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Zhou-Hua PAN
Channelrhodopsin 2 vs Blindness

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Meet the Mind-Controlling Algae Protein That Could Cure Blindness

by Shelly Fan

It sounds completely crazy: as early as next year, using gene therapy scientists hope to restore sight in the blind by giving their eyes additional "light sensors."

We're not talking about bionic eyes: instead of implantable electronics, scientists are turning to a protein called channelrhodopsin-2. You've probably heard of this protein before — it's the same magical switch that, in response to light, can turn a gentle mouse aggressive, shut down obsessive grooming behavior, and implant false memories in unsuspecting mice.

What does a mind-controlling protein have to do with restoring vision?
Meet the protein that sparked a neuroscience revolution

The answer lies in how channelrhodopsin-2 works. The protein comes from the lowly green algae, which uses it to seek out sunlight for photosynthesis.

At its core, channelrhodopsin-2 is a light-sensitive protein tunnel that sits on the surface of cells. Normally the tunnel is completely cinched up, which allows a cell to maintain a steady interior environment.

However, when a certain wavelength of light hits the protein, the tunnel temporarily flashes open, much like a camera shutter. When open, the protein is like a highway, shuttling ions into the cell — the same biophysical process that makes a neuron burst with activity.

About a decade ago, neuroscientists realized that they could stick the protein into mouse neurons that were previously impervious to light. By using sophisticated genetic tools, the protein could be restricted to certain types or populations of neurons, rather than huge chunks of the brain.

Then, by shining light through an implanted fiber optic laser, researchers can artificially activate select networks of neurons. The results are nothing short of science fiction: a flash of light, and a mouse — going about its business as usual — might, for example, suddenly freeze in place as if terrorized. Turn the light off, and it'll revert back to its normal happy-go-lucky state, seemingly unaware that anything strange just happened. Hence, the "mind control" part.

The scientists dubbed this powerful new technique optogenetics, and hundreds — if not thousands — of labs around the world are now using this technique to explore the intricate

neural connections in the brain.

Adding backup hardware to the human eye

The brain-bending powers of channelrhodopsin-2 are so mind-boggling that it's easy to forget the simple nature of the protein: it senses light, and transmits that information through electricity to higher processing centers.

Broadly speaking, human eyes work similarly. Light passes through the length of our eyeballs and falls on the back of the retina, activating light-sensitive proteins called photoreceptors (these are shaped like rods and cones). The photoreceptors transmit light information through two filter layers — ganglion and bipolar cells — that process the electric signals and send them to visual areas of the brain.

In many eye disorders, such as retinitis pigmentosa or macular degeneration, the rods and cones gradually die off. This leads to progressively failing vision, and — without a cure — eventually turns one out of four sufferers legally blind.

These are cold, brutal diseases, but there is one silver lining: they leave ganglion and bipolar cells intact and still able to still communicate with the brain.

The obvious treatment would be to introduce human hardware back into the human retina through gene therapy. Yet, human light-sensitive proteins are notoriously hard to engineer. To function normally, they have to be tightly coupled to many other supporting proteins. This means scientists would have to get multiple genes at the right ratio and levels into the retina — a feat that is currently impossible.

Channelrhodopsin-2, on the other hand, works just by itself.

In 2006, Dr. Zhou Hua Pan, a researcher at Wayne State University, decided to stick the protein into mice that were genetically engineered for photoreceptor degeneration. It worked on the first try; in less than three months after a single treatment, the mice passed every vision test the scientists could throw at them.

"It worked perfectly, even in the very beginning," Pan told Wired. "That was basically just really, really lucky."

Pan's success did not escape the notice of the biotech industry. In 2009, RetroSense Therapeutics, a startup located a short drive away from Wayne State, leased the eye-wiring tech from Pan in a bid to bring it to human trials. Last month, the FDA gave its nod of approval: as early as this fall, the company will start installing channelrhodopsin-2 into the retinas of 15 patients blinded by retinitis pigmentosa through gene therapy.

The company is aiming for retinal ganglion cells, which are spared by the disease even in advanced stages. In essence, by giving these "middle men" the ability to sense light, scientists hope to circumvent the need for rods and cones.

A long road ahead to color

To be clear, as promising at it is, the algae protein can't restore human vision to all its colorful vividness.

The photoreceptors in our eyes, optimized by eons of evolution, operate over a wide range of light intensities and wavelengths — we get to see everything from pale starlight blinking in an

indigo sky to glaring sunlight above white hot sand, and all the colors in between.

In contrast, channelrhodopsin-2 paints a dim monochromatic landscape. The protein is 2,000 times less sensitive to light than our retinal cones. It only responds to a very narrow set of wavelengths — and thus colors — of light, a far cry from our normal eyesight. In a way, going from rods and cones to channelrhodopsin-2 is like going from an expensive 20-gear road bike to a fixie. It works, but it's not optimal.

Ganglion cells also don't normally deal with light — they generally process electrical signals that come from rods and cones. Whether raw light signals work as well as pre-processed electrical information from photoreceptors is still up in the air. If the gene therapy successfully delivers channelrhodopsin-2 to these cells, the brain will have some serious rewiring to do before it can interpret these strange new signals.

Even with these caveats, the therapy may be a game changer. A decade ago, channelrhodopsin-2 dramatically changed the face of neuroscience — here's to hoping it'll spark another revolution soon.

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http://news.yahoo.com/scientists-may-finally-discovered-cure-154544209.html
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Scientists May Have Finally Discovered a Cure for Blindness

by

Trace William Cowen

When Wayne State University researcher Dr. Zhou-Hua Pan placed a light-sensitive green algae protein into blind mice in 2006, he was amazed to find that it restored the subjects' vision almost immediately. Fast forward to 2015, the year of many great things, and that protein is now the subject of a forthcoming set of human trials aimed at unveiling a potential cure for blindness in humans.

RetroSense Therapeutics, the company who leased the research from Dr. Zhou-Hua Pan and recently received approval from the Food and Drug Administration to administer human trials, is expected to begin testing the protein on 15 patients by the end of the year. According to Singularity Hub, channelrhodopsin-2 is the same "magical switch" protein already famous for its ability to "turn a gentle mouse aggressive, shut down obsessive grooming behavior, and implant false memories in unsuspecting mice."

The protein is placed directly into the retina using gene therapy, allowing the rod-and-cone system to be bypassed entirely and giving the eye's ganglion cells the ability to sense light on their own. Though some levels of colorblindness may persist even with successful implementation of the forthcoming human trials, some researchers speculate that the human brain could potentially make adjustments in order to counteract the color loss.

Keep up the good work, science. We're all counting on you.

IDENTIFICATION OF CHANNELRHODOPSIN-2 (CHOP2) MUTATIONS AND METHODS OF USE.

The invention provides compositions and kits including at least one nucleic acid or polypeptide molecule encoding for a mutant ChR2 protein. Methods of the invention include administering a composition comprising a mutant ChR2 to a subject to preserve, improve, or restore phototransduction. Preferably, the compositions and methods of the invention are provided to a subject having impaired vision, thereby restoring vision to normal levels.

FIELD OF THE INVENTION

[0004] This invention relates generally to the field of molecular biology. Mutations in the Channelopsin-2 (Chop2) gene are identified. Compositions comprising a mutant Chop2 gene are used in therapeutic methods to improve and restore vision loss.

BACKGROUND OF THE INVENTION

[0005] The retina is composed of photoreceptors (or photoreceptor cells, rods and cones). Photoreceptors are highly specialized neurons that are responsible for phototransduction, or the conversion of light (in the form of electromagnetic radiation) into electrical and chemical signals that propagate a cascade of events within the visual system, ultimately generating a representation of our world.

[0006] Photoreceptor loss or degeneration severely compromises, if not completely inhibits, phototransduction of visual information within the retina. Loss of photoreceptor cells and/or loss of a photoreceptor cell function are the primary causes of diminished visual acuity, diminished light sensitivity, and blindness. There is a long-felt need in the art for compositions and method that restore photosensitivity of the retina of a subject experiencing vision loss.

SUMMARY OF THE INVENTION

[0007] The invention provides a solution for the long-felt need for a method of restoring and/or increasing the light sensitivity of photoreceptor cells by expression of advantageous mutations, and/or combinations thereof, of the Channelopsin-2 (Chop2) gene, and subsequently providing methods for Channelopsin-2 (Chop2)-based gene therapy.

[0008] Channelopsin-2 (Chop2)-based gene therapy offers a superior strategy for restoring retinal photosensitivity after photoreceptor degeneration. The protein product of the Chop2 gene, when bound to the light-isomerizable chromophore all-trans-retinal, forms a functional light-gated channel, called channelrhodopsin-2 (ChR2). Native ChR2 shows low light sensitivity. Recently, two mutant ChR2s, L132C and T159C, were reported to markedly increase their light sensitivity (Kleinlogel et al. (2011) Nat. Neurosci. 14:513-8; Berndt et al. (2011) Proc Natl Acad Sci USA. 108:7595-600; Prigge et al. (2012) J Biol. Chem. 287(38)3104:12; the contents of each of which are incorporated herein in their entireties). The properties of these two ChR2 mutants (i.e., L132C and T159C) were examined and compared with a number of double mutants at these two sites to identify suitable candidates for therapeutic methods. Compositions comprising one or more of these mutations are provided to a subject in need thereof for the purpose of restoring vision. Specifically, desired mutations in the Chop2 gene are introduced to a cell and/or integrated into the genomic DNA of a cell to improve or restore vision. Desired mutations in the Chop2 gene that are introduced to a cell to improve or restore vision may also remain episomal, not having integrated into the genomic DNA.

[0009] Mutations at the L132 or T159 amino acid positions of Chop2 (and therefore, the

resulting ChR2) markedly lower the threshold light intensity that is required to elicit the ChR2-mediated photocurrent. Double mutants at the amino acid positions L132 and T159 further increase the photocurrent at low light intensities, exceeding that of either of the corresponding single mutations. Retinal ganglion cells expressing the double mutants at the L132 and T159 positions can respond to light intensities that fall within the range of normal outdoor lighting conditions but should still maintain adequate, and high temporal resolution that are suitable for restoring useful vision. Thus, mutant Chop2 protein of the present invention that form mutant ChR2s having improved light sensitivity are used alone or in combination to restore or improve vision.

[0010] Specifically, the invention provides an isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L). In certain embodiments of the isolated polypeptide molecule, the amino acid at position 132 is cysteine (C) or alanine (A). When the amino acid at position 132 is cysteine (C), the polypeptide molecule may comprise or consist of SEQ ID NO: 13. When the amino acid at position 132 is alanine (A), the polypeptide molecule may comprise or consist of SEQ ID NO: 20.

[0011] The invention provides an isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 159 of SEQ ID NO: 26 is not a threonine (T). In certain embodiments of the isolated polypeptide molecule, the amino acid at position 159 is cysteine (C), serine (S), or alanine (A). When the amino acid at position 159 is cysteine (C), the polypeptide molecule may comprise or consist of SEQ ID NO: 14. When the amino acid at position 159 is serine (S), the polypeptide molecule may comprise or consist of SEQ ID NO: 17. When the amino acid at position 159 is alanine (A), the polypeptide molecule may comprise or consist of SEQ ID NO: 23.

[0012] The invention provides isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T). In certain embodiments of the isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T), the amino acid at position 132 is cysteine (C), and the amino acid at position 159 is cysteine (C). In a preferred embodiment of this isolated polypeptide molecule, the polypeptide molecule comprises or consists of SEQ ID NO: 16. The invention provides an isolated nucleic acid molecule that encodes for the isolated polypeptide comprising or consisting of SEQ ID NO: 16. Preferably, the isolated nucleic acid molecule that encodes for the isolated polypeptide comprising or consisting of SEQ ID NO: 16, is a nucleic acid molecule that comprises or consists of SEQ ID NO: 15.

