Gene Therapy for Blindness

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Abstract

Sight-restoring therapy for the visually impaired and blind is a major unmet medical need. Ocular gene therapy is a rational choice for restoring vision or preventing the loss of vision because most blinding diseases originate in cellular components of the eye, a compartment that is optimally suited for the delivery of genes, and many of these diseases have a genetic origin or genetic component. In recent years we have witnessed major advances in the field of ocular gene therapy, and proof-of-concept studies are under way to evaluate the safety and efficacy of human gene therapies. Here we discuss the concepts and recent advances in gene therapy in the retina. Our review discusses traditional approaches such as gene replacement and neuroprotection and also new avenues such as optogenetic therapies. We conjecture that advances in gene therapy in the retina will pave the way for gene therapies in other parts of the brain.

Contents

AN EYE-OPENER: THE TRANSLATIONAL SUCCESS OF *RPE65* GENE REPLACEMENT

Over the past decade, considerable progress has been made in gene therapy for monogenic inherited blinding diseases, as epitomized by the advances achieved in the treatment of one form of Leber congenital amaurosis (LCA). LCA is the most severe retinal dystrophy and leads to a major visual impairment at birth or before the age of one year. LCA is caused by mutations in any one of at least 17 genes encoding proteins involved in a variety of retinal functions (den Hollander et al. 2008, Falk et al. 2012). To date, a form of LCA that is caused by loss-of-function mutations in the retinal pigment epithelium (RPE)-specific *RPE65* gene is the most extensively studied, both in animal models and in humans. In animal models, proof-of-principle for gene-replacement therapy was first demonstrated by a team led by Jean Bennett. The team restored visual function to Briard dogs affected by naturally occurring *RPE65* mutations using adeno-associated virus (AAV)-mediated delivery of *RPE65* to the eye (Acland et al. 2001). Three years after a single subretinal administration of AAV-*RPE65*, retinal responses remained stable, providing evidence of long-term restoration of photoreceptor function (Acland et al. 2005). Remarkably, the improvements have remained stable for more than ten years. In humans, phase I clinical trials simultaneously conducted by three different groups demonstrated that AAV-mediated *RPE65* gene therapy was safe and led to a slight improvement in vision, mostly under dark-adapted conditions (Bainbridge et al. 2008, Hauswirth et al. 2008, Maguire et al. 2008). Investigators have subsequently reported stable clinical benefits in more than 30 patients, with improvement in bright and dim light vision and no severe adverse effects (Cideciyan et al. 2009a,b; Simonelli et al. 2010). Moreover, readministration of AAV-*RPE65* to the contralateral eye in three LCA patients 1.7 to 3.3 years after the initial injection of *RPE65* gene–based treatment in

one eye was shown to be safe and effective (Bennett et al. 2012). The pioneering *RPE65* gene therapy trials provided promise for gene therapy in different forms of LCA (Pawlyk et al. 2005, Sun et al. 2010) and other retinal diseases.

WHY THE EYE?

The number of visually impaired people throughout the world is estimated to be 285 million, of whom 39 million are legally blind (Mariotti 2012). Most of these patients suffer from diseases that affect cell types in the eye. The eye has unique characteristics, compared with other tissues and organs, that make it particularly suited for gene therapy (Bainbridge et al. 2006). First, it is a small, closed compartment. Because of this, a long-lasting, high viral concentration can be achieved by injecting only a small amount of a virus, and systemic dissemination and risk for adverse systemic effects are minimal. Owing to internal compartmentalization within the eye, a virus can be selectively delivered to different ocular structures, such as the anterior chamber, the vitreous cavity, or the subretinal space. Second, most cell types of the eye are stable, and many of them are evolutionarily highly conserved across mammals and even across vertebrates. Since most cell types do not divide, the risk for malignant transformation is reduced, and one can achieve sustained gene expression because transgenes are not diluted in cell division. Nevertheless, division of glial cells is a possibility (Bhatia et al. 2011); therefore, the use of vectors that do not integrate into the genome is desirable. Because gene expression in cell types is often conserved, one can screen cell-type targeting vectors in nonprimate mammals before testing them in primates. Third, the eye is partially shielded from the actions of the immune system by a blood-retinal barrier (Streilein et al. 1997). This feature, together with a gamut of others, including the local inhibition of immune responses and the systemic induction of immunosuppressive regulatory T-cells by eye-specific mechanisms, contributes to a phenomenon known as ocular immune privilege (Caspi 2010, Streilein 2003), which ensures partial protection against immune responses directed against gene products and vector antigens. Fourth, numerous animal models of inherited retinal diseases have already been developed in rodents, cats, and dogs, which facilitates preclinical assessment of therapeutic efficacy (Fletcher et al. 2011). Fifth, the optical transparency of the eye, together with recent advances in in vivo imaging techniques such as scanning fluorescent ophthalmoscopy, optical coherence tomography, autofluorescence, and adaptive optics, allows not only for direct noninvasive visualization of reporter gene expression from targeting viruses in animal models but also for accurate evaluation of the gene therapy outcomes in both animal models and human patients. Sixth, the untreated contralateral fellow eye is potentially useful as a control in clinical trials.

THE RETINA: AN IMAGE-PROCESSING MACHINE

The retina can be viewed as a parallel image processor that acquires images via a mosaic of photoreceptors and that extracts various visual features from the acquired images (Azeredo da Silveira & Roska 2011, Gollisch & Meister 2010, Masland 2001, Wässle 2004) (**Figure 1**). Rod photoreceptors respond directly to light at lower intensities and cone photoreceptors at higher intensities. The cellular infrastructure that underlies parallel processing consists of mosaics of local neuronal circuits. The retina has ~20 such circuit mosaics, built from more than 60 cell types, that independently extract different features from the visual world. Each mosaic has an associated mosaic of output cells, the ganglion cells, which relay the computed feature to higher brain centers. Here we briefly summarize current knowledge about the characteristics of the retinal circuit that lead to a better understanding of different approaches in retinal gene therapy. Each cone in the retina is connected to ~10 types of cone bipolar cells, and each of these bipolar cells is connected to

