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By

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Abstract

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Optogenetics, the ability to control cells with light, has revolutionized neuroscience by endowing researchers with hyper precise tools which can dissect neural circuits. However, the use of optogenetic effectors is not limited to academic studies on neurons. This dissertation examines two alternative applications of optogenetics: to study glia-neuron interactions and to therapeutically restore light sensitivity to degenerate retinas. In order express these optogenetic effectors, Adeno-Associated Virus (AAV) was used to deliver transgenes.

Over the course of these studies, the ability to specifically target certain types of cells became paramount. Specifically, special AAV capsid variants had to be used in order to transduce Müller glia, the principal glia cell of the retina. The capsid variants, 7m8 and ShH10, are not specific for Müller cells, so different glial promoters were investigated. Two promoters evaluated, GLAST and gfaABC1D, had unexpected expression profiles. Although GLAST is a Müller cell specific protein, the GLAST promoter was unable to restrict transgene expression to Müller glia. However, the smaller the gfaABC1D promoter was able to completely restrict transgene expression to retinal glia in both health and disease.

With new methods to selectively transduce Müller glia, their role in glutamate uptake was investigated. The electrogenic transporters used to transport glutamate against its concentration gradient require a hyperpolarized membrane potential made possible through high expression of potassium channels. If glutamate uptake was dependent on Müller cell membrane potential, then transient depolarization of the Müller glia should inhibit glutamate uptake. To transiently depolarize Müller cells, they were targeted to express a bistable channelrhodopsin2 (ChR2) mutant (C128S/D156A, “BiChR2”). The effect of BiChR2 induced Müller glia depolarization on the retinal light response was investigated by examining the electroretinogram (ERG). While there were no significant changes to the ON-bipolar cell generated ERG B-wave, subtle temporal changes in arose that should be investigated further.

Optogenetics have a therapeutic potential to restore vision in degenerate retinas. People with late stage retinal degenerative disease lose sight as a consequence of photoreceptor death. However, other retinal cells remain. Endowing the surviving retinal cells with the ability to sense light through optogenetics could restore sight. The effectiveness of optogenetics tools used for vision restoration were analyzed based on their light and temporal sensitivity.

For Spencer McRae Baker

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Table of Contents

Abstract……...1

Dedication Page.…….....i

Acknowledgements.…….....ii

Table of Contents.……...iii

List of Figures and Tables.…….……..v

Main Text……...1-52

Chapter 1: Introduction……...1-11

The Retina……1

Müller Glia……...3

Retina Disease……..4

Targeting Müller cells……..5

Optogenetic Applications in Müller cells……6

Optogenetics for Vision Restoration……7

References……7

Chapter 2: Targeting Müller cells in Health and Disease……...12-28

Abstract……..13

Introduction……13

Methods and Materials……...14

Results……15

Discussion……..23

Supplementary Material…….24

References……..26

Chapter 3: Optogenetic Applications in Müller cells…….…….29-41

Abstract……..30

Introduction……30

Methods and Materials……...31

Results……33

Discussion……..36

Supplementary Material…….37

References……..40

Chapter 4: Innovative Optogenetic Strategies for Vision Restoration……..41-52

Abstract……..42

Introduction……42

Ion Channels……..44

GPCRs……...46

Delivery Methods……..47

Multi-Effector Therapy……..48

Conclusions……49

References……..49

List of Figures and Tables

Chapter 1: Introduction

Figure 1.1……2

Figure 1.2……6

Chapter 2: Targeting Müller cells in Health and Disease

Figure 2.1……...16

Figure 2.2……...17

Figure 2.3……...20

Figure 2.4……...21

Figure 2.5……...22

Supplementary Table 2.1……...24

Supplementary Figure 2.1……25

Chapter 3: Optogenetic Applications in Müller cells

Figure 3.1……...34

Figure 3.2……...35

Figure 3.3……..36

Figure 3.4……..37

Figure 3.5……..38

Supplementary Table 3.1……...39

Chapter 4: Innovative Optogenetic Strategies for Vision Restoration

Figure 4.1……...43

Figure 4.2……...45

Chapter 1

Introduction

The Retina

The retina is the thin piece of light sensitive neural tissue at the back of the eye. Responsible for converting light into electrical signals, the retina processes and sends visual perception information to the brain. This tissue has a simple laminar organization with distinct cell specific layers (Fig. 1.1). Facing the vitreous, the Ganglion Cell Layer (GCL) contains ganglion cells, which directly innervate the brain with their long axons, and some amacrine cells. In the Inner Nuclear Layer (INL) amacrine cells, bipolar cells, and horizontal cells can be found along with Müller cell somas. Between the GCL and the INL is the Inner Plexiform Layer (IPL), which contains the synaptic meshwork that allows the INL and GCL to communicate. Likewise, above the INL is the Outer Plexiform Layer (OPL) where INL cells synapse onto Outer Nuclear Layer (ONL) photoreceptors.

Counterintuitively, light must pass through all the retinal layers to reach the light sensitive photoreceptors. There are two types photoreceptors, rods and cones, with the rods responsible for low light vision and the cones responsible for high acuity bright color vision. Rods and cones use special GPCRs that are associated with the chromophore 11-*cis* retinal to initiate the light response[1]. The chromophore isomerizes when exposed to light into all-*trans* retinal, which causes activation of the opsin GPCR leading to a cascade of biochemical events that initiate the light response. Rods employ the hyper-light sensitive rhodopsin[2], while human cones can contain one of three different cone-opsins: long, medium, and short wavelength (mice and most other mammals only have two, long-medium and short wavelength cones)[3].

Light activation of these opsins hyperpolarizes the membrane potential of photoreceptors. In the dark, photoreceptors are constantly depolarized and release glutamate onto the OPL. Light induced isomerization of 11-*cis* retinal to all-*trans* retinal causes the opsin to become active, which in turn activates its associated G-protein, transducin. The transducin heterotrimer dissociates with the alpha subunit activating phosphodiesterase (PDE) which lowers the concentration of cGMP. The lower concentration of cGMP closes cyclic nucleotide gated channels, hyperpolarizing the cell and preventing the release of neurotransmitter[1].

Photoreceptors release neurotransmitter onto horizontal and bipolar cells in the OPL. Horizontal cells provide sign inverting inhibitory feedback onto photoreceptors initiating the generation of inhibitory surround of receptive fields as well as contributing to light adaptation, gain control, and color opponency in downstream ganglion cells. Bipolar cells process visual information by generating signals in response to the increases or decreases in light intensity[5]. While there are a variety of bipolar cell types that can be identified by sublaminar positioning and morphology, they can generally be divided into two types of cells: “ON” or “OFF”. ON bipolar cells are “on” or depolarized when the photoreceptor it synapses with becomes inhibited by light. To do this, they employ a special metabotropic GPCR, mGluR6, which depolarizes the ON bipolar cell in the absence of glutamate[6–8]. Conversely, OFF bipolar cells use a variety of ionotropic glutamate receptors to become depolarized when their upstream photoreceptor is in the dark and releasing glutamate[9,10].

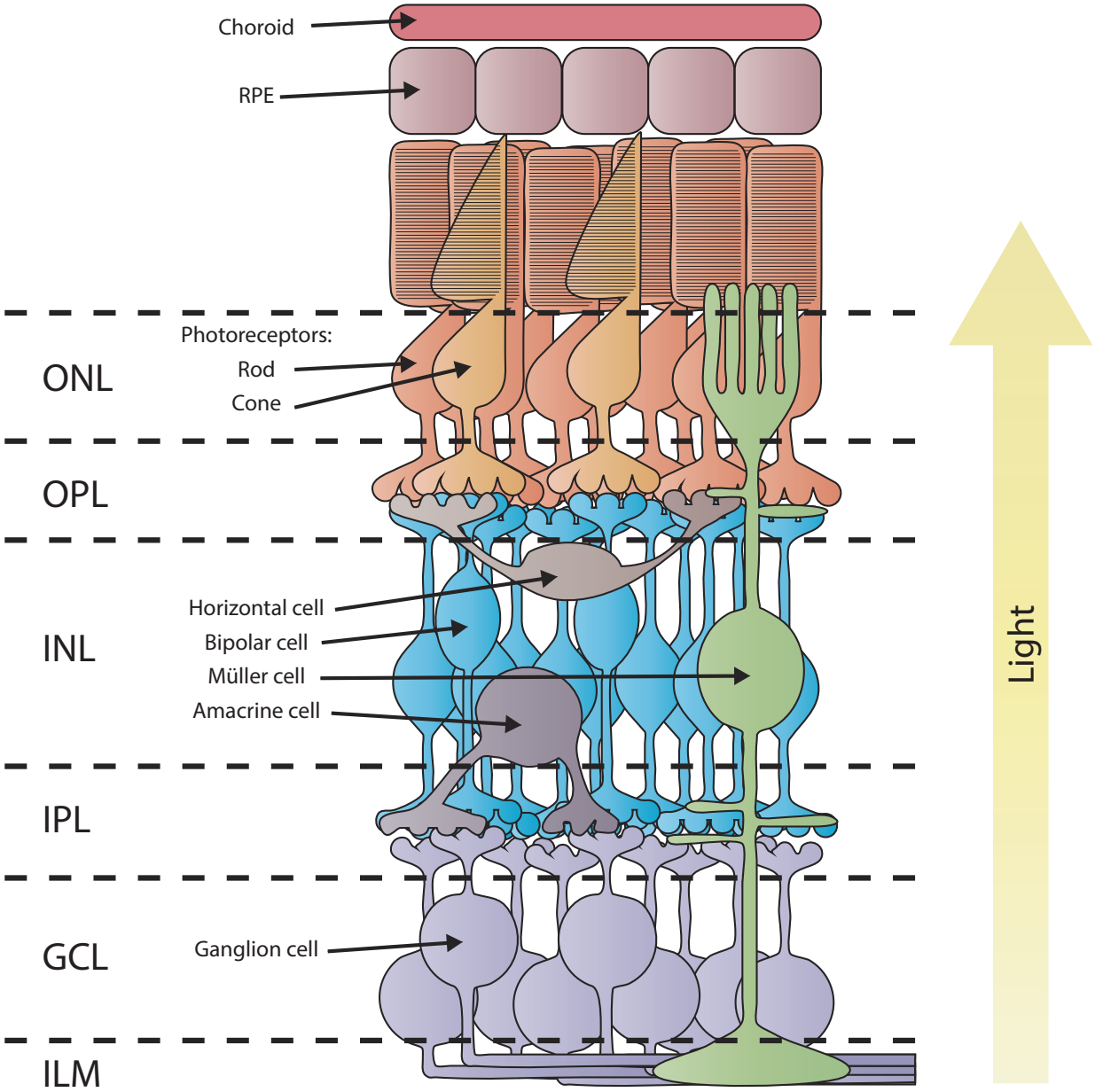

Figure 1.1. Diagram of the mammalian retina. The retina is a laminar neural tissue at the back of the eye. The Inner Limiting Membrane (ILM) faces the vitreous cavity of the eye and is partially composed of Müller end feet and ganglion cell axons. Ganglion cells and some amacrine cells can be found in the Ganglion Cell Layer (GCL). The Inner Plexiform Layer (IPL) contains the synapses that connect the GCL and Inner Nuclear Layer (INL) cells. The INL contains amacrine, bipolar, and horizontal cells along with Müller cell somas. The Müller cells span the entire length of the retina with processes that innervate the plexiform layers. The Outer Plexiform Layer (OPL) connects the INL to the Outer Nuclear Layer (ONL) which contains the photoreceptor nuclei. Of the two types of photoreceptors, the rods are responsible for low light vision and cones are responsible for high light, high acuity vision. The photoreceptor outer segments are continuously phagocytosed by Retinal Pigmented Epithelium (RPE) cells. Above the RPE lays the choroid, the vascular layer that supplies most nutrients to the outer retina.

Rods and cones synapse onto rod and cone bipolar cells respectively. While cone photoreceptors can synapse onto ON and OFF cone bipolar cells, the rod photoreceptor only has the rod ON bipolar cell. The rod light perception circuit co-opts the cone circuit via AII and A17 amacrine cells to contribute to cone ON and OFF pathways[11,12]. Certain amacrine cells also help generate other retina circuit level visual processing like direction selectivity.

The final recipients for visual information in the retina are the retinal ganglion cells. There are over 30 types of mouse retinal ganglion cells that can detect ON, OFF and ON/OFF center surround receptive fields generated by inner retina processing by bipolar, amacrine, and horizontal cells[13,14]. The ganglion cell bodies can be found in the GCL, but their long axons bundle at the optic nerve head and travel to central targets in the brain, including the lateral geniculate nucleus of the thalamus.

Müller glia

Maintaining and supporting this highly active neural tissue are the Müller cells. Spanning the entire thickness of the retina, Müller glia are the principal glia cell of the retina. Functionally similar to Central Nervous System (CNS) astrocytes, Müller cells provide nutrients, remove waste, recycle neurotransmitter, maintain ion homeostasis, and provide neurotrophic support[15]. All the retinal neurons are directly embedded in this radial glia cell which provides an elastic structure.

Neurons require vast metabolic support to maintain their membrane potential via the active transport of ions against their concentration gradient. The brain by weight is the most metabolically active organ consuming 20% of the body's energy[16]; and the retina exceeds the brain in oxygen consumption[17]. Most neurons in the retina use oxidative metabolism to produce ATP to maintain their membrane potential and perform other necessary cellular activities. The outer retina draws most of its nutrients from the choroidal blood supply behind the Retinal Pigmented Epithelium (RPE) (Fig. 1.1). In vascularized mammalian retinas, the inner retina is fed by the central retinal artery which is a branch of the ophthalmic artery and also feeds the choroidal capillaries[17]. Müller cells ensheath these smaller intraretinal capillaries, drawing nutrients for themselves and their neural brethren. Unlike their neural counterparts, Müller cells have few mitochondria and mainly rely on glycolysis for energy. The lactate metabolic product is shuttled to surrounding neurons who convert it to pyruvate to generate energy[17].

Linked to metabolism is the glutamate-glutamine cycle, which Müller cells also regulate. Neurons rely on astrocytic glia like Müller cells for certain neurotransmitter precursors, like glutamine to make the most common excitatory neurotransmitter, glutamate. Neurons lack the ability to produce glutamate, the most common excitatory neurotransmitter in the CNS, and rely on astrocytic glia like Müller cells for neurotransmitter production. Astrocytes and Müller cells electrogenically take up synaptic glutamate and immediately convert it to glutamine, which is then shuttled back to the neurons which convert it back to glutamate[15,18–20]. Since photoreceptors are constantly releasing glutamate in the dark, it is important for excess glutamate to be taken up by Müller cells to prevent excitotoxicity[21].

Neurotransmitter reuptake is also linked to another important role for Müller cells, ion homeostasis[22]. Müller cells contain many potassium ion channels, most notably the inward

rectifying channel Kir4.1, which enables them reset extracellular ion concentrations after changes in neural polarization[23]. Since the retinal neurons change their membrane potential at a high rate, it is important for Müller cells to take up released potassium and redistribute it. Müller cells' high permeability for potassium endows them with a the strongly hyperpolarized membrane potential of -80 mV. This hyperpolarization facilitates neurotransmitter reuptake, since it is an electrogenic process. During disease states Müller cells often down- or mis-regulate their potassium channels[24], depolarizing their membrane potential inhibiting neurotransmitter reuptake[15,25], which can result in excitotoxicity further exacerbating the disease state[26].

Retinal Disease

According to WHO, over 253 million people have vision impairment. After refractive errors and cataracts, retinal diseases like Age-related Macular Degeneration (AMD), glaucoma, and diabetic retinopathy are the most common causes of vision impairment and blindness worldwide[27]. There are additional genetic diseases like Retinitis Pigmentosa (RP) that affect smaller populations, but with a similar devastating phenotype. The pathogenesis for most retinal degenerative diseases is photoreceptor death. Certain diseases specifically target a type of photoreceptor, while others destroy all photoreceptors unbiasedly.

Patients with RP lose sight due to rod cell death, with most causative mutations in the rods themselves[28]. Additionally, mutations in the phototransduction cascade, cytoskeletal structure, maintenance of cilia, signaling, cell-cell interaction or synaptic interaction can cause photoreceptor death. RPE mutations interrupting vitamin A metabolism or phagocytosis also cause rod cell death[29]. Most often rods die in the peripheral retina with vision loss encroaching towards the center[30].

Compared to RP, vision loss in AMD occurs in the opposite fashion with macular cone photoreceptors dying first causing central vision loss that gradually increases towards the periphery. This is caused by deposits in Bruch's membrane, termed drusen, that inhibit nutrient delivery from the choroid. The majority of macular degeneration cases are the "dry" form characterized by drusen aggregates. The disease can progress to the exudative "wet" form characterized by neovascularization[31,32].

Another disease with impaired retinal nutrient delivery is diabetic retinopathy. For people with severe diabetes, retinal tissue becomes hypoxic due to a weakened circulatory system. In an effort to enhance nutrient delivery, the retina grows new leaky blood vessels that can hemorrhage, further distorting vision[33].