[0013] In certain embodiments of the isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T), the amino acid at position 132 is cysteine (C) and the amino acid at position 159 is serine(S). The isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T), may comprise or consist of SEQ ID NO: 19. Alternatively, or in addition, the isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T), wherein the amino acid at position 132 is cysteine (C) and wherein the amino acid at position 159 is serine(S) may comprise or consist of SEQ ID NO: 19. The invention provides an isolated nucleic acid molecule that encodes for the isolated polypeptide that comprises or consists of SEQ ID NO: 19. Preferably, the nucleic acid molecule comprises or consists of SEQ ID NO: 18.

[0014] In certain embodiments of the isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T), the amino acid at position 132 is alanine (A) and the amino acid at position 159 is cysteine (C). The isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T) may comprise or consist of SEQ ID NO: 22. Alternatively, or in addition, the isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T), wherein the amino acid at position 132 is alanine (A) and wherein the amino acid at position 159 is cysteine (C) may comprise or consist of SEQ ID NO: 22. The invention provides an isolated nucleic acid molecule that encodes for the isolated polypeptide that comprises or consists of SEQ ID NO: 22. Preferably, this nucleic acid molecule comprises or consists of SEQ ID NO: 21.

[0015] In certain embodiments of the isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T), the amino acid at position 132 is cysteine (C) and the amino acid at position 159 is alanine (A). The isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T) may comprise or consist of SEQ ID NO: 25. Alternatively, or in addition, the isolated polypeptide molecule comprising or consisting of SEQ ID NO: 26 in which the amino acid at position 132 of SEQ ID NO: 26 is not leucine (L) and the amino acid at position 159 is not threonine (T), wherein the amino acid at position 132 is cysteine (C) and wherein the amino acid at position 159 is alanine (A) may comprise or consist of SEQ ID NO: 25. The invention provides an isolated nucleic acid molecule that encodes for the isolated polypeptide that comprises or consists of SEQ ID NO: 25. Preferably, this nucleic acid molecule comprises or consists of SEQ ID NO: 24.

[0016] The invention provides any one of the isolated polypeptide molecules described herein, wherein the polypeptide molecule encodes for a mutant Chop2 protein that forms a mutant ChR2, which elicits a current in response to a threshold intensity of light that is lower than the threshold of a wild type ChR2 protein. Moreover, the current conducts cations. Exemplary cations include, but are not limited to, H<+>, Na<+>, K<+>, and Ca<2+> ions. The ChR2 wild type and mutant proteins described herein non-specifically conduct cations. Consequently, the current conducts one or more of the following: H<+>, Na<+>, K<+>, and Ca<2+> ions.

[0017] The invention provides any one of the isolated polypeptide molecules described herein further comprising a pharmaceutically acceptable carrier. The invention also provides a composition comprising at least one isolated polynucleotide molecule described herein. The composition may further comprise a pharmaceutically-acceptable carrier.

[0018] The invention provides an isolated nucleic acid molecule that encodes for any of the isolated polypeptides described herein. Moreover, the isolated nucleic acid molecule may further include a pharmaceutically acceptable carrier. The invention also provides a composition comprising at least one isolated nucleic acid molecule described herein. The composition may further comprise a pharmaceutically-acceptable carrier.

[0019] The invention provides a cell, wherein the cell has been contacted with or comprises an isolated polypeptide molecule of the invention. Moreover, the invention provides a cell, wherein the cell has been contacted with or comprises an isolated nucleic acid molecule that

encodes for an isolated polypeptide molecule of the invention. The invention provides, a composition comprising, consisting essentially of, or consisting of a cell that comprises an isolated polypeptide molecule of the invention or a nucleic acid molecule that encodes for an isolated polypeptide molecule of the invention. Cells of the invention may be contacted with the isolated polypeptide or an isolated nucleic acid encoding the polypeptide in vitro, ex vivo, in vivo, or in situ. In certain embodiments of the invention, the cell is a photoreceptor; a horizontal cell; a bipolar cell; an amacrine cell, and, especially, an AII amacrine cell; or a retinal ganglion cell, including a photosensitive retinal ganglion cell. Preferably, the cell is a retinal ganglion cell, a photosensitive retinal ganglion cell, a bipolar cell, an ON-type bipolar cell, a rod bipolar cell, or an AII amacrine cell. In certain aspects of the invention, the cell is a photoreceptor, a bipolar cell, a rod bipolar cell, an ON-type cone bipolar cell, a retinal ganglion cell, a photosensitive retinal ganglion cell, a horizontal cell, an amacrine cell, or an AII amacrine cell.

[0020] The invention provides a method of improving or restoring vision, comprising administering to a subject any one of the compositions described herein. The invention further provides a prophylactic method of preserving vision, comprising administering to a subject any one of the compositions described herein.

[0021] The methods described herein may also be applied to those subjects who are healthy, blind (in part or in total), and/or those subjects with retinal degeneration (characterized by a loss of rod and/or cone photoreceptor cells), but may be dependent upon the activity of photosensitive retinal ganglion cells for a determination of ambient light levels. For example, the methods described herein can be used to preserve, improve, or restore the activity of a photosensitive retinal ganglion cell that mediates the transduction of light information for synchronizing circadian rhythms to the 24-hour light/dark cycle, pupillary control and reflexes, and photic regulation of melatonin release.

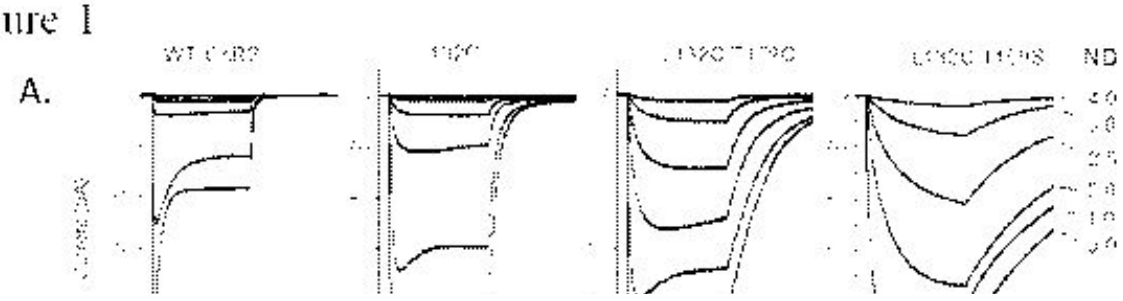
[0022] In certain embodiments of the methods of the invention, the subject may have normal vision or impaired vision. Alternatively, or in addition, the subject may be at risk for developing an ocular disease that leads to impairment of vision. For example, the subject may have a family history of, ocular disease, including, macular degeneration and retinitis pigmentosa. The subject may be at risk for incurring an eye injury that causes damage to photosensitive cells in the retina. The subject may have a genetic marker or genetic/congenital condition that results in impaired vision, low vision, legal blindness, partial blindness, or complete blindness. Subjects may have a refractive defect that results in myopia (near-sightedness) or hyperopia (far-sightedness).

[0023] Compositions of methods of the invention may be administered to a subject either systemically or locally. A preferred route of local administration is intravitreal injection.

[0024] Other features and advantages of the invention will be apparent from and are encompassed by the following detailed description and claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1



B.

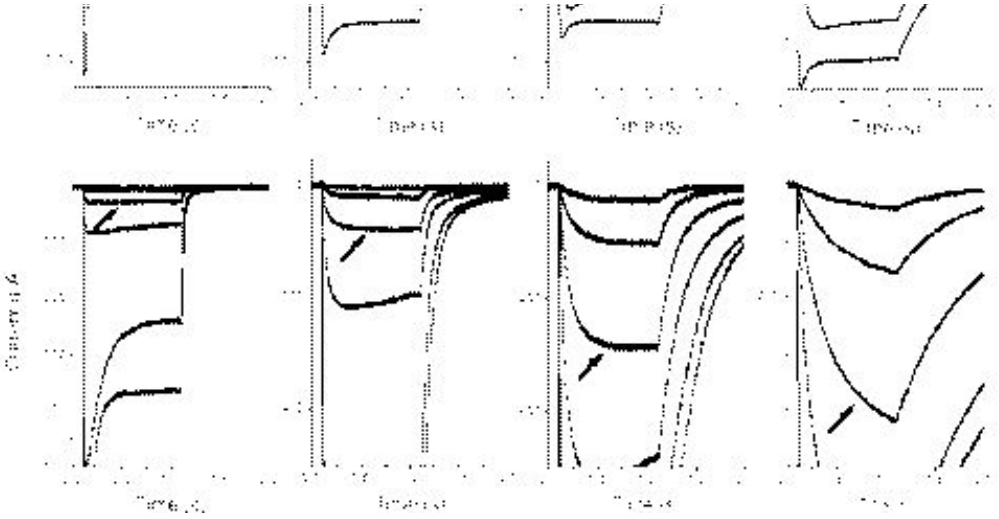


Figure 2

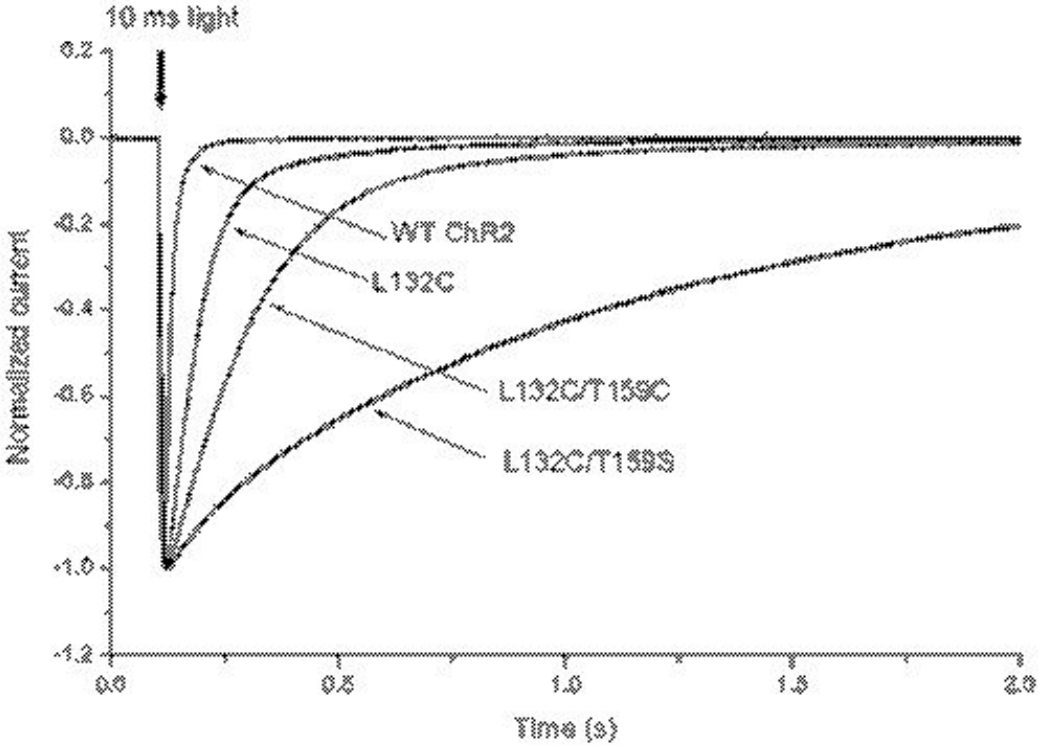
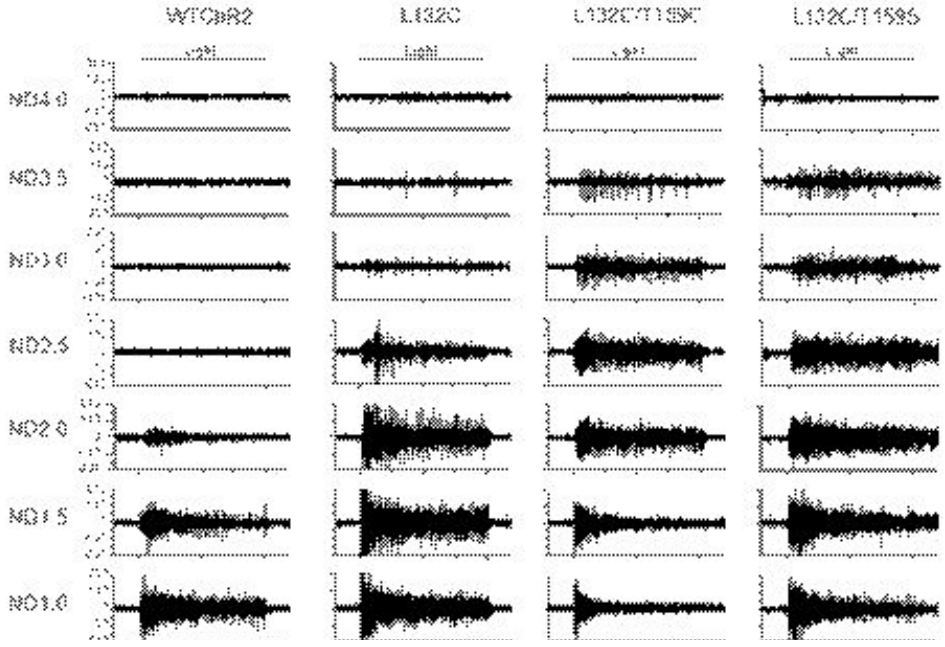