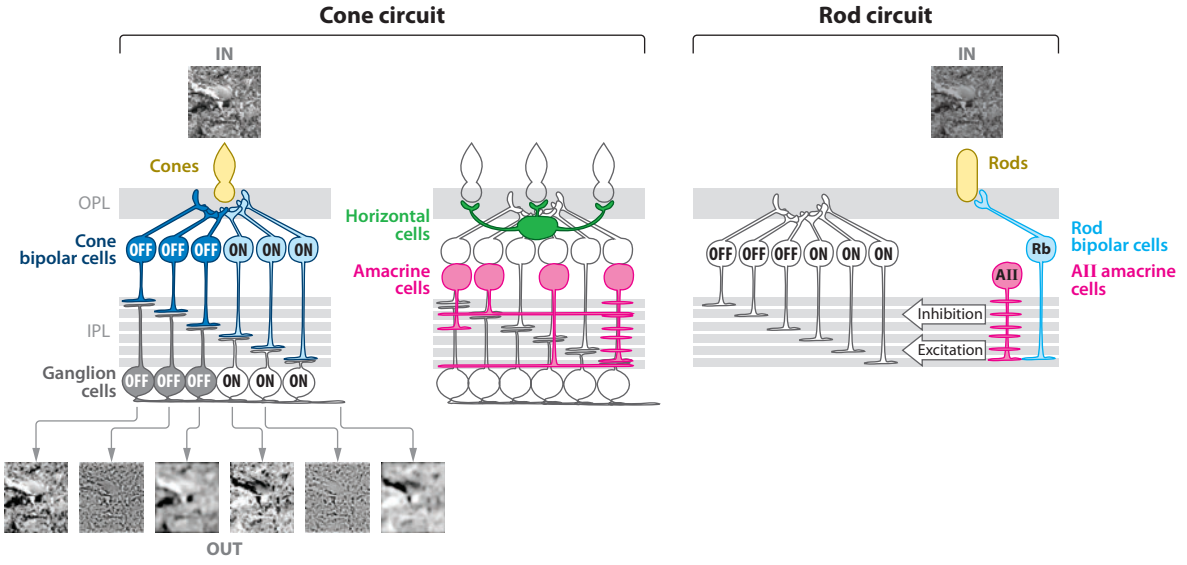

Figure 1

Basic elements of cone- and rod-driven circuits. (*Left*) Cone circuit, (*right*) rod circuit. ON and OFF refer to cells that are activated by light contrast increments and decrements, respectively. For rods, only the rod-rod bipolar (Rb) circuit is shown. This rod circuit joins the cone circuit at the level of cone bipolar cell axon terminals. The AII amacrine cell is excitatory toward ON cone bipolar cells and inhibitory toward OFF cone bipolar cells. IPL, inner plexiform layer; OPL, outer plexiform layer.

several types of ganglion cells. Cones, bipolar cells, and ganglion cells use the excitatory neurotransmitter glutamate to communicate. The axon terminals of bipolar cells and the dendrites of ganglion cells are restricted to narrow laminae in the inner retina, forming ~10 layers. Communication between cones and bipolar cells is modified by the inhibitory horizontal cells, and communication between bipolar cells and ganglion cells is modified by a large variety of inhibitory amacrine cells. Cones respond to light by lowering their membrane voltage; i.e., they hyperpolarize. Half of the cone bipolar cells also hyperpolarize (OFF cells), whereas the other half increase their membrane voltage, depolarizing when light intensity increases (ON cells). The polarity of the ganglion cell responses is determined by the polarity of the bipolar cells from which they receive input. Each rod is connected to a special bipolar cell type called the rod bipolar cell. Rod bipolar cells "talk" to the so-called AII amacrine cells, which then provide excitatory input to the axon terminals of ON cone bipolar cells and inhibitory input to OFF cone bipolar cell terminals. Rods are hyperpolarized by light, whereas rod bipolar cells and AII amacrine cells are depolarized: These are therefore ON cells. The key point here is that AII amacrine cells can modulate both ON and OFF cone bipolar cells, but with opposite effects. The retina also incorporates different glial cell types, the most well studied being the Muller cells, which have important roles in a variety of homeostatic processes as well as in responses to injury and disease (Bringmann & Wiedemann 2012). Retinal cells are arranged in mosaics, covering the entire retina. The only exception to the mosaic arrangement is a special area of the retina in some primates and in a few predatory birds and reptiles. This area is called the fovea (Hendrickson 1992) and is the place with the highest cone density. The human fovea, also called macula, has no rods within its center, and the only cellular compartment that is organized

in a mosaic fashion is the cone outer segment. Foveal cone cell bodies are piled on top of each other, whereas cell bodies of all other cell types are shuffled to the side, forming a concentric ring of cell bodies (Hendrickson 1992).

TARGETING RETINAL CELL TYPES: THE PROBLEM OF DIVERSITY

Gene therapy appears to be conceptually simple: A gene is delivered to a tissue where the lack of function of the mutated gene leads to loss of tissue function. However, when the target tissue has tens of different cell types, and only a few of them express the disease-associated gene, cell-type targeting is needed. One cannot treat a part of the brain the same way as one would tissues with simple architectures, such as the liver, which is made up of only a few cell types. Achieving cell-type-restricted gene expression is a largely unsolved problem (Busskamp et al. 2012, Busskamp & Roska 2011). The key for targeting lies in two components: one that permits the efficient entry and intracellular transport of the vector to a specific cell type and a second that restricts expression to a given cell type. This second factor often takes the form of a cell-type-specific promoter. In addition to cell targeting, two other very important variables to control for are the desired gene-expression level and its cell-to-cell variation. First we discuss permissive factors for gene delivery, followed by strategies to restrict expression, and then we comment on controlling the mean and variance of gene-expression levels.

Permissive Factors

Gene delivery can be based on viral or nonviral vectors. Viral vectors are engineered from adeno-associated viruses (AAVs), lenti viruses (LVs), and herpes viruses (HVs) (Colella & Auricchio 2012). Nonviral methods include gene delivery based on naked DNA, oligonucleotides, DNA enclosed in cationic liposomes, and DNA associated with polymers (Li & Huang 2000). The most widely used vectors for retinal gene delivery are AAVs (Colella & Auricchio 2010). AAVs can be modified to fine-tune a number of permissive components. These components do not give specificity to the AAV, in terms of targeting specific cell types, but do permit high expression levels or expression in a particular time frame. Permissive components include capsid types responsible for viral entry, capsid residues responsible for intracellular AAV processing, and single- or double-stranded AAV forms, as well as the sequence of the gene of interest. An additional permissive property is the site of injection in the eye: whether it be the anterior chamber, vitreous cavity, or subretinal space. AAVs are available with more than 100 different natural capsids, and thousands of genetically modified capsids have been created through rational mutagenesis of the capsid (Petrs-Silva et al. 2009, 2011; Zhong et al. 2008) or selected through combinatorial screens (Bartel et al. 2012, Dalkara et al. 2011, Klimczak et al. 2009). AAVs with different capsids are called serotypes. Each serotype has a different efficiency for infecting retinal or other ocular cell types, a property called tropism (Colella & Auricchio 2010). Mutant capsids sometimes allow better transduction efficiencies or different tropisms over the natural variants. Unfortunately, the tropism of a serotype is species dependent. Therefore, all serotypes must be tested in primates in vivo and, ideally, ex vivo in human retinal explants. However, we do not know for certain if any of these tests reliably predict good in vivo expression in humans: First, it is not clear if nonhuman primates have the same capsid-cell-type interactions as humans do; and second, due to dilution and other factors, ex vivo tests do not necessarily reflect the in vivo situation. Recent studies have found that some capsid residue (tyrosine) mutations increase gene expression by decreasing the intracellular elimination of AAV particles. These capsid modifications can be fashioned regardless of the capsid type used and could improve gene expression. The slow onset (~3 weeks) of transgene expression is considered to be a limitation of AAVs, but it could be circumvented by using self-complementary

