Review

The Healthy and Diseased Retina Seen through Neuron-Glia Interactions

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The Healthy and Diseased Retina Seen through Neuron-Glia Interactions

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Abstract: The retina is the sensory tissue responsible for the first stages of visual processing, with a conserved anatomy and functional architecture among vertebrates. To date, retinal eye diseases, such as diabetic retinopathy, age-related macular degeneration, *retinitis pigmentosa*, glaucoma and others, affect nearly 170 million people worldwide, resulting in vision loss and blindness. To tackle retinal disorders, the developing retina has been explored as a versatile model to study intercellular signaling, as it presents a broad neurochemical repertoire. Retina, dissociated and arranged as typical cultures, as mixed or neuron- and glia-enriched, and/or organized as neurospheres or as organoids are valuable to understand both neuronal and glial compartments which have contributed to reveal roles and mechanisms of transmitter systems as well as antioxidants, trophic factors, and extracellular matrix proteins. Overall, contributions in understanding neurogenesis, tissue development, differentiation, connectivity, plasticity, and cell death are widely described. A complete access to the genome of several vertebrates, as well the recent transcriptome at the single cell level at different stages of development also anticipates future advances in providing cues to target blinding diseases or retinal dysfunctions.

Keywords: retina; signaling; disease; neuron; glia

Introduction

The retina is the sensory tissue responsible for the first stages of visual processing. Retinal organization is as complex as other regions of the central nervous system (CNS), with quick and easy access, and a wide neurochemical repertoire such as in the brain. For these reasons, the retina is extensively used as a model for studying development and diseases [1–4] with several advantages whether used *in vivo*, *ex vivo* (as explants), or *in vitro*. Retina can be dissociated to generate different cell cultures, such as: (i) mixed cultures, (ii) neuron-enriched cultures, (iii) purified as Müller glia cultures, and (iv) neurospheres [5,6]. Recently, functional platforms originated from stem cell

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organoids are being engineered to mitigate ocular diseases [7]. Neurogenesis and tissue development is widely described in the embryonic and mature retina [8]. Access to the complete genome of several vertebrates including Mus musculus and *Gallus gallus*, as well as the transcriptome of individual cells at different stages of development are available [9,10].

All this information provides useful tools to translate into experimental strategies. Not less important, the cells that make up the retina express most of the transmitters and modulators present in other regions of the CNS. These advantages and the importance of the retina as a key sensory tissue, together with the fact that most diseases that cause blindness are consequences of retinal dysfunction, make the retina a fascinating model for the analysis of neural structure, function, development, and diseases [11].

Organization of the retina

The basic plan of the retina organization is a highly conserved structure among all vertebrates. Five types of neurons are organized in three cell layers (nuclear) separated by two layers of synapse contacts (plexiform). The photoreceptors are in the outer nuclear layer (ONL), three types of interneurons (bipolar, amacrine, and horizontal cells) are in the inner nuclear layer (INL), and finally, the retinal ganglion cells and displaced amacrine cells are in the ganglion cell layer (GCL). Photoreceptors capture light and transform it into electrochemical signals. They make synapses with bipolar and horizontal cells in the outer plexiform layer (OPL), while ganglion cells make synapses with bipolar cells and amacrine cells in the inner plexiform layer (IPL). The axons of ganglion cells project from the eye and form the optic nerve, which will communicate with other brain regions to continue visual processing. Each class of cells are linked with specific connectivity patterns that generate ganglion cells with different sensitivities for stimuli such as edges, color contrasts, and moving or stationary objects. In addition, the retina also contains glial cells, the major one being the Müller glia, which interact symbiotically with all layers of the retina, potentially communicating with all cellular types. Müller glia intimate interaction with retinal neurons and microglia through secreted factors, including neurotransmitters, has been investigated in physiological and pathophysiological contexts [12]. Moreover, Müller glia role as an endogenous regenerative cell source in teleost fish and as a potential target for the development of new regenerative approaches in mammals have also received attention [13]. In addition, astrocytes which are found mostly in the nerve fiber layer and microglia, which invade the retinal tissue during the embryonic period are also key players in retinal homeostasis and in diseases [14].

Despite the retina's plan being preserved, the proportion and characteristics of cell types and subtypes and connectivity patterns vary among species. Birds are highly visual, with relatively large eyes compared to their skull, with sophisticated and high-acuity retinas [15]. For example, while most mammals have two types of cone photoreceptors, most birds are tetrachromatic, with cones sensitive to red, green, blue, and ultraviolet light [16].

The synapses between photoreceptors, bipolar, and ganglion cells are defined as the vertical pathway, and communication is mainly done through glutamatergic release in mature tissue. The communication of these cell types with horizontal and amacrine cells is defined as the horizontal pathway and it works mainly through inhibitory (GABAergic) synapses. In addition, there is an inhibitory structure endorsed by the GABAergic (and/or glycinergic) system in the horizontal pathway, mediated by horizontal and amacrine cells, which modulate neuronal excitability, vertical pathway, responsible for light preprocessing, such as contrast and approach sensitivity [17,18]. Although GABA and glutamate may be considered the main neurotransmitters the vertebrate retina presents most of the known neurotransmitters and also multiple neuropeptides [19]

Diverse messengers as acetylcholine, ATP, dopamine, adenosine and serotonin, peptides such as PACAP (Pituitary Adenylyl Cyclase polypeptide) and VIP (Vasoactive Intestinal Peptide), and lipid endocannabinoids (eCB) are present in the retina (Figure 1). The production and release of these molecules influence the functioning control of cell cycle and neuron differentiation throughout development to provide a mature retinal circuitry [20,21].

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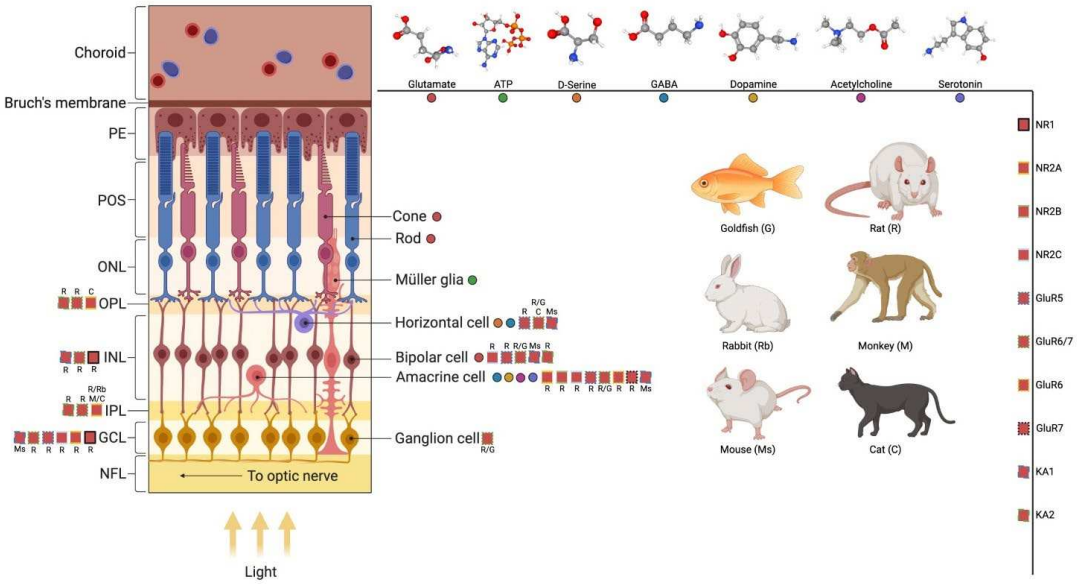

Figure 1. Comparative analysis of the vertebrate retina, selective mediators, and glutamate receptor expression across species. A comprehensive illustration of the retinal structure, neurotransmitter distribution, and glutamate receptor subunit expression across various species. The left side of the image presents a detailed cross-sectional view of the retina, delineating its layered architecture and the cellular components within each layer. The layers are sequentially labeled from the outermost to the innermost as follows: Choroid, Bruch's membrane, Pigment epithelium (PE), Photoreceptor outer segments (POS), Outer nuclear layer (ONL), Outer plexiform layer (OPL), Inner nuclear layer (INL), Inner plexiform layer (IPL), Ganglion cell layer (GCL), and Nerve fiber layer (NFL). The direction of light entering the retina is indicated by yellow arrows at the bottom, pointing towards the nerve fiber layer. Cell types within the retina are represented by distinct symbols: cones and rods (photoreceptors), Müller glia, horizontal cells, bipolar cells, amacrine cells, and ganglion cells. These symbols are color-coded and positioned to reflect their location within the retinal layers, illustrating the complex interplay of cells involved in visual processing. The right side of the image features a chart displaying the molecular structures of various neurotransmitters, including glutamate, ATP, D-serine, GABA, dopamine, acetylcholine, and serotonin. These neurotransmitters play pivotal roles in retinal signal transduction and are essential for the proper functioning of the visual system. Below the transmitter chart, a key indicates the expression of different glutamate receptor subunits (NR1, NR2A, NR2B, NR2C, GluR5, GluR6/7, GluR6, GluR7, KA1, KA2) across six species: goldfish (G), rat (R), rabbit (Rb), monkey (M), mouse (Ms), and cat (C). Each species is represented by an icon and a corresponding initial, providing a comparative view of glutamate receptor diversity and its potential impact on visual processing across different vertebrates.

Neurotransmitters

Glutamate

Glutamatergic communication is essential to the retina, present early in development, and its dysfunctions are implicated in several disorders. Excitotoxicity, which occurs by the increased influx of calcium through ionotropic glutamate receptors (iGluRs), is often implicated in neuronal damage. Studies with cells or retinospheroids have shown that relatively short incubation periods (between 30 min and 2 hours), are enough to induce neuronal death, usually observed between 8 and 24 h after exposure, with a few dying numbers after 48 h. Calcium influx through iGluRs correlates with cell death [22]. In this sense, development of retinal degeneration strongly implicates in excitotoxicity, but also in inflammation and oxidative stress, as underlying mechanisms in glaucoma, in retinitis pigmentosa, diabetic retinopathy and retinal ischemia, among other diseases [23,24]. These effects are

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largely explained by the increase in the extracellular levels of excitatory amino acids (EAA), leading to retinal remodeling. Indeed, exposure to EAA or its analogues, depending on concentration and exposure time, leads to retinal cell death in two-dimension cultures, organotypic cultures and/or in *ex vivo* models [22,25]. Intravitreal administration of NMDA, an agonist of a receptor subtype of glutamate, is used to induce retinal excitotoxicity [26,27]. The NMDA receptor is also involved in synaptic plasticity, memory, and learning, to name a few physiological tasks. Although not exclusively, it has been shown as an essential mediator involved in ischemia and cell death in the last decades [28,29].

Calcium mobilization is essential in a myriad of phenomena since a cell is born, including growth, proliferation, cytoskeletal remodeling, adhesion cell, transcription of genes, but also in the activation of proteases, such as caspases, and in the induction of cell death [30]. Therefore, the availability of Ca^{2+} is highly regulated by the action of proteins that regulate its levels and cell imaging has been used as a reliable method to study neuron-glial circuits in the last 4 decades [31]. Excessive calcium entry induces excitotoxicity which leads to death through the activation of calcium-dependent enzymatic systems, such as nitric oxide neuronal synthase (nNOS), calpains and phospholipases [32]. Excessive disruption of calcium causes disturbance and loss of mitochondrial potential ($\Delta\Psi$), activating death programmed and unscheduled pathways [33]. In particular, GluN2B subunit is linked to PSD-95 through its carboxyl terminus, which couples to nitric oxide neuronal synthase (nNOS) which, from the excessive activation of the receptor, produces pro-death signals, leading to a reduction in CREB activity [25,33,34]. Due to the rich presence of iGluRs, the retina tissue is highly vulnerable to excitotoxicity, a mutual mechanism for diseases including hypoglycemia, hypoxia, ischemia, and chronic neurodegenerative diseases [35]. On the other hand, excessive activation of AMPA receptors also have been correlated to ischemia-like insult to Retinal ganglion cells (RGCs) [36], and its control might be a relevant therapeutic target in ocular neuropathies [35].

NMDAR stimulation activates the enzyme calcium calmodulin kinase III (CaMKIII) [37,38], currently known as eukaryotic Elongation Factor 2 kinase (eEF2K) due to the recognition that its only activity is the phosphorylation of Thr-56 of the translation factor eEF2 [39–41]. eEF2 mediates translocation of peptidyl-tRNA from the ribosomal A-site to P-site by GTP hydrolysis, consuming a significant amount of energy. When phosphorylated, this factor hinders the elongation phase of protein synthesis, blocking the growth of the polypeptide chain [42,43]. As a calcium-calmodulin complex (Ca^{2+}-CaM)-dependent kinase, this enzyme has been implicated in several signaling processes, which require the rapid and transient inhibition of protein synthesis. Interestingly, over short timescales, changes in neuronal protein synthesis can occur completely independently of new transcription, for example, in response to stimuli in synaptosomes [44], isolated axons [45], and in dendritic spines [46].