Figure 3



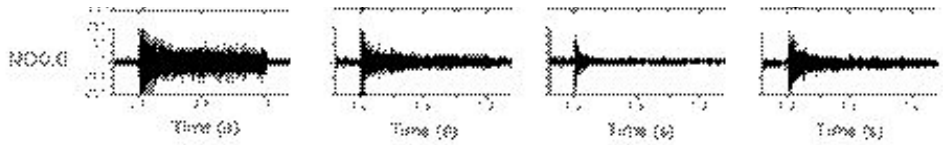


Figure 4a

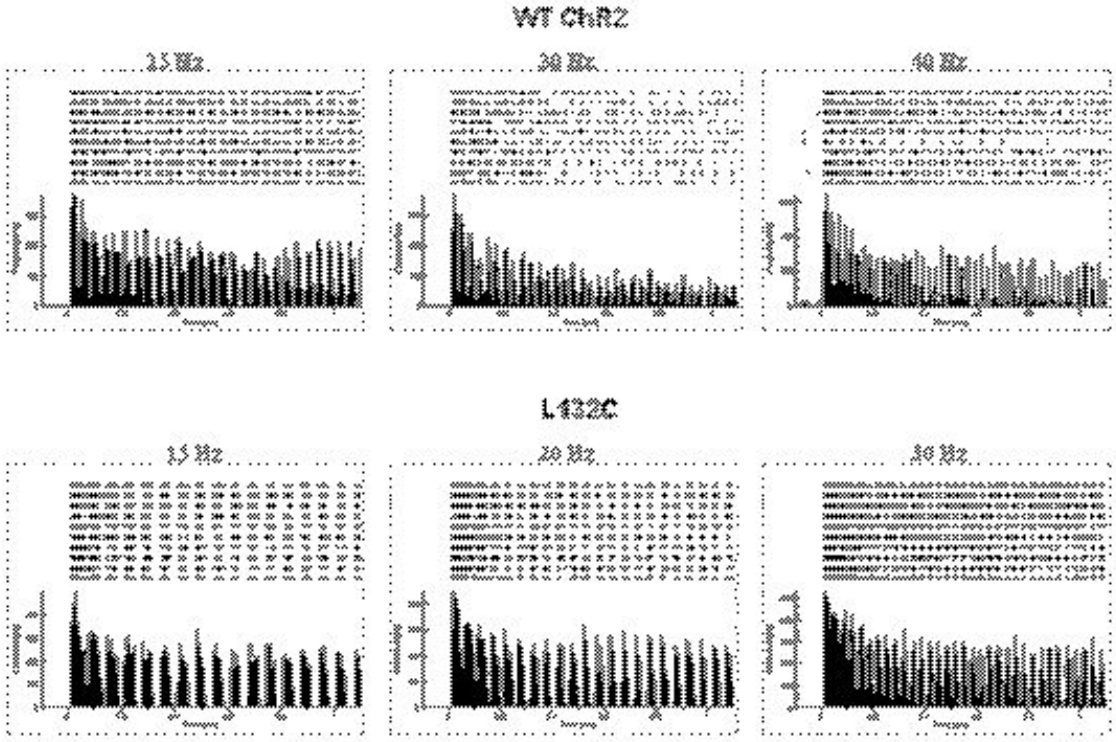
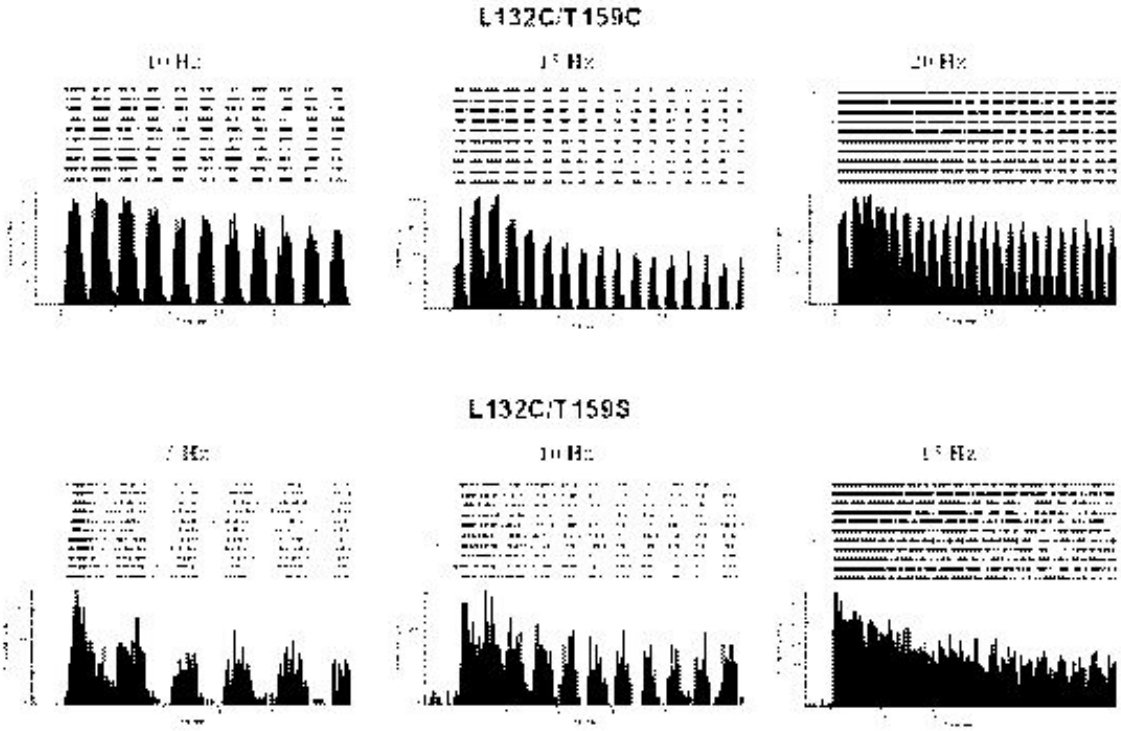


Figure 4b



[0025] **FIG. 1** shows representative recordings of the light-evoked currents from wild-type (WT) ChR2, L132C, L132C/T159C, and L132C/159S mutants in HEK cells for comparison of their light sensitivity (A). The light stimuli (photons/cm<2>.s at 460 nm) were generated by a xenon arc lamp and attenuated by neutral density filters: ND4.0 (2.8×10<14>), ND3.0 (1.4×10<15>), ND2.5 (4.8×10<15>); ND2.0 (1.6×10<16>), ND1.0 (1.3×10<17>), ND0 (1.2×10<18>). (B) The same current traces are shown at a different current scale. The traces

pointed by arrows are evoked by the same light intensity (ND2.5).

[0026] **FIG. 2** shows representative recordings of the light-evoked currents from wild-type (WT) ChR2, T159C, L132C, L132C/T159C, and L132C/T159S mutants to a 10 ms light pulse (1.2×10<18 >photons/cm<2>/s at 460 nm) in HEK cells for comparison of their deactivation time course (decay time course after light off).

[0027] **FIG. 3** shows representative multichannel array recordings of WT ChR2, L132C, L132C/T159C, and L132C/T159S mediated spiking activities from retinal ganglion cells in retinal whole-mounts for comparison of their light sensitivity. Light stimuli (photons/cm<2>/s) was generated by a 473 nm blue laser and attenuated by neutral density filters: ND0 (6.3×10<16>), ND1.0 (7.4×10<15>), ND1.5 (2.7×10<15>), ND2.0 (7.3×10<14>), ND2.5 (3.2×10<14>), ND3.0 (8.5×10<13>), ND3.5 (3.8×10<13>), and ND4.0 (9.5×10<12>).

[0028] **FIG. 4** shows representative multichannel array recordings of WT ChR2, L132C, L132C/T159C, and L132C/T159S mediated spiking activities from retinal ganglion cells in retinal whole-mounts for comparison of their temporal dynamics. In each panel, the raster plots of 10 consecutive light-elicited spikes originated from a single neuron (top) and the averaged spike rate histograms (bottom) are shown. Light pulses at different frequency was generated by a 473 nm blue laser with intensities about one log unit above the threshold intensity of each mutant. Recordings of WT ChR2 and L132C are shown in (A), and recordings of L132C/T159C and L132C/T159S are shown in (B).

DETAILED DESCRIPTION

Visual System

[0029] The central nervous system mediates vision (also referred to herein as sight) through specialized cells and unique methods of signal transduction present in the visual system. The principle responsibility of the visual system is to transform light, in the form of electromagnetic radiation, into a representation or image of the surrounding world. In addition to the “visual” function of this system, the visual system also regulates the pupillary light reflex (PLR), circadian photoentrainment to periodic light/dark cycles, and release of the hormone melatonin.

[0030] The cells of the retina are the first cells of the visual or nervous system to encounter light (electromagnetic radiation of varying wavelengths and intensities). Photons travel through the cornea, pupil, and lens before reaching the retina. The retina has a unique structure because the photoreceptor cells that directly absorb photons are located in the outer layer of the retina. Photons that traverse the lens first encounter an inner layer of retinal ganglion cells (a minority of which are photosensitive through the expression of the opsin, melanopsin) and an intermediate layer of bipolar cells before reaching the outer layer of photoreceptor cells (also known as rods and cones). Rod photoreceptors operate in dim illumination condition (scotopic vision) while cone photoreceptors operate in bright illumination conditions (photopic vision) responsible for color vision. Cone photoreceptors synapse directly onto ON- and OFF-type cone bipolar cells, which in turn, synapse directly onto ON- and OFF-type retinal ganglion cells. Rod photoreceptors synapse to rod bipolar cells (a unique type of bipolar cells, which is ON-type), which synapse to AII amacrine cells. The AII amacrine cells then relay the visual signals to ON-type cone bipolar cells through gap junction and to OFF-type cone bipolar cells as well as OFF ganglion cells through inhibitory glycinergic synapses. Retinal ganglion cells are responsible for relating visual information to neurons of the brain.

