF levels are present in the ocular fluid from patients with active new vessel formation compared with no rise in patients with NPDR or inactive PDR, suggesting that VEGF stimulates neovascularisation[88]. Data from major randomized controlled clinical trials investigating the use of anti-VEGF therapy for DME provide robust evidence that intraocular administration of anti-VEGF agents is better than laser therapy in preserving and improving vision[89-91]. Anti-VEGF drugs cause regression of PDR, albeit temporarily because the effects last only a few weeks, making repeat injection necessary. Anti-VEGF drugs have fewer adverse effects. They do have to be given by injection into the eye[84] and, as a consequence of the surgery, may lead to local complications such as endophthalmitis and retinal detachment. It must be taken into account that long-term intravitreous injections of VEGF inhibitors could lead to neurodegeneration of the remaining healthy retina and an increased risk of circulation disturbances in the choriocapillaris[92, 93]. In addition, although delivered within the vitreous, anti-VEGF drugs could pass into the systemic circulation, which could potentially result in hypertension, proteinuria, increased cardiovascular events and impaired wound healing[69, 94].

Sustained-release low-dose delivery via dexamethasone or fluocinolone acetonide implant will limit frequent intravitreal injection and possibly reduce the costs associated with intravitral anti-VEGF therapy[95]. Long-acting steroids have significant adverse effects, notably causation or acceleration of cataracts in the eye, and also raised intraocular pressure that can lead to glaucoma[84].

2. HUMAN EYE

The human eye is composed by several structures[96] (Figure 3).

- The **pupil** allows the entrance of light to the eye. It appears dark due to the absorbing pigments in the retina.
- The **iris**, it is a circular muscle that controls the size of the pupil in order to control the light that enters to the retina depending on environmental conditions.
- The **cornea** is a transparent external surface that covers both, the pupil and the iris. This is a powerful lens of the optical system of the eye and allows, together with the **crystalline lens** the production of a sharp image at the retinal photoreceptor level.
- The **sclera** forms part of the supporting wall of the eyeball. The sclera is continuous with the cornea. Furthermore it is the external covering of the eye. The sclera is also in continuity with the dura of the central nervous system.

The eye can be divided into three different layers:

- The external layer, formed by the sclera and the cornea
- The intermediate layer, divided into two parts:
    - Anterior, composed by iris and ciliary body
    - Posterior, composed by the choroid
- The internal layer, the retina which is the sensory part of the eye

Also, the human eye has three chambers of fluid:

- Anterior chamber, between cornea and iris. It is filled with aqueous humour.
- Posterior chamber, between iris, zonule fibers and lens. Also filled with aqueous humour.
- Vitreous chamber, between the lens and the retina. Filled with vitreous humour, with is a more viscous fluid.

Light rays are focused through the transparent cornea and lens upon the retina. The central point for image focus, which is called the visual axis, in the human retina, is the **fovea**. Here a maximally focussed image initiates resolution of the finest detail and direct transmission of that detail to the brain for the higher operations needed for perception. Slightly more nasally than the visual axis is the optic axis projecting closer to the optic nerve head. The optic axis is the longest sagittal distance between the front or vertex of the cornea and the furthest posterior part of the eyeball. It is about the optic axis that the eye is rotated by the eye muscles. Some vertebrate retinas have instead of a fovea, another specialization of the central retina, known

as *area centralis* or visual streak, but this is not the case of the mouse which has a cone concentration about 3% in all areas of the retina[97].

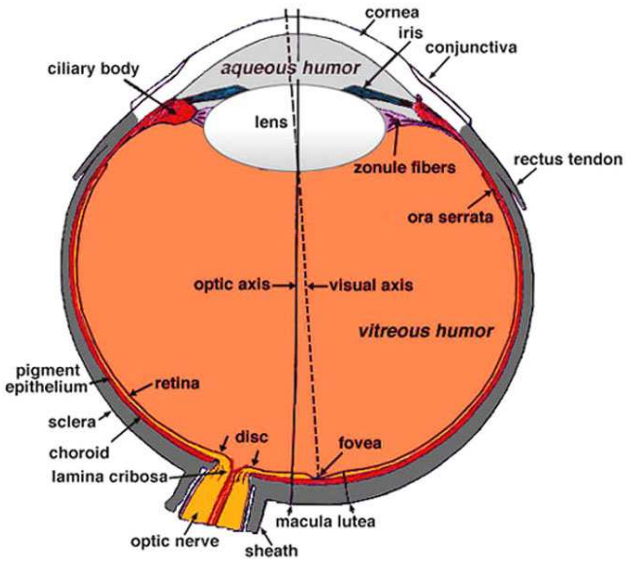

Figure 3. Sagital section of the human eye. Extracted from Kolb, 2005.

3. RETINA

The retina is the part of the eye sensible to the light. It is approximately 0.5 mm thick and lies in the back of the eye (Figure 4).

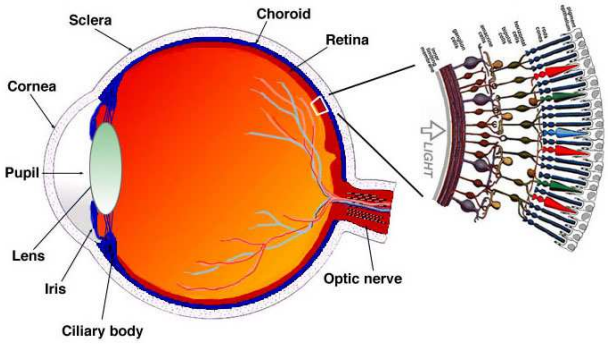

Figure 4. Section through the human eye with schematic enlargement of the retina. Extracted from Kolb, 2005.

A radial section of the retina shows that the ganglion cells (the output neurons of the retina) are located closest to the lens and front of the eye, and the photosensors (the rods and cones) lie against the pigment epithelium and choroid. Light must, therefore, travel through the thickness of the retina before striking and activating the rods and cones. Then, the absorption of photons by the visual pigment of the photoreceptors is translated into a biochemical message first, and, after that, to an electrical message that can stimulate all the succeeding neurons of the retina. The retinal message concerning the received light input and some preliminary organization of the visual image into several forms of sensation are transmitted to the brain from the spiking discharge pattern of the ganglion cells.

The optic nerve contains the ganglion cell axons running to the brain and, additionally, incoming blood vessels that open into the retina to vascularize the retinal layers and neurons.

3.1 RETINAL STRUCTURE IN MAMMALS

All vertebrate retinas are composed of three layers of nerve cell bodies and two layers of synapses.

In figure 5 are shown the functional components of the retina, set on layers from the outermost retina to the innermost:

1) **Pigment epithelium (RPE)** is a pigmented cell layer just outside the neurosensory retina that nourishes retinal visual cells.
2) **Photoreceptors outer segments (POS)** is the part of the photoreceptor filled with stacks of membranes containing the visual pigment molecules such as rhodopsins.
3) **Outer limiting membrane (OLM)** is formed from adherent junctions between Müller cells and photoreceptor cell inner segments.
4) **Outer nuclear layer (ONL)**, composed by cellular bodies of cones and rods.
5) **Outer plexiform layer (OPL)**. Here occur the connections between rods and cones vertically running with bipolar cells and horizontally orientated rods and cones with horizontal cells.
6) **Inner nuclear layer (INL)** contains the cell bodies of bipolar, horizontal and amacrine cells.
7) **Inner plexiform layer (IPL).** In this layer is where the vertical-information-carrying nerve cells, the bipolar cells, connect to ganglion cells. In addition, different varieties of horizontally- and vertically-directed amacrine cells somehow interact in further networks to influence and integrate the ganglion cell signals.

8) **Ganglion cell layer (GCL)**, contains cell bodies of ganglion cells and displaced amacrine cells.
9) **Retinal Nerve fiber layer (RNFL)** is formed by retinal ganglion cell axons.
10) **Inner limiting membrane (ILM)** is composed of laterally contacting Müller cell end feet and associated basement membrane constituents.

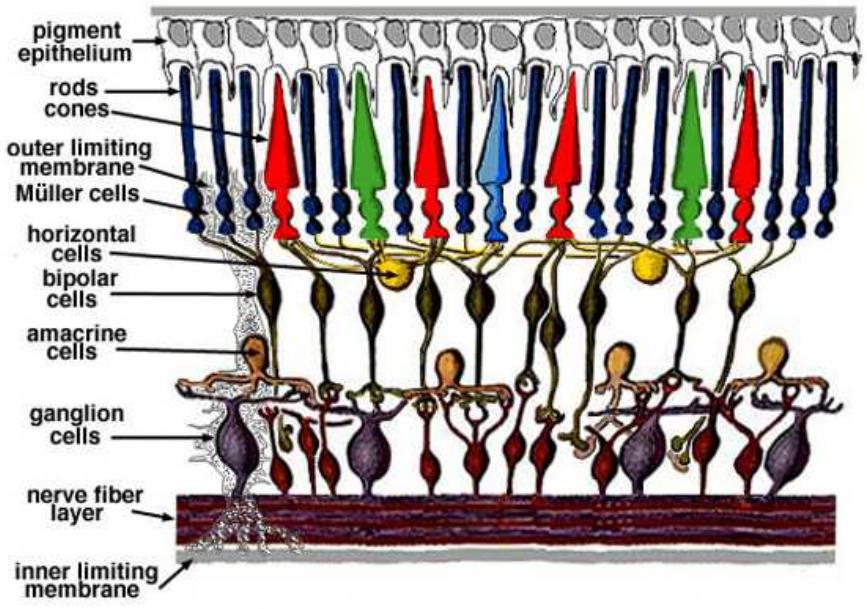

Figure 5. Portion of human retina. Extracted from Kolb, 2005.

The OLM forms a barrier between the subretinal space. In this layer the inner and outer segments of the photoreceptors project in order to be in close association with the RPE. The ILM is the inner surface of the retina bordering the vitreous humor and thereby forming a diffusion barrier between neural retina and vitreous humor.

There are two sources of blood supply to the mammalian retina: the central retinal artery and the choroidal blood vessels. The choroid receives the greatest blood flow (65-85%) and is vital for the maintenance of the outer retina (particularly the photoreceptors) and the remaining 20-30% flows to the retina through the central retinal artery from the optic nerve head to nourish the inner retinal layers. The central retinal artery has 4 main branches in the human retina[96]. The arterial intraretinal branches then supply three layers of capillary networks:

- The radial peripapillary capillaries.
- An inner layer of capillaries.
- An outer layer of capillaries.

The precapillary venules drain into venules and through the corresponding venous system to the central retinal vein.

3.2 BLOOD RETINAL BARRIER

The BRB consists of inner (iBRB) and outer (oBRB) components and plays by itself a fundamental role in the microenvironment of the retina and retinal neurons. The BRB regulates fluids and molecular movement between the ocular vascular beds and retinal tissues and prevents leakage into the retina of macromolecules and other potentially harmful agents[98].

In both iBRB and oBRB, the cell TJs restrict paracellular movement of fluids and molecules between blood and retina, and the endothelial cells and RPE cells actively regulate inward and outward movements. As a result, the levels in the blood plasma of aminoacids or fatty acids fluctuate over a wide range while their concentrations in the retina remain relatively stable.

3.2.1 OUTER BLOOD RETINAL BARRIER

The oBRB is established by the tight junctions (TJs) between neighboring retinal pigment epithelial (RPE) cells[99]. RPE is located between the outer segments of the photoreceptors and the choroid, which provides blood supply. It is a monolayer of cells hexagonally packed, connected by tight-junction and contains pigment granules and organelles for the digestion of photoreceptor outer segment membranes into phagosomes. In the apical membrane it faces the subretinal space which is occupied by an extracellular matrix that allows interaction between the RPE and the light sensitive outer segments of the photoreceptors. With its basolateral membrane, the RPE is in contact with Bruch's membrane which also represents an interaction matrix for the RPE with blood flow and the fenestrated vessels of the choroid [100, 101]. The metabolic relationship of the RPE apical villi and the photoreceptors is considered to be critical for the maintenance of visual function[98].

3.2.2 INNER BLOOD RETINAL BARRIER

The inner blood retinal barrier is established by the TJs between neighboring retinal endothelial cells. These specialized TJs restrict the diffusional permeability of the retinal endothelial layer to values in the order of 0.14×10^{-5} $cm*s^{-1}$ [102]. The retinal endothelial layer functions as an epithelium and in this way is directly associated with its differentiation and with the polarization of the BRB function. This continuous endothelial cell layer, which forms the main structure of the iBRB, rests on a basal lamina that is covered by the processes of astrocytes and Müller cells. Pericytes are also present, encased in the basal lamina, in close contact with the endothelial cells, but do not for a continuous layer and, therefore, do not contribute to the diffusional barrier[103]. Astrocytes,

Müller cells, and pericytes are considered to influence the activity of the retinal endothelial cells and of the iBRB by transmitting to endothelial cells regulatory signals indicating the changes in the microenvironment of the retinal neuronal circuitry[98]. The microvascular hemodynamic response is known as neurovascular coupling, and it refers to the regulation of blood flow at the retinal level in response to neuronal activity or metabolic demands. An increase of neural activity leads to retinal arterial and venous dilatation but this response is impaired in the early stages of DR[104, 105]. The flicker-ERG stimulation (30 Hz frequency) is used to study this process[105].

3.3 RETINAL CELL TYPES

3.3.1 PHOTORECEPTORS

Photoreceptors are the primary recipients of visual stimulation. They are the neurons in charge of converting light stimuli into signals that can stimulate biological processes. Photoreceptor protein in the cell absorb photons, triggering a change in the cell's membrane potential.

There are two types of photoreceptors: cones, which are responsible for color vision and rods which are in charge of night vision. Three types of cone photoreceptor are present in the human retina; however mouse retina has two types of cones. A single type of rod photoreceptor is present in the normal mammalian retina.

Cones are robust conical-shaped structures that have their cell bodies situated in a single row right below the OLM and their inner and outer segments protruding into the subretinal space towards the RPE. On the other hand, rods are slim rod-shaped structures with their inner and outer segments filling the area between the larger cones in the subretinal space and stretching to the pigment epithelium cells. Apical processes from the pigment epithelium envelope the outer segments of both rods and cones.

The photoreceptor consists of (see figure 6):

- Outer segment, filled with stacks of membranes containing the visual pigment molecules such as rhodopsin.
- Inner segment containing mitochondria, ribosomes and membranes where opsin molecules are assembled and passed to be part of the outer segment discs.
- Cell body containing the nucleus of photoreceptor cell.
- Synaptic terminal where neurotransmission to second order neurons occurs.

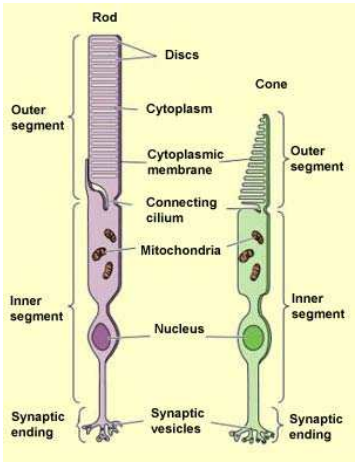

Figure 6. Rod and cone structure. Extracted from thebrain.mcgill.ca.

Vertebrate photoreceptors can respond to light because they contain a visual pigment embedded in the bilipid membranous discs that make up the outer segment. The visual pigment consists of a protein called opsin and a chromophore derived from vitamin A, known as retinal. Light transduces the visual pigment via the following enzyme cascade: photons - rhodopsin - activated rhodopsin (metarhodopsin II) - a GTP binding protein (transducin) - an enzyme hydrolyzing cGMP (cGMP-phosphodiesterase) - closes a membrane bound cGMP-gated cation channel[104, 106].

In the dark, steady current flows into the open channels, carried mainly by Na^+ ions, constituting a “dark current” that partially depolarizes the photoreceptor cell. Thus, the depolarized photoreceptor releases neurotransmitter (glutamate) from its synaptic terminals onto second-order neurons in the dark. On light stimulation the rhodopsin molecules are isomerized to the active form leading to closure of the cation channels of the photoreceptor membrane, stopping the dark current and causing the photoreceptor cell membrane to hyperpolarize and cease neurotransmitter release to second-order neurons[107, 108].

3.3.2 BIPOLAR CELLS

Bipolar cells link the primary sensory neurons to long projection neurons (retinal ganglion cells). They receive synaptic input from either rods or cones, but not both, and they are designated rod bipolar or cone bipolar cells respectively. There are roughly 10 distinct forms of cone bipolar cells, however, only one rod bipolar cell, due to the rod receptor arriving later in the evolutionary history than the cone receptor. Then, these cells carry out the first elementary operations of the visual system[107].

Most mammalian retinas are dominated by rod photoreceptors, rendering the rod bipolar cell (RBC) the most numerous type of bipolar cell. The convergence of multiple rods onto individual RBCs is the low-light rod pathway is higher than in the cone pathway and provides a high signal-to-noise ratio[109].

3.3.3 GANGLIONAR CELLS

Ganglion cells are the final output neurons of the vertebrate retina. The ganglion cell collects the electrical messages concerning the visual signal from the two layers of nerve cells preceding it in the retinal wiring scheme. A great deal of preprocessing has been accomplished by the neurons of the vertical pathways (photoreceptor to bipolar to ganglion cell chain), and by the lateral pathways (photoreceptor to horizontal cell to bipolar to amacrine to ganglion cell chain) before presentation to the ganglion cell and so it represents the ultimate signaler to the brain of retinal information. Ganglion cells are larger on average than most preceding retinal interneurons and have large diameter axons capable of passing the electrical signal, in the form of transient spike trains, to the retinal recipient areas of the brain many millimeters or centimeters distant from the retina. The optic nerve collects all the axons of the ganglion cells and this bundle of more than a million fibers then passes information to the brain for sorting and integrating into further information processing channels[110, 111].

3.3.4 AMACRINE CELLS

Amacrine cells of the vertebrate retina are interneurons that interact at the second synaptic level of the vertically direct pathways consisting of the photoreceptor-bipolar-ganglion cell chain. These cells serve to integrate, modulate and interpose a temporal domain to the visual message presented to the ganglion cell[112].

Amacrine cells constitute the most diverse group of cell types within the retina with respect to morphology, size and animal coverage. Cell bodies of amacrine cells are placed in the innermost layer of the inner nuclear layer, in the middle of the inner plexiform layer (A1 amacrine cell), and in the ganglion cell layer (displaced amacrine cells)[113].

Amacrine cells make numerous synapses onto ganglion cell dendrites, bipolar cell terminals, interplexiform processes and other amacrine cell processes[114]. Electron microscopy has shown that amacrine cell processes have characteristics of both axons and dendrites. Thus, amacrine cell processes can be both pre-and postsynaptic over a very short portion of their lenght[115]. Most amacrine cells are inhibitory neurons in the vertebrate retina, containing the common inhibitory neurotransmitters GABA or glycine. There are, at least, two types of synaptic arrangements.

First, a postsynaptic amacrine cell process makes a synapse back onto the bipolar terminal a short distance away, these are called reciprocal synapses. Second type of arrangement, one amacrine cell process makes a synapse onto an adjoining amacrine cell process, which makes a nearby synapse onto a third element (a ganglion cell dendrite, a bipolar cell terminal, or another amacrine cell processes). These are called serial synapses. Another kind of communication between amacrine cells processes are gap junctions that serve to extend the receptive field size of the cells[113].

3.3.5 HORIZONTAL CELLS

Horizontal cells together with amacrine cells, are retinal interneurons that lie within the INL[116]. Horizontal cells provide inhibitory feedback to rods and cones and possibly to the dendrites of bipolar cells. Their function is to provide a mechanism of local gain control to the retina. The horizontal cell, which has a moderately wide lateral spread and is coupled to its neighbors by gap junctions, measures the average level of illumination falling upon a region of the retinal surface. Then, it subtracts a proportionate value from the output of the photoreceptors. This serves to hold the signal input to the inner retinal circuitry within its operating range, an extremely useful function in a natural world where any scene may contain individual objects with brightness that varies across several orders of magnitude[117].

3.3.6 GLIAL CELLS

The neural retina contains, in addition to microglial cells, two forms of neuron-supporting macroglial cells, astrocytes and Müller (radial glial) cells[118].

- Müller cells

The Müller cell is the predominant glial element in the retina, representing 90% of the retinal glia[119]. They form architectural support structures stretching radially across the thickness of the retina and are limits of the retina at the outer and inner limiting membrane, respectively[120]. Müller cells interact with most, if not all neurons in the retina, resembling a symbiotic relationship[121-123]. They have a wide range of functions, all of which are vital to the health of the retinal neurons. Müller cells supply end products of anaerobic metabolism (breakdown of glycogen) to fuel aerobic metabolism in the nerve cells; they mop up neuronal waste products, such as carbon dioxide and ammonia, and recycle spent amino acid transmitters; protect neurons from exposure to excess neurotransmitters such as glutamate, using well-developed uptake mechanisms to recycle this transmitter. They are particularly characterized by the presence of high concentrations of glutamine synthase. An unbalance in the glutamate recycling

could lead to an improper clearance of synaptic glutamate. This fact together with the overactivation of glutamate receptors in these cells leads to the so called glutamate excitotoxicity, it will be further discussed below and it is an essential point in the present thesis Müller cells may be involved in both phagocytosis of neural debris and release of neuroactive substances such as GABA, taurine, and dopamine; they are thought to synthesize retinoic acid from retinal; they control homeostasis and protect neurons from deleterious changes in their ionic environment by taking up extracellular K^+ and redistributing it[120]; They contribute to the generation of the electroretinogram (ERG) b-wave[124, 125] and the scotopic threshold response[126]. They do so by regulation of K^+ distribution across the retinal vitreous border, across the whole retina, and locally in the inner plexiform layer of the retina[120]. It will be further discussed below but it is interesting to mention that Müller cells play an important role in the retinal neurodegeneration due to the many functions they carry to maintain the retinal homeostasis.

- Astrocytes

Named on the basis of their stellate shape, astrocytes are almost exclusively confined to the innermost retinal layers. Intermediate filament fill their processes, and thus they stain dramatically with antibodies against GFAP[127]. Astrocytes are arranged over the surface of the ganglion cell axon bundles as they course into the optic nerve head, forming a tube through which the axons run. Blood vessels running in and among the ganglion cell bundles are also covered by both processes and even an occasional cell body of an astrocyte[120]. Some of the beneficial roles fulfilled by astrocytes include neurotrophic support, enhanced mechanical support for degeneration axons, and the maintenance of the blood-retina barrier[119].

- Microglia

Resting microglial cells are scattered throughout the retina, forming a network of potential immunoeffector cells. They are considered to play a critical role in host defense against invading microorganisms, immunoregulation and tissue repair. Microglial cells have the capability of both presenting antigen and phagocytosing debris. Microglial cells are in a resting state in the normal adult retina, but can become activated by various stimuli, including nerve degeneration, inflammation and traumatic nerve lesions[128]. In the resting state they have a ramified appearance with a small cell body and rather large nucleus. In the activated state the cell morphology is more amoeboid and they exhibit pseudopodia[129]. In healthy conditions microglia are found at variable densities in the different retinal layers, including NFL, GCL, IPL, INL and OPL[119].

Figure 7 summarizes and represents the cellular components of the retina explained above.

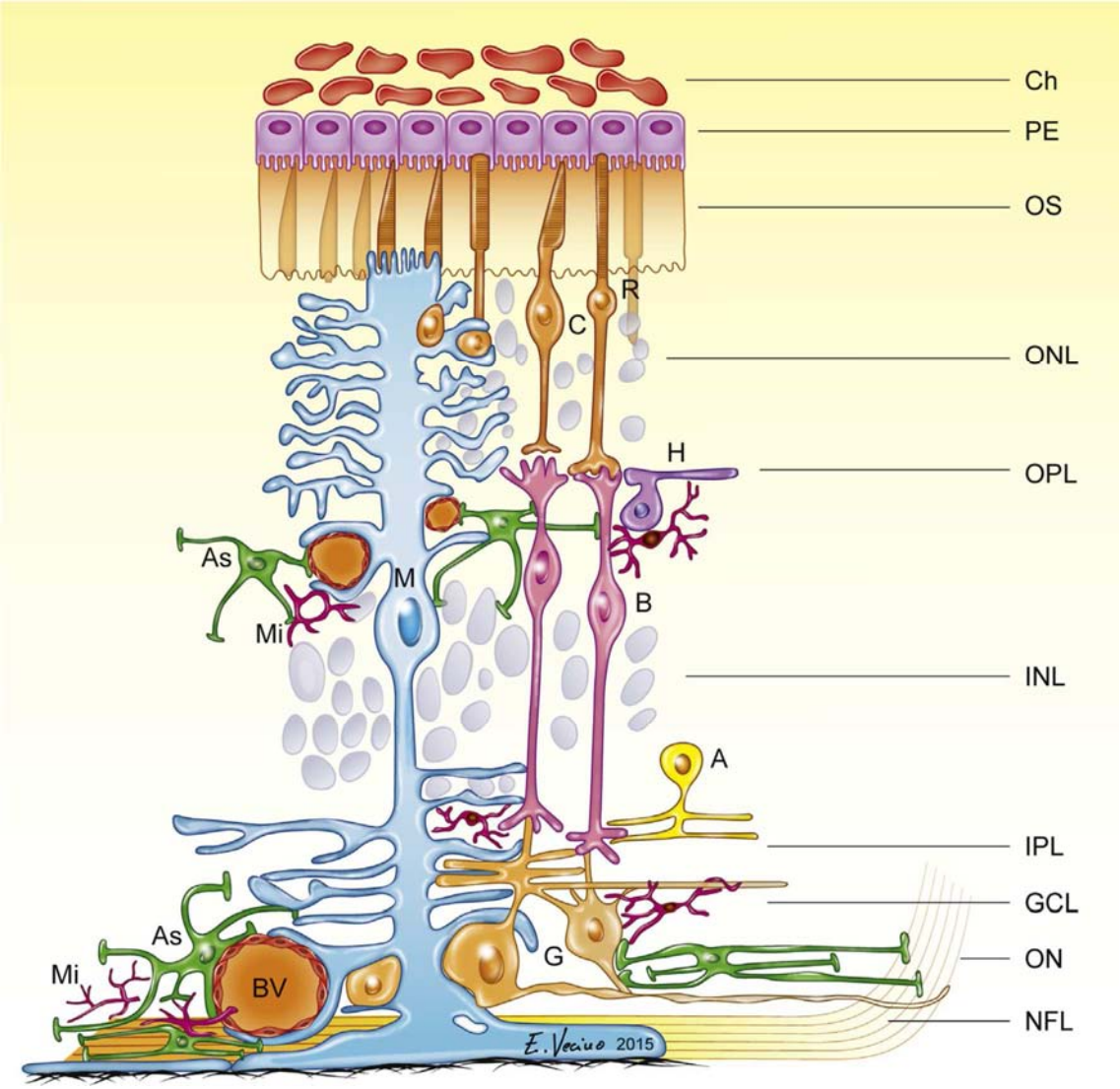

Figure 7. Schematic drawing of the cellular components of the retina: glia and neurons. (BV) blood vessels, (A) amacrine cells, (AS) astrocytes in green, (B) bipolar cells, (C) cones, (G) ganglion cells, (H) horizontal cells, (M) Müller cells in blue, (Mi) microglia in red, (R) rods, (C) cones. Extracted from Vecino, 2015

3.4 PHOTOTRANSDUCTION

In photoreceptors, light causes a graded change in membrane potential. This fact leads to a change in the rate of transmitter released onto postsynaptic neurons. Shining light on a photoreceptor causes membrane hyperpolarization.

In the dark, the membrane potential of a rod cell is about −30 mV, considerably less than the resting potential (−60 to −90 mV) typical of neurons and other electrically active cells[130].

Progressive increases in the intensity of illumination cause the potential across the receptor membrane to become more negative, a response that saturates when the membrane potential reaches about -65 mV. There is a consistent relationship between luminance changes and the rate of transmitter release from the photoreceptor terminals. Transmitter release from synaptic terminals of the photoreceptor is dependent on voltage-sensitive Ca^{2+} channels in the terminal membrane. Then, in the dark, when photoreceptors are relatively depolarized, the number of open Ca^{2+} channels in the synaptic terminal is high, and the rate of transmitter release is correspondingly great. The effect of light is to close these channels, causing the membrane potential to become more negative (hyperpolarized) and the rate of transmitter release is reduced[130].

The degree of inside negativity of photoreceptors in the dark is due to the presence of ion channels in the outer segment that allows that Na^+ and Ca^{2+} ions flow into the cell, leading to a relatively depolarized state. The probability these channels in the outer segment being open or closed is regulated in turn by the levels of nucleotide cyclic guanosine monophosphate (cGMP). In darkness, high levels of cGMP in the outer segment keep the channels open. In the light, cGMP levels drop and some of the channels close, leading to hyperpolarization of the outer segment membrane and ultimately the reduction of transmitter release at the photoreceptor synapse (Figure 8)[131].

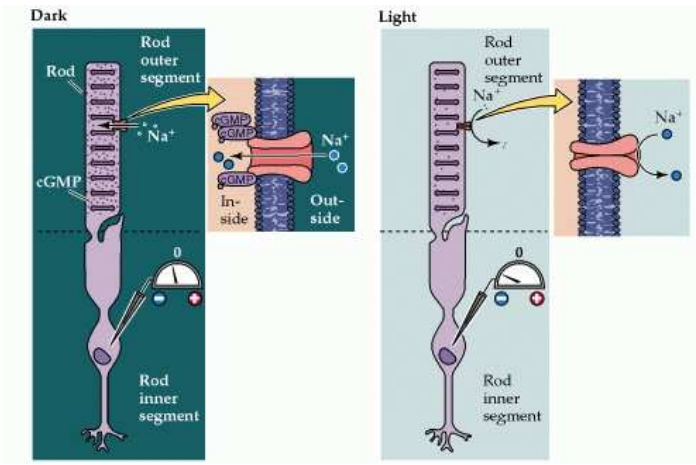

Figure 8. Phototransduction. Cyclic GMP-gated channels in the outer segment membrane are responsible for the light-induced changes in the electrical activity of photoreceptors. Extracted from Neuroscience 2nd edition.

3.5 ELECTROPHYSIOLOGY

The global or full-field electroretinogram is a widely used electrophysiologic test of retinal function. It is a mass electrical response of the retina to a photic stimulation[132].

The basic method consists on stimulating the eye with a bright light source such as a flash originated by LEDs. The flash of light evokes a biphasic waveform recordable at the cornea (example in figure 9). The a-wave and the b-wave are the two components that are most often measured. The a-wave is the first large negative component, followed by the b-wave which is corneal positive and usually larger in amplitude[133].

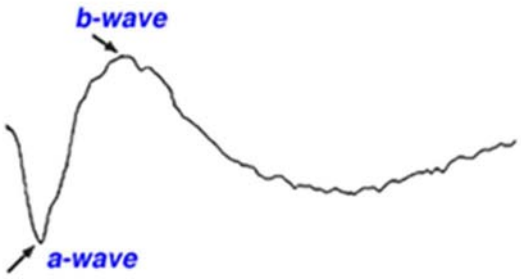

Figure 9. Basic form of an electroretinogram. Extracted from Creel, 2005.

The two main measures that are taken from the ERG are shown in figure 10 and consist of[133]:

- The amplitude (a) from the baseline to the negative bottom of the a-wave, and the amplitude of the b-wave measured from the lowest point of the a-wave to the following peak of the b-wave.
- The time (t) from flash onset to the base of the a-wave and the time from the flash onset to the peak of the b-wave (figure 8). These times are referred to as “implicit time” (IT).

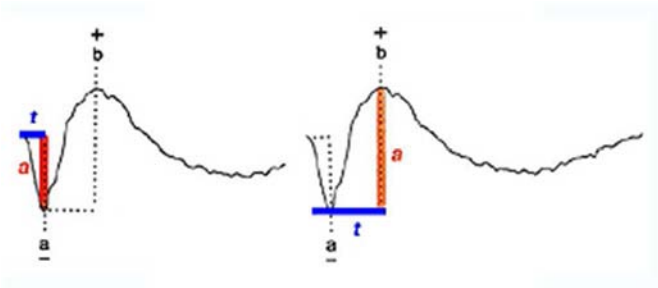

Figure 10. Amplitude and implicit time measurements. a: amplitude and t: implicit time. Extracted from Creel, 2005.

The a-wave reflects the physiology of the photoreceptors in the outer retina[133]. Light absorbance by the visual pigment molecules in the outer segments of the photoreceptors reduces the "dark" current. The current is expressed as a negative wave when recorded from the vitreous or the cornea[134].

The exact source of the ERG b-wave is still under dispute. The major contribution comes from light-induced activity in ON-center bipolar cells. The extracellular currents that generate the b-wave either originate directly in these cells or reflect potassium-induced changes in the membrane potential of the Müller cells enveloping them. The b-wave is telling us about light-induced electrical activity in retinal cells post-synaptic to the photoreceptors. The b-wave is also affected by OFF-center bipolar cells and by light-induced activity in third-order retinal neurons (amacrine and ganglion cells)[134].

The oscillatory potentials (OPs) consist of a series of high-frequency, low-amplitude wavelets, superimposed on the b-wave, that occur in response to a strong stimulus (Figure 11). OPs are present under light- and dark-adapted conditions, with contributions from both rod-and cone-driven signals. The number of OPs induced by a flash of light varies between four to ten, depending upon species and stimulus conditions. OPs are generated in the proximal retina, amacrine and retinal ganglion cells are involved in its generation[135]. OPs are significantly attenuated in various retinal degenerations including DR[133].

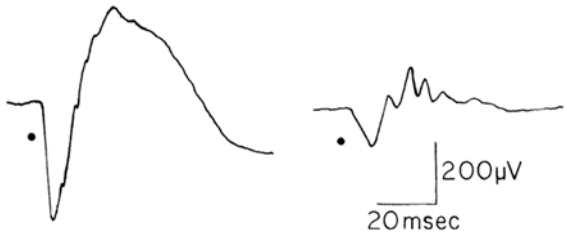

Figure 11. Oscillatory potential (OP) in ERG. OPs (right panel) are isolated from the flash ERG illustrated in the left panel. Extracted from Qian, 2011.

Two other waveforms that are sometimes recorded in the clinic are the c-wave originating in the pigment epithelium[136] and the d-wave indicating activity of the OFF bipolar cells (figure 12). Moreover, using different rates (flicker) of stimulus presentation also allows rod and cone

contributions to the ERG to be separated. Even under ideal conditions rods cannot follow a flickering light up to 20 per second whereas cones can easily follow a 30 Hz flicker, which is the rate routinely used to test if a retina has good cone physiology[133], it is also a useful tool to evaluate the neuro-vascular response of the retina[67, 137]. Neurovascular coupling is a physiological process adjusting the nervous microcirculation blood flow in response to neuronal activity. The flicker-ERG stimulation (30 Hz frequency) is used to study this process[3].

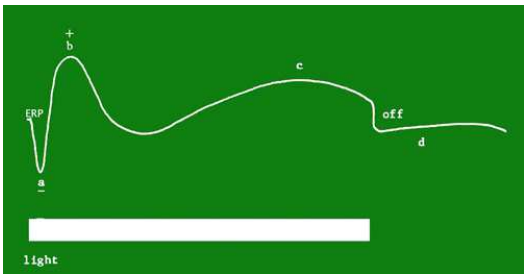

Figure 12. The figure shows all components of ERG when there is a long pulse of light as a stimulus. Extracted from Creel, 2005.

4. PATHOGENIC MECHANISMS INVOLVED IN RETINAL NEURODEGENERATION

4.1 METABOLIC PATHWAYS TRIGGERED BY HYPERGLYCEMIA

While most cells are able to reduce the transport of glucose inside the cell when they are exposed to hyperglycemia, so that their internal glucose concentrations remains constant, other cells are damaged by hyperglycemia because those cannot do reduce the transport of glucose efficiently. Therefore, diabetes selectively damages cells, like endothelial cells, whose glucose transport rate does not decline rapidly as a result of hyperglycemia, leading to high glucose inside the cell[138].

The tissue-damaging effects of hyperglycemia involve several mechanisms going on inside the cell.

The first mechanism discovered was the increased flux through the polyol pathway[139]. The enzyme aldose reductase has a central role in the polyol pathway (Figure 13). The normal function of aldose reductase is to reduce toxic aldehydes in the cell to inactive alcohols. This enzyme has a low affinity (high K_m) for glucose, and at the normal glucose concentration, metabolism of glucose by this pathway represents a very small percentage of total glucose use[140]. But when the glucose concentration in the cell becomes too high, aldose reductase also reduces glucose to sorbitol, which is later oxidized to fructose by sorbitol dehydrogenase (SDH). In the process of reducing high intracellular glucose to sorbitol, the aldose reductase consumes the cofactor NADPH[141]. However, NADPH is also an essential cofactor for regenerating a critical intracellular antioxidant, reduced glutathione (GSH). Hence, the cell cannot regenerate GSH due to the lack of NADPH, thereby the polyol pathway increases susceptibility to intracellular oxidative stress[138].

Moreover, sorbitol is impermeable to cellular membranes, it accumulates within the cell, and this is followed by the slow metabolism of sorbitol to fructose[142, 143]. Because of this, sorbitol is also thought to damage the cell by inducing osmotic stress[144]. In addition, the fructose produced by the polyol pathway can be phosphorylated to fructose-3-phosphate which in turn can be degraded to 3-deoxygucolsone, both of which are strong glycating agents and can result in the production of AGEs[145].

Increased intracellular formation of advanced glycation end-products (AGE) is found in diabetic retinal vessels[146, 147] and renal glomeruli[148]. AGEs are a heterogeneous group of molecules formed from the nonenzymatic reaction of reducing sugars with free amino groups of proteins, lipids and nucleic acids[149]. Intracellular hyperglycemia is the primary initiating event in the formation of both intracellular and extracellular AGEs[150].

AGEs can arise from intracellular auto-oxidation of glucose to glyoxal[151], decomposition of the

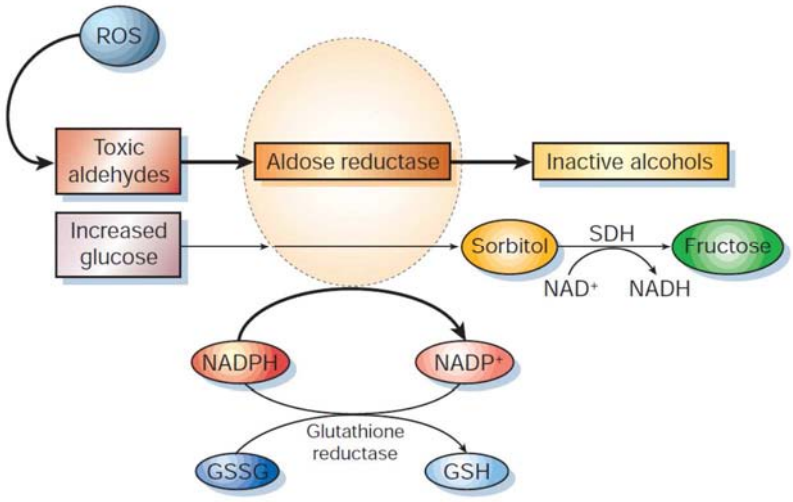

Figure 13. Aldose reductase and the polyol pathway. Extracted from Brownlee, 2001.

Amadori product (glucose-derived 1-amino-1-deoxyfructose lysine adducts) to 3-deoxyglucosone, and fragmentation of glyeraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal[152]. These reactive intracellular dicarbonyls (glyoxal, methylglyoxal and 3-deoxyglucosone) react with amino groups of intracellular and extracellular proteins to form AGEs[140].

The rate of AGE formation and accumulation depends on many factors such as the nature of the reducing sugar, availability of metal ions, redox balances, and longevity of the modified protein[147]. AGE formation may lead to protein cross-linking, which may result in abnormal tertiary structure and alter enzymatic activity, receptor recognition, and normal protein clearance[153].

Production of intracellular AGE precursors damages target cells by three general mechanisms:

- Intracellular proteins modified by AGEs have altered function.
- Extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with the receptors for matrix proteins on cells.

- Plasma proteins modified by AGE precursors bind to AGE receptors on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of reactive oxygen species. This AGE receptor ligation activates the pleoiotropic transcription factor NF-κB, causing pathological changes in gene expression. As it is known that transcription factor NF-κB itself activates many proinflammatory genes in the vasculature[154].