It has been reported that the expression of some synaptic proteins, such as the alpha subunit of CaMKII in isolated synaptosomes [47] and brain-derived neurotrophic factor (BDNF) [48], paradoxically increases with NMDAR/Ca^{2+}-CaM/eEF2K activation, despite this pathway inhibiting general protein synthesis, an effect that is not yet completely understood. Activation of the NMDAR/ Ca^{2+}-CaM/eEF2K pathway was described to enhance the availability of intracellular free L-arginine contributing to increased NO synthesis by nNOS [37,38]. Therefore, NMDA-triggered Ca^{2+} signaling could operate in two different ways to increase NO production: 1) by activating nNOS directly and 2) by supplying the nNOS substrate, L-Arg. According to these models, protein synthesis could play an active role in the regulation of L-Arg "pools" and the synthesis of NO in neurons.

Furthermore, eEF2 phosphorylation mediated by NMDAR activation was clearly associated with CREB activation in the retina, an event that appears to depend on the increase in free L-arginine and the activation of nNOS [38]. Increased L-arginine has also been described during pharmacological treatments with cycloheximide or anisomycin (CHX or ANISO), where the PKG-dependent NO signaling pathway (canonical pathway) was shown to activate ERK and AKT [37,49]. It is known that both CREB, AKT, and ERK are widely associated with promoting survival, neuronal growth, synaptic plasticity, response to stress, and learning and memory [50–52].

γ-Aminobutyric acid (GABA)

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GABA is widely reported as the main inhibitory transmitter of the mature CNS of vertebrates [53–55], being estimated that around a third of all neurons are GABAergic [56]. $GABA^+$ cells are mainly interneurons which are responsible for controlling the excitability of the local circuitry [57,58]. GABA is found in the retina of several vertebrates [54], present in subpopulations of horizontal, amacrine, and ganglion cells and in Müller glia in the avian retina [59–64]

GABA is mainly synthesized from glutamate via glutamate carboxylase (GAD) and stored in vesicles by the vesicular GABA transporter (VGAT) until its release at the synaptic cleft [65]. GAD has two isoforms named after their molecular weight GAD65 and GAD67 [66], which are rate-limiting enzymes that keep GABA levels [67]. GAD65 has been reported as a specialized and ready-to-synthesize GABA under short-term demand. It is found mostly on nerve terminals and has a readily inducible state which depends on neuronal activity [68]. It has also been described as essential for neuroprotection and development [65,69]. GAD67 has a more dispersed distribution on GABAergic neurons while mostly fully activated [68], and may also be responsible for glial GABA synthesis [70]. Recently it has been also described that glial GABA synthesis is generated by diamine oxidase (DAO) and aldehyde dehydrogenase A1 (Aldh1a1) [71]. In the enteric nervous system [72] and midbrain dopaminergic neurons GABA is suggested also to be produced by putrescine via ornithine decarboxylase [73,74] and diamine oxidase (DAO) [75]. Interference with GABA synthesis and uptake is linked to pathologies such as schizophrenia [76]. Interestingly, retinal glia is highly involved in glutamate and GABA uptake in the retina and Müller cells are affected by diabetes, turning into a reactive state and incapable of an efficient antioxidant control [24,77].

GABA receptors are classified as GABAA, GABAB, and GABAC. GABAA and GABAC are ligand-gated ion channels permeable selectively to Cl^- with GABAA being also permeable to bicarbonate (HCO3-) to a lesser extent; and GABAB is a G-protein coupled receptor [55,78].

After release, GABA is cleared from the synaptic cleft by re-uptake by its high-affinity GABA transporters (GATs) in a Na^+ and Cl^- symport with substrate-dependent and ligand-gated ion channel properties [79]. The transporters are present in presynaptic terminals of GABAergic neurons and glial cells [80,81]. The GATs belong to the sodium symporters family, also known as the solute carrier 6 (SLC6) family [79,82] and are responsible for GABA uptake from the extracellular environment in favor of the Na^+ gradient maintained by the Na^+/K^+ ATPase pump, however it can also release GABA through a transport gradient reversal mechanism in the retina [83,84]. In mammals, but not in avian retina, Müller glia uptake and recycle GABA. Similar reversal mechanisms were described for other neurotransmitter transporters such as for dopamine, glutamate, serotonin, and glycine [85–87].

GABA is released into the synaptic cleft in retina when stimulated by depolarization and exerts its effects pre- and post-synaptically via ionotropic ($GABA_A$ and $GABA_C$ receptors) and metabotropic ($GABA_B$) receptors. In the retina, activation of iGluR in amacrine and horizontal cells promotes GABA release [88]. Dopamine inhibits the release of GABA induced by NMDA, but not by kainate, which effect could act directly in or near the NMDA receptor complex through mechanisms that seem not to involve known dopaminergic receptor systems [83,89,90]. NO, an endogenous mediator in the retina, might regulate the GABA release in a biphasic manner. Low and moderate NO production inhibit basal GABA release, mainly from amacrine cells and ganglionic cell layer (GCL) cells, while NMDA or L-arginine (at high concentration) induce a NO-dependent increase in GABA release in GCL cells [91]. The GABAergic system delineates an important physiological significance to modulate and contribute to control of sensory inputs in retinal function.

In the chicken retina, GAT-1 is responsible for about 90% of GABA uptake [92,93]. This transport is dependent on Na^+ and Cl^- and independent of Ca^{2+} [82,94] indicating that in the retina GABA, release is mainly mediated by receptor reversal and not exocytosis [61].

Additionally, in the chick retina model, it is known that several neurotransmitters and drugs might modulate the release of GABA, including glutamate, via NMDA and non-NMDA receptors [61,83,88,90,95,96] and aspartate, via selective activation of NMDA receptors [90,97]; ethanol [98], dopamine [90] and adenosine receptors, via protein kinase C (PKC) [99] and via A_1R blockade with caffeine [61,95].

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Dopamine

Dopamine is known as one of the main mediators in the vertebrate retina present in amacrine cell bodies and processes [100]. The synthesis of dopamine, as well as the other catecholamines depends on tyrosine hydroxylase (TH), the limiting enzyme for the synthesis of catecholamines and its cofactor tetrahydropterin, converting L-tyrosine to L-DOPA (3,4 dihydroxy-phenylalanine). After L-DOPA synthesis, it is rapidly metabolized to dopamine by aromatic amino acid decarboxylase (AADC) or by dopa decarboxylase (DDC), also capable of decarboxylating the amino acid tryptophan.

The change in endogenous levels of dopamine may be correlated with disorders such as myopia, since during development dopaminergic signaling regulates visual acuity and its modulation depends on several factors such as visual stimuli or chemical mediators [101]. Changes in the dopaminergic system are correlated with several effects, such as modification of neurogenesis, reduction of filopodial activity, neuritic retraction, reduction of conductance of GAP junctions, inhibition of GABA release, reduction of apoptosis and regulation of spontaneous neural activity [102]. These functions seem to be linked mainly to D1 receptors mediated effects [103].

Although noradrenaline and adrenaline also act as neurotransmitters in the mammalian retina, studies carried out in the chick retina demonstrate the absence of the noradrenaline-producing enzyme, dopamine-beta-hydroxylase, characterizing an absence of noradrenaline and adrenaline synthesis in this model being present, therefore, only the dopaminergic system [104]. Concomitantly, the retinal pigment epithelium (RPE) is capable of replenishing L-DOPA and synthesizing dopamine, due to the expression of DDC [105]; however, it does not express DAT, indicating the existence of another mechanism of dopamine transport through the membrane [106].

Dopamine receptors have been described in early stages of embryonic chick development [107] and has key roles in mature neurons. D1 receptors can be classified into subtypes D1A and D1B, which have different roles during differentiation [108,109]. A transient dopamine receptor controls the effects of dopamine on the morphology and motility of cultured retina neurons [110]. Indeed, a transient β1 adrenergic receptor was also found in the avian retina, through detection of mRNA and β1 adrenergic receptor protein in post-hatched tissue [111]. It was shown that norepinephrine cross-reacts with D1 dopaminergic receptor like dopamine in the embryonic retina, but as the retina matured, selective D1 receptor activation by dopamine or β1-like adrenergic receptors occurs in the mature tissue [111].

Components of the dopaminergic system are detected throughout the differentiation of amacrine cells in embryonic chick at E3-8, functional DAT is around E8, and D1 receptor at E7. These structures appear before the first spontaneous electrical activity in the developing retina (E8-E11). Additionally, TH (Tyrosine hydroxylase) which is one of the most important molecular components to characterize the dopaminergic phenotype has been reported to appear later in development in the chicken retina (E12). After maturation of the dopaminergic system, dopamine levels increase at around E15, which coincides with the peak of the A1 adenosine receptor density (E15) and a peak of intracellular cAMP accumulation (E16) by exogenous dopamine activation [59,102,105,111]. It is also known that during this period, a dopaminergic stimulus will promote an increase in cAMP and that stimulation with adenosine agonists will be able to partially inhibit this increase. The A2 adenosine receptor is expressed on E14 while A1 is present from E11 onwards [112]. The increase in cAMP levels is one of the factors for the increased differentiation of TH-positive cells [113]. Activation of PAC1 receptors by PACAP generates an increase in cAMP levels in chick retina cultures and modulates the expression of TH-positive neurons [114].

Among endogenous/exogenous factors specifically involved with the differentiation of dopaminergic cells are drugs that increase cAMP levels [113].

Endocannabinoid system

The endocannabinoid system controls neural excitability, mainly through the modulation of glutamate and GABA release, suggesting a relevant role in the process of visual encoding. In this sense, it has been identified as the main circuit breaker in the nervous system [115], known to be

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involved in the modulation of synaptic transmission and plasticity [116] and in several physiological processes, from embryogenesis to late development and homeostasis maintenance in the mature tissue [117]. It is commonly acknowledged as the most abundant synaptic system in the brain [118], present early in development in neurons and glial cells [119]. This also happens in the retina, where several markers (receptor, enzymes and transporters) have been functionally characterized [120,121], in addition to its messengers (anandamide, 2-aracdonoyil glycerol and others), which are involved in visual processes [119,122,123] and in pathophysiological conditions affecting the ocular system, such as in glaucoma or diabetic retinopathy [124–127]. The expression of cannabinoid receptors (CB1 and CB2), as well as TRPV channels in the vertebrate retina starts early during retina development.

The presence of the endocannabinoid system in the retina began to be investigated by the end of the 1990's. Initially, Schlicker demonstrated that cannabinoid agonists were capable of inhibiting dopamine release in guinea-pig retinas [128]. Buckley and coworkers observed that CB1 mRNA was detectable since E11 during rat embryogenesis [129]. Retinal development begins around E12 with ectoderm invagination in rats, which generates the lens vesicle and the inner neuronal layer of the optic cup (future neuronal layer of the retina) [130]. CB1 mRNA is detectable in the inner layer since E12, and in E13 it is already present in the retina, showing the importance of this system for development [131].

CB1 is highly conserved in the mature retina among vertebrates as it was identified in rhesus monkeys, mice, rats, chicks, goldfish, and tiger salamanders, to name a few [132]. These receptors are generally located at the synaptic layers, the inner and outer plexiform layers, in cones and/or rods, amacrine, and ganglion cells [133]. Other elements of the system are also found in ocular tissue, such as ligands and enzymes involved in the synthesis and degradation of endocannabinoids [134]. Functionally, it has been shown that cannabinoid agonists decrease the amplitude of voltage-gated L-type calcium channel currents in retinal bipolar cells, indicating their role in neuronal communication [133]. They also modulate calcium shifts in avian retinal Müller cells induced by ATP, but not in depolarized neurons [121]. Indeed, cannabinoid CB1 and purinergic P2X7 receptors have a role in avian retinal progenitors [135]. Besides, some aspects of retinal processing, such as modulation of response strength to visual stimulation, receptive field organization, and contrast sensitivity are also modulated by tonic endocannabinoid release in retina [136].

In the retina, CB1 is detected in ganglion cells since embryonic day 3 (~E3) [137]. Corroborating with their results, Leonelli and coworkers showed the presence of the CB1 receptor in the retinotectal system using conventional immunoperoxidase protocols. In their study, weak CB1 labeling could be detected since E4 in the retina and optic tectum, with the signal raising over development [138]. The eCB system is classically composed of cannabinoid receptors, endogenous cannabinoids (eCB), and the enzymes responsible for their synthesis and degradation. It is known that there are two major types of receptors, cannabinoid receptors type 1 (CB1) and 2 (CB2), both receptors coupled to G protein and involved in several cell signaling systems [139]. The activation of CB1 and CB2 is classically followed by the reduction of intracellular levels of cAMP, a consequence of the inhibition of the enzyme adenylate cyclase by the involvement of the Gi protein [140] among other elements.