Phototransduction

[0031] Within the retina, photoreceptor cells absorb photon particles and transform the raw data of light frequency and wavelength into chemical and subsequently electrical signals that propagate this initial information throughout the visual and nervous systems. Specifically, an opsin protein located on the surface of a photoreceptor (rod, cone, and/or photosensitive retinal ganglion cell) absorbs a photon and initiates an intracellular signaling cascade, which results in the hyperpolarization of the photoreceptor. In the dark, the opsin proteins absorb no photons, the photoreceptors are depolarized. The visual signals of photoreceptors then relay through bipolar cells, amacrine cells, and ganglion cells to the high visual centers in the brain. Specifically, when rod and cone photoreceptors are depolarized (in the dark), they cause the depolarization of rod bipolar cells and ON-type cone bipolar cells, but the hyperpolarization of OFF-type cone bipolar cells, which in turn cause the depolarization of AII amacrine cells and the increase of the spiking of ON-type retinal ganglion cells and the decrease of the spiking of OFF-type retinal ganglion cells. The opposite happens (to rod, ON- and OFF-bipolar cells, AII amacrine and ON- and OFF-ganglion cells), when rod and cone photoreceptors are hyperpolarized (in response to light).

[0032] Light information is processed and refined significantly by the actions of photoreceptors, bipolar cells, horizontal cells, amacrine cells, and retinal ganglion cells. To add to the complexity of this system, photoreceptors are found in three main varieties, including rods, cones (of which three types respond most strongly to distinct wavelengths of light), and photosensitive retinal ganglion cells. Thus, a first layer of information processing occurs at the level of the photoreceptors which respond differentially to certain wavelengths and intensities of light. Bipolar cells of the retina receive information from both photoreceptor cells and horizontal cells. Horizontal cells of the retina receive information from multiple photoreceptor cells, and, therefore, integrate information between cell types and across distances in the retina. Bipolar cells further integrate information directly from photoreceptor cells and horizontal cells by producing mainly graded potentials to retinal ganglion cells, although some recent studies indicate that some bipolar cells can generate action potentials. Cone bipolar cells synapse on retinal ganglion cells and amacrine cells while rod bipolar cells synapse only to AII amacrine cells. Similar to horizontal cells, most amacrine cells integrate information laterally within the retina. Unlike horizontal cells, most amacrine cells are inhibitory (GABAergic) interneurons. Amacrine cells are also more specialized than horizontal cells, because each amacrine cell specifically synapses on a particular type of bipolar cell (one of the ten varieties of bipolar cell). Particularly, the AII amacrine cell is a critical relay neuron in the rod pathway (under scotopic vision when cone photoreceptors do not respond). The AII amacinre cells receive synaptic inputs from rod bipolar cells and then piggy-back the signals to cone pathway through ON- and OFF-cone bipolar cells to ON- and OFF-ganglion cells as described above. Therefore, expression of Chop2, and the resulting formation of ChR2, in rod bipolar cells or AII amacrine cells can create both ON and OFF responses in retinal ganglion cells. Furthermore, retinal ganglion cells integrate information from bipolar cells and from amacrine cells. Although retinal ganglion cells vary significantly with respect to size, connectivity, and responses to visual stimulation (e.g. visual fields), all retinal ganglion cells extend a long axon into the brain. Except for a minute portion of the retinal ganglion cells that transduce non-visual information regarding the pupillary light reflex and circadian entrainment, the totality of axons extending from the retinal ganglion cells form the optic nerve, optic chiasm, and optic tract of the central nervous system. Consequently, a significant amount of information processing occurs in the retina itself.

[0033] Photoreceptor cells express endogenous opsin proteins, such as rhodopsin. The mutant Chop2 proteins of the invention may be expressed in any cell type, and form functional ChR2 channels. Preferably, the cell is a retinal cell. Exemplary cells, include, but are not limited to, photoreceptor cells (e.g., rods, cones, and photosensitive retinal ganglion cells), horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells.

Channelopsin-2 (Chop2)

[0034] Channelopsin-2 (Chop2) was first isolated from the green algae, Chlamydomonas reinhardtii. Channelopsin-2 is a seven transmembrane domain protein that becomes photo-switchable (light sensitive) when bound to the chromophore all-trans-retinal. Chop2, when linked to a retinal molecule via Schiff base linkage forms a light-gated, nonspecific, inwardly rectifying, cation channel, called Channelrhodopsin-2 (Chop2 retinalidene, abbreviated ChR2).

[0035] As referred to herein, "channelopsin-2" or "Chop2" refers to the gene that encodes channelopsin-2, which then forms Channelrhodopsin-2 (ChR2) once bound to retinal. Gene constructs of the present invention refer primarily to channelopsin-2 (i.e., without the retinal), and all Chop2 variants disclosed herein form functional channelrhodopsin-2 variants. The methods disclosed herein may include delivering Chop2 to cells without exogenous retinal. It is understood that upon expression of Chop2 in cells (i.e., retinal neurons), endogenously available retinal binds to the wild-type Chop2 or the Chop2 mutants of the present invention to form functional light-gated channels, WT ChR2 or mutant ChR2. As such, Chop2 proteins, as referred to herein, can also be synonymous with ChR2.

[0036] As used herein, "channelrhodopsin-2" or "ChR2" refers to the retinal-bound functional light-sensitive channel. In one embodiment, the bound retinal may be provided exogenously. In a preferred embodiment, the bound retinal is provided from endogenous levels available in the cell. The present invention also encompasses the functional channelrhodopsin-2 channels formed by the polypeptides and polynucleotides encoding the Chop2 mutants described herein.

[0037] Upon illumination by the preferred dose of light radiation, ChR2 opens the pore of the channel, through which H<+>, Na<+>, K<+>, and/or Ca<2+> ions flow into the cell from the extracellular space. Activation of the ChR2 channel typically causes a depolarization of the cell expressing the channel. Depolarized cells produce graded potentials and or action potentials to carry information from the Chop2/ChR2-expressing cell to other cells of the retina or brain.

[0038] The wild type form of ChR2 or mutant ChR2s with high temporal resolution have become a central focus of neuroscience research. When expressed in a mammalian neuron, ChR2 mediates light-controlled depolarization of in vitro or ex vivo cultures. Wild type ChR2s or mutant ChR2s with high temporal resolution (the latter usually display low light sensitivity) presents several challenges that must be addressed to enable their use for the purpose of vision restoration. For the purpose of vision restoration, the ChR2 with high light sensitivity rather than high temporal resolution is desired.

[0039] Wild type ChR2 proteins require illumination from high blue light intensities for full activation (i.e. 10<18>-10<19 >photons s<-1 >cm<-2 >at a wavelength of 480 nm). Continuous illumination of this type can damage cells.

[0040] The kinetics of the wild type ChR2 protein is suboptimal for maximizing channel efficacy. Efficacy can be increased by modifying one or more amino acids of the wild type ChR2 protein either to prolong the open state of the channel or increase the unit conductance of the channel, or both. The single-channel conductance of wild-type ChR2 is small. Thus, neuronal activation in vivo would either require high expression of the wild type channel or very intense activation with the preferred wavelength of blue-light. A simpler solution may be found by altering the channel conductance or to prolong the channel open time. Either one of these mechanisms and, in particular, the combination of these mechanisms, enable lower and safer light intensities to be used to achieve the same level of cellular depolarization.

[0041] For example, mutant ChR2 proteins of the invention achieve greater light sensitivity

through the prolongation of the channel open state. Consequently, each mutant ChR2 channel conducts a greater photocurrent than a wild type ChR2 channel when activated by the same light intensities. Therefore, the mutant channels are activated by light intensities that are lower than those required for activation of the wild type ChR2 channels. Quantitatively, detectable spiking activity of retinal ganglion cells expressing mutant ChR2 proteins can be elicited by a light intensity that is 1.5-2 log units lower than the light intensity required to elicit spiking activity from retinal ganglion cells expressing wild type ChR2. Thus, the light intensities required to activate the mutant ChR2 proteins are close to or fall within the range of normal outdoor lighting conditions.

[0042] The following sequences provide non-limiting examples of wild type and mutant Chop2 proteins, and polynucleotides encoding said WT and mutant Chop2 proteins of the invention, and forming WT and mutant ChR2s of the invention.

[0043] A wild type (WT) Chop2 of the invention may be encoded by the following Chlamydomonas reinhardtii chlamyopsin 4 light-gated ion channel (COP4) mRNA sequence (GenBank Accession No. XM—001701673, and SEQ ID NO: 1):

1 gcagcaccat acttgacatc tgtcgccaag caagcattaa acatggatta tggaggcgcc
61 ctgagtgccg ttgggcgcga gctgctattt gtaacgaacc cagtagtcgt caatggctct
121 gtacttgtgc ctgaggacca gtgttactgc gcgggctgga ttgagtcgcg tggcacaaac
181 ggtgcccaaa cggcgtcgaa cgtgctgcaa tggcttgctg ctggcttctc catcctactg
241 cttatgtttt acgcctacca aacatggaag tcaacctgcg gctgggagga gatctatgtg
301 tgcgctatcg agatggtcaa ggtgattctc gagttcttct tcgagtttaa gaacccgtcc
361 atgctgtatc tagccacagg ccaccgcgtc cagtggttgc gttacgccga gtggcttctc
421 acctgcccgg tcattctcat tcacctgtca aacctgacgg gcttgtccaa cgactacagc
481 aggcgcacca tgggtctgct tgtgtctgat attggcacaa ttgtgtgggg cgccacttcc
541 gccatggcca ccggatacgt caaggtcatc ttcttctgcc tgggtctgtg ttatggtgct
601 aacacgttct ttcacgctgc caaggcctac atcgagggtt accacaccgt gccgaagggc
661 cggtgtcgcc aggtggtgac tggcatggct tggctcttct tcgtatcatg gggtatgttc
721 cccatcctgt tcatcctcgg ccccgagggc ttcggcgtcc tgagcgtgta cggctccacc
781 gtcggccaca ccatcattga cctgatgtcg aagaactgct ggggtctgct cggccactac
841 ctgcgcgtgc tgatccacga gcatatcctc atccacggcg acattcgcaa gaccaccaaa
901 ttgaacattg gtggcactga gattgaggtc gagacgctgg tggaggacga ggccgaggct
961 ggcgcggtca acaagggcac cggcaagtac gcctcccgcg agtccttcct ggtcatgcgc
1021 gacaagatga aggagaaggg cattgacgtg cgcgcctctc tggacaacag caaggaggtg
1081 gagcaggagc aggccgccag ggctgccatg atgatgatga acggcaatgg catgggtatg
1141 ggaatgggaa tgaacggcat gaacggaatg ggcggtatga acgggatggc tggcggcgcc
1201 aagcccggcc tggagctcac tccgcagcta cagcccggcc gcgtcatcct ggcggtgccg
1261 gacatcagca tggttgactt cttccgcgag cagtttgctc agctatcggt gacgtacgag
1321 ctggtgccgg ccctgggcgc tgacaacaca ctggcgctgg ttacgcaggc gcagaacctg
1381 ggcggcgtgg actttgtgtt gattcacccc gagttcctgc gcgaccgctc tagcaccagc
1441 atcctgagcc gcctgcgcgg cgcgggccag cgtgtggctg cgttcggctg ggcgcagctg
1501 gggcccatgc gtgacctgat cgagtccgca aacctggacg gctggctgga gggcccctcg
1561 ttcggacagg gcatcctgcc ggcccacatc gttgccctgg tggccaagat gcagcagatg
1621 cgcaagatgc agcagatgca gcagattggc atgatgaccg gcggcatgaa cggcatgggc
1681 ggcggtatgg gcggcggcat gaacggcatg ggcggcggca acggcatgaa caacatgggc
1741 aacggcatgg gcggcggcat gggcaacggc atgggcggca atggcatgaa cggaatgggt
1801 ggcggcaacg gcatgaacaa catgggcggc aacggaatgg ccggcaacgg aatgggcggc
1861 ggcatgggcg gcaacggtat gggtggctcc atgaacggca tgagctccgg cgtggtggcc
1921 aacgtgacgc cctccgccgc cggcggcatg ggcggcatga tgaacggcgg catggctgcg
1981 ccccagtcgc ccggcatgaa cggcggccgc ctgggtacca acccgctctt caacgccgcg
2041 ccctcaccgc tcagctcgca gctcggtgcc gaggcaggca tgggcagcat gggaggcatg
2101 ggcggaatga gcggaatggg aggcatgggt ggaatggggg gcatgggcgg cgccggcgcc
2161 gccacgacgc aggctgcggg cggcaacgcg gaggcggaga tgctgcagaa tctcatgaac

