Review

Neurodegeneration in the diabetic eye: new insights and therapeutic perspectives

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Diabetic retinopathy (DR), one of the leading causes of preventable blindness, has been considered a microcirculatory disease of the retina. However, there is emerging evidence to suggest that retinal neurodegeneration is an early event in the pathogenesis of DR, which participates in the development of microvascular abnormalities. Therefore, the study of the underlying mechanisms leading to neurodegeneration and the identification of the mediators in the crosstalk between neurodegeneration and microangiopathy will be essential for the development of new therapeutic strategies. In this review, an updated discussion of the mechanisms involved in neurodegeneration, as well as the link between neurodegeneration and microangiopathy, is presented. Finally, the therapeutic implications and new perspectives based on identifying those patients with retinal neurodegeneration are given.

Diabetic retinopathy and visual impairment

Diabetic retinopathy (DR) is the leading cause of visual impairment and preventable blindness [1,2], representing a significant socioeconomic cost for healthcare systems worldwide [3–5]. DR prevalence in the diabetic population is approximately one-third, and one-tenth of these sufferers have vision-threatening states such as diabetic macular edema (DME; see Glossary) or proliferative diabetic retinopathy (PDR) [2]. In addition, given that DR is the most common complication of diabetes and diabetes is expected to increase from 366 million in 2011 to 552 million in 2030, DR will become a serious problem in the future [6]. The potentially substantial worldwide public health burden of DR highlights the importance of searching for new approaches beyond current standards of diabetes care.

Glossary

Diabetic macular edema (DME): abnormal accumulation of extravascular fluid in the macula that leads to its thickening. This is the main cause of vision loss in diabetics.

Diabetic retinal neurodegeneration: a progressive degenerative process in the retina induced by diabetes and characterized by neural apoptosis and reactive gliosis.

Early stages of diabetic retinopathy (DR): nonproliferative DR or even a previous stage in which microvascular abnormalities cannot be detected by ophthalmoscopic examination but mfERG or FD-OCT reveal some abnormalities.

Frequency domain optical coherence tomography: optical coherence tomography is one of a class of optical tomographic techniques. A relatively recent implementation of optical coherence tomography, frequency-domain optical coherence tomography (FD-OCT), provides advantages regarding signal-to-noise ratio, permitting faster signal acquisition. In FD-OCT broadband interference is acquired with spectrally separated detectors (either by encoding the optical frequency in time with a spectrally scanning source or with a dispersive detector, such as a grating and a linear detector array). This technique is of particular interest to evaluate the changes in the nerve fiber layer, ganglion cell density, photoreceptor abnormalities, retinal thickness, and quantification of extracellular space of the retina.

Glial cells: cells essential for supporting retinal neurons. There are two main types: macroglia (Müller cells and astrocytes) and microglia. Under normal conditions, Müller cells support neuronal activity and the integrity of the blood–retinal barrier (BRB), whereas gliotic alterations of Müller cells under pathological conditions may contribute to retinal neurodegeneration and edema formation.

Glutamate excitotoxicity: aberrant accumulation of extracellular glutamate that leads to overactivation of ionotropic glutamate receptors, mainly alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors. This phenomenon has a key role in the pathogenesis of neurodegeneration in the diabetic retina.

Multifocal electroretinogram (mfERG): this technique allows local ERG responses to be recorded simultaneously from multiple regions of the retina. It is used to evaluate discrete functions across large areas of the retina and to diagnose and study retinal diseases. It uses the same electrodes and amplifiers as conventional ERG recording, and provides a topographical measure of retinal activity. Because mfERG shows good repeat reliability, it can be used to follow the progression of retinal diseases and permit prediction as to which retinal locations will develop new retinopathy signs in the near future.

Nonproliferative DR: when the first lesions can be detected under ophthalmologic examination (microaneurisms, microhemorrhages, and hard exudates). This is also called background retinopathy.

Proliferative diabetic retinopathy (PDR): advanced stage of DR marked by new vessel growth on the retina or by fibrous tissue proliferation.

Reactive gliosis: the general response to injury of glial cells. It is characterized by upregulation of proinflammatory cytokines and various kinds of molecules. One of the most characteristic is glial fibrillary acidic protein (GFAP). This intermediate filament protein is expressed in the normal retina mostly by astrocytes and minimally by Müller cells. In diabetes, Müller cells acquire prominent GFAP immunoreactivity, whereas astrocytes progressively lose GFAP immunoreactivity and may also decrease in number.

Retinal neurovascular coupling: refers to the regulation of blood flow at the retinal level in response to neural activity or metabolic demands. An increase of neural activity leads to retinal arterial and venous dilation but this response is impaired in the early stages of diabetic retinopathy.