Regarding function, Warrier and Wilson demonstrated that eCB play a modulatory role in regulating the release of neurotransmitters from embryonic retinal amacrine cells, indicating their involvement in fine-tuning synaptic transmission during the developmental stages of the visual system [141]. Chaves and colleagues explored the consequences of retinal removal on the expression of cannabinoid CB1 receptors in the optic tectum of chick brains [142]. Adult chicks were used in experiments conducted at various time intervals post-retinal lesion (ranging from 2 to 30 days). Notably, the study revealed no evidence of cell death in the deafferented tectum within the first 30 days post-lesion, although Fluoro-Jade B staining did indicate degenerating axons and terminals. Retinal ablation led to an increase in CB1 receptor protein levels in the optic tectum, as well as in other retinorecipient visual areas, coinciding with heightened MAP-2 staining and suggesting dendritic remodeling. However, CB1 receptor mRNA levels remained unaltered following retinal removal. These results imply that CB1 receptor expression in visual structures of the adult chick brain may be negatively regulated by retinal innervation. The increased CB1 receptor expression observed

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after retinal removal suggests that these receptors are not presynaptic in retinal axons projecting to the tectum, pointing to a potential role of the cannabinoid system in plasticity processes ensuing after retinal lesions.

Cannabinoid receptors and TRPA1 were also explored in the context of retinal ischemia, a condition marked by inadequate blood flow to the retina, often associated with vision loss and a lack of effective treatments. The research by Araújo et al. explored the use of cannabinoid system modulation to mitigate cell death triggered by acute ischemia in an avascular (chick) retina [143]. A combination of WIN 55212-2 (a cannabinoid receptor agonist) and cannabinoid receptor antagonists (AM251/O-2050 or AM630) was shown to reduce the release of lactate dehydrogenase (LDH) induced by retinal ischemia in an oxygen and glucose deprivation (OGD) model. Surprisingly, administering any of these drugs individually did not prevent LDH release during OGD. This suggests that the increased availability of eCB combined with cannabinoid receptor antagonists has a neuroprotective effect in the context of retinal ischemia. The study also explored the involvement of TRPA1 receptors in retinal cell death during ischemic events. TRPA1 levels increased after OGD. Notably, selective activation of TRPA1 did not worsen LDH release during OGD, while blocking TRPA1 completely prevented LDH leakage under ischemic conditions. This indicates that TRPA1 activation plays a critical role in inducing cell death during ischemia. The study suggests that cannabinoid metabotropic cannabinoid receptors, including type 1 and type 2, are not associated with cell death during the early stages of ischemia, pointing to the potential utility of targeting TRPA1 for neuroprotective strategies in the context of retinal ischemia. Overall, this research offers insights into potential mechanisms underlying neuroprotection during retinal ischemia and identifies TRPA1 as a promising target for future neuroprotective interventions in this condition.

WIN 55,212-2 was also shown to decrease cAMP production in cultured avian embryonic retinal cells under basal conditions. WIN had an impact on glial cells, reducing calcium levels evoked by ATP but not affecting calcium shifts in neuronal cells activated by KCl. Furthermore, WIN inhibited GABA release induced by KCl or L-Aspartate in amacrine cells but had no effect on GABA release in an oxygen-glucose deprivation (OGD) condition. This research underscores the crucial role of cannabinoid receptors in regulating signaling during synapse formation in the avian retina during critical embryonic stages, providing valuable insights into the expression and functions of CB1 and CB2 receptors in retinal cells, particularly their influence on cell excitability and GABA release [144–146]. In the avian retina, progenitor emergence around the first embryonic week is modulated by cannabinoid receptor activation [(by the CB1/CB2 agonist WIN 5212-2 (WIN)] [135,147]. In our hands, retinal cells in culture respond selective to KCl and/or AMPA (neurons) or ATP (glia) while progenitor cells were activated by muscimol or GABA [135,148].

We have previously shown that chronic incubation of retinal cells in culture with WIN, selective decreases calcium response to ATP, but not to KCl, suggesting that somehow glial cells, but not neurons, are modulated by cannabinoid receptor activation [121]. Therefore, in addition to regulate cAMP production, [(3)H]-GABA release induced by KCl or L-ASP or [(3)H]-D-ASP release by KCl in cultured avian retinal cells [121], WIN also decreases the number of glial cells that responded with Ca(2+) shift levels evoked by ATP, but did not altered neuronal cells activated by KCl [121]. Therefore, cannabinoid receptors function as regulators of avian retina signaling at critical embryonic stages during synapse formation.

The cannabinoid agonist WIN 55,212-2 was also used to investigate the developmental properties of the retinal glial progenitor cells. The findings from Freitas et al. indicate that WIN treatment leads to a reduction in [^{3}H]-thymidine incorporation and a decrease in the number of proliferating cell nuclear antigen-positive nuclei (PCAN$^+$) counts, suggesting that activation of cannabinoid receptors hampers the proliferation of cultured retinal progenitors [135]. Additionally, WIN treatment reduces retinal cell viability, an effect that can be blocked by CB1 and CB2 receptor antagonists, as well as the P2X7 receptor antagonist A438079. This implicates the P2X7 nucleotide receptor in cannabinoid-mediated cell death. Moreover, WIN induces an increase in mitochondrial superoxide and enhances the P2X7 receptor-mediated uptake of sulforhodamine B in cultured cells. While a substantial proportion of cultured cells respond to glutamate, GABA, and high potassium

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(KCl) with intracellular calcium shifts, only a few cells respond to the activation of P2X7 receptors by ATP. Remarkably, treatment with WIN decreases the number of cells responding to glutamate, GABA, and KCl, but significantly increases the number of cells responding to ATP, suggesting that activation of cannabinoid receptors primes P2X7 receptor-mediated calcium signaling in retinal progenitors in culture.

Campbell and colleagues also investigated the involvement of the eCB system in the proliferation of progenitor-like cells in the retina [149]. Their research involves a comprehensive characterization of the expression patterns of eCB-related genes in both chick and mouse models. The findings reveal that CNR1, the eCB receptor, and enzymes related to eCB metabolism are expressed in MG and inner retinal neurons. In the chick model, intraocular injections of cannabinoids, specifically 2-AG and AEA, were shown to stimulate the formation of MG-derived progenitor cells (MGPCs). The study also demonstrates that pharmacological agents targeting the eCB system can significantly influence glial reactivity and the capacity of MG to transition into MGPCs. Moreover, in damaged mouse retinas where MG activates NFkB signaling, activation of CNR1 was observed to decrease NFkB activity, whereas CNR1 inhibition increased NFkB signaling, with no discernible impact on neuronal cell death levels. Interestingly, the research reveals that retinal microglia, immune cells in the retina, appear to be largely unaffected by alterations in eCB signaling in both chick and mouse retinas.

These results underscore the influence of the eCB system on MG reactivity and the formation of proliferating MGPCs in the retina, shedding light on potential implications for retinal health and therapeutic strategies, especially regarding glial responses to injury.

TRP Channels

Transient receptor potential (TRP) channels constitute a superfamily of cation-permeable ionotropic receptors initially identified in the visual system of spontaneous mutants of Drosophila melanogaster. The electroretinogram of these flies revealed a loss in the sustained depolarizing response of photoreceptors to light stimuli, contrasting with the sustained responses observed in normal flies [150]. This discovery led to the identification of a receptor named TRPC1 (canonical), encouraging further exploration and characterization of other members within this superfamily, proving pivotal in various physiological contexts [151]. Diverse in structure and activation mechanisms, TRP channels represent the second-largest class of ionotropic receptors described [152]. Based on their primary structural similarities, these channels have been classified into seven subfamilies: TRPA (ankyrin), TRPC, TRPM (melastatin), TRPML (mucolipin), TRPN (no mechanoreceptor C potential), TRPP (polycystin), and TRPV (vanilloid). These channels exhibit sensitivity to various stimuli, including temperature, pH, osmolarity, inflammation, membrane stretch, inorganic ions (Ca^{2+}, Mg^{2+}), phosphorylation, lipids (e.g., AEA [153], and their metabolites (e.g., arachidonic acid; epoxyeicosatrienoic acid) [154]. This diversity equips cells to detect subtle variations in both external and internal environments [155].

TRP channels have been identified in the retinas of various animals, playing crucial roles in the lower visual coding process [156]. mRNA for members of all subfamilies has been detected in the mouse retina [157]. However, the precise cell-specific localization of TRPs poses a challenge due to the limited availability of specific antibodies [157–161].

TRPC1-6 channels, excluding TRPC2, have been identified in the retina, with some associated with specific functions. TRPC1 expression is noted in rods, plexiform layers, INL, and vascular cells, influencing phototransduction, angiogenesis, and synaptic activity [162,163]. TRPC3 is present in vascular endothelium, while TRPC4 is found in Müller glia, potentially impacting angiogenesis and synaptic activity [162,164]. TRPC5 exhibits developmental expression in amacrine cells and Müller glia, later localizing in INL cells such as bipolar cells, horizontal cells, amacrine cells, displaced RGCs, Müller glia, and in both plexiform layers. TRPC5 influences GABA release of GABA by amacrine cells, in the control of RGC axon length and perhaps angiogenesis [163,165]. TRPC6 is found in Müller glia, RGC, and vascular endothelium, participating in neuroprotection, angiogenesis regulation, and potentially myogenic vasoconstriction [156,162–165] . TRPM1-3 and 7 channels are also present in the

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retina, with TRPM1 being the most extensively studied. TRPM1 is in rod and cone ON bipolar cells, contributing to several functions including the depolarization of ON bipolar cells in response to light, regulation of RGC activity, development of rod bipolar cells, and establishment of synaptic connections with an amacrine cell subtype [166–170]. TRPM2 is detected in the RPE and possibly in microglia, playing a role in neuronal survival and potentially responding to oxidative stress [171,172]. TRPM3 is found in RGC and Müller glia, regulating the spontaneous activity of developing circuits [173]. TRPM7 is identified in vascular smooth muscle [156,174].

TRPA1 is in Müller glia, horizontal cells, amacrine cells, and RGC, influencing redox balance and mediating neuronal damage [175]. TRPP1 is found in vascular smooth muscle cells, hypothesized to play a role in myogenic vasoconstriction [174]. The location and function of the TRPML subfamily remain uncertain [156].

Location and function of TRP vanilloids in the retina

Except for TRPV3, all members of the TRPV subfamily (1-6) are present in the retina, with TRPV1 and TRPV4 exhibiting the most substantial evidence regarding their localization, function, and potential for neuroprotection [156]. TRPV1 is a channel primarily permeable to Ca2+ and Na+ cations, responsive to certain vanilloids found in peppers, such as capsaicin and piperine. This channel serves as an information conduit for cells, participating in the sensory transduction of pain, touch, light, temperature (42ºC, [176], osmolarity, pheromones, acidity (pH 6.5;[177]), inflammation, and taste [178,179]. Additionally, endogenous molecules like the endocannabinoids anandamide (AEA) and N-arachidonoyl dopamine (NADA), as well as exogenous molecules like the phytocannabinoid cannabidiol (CBD) [180], and vanilloids like capsaicin and piperine, also modulate TRPV1.

TRPV1 is distributed in photoreceptors, horizontal cells, bipolar cells, amacrine cells, microglia, some RGCs, vascular endothelium, and vascular smooth muscle. This receptor has been implicated in various functions, including the modulation of synaptic transmission, regulation of RGC function and survival, release of endocannabinoids, and control of angiogenesis. Additionally, it might be involved in lateral inhibition and purinergic control of basal vascular tone [156,159,181–184]. TRPV1's presence in the OPL [185] and photoreceptors [186] has been associated with postsynaptic transmission to bipolar and horizontal cells. This is intriguingly accompanied by the paradoxical absence of changes in a (outer retina) and b (inner retina) waves of the photopic and scotopic electroretinogram in TRPV1 knockout animals [187]. Colocalization of TRPV1 in the IPL with synaptophysin suggests a role in presynaptic potential toward the GCL [184]. Moreover, the diffuse localization of TRPV1 in the OPL also implies its presence in Müller glial processes and/or resident microglia [186,188].