2221 gagatcaatc gcctgaagcg cgagcttggc gagtaaaagg ctggaggccg gtactgcgat
2281 acctgcgagc tcgcgcgcct gactcgtcgt acacacggct caggagcacg cgcgcgtgga
2341 cttctcaacc tgtgtgcaac gtatctagag cggcctgtgc gcgaccgtcc gtgagcattc
2401 cggtgcgatc ttcccgcctt cgcaccgcaa gttcccttcc tggccctgct gcgcctgacg
2461 catcgtccga acggaagggc ggcttgatca gtaaagcatt gaagactgaa gtcgtgcgac
2521 cgtagtgcta tggctctgca cgtaagtggg cgctgccctg cttactacgc attgcccaag
2581 actgcttcct tttggtggcc gaggccctgg tcccacatca ttcatttgca taacgtactg
2641 tttagttaca tacgctttgc ttaacctcga caattgcaac atgggctgag agtccgtacg
2701 gcggctatgg acgaaggtgt tatcggatgt gattaggaat ctcggttgaa aggcttcgag
2761 aaagtgagct tcatctgtgg cttctgttgg ggtcatcaag aagaacgacg gtaaggcaaa
2821 cgaggtaaaa gtggcacgtc tttgtgcaca acgggcccgt ggagagtggg ggagtgcatg
2881 tgtgcggtcc taacacgcga gtgcaaagcg ggcttttctg gagctgggtt acggtctggc
2941 tcggcaactg ctctgtgttt taaccacagc ttcggaagtc tgggtatgtt ttgttggcag
3001 aaacatttgg gtaacttgag ggtgattcgt ctggagtcgg acaacatggc tgccgtccgt
3061 gtgcagggac ggtaatcaat gagctggagc tgtgatgctc accacacgtt gcatacccct
3121 gcttacaaaa acactttgat gtcgtggcca aactatgcgt gagcaaagag ttaaagaggc
3181 atgagtgcat ggttgcggac gtgcgcaaca attgcatcaa gtatttgacg ccttcaagcc
3241 aacaagtgcg cgcgcggcaa cttgattaac acgccggacg cagtggtggg ggcgtgtaca
3301 gtgtttatga gctgccattc tgcgatccgt agtgttaggt tgcgtgtgac gccgcgcggc
3361 tgtgggccct tacatggaga gttgggtgct tcaccacacg gttggcgccg ctgaagggtg
3421 tgctatgttt tggtaaagcc ggggccctga agaccgcaac cgtagaaccg tactgaaagg
3481 gtgtcagccc ggggtaactg gatgccctgg gacatagcta ttaatgttga agtgaagccg
3541 tcaagccgag tgccgtgcgc cgctgtatca ccaaggcccg tccta

[0044] A wild type (WT) ChR2 of the invention may be encoded by the following Chlamydomonas reinhardtii chlamyopsin 4 light-gated ion channel (COP4) amino acid sequence (GenBank Accession No. XP—001701725, and SEQ ID NO: 2):

1 mdyggalsav grellfvtnp vvvngsvlvp edqcycagwi esrgtngaqt asnvlqwlaa
61 gfsilllmfy ayqtwkstcg weeiyvcaie mvkvilefff efknpsmlyl atghrvqwlr
121 yaewlltcpv ilihlsnltg lsndysrrtm gllvsdigti vwgatsamat gyvkviffcl
181 glcygantff haakayiegy htvpkgrcrq vvtgmawlff vswgmfpilf ilgpegfgvl
241 svygstvght iidlmskncw gllghylrvl ihehilihgd irkttklnig gteievetlv
301 edeaeagavn kgtgkyasre sflvmrdkmk ekgidvrasl dnskeveqeq aaraammmmn
361 gngmgmgmgm ngmngmggmn gmaggakpgl eltpqlqpgr vilavpdism vdffreqfaq
421 lsvtyelvpa lgadntlalv tqaqnlggvd fvlihpeflr drsstsilsr lrgagqrvaa
481 fgwaqlgpmr dliesanldg wlegpsfgqg ilpahivalv akmqqmrkmq qmqqigmmtg
541 gmngmgggmg ggmngmgggn gmnnmgngmg ggmgngmggn gmngmgggng mnnmggngma
601 gngmgggmgg ngmggsmngm ssgvvanvtp saaggmggmm nggmaapqsp gmnggrlgtn
661 plfnaapspl ssqlgaeagm gsmggmggms gmggmggmgg mggagaattq aaggnaeaem
721 lqnlmneinr lkrelge

[0045] A wild type (WT) Chop2 of the invention may be encoded by the following Chlamydomonas reinhardtii retinal binding protein (cop4) gene sequence (GenBank Accession No. AF461397, and SEQ ID NO: 3):

1 gcatctgtcg ccaagcaagc attaaacatg gattatggag gcgccctgag tgccgttggg
61 cgcgagctgc tatttgtaac gaacccagta gtcgtcaatg gctctgtact tgtgcctgag
121 gaccagtgtt actgcgcggg ctggattgag tcgcgtggca caaacggtgc ccaaacggcg
181 tcgaacgtgc tgcaatggct tgctgctggc ttctccatcc tactgcttat gttttacgcc
241 taccaaacat ggaagtcaac ctgcggctgg gaggagatct atgtgtgcgc tatcgagatg
301 gtcaaggtga ttctcgagtt cttcttcgag tttaagaacc cgtccatgct gtatctagcc
361 acaggccacc gcgtccagtg gttgcgttac gccgagtggc ttctcacctg cccggtcatt
421 ctcattcacc tgtcaaacct gacgggcttg tccaacgact acagcaggcg caccatgggt

481 ctgcttgtgt ctgatattgg cacaattgtg tggggcgcca cttccgccat ggccaccgga
541 tacgtcaagg tcatcttctt ctgcctgggt ctgtgttatg gtgctaacac gttctttcac
601 gctgccaagg cctacatcga gggttaccac accgtgccga agggccggtg tcgccaggtg
661 gtgactggca tggcttggct cttcttcgta tcatggggta tgttccccat cctgttcatc
721 ctcggccccg agggcttcgg cgtcctgagc gtgtacggct ccaccgtcgg ccacaccatc
781 attgacctga tgtcgaagaa ctgctggggt ctgctcggcc actacctgcg cgtgctgatc
841 cacgagcata tcctcatcca cggcgacatt cgcaagacca ccaaattgaa cattggtggc
901 actgagattg aggtcgagac gctggtggag gacgaggccg aggctggcgc ggtcaacaag
961 ggcaccggca agtacgcctc ccgcgagtcc ttcctggtca tgcgcgacaa gatgaaggag
1021 aagggcattg acgtgcgcgc ctctctggac aacagcaagg aggtggagca ggagcaggcc
1081 gccagggctg ccatgatgat gatgaacggc aatggcatgg gtatgggaat gggaatgaac
1141 ggcatgaacg gaatgggcgg tatgaacggg atggctggcg gcgccaagcc cggcctggag
1201 ctcactccgc agctacagcc cggccgcgtc atcctggcgg tgccggacat cagcatggtt
1261 gacttcttcc gcgagcagtt tgctcagcta tcggtgacgt acgagctggt gccggccctg
1321 ggcgctgaca acacactggc gctggttacg caggcgcaga acctgggcgg cgtggacttt
1381 gtgttgattc accccgagtt cctgcgcgac cgctctagca ccagcatcct gagccgcctg
1441 cgcggcgcgg gccagcgtgt ggctgcgttc ggctgggcgc agctggggcc catgcgtgac
1501 ctgatcgagt ccgcaaacct ggacggctgg ctggagggcc cctcgttcgg acagggcatc
1561 ctgccggccc acatcgttgc cctggtggcc aagatgcagc agatgcgcaa gatgcagcag
1621 atgcagcaga ttggcatgat gaccggcggc atgaacggca tgggcggcgg tatgggcggc
1681 ggcatgaacg gcatgggcgg cggcaacggc atgaacaaca tgggcaacgg catgggcggc
1741 ggcatgggca acggcatggg cggcaatggc atgaacggaa tgggtggcgg caacggcatg
1801 aacaacatgg gcggcaacgg aatggccggc aacggaatgg gcggcggcat gggcggcaac
1861 ggtatgggtg gctccatgaa cggcatgagc tccggcgtgg tggccaacgt gacgccctcc
1921 gccgccggcg gcatgggcgg catgatgaac ggcggcatgg ctgcgcccca gtcgcccggc
1981 atgaacggcg gccgcctggg taccaacccg ctcttcaacg ccgcgccctc accgctcagc
2041 tcgcagctcg gtgccgaggc aggcatgggc agcatgggag gcatgggcgg aatgagcgga
2101 atgggaggca tgggtggaat ggggggcatg ggcggcgccg gcgccgccac gacgcaggct
2161 gcgggcggca acgcggaggc ggagatgctg cagaatctca tgaacgagat caatcgcctg
2221 aagcgcgagc ttggcgagta a

[0046] A wild type (WT) Chop2 of the invention may be encoded by the following Chlamydomonas reinhardtii retinal binding protein (cop4) amino acid sequence (GenBank Accession No. AAM15777, and SEQ ID NO: 4):

1 mdyggalsav grellfvtnp vvvngsvlvp edqcycagwi esrgtngaqt asnvlqwlaa
61 gfsilllmfy ayqtwkstcg weeiyvcaie mvkvilefff efknpsmlyl atghrvqwlr
121 yaewlltcpv ilihlsnltg lsndysrrtm gllvsdigti vwgatsamat gyvkviffcl
181 glcygantff haakayiegy htvpkgrcrq vvtgmawlff vswgmfpilf ilgpegfgvl
241 svygstvght iidlmskncw gllghylrvl ihehilihgd irkttklnig gteievetiv
301 edeaeagavn kgtgkyasre sflvmrdkmk ekgidvrasl dnskeveqeq aaraammmmn
361 gngmgmgmgm ngmngmggmn gmaggakpgl eltpqlqpgr vilavpdism vdffreqfaq
421 lsvtyelvpa lgadntlalv tqaqnlggvd fvlihpeflr drsstsilsr lrgagqrvaa
481 fgwaqlgpmr dliesanldg wlegpsfgqg ilpahivalv akmqqmrkmq qmqqigmmtg
541 gmngmgggmg ggmngmgggn gmnnmgngmg ggmgngmggn gmngmgggng mnnmggngma
601 gngmgggmgg ngmggsmngm ssgvvanvtp saaggmggmm nggmaapqsp gmnggrlgtn
661 plfnaapspl ssqlgaeagm gsmggmggms gmggmggmgg mggagaattq aaggnaeaem
721 lqnlmneinr lkrelge

[0047] A wild type (WT) Chop2 of the invention may be encoded by the following Chlamydomonas reinhardtii sensory opsin B (CSOB) mRNA sequence (GenBank Accession No. AF508966, and SEQ ID NO: 5):