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Current treatment of DR

The tight control of blood glucose levels and blood pressure are essential in preventing DR development or arresting its progression. The present standard of care of newly diagnosed DR relies on laser photocoagulation, the efficacy of which has been widely demonstrated [7]. However, laser treatment is not uniformly successful in halting visual decline and is associated with side effects such as moderate visual loss, diminished visual field, reduced color vision, and reduced contrast sensitivity [7]. Intravitreal corticosteroids have been successfully used in eyes with persistent DME and loss of vision following the failure of conventional treatment. However, reinjections are commonly needed, and there are substantial adverse effects such as infection, glaucoma, and cataract formation [8].

Therapies targeting vascular endothelial growth factor (VEGF) are revolutionizing the treatment of DR. Trials of these therapies provide robust evidence that intraocular administration of anti-VEGF agents is better than laser therapy in preserving and in improving vision for patients with DME [9–12]. Among the four anti-VEGF agents (ranibizumab, bevacizumab, pegaptanib, and aflibercept), ranibizumab has been the one most thoroughly tested. In addition, laser treatment and anti-VEGF injections could be combined. In this regard, it has been reported that, in those patients receiving combined ranibizumab and laser therapy, the best long-term visual outcome could be achieved with the initiation of injections followed by deferred laser therapy 6 months later [13]. At present, the role of ocular anti-VEGF therapy for PDR is less clear, although nationwide studies by groups such as the Diabetic Retinopathy Clinical Research Network (DRCRnet) are underway to address this issue. The main problem with intravitreous injections of anti-VEGF agents is that it is an invasive procedure and could even have deleterious effects for the remaining healthy retina. This is especially important in diabetic patients in whom long-term administration is to be expected. Apart from local side effects, anti-VEGF agents can also produce systemic complications due to their capacity to pass into systemic circulation [14]. A recent meta-analysis concluded that ocular and non-ocular side effects of anti-VEGF therapy for treatment of DME are rare [15]. However, it should be noted that most data were obtained at one year and, therefore, long-term confirmation is needed.

Vitreoretinal surgery is an expensive treatment that should be carried out only by vitreoretinal specialists experienced in the procedure and it is normally reserved for the ultimate blinding complications of PDR [16]. In summary, current treatments for DR are applicable only at advanced stages of the disease and are associated with significant adverse effects. Therefore, new pharmacological treatments for the early stages of the disease are needed.

Neurodegeneration in the diabetic retina: morphological and functional features

The general structure of the retina, its main components, and its vascular supply are summarized in Box 1. The metabolic pathways triggered by hyperglycemia such as the polyol and hexosamine pathways, the *de novo* synthesis of diacylglycerol-protein kinase C (DAG-PKC), and the production of free radicals and advanced glycation end-products (AGEs) are crucial in the development of DR [17]. In addition, there is growing evidence that inflammatory mechanisms also have an important role in its development [18]. The activation of all these pathways leads to abnormalities in the neural retina (retinal neurodegeneration) and the capillary bed located in the inner retina (microangiopathic injury) (Figure 1). Although microcirculatory impairment is the classic hallmark of DR, there is emerging evidence to suggest that retinal neurodegeneration is an early event in the pathogenesis of DR [19–33]. The main histological and functional abnormalities are summarized below.

Histological findings and signaling pathways leading to apoptosis

Neural apoptosis and reactive gliosis are the most important histological features of DR. Retinal ganglion cells

Box 1. General structure of the retina and its vascular supply

The retina is a structure with several layers of neurons interconnected by synapses (neuroretina) and a monolayer of pigmented cells called the retinal pigment epithelium (RPE) situated between the neuroretina and the choroids, which is essential to nourish the neuroretina [172]. The only neurons that are directly sensitive to light are the photoreceptor cells. These mainly comprise two types: the rods and cones. Rods function mainly in dim light and provide black-and-white vision, whereas cones support daytime vision and the perception of color. A third, much rarer type of photoreceptor, the photosensitive ganglion cell, is important for reflexive responses to bright daylight. Neural signals from the rods and cones undergo processing by other neurons of the retina. The output takes the form of action potentials in retinal ganglion cells (RGCs), axons of which form the optic nerve.

The retina in humans and most mammals receives its oxygen from a dual blood supply. The choroid, lying immediately behind the retina, supports the outer retina, and retinal circulation primarily supports the inner retina. This complex dual vascular system provides oxygen and nutrients to the highly metabolically active neural retina, a tissue that has higher oxygen consumption per unit weight of tissue than any other human tissue [173]. The presence of a dual circulation makes retinal oxygenation unique [84]. The photoreceptors and the greater portion of the outer plexiform layer receive nourishment from the choriocapillaris indirectly, whereas the inner retinal layers are supplied by the superficial and deep capillary plexuses formed by branches of the central artery of the retina. The delicate balance between oxygen supply and consumption in the retina places the retina at particular risk of ischemic damage, and retinal ischemia is thought to have a major role in many retinal diseases including diabetic retinopathy (DR). The outer retina is more resistant to hypoxic stress whereas inner layers of the retina are known to show the highest sensitivity to hypoxic challenges [85]. In particular, RGCs have been reported to be highly sensitive to acute, transient, and mild systemic hypoxic stress [174], as well as to the neurodegenerative process that occurs in diabetes [34].