Animal models simulating RGC degeneration, such as those for glaucoma, hold the potential to elucidate the role of TRPV1 and eventually offer insights for the development of neuroprotective strategies targeting TRPV1. In glaucoma models induced by elevated intraocular pressure, TRPV1 expression in RGCs increases. Conversely, TRPV1 antagonism using iodoresiniferatoxin enhances RGC density and diminishes apoptosis induced by high hydrostatic pressure [186], indicating a promising avenue for neuroprotection. It is intriguing to observe that TRPV1 undergoes diverse modulation across different cell types and species. Its modulation by exogenous agents like capsaicin and CBD provides valuable insights into the pharmacology governing the effects of this channel. CBD, by displacing capsaicin from the TRPV1 receptor and acting as its agonist, elevates intracellular Ca^{2+} levels [189]. This interaction, linked to the desensitization and internalization of TRPV1 [190], positions CBD as a potential pharmacotherapeutic treatment in conditions where TRPV1 inhibition is crucial, such as pain, epilepsy [191] and potentially glaucoma. TRPV2 is present in bipolar cells, amacrine cells, RGC, vascular smooth muscle cells, and in some somatostatin and P2X7 positive cells. Its function is associated with the regulation of vascular tone and the permeability of the blood-retinal barrier [192,193].

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Adenosine

Adenosine is an important neuromodulator in the CNS [194,195] and regulates adenylyl cyclase activity through distinct G protein-coupled receptors named A1, A2a, A2b and A3 which are present in the retina of several species [196–200]. A1 receptors are expressed since early development of chicken retina modulating dopamine-dependent cyclic AMP accumulation [112,201], while A2 receptors appear in late stages of retina development promoting direct adenylyl cyclase activation [202]. Adenosine and adenosine transporters and receptors are also expressed in mixed neuronal-glial cultures of developing chicken retina cells [203,204] and it was demonstrated that A1 receptor expression is dependent on cell aggregation and cyclic AMP accumulation induced by activation of A2a receptors [205]. In purified retinal neuronal cultures obtained from E8 embryos, long term activation of A2a receptors regulates the survival of neurons as well as photoreceptors [206], and protects neurons from glutamate excitotoxicity [207]. However, in cultures from E6 embryos, adenosine promotes cell death when added in the first day of culture and this effect depends on A2a receptors modulating CREB inhibition through a PKC pathway. On the other hand, the survival effect in E8 cultures is mediated by a cyclic AMP/PKA pathway and CREB activation, then demonstrating a shift of signaling pathways modulated by A2a receptors during chick retina development [208]. Uptake and release mechanisms for adenosine were also described in chick retinal cultures [204,209], and a calcium-dependent release of purines were described in these cultures [209] when submitted to depolarization or stimulated with glutamate [210]. Interestingly, the release of purines was found also to be mediated by transporters in a calcium/CAMK II-dependent way [210]. Our recent data show the presence of adenosine A3 receptors modulating the release of ascorbate in cultures of chick retinal cells [199].

Neuropeptides: PACAP

Pituitary Adenylyl Activating Polypeptide (PACAP) is a neuropeptide which contains 27 or 38 amino acids and belong to the same family of the Vasoactive Intestinal Peptide (VIP), with which shows high homology. This leads to common receptors which are activated by these peptides: PAC1, VPAC1 and VPAC2 and these are coupled to one or more signaling pathways depending on the isoform [211–213]. Earlier evidence of potential roles for PACAP signaling in the retina were proposed by Onali and Lianas [214] who showed that PACAP efficiently induced adenylyl cyclase activation in the retina of various species. After that, many research groups have described critical roles for PACAP signaling in retina development, as well as in mature retina, mostly with neuroprotective roles [215,216]. When studying the potential effects of PACAP in retina development we showed that it does induce cell cycle exit of late retinal progenitors from rats through the downregulation of Cyclin D1 [217], which correlated to the transient induction of Klf4 [218]. PACAP also contributed in the developing avian retina for the acquisition of the dopaminergic phenotype, defined by the expression of tyrosine hydroxylase [114], and interestingly, although the response to PACAP is less potent throughout development when cAMP accumulation is measured, this desensitization may be reversed through the use of a PACAP antagonist (PACAP6-38), leading to a two-fold increase in the number of tyrosine hydroxylase positive cells [219].

PACAP has also been shown to have, neuroprotective and regenerative properties [215,216]. Protective effects of PACAP were described in various developmental stages, cell types and disease models. In the neonatal retina we showed a protective effect in both postmitotic undifferentiated cells and developing photoreceptors. In postmitotic precursors the effect was dependent on cAMP/PKA signaling and we detected that CREB was activated as early as 5 min after treatment [220,221]. Denes and colleagues [221] also showed that the PACAP contributed to the generation of horizontal cells in the postnatal rat retina through the induction of cell proliferation.

In disease models, the evidence of neuroprotective effects is abundant. In the intraocular hypertension ischemia-reperfusion model, one experimental model for glaucoma, intravitreal injection of PACAP protected retinal ganglion cells in the fM and pM ranges with bell like curves. The effect showed to be dependent on cAMP/PKA and MAPK pathways [222]. In an ischemia model of bilateral common carotid artery occlusion, Danyadi et al suggested functional recovery based on

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electroretinographic measurements (ERG) [223]. In a model of oxygen-induced retinopathy (OIR) used to reproduce the retinopathy of prematurity (ROP) it was shown a protective effect for PACAP applied intravitreally in the extent of avascular area [224]. When the same model of retinopathy was applied to wild type (WT) and PACAPKO mice, the authors showed differences in retinal vasculature, with enhanced avascular area, and an impact on ERG [225] reinforcing that absence of PACAP increases the vulnerability to stressors. Patko and coworkers [226] also showed effects of PACAP applied in eye drops in the preservation of retinal vasculature on a glaucoma model with increase in intraocular pressure induced by microbeads injection. In this study they also demonstrated that PACAP blocked the change in the thickness of retinal nerve fiber layer (RNFL) and total thickness of the retina [226]. PACAP also showed protective effect on UV-A-induced lesions which lead to severe degeneration of photoreceptors and also impact inner nuclear layers and plexiform layers [227]. Evidence also accumulate on the protective effect of PACAP in neurodegeneration of metabolic origin, in particular diabetic retinopathy as reviewed by Gabriel and colleagues [228].

Interestingly, Wang et al used an exosome-mediated strategy for PACAP delivery in a model of traumatic optic neuropathy and showed a protective effect for RGCs, with as increase in the RNFL thickness and regeneration of axons as well as enhanced optic nerve function [229]. Recently, Van and coworkers tested if PAC1 receptors are critical to retinal protect neurons in a cell-autonomous manner, using adeno-associated virus (AAV2) to deliver Cre recombinase to the retina of mice harboring floxed PAC1 alleles.

Mice were challenged with a chronic experimental autoimmune encephalomyelitis (EAE), which recapitulates major features of Multiple Sclerosis (MS) and associated optic neuritis. Deletion of PAC1 in control conditions resulted in a deficit of retinal ganglion cells (RGCs) and dendrites, which unexpectedly suggests a homeostatic role of PAC1. In addition, absence of PAC1 resulted in increased EAE-induced loss of a subpopulation of RGCs which had been previously described as more vulnerable in glaucoma models. Damage to axons and increased recruitment of microglia/macrophages to optic nerve was also described [230].

Nitric Oxide

Nitric oxide (NO) is a gaseous signal that serves as a key regulator of various physiological processes within the retina, including transmission, vascular regulation, and immune responses [231,232]. Its production occurs through the enzyme nitric oxide synthase (NOS), which catalyzes the reaction of L-arginine, NADPH, and oxygen to form NO, citrulline, NADP +, and H_2O [233]. In the retina, the presence of L-arginine transport systems has been described and linked to NO production since the early developmental stages, as demonstrated in chick retinal cultures [234]. Among several cell types, Müller cells uptake and deliver L-arginine to neuronal NO-synthesis demand in retina [37,234], and astroglia in cortex [235–237]. There are three isoforms of NOS, two of which are constitutive and calcium-calmodulin-dependent: the neuronal (nNOS or NOS-1) [238] and the endothelial isoform (eNOS or NOS-3) [239]. Alternatively, the constitutively isoform binds calmodulin and calcium-independent inducible isoform (iNOS or NOS-2) [240]. Besides to calmodulin, four more cofactors are required for enzymatic catalysis - flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme and tetrahydrobiopterin (BH4) [233].

The expression of nNOS is primarily localized in neurons, including those within the retina. Its widespread distribution and high activity would immediately provide a basis for concluding that NO should be involved in several functions within the CNS [241,242]. Pioneer works established a clear linkage between NMDA-type glutamate receptors, NO and cGMP production in the CNS [243,244]. Functionally, NO in neurons may require a physical coupling between nNOS and NMDA receptors to compartmentalize the influx of Ca^{2+} from the channel pore to calmodulin [245]. nNOS possesses a PDZ domain, which interacts with proteins such as PSD-95 (postsynaptic density protein-95), a scaffold protein located in the postsynaptic region of neuronal cells [246].

In retina, nNOS is localized in specific retinal cells [247–250]. This isoform was predominantly found in puncta in the IPL, in amacrine cells, and in GCL. For detailed review see [251]. Three main

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types of nNOS-positive amacrine cells have been identified, one of which is referred to as displaced amacrine (adjacent to the ganglion cell layer). All amacrine NOS-positive are GABAergic cells and express the GABA-synthesizing enzymes GAD-65 and GAD-67 [252,253]. These cells receive synaptic input from cone bipolar cells and various other amacrine. They also form synapses with ganglion cells, as well as with bipolar cells [251].

The nNOS distribution play a pivotal role in neurotransmission, synaptic modulation, and other neuronal functions within the retina. NO significantly influences neurotransmission and the modulation of signal transmission between retinal cells. It also impacts the release of neurotransmitters and synaptic plasticity [233,254]. This contribution is instrumental in the regulation of visual signal processing and adaptation to changing light conditions. For example, it has been demonstrated that light stimulation can provoke depolarizing inward currents in amacrine cells with transient increase in intracellular calcium levels mediated by voltage-dependent channels, which would trigger the activation of nNOS [255]. The fluorescence technique using 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) has been applied to visualize NO synthesis in the retina. This technique is useful to correlates the expression of NOS enzyme with NO production. However, it is crucial to bear in mind that DAF-NO adducts may reflect the diffusibility of NO, given that the DAF probe can permeate various cell types and primarily serve as a target for NO trapping, rather than specifically identifying the cell type responsible for NO synthesis. In any case, most authors agree that there is a good correlation between the location of nNOS and the possible radial distance for the action of NO detected by DAF-NO adducts, which becomes a useful tool for understanding the physiology of NO retina [256,257].

Hence, physiologically, NO synthesis in the retina is regulated by light exposure and the extent of visual adaptation [258–260]. There is evidence that NO production in cone cells increases their responses to light during adaptation [261]. NO also appears to reduce the coupling of gap junctions between horizontal cells, or even decrease the conductance of gap junctions between bipolar cells and amacrine cells of the AII subtype [262,263].

Moreover, soluble guanylate cyclase (sGC), the canonical receptor for NO (in its free radical form), exhibits high expression levels in the inner retina, but its presence in outer retina is subject to controversy. While some authors found a very limited expression [264], others identified its presence in outer nuclear layers and in both plexiform layers [256]. Strong immunostaining was observed in specific subgroups of bipolar and amacrine cells, with relatively weaker staining in rod bipolar cells in specific ON cone bipolar cells and, to a lesser extent, in OFF and rod bipolar cells, as well as certain ganglion cells [264]. NO doners were able in enhance cGMP, detected through cGMP immunocytochemistry visualization in the IPL and OPL and select amacrine cells, bipolar cells, and somata in the GCL [256]. Photoreceptors, horizontal cells, and Müller cells appear to not show immunoreactivity for sGC [264]. Photoreceptors, horizontal cells, and Müller cells appear to not show immunoreactivity for sGC [264].

Canonical NO signaling has been shown to modulate a variety of channels and receptors, including Ca^{2+} channels [265], GABA A receptors [266] and AMPA/kainate receptors [267,268].

NO can also regulate the release of several neuromodulators, as well as activity of transcription factors in the chick retina. For example, it was shown that NMDA receptor stimulation could stimulate glutamate, GABA, and glutamine release in the retina, through a mechanism entirely dependent on NO [269,270]. In the adult turtle retina, NO can also stimulate GABA release through cGMP-dependent mechanisms, which involves the reversal of GABA transporters (GAT) in horizontal cells. This process is dependent on calcium ions in the inner plexiform layer [271].

It has been demonstrated in different animal models (bovine, rabbit, and carp) that NO inhibits depolarization-stimulated dopamine release in retinal cells. Furthermore, this NO modulation of dopamine release may represent a sophisticated and high-level function in the process of light transduction within the retina, as dopamine is a recognized neurotransmitter associated with light adaptation [260].