Receptors for AGE (RAGE) are expressed in almost all of the retinal cells, although the highest expression levels seem to be in Müller cells[155, 156]. RAGE expression in the retina has been predominantly localized to glia in the inner retina and is elevated in diabetic conditions. The activation of RAGE in retina by local or circulating ligands such as AGEs and S100B leads to changes in the activities of various retinal cells including Müller glia, retinal microglia, RPE, and retinal endothelial cells[153].

PKC activation: Intracellular hyperglycemia increases the amount of the lipid second messenger diacylglycerol (DAG) and DAG activates the PKC family. This fact has been observed in culture vascular cells and in retina and glomeruli of diabetic animals[157, 158]. The isoforms β- and δ- are the ones that are activated primarily, but increases in other isoforms have also been found in the retina, such as PKC-α and –ε[157]. Hyperglycemia can activate PKC isoforms indirectly by ligation of AGE receptors and also by increased activity of the polyol pathway[159, 160] because it increases reactive oxygen species (ROS) [140].PKC is involved in a great number of physiological processes, because of that, its upregulation contributes to the pathogenesis of DR by inducing enhanced release of angiogenic factors, endothelial and leukocyte dysfunction leading to capillary occlusion and leukostasis, changes in blood flow to the retina, and differential synthesis of extracellular matrix (ECM) proteins and ECM remodeling. As a result, the PKC pathway directly influences other pathways such as inflammation, neovascularisation, and aberration of hemodynamics, what additionally contribute to the pathogenesis and progression of DR[149].

Increased hexosamine pathway activity. When glucose is high inside a cell, the majority of that glucose is metabolized through glycolysis. The first step is converting it to glucose-6 phosphate, then fructose-6 phosphate, and follows through the rest of the glycolytic pathway. However, some of the fructose-6-phosphate is diverted from glycolysis to provide substrate for the enzyme glutamine:fructose 6-phosphate amidotransferase (GFAT), and is converted to glucosamine 6-phosphate, which is subsequently converted to uridine diphosphate-N acetylglucosamine (UDPGlcNAc). UDPGlcNAc then attaches to the specific serine and threonine residues in transcription factors, leading to posttranslational modification of cytoplasmic nuclear proteins. The overmodification of this glucosamine frequently results in pathologic

changes in gene expression[161-163]. In the pathogenesis of DR, activation of hexosamine pathway is associated with the apoptosis of endothelial cells and neurons[164].

4.2 ROLE OF OXIDATIVE STRESS

Oxidative stress is the consequence of the generation of excessive reactive oxygen species (ROS) production by a cell and the suppression of the capacity to defend against them, therefore it may be defined as an imbalance between the level of ROS or oxygen radicals and the antioxidant defenses in a biological system[165]. Clinical and experimental evidence has clearly demonstrated that diabetes increases oxidative stress[138, 165, 166], superoxide levels are elevated in the retina of diabetic rats and in retinal cells incubated in high glucose media[167-169].

Oxidative stress induced by hyperglycemia. Hyperglycemia increases the production of ROS in mitochondria. Mitochondria are the principal endogenous source of superoxide.

There are four protein complexes in the mitochondrial electron transport chain, namely complex I, II, III and IV (Figure 14). Glucose metabolization through the tricarboxylic acid cycle (TCA) generates electron donors. The main electron donor is NADH, which gives electrons to complex I. The other electron donor generated by the TCA cycle is $FADH_2$, formed by succinate dehydrogenase, which donates electrons to complex II. Electrons from both these complexes are passed to coenzyme Q, and then from coenzyme Q they are transferred to complex III, cytochrome-C, complex IV and finally to molecular oxygen, which they reduce to water[138]. This system allows regulating precisely the level of ATP. Part of the energy of those electrons is used to pump protons across the membrane at complexes I, III, and IV. This generates a voltage across the mitochondrial membrane. The energy from the voltage gradient drives the synthesis of ATP by ATP synthase[170, 171]. Alternatively, uncoupling proteins (UCPs) can lower the voltage gradient to generate heat as a way of keeping the rate of ATP generation constant[140].

In cells that live in the diabetic milieu, with high glucose inside, there is more glucose being oxidized in the TCA cycle, which leads to more electron donors (NADH and $FADH_2$) into the electron transport chain. As a result, the voltage gradient across the mitochondrial membrane increases until a critical threshold is reached. Then, electron transfer inside complex III is blocked[172], causing the electrons to back up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating superoxide. The mitochondrial isoform of the enzyme superoxide dismutase (SOD) degrades this oxygen free radical to hydrogen peroxide, which is then converted to H_2O_2 and O_2 by other enzymes.

The unifying model proposed by Brownlee[138] is based on the observation that hyperglycemia induced overproduction of mitochondrial superoxide induces a 66% decrease in the glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH) activity[173] (Figure 15).

When GAPDH is inhibited, the levels of all the glycolytic intermediates which are upstream of GAPDH increase. Increased levels of the upstream glycolytic metabolite glyceradehyde-3-phosphate activate two of the four pathways. It activates the AGE pathway because the major intracellular AGE precursor methylglyoxal is formed from glyceraldehydes-3 phosphate. It also activates the classic PKC, diacylglycerol is also formed from glyceraldehydes-3 phosphate.

Further upstream, the levels of the glycolytic metabolite fructose-6 phosphate increase. This fact leads to increased flux through the hexosamine pathway. Finally, inhibition of GAPDH increases levels of glucose. In this way, there is an increase in flux through the polyol pathway.

To sum up, when intracellular hyperglycemia develops in target cells of diabetic complication, it causes increased mitochondrial production of ROS. The ROS cause strand breaks in nuclear DNA, which activates poly(ADP-ribose) polymerase (PARP). PARP then modifies GAPDH, thereby reducing its activity. Finally, decreased GAPDH activity activates the polyol pathway, increases intracellular AGE formation, activates PKC and subsequently NFκB, and activates hexosamine pathway flux.

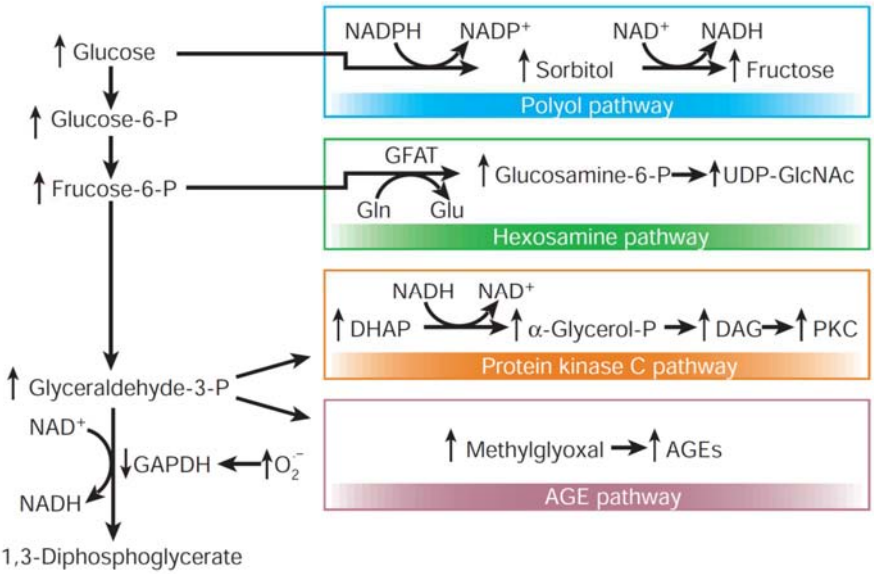

Figure 15. Mitochondrial overproduction of superoxide activate four major pathways of hyperglycemic damage by inhibiting GAPDH. Extracted from Brownlee, 2001.

To counterbalance ROS damage exist endogenous antioxidant defense mechanisms, including enzymatic and nonenzymatic pathways. However, it has been demonstrated that in diabetes the activities of antioxidant defense enzymes responsible for scavenging free radicals and maintaining redox homeostasis such as SOD, glutathione reductase, glutathione peroxidase, and catalase and vitamins A, C and E are diminished in the retina[174, 175].

Unstable ROS molecules also generate damage by reacting with structural components in the cell, such as neutral lipids and phospholipids of the membranes, via a process known as lipid peroxidation[176]. Increased lipid peroxidation in diabetes generates reactive aldehydes, such as 4-hydroynonenal, malondialdehyde and acrolein, and these aldehydes further react with proteins generating relatively stable advanced lipoxidation end-products (ALE). ALEs are thought to play a role in the pathogenesis of DR-related microvascular dysfunction, possibly via impairing normal retinal Müller glia function[177, 178]. Lipid peroxidation changes the fluidity of cell membranes, reduces the capacity to maintain defined ion gradients and also increases membrane permeability. Consequently, lipid peroxidation leads to a loss of intracellular proteins, reduces Ca^{2+} transport across the cell and endoplasmic reticulum membranes, altering mitochondrial voltage channels, and cell function[176, 179]. Moreover, the retina is the only neural tissue directly exposed to light, thus resulting in the photo-oxidation of many lipids that become extremely toxic to retinal cells[180].

Recently, it has been demonstrated that diabetes also increases arginase activity in the retina. This leads to a decreased arginine supply for nitric oxide syntase (NOS), and reduces nitric oxide levels, increasing superoxide generation[181]. Superoxide reacts with nitric oxide, generating peroxynitrite, increased retinal peroxynitrite levels are implicated in vascular and neural damage associated with DR[181, 182].

Oxidative stress can result in direct, free radical-based DNA damage, but can also trigger redox pathways required for transcriptional activation. NFκB in retinal pericytes is extremely sensitive to the redox status of the cells, and normally remains in an inactive form, as it is bound with an inhibitory IκB protein[176]. Several inflammatory stimuli, such as TNFα, and also elevated levels of oxidative stress can promote specific signal transducing pathways to enable phosphorylation of the IκB and subsequent degradation by the 26S proteosome[183]. The phosphorylation of IκB releases NFκB from IκB protein and permits NFκB to translocate to the nucleus[184]. Many target genes related to pro-inflammatory response are cyclically activated by NFκB. The imbalance between NFκB and IκB has several consequences, such as hyper-inflammation and loss of cell repair and function, which lead to apoptosis and DR disease evolution[176, 185]. Since DR shares

similarities with a low grade chronic inflammatory disease, and the levels of cytokines in the retina, and leukostasis is increased[186]. ROS, in the same way as for NFκB, are considered a strong stimulus for the release of the cytokines, for example, interleukin-1β itself can trigger signaling cascades resulting in excessive ROS[187]. Experimental evidence suggests that ROS seem to have paradoxic effects on NF-*κ*B regulation. ROS can either activate or inhibit NF-*κ*B activity, depending on the level of ROS, types of stimuli, and cell types. Moderate increase of ROS often leads to NF-*κ*B activation, which requires sequential steps in the cytosol and nucleus. Conversely, severe increase of ROS could inactivate NF-*κ*B, leading to cell death[188].

4.3 INFLAMMATION

As said before, DR exhibits characteristics of low-grade chronic inflammation[189]. There are changes in the expression of inflammatory transcripts, and simultaneously there are changes in retinal permeability and apoptosis[190]. Hyperglycemia, oxidative stress, AGE formation, and hypertension all contribute to inflammation, nevertheless, the inflammatory response itself spreads these pathways further, through cytokines, adhesion molecules, VEGF signaling, enhanced RAGE expression, changes in NO regulation and NF-κB signaling[149].

In the vitreous of patients with DR[191-194], proinflammatory cytokines, as TNF, IL-1β, IL-6, chemokines and adhesion molecules have been found increased. Among the effects of these molecules in the retina are increased endothelial cell permeability, breakdown of BRB and induction of leukocyte adhesion to vascular walls[196-198].

Alterations in the morphology of retinal microglia have been observed. These changes include shortening and decreased ramification of microglial membrane processes[199, 200]. Diminished ramification of microglia may indicate inflammatory activation of these cells[29, 199, 201, 202]. Recently, SD-OCT documented discrete hyperreflective spots, which may correspond to aggregates of activated microglia that increase with the clinical progression of DR[203, 204]. Several animal studies have suggested that these cytokines may contribute to microvascular complications and apoptosis during DR. The microglia which progresses to a fully activated inflammatory state produce numerous cytokines, including IL-6, IL-1, TNF, and MCP-1[205].

Genomic assessment of whole retinas of diabetic rats identified increased expression of the inflammatory genes CCL2, ICAM-1, STAT3, CCR5 and CD44[190], and Müller cells isolated from diabetic retinas of streptozotocin-diabetic rats had increased expression of several genes associated with immune function and inflammation[206].

In addition, it has been demonstrated that upregulation of the RAGE has a key role in the hyperglycemia-induced activation of Müller glia and downstream cytokine production in the context of DR[156, 207].

The mechanism by which these cytokines could contribute to retinal vascular and neural apoptosis is not yet clear but may imply the induction of glutamate excitotoxicity, oxidative stress or mitochondrial dysfunction[208-210].

4.4 GLUTAMATE EXCITOTOXICITY

Glutamate is the major excitatory neurotransmitter in the retina and, as said in the phototransduction section, it is used in the forward transmission of visual signals by photoreceptors, bipolar, and ganglion cells.

There are two classes of glutamate receptors, metabotropic and ionotropic. There are at least eight metabotropic glutamate receptors (mGluR). The metabotropic receptors are G-protein coupled receptors. These are subdivided into three subclasses. Type I metabotropic receptors are associated with intracellular phosphotidyl inositol metabolism. Type II and III receptors are associated with an inhibitory cAMP cascade as well as other postsynaptic cascades that lead to the release of Ca^{2+} from intracellular stores.

The ionotropic receptors work via ion channels. There are three subclasses of ionotropic receptors: N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainite type receptors. The NMDA receptors are the ones that are most associated with excitatory neurotoxicity and calcium entry into cells. The calcium entry causes release of caspases from the mitochondria leading to apoptosis[211].

In the neural retina, photoreceptors, neurons, and macroglial cells express high-affinity glutamate transporters (GLT)[212]. In the inner retina, Müller cells are responsible for the majority of glutamate uptake[213, 214]. The transport into Müller cells is reduced under pathological conditions, and, as a result, more glutamate is transported into inner retinal neurons[215, 216]. The bulk of glutamate released from photoreceptor terminals, in the outer retina, has been suggested to be removed by presynaptic transporters of photoreceptor cells[217] and probably by postsynaptic transporters at horizontal and bipolar cells[218].

There are five known high-affinity excitatory amino acid transporters for glutamate: EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), EAAT4, and EAAT5[219-223]. Uptake of glutamate into astrocytes and Müller cells is mediated by GLAST and GLT1 and into neuron by EAAC1, EAAT4, and EAAT5, the latter is primarily found in the retinal photoreceptor cells. Moreover, there are

glutamine transporters that need to be synchronized to transport glutamine from astrocytes into the neurons[211].

The clearance of synaptic glutamate by Müller cells is required for the prevention of neurotoxicity. Because of the significant neurotoxic effects of glutamate, glutamate concentrations have to be very closely regulated in the synapse.

Glutamate is taken up by Müller cells and there it is amidated to glutamine by the enzyme glutamine synthetase (see figure 16). Glutamine can be also transaminated to alpha-ketoglutarete which is released and taken up by neurons as a substrate for their oxidative metabolism, loaded into secretory vesicles or used for the production of glutathione. Glutamine synthetase is localized in the cytosol of astrocytes and Müller cells, in the neural retina[224]. Glutamine is released from Müller cells and taken up by neurons as a precursor for the synthesis of glutamate and GABA[225]. Otherwise, glutamine is transported into the mitochondria of Müller cells. The shuttle of glutamate and glutamine, respectively, between neurons and Müller cells is known as glutamate-glutamine cycle[226].

A small amount of glutamate actually escapes from the synaptic space and may have significant peripheral effects[227]. The amount that escapes appears to increase in pathologic conditions[211].

The rate of the glutamate uptake by Müller cells is enhanced by the glutamine synthetase activity[228]. Also, the expression of glutamine synthetase is regulated by glutamate[226].

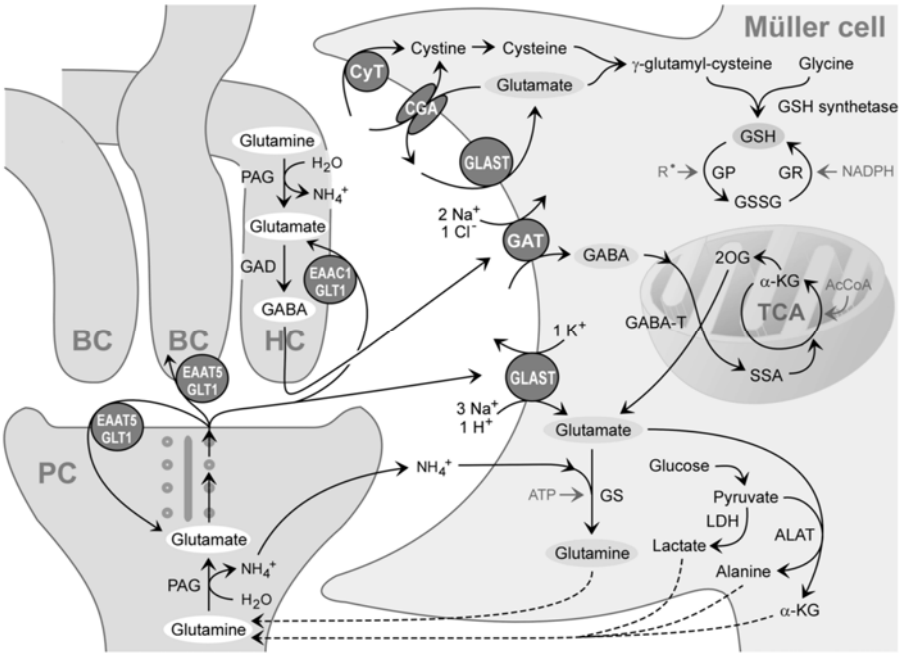

Figure 16. Recycling of neurotransmitters in the outer plexiform layer of the mammalian retina. Extracted from Bringmann, 2013.

The expression and activity of GLAST in Müller cells is regulated by the availability of glutamate[226]. Extracellular glutamate increases the expression of GLAST[229], whilst extended exposure to high glutamate induces a time-dependent internalization of the transporters[230]. An enhanced expression or activity of GLAST in Müller cells was observed under certain pathological conditions such as ischemia[231,232]. Other factors that increase GLAST expression are: cyclic AMP[233] and neurotrophic factors for example, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor (BDNF), and pigment epithelium-derived factor (PEDF)[234-236].

Elevated extracellular glutamate causes neuronal loss in many retinal disorders including DR, glaucoma, ischemia, and inherited photoreceptor degeneration[237-239]. Elevated levels of glutamate have been found in the vitreous fluid of patients with proliferative DR[211, 240], as well as in the retina of experimental models of diabetes[237, 241, 242].

Müller cells' glutamate uptake block the neurotoxic effect of the transmitter[243]. Therefore, a malfunction of the glial glutamate transport contributes to the increase in extracellular glutamate up to excitotoxic levels.

There is controversy about the alterations in the expression of GLAST. Some authors have found that the expression of GLAST is not significantly altered in diabetes and retinal ischemia[215, 244-246], but its efficiency in glutamate transport into Müller cells is reduced[215, 247]. A high amount of glutamate is transported into photoreceptor, bipolar and ganglion cells under these situations[215]. Nevertheless, our group and other authors found that there does exist a downregulation in GLAST expression in DR[248, 249]. These divergences may be due to the model used in the study.

Glutamate transporters are electrogenic and utilize energy stored in the transmembrane potential and the Na^+/K^+-ion concentration gradients to accumulate glutamate in the cell[250]. The efficiency of the electrogenic glutamate transport is decreased by the depolarization of Müller cells[251]. This fact can be induced by inflammatory lipids such as arachidonic acid and prostaglandins which are produced under oxidative stress conditions[252, 253]. These inflammatory mediators inhibit the sodium-potassium-ATPase, resulting in cellular depolarization[254, 255].

GABA is the major inhibitory neurotransmitter in the brain and retina. It is synthesized in the presynaptic axons of certain neurons via the use of glutamate decarboxylase (GAD). GABA is metabolized in the interior of Müller cells, where mitochondrial enzyme GABA transaminase catalyzes the formation of glutamate from 2-oxoglutarate, coupled to a conversion of GABA to succinate semialdehyde[226]. The GABA transaminase reaction has high efficiency, as a consequence, Müller cells display a very low level of intracellular GABA[256]. Under diabetic and ischemic condition, GABA rapidly accumulates in Müller cells[257, 258], due to a decrease in the GABA transaminase activity[257, 259].

Neuronal excitation and the defense against oxidative stress are linked by the uptake of GABA and glutamate by Müller cells. Müller cells provide photoreceptors and neurons with an antioxidative environment[118]. The major glia-derived antioxidant is reduced glutathione produced from glutamate, cysteine and glycine[260]. Under normal conditions, retinal glutathione is concentrated in glial and horizontal cells[260, 261]. In response to oxidative stress, glutathione is rapidly released from Müller cells and provided to neurons[261], where it acts as cofactor of enzymes which remove toxic peroxides and regulate protein function through thiolation and dethiolation. The production of glutathione in Müller cells is critically dependent on the availability of extracellular glutamate and cysteine[226].

Diabetes alters the equilibrium of glutamate and glutamine between neurons and glial cells by several potential mechanisms[40]:

1- Increase of glutamate production by glial cells due to the loss of the Müller cell-specific enzyme glutamine synthetase[237, 241].
2- Reduction in the retinal ability to oxidize glutamate to alfa-ketoglutarate[262-264].
3- Impairment of glutamate uptake by glial cells[247].

Therefore, diabetes may have a negative effect on glutamate uptake and metabolism, leading to a potential accumulation of extracellular glutamate, resulting in the so-called "excitotoxicity". The excitotoxicity of glutamate has as a consequence the over-activation of ionotropic glutamate receptors, mainly AMPA and NMDA, which have been found to be overexpressed in streptozotocin-induced diabetic rats[265, 266]and in human retinas[267]. This fact causes an uncontrolled intracellular calcium response in postsynaptic neurons[268, 269] and the cell death[211]. There are at least two mechanism involved in glutamate-induced apoptosis: caspase3-dependent pathway and caspase-independent pathway involving calpain and mitochondrial apoptosis-inducing factor[270].

4.5 IMBALANCE IN THE RETINAL PRODUCTION OF NEUROPROTECTIVE FACTORS

The retina synthesizes neuroprotective factors which counteract the deleterious effects of neurotoxic factors involved in neurodegeneration[105]. The loss of the neuroprotective factors or the reduction of their effectiveness is essential for the development of retinal neurodegeneration. These neuroprotective factors are: somatostatin (SST), pigment epithelial derived factor (PEDF), Erythropoietin (Epo), Interphotoreceptor retinoid-binding protein (IRBP), among others.

Somatostatin

SST immunoreactivity has been found in the mammalian neuroretina, mainly in GABAergic amacrine cells[271, 272]. However, our group has reported that SST expression is higher in the RPE than in the neuroretina from human eye donors[42]. The amount of SST produced by the human retina is significant as it is reflected by the strikingly high levels found in the vitreous fluid. Surprisingly, intravitreal SST levels are four-fold higher than plasma levels in non-diabetic subjects[273, 274] , suggesting that SST exerts an important role in retinal homeostasis.
Apart from SST, SST receptors (SSTRs) are also expressed in the retina. SSTR1 and SSTR2 are the most widely expressed[275]. The production of both SST and its receptors simultaneously suggests an autocrine action in the human retina. SST acts as a neuromodulator and angiostatic factor[276]. In addition, SST has been involved in the transport of water and ions. Various ion/water

transport systems are located on the apical side of the RPE, adjacent to the subretinal space, and, indeed, a high expression of SSTR2 has been shown in this apical membrane of the RPE[277]. In the diabetic retina, there is a downregulation of SST[42]. The lower production of SST in the retina is associated with a dramatic decrease of intravitreal SST levels in both diabetic patients with PDR[273, 274] and with DME[237]. Moreover, cortistatin (CST), a neuropeptide with strong structural and functional similarities to SST, is also downregulated in DR[43].

As a result, the physiological role of SST in preventing both fluid accumulation within the retina and neovascularization could be reduced, and consequently the development of DME and PDR is favored. For all these reasons, SST replacement treatment can be considered a new target not only for preventing the neurodegenerative process but also for more advanced stages of DR such as DME and PDR[278].

There is mounting evidence suggesting that SST could play a key role in preventing the main pathogenic mechanisms involved in DR: neurodegeneration, neovascularization, and vascular leakage. It is worth mentioning that, our team demonstrated that treatment with SST eye drops in streptozotocin-induced diabetic rats prevented ERG abnormalities, glial activation, apoptosis, and the misbalance between proapoptotic and survival signaling detected in these rats. In addition, SST eye drops inhibited glutamate accumulation in the retina and GLAST downregulation induced by diabetes mellitus. Our conclusion was that topical administration of SST has a potent effect in preventing retinal neurodegeneration induced by diabetes mellitus[248]. To date, the use of **eye drops** has not been considered a good route for the administration of drugs addressed to preventing or arresting DR. This is because it is generally assumed that they do not reach the posterior chamber of the eye (i.e., the vitreous and the retina). However, this is a misleading concept and there is emerging experimental evidence that eye drops are useful in several diseases of the retina including DR[279]. In this regard, the work previously mentioned demonstrated that SST reaches the vitreous and the retina after topical administration[248]. In addition, a multicentric, phase II-III, randomized controlled clinical trial (EUROCONDOR-278040. EudraCT: 2012-001200-38) to assess the efficacy of brimonidine and SST administered topically (eye drops) to prevent or arrest retinal neurodegeneration and its effect on the development and progression of microvascular impairment was approved by the European Commission in the setting of the FP7-HEALTH.2011. This trial started in February 2013, and the final results should be available in 2016[280,40].

Pigment epithelial derived factor

PEDF was first purified from human RPE cells and it was described as a neurotrophic factor with neuroprotective properties[281]. PEDF exerts anti-inflammatory functions, attenuating the expression of chemical mediators, such as vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNFα), and intercellular adhesion molecule 1(ICAM-1) in retinal vascular ECs[282, 283]. A part from its neurotrophic and neuroprotective properties, PEDF is among the most important natural inhibitors of angiogenesis and it is downregulated in DR. In addition, it has recently been suggested that PEDF might upregulate GS and GLAST expression and decrease glutamate levels by suppressing IL-1β which is an inflammatory factor. The same effects have been found under hypoxia conditions. In these functions may underlie the neuroprotective effects of PEDF[284, 285].

Erythropoietin

Epo and its receptor (Epo-R) are both synthesized by the human retina, mainly in RPE[286, 287]. Epo is a potent neuroprotective factor[288-290]. In the vitreous fluid of diabetic patients[287] our group has found high levels of these molecule. Moreover, Epo is a potent physiological stimulus for the mobilization of endothelial progenitor cells (EPCs) toward injured retinal sites, thus participating in the remodeling of the damaged tissue[291]. The downregulation of neuroprotective factors occurs simultaneously with the overexpression of Epo and, therefore, it is possible that this counteracts the reduction of the neuroprotective factors. However, in advanced stages of DR the elevated levels of Epo could favor neovascularization, thus contributing to PDR development[105, 291, 292]. In streptozotocin-induced diabetic rats, exogenous erythropoietin administration by intravitreal[293] or intraperitoneal injection[294, 295] prevents structural vascular and neural damage in early diabetes. Nevertheless, in advanced stages of the disease elevated levels of Epo could enhance the effects of VEGF, thus contributing to neovascularization and, in consequence, worsening proliferative DR[40, 105].

Interphotoreceptor retinoid-binding protein

IRBP is an interphotoreceptor matrix glycolipoprotein secreted by photoreceptors that plays an essential role in photoreceptor survival[296, 297]. In addition to participate in the visual cycle, IRBP is important in fatty acid transport and it is vital to the maintenance of the photoreceptors[298, 299]. The absence of IRBP in the interphotoreceptor matrix resulted in significant increases in TNFα, TNFR1 and RIP kinases, the intracellular key mediators of TNF-induced cellular necrosis[300].

Our group has found that vitreous IRBP concentration is reduced in diabetic patients, and both IRBP mRNA and protein content are significantly lower in the retinas from diabetic donors than in those from non-diabetic donors[44, 301]. Besides, there is a relation between the levels of IRBP and the neurodegeneration found in the retinas from diabetic patients. When the levels of IRBP are low, the neurodegeneration found in the retinas from diabetic patients is high[44]. Furthermore, a dose-dependent downregulation of IRBP was detected with glucose, TNFα and IL1β in cultures of Y79 human retinoblastoma cells[44]. Therefore IRBP downregulation is associated with retinal neurodegeneration[44, 69].

Other neuroprotective factors

Other factors found in the eye that exhibit neuroprotective properties in experimental models of diabetes include brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Levels of BDNF are reduced in animal models of diabetes[302] and this reduction is correlated with amacrine cell degeneration. Intravitreal administration of BDNF to diabetic rats reverses amacrine cell degeneration[302] and overexpression of BDNF in diabetic rats enhances ganglion cell survival and function[303]. NGF was implicated in the pathogenesis of DR when it was demonstrated that treatment of diabetic rats with NGF prevents ganglion cell apoptosis[304]. In human diabetic donors in streptozotocin-diabetic rats has been found that levels of NGF are also reduced[305] in association with retinal neurodegeneration[306].

Our group has made important contributions to the determination of insulin-like growth factor-1 (IGF-1) in the human diabetic eye. It was demonstrated that there is an increase in the vitreous levels of IGF-1 in PDR patients due to serum diffusion caused by BRB disruption. Also it was found that there is a deficit in the ocular production of this molecule in diabetic patients[307-309].

5. MOUSE MODELS FOR THE STUDY OF DIABETIC RETINOPATHY

Although a lot of important information or clues on the development of DR can be obtained from human studies, the mechanisms of DR development are still being elucidated.

Animal models enable us to have a more comprehensive understanding of the etiology of the disease at a molecular level in a controlled manner. Moreover, animal models allow us to increase our knowledge in developing drug screening[310].

To date, several species including mice, rats, cats, dogs, pigs and non-human primates, have been used as models to provide valuable information on the cellular and molecular aspects of

pathogenesis of DR. The most used models are rodent models, and they have been widely used for studying the molecular mechanisms underlying the pathogenesis of DR.

The main advantages of rodent models are that they are easy to handle, relatively inexpensive, have short reproductive cycles and have a similar genetic background to humans, allowing experimental results to be extrapolated.

Rodent models of DR vary with respect to species (predominantly rat or mouse), strain, method used for diabetes induction and duration of diabetes. Diabetes in animals is usually induced with chemicals such as streptozotocin (STZ) or alloxan, by surgical pancreatectomy, or spontaneously by selective breeding or genetic manipulation[311].

Advanced techniques of genetic manipulation, such as tissue specific transgenic expression and targeted gene knockout, have increased the relative importance of mouse models for experiments that specifically require genetically engineered models. Thus, these animals provide a remarkable platform to investigate the pathogenesis of at least the early stages of the retinopathy, because genetic alterations of selected metabolic and pathophysiological mechanisms are now possible[311].

However, a major criticism of using rodents to model DR is that they might not exactly mirror the human condition, especially with regard to the extent of pathology. Rodent models reproduce most aspects of the early stages of DR but have not been found to reproducibly develop the late, neovascular stage of the disease, probably owing to the short lifespan of the animals and thus the shorter duration of diabetes.

Mice have been routinely used in many in vivo studies since they are small in size and therefore easy to handle and inexpensive to house. They also have relatively short life span that allows a shorter experimental turnover time. Indeed, mechanistic studies of DR have been carried out extensively in mice as these models share similar symptoms of early DR as in human. More importantly, the availability of a collection of transgenic and knockout mice allows researchers to study the role of particular genes, which may even be cell type specific, in the development and pathophysiological[310].

Mice have been used less frequently as models in studies of DR[312]. This is because they are more resistant to the STZ effect, have lower eye cups and present a lower degree of lesions compared

to rats[313]. This relative protection in developing pathological lesions related to diabetes can be partly attributed to a lower activity of aldose reductase compared to rats[31].

5.1 TYPE 1 DM MODELS

5.1.1 PHARMACOLOGICALLY INDUCED (STZ)

Type 1 diabetes can be induced in mice by injection of chemicals, including streptozotocin and alloxan, both of which are toxic and destroy the β-cells in the pancreatic islets.

Streptozotocin is a potent neurotoxic agent. STZ induces toxicity related to the glucose moiety in its chemical structure, which enables STZ to enter into cells via the GLUT-2[314, 315]. STZ functions as a DNA synthesis inhibitor in both bacterial and mammalian cells. STZ exerts beta-cell toxicity mostly by causing alkylation of DNA, which triggers activation of poly ADP-ribosylation consequently leading to depletion of cellular NAD+ and ATP, and damaging the main beta-cell function – insulin production and secretion. STZ action in β-cells is accompanied by characteristic alterations in blood insulin and glucose concentrations[316].

Since STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells, it was proposed that this molecule contributes to STZ-induced DNA damage. Moreover, STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells. The synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ[317].

STZ is a toxic drug itself and it has been one of the chemical agents for the induction of diabetes in experimental animals. However, there are evidences that point out STZ to be also neurotoxic. Park et al. [318] noted a temporary increase in inner retinal thickness at 1 week after STZ injection, possibly reflecting swelling caused by neurotoxicity that recovers after STZ is cleared from the circulation. In addition, Phipps et al. [319] observed retinal neuronal dysfunction, an immediate decrease in the ERG waveform, as early as 2 days after STZ injection.

Compelling evidence demonstrates that single or multiple injections of low doses of STZ into the lateral cerebral ventricles produce neurochemical and brain glucose metabolism changes, as well as long-term and progressive deficits in learning, memory and cognitive behavior, which resemble features of Alzheimer's disease (AD) patients. Moreover, direct histopathological evidence of specific neurotoxic damage caused by intracerebroventricular STZ administration to axons and myelin in the fornix, anterior hippocampus and periventricular structures that are essential for learning and spatial memory have been reported [320]. Pathological changes to the

brain after an intraventricular injection of STZ are very similar to the neurodegeneration reported in DR[321].

STZ toxicity is dependent on glucose transporter GLUT-2, through which it enters the cell. For long time it has been demonstrated the presence of GLUT-2 in the rat retina [322]. It has been demonstrated that GLUT-2 is expressed in Müller cells, on the apical surfaces that face the inter-photoreceptor space. The importance of GLUT-2 glucose transporter in this process is also shown by the observation that STZ damages other organs expressing this transporter, particularly kidney and liver [317].

Streptozotocin is a potent neurotoxic agent and is able to produce neuronal degeneration[320, 321]. Therefore, neurodegeneration observed in rodents with STZ-DM could be due to STZ itself rather than the metabolic pathways related to diabetes.
STZ-diabetic mouse present alterations in electroretinogram measurements, vascular leakage, leukostasis, neural apoptosis and Müller cell activation[27, 237], features observed in human DR, however they lack symptoms of neovascularisation, which are used in the clinical staging of human DR[323].

5.1.2 MODELS CARRYING ENDOGENOUS MUTATION

INS2^{Akita}

These mice carry a point mutation in the insulin2 gene, which results in a single amino acid substitution that causes misfolding of the insulin protein which accumulates in the pancreatic β-cells, and ultimately leads to cell death.

Cellular lesions, such as reactive microglia, are evidenced as early as 8 weeks of diabetes[199]. By 12 weeks of onset of hyperglycemia, immunological studies showed abnormal swelling in somas, axons and dendrites of RGCs, and the number of these cells was reduced in the peripheral region[324]. It has been reported that the number of RGCs was significantly reduced in the peripheral regions after 22 weeks of hyperglycemia[325]. Moreover, a thinning in the IPL and INL has been shown in this model after 6 months of hyperglycemia, which may be due to the decrease of the cholinergic and dopaminergic amacrine cells[324]. A decrease of a- and b-wave amplitudes in the ERG after 8 months indicates functional problem associated with the cellular defects or degeneration[326].

On the other hand, another study showed that despite 10 months of diabetes this mouse model did not show loss of optic nerve axons (thus suggesting no loss of retinal ganglion cells). Likewise,

other parameters of cell death (terminal transferase deoxyuridine triphosphate nick-end labeling and annexin V labeling) did not suggest ganglion cell death[327]. In addition, recently it has been demonstrated that the classical clinical correlates of human DR are absent in $Ins2^{Akita}$ mice up to 6 months of age suggesting that either the histopathological processes underlying the development of DR in this model require longer than 5 months of hyperglycemia to result in disruption of retinal architecture[323].

Non-obese diabetic (NOD) mice

Non-obese diabetic mice spontaneously develop type 1 diabetes owing to autoimmune destruction of insulin-producing pancreatic β-cells by CD4+ and CD8+ T cells[328]. The onset of hyperglycemia in these animals is 12 weeks of age, and after four weeks of hyperglycemia apoptosis of pericytes, endothelial cells and RGCs, perivascular edema, and retinal capillary basement membrane thickening have been reported[310, 329]. Studies have shown the loss of retinal microvessels, reduced perfusion of the retina and disordered focal proliferation of vessels in NOD mice[330, 331]. Only few reports on DR have been published using this model[311] and they are mainly focused on advanced stages of the disease.

5.2 TYPE 2 DM MODELS

5.2.1 MODELS CARRYING ENDOGENOUS MUTATION

KKA^{y}

The KK mouse strain exhibits glucose intolerance and insulin resistance, and becomes obese with aging. Pericyte ghosts, acellular capillaries with occasional microaneurysms have been reported between 20 and 64 weeks of age. Lethal yellow (A^{y}) is a mutation at the mouse agouti locus in chromosome 2 that causes a number of dominant pleiotropic effects, including a completely yellow coat color, obesity, and insulin-resistant type 2 diabetic condition[332].

KKAy mouse is a combined model made by the introduction of the yellow obese gene, A^{y} , into the KK mouse. KKA^{y} mice are characterized by early onset and prolongation of severe levels of hyperinsulinemia, hyperglycemia, obesity and yellow coat color, accompanied by pathological changes in a variety of tissues.

It has been reported that after 1 month of diabetes, the numbers of apoptotic cells in the retinal ganglion cell and inner nuclear layers were significantly greater in the diabetic KKA^{y} mice than in the control group, and the rate of cell death increased with the duration of diabetes[333]. After 3 months of diabetes, the major changes in the retinal capillaries involved in mitochondria, with

endothelial cell hyperplasia, basement membrane thickening, and some edema and vacuolar degeneration of capillary cells[334].

Food intake is important in determining the severity of the diabetic phenotype and restriction of energy intake reduces both the obesity and hyperglycemia seen in this strain of mice[335, 336].

The diabetic characteristics of KKA^{Y} mice revert to normal at the age of 40 weeks. This amelioration is not associated with an improvement in insulin resistance or glucose metabolism[337, 338]. Due to the limited retinopathologic findings and the uncertain etiology of this model, it is not popularly used in DR studies.

db/db

This is the model used in the present study.

The diabetes (db/db) mutation in mouse was first described by Hummel and coworkers[339] in 1966. In their study they concluded that this mutation was inherited as a unit autosomal recessive and that it was characterized by a metabolic disturbance resembling diabetes mellitus in human. They also described abnormal deposition of fat at 3 to 4 weeks of age, followed shortly by hyperglycemia, polyuria, and glycosuria. Accompanying morphological changes in the islets of Langerhans suggested neogenesis to compensate for insulin resistance. This characterization was followed by others and finally in 1996 the specific mutation in the diabetes gene was fully characterized: it was found that a point mutation changing G→T resulted in the production of an abnormally spliced mRNA for the leptin receptor[340]. This fact leads to a defective signal transduction[341]. The db/db mouse develops obesity, insulin resistance, hyperglycemia and resistance to leptin. There is also a marked hyperinsulinemia, indicating that db/db mice do not have absolute insulin deficiency but instead display insulin resistance. Furthermore, at 15 weeks there is a decrease in plasma insulin, suggesting beta-cell failure[342]. Hyperglycemia and obesity continue to worsen throughout the life of these mice[343]. The percentage of mortality is 20% at 5 months and 75% at 12 months[344]. Thus, the db/db mouse is a model for severe type 2 diabetes associated with obesity.