Retinal neural tissue is protected from potential harmful molecules in the circulation by the inner blood–retinal barrier (BRB), which is constituted of endothelial cells, and the outer BRB, which is constituted of RPE. Tight junctions between neighboring endothelial cells and RPE cells are essential for the strict control of fluid and solutes that cross the BRB, as well as preventing the entrance of toxic molecules and plasma components into the retina.

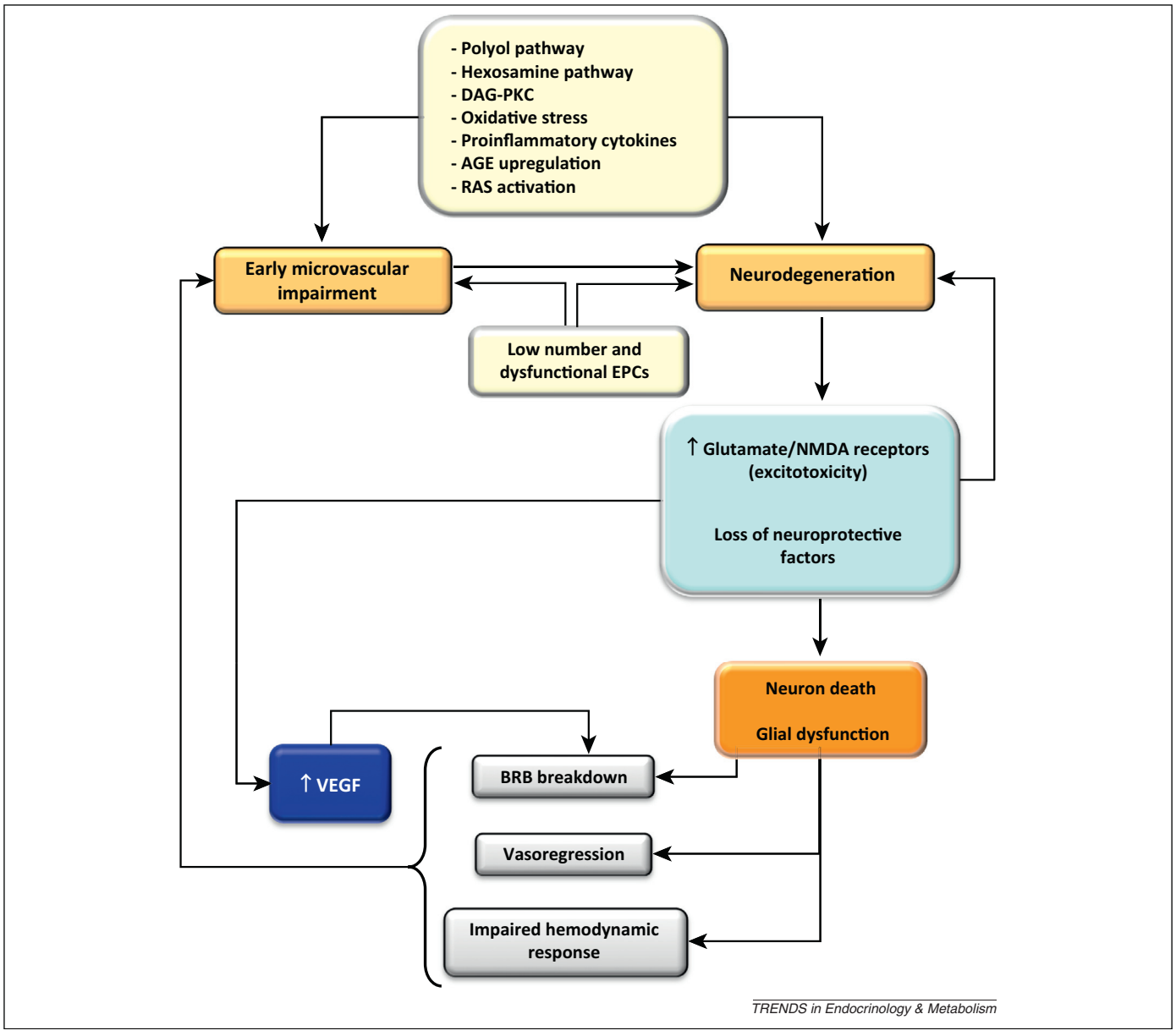

Figure 1. Schematic representation of the main mechanisms leading to diabetic retinopathy (DR). The hallmarks of neurodegeneration are neuronal apoptosis and glial dysfunction, whereas blood–retinal barrier (BRB) breakdown, vasoregression, and altered microvascular hemodynamic response (impaired neurovascular coupling) are the main features of early microvascular abnormalities. The accumulation of glutamate and the loss of neuroprotective factors trigger vascular endothelial growth factor (VEGF) activation, which has a key role in the disruption of the BRB. The loss of neurons and glial dysfunction participate in BRB breakdown, vasoregression, and impaired neurovascular coupling. Finally, the reduced and dysfunctional endothelial progenitor cells (EPCs) reported in diabetes lead to an impairment of the remodeling capacity, thus contributing to microangiopathy and neurodegeneration. Abbreviations: AGE, advanced glycation end-products; DAG-PKC, diacylglycerol-protein kinase C; NMDA, *N*-methyl-D-aspartate; RAS, renin–angiotensin system.