The presence of the sodium-dependent ascorbic acid transporter (SVCT-2) has been demonstrated in the INL of rat retinas [272], as well as in cultured chick retinal cells and post-hatched

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chick retinas [273,274]. NO-donors (SNAP and Noc-5), as well as L-arginine, stimulate ascorbic acid uptake in cultured retinas through the canonical pathway [90]. Interestingly, it was observed that this stimulation occurred through an increase in the Vmax for ascorbic acid uptake, suggesting that NO can modulate the levels of active SVCT-2 transporters on the membrane in vitro and ex vivo. This hypothesis gained strength as it was detected through qRT-PCR, western blotting, and immunocytochemistry analyses that NO increased SVCT-2 transcription and expression through its classical sGC/cGMP/PKG pathway [90]. This effect appears to occur through NF-κB activation since its inhibitors (PDTC and sulfasalazine) completely blocked NO- or L-Arg-induced SVCT-2 expression and ascorbic acid uptake [273].

It has also been described that NO can activate the phosphorylation of the transcription factor CREB through glutamatergic signaling. Both AMPA [275] and NMDA [38] ionotropic receptors have been implicated in this effect, which has been described as occurring via the canonical PKG-dependent pathway. Interestingly, it has been demonstrated that NO can also mediate CREB phosphorylation in Müller glia, through a mechanism involving PKG and ERK-II in an evident neuron-glia crosstalk [275]. In addition, it also has been demonstrated that NO is involved in extensive cell death during early stages of retinal development (E6), while in subsequent stages (E8) NO significantly reduces apoptosis. In this study, NO significantly decreased nuclear phospho-CREB staining in E6, while robustly enhancing CREB phosphorylation in the nuclei of E8 neurons. The ability of NO to differentially regulate CREB during retinal development depended on the capacity of PKGII to decrease (E6) or increase (E8) nuclear AKT activation. These data demonstrate that NO/PKGII-mediated signaling may function to control the viability of neuronal cells during early retinal development through the AKT/CREB activity [254]. Moreover, despite the well-known neurotoxic actions of NO synthesis, this messenger is clearly associated with neuroprotective effects in the retina [233].

Finally, even though NO signaling primarily occurs through nNOS, the retina also expresses the eNOS and iNOS isoforms. As is well-known, NO is a potent vasodilator and, in the retina, this property is vital for regulating blood flow to meet the metabolic demands of retinal cells. When there is an increased need for oxygen and nutrients, such as during increased neuronal activity, NO is released to dilate blood vessels, ensuring an adequate supply of resources to the retinal tissue. Conversely, reduced production or availability of NO can lead to impaired blood flow regulation and potential retinal ischemia [231]. iNOS is typically not present at baseline in healthy retinal tissue but can be induced in response to inflammatory and immune stimuli. Its expression is induced by various immune and inflammatory signals, and its activity leads to the production of NO. In the retina, iNOS-derived NO is involved in immune responses and can modulate the inflammatory environment during retinal diseases or injuries. NO can have both protective and harmful effects in the retina, depending on the context. It can contribute to the regulation of immune responses during retinal pathologies, such as diabetic retinopathy, uveitis, or glaucoma [232].

In summary, NOS enzymes, including eNOS, nNOS, and iNOS, are responsible for synthesizing nitric oxide in the retina, as well as its associated tissues. Each isoform has a specific cellular distribution and function, contributing to various physiological processes in visual function such as neurotransmission, vascular regulation, and immune responses. The balanced activity of these NOS enzymes is essential for maintaining retinal function and responding to changing conditions and challenges [232].

Gliotransmitters

In the retina, ATP can be released by both vesicular and channel-mediated mechanisms. While vesicular storage and release of nucleotides is mediated by the Vesicular Nucleotide Transporter protein (VNUT) that is expressed in photoreceptors, bipolar and amacrine cells, Müller glia and astrocytes in the mouse retina [276] nucleotides can be released by channels such as pannexin hemichannels from ganglion cells [277]. Several stimuli, including glutamate, tonicity changes, ischemia, growth factors or purines induces channel-mediated ATP release from RPE cells [278].

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Moreover, either channel or vesicular nucleotide release from Müller glia can be triggered by mechanical/osmotic or neurochemical stimuli such as glutamate or nucleotides themselves [279–282].

Nucleotide receptors

The retina expresses several G-protein coupled P2Y receptors that are mainly coupled to calcium mobilization. While P2Y1 receptor is the main P2Y receptor in this tissue, P2Y2, P2Y4 and P2Y6 receptors were also detected [283]. Direct evidence for the P2Y11, P2Y12 and P2Y13 receptors is still missing. However, expression of mRNA for P2Y12 receptors in the post-natal rat retina [284] as well as the blockade of glial proliferation by a P2Y13 specific antagonist [285] was obtained.

Many P2X receptors that are ion-channels are also expressed consistently in the retina. All P2X1-7, except P2X6, are well expressed, the P2X7 being the best characterized subtype in the retina of several species.

Nucleotides and retinal cell proliferation

A major effect of nucleotides in the developing retina is the stimulation of progenitor's proliferation. Activation of P2Y2/4 receptors by ATP or UTP induces proliferation of progenitors that will generate photoreceptors, amacrine, ganglion and horizontal cells [286–288]. Activation of ADP-sensitive receptors induces the proliferation of late developing glial/bipolar progenitors [289,290] by stimulating their entry in the S phase of the mitotic cycle [291].

Nucleotide-dependent proliferation of retinal progenitors is associated with the formation of inositol phosphates [292], Ca^{2+} mobilization from intracellular stores and its capacitive entry that occurs as early as the embryonic day 3 in the chick embryo retina [286,293,294]. These responses decrease as progenitors exit cell cycle and begin to differentiate [295], responses that, similar to ATP-induced increase in [^{3}H]-thymidine incorporation, are decreased by conditioned medium obtained from postmitotic retinal cells in culture [290].

ADP-mediated increase in cell proliferation is inhibited by MEK inhibitors in the developing chick retina [289,292] and ADP activates ERK pathway over the neuroblastic layer where BrdU labeled glia progenitors are located [292]. PI3K/Akt is another signaling pathway associated with nucleotide-induced proliferation of retinal progenitors [296] and Müller cells from the adult retina [297,298]. In retina cell cultures, ADP or ATP induces the phosphorylation of Akt that increases cyclin D1 involved in the progression of cells through the G1 phase of the cell cycle [296]. Phosphorylated Akt is also observed in retinal progenitors during mitosis and is required for expression of CDK1 that controls the transition of progenitors from G2 phase to mitosis [299].

ADP phosphorylates cyclic nucleotide responsive element binding protein (CREB) through an ERK dependent mechanism is also required for the proliferation of retinal glial progenitors in culture [285].

The nucleotide receptor subtype(s) involved in the proliferation of glial progenitors is still poorly defined [300]. Knocking down P2Y1 receptor expression decreases eye formation in frog tadpoles and more than 80% of glial progenitors of the newborn mouse retina express P2Y1 receptors [291]. Injection of the P2Y1 receptor antagonist MRS2179 in the eyes of newborn rats decreases the number of BrdU positive progenitors [284]. However, eye formation and retina function were shown not to be affected in P2Y1 knockout mice [301], suggesting that other receptor subtypes may operate in the absence of the P2Y1 receptor in the developing retina. Either P2Y1 or P2Y13 receptor antagonists prevent ADP-induced proliferation of retinal glial progenitors in culture and stimulation of only the P2Y1 receptor does not induce their proliferation [285], suggesting that both receptors participate in the proliferative response of chick retinal glial progenitors in culture.

In the newborn rat retina, blockade of P2Y12 receptors induces an increase in cyclin D1 and a decrease in p57 protein. Since P2Y12 inhibition does not affect S phase of the cell cycle and induces the death of cyclin D1 positive cells, activation of these receptors seems to be required for the exit of late developing retinal progenitors from the cell cycle [284].

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Nucleotides and retinal cell migration

Damaged mammalian retina has low capacity to regenerate and de-differentiated glia contributes to the formation of glial scars. In rabbits, after retinal detachment, Müller cells migrate to the outer retina, undergo mitosis and some cells grow beyond the OLM, forming glial scars in the subretinal space [302]. ATP may contribute to the formation of glial scars by regulating both proliferation and migration of Müller cells [303]. Accordingly, activation of UTP-sensitive P2Y2/4 receptors induces the growth of glial cells through a mechanism involving PI3K, SRC and FAK signaling pathways in mechanically scratched retinal cultures [304] . When purified retina glial cultures are used, both cell adhesion and migration are decreased by P2 receptor antagonists [304].

Nucleotides and the induction of cell death in the retina

Activation of cytotoxic mechanisms by nucleotides in the developing retina was demonstrated in newborn rats and in developing avian retinal cells in culture [305,306]. Application of ATP to isolated rat retinas induces the death of cholinergic amacrine cells that express P2X7 receptors. In developing avian retinal cells in culture, P2X7 receptor-induced death of neuroblasts is dependent on the presence of glial cells and can be blocked by glutamate receptor antagonists.

Nucleotide-induced cytotoxic mechanisms were also demonstrated in mature retinal ganglion cells and photoreceptors. P2X7 receptor-induced death of rat retinal ganglion cells in culture was clearly demonstrated by [307]. The sustained stimulation of these cells with the P2X7 agonist Bz-ATP provokes large increases in intracellular calcium followed by their death. Ganglion cell death induced by nucleotides is blocked by P2X7 receptor antagonists and is also observed in the retina in vivo [308].

P2X7 receptors were implicated in the death of retinal neurons promoted by several kinds of injury. Hypoxia induces a significant increase in the death of retinal neurons in culture that can be prevented by the P2X7 receptor antagonists BBG and oxidized ATP [309]. High pressure transients applied to rat retinas or oxygen/glucose deprivation in human retinas induce significant damage to retinal ganglion cells that is prevented by apyrase and P2X7 receptor antagonists [305,310]. Accordingly, increase in intraocular pressure or activation of Müller cells activates microglia after ATP release and activation of P2X7 receptor in these cells [311,312]. In rats, optic nerve crush (ONC) causes retinal ganglion cell death that is significantly attenuated when P2X7 receptor antagonists are applied during 7 days after the injury [313]. In this specie, intravitreal injection of an agonist of metabotropic glutamate receptors induces Müller cell gliosis with increased ATP released from these activated cells and increased death of ganglion cells that is partially blocked by the application of the P2X7 receptor antagonist BBG, indicating that reactivation of retinal glial cells can induce the death of ganglion cells through the release of excessive ATP and activation of P2X7 receptors [314]. Interestingly, in this model, glia activation induces the upregulation of P2X7 receptor in ganglion cells through a mechanism dependent on ATP released from the activated glia, indicating that gliosis may potentiate the deleterious effect ATP by upregulating P2X7 receptor expression in ganglion cells [314] Upregulation of P2X7 receptor expression in these cells is also observed in rat retinas from eyes submitted to elevated intraocular pressure (IOP) [309] and at the early stages of development of the retina of *rds* mice, a murine model of *retinitinis pigmentosa* disease [315].

Death of retinal photoreceptors induced by P2X7 receptor activation was also demonstrated. Intravitreal injection of ATP causes consistent apoptosis of photoreceptors in the rat retina, an effect that is significantly reduced by P2X7 receptor antagonists [316,317]. ATP released in the subretinal space after retinal detachment promotes pyroptosis of microglia through P2X7 receptor activation, leading to photoreceptors death [318]. A P2X7 antagonist also slows photoreceptor degeneration in the retina of *rd1* mouse model of *retinitis pigmentosa* [316]. In retinas from humans with age-related macular degeneration (AMD), photoreceptor cell apoptosis is also via P2X7 receptor activation [317].

P2X7 glial receptors and retinal development

Activation of the purinergic P2X7 ionotropic receptor increases calcium influx in most of glia cells, which are highly located in Müller glia, astrocytes, microglia and oligodendrocytes [144–146].

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In the avian retina, progenitor emergence around the first embryonic week is modulated by cannabinoid receptor activation [(by the CB1/CB2 agonist WIN 5212-2 (WIN)] [135]. Indeed, progenitor's proliferation decreased as assayed through [(3)H]-thymidine incorporation when cultures were incubated with 0.5-1.0 μM WIN. In addition, the same effect was shown in the presence of URB602 and URB597, inhibitors of the monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), respectively [135,147]. In our hands, retinal cells in culture respond selective to KCl and/or AMPA (neurons) or ATP (glia) while progenitor cells were activated by muscimol or GABA [135,148].

Antioxidants

Glutathione

Glutathione (GSH) is a tripeptide with essential redox duties in the CNS, found at higher concentration in glia cells [319], especially in retinal Müller glia, compared to neuronal compartment which allocates ascorbate as the main antioxidant controlling biochemical processes such as protein folding and maintenance of the redox state by disulfide exchanges, and gene expression regulation [320]. It is suggestive that neurodegenerative diseases may lower the GSH/GSSG ratio, altering the levels of these peptides, and misexpress certain enzymes associated with the biosynthesis of GSH [320,321]. A low GSH/GSSG ratio leads to mitochondrial dysfunction. Nevertheless, the relationship between GSH and the glutamate system in the pathogenesis of nervous system diseases varies from synergism to antagonism [322]. Increasing GSH/GSSG levels systemically is obtained with administration of N-acetylcysteine (NAC). It is important to highlight that in the chicken embryo retina, ́GSH induces calcium influx in cultured Müller glia, but not in neurons [323,324].