The first description of DR in the db/db mouse's eye was made on 1989 by Midena and collegues[345]. They found, in mice from 12 to 64 weeks of age, a marked increase in the ratio of endothelial cells to intramural pericytes in diabetic mice compared to controls. This increase resulted from a selective and highly significant loss of pericytes in db/db mice. They also observed acellular capillaries. All these features worsened with aging.

Nearly a decade later Clements et al. [346] found that db/db mice of 22 weeks of age had basement membrane thickening and accumulation of basement membrane material in the capillaries of the ONL, compared with their non-diabetic littermates. Furthermore, they found that treatment with monoclonal antibodies that specifically recognized Amadori-modified glycated albumin ameliorated these abnormalities without altering blood glucose levels.

In 2003 it was described an increase in microvascular blood flow in the retina of these mice at 18 weeks of age, but this was not found in 8 weeks old mice[347].

Cheung and coworkers[348] deepened in the study of db/db mice and found that these mice expressed elevated levels of aldose reductase (AR). AR is expressed in astrocytes, Müller cells, retinal ganglion cells, neurons in the INL, and capillary pericytes in db/db mouse. This is a very interesting feature that not all rodent models express; the increased expression of AR further exacerbates the toxic effect of hyperglycemia. In their study 60 weeks old db/db mice showed pericyte loss, breakdown of BRB, apoptosis and proliferation of retinal capillaries, a more advanced feature of DR. Moreover, they demonstrated the presence of nitrosative-oxidative stress in those retinas, by showing an increase in the presence of nitrotyrosine and PARP. They also generated a transgenic model knocking out the AR gene in db/db mouse. AR deficiency resulted in decrease of vascular leakage, prevention of apoptosis and oxidative stress, prevention in the loss of pericytes. In that way, they demonstrated that AR plays a crucial role in the development of diabetic complications.

Analysis of the vitreous fluid of these mice demonstrated that, in comparison to their db/+ littermates, db/db had decreased levels of PEDF and increased VEGF, and also elevated concentrations of lipid peroxide products and type IV collagen at 18-20 weeks of age[349]. All these features had been found in the vitreous fluid of human patients with DR[350, 351]. Interestingly, at this age retinal microvessel proliferation has not been observed[345, 346].

Tang and coworkers[352] studied the effect of 8 weeks of treatment with dietary wolfberries in db/db mice starting at the age of 6 weeks. At the end of the study they found that 14 weeks old db/db mice had the whole central retina significantly thinner than WT mice, and a thinning in INL and photoreceptor layers. Moreover at this age db/db mice displayed less cells in GCL than WT mice. When they analyzed the possible causes found that biomarkers of ER stress (BiP, PERK, ATF6, and active caspase-12) were elevated. Oxidative stress was also present because Mn SOD and thioredoxin were inhibited in retina of db/db mice.

The first study to show results about retinal function of db/db mice was published in 2012[353]. The authors demonstrated a gradually deteriorated function in db/db mice under omega-6PUFA-rich diet. The ERG assessment was made at 9, 14 and 26 weeks of age and indicated that omega-3PUFAs preserve retinal function. But this study did not show the functional alterations in comparison to db/db mice without treatment.

He and coworkers studied Antioxidant Response Element (ARE) - regulated pathways in db/db mice. They described that the expression of HO-1 (an ARE-mediated antioxidant) was in the early diabetic retina in response to hyperglycemia, and this upregulation is mediated partially by NADPH oxidase. However, the persistence of hyperglycemic insults diminished the expression of HO-1, suggesting that long-term hyperglycemia leads to an increase in ROS generation and decreased antioxidant capacity[354].

A proteomic study in the retina of db/db mice was made by Li et al.[355] in mice starting at 8 weeks of age a treatment with metformin for 2 weeks, and the analysis was performed at 10 weeks. They found alterations in VGLUT1, which is responsible for loading glutamate into synaptic vesicles, it was found to be differentially abundant in db/db mice and was not normalized by metformin. The decrease in Slc17a7/VGLUT1 was confirmed by transcriptomic and immunocytochemical analysis.

All these data point out that db/db mice manifest biochemical abnormalities that reflect developing retinopathy without relying on exogenous induction of diabetes and/or hypoxia[349]. Therefore, it seems appropriate for investigating the underlying mechanisms of retinal neurodegeneration associated with diabetes and for testing new drugs. However, the characterization of the retinal neurodegenerative process and its functional consequences in db/db mice needs to be completed.

6. TARGETING NEUROPROTECTION. POTENTIAL NEUROPROTECTIVE TREATMENTS

6.1 FENOFIBRATE

Fenofibrate is a fibric acid derivative introduced in clinical practice on 1975. It is conventionally used to treat hypertriglyceridemia, low HDL-C levels, and as an adjunct to statins in dyslipidemia (Figure 17).

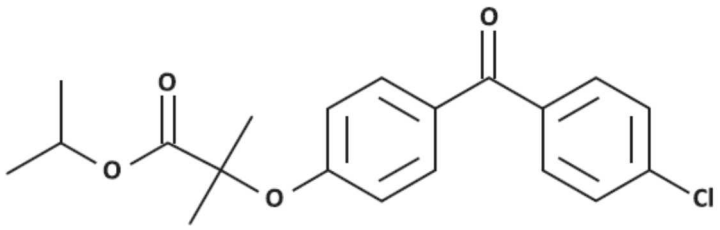

Figure 17. Chemical structure of fenofibrate. Lines indicate carbon bonds. O, oxygen; Cl, chlorine. Extracted from Noonan et al., 2013.

6.1.1PHARMACOKINETIC PROPERTIES

Fenofibrate is rapidly absorbed following oral administration and converted by esterases to its active metabolite fenofibric acid (FA)[356], which is a peroxisome proliferator-activated receptor-α (PPARα) agonist. After administration of 200 mg/day of the capsule formulation of micronized fenofibrate, the mean plasma concentration of fenofibric acid is 15 mg/mL; the maximum plasma concentration (C_{max}) occurs a mean 5 hours after dosing. Approximately 99% of fenofibric acid is bound to plasma albumin[357]. It has an elimination half-life of 20 hours, allowing a daily administration. Following oral administration of fenofibrate and its metabolism to fenofibric acid, no unchanged fenofibrate is detectable in plasma. The drug is mainly excreted in the urine as fenofibric acid and its glucoronide conjugate and feces, at 6 days is up to 93%[357].

6.1.2 PHARMACODYNAMIC PROPERTIES

Three major types of PPAR have been identified: PPAR-alpha, PPAR-gamma and PPAR-beta/delta. They are codified by different genes and have a distribution pattern variable depending on the tissue. In the nucleus they act as transcription factors and modulate gene expression related to glucose and lipid metabolism, adipogenesis, inflammation and oxidative stress[358].

Fenofibrate is a PPARα agonist. PPARalpha is highly expressed in tissues with elevated mitochondrial and peroxisomal fatty-acid beta-oxidation rates, such as liver, heart muscle, kidney, skeletal muscle, brown fat, and retina. PPARalpha is also present in monocytes, macrophages and endothelial cells[359].

The main natural ligands of PPARalpha are fatty acids and endogenous eicosanoids. Physiological concentrations of diet-derived unsaturated fatty acids also activate PPARalpha. In addition, PPARalpha are strongly stimulated by synthetic molecules such as the fibrates. The main effects of PPARalpha stimulation in humans are decreasing triglycerides, shifting low-density lipoprotein cholesterol to larger particles, and increasing high-density lipoprotein cholesterol particles[358].

Bound PPARα undergoes a conformational change to form a heterodimer complex with another nuclear receptor, the retinoid X receptor. The PPARα-retinoid X receptor complex then binds with specific DNA peroxisome proliferator response elements to activate or repress target gene transcription (Figure 18).

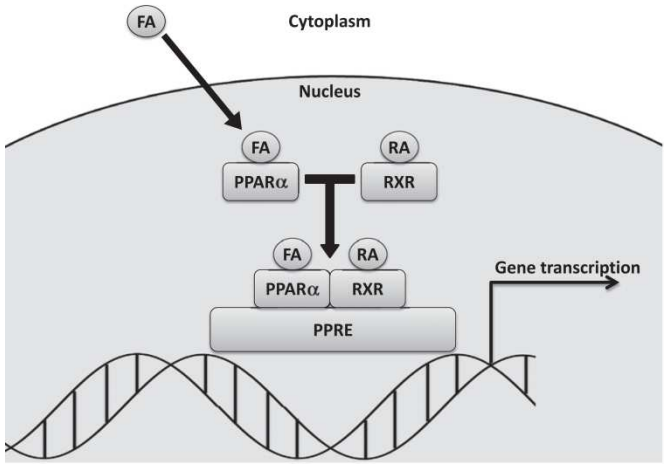

Figure 18. Classical fenofibrate signaling pathway. Extracted from Noonan et al., 2013.

6.1.3 MECHANISMS OF ACTION

Lipid effects:

Activated PPARalpha lowers free fatty acids by upregulating synthesis of proteins responsible for fatty acid transport and beta-oxidation, which inhibits the formation of triglycerides and VLDL. Triglyceride levels are further reduced due to upregultation of the synthesis of lipoprotein lipase and apolipoprotein (Apo)-V and downregulation of Apo-CIII. As a consequence of these

changes, there is a shift in the balance of LDL species from small, dense particles toward larger ones, that are more easily cleared by the LDL receptor and less likely to become oxidized. In addition, increased Apo-AI and Apo-AII expression increases vasoprotective HDL-C levels and facilitates reverse cholesterol transport[360, 361]. In this regard, our group has demonstrated that Apo-AI is overexpressed in the retina of diabetic patients[301, 362]. Moreover, Apo-AI is a key factor for the intraretinal transport of lipids, thus preventing lipid deposition and lipotoxicity, and is also a potent scavenger of reactive oxygen species. For these reasons the high Apo-A1 content found in diabetic retinas is seen as a protective mechanisms against lipid deposition (hard exudates) and against oxidative stress[359, 362].

No-lipidic mechanisms:

There are several nonlipidic mechansims by which FA could exert its beneficial effects in preventing or arresting DR.

- *Neuroprotective effect*: in experimental models of cerebral ischaemia and neurodegenerative diseases, PPARalpha activation had a neuroprotective effect, independent of lipid metabolism. Antioxidant, anti-inflammatory, and antiapoptotic properties of fenofibrate have been implicated in this effect[363]
- *Antiapoptotic activity*: FA exerts a protective effect on retinal microvasculature, supressing apoptosis and stimulating phosphorilation of endothelial nitric oxide synthase (eNOS), and therefore producing nitric oxide (NO). Moreover, FA prevents apoptosis of cultured human retinal endothelial cells induced by serum deprivation through a PPAR-independent but AMPK-activated-dependent pathway[364-366]. Furthermore, in a RPE cell line our group has been observed that FA exerts a dual protective effect, by down-regulation of stress-mediated signaling and induction of survival and autophagy pathways[367].
- *Antioxidant and anti-inflammatory activity*: FA through PPARalpha activation stimulates expression and activation of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase[363]. PPARalpha activation induces apoptosis of human monocyte-derived macrophages and inhibits the expression of vascular cell adhesion molecules on the endothelium[368]. This is thought to be relevant in the prevention of leukostasis[359]. Our group has demonstrated that FA prevents the deletereous action of IL-1β in the disruption of the BRB by supressing AMPK activation[369]. Fenofibrate reduces systemic inflammation and increases plasmatic levels of adiponectin, which has a protective effect on retinal blood vessels by

modulating TNF-α pathway[370, 371]. In addition, the anti-inflammatory effects of FA are done through inhibition of NF-κB activity[372], avoiding the increase in the expression of IL-6 and COX-2 induced by IL-1[373, 374]. Several studies have demonstrated that fenofibrate is able to inhibit Wnt pathway avoiding LRP6 phosphorylation and β-catenin accumulation. In DR, hyperglycemia and oxidative stress activate Wnt pathway, leading to an increase in ROS production and stimulating proangiogenic genes transcription. The beneficial effects of fenofibrate on Wnt pathway inhibition are due to PPARalpha activation[361, 375].

- *Antiangiogenic activity*: fenofibrate inhibited basic fibroblast growth factor-induced proliferation of bovine capillary endothelial cells VEGF-induced human umbilical vein endothelial cell proliferation and migration[376]. Moreover, fenofibrate reduced human umbilical vein endothelial cell expression of VEGF receptor-2 via PPARalpha-dependent inhibition of Sp-1[377]. In experiments treating two murine models of type 1 DM with fenofibrate (orally and intravitreally) the authors found an improvement in leukostasis and vascular permeability, as well as a reduction in adhesion molecules and VEGF overexpression[378].

6.1.4 CLINICAL TRIALS

Two major clinical trials showed that DR progression was significantly reduced by fenofibrate: the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) [379, 380] and the Action to Control Cardiovascular Risk in Diabetes (ACCORD)-Eye[381].

The FIELD study

It was a placebo-controlled trial conducted on 9795 participants with type 2 diabetes aged between 50 and 75, which had not received a statin treatment at baseline. This study was primarily designed to assess treatment effects on cardiovascular outcomes. The average follow-up was 5 years. The study showed no significant effect with fenofibrate (200 mg/day) on major coronary events (11% reduction vs. placebo; p=0.16). Nevertheless, fenofibrate treatment did reduce significantly total cardiovascular events (from 13.9% to 12.5% placebo vs. fenofibrate; p=0.035). The 8% of total participants had DR (PDR or DME) at baseline, and the evaluation of fenofibrate effect on DR progression and the need of laser photocoagulation were included as tertiary objective. A percentage of 3.6 of participants treated with fenofibrate versus 5.2% in the placebo group required laser therapy over 5 years (p=0.0003). In patients suffering from DME the reduction was of 30% (2.2% vs. 1.5%; p=0.015). They did not observe significant differences in lipid concentration between patients needing laser therapy and those who did not need it,

this fact suggest that the beneficial effects of fenofibrate, evident since 8 months of treatment, are independent of its hypolipemiant action[379].

The FIELD study incorporated an ophthalmology sub-study including 1012 participants without preexisting clinically significant retinopathy, DME, or history of laser treatment[380]. DR progression, defined by two steps of the Early Treatment Diabetic Retinopathy Study (ETDRS) scale, the primary endpoint of the sub-study, was significantly reduced (79%) in participants with preexisting DR, but not in those without DR.

However, there are some weaknesses in this study. It should be noted that retinal photographs were not collected routinely in the main study, given that the initial status of the retina (the presence and degree of DR) is the main determinant of laser requirement during follow-up, knowledge of the status of the retina at study entry is a key piece of information to confirm the results. Second, the criteria followed by the participating centers to indicate laser treatment were not defined at entry and were therefore presumably heterogeneous. And third, absolute event rates over the 6-year follow up period were very low, which precludes definitive conclusions[382].

ACCORD-Eye study

The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial was a large, randomized, controlled trial of the effects of intensive versus standard degrees of glucose lowering, intensive versus standard degrees of systolic blood pressure lowering, and the addition of a fibrate versus placebo to a statin on the 5-year incidence of serious cardiovascular outcomes in 10251 people with type 2 diabetes and other cardiovascular risk factor[381]. ACCORD participants who did not have a history of proliferative DR treated with laser photocoagulation or vitrectomy were eligible to also participate in the ACCORD Eye Study (n=3369). In this subgroup of participants, the effect of the interventions on retinal pathology at baseline and after 4 years of follow-up was assessed. Fenofibrate treatment was associated with a 40% decrease in DR progression over 4 years (from 10.2 to 6.5 %, p=0.006). These findings are consistent with the results of the FIELD study, despite somewhat stronger criteria for the DR outcome (≥3 steps of the ETDRS scale or proliferative DR requiring either laser treatment or vitrectomy). Notably, the beneficial effects were even higher than in the group allocated to intensive glycemic control, in which a 30 % reduction of DR progression was detected. Once again, the benefit was greater in participants with evidence of retinopathy at baseline (absolute risk reductions 6.9 vs. 0.2 % in those without DR at baseline)[383].

6.2 GLP-1

Glucagon Like Peptide-1 (GLP-1) is a member of the "glucagon peptide family" and is derived from the expression of preproglucagon gene located on chromosome 17. The gene product is acted upon by a specific propeptide convertase (PC) that cleaves propeptide and proprotein substrates at the C-terminus to generate biologically active peptides. PC1/3 is particularly involved in the generation of GLP-1 and GLP-2 in the intestinal L cells.

GLP-1 comprises 30 aminoacid. It further undergoes amidation at the carboxyl-terminus. This C-terminal amidation along with the histidine at position 7 of GLP-1 are very important for its insulinotropic action and probably also for glucagon-inhibiting activity (Figure 19). Amidation, in addition, has been shown to prolong the survival of GLP-1 in the blood stream[384].
GLP-1 enters into circulation in response to the absorption of glucose and other nutrients and powerfully augment glucose-induced insulin secretion[385].

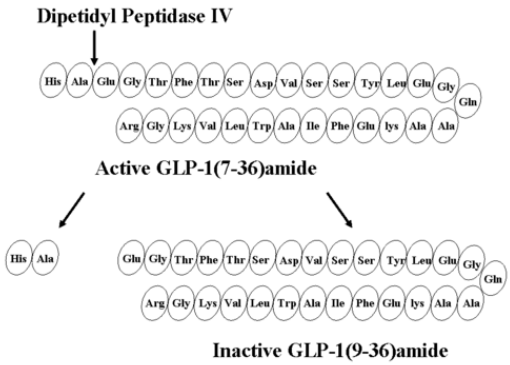

Figure 19. Chemical structure of GLP-1. Extracted from Kim et al. 2015.

6.2.1 REGULATION AND BIOLOGICAL ACTIVITY OF GLP-1

In humans, GLP-1 is secreted by L-cells mainly located in the distal jejunum, ileum, and colon[385]. Two biologically active and equipotent forms of truncated GLP-1 are released: GLP-1 (7–36) amide, 80% of circulating GLP-1 and GLP-1 (7–37) amide. It has been recognized that the posttranslational processing of preproglucagon differs in a tissue-specific manner. GLP-1 (1–36 amide) and GLP-1 (1–37) are minor forms and are believed to be inactive[386].

Plasma levels of GLP-1 increase within minutes of eating, which suggest that endocrine and neural signals provoke secretion of GLP-1 before absorbed nutrients directly stimulate L-cells[387].

GLP-1 is secreted in a biphasic pattern with early phase beginning with 5-15 min and prolonged second phase observed from 30 to 60 min of meal ingestion[388]. GLP-1 is rapidly inactivated via enzymatic cleavage, between positions 8 and 9, by dipeptidyl peptidase-4 (DPP-4). More than 50% of secreted GLP-1 is metabolized even before it reaches the systemic circulation. The half-life of circulating GLP-1 is less than two minutes[385].

GLP-1 activity is mediated by the GLP-1 receptor, which is expressed in pancreatic islet cells, gastrointestinal tract, heart, lung, blood vessels, kidney and central nervous system (CNS)[389]. Stimulation of the GLP-1 receptor activates various intracellular signaling pathways including those regulating insulin secretion and biosynthesis, β-cell proliferation and neogenesis, and inhibition of apoptosis[385, 390-392].

In the pancreatic beta-cell, receptor-binding of GLP-1 in the presence of elevated glucose concentration leads to stimulation of insulin secretion[393]. In the pancreatic alpha cell, GLP-1 robustly inhibits glucagon secretion through a direct mechanism[394] or mediated by local stimulation of insulin and somatostatin[395]. Furthermore, GLP-1 decreases gastrointestinal motility and thereby induces delayed and protracted entry of nutrients to the absorbing parts of the gastrointestinal tract[396]. This effect is a key mediator for the normalization of postprandrial glucose (PPG) excursions, and potentially more important than the insulinotropic effects of GLP-1 for maintaining PPG homeostasis. GLP-1 also promotes satiety presumably mediated by a combination of central and peripheral mechanisms[397]. Additionally GLP-1 has been shown to increase resting energy expenditure and lower plasma concentrations of free fatty acids[398]. Moreover, GLP-1 seems to reduce systolic blood pressure and may even exert protective effects on the myocardium[399].

6.2.2 GLP-1 RECEPTOR: STRUCTURE AND EXPRESSION

In humans, GLP-1R is a 463 amino acid G protein-coupled receptor belonging to the secretin-like family. Structural characteristics of this receptor family include: a relatively long, extracellular N-terminal domain responsible for high affinity binding of endogenous peptide ligands; six highly conserved cysteine residues in the extracellular domain that form three conserved disulphide bridges; an amino terminal signal peptide, several N-linked glycosylation sites and, seven transmembrane bundle[400].

Expression of GLP-1R has been demonstrated in pancreatic islets of rodents and humans, GLP-1R is expressed on beta cells. Within the CNS mRNA for the GLP-1R can be detected in the thalamus, hypothalamus and brainstem in rodents and humans[401, 402]. Moreover, it has been

found in kidney and lung, although expression in the human lung is restricted to small vessels[403]. It has been recently published that GLP-1 protects the cardiac microvessels against oxidative stress, apoptosis, and the resultant microvascular barrier dysfunction in diabetes, contributing to improvement of cardiac function and cardiac glucose metabolism[404, 405]. GLP-1R has been demonstrated to be localized in the cardiac atria[406] as well as arteries and arterioles in the left ventricle[407] .

GLP-1R expression has also been found in retinas from rats[408]. Fan and coworkers[409] demonstrated that GLP-1R was expressed throughout the entire retina (in GCL, IPL, INL, OPL, and in the inner segment). In addition they found expression of this receptor in a primary cell culture of rat retinal Müller cells. Other authors found that glial cells do not express GLP-1 receptors unless they are activated in an inflammation response[410].

GLP-1R expression has also been found in the retina of chicken[411], and in ARPE-19 cells (an immortalized line of human RPE)[412], but no data are available in the human retina or in db/db mouse retina.

6.2.3 MOLECULAR MECHANISMS UNDERLYING GLP-1R PHYSIOLOGY

GLP-1 like insulin and IGF-1 activates second messenger signaling pathways that are commonly linked to growth factor signaling[413].

Activation of GLP-1R activates a G-protein, which in turn activates the Adenylate cyclase (AC) system[414]. The Gα subunit of the GLP-1 receptor stimulates AC, which leads to an increase in intracellular cAMP and activation of protein kinase A (PKA). PKA activity can increase vesicle release in beta-cells to enhance glucose-stimulated insulin secretion. ADP that is also produced by AC during cAMP production acts on ATP-sensitive K+ channels. This leads to a slow depolarization of the cell membranes following closure of K+ channels and reduced re-polarization of neuronal membranes[415]. This can increase the opening of voltage-dependent L-type Ca^{2+} channels and increases cytosolic Ca^{2+}, which in turn acts as a second messenger. One of the effects that increase cytosolic Ca^{2+} can have is the increase of transmitter release[416]. Activation of GLP-1Rs also leads to an increase in cytosolic Ca^{2+} levels as a result of activation of voltage-dependent L-channels following phosphorylation of PKA and/or mobilization of intracellular Ca^{2+} stores. Also can activate intracellular Ca^{2+} stores an increase in phosphatidylinositol 3-kinase (PI3K) levels via G-protein activation[417]. It has been proposed that the fast action of GLP-1 on synaptic transmission is mediated by these fast, ion channel and vesicle release-dependent processes[418]. The G-protein-dependent increase in PI3K levels also activates MAPK (mitogen-activated protein kinase). This pathway activates gene expression,

which controls the expression of peptides that are required for cell growth, repair and differentiation of beta-cells and also in neuronal cells. Inhibition of PI3K (with LY294002) or MAPK (with PD98059) reduced GLP-1-stimulated neurite outgrowth. The activation of PI3K by GLP-1 and downstream transcription factors regulate expression of the genes that encode insulin, beta-cell growth and differentiation phenotype[419, 420]. cAMP activates multiple intracellular messenger systems via PKA or independently of PKA activation (Figure 20).

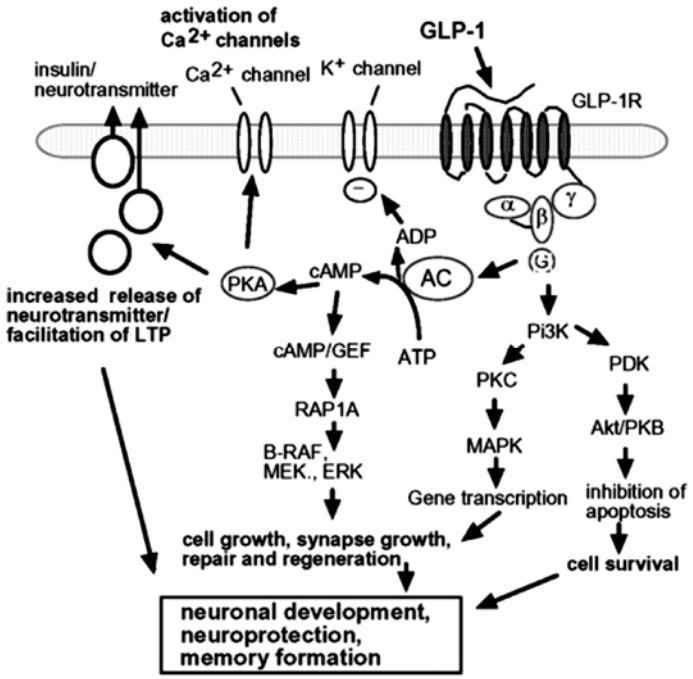

Figure 20. Roles and functions of GLP-1R. Extracted from Hölscher, 2011.

6.2.4 GLP-1 EXERTS NEUROPROTECTIVE EFFECTS

GLP-1 exerts neuroprotective effects in both, central and peripheral nervous system. GLP-1 penetrates the BBB after intravenous injection by simple diffusion[421]. Diferent studies have shown that activation of GLP-1R is associated with a number of cellular survival and plasticity phenomena, including islet-cell neogenesis, enhanced learning, protection against apoptosis, and cell death secondary to oxidative insult[422]. In 2003 was demostrated that GLP-1 enhanced associative spatial learning and these effects were blocked by a GLP-1R antagonist. An increase in GLP-1R expression through hyppocampal gene transfer potently enhanced learning and memory[423]. Perry and coworkers demonstrated that GLP-1 and exendin-4 (a longer acting,

naturally occurring analogue of GLP-1 that binds GLP-1R) protected cultured hippocampal neurons against glutamate-induced death and attenuated the basal forebrain cholinergic deficit in a well-defined rodent model of neurodegeneration[424]. These authors also reported that GLP-1 can reduce the levels of amyloid-beta peptide (Aβ) in the brain in vivo and can reduce the levels of amyloid precursor protein (APP) in cultured neuronal cells. Moreover, GLP-1 and exendin-4 protect cultured hippocampal neurons against death induced by an oxidative insult[425]. McClean and collegues, in 2010, showed that liraglutide (a protease resistant GLP-1 analogue) has a fast acting facilitatory effect on LTP (long term potentiation of synaptic transmission). This means that the GLP-1 receptor agonists they tested had pronounced effects on synaptic activity and support the concept that GLP-1 is a neurotransmitter and plays an important role in modulating neuronal activity[426]. Recently, liraglutide has been demonstrated to have neuroprotective effects also in traumatic brain injury (TBI)[427]. Pretreatment with liraglutide rescued neuronal cells from oxidative stress- and glutamate excitotoxicity-induced cell death. Liraglutide produced neurotrophic and neuroprotective effects likely involving the cAMP/PKA/pCREB pathway. These findings in cell culture were translated in a TBI mouse model. Post-treatment with a clinically relevant dose of liraglutide for 7 days in mice ameliorated memory impairments caused by TBI.

Conversely, failure of GLP-1R function impairs synaptic plasticity as well as some forms of learning, cognition and memory formation. It is expemplified by experiments knocking out GLP-1R in mice or by administrating GLP-1R antagonists[423, 426, 428].

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 been shown that intravitreal injections of exendin-4 (a GLP-1R agonist) prevented ERG abnormalities (reduction in b-wave amplitude and OPs amplitude) in STZ-induced diabetic rats one month after treatment. It also prevented the cell loss in ONL and INL and retinal thickness. They found a decrease in the concentration of glutamate in the retinas of exendin-4 treated rats. Consistent with these changes, GLP-1R and GLAST expression were reduced in diabetic retinas but upregulated in exendin-4 treated rats. They found that this neuroprotective effect was transient because 3 month after injection they did not find any improvement[429], so it seems to have an acute effect. A very similar effect was found by Fand et al.[430] when they injected the same analog of GLP-1 intravitreally in Goto-Kakizaky rats, a prevention in the reduction of b-wave and oscillatory potential amplitudes and retinal cell loss. They also found a mantained Bcl-2/Bax and Bcl-xL/Bax ration balances, and reduced reactive gliosis.

6.2.5 GLP-1 RECEPTOR AGONISTS

In 2005, the glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists were introduced into clinical practice, and since 2009 they have been part of the joint position statements on treatment of T2D by the European Association for the Study of Diabetes (EASD) and the American Diabetes Association (ADA) [431, 432]. The GLP-1R agonists target a broad spectrum of the multifaceted pathophysiology of T2D. They improve glucose homeostasis without risk of hypoglycemia, facilitate body weight loss, and exert effects on cardiovascular parameters of potential benefit.

GLP-1R agonists are designed to overcome the rapid degradation by the enzyme DPP-4. Thereby, they are being developed under two strategies. One strategy exploits the structure of native GLP-1, with few amino acid alterations that protect the molecule from being degradated by DPP-4. The other strategy exploits a naturally occurring protein, exendin-4 (originally isolated from the saliva of the lizard *Heloderma suspectum*), which activates the GLP-1R with equal potency as native GLP-1[399]. Based on their pharmacokinetic profiles the analogues can be divided into short-acting or continuous-acting GLP-1R agonists. The short-acting GLP-1R agonists (exenatide and lixisenatide) are despite being resistant to degradation by DPP-4 still subject to renal elimination, which confers a plasma half-life of approximately 2 to 4 hours for these agents[433, 434]. The modifications applied to make the peptide continuous-acting include (see Figure 21):

- Incorporation of the molecules in injectable microspheres (exenatide once weekly).
- Fusion with larger carrier molecules like albumin (albiglutide).
- Attachment of a fatty-acid side-chain which allows reversible binding to albumin (liraglutide and semaglutide).

The longer half-life enables these compounds to be administered in larger intervals while at the same time reduce fluctuations of plasma peptide levels, leading to continuous activation of GLP-1R.

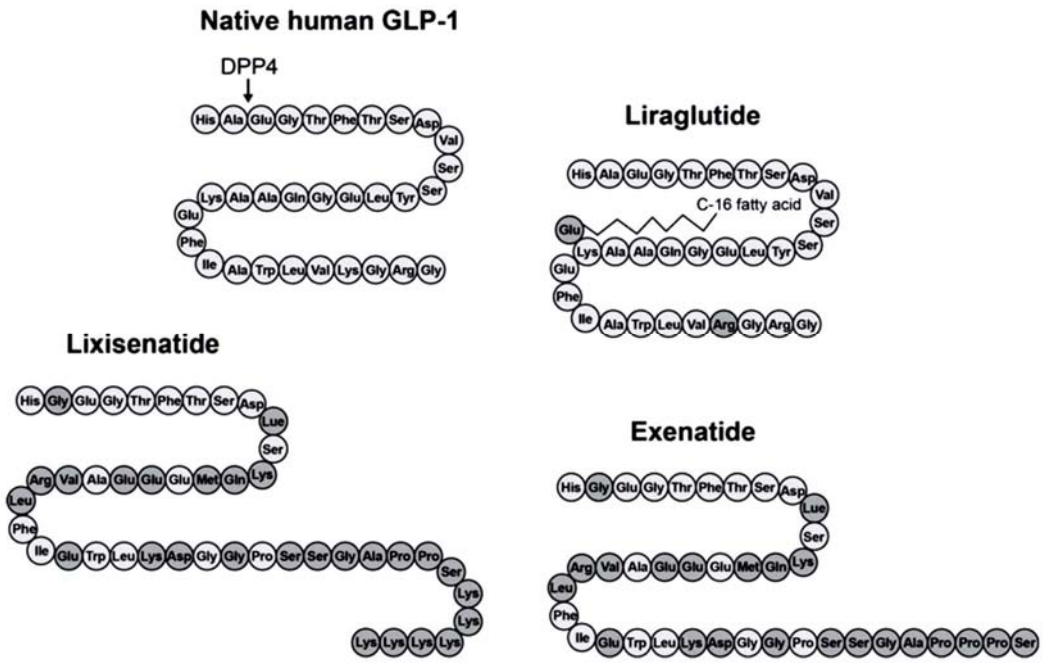

Figure 21. Structure of GLP-1 agonists. Dark grey circles are differences vs. native GLP-1. Extracted from Bolli, 2014.

A brief explanation of the GLP-1 receptor agonists used for the research in this thesis are commented below:

Exenatide: Exenatide is a synthetic version of exendin-4 which has a low (53%) amino acid sequence homology with human GLP-1[435]. Exenatide is primarily cleared in the kidneys by glomerular filtration[433], and the half-life after subcutaneous injection is approximately 2 to 3 hours with detectable plasma concentrations up to 10 hours after injection.

Lixisenatide: Lixisenatide is based on exendin-4, but with a deletion of a proline and an addition of six lysine amino acids at the C-terminus[436]. These modifications lead to a half-life of 2 to 3 hours after subcutaneous injection.

Liraglutide: The structure of liraglutide is based on native GLP-1 with an Arg34Lys substitution and an addition of a 16 carbon fatty-acid side-chain at Lys26. This results in 97% homology with native GLP-1[437]. The fatty-acid side-chain enables the compound to be bound non-covalently to albumin and ensures that only 1-2% of liraglutide is circulating as free peptide in plasma after subcutaneous injection. As a result, the half-life of liraglutide is approximately 11 to 15 hours and a once-daily dosing ensures continuous exposure of the GLP-1Rs[399].

As said before, GLP-1 mimetics can protect cultured neurons from stressors, reduce apoptosis and enhance cell division[424, 438] . Furthermore, these drugs can cross the BBB[439].

On the other hand, there are other GLP-1R agonists available such as albiglutide and dulaglutide. Albiglutide has two copies of GLP-1 in series, each with an Ala8Gly substitution, and this molecule is fused to albumin[440], which should hamper the ability to cross the blood-brain barrier for entry into the CNS[441]. Dulaglutide has two copies of a GLP-1 analogue (with amino acid substitutions Ala8Gly, Gly22Glu and Arg36Gly) covalently linked to an Fc fragment of human IgG4[442, 443].

HYPOTHESIS AND OBJECTIVES

Diabetes is a pandemic disease affecting the first world and westernized countries. Among diabetic patients the overwhelming majority suffer from type 2 diabetes. DR is the most common complication of diabetes and one of the leading causes of preventable blindness.

There is a need to find new therapeutic strategies to stop the progression of DR in its early stages, when patients do not present symptoms but the neurodegenerative process is already present. To achieve that goal it is necessary to have a good animal model where potentially neuroprotective drugs could be tested on and that help us to understand their mechanism of action.

As said before, in recent years the db/db mouse has been used as a spontaneous diabetic model of type 2 diabetes to investigate the pathogenesis of DR. Several authors have reported the presence of retinal neurodegeneration in this model. However, the characterization of the retinal neurodegenerative process and its functional consequences in db/db mice are not yet elucidated. In addition, it should be clarified whether that neurodegeneration is due to genetic factors or whether it is due to diabetes. It is essential to determine if db/db mouse is an appropriate model for investigating the underlying mechanisms of diabetes-induced retinal neurodegeneration and for testing neuroprotective drugs.

Fenofibrate has been demonstrated, by two major clinical trials, to arrest the progression of DR in type 2 diabetic patients. However, the neuroprotective effect of fenofibrate in the setting of DR has never been evaluated.

GLP-1 exerts neuroprotective effects in both the central and the peripheral nervous system. 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. GLP-1 acts through GLP-1R, and expression of that receptor has been found in retinas from rats, chickens and ARPE-19 cells. However, it has never been examined in human or mouse retina.

Based on what has been explained, the objectives of the present work have been:

CHAPTER ONE: *The db/db mouse: a useful model for the study of diabetic retinal neurodegeneration.*

1. To characterize the neurodegenerative process that occurs in the retinal of db/db mice.
2. Examine the morphological, biochemical and functional abnormalities in a sequential manner (8, 16, 24 weeks).

3. Determine if lowering blood glucose levels arrest the neurodegenerative process and discard that the abnormalities found have a genetic origin.
4. To perform a transcriptomic analysis in 8-week old diabetic mice to identify new potential causative candidates of DR.

CHAPTER TWO: *Effect of fenofibrate on retinal neurodegeneration in an experimental model of type 2 diabetes.*

1. To evaluate the potential retinal effect of fenofibric acid in preventing retinal neurodegeneration in the db/db mouse.
2. Discard that the beneficial effects of FA are due to its lipid-lowering action. This will be achieved by applying a short-term treatment.
3. Determine whether FA action is mediated by an increase in glutamate clearance, thus preventing glutamate accumulation.

CHAPTER THREE: *Topical administration of GLP-1 receptor agonists prevents retinal neurodegeneration in experimental diabetes.*

1. To examine the expression and content of GLP-1R in human and in db/db mice retinas.
2. To determine the retinal neuroprotective effects of systemic administration. To distinguish whether the neuroprotective effects of GLP-1R agonist are independent of its glucose lowering effect by means of topical administration (eye drops) of GLP-1R agonists in db/db mice.
3. Demonstrate that GLP-1R agonists topically administered reach the mouse retina.
4. To assess whether there are differences in the neuroprotective action among several GLP-1R agonists topically administered.
5. Perform a dose-efficacy study of topical administration.
6. Demonstrate that the specific response passes through activation of GLP-1R.
7. Determine whether GLP-1R agonist's effects are mediated by glutamate pathway.
8. Study the effect of GLP-1R agonists on proapoptotic/proinflammatory markers and survival pathways.

RESULTS

CHAPTER I

The db/db mouse: a useful model for the study of diabetic retinal neurodegeneration.

In order to characterize the sequential events that are taking place in retinal neurodegeneration in the db/db mouse, a spontaneous model of type 2 diabetes, the analysis was performed at different ages, 8, 16 and 24 weeks. Db/db mice were hyperglycaemic at 4 weeks of age (>250 mg/dL), whilst the control group (db/+) kept glucose levels below 150 mg/dL during the follow-up. We observed that hyperglycaemia runs in parallel with a significant increase of weight.

Mouses' retinas were functionally analysed by means of ERG. Major changes were found in the b-wave implicit time, which was significantly increased in diabetic mice at all flash intensities tested when compared with non-diabetic mice at 16 and 24 weeks. However, at 8 weeks of age the delay was not significant. In addition, b-wave amplitude was significantly reduced in diabetic mice in comparison with controls at 16 and 24 weeks, but not at 8. The a-wave was also affected but less markedly than b-wave. The a-wave amplitude was significantly reduced and at 16 and 24 weeks of age the implicit time was increased. The OPs in db/db mice presented a significant increase of implicit time and decreased amplitude at 16 and 24 weeks.

Morphological alterations were evaluated post-mortem staining with haematoxylin and eosin the fixed tissue. Total retinal thickness was significantly decreased in diabetic mice in comparison with non-diabetic mice at 16 and 24 weeks. Furthermore, a thinning in both ONL and INL as well as a reduction in the number of cells in the GCL was observed in diabetic mice in comparison to db/+ at 8, 16 and 24 weeks.

Histological markers of neurodegeneration (glial activation and apoptosis) were evaluated using immunofluorescence techniques. A significant increase in TUNEL-positive cells and caspase-3 was observed in diabetic mice in comparison to retinas from non-diabetic mice at all ages. By electron microscopy we found DNA fragmentation in photoreceptors from db/db mice in comparison with non-diabetic mice. We also found reactive gliosis in diabetic mice characterized by upregulation in GFAP staining. All these features of neurodegeneration were more intense at 16 than at 8 weeks, but no significant differences between 16 and 24 were observed.

Moreover, markers of glutamate pathway were studied. Glutamate levels were determined by HPLC and the expression of glutamate/aspartate transporter (GLAST) was also assessed. Glutamate levels were higher in diabetic mice in comparison with non-diabetic mice at 8, 16 and 24 weeks. The increase in glutamate levels ran in parallel with a decrease in GLAST content in the retinas of diabetic mice.

To determine whether the responsible for neurodegeneration was the mutation that these mice carry an additional interventional study to lower blood glucose levels was performed. A group

of db/db mice was fed with a restrictive diet for 15 days. These mice presented lower weight and blood glucose levels than *ad libitum* fed mice. We found significantly lowered levels of GFAP, lower ratio of apoptosis and that the ERG abnormalities were significantly arrested.