Neovascularization is a common symptom of a variety of retinal diseases including wet AMD, diabetic retinopathy, and retinopathy of prematurity. Generally, nutrient delivery is inhibited resulting in tissue hypoxia. The highly metabolically active retina releases angiogenic factors allegedly in the hope that blood vessel growth can deliver much needed nutrients and oxygen. However, the new weak and leaky blood vessels can hemorrhage or cause the retina to detach from the back of the eye further distorting vision and killing cells. Certain diffusible factors like Vascular Endothelial Growth Factor (VEGF) promote angiogenesis and have been the focus for anti-angiogenic therapies[34]. Antibody therapies targeted to VEGF, like Lucentis® (ranibizumab), Eylea® (aflibercept), and Avastin®, have enjoyed clinical success for treating

wet AMD. As the relay point between the vasculature and the inner retina[35], Müller cells are responsible for regulating agiogenesis[36,37] and could be effective targets to knockdown pro-angiogenic factors like VEGF[38] or could be used to produce anti-angiogenic factors[39].

Targeting Müller cells

Due to their important homeostatic roles and ability to interact with retinal neurons and the vasculature, Müller cells are an attractive target for retina therapeutics. However, only certain small molecule drugs can pass the Blood Retina Barrier (BRB), making most therapeutic interventions inherently invasive. For example, the anti-VEGF therapies have to be directly injected into the eye repeatedly for the rest of the patient's life. Instead of directly injecting laboratory produced proteins or RNA, therapeutic DNA can be delivered to cells so that the patient makes their own biological therapeutic product. In order to get the large and negatively charged DNA macromolecule past the cell's lipid membranes, recombinant virus infection has been shown to be the most effective[40–44].

Recently, the FDA approved the first gene therapy using adeno-associated virus (AAV)[45]. Certain Leber's Congenital Amaurosis (LCA) patients were unable to see because of mutation in RPE65, an important enzyme that converts all-*trans* retinal back to 11-*cis* retinal in the RPE[46]. Subretinal injection of a new correct version of RPE65 packaged in AAV2 restored sight for these patients. The safe, stable, long-term expression of this therapeutic transgene underlies the safety and efficacy of AAV as a treatment vector[47–49].

AAV is a small icosahedral dependovirus with a 4.7 kb genome flanked by two Inverted Terminal Repeats (ITRs)[50]. The ITRs form a hairpin loop structure that is important for packaging the genetic cargo and later for stable heterologous expression. Wildtype AAV integrates its genome into a specific region of human chromosome 19 (Ch19) (19q13.3-qter), but recombinantly produced AAV (rAAV) DNA forms episomes after second strand synthesis that can concatemerize via its ITRs. Episomal transgene expression is stable in post mitotic cells like neurons and glia and can potentially persist for the life of the organism[51]. This way a single administration of AAV can potentially cure or treat a disease with one administration.

Müller cells are an advantageous gene therapy target for retinal degenerative diseases because they are one of the last surviving cell types and they span the entire retina, interacting with all the retinal neurons. Müller cells could be used to deliver diffusible neuroprotective compounds like neurotrophic factors to promote retinal survival[52]. They can be used to inhibit angiogenesis in neovascular disorders[38,39]. Finally, they can also be targets for certain gene replacement therapies[53].

Unfortunately, naturally occurring AAV serotypes are inefficient at transducing Müller cells. Interestingly, when injected from the vitreous Müller cells are one of the first cell types AAV encounters, with their endfeet composing the ILM (Fig. 1.1). How AAV bypasses Müller cells but infects other retinal neurons remains a mystery. However, recently new AAV serotypes have been developed by directed evolution. These new variants, ShH10[54] and 7m8[55], are able to effectively infect Müller cells and other retinal neurons from the vitreous.

Chapter 2 of this dissertation explores how to achieve Müller specific expression using these new virus serotypes and cell type specific promoters.

Optogenetics in Müller cells

During retinal degeneration, Müller cells often become depolarized upon decreased Kir4.1 and other potassium channels' current[23,24,56]. Their lowered membrane potential can inhibit their electrogenic neurotransmitter exchange transporters, reducing uptake of compounds like glutamate and GABA[57]. It is thought that glutamate induced excitotoxicity due to inefficient Müller reuptake contributes to and exacerbates retinal degeneration[15].

A noninvasive method to determine if Müller neurotransmitter reuptake significantly contributes to retinal signaling is by analysis of the electroretinogram (ERG). Analogous to the cerebral electroencephalogram (EEG), the ERG measures the retinal light response. It has a characteristic and well understood waveform (Fig. 1.2). After the light flash, there is a negative slope attributed to the hyperpolarization of the photoreceptors called the A-wave. The light stops the photoreceptors from releasing glutamate, which activates the ON bipolar cells. Since the majority of bipolar cells are ON bipolar cells, the *en masse* depolarization of the ON bipolar cells generates the large positive slope called the B-wave. There are additional oscillatory potentials between the A- and B-waves attributed to amacrine cells.

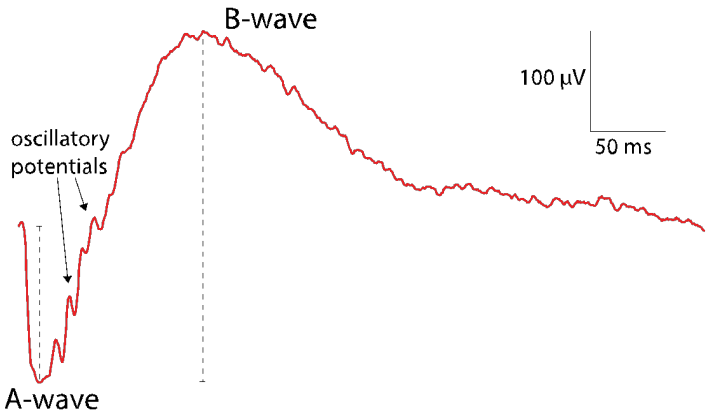

Figure 1.2. Example of a Typical Mouse Electroretinogram Waveform. The trace begins with a flash of light. The initial downward slope is attributed to the hyperpolarization of the photoreceptors and is known as the A-wave. The cessation of glutamate release hyperpolarizes the ON-bipolar cells, generating the large positive slope known as the B-wave. Between the A-wave and B-wave are oscillatory potentials which are attributed to amacrine cells.

If Müller cells clear glutamate from the synaptic cleft between photoreceptors and bipolar cells, there should be a reduction in the ERG B-wave if this Müller process is somehow inhibited. Indeed, when the main GLutamate-ASpartate Transporter (GLAST) was pharmacologically blocked, the ERG B-wave was significantly reduced as compared to control

contralateral eyes[58]. Similarly, Kir4.1 KO also decreases the ERG B-wave when compared to wildtype litter mates[23].

If the membrane potential of Müller cells truly affects neurotransmitter reuptake, transient depolarization in healthy retina tissue should similarly decrease the ERG B-wave. To achieve transient depolarization, Müller cells were infected with AAV carrying optogenetic ion channel transgenes. Optogenetic ion channels like Channelrhodopsin2 (ChR2) open and close in response to light[59]. Chapter 3 of this dissertation examines the electrophysiological implications of expressing and activating the optogenetic ion channels in Müller cells.

Optogenetics for vision restoration

Optogenetic effectors are invaluable neuroscience research tools that enables researchers to dissect circuits to study neural (and glial) interactions. Beyond basic research applications, light sensitive proteins have therapeutic potential. In the degenerate retina lacking light sensitive photoreceptors, the remaining cells can be endowed with light sensitivity through heterologous expression of optogenetic proteins. Restoring light sensitivity to the retina is an ambitious project that has quantitative limitations concerning the light required to activate the newly light sensitive cells and the amount of time it takes these cells to react.

Chapter 4 of this dissertation reviews the latest attempts at optogenetic vision restoration and compares the photo- and temporal sensitivity of the different effectors used.

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Chapter 2

Targeting Retinal Müller Glia in Health and Disease

work done in collaboration with Cécile Fortuny and John G. Flannery

Abstract

The ability to target certain types of cells is a basic requirement for many gene therapies. In the retina, Müller glia-based gene therapies hold promise to alleviate or cure many retinal degenerative disorders including retinitis pigmentosa, macular degeneration, and diabetic retinopathy. Adeno-associated virus (AAV) is currently the most popular gene delivery vector and has demonstrated efficacy in the retina. However, AAV transduction of Müller glia has remained difficult until the development of the 7m8 and ShH10 serotypes. Both serotypes can infect Müller glia from the vitreous, but also infect all other retinal cell layers. In order to achieve specific glia expression, certain promoters must be employed. Here, the GLAST and gfaABC1D promoters were evaluated for their ability to restrict eGFP expression to Müller glia in both wildtype and rd10 retinal degenerative mice. The GLAST promoter targets Müller glia strongly, but also has off-target expression in retinal ganglion cells, while the gfaABC1D promoter was able to completely restrict expression to glia in the retina.

Introduction

As gene therapy has become a realistic method to treat neurodegenerative diseases, the ability to target and deliver therapeutic DNA to specific cells is now paramount. Adeno-associated virus (AAV) vectors are among the most promising delivery mechanisms, with FDA approval of one AAV-based gene therapy[1] and many other clinical trials currently underway[2]. AAV is a small icosahedral dependovirus with a 4.7 kB genome. When recombinantly produced, AAV does not integrate its DNA into chromosomes, but rather concatermerizes to form a circular episome in the host cell's nucleus[3]. Targeting post-mitotic cells, like most found in the CNS and retina, circumvents dilution of these non-Mendelian genetic elements and permits stable, long-term transgene expression. The retina has been the focus of much AAV research being both amendable to AAV infection, and external providing ease to access and assay. Similar to the brain but smaller and well characterized, the retina is an ideal tissue to research therapies that could later be applicable to the broader CNS. Further, blindness from retinal degeneration impacts millions of people and causes significant economic losses worldwide[4].

There are over 300 different genetic mutations that cause retinal cell death leading to blindness[5]. Over 95% of these mutations affect the photoreceptors, resulting in their death and consequential loss of vision. Regardless of genetic mutation, however, the principal glia cell, the radial Müller cell, survive and may be the key for tissue rescue.

Müller cells are advantageous targets for gene therapy because of their role in maintaining tissue homeostasis, their intricate relationship with the vasculature, and their presence throughout the retina. Similar to astrocytes, Müller cells provide metabolic support, maintain extracellular ion homeostasis, remove waste, and produce neurotrophic factors[6]. Neurotrophic factors like CNTF[7], GDNF[8], and BDNF[9] are normally produced by Müller cells in the retina and support retinal neuron health and survival. Their overexpression or exogenous application has also been shown to have promising rescue effects[10–13], with CNTF implants currently in clinical trials for a variety of retinal diseases[2,14]. AAV-based increased expression of these neurotrophic factors by Müller cells could mitigate the effects of many diseases. This therapeutic option is particularly attractive because one therapy could potentially be used for many different diseases.

One potential function of targeting Müller cells is to prevent neovascularization, the pathological growth of new leaky blood vessels that can cause retinal detachment. For patients with the wet form of age-related macular (AMD) or diabetic retinopathy, genetically targeting Müller cells

to knockdown pro-angiogenic proteins like VEGF [15] or overexpress anti-angiogenic proteins like PEDF[16] could prevent neovascularization in these disease states.

Müller glia also hold promise in regenerative medicine. Müller cells are derived from the same neuroprogenitor pool as the other retinal cells, and in certain species like Zebrafish Müller cells have been shown to transdifferentiate into photoreceptors and other retina cells upon injury[17]. With progress being made in dedifferentiating and transdifferentiating retinal glia into photoreceptors in mammals, it is important to be able to specifically target Müller cells.

Müller cells are difficult to transduce with naturally occurring AAV serotypes despite being one of the first cells the virus encounters from the vitreous. Previously, AAV2/6 was shown to be the most effective at infecting Müller cells, with 22% of the cells infected being these glia[18]. However, new viral variants have been engineered that achieve higher Müller cell transduction and panretinal expression from intravitreal injections. The 7m8 serotype has a broad tropism and can infect any retinal cell[19], while ShH10 prefers Müller cells but can also infect other retinal cells[20]. Since neither of these capsids specifically transduce Müller cells alone, a promoter must be used to restrict expression. The GLAST[21] and gfaABC1D[22] promoters were evaluated for their ability to selectively infect Müller cells when packaged inside 7m8 or ShH10. Of these promoters, the GLAST promoter was surprisingly non-specific, while the gfaABC1D promoter was able to achieve complete glia selectivity in the retina.

Methods and Materials

Generation and Purification of AAV Vectors

The pAAV-GLAST-eGFP vector was cloned by inserting the GLAST promoter from the GLASTp-dsRed2 vector from addgene (addgene.org/17706) into the backbone of a pAAV-CAG-EGFP cassette, containing inverted terminal repeats (ITRs), the simian virus 40 (SV40) polyadenylation signal and the woodchuck post-transcriptional regulatory element (WPRE). The AAV-GFAP-eGFP vector was purchased from addgene (addgene.org/50473). Endotoxin free AAV plasmids were co-transfected with pHelper and 7m8 or ShH10-Y445F capsid plasmids into HEK293T cells. After three day, cells were harvested and spin down at 1000rpm for 10 minutes. The supernatant was then collected and resuspended in PEG 8000 (2.5 M NaCl) to precipitate virus at 4 ºC for 2 hours and then pelleted (4000 rpm for 20 min at 4 ºC). Cells were lysed in AAV lysis media (0.15 NaCl, 50 mM Tris HCl, 0.05% Tween, pH 8.5) by three consecutive freeze/thaws and then treated with Benzonase (250 U/µl Novagen #71205-3) for 30 mins at 37 ºC. The media pellet was resuspended with the crude lysate and incubated at 4 ºC overnight. The lysate was then spun down at 4000 rpm for 20 min at 4 ºC. The supernatant was loaded onto an iodixanol density gradient (Opti-prep) and centrifuged for 60 min in a Beckman XL-100K ultracentrifuge at 69000 rpm at 18°C. Fractions containing the viral vectors were collected and concentrated using Amicon Ultra-15 Centrifugal Filter Units. Viral titers were quantified by qPCR and all viral stocks with titers above 1×10^{12} genome copies/ml were stored at 4 °C.

Animals and Intravitreal Injections

All procedures concerning animals adhered to the ARVO statement for the use of animals in ophthalmic and vision research as well as in accordance with USDA Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals, UC Berkeley Association for Assessment and Accreditation of Laboratory Animal Care, International, and UC Berkeley Animal Care and Use Committee. Wild-type (WT) mice (C57Bl6J) and rd10 (B6.CXB1-*Pde6b*^{rd10}*/J*) mice were purchased from Jackson Laboratories and used for all experiments. For intraocular intravitreal injections, rd10 mice were injected at 2 months of age to ensure adequate degeneration. Mice were anesthetized with

ketamine (58 mg/kg) and xylazine (6.5 mg/kg) by intraperitoneal injection. The topical anesthetic proparacaine (0.5%) were applied to the eyes, and the pupils were dialed with phenylephrine (2.5%) and tropicamide (1%). An ultrafine 30 1/2-gauge disposable needle was passed through the sclera, at the equator and next to the limbus, to create a small hole into the vitreous cavity. Two µl of virus with a titer $\geq 1\times10^{12}$ vg/ml of virus was then injected into the vitreous with direct observation of the needle above the optic nerve.

Fundus Photography

Transgene expression was assessed one to eight weeks after injections using a fundus camera (Retcam II; Clarity Medical Systems Inc., Pleasanton, CA) equipped with a wide angle 130° retinopathy of prematurity (ROP) lens to monitor eGFP expression in live, anesthetized mice. Pupils were dilated for fundus imaging with phenylephrine (2.5%) and Tropicamide (1%).

Immunohistochemical analysis, confocal microscopy, and cell counting

Mice were euthanized at 3-4 weeks after injection using CO_2 inhalation followed by cervical dislocation. Enucleated eyes were placed in 10% formalin overnight and then dissected and rinsed in PBS. Retinas were embedded in 5% agarose and sectioned at 125 uM. The sections were blocked for ≥2 hours at room temperature in blocking buffer (1% normal goat serum, 1% FBS, 0.5% Triton-X 100) before antibody labelling overnight. The antibodies used were: rabbit anti-GS (Sigma G-2781, 1:1000), rabbit anti-PKC alpha (abcam ab32376, 1:500), mouse anti-GFAP (Sigma G3893, 1:500) Alexa Fluor 594 goat anti-rabbit (Invitrogen, 1:2000), Alexa Fluor 633 goat anti-mouse (Invitrogen, 1:2000). Images were taken on a Zeiss LSM 710 laser scanning confocal microscope (NIH Grant 1S10RR026866-01). Images were analyzed on FIJI[23] from ImageJ[24]. For each virus and promoter combination, three retinas were counted with five images of each retina.

qRT-PCR Analysis of Muller Glia cell markers in the healthy and degenerate retina

Animals were euthanized and retinas (n=4 each) from P20, P85 and P365 WT and rd10 mice were collected. RNA was extracted from tissue using the RNeasy Mini Qiagen kit, and subjected to DNAse digestion. The resulting RNA was used to synthetize cDNA. qRT-PCR samples were run in triplicate using a collection of primers targeting Muller Glia gene markers (GS, GLAST, GFAP) as well as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the rhodopsin gene as an internal control of retinal degeneration. The relative standard curve method was used to calculate fold difference in mRNA expression normalized to the P20 WT eyes. Primer sequences can be found in Supplemental Table 2.1.