(RGCs), located in the inner retina, are the retinal neurons in which the apoptotic process related to diabetes is first detected [34]. RGC loss results in a reduction in the thickness of the retinal nerve fiber layer, and has been detected in rats with streptozotocin (STZ)-induced diabetes, and diabetic patients without or with only minimal DR, by using scanning laser polarimetry or optical coherence tomography (OCT) [35–40].

Neural apoptosis is accompanied by changes in glial cells (astrocytes and Müller cells) known as 'reactive gliosis' (Box 2). At present it is unknown whether neural apoptosis or reactive gliosis is first in the neurodegenerative process that occurs in the diabetic retina. It should be noted that glial cells are not neurons although they are essential for their survival. Therefore, the eventual demonstration that reactive gliosis antedates neural apoptosis would argue against the primary hypothesis that neuronal damage is the first event in DR. It is worth mentioning that the main features of retinal neurodegeneration have been found in the retinas of diabetic donors without any microcirculatory abnormalities during ophthalmoscopic examinations performed the year before death [41–43]. In addition, it has been recently demonstrated that an imbalance between proapoptotic and survival signaling exists in the neuroretinas of diabetic patients in the early stages of DR [44].

Box 2. Glial cells in the retina and main consequences of reactive gliosis

There are two types of glial cells in the retina: macroglia and microglia. The predominant cell type of macroglia is the Müller cell, which is unique to the retina. Müller cells are spindle-shaped and span the entire retina from the outer limiting membrane to the retinal ganglion cells. The second type is the astrocyte, which migrates into the retina along the optic nerve during development. Astrocytes are less abundant than Müller cells and form a monolayer at the inner limiting membrane. One of the most significant characteristics of reactive gliosis is glial acidic fibrilar protein (GFAP) overexpression. Retinal astrocytes normally express GFAP, whereas Müller cells do not. However in diabetes an aberrant expression of GFAP is shown by Müller cells [175]. Because Müller cells produce factors capable of modulating blood flow, vascular permeability, and cell survival, and their processes surround all the blood vessels in the retina, it seems that these cells have a key role in the pathogenesis of retinal microangiopathy in the diabetic eye [176]. Diabetes also induces activation of microglial cells, the main resident sentinel immune cells located in the inner part of the retina, which then migrate to the subretinal space and release cytokines that contribute to neuronal cell death [177]. Recent data show that the physiological transcellular migration of activated microglia through the retinal pigment epithelial (RPE) cells in the retina is modulated by chronic hyperglycemia leading to subretinal accumulation of activated microglia and/or macrophages [178]. Activated microglial cells adjacent to the vessels also appear to have a key role in vasoregression, the vascular hallmark of the early stages of diabetic retinopathy (DR) [30].

Electroretinogram abnormalities

The electroretinogram (ERG), which measures the electrical responses of various cell types in the retina, identifies several abnormalities in retinal function in type 1 diabetic patients and rats without evidence of microvascular abnormalities [45–48]. The use of multifocal ERG (mfERG) has provided compelling evidence suggesting a direct link between neural dysfunction and vascular abnormalities in DR. Thus, a delayed mfERG implicit time (mfERG-IT) predicts the development of early microvascular abnormalities [49,50]. The implicit time in mfERG is spatially associated with retinopathy, correlates with retinopathy severity, and is a predictor for the development of visible vascular abnormalities over a 1-year [51,52] and a 3-year period [53]. Nevertheless, it should be noted that electrophysiological evaluation could be a more sensitive method for detecting the impairment of neuron function than standard ophthalmologic evaluation (i.e., retinography) for detecting structural microvascular abnormalities.

Impairment of neurovascular coupling

Neurovascular coupling is the intrinsic physiological mechanism by which neural activity is coupled to blood flow and metabolism, thus enabling the retina to regulate blood flow in response to neural activity or metabolic demands. Visual stimulation is a powerful modulator of retinal and optic nerve blood flow [54], and flicker light stimulation (intermittent flash) has been used to investigate this process because it increases neural activity. This increase of neural activity leads to retinal arterial and venous dilation [55].