Furthermore, in order to define gene expression changes associated with early DR a transcriptome analysis was performed. It consisted on a genome-wide expression profiling analysis on total RNA isolated from retinas of 8-week old diabetic and control mice. Downregulated genes fitted into categories related to synaptic transmission, with a particular abundance of terms related to glutamate transport and metabolism. The upregulated genes corresponded to categories related to mitochondrial respiration and oxidative stress. These results were confirmed by Real time quantitative PCR and suggest that glutamate signalling and metabolism is altered in diabetic mice. The increase in the expression of genes encoding for proteins of the respiratory chain ran in parallel with an increase in the expression of UCP2, a protein involved in the control of mitochondria-derived ROS production.

This work demonstrates that db/db mouse reproduces the features of the neurodegenerative process that occurs in the human diabetic eye. Therefore, it is an appropriate model for investigating the underlying mechanisms of diabetes-induced retinal neurodegeneration and for testing new neuroprotective drugs.

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The db/db Mouse: A Useful Model for the Study of Diabetic Retinal Neurodegeneration

Patricia Bogdanov[1,2☯], Lidia Corraliza[1,2☯], Josep A. Villena[2,3], Andrea R. Carvalho[4,5], José Garcia-Arumí[4,5], David Ramos[6], Jesús Ruberte[6], Rafael Simó[1,2], Cristina Hernández[1,2]*

1 Diabetes and Metabolism Research Unit, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain, **2** Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III (ISCIII), Madrid, Spain, **3** Laboratory of Metabolism and Obesity, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Spain, **4** Ophthalmologic Research Laboratory. Vall d'Hebron Research Institute. Universitat Autònoma de Barcelona, Barcelona, Spain, **5** Red Temática de Investigación en Oftalmolología (OFTARED), Instituto de Salud Carlos III (ISCIII), Madrid, Spain, **6** Department of Animal Health and Anatomy, Faculty of Veterinary Sciences, Universitat Autònoma de Barcelona, Barcelona, Spain

Abstract

Background: To characterize the sequential events that are taking place in retinal neurodegeneration in a murine model of spontaneous type 2 diabetes (db/db mouse).

Methods: C57BLKsJ-db/db mice were used as spontaneous type 2 diabetic animal model, and C57BLKsJ-db/+ mice served as the control group. To assess the chronological sequence of the abnormalities the analysis was performed at different ages (8, 16 and 24 weeks). The retinas were evaluated in terms of morphological and functional abnormalities [electroretinography (ERG)]. Histological markers of neurodegeneration (glial activation and apoptosis) were evaluated by immunohistochemistry. In addition glutamate levels and glutamate/aspartate transporter (GLAST) expression were assessed. Furthermore, to define gene expression changes associated with early diabetic retinopathy a transcriptome analyses was performed at 8 week. Furthermore, an additional interventional study to lower blood glucose levels was performed.

Results: Glial activation was higher in diabetic than in non diabetic mice in all the stages ($p<0.01$). In addition, a progressive loss of ganglion cells and a significant reduction of neuroretinal thickness were also observed in diabetic mice. All these histological hallmarks of neurodegeneration were less pronounced at week 8 than at week 16 and 24. Significant ERG abnormalities were present in diabetic mice at weeks 16 and 24 but not at week 8. Moreover, we observed a progressive accumulation of glutamate in diabetic mice associated with an early downregulation of GLAST. Morphological and ERG abnormalities were abrogated by lowering blood glucose levels. Finally, a dysregulation of several genes related to neurotransmission and oxidative stress such as UCP2 were found at week 8.

Conclusions: Our results suggest that db/db mouse reproduce the features of the neurodegenerative process that occurs in the human diabetic eye. Therefore, it seems an appropriate model for investigating the underlying mechanisms of diabetes-induced retinal neurodegeneration and for testing neuroprotective drugs.

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* E-mail: cristina.hernandez@vhir.org

☯ These authors contributed equally to this work.

Introduction

Diabetic retinopathy (DR) is the most common complication of diabetes and one of the leading causes of preventable blindness [1]. Current treatments for DR are applicable only at advanced stages of the disease and are associated with significant adverse effects [2,3]. Therefore, new pharmacological treatments for the early stages of the disease are needed. However, the mechanisms involved in the onset of DR are still poorly understood.

Emerging evidence suggests that retinal neurodegeneration is an early event in the pathogenesis of DR [4–8] which participates in the microcirculatory abnormalities that occur in DR [9–13]. Consequently, new therapeutic strategies based on neuroprotection have been proposed [14–15].

The experimental model currently used to study retinal neurodegeneration in DR is the rat with streptozotocin-induced diabetes (STZ-DM). However, since STZ is neurotoxic itself [16], a debate has arisen regarding the appropriateness of this model for examining retinal neurodegeneration shortly after STZ adminis-

tration. A second rodent model, the Ins2Akita (Akita) mouse, which contains a dominant point mutation in the gene encoding for insulin-2 that induces spontaneous type 1 diabetes in the B6 mouse strain, reproduces some findings of the neurodegenerative process that occurs in the human diabetic retina [17]. However, both STZ-DM and Akita mouse are models of type 1 diabetes and further characterization of the neurodegenerative process in type 2 models is needed.

In recent years the C57BL/KsJ-*db/db* mouse has been used as a spontaneous diabetic model of type 2 diabetes to investigate the pathogenesis of DR [17–23]. The C57BL/KsJ-*db/db* mouse carries a mutation in the leptin receptor gene and is a well-established model of obesity-induced type 2 diabetes. Several authors have reported the presence of retinal neurodegeneration (apoptosis, glial activation and retinal thinning) in this model [19,22]. Therefore, C57BL/KsJ-*db/db* seems appropriate for investigating the underlying mechanisms of retinal neurodegeneration associated with diabetes and for testing new drugs. However, the characterization of the retinal neurodegenerative process and its functional consequences in db/db mice is far from being completed. In addition, whether neurodegeneration can be attributed to genetic factors rather than to diabetes is a question which remains to be elucidated.

In the present study we have characterized the neurodegenerative process that occurs in the retina of C57BL/KsJ-*db/db* mice by examining morphological, biochemical and functional abnormalities in a sequential manner (8, 16, 24 weeks). Moreover, a transcriptomic analysis in 8-week old diabetic mice was performed to identify new potential causative candidates of DR. In addition, we have demonstrated that the neurodegenerative process is significantly arrested after blood glucose levels have been lowered. Overall, our results suggest that C57BL/KsJ-*db/db* reproduces the neurodegenerative features that occur in the human diabetic eye, and is an appropriate experimental model for studying the mechanisms involved in diabetes-induced retinal neurodegeneration.

Methods

Animals

A total of 90 C57BL/KsJ-*db/db* male mice obtained from Harlan Laboratories, Inc. were divided into two groups: 45 non-diabetic (db/+) and 45 diabetic mice (db/db). To assess the chronological sequence of the retinal abnormalities associated with diabetes, 15 diabetic mice (db/db) were compared with 15 age-matched non-diabetic mice (db/+) at different ages (8, 16 and 24 weeks). Blood glucose concentrations were measured from the tail vein (glucose assay kit; Abbott). Mice were housed under controlled conditions of temperature (20°C) and humidity (60%) with a 12-hour light/dark cycle and had free access to food and water.

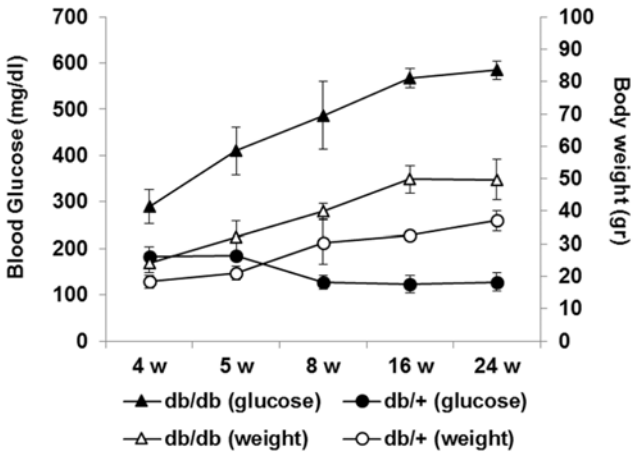


Figure 1. Blood glucose levels (black marks) and body weight (white marks) in db/+ (circles; n = 12) and db/db mice (triangles; n = 12). Values are expressed as mean ± SD. *p<0.05; **p<0.001.
doi:10.1371/journal.pone.0097302.g001

Interventional study. Diabetic mice (db/db) 8 weeks old received diet *ad* libitum (n = 10) or restrictive diet (normal chow diet restricted to 60% of total daily calories; n = 10) for 15 days. Ten non-diabetic mice matched by age served as control group. At day 15 the animals were euthanized by cervical dislocation and the eyes enucleated.

This study was approved by the Animal Care and Use Committee of VHIR (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).

Electroretinography

Before retinal electrophysiological tests, the animals were dark-adapted (12 hours overnight) and anesthetized under a dim red light with a 2% isoflurane/O2 mixture. Pupils were dilated with topic 1% tropicamide and ciclopegic was applied on the corneal surface. Full field electroretinography (ERG) recordings were measured using an HMsERG (Ocuscience) with two recording channels. Recordings were measured from corneal electrodes attached to the corneas by a lens embedded in 1% methylcellulose to avoid cornea dehydratation. Two needle probes were inserted for reference subcutaneously between each jaw and a grounding probe were inserted into the base of the tail. Then their noses were inserted into a mini Ganzfeld flash photo-stimulator with white LED.

Scotopic ERG stimuli were simultaneously recorded from both eyes of dark-adapted (12–16 hours) mice. Light stimuli were delivered via a Ganzfeld light source with flash intensities from 30 to 30000 mcd.s/m^{-2}. Responses were amplified 5000X, high-pass filtered with a 10-Hz cutoff frequency, and low-pass filtered at 300 Hz using an amplifier. The ERG voltage and stimulus-monitor signals were digitalized with hardware (HMsERG) and software (HMsERG View) from Ocuscience. Data were recorded at either 0.2 or 0.5 ms/pt. A stimulus set consisted of 3 to 20 responses at the same wavelength and intensity of light. The oscillatory potentials (OPs) were isolated by a band-pass filtering the retinal response between 34 and 300 Hz. We chose 34 Hz as a cutoff frequency to avoid any loss of signal power, especially for the slower OPs of diabetic animals. OPs were isolated for a light stimulus of 3000 mcd.s/m^{-2}.

The amplitude and implicit time of the ERG a-and b-waves were measured at the maximum negative and positive peaks of the recordings with respect to the baseline before stimulation. As recommended by the ISCEV (International Society for Clinical Electrophysiology of Vision) [24] OP amplitudes were measured from the negative peak to the next positive peak whereas OP latencies were measured at the positive peaks. We added up OP amplitudes (ΣOP amplitude) and implicit time (ΣOP implicit time) for the first 5 OPs.

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

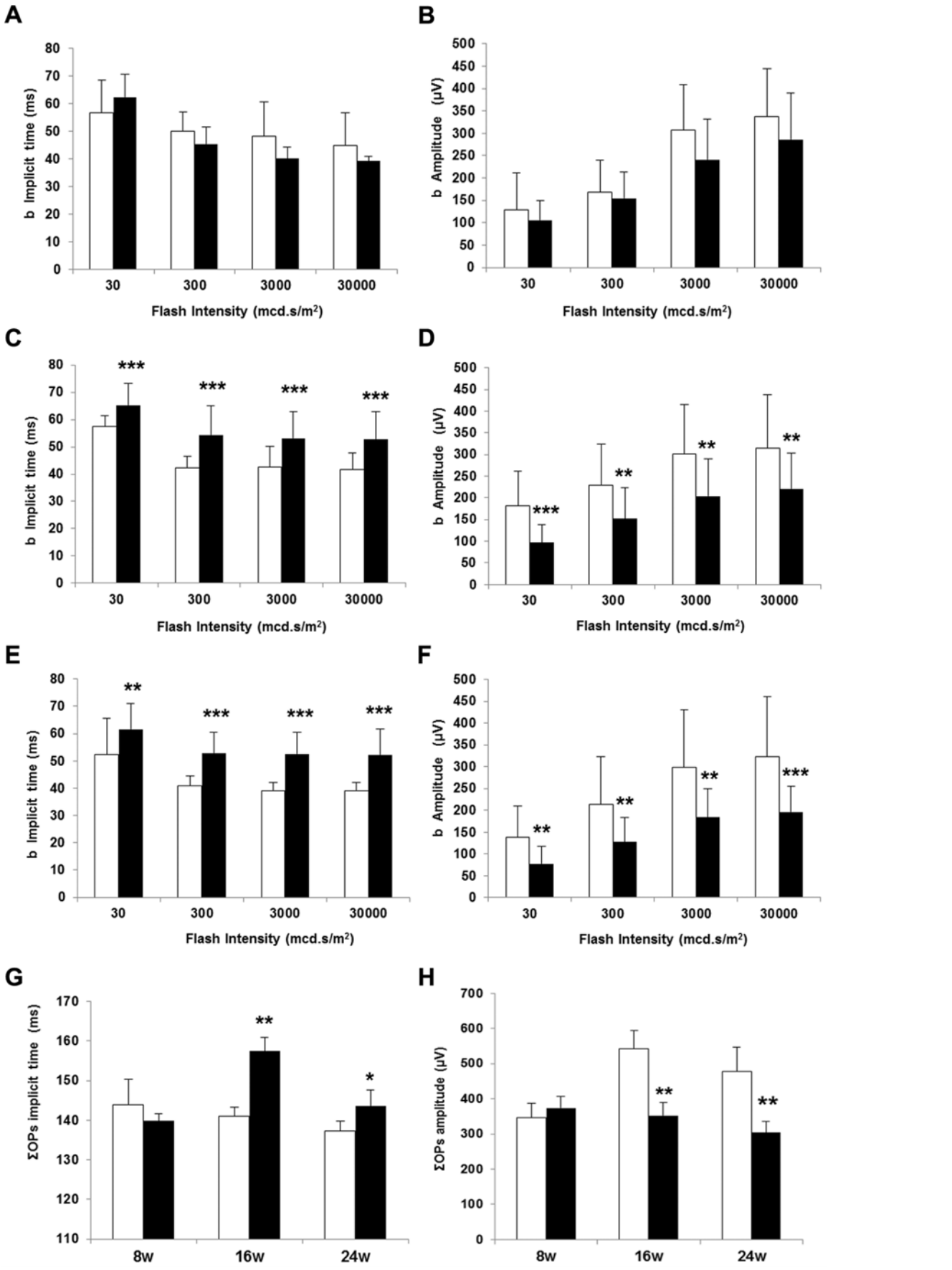

A
b Implicit time (ms)
Flash Intensity (mcd.s/m²)
B
b Amplitude (μV)
Flash Intensity (mcd.s/m²)
C
b Implicit time (ms)
Flash Intensity (mcd.s/m²)
D
b Amplitude (μV)
Flash Intensity (mcd.s/m²)
E
b Implicit time (ms)
Flash Intensity (mcd.s/m²)
F
b Amplitude (μV)
Flash Intensity (mcd.s/m²)
G
ΣOPs implicit time (ms)
8w
16w
24w
H
ΣOPs amplitude (μV)
8w
16w
24w

Figure 2. b-wave implicite time (ms) in non diabetic and diabetic mice at 8 (A), 16 (C) and 24 weeks (E). ΣOPs implicit time (ms) (G). b-wave amplitude (μV) in non diabetic and diabetic mice at 8 (B), 16 (D) and 24 weeks (F). ΣOPs amplitude (μV) (H). White bars: non diabetic mice. Black bars: diabetic mice. Data are expressed as mean ± SD. *p<0.05; **p<0.01;***p<0.001.
doi:10.1371/journal.pone.0097302.g002

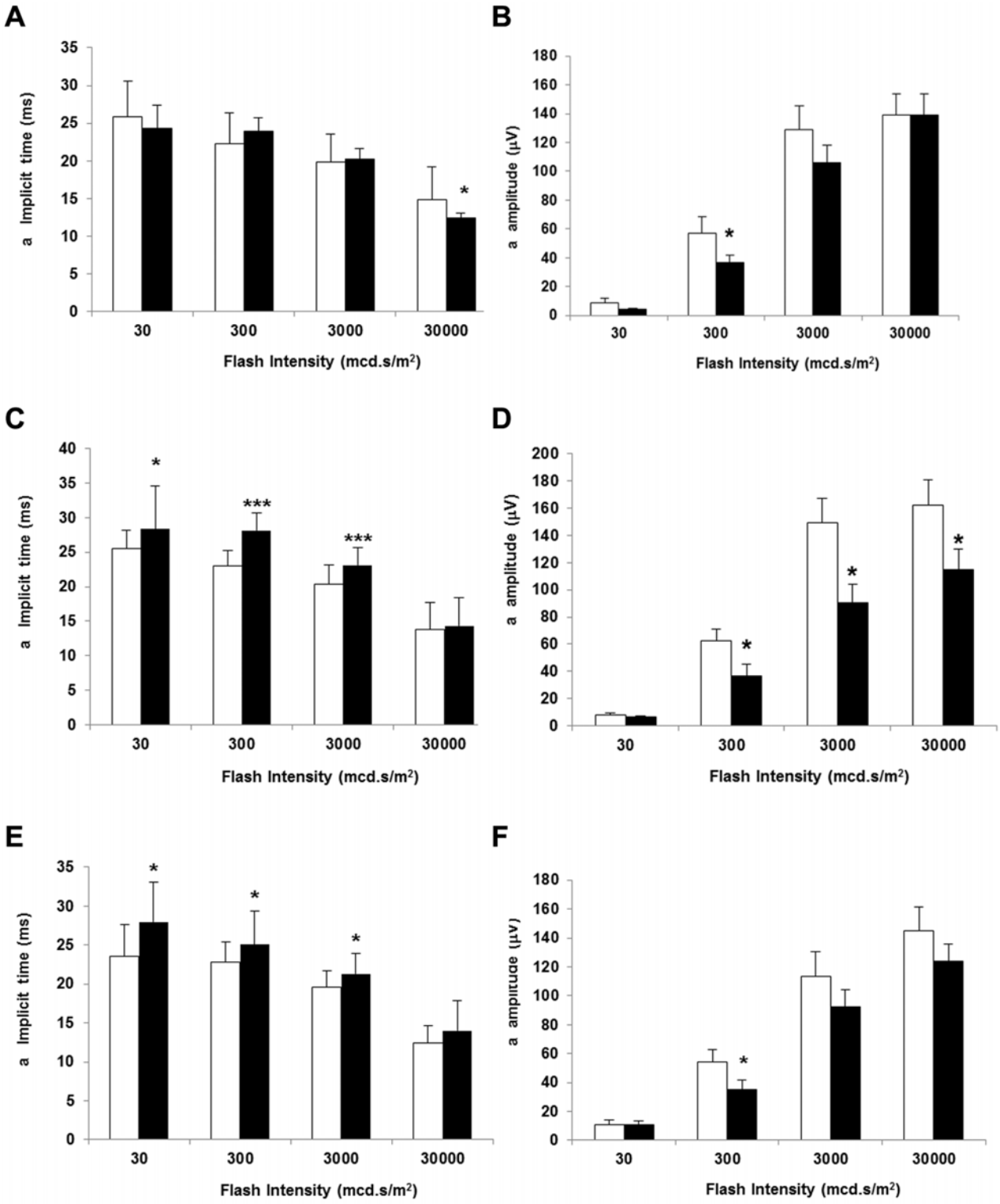


Figure 3. a-wave implicit time (ms) in non diabetic and diabetic mice at 8 (A), 16 (C) and 24 weeks (E). a-wave amplitude (μV) in non diabetic and diabetic mice at 8 (B), 16 (D) and 24 weeks (F). White bars: non diabetic mice. Black bars: diabetic mice. Data are expressed as mean ± SD. *p<0.05.
doi:10.1371/journal.pone.0097302.g003

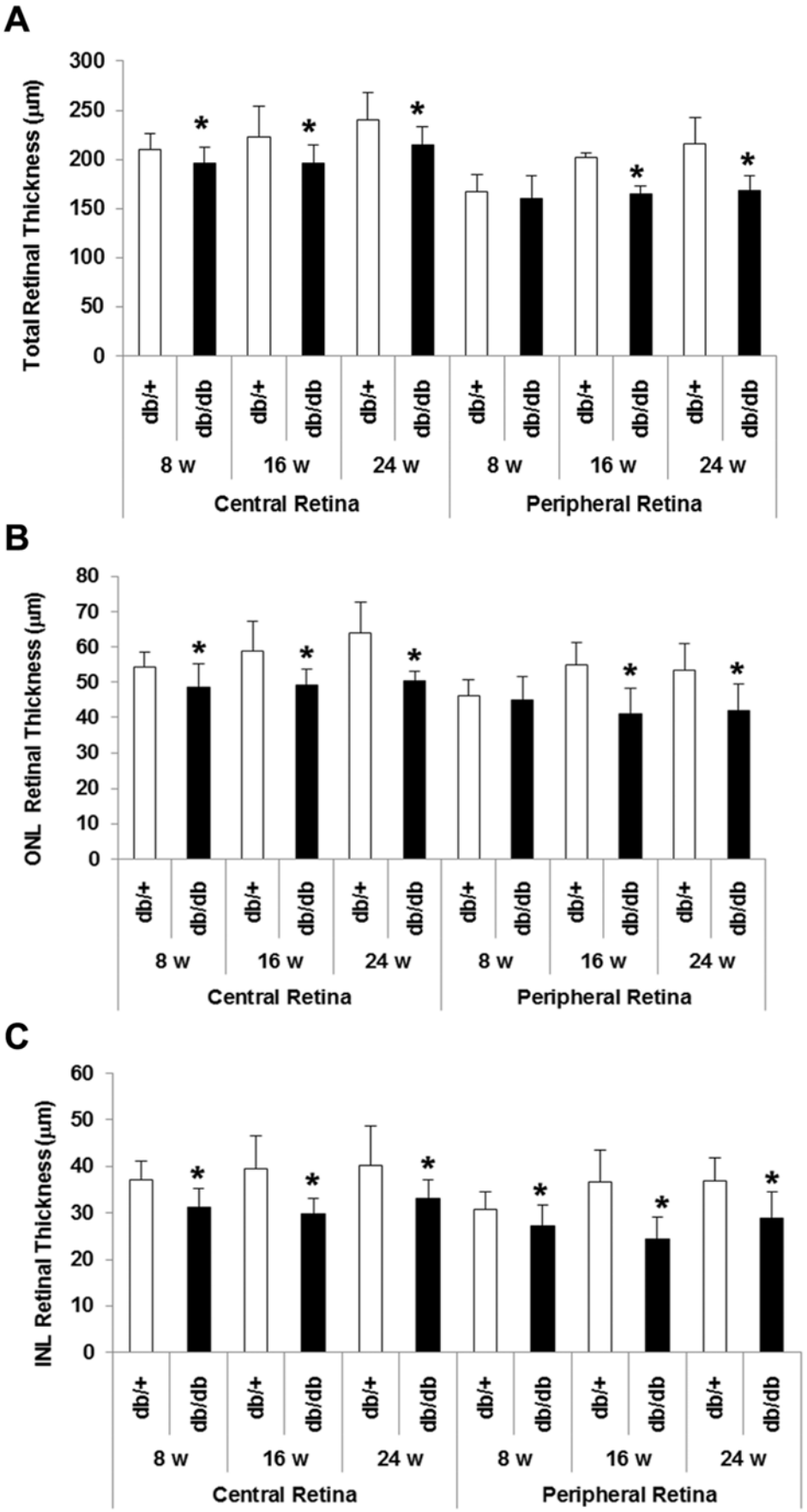

A
Total Retinal Thickness (μm)
300
250
200
150
100
50
0
db/+
db/db
8 w
16 w
24 w
Central Retina
Peripheral Retina
B
ONL Retinal Thickness (μm)
80
70
60
50
40
30
20
10
0
C
INL Retinal Thickness (μm)
60
50
40
30
20
10
0

Figure 4. Thickness of total retina (A), outer nuclear layer (B) and inner nuclear layer (C). The measurements have been performed in the central retina and in peripheral retina. Results are expressed as mean ± SD. *p<0.05 between non diabetic (white bars) and diabetic (black bars) mice.
doi:10.1371/journal.pone.0097302.g004

stored at −80°C for protein assessments. The other eye was flash frozen in Tissue Freezing Medium (TFM, Electron Microscopy Sciences), by immersion in liquid nitrogen, and cryosectioned at 8 µm through the dorsal/ventral plane. Sections were mounted on slides and stored at −80°C. These sections were prepared for the assessment of retinal morphology, evaluation of GFAP and TUNEL immunoreactivity. For caspase-3 immunohistochemistry, the eyes were fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Ocular globes embedded in paraffin were sectioned (3 µm) along the eye axis.

Neurodegeneration Measurements

Retinal morphometry. Microscopic evaluation of retinas included scanning tissue sections to evaluate morphology followed by systematic morphometric analysis. The sections were stained with hematoxylin and eosin (H&E). Images of H&E sections were captured with a microscope (Olympus, Lake Success, NY) using the program Image J for quantification. The measurements were taken at two peripheral and three central regions of the retina and were examined to ensure similar locations of measurements for all eyes. Sections through the posterior eye segment were defined as central retina when the plane passed through the optic nerve or at less than 300 µm from the optic head rim. The remaining sections from both sites of the optic nerve were considered as peripheral

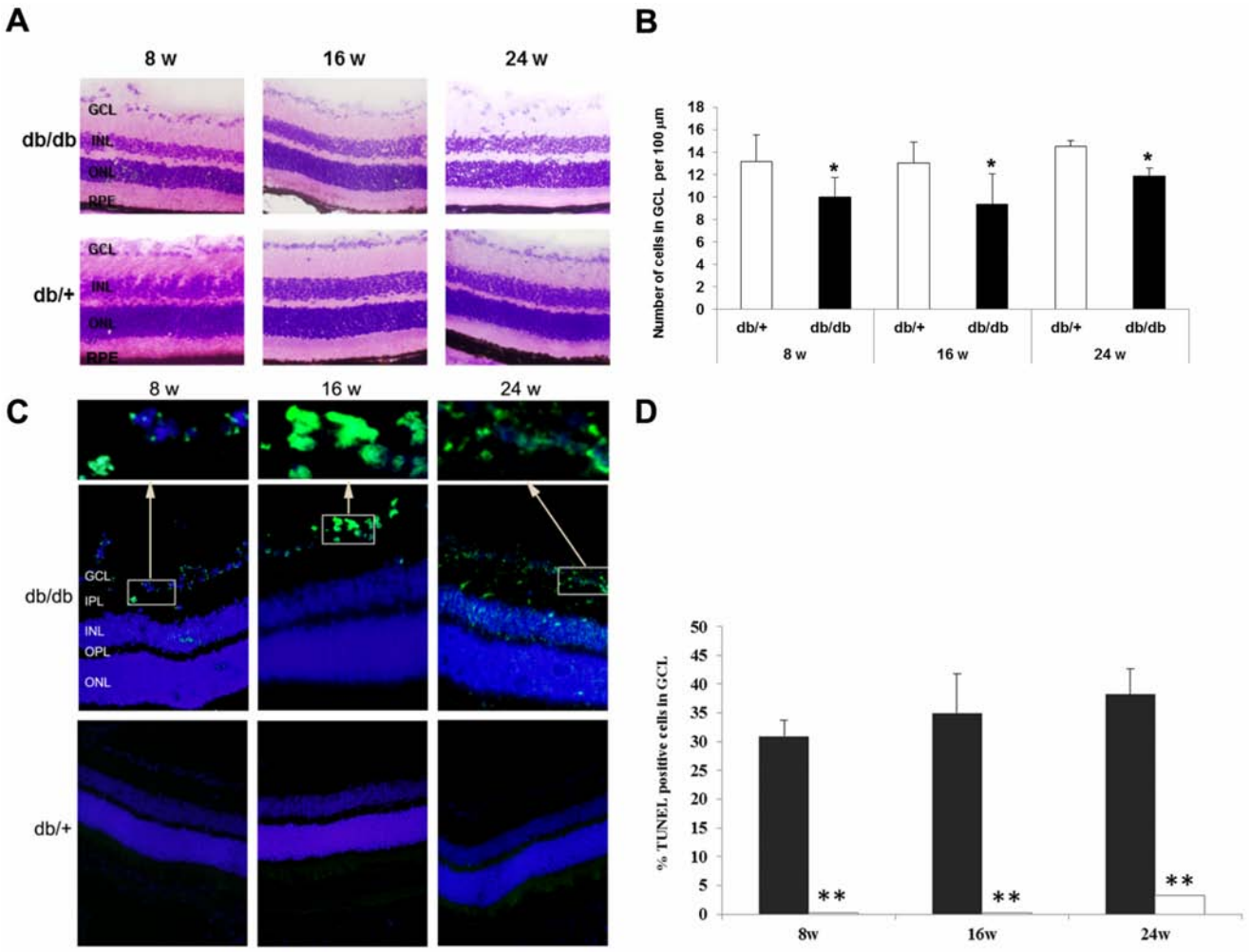


Figure 5. Images demonstrating the progressive loss of cells in retinal ganglion layer (GCL). A) Hematoxylin and eosin stained central retina in a representative case of a diabetic mouse (upper panel) and a non-diabetic mouse (lower panel) of 8, 16 and 24 weeks old. In the diabetic retina a loss of cells in GCL was observed. B) Cell number in GCL in control and diabetic mice in central retina. C) Comparison of TUNEL immunofluorescence (green) between representative samples from a diabetic (upper panel) and a non-diabetic mouse (lower panel) at 8 weeks, 16 and 24 weeks. Nuclei were labeled with Hoechst (blue). D) Percentage of TUNEL positive cells in the GCL in non-diabetic and diabetic mice at 8, 16 and 24 weeks (upper panel). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Results are expressed as mean ± SD. White bars: non-diabetic mice; Black bars: diabetic mice. *p<0.05, **p<0.001 between non-diabetic and diabetic mice.
doi:10.1371/journal.pone.0097302.g005

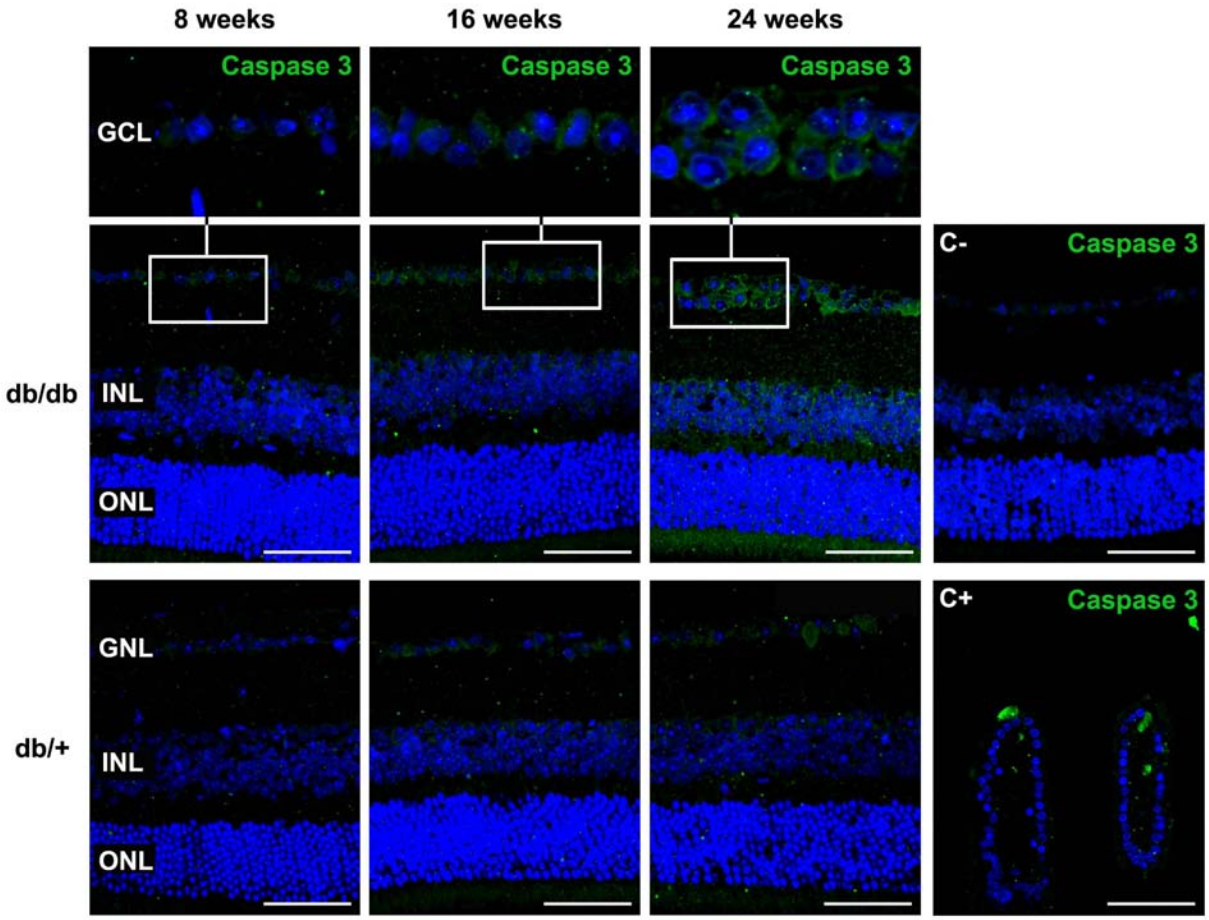


Figure 6. Comparison of cleaved caspase-3 immunofluorescence (green) between representative samples from a diabetic (upper panel) and a non-diabetic mouse (lower panel) at 8, 16 and 24 weeks. Insets show the increased expression of cleaved caspase-3 in cells residing in the ganglion cell layer (GCL) in db/db mice. Nuclei counterstained with Hoescht (blue). ONL: outer nuclear layer; INL: inner nuclear layer; C-: negative control; C+: positive control mouse jejunal villi. Scale bars: 40 μm.
doi:10.1371/journal.pone.0097302.g006

retina. Image analysis of ten sections of each region were used to quantify total retinal thickness, the thickness of the inner nuclear layer (INL), outer nuclear layer (ONL) and cell number per mm^2 in ganglion cell layer (GCL). These measurements were performed by two of the investigators (P.B and L.C).

Immunohistochemical analysis for glial activation assessment. Glial activation was evaluated by fluorescence

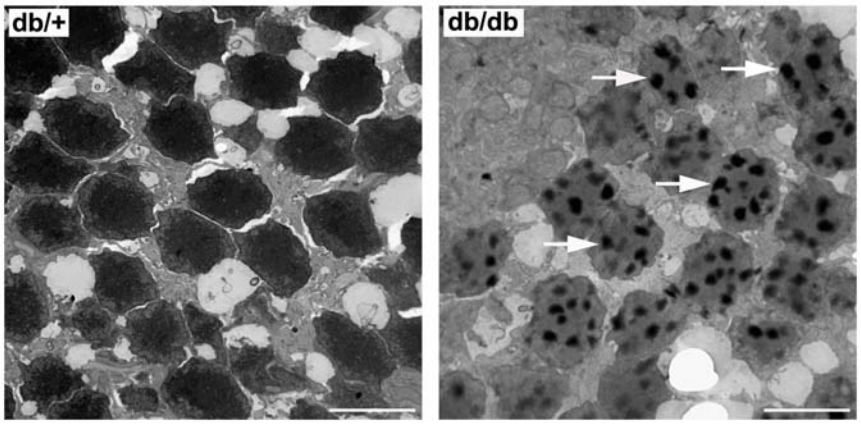
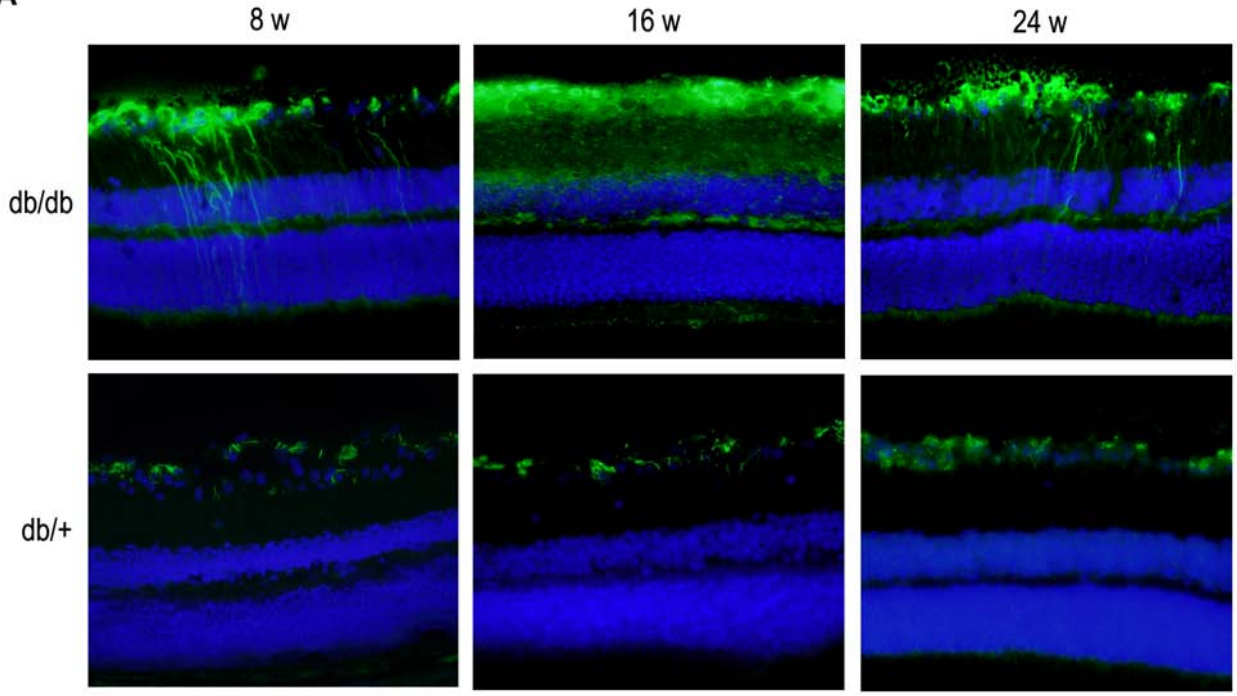


Figure 7. Transmission electron micrographs of photoreceptors in a representative case of a non-diabetic (left panel) and a diabetic mouse (right panel) 8 weeks old. Arrows indicate nuclear fragmentation. Scale bars: 6.71 μm.
doi:10.1371/journal.pone.0097302.g007

A



B

	% POSITIVE GFAP LABELING					
	8 w		16 w		24 w	
score	db/db	db/+	db/db	db/+	db/db	db/+
1	0	67	0	83	0	100
2	16	33	0	17	0	0
3	50	0	0	0	0	0
4	17	0	0	0	0	0
5	17	0	100	0	100	0

Figure 8. Glial activation. A) Comparison of GFAP immunofluorescence (green) between representative samples from a diabetic (upper panel) and a non-diabetic mouse (lower panel) at 8 weeks, 16 and 24 weeks. In the diabetic retina, the Müller cells' endfeet show abundant GFAP immunofluorescence and the radial processes stain intensely throughout both the inner and outer retina. Nuclei were labeled with DAPI (blue). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. B) Quantification of glial activation based on extent of GFAP staining.
doi:10.1371/journal.pone.0097302.g008

microscopy using specific antibodies against GFAP (Glial fibrillar acidic protein). Sections were fixed in acid methanol (−20°C) for 2 min, followed by three washes with PBS, 5 min each. Sections were permeabilized with TBS-Triton X-100 0,025% and were incubated in blocker (1% BSA, and 10% goat serum in PBS) for 2 hours at room temperature. Sections were then incubated with rabbit anti- GFAP (Abcam Ltd, Cambridge, U.K.) (1:500 dilution prepared in blocking solution) overnight at 4°C in a humid atmosphere. After three washes in PBS, 5 min each, the sections were incubated with secondary antibody Alexa 488 goat-anti-rabbit (Life Technologies S.A, Madrid, Spain) (1:200 dilution prepared in blocking solution). The sections were washed three times in PBS, counterstained with Hoesch and mounted with Mounting Medium Fluorescence (Prolong, Invitrogen) and mounted with a coverslip. Comparative digital images from diabetic and control samples were recorded with an Olympus microscope using identical brightness and contrast settings.