1 ttgacatctg tcgccaagca agcattaaac atggattatg gaggcgccct gagtgccgtt

61 gggcgcgagc tgctatttgt aacgaaccca gtagtcgtca atggctctgt acttgtgcct
121 gaggaccagt gttactgcgc gggctggatt gagtcgcgtg gcacaaacgg tgcccaaacg
181 gcgtcgaacg tgctgcaatg gcttgctgct ggcttctcca tcctactgct tatgttttac
241 gcctaccaaa catggaagtc aacctgcggc tgggaggaga tctatgtgtg cgctatcgag
301 atggtcaagg tgattctcga gttcttcttc gagtttaaga acccgtccat gctgtatcta
361 gccacaggcc accgcgtcca gtggttgcgt tacgccgagt ggcttctcac ctgcccggtc
421 attctcattc acctgtcaaa cctgacgggc ttgtccaacg actacagcag gcgcaccatg
481 ggtctgcttg tgtctgatat tggcacaatt gtgtggggcg ccacttccgc catggccacc
541 ggatacgtca aggtcatctt cttctgcctg ggtctgtgtt atggtgctaa cacgttcttt
601 cacgctgcca aggcctacat cgagggttac cacaccgtgc cgaagggccg gtgtcgccag
661 gtggtgactg gcatggcttg gctcttcttc gtatcatggg gtatgttccc catcctgttc
721 atcctcggcc ccgagggctt cggcgtcctg agcgtgtacg gctccaccgt cggccacacc
781 atcattgacc tgatgtcgaa gaactgctgg ggtctgctcg gccactacct gcgcgtgctg
841 atccacgagc atatcctcat ccacggcgac attcgcaaga ccaccaaatt gaacattggt
901 ggcactgaga ttgaggtcga gacgctggtg gaggacgagg ccgaggctgg cgcggtcaac
961 aagggcaccg gcaagtacgc ctcccgcgag tccttcctgg tcatgcgcga caagatgaag
1021 gagaagggca ttgacgtgcg cgcctctctg gacaacagca aggaggtgga gcaggagcag
1081 gccgccaggg ctgccatgat gatgatgaac ggcaatggca tgggtatggg aatgggaatg
1141 aacggcatga acggaatggg cggtatgaac gggatggctg gcggcgccaa gcccggcctg
1201 gagctcactc cgcagctaca gcccggccgc gtcatcctgg cggtgccgga catcagcatg
1261 gttgacttct tccgcgagca gtttgctcag ctatcggtga cgtacgagct ggtgccggcc
1321 ctgggcgctg acaacacact ggcgctggtt acgcaggcgc agaacctggg cggcgtggac
1381 tttgtgttga ttcaccccga gttcctgcgc gaccgctcta gcaccagcat cctgagccgc
1441 ctgcgcggcg cgggccagcg tgtggctgcg ttcggctggg cgcagctggg gcccatgcgt
1501 gacctgatcg agtccgcaaa cctggacggc tggctggagg gcccctcgtt cggacagggc
1561 atcctgccgg cccacatcgt tgccctggtg gccaagatgc agcagatgcg caagatgcag
1621 cagatgcagc agattggcat gatgaccggc ggcatgaacg gcatgggcgg cggtatgggc
1681 ggcggcatga acggcatggg cggcggcaac ggcatgaaca acatgggcaa cggcatgggc
1741 ggcggcatgg gcaacggcat gggcggcaat ggcatgaacg gaatgggtgg cggcaacggc
1801 atgaacaaca tgggcggcaa cggaatggcc ggcaacggaa tgggcggcgg catgggcggc
1861 aacggtatgg gtggctccat gaacggcatg agctccggcg tggtggccaa cgtgacgccc
1921 tccgccgccg gcggcatggg cggcatgatg aacggcggca tggctgcgcc ccagtcgccc
1981 ggcatgaacg gcggccgcct gggtaccaac ccgctcttca acgccgcgcc ctcaccgctc
2041 agctcgcagc tcggtgccga ggcaggcatg ggcagcatgg gaggcatggg cggaatgagc
2101 ggaatgggag gcatgggtgg aatgggggggc atgggcggcg ccggcgccgc cacgacgcag
2161 gctgcgggcg gcaacgcgga ggcggagatg ctgcagaatc tcatgaacga gatcaatcgc
2221 ctgaagcgcg agcttggcga gtaaaaggct ggaggccggt actgcgatac ctgcgagctc
2281 gcgcgcctga ctcgtcgtac acacggctca ggagcacgcg cgcgtggact tctcaacctg
2341 tgtgcaacgt atctagagcg gcctgtgcgc gaccgtccgt gagcattccg gtgcgatctt
2401 cccgccttcg caccgcaagt tcccttcctg gccctgctgc gcctgacgca tcgtccgaac
2461 ggaagggcgg cttgatcagt aaagcattga agactgaagt cgtgcgaccg tagtgctatg
2521 gctctgcacg taagtgggcg ctgccctgct tactacgcat tgcccaagac tgcttccttt
2581 tggtggccga ggccctggtc ccacatcatt catttgcata acgtactgtt tagttacata
2641 cgctttgctt aacctcgaca attgcaacat gggctgagag tccgtacggc ggctatggac
2701 gaaggtgtta tcggatgtga ttaggaatct cggttgaaag gcttcgagaa agtgagcttc
2761 ttctgtggct tctgttgggg tcatcaagaa gaacgacggt aaggcaaacg aggtaaaagt
2821 ggcacgtctt tgtgcacaac gggcccgtgg agagtggggg agtgcatgtg tgcggtccta
2881 acacgcgagt gcaaagcggg cttttctgga gctgggttac ggtctggctc ggcaactgct
2941 ctgtgtttta accacagctt cggaagtctg ggtatgtttt gttggcagaa acatttgggt
3001 aacttgaggg tgattcgtct ggagtcggac aacatggctg ccgtccgtgt gcagggacgg
3061 taatcaatga agctgaagct gtgatgctca ccacacgttg catacccctg cttacaaaaa
3121 cactttgatg tcgtggccaa actatgcgtg agcaaagagt taaagaggca tgagtgcatg
3181 gttgcggacg tgcgcaacaa ttgcatcaag tatttgacgc cttcaagcca acaagtgcgc
3241 gcgcggcaac ttgattaaca cgccggacgc agtggtgggg gcgtgtacag tgtttatgag

3301 ctgccattct gcgatccgta gtgttaggtt gcgtgtgacg ccgcgcggct gtgggccctt
3361 acatggagag ttgggtgctt caccacacgg ttggcgccgc tgaagggtgt gctatgtttt
3421 ggtaaagccg gggccctgaa gaccgcaacc gtagaaccgt actgaaaggg tgtcagcccg
3481 gggtaactgg atgccctggg acatagctat taatgttgaa gtgaagccgt caagccgagt
3541 gccgtgcgcc gctgtatcac caaggcccgt ccaaaaaaaa aaaaaaaaaa aaaaaaaaa

[0048] A wild type (WT) Chop2 of the invention may be encoded by the following Chlamydomonas reinhardtii sensory opsin B (CSOB) amino acid sequence (GenBank Accession No. AAM44040, and SEQ ID NO: 6):

61 gfsilllmfy ayqtwkstcg weeiyvcaie mvkvilefff efknpsmlyl atghrvqwlr
121 yaewlltcpv ilihlsnltg lsndysrrtm gllvsdigti vwgatsamat gyvkviffcl
181 glcygantff haakayiegy htvpkgrcrq vvtgmawlff vswgmfpilf ilgpegfgvl
241 svygstvght iidlmskncw gllghylrvl ihehilihgd irkttklnig gteievetlv
301 edeaeagavn kgtgkyasre sflvmrdkmk ekgidvrasl dnskeveqeq aaraammmmn
361 gngmgmgmgm ngmngmggmn gmaggakpgl eltpqlqpgr vilavpdism vdffreqfaq
421 lsvtyelvpa lgadntlalv tqaqnlggvd fvlihpeflr drsstsilsr lrgagqrvaa
481 fgwaqlgpmr dliesanldg wlegpsfgqg ilpahivalv akmqqmrkmq qmqqigmmtg
541 gmngmgggmg ggmngmgggn gmnnmgngmg ggmgngmggn gmngmgggng mnnmggngma
601 gngmgggmgg ngmggsmngm ssgvvanvtp saaggmggmm nggmaapqsp gmnggrlgtn
661 plfnaapspl ssqlgaeagm gsmggmggms gmggmggmgg mggagaattq aaggnaeaem
721 lqnlmneinr lkrelge

[0049] A wild type (WT) Chop2 of the invention may be encoded by the following Chlamydomonas reinhardtii acop2 mRNA for archaeal-type opsin 2 nucleic acid sequence (GenBank Accession No. AB058891, and SEQ ID NO: 7):

1 catctgtcgc caagcaagca ttaaacatgg attatggagg cgccctgagt gccgttgggc
61 gcgagctgct atttgtaacg aacccagtag tcgtcaatgg ctctgtactt gtgcctgagg
121 accagtgtta ctgcgcgggc tggattgagt cgcgtggcac aaacggtgcc caaacggcgt
181 cgaacgtgct gcaatggctt gctgctggct tctccatcct actgcttatg ttttacgcct
241 accaaacatg gaagtcaacc tgcggctggg aggagatcta tgtgtgcgct atcgagatgg
301 tcaaggtgat tctcgagttc ttcttcgagt ttaagaaccc gtccatgctg tatctagcca
361 caggccaccg cgtccagtgg ttgcgttacg ccgagtggct tctcacctgc ccggtcattc
421 tcattcacct gtcaaacctg acgggcttgt ccaacgacta cagcaggcgc accatgggtc
481 tgcttgtgtc tgatattggc acaattgtgt ggggcgccac ttccgccatg gccaccggat
541 acgtcaaggt catcttcttc tgcctgggtc tgtgttatgg tgctaacacg ttctttcacg
601 ctgccaaggc ctacatcgag ggttaccaca ccgtgccgaa gggccggtgt cgccaggtgg
661 tgactggcat ggcttggctc ttcttcgtat catggggtat gttccccatc ctgttcatcc
721 tcggccccga gggcttcggc gtcctgagcg tgtacggctc caccgtcggc cacaccatca
781 ttgacctgat gtcgaagaac tgctggggtc tgctcggcca ctacctgcgc gtgctgatcc
841 acgagcatat cctcatccac ggcgacattc gcaagaccac caaattgaac attggtggca
901 ctgagattga ggtcgagacg ctggtggagg acgaggccga ggctggcgcg gtcaacaagg
961 gcaccggcaa gtacgcctcc cgcgagtcct tcctggtcat gcgcgacaag atgaaggaga
1021 agggcattga cgtgcgcgcc tctctggaca acagcaagga ggtggagcag gagcaggccg
1081 ccagggctgc catgatgatg atgaacggca atggcatggg tatgggaatg ggaatgaacg
1141 gcatgaacgg aatgggcggt atgaacggga tggctggcgg cgccaagccc ggcctggagc
1201 tcactccgca gctacagccc ggccgcgtca tcctggcggt gccggacatc agcatggttg
1261 acttcttccg cgagcagttt gctcagctat cggtgacgta cgagctggtg ccggccctgg
1321 gcgctgacaa cacactggcg ctggttacgc aggcgcagaa cctgggcggc gtggactttg
1381 tgttgattca ccccgagttc ctgcgcgacc gctctagcac cagcatcctg agccgcctgc
1441 gcggcgcggg ccagcgtgtg gctgcgttcg gctgggcgca gctggggccc atgcgtgacc
1501 tgatcgagtc cgcaaacctg gacggctggc tggagggccc ctcgttcgga cagggcatcc
1561 tgccggccca catcgttgcc ctggtggcca agatgcagca gatgcgcaag atgcagcaga