The mechanisms underlying neurovascular coupling are complex and multifactorial and different types of cells (neural cells, glial cells, endothelial cells, and perycites) and several vasoactive mediators are involved [54,56–58]. Nitric oxide (NO) released from neural cells and endothelial cells seems to participate in vasodilation [56]. In fact, there is evidence that blocking NO synthase alters neurovascular coupling [59,60]. However, when NO levels are raised experimentally, vasodilatation is reduced due to NO inhibition of the synthesis of vasodilators synthesized by glial cells [57]. Therefore, NO could be contemplated as a modulator rather than a direct mediator of the response [57,58]. Apart from NO, there are other vasodilating mediators in the retina such as prostanoids, adenosine, ADP, ATP, lactate, glutamate, gamma-aminobutyric acid (GABA), taurine, adrenomedullin (AM), calcitonin gene-related peptide, atrial natriuretic peptide, brain-derived peptide, C-type natriuretic peptide, and retinal relaxing factor [61]. Glial cells have an essential role in the hemodynamic response by means of the production of vasoactive factors [56–58,62,63]. Interestingly, it has been reported that glial-induced vasodilating prostanoids (i.e., epoxygenase metabolites) are active at low NO concentrations, whereas vasoconstricting prostanoids (i.e., 20-hydroxyeicosatetraeonic acid) are predominant at higher NO concentrations [56]. In addition, most of these molecules can increase or decrease blood flow depending on local oxygen concentration, but how this switch occurs is unclear [57].

Flicker-induced retinal diameter change has been shown to deteriorate early in diabetic patients, and it has been reported in diabetic patients without structural microvascular abnormalities in the retina [64–66]. Moreover, in diabetic patients without or with mild DR, flicker-induced vasodilatation is already abnormal when vascular function to exogenous NO is still normal, thus suggesting that this abnormal response in diabetes is not the consequence of generally reduced retinal vascular reactivity [67]. Furthermore, it has recently been reported that flicker-induced vasodilatation can be impaired when pattern ERG is still normal in type 1 diabetic patients, thus suggesting that neurovascular impairment precedes neural dysfunction [68]. All these findings reinforce the concept that the neurovascular unit is altered at very early stages of DR [69], and further studies addressed at dissecting the relative contributions of neural or glial signaling are needed.

Mediators of retinal neurodegeneration

In this review we will focus on three of the most important mechanisms in the neurodegenerative process that occurs in DR: extracellular glutamate accumulation; oxidative stress; and reduction of neuroprotective factors synthesized by the retina.

Extracellular glutamate accumulation

Glutamate is the major excitatory neurotransmitter in the retina and it is elevated in the extracellular space in experimental models of diabetes [70–72], as well as in the vitreous fluid of diabetic patients with PDR [73,74]. This extracellular and synaptic excess of glutamate leads to overactivation of ionotropic glutamate receptors, mainly alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors, which results in an uncontrolled intracellular calcium response in postsynaptic neurons and cell death [75,76]. This deleterious effect of glutamate on retinal neurons is

Box 3. Mechanisms accounting for extracellular accumulation of glutamate in diabetic retinopathy

The main mechanisms involved in extracellular accumulation of glutamate in diabetic retinopathy (DR) are the following: (i) reduction of the Müller cell specific enzyme glutamine synthetase, which converts glutamate to glutamine [70,71]; (ii) decrease in the retinal ability to oxidize glutamate to alpha-ketoglutarate [72]; (iii) impairment of glutamate uptake by the glial cells – an essential step in the regulation of extracellular glutamate is the transport of this amino acid into Müller cells through the high-affinity L-glutamate/L-aspartate transporter (GLAST), which has been compromised in the diabetic retina [83].

known as 'excitotoxicity'. The reasons why diabetes facilitates extracellular accumulation of glutamate are discussed in Box 3.

Oxidative stress

The aberrant production of the mitochondria-derived reactive oxygen species (ROS) to increase the level of oxidative stress is crucial for DR development. Mitochondria account for the bulk of endogenously formed ROS in most cells, and due to their high reactivity and local production the mitochondrial components (mainly mtDNA) are also the first to be exposed and damaged by ROS. This may cause mitochondrial energy production to drop below that required for cellular functioning, leading to loss of tissue function contributing to the onset and/or progression of retinal degeneration [77]. To counterbalance ROS damage, endogenous antioxidant defense mechanisms, including enzymatic and nonenzymatic pathways, exist. Common antioxidants include the vitamins A, C, and E, glutathione (GSH), and the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx), for a review see [78].

The retina is the only neural tissue that has direct exposure to light, thus resulting in the photo-oxidation of many lipids that become extremely toxic to retinal cells [79]. In this regard it has been shown that glutathione is depleted in the retinas but not in the brain of rats after 2 months of STZ-induced diabetes [80].