To evaluate the degree of glial activation we used a scoring system based on extent of GFAP staining previously used [25]. 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).

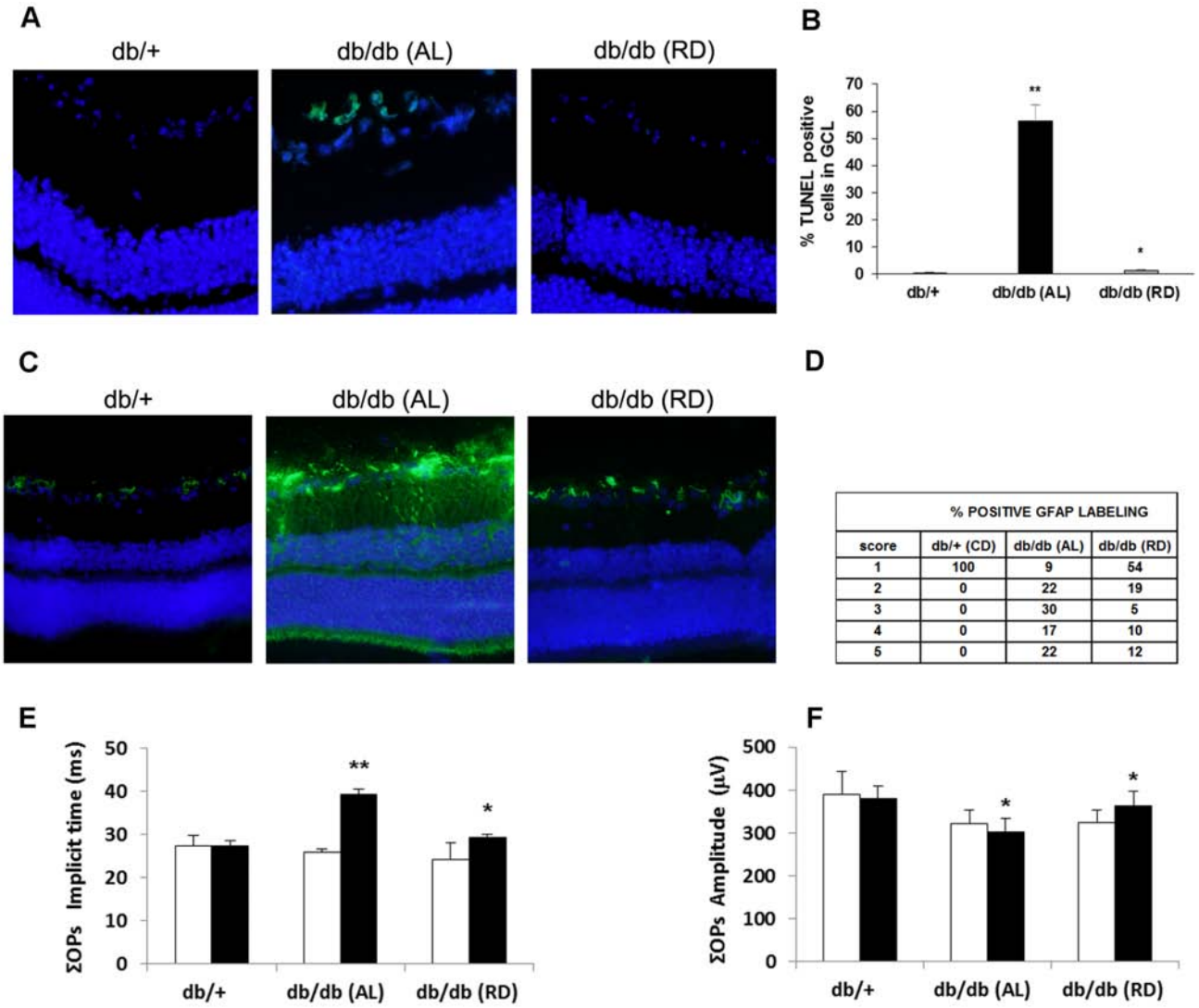


Figure 9. Beneficial effects of lowering blood glucose by using restrictive diet on retinal neurodegeneration in db/db mice. A) Comparison of TUNEL immunofluorescence (green) between representative samples from a diabetic mouse (db/db) *ad libitum* (AL) feeding (upper panel), a db/db mouse under dietary restriction (RD) (middle panel) and a control (db/+) mouse (lower panel) at 10 weeks. B) Percentage of TUNEL positive cells in the GCL in db/db mice ad libitum feeding (black bars), db/db mice under dietary restriction (gray bars) and control (db/+) mice (white bars) at 10 weeks. n = 10 each group. *$p<0.05$ in comparison with db/db feeding AL. **$p<0.01$ in comparison with db/+. C) Comparison of GFAP immunoreactivity (green) in the central retina between representative samples from a diabetic mouse (db/db) AL feeding (upper panel), a db/db mouse under dietary restriction (middle panel) and a control (db/+) mouse (lower panel) at 10 weeks. Nuclei were labeled with Hoechst (blue). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. D) Quantification of glial activation based on scoring system (see text) (n = 10 each group). E) ΣOPs implicit time (ms) and F) ΣOPs amplitude (μV). White bars: before diet intervention. Black bars: after diet intervention. Data are expressed as mean ± SD. *$p<0.05$; **$p<0.01$.
doi:10.1371/journal.pone.0097302.g009

Immunohistochemical analysis for apoptosis assessment. Apoptosis was evaluated using the TUNEL (Terminal Transferase dUTP Nick-End Labeling) method coupled with fluorescein (DeadEnd Fluorometric TUNEL System kit; PROMEGA, USA) with Hoechst 33342, Trihydrochloride, Trihydrate (Molecular Probes) staining. Cryosections of retina were permeabilised by incubation for 2 min on ice with 0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared. Apoptotic cells were identified using green fluorescence [Alexa Fluor 594 goat-anti-rabbit (Invitrogen) (1:200 dilution prepared in blocking solution with 5% BSA)]. 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.

Immunohistochemistry for caspase-3. Paraffined sections were rehydrated and washed in 0.01-M phosphate buffered saline (PBS). Then, they were incubated over night at 4°C with a rabbit anti-cleaved caspase-3 antibody (Cell Signalling Technology, Inc., Danvers, USA) at 1:300 dilution. Then, ocular sections were washed in PBS and incubated with the specific secondary antibody biotinylated anti-rabbit IgG (1:100) (Vector Laboratories, Burlingame, USA). Once washed in PBS, a streptavidin Alexa Fluor 488 conjugate (Molecular Probes-Life Technologies, Grand Island, USA) at 1:100 dilution was used to detect cleaved caspase-3 immunolabelling; the incubation was made over night at 4°C.

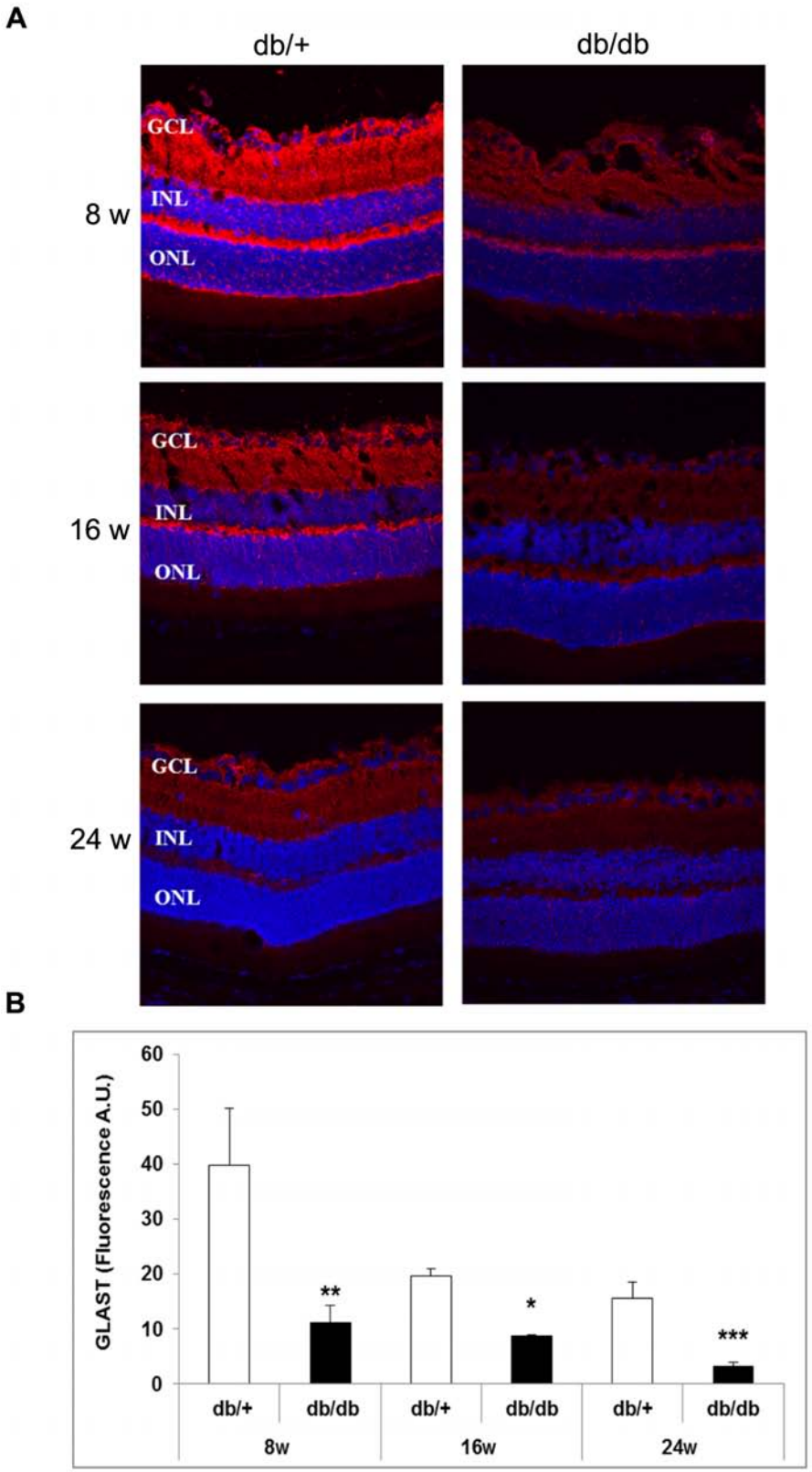


Figure 10. Comparison of GLAST immunofluorescence between representative samples from non-diabetic and diabetic mice. A) GLAST immunofluorescence (red) of representative samples from non-diabetic and diabetic mice at 8 weeks, 16 and 24 weeks. Nuclei were labeled with Hoechts (blue). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. B) Quantification of GLAST immunofluorescence in non-diabetic (white bars) and diabetic mice (black bars). A.U.: arbitray units. *$p<0.05$; **$p<0.001$ between non-diabetic and diabetic mice.
doi:10.1371/journal.pone.0097302.g010

Table 1. Genes up-regulated in retinas of diabetic mice.

ID	Symbol	Gene name	LogFC	P Value
71760	Agxt2l1	Alanine-glyoxylate aminotransferase 2-like 1	0.862	2.9E-07
19878	Rock2	Rho-associated, coiled-coil containing protein kinase 2	0.819	1.2E-06
66594	Uqcr11	Ubiquinol-cytochrome c reductase, complex III subunit XI	0.815	4.9E-06
11811	Apobec2	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 2	0.791	8.0E-08
20341	Selenbp1	Selenium binding protein 1	0.769	1.8E-04
57441	Gmnn	Geminin, DNA replication inhibitor	0.706	2.5E-05
15122	Hba-a1	Haemoglobin alpha, adult chain 1/2	0.691	1.4E-03
16668	Krt18	Keratin 18	0.691	9.3E-09
11815	Apod	Apolipoprotein D	0.677	5.4E-03
110880	Scn4a	Sodium channel, voltage-gated, type IV, alpha subunit	0.599	7.8E-08
19872	Rny1	RNA, Ro-associated Y1	0.591	2.9E-04
20557	Slfn3	Schlafen Family Member 12	0.577	2.4E-05
14719	Got2	Glutamic-oxaloacetic transaminase 2	0.530	2.1E-03
12865	Cox7a1	Cytochrome c oxidase subunit VIIa polypeptide 1	0.511	6.7E-04
56338	Txnip	Thioredoxin interacting protein	0.509	6.5E-05
83554	Fstl3	Follistatin-like 3	0.498	1.3E-05
12925	Crip1	Cysteine-rich protein 1	0.496	1.0E-06
319178	Hist1h2bb	Histone cluster 1, h2bb	0.486	3.3E-05
11303	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	0.485	1.7E-06
17873	Gadd45b	Growth arrest and DNA-damage-inducible, beta	0.467	8.2E-03
19725	Rfx2	Regulatory factor x,2	0.449	9.6E-04
66929	Asf1b	ASF1 anti-silencing function 1 homolog B	0.447	2.4E-05
70893	Glb1l3	Galactosidase, beta 1 like 3	0.441	9.1E-05
11830	Aqp5	Aquaporin 5	0.439	7.1E-03
30794	Pdlim4	PDZ and LIM domain 4	0.437	1.2E-05
19784	Rprl2	Ribonuclease P RNA-like 2	0.427	8.3E-04
75316	Taf1d	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D	0.418	4.5E-05
11927	Atox1	ATX1 (antioxidant protein 1) homolog 1	0.399	3.6E-05
109900	Asl	Argininosuccinatelyase	0.397	1.3E-06
108015	Chrnb4	Cholinergic receptor, nicotinic, beta polypeptide 4	0.393	1.5E-03
15561	Htr3a	5-hydroxytryptamine (serotonin) receptor 3A	0.388	6.0E-04
319155	Hist1h4c	Histone cluster 1, H4c	0.387	1.1E-03
12306	Anxa2	Annexin A2	0.387	4.3E-04
11854	Rhod	Ras homolog gene family, member D	0.386	2.8E-04
18129	Notch2	Notch gene homolog 2 (Drosophila)	0.385	1.6E-05
77462	Tmem116	Transmembrane protein 116	0.380	9.3E-05
70807	Arrdc2	Arrestin domain containing 2	0.378	1.1E-03
11853	Rhoc	Ras homolog gene family, member C	0.372	2.9E-05
227292	Ctdsp1	CTD small phosphatase 1	0.372	1.6E-03
171209	Accn3	Amiloride-sensitive cation channel 3	0.371	2.8E-03
69875	Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11	0.370	3.6E-06
65972	Ifi30	Interferon, gamma-inducible protein 30	0.368	5.5E-04
74760	Rab3il1	RAB3A interacting protein (rabin3)-like 1	0.363	1.2E-04
263406	Plekhg3	Pleckstrin homology domain containing, family G member 3	0.362	6.9E-05
71660	Rarres2	Retinoic acid receptor responder (tazarotene induced) 2	0.355	6.1E-03
212974	Athl1	ATH1, acid trehalase-like 1	0.354	1.2E-03
12036	Bcat2	Branched chain amino-acid transaminase 2	0.354	1.2E-05
68337	Crip2	Cysteine-rich protein 2	0.350	5.4E-03
22228	Ucp2	Uncoupling protein 2	0.349	3.8E-03
66230	Mrps28	Mitochondrial ribosomal protein S28	0.349	1.2E-03

Nuclear counterstaining with Hoescht stain solution (Sigma-Aldrich Chemie, Buchs, Switzerland) was performed for microscopic analysis with the laser scanning confocal microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany). Negative control was carried out by omitting the primary antibody. Specific labeling of cleaved caspase-3 antibody in mouse jejunal paraffin sections was used as positive control.

Transmission electron microscopy analysis. One mm^3 retinal fragments were dissected from four 8 week-old db/db mice. Retinas from four age-matched db/+ mice were used as control retinas. Retinal fragments were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde, post fixed in 1% osmium tetroxide, stained in aqueous uranyl acetate, dehydrated and embedded in spurr resin. Ultrathin sections (70 nm) were stained with lead citrate and examined under transmission electron microscopy (Jeol 1400; Jeol Ltd., Tokyo, Japan).

Glutamate Quantification

Quantification of glutamate was performed by liquid-chromatography coupled to mass spectrometry (LC-MS/MS). Chromatographic separation was performed on an Agilent 1200 series (Waldbronn, Germany) using an Ascentis Express HILIC column, 50×2.1 mm with 2.7 Rm particle size from Supelco (Belfonte, PA) maintained at 25°C throughout the analysis, a mobile phase acetonitrile and water (50 mM ammonium acetate) with a flow rate of 0.6 mL min-1. The volume injected was 10 RL. The mobile phase involved a gradient starting at 87% of ACN which was maintained for 3 minutes. Then, from min 3 to 10 the ACN content was decreased to 20% and increased again to 87% at min 12.5. Glutamate was eluted at 6.5 minutes. The mass detection system was an Agilent 6410 Triple Quad (Santa Clara, CA) using positive electrospray ionization with a gas temperature of 350°C, gas flow rate of 12 L min-1, nebulizer pressure of 45 psi, capillary voltage of 3500 V, fragmentor of 135 V and collision energy of 10 V.

Immunohistochemistry for GLAST

Glutamate/aspartate transporter (GLAST) and L-glutamate was evaluated by fluorescence microscopy using specific antibodies. Sections were incubated in blocking solution (3% BSA, Tween 0,05% PBS) for 1 h. at room temperature followed by incubation with primary antibody rabbit anti-GLAST (EAAT1) (1:100, Abcam ab416, Cambridge, UK). After washing, sections were incubated with a fluorescent anti-rabbit ALEXA 594 as a secondary antibody (Life Technologies S.A, Madrid, Spain) in blocking solution for 1 h, washed, nuclei were stained with Hoechst and mounted in Mounting Medium Fluorescence (Prolong, Invitrogen) with a coverslip. Fluorescence intensity of images was quantified by ImageJ.

DNA Microarrays

Gene expression profiling analysis in retinas of 8-week old diabetic (db/db) mice and non-diabetic (db/+) controls was performed using Mouse Gene 1.0 ST DNA arrays (Affymetrix, UK). For this purpose, total RNA was first isolated from retinas (n = 4/group) using the RNeasy Mini Kit (Qiagen, Germany) and 200 ng were then used to synthesize sense ssDNA with the Ambion WT Expression Kit (Life Technologies, UK). Next, ssDNA was fragmented, labeled and hybridized onto DNA microarrays using the GeneChip WT Terminal Labeling and Hybridization Kit and the GeneTitan platform (Affymetrix, UK), following the manufacturer's instructions. Microarray Analysis Suite 5.0 software was used to process the microarray images and analysis of the data obtained was performed by the Statistics and Bioinformatics Unit of the Vall d'Hebron-Research Institute using the open source software Bioconductor.

Gene Expression

Real-time quantitative PCR was used to quantify relative transcript levels in retinas of 8-week old diabetic and control mice. 400 ng of total RNA were used to synthesize cDNA by using SuperScript II reverse transcriptase (Life Technologies, USA) and oligo(dT) primers. Quantitative PCR was performed by using gene-specific primers and SYBR Green in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, UK). Relative mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ threshold method, using cyclophilin A as a reference gene [26].

Statistical Analysis

Normal distribution of the variables was evaluated using the Kolmogorov-Smirnov test. Comparisons of continuous variables were performed using the paired and unpaired Student t-test. Levels of statistical significance were set at $p<0.05$.

Results

Blood Glucose Levels

The diagnosis of diabetes was based on blood glucose levels. The mice with blood glucose level greater than 250 mg/dl were confirmed as diabetic mice. In the control group (db/+) blood glucose levels were <150 mg/dl during the follow-up whilst all db/db mice presented blood glucose >250 mg/dl at 4 weeks of age. Animal body weight and glucose levels are shown in Figure 1. We observed that hyperglycemia in diabetic mice runs in parallel with a significant increase of weight.

ERG Abnormalities

The average b-wave implicit times and b-wave amplitudes as a function of flash intensity are presented in Figure 2 (A–F). The b-wave implicit time significantly increased in diabetic mice at all flash intensities tested when compared with non-diabetic mice at 16 and 24 weeks. However, the implicit time was not significantly delayed in diabetic mice at 8 weeks of age. In addition, b-wave amplitude was significantly reduced in diabetic mice in comparison with non diabetic mice at 16 and 24 weeks but not at 8 weeks. The changes observed in amplitude and implicit time of the a-wave in diabetic mice in comparison with non-diabetic mice were less marked than that observed in b-wave (Figure 3).

The OPs amplitudes measured under scotopic conditions and the corresponding implicit times are shown in Figure 2 (G, H). We detected statistically significant differences (increase of implicit time and decrease of amplitude) at 16 and 24 weeks.

Retinal Morphometry

Measurements of retinal thickness in diabetic and in non diabetic mice at 8, 16 and 24 weeks are shown in Figure 2. Since postnatal growth of retinal thickness exists in C57BL mice until month 6 [27,28], a progressive increase in the thickness of retinas from non-diabetic mice was observed whereas this was not the case in diabetic mice. Total retinal thickness (measured from inner limiting membrane to Bruch's membrane) in both central and peripheral retina was significantly decreased in diabetic mice in comparison with non-diabetic mice at 16 and 24 weeks (Figure 4). Furthermore, a thickening in both ONL and INL (Figure 4) as well as a reduction in the number of cells in the GCL (Figure 5B) was observed in diabetic mice in comparison with non-diabetic mice at 8, 16 and 24 weeks.

Table 2. Genes down-regulated in retinas of diabetic mice.

ID	Symbol	Gene name	LogFc	P value
12182	Bst1	Bone marrow stromal cell antigen	−0.495	9.0E-06
219257	Pcdh20	Protocadherin	−0.440	4.0E-04
319554	Idi1	Isopentenyl-diphosphate delta isomerase 1	−0.438	6.8E-05
12293	Cacna2d1	Calcium channel, voltage-dependent, alpha 2/delta subunit 1	−0.437	4.0E-04
331487	Uprt	Uracil phosphoribosyltransferase (FUR1) homolog	−0.435	8.6E-04
83924	Gpr137b	G protein-coupled receptor 137B	−0.407	1.9E-03
66917	Chordc1	Cysteine and histidine-rich domain (CHORD) containing	−0.399	3.3E-04
66637	Tsen15	tRNA splicing endonuclease 15 homolog	−0.398	7.9E-03
20383	Srsf3	Terine/arginine-rich splicing factor 3	−0.390	5.4E-03
26557	Homer2	Homer homolog 2	−0.390	1.3E-03
67681	Mrpl18	Mitochondrial ribosomal protein L18	−0.383	9.5E-03
217951	Tmem196	Transmembrane protein	−0.383	9.9E-04
105727	Slc38a1	Solute carrier family 38, member 1	−0.362	4.7E-04
74182	Gpcpd1	Glycerophosphocholine phosphodiesterase GDE1 homolog	−0.356	5.7E-06
229279	Hnrnpa3	Heterogeneous nuclear ribonucleoprotein A3	−0.352	9.5E-05
21333	Tac1	Tachykinin, precursor 1	−0.352	5.2E-03
14405	Gabrg1	Gamma-aminobutyric acid (GABA) A receptor, gamma 1	−0.352	4.2E-03
102910	Armcx4	Armadillo repeat containing, X-linked 4	−0.351	9.7E-05
59058	Bhlhe22	Basic helix-loop-helix family, member e22	−0.351	1.9E-03
14401	Gabrb2	Gamma-aminobutyric acid (GABA) A receptor, beta 2	−0.349	5.6E-05
57329	Otor	Otoraplin	−0.345	1.8E-03
70930	Nol8	Nucleolar protein 8	−0.343	3.1E-04
70620	Ube2v2	Ubiquitin-conjugating enzyme E2 variant 2	−0.342	9.7E-04
331487	Uprt	Uracil phosphoribosyltransferase (FUR1) homolog	−0.342	1.2E-03
56353	Rybp	RING1 and YY1 binding protein	−0.341	9.6E-03
71599	Senp8	SUMO/sentrin specific peptidase family member 8	−0.339	8.9E-03
15289	Hmgb1	High mobility group box 1	−0.334	6.3E-03
15505	Hsph1	Heat shock 105 kDa/110 kDa protein	−0.333	1.1E-03
93739	Gabarapl2	Gamma-aminobutyric acid (GABA) A receptor-associated protein-like 2	−0.331	7.1E-03
668923	Zfp442	Zinc finger protein 442	−0.328	6.0E-03
72289	Malat1	Metastasis associated lung adenocarcinoma transcript 1	−0.325	1.1E-04
14009	Etv1	Ets variant 1	−0.325	6.7E-03
11798	Xiap	Apoptotic suppressor protein	−0.323	2.5E-04
17968	Ncam2	Neural cell adhesion molecule 2	−0.320	6.1E-04
76184	Abca6	ATP-binding cassette, sub-family A (ABC1), member 6	−0.314	1.4E-03
12300	Cacng2	Calcium channel, voltage-dependent, gamma subunit 2	−0.314	4.8E-03
109905	Rap1a	RAP1A, member of RAS oncogene family	−0.314	8.5E-04
71206	Katnal2	Katanin p60 subunit A-like 2	−0.312	4.3E-03
20541	Slc8a1	Solute carrier family 8, member 1	−0.312	3.1E-03
14417	Gad2	Glutamate decarboxylase 2	−0.311	6.6E-03
14823	Grm8	Glutamate receptor, metabotropic 8	−0.310	2.0E-03
14799	Gria1	Glutamate receptor, ionotropic, AMPA 1	−0.310	2.8E-04
19128	Pros1	Protein S	−0.309	1.1E-03
320772	Mdga2	MAM domain containing glycosylphosphatidylinositol anchor 2	−0.307	2.9E-04
243382	Ppm1k	Protein phosphatase 1K	−0.306	1.5E-03
246229	Bivm	Basic, immunoglobulin-like variable motif containing	−0.305	8.1E-05
228942	Cbln4	Cerebellin 4	−0.304	1.4E-03
11658	Alcam	Activated leukocyte cell adhesion molecule	−0.303	1.8E-03
18718	Pip4k2a	Phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	−0.302	2.9E-04
18231	Nxph1	Neurexophilin 1	−0.302	8.0E-04

doi:10.1371/journal.pone.0097302.t002

Neurodegeneration Features

A significant increase in TUNEL-positive inmunofluorescence was observed in diabetic mice in comparison with retinas from non diabetic mice at 8, 16 weeks and 24 weeks (Figure 5C). Since the TUNEL-positive cells were mainly localized in the GCL, we also counted the percentage of apoptotic cells in this layer, and a significant increase was found in diabetic mice in comparison with non-diabetic mice at 8, 16 and 24 weeks (Figure 5D). In addition, activated caspase-3 was found significantly higher in the retina of db/db mice in comparison with non-diabetic mice at 8, 16 and 24 weeks (Figure 6).

Since in the ERG measurements we found a-wave abnormalities, which mainly indicate photoreceptor impairment, we wanted to examine whether apoptosis was also present in photoreceptors. For this purpose transmision electron microsocopy was used and striking DNA fragmentation was found in photoreceptors from db/db mice in comparison with non-diabetic mice (Figure 7).

As expected, in non-diabetic mice GFAP expression was confined to the retinal GCL (GFAP score $\leq$2) (Figure 8). In contrast, in diabetic mice we observed the ''reactive'' diabetic phenotype characterized by upregulation of GFAP in Müller cells (GFAP score $\geq$2 at week 8 and score = 5 at 16 and 24 weeks).

All these features of neurodegeneration were more intense at 16 than at 8 weeks, but no significant differences between 16 and 24 weeks were observed.

Table 3. Gene enrichment analysis of genes differentially expressed in retinas of diabetic mice.

Ontology	GO ID	GO Term	*P* value (P<1E-03)
Down-regulated genes			
MF	GO:0003723	RNA binding	6.18E-08
MF	GO:0004971	Alpha-amino-3-hydroxy-5-methyl-4 isoxazole propionate selective glutamate receptor activity	1.38E-05
MF	GO:0005488	Binding	2.8E-05
MF	GO:0022891	Substrate-specific transporter activity	2.1E-04
MF	GO:0005313	L-glutamate transporter activity	2.7E-04
MF	GO:0046943	Carboxylic acid transporter activity	4.8E-04
BP	GO:0007268	Synaptic transmission	2.4E-06
BP	GO:0007268	Transmission of nerve impulse	2.98E-06
BP	GO:0035637	Multicellular organismal signaling	2.98E-06
BP	GO:0015931	Nucleobase-containing compound transport	1.0E-04
BP	GO:0044237	Cellular metabolic process	1.0E-04
BP	GO:0050657	Nucleic acid transport	1.9E-04
CC	GO:0043005	Neuron projection	3.1E-07
CC	GO:0045202	Synapse	5.5E-06
CC	GO:0005622	Intracellular	5.6E-06
CC	GO:0044456	Synapse part	5.9E-06
CC	GO:0032279	Asymmetric synapse	2.2E-05
CC	GO:0044424	Intracellular part	2.4E-05
Up-regulated genes			
MF	GO:0003735	Structural constituent of ribosome	8.1E-05
MF	GO:0015078	Hydrogen ion transmembrane transporter activity	3.2E-04
MF	GO:0004364	Glutathione transferase activity	6.6E-04
MF	GO:0019843	rRNA binding	7.7E-04
BP	GO:0006979	Response to oxidative stress	6.3E-05
BP	GO:0007589	Body fluid secretion	1.4E-04
BP	GO:0016049	Cell growth	4.6E-04
BP	GO:0034599	Cellular response to oxidative stress	6.3E-04
BP	GO:0090208	Positive regulation of triglyceride metabolic process	7.2E-04
BP	GO:0070301	Cellular response to hydrogen peroxide	8.5E-04
CC	GO:0005840	Ribosome	1.4E-07
CC	GO:0070469	Respiratory chain	5.8E-06
CC	GO:0022626	Cytosolic ribosome	1.0E-05
CC	GO:0044429	Mitochondrial part	1.3E-05
CC	GO:0044444	Cytoplasmic part	1.3E-05
CC	GO:0005743	Mitochondrial inner membrane	1.4E-05

Only the six most significant gene ontology (GO) terms in each category are shown (MF, molecular function; BP, biological process; CC, cellular compartment).
doi:10.1371/journal.pone.0097302.t003

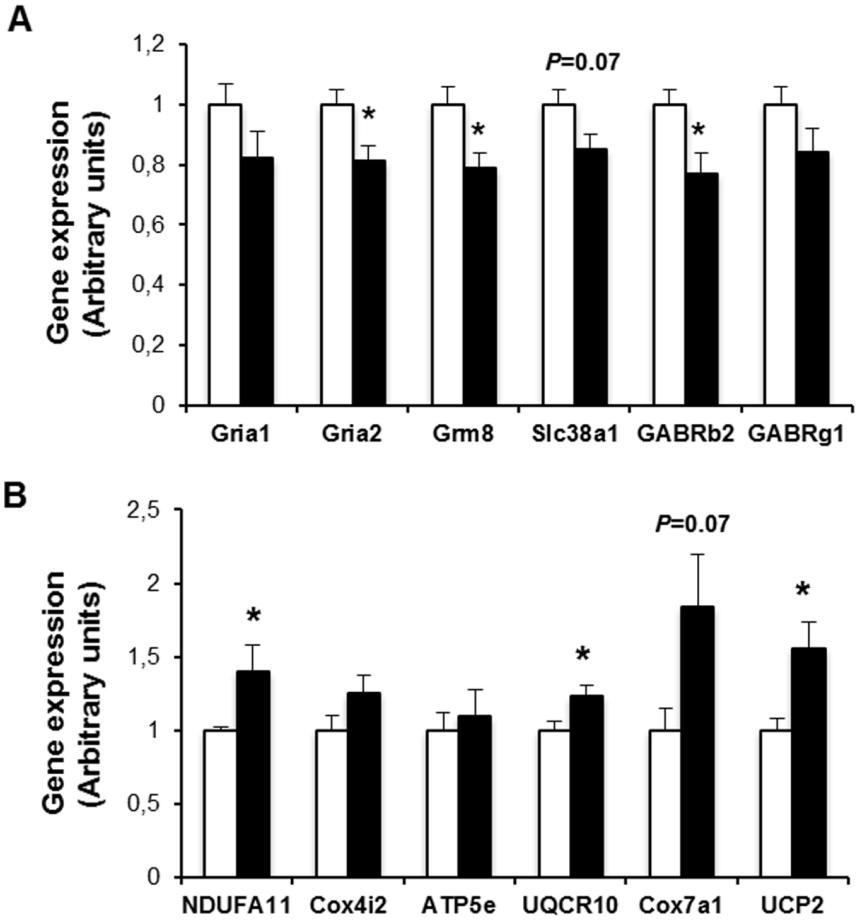


Figure 11. Relative expression of genes involved neurotransmission (A) and mitochondrial function (B) in retinas of diabetic mice (black bars) and control non-diabetic mice (white bars) was assessed by real-time quantitative PCR. Results are expressed as mean ± SEM, n = 9–10/group. *p<0.05.
doi:10.1371/journal.pone.0097302.g011

Effect of Lowering Blood Glucose on Retinal Neurodegeneration

As expected, db/db mice fed for 15 days with restrictive diet presented lower weight and blood glucose levels than those mice fed with *ad libitum* diet (weigth: 32.5±2.8 gr vs. 42.2±2.5 gr; p< 0.05; blood glucose: 318±27 mg/dL vs. 536±29 mg/dL, p< 0.01). At this point (after 15 days of dietary restriction) a significantly lower GFAP immunofluorescence and a lower ratio of apoptosis in GCL were detected (Figures 9 A–D). Finally, ERG abnormalities were significantly arrested (Figure 9 E–F).

Markers of Glutamate Pathway

Glutamate levels (μM/g total protein) were higher in diabetic mice in comparison with non diabetic mice at 8 weeks (60±8 *vs.* 38±6; p<0.05), 16 weeks (108±12 vs. 56±10; p<0.05) and 24 weeks (125±11 vs. 57±9; p<0.05). The progressive increase of glutamate levels in diabetic mice run in parallel with a decrease in GLAST content. GLAST was significantly decreased in diabetic mice in comparison with the non diabetic mice (Figure 10).

Transcriptomic Analysis

To unravel the molecular mechanisms involved in early retinal neurodegeneration besides glutamate and its metabolic pathways, we performed a genome-wide expression profiling analysis on total RNA isolated from retinas of 8-week old diabetic (db/db) and control littermate mice. After filtering for redundant and non-annotated gens, we found that 657 genes were differentially expressed in retina of diabetic mice (P<0.01). Of these genes, we found 316 genes to be up-regulated and 341 down-regulated. The main genes up-regulated and down-regulated are listed in Table 1 and Table 2, respectively. To gain insight into the biological function of the genes differentially expressed in retinas of diabetic mice, a gene enrichment analysis was performed. Interestingly, we found that Gene Ontology (GO) terms significantly enriched among the down-regulated genes fitted into categories related to synaptic transmission, with a particular abundance of terms related to glutamate transport and metabolism (Table 3). On the other hand, the GO terms significantly over-presented among the up-regulated genes corresponded to categories related to mitochondrial respiration and oxidative stress (Table 3).

Real time quantitative PCR confirmed an aproximately 20% reduction in the expression of genes related to neurotransmission, such as inotropic glutamate receptor-1 and -2 (Gria-1 and Gria-2), metabotropic glutamate receptor 8 (Grm8), glutamine transporter (Slc38a1) or the subunits β2 and γ1 of the gamma-aminobutyric acid A receptor (Figure 11A). These results suggest that glutamate signaling and metabolism is altered in diabetic mice.

Interestingly, we have also found that some mitochondrial genes, most of them encoding for proteins of the respiratory chain, were mildly increased in retinas of diabetic mice (Figure 11B). A

parallel increase in the expression of *UCP2*, a protein involved in the control of mitochondria-derived reactive oxygen species (ROS) production, was observed (Figure 11B).

Discussion

Neurodegeneration is an early event and plays a crucial role in the pathogenesis of DR. In fact, the main features of retinal neurodegeneration (apoptosis and glial activation) have been found in the retina of diabetic donors without any microcirculatory abnormalities appearing in the ophthalmologic examinations performed during the year before death [6,29]. In addition, there are several pieces of evidence to suggest that neurodegeneration participates in early microvascular abnormalities that occur in DR [9–11,13,15,30–32]. In this regard, blood-retinal barrier (BRB) breakdown as the result of VEGF upregulation produced by glutamate-induced excitotoxicity has been well documented [9,11,12], and a cross-talk between neurodegeneration and vasoregression has been reported [10,13]. In addition, the impairment of neurovascular coupling which can be detected in diabetic patients without structural abnormalities [32,33] seems primarily mediated by ganglion cell damage [34]. Finally, it has been shown that a delayed multifocal ERG implicit time predicts the development of early microvascular abnormalities [29,35–37]. Therefore, it is reasonable to hypothesize that therapeutic strategies based on neuroprotection will be effective in preventing or arresting DR development. However, the morphological and functional characterization of neurodegeneration in a spontaneous diabetic model is a challenge that had to be met. In the present study we have evaluated the neurodegenerative process in retinas of db/db mice and we have found the same features that occur in retinas from diabetic donors. These findings permit us to propose the db/db mouse as a good model for DR and appropriate for testing neuroprotective drugs.

Mice are more resistant to the STZ effect and present a lower degree of retinal lesions compared to rats. Nevertheless, because of its great potential for genetic manipulation, the mouse offers a unique opportunity to study the molecular pathways involved in disease development. In the present study we have found that the spontaneous development of diabetes in db/db mice results in a progressive thicknness of the neuroretina in comparison with non-diabetic mice, mainly due to the apoptosis of the ganglion cell layer (GCL). In this regard, we found a 24% reduction in the number of cell bodies in the GCL at 8 weeks (after 4 weeks of hyperglycemia). This reduction increased at 16 weeks to 29%. These results are in agreement with those recently reported by Tang et al [22] showing that ganglion cell number and total retinal thickness are decreased in db/db mice compared with wild-type controls. In addition, it should be mentioned that, as occurs in db/db mice, GCL has been the layer with the highest rate of apoptosis in human retinas [6,7,38]. Noteworthy, thinning of the GCL has been found in diabetic patients with no or only minimal DR [6,39–41]. Apart from the loss of cells in the GCL we also found a thinning of the ONL in db/db mice, which had already been found at 8 weeks. In this regard we demonstrated DNA nuclear fragmentation in photoreceptors by using transmision electron microscopy, thus clearly indicating the presence of apoptosis. This finding is consistent with the impairment of a-wave observed in the ERG studies.

Neural apoptosis is accompanied by changes in both types of glial cells (microglia and macroglia), the most representative being those occurring in Müller cells, the predominant type of macroglial cells. Retinal astrocytes normally express GFAP, while in Müller cells this expression is much lower. However, in diabetes an aberrant overexpression of GFAP is shown by Müller cells [42]. Cheung et al [19] have previously reported that GFAP and cleaved caspase 3 labeling were increased in the retina of db/db mice compared with non-diabetic controls. In the present study we have found a significant overexpression of GFAP at 8 weeks, thus running in parallel with the apoptotic changes. This is a significant feature that has not been found in other spontaneous diabetic mice such as the $Ins2^{Akita}$ mouse model [43].

In order to explore whether neurodegeneration detected in homozygous db/db animals is of a genetic nature or related to hyperglycemia we examined the effect of lowering blood glucose levels on retinal neurodegeneration. We found a significant reduction of the most important features of neurodegeneration (apoptosis, glial activation, ERG abnormalities) by lowering blood glucose levels in db/db mice. Therefore, diabetes is the main reason accounting for the retinal neurodegeneration that occurs in db/db mice.

It is possible that glial activation is a consequence of neural death. In fact we have recently found the activation of the Fas/FasL death receptor pathway in the diabetic eye which can induce the secretion of pro-inflammatory cytokines, thus leading to neuroinflammation and glial activation [44]. On the other hand, Müller cells produce factors capable of modulating blood flow, vascular permeability, and cell survival. Therefore, these cells could play a primary role in accounting for neural death. Further studies to unravel the hierarchical role of apoptosis and glial activation in the neurodegenerative process of DR are needed.