1621 tgcagcagat tggcatgatg accggcggca tgaacggcat gggcggcggt atgggcggcg
1681 gcatgaacgg catgggcggc ggcaacggca tgaacaacat gggcaacggc atgggcggcg
1741 gcatgggcaa cggcatgggc ggcaatggca tgaacggaat gggtggcggc aacggcatga
1801 acaacatggg cggcaacgga atggccggca acggaatggg cggcggcatg ggcggcaacg
1861 gtatgggtgg ctccatgaac ggcatgagct ccggcgtggt ggccaacgtg acgccctccg
1921 ccgccggcgg catgggcggc atgatgaacg gcggcatggc tgcgccccag tcgcccggca
1981 tgaacggcgg ccgcctgggt accaacccgc tcttcaacgc cgcgccctca ccgctcagct
2041 cgcagctcgg tgccgaggca ggcatgggca gcatgggagg catgggcgga atgagcggaa
2101 tgggaggcat gggtggaatg gggggcatgg gcggcgccgg cgccgccacg acgcaggctg
2161 cgggcggcaa cgcggaggcg gagatgctgc agaatctcat gaacgagatc aatcgcctga
2221 agcgcgagct tggcgagtaa aaggctggag gccggtactg cgatacctgc gagctcgcgc
2281 gcctgactcg tcgtacacac ggctcaggag cacgcgcgcg tggacttctc aacctgtgtg
2341 caacgtatct agagcggcct gtgcgcgacc gtccgtgagc attccggtgc gatcttcccg
2401 ccttcgcacc gcaagttccc ttcctggccc tgctgcgcct gacgcatc

[0050] A wild type (WT) Chop2 of the invention may be encoded by the following Chlamydomonas reinhardtii acop2 mRNA for archaeal-type opsin 2 amino acid sequence (GenBank Accession No. BAB68567, and SEQ ID NO: 8):

1 mdyggalsav grellfvtnp vvvngsvlvp edqcycagwi esrgtngaqt asnvlqwlaa
61 gfsilllmfy ayqtwkstcg weeiyvcaie mvkvilefff efknpsmlyl atghrvqwlr
121 yaewlltcpv ilihlsnltg lsndysrrtm gllvsdigti vwgatsamat gyvkviffcl
181 glcygantff haakayiegy htvpkgrcrq vvtgmawlff vswgmfpilf ilgpegfgvl
241 svygstvght iidlmskncw gllghylrvl ihehilihgd irkttklnig gteievetlv
301 edeaeagavn kgtgkyasre sflvmrdkmk ekgidvrasl dnskeveqeq aaraammmmn
361 gngmgmgmgm ngmngmggmn gmaggakpgl eltpqlqpgr vilavpdism vdffreqfaq
421 lsvtyelvpa lgadntlalv tqaqnlggvd fvlihpeflr drsstsilsr lrgagqrvaa
481 fgwaqlgpmr dliesanldg wlegpsfgqg ilpahivalv akmqqmrkmq qmqqigmmtg
541 gmngmgggmg ggmngmgggn gmnnmgngmg ggmgngmggn gmngmgggng mnnmggngma
601 gngmgggmgg ngmggsmngm ssgvvanvtp saaggmggmm nggmaapqsp gmnggrlgtn
661 plfnaapspl ssqlgaeagm gsmggmggms gmggmggmgg mggagaattq aaggnaeaem
721 lqnlmneinr lkrelge

ChR2 Mutants

[0051] The present invention provides Chop2 mutants wherein one or more amino acids are mutated. In some embodiments, the Chop2 is the full-length polypeptide, such as SEQ ID NOs: 2, 4, 6, and 8, with at least one amino acid mutation. In some embodiments, the mutation is at amino acid 132 and/or amino acid 159. In some preferred embodiments, the amino acid at position 132 is mutated from a leucine to a cysteine or an alanine. In some preferred embodiments, the amino acid at position 159 is mutated from a threonine to an alanine, a cysteine, or a serine. In all embodiments, the Chop2 mutants form a functional ChR2 channel.

[0052] The present invention also encompasses Chop2 proteins and nucleic acids that encode a biologically active fragment or a conservative amino acid substitution or other mutation variant of Chop2. Non-limiting examples of useful fragments include polypeptides encoding amino acids 1-315 of the wild-type Chop2, i.e., SEQ ID NO: 26, wherein at least one amino acid is mutated or conservatively substituted, for example at amino acid positions 132 and/or 159. Smaller fragments of wild-type Chop2, wherein at least one amino acid is mutated or conservatively substituted (i.e., at amino acid positions 132 and/or 159) may also be useful in the present invention. Accordingly, Chop2 polypeptides and nucleic acids of the present invention further include, but are not limited to, biologically active fragments encoding amino acids 1-315, 1-310, 1-300, 1-275, 1-250, 1-225, 1-200, 1-175, or 1-160 of the wild-type Chop2, wherein at least one amino acid is mutated or conservatively substituted, for example

at amino acid positions 132 and/or 159. In other embodiments, the Chop2 polypeptides and nucleic acids of the present invention can be up to, or about, 315 amino acids long, 310 amino acids long, 300 amino acids long, 275 amino acids long, 250 amino acids long, 225 amino acids long, 200 amino acids long, 175 amino acids long, or 160 amino acids long.

[0053] A single mutant Chop2 of the invention may be encoded by the following Synthetic construct hVChR1-mKate-betahChR2(L132C) gene sequence (GenBank Accession No. JN836746, and SEQ ID NO: 9) with the following annotations, GFP sequence is in bold, L132C Chop2 sequence is underlined::

1 atggattacc ctgtggcccg gtccctgatt gtaagatacc ccaccgatct gggcaatgga
61 accgtgtgca tgcccagagg acaatgctac tgcgaggggt ggctgaggag ccggggcact
121 agtatcgaaa aaaccatcgc tatcaccctc cagtgggtag tgttcgctct gtccgtagcc
181 tgtctcggct ggtatgcata ccaagcctgg agggctacct gtgggtggga ggaagtatac
241 gtggccctga tcgagatgat gaagtccatc atcgaggctt tccatgagtt cgactcccca
301 gccacactct ggctcagcag tgggaatggc gtagtgtgga tgagatatgg agagtggctg
361 ctgacctgtc ccgtcctgct cattcatctg tccaatctga ccgggctgaa agatgactac
421 tccaagagaa caatgggact gctggtgagt gacgtggggt gtattgtgtg gggagccacc
481 tccgccatgt gcactggatg gaccaagatc ctctttttcc tgatttccct ctcctatggg
541 atgtatacat acttccacgc cgctaaggtg tatattgagg ccttccacac tgtacctaaa
601 ggcatctgta gggagctcgt gcgggtgatg gcatggacct tctttgtggc ctgggggatg
661 ttccccgtgc tgttcctcct cggcactgag ggatttggcc acattagtcc ttacgggtcc
721 gcaattggac actccatcct ggatctgatt gccaagaata tgtgggggt gctgggaaa
781 tatctgcggg taaagatcca cgagcatatc ctgctgtatg gcgatatcag aaagaagcag
841 aaaatcacca ttgctggaca ggaaatggag gtggagacac tggtagcaga ggaggaggac
901 gggaccgcgg tcgccaccat ggtgtctaag ggcgaagagc tgattaagga gaacatgcac
961 atgaagctgt acatggaggg caccgtgaac aaccaccact tcaagtgcac atccgagggc
1021 gaaggcaagc cctacgaggg cacccagacc atgagaatca aggtggtcga gggcggccct
1081 ctccccttcg ccttcgacat cctggctacc agcttcatgt acggcagcaa aaccttcatc
1141 aaccacaccc agggcatccc cgacttcttt aagcagtcct tccctgaggg cttcacatgg
1201 gagagagtca ccacatacga agacgggggc gtgctgaccg ctacccagga caccagcctc
1261 caggacggct gcctcatcta caacgtcaag atcagagggg tgaacttccc atccaacggc
1321 cctgtgatgc agaagaaaac actcggctgg gaggcctcca ccgagatgct gtaccccgct
1381 gacggcggcc tggaaggcag agccgacatg gccctgaagc tcgtgggcgg gggccacctg
1441 atctgcaact tgaagaccac atacagatcc aagaaacccg ctaagaacct caagatgccc
1501 ggcgtctact atgtggacag aagactggaa agaatcaagg aggccgacaa agagacctac
1561 gtcgagcagc acgaggtggc tgtggccaga tactgcgacc tccctagcaa actggggcac
1621 aaacttaatt gcctgcagga gaagaagtca tgcagccagc gcatggccga attccggcaa
1681 tactgttgga acccggacac tgggcagatg ctgggccgca ccccagcccg gtgggtgtgg
1741 atcagcctgt actatgcagc tttctacgtg gtcatgactg ggctctttgc cttgtgcatc
1801 tatgtgctga tgcagaccat tgatccctac acccccgact accaggacca gttaaagtca
1861 ccgggggtaa ccttgagacc ggatgtgtat ggggaaagag ggctgcagat ttcctacaac
1921 atctctgaaa acagctctag acaggcccag atcaccggac gtccggagac tgagacattg
1981 ccaccggtgg actacggggg ggccctgagc gctgtgggca gagaactcct gttcgtgaca
2041 aatccagtcg tggtgaacgg ctccgtactc gtacccgagg atcagtgcta ttgcgcagga
2101 tggatcgaga gcagaggcac aaacggcgca cagactgcat ccaacgtgct ccagtggttg
2161 gccgcaggct tttccattct cctgctcatg ttttacgcct accagacttg gaagtccaca
2221 tgtggctggg aggaaatcta cgtgtgtgca atcgaaatgg tgaaggtgat cctggagttt
2281 ttcttcgaat ttaaaaaccc aagcatgctg tacctggcta ctggccacag agtgcagtgg
2341 ctgcggtatg ccgaatggct gctgacttgc ccagtgattt gcatccacct gtccaacctg
2401 actgggctgt ctaacgatta cagtaggaga acaatgggac tgctcgtatc cgacatcggc
2461 actatcgtat ggggcgcaac tagtgccatg gccactggat acgtgaaagt gatcttcttc
2521 tgcctgggac tctgctacgg agcaaacaca ttttttcatg ccgcaaaagc atatatcgag
2581 gggtatcata ccgtcccaaa gggccggtgt agacaagtgg tgactggcat ggcttggctg

2641 ttcttcgtgt cctgggggat gtttcccatc ctctttatcc tgggcccaga aggcttcggg
2701 gtgctgagtg tgtatggcag taccgtagga cacactatca ttgacctgat gagcaaaaac
2761 tgctgggggc tgctcggcca ctacctgaga gtactcatcc acgagcatat cctgattcat
2821 ggcgatatcc ggaaaactac caagctcaat atcgggggca ccgagattga agtggagaca
2881 ctcgtggagg acgaggccga ggccggagca gtgaacaaag gcactggcaa gtatgcctcc
2941 agagaatcct ttctggtgat gcgggacaaa atgaaggaga aaggcattga tgtacggtgc
3001 agtaatgcca aagccgtcga gactgatgtg tag

[0054] A single mutant ChR2 of the invention may be encoded by the following Synthetic construct hVChR1-mKate-betahChR2(L132C) amino acid sequence (GenBank Accession No. AER29839, and SEQ ID NO: 10) with the following annotations, GFP sequence is in bold, L132C Chop2 sequence is underlined:

1 mdypvarsli vryptdlgng tvcmprgqcy cegwlrsrgt siektiaitl qwvvfalsva
61 clgwyayqaw ratcgweevy valiemmksi ieafhefdsp atlwlssgng vvwmrygewl
121 ltcpvllihl snltglkddy skrtmgllvs dvgcivwgat samctgwtki lfflislsyg
181 mytyfhaakv yieafhtvpk gicrelvrvm awtffvawgm fpvlfllgte gfghispygs
241 aighsildli aknmwgvlgn ylrvkihehi llygdirkkq kitiagqeme vetlvaeeed
301 gtavatmvsk geelikenmh mklymegtvn nhhfkctseg egkpyegtqt mrikvveggp
361 lpfafdilat sfmygsktfi nhtqgipdff kqsfpegftw ervttyedgg vltatqdtsl
421 qdgcliynvk irgvnfpsng pvmqkktlgw eastemlypa dgglegradm alklvggghl
481 icnlkttyrs kkpaknlkmp gvyyvdrrle rikeadkety veqhevavar ycdlpsklgh
541 klnclqekks csqrmaefrq ycwnpdtgqm lgrtparwvw islyyaafyv vmtglfalci
601 yvlmqtidpy tpdyqdqlks pgvtlrpdvy gerglqisyn isenssrqaq itgrpetetl
661 ppvdyggals avgrellfvt npvvvngsvl vpedqcycag wiesrgtnga qtasnvlqwl
721 aagfsilllm fyayqtwkst cgweeiyvca iemvkvilef ffefknpsml ylatghrvqw
781 lryaewlltc pvicihlsnl tglsndysrr tmgllvsdig tivwgatsam atgyvkviff
841 clglcygant ffhaakayie gyhtvpkgrc rqvvtgmawl ffvswgmfpi lfilgpegfg
901 vlsvygstvg htiidlmskn cwgllghylr vlihehilih gdirkttkln iggteievet
961 lvedeaeaga vnkgtgkyas resflvmrdk mkekgidvrc snakavetdv