There is emerging evidence that oxidative stress can damage neural (in particular RGCs) and microvascular retinal cells [81,82]. One of the mechanisms might be through the impairment of L-glutamate/L-aspartate transporter (GLAST) [83], thus favoring excitotoxicity. Finally, it should be mentioned that ischemia results in Ca^{2+} influx through the voltage-dependent Ca^{2+} channels followed by Ca^{2+}-dependent glutamate release, which further increases the extracellular accumulation of glutamate [84]. In addition, glutamate toxicity results in glutathione depletion, thus contributing to oxidative stress [85]. All these findings point to oxidative stress as an underlying mechanism linking neurodegeneration with early microvascular abnormalities.

Imbalance in the retinal production of neuroprotective factors

The retinal production of several neuroprotective factors such as pigment epithelial-derived factor (PEDF), somatostatin (SST), and interstitial retinol-binding protein (IRBP) is lower in the retina of diabetic patients compared with nondiabetic subjects. Downregulation of these factors may compromise the neuroprotection against neurotoxic factors involved in neurodegeneration.

PEDF is mainly synthesized by the retinal pigment epithelium (RPE) and has a key role in retinal homeostasis because of its antiangiogenic and neuroprotective actions. PEDF prevents oxidative stress and glutamate excitotoxicity [86,87]. Therefore, the PEDF downregulation that occurs in the diabetic retina seems crucial in favoring neurodegeneration and could also mediate early microvascular abnormalities.

SST also has antiangiogenic and neuroprotective properties [88] and, as occurs with PEDF, it is mainly synthesized by RPE [41]. In PDR and DME there is a lower production of SST, which results in a significant decrease of its intravitreal levels [89–91]. In addition, the downregulation of SST production by the human retina occurs at very early stages of DR and is associated with retinal neurodegeneration [41]. Cortistatin (CST), a neuropeptide with strong structural and functional similarities to SST, is also downregulated in DR [42].

IRBP is a glycoprotein synthesized by the photoreceptors and extruded into the interphotoreceptor matrix that fills the subretinal space [92,93]. Apart from participating in the visual cycle, IRBP is important in fatty acid transport and is essential to the maintenance of the photoreceptors [94–99]. A low expression and content of IRBP has been reported in the retinas from diabetic donors at very early stages of DR, and this downregulation was associated with retinal neurodegeneration [43].

Apart from the downregulation of natural neuroprotective factors produced by the retina, an upregulation of neurotrophic and survival factors such as VEGF and erythropoietin (Epo) also exist in the diabetic retina. Notably, this overexpression is already detected in the early stages of DR where ischemia is not a predominant event [100–103].

VEGF is a well-known pathogenic factor for DME and PDR but it is required for normal vascular development and has an essential role in maintaining the integrity of endothelial cells via antiapoptotic signaling [104,105]. In addition, VEGF also has significant neuroprotective properties [105–107]. In this regard, a dose-dependent decrease in ganglion cells has been reported following the injection of an antibody that blocks all VEGF isoforms in rats [108]. However, other experimental studies have not found significant neural damage in VEGF knockout mice [109] or after blocking the phosphorylation of VEGF receptors in transgenic mice with sustained expression of VEGF in the photoreceptors [110]. Therefore, further research to clarify the role of VEGF on retinal neuroprotection is needed.

Epo and its receptor (Epo-R) are both synthesized by the human retina (mainly in RPE) [81]. Epo is a potent neuroprotective factor [111–113], and strikingly high levels have been found in the vitreous fluid of diabetic patients [102]. Apart from neuroprotection, 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 [114].

The overexpression of VEGF and Epo coincides with the downregulation of neuroprotective factors and, therefore,

it is possible that this counteracts the reduction of the neuroprotective factors mentioned above. However, in advanced stages of DR the elevated levels of either VEGF or Epo could favor neovascularization, thus contributing to PDR development [114,115]. In addition, Epo could enhance the effects of VEGF. Therefore the overexpression of VEGF and Epo might act as a double-edged sword in the pathogenesis of DR.

Other neuroprotective factors such as insulin [116], neuroprotectin D1 (NPD1) [117], brain-derived neurotrophic factor (BDNF) [118], glial cell line derived neurotrophic factor (GDNF) [119], ciliary neurotrophic factor (CNTF) [120], nerve growth factor (NGF) [121], and AM [122] might also be involved in the neurodegenerative process that occurs in DR.

Other contributing factors

A large body of evidence supports the role of inflammation in the pathogenesis of DR [18,123,124]. An emerging issue in DR research is the focus on the mechanistic link between the activation of subclinical inflammation and neurodegeneration. In this regard, it has been shown that Müller cells show inflammation-linked responses when exposed to the diabetic milieu [125,126]. In addition, it has recently been demonstrated that upregulation of the receptor for AGEs (RAGE) has a key role in the hyperglycemia-induced activation of Müller glia and downstream cytokine production in the context of DR [127,128]. The mechanism by which these cytokines contribute to neural apoptosis is not clear but may involve the induction of excitotoxicity, oxidative stress, or mitochondrial dysfunction [33]. Finally, experimental research strongly suggests that renin–angiotensin system (RAS) activation has an essential role in the retinal neurodegeneration induced by diabetes [129–132].