Results

Intravitreal injections of 7m8 and ShH10 both effectively infect Müller cells

Until the development of the 7m8 and ShH10 AAV serotypes, it was difficult to infect Müller glia from intravitreal injections[19,20]. In order to evaluate both vectors ability to infect Müller glia and retinal cells in general, eGFP under the under the ubiquitous CAG promoter was packaged into each serotype and intravitreally injected into the eyes of adult C57Bl6J mice. Fundus photography (Fig 2.1. A&B) shows widespread eGFP expression steaming from the optic nerve head spreading out towards the periphery. Quantification of by fundus fluorescence showed that 7m8 achieved significantly greater overall panretinal expression than ShH10 (Fig. 2.1F). While both serotypes show the greatest eGFP expression along the retinal vasculature, eGFP expression was more absent between veins for ShH10 than for 7m8 which was able to achieve a more even expression profile.

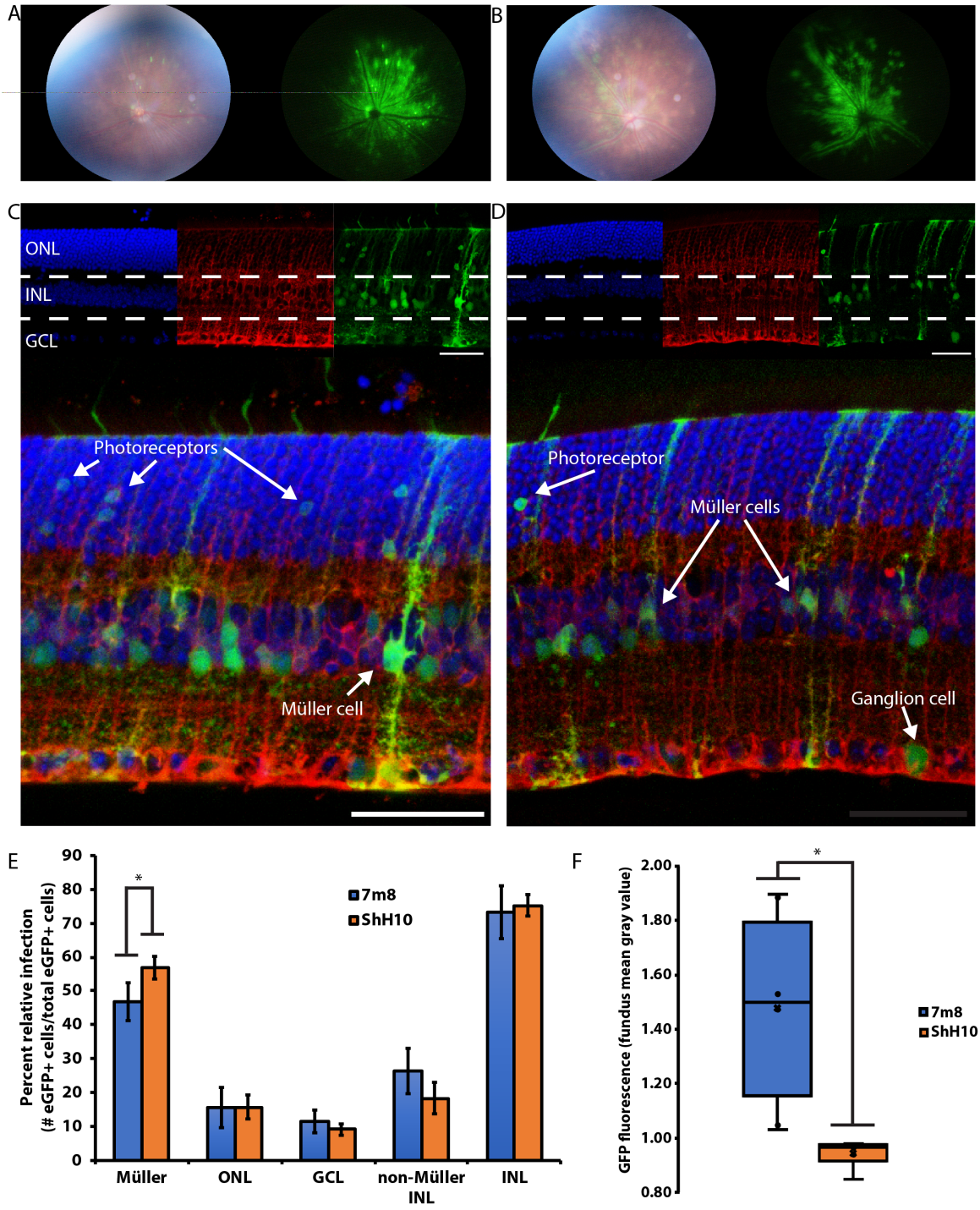


Figure 2.1. 7m8 and ShH10 infect Müller glia and other retinal cell types from the vitreous. Expression profiles of 7m8 and ShH10 were established by packaging eGFP under the ubiquitous CAG promoter into both virus capsids and evaluating retinal fluorescence. Eye fundus images from 7m8(**A**) and ShH10(**B**) intravitreally injected adult C57Bl6J eyes show pan retinal eGFP (green) expression (left). Sections from 7m8 (**C**) and ShH10 (**D**) injected eyes were used to identify cell types for quantification (**E**). Retinal cell layers were determined by DAPI staining (blue) and eGFP+ cells (green) were identified as Müller cells based on co-labeling with GS (red). Both viruses were able to infect all retinal layers. ShH10 infected significantly more Müller cells than 7m8 (*=p>0.05). The scales bar represents 50 µm. (**F**) Quantification of eGFP fluorescence from fundus images (n=10) from 7m8 and SHH10 infected eyes imaged at identical light intensities (*=p>0.05).

Both vectors were able to transduce most of if not all retinal cell types (Fig. 2.1 C,D,E). Both vectors were able to infect Outer Nuclear Layer (ONL) photoreceptors at similar rates (15.4 ± 6.0% of eGFP+ cells for 7m8 and 15.7 ± 3.4% for ShH10), which was a surprising result. While 7m8 was specifically developed to infect photoreceptors from the vitreous[19], ShH10 previously has not been shown to infect them. Both serotypes also infected Ganglion Cell Layer (GCL) cells at a similar rate (11.4 ± 3.4% of eGFP+ for 7m8 and 9.1 ±1.6% for ShH10). Being mostly composed of ganglion cells, the GCL is the closest layer to the vitreous where the virus is administered. The cell bodies of the Müller cells lay in the Inner Nuclear Layer (INL) with the cell bodies of amacrine cells, bipolar cells, and horizontal cells. Both viruses primarily infected cells in the INL at the similar rates (~73% of the cells infected). Identified by glutamine synthetase (GS) staining, Müller cell bodies are radial and span the entire retina with irregularly shaped somas (Fig. 2.1C&D). Counter staining with PKCα illuminates the oblong bipolar cells closer to the ONL and to a lesser extent amacrine cells whose round somas are closer to the GCL (Supplementary Fig. 2.1 A&B). ShH10 displayed a significantly greater Müller cell tropism than 7m8, with Müller cells being 57.0 ± 3.4% of the cells infected by ShH10, while 46.7 ± 3.0% of the cells 7m8 infected were Müller glia (p=0.017, Fig. 2.1E). Of the remaining non-Müller INL, predominately amacrine cells were infected followed by bipolar cells.

Generally, while ShH10 is more selective than 7m8 for Müller glia, 7m8 was able to achieve greater overall infection. Both virus serotypes infect all retinal cell layers at a similar rate, with ShH10 displaying greater affinity for Müller cells within the INL.

Physical and transcriptional alterations in the rd10 degenerative model affect AAV infectivity

Most future gene therapy applications in the retina will be in diseased tissue. Other than the loss of photoreceptors, there is profound physical and transcriptional remodeling in degenerative retinas. Viral serotype infectivity profiles may change due to changes in the retina and Müller cells themselves. The rd10 mouse degeneration model was used to assess changes in transcription, expression and transduction in retinal disease. In this model, a mutation in exon 13 of the beta subunit of rod phosphodiesterase (PDE)[25] slowly kills the rod photoreceptors; closely mimicking the human disease, retinitis pigmentosa (RP). After 90 days degeneration plateaus, with a thin layer of degenerating cone photoreceptors persisting in a greatly reduced ONL.

Compared to age matched wildtype controls, Müller glia upregulate key transcripts related to important homeostatic activities in the rd10 degeneration model (Fig. 2.2A). As the rod photoreceptors die, as demonstrated by the loss of rhodopsin (RHO) transcripts, transcripts for important Müller cell proteins products like GS, GLutamate-ASparate Transporter (GLAST), and GFAP are elevated. GS and GLAST are both involved in glutamate metabolism and their expression are co-regulated and are often upregulated in disease states like RP[26]. GFAP is not normally expressed by Müller cells, a major difference between them and astrocytes, but in disease the intermediate filament's expression in greatly increased[27]. As compared to age-matched WT controls, 60 day old rd10 mice Müller cells express GFAP as evidenced by co-labeling with GS, while the WT controls only express GFAP outside the Inner Limiting Membrane (ILM) where astrocytes lie (Fig. 2.2B&C).

Degeneration induces global retinal changes that can affect AAV infectivity through gross changes in retinal architecture and transcriptional changes in individual cells. Changes in Müller cell expression may affect serotype infectivity through upregulation of the intermediate GFAP, which is associated with glial scar production and hardening of the ILM[6,28]. 7m8 and ShH10's ability to intravitreally infect Müller and other retina cells was evaluated in the rd10 degeneration model through eGFP fluorescence induced by the CAG promoter (Fig. 2.2D-F).

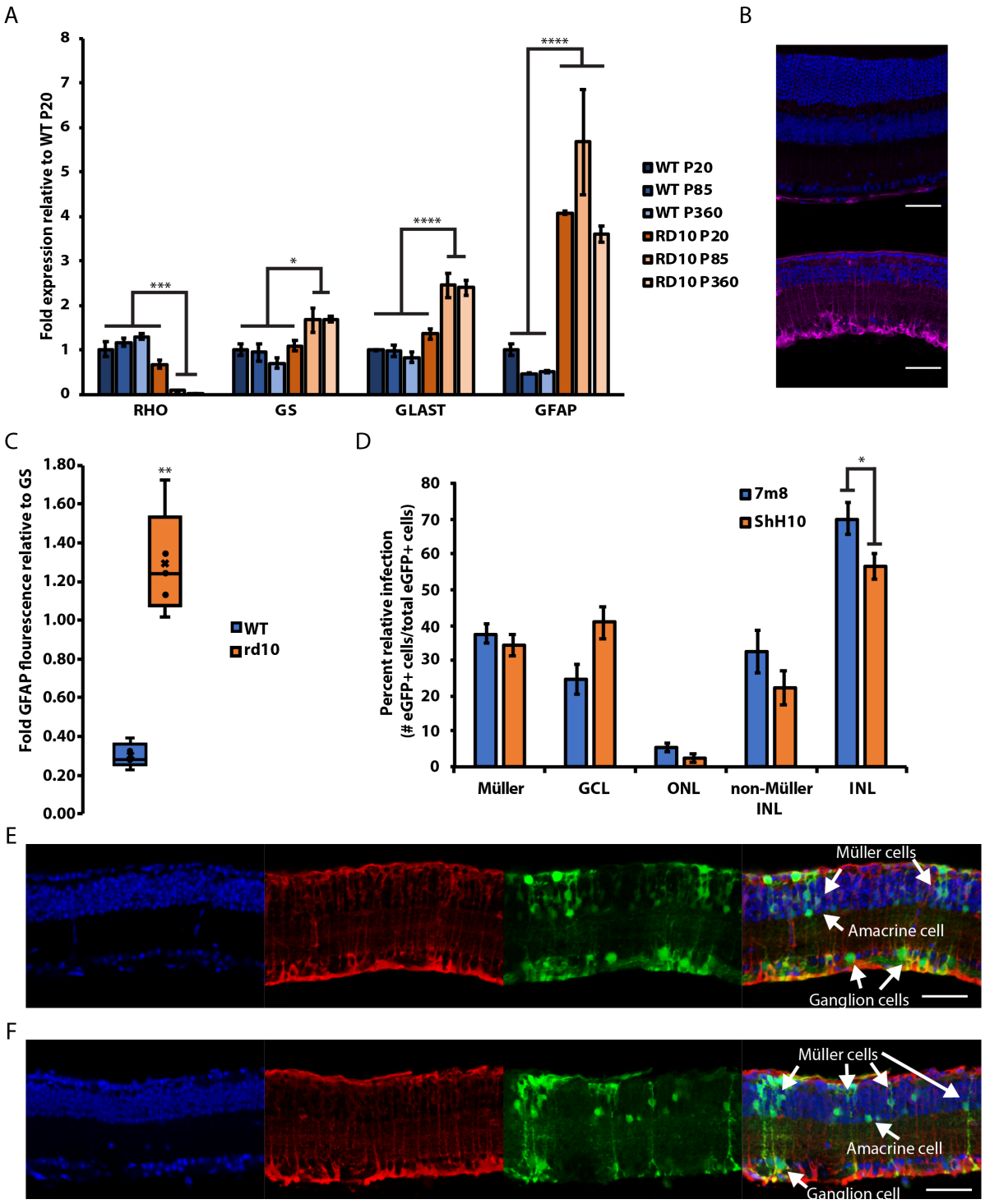


Figure 2.2. Physical and transcriptional alterations in the rd10 degenerate model affect 7m8 and ShH10 retinal infectivity. The rd10 mouse retinal degeneration model gradually degenerates after eye opening at P15. (**A**) RT-qPCR analysis from RNA extracted from wildtype and rd10 retinae at P20, P85, and P360 (normalized to GAPDH). (**B**) P60 retina from wildtype (top) and rd10 (below). The remnants of the ONL is a single layer of DAPI labeled nuclei (blue) above the INL. GFAP staining (magenta) increases upon degeneration. (**C**) Quantification of B, (**=p<0.005). (**D**) Quantification of 7m8- and ShH10-CAG-eGFP sections (E&F respectively, eGFP in green), which were stained with GS (red) to localize Müller cells and DAPI (blue) to identify retinal layers. The scale bars represent 50 µm.

Unsurprisingly, both viral capsids had a reduction in ONL infection, mostly due to a lack of photoreceptors. Occasional lingering cone photoreceptors were infected by both serotypes at low rates (5.4 ± 1.4% of eGFP+ for 7m8 and 1.37 ± 1.0% for ShH10, Fig. 2.2D).

Both serotypes also had reduced Müller and INL infection rates as compared to healthy retinae. Müller cells were only 36.5% ± 3.6% of the eGFP positive cells from 7m8 infection (Fig. 2.2D&E), down from 46.7 ± 3.0% in wildtype eyes (Fig. 2.1E). ShH10 also saw about a 10% reduction in specificity with 34.5 ± 3.0% of the eGFP+ cells from the ShH10 infection being Müller glia (Fig. 2.3D&F). 7m8 was better able at infecting the INL than ShH10 (70.0 ± 4.5% for 7m8 and 56.7 ± 3.6% for ShH10). The reduction in Müller and INL eGFP expression was compensated for in the GCL, which saw 24.6 ± 4.2% of eGFP expression for 7m8 and 40.83 ± 4.5% for ShH10.

The decrease of viral tropism for INL cells in the rd10 degenerate model demonstrates how non-ONL cells and the rest of the retina change in degeneration. The number of INL cell bodies do not decrease in this degeneration, but their viral transduction was greatly reduced. This limited viral penetration resulted in higher GCL transduction for both serotypes. Regardless of cause, the degenerate retina displays different infectivity profiles than wildtype and thus needs to be examined when evaluating AAV and promoter expression profiles.

The GLAST promoter improves Müller cell specificity

Specific Müller glia expression is necessary for the treatment of certain retinal diseases. While off-target production of neurotrophic factors could potentially be tolerated, therapies aimed at trans- or dedifferentiation require highly specific vectors. Due to unusual GFAP regulation Müller glia, alternative promoters from the full length GFAP promoter (gfa2) were explored.

The 2.1 kB GLAST promoter was chosen as a means to restrict expression to Müller glia because GLAST is specific to Müller cells in the retina and has been used to the past to direct glia specific expression[21,29]. GLAST driven eGFP expression when delivered by 7m8 or ShH10 greatly restricts eGFP expression Müller cells in both wildtype and rd10 mice (Fig. 2.3). The promoter eliminated all ONL expression and greatly limited INL expression to Müller cells. Müller cells were by far the most commonly infected cell by both serotypes in healthy and diseased retinae. In adult C57Bl6J, 7m8-GLAST (Fig. 2.3A) was able to achieve 83.0 ± 5.8% Müller specificity, and ShH10-GLAST (Fig. 2.3C) attained a similar rate of 84.3 ± 3.8% (Fig. 2.3E). 7m8 was not able to maintain this selectivity in the degenerate model (Fig. 2.3C) and its Müller selectivity dropped to 67.1 ± 4.8% in rd10 mice. ShH10 performed similarly in the rd10 (Fig. 2.3D) mice as compared to wildtype, with 79.3 ± 5.8% Müller cell specificity.