We have also examined the functional consequences of retinal neurodegeneration by means of sequential ERG. The abnormalities here reported in db/db mice in b-wave and OPs (reduced amplitude and prolonged implicit time) were similar to those described in the early stages of DR in diabetic patients [45,46]. The type of ERG abnormality could help to identify anatomically the location of retinal damage. In this regard, the OPs have been shown to be derived from the inner plexiform layers involving the axon terminals of the bipolar cells, the processes of the amacrine cells, and the dendrites of the ganglion cells [47]. By contrast, changes in b-wave amplitudes and implicit times are consistent with defects in the mid-retinal layer.

Nevertheless, ERG abnormalities in the diabetic retina are not only due to apoptosis of retinal neurons. In this regard, both glial activation and changes in blood glucose levels have been involved in the ERG abnormalities observed in diabetic patients without cellular loss or major structural damage [48,49].

In the present study we found a progressive increase of glutamate accumulation in diabetic mice. Glutamate accumulation in extracellular space and the overactivation of glutamate receptors (“excitotoxicity”) plays an important role in retinal neurodegeneration. In fact, elevated levels of glutamate in the retina have been found in experimental models of diabetes [50–52], as well as in the vitreous fluid of diabetic patients [53]. Glutamate transporters are essential for keeping the extracellular glutamate concentration below neurotoxic levels [54]. In this regard, the glial GLAST, the main glutamate transporter expressed by Müller cells, is the most dominant glutamate transporter, accounting for at least 50% of glutamate uptake in the mammalian retina [55]. A reduction of GLAST expression in rat retinas with diabetes induced by STZ has been reported [56,57]. However, to the best of our knowledge GLAST expression in db/db mice mouse has not previously been examined. We observed a significant reduction of GLAST in diabetic (db/db) mice in comparison with non-diabetic (db/+) mice. This finding could contribute to the glutamate accumulation that leads to neurodegeneration in db/db mice. It is worth

mentioning that we did not find any differences in GLAST mRNA levels between diabetic and non-diabetic mice, thus suggesting that the differences in GLAST could be attributed to post-translational changes.

To the best of our knowledge this is the first transcriptomic analysis performed in the db/db model. We found a downregulation of several genes related to glutamate metabolism (glutamate receptors and glutamate transporters) in early stages of DR in db/db mouse. This finding could lead to the observed extracellular glutamate accumulation, thus inducing excitotoxicity. As previously commented, it has been reported that diabetes downregulates genes implicated in glutamate transporter in animal models. However, the information on genes related to glutamate receptors is controversial and mainly focused in STZ-diabetes induced animal models [58–61]. Furthermore, we found a simultaneously upregulation of mitochondrial UCP2 that could be contemplated as a mechanism to mitigate oxidative stress. This finding links glutamate excitotoxicity and oxidative stress and suggests that a vicious cycle involving glutamate excitotoxicithy, oxidative stress and mitochondrial dynamics reported in a non-diabetic model of retinal neurodegeneration could also be applied to diabetic retina [62–65].

In conclusion, the db/db mouse reproduces the features of the neurodegenerative process that occurs in the human diabetic eye. Therefore, it seems an appropriate model for investigating the underlying mechanisms of diabetes-induced retinal neurodegeneration and for testing new neuroprotective drugs.

Author Contributions

Conceived and designed the experiments: PB LC RS CH. Performed the experiments: PB LC JV AC JG DR JR. Analyzed the data: PB LC JV RS CH DR JR. Contributed reagents/materials/analysis tools: JG RS CH JR. Wrote the paper: PB RS CH.

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CHAPTER II

Effect of fenofibrate on retinal neurodegeneration in an experimental model of type 2 diabetes.

In order to evaluate the potential effect of fenofibric acid in preventing retinal neurodegeneration in db/db mice we evaluated 24 diabetic mice aged 8 weeks. These mice were divided into two groups assigned to daily oral treatment with FA (100 mg/kg/day) for one week. As control group, 10 non-diabetic mice (db/+) were used. Blood glucose and triglycerides levels were measured at baseline and at the end of the study. After one week of treatment with FA db/db mice showed lowered triglyceride levels than the vehicle group but did not reach statistical significance. As expected, blood glucose levels did not differ between diabetic groups.

Functional abnormalities were assessed by electroretinography and the results show that after one week of follow up, FA treatment prevents the decrease in oscillatory potential amplitude observed in diabetic mice treated with vehicle. Moreover the b-wave implicit time tends to increase in diabetic mice receiving vehicle. Furthermore, in diabetic mice treated with FA, a significant reduction in b-wave implicit time was observed.

Retinal neurodegeneration measurements included a morphometric analysis, glial activation assessment by evaluating GFAP expression by immunofluorescence and western blot, and a TUNEL assay to determine the apoptosis rate. FA administration for one week resulted in significant decrease in reactive gliosis. Western blot for GFAP pointed out the same direction, a higher content of GFAP in protein retinal extracts from vehicle mice in comparison to FA-treated mice. Apoptosis rate was lowered after one week of FA treatment, besides, there was a prevention in the reduction of number of cells in the GCL in db/db mice treated with vehicle.

GLAST expression is downregulated in diabetes, one possible mechanism fenofibrate could act through is preventing glutamate accumulation. GLAST was assessed by immunofluorescence. This transporter was downregulated in the retinas of diabetic mice treated with vehicle in comparison to non-diabetic mice. Moreover, in diabetic mice treated with FA, this downregulation of GLAST induced by diabetes was prevented.

This article demonstrates that FA has a neuroprotective action in db/db mice, and that its effect occurs very shortly after administration. One of the mechanisms involved in the neuroprotective effect of FA could be the eventual reduction of glutamate excitotoxicity by increasing glutamate uptake through upregulating GLAST.

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ORIGINAL ARTICLE

Effect of fenofibrate on retinal neurodegeneration in an experimental model of type 2 diabetes

Patricia Bogdanov · Cristina Hernández · Lidia Corraliza · Andrea R. Carvalho · Rafael Simó

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Abstract There is now consistent evidence from two major clinical trials (the Fenofibrate Intervention and Event Lowering in Diabetes and the Action to Control Cardiovascular Risk in Diabetes Eye) that fenofibrate arrests the progression of diabetic retinopathy in type 2 diabetic patients. However, the underlying mechanisms of this beneficial effect remain to be elucidated. The aim of the study was to evaluate the potential effect of fenofibric acid (FA), the active metabolite of fenofibrate, in preventing retinal neurodegeneration in an experimental mouse model of type 2 diabetes. For this purpose, we evaluated a total of 24 diabetic mice (db/db) aged 8 weeks that were randomly assigned to daily oral treatment (by gavage) with FA (100 mg/kg/day) ($n = 12$) or vehicle ($n = 12$) for 1 week. Ten non-diabetic mice (db/+) were used as control group. Retinal neurodegeneration was evaluated by measuring glial activation (immunofluorescence and Western blot) and apoptosis. Glutamate/aspartate transporter (GLAST) was assessed by immunofluorescence. Functional abnormalities were assessed by electroretinography (ERG). We observed that diabetic mice presented significantly higher glial activation and apoptosis in ganglion cell layer (GCL) than in age-matched non-diabetic mice. Treatment with FA resulted in a significant decrease in both glial activation and the rate of apoptosis in GCL in comparison with diabetic mice treated with vehicle. In addition, FA prevented GLAST downregulation induced by diabetes. Furthermore, a significant improvement of ERG parameters (oscillatory potential amplitudes and b-wave implicit time) was observed. We conclude that FA prevents retinal neurodegeneration induced by diabetes. Our results suggest that neuroprotection is one of the underlying mechanisms by which fenofibrate exerts its beneficial actions in diabetic retinopathy.

Keywords Diabetic retinopathy · Diabetic mouse model · Fenofibrate · Neurodegeneration

Managed by Massimo Federici.

Patricia Bogdanov and Cristina Hernández have contributed equally to this work.

P. Bogdanov · C. Hernández · L. Corraliza · R. Simó (✉)
Diabetes and Metabolism Research Unit, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Pg. Vall d'Hebron 119-129, 08035 Barcelona, Spain
e-mail: rafael.simo@vhir.org

P. Bogdanov · C. Hernández · L. Corraliza · R. Simó
Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III (ISCIII), Madrid, Spain

A. R. Carvalho
Ophthalmologic Research Laboratory, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain

A. R. Carvalho
Red de Investigación Colaborativa en Oftalmología, ISCIII, Madrid, Spain

Abbreviations

ACCORD	The Action to Control Cardiovascular Risk in Diabetes
DR	Diabetic retinopathy
FA	Fenofibric acid
ERG	Electroretinography
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GCL	Ganglion cell layer
GLAST	Glutamate–aspartate transporter
GFAP	Glial fibrillar acidic protein

H&E	Hematoxylin and eosin
ISCEV	International Society for Clinical Electrophysiology of Vision
Lp-PLA2	Lipoprotein-associated phospholipase A2
OIR	Oxygen-induced retinopathy
ONL	Outer nuclear layer
OPs	Oscillatory potentials
PPAR	Peroxisome proliferator-activated receptor
RPE	Retinal pigment epithelium
TUNEL	Terminal Transferase dUTP Nick-End Labeling

Introduction

Diabetic retinopathy (DR) is the most common complication of diabetes and one of the leading causes of preventable blindness [1]. Current treatments for DR are applicable only at advanced stages of the disease and are associated with significant adverse effects [2, 3]. Therefore, new pharmacological treatments for the early stages of the disease are needed.

Fenofibrate is a peroxisome proliferator-activated receptor (PPAR)-α agonist indicated for the treatment for hypertriglyceridemia and mixed dyslipidemia [4]. Apart from its lipid-lowering action, data from two major clinical trials, the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) [5] and the Action to Control Cardiovascular Risk in Diabetes (ACCORD) Eye [6] studies, provide robust evidence of the beneficial effects of fenofibrate in arresting the progression of DR. In this regard, fenofibrate has been recommended as an adjunctive treatment for type 2 diabetic patients with early DR to delay progression [7, 8]. The mechanisms by which fenofibrate exerts its beneficial action in DR are just beginning to be elucidated. Among the potential mechanisms of action, both lipidic and non-lipidic mechanisms have been involved. However, the non-lipidic mechanisms seem to have more relevance given that in the above-mentioned studies there was no relationship between the lipid-modifying effects of fenofibrate and the incidence or progression of DR [7, 8].

It has been reported that fenofibrate prevents the apoptosis of human retinal endothelial cells induced by serum deprivation through a PPARα-independent but AMP-activated protein kinase-dependent pathway [9]. In addition, we have previously reported that fenofibric acid (FA), the active metabolite of fenofibrate, prevents the disorganization of tight junction proteins and hyperpermeability provoked by the diabetic milieu in retinal pigment epithelium (RPE) [10]. Moreover, FA downregulates the overexpression of fibronectin and collagen IV in the basement membrane of RPE, thus preventing the increase in permeability associated with the aberrant accumulation of these basement membrane components [11]. Furthermore, we also demonstrated that FA elicits a dual protective effect in RPE by the downregulation of stress-mediated signaling and the induction of autophagy and survival pathways [12]. Recently, Chen et al. [13] demonstrated that fenofibrate significantly ameliorated retinal vascular leakage and leukostasis in type 1 murine diabetic models, as well as retinal neovascularization in the oxygen-induced retinopathy (OIR) rat model.

Diabetic retinopathy (DR) is considered a microcirculatory disease of the retina. However, increasing evidence suggests that retinal neurodegeneration is an early event in the pathogenesis of DR, which participates in microcirculatory abnormalities [14–16]. In fact, the main features of retinal degeneration (apoptosis and glial activation) have been found in the retinas of human diabetic donors without any microcirculatory abnormalities appearing in ophthalmoscopic examinations performed during the year before death [17–19]. Glutamate accumulation in extracellular space and the overactivation of ionotropic glutamate receptors ("excitotoxicity") play an important role in retinal neurodegeneration. Glutamate transporters are essential for terminating synaptic transmission as well as for keeping the extracellular glutamate concentration below neurotoxic levels [20]. The transport of glutamate from the extracellular into Müller cells, which is largely mediated by the glutamate–aspartate transporter (GLAST), is impaired in diabetes, thus favoring "excitotoxicity" [21].

In recent years, PPARα activation has been shown to have a neuroprotective effect in experimental models of cerebral ischemia and neurodegenerative diseases [22]. However, the neuroprotective effect of fenofibrate in the setting of DR has never been evaluated.

On this basis, the aim of the present study was to evaluate the potential direct retinal effect of FA in preventing retinal neurodegeneration in the db/db mouse, an experimental model of type 2 diabetes. For this reason, a short-term treatment with FA was intentionally planned. By selecting a longer period of treatment, it could be very difficult to know whether the neuroprotective effect was a direct action of FA or it was mediated by its systemic lipid-lowering effect. Finally, the possibility that FA acts through increasing glutamate clearance by GLAST thus preventing glutamate accumulation was also explored.

Materials and methods

Animals

To test the effect of FA, the db/db mouse was used. This mouse carries a mutation in the leptin receptor gene and is

a model for obesity-induced type 2 diabetes. A total of 24 male db/db (BKS.Cg- + *Lepr* ^{db}/+ *Lepr* ^{db}/OlaHsd) mice aged 8 weeks were purchased from Harlan Laboratories, Inc. In addition, ten non-diabetic (db/+) mice matched by age were used as control group.

The mice were housed under controlled conditions of temperature (20 °C) and humidity (60 %) with a 12-h light/dark cycle and free access to food and water. Mice were euthanized by cervical dislocation, and the eyes were immediately enucleated. The neuroretina from one of the eyes was extracted and frozen in liquid nitrogen and stored at −80 °C for protein assessments. The other eye was flash frozen in Tissue Freezing Medium (TFM^{TM}, Electron Microscopy Sciences), by immersion in liquid nitrogen, and cryosectioned at 8 μm through the dorsal/ventral plane. Sections were mounted on slides and stored at −80 °C.

Interventional study

The diabetic mice were randomly assigned to daily oral treatment (by gavage) with FA (100 mg/Kg/day) ($n = 12$) or vehicle ($n = 12$) for 1 week. The FA was dissolved in 0.2 % carboxymethylcellulose sodium salt (CMC) + 0.4 % Tween 80)/saline solution. Blood glucose (Optium. Abbot, Berkshire, UK) and triglycerides (Accutrend Plus. Roche, Vienna, Austria) levels were measured at baseline and at the end of the study. After treatment, the animals were euthanized and the eyes enucleated.

Retinal neurodegeneration measurements

Microscopic evaluation of retinas included scanning tissue sections to evaluate morphology followed by systematic morphometric analysis. The sections were stained with hematoxylin and eosin (H&E). Images of H&E sections were captured with a microscope (Olympus, Lake Success, NY) using the program ImageJ for quantification. The measurements were taken at two peripheral and three central regions of the retina and were examined to ensure similar locations of measurements for all eyes. Sections through the posterior eye segment were defined as central retina when the plane passed through the optic nerve or at <300 μm from the optic head rim. The remaining sections from both sites of the optic nerve were considered as peripheral retina. Image analyses of ten sections of each region were used to quantify cell number per mm^2 in ganglion cell layer (GCL).

Measurements of glial activation

Glial fibrillar acidic protein (GFAP) immunohistochemistry Glial activation was evaluated by fluorescence microscopy using specific antibodies against glial fibrillar acidic protein (GFAP). Sections were fixed in acid methanol (−20 °C) for 2 min, followed by three washes with PBS, 5 min each. Sections were permeabilized with TBS-Triton X-100 0.025 % and were incubated in blocker (1 % BSA, and 10 % goat serum in PBS) for 2 h at room temperature. Sections were then incubated with rabbit anti-GFAP (Abcam Ltd, Cambridge, UK) (1:500 dilution prepared in blocking solution) overnight at 4 °C in a humid atmosphere. After three washes in PBS, 5 min each, the sections were incubated with secondary antibody Alexa 488 goat-anti-rabbit (Invitrogen) (1:200 dilution prepared in blocking solution). The sections were washed three times in PBS, counterstained with Hoesch, and mounted with Mounting Medium Fluorescence (Prolong, Invitrogen) and mounted with a coverslip. Comparative digital images from diabetic and control samples were recorded with an Olympus microscope using identical brightness and contrast settings.

To evaluate the degree of glial activation, we used a scoring system based on the extent of GFAP staining previously described [23]. This 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 outer nuclear layer (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).

Western blot analysis of GFAP Neuroretinas were extracted in 50 μL of lysis buffer (RIPA buffer: PMSF, 1 mmol/L; Na3VO4, 2 mmol/L; NaF, 100 mmol/L; and containing 1× protease inhibitor cocktail (Sigma, St Louis, MO). A total of 20 μg protein was resolved by 10 % (vol./vol.) SDS–PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blots were probed with rabbit anti-GFAP (Abcam Ltd, Cambridge, UK) diluted 1:10,000. The same blot was stripped and reblotted with a rabbit primary antibody specific to CypA (Enzo Life Sciences Inc., Framingdale, NY, USA) to normalize protein levels. Densitometric analysis of the autoradiographs was performed with ImageJ software. Results are presented as densitometry arbitrary units.

Immunohistochemical analysis for apoptosis assessment

The Terminal Transferase dUTP Nick-End Labeling (TUNEL) staining was carried out using the DeadEnd Fluorometric TUNEL System kit (PROMEGA, Madison, WI, USA). Cryosections of retina were permeabilized by incubation for 2 min on ice with 0.1 % Triton X-100 in 0.1 % sodium citrate, freshly prepared. The secondary antibody was Alexa 594 goat-anti-rabbit (Invitrogen, San

Diego CA, USA). For evaluation by fluorescence microscopy, the excitation wavelength was 488 nm and detection in the range of 515–565 nm (green) was used.

Immunohistochemistry for GLAST

Sections of 4 μm were deparaffined in xylene and hydrated in a graded ethanol series. Then, sections were antigen retrieval (sodium citrate 10 mM, pH 6.0) and washed in PBS. Next, sections were incubated in blocking solution (2 % BSA, Tween 0.05 % PBS) for 1 h at room temperature followed by incubation with primary antibody anti-GLAST (1:200, Abcam, Cambridge, UK). After washing, sections were incubated with a fluorescent anti-rabbit Alexa 488 as a secondary antibody (Life Technologies S.A., Madrid, Spain) in blocking solution for 1 h, washed, and mounted in Vectashield (Vector Labs, Vector Laboratories; Burlingame, CA, USA). Hoescht was used for nuclear staining. Quantification of fluorescence intensity of images was performed as previously explained for GFAP.

Electroretinography

Before retinal electrophysiological tests, the animals were dark-adapted (12 h overnight) and anesthetized under a dim red light. Mice were anesthetized using a gas chamber with 2 % isoflurane/O2 mixture and maintained with the same 2 % isoflurane/O2 mixture under mice nasal gas masks. Pupils were dilated with topic 1 % tropicamide and ciclopegic was applied on the corneal surface. Full-field electroretinography (ERG) recordings were measured using an HMsERG (Ocuscience, MI, USA) with two recording channels. Scotopic ERG stimuli were simultaneously recorded from both eyes of dark-adapted (12–16 h) mice. The amplitude and implicit time of oscillatory potentials (OPs) and b-wave were measured as recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV) [24]. We added up OP amplitudes (ΣOP amplitude) and implicit time (ΣOP implicit time) for the first 5 OPs. The ERG recordings were performed at baseline and after a week of either FA or vehicle administration.

Statistical analysis

Data are presented as mean ± SD. Comparisons of continuous variables were performed using the paired and unpaired Student's *t* test. Levels of statistical significance were set at $p < 0.05$.

Results

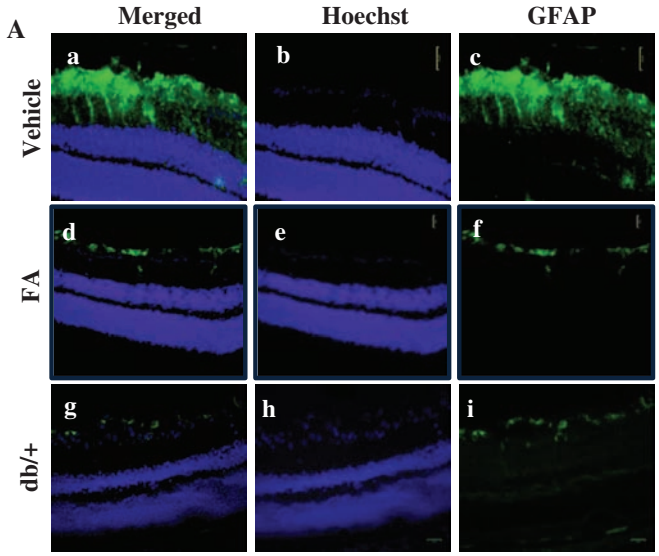
Blood triglycerides levels at the end of the study were lower in the db/db group treated with FA in comparison with the db/db group treated with vehicle but did not reach statistical significance (144 ± 15 mg/dL vs. 210 ± 11 mg/dL; $p = 0.06$). As expected, blood glucose concentration at the end of treatment was similar in db/db mice treated with FA than in db/db mice treated with vehicle (407 ± 36 mg/dL vs. 432 ± 41 mg/dL; $p =$ n.s).

Morphological features of retinal neurodegeneration

As expected, in non-diabetic mice, GFAP expression was confined to the retinal ganglion cell layer (GCL), and therefore, the GFAP score was ≤2 (Fig. 1a, b). The diabetic mice treated with vehicle presented significant higher GFAP expression than non-diabetic mice matched by age (9 week olds). Thus, 84 % of diabetic mice presented a GFAP score ≥3. FA administration for one week resulted in a significant decrease in reactive gliosis, and the GFAP score of the mice treated with FA was <3 in all cases (Fig. 1b). Consistently, with the immunochemistry data, the Western blot analyses showed a higher GFAP content (densitometric arbitrary units) in the retinas from diabetic mice treated with vehicle in comparison with the retinas from diabetic mice treated with FA (0.68 ± 0.42 vs. 0.18 ± 0.29; $p < 0.05$) (Fig. 1c, d).

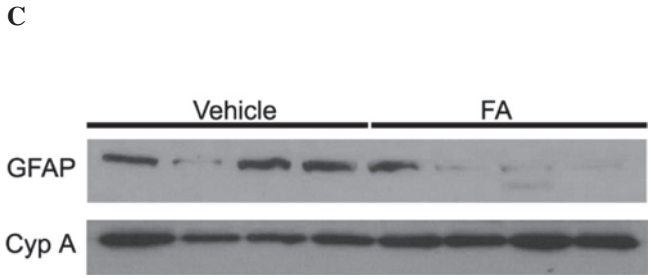
The apoptosis rate in the GCL was significantly higher in diabetic mice treated with vehicle than in non-diabetic mice (46.2 % ± 8.2 vs. 0.5 % ± 0.1; $p < 0.05$) (Fig. 2a, b). FA administration for one week resulted in a significant prevention of GCL apoptosis. Thus, the rate of apoptosis was significantly lower in diabetic mice treated with FA than in mice treated with vehicle (17.4 % ± 4.4 vs. 46.2 % ± 8.2; $p < 0.05$) (Fig. 2a, b). Furthermore, the reduction in the number of cells in the GCL in db/db mice treated with vehicle in comparison with non-diabetic mice (9.69 ± 0.26 cells/100 μm vs. 14.47 ± 0.47 cells/100 μm; $p < 0.05$) was prevented in db/db mice treated with FA (db/db-FA: 13.40 ± 0.28 cells/100 μm vs. db/db-vehicle: 9.69 ± 0.26 cells/100 μm; $p < 0.05$) (Fig. 3a, b).

Finally, GLAST immunofluorescence (Fig. 4a) was downregulated in the retinas of diabetic mice treated with vehicle in comparison with non-diabetic mice (11.1 ± 2.2 arbitrary units/area vs. 35.3 ± 1.7 arbitrary units/area; $p < 0.05$). In diabetic mice treated with FA, this downregulation of GLAST induced by diabetes was prevented (db/db-FA: 47.0 ± 3.5 arbitrary units/area vs. non-diabetic mice: 35.3 ± 1.7 arbitrary units/area; $p =$ n.s) (Fig. 4b).



B

score	% positive GFAP labeling		
	db/db vehicle	db/db FA	db/+
1	0	67	89
2	16	33	11
3	50	0	0
4	17	0	0
5	17	0	0



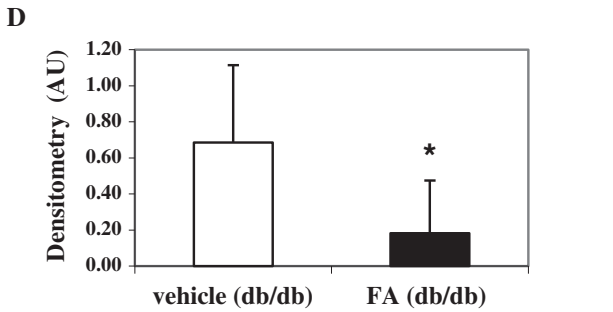


Fig. 1 **a** GFAP immunoreactivity (*green*) in the central retina in representative samples from a diabetic mouse (db/db) treated with vehicle (*upper panels*), a diabetic mouse (db/db) treated with FA (*middle panels*) and a non-diabetic mouse (db/+) (*lower panels*). **b** Quantification of glial activation based on the extent of GFAP staining. **c** Western blotting of GFAP in diabetic mouse (db/db) treated with vehicle or FA. **d** Autoradiograms were quantified by scanning densitometry. Results are expressed as arbitrary units (AU)

Electroretinographic evaluation

After one week of follow-up, FA treatment prevents the decrease in oscillatory potential amplitude observed in diabetic mice treated with vehicle (Fig. 5a). The b-wave implicit time tends to increase in diabetic mice receiving vehicle (Fig. 5b). By contrast, in diabetic mice treated with FA, a significant reduction in b-wave implicit time was observed (Fig. 5c).

Discussion

In the present study, we provide first evidence that FA exerts neuroprotection in an experimental model of DR. Therefore, this effect could be added to the mechanisms by which fenofibrate exerts its beneficial actions in DR.

In recent years, the C57BL/KsJ-*db/db* mouse has been used as a spontaneous diabetic model of type 2 diabetes to investigate the pathogenesis of DR [25–27]. The C57BL/KsJ-*db/db* mouse carries a mutation in the leptin receptor gene and is a model for obesity-induced type 2 diabetes. It develops hyperglycemia as a result of excessive food consumption. Several authors have reported the presence of retinal neurodegeneration (apoptosis, glial activation, and retinal thinning) in this model [25, 26]. We have previously reported that the sequence of retinal neurodegenerative features observed in the db/db mouse is similar to that described in the early stages of DR in diabetic patients [28]. Therefore, it seems an appropriate model for testing the effect of FA on the retinal neurodegeneration that occurs in the diabetic eye. In the present study, we confirm that histological hallmarks of retinal neurodegeneration (apoptosis of ganglion cells and glial activation) as well functional abnormalities appear very early after diabetes onset. In addition, as recently reported in type 1 diabetic models (streptozotocin-induced diabetic rats) [29, 30], we found that diabetes induces a downregulation of GLAST in db/db mice.

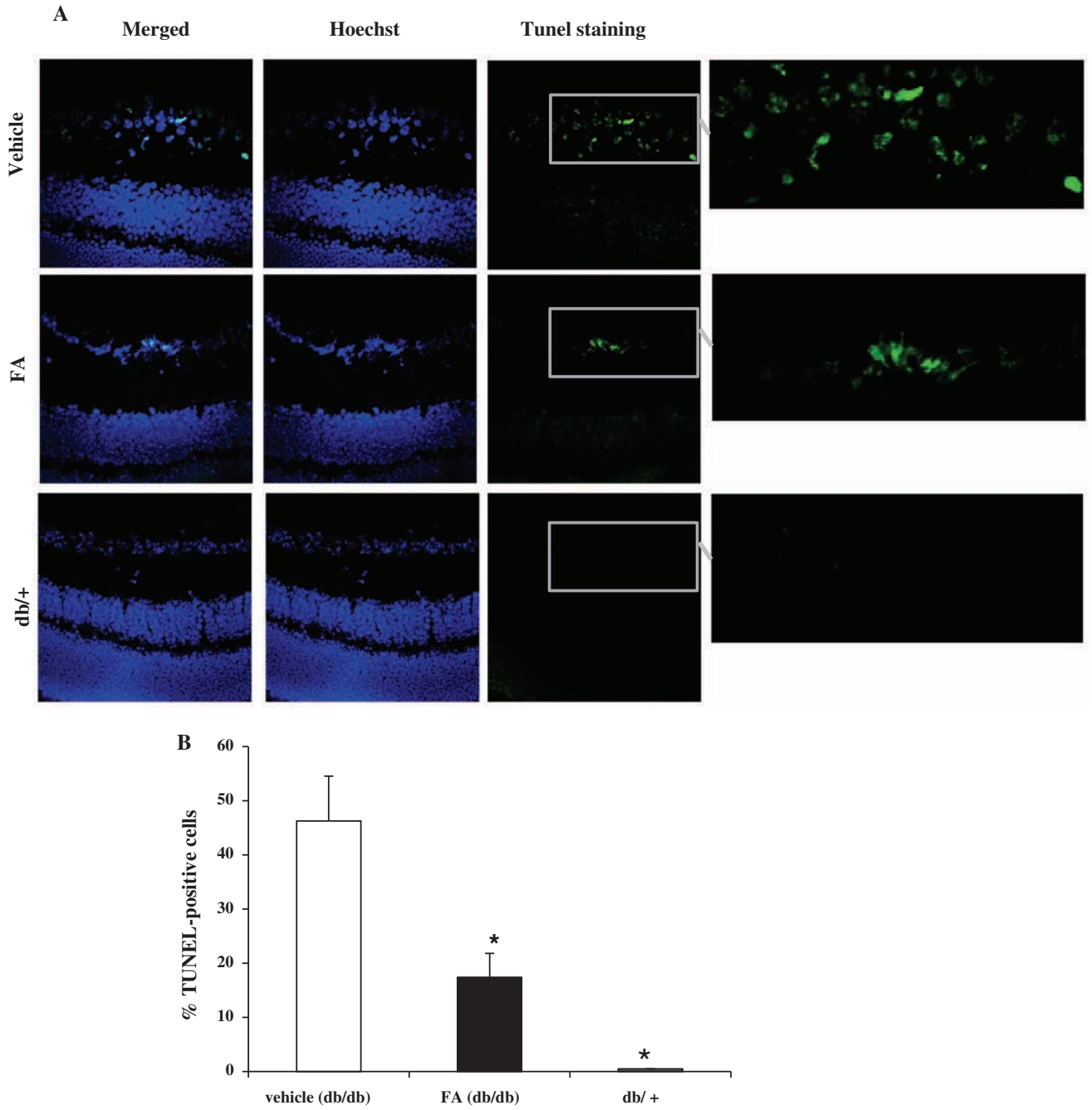


Fig. 2 **a** TUNEL immunofluorescence (*green*) from a db/db mouse treated with vehicle (*upper panels*); a db/db treated with FA (*middle panels*) and a non-diabetic mouse (db/+) (*lower panels*). **b** Percentage of TUNEL-positive cells in the GCL in db/db mice treated with vehicle (*white bars*; $n = 12$), db/db mice treated with FA (*black bars*; $n = 12$) and db/+ mice (*gray bars*; $n = 10$). Data are expressed as mean ± SD. $*p < 0.05$ in comparison with db/db mice treated with vehicle

In the present study, we demonstrated that a short-term administration of FA significantly abrogates reactive gliosis and apoptosis, as well as ERG abnormalities, in diabetic mice. Notably, after one week of FA administration, reactive gliosis was very similar to that observed in non-diabetic mice and the rate of apoptosis in GCL was strongly reduced.

Although the analysis of the mechanisms underlying the neuroprotection conferred by FA was beyond the scope of this study, our results suggest that FA could prevent glutamate accumulation by abrogating the downregulation of GLAST induced by diabetes. The excitotoxicity induced by glutamate accumulation plays an essential role in neurodegeneration. In fact, elevated

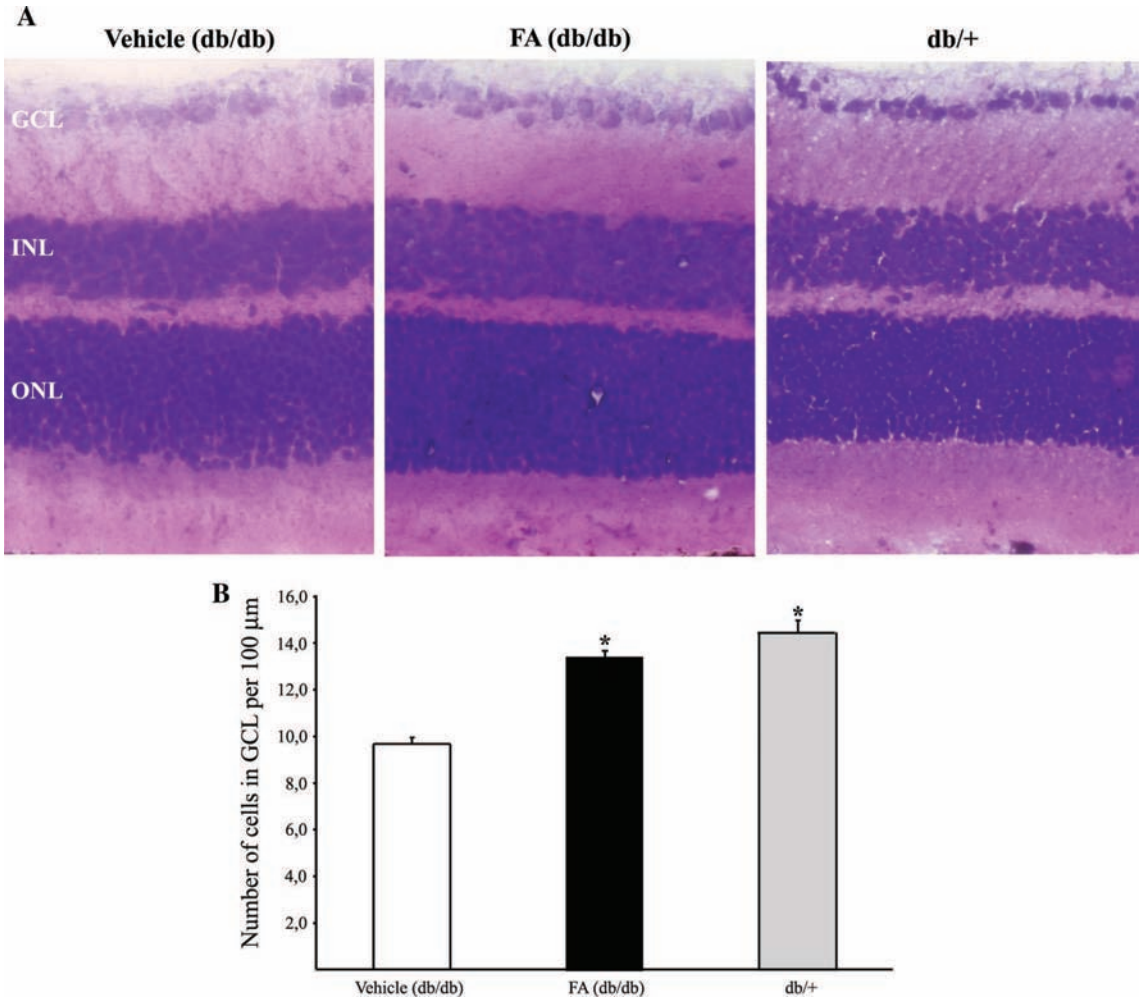


Fig. 3 **a** Hematoxylin and eosin-stained central retina in a representative case of a db/db mouse treated with vehicle, a db/db mouse treated with FA, and a non-diabetic mouse (db/+). **b** Cell number in GCL in diabetic mice treated with vehicle (*white bar*), diabetic mice treated with FA (*black bar*), and non-diabetic mice (*gray bar*). *GCL* ganglion cell layer, *INL* inner nuclear layer, *ONL* outer nuclear layer. Data are expressed as mean ± SD. *$p < 0.05$ in comparison with db/db mice treated with vehicle

levels of glutamate in the retina have been found in experimental models of diabetes [31–33], as well as in the vitreous fluid of diabetic patients [34, 35]. Glutamate transporters are essential for keeping the extracellular glutamate concentration below neurotoxic levels [20]. In this regard, the glial glutamate/aspartate transporter (GLAST), the main glutamate transporter expressed by Müller cells [36], is the most dominant glutamate transporter, accounting for at least 50 % of glutamate uptake in the mammalian retina [37]. Recently, it has been reported that the neuroprotective effect of somatostatin and glial cell line-derived neurotrophic factor (GDNF) in diabetic rats (streptozotocin-induced diabetes) was related to upregulation of GLAST [29, 30]. Our results suggest that this mechanism also participates in the neuroprotective effect of fenofibrate.

In the experimental models of cerebral ischemia and neurodegenerative diseases, PPARα activation had a neuroprotective effect, independent of lipid metabolism [19]. Antioxidant, anti-inflammatory, and anti-apoptotic properties of fenofibrate have been implicated in this effect [38]. In addition, it has been shown that fenofibrate reduces lipoprotein-associated phospholipase A2 (Lp-PLA2). Lp-PLA2 has a prominent pro-inflammatory effect and, as occurs with glutamate, induces cell death in the brain, and consequently, similar effects might also occur in the retina [39]. Therefore, the capacity of fenofibrate to lower PLA2 may also be involved in its eventual neuroprotective effect at retinal level. Specific studies to confirm this hypothesis are needed.

The FIELD [5] and ACCORD Eye [6] studies have shown that the greatest benefit with fenofibrate was

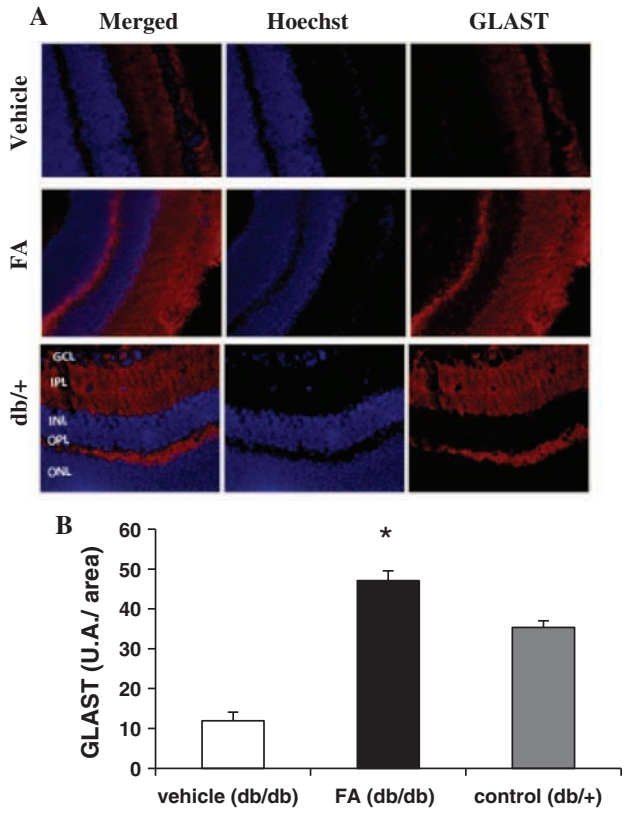


Fig. 4 **a** GLAST immunofluorescence (*red*) from a db/db mouse treated with vehicle (*upper panels*); a db/db treated with FA (*middle panels*) and a non-diabetic mouse (db/+) (*lower panels*). **b** Quantification of GLAST immunofluorescence in db/db mice treated with vehicle (*white bars*; $n = 12$), db/db mice treated with FA (*black bars*; $n = 12$) and db/+ mice (*gray bars*; $n = 10$). Data are expressed as mean ± SD. $^{*}p < 0.05$ in comparison with db/db mice treated with vehicle

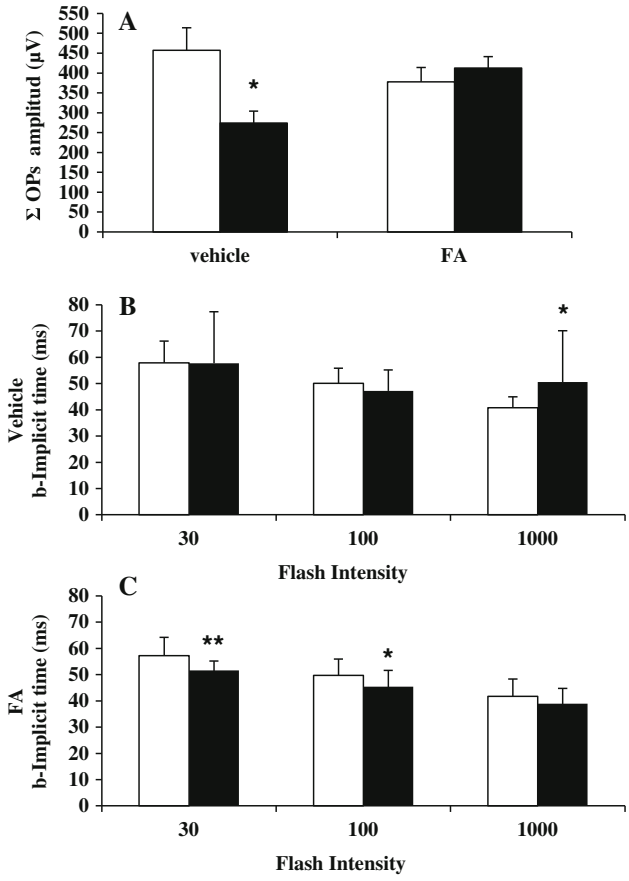


Fig. 5 **a** ΣOPs amplitude (μV) in db/db mice treated with vehicle or fenofibrate at baseline (*white bars*) and after 1 week of treatment (*black bars*). **b** b-wave implicit time in db/db mice treated with vehicle and **c** b-wave amplitude in db/db mice treated with FA at baseline (*white bars*) and after 1 week of treatment (*black bars*). Flash intensity units are mcd s/m^{-2}. Data are expressed as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$ between baseline and after treatment (FA or vehicle)

observed in those patients with preexisting DR. However, our results suggest that fenofibrate could be useful for treating neurodegeneration and, therefore, could be recommended even before microangiopathic abnormalities appear in the fundoscopic examination. However, a clinical trial specifically addressed to prove this concept is needed.