Mechanisms linking retinal neurodegeneration with microvascular abnormalities

Emerging evidence suggests that neurodegeneration participates in early microvascular changes that occur in DR such as the breakdown of the blood–retinal barrier (BRB) [28,133,134], vasoregression [30], and the impairment of neurovascular coupling [64,135]. The main mechanisms linking neurodegeneration and microvascular abnormalities are summarized in Figure 1.

The relationship between the excitotoxicity mediated by glutamate and the breakdown of the BRB induced by VEGF is one of the most interesting pathways linking neurodegeneration with vascular impairment. In this regard, it has been demonstrated that hyperglycemia induces an increase in extracellular glutamate, and the subsequent overactivation of NMDA receptors mediates VEGF production and BRB breakdown [29,31]. Glial dysfunction also has an essential role in this pathophysiological event [136]. Finally, the loss of PEDF and SST can contribute to the disruption of the BRB directly or through the upregulation of VEGF [87,137–143].

Vasoregression is the primary response of retinal microvessels to chronic hyperglycemia and is characterized by the loss of perycites followed by the formation of acellular, nonperfused capillaries [144]. The relationship between neurodegeneration and vasoregression has recently been reported by Feng *et al.* [30] who characterized a transgenic rat with a defect in a cilia gene that mimics the specific neurodegenerative features observed in DR. Interestingly, in this model, primary neuronal degeneration was followed by vasoregression. In addition, activated microglial cells close to the vessels undergoing vasoregression seem to have played an essential part in this process [30].

The impairment of neurovascular coupling is an early event in DR. However, little is known about the underlying mechanisms linking retinal neurodegeneration and the dysfunction of the neurovascular unit. As previously mentioned, glial cells have a key role in the hemodynamic response that governs the neurovascular coupling. In fact, activated glia can produce either dilatation or constriction evoked by the same stimulus but under different NO concentrations. In addition, glial cells, by means of increasing Ca^{2+}, can increase NO production, which in turn modulates the vascular response [56]. Recent studies using adult neurodegenerative animal models such as the hypertensive transgenic TGR [CMV-PKD2(1/703)HA] rat, imply early activation of the innate immune and complement systems, as well as microglia playing a part in the damage to the retinal neurovascular unit [145].

Another point of interest is the potential of EPCs to promote vascular repair, thus preventing ischemic injury to various tissues including the retina. It has been observed that bone marrow (BM)-derived EPCs from diabetic patients are dysfunctional, producing fewer endothelial cells with reduced proliferative and migratory potential [146,147]. Therefore, these dysfunctional EPCs could be one of the mechanisms involved in vasoregression and the neurodegenerative process that occur in the early stages of DR. Other factors involved in the crosstalk between neurodegeneration and vascular abnormalities are oxidative stress [81,132,133], upregulation of the RAGE [127–129,148], and RAS activation [130–133].

At present, there is enough information available to suggest that neural apoptosis precedes overt vascular abnormalities. However, subtle undetected vascular defects might exist before or at the same time that the retinal neurodegenerative process occurs in DR, and these vascular changes may have secondary repercussions for neurons. In this regard, in nondiabetic mouse and cell culture models, Ye *et al.* [149] showed that the loss of Fz4 signaling results in defective vascular growth and the loss of intraretinal capillaries, which in turn leads to reversible silencing of retinal neurons. In addition, it has recently been reported that endothelin-1 (ET-1), a potent vasoconstrictor, is overexpressed in endothelial cells in the setting of DR and might contribute to increased glutamate-induced neurotoxicity in neural cells [150]. In this regard, it has been found that endothelin B receptors contribute to retinal ganglion cell loss in rat models of glaucoma and optic nerve injury [151,152], and a progressive retinal neurodegeneration has been found in transgenic mice with overexpression of ET-1 in vascular endothelial cells [153]. Finally, as previously mentioned, it has recently been reported that neurovascular dysfunction precedes alterations in ERG in type 1 diabetic patients [68]. Nevertheless, specific studies aimed at examining, with appropriate methodology, the neural repercussions of early vascular

impairment in DR are needed. An interesting possibility is that the primary event and the predominant disease mechanism may be different in different patients, resulting in different phenotypes of DR progression [154].

Therapeutic implications

Treatment based on neuroprotection opens up a new approach for preventing or arresting DR development. By reducing neurodegeneraton, apart from sparing neuron loss, the putative mechanisms triggered by neurodegeneration such as the breakdown of the BRB, vasoregression, and the impairment of neurovascular coupling would be abrogated, thus reducing the contribution of neurodegeneration to microvascular impairment.