AAVs (scAAVs), which ensure more rapid transgene expression (<1 week). It is not clear if, in the long term, scAAVs express more, and a limitation of scAAVs is that they can package only half the amount (2.4 kb) of DNA compared with normal AAVs (4.8 kb). The site of injection is an important determinant of the number of AAV particles that reach a given cell type. For retinal delivery, AAVs can be injected into the subretinal or the intravitreal space. When a normal AAV is injected into a primate eye intravitreally, the cells in the fovea are densely labeled but cells outside the fovea are sparsely transduced (Ivanova et al. 2010, Yin et al. 2011; D. Dalkara & J. Flannery, personal communication). This difference of infectivity in the fovea versus periphery is likely due to the very thick inner limiting membrane that forms a barrier between the intravitreal space and the retina. Subretinal injection leads to high-density labeling around the injection site. New AAV variants, with modified capsids, may be able to infect densely from the vitreal side outside of the fovea, but this has not yet been demonstrated in primates. Intravitreal injections have one more limitation: The intravitreal space is large and not well defined because the vitreous body that occupies this space is heterogeneous and changes in consistency with the subject's age, being gelatinous in young people and liquid in older people. The vitreous body can be removed, leaving an aqueous space several milliliters in size that could be filled either with a very large amount of virus or with a smaller amount, leading to significant AAV dilution. An advantage of intravitreal injection is that it is simple, and no detachment of the neural retina from the pigment epithelium is created. Subretinal injection is more difficult, leading to a temporary separation of the pigment epithelium from the photoreceptors; however, AAV concentrations can be tightly controlled. In gene-replacement therapy, where the photoreceptor outer segments are intact and need to interact with the pigment epithelium for outer segment renewal, subretinal injections must be done with care. We argue, however, that when the rods and cones do not respond to light, and therefore their interaction with the pigment epithelium may not be as important, subretinal injections are the preferred method. A limitation of AAVs is the restricted length of DNA (a total of 4.8 kb) that can be packaged into these vectors. Possible solutions to this limitation, as yet still theoretical, would be either to generate dual AAV vectors, each carrying one half of the transgene, which then reassembles in vivo or the packaging of oversized genomes (Colella & Auricchio 2012).

Restrictive Factors

The specificity of AAVs can be controlled by cell-type-specific promoters (Busskamp et al. 2012). The notion "specific promoter" must be treated with caution. First, gene expression driven by a specific promoter is likely detectable in nontargeted cell types. The ratio of expression between target and off-target cell types and the threshold for the biological effect are the relevant quantities to describe specificity. Second, whether a promoter drives specific expression in a cell type depends on the method of expression. The same promoter could behave differently when used in a transgenic animal, when electroporated, or when expressed from a virus. This variance is because the specific promoter is simply a DNA sequence that binds a combination of transcription factors, some of which are cell-type specific. However, when surrounded by different sequences in a viral or transgenic context, the binding can be modified. Using different expression methods, the copy number of the promoter-gene construct also varies significantly. Specificity and expression also depend on the species. Although finding promoters based on the gene-expression patterns of different retinal cell types (Siegert et al. 2012) and based on sequence conservation in mammals is a rational starting point, the process to select the stretch of DNA that fits the AAV and confers specificity is still trial and error. A key point here is that, ideally, the promoter should be tested in combination with all the other elements of the targeting vector in

the final formulation, both in vivo in nonhuman primates and ex vivo in postmortem human retinas. Specificity could also be achieved by sensing the cell's gene-expression state combinatorially using cell-type classifiers built by synthetic biology approaches (Benenson 2012).

Control of Mean and Variance of Gene Expression

The number of AAV particles present in the different cells varies, even within cells of the same type. To our knowledge, none of the present preclinical or clinical gene-therapy trials controls for the variance of gene expression due to copy number variation. In some instances, the precise level of expression is not as important; the replacement of defective enzymes could be one such case. However, when precise stoichiometry is needed, regulation of the mean and variance of gene expression could be critical. The mean expression can be tuned using promoters of different strengths as well as using different combinations of permissive factors, whereas the control of cell-to-cell variation ("noise") in gene expression may require insights from synthetic biology (Benenson 2012).

FORMS OF GENE THERAPY

Gene therapy for hereditary eye diseases may take one of two different forms: One depends on the mutated gene or even on the mutation itself; the other is independent of the mutated gene and depends only on the structural and functional state of the retinal circuit. The first form is called gene supplementation or gene replacement. Although this approach is the most logical and straightforward to correct a genetic disease of the eye, one challenge in gene-replacement strategies is the large number of affected genes. Hundreds of disease-causing gene mutations and dozens of mutated genes have been reported. A second challenge is the limited packaging capabilities of gene-delivery vectors suitable for use in humans. Only a few disease-associated genes fit into these vectors. A third challenge is that the cell type that expresses the mutated gene must be alive at the time of therapy. A fourth challenge concerns dominant mutations in which the mutated allele is toxic to the cell. The eye diseases most suited to gene supplementation are recessive hereditary diseases caused by mutations in small genes in long-surviving retinal cell types such as the retinal pigment epithelium.

A number of mutation-independent approaches either attempt to slow down retinal degeneration (neuroprotection) or do not interfere with the intrinsic progression of retinal degeneration, but attempt to restore photosensitivity by creating new photosensors and coupling them to the remaining retinal circuitry. For this latter strategy, researchers have considered three approaches: Electronic implants (Humayun et al. 2012, Zrenner et al. 2011) gather light using a technology similar to that used by video cameras and communicate pixel intensity to retinal cells via injected currents; stem cell approaches (Ong & da Cruz 2012, Singh & MacLaren 2011, Tibbetts et al. 2012) attempt to derive new photoreceptor cells, which must then be integrated into existing retinal circuits; and finally, optogenetic approaches (Busskamp et al. 2012, Busskamp & Roska 2011) can be used to target light sensors genetically to strategically important retinal cells. Either nonphotoreceptor cells are turned into photosensors (Bi et al. 2006, Lagali et al. 2008), or the photosensitivity of native photoreceptors compromised by disease is restored (Busskamp et al. 2010). The key point in optogenetic therapy is that retinal cells are already connected to other retinal circuit elements, and therefore a critical technological problem, namely how to connect photosensors to existing retinal circuits in a biologically relevant way, has already been solved. Here we discuss neuroprotective and optogenetic therapies. Stem cell (Ong & da Cruz 2012, Singh & MacLaren 2011, Tibbetts et al. 2012) and electronic implant approaches (Weiland et al. 2005, Zrenner et al. 2011) have been reviewed elsewhere.