In conclusion, FA has a neuroprotective action in db/db mice, and this effect occurs very shortly after its administration. The eventual reduction of glutamate excitotoxicity by increasing glutamate uptake through upregulating GLAST could be one of the mechanisms involved in the neuroprotective effect of FA. Specific experimental studies addressed to unravelling the underlying mechanisms and a pilot clinical trial addressed to confirming fenofibrate as a neuroprotective agent seem warranted.

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Conflict of interest None.

Human and animal rights This article does not contain any studies with human subjects performed by any of the authors. All the animal experiments were performed in accordance with the protocol approved by the Animal Care and Use Committee of Vall d'Hebron

Research Institute (VHIR) and Association for Research in Vision and Ophthalmology (ARVO).

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CHAPTER III

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

To determine the expression of GLP-1 and GLP-1R in human retinas, 8 retinas from diabetic donors and 8 non-diabetic donors, matched by age and gender, were obtained from Tissue Bank of our centre. GLP-1 was expressed in human retinas, mainly in GCL. Interestingly, we found lower levels of mRNA and protein content in the diabetic retinas in comparison with non-diabetic controls. GLP-1R was expressed in human retina at levels even higher than those observed in human bowel and liver. Nevertheless, no significant differences were found between diabetic and non-diabetic donors. Both GLP-1 and its receptor are expressed mainly in the GCL. Moreover, we determined that GLP-1R is also expressed in the retina of db/db mice as well as in non-diabetic mice (db/+), and its abundance is independent of the presence of diabetes.

To determine whether systemic administration of GLP-1R agonist (liraglutide 400 µg/kg/day) had neuroprotective effects *per se* and not for its lowering glucose levels action a group of 8 weeks old db/db mice were fed with restrictive diet (restricted to 60% of total daily calories) in order to lower weight and blood glucose levels. In that way, we could have a control group for systemic treatment with liraglutide. Treatments lasted for 15 days. In comparison with the placebo group, the 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. Vehicle-treated mice presented high levels of glial activation, they had prominent expression of GFAP through the entire retina. In contrast, mice treated with liraglutide presented significantly a lower GFAP immunofluorescence score than the vehicle group and similar to non-diabetic mice. Interestingly, the prevention of glial activation with liraglutide tended to be higher than the obtained with restrictive diet, even though liraglutide presented a lower reduction in blood glucose levels. Liraglutide treated mice presented lower levels of apoptosis than vehicle treated mice. No differences in the percentage of apoptotic cell were observed between diabetic mice treated with liraglutide in comparison with those treated with restrictive diet.

Although systemic treatment with GLP-1R agonist is useful preventing neurodegeneration, it is very difficult to know whether the beneficial effect is due to GLP-1R activation or the result of its hypoglycaemic action. Since eye drop administration is unlikely to reduce blood glucose levels and is a non-invasive technique of administration four GLP-1R agonists were tested in that way. We observed that liraglutide administered by eye drops was able to reach the retina. We found a significant increase of GLP-1 immunofluorescence in the retina two hours after topical administration. Although liraglutide topically administered was not able to lower blood glucose levels, it prevented the morphological and functional neurodegenerative abnormalities caused

by diabetes (apoptosis and glial activation) to the same level than systemic treatment. All GLP-1R agonists topically administered (native GLP-1, exenatide and lixisenatide) were as effective as liraglutide in preventing glial activation and apoptosis.

Treatment with liraglutide, systemic or topically administered, prevented ERG abnormalities induced by diabetes. Treatment resulted in prevention of the decrease in amplitude of a-wave and b-wave and OPs observed in diabetic mice treated with vehicle.

A dose-dependent absorption and dose-efficacy study of topical administration of liraglutide was performed. We observed an accumulation of liraglutide in the retina and that it was dependent on the dose administered. Also it was observed that eye drops at concentrations of 2 μg/μl (200 μg/kg/day) had slightly lower effectiveness in preventing glial activation and apoptosis than at 4 μg/μl (400 μg/kg/day), and that the dose of 0.40 μg/μl (40 μg/kg/day) was significantly less effective than 2μg/μl (200 μg/kg/day).

To demonstrate the specific response of GLP-1R activation, cAMP was measured as it is the result of increased activity of adenylyl cyclase, and secondary messenger of GLP-1R. An increase in cAMP content was found in liraglutide treated mice in comparison to vehicle. Moreover, pAKT/AKT ratio was also increased in liraglutide treated mice demonstrating an increase in phosphatidylinositol 3-kinase downstream pathway.

The effect of liraglutide on retinal glutamate and GLAST content was analysed. Liraglutide was able to prevent the increase of glutamate levels induced by diabetes and no differences in glutamate content were found between systemic or topically administered treatment. GLAST content was downregulated in retinas from diabetic mice treated with vehicle. In liraglutide treated mice, by the two routes of administration, there was prevention in GLAST downregulation.

Both liraglutide treatments 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.

In order to assess the effect of GLP-1R agonists on early microvascular impairment we examined the albumin leakage, as well as VEGF and IL-1β because are two of the most important players in the BRB breakdown. Overexpression of VEGF and IL-1β was observed in diabetic mice and it was associated with albumin leakage, showing that disruption of BRB was present. These abnormalities were prevented by topical administration of eye drops containing liraglutide and also when the administration was systemic.

This work concludes that GLP-1R agonists by means of a significant reduction of extracellular glutamate and an increase of prosurvival signalling are useful for preventing retinal neurodegeneration. This drug could be regarded as a useful tool for the treatment of early stages of DR.

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

Short running title: GLP-1 and retinal neurodegeneration

Cristina Hernández* [1,2], Patricia Bogdanov* [1,2], Lidia Corraliza [1,2], Marta García-Ramírez[1,2], Cristina Solà-Adell[1,2], José A. Arranz [3], Ana I. Arroba [2,4], Angela M.Valverde [2,4], Rafael Simó[1,2]

* These authors contributed equally

[1]Diabetes and Metabolism Research Unit. Vall d'Hebron Research Institute. Universitat Autònoma de Barcelona, Spain. [2]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), ISCIII, Spain. [3]Unidad de Metabolopatías. Laboratorios Clínicos. Hospital Universitario Vall d'Hebron, Barcelona, Spain. [4]Instituto de Investigaciones Biomédicas Alberto Sols (CSIC/UAM), Madrid, Spain.

Corresponding author:

Cristina Hernández, MD, PhD

Diabetes and Metabolism Research Unit. Vall d'Hebron Research Institute. Universitat Autònoma de Barcelona.

Pg. Vall d'Hebron 119-129, 08035 Barcelona, Spain.

e-mail: cristina.hernandez@vhir.org

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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 electrophysological 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 exicitotoxicity 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 –80ºC. 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′-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 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 protassium 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 –80ºC for protein assessments. The other eye was fixed in 4% paraformaldehyde within approximately 6 hours after enucleation. Immunohistochemical studies were done on paraffined 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 (Glial 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 Hoestchst 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 reblotted 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 immunoflurescence between subcuteneous vs. topical liraglutide administration.

Effect of GLP-1R agonist on proapoptotic/survvival 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.

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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). Apotosis: 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-

way ANOVA and Bonferroni multiple comparison test were used. * $p<0.05$ in comparison with the other groups.

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. Apotosis: 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.

FigureS5. A) ERG traces in response to stimulus intensity of 40 candela (cd)s/m2 in a representative non-diabetic mouse (dark blue), a db/db mouse treated with vehicle (red), a db/db treated with liraglutide by subcutaneous route (grey) and a db/db mouse treated by topical ocular route (green). 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 implicite time. We added up OP implicit time (ΣOP implicit time) for the first 5 OPs. White columns: baseline. Black columns: at day 14. n = 10 mice per group. Results are mean ± SD. Differences between final and baseline ERG parameters in each group were assessed by paired Student t test. * $p<0.05$.

FigureS6. 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).

FigureS7. A) Retinal concentration of glutamate measured by HPLC in the following groups: db/db mice treated with vehicle (black bars), db/db mice treated subcutaneously with liraglutide (chessboard bars), db/db mice treated with liraglutide eye drops (vertical stripes bars) and non-diabetic mice (white bars). B) Comparison of GLAST immunofluorescence

(red) between representative samples from a db/db mouse treated with vehicle, a db/db mouse treated with liraglutide sc, a db/db mouse treated with liraglutide eye drops and a non-diabetic mouse. Nuclei were labeled with Hoechst (blue). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. C) Quantification of GLAST 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. * $p<0.001$ in comparison with the other groups.

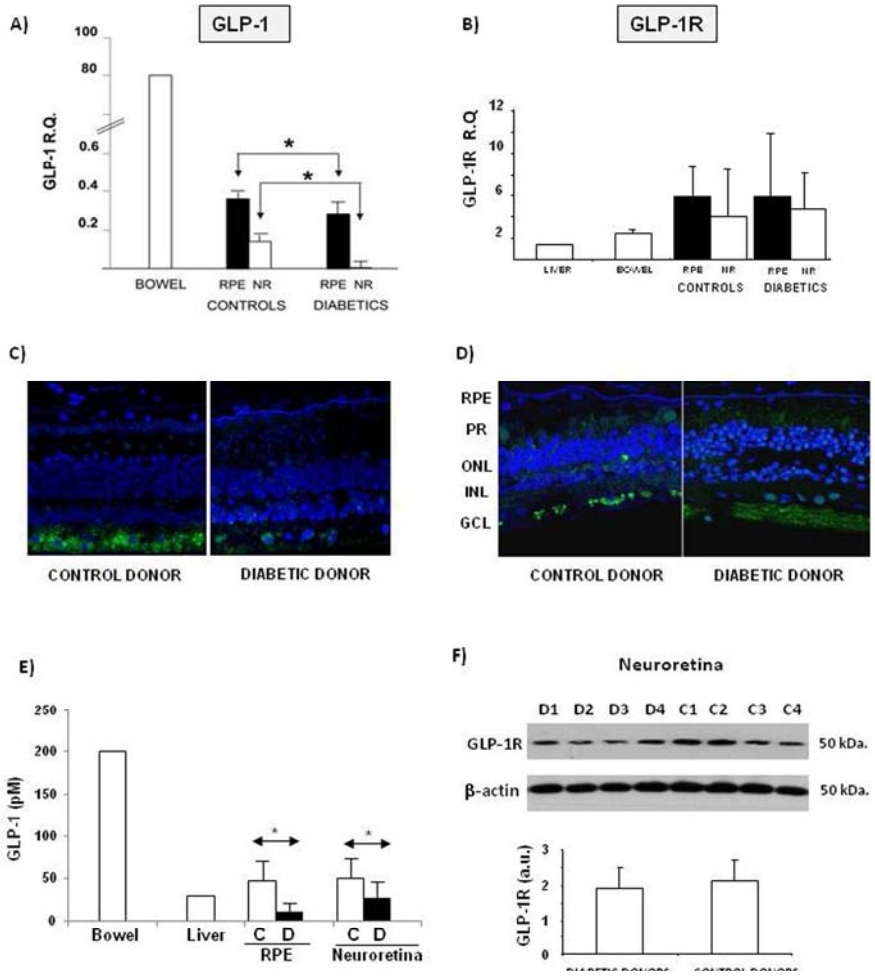
Figure 8. VEGF (red) (A), IL-1b (green) (B) and albumin (red) (C) immunofluorecence from a representative case of a diabetic mouse treated with vehicle (D-Sham) (left image), a diabetic mouse treated with Liraglutide (D-lira eye drop) (central image) and a non-diabetic mouse (control db/+) (right image).

FIGURESLEGENDSSSUPPLEMENTALSMATERIALS

FigureS1. A) Comparison of GLP-1R levels between 3 diabetic (db/db) and 3 non-diabetic mice (db/+) measured by Western blot. B) Autoradiograms were quantified by scanning densitometry. Results (mean ± SD) are expressed as arbitrary units of protein expression. n = 6 mice per group.

FigureS2. A) Evolution of body weight showing that only restrictive diet was able to avoid the progressive increase of weight. B) Blood glucose levels over time showing that only restrictive diet and liraglutide administered subcutaneously were able to reduce their levels. The effect of restrictive diet was more important than observed with subcutaneous liraglutide. It should be emphasized that topical administration of liraglutide was unable to reduce blood glucose levels. db/+ mice (white circles), db/db mice treated with vehicle (black squares), db/db treated with restrictive diet (black circles), db/db treated with subcutaneous liraglutide (white squares), and db/db mice treated with eye drops containing liraglutide (black triangles). n = 10 mice per group.

FigureS3. A) Western blot analysis of proteins from apoptotic (caspase 8, Bax, p53, BclxL) and neuroinflammatory (iNOS, FasL) pathways. B) Western blotting quantification. Data are densitometry units normalized to average controls (non-diabetic mice) protein expressions and are expressed as means ± SD. n = 6 mice per group. * $p<0.001$ in comparison with the diabetic group treated with vehicle (D-Sham). **S**



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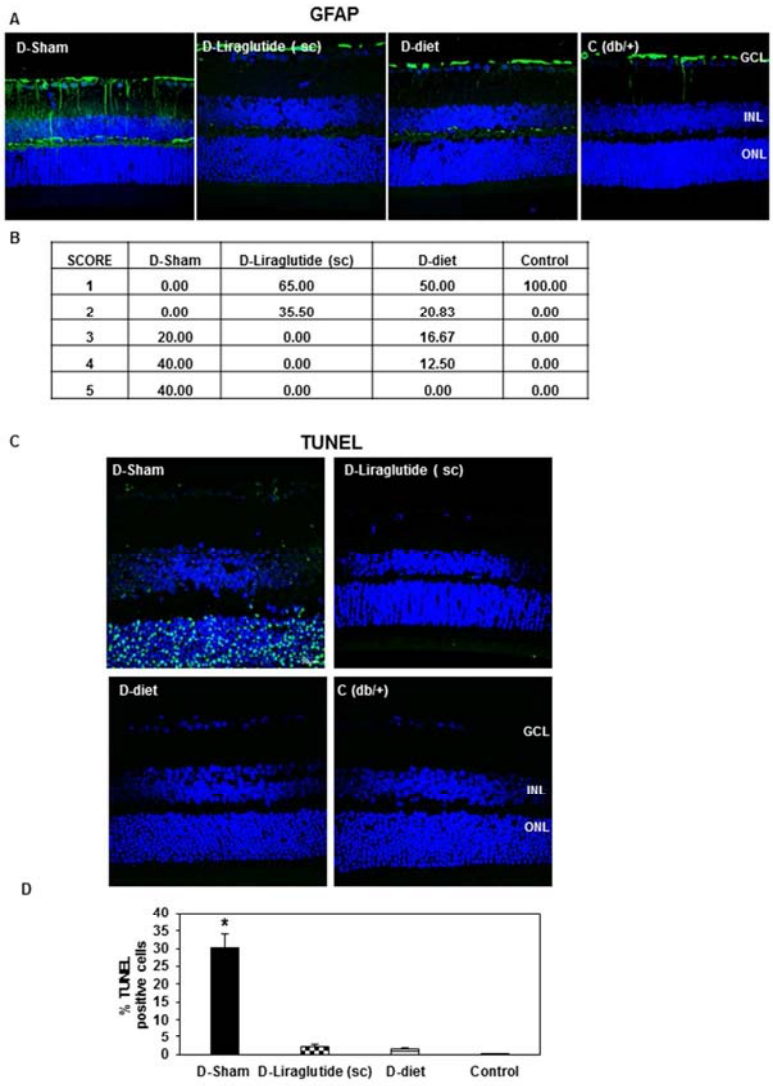

SCORE	D-Sham	D-Liraglutide (sc)	D-diet	Control
1	0.00	65.00	50.00	100.00
2	0.00	35.50	20.83	0.00
3	20.00	0.00	16.67	0.00
4	40.00	0.00	12.50	0.00
5	40.00	0.00	0.00	0.00

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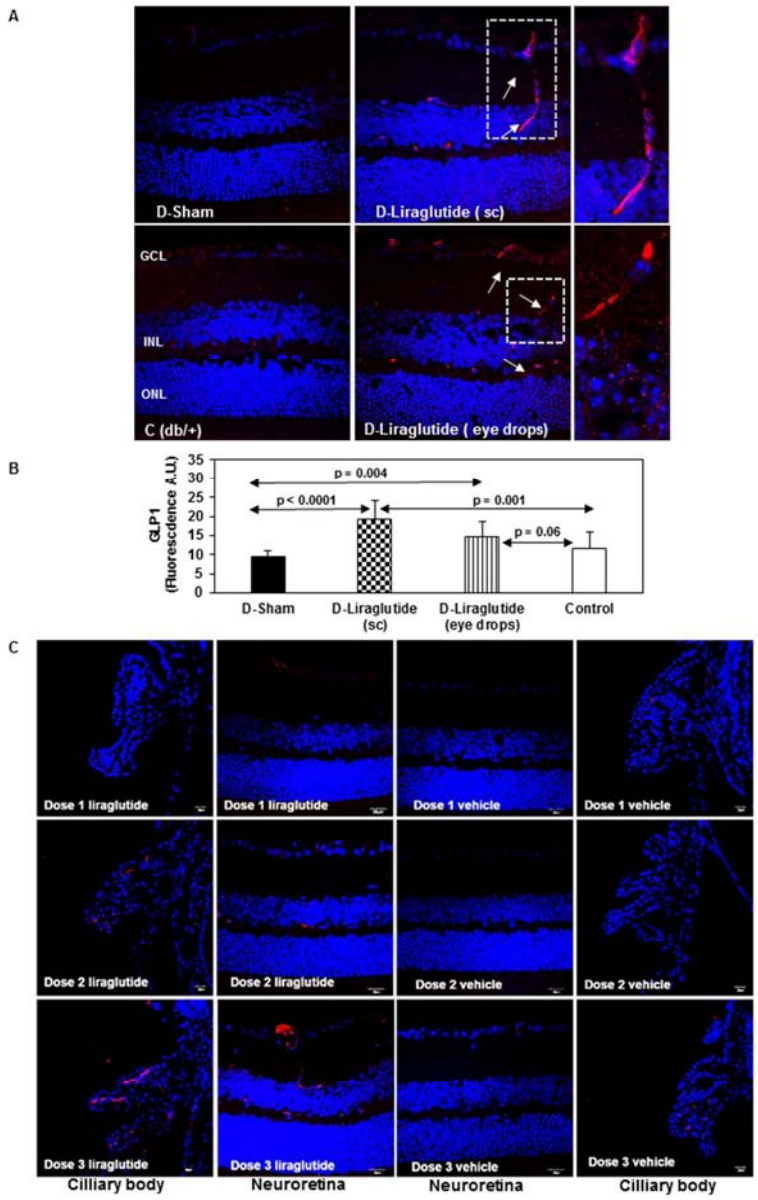

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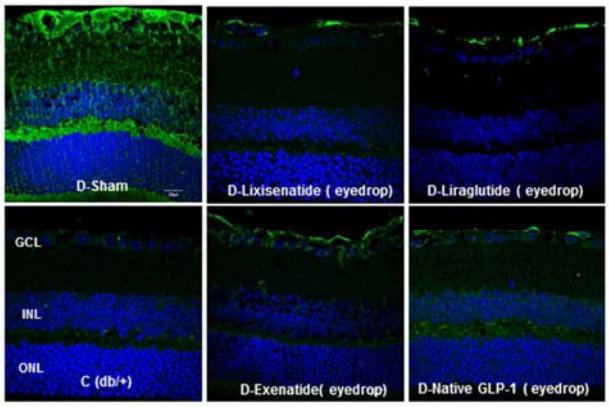
60x88mm (300 x 300 DPI)

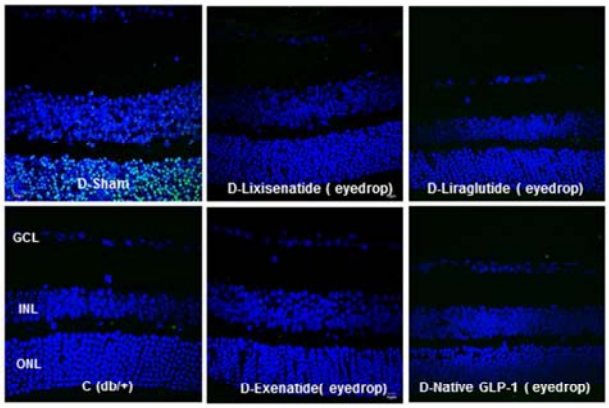
A



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, 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. Apotosis: 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.
60x88mm (300 x 300 DPI)

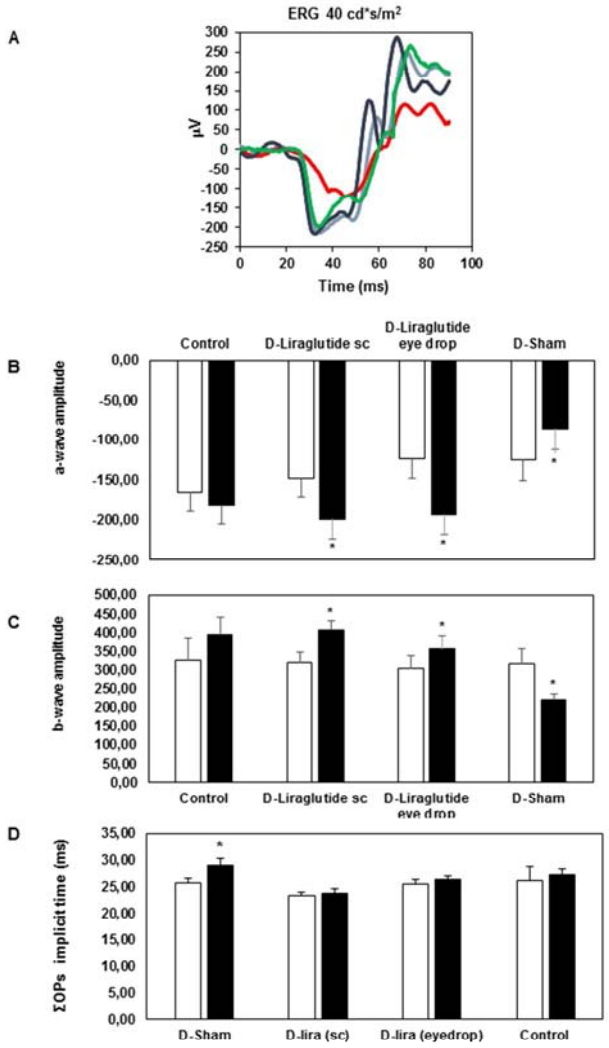


Figure 5. A) ERG traces in response to stimulus intensity of 40 candela (cd)s/m2 in a representative non-diabetic mouse (dark blue), a db/db mouse treated with vehicle (red), a db/db treated with liraglutide by subcutaneous route (grey) and a db/db mouse treated by topical ocular route (green). B) Prevention 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 implicite time. We added up OP implicit time (ΣOP implicit time) for the first 5 OPs. White columns: baseline. Black columns: at day 14. n = 10 mice per group. Results are mean ± SD. Differences between final and baseline ERG parameters in each group were assessed by paired Student t test. * p<0.05.
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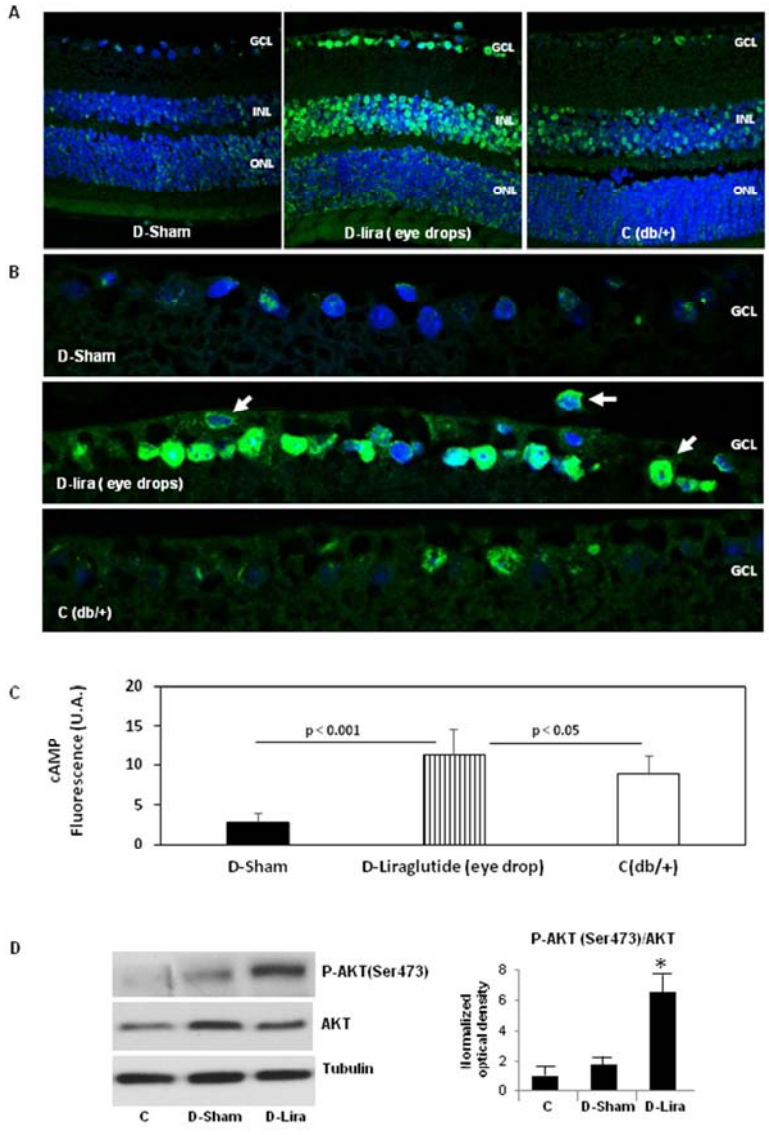

Figure 6. A) Comparison of cAMP immunofluorescence (green) between representative retinal samples from a db/db mouse treated with sham, a db/db mouse treated with liraglutide eye drops, and a non-diabetic mouse. Nuclei were labeled with Hoechst (blue). B) Detailed images of GCL showing the expression 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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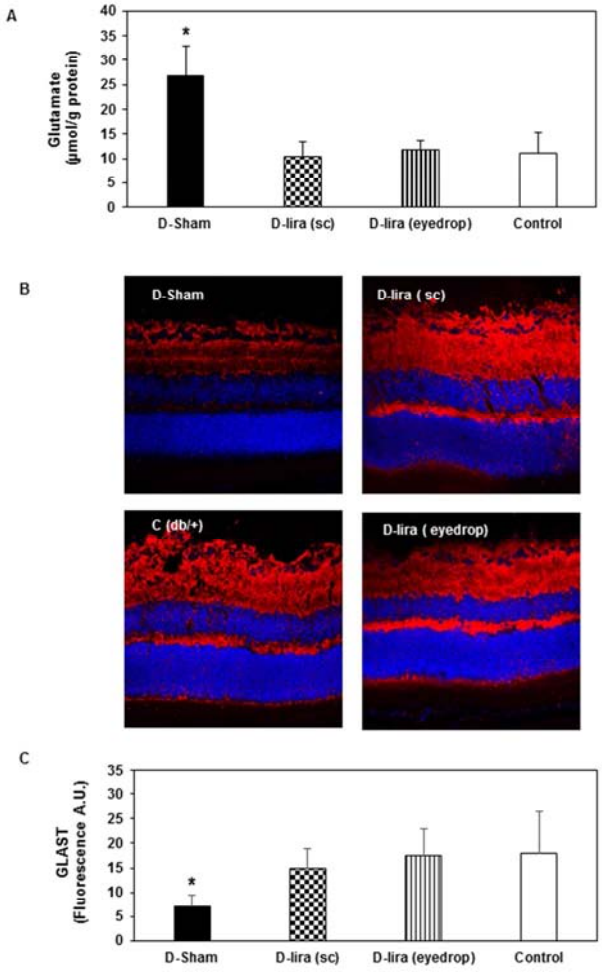

Figure 7. A) Retinal concentration of glutamate measured by HPLC in the following groups: db/db mice treated with vehicle (black bars), db/db mice treated subcutaneously with liraglutide (chessboard bars), db/db mice treated with liraglutide eye drops (vertical stripes bars) and non-diabetic mice (white bars). B) Comparison of GLAST immunofluorescence (red) between representative samples from a db/db mouse treated with vehicle, a db/db mouse treated with liraglutide sc, a db/db mouse treated with liraglutide eye drops and a non-diabetic mouse. Nuclei were labeled with Hoechst (blue). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. C) Quantification of GLAST 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. * $p<0.001$ in comparison with the other groups.
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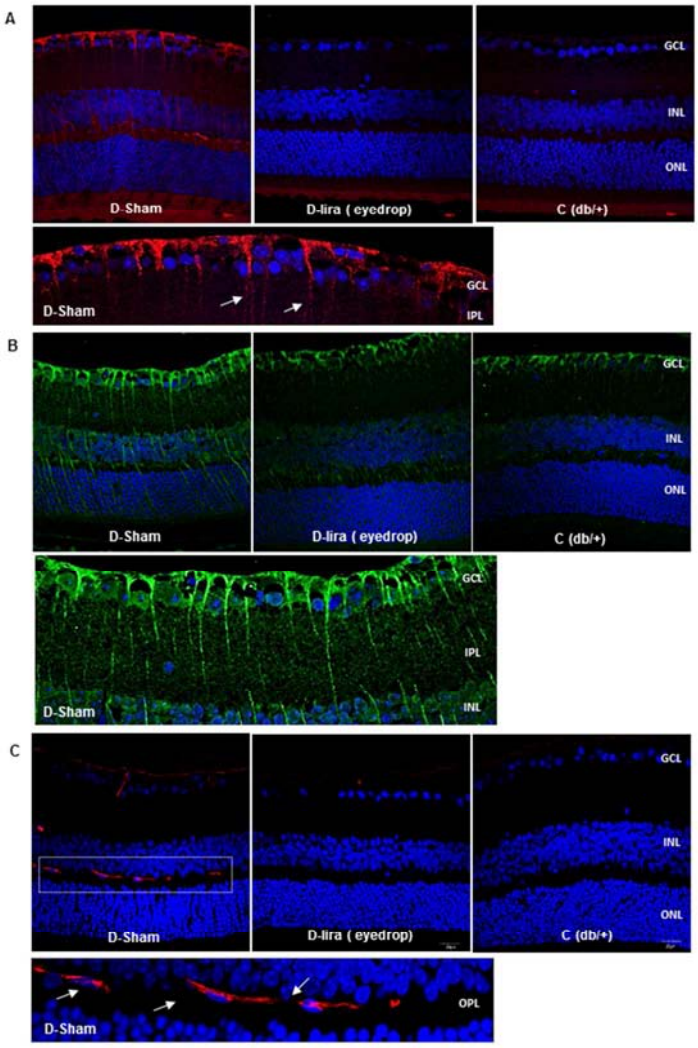

Figure 8. VEGF (red) (A), IL-1b (green) (B) and albumin (red) (C) immunofluorecence from a representative case of a diabetic mouse treated with vehicle (D-Sham) (left image), a diabetic mouse treated with Liraglutide (D-lira eye drop) (central image) and a non-diabetic mouse (control db/+) (right image).
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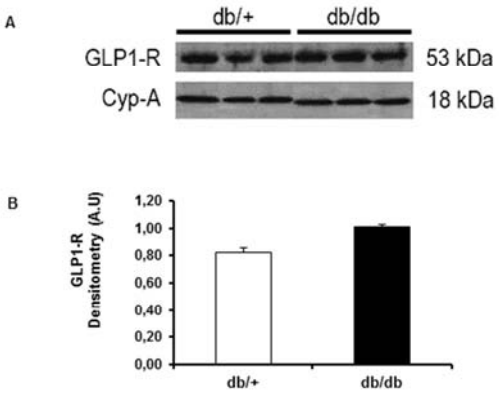

Figure 1. A) Comparison of GLP-1R levels between 3 diabetic (db/db) and 3 non-diabetic mice (db/+) measured by Western blot. B) Autoradiograms were quantified by scanning densitometry. Results (mean ± SD) are expressed as arbitrary units of protein expression. n = 6 mice per group.
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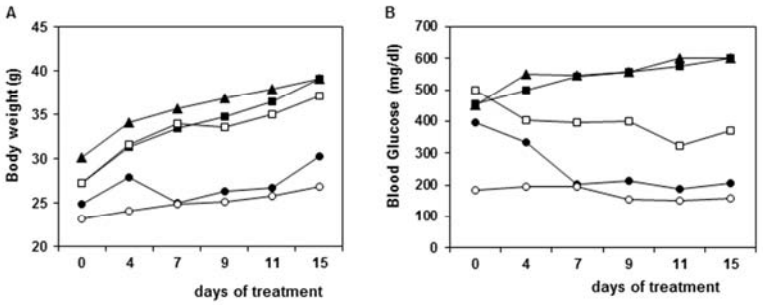

Figure 2. A) Evolution of body weight showing that only restrictive diet was able to avoid the progressive increase of weight. B) Blood glucose levels over time showing that only restrictive diet and liraglutide administered subcutaneously were able to reduce their levels. The effect of restrictive diet was more important than observed with subcutaneous liraglutide. It should be emphasized that topical administration of liraglutide was unable to reduce blood glucose levels. db/+ mice (white circles), db/db mice treated with vehicle (black squares), db/db treated with restrictive diet (black circles), db/db treated with subcutaneous liraglutide (white squares), and db/db mice treated with eye drops containing liraglutide (black triangles). n = 10 mice per group.
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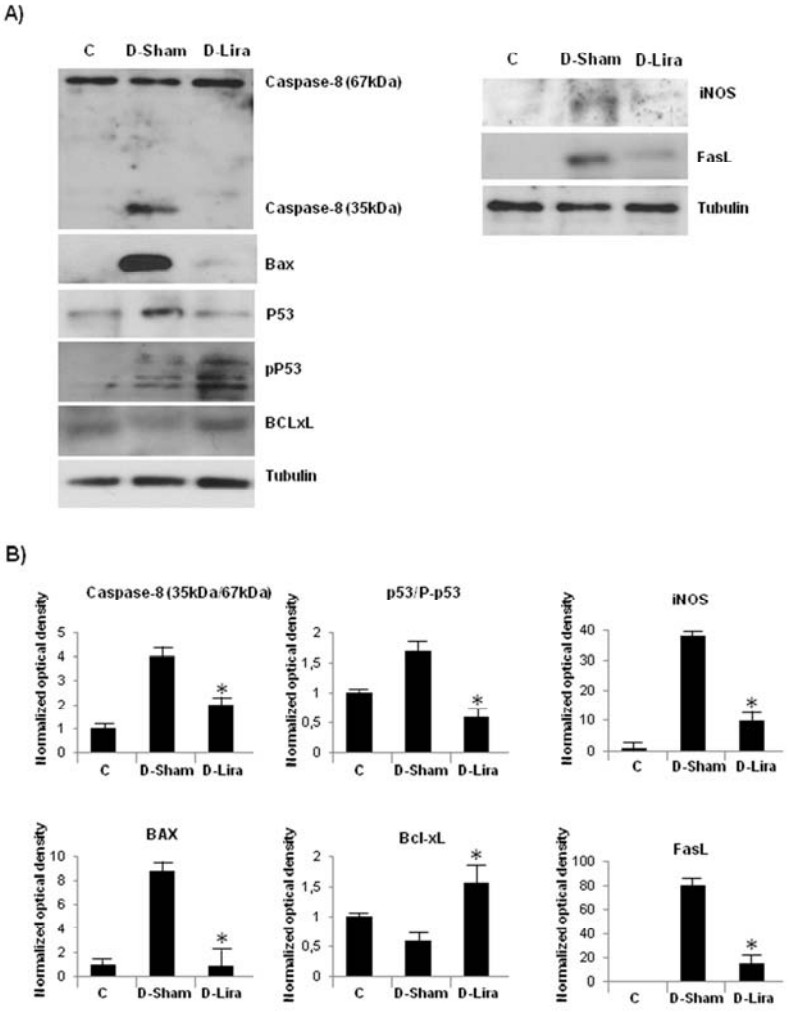

Figure 3. A) Western blot analysis of proteins from apoptotic (caspase 8, Bax, p53, BclxL) and neuroinflammatory (iNOS, FasL) pathways. B) Western blotting quantification. Data are densitometry units normalized to average controls (non-diabetic mice) protein expressions and are expressed as means ± SD. n = 6 mice per group. * p<0.001 in comparison with the diabetic group treated with vehicle (D-Sham).
190x254mm (96 x 96 DPI)

Table 1. Clinical characteristics of diabetic and non diabetic donors

	Diabetic donors N= 8	Non diabetic donors N= 8	p
Age (years)	67.4±8.3	66.9 ± 10.3	n.s
Gender (M/F)	5/3	4/4	n.s
Type of diabetes (1/2)	0/8	-	
Diabetes duration (years)	6.2 ± 4.5	-	
A1c (%)*	7.4 ± 1.3	-	
Cause of death			
- cardiovascular disease	6	5	
- cancer	1	1	
- other	1	2	

*: last A1c before death.