Unexpectedly, ganglion cells were also transduced comprising 13.0 ± 3.6% of cells infected by 7m8 (Fig. 2.3A) and 13.7 ± 2.0% for ShH10 (Fig. 2.3C) in wildtype. In the rd10 degenerative model, the ganglion cell misexpression rate rose to 30.4 ± 2.6% for 7m8 (Fig. 2.3B) and 19.2 ± 6.9% for ShH10 (Fig. 2.3D). Ganglion cells are not known to express GLAST, but can misexpress certain glutamate transporters under certain disease conditions[30]. Regardless of the causative reason, the GLAST promoter does not completely restrict eGFP expression to Müller glia. Further, the promiscuity of the promoter increases in degeneration, demonstrating how changes in disease states can alter not only viral penetration but also promoter performance.

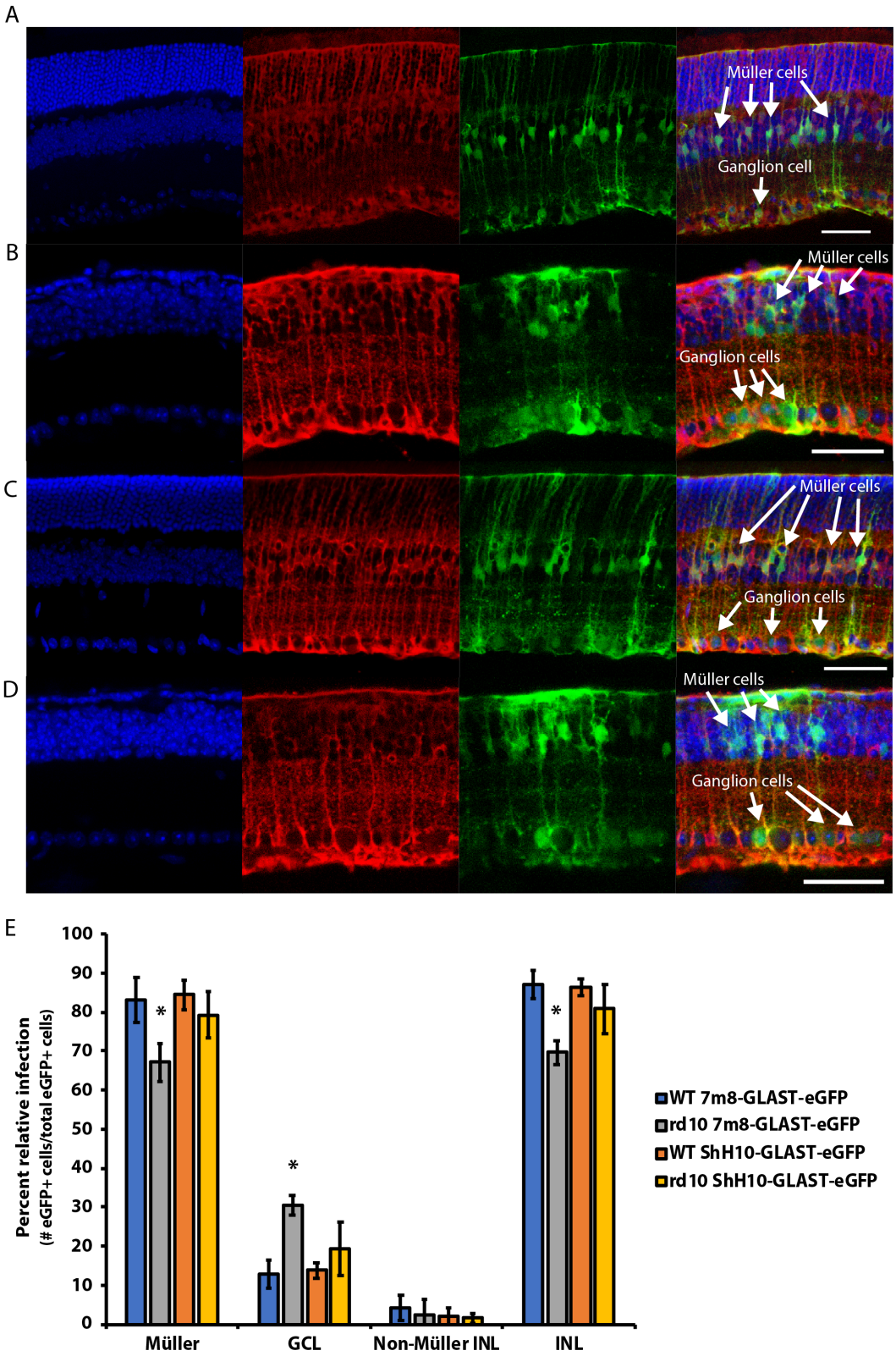


Figure 2.3. The GLAST promoter partially restricts eGFP expression to Müller glia when intravitreally delivery by 7m8 or ShH10. C57Bl6J (**A**&**C**) and rd10 mice (**B**&**D**) were injected with 7m8-GLAST eGFP (**A**&**B**) and ShH10-GLAST-eGFP (**C**&**D**). Müller cells were identified by eGFP (green) colabeling with GS (red). DAPI labeled cell nuclei (blue). The scale bars represents 50 µm. (**E**) Quantification of retinal sections (*=p<0.05).

The gfaABC1D promoter completely restricts eGFP expression to Müller glia.

The gfaABC1D promoter is a small 681 bp promoter composed of protein binding regions from the gfa2 promoter[22]. Its small size makes it very amendable to AAV use, which is restricted to a 4.9 kB carrying capacity. When driving eGFP expression, gfaABC1D was able to completely restrict eGFP expression to Müller cells (Fig. 2.4). Regardless of serotype or disease state, eGFP expression was only observed in Müller glia. This result was surprising considering the unusual regulation of GFAP by Müller glia. Müller glia do not expression GFAP normally, but greatly upregulate it during disease states (Fig. 2.2A-C). Intuitively, the gfaABC1D promoter, based off the full-length GFAP promoter gfa2, should not drive eGFP expression effectively in healthy retina. However, the gfaABC1D was able to robustly drive eGFP expression in both C57Bl6 mice and rd10 degenerate mice.

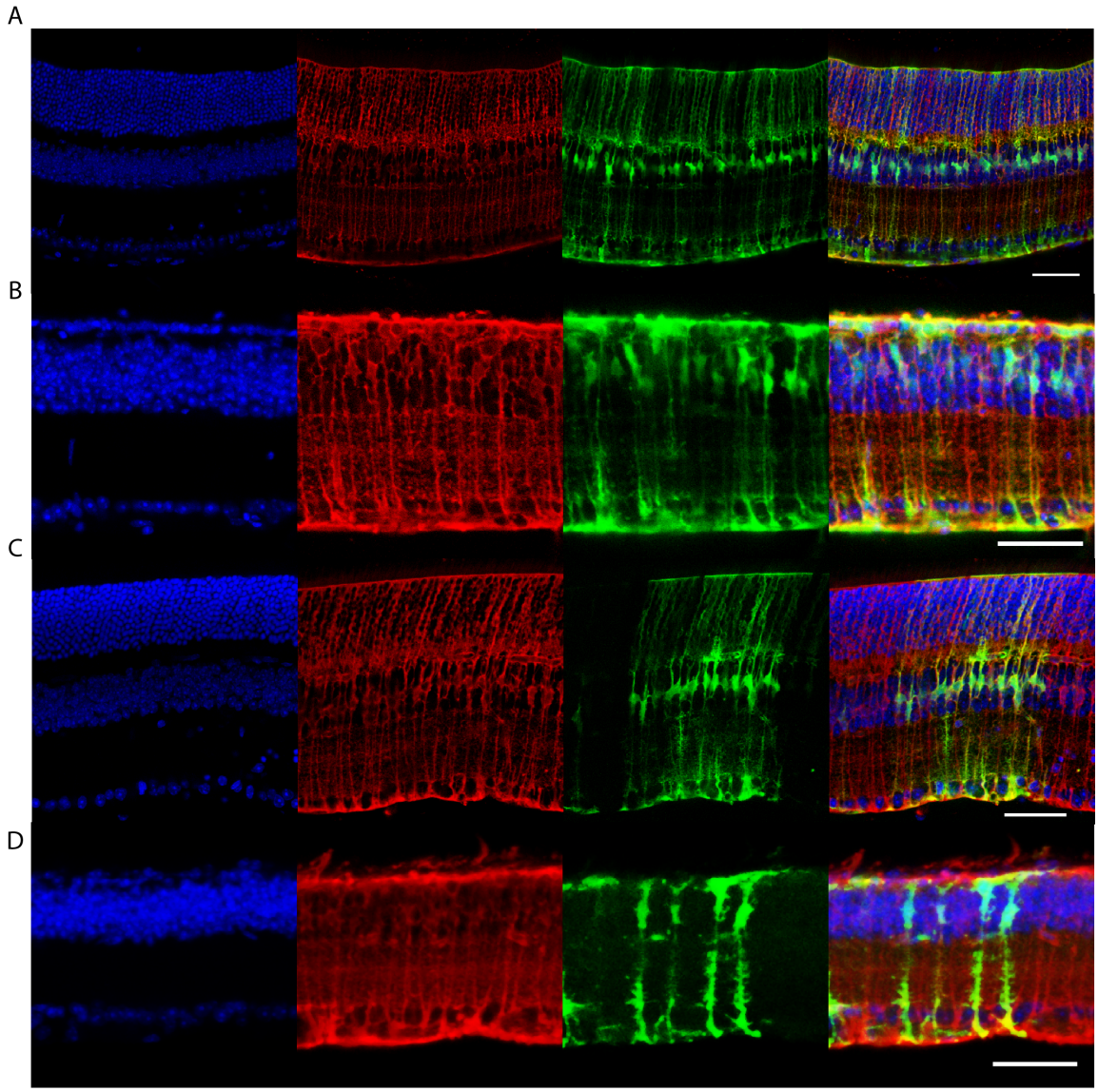


Figure 2.4. The gfaABC1D promoter completely restricts eGFP expression to Müller glia. 7m8-gfaABC1D-eGFP (A&B) and ShH10- gfaABC1D-eGFP (C&D) were intravitreally injected into C57Bl6J (A&C) and rd10 (B&D) mice. Infected Müller cells were identified by colabeling with eGFP (green) and GS (red). Cell nuclei were labeled with DAPI (blue). The scale bars represent 50 µm.

Promoter Analysis

The promoters evaluated here, GLAST and gfaABC1D, presented unpredicted expression profiles. GLAST is a Müller specific protein, but the promoter permitted GCL off-target expression. The gfaABC1D promoter was able to drive expression of eGFP in both healthy and diseased retinas when it should only work in disease. Eukaryotic gene regulation is incredibly complex that often involves genetic elements kilobases away from the immediate upstream promoter driving expression. It is not surprising that heterologous expression systems using limited regulatory elements would not be able to attain identical expression patterns as the regulatory system it was based off of. To further examine what immediate genetic elements drove the specific expression patterns described here, Genomax software (Intrexon Bioinformatics) was used to identify potential transcription binding sites. Of the hundreds of potential binding sites identified, a few pro-glia, anti-neuron, and pro-neuron transcription factors were selected as highly likely to direct this expression pattern (Fig. 2.5).

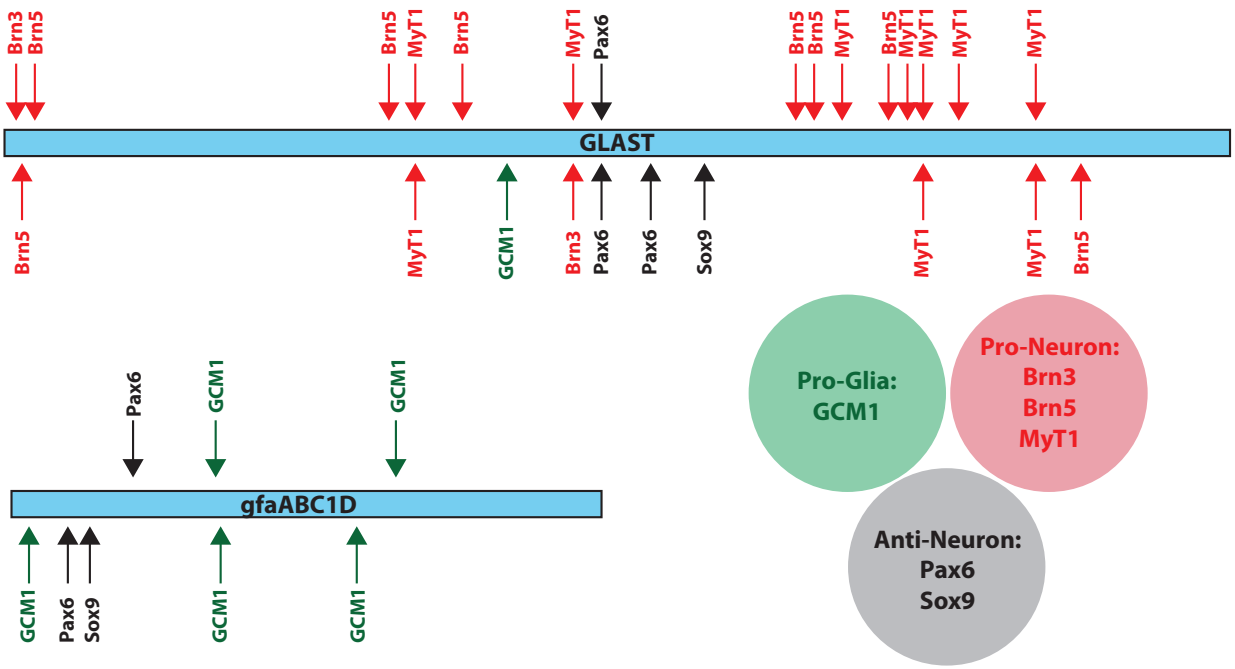


Figure 2.5. Diagram of potential pro-glia and pro-neuron transcription binding sites in the GLAST and gfaABC1D promoters. Pro-glia transcription factors are listed in black and pro-neuron transcription factors are listed in red.

Glia Cell Missing homolog 1 (GCM1) is a transcription factor to associated with early neural development, specifically driving the glia cell fate. When knocked out in drosophila, glia fail to differentiate and are replaced by neurons. Further, ectopic GCM expression also drives a pro-glia cell fate in presumptive neurons[31]. Both GLAST and gfaABC1D contained binding sites for GCM1, but there were four binding sites in the gfaABC1D promoter and only one in GLAST (Fig. 2.5). This pro-glia binding site could push for strong glia expression, especially for gfaABC1D.

Both promoters had binding sites for transcription factors that were categorized as anti-neural. Of note are Pax6 and Sox9, transcription factors expressed by retinal progenitor cells and some adult Müller glia[32,33]. In development, these transcription factors are turned off when the cell is terminally differentiating into a retinal neuron. Their presence would implicate an inhibition of neuronal expression for these promoters.

Brn3 and Brn5 are both transcription factors known to be expressed by retinal ganglion cells[34,35]. Both proteins have many binding sites on the GLAST promoter, but none appear on the gfaABC1D promoter. Similarly, the GLAST promoter contains sites for MyT1, Myelin Transcription Factor 1, which promotes neurogenesis over proliferation in the developing retina[36], whereas the gfaABC1D promoter does not. The presence of these pro-neural, and in the case of Brn3 and Brn5 specifically pro-ganglion, transcription factors in the GLAST promoter could explain its eGFP expression in the GCL and the lack thereof for the gfaABC1D promoter.

Discussion

Müller cells are attractive therapeutic targets for treating retinal disease due to their role in homeostasis and ability to survive in degeneration. They can be used to supply trophic factors to help neural survival, to inhibit angiogenesis, or to de- and/or transdiffentiate into other retinal neurons like photoreceptors. Until recently, naturally occurring AAV serotypes were inefficient at transducing Müller glia from the vitreous. With the engineered capsids 7m8 and ShH10, intravitreal Müller cell transduction is now possible (Fig. 2.1). Both capsid variants are able to infect Müller cells at a high rate (46.7 ± 3.0% for 7m8 and 57.0 ± 3.4% by ShH10). However, physical and transcriptional changes that arise during degeneration reduce Müller and INL viral infection for both serotypes (Fig. 2.2).