GSH has been investigated for its potential roles as both an antioxidant and a signaling molecule. A study using embryonic avian retinal cells, including mixed retinal cells and purified Müller glia cells in culture investigated the effects of GSH on calcium shifts in these cells. As shown, GSH induces calcium shifts exclusively in glial cells, later identified as 2M6-positive cells, while neurons respond to KCl [324]. In addition, P2X7 receptor is involved in the effects of GSH on Müller glia. Intriguingly, GSH's oxidized form, GSSG, fails to induce calcium mobilization in glial cells, underscoring the specific importance of GSH's antioxidant and structural properties in elevating cytoplasmic calcium levels. Additionally, a short GSH pulse was found to protect Müller glia from oxidative damage caused by hydrogen peroxide (H_2O_2).

GSH was also shown to induce GABA release from various retinal cell cultures, including Müller cells, which can be inhibited by the P2X7 blocker BBG or in the absence of sodium [145]. Moreover, GSH induces propidium iodide uptake in Müller cells in culture, and this effect is mediated by the P2X7 receptor. Overall, the study suggests that GSH, in addition to its well-established antioxidant role, functions as a signaling molecule, particularly in Müller glia, regulating calcium shifts and GABA release.

The signaling properties attributed to GSH may be further corroborated by evidence showing high concentrations of the molecule in the retinas of chicks and other model animals [319]. Pow and Crook showed that rabbit Müller cells were strongly immunoreactive for GSH, while neurons presented low or undetectable levels of the molecule [325]. Although glial GSH was shown to be relevant for neuronal protection during stress [326], there is evidence to support the idea that the elevated GSH concentrations found in the retina are not directed to enhance cell survival [327]. In fact, Castagné and Clarke showed that inhibition of GSH synthesis by L-buthionine-[S,R]-sulfoximine can diminish cell death retinal cell death [328].The signaling properties attributed to GSH may be further corroborated by evidence showing high concentrations of the molecule in the retinas of chicks and other model animals [319]. Pow and Crook showed that rabbit Müller cells were strongly immunoreactive for GSH, while neurons presented low or undetectable levels of the molecule [325]. Although glial GSH was shown to be relevant for neuronal protection during stress [326], there is evidence to support the idea that the elevated GSH concentrations found in the retina are not directed

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Vitamin C

Vitamin C, made up of its oxidizing and reducing components ascorbate (AA) and dehydroascorbate (DHA) respectively, is essential for multiple physiological functions. Many mammals are capable of synthesizing vitamin C from glucose but, however, humans do not have the last enzyme responsible for its biosynthesis [329]. Because of this, vitamin C must be ingested through food and supplements. Once absorbed, vitamin C will be distributed to tissues through its transporters, which are of two types: sodium-dependent vitamin C transporters (SVCT), which transport AA, and glucose transporters, which transport DHA [330]. High concentrations of vitamin C are found in the brain, mainly in neuronal cells [331]. Among the physiological processes, vitamin C acts as an enzymatic cofactor in the conversion of dopamine to noradrenaline [332], acts as a reducing agent and scavenger of oxygen and nitrogen free radicals generated during cellular metabolism, increases synaptic activity [333], participates in the formation of the myelin sheath for Schwann cells [334] and acts as a neuromodulator in the Nervous System. Because neurodegenerative diseases are associated with high levels of oxidative stress, AA has been associated as an important therapeutic agent in neurodegenerative diseases. Studies show that the pathophysiological processes of neurodegenerative diseases and neuropsychiatric disorders are improved with nutritional interventions. Among them, the association of treatments with AA has presented a promising scenery. The anti-inflammatory, antioxidant and antiexcitotoxic role of ascorbate is believed to be responsible for its protective actions [335–338].

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Reciprocal interactions between retinal transmitters

Due to the organization of retinal tissue and the massive presence of different types of synapses, especially in plexiform layers, it is highly expected an extensive interaction between these modulatory systems. In many cases, interactions are reciprocal and show distinct levels of complexity during development. Bellow you can find some examples of these interactions in the chicken retina.

Dopamine and adenosine

Dopamine promotes the accumulation of cAMP in developing chicken retina since embryonic day 7 (E7), the maximal effect being observed in E8 and decreasing in subsequent days. The stimulation level in post-hatched retinas (PH) is low [339]. By the other hand, adenosine promotes

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cAMP accumulation in this tissue only after E13, increasing up to E17 and attaining low levels in PH, similarly to what happens with the dopamine stimulus [202]. Interestingly, adenosine can inhibit cAMP accumulation induced by dopamine since early developmental stages when direct stimulation with adenosine is no longer observed [112]. This inhibitory effect is mediated by A1 receptors which are present in early embryonic stages [201]. The mechanism of inhibition as well the functional and embryological consequences remain to be investigated.

Glutamate and adenosine

Glutamate is a major excitatory neurotransmitter in the retina, including the chicken retina [340], where it was found to regulate the release of adenosine, GABA, and vitamin C [94,210,274]. Activation of ionotropic glutamate receptors as AMPA, kainite and NMDA receptors is able to promote a dose-dependent release of purines in cultures of chick retinal cells. Interestingly, this release was shown to be calcium-dependent but also mediated by nucleoside transports and is regulated by a calmodulin-dependent kinase type II (CAMKII) mechanism. Adenosine, but not GABA or choline uptake in the cultures, is also modulated by CAMKII, supporting the hypothesis that the enzyme directly or indirectly modulates nucleoside transport [18].

Glutamate and vitamin C

Glutamate is also able to induce ascorbate (AA) release in cultures of developing chick retinal cells [274]. As stated in a previous section, ascorbate transport is mediated by the sodium-dependent vitamin C transporter (SVCT) which is expressed in the chicken retina as well as in retinal neurons in culture. The release by glutamate is dependent on the presence of sodium ions and blocked by sulfinpirazone, an SVCT inhibitor, indicating that it is mediated by the SVCT working in a opposite direction. The hypothesis is that glutamate activates ionotropic receptors allowing sodium ions entry and its accumulation in the vicinity of SVCT, producing its functioning in the release direction [274]. Interestingly, released AA inhibits glutamate transport through the excitatory amino acid transporter type 3 (EAAT3) present in neurons, promoting an accumulation of extracellular glutamate, activation of NMDA and AMPA receptors, and consequent activation of signaling pathways leading to CREB stimulation [341].

Glutamate and GABA

The interactions between the major amino acids glutamate and GABA were described in the CNS, including in the retina where disturbances in the balance of these two neurotransmitters are involved in neurodegeneration and aging [342]. As stated above, activation of glutamate ionotropic receptors or depolarization with veratridine promotes a transporter-mediated release of GABA in cultures of chicken retina cells [94,343]. Interestingly, the transport of GABA by glial cells is regulated by a glutamatergic input, suggesting an interplay between neurons and glial cells in the retina [344]. In addition, GABA and glutamate regulate Glutamate dehydrogenase (GAD) expression, the main synthezing enzyme responsible for GABA synthesis, in cultured retinal cell [345], indicating a strong interaction between these neurotransmitters in the retina.

Dopamine and glutamate

Dopamine regulates Src kinase activity in cultured chicken retinal cells through D1 receptors, accumulation of cyclic AMP and activation of PKA, which phosphorylates

the C-terminal domain Src kinase (CSK) at the position serine 364 [346] . Stimulation of CSK then leads to Src phosphorylation at the inhibitory domain tyrosine 527 and consequent inhibition of Src kinase activity [347]. It is well known that Src phosphorylates the N2B subunit of NMDA receptor at Tyrosine 1472 thus regulating receptor function [348] . We were able to show that activation of dopamine D1 receptors inhibits NMDA receptor function through this pathway in the retina, showing a possible important pathway linking activation of dopamine receptors and inhibition of glutamate NMDA receptors [346].

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Endocannabinoid and dopamine

Dopamine is found in amacrine retinal cells very early in development, around embryonic day 8 [102]; on the other hand, cannabinoid receptors also emerge early in embryonic stages [120], which control excitability and the levels of second messengers as cAMP or calcium signaling during development. CB1 receptor is highly expressed from embryonic day 5 (E5) until post-hatched day 7 (PE7), decreasing its levels throughout development. While CB1 is heavily located in the GCL and inner plexiform layer (IPL), the CB2 receptor is primarily placed in the inner plexiform layer (IPL) at PE7. Cannabinoid CB1 and CB2 are found in both neurons and glial cells, but MAGL, the enzyme that degrades 2-AG, is only expressed in Müller glia [120]. Tyrosine hydroxylase (TH), the regulatory enzyme that synthesizes catecholamines, are found in amacrine cells that also express both CB1 and D1 receptor. As cyclic AMP (cAMP) is a signaling messenger increased by D1 activation and decreased by CB1 activation, this seems to be an important relay to regulate retina signaling and development [120]. Indeed, neurite outgrowth has been shown to be modulated by cAMP not only in the retina, but in the entire CNS [349,350]. In conclusion, a relationship between the endocannabinoid and dopaminergic systems is found in the avian retina development that defines cAMP accumulation via D1 receptor activation and may influence embryological parameters during avian retina differentiation [121].

Dopamine, glutamate, and vitamin C

Dopamine is also able to promote AA release in chick retina cultures, an effect promoted by stimulation of D1 receptors, accumulation of cyclic AMP and activation of EPAC 2 [351]. Interestingly, AA release is mediated by SVCT since is sodium-dependent and blocked by sulfinpirazone. However, more recent evidence indicates that the release of AA induced by dopamine is mediated by glutamate and activation of AMPA receptors but not NMDA receptors [352]. These results point to the existence of neuronal circuits comprising dopaminergic, glutamatergic, and AA releasing cells in the retina.

Adenosine, vitamin C and nitric oxide

As described above, glutamate can release purines (including adenosine) in the retinal cultures [210] and adenosine regulates the cyclic AMP accumulation induced by dopamine in the retina [112]. Dopamine also promotes the release of adenosine [353] as well as a glutamate-mediated vitamin C release [352]. Recent work shows that adenosine acting on A3 receptors promotes the release of AA and controls the redox balance in retinal neurons in culture [199]. Nitric oxide is also another important neuromodulator in the retina, specially linked to activation of glutamate receptors. For example, nitric oxide regulates the SVCT in retinal cultures increasing its expression through an NFkB- dependent mechanism [273]. Many effects of glutamate mediated by ionotropic receptors also involve nitric oxide production [354]. Indeed, some effects of vitamin C are mediated through accumulation of glutamate and production of nitric oxide. These findings clearly indicate the existence of reciprocal interactions among different neurotransmitters and neuromodulators in the retina.

The Diseased Retina

Retinal diseases encompass a diverse range of ocular disorders that have a profound impact on visual health and patient well-being. They can range from genetic to non-genetic disorders, and degenerative conditions that can lead to varying degrees of vision impairment and, in some cases, even blindness. Genetic retinal degenerations represent a significant subset of retinal diseases with an incidence of approximately 1 in 3000 individuals (https://web.sph.uth.edu/RetNet/)[355], more than 2.5 million people worldwide, posing a significant burden on global eye health. As our understanding of the genetic basis of retinal diseases continues to expand, the identification of causative genes and genetic variants has been increasing, and, today, we have 341 genes with causative variants identified, presenting highly distinct disease courses and phenotypes [356].

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Although an extensive genetic landscape has already been mapped, the mutations responsible for 30% to 50% of cases of inherited retinal diseases remain undisclosed [357].

Non-genetic retinal diseases constitute a diverse array of ocular disorders with a global impact, often regardless of an individual's genetic constitution. These conditions can stem from a variety of factors, including the natural aging process, lifestyle choices, infections, and environmental influences. Age-related macular degeneration (AMD) stands as one of the most prevalent non-genetic retinal diseases, impacting 196 million people in 2020, with a prediction to increase to 288 million people worldwide in 2040, particularly among the elderly population [358]. It leads to central vision loss through a combination of the accumulation of deposits (drusen) and, in the non-neovascular form, retinal pigment epithelium abnormalities or, in the neovascular form, abnormal blood vessel growth, both in the macula, leading to damage and impairment of the central vision [359]. Similarly, diabetic retinopathy, intricately tied to diabetes, is another widespread non-genetic condition characterized by the deterioration of retinal blood vessels, potentially leading to severe vision loss if left untreated [360]. Retinopathy of prematurity (ROP), predominantly affecting premature infants due to excessive oxygen exposure during early medical care, is another example, known for its capacity to induce vision problems or even blindness [361]. Additionally, glaucoma, often associated with elevated intraocular pressure, is a global illustration of non-genetic ocular diseases [362].[362]. These various non-genetic retinal diseases underscore the importance of regular eye examinations and proactive healthcare measures to prevent or manage vision impairment on a global scale.