[0055] A single mutant Chop2 of the invention may be encoded by the following Synthetic construct hVChR1-mKate-betahChR2(L132C) gene sequence (GenBank Accession No. 1N836745, and SEQ ID NO: 11) with the following annotations, GFP sequence is in bold, L132C Chop2 sequence is underlined:

1 atggattacc ctgtggcccg gtccctgatt gtaagatacc ccaccgatct gggcaatgga
61 accgtgtgca tgcccagagg acaatgctac tgcgaggggt ggctgaggag ccggggcact
121 agtatcgaaa aaaccatcgc tatcaccctc cagtgggtag tgttcgctct gtccgtagcc
181 tgtctcggct ggtatgcata ccaagcctgg agggctacct gtgggtggga ggaagtatac
241 gtggccctga tcgagatgat gaagtccatc atcgaggctt tccatgagtt cgactcccca
301 gccacactct ggctcagcag tgggaatggc gtagtgtgga tgagatatgg agagtggctg
361 ctgacctgtc ccgtcctgct cattcatctg tccaatctga ccgggctgaa agatgactac
421 tccaagagaa caatgggact gctggtgagt gacgtggggt gtattgtgtg gggagccacc
481 tccgccatgt gcactggatg gaccaagatc ctctttttcc tgatttccct ctcctatggg
541 atgtatacat acttccacgc cgctaaggtg tatattgagg ccttccacac tgtacctaaa
601 ggcatctgta gggagctcgt gcgggtgatg gcatggacct tctttgtggc ctgggggatg
661 ttccccgtgc tgttcctcct cggcactgag ggatttggcc acattagtcc ttacgggtcc
721 gcaattggac actccatcct ggatctgatt gccaagaata tgtggggggt gctgggaaat
781 tatctgcggg taaagatcca cgagcatatc ctgctgtatg gcgatatcag aaagaagcag
841 aaaatcacca ttgctggaca ggaaatggag gtggagacac tggtagcaga ggaggaggac
901 gggaccgcgg tcgccaccat ggtgtctaag ggcgaagagc tgattaagga gaacatgcac
961 atgaagctgt acatggaggg caccgtgaac aaccaccact tcaagtgcac atccgagggc
1021 gaaggcaagc cctacgaggg cacccagacc atgagaatca aggtggtcga gggcggccct

1081 ctccccttcg ccttcgacat cctggctacc agcttcatgt acggcagcaa aaccttcatc
1141 aaccacaccc agggcatccc cgacttcttt aagcagtcct tccctgaggg cttcacatgg
1201 gagagagtca ccacatacga agacgggggc gtgctgaccg ctacccagga caccagcctc
1261 caggacggct gcctcatcta caacgtcaag atcagagggg tgaacttccc atccaacggc
1321 cctgtgatgc agaagaaaac actcggctgg gaggcctcca ccgagatgct gtaccccgct
1381 gacggcggcc tggaaggcag agccgacatg gccctgaagc tcgtgggcgg gggccacctg
1441 atctgcaact tgaagaccac atacagatcc aagaaacccg ctaagaacct caagatgccc
1501 ggcgtctact atgtggacag aagactggaa agaatcaagg aggccgacaa agagacctac
1561 gtcgagcagc acgaggtggc tgtggccaga tactgcgacc tccctagcaa actggggcac
1621 aaacttaatt gcctgcagga gaagaagtca tgcagccagc gcatggccga attccggcaa
1681 tactgttgga acccggacac tgggcagatg ctgggccgca ccccagcccg gtgggtgtgg
1741 atcagcctgt actatgcagc tttctacgtg gtcatgactg ggctctttgc cttgtgcatc
1801 tatgtgctga tgcagaccat tgatccctac acccccgact accaggacca gttaaagtca
1861 ccgggggtaa ccttgagacc ggatgtgtat ggggaaagag ggctgcagat ttcctacaac
1921 atctctgaaa acagctctag acaggcccag atcaccggac gtccggagac tgagacattg
1981 ccaccggtgg actacggggg ggccctgagc gctgtgggca gagaactcct gttcgtgaca
2041 aatccagtcg tggtgaacgg ctccgtactc gtacccgagg atcagtgcta ttgcgcagga
2101 tggatcgaga gcagaggcac aaacggcgca cagactgcat ccaacgtgct ccagtggttg
2161 gccgcaggct tttccattct cctgctcatg ttttacgcct accagacttg gaagtccaca
2221 tgtggctggg aggaaatcta cgtgtgtgca atcgaaatgg tgaaggtgat cctggagttt
2281 ttcttcgaat ttaaaaaccc aagcatgctg tacctggcta ctggccacag agtgcagtgg
2341 ctgcggtatg ccgaatggct gctgacttgc ccagtgattc tgatccacct gtccaacctg
2401 actgggctgt ctaacgatta cagtaggaga acaatgggac tgctcgtatc cgacatcggc
2461 actatcgtat ggggcgcaac tagtgccatg gccactggat acgtgaaagt gatcttcttc
2521 tgcctgggac tctgctacgg agcaaacaca ttttttcatg ccgcaaaagc atatatcgag
2581 gggtatcata ccgtcccaaa gggccggtgt agacaagtgg tgactggcat ggcttggctg
2641 ttcttcgtgt cctgggggat gtttcccatc ctctttatcc tgggcccaga aggcttcggg
2701 gtgctgagtg tgtatggcag taccgtagga cacactatca ttgacctgat gagcaaaaac
2761 tgctgggggc tgctcggcca ctacctgaga gtactcatcc acgagcatat cctgattcat
2821 ggcgatatcc ggaaaactac caagctcaat atcgggggca ccgagattga agtggagaca
2881 ctcgtggagg acgaggccga ggccggagca gtgaacaaag gcactggcaa gtatgcctcc
2941 agagaatcct ttctggtgat gcgggacaaa atgaaggaga aaggcattga tgtacggtgc
3001 agtaatgcca aagccgtcga gactgatgtg tag

[0056] A single mutant Chop2 of the invention may be encoded by the following Synthetic construct hVChR1-mKate-betahChR2(L132C) amino acid sequence (GenBank Accession No. AER29838, and SEQ ID NO: 12) with the following annotations, GFP sequence is in bold, L132C Chop2 sequence is underlined:

1 mdypvarsli vryptdlgng tvcmprgqcy cegwlrsrgt siektiaitl qwvvfalsva
61 clgwyayqaw ratcgweevy valiemmksi ieafhefdsp atlwlssgng vvwmrygewl
121 ltcpvllihl snltglkddy skrtmgllvs dvgcivwgat samctgwtki lfflislsyg
181 mytyfhaakv yieafhtvpk gicrelvrvm awtffvawgm fpvlfllgte gfghispygs
241 aighsildli aknmwgvlgn ylrvkihehi llygdirkkq kitiagqeme vetlvaeeed
301 gtavatmvsk geelikenmh mklymegtvn nhhfkctseg egkpyegtqt mrikvveggp
361 lpfafdilat sfmygsktfi nhtqgipdff kqsfpegftw ervttyedgg vltatqdtsl
421 qdgcliynvk irgvnfpsng pvmqkktlgw eastemlypa dgglegradm alklvggghl
481 icnlkttyrs kkpaknlkmp gvyyvdrrle rikeadkety veqhevavar ycdlpsklgh
541 klnclqekks csqrmaefrq ycwnpdtgqm lgrtparwvw islyyaafyv vmtglfalc
601 yvlmqtidpy tpdyqdqlks pgvtlrpdvy gerglqisyn isenssrqaq itgrpetet
661 ppvdyggals avgrellfvt npvvvngsvl vpedqcycag wiesrgtnga qtasnvlqwl
721 aagfsilllm fyayqtwkst cgweeiyvca iemvkvilef ffefknpsml ylatghrvqw
781 lryaewlltc pvilihlsnl tglsndysrr tmgllvsdig tivwgatsam atgyvkviff
841 clglcygant ffhaakayie gyhtvpkgrc rqvvtgmawl ffvswgmfpi lfilgpegfg

901 vlsvygstvg htiidlmskn cwgllghylr vlihehilih gdirkttkln iggteievet
961 lvedeaeaga vnkgtgkyas resflvmrdk mkekgidvrc snakavetdv

[0057] A L132C single mutant Chop2 of the invention may be encoded by the following amino acid sequence (positions 132 underlined and bolded, SEQ ID NO: 13):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV ICIHLSNLTG LSNDYSRRTM GLLVSDIGTI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0058] A T159C single mutant Chop2 of the invention may be encoded by the following amino acid sequence (positions 159 underlined and bolded, SEQ ID NO: 14):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV ILIHLSNLTG LSNDYSRRTM GLLVSDIGCI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0059] A L132C/T159C double mutant Chop2 of the invention may be encoded by the following nucleotide sequence (SEQ ID NO: 15):

1 atggactacg ggggggctct gtctgctgtc gggagggaac tgctgtttgt gactaaccct
61 gtcgtcgtga acgggagtgt gctggtccct gaggaccagt gctactgtgc cggctggatc
121 gaatcacgcg gaaccaacgg ggcccagaca gctagcaatg tgctgcagtg gctggccgct
181 gggtttagta tcctgctgct gatgttctac gcctatcaga cttggaagtc aacctgcggc
241 tgggaggaaa tctacgtgtg cgctattgag atggtgaaag tgatcctgga gttcttcttc
301 gagttcaaga acccaagcat gctgtacctg gctactggac accgagtgca gtggctgaga
361 tatgcagaat ggctgctgac atgccccgtc atctgcattc acctgtccaa cctgacaggc
421 ctgagcaatg actactccag gagaactatg ggactgctgg tgtccgacat cggctgcatt
481 gtctggggag caacttctgc tatggcaacc ggatacgtga aggtcatctt tttctgcctg
541 gggctgtgct atggcgcaaa tacctttttc cacgcagcca aggcctacat tgaggggtat
601 cataccgtgc caaaaggccg gtgccgacag gtggtcacag gaatggcttg gctgtttttc
661 gtctcttggg gaatgtttcc catcctgttc attctggggc ctgaagggtt cggcgtgctg
721 tctgtctacg gaagtacagt ggggcatact atcattgacc tgatgtccaa aaactgttgg
781 ggcctgctgg gacactatct gagagtgctg atccacgagc atatcctgat tcatggcgat
841 attcggaaga ccacaaaact gaatatcggc ggaaccgaga ttgaagtgga aacactggtg
901 gaagacgagg ctgaggctgg ggctgtgaac aaggggactg gcaaa

[0060] A L132C/T159C double mutant Chop2 of the invention may be encoded by the

following amino acid sequence (positions 132 and 159 underlined and bolded, SEQ ID NO: 16):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV ICIHLSNLTG LSNDYSRRTM GLLVSDIGCI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETL
301 EDEAEAGAVN KGTGK

[0061] A T159S single mutant Chop2 of the invention may be encoded by the following amino acid sequence (positions 159 underlined and bolded, SEQ ID NO: 17):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV ILIHLSNLTG LSNDYSRRTM GLLVSDIGSI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0062] A L132C/T159S double mutant Chop2 of the invention may be encoded by the following nucleotide sequence (SEQ ID NO: 18):

1 atggactacg ggggggctct