In order to specifically direct transgene expression to Müller cells, the GLAST and gfaABC1D promoters were evaluated. The 2.2 GLAST promoter was able to reduce off-target cell expression in the INL and ONL, but still induced expression in ganglion cells (Fig. 2.3). While GLAST is a Müller specific protein in the retina, the immediate upstream promoter region of this protein does not drive transgene expression in Müller cells alone. Brn3, Brn5, and MyT1 transcription binding sites in the GLAST promoter could explain its promiscuity (Fig. 2.5). Brn3 is an established ganglion cell marker and Brn5 is also known as a ganglion specific transcription factor in the retina. MyT1 is a pro-neural transcription factor, so a promoter containing its binding sites could drive neural expression

While the GLAST promoter is not entirely specific for Müller glia, it still holds therapeutic value. There are a variety of therapeutic strategies in which one would want to target both Müller glia and ganglion cells. For example, Müller and ganglion cell mediated expression of BDNF could be an efficacious treatment for glaucoma, a retinal disease that primarily affects the GCL[9,13].

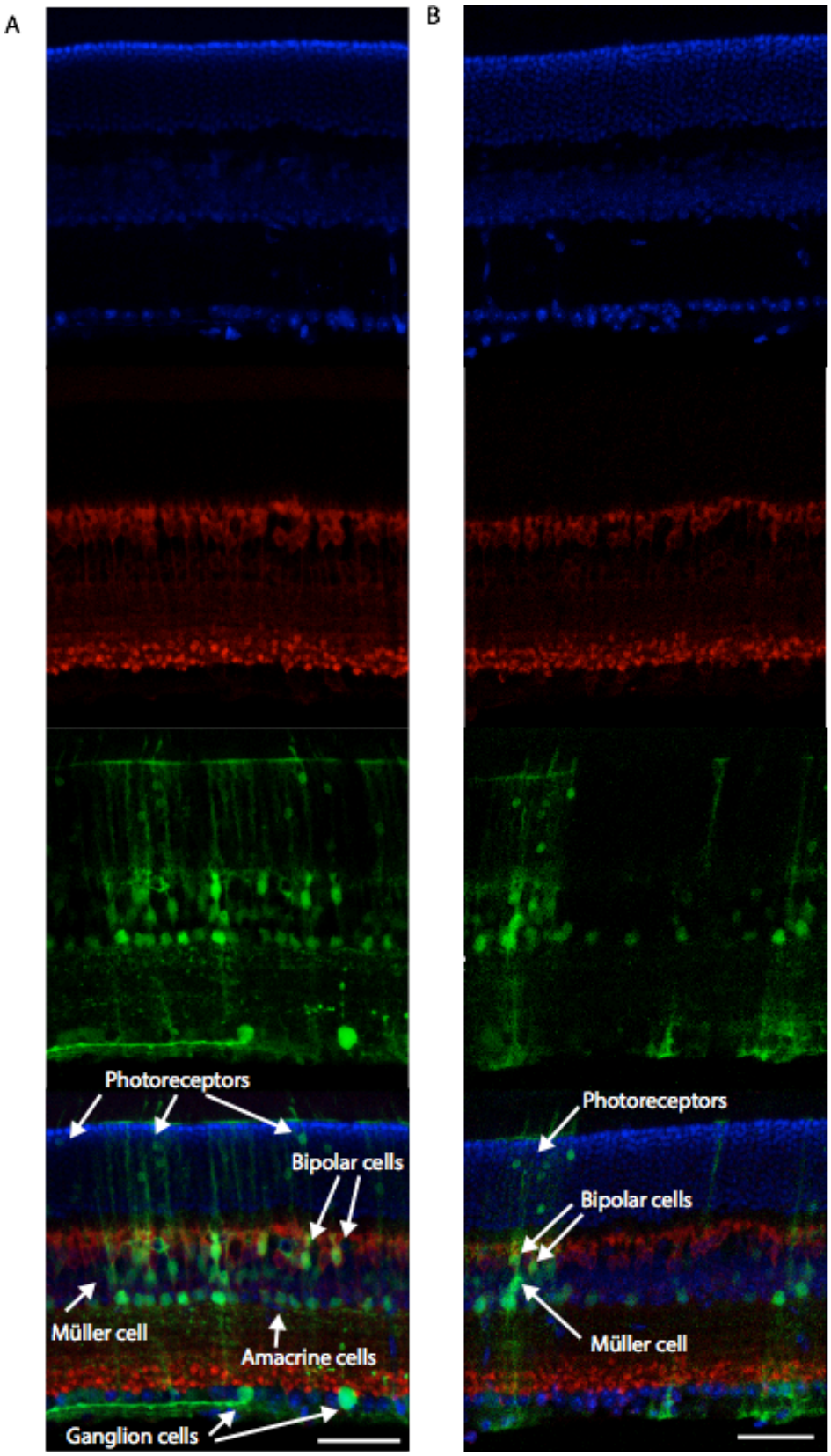
For other therapies like regenerating photoreceptors from Müller cells, cell specific targeting is essential. Thankfully, the gfaABC1D promoter was able to induce Müller glia specific expression in both wildtype and disease (Fig. 2.4). Interestingly, even though this promoter is based off of the GFAP (gfa2) promoter, its expression profile does not match that of GFAP in the retina. Müller glia only express GFAP in disease or stress states, but the gfaABC1D promoter was able to induce eGFP expression in both health and disease. Since this promoter is only a fraction of the full-length promoter, there are regulatory mechanisms applying to transgene expression. The small size of gfaABC1D also makes it incredibly amendable for AAV use. AAV has a small carrying capacity of 4.9 kB, so a 681 bp promoter that can drive cell specific expression is incredibly valuable.

For the first time Müller cells can be specifically transduced from the vitreous using AAV. When packaged into either the 7m8 or ShH10 AAV capsid variants, the gfaABC1D promoter can drive Müller glia specific transgene expression.

Supplementary Material

Supplementary Table 1. qPCR Primers

Name	Sequence
GFAP F	AAG CTC CAA GAT GAA ACC AAC
GFAP R	GGC CAC ATC CAT CTC CA
GLAST F	AAA CCG GAG AAA CCC GTG
GLAST R	TGA GCC CAG GGA GAT GGA TA
GS (“GLUI”) F	GGA TAG CCC GTT TTA TCT TGC
GS (“GLUI”) R	GTG GTA CTG GTG CCT CTT GC
mGAPDH 3’	GGA TGC AGG GAT GAT GTT CT
mGAPDH 5’	AAC TTT GGC ATT GTG GAA GG
Rhodopsin F	CAA GAA TCC ACT GGG AGA TGA
Rhodopsin R	GTG TGT GGG GAC AGG AGA CT



Supplementary Figure 2.1. Intravitreally delivered 7m8 and ShH10 eGFP expression profiles driven by the CAG promoter. eGFP expression (green) was countered stained with PKCalpha (red) to identify non-Müller INL cell somas belong to bipolar cells and amacrine cells. Cell nuclei were stained with DAPI (blue). Scar bars represent 50 µm.

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Chapter 3

Optogenetic Applications in Retinal Müller Glia

work done in collaboration with John G. Flannery

Abstract

Müller glia are responsible for a variety of important homeostatic functions in the retina, including neurotransmitter uptake and ion homeostasis. Their ability to efficiently take up glutamate may depend on the proper expression of ion channels related to potassium homeostasis. In the retina, glutamate is constantly being released in the dark by photoreceptors, which inhibits ON bipolar cells and excites OFF bipolar cells. When light inhibits photoreceptor glutamate release, Müller glia help clear synaptic glutamate which enables ON bipolar cells to depolarize. If glutamate uptake is inhibited in Müller cells, the B-wave of the electroretinogram is reduced due to inhibition of ON-bipolar cells. To evaluate how depolarizing the membrane potential of Müller glia affects glutamate uptake, Müller glia were transduced with the AAV capsid variant ShH10 carrying a bistable ChR2 mutant (C128S/D156A, "BiChR2") under the retinal glia specific gfaABC1D promoter. Although there were no significant changes to the ERG, subtle differences arose that could indicate glial effects on the light response.

Introduction

Glia like retinal Müller cells and CNS astrocytes provide invaluable homeostatic support to neurons. One of these essential roles is taking up excess neurotransmitter after synaptic transmission. Thought to aid in temporal precision by quickly terminating neurotransmission, glia rapidly take up neurotransmitter by a variety of mechanisms. In the retina, Müller cells use the glutamate transporter GLAST (or EAAT1) to electrogenically take up glutamate[1,2].

Efficient glutamate uptake is especially important in the retina at the photoreceptor-bipolar cell synapse due to continuous release of glutamate by photoreceptors in the dark. Light inhibits photoreceptor glutamate release, and the absence of glutamate is what depolarizes the most common bipolar cell, the ON-bipolar cell. Without efficient glutamate removal, light induced ON-bipolar cell depolarization could be inhibited.

If Müller glutamate uptake indeed shapes and terminates neurotransmission in the retina, then *en masse* electrical recordings could indicate a phenotypic change upon knockout or inhibition of GLAST. For the retina, the electroretinogram (ERG) can provide this valuable information.

The ERG measures the electrical response of retinal cells to light and has a characteristic waveform with an "A-wave" and "B-wave." The negative slope from the A-wave is attributed to the hyperpolarization of the photoreceptors upon light stimulation. The subsequent absence of glutamate causes the ON bipolar cells to depolarize, generating the very large and positive B-wave. If glutamate uptake is inhibited in some way in this synapse, then the excess glutamate should inhibit these ON bipolar cells resulting in a reduced of B-wave amplitude.

In a GLAST knockout mouse model, Harada *et al.* observed this phenomenon of a reduced B-wave as compared to wildtype littermates[1]. Similarly, when the Tse *et al.* group injected the a GLAST inhibitor into a wildtype mouse eye, the injected eye showed a lower B-wave amplitude as compared to the uninjected control contralateral eye[3].

While essential for retinal glia glutamate uptake, GLAST is not able to function alone and requires the proper functioning of other Müller proteins and processes to do its job. Beyond taking up neurotransmitter, Müller cells have a myriad of other important homeostatic roles like providing nutrients and maintaining ion homeostasis[4]. These roles are not fulfilled individually but are interictally intertwined. For example, in the retina Müller cells are able to electrogenically take up glutamate partially due to the hyperpolarized membrane potential they have to maintain potassium homeostasis[5].

Müller cells have a hyperpolarized membrane potential of -80 mV due to high expression of leaky potassium ion channels, particularly the inward rectifying potassium channel Kir4.1[6]. The expression of Kir4.1 and generation of a hyperpolarized membrane potential is one of the last characteristics to emerge during retinal and Müller development, occurring at P15 in mice[7,8]. In disease, the down and mis-regulation of Kir4.1 and loss of the hyperpolarized membrane potential is one of the first changes that occur in Müller glia[9].

Loss of membrane potential affects basic homeostatic roles for Müller glia in the retina, which could exacerbate disease states. GLAST is able to move glutamate against its concentration gradient by co-transporting three Na+ and one H+, and counter-transporting one K+[10]. However, lower membrane potentials may not support this electrogenic process inhibiting glutamate uptake, and potentially resulting in excitotoxicity[4]. Glutamate disregulation can also affect other homeostatic processes like the glia-neuron glutamate cycle and ammonia metabolism[11].

If the membrane potential of Müller cells is vital to neurotransmitter reuptake, then depolarization of Müller cells should inhibit GLAST and glutamate uptake. Here we attempt to transiently depolarize Müller cells in healthy mouse retinae to see if this membrane potential is critical for glutamate uptake. To do this we expressed optogenetic ion channel ChR2(C128S/D156A)[12,13], a "bistable" ChR2 mutant (BiChR2), in mouse Müller cells using Adeno-Associated Virus (AAV) capsid variant ShH10[14] and the gfaABC1D promoter[15]. Changes in the light response upon BiChR2 activation were assessed by ERG *in vivo*. Despite stable transgene expression, no significant changes in the light response developed. However, small changes were observed that could become more profound with certain optimizations in potential future experiments.

Methods and Materials

Generation and Purification of AAV Vectors

The pAAV-gfaABC1D-eGFP vector was purchased from addgene (addgene.org/50473). pAAV-gfaABC1D-BiChR2-Venus was cloned by inserting C128S and D156A point mutations (QuikChange Lightning Multi Site-Directed Mutagenesis Kit, Agligent) into ChR2-Venus from pCAGGS-ChR2-Venus (addgene.org/15753). ChR2(C128S/D156A)-Venus was then inserted into the backbone of the pAAV-gfaABC1D-eGFP vector. Endotoxin free AAV plasmids were co-transfected with pHelper and 7m8 or ShH10-Y445F capsid plasmids into HEK293T cells. After three days, cells were harvested and spin down at 1000rpm for 10 minutes. The supernatant was then collected and resuspended in PEG 8000 (2.5 M NaCl) to precipitate virus at 4 ºC for 2 hours and then pelleted (4000 rpm for 20 min at 4 ºC). Cells were lysed in AAV lysis media (0.15 NaCl, 50 mM Tris HCl, 0.05% Tween, pH 8.5) by three consecutive freeze/thaws and then

treated with Benzonase (250 U/µl Novagen #71205-3) for 30 mins at 37 ºC. The media pellet was resuspended with the crude lysate and incubated at 4 ºC overnight. The lysate was then spun down at 4000 rpm for 20 min at 4 ºC. The supernatant was loaded onto an iodixanol density gradient (Opti-prep) and centrifuged for 60 min in a Beckman XL-100K ultracentrifuge at 69000 rpm at 18°C. Fractions containing the viral vectors were collected and concentrated using Amicon Ultra-15 Centrifugal Filter Units. Viral titers were quantified by qPCR and all viral stocks with titers above 1×10^{12} genome copies/ml were stored at 4 °C.

Animals and Intravitreal Injections

All procedures concerning animals adhered to the ARVO statement for the use of animals in ophthalmic and vision research as well as in accordance with USDA Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals, UC Berkeley Association for Assessment and Accreditation of Laboratory Animal Care, International, and UC Berkeley Animal Care and Use Committee. Wild-type (WT) mice (C57Bl6J) were purchased from Jackson Laboratories and used for all experiments. Mice were anesthetized with ketamine (58 mg/kg) and xylazine (6.5 mg/kg) by intraperitoneal injection. The topical anesthetic proparacaine (0.5%) were applied to the eyes, and the pupils were dialed with phenylephrine (2.5%) and tropicamide (1%). An ultrafine 30 1/2-gauge disposable needle was passed through the sclera, at the equator and next to the limbus, to create a small hole into the vitreous cavity. Two µl of virus with a titer $\geq1\times10^{12}$ vg/ml of virus was then injected into the vitreous with direct observation of the needle above the optic nerve.

Fundus Photography

Transgene expression was assessed one to eight weeks after injections using a fundus camera (Retcam II; Clarity Medical Systems Inc., Pleasanton, CA) equipped with a wide angle 130° retinopathy of prematurity (ROP) lens to monitor eGFP expression in live, anesthetized mice. Pupils were dilated for fundus imaging with phenylephrine (2.5%) and Tropicamide (1%).

Immunohistochemical analysis, confocal microscopy, and cell counting

Mice were euthanized at 3-4 weeks after injection using CO_2 inhalation followed by cervical dislocation. Enucleated eyes were placed in 10% formalin overnight and then dissected and rinsed in PBS. Retinas were embedded in 5% agarose and sectioned at 125 uM. The sections were blocked for ≥2 hours at room temperature in blocking buffer (1% normal goat serum, 1% FBS, 0.5% Triton-X 100) before antibody labelling overnight. The antibodies used were: rabbit anti-GS (Sigma G-2781, 1:1000), Alexa Fluor 594 goat anti-rabbit (Invitrogen, 1:2000). Images were taken on a Zeiss LSM 710 laser scanning confocal microscope (NIH Grant 1S10RR026866-01). Images were analyzed on FIJI[16] from ImageJ[17].

Electroretinography

Mice were anesthetized followed by pupil dilation. Mice were placed on a 37°C heated pad and contact lenses were positioned on the cornea of both eyes. A reference electrode connected to a splitter was inserted into the forehead and a ground electrode was inserted in the tail. For scotopic ERGs, mice were dark-adapted for 2 hours before anesthetization and all procedures were performed in scotopic conditions. ERGs were recorded (Espion E2 ERG system; Diagnosys LLC, Littleton, MA) in response to five light flash intensities ranging from 0.1-1.0 cd*s/m^2 on a dark background. Each stimulus was presented in series of three. For photopic ERGs the animal was first exposed to a rod saturating background for 5 minutes. Stimuli ranging from 0.125-25 cd*s/m^2 were presented 25 times on a lighted 30 cd*s/m^2

background. Stimulus intensity and timing were computer controlled. ERG amplitudes and peak times were compared using a student's t-test.

qRT-PCR Analysis of Muller Glia cell markers

Animals were euthanized and retinas (n=5 each) from ShH10(Y445F)-gfaABC1D-BiChR2-Venus and ShH10(Y445F)-gfaABC1D-eGFP injected mice were collected. RNA was extracted from tissue using the RNeasy Mini Qiagen kit and subjected to DNAse digestion. The resulting RNA was used to synthetize cDNA. qRT-PCR samples were run in duplicate using a collection of primers targeting Muller Glia gene markers (GS, GFAP, GLAST, Kir4.1) as well as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the rhodopsin (Rho) gene as an internal control of retinal health. Primer sequences can be found in Supplementary Table 3.1.