Which kind of gene therapy can be performed depends on the stage of retinal degeneration. **Figure 2** correlates the stage of degeneration with the possible forms of gene

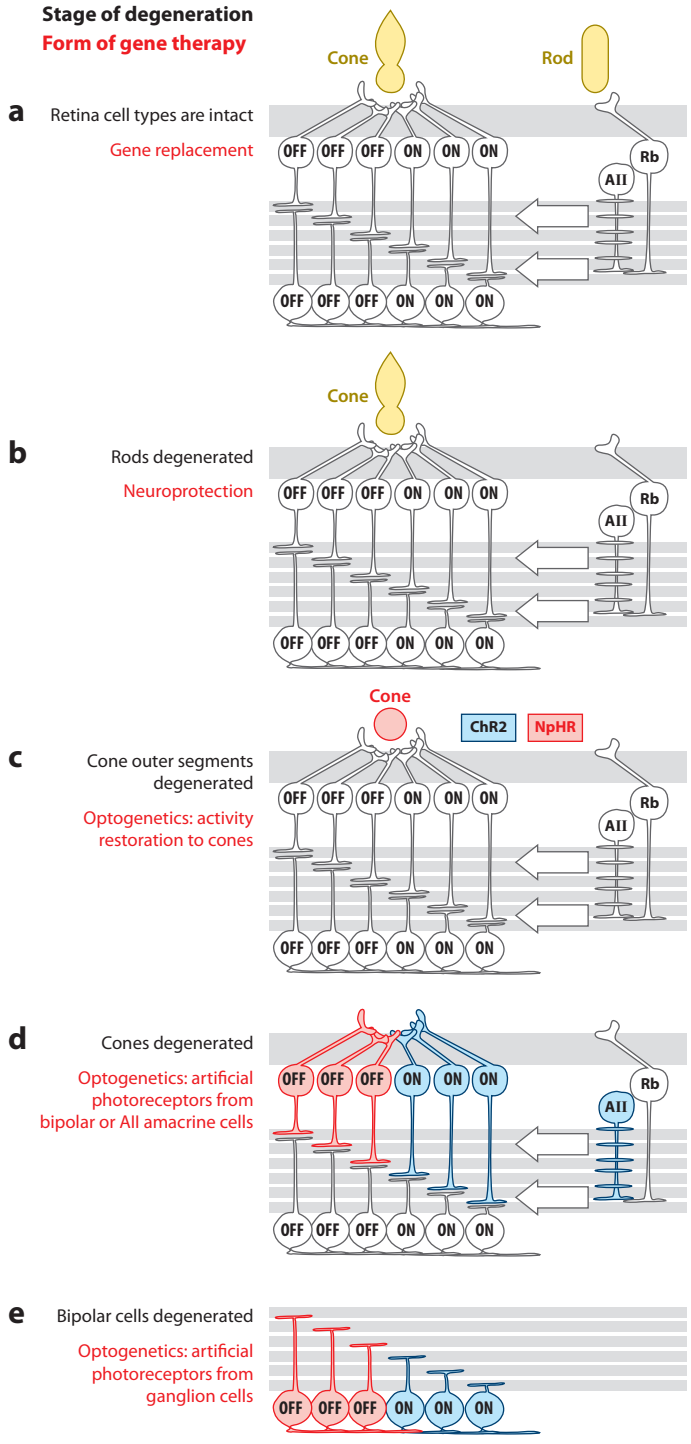

Figure 2

Stages of retinal degeneration and the forms of gene therapy that can be used in that stage.

therapy. Note that to what extent a stage at which no cones are left or only ganglion cells remain does exist in patients is not clear (**Figure 2**).

GENE-REPLACEMENT THERAPIES

Hereditary eye diseases can affect only the eye (nonsyndromic) or can affect other organs in addition to the eye (syndromic).

Nonsyndromic Retinitis Pigmentosa

Retinitis pigmentosa (RP) refers to a diverse group of progressive, hereditary diseases that often lead to incurable blindness and affect two million people worldwide. RP commonly starts with night blindness in young adults, reflecting early degeneration of the highly sensitive rod photoreceptors. This symptom is followed by a delay with a progressive decline in daylight central vision, owing to loss of function of the less-sensitive cone photoreceptors. Mutations in more than 44 genes have been demonstrated in different forms of RP, but in ~50% of cases the mutation has not yet been identified. Most known RP genes are expressed in rods or retinal pigment epithelium, and in such cases, degeneration of cones is thought to be a secondary consequence of the death of the rods.

In nonsyndromic, recessive RP, retinal pigment epithelium and photoreceptor cells are the targets of a large proportion of gene-replacement studies (Ali et al. 2000, Bennett et al. 1996, Takahashi et al. 1999, Vollrath et al. 2001). Recessive RP is particularly suitable for gene-replacement therapy if the gene of interest fits the targeting vector and if the cells involved, usually rods, are alive at the time of therapy.

The mutational heterogeneity (see **https://sph.uth.tmc.edu/retnet/**), together with the molecular gain-of-function leading to degeneration, represents a significant obstacle to the development of gene therapies for dominantly inherited RP. Several new studies try to address simultaneous silencing of the effect of dominant mutations and the

substitution for normal function (Baulcombe 2002, Chadderton et al. 2009, Farrar et al. 2011, Mao et al. 2012, Tam et al. 2008).

Syndromic Retinitis Pigmentosa

The most frequent forms of syndromic RP are Usher syndrome and Bardet-Biedl syndrome. There is, as yet, no effective cure or prevention of the vision loss associated with these diseases, but recent studies have provided potential strategies for therapy of the retinal phenotype.

Bardet-Biedl syndrome affects the function of nonmotile cilia and leads to obesity, RP, the formation of extra digits, hypogonadism, and kidney problems (Forsythe & Beales 2012). The first successful gene-based treatment of Bardet-Biedl syndrome–associated retinal degeneration was demonstrated in a *Bbs4*-null mouse model, where an AAV-based vector expressing BBS4 prevented photoreceptor death, improved retinal function, and visually evoked behavior responses, providing hope for a promising future therapy in humans (Simons et al. 2011).