In the face of the challenges posed by neurodegenerative conditions leading to blindness, the field of ophthalmology and vision science continues to push the boundaries of medical research. The convergence of genetics, innovative therapies, and cutting-edge technologies offers a ray of hope for those affected by these diseases. The relentless pursuit of new treatments, including gene therapies, stem cell transplantation, and precision medicine, is promising. These advancements hold the potential to not only slow the progression of vision loss but also, in some cases, to restore sight. All these new and innovative therapies are on the cusp of revolutionary breakthroughs that may one day conquer the challenges posed by neurodegeneration and blindness, offering a brighter future for those impacted by these conditions.

Glaucoma

Glaucoma is an optic neuropathy characterized by an insidious onset and gradual progression that comprises a group of neurodegenerative diseases marked by structural damage to the optic nerve with axonal loss and retinal ganglion cell (RGC) degeneration by apoptosis [363]. It is the leading cause of blindness around the globe and the elderly are more susceptible to develop the disease. Aging populations have been increasing in both developed and in development countries, and the rise of diseases associated with aging can impact the quality of life of individuals and economic growth. Today glaucoma is considered a major public health problem and the 3rd main cause of long-term disabilities experienced by individuals [364]. Its prevalence varies by geographic region and demographic factors, with higher rates observed among people of African, Asian, and Hispanic descent. The number of people with glaucoma worldwide is estimated to be 80 million, increasing to 111.8 million in 2040 [365]. In Brazil, the cases rose from 900,000 in 2010 to 2.5 million in 2020 [366]. Alarmingly, up to 40% of glaucoma patients can progress to blindness. Glaucoma is highly heritable, and a true family history of glaucoma increases the risk in a first-degree relative nearly eight times compared with the general population [367]. Elevated intraocular pressure is a major risk factor in glaucoma. It is widely believed to contribute to the compression and damage of the optic nerve fibers, thereby accelerating the degeneration of RGCs. However, it is important to note that not all individuals with high IOP develop glaucoma, and, conversely, glaucoma can occur in those with normal IOP, suggesting that other factors play a crucial role [368]. However, existing treatment paradigms focus on reducing intraocular pressure, mainly through the daily use of eye drops, sometimes associated with side effects or invasive surgical interventions [369,370]. The frequent inadequacy of ocular pressure-lowering approaches, substantial rates of treatment non-adherence

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affecting 30-70% of patients, and resulting financial burdens indicate the need for long-lasting, neuroprotective therapies.

The increased pressure induced by glaucoma can cause ischemic events in the retina, and in literature there are some evidence of cannabinoids mediating or regulating damage after this events. Blockage of CB1 and CB2 in an ex vivo model of ischemia can decrease the damage induced by oxygen and glucose deprivation (OGD) [143]. In an interesting way, using the same model, blockage of TRPA1 (Transient receptor ankyrin 1), a channel that can be activated by cannabinoids and their substrates, inhibit the damage induced by the OGD [143]. After the role of TRPA1 on an acute model of glaucoma has been shown, it was also demonstrated that TRPA1-/- knockout mice show a complete blockade of retinal damage (inhibiting the decrease of retinal thickness, increase of oxidative stress, increase of caspase activity) in a model of increased intraocular pressure and 2 or 7 days of reperfusion [371].

Diabetic retina

Diabetes Mellitus represents a major public health problem. In 2017, the International Diabetes Federation estimated that 425 million people had diabetes and expected the number of people affected to be approximately 629 million by 2045. Diabetic retinopathy (DR) is a microvascular complication of diabetes mellitus and is the most common cause of blindness affecting the working age population [372–374]. The increasing knowledge of the pathophysiology of the disease allowed the identification at a more and more early stage, increasing the possibility of treatment. Around 126.6 million people worldwide were affected by the condition in 2011, and this number is expected to rise to 191 million by 2030 [375], with 56.3 million of them at high risk of visual impairment, a group that includes individuals with proliferative diabetic retinopathy and diabetic macular edema [376], making the disease a major burden on the healthcare system, with a predicted increase in the number of people suffering from visual impairment [377].

Diabetic retinopathy is clinically characterized by vascular alterations, and it is divided into two stages, non-proliferative (NPDR), which represents the initial stage of DR, when microaneurysms, small hemorrhagic spots and exudates can already be observed on fundus examination, but the patient may be asymptomatic, and proliferative (RDP), a more advanced stage characterized by neovascularization and greater visual consequences with the expansion of poorly formed vessels into the vitreous, increased risk of retinal detachment and formation of macular edema [374].

However, many studies have been demonstrating that DR is a neurovascular disease, with changes in neuronal morphology, reduction of synaptic proteins, changes in neurotransmitter systems, and neuronal death in the retina [378–383], for review [378,384]; even before vascular alterations occur and can be clinically detected [385]; for review [386]. Early changes in electrical activity, thickness of the human retina, electrophysiological and visual perceptual changes prior to vascular changes have also been shown [77,387] for review [386]. Retinal pigmented epithelium cells (RPE) also showed oxidative stress [4,388] tight junction destruction [389–391] and apoptosis [392]. Therefore, all cell types that constitute the retina are affected by persistent hyperglycemia in diabetes and contributed to the disastrous outcome.

Several pathways have been associated with the development of DR induced by hyperglycemia/diabetes: formation of advanced glycated end-products (AGEs), increase in the polyol pathway, diacylglycerol/PKC and hexosamine pathway. However, they all have a common axis of activation: oxidative stress [393–395]. Therefore, antioxidative stress has become a promising strategy for the treatment of DR [395]. Although there are several preclinical studies showing promising results with antioxidants, clinical trials have been scarce with controversial outcomes [24] for a brief review). Nuclear factor erythroid 2-like 2 (Nrf2) / Kelch-like ECH-associated protein 1 (Keap1) pathway is a crucial pathway to fight oxidative stress. Nrf2 is a transcription factor binds to a specific promoter region, the antioxidant-responsive element (ARE), stimulating the transcription of several cytoprotective genes that triggers a cellular antioxidant response, crucial pathway to fight oxidative stress. In physiological conditions, Keap1 binds to Nrf2 leading to ubiquitination and degradation of Nrf2, which controls the levels of this transcription factor. With the increase in reactive oxidative

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species (ROS), Nrf2 dissociates from Keap1 and translocates to the nucleus, stimulating the transcription of ARE-containing genes. Therefore, the oxidative stress stimulates the increase in Nrf2 stability and nucleus levels, which can induce an antioxidant response. However, it has been systematically shown, in vitro and in vivo studies, that exposure to high glucose induces a decrease in Nrf2 levels, particularly in the nucleus, but also total Nrf2, in all types of retinal cells: RPE cells, mainly investigated in ARPE-19 cells, [396–398] endothelial cells, mainly HREC cells [399], Müller cells [24,400], and ganglion cells [401] . In vivo experiments also show that diabetes decreases Nrf2 retinal content even in early periods of diabetes, before vascular alterations occur [382,383,402,403]. As mentioned, Nrf2 controls the gene transcription of several antioxidant signals, some of them crucial to glutathione (GSH) generation, such as glutathione peroxidase and catalytic subunit (xCT) of the transport X_c^- system [24] for review). This transporter uptakes cystine, important and limiting precursor to the GSH synthesis [404]. So, a decrease in Nrf2 in high glucose condition, in retinal cell culture, or diabetes leads to a reduction in glutathione peroxidase, X_c^- system and, consequently, glutathione retinal levels as well as in different retinal cell types (RPE, endothelial, Müller, ganglion cells) [405].

Retinas from diabetic animals have less Nrf2 bound to the promoter of the catalytic subunit of the x_c^- system (xCT) since early stages of diabetes [383], which could explain the lower expression of xCT. Besides, Nrf2 also controls the gene transcription of other antioxidant important enzymes, such as hemoxigenase, NQO1, catalase, which are also decreased in retinal/cell types in diabetic retinas or cultures exposed to high glucose in a Nrf2-dependent mode [406]. Accordingly, an increase in oxidative stress, followed and dependent of Nrf2 reduction, is observed in the retina of diabetic animals as well as in the cell types. Although high glucose exposure and diabetes induce oxidative stress, which activate Nrf2 pathway, most of the studies shows that the maintenance of the hyperglycemia induces the reduction of Nrf2, hampering the cell capacity to fight oxidative stress and leading to cell death through ferroptosis [397] and apoptosis [405]. The apparently contradictory effect lies on a lot more complex Nrf2-regulating signaling pathways. It has been shown that Nrf2 is closely regulates by Akt/GSK3 pathway [407,408]. Nrf2 degradation/nuclear extrusion is activated by GSK3b, which is blocked by Akt phosphorylation and inhibition of GSK3 [407,408]. Several studies report a reduction in Akt activation in high glucose or diabetes conditions [396,401] which can be via the classical PTEN/Akt pathway. PTEN activity is increased in hyperglycemia/diabetes, decreasing activated-Akt level [396]. In addition, it was shown that hyperglycemia increases PP2A activity, which dephosphorylate Akt and stimulate GSK3b [409]. Hyperglycemia/oxidative stress stimulates the regulated in development and DNA damage 1 (REDD1), a stress induced protein that promotes the association of PP2A and Akt, decreasing Akt phosphorylation/activity and the Akt-induced inhibition of GSK3b.

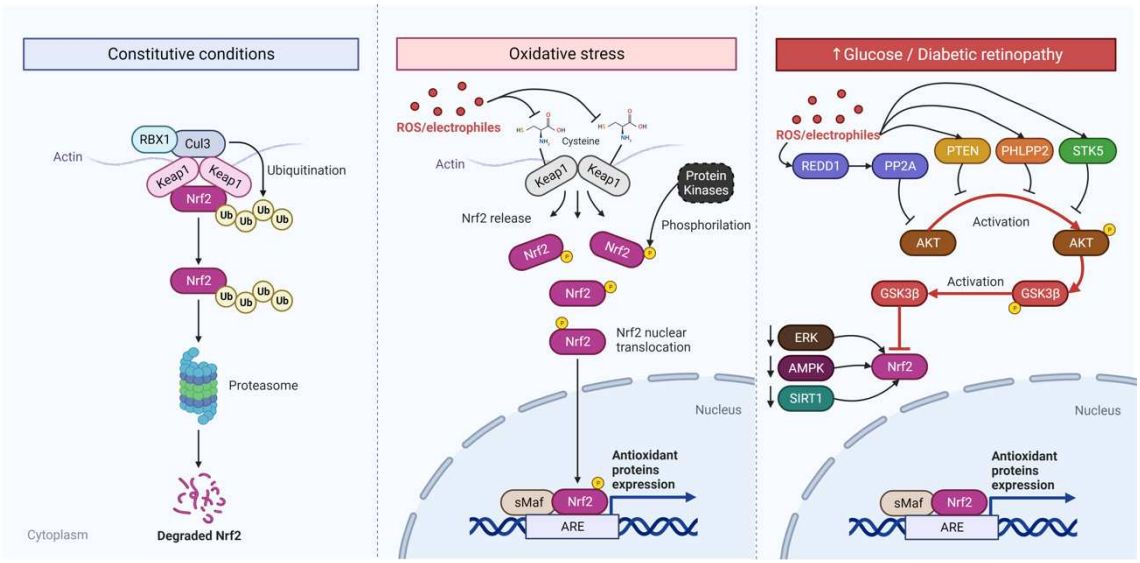

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Figure 2. Regulatory pathways in retinal stress response and diabetic retinopathy progression. Cellular mechanisms of the retinal response to stress under constitutive conditions, oxidative stress, and in the context of high glucose (HG) or diabetic retinopathy (DR). The left panel depicts the constitutive degradation pathway of the transcription factor Nrf2, which is bound by the Kelch-like ECH-associated protein 1 (Keap1) and targeted for ubiquitination and subsequent proteasomal degradation under normal conditions. The central panel shows the response to a light to mild oxidative stress, where reactive oxygen species (ROS) or electrophiles modify cysteine residues on Keap1, leading to the release of Nrf2. Nrf2 can also be phosphorylated by protein kinases, which promotes its translocation into the nucleus. Once in the nucleus, Nrf2 binds to antioxidant response elements (ARE) in the DNA, leading to the expression of proteins of antioxidant response. The right panel focuses on the molecular pathways involved in diabetic retinopathy, a condition characterized by increased glucose levels that lead to retinal damage. Here, the diagram outlines the interplay between ROS/electrophiles and various signaling molecules, including REDD1, PP2A, PTEN, and PHLPP2, all of them are increased by HG/DR, inhibiting AKT. Activated AKT (p-AKT) phosphorylates and inactivates GSK3β, which in turn affects Nrf2 activity. Additionally, the diagram indicates the involvement of ERK, AMPK, and SIRT1, which positively regulates Nrf2, but are all decreased in HG/DR, inhibiting Nrf2-activated antioxidant response.