Results

AAV Mediated BiChR2 Expression in Müller cells

To selectively depolarize Müller glia with a light sensitive protein, the AAV capsid variant ShH10[14] was used to deliver the BiChR2-Venus transgene under the retinal glia specific gfaABC1D promoter[15]. In the contralateral control eye, eGFP was delivered by the same capsid and promoter combination. BiChR2-Venus and eGFP expression was assessed by fundus fluorescence *in vivo* (Fig. 3.1A&B). Both vectors achieved pan retinal fluorescence, with the strongest expression near the optic nerve head and along the vasculature.

BiChR2-Venus and eGFP fluorescence was restricted to Müller glia (Fig. 3.1C&D). Müller cells were identified by glutamine synthetase (GS) staining. While both BiChR2-Venus and eGFP expression was restricted to Müller cells, their expression profiles differed, most likely due to different localizations of BiChR2-Venus and eGFP. BiChR2 is fused to the fluorophore Venus, so the fluorescent protein is going to be membrane-bound. As compared to the cytosolic expression of eGFP (Fig. 3.1C), BiChR2-Venus expression is increased in the Inner Plexiform Layer (IPL) due to the presence of fine Müller glia processes and thus increased surface area (Fig. 3.1D). In contrast, the soma of the BiChR2-Venus expressing Müller glia are not nearly as fluorescent as the eGFP expressing cells since the membrane-bound construct doesn't fill the inside space like eGFP does.

These slight differences in fluorescence expression indicate proper transgene expression and localization, with the ion channel integrated into the membrane and the cytosolic protein filling the inside of the cells.

ERG Recordings

To assess the extent to which BiChR2 activation in Müller cells affects the light response, a photopic ERG protocol was developed (Fig. 3.2). Light adapted mice were exposed to a series of light flashes to record a baseline response before BiChR2 activation (Fig. 3.2A). These light flashes were a thousand times less than the minimum requirements to activate a light sensitive microbial ion channel like BiChR2. The photopic protocol was repeated after bright 465 nm light stimulation (26475 cd*s/m^2) was used to activate BiChR2. Finally, a recovery recording followed this experimental condition where BiChR2 was inactivated by bright 520 nm light (24124 cd*s/m^2).

Averaged ERG waves showed that the B-wave was slightly lower regardless of experimental condition for eyes expressing BiChR2 in Müller cells compared to the eGFP control eye (Fig. 3.2A). Additionally, the oscillatory potential before the peak of the B-wave was lower for the BiChR2 expressing eyes than eGFP eyes. While this difference is not significant, it is somewhat troubling. In ERG recordings before injections, this difference was not observed (Fig. 3.3). This (non-significant) difference was also observed in scotopic ERG recordings (Fig. 3.4).

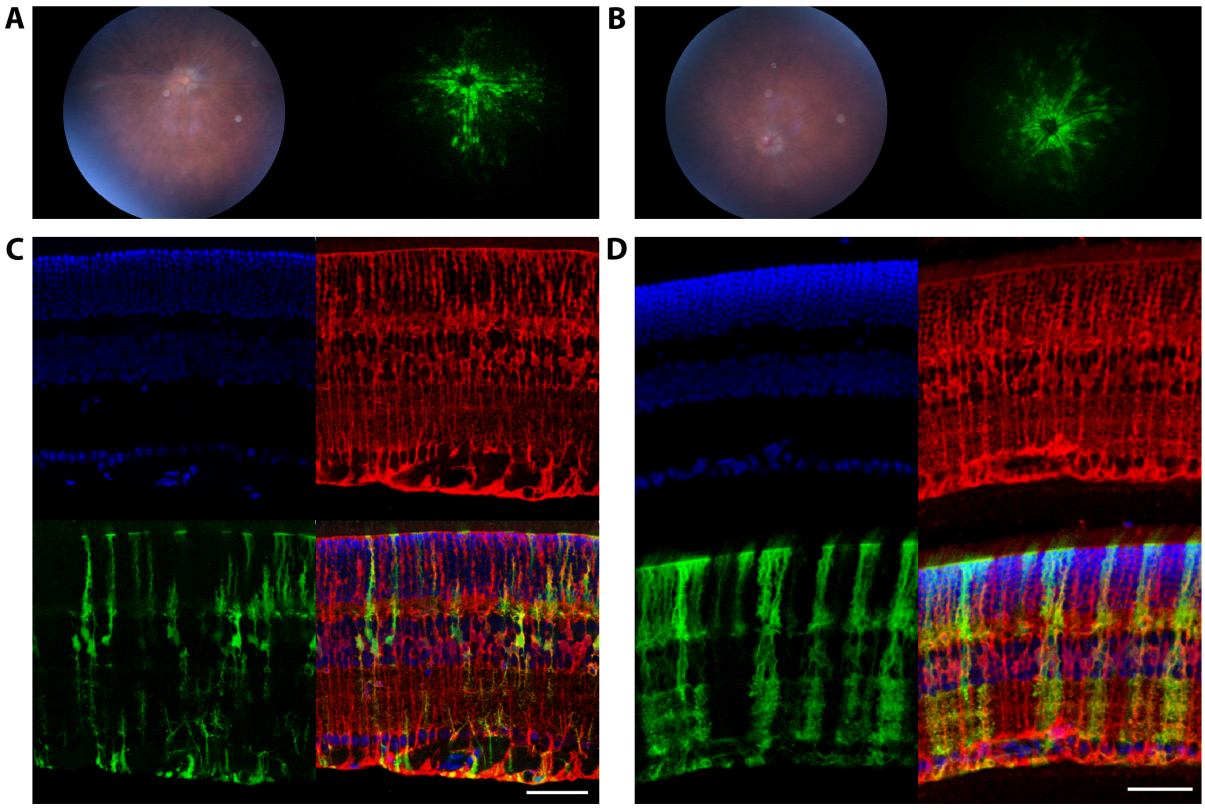

Figure 3.1. BiChR2 and eGFP are selectively expressed by Müller glia when delivered by ShH10 under the gfaABC1D promoter. (**A**&**B**) Example fundus photography of ShH10-gfaABC1D-eGFP (**A**) and ShH10-gfaABC1D-BiChR2-Venus (**B**) injected retinae. On the right is the white-field image, and on the left is fluorescence. (**C**&**D**) Retinal sections from mice intravitreally injected with ShH10-gfaABC1D-eGFP (**C**) and ShH10-gfaABC1D-BiChR2-Venus (**D**). Cell nuclei were stained with DAPI (blue), and Müller cells were identified by GS staining (red). Transgenic fluorescence (green) was localized to Müller glia for both constructs. Scale bars represent 50 µm.

To ensure that this slight difference in ERG waveform was not due to a stress state induced through BiChR2-Venus expression, RNA was collected from retinae injected with ShH10-gfaABC1D-BiChR2-Venus was ShH10-gfaACB1D-eGFP and the expression of Müller specific transcripts related to health and disease were evaluated (Fig. 3.5). There were no

observed transcriptional differences between BiChR2-Venus and eGFP eyes, indicating normal retinal health.

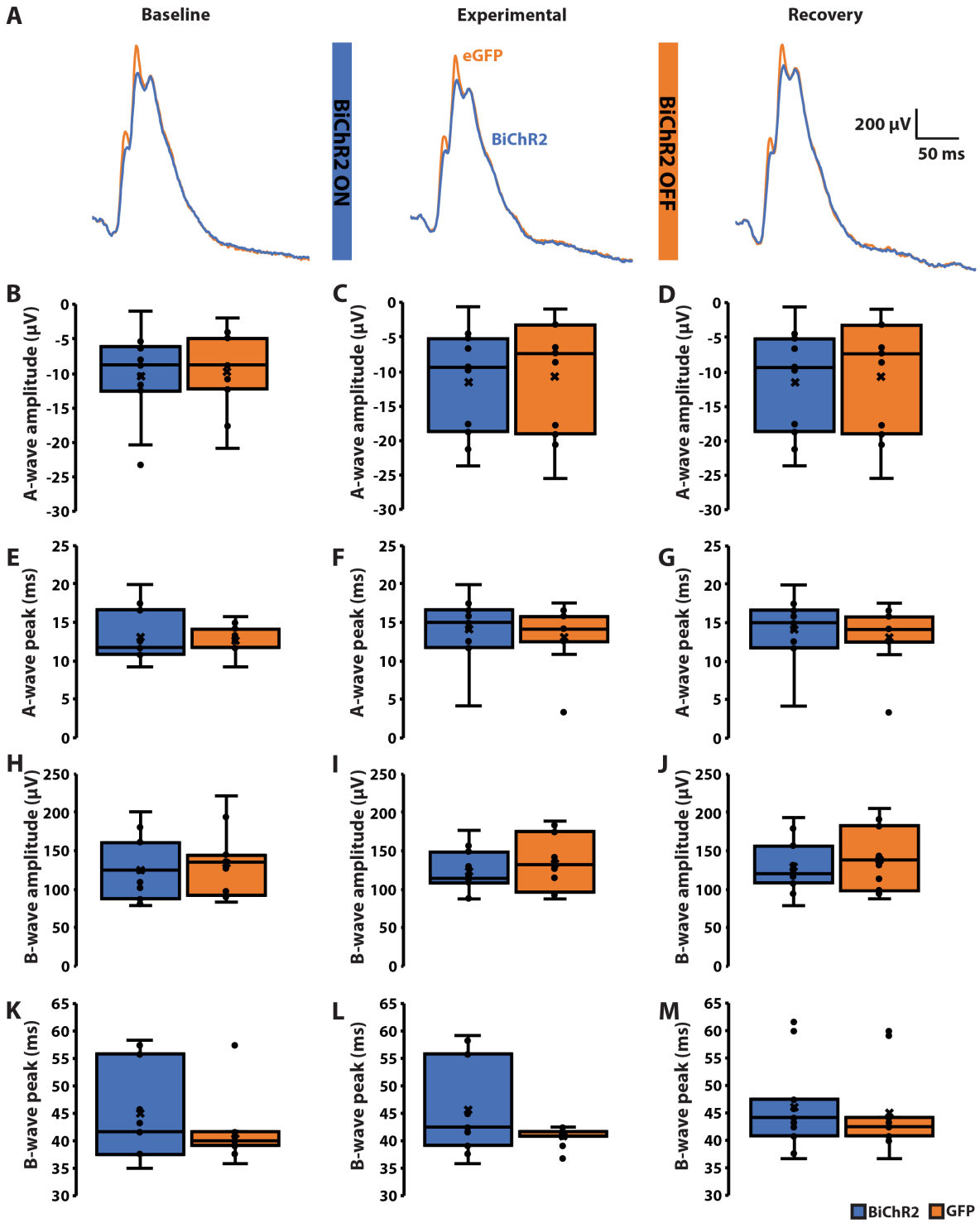

Figure 3.2. Effect of Müller BiChR2 expression on the ERG. (**A**) Average 25 cd*s/m^2 photopic ERG traces from three conditions: a baseline recording, an experimental recording with BiChR2 turned on, and a recovery recording with BiChR2 inactivated. (**B-D**) A-wave amplitude measurements from the mice averaged in **A**. (**E-G**) The time taken to reach the peak of the A-

wave across the three different conditions. (**H-J**) The amplitude of the ERG B-wave from the three different conditions. (**K-M**) The time taken to reach the peak of the B-wave. Error bars = SD, n=10.

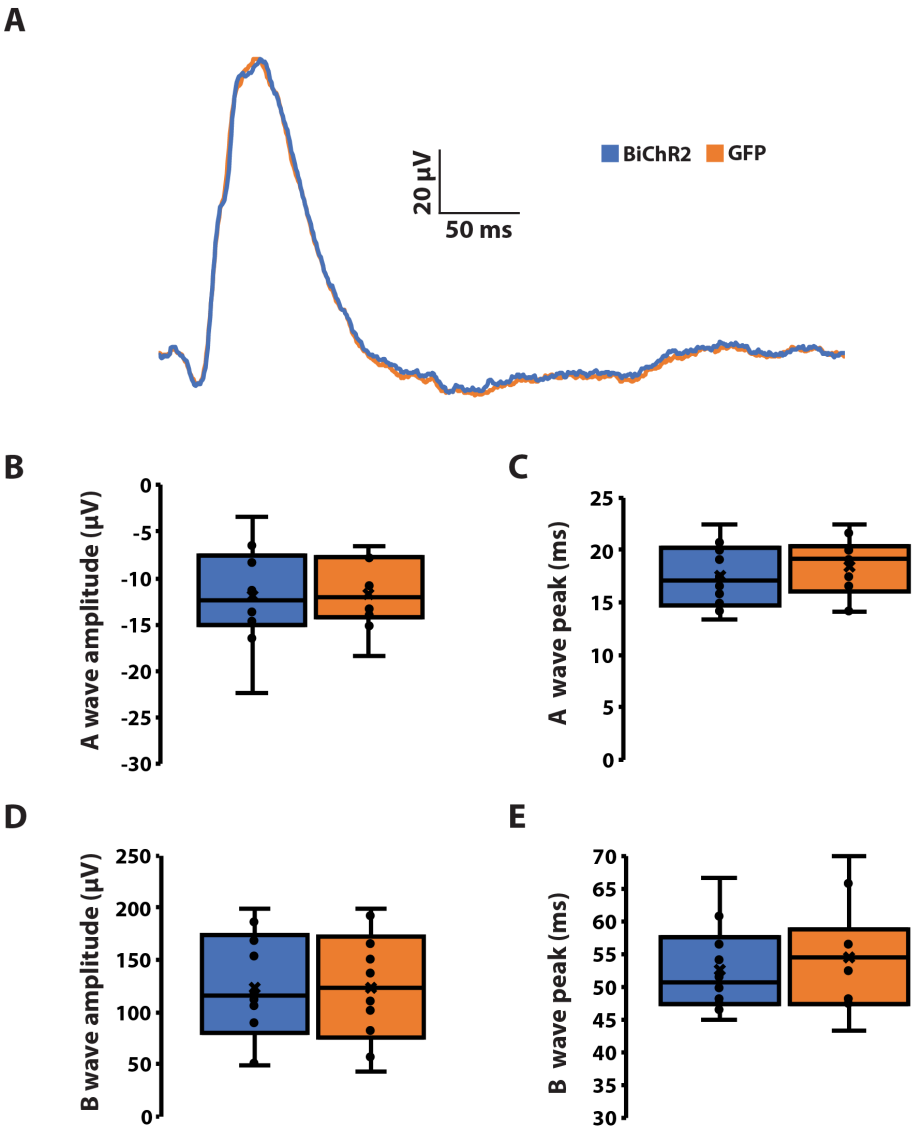

Figure 3.3. Pre-injected photopic ERG shows no difference between eyes. Average Photopic ERG trace from C57Bl6J mice before they were injected with ShH10-gfaABC1D-BiChR2 and ShH10-gfaABC1D-eGFP (n=10).

Also inferring normal retinal health, the A-wave remained constant across conditions (Fig. 3.2B-G). Since the A-wave is generated by the hyperpolarization of the photoreceptors in response to light, there should be no change to the A-wave regardless of BiChR2 expression or activation. The A-wave amplitude was similar for both the BiChR2 and eGFP eyes and did not change upon BiChR2 activation or inactivation (Fig. 3.2B-D). Similarly, the time it took to reach the A-wave peak was consistent between eyes and conditions (Fig. 3.2E-G).

The difference between B-wave amplitudes did not differ significantly between eyes across the conditions (Fig. 3.2H-J), even though the averaged waveforms (Fig. 3.2A) imply some kind of difference. While not significant, the amount of time to reach the peak of the B-wave has a much greater range for the BiChR2 expressing eyes than the eGFP expressing eyes (Fig. 3.2K&L). Interestingly, the range of time to reach peak is reduced for the BiChR2 eye upon BiChR2 inactivation (Fig. 3.2M). The large range of time to reach the B-wave peak for the BiChR2 expressing eye could indicate a loss of temporal precision due to inhibited glutamate uptake, but this conclusion is hard to make with such a limited phenotypic impact.