Supplementary table 2. Description of antibodies used in western blotting

Antibody	Reference	Manufacture	Host
NOS-2 (iNOS)	sc-650	Santa Cruz Biotechnology (Palo Alto, CA)	Rabbit
Caspase-8(p18) H-134	sc-7890	Santa Cruz Biotechnology (Palo Alto, CA)	Rabbit
Bax (N-20)	sc-493	Santa Cruz Biotechnology (Palo Alto, CA)	Rabbit
AKT1/2/3 (H-136)	sc-8312	Santa Cruz Biotechnology (Palo Alto, CA)	Rabbit
Fas Ligand	ab-68338	Abcam, Cambrigde, UK	Rabbit
Phospho-p53(S15)	ab-1431	Abcam, Cambrigde, UK	Rabbit
p53	ab-28	Abcam, Cambrigde, UK	Mouse
BcL-xL	610211	BD Transduction Laboratories	Rabbit
phospho-AKT (Ser473)	#9272	Cell Signaling Technology (MA, USA)	Rabbit
α-Tubulin (Clone TUB 2.1)	T5201	Sigma Chemical Co. (St Louis, MO)	Mouse

SUPPLEMENTARY DATA

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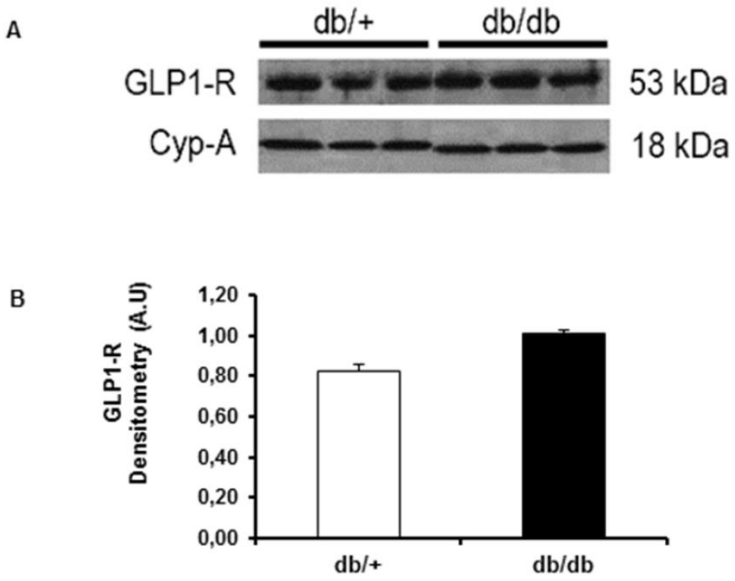

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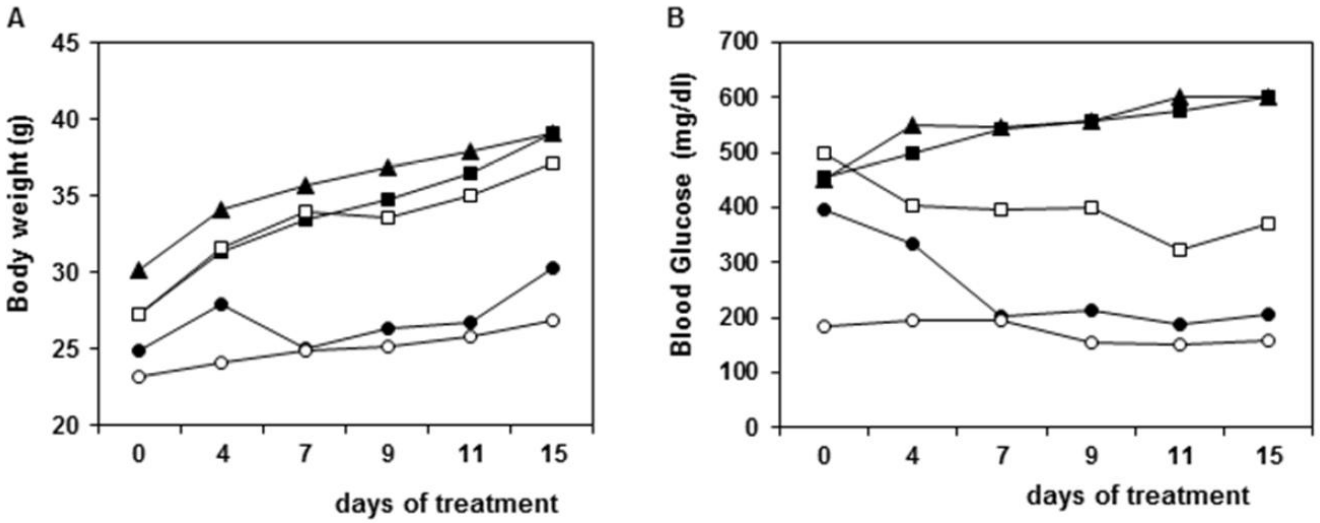

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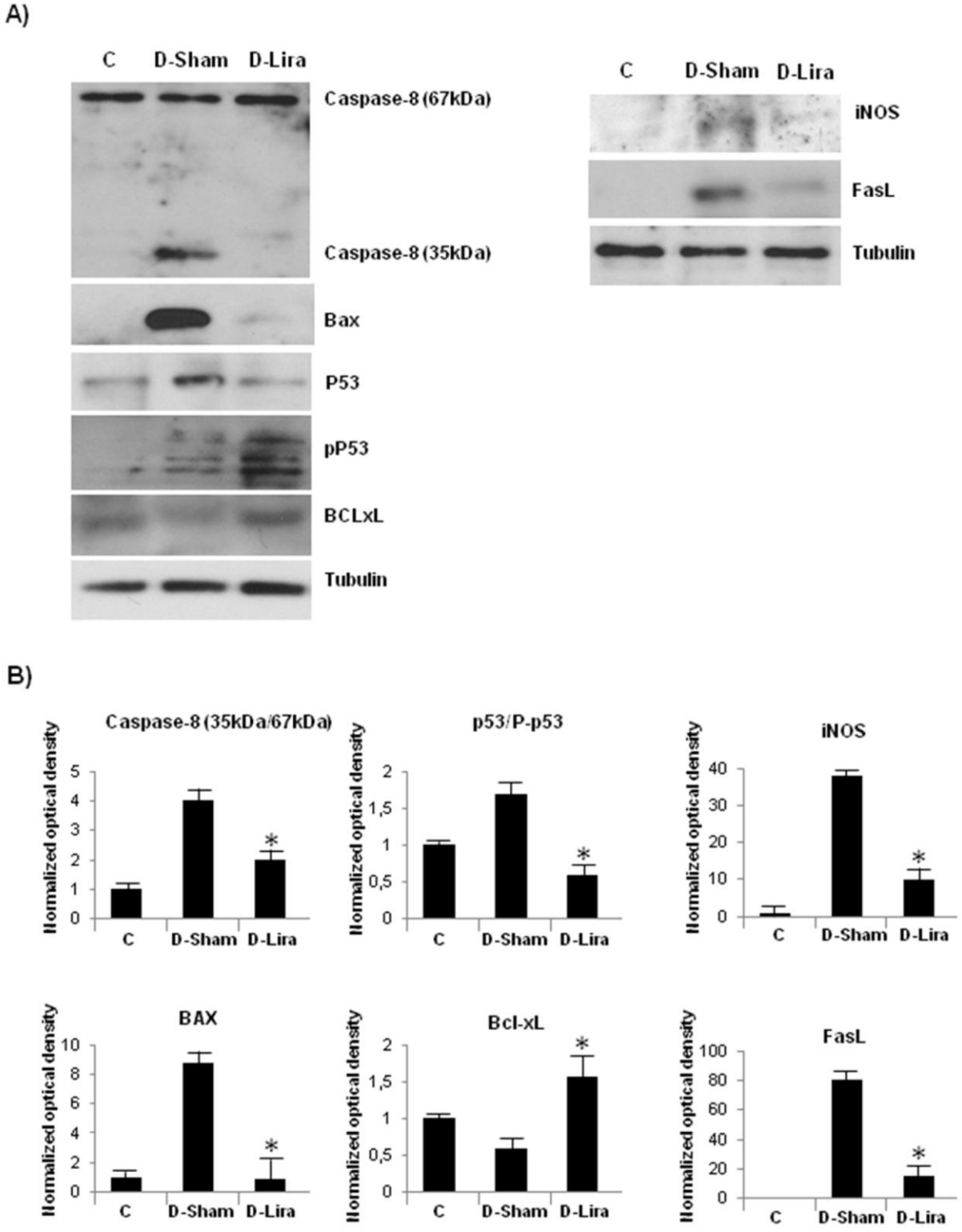

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DISCUSSION

NEURODEGENERATION AS AN EARLY EVENT IN DIABETIC RETINOPATHY

Neurodegeneration is an early event in the pathogenesis of DR and plays a crucial role in the pathogenesis of DR. It has been reported that diabetic patients, even in the absence of clinically evident vascular lesions or macular oedema, show a wide variety of visual function abnormalities. In addition, the main features of retinal neurodegeneration, such as apoptosis and glial reactivity, become evident in the retina of diabetic patients without microcirculatory abnormalities in the examination performed during the year before death[42, 44].

Abnormalities in retinal electrophysiological activity can be measured before clinically detectable vascular lesions associated with DR[30]. Degeneration of the inner nuclear layer of the retina, and apoptotic degeneration of retinal ganglion cells (RGCs) in retina from diabetic patients have also been described.

Elevated glutamate concentrations in the vitreous humour have been demonstrated in patients with DR[240]. The increase in vitreoretinal glutamate levels can be due to oxidative stress-induced damage and dysfunction of glutamate transporter on Müller cells[247], but there is a possibility that other mechanisms also contribute to this fact, i.e., decreased metabolism in the cycle glutamate-glutamine[237] and increased permeability of the BRB allowing the entrance of plasma glutamate. Blocking the NMDA receptor channel, with the uncompetitive agonist memantine, in streptozotocin- induced diabetic rats, results in prevention of increase in VEGF expression and also prevent the BRB breakdown[34]. Chronic treatment with memantine also improves retinal function and reduces RGC loss. These results may suggest that overreaction of NMDA receptors by elevated extracellular glutamate mediates the VEGF production, BRB breakdown and RGC damage observed in DR.

Neurovascular coupling is the process that enables the retina to regulate blood flow in response to neuronal activity. Flicker light stimulation induces increased neuronal activity that leads to retinal arterial and venous dilatation because of the release of vasodilating factors, especially nitric oxide, from neuronal cells and endothelial cells. Flicker-induced retinal diameter change has been shown to deteriorate early in patients with diabetes[444-448]. Lecleire-Collet and colleages[67] found a significant reduction of the flicker-induced response in the retinal arteries and veins of normotensive patients with diabetes with no clinically detectable DR. Demonstrating that neural and neurovascular dysfunctions are early events and both of which precede the onset of clinically detectable DR. This impairment in neurovascular coupling seems to be primarily mediated by ganglion cell damage[449].

The amplitude of the b-wave of the scotopic full-field (flash) ERG, reflecting largely the activity of the bipolar cells, and the implicit time of the OPs, manifestations of feedback between the amacrine and bipolar cells and/or feedback from ganglion cells to amacrine cells, are abnormal in diabetes in the absence of visible fundus signs of retinopathy. These findings suggest that function of neuronal components of the middle and inner retinal layers is altered in diabetes prior to the development of retinopathy.

In patients with minimal to moderate NPDR, additional ERG measures are altered. Among them, increased scotopic b-wave implicit time, nonselective reduction of scotopic OP amplitudes, and increased implicit time and decreased amplitude of the photopic 30 Hz flicker response, reflecting cone disfunction[450-452]. These findings suggest that photoreceptor abnormalities occur in the presence of retinopathy[453].

Delayed multifocal ERG implicit time has been demonstrated to predict the development of early microvascular abnormalities. The mfERG has proven to sensitively detect early functional change, to provide an index of retinal status, and predict which retinal locations will develop new retinopathy signs in the near future. Analysis of ITs revealed that local “standard” mfERGs are abnormal in eyes of diabetic subjects without retinopathy and, to a greater degree, in eyes with mild or moderate NPDR. Abnormal mfERG IT are locally predictive of the development of new DR over one and two years, and these functional abnormalities are spatially associated with retinopathy once it is present[453-455].

Current treatments of DR are aimed at slowing progression of sight loss once structural damage to the retina is funduscopically obvious. For all that has been discussed above is reasonable to hypothesize that therapeutic strategies based on neuroprotection will be effective in preventing or arresting DR development.

However, the morphological and functional characterization of neurodegeneration in a spontaneous diabetic model is a challenge that had to be met. In the present thesis, the neurodegenerative process in retinas of db/db mice has been evaluated.

CHARACTERIZATION OF A DIABETIC MOUSE MODEL TO STUDY EARLY STAGES OF DIABETIC RETINOPATHY

The present thesis demonstrates that the C57BL6-KSJ-db/db mouse reproduces the early neurodegenerative process that takes place in the human diabetic eye.

Considering the characteristics related to neurodegeneration, Cheung and coworkers[348] previously demonstrated that this model presented increased GFAP and expression of cleaved caspase-3 at 60 weeks of age. Our findings indicate that these changes occur as early as 8 weeks of age, and that GFAP overexpression occurs in parallel with the apoptotic changes. This is a significant feature that has not been found in other spontaneous diabetic mice such as the Ins2^{Akita}[199].

It has been demonstrated that inner retinal layers in the pericentral and peripheral area of the macula in type 2 diabetic patients with minimal DR are thinner compared to normal controls[456]. Increased apoptosis goes along with thinning in the ganglion cell layer and it has also been described in patients suffering from diabetes but with minimal DR[46-48]. In the present study, we have found that the spontaneous development of diabetes in db/db mice results in a progressive thinning of the neuroretina in comparison with non-diabetic mice, mainly due to apoptosis in the ganglion cell layer. After four weeks of hyperglycemia, at 8 weeks of age, these mice presented a 24% of reduction in the number of cell nuclei in the GCL. This reduction increased at 16 weeks to 29%, as occurs in human retinas. GCL has been the layer with the highest rate of apoptosis in human retinas[39, 42, 457]. We also found thinning of the ONL from 8 weeks of age. In addition, we demonstrated the presence of DNA fragmentation in this layer by electron microscopy, and together with the alterations in the electroretinogram a-wave indicate the presence of apoptosis in photoreceptors.

Electrophysiological determinants provide indications of pathogenic changes in the earliest stages of human DR, suggesting that initial changes in neural function of the retina may contribute to deterioration of vascular integrity[28]. It has been demonstrated that multifocal ERG can quantify changes in retinal function in absence of clinical features[458]. OPs in the electroretinogram begin to change before the onset of non-proliferative retinopathy[459, 460]. The abnormalities here reported in db/db mice, reduced amplitude and prolonged implicit time in b-wave and OPs are similar to those described in the early stages of DR in diabetic patients[62, 450]. The type of ERG abnormality could help to identify anatomically the location of retinal damage. OPs have been shown to be derived from the inner plexiform layers involving the axon terminals of the bipolar cells, the processes of amacrine cells and dendrites of ganglion cells[461]. By contrast, changes in b-wave amplitudes and implicit times, as stated in the introduction section, come from bipolar cells and also reflect the potassium-induced changes in the membrane potential of Müller cells, so, b-wave variations indicate impairment in the mid-retinal layer.

As glia supports the functions of neurons and endothelial cells, glial reactive changes affect the function and survival of vascular and neuronal elements of the retina. For instance, Müller cells regulate the extracellular environment of neurons in the retina by clearing glutamate from synaptic clefts and by providing glutamine to neurons for the resynthesis of glutamate to be used in neurotransmission[28]. Lieth et al.[237] demonstrated altered disposal of glutamate by glial cells in the diabetic retina and that it is accompanied by an overall increase in total glutamate level in the retinal tissue. Two decades ago was firmly demonstrated that an excess of glutamate is toxic for retinal neurons[462, 463]. Excitotoxicity due to overstimulation of NMDA receptors results in increased cytosolic calcium concentrations leading to activation of a number of enzymes including phospholipases, endonucleases, and proteases such as calpain. These enzymes go on to damage cell structures such as components of the cytoskeleton, membrane and DNA. Glutamate transporters are essentials for the maintenance of extracellular glutamate concentration below neurotoxic levels[226, 464]. In this regard, the glial GLAST, is the main glutamate transporter expressed by Müller cells, and is the most dominant glutamate transporter accounting for at least 50% of glutamate uptake in the mammalian retina[465]. A reduction of GLAST expression was found in rat retinas with diabetes induced by STZ[248, 429]. However, to the best of our knowledge, there were no descriptions about the role of GLAST in db/db mouse's retina. We observed a significant reduction of GLAST in diabetic (db/db) mice in comparison with non-diabetic (db/+) mice. This finding may indicate that there is glutamate accumulation that leads to neurodegeneration in the retina of db/db mice. It is worth mentioning that we did not find any differences in GLAST mRNA levels between diabetic and non-diabetic mice, thus suggesting that the differences in GLAST could be attributed to post-translational changes.

On the other hand, it is possible that glial activation is a consequence of neuronal death. In fact, our group has recently demonstrated in human retinas that there is an increase in proapoptotic molecules found in the Fas/FasL death receptor pathway (FasL, active caspase-8, truncated Bid, Bim, and active caspase-3) expression in comparison to control neuroretinas. Otherwise, there were no significant differences in the expression of the antiapoptotic markers[466].

To determine whether the neurodegeneration found in the db/db mice is due to hyperglycaemia and not to the mutation in the leptin receptor gene we examined the effect of lowering blood glucose levels on retinal neurodegeneration. The result was a significant reduction of the most important features of neurodegeneration such as apoptosis, glial activation and ERG abnormalities by lowering blood glucose levels in db/db mice. From these facts, we conclude

that diabetes is the main reason accounting for the retinal neurodegeneration that occurs in db/db mice.

The transcriptomic analysis revealed a downregulation of several genes related to glutamate metabolism, glutamate receptors and glutamate transporters, in early stages of DR in db/db mouse. This fact might lead to the observed extracellular glutamate accumulation, thus inducing excitotoxicity. This finding is in line with that made by Ly et al.[355], they found in a proteomic analysis of db/db mice retinas alterations in the expression of VGLUT1, which is responsible for loading glutamate into synaptic vesicles. Both findings point out alterations in the metabolism of glutamate. Information on genes related to glutamate receptors is controversial and mainly focused on STZ diabetic rats. Ng and coworkers[265] found upregulation of glutamate receptors (NMDAR1 GluR2/3) immunoreactivities in ganglion, amacrine and bipolar cells as well as in the IPL and OPL one month after diabetes induction by STZ and this fact was enhanced at 4 months. Concomiantly increased was the immunoreactivity of calcium-binding proteins (calbindin and parvalbumin). These results suggested that upregulation of glutamate receptors and calcium-binding proteins could reflect changes of the glutamate and calcium metabolism in the diabetic retina. On the other hand, in STZ-induced diabetic rats after 12 weeks of diabetes, significant changes in the transcriptional expression of genes related to ionotropic glutamate neurotransmission were found[467]. Most genes with decreased transcript levels were expressed by RGC, which include glutamate transporters and ionotropic glutamate receptors. They also found decreased expression of GLAST (SLC1A3) and suggest an impaired function of Müller cells. Another study, also performed in STZ-induced diabetic rats, found that the transcript levels of most ionotropic glutamate receptor subunits were not significantly changed in the retinas of diabetic rats, as compared to age-matched controls but protein levels of AMPA, kainate, and NMDA receptors were found to be altered[266]. Silva and colleges[468], found in the same model a decrease in GLAST protein content in diabetic retinas accompanied by an increase in glutamine synthase expression. Taken together, these results provide robust evidence of altered glutamate metabolism and lowered GLAST expression in the diabetic retina.

Furthermore, the transcriptomic analysis showed a simultaneous upregulation of mitochondrial UCP2 that could be contemplated as a mechanism to mitigate oxidative stress. As it has been explained in the introduction section, UCP2 is known to compensatively dissipate mitochondrial membrane potential and to uncouple oxidative respiration, in order to generate heat as a way of keeping the rate of ATP generation constant[140]. It has been found in STZ-induced diabetic rats and in bovine retinal endothelial cells (BREC) cultures, which the increase in UCP2 expression resulted in a decrease in total ROS production[451]. Furthermore, another study[469] performed in

BREC demonstrates that treatment with an ROS scavenger completely blocked the insulin-induced UCP2 upregulation. Hyperglycaemic state results inevitably in an increase in the flux of electrons through the electron transport chain and the buffering system results in an increase of UCP2 protein. On the other hand, the presence of that a vicious cycle involving glutamate excitotoxicity, oxidative stress and mitochondrial dynamics, has been reported in a non-diabetic model of retinal neurodegeneration[470, 471]. In a model of glaucoma the protective effect of treatment with memantine in RGCs was mediated by regulation of proteins that control the fusion/fission rate of mitochondria. Alterations in the expression and distribution of these proteins (OPA1 and Dnm1) may be an important component of a biochemical cascade leading to glutamate excitotoxicity-mediated mitochondrial dysfunction in RGC death in glaucoma. In this regard, Nguyen et al.[472] have reported that mutations in OPA1 in turn lead to the upregulation of NMDA glutamate receptors as well as the downregulation of mitochondrial SOD2 in the retina of heterozygous Opa1$_{enu/þ}$ mice. These findings indicate that not only glutamate excitotoxicity influences the mitochondrial fission/fusion balance, but also that altered mitochondrial fusion affects glutamate excitotoxicity.

Taken together our results point out that db/db mouse is an appropriate model that reproduces the neurodegenerative features found in retinas of diabetic patients.

EVALUATION OF THE PONTENTIAL EFFECT OF FENOFIBRIC ACID IN PREVENTING RETINAL NEURODEGENERATION IN A MODEL OF TYPE 2 DIABETES

This study provides evidence that FA exerts neuroprotection in an experimental model of DR. Here we confirm that histological hallmarks of retinal neurodegeneration (apoptosis of ganglion cells and glial activation) as well as functional abnormalities appear very early after diabetes onset. The abnormalities include alterations in glutamate metabolism, for instance downregulation of GLAST.

Short term administration of FA significantly abrogates reactive gliosis and apoptosis. Notably, after one week of FA administration, reactive gliosis was very similar to that observed in non-diabetic mice and the rate of apoptosis in the GCL was strongly reduced. Moreover, ERG abnormalities found in diabetic mice, namely decreased OPs amplitude and increased b-wave implicit time, were avoided. To the best of our knowledge, this is the first time that electrophysiological response on the retina is recorded after fenofibrate treatment.

Previously, it has been reported that fenofibrate prevents apoptosis of human retinal endothelial cells[364]. In addition, in cultures of human RPE cells, our group has reported that FA prevents the disorganization of tight junction proteins and hyperpermeability caused by the diabetic milieu[369]. Moreover, in human RPE cells cultured in conditions mimicking the diabetic milieu, FA downregulated the overexpression of basement membrane components (fibronectin and collagen IV), thus, preventing the increase of permeability associated with the accumulation of these basement membrane proteins[473]. Furthermore, our group also showed that FA exerts a dual protective effect in RPE. In ARPE-19 cell cultures, hyperglycaemia and hypoxia triggered the phosphorylation of the endoplasmic reticulum stress markers PERK and eIF2α and the induction of the pro-apoptotic transcription factor CHOP. Under these conditions, ROS were elevated and the integrity of tight junctions was disrupted. When treated with FA, ARPE-19 cells were protected against these deleterious effects induced by hyperglycaemia and hypoxia. FA increased insulin-like growth factor I receptor (IGF-1R)-mediated survival signalling in cells cultured under hyperglycaemia and hypoxia, thereby suppressing caspase-3 activation and down-regulation of Bcl-X_L. Moreover, FA increased LC3-II, an autophagy marker. FA has a dual protective effect in RPE by downregulation of stress-mediated signalling and induction of autophagy and survival pathway[367].

Chen et al.[378] have reported that oral administration of fenofibrate, for 3 and 7 weeks respectively, significantly ameliorated retinal vascular leakage and leukostasis in STZ-induced diabetic rats and in Akita mice. Favourable effects on DR were also achieved by intravitreal injection of fenofibrate. Furthermore, PPAR-α knockout abolished the fenofibrate-induced downregulation of VEGF and reduction of retinal vascular leakage in DR model. Ding and collegues[474] fed STZ-induced diabetic mice for 12 weeks with a diet containing 0.06% fenofibrate. Treatment significantly ameliorated retinal acellular capillary formation and pericyte loss. In contrast, PPAR-α (-/-) mice with diabetes developed more severe retinal acellular capillary formation and pericyte dropout, compared with diabetic WT mice. Furthermore, PPAR-α knockout abolished the protective effect of fenofibrate against diabetes-induced retinal pericyte loss. These results suggest that the beneficial effects that fenofibrate exerts in these models (reduction of vascular permeability, and VEGF expression, reduction of pericyte loss) pass through PPAR-α activation.

A recent study[475] demonstrates that oral treatment with fenofibrate during 5 days protected against increased activation of caspase-3 and reduced neuronal damage in a model of Parkinson Disease. Fenofibrate treatment, in that model, also protected against hypolocomotion, depressive-like behaviour, impairment of learning and memory and dopaminergic

neurodegeneration found after the infusion of neurotoxins that induce Parkinsonism in rats. The authors state that this effect is due to the inhibition of NF-κB by fenofibrate. NF-κB is related to the production of inflammatory mediators involved in neuroinflammation. On this subject, our group has published relevant data concerning NF-κB and fenofibrate in an *in vitro* model of diabetic oBRB. It was found that FA prevented RPE monolayer disruption, and the consequent hyperpermeability induced by IL-1β, through inhibition of NF-κB. This effect was due to PPAR-α activation and was associated with a significant downregulation of the expression of proinflammatory cytokines (IL-6, IL-8 and MCP-1). Therefore, the anti-inflammatory effects of FA through inhibition of NF-κB activity play a key role in the beneficial effect of fenofibrate[372].

Nevertheless, it seems that fenofibrate could trigger other pathways. A study[476] performed in a model of OIR demonstrated that fenofibrate decreases retinal apoptosis and oxidative stress in a PPAR-α dependent manner, while, knockout for PPAR-α showed increased retinal cell death and glial activation in comparison with WT OIR mice. The authors observed that nuclear hypoxia-inducible factor-α (HIF-1α) and nicotine adenine dinucleotide phosphate oxidase-4 (Nox 4) were increased in OIR retinas and downregulated by fenofibrate. They conclude that PPAR-α activation has a potent antiapoptotic effect in the ischemic retina and this protective effect is mediated in part though downregulation of HIF-1α/Nox4 and consequently alleviation of oxidative stress. Even though in a model of early DR we would not expect the presence of hypoxia because it is a typical trait of more advanced stages, it has been demonstrated[477] that HIF-1α is incremented and stabilized due to hyperglycemia. Taking these data into account fenofibrate could have a positive effect through this pathway.

Cho and coworkers[478] studied the effect of fenofibrate treatment in diabetic peripheral neuropathy (DPN) in db/db mice. They demonstrated that fenofibrate treatment ameliorated neural and endothelial damage by activating the PPAR-α-AMPK-PGC-1α-eNOS pathway, leading to the subsequent activation of downstream molecules such as PI3K and those involved in Akt-eNOS-NOx signaling. Through this pathway, PPAR-α attenuates vascular damage in various ways, including reduction of lipotoxicity, inflammation, generation of reactive oxygen species, endothelial dysfunction, and angiogenesis in DPN. PGC-1α is a metabolic sensor whose role is to stimulate angiogenesis in ischemic tissue. Cho's work demonstrated that diabetes decreased PGC-1α expression levels and suppressed AMPK-Akt-eNOS signaling. Since AMPK has demonstrated to be involved in the response of ARPE cells to fenofibrate treatment[369], this pathway could be involved in the neuroprotective effects that exerts fenofibrate in DR. Further studies are needed to confirm this hypothesis.

The results of the present work suggest that FA could prevent glutamate accumulation by abrogating the downregulation of GLAST induced by diabetes. As it has been discussed in the previous section, glutamate accumulation plays an essential role in neurodegeneration. Our results indicate that this mechanism also participates in the neuroprotective effect of fenofibrate. To the best of our knowledge, this is the first time that data linking GLAST expression and fenofibrate is published. On the other hand, it has been reported that the neuroprotective effect of somatostatin and glial cell line-derived neurotrophic factor (GDNF) in diabetic rats (streptozotocin-induced diabetes) is related to upregulation of GLAST[248, 479]. Our results suggest that this mechanism also participates in the neuroprotective effect of fenofibrate.

The FIELD[380] and ACCORD Eye[381] studies have shown that the greatest benefit with fenofibrate was observed in those patients with pre-existing DR. However, our results suggest that fenofibrate could be useful for treating neurodegeneration and, therefore, could be recommended even before microangiopathic abnormalities appear in the funduscopic examination. Nevertheless, clinical trials specifically addressed to prove this concept are needed.

In conclusion, FA has a neuroprotective action in db/db mice, and this effect occurs very shortly after its administration. The eventual reduction of glutamate excitotoxicity by increasing glutamate uptake through upregulating GLAST could be one of the mechanisms involved in the neuroprotective effect of FA.

EVALUATION OF THE POTENTIAL EFFECT OF GLP-1R AGONISTS IN PREVENTING RETINAL NEURODEGENERATION IN A MODEL OF TYPE 2 DIABETES

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 liver or bowel. However, we did not find any difference in GLP-1R, neither at protein or mRNA level, between diabetic donors and non-diabetic donors. However, in a recent study performed in ARPE-19 cells and STZ-induced diabetic rats, the authors found decreased expression of GLP-1R in diabetic conditions[480]. It seems that these models do not reproduce what we have found in retinas and RPE from human donors. On the other hand, we have found that GLP-1 was downregulated in the diabetic retinas. Furthermore, in this study, we provide evidence that GLP-1R agonists prevent retinal neurodegeneration independently of their hypoglycaemic action.

It has been reported that GLP-1 and several longer-lasting protease-resistant GLP-1R agonists cross the blood-brain barrier and exert a neuroprotective action in the brain of the mouse models of several neurodegenerative diseases, such as Alzheimer disease (AD). GLP-1R agonists such as liraglutide or exendin-4 have profound effects on memory formation and on synaptic plasticity in the brain[481]. These mimetics can cross the blood-brain barrier[421, 482] a property that is of vital importance if they are to be used to treat neurodegenerative disorders. Therefore, it can be assumed that circulating liraglutide reaches the retina. Other growth factors have shown similar neuroprotective properties. However, most neuroprotective growth factors such as NGF and BDNF do not cross the blood-brain barrier and therefore have no protective effect in CNS when injected peripherically[418]. In this regard, several clinical trials aimed at exploring the effects of GLP-1R agonists in preventing the development of AD are in progress[483-485].

In this study, we demonstrated that systemic treatment with the GLP-1R agonist liraglutide prevents retinal neurodegeneration in diabetic mice. However, given that liraglutide exerts a hypoglycaemic 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 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. Recently, it has been published[486] that Pro-GLP-1 (another long-lasting GLP-1R agonist) was significantly neuroprotective in C57BL/6 mice subjected to middle cerebral artery occlusion (MCAO). However, Pro-GLP-1 had no effect on blood glucose and insulin levels, which indicated that neuroprotection was mediated by the activation of GLP-1R in the brain. In cultured cortical neurons, treatment with this agonist, attenuated apoptosis induced by oxygen-glucose deprivation. The neuroprotective effects of Pro-GLP-1 were blocked by selective GLP-1 receptor antagonist and knockdown of GLP-1R with shRNA. 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 signalling is a relevant mechanism[487].

Procaspase activation can be triggered from outside the cell by activation of death receptors on the cell surface. For example, killer lymphocytes can induce apoptosis by producing Fas Ligand (FasL), which binds to the death receptor protein Fas on the surface of the target cell[488]. The clustered Fas proteins then recruit intracellular adaptor proteins that bind and aggregate procaspase-8 molecules, which cleave and activate one another. The activated caspase-8 molecules then activate downstream procaspases to induce apoptosis. This process is known as the extrinsic pathway of apoptosis[489]. Our results show that this pathway is activated in the neuroretina of db/db mice, since we found increase of caspase-8 and FasL. Moreover, we found that liraglutide treatment, orally or systemically administered, was able to prevent the diabetes-induced increase of these molecules.

The intrinsic apoptotic pathway consists on mitochondria being induced to release the electron carrier protein cytochrome c into the cytosol, where it binds and activates an adaptor protein called Apaf-1. This mitochondrial pathway of procaspase activation is recruited in most forms of apoptosis to initiate or to accelerate and amplify the caspase cascade. DNA damage can trigger apoptosis. This response usually requires p53, which can activate the transcription of genes that encode proteins that promote the release of cytochrome c from mitochondria. These proteins belong to the Bcl-2 family. p53 is a factor that participates in hypoxic and oxidative-stress-mediated retinal cell death. During oxidative stress, p53 expression is increased, translocates to the nucleous, and binds to p53 response elements (p53RE) in the promoter region of its target genes[490]. Our results demonstrate that liraglutide, systemically or topically administered was able to prevent diabetes-induced retinal activation of p53. GLP-1R signaling negatively regulates p53 signaling in PC12 cells[491]. Kim and colleages[480] demonstrated in ARPE-19 cell cultures that GLP-1R knockdown increases p53 expression and intracellular ROS generation. Evidences about the effects of liraglutide on p53 expression have been recently published by Cheng and coworkers[492] in a murine model of myocardial ischemia/reperfusion, they found an amelioration of myocardial damage when animals were treated with liraglutide. Interestingly, the authors have also found a downregulation of caspase-3 and p53 expression in liraglutide-treated animals. Moreover, they demonstrate a decrease in oxidative stress by upregulation of SOD and decrease in MDA concentration. Therefore, it is an important finding that GLP-1R activation by liraglutide inhibits p53 upregulation, and in consequence, inhibits downstream activation of apoptosis pathways in the diabetic retina.

The Bcl-2 family of intracellular proteins helps regulate the activation of procaspases. Some members of this family, like Bcl-2 itself or Bcl-X_L, inhibit apoptosis, at least partly by blocking the release of cytochrome c from mitochondria. Other members of the Bcl-2 family are not death

inhibitors, but instead promote procaspase activation and cell death. Some of these apoptosis promoters, such as Bad, function by binding to and inactivating the death-inhibiting members of the family, whereas others, like Bax and Bak, stimulate the release of cytochrome c from mitochondria[488, 493]. Our group made an important contribution in this field demonstrating for first time activation of the death receptor pathway in retinas from human non-diabetic donors and type 2 diabetic donors with mild-non proliferative DR[466]. It was demonstrated that in retinas from diabetic donors there was elevation in the expression of FasL and activation of caspase-8 in comparison to non-diabetic donors. Moreover, this study showed that the apoptotic signals emerging from the death receptor pathway were amplified to additional activation of the intrinsic apoptotic signaling pathway resulting in stronger activation of the executer caspase-3. The present study demonstrates that liraglutide treatment was able to prevent the diabetes-induced increase of Bax/Bcl-X_L ratio. In the same direction that our results, it has been demonstrated that GLP-1R knockdown in ARPE-19 cells cultured in hyperglycemia conditions results in an increase of p53-mediated Bax expression leading to RPE cell apoptosis[480]. Liu and coworkers[494] have recently demonstrated on a mouse model of Parkinson Disease that intraperitoneal injections of lixisenatide and liraglutide for 14 days resulted in a reduction of motor impairment and one of the mechanisms involved was a reduction of Bax/Bcl-X_L ratio. Briyal and collegues[495] have also pointed out a prevention in Bax increased expression in brains of STZ-induced diabetic rats treated with liraglutide. They pretreated the diabetic rats with liraglutide for 14 days and then induced middle cerebral artery occlusion (MCAO) and found that Bcl-2 expression was decreased and Bax expression was increased in vehicle-treated MCAO rats compared to sham. In addition, the authors found that liraglutide pretreatment increased the expression of Bcl-2 and decreased Bax expression in MCAO rats. The beneficial effects of liraglutide pretreatment were associated with inhibition of oxidative stress and neuronal apoptosis-related pathways, such as a reduction of the MDA content, elevation of GSH and SOD activities. Moreover, liraglutide pretreatment had similar effects on both diabetic and non-diabetic rats. Hence, there are evidences pointing liraglutide treatment to prevent the increase of Bax/Bcl-X_L ratio, and therefore, preventing the activation of the intrinsic apoptotic pathway.

Two major isoforms of nitric oxide synthase have been identified: constitutive and inducible nitric oxide synthase (iNOS). The inducible isoform is calcium-independent and calmodulin-independent and is expressed in response to stimulation by cytokines and endotoxins that include interleukin-1, tumour necrosis factor-α, and lipopolysaccharide. The constitutive nitric oxide synthase generates low levels of nitric oxide for short periods. This nitric oxide is thought to be important in signal transduction mechanisms, including the regulation of basal vascular

tones and blood pressure, and is involved in neurotransmission in the CNS. In contrast, iNOS is capable of producing a large, continuous flux of nitric oxide. The relatively large amount of nitric oxide that can be synthesized by inducible nitric oxide synthase over a sustained period exerts cytotoxic and cytostatic effects and has been implicated in diverse functions associated with inflammation and injury[496]. It has been [497] found increased levels of iNOS in the retina of human diabetic donors suffering from non-proliferative DR. They state that based on morphology and GFAP immunoreactivity, iNOS appeared to localize to Müller cells. Our results demonstrate a reduction in iNOS protein content in the retinas of liraglutide-treated mice. This fact shows that liraglutide could also have effects on neuroinflammation. In fact, in a mouse model of AD Alzheimer's disease liraglutide was demonstrated to have neuroprotective and anti-inflammatory properties[498]. However, in the latter, the amount of iNOS was not reduced, but as the total amount of nitrite was, this indicated that the synthesis of NO by iNOS was reduced.

Glutamate is the major excitatory neurotransmitter in the retina. As it has been discussed above, 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. This extracellular and synaptic excess of glutamate leads to overactivation of ionotropic glutamate receptors, which results in an uncontrolled intracellular calcium response in postsynaptic neurons and cell death. Our results suggest that liraglutide could prevent glutamate accumulation by abrogating the downregulation of GLAST induced by diabetes.

From the clinical point of view, the early identification of neurodegeneration will be crucial for implementing an early treatment based on drugs with neuroprotective effect. However, at this 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, namely eye drops, in the early stages of DR. It has been described that there are some molecules (such as brimonidine, dexamethasone, and memantine, amongst others) with the ability to reach the retina or other structures of the posterior chamber after topical administration[248, 499-502]. 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. Eye drop administration has important advantages, i.e. ease of application and the possibility of their being self-administered. Moreover, 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 (type 1 diabetic patients or type 2 diabetic patients with insulinopenia). However, clinical trials are needed to determine whether topical drug administration might present as positive results in diabetic humans as it presents in rodents.

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 GCL, and, therefore, could exert significant autocrine/paracrine actions. The presence of autocrine regulation, that is to say enhanced GLP-1 secretion following activation of the GLP-1R on GLP-1-producing cells, of glucose-induced GLP-1 secretion has been reported in L-cells[503] culture and in the intestine of mice[504]. 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.

In the present study 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. Focussing on the effect of liraglutide on microvasculature, Kelly and coworkers[505] have recently published that liraglutide treatment for eight weeks restored cerebral, splenic, and renal vascular architecture in a mouse model of AD. Their model, APP/PS1 mice, showed microangiopathies including cerebral microaneurysms, intracerebral microvascular leakage, extravasation from renal glomerular microvessels, and significant reduction in splenic sinus density. Systemic administration of liraglutide reduced the incidence of cerebral microaneurysms and leakage, restored renal microvascular architecture and significantly increased both splenic venous sinus number. These findings give us another mechanistic reason why GLP-1R agonists can be useful for preventing not only neurodegeneration but also early microvascular impairment.

Finally, 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 signalling 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.

In conclusion, GLP-1R agonists by means of a significant reduction of extracellular glutamate and an increase of prosurvival signalling 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 early stages of DR.

CONCLUSIONS

1- The spontaneous development of diabetes in db/db mice results in a progressive retinal neurodegeneration characterized by apoptosis and reactive gliosis. The neurodegenerative features found in this model are already present at 8 weeks age.

2- The morphologic and functional retinal abnormalities in db/db mice are similar to those described in the early stages of DR in diabetic patients. Diabetes is the main reason accounting for the retinal neurodegeneration that occurs in db/db mice and cannot be attributed to the mutation of leptin receptor gene.

3- db/db mouse is an appropriate model for investigating the underlying mechanisms of diabetes-induced retinal neurodegeneration and for testing neuroprotective drugs.

4- Altered glutamate metabolism and mitochondrial oxidative stress are mechanisms involved in the neurodegenerative process.

5- Short term administration of fenofibric acid, the active metabolite of fenofibrate, abrogates the main neurodegenerative features in db/db mice.

6- The reduction of excitotoxicity induced by glutamate is one of the mechanisms involved in the beneficial effects of fenofibrate in the early stages of DR.

7- GLP-1 and GLP-1R are expressed in human retina as well as in the retina of db/db mice. The local production of GLP-1 colocalized with its receptor in the retina suggests an autocrine/paracrine action.

8- Topical ocular administration of GLP-1R agonist prevents retinal neurodegeneration in db/db mice. Thus, GLP-1R agonists have a direct neuroprotective effect in the retina, independent of their ability of lowering blood glucose levels or increasing insulin secretion.

9- GLP-1R agonists activate AKT pathway, prevent diabetes-induced retinal activation of p53, FasL and iNOS. Glutamate metabolism amelioration is also among the mechanisms triggered by GLP-1R activation. Moreover, GLP-1R agonists prevent the diabetes induced vascular leakage in db/db mice.

10- 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. Topical administration of GLP-1R agonists could open up a new approach in the treatment of the early stages of DR.

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