In diabetes, or high glucose conditions, REDD1 levels augment and induces Nrf2 degradation through GSK3 activation [410,411], hampering the Nrf2-induced antioxidant response. REDD1 is also directly activated by oxidative stress, generating positive feedback, worsening the oxidative stress [411]. The Akt inhibition by REDD1 leads to a decrease in the activity of mTOR, which disinhibited 4E-BP1 that represses the VEGF mRNA translation [412].

Since hyperglycemia increases REDD1, a consequently increase in oxidative stress and VEGF levels is seen, contributing to angiogenesis and cell death [413].

Finally, in ARPE-19 cells, Nrf2 can be also positively regulated by SIRT-1 and AMPK [397,414]. Therefore, hyperglycemia in diabetes, or high glucose exposure in vitro, can induce, by different mechanisms, a decrease in Nrf2 levels and an impairment in the antioxidant Nrf2-stimulated response.

The inflammatory component also appears to be a central event in the progression of DR. The increase in the expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin added to higher rates of leukocyte adhesion and leukostasis are phenomena observed in animal models of diabetes and human patients, and are associated with damage to the blood-retinal barrier and the loss of endothelial cells [415–419]. Increased expression of chemokines such as MCP-1, MIP-1α and MIP-1β, and cytokines such as TNF-α, IL-6, IL-8 and IL-1β also appear to be involved in the pathogenesis of DR [420–423]. Glial cells in the retina such as astrocytes, Müller glia and microglia orchestrate the inflammatory reaction, producing and releasing the aforementioned factors [424,425]. Importantly, it has been shown that the increase in inflammatory cytokines, mainly TNF-α, IL-6, and IL1-β, occurs due to Nrf2 decrease induced by high glucose or hyperglycemia in diabetic animals, and preventing Nrf2 inactivation prevents the inflammatory response [405,426].

Because of the crucial role of Nrf2 to control oxidative stress and, consequently, inflammation and cell death signaling pathways, like ferroptosis and apoptosis, preventing, acting directly or indirectly, the reduction of Nrf2 protects retinal cells from degeneration [397,401,405,426]. The present medical treatments for DR include glycemic control, laser therapy, glucocorticoid therapy, anti-VEGF intraocular injections, etc., but none of them cure neither stops the disease progression and all combat the vascular alterations seen in a very advance stage of the disease [427]. Although the available treatments are important to ameliorate the clinical deficits, new approaches, especially those that can be used earlier, are necessary to improve treatment and avoid blindness.

Drugs that inhibit important pathways for the progression of this disease have been tested for its treatment. Examples of this were tests with aminoguanidine, an inhibitor of the advanced glycation species pathway [428], to inhibit inflammatory pathways and the systemic administration of several antioxidants [429–431]. Other drugs that have been tested include other antiangiogenic drugs that modulate other factors such as PDGF, b-FGF, Ang-1, 2, and the Ang-1,2 receptor, Tie2,

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drugs with anti-inflammatory effects such as corticosteroids (show improvements in symptoms of diabetic retinopathy but can cause increased intraocular pressure and cataracts) and integrin and interleukin 6 inhibitors, alpha-lipoic acid (mitochondrial antioxidant), lutein (carotenoid with antioxidant action), ARA290 (peptide derived from erythropoietin) and darapladib (phospholipase A2-associated lipoprotein inhibitor – LpPLA-2) [374]. However, the results of these clinical studies were inconclusive or suspended due to side effects.

Recently, several preclinical studies have been focusing in searching substances that inhibit the impairment in Nrf2 pathway induced by diabetes in neural and vascular retinal cells: Acteoside [432], Maslinic Acid [4], Astragaloside-IV [397], Urolithin A [426], Hydroxysafflor yellow A [396], Carnosol [399], Astaxanthin [402], amygdalin [433], among others. However, as for other previous approaches, it will be critical to investigate the protective ability of these agents in diabetic patients and the potential of deleterious side effects.

Investigation of innovative therapeutic strategies

Gene therapy and the future of vision recovery

Gene therapy is a groundbreaking medical field that has achieved remarkable progress over the past two decades, providing newfound hope for the treatment of previously untreatable and hereditary diseases. Among the numerous applications within gene therapy, retina gene therapy emerges as a particularly promising avenue, offering a potential solution to a broad spectrum of ocular disorders and vision impairments. The eye, with its unique characteristics, presents a compelling organ for gene therapy. Its privileged immune status makes it an ideal candidate for genetic interventions, decreasing potential immune responses to gene therapy treatments [434]. Moreover, the eye's accessibility for medication delivery is unmatched, allowing for targeted and minimally invasive interventions. It is a critical consideration given the complex structure and sensitivity of ocular tissues. Among these tissues, the retina is a primary candidate for gene therapy. Its visibility enables precise monitoring and evaluation, which is essential for assessing the effectiveness of gene therapy. The absence of lymphatic vessels, a direct blood network in the outer layers, and a lack of cell division post-birth make the retina an ideal canvas for achieving sustained transgene expression.

In recent years, substantial improvements have been made in identifying the genes responsible for genetic retinal diseases [358], thanks to advanced techniques like next-generation sequencing, single nucleotide polymorphism microarrays, and comparative genomic hybridization. This burgeoning genetic knowledge has laid the foundation for more precise therapeutic interventions, moving beyond symptom management toward targeting the root causes of these conditions.

The advent of gene therapies has ushered in a new era of hope for individuals afflicted by monogenic eye diseases. Ophthalmology has been at the forefront of gene therapy research, capitalizing on the eye's unique characteristics for effective gene delivery. In 2017, the approval of voretigene neparvovec-rzyl (Luxturna) marked a significant milestone, becoming the first FDA-approved in vivo gene therapy for RPE65-associated biallelic variants. Luxturna belongs to the concept of gene replacement or augmentation in which a functional copy of a damaged, non-functional gene is added to augment the production of functional protein, being a natural fit to inherited retinal diseases caused by loss-of-function mutations. The achievement with Luxturna has not only transformed the treatment landscape but has also ignited a flurry of research activities in the field of ocular gene therapy [435].

Subsequently, the field expanded its horizons, recognizing that many complex diseases are influenced not only by the primary disease-causing gene but also by modifier genes that can either exacerbate or mitigate the condition's effects [436,437]. The concept of modifier gene therapy is now at the forefront of research, aiming to fine-tune the treatment of multifactorial disorders like glaucoma, diabetic retinopathy, macular degeneration, between others, by targeting genes that play a pivotal role in disease progression. OCU400 (Ocugen Inc.) is a modifier gene therapy to treat people with inherited retinal diseases, Retinitis Pigmentosa, caused by a broad range of gene mutations, and

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is currently in clinical trial phase 2. The therapeutic candidate is an adeno-associated virus serotype 5 (AAV5) containing the gene for human nuclear hormone receptor NR2E3 and as a modifier gene therapy, it expands the patient reach treating multiple mutations with a single product instead of developing a product for every mutation, and potentially decreasing costs [438]. This evolution signifies a shift towards more precise and personalized approaches, with the goal of not only treating symptoms but also addressing the root causes of complex diseases, ultimately paving the way for more effective and tailored therapeutic interventions.

Gene therapies using CRISPR/Cas9 technology are also in clinical trials. In these gene editing therapies, mutations in a gene are corrected or expression of the mutated protein is reduced to alter a diseased state. In early 2020, an open-label, single ascending dose study started to enroll LCA10 patients to test CRISPR-Cas9 gene edition to correct the IVS26 mutation (NCT03872479), by delivering a highly specific small guide-RNAs to the gene CEP290, along with SaCas9 under control of a photoreceptor-specific GRK1 promoter, packaged into an AAV5 vector into the subretinal space [439]. In 2022 the developers released some results in which 3 out of 14 patients showed clinically meaningful improvements in best-corrected visual acuity. The results provided a proof of concept that CRISPR-based gene editing can be safely delivered to the retina, however the developers have made the decision to pause enrollment while looking for partners to continue the studies.

In advanced cases of retinal degeneration in which the photoreceptors are very compromised, optogenetics come as an innovative tool involving the delivery of light-sensitive microbial opsins to the remaining retinal cells using gene therapy [440] With optogenetics, it is possible to treat the disease independent of the underlying gene defect. It provides new photosensitive genes, such as channel rhodopsin, halorhodopsin, and melanopsin, to the retina's output cells, the ganglion cells, or bipolar cells, adding the light-activity to these cells in their existing neural networks [441,442]. Promising results in preclinical rodent and nonhuman primate models, led to different clinical trials (NCT05417126, NCT04945772, NCT04945773, NCT02556736, NCT03326336) less than a decade after the first attempt at visual restoration using this approach. However, optogenetics still require optimization to allow for complex visual processing and to increase the sensitivity of the photosensitive proteins that are currently in use.

The future of vision recovery through retinal gene therapy holds great promise, but also presents a complex landscape of challenges. Advances in vector design are expected to prioritize reducing immunogenicity, enhancing target specificity, and improving transduction efficiency, with potential shifts towards less immunogenic vector options. Overcoming the payload size limitations of vectors may involve innovative strategies, such as non-viral techniques or dual/triple transduction methods. While the retina's immune privilege makes it an ideal candidate for gene therapies, it introduces unique obstacles, including the identification of disease-causing genes, precise delivery, optimal administration routes, clinical feasibility, and managing immune responses. Additionally, physically delivering therapeutic products to the delicate and isolated retinal tissue remains a formidable challenge. Nevertheless, ongoing research and a multitude of creative approaches demonstrate the determination of the scientific community to unlock the full potential of retinal gene therapy, offering hope for the restoration of vision and a brighter future for individuals with retinal diseases.

Cell reprogramming

Diseases that affect the retina usually lead to visual loss, which is very debilitating. Many research groups are then investing on the study of innovative therapeutic approaches to stop or delay disease progression, protect the affected cell populations or even to promote the regeneration of the retina for the reversal of progressive blindness. The regenerative approaches are directed either to the generation of new neurons *ex vivo* for transplantation [443], or to the generation of the affected neurons from endogenous cell sources, such as Müller glia (MG) [444]. In teleost fish MG acts as a multipotent stem cell which, in response to damage, dedifferentiate generating MG-derived progenitors which then give rise to all retinal cell types [445]. However, this regenerative potential is virtually absent in mammalian retinas [444,446]. Recently, Hoang at al described the differential activation of signaling pathways in response to damage in fish, chick, and mice retinas [447]. They

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showed that Nuclear Factor I (NFI) transcription factors maintain and restore quiescence in mammalian MG while in zebrafish and chick they are essential for regeneration when MG transit from quiescence to reactive [447].

Many research groups are then investing in identifying and testing reprogramming strategies to reactivate the regenerative potential of Müller glia mainly through the modulation of the expression of specific transcription factors. Using transgenic mice to overexpress the proneural transcription factor Ascl1 alone and in combination to damage, Dr Reh's group showed the generation of new neurons from MG, which are mostly bipolar cells [448,449]. In addition, when Ascl1 was used together with a histone deacetylase inhibitor they were able to generate bipolar cells from MG of adult damaged retinas, and these new neurons formed synaptic connections [450]. Recently, Todd and coworkers generated Retinal ganglion-like cells (RGC-like cells) with the combination of Ascl1 and another proneural bHLH factor: Atoh1 [451]. And when Pou4f2 and Isl1 were added to this equation more molecular characteristics of RGCs [452] were obtained, although no demonstration of the ability of these cells to project axons to brain targets were shown.

On the other hand, studies using AAV vectors have also presented data on the generation of retinal ganglion cells, upon combined coexpression of Math5/Atoh7 and Brn3b/Pou4f2 [453] and downregulation of Ptbp1 [454]; or photoreceptors through two AAV injections, one with beta-catenin to stimulate proliferation and the other with Otx2, Crx and Nrl [455]. However, demonstration of the lack of specificity of alleged glial promoters raised concerns on some of these studies and highlighted the need for proper strategies for tracing MG as the cell of origin in protocols for MG to neuron reprogramming [456–459]. Groups are also searching for alterative or complementary tools for MG reprogramming such as investigating ways to identify novel cell-type specific regulatory regions regions to drive gene expression [460], modifications in AAV- carried sequences testing modifications in AAV-carried sequences [461], or screening compounds to increase neurogenic reprogramming of MG [462]. Great advance was obtained in the last decades on the identification of critical approaches necessary to obtain reliable information which could work as proof of principle for the investment on regenerative strategies for ocular diseases [463] .

However, many challenges are still ahead, as emphasized by alliances of investigators who are working in collaborative networks to promote advances in this field [464]. Even though relevant data accumulated it is essential to guarantee that translational approaches are designed to promote the generation and integration of new neurons in the retinal tissue to yield restoration of lost functions.

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