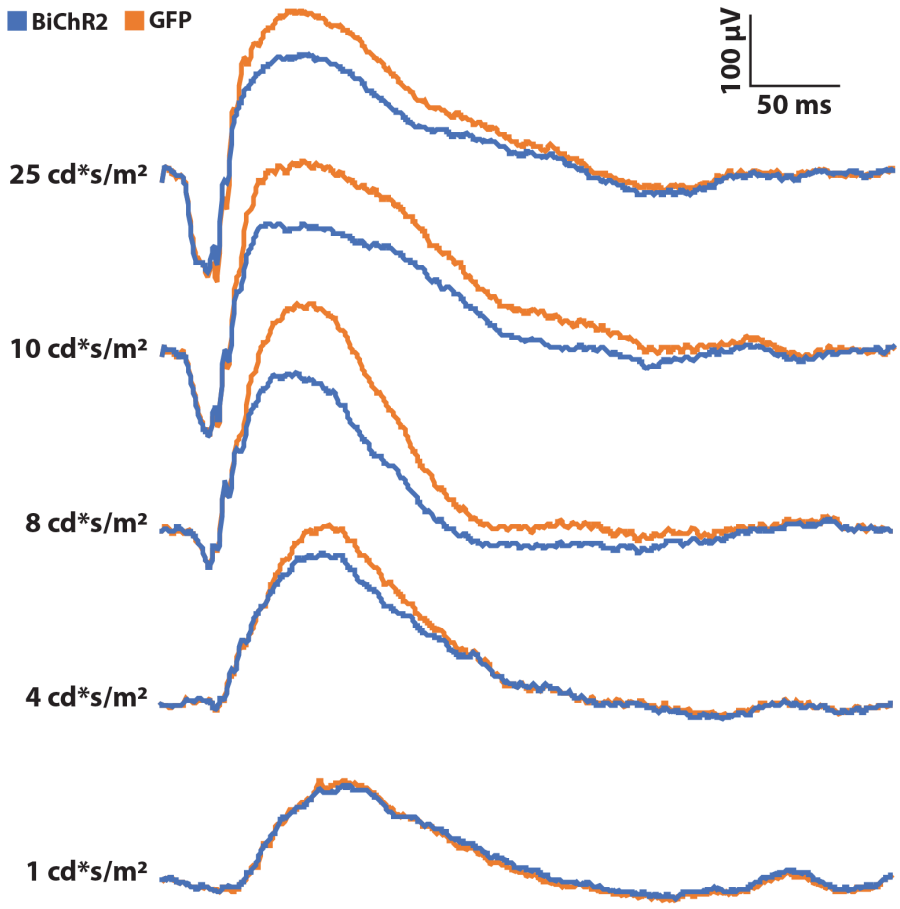

Figure 3.4. Retinas with Müller cells expressing BiChR2 have a lower scotopic ERG B-wave. Average Scotopic ERG traces from C57Bl6J mice injected with ShH10-gfaABC1D-BiChR2 and ShH10-gfaABC1D-eGFP (n=5).

Discussion

Müller glia are responsible for a variety of important homeostatic functions in the retina that are interrelated. ERG recordings of mice expressing the depolarizing optogenetic ion channel BiChR2 in Müller cells were used to examine the relationship between glial membrane potential and glutamate uptake.

Müller glia were transduced with the AAV capsid variant Shh10 carrying either BiChR2-Venus or eGFP under the control of the retinal glia specific gfaABC1D promoter. The ShH10 capsid with the gfaABC1D promoter was able to promote strong and Müller cell specific expression (Fig. 3.1). Both transgene products localized properly, with the fluorophore-tagged ion-channel BiChR2-Venus displaying fluorescence on the membrane and the cytosolic eGFP protein product accumulating inside the cells.

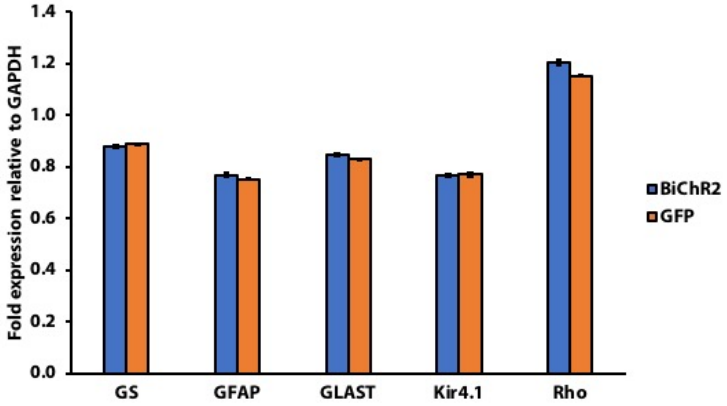

Figure 3.5. Retinas with Müller cells expressing BiChR2 do not different transcriptionally from retinas with Müller cells expressing eGFP. RT-qPCR analysis of RNA from retinas infected with ShH10-gfaABC1D-eGFP or ShH10-gfaABC1D-BiChR2 (normalized to GAPDH).

From ERG recordings, there was no significant difference between BiChR2 expressing eyes and eGFP control eyes regardless of BiChR2 activation (Fig. 3.2). The A-wave remained the same in terms of both amplitude and timing for all conditions and constructs. The B-wave for BiChR2 expressing eyes was slightly reduced compared to eGFP control eyes (Fig. 3.2A). While there are no obvious signs of stress in the Müller cells (Fig. 3.5), that does not preclude any other defects. Insertion of a new membrane protein like BiChR2-Venus could disrupt important membrane associated macromolecular complexes or prevent proper insertion of other membrane bound proteins. To alleviate some of this potential stress, future experiments could try multicistronic transgene cassettes so that the optogenetic ion channel isn't physically tethered to the fluorophore, reducing protein insertion into the membrane.

The slight reduction in the ERG B-wave for BiChR2 expressing Müller cells could be due to a loss of temporal precision (Fig. 3.2K&L). There was larger range of time to reach the peak of the B-wave for the BiChR2 eyes than their control counter parts. Additionally, this difference was reduced when the BiChR2 was inactivated (Fig. 3.2M). In future experiments, greater transgene expression could enhance these subtle differences through use of more efficient AAV capsid variants like 7m8.

Further experiments need to be performed to evaluate the extent of depolarization in Müller cells upon BiChR2 activation. The lack of change of the ERG B-wave upon BiChR2 activation might mean the Müller cells are not sufficiently depolarized. Being essential players in ion homeostasis, it is possible these cells can rapidly adapt and correct ionic perturbations like those induced by BiChR2 activation. Direct electrophysiological recordings from these cells

could shed light on these issues. While the extent of BiChR2 activation in Müller glia is still unknown, future prospects on optogenetic applications in Müllers are promising and exciting.

Supplementary Material

Supplementary Table 3.1. qPCR Primers

Name	Sequence
GFAP F	AAG CTC CAA GAT GAA ACC AAC
GFAP R	GGC CAC ATC CAT CTC CA
GLAST F	AAA CCG GAG AAA CCC GTG
GLAST R	TGA GCC CAG GGA GAT GGA TA
GS F	GGA TAG CCC GTT TTA TCT TGC
GS R	GTG GTA CTG GTG CCT CTT GC
mGAPDH 3’	GGA TGC AGG GAT GAT GTT CT
mGAPDH 5’	AAC TTT GGC ATT GTG GAA GG
Kir4.1 F	CCA GGA AAG CTG AAC CCA CT
Kir4.1 R	CTG CCT CAG CCC AAA CCA TA
Rhodopsin F	CAA GAA TCC ACT GGG AGA TGA
Rhodopsin R	GTG TGT GGG GAC AGG AGA CT

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Chapter 4

Innovative Optogenetic Strategies for Vision Restoration

work done in collaboration with John G. Flannery and published in Frontiers in Cellular Neuroscience

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Abstract

The advent of optogenetics has ushered in a new era in neuroscience where spatiotemporal control of neurons is possible through light application. These tools used to study neural circuits can also be used therapeutically to restore vision. In order to recapitulate the broad spectral and light sensitivities along with high temporal sensitivity found in human vision, researchers have identified and developed new optogenetic tools. There are two major kinds of optogenetic effectors employed in vision restoration: ion channels and G-protein coupled receptors (GPCRs). Ion channel based optogenetic therapies require high intensity light that can be unsafe at lower wavelengths, so work has been done to expand and red-shift the excitation spectra of these channels. Light activatable GPCRs are much more sensitive to light than their ion channel counterparts but are slower kinetically in terms of both activation and inactivation. This review examines the latest optogenetic ion channel and GPCR candidates for vision restoration based on light and temporal sensitivity.

Introduction

Shortly after the popularization of optogenetic tools in neuroscience began in the early 2000s[1–3], researchers began evaluating their use in a novel *in vivo* application: vision restoration. In America, over 1,000,000 people are currently considered blind, with that number expected to double by 2030[4]. Most patients suffering from retinal degenerative diseases, like retinitis pigmentosa and macular degeneration, often first lose their light sensitive photoreceptors, the rods and cones, leaving the remaining retinal tissue light insensitive (Fig. 4.1). However, the surviving cells can retain functionality and connections to the brain long after photosensitivity disappears. For decades researchers have attempted to activate this remaining tissue with prosthetic electrical stimulation[5]. With the advent of optogenetics, photosensitivity and vision can be restored at cellular resolution.

The first successful attempt at bestowing light sensitivity to non-photoreceptor retinal cells with optogenetic tools was in 2006[6]. In this pioneering work they heterologously expressed a microbial opsin, channelrhodopsin-2 from *Chlamydomonas reinhardtii* (ChR2) (Fig. 4.2A), in thalamic projecting retinal ganglion cells (RGCs) (Fig. 4.1B) via adeno associated virus (AAV) serotype 2. This groundbreaking paper showed that endowing surviving retinal cells with light sensitive proteins can restore light responses both retinally and cortically. Furthermore, it was one of the first papers to use AAV as a retina delivery vector and to demonstrate long term expression and safety. For over a decade researchers have been improving upon this basic method of virally expressing optogenetic proteins in surviving retinal cells, using new effectors to improve light and temporal sensitivity.

The ambitious aim to cure blindness optogenetically has driven light sensitive protein development, benefiting neuroscience as a whole with new effectors. Human vision has broad spectral (400-700 nm) and light sensitivity (10^4 to 10^{16} photons cm^{-2} s^{-1}) with high temporal resolution (up to 60 Hz for long cone opsins)[7]. In order to restore sight, researchers have had to step beyond ChR2 to find and engineer new optogenetics that can better recapitulate human vision. In this review we will compare the latest optogenetic ion-channel and G-protein coupled receptor (GPCR) based technologies in their effectiveness at restoring sight to blind retinas based on the amount of light required for activation and temporal sensitivity.

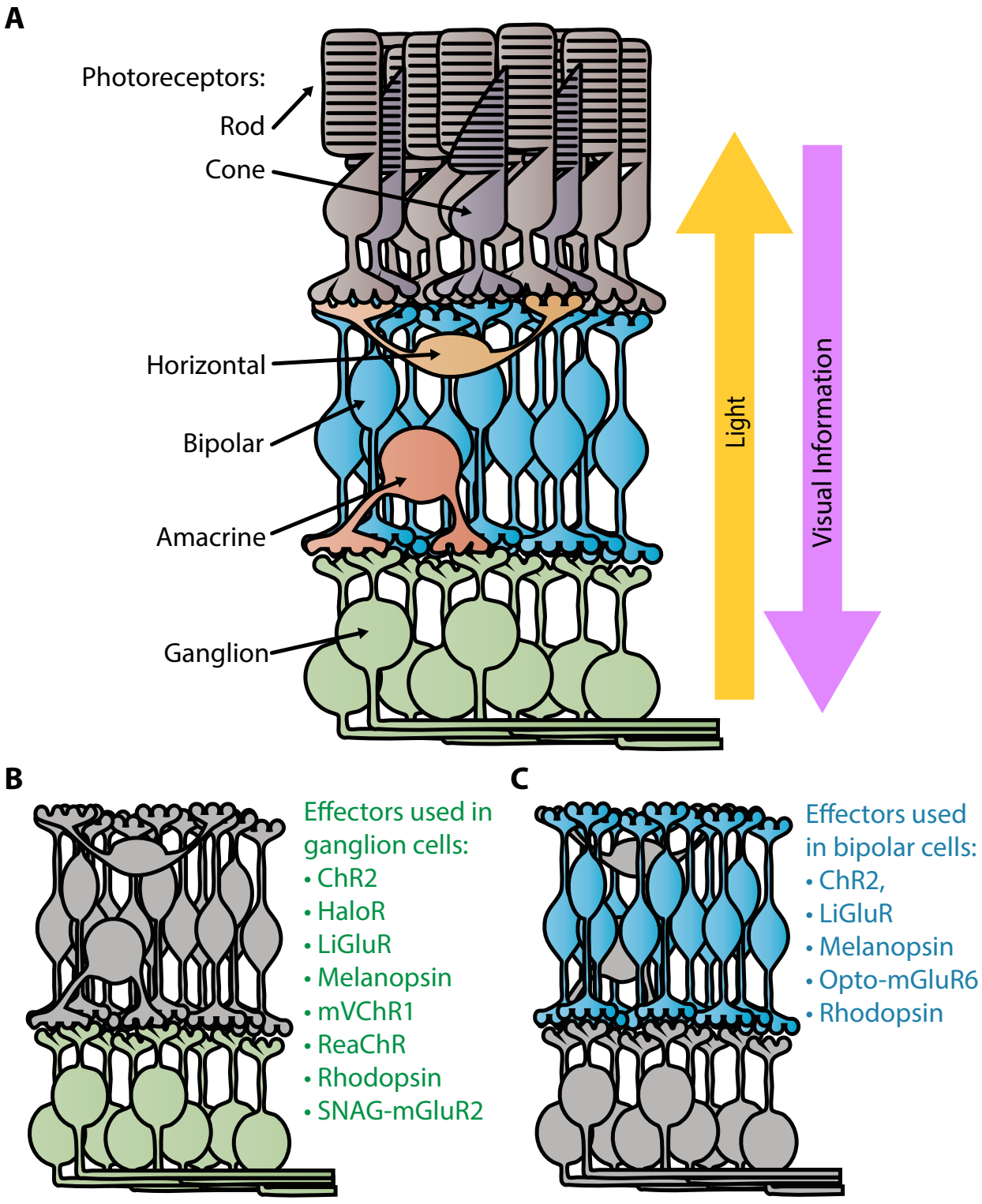

Figure 4.1. Retina schematic. (A) Diagram of a normal healthy retina. Light passes through the retina, entering through the retinal ganglion cell layer to reach the light sensitive photoreceptors, the rods and cones, in the outer retina. Visual information is sent from the photoreceptors to the bipolar cells where the ON/OFF processing begins. Ganglion cells are the terminal retinal signal recipients and they relay onto neurons in the lateral geniculate nucleus in the thalamus. **(B)** and **(C)** depict the degenerate retina without photoreceptors. **(B)** lists the optogenetic therapies that have been tested in ganglion cells[6,8,11,16,20,24,55], while **(C)** lists those tested in bipolar cells[13,23,25,26,36,56].

Ion channels

The main advantage of optogenetic ion channel based therapies is temporal sensitivity. Photoactivation allows ions to flow through the channel activating or inhibiting the neural host. Upon light stimulus removal, inactivation is quick. With an opening and closing rate on the order of milliseconds, optogenetic ion channels have the potential for successive high frequency stimulation required for normal human vision (Fig. 4.2D). However, light sensitivity is sacrificed for temporal sensitivity, with most channels requiring at least 10^{15} photons cm^{-2} s^{-1} to activate their neural hosts (Fig. 4.2C)[6,8]. This amount of light is dangerous at shorter wavelengths, like the 470 nm that maximally activates ChR2[9]. Of growing interest is "red-shifting" these ion channels to safer long wavelengths.

One such ion channel that was successfully red-shifted is the modified mammalian ion channel LiGluR (Fig. 4.2A)[10]. Based on the human ionotropic glutamate receptor 6, LiGluR has a mutated cysteine residue that allows Maleimide-Azobenzene-Glutamate (MAG), a photoswitchable tethered ligand (PTL), to covalently bind to the outside of the ion channel. Certain wavelengths of light isomerize the azobenzene from trans to cis, forcing the distal glutamate into the protein's binding pocket opening the ion channel. The first generation of MAG, MAG0, was bistable and used 380 nm light to open and 500 nm to close. While this ultraviolet bistable channel was able to restore the visual response[11], including the pupillary response, the amount and wavelengths of light required are dangerous to humans. To circumvent these problems, a second generation MAG, MAG460, was developed[12]. No longer bistable and activated at 460 nm (Fig. 4.2B), MAG460 was also able to restore the visual response in both mice and dogs[13]. While the LiGluR-MAG460 system requires a similar amount and spectrum of light as ChR2 (Fig. 4.2C & D), its modular design is advantageous. Since LiGluR requires the PTL to be delivered in trans, patients would have the option of which one to have delivered and when, which would be especially beneficial if other PTLs with different activation spectrums are developed in the future.

Currently, in order to achieve activation using wavelengths above 550 nm, different opsins are required. In 2008 the Deisseroth group discovered the red-shifted ChR2 ortholog VChR1 from *Volvox carteri*[14]. This cation channel's excitation spectrum is red shifted approximately 70 nm when compared to ChR2, with a peak excitation at 535 nm and a capacity to produce spiking at 589 nm. While this red-shifted spectrum was promising, this channel does not express well due to inefficient plasma membrane integration.

Various groups have tackled this expression problem by generating chimeras with other microbial ion channels. Of these chimeric microbial channels, ReaChR[15] and mVChR[16] have demonstrated vision restoration efficacy in rodents, and in the case of ReaChR, primates (Figs. 4.1B & 4.2A)[8]. ReaChR, which used the N-terminus of ChiEF to facilitate membrane trafficking, the transmembrane domain from VChR2 to increase expression, and a L171I point mutation to reduce desensitization above 600 nm, has a red-shifted activation spectrum with a peak excitation ~600 nm and is capable of generating photocurrent at 630 nm (Fig. 4.2B)[15]. In mice this channel could produce spiking frequencies up to 30 Hz in ganglion cells and 22 Hz in macaque retinal explants[8]. Considering that film often uses 24 frames per second, the temporal sensitivity of ReaChR seems sufficient for vision rescue. While ReaChR still requires light on the same order of magnitude as ChR2 at 10^{15} photons cm^{-2} s^{-1} (Fig. 4.2C), light at this portion of the spectrum is safe up to $10^{17\text{-}18}$ photons cm^{-2} s^{-1} [9].