The reduction of oxidative stress and the administration of neuroprotective agents are among the most important therapeutic strategies based on neuroprotection. There are several pharmacological studies showing that reducing oxidative stress may be an effective approach to slow neurodegeneration in experimental DR [132,155,156]. In addition, it has been demonstrated that a deficit of ascorbic acid (AA) is the main metabolic fingerprint of the vitreous fluid of PDR patients in a 1H-NMR metabonomic approach [157]. AA, a potent antioxidant, participates in neuropeptide production and inhibits VEGF. Therefore, it seems reasonable to design strategies to increase AA levels in the diabetic retina.

Neuroprotective factors such as PEDF [66,67,158,159], SST [160], NGF [122], BDNF [161], and Epo [162–164] have been used in experimental DR. Intraocular gene transfer of PEDF significantly increases neuroretinal cell survival after ischemia-reperfusion injury [158]. In addition, intravitreal injections of PEDF prevent neuronal derangements and vascular hyperpermeability in early DR [159]. SST and SST analogs administered intravitreally protect the retina from AMPA-induced neurotoxicity [160]. Hammes *et al.* [122] showed that treatment of diabetic rats with NGF prevents the apoptosis of ganglion cells and Müller cells. Gong *et al.* [161] demonstrated that intravitreous injections of adeno-associated virus plasmid carrying and expression cassette of BDNF (pAAV-BDNF) reduced the apoptosis of RGCs and improved their function in STZ-induced diabetic rats. These promising results suggest that enhancing the expression and function of the neuroprotective factors synthesized by the retina could be a therapeutic target in DR.

Exogenous Epo and Epo-derived peptide administration by intravitreal [162] or intraperitoneal injection [163] protects against neuroglial and vascular degeneration in diabetic rats [164]. The potential advantages of Epo or EpoR agonists in the treatment of DR include neuroprotection, vessel stability, and increase of tissue repair by the recruitment of EPCs toward the pathological area. Nevertheless, in advanced stages the elevated levels of Epo could enhance the effects of VEGF, thus contributing to neovascularization and, as a consequence, worsening PDR [114,115].

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. Emerging experimental evidence indicates that many drugs can reach the retina in pharmacological concentrations [165]. In fact, the neuroprotective effects of topical administration of brimonidine, NGF, PEDF, insulin, and SST have already been reported in experimental models [120,133,166–170]. In addition, the topical administration of drugs limits their action to the eye and minimizes the associated systemic effects, resulting in higher patient compliance [171]. Therefore, topical therapies could revolutionize the care of diabetic patients. However, clinical trials to test the safety and effectiveness of neuroprotective agents are needed. In this regard, it is worth mentioning that the first clinical trial on this issue is already ongoing. A multicenter, Phase II–III, randomized controlled clinical trial (EUROCONDOR-278040) to assess the efficacy of two neuroprotective agents (SST and brimonidine) administered topically to prevent or arrest DR was approved by the European Commission in the setting of FP7-HEALTH-2011. The first results should be obtained at the end of 2015.

Concluding remarks and new perspectives in clinical practice

Neurodegeneration is an early event in the pathogenesis of DR and, therefore, it is reasonable to propose therapeutic strategies based on neuroprotection as a new and targeted approach for treating the early stages of DR. The current methods to identify the presence of neurodegeneration are mfERG and frequency domain optical coherence tomography (FD-OCT). These methods permit us to detect morphological and functional abnormalities even before microvascular abnormality can be observed under ophthalmoscopic examination. In addition, the functional abnormalities indicative of neuroretinal damage detected by mfERG could be correlated with structural changes occurring in the retina using FD-OCT. It will be of particular interest to evaluate changes in the nerve fiber layer, ganglion cell density, photoreceptor abnormalities, retinal thickness, and the quantification of the extracellular space of the retina. In fact, based on these two examinations it should be possible to identify a phenotype of diabetic patients in which neurodegeneration has a key role in the development of DR and, therefore, those patients in whom neuroprotection should be more effective. However, standardization of these methods and cost-effectiveness studies are still required before their widespread use in clinical practice can be recommended.

The possibility of using topical therapy for delivering neuroprotective agents opens up a new and safe strategy for the treatment of the early stages of DR. Another advantage of targeting neurodegeneration is the feasibility in monitoring the effect of neuroprotective drugs by using FD-OCT and/or mfERG. This approach would enable us to obtain valid results in a short-term follow-up using a reasonable number of patients. This is important because, at present, two important limiting factors of clinical trials testing new drugs for DR treatment are the requirement of prohibitive sample sizes and follow-up times to assess a disease with relatively low occurrence rates, as measured by conventionally used outcomes.

In conclusion, the central role of neurodegeneration in the pathogenesis of DR is a solid basis for proposing neuroprotection as an effective strategy for preventing or arresting DR. However, clinical trials to determine not only the effectiveness and safety but also the compliance of a noninvasive route to administer these drugs, as well as a standardization of the methods for monitoring neurodegeneration such as mfERG and FD-OCT, are needed.

Appendix

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