Usher syndrome is manifested in deafness and blindness. On the basis of symptom onset and severity, the disease is categorized into three groups: Usher I, II, and III. In Usher I syndrome, one of the major hurdles in the design, development, and validation of gene therapy is the discovery that those mutations that cause retinal degeneration in humans cause little or no retinal degeneration in mice. Sahly et al. (2012) recently reported clues to understanding this discrepancy and the function of Usher proteins in the primate retina. Despite the mild retinal phenotype in mice, Hashimoto et al. (2007) demonstrated the retinal expression of an Usher I gene, *MYO7A*, and the correction of some cellular defects. Owing to the large size of *MYO7A* (~7 kb), the researchers used LV for gene delivery. Researchers in this field still intensely debate the effectiveness of LV variants for transducing adult photoreceptors in vivo. Usher II mouse models better resemble the human phenotype, and a recent study using an AAV-based delivery system has demonstrated successful and specific expression of one of the Usher II genes, *DFNB31*, in both rods and cones (Zou et al. 2011).

Other Hereditary Retinal Diseases Amenable to Gene-Replacement Therapy

Although gene-therapy prospects for retinal degeneration have been most intensively studied in LCA and RP, studies have also demonstrated successful gene therapy for other degenerative conditions of the retina.

Achromatopsia is a genetic disorder of cones caused by mutations in a number of cone-expressed genes: It leads to the loss of cone function, but the cells often remain alive for a long time. In the Gnat2$^{-/-}$ mouse model of achromatopsia, the AAV-mediated *GNAT2* gene therapy targeted the cones and rescued the cone-mediated electroretinogram (ERG) and visual acuity (Alexander et al. 2007). In the Cngb3$^{-/-}$ mouse model, the AAV-delivered gene driven by a cone arrestin promoter showed a similar effect (Carvalho et al. 2011). Researchers have found that the robustness and stability of the observed treatment effect were independent of mutation but dependent on both promoter type and age. Komaromy et al. (2010) achieved a stable therapeutic effect (for at least 33 months) in younger animals. Because mutations in the *CNGB3* gene are the most prevalent cause of achromatopsia, accounting for more than 50% of all known cases of this disease, this study provides proof-of-concept for a potential therapy to treat the biggest subset of patients.

Stargardt disease, which affects cones and rods, is most often manifested in early-onset macular degeneration. In Abca4$^{-/-}$ mice, a model of the most common human recessive Stargardt disease, intraocular administration of an AAV encoding *ABCA4* resulted in protein localization to rod outer segments and stable morphological and functional improvement of the phenotype in one study (Allocca et al. 2008). The large size of the *ABCA4* gene (~7 kb) led other groups to use LVs. In the same mouse

model, LVs ensured high transduction efficiency in rods and cones and significant reduction of lipofuscin pigment A2E accumulation, suggesting that LV gene therapy is potentially an efficient tool to treat ABCA4-associated diseases (Kong et al. 2008).

X-linked juvenile retinoschisis (XLRS) is another hereditary disease that leads to macular degeneration, although in some cases the peripheral retina is also affected. The gene that is responsible for most cases of XLRS is *RS1*, which codes for the protein retinoschisin. The lack of retinoschisin causes small tears between the layers of the retina. In the *Rs1h*$^{-/-}$ mouse model of XLRS, AAV-mediated intravitreal delivery of the normal *RS1* gene reduced the structural and functional loss of the retina when evaluated at 14 months of age; substantial (but variable) long-term rescue ERG amplitudes and waveforms were also reported (Kjellstrom et al. 2007, Min et al. 2005, Park et al. 2009).

Leber's hereditary optic neuropathy (LHON) is a mitochondrial disorder affecting ganglion cells that results in severe and usually irreversible visual loss in one eye. With some delay the fellow eye frequently suffers a similar loss of function. Unlike most retinal degenerations, which result in slow, progressive loss of vision over many years, LHON progresses quickly, although the timing of vision loss in the first eye is not predictable. Because vision loss in the second eye is highly likely a few months after the vision loss in the first eye, genetic correction of the mitochondrial defect for the second eye during this time window is logical. Investigators have recently undertaken important steps toward gene therapy for LHON. In a rat model, *ND1* expression restored vision, and signs of recovery appeared as early as 1–2 weeks after AAV-mediated *ND1* delivery into the superior colliculus (Marella et al. 2010). It should be emphasized that few practical methods for delivering genes to the mitochondria are currently available. To address this, Manfredi et al. (2002) developed an approach termed allotropic expression. Rescue of optic neuropathy (Ellouze et al. 2008) in an induced rat model of LHON suggested that allotropic *ND4* gene therapy could be effective in LHON patients with an *ND4* mutation. The authors optimized the allotropic expression for the mitochondrial genes *ATP6*, *ND1*, and *ND4* and obtained a complete and long-lasting rescue of mitochondrial dysfunction in human fibroblasts in which these genes were mutated. The same group has recently reported a substantial and long-lasting protection of retinal ganglion cell and optic nerve integrity after intravitreal administration of an AAV vector containing the full-length open reading frame and the 3′ untranslated region of the *AIF1* (apoptosis-inducing factor 1) gene in a spontaneous model of optic atrophy, the Harlequin mouse (Bouaita et al. 2012).

NEUROPROTECTION

Gene-therapy strategies can be used not only to correct the gene defect, but also to delay the degeneration independent of the mutation. One of the most frequently studied neuroprotective methods in animal models is the local expression of neurotrophic proteins to promote the survival of photoreceptors and the retinal pigment epithelium. Neuroprotection offers the possibility to treat not only genetic diseases but also a range of conditions, including acquired ocular disorders. Among the most extensively studied neuroprotective factors are ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (FGF), and glial cell–derived neurotrophic factor (GDNF) (Barnstable & Tombran-Tink 2006).

An attractive candidate to prevent and treat retinal degeneration is the protein rod-derived cone viability factor (RdCVF), specifically expressed and secreted by photoreceptors (Léveillard et al. 2004). RdCVF is a product of the nucleoredoxin-like 1 (*NXNL1*) gene homologous to the family of thioredoxins known to possess strong antioxidative properties. This trophic factor has directly induced cone survival in animal models of recessive and dominant RP, and studies have documented functional rescue independent of the

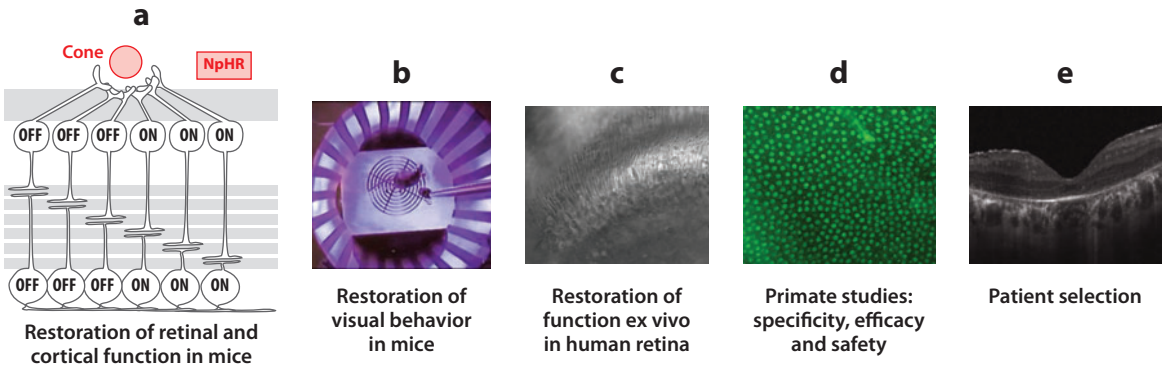

Figure 3

Translational route for one of the optogenetic therapies where cone activity is restored by the expression of halorhodopsin (NpHR).

mechanism and extent of rod degeneration. In treated animals, the functional effect observed appears to be related to the maintenance of cone outer segments (Yang et al. 2009). The gene encoding RdCVF also encodes for a second product with the characteristics of a thioredoxin-like enzyme, suggesting that RdCVF may play a physiological signaling role. It may be involved in the maintenance of photoreceptors by both autocrine and paracrine mechanisms, of importance during aging and exposure to oxidative stress. The two forms of RdCVF could be of particular interest as a therapeutic modality to prevent the secondary degeneration of cones in RP and, thereby, save vision (Fridlich et al. 2009, Léveillard et al. 2004, Léveillard & Sahel 2010).

Metabolic dysregulation and oxidative stress correlate with loss of visual activity, particularly in cones, and novel approaches are designed to modulate these pathways (Punzo et al. 2009, 2012). In addition, gene-based delivery can also be used to bring antiapoptotic, anti-inflammatory, or antiangiogenic molecules. Clinical trials are currently under way to investigate the effectiveness of the various neuroprotective therapies.

OPTOGENETIC THERAPY

Optogenetic therapy is relevant for treating forms of blindness in which some part of the retinal circuitry remains intact. Most diseases that fall into this category affect photoreceptors, rods, and cones. Initial attempts to restore vision using optogenetic methods have focused on RP, which is a collection of hereditary diseases that affect mostly rod-expressed genes and lead to rod degeneration. Later in the course of disease, due to a process that is not well understood, the cones also degenerate or lose their outer segments and become light insensitive, although, in most cases, they do not express the gene affected by the mutation. Optogenetic therapy is in the stage of preclinical studies. A translational route for optogenetic therapies is shown in **Figure 3**.

Optogenetic Tools

The two most well-known optogenetic tools are channelrhodopsin-2 (ChR2) (Boyden et al. 2005, Nagel et al. 2003), from the algae *Chlamydomona reinhardtii*, and halorhodopsin (NpHR) (Zhang et al. 2007), from the archaebacterium *Natronomas pharaonis*. These proteins are photosensitive and can be activated at specific light wavelengths. ChR2, a nonselective cation channel, provides neurons with excitatory currents and can therefore be used to activate cells (optogenetic activator), whereas NpHR, a chloride pump, generates inhibitory currents, which subsequently inactivate cells (optogenetic inhibitor). In recent years, a

large number of mutant channelrhodopsins have been produced, with different functional properties, and new light-gated pumps have been isolated from different species (Chow et al. 2012; Kleinlogel et al. 2011a,b; Prigge et al. 2012; Tye & Deisseroth 2012). A key property of these light-gated proteins is that in most animals, including mammals, they do not require any externally supplied cofactors. Both optogenetic activators and inhibitors can be used to restore retinal photosensitivity because certain retinal cells, such as ON bipolar and ON ganglion cells, are naturally activated by light, whereas photoreceptors, OFF bipolar cells, and OFF ganglion cells are naturally inhibited by light (Masland 2001, Wässle 2004).

Apart from natural light-gated channels, pumps, and their mutants, synthetic light-gated actuators have also been described using azobenzene as the light sensor (Fehrentz et al. 2011, Kramer et al. 2009). One version of this approach uses a genetically expressed channel in combination with an organic component (Caporale et al. 2011). The second approach uses only an organic molecule to modulate intrinsic channels expressed in retinal neurons (Polosukhina et al. 2012). Finally, researchers have used intrinsic light-sensitive proteins, such as melanopsin (Lin et al. 2008). Melanopsin is expressed in intrinsically photosensitive retinal ganglion cells, which participate in non-image-forming visual functions such as the entrainment of the circadian rhythm and the pupillary reflex (Schmidt et al. 2011).

Functional State of the Retinal Circuit in Blinding Diseases

A large body of work has detailed the changes in the retinal architecture in the rd1 mouse model of RP (Jones et al. 2012, Marc et al. 2003). These changes are mostly localized to the rod circuitry, the cones, and, to a lesser degree, the cone circuitry. The degeneration is progressive: Despite complete blindness, the state of the retinal circuit is different in two-month-old and one-year-old mice. Work on a different mouse model, *rd10*, has revealed a different time course for the disease (Gargini et al. 2007, Phillips et al. 2010). From research on the anatomy of RP mouse models, we can draw at least two conclusions. First, the time course of degeneration can be different depending on the mutation. Second, the state of the retina can be different within a group of individuals affected by the same mutation depending on the time of intervention. Work on the anatomy of postmortem human retinas from RP patients (Milam et al. 1998) has revealed interesting details about the changes in human retinal architecture. Notably, in all foveal regions examined, at least one layer of cone cell bodies was alive, but these dormant cones lacked outer segments (Milam et al. 1998). The existence of dormant cones is not specific to the human fovea: Work by LaVail and his colleagues revealed surviving cones in retinal degenerations at stages when no visual function could be detected (LaVail 1981). Despite all this important RP research, the state of the retina of a particular patient at a given time cannot be predicted. In vivo imaging, including optical coherence tomography, autofluorescence, and adaptive optics, allows clinicians to assess each patient and, therefore, decide which optogenetic strategy is most appropriate.

Optogenetic Strategies

A basic question when choosing a strategy is whether to express the optogenetic sensor randomly, using broad-spectrum promoters (Caporale et al. 2011, Isago et al. 2012, Ivanova et al. 2010, Ivanova & Pan 2009, Lin et al. 2008, Nirenberg & Pandarinath 2012, Thyagarajan et al. 2010, Tomita et al. 2009, 2010), or to target certain cell types using cell-type-specific promoters (Busskamp et al. 2010, Doroudchi et al. 2011, Lagali et al. 2008). At least two factors affect this decision. The first is safety: After an injection into the eye, some viral particles will appear in the blood. Broad promoters may drive gene expression in many tissues, including reproductive organs. Special care is needed in preclinical tests to assess this possibility. Furthermore, if ganglion cells express

the sensor, without a dendritic localization signal, the antigen will be present not only in the eye but also in the brain regions where the ganglion cells project (Bi et al. 2006). If there is an immune reaction, dealing with the eye alone is simpler than having to treat higher brain regions. Second, with respect to retinal function, broad promoters will make most cells that take up the virus light sensitive. This way, the function of any remaining retinal circuit is lost and the retinal output will simply signal changes in light intensity, just as do the intrinsically photosensitive ganglion cells but more quickly. Furthermore, unless the sensor is localized to the dendrites (Greenberg et al. 2011), retinal ganglion cell axons will be light sensitive; local light stimulation will therefore activate both local retinal cells and any retinal cells whose axons cross the stimulation point, which may result in the patient seeing randomly distributed dots instead of one localized spot. Finally, investigators have shown that broad promoters injected into primate eyes mostly drive expression in the fovea (Ivanova et al. 2010, Yin et al. 2011), where ganglion cells and bipolar cells are not organized in mosaics, which prevents the projected image from forming regular neural images. Despite these problems, some broad promoters are strong and often work across species; therefore, some light sensitivity will be conferred to patients. Because they are highly adaptive, the higher visual centers will likely use all possible information present in the concerted activity of ganglion cells to extract information about the visual world.

When the goal of therapy is to restore biologically relevant function, specificity at the periphery of visual circuits is more important than at other sensory circuits such as auditory circuits. This is because the physical arrangement of the front circuitry for audition is different from that for vision. In the auditory system, only the first synapse, between sensory and secondary cells, is positioned at the periphery. Therefore, only a limited amount of neuronal processing occurs in the inner ear. In the visual system, the second synapse is also embodied in the retina, and this second synapse is where complex neuronal computations, among amacrine, bipolar, and ganglion processes, are performed at parallel sites. The effect of nonspecific stimulation of the inner ear is similar to that of cell-type-specific cone or bipolar cell stimulation in the retina.

To restore biologically appropriate neuronal activity to the retina, using cell-type-targeted treatments, the key principles for designing the strategy are as follows: First, the closer to the photoreceptors we stimulate cells in the neuronal chain, the more natural the retinal processing will be; second, the optogenetic sensor-evoked activity should match the natural activity of the stimulated cell. ON cells are activated by light, whereas OFF cells are inhibited by light. However, the cell types that can be stimulated depend on which cells are alive in the patient.

At least four different strategies can be used, dictated by the functional organization of the mammalian retina. First, if cones are still alive but are nonfunctional, i.e., they lack outer segments, they can be targeted with optogenetic inhibitors (Busskamp et al. 2010). Second, ON (Lagali et al. 2008) and OFF bipolar cells can be targeted with activators and inhibitors, respectively. Third, AII amacrine cells, which could drive both the ON and OFF systems, can be targeted with activators. Fourth, ON and OFF ganglion cells can be targeted with activators and inhibitors, respectively.

Each strategy has its own advantages and disadvantages. Some of the limitations are "dynamic"; e.g., a lack of promoter or suitable serotype could be a problem today but be solved in the near future. Others are dictated by biology and therefore are unlikely to be resolved easily. One example is the special structure of the fovea. As described earlier, the fovea, which is the place for high-resolution color vision and is responsible for most of our visual perception, has a specialized structure in which the ganglion cells and the bipolar cells are not organized in special mosaics but are piled on top of each other in a ring around the fovea. As a consequence, if the ganglion or bipolar cells become light sensitive, any projected image will be

severely distorted. The distortion itself could be corrected to some degree, but because the cells pile up, a single image pixel will illuminate many cells with unknown topology. The only cells that are organized in a mosaic are the cones or, more precisely, the cone outer segments. The cone cell bodies are also piled on top of each other but more regularly than bipolar and ganglion cells: If the outer segments of two cones are close, the cell bodies are also close. It is the axons of the cones that break the spatial continuity. From these arguments, one may conclude that, in the fovea, the only cell type that can be stimulated with an image is the cones; however, even for these cells, the light sensitivity of their axons may cause image irregularities. Furthermore, because cone cell bodies are also piled on top of each other, once the outer segments are lost, it is impossible to regain the same resolution as that in healthy humans. For optogenetically transduced cones with no outer segments, the physical resolution is dictated by the size of the cone cell bodies and is further diminished by the light-sensitive axons. However, the resolution probably will not decrease with the further loss of cone cell bodies until only one layer of cones is left; this situation was observed in all postmortem examinations of human RP retinas.

Goggles, Adaptation, and Sensitivity

Whereas rods and cones can adapt to intensity distributions across eight decades, optogenetic sensors respond in a narrow range of intensities across only two decades. One possibility to increase the dynamic range of optogenetically transformed cells is to express multiple sensors with different light sensitivities. A more practical solution is the use of an external device, embedded in goggles, which acquires images across a large range of intensities, ranging from dim indoor environments to bright outdoors, and projects an image with the intensity distribution of the light-sensitized retinal cell type. Such goggles are currently under development (Grossman et al. 2010). The need for goggles is independent of the sensitivity of the optogenetically transduced cell. Using the vectors and optogenetic sensors that have already been described, the sensitivity of transduced cells is low. However, recent developments in both sensors and vectors have resulted in new vectors, leading to significantly increased sensitivities (Kleinlogel et al. 2011a).

Sophisticated Control: Color and Fine Control of Cellular State

Optogenetic vision restoration could be qualitatively improved in at least two ways. First, none of the currently available approaches allows for color perception. Introducing sensors with different optimal wavelengths could enable at least rudimentary color discrimination. Second, the state of the optogenetically transduced cells is unknown; therefore, optogenetic stimulation with one polarity, activation or inhibition, may use only a fraction of the cells' dynamic range. As an example, a normal cone in the dark is depolarized and constantly releases glutamate. When the cone is illuminated with light, hyperpolarization occurs and glutamate release ceases. Cones can modulate the release of glutamate within a given voltage range and can therefore transmit information to the next cells, the bipolar cells. Imagine cones in a blind patient being transduced with NpHR and then stimulated with red light. As a response, the cone cells will hyperpolarize, but whether this process will modulate glutamate release will depend on the dark voltage of the diseased cones. In *rd1* mice, cones are depolarized enough to modulate glutamate release (Busskamp et al. 2010). The best way to drive cones through their full dynamic range would be to modulate the dark voltage of the cones, for example, by expressing a highly sensitive but slow-acting ChR2 together with NpHR. The dark voltage could be modulated using background blue light, and the light responses could be modulated with red light. This kind of push-pull technique would allow complete control of the cones, independent of their voltage state: This is important because the voltage state may change during the course of the disease.

Potential Complications: Immune Reaction, Eye Movement, Photophobia, and Interference with Remaining Central Vision

Restoring optogenetic vision presents at least four potential complications. First, an immune reaction against the sensor is a potential danger. In our preliminary preclinical work, we found no sign of inflammation or other forms of immune reaction. A number of people are naturally infected by AAVs and therefore have antibodies against particular serotypes. These serotypes could be neutralized during gene therapy; therefore, prescreening of patients may be necessary. Second, the presence of uncontrolled eye movement in some patients may interfere with image projection to the region surrounding the optogenetically transduced area. Third, some RP patients experience discomfort or pain in the eyes when exposed to higher light intensities, a symptom called photophobia. These last two difficulties can be controlled for by careful patient selection. A fourth possible complication is the pupillary reflexes mediated by intrinsically photosensitive ganglion cells. The peak sensitivity of the melanopsin-mediated response in these ganglion cells is ~490 nm (Berson et al. 2002); therefore, the more red shifted the sensor is, the less pupillary constriction it causes. A more conceptual difficulty is that many patients have some remaining central vision. The development of optogenetic sensors in the near-infrared region of the electromagnetic spectrum could enable the use of these remaining natural sensors together with injected optogenetic sensors.

ASSESSING ELIGIBILITY FOR GENE THERAPY

The advances in gene therapy in animal models require investigators to identify patients who would benefit from these treatments. Key steps for this process are human genetics and in vivo noninvasive imaging of the retina in patients.

Modern optical coherence tomography (OCT) devices can produce high-resolution images of the retina and optic nerve in a noninvasive manner. OCT is used to examine the retinal architecture and, in particular, the photoreceptor inner/outer segment border. OCT is particularly useful to correlate the retinal sensitivity and outer retina structure. With this technique, researchers can evaluate the preservation of the cone outer segments when the rods are already lost. For example, patients with existing outer segments would be eligible for treatment with neuroprotective agents such as RdCVF. Individuals with visual acuity below light perception and no visual field but with a preserved layer of cone bodies still shown on the OCT could be eligible for optogenetic functional restoration of cones (**Figure 3**). Autofluorescence imaging indicates the status of the interactions between photoreceptor and pigment epithelium cells.

The retinal imaging devices that are currently available do have limits, however. Irregular optical defects (ocular wave-front aberrations) may limit the resolution of these devices. The newly introduced adaptive optics-based laser-imaging technologies can correct the irregular optical defects and provide a retinal image with a lateral resolution of 2.5–3 μm, making it possible to visualize human cones noninvasively not only in the macula but all over the retina in a few minutes. Adaptive optics imaging to assess photoreceptor mosaic structure directly may have important implications for establishing functional correlates and studying gene-therapy outcomes.

In connection with such imaging studies, functional investigations should extend beyond testing visual acuity. Several protocols for visual field testing allow regional sensitivity thresholds to be determined. Microperimetry provides a direct mapping of function by projecting stimuli of variable size and luminance while observing the back of the eye, known as the fundus. Once an accurate map of retinal function has been obtained, together with high-resolution imaging, it will be easier to select patients for trials, and investigators can

Figure 4

Rehabilitation using the combination of real and virtual visual elements. To implement innovative adaptive rehabilitation strategies and to demonstrate the functional benefit of different visual restoration approaches relevant to real-life situations, new rehabilitation and testing platforms are being developed. The picture shows the Streetlab platform at the Vision Institute, Paris, which integrates real and virtual-reality visual elements to provide a place for rehabilitation as well as quantifiable tests for visual functions that are useful for performing tasks in everyday life.

identify the area of injection to optimize the ratio between the expected functional benefits and potential hazards resulting from subretinal injections.

Functional studies are also of paramount importance to establish the actual benefit of these novel therapies. Although visual acuity represents the gold standard for regulatory and funding bodies, no significant changes may be measured, although the visual field may stabilize or improve. In the LCA trials, even though visual acuity did not improve, testing for mobility in dark and even lighted environments indicated an obvious improvement (fewer bumps, shorter time to target). Because most quality-of-life questionnaires may not detect such changes, it is timely to develop standardized mobility and task-related tests that would provide both sensitivity and reliability (**Figure 4**).

REHABILITATION

Rehabilitation is an important component of vision restoration for patients who become blind both before and after the critical period of vision. Following rehabilitation in patients who became blind after the critical period, vision restoration using retinal prosthesis has led to some visual experience, including the localization of light sources and objects, and in some cases to the recognition of shapes and letters (Humayun et al. 2012, Zrenner et al. 2011). Even in early-onset blindness caused by LCA2, vision restoration using gene therapy and rehabilitation led to a measurable retinotopic map in the visual cortex (Ashtari et al. 2011, Sahel 2011). Finally, studies have demonstrated that the visual cortex can be activated in congenitally blind patients by stimulating other sensory pathways, showing the plasticity of the cortex (Pascual-Leone et al. 2005, Reich et al. 2012). Upon vision restoration to blind patients, innovative rehabilitation programs can recruit brain plasticity to teach the brain the novel "language" that the restored retina uses to communicate visual input (**Figure 4**).

CONCLUSION

Our capability to perform ocular gene therapy has increased substantially in the past decade owing to the enormous progress made in uncovering novel genetic causes and risks in blinding eye diseases, in developing and analyzing animal models, in developing in vivo imaging

modalities in human patients, and in refining gene-delivery tools. Despite all this progress, many questions still remain unanswered: how to choose promoters and prepare vectors for clinical use; how to decide on the volume of injection and the location of intraocular vector administration; whether to use gene-replacement or rather mutation-independent gene therapy; how to choose and standardize patients for a given therapy; and how to evaluate visual function before and after gene therapy. To provide the most relevant therapy, we need further improvement in our understanding of genotype-phenotype correlations and in the diagnosis of the functional status of retinal cells in vivo in patients. The enthusiasm to provide therapy for such a major unmet medical need propels the field of ocular gene therapy forward to answer these questions. We believe that the recent major advances in gene therapy for the eye will pave the way for gene therapies in other parts of the brain.

DISCLOSURE STATEMENT

Both authors are among the founders of Gensight, Inc., a new company that specializes in innovative retinal gene therapy applications such as optogenetic vision restoration. José-Alain Sahel is also a consultant for Sanofi and a founder of Pixium Vision, a new company dedicated to the development of artificial retinas.

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