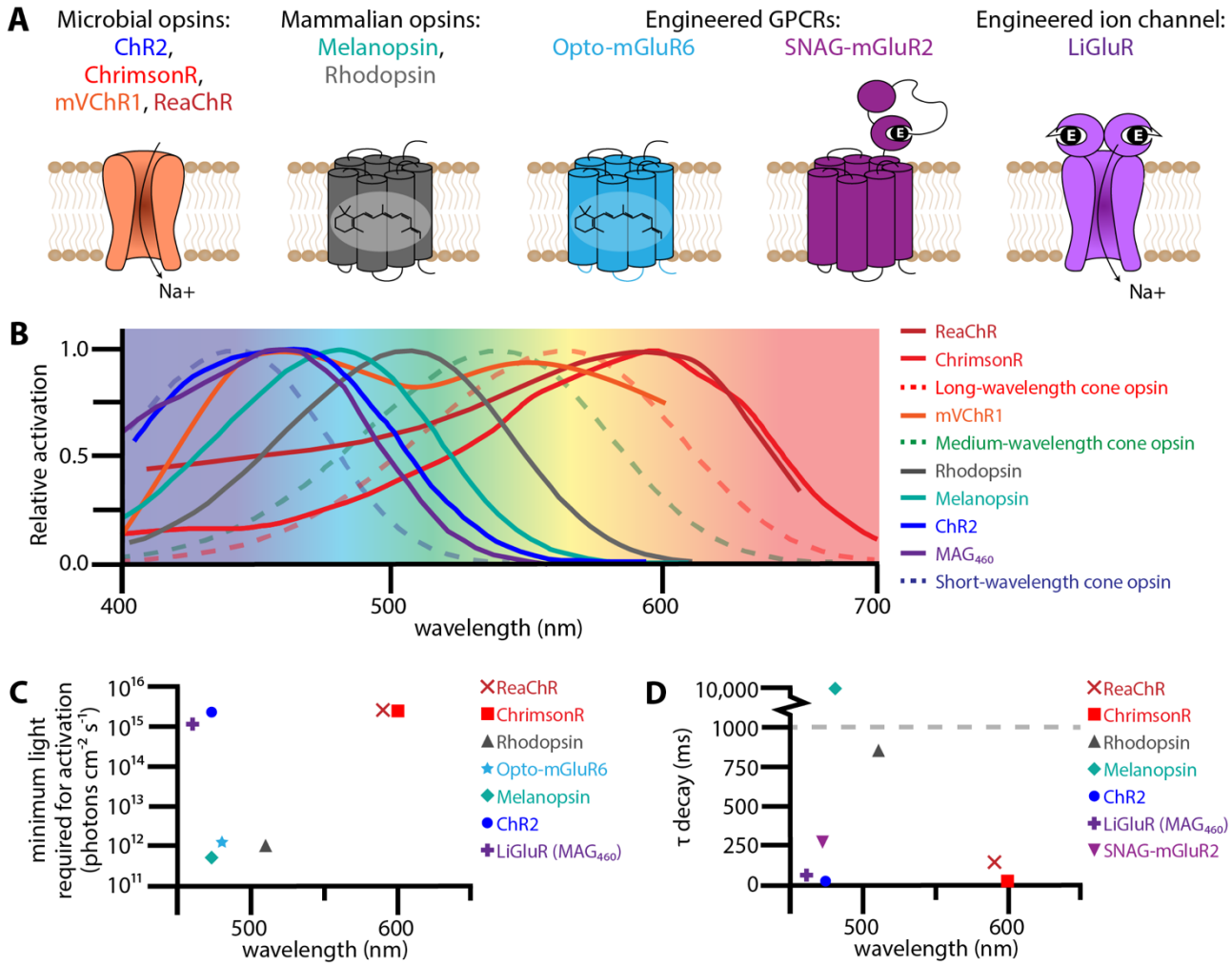

Figure 4.2. Therapeutic optogenetic effectors used to restore the visual response in degenerate retinas. (A) Structural diagrams of optogenetic microbial opsins, mammalian opsins, GPCRs, and ion-channels. The microbial opsins are all sodium permeable ion channels. The mammalian opsins, melanopsin and rhodopsin, are GPCRs with six transmembrane domains containing the chromophore 11-cis retinal. The engineered GPCR Opto-mGluR6 is comprised of the transmembrane domains from melanopsin with the intracellular loops from mGluR6. SNAG-mGluR2 is mGluR2 with a N-terminal SNAP-tag that tethers the PORTL BGAG. Upon light stimulation, the azobenzene in BGAG isomerizes allowing the distal glutamate to bind to the active site of mGluR2. The engineered ion channel LiGluR is iGluR6 with a cysteine mutation that allows for the covalent binding of the photoswitch MAG. Light isomerizes the azobenzene in MAG forcing the glutamate into the binding pocket. **(B)** Excitation spectra for optogenetic effectors used for vision rescue (solid lines) and human cone opsins (dotted lines). **(C)** The minimum light required for activation for various optogenetic effectors when used for vision rescue plotted against wavelength. **(D)** The τ decay constant plotted against wavelength for various optogenetic effectors. The excitation spectra, minimum light requirements, and τ decay constants were collected from the following publications: 8,13,16,20,23,24,36,57,58.

The other red-shifted chimeric channel, mVChR1, is a fusion of VChR1 and the N-terminus *Chlamydomonas* channelrhodopsin-1 (not to be confused with C1V1) and has the largest activation spectrum of the ion channel based opsins tested thus far, ranging from 468 to 640 nm (Fig. 4.2B)[16]. The conductance of mVChR1 is not as efficient as ChR2 and it also requires a similar amount of light

to open the channel. However, it is the only ion channel responsive to light throughout the whole human visual spectrum, making it a promising gene therapy candidate. To further improve spectral sensitivity, the same group used both mVChR1 with ChR2 to restore vision in blind mice[17]. Unsurprisingly, the use of multiple opsins generated greater light responses across the spectrum than one opsin alone.

In 2014 another red-shifted microbial ion channel was discovered in *Chlamydomonas noctigama*, termed ChrimsonR (Fig. 4.2A)[18]. With a similar activation spectrum and light requirements of ReaChR but with a faster deactivation time constant (Fig. 4.2B, C, D), ChrimsonR is another strong vision restoration gene therapy candidate and is currently in clinical trials[19].

Even though the red-shifted opsin variants use a safer wavelength of light, they still require extremely bright light on the order of 10^{15} photons cm^{-2} s^{-1} (Fig. 4.2C). Since the light is the direct effector of these ion channels, there is no way to increase light sensitivity of the system other than increasing the light sensitivity of the opsin itself. Currently, the best strategy to increase light sensitivity is to use a light sensitive effector with the capacity to amplify the light response, like GPCRs.

GPCRs

While there have been great improvements in spectral sensitivity and conductance for light activated ion channels, they still pale in comparison to the signal generated by optogenetic GPCRs. The pay-off for this increased light sensitivity is a loss in temporal sensitivity, with optical GPCRs lagging behind their ion channel in terms of both activation and inactivation[20–24]. However, the slower kinetics of the optogenetic GPCR could be well tolerated due the loss of a synaptic layer. Compared to wild type, the light signal was able to reach V1 faster for the optogenetic ion channel gene therapies by tens of milliseconds[6,8,11,25,26]. The loss of the photoreceptors and their synapse means that there are fewer cells the light signal has to pass between before reaching the brain in these animals with restored vision. Due to this, the slowness of the GPCRs can be partially compensated for by signal generation in downstream cells.

The first GPCR to be adapted for vision restoration was the light sensor for intrinsically photosensitive retinal ganglion cells, melanopsin (Fig. 4.2A)[20]. Responsible for the pupillary light response and maintenance of circadian rhythms[27], melanopsin is a clear candidate for vision restoration due to its established ability to generate light responses in non-photoreceptor retinal cells. When delivered intravitreally to the ganglion cell layer[20], or subretinally to outer retinal cells [21], melanopsin treated retinas are three fold more light sensitive than any microbial opsin, only requiring 10^{12} photons cm^{-2} s^{-1} to generate a signal (Fig. 4.2C). While melanopsin treated mice are able to perform some basic light response behavior and show an increased pupillary light response, the GPCR's kinetics are incredibly slow. It takes hundreds of milliseconds to several seconds to activate melanopsin, and even longer for it to turn off (Fig. 4.2D)[21]. This slow response time is sufficient for basic perception (i.e. is it daytime or not), but makes it a poor tool for the high acuity vision associated with humans.

Another candidate for vision rescue is rhodopsin, the exceedingly light sensitive GPCR found in rod photoreceptors[22,23]. Rod photoreceptors are capable of responding to a single photon thanks to the phototransduction cascade. Light isomerizes the chromophore 11-cis retinal into all-trans retinal, inducing a conformational change in the GPCR which activates its G-protein, transducin. The α-subunit of transducin dissociates and activates phosphodiesterase (PDE), which lowers the

concentration of cGMP, closing cyclic nucleotide gated channels and hyperpolarizing the cell. When expressed in non-photoreceptor cells, rhodopsin has a similar light sensitivity to melanopsin of 10^{12} photons cm^{-2} s^{-1} (Fig. 4.2C), but importantly responds to light ten times faster than melanopsin (Fig. 4.2D)[22,23]. However, when compared to rod photoreceptors, rhodopsin activation in ganglion cells or bipolar cells is much slower. When heterologously expressed, it is unlikely that the time to isomerize 11-cis or activate rhodopsin itself has changed, but rather the lack of other phototransduction cascade proteins increases cellular response times. Rod photoreceptors have specialized discs which contain all the phototransduction cascade proteins to promote efficient signaling. While other cells lack this specific structure and phototransduction cascade, photoactivation of other existing signaling cascades could improve temporal sensitivity.

What is impressive is that the rhodopsin protein is photosensitive at all. Many believed that rhodopsin outside a photoreceptor would be unable to attain its 11-cis retinal chromophore. 11-cis is tightly regulated being recycled from all-trans in retinal pigment epithelium (RPE) and Müller cells, which have developed specialized mechanisms to deliver the incredibly photosensitive pigment to photoreceptors[28]. While some teams have had to supply the 11-cis for the multielectrode array (MEA) experiments, *in vivo* assays demonstrated effective iterative activation of rhodopsin without the use of exogenous chromophore[22]. Perhaps in the degenerate retina, the RPE and Müller cells still produce 11-cis and can aberrantly deliver it. Considering that photoreceptors contain approximately a thousand discs each with thousands of rhodopsin molecules[29], the concentration of rhodopsin ectopically expressed in ganglion cells or bipolar cells would pale in comparison to wild type levels, perhaps low enough to ensure chromophore delivery despite being non-target cells. Furthermore, there might be alternative chromophore delivery mechanisms. It was recently determined that melanopsin also uses 11-cis[30], so whatever mechanism delivers the 11-cis to melanopsin could potentially also deliver it to rhodopsin when expressed in ganglion or bipolar cells.

Recently, optically controlled GPCRs have been engineered by multiple groups[31–36]. Unlike melanopsin or rhodopsin, these GPCRs have been constructed or modified to become light sensitive. One particularly interesting candidate, Opto-mGluR6, is a chimeric protein composed of the chromophore-adhering transmembrane domains of melanopsin with the regulatory transmembrane domains of mGluR6, the ON-bipolar specific GPCR (Fig. 4.2A)[36]. Opto-mGluR6 is the most light sensitive construct tested so far, eliciting a light response at $5x10^{11}$ photons cm^{-2} at 473 nm (Fig. 4.2C). In degenerate animals expressing Opto-mGluR6 in their bipolar cells (Fig 4.1C), the light responses generated signals in bipolar cells and ganglion cells had similar timing to photoreceptor evoked light responses in wild type animals. Expressing a photoactivable version of the naturally occurring GPCR in the target cell type is an enticing goal, however, the Opto-mGluR6 group had to resort to transgenic animals to show any function or behavior. In order for any of these therapies to be a viable option for people, non-transgenic routes must be pursued.

Delivery methods

The goal of these ambitious projects is to cure blindness in humans. While transgenic animals are an invaluable laboratory tool, they are not applicable to humans. Viral transduction is currently the best method to constitutively express heterologous proteins in mammals. Other methods like electroporation have been shown to successfully deliver transgenes in mice[37], but the AAV method has been demonstrated to be safe, effective, and long lasting, as evidenced by the recent FDA approval for the first vision restoration gene therapy for LCA2[38–41].

The current trend in many laboratories is to try and recapitulate the ON/OFF light response by infecting ON-bipolar cells with improved viral capsids and promoters (Fig. 4.1C)[13,22,23,26,36,42,43]. The

hypothesis is that infecting the furthest upstream cells preserves the circuitry and processing resulting in a better final signal sent to the brain. By using the mGluR6 promoter, the ON-bipolar cell specific metabotropic glutamate receptor, optogenetic gene expression is limited to these upstream cells. ChR2[26,42,44], LiGluR[13], rhodopsin[22,23], and Opto-mGluR6[36] mediated light activation of bipolar cells (Fig 4.1C) can produce ON and OFF responses in ganglion cells and diverse responses in V1, unlike the simple ON response produced by photosensitive ganglion cells.

While this is an admirable goal, bipolar cells are one of the hardest cell types to infect in the retina[45]. Laboratories have engineered new and improved AAV variants with unprecedented retinal penetration[46], and even the best capsid variants do not efficiently transduce bipolar cells. AAV never achieves complete infection and is normally "patchy", so complete restoration of a receptive field looks unlikely. Furthermore, the mGluR6 promoters used to restrict expression to the ON-bipolar cells may not work in many retinal dystrophies due to dis- and downregulation of mGluR6[47]. mGluR6 is tonically activated by the continuous release of glutamate by photoreceptors in the dark. Upon photoreceptor cell death, mGluR6 is no longer stimulated and the ON-bipolar cells undergo transcriptional changes that limit mGluR6 expression and potentially other genes under that promoter[48].

Instead of trying to restrict expression to bipolar cells, it might be more advantageous to use ubiquitous promoters that would allow expression in bipolar cells and other retinal cells to increase light sensitivity. This way some greater processing is preserved allowing for the generation of ON and OFF light responses, while generally increasing the light sensitivity of the retina as a whole.

Multi-effector therapy

Bipolar cells direct the ON and OFF pathways in healthy tissue, but that does not mean that bipolar derived signal is the only way to generate ON and OFF signals. There are a variety of inhibitory optogenetic ion channels[49–51], pumps[52–54], and GPCRs[31,33] that could emulate an "OFF" response. This was first demonstrated by Zhang et al. in 2009 by combining the inhibitory ion pump HaloR with ChR2 to produce ON and OFF and ON/OFF light responses. While HaloR requires twenty times more light than ChR2, which already requires an unsafe amount of light, this study importantly shows that the retina can produce multiple types of light responses without bipolar cell transduction.

Recently, Berry et al. improved upon Zhang's original work by using their engineered SNAG-mGluR2 and LiGluR combination therapy[24]. Similar to LiGluR, SNAG-mGluR2 is a modified version of the metabotropic glutamate receptor 2 (mGluR2) with a N-terminal SNAP tag that allows stable conjugation of the azobenzene-glutamate photoswitch by a selective benzylguanine-reactive group (Fig. 4.2A)[31]. SNAG-mGluR2 is one of the fastest optogenetic GPCRs with kinetics on the order of hundreds of milliseconds (Fig. 4.2D), but it unfortunately requires a similar amount of light as ChR2 (Fig. 4.2C). The combination of the excitatory LiGluR ion channel and inhibitory SNAG-mGluR2 GPCR generates diverse light responses including ON, OFF and ON/OFF responses. These diverse responses improved visual behavior in treated mice compared to LiGluR or SNAG-mGluR2 alone. While this combination therapy still requires bright light, it importantly shows that multi-effector therapy has the potential to restore complex and diverse cellular light responses similar to natural vision.

Conclusions

The audacious goal of genetically restoring vision to the blind is now possible. Newly discovered and developed optogenetics have improved upon the original ChR2 studies. By red-shifting ion channels, researchers have made safer alternatives with broader spectrums. And new GPCRs are gaining speed to allow for the temporal precision required for high acuity vision. The new trend to use multiple effectors to generate diverse responses should be expanded further. Humans use three different cone opsins and one rod opsin to generate vivid visual perception. With so many optogenetics with diverse excitation spectra, combination therapies using multiple effectors producing excitatory and inhibitory responses at different wavelengths could generate the complex visual information comparable to responses naturally derived in healthy tissue. With new and innovative therapies constantly being developed, vision restoration is within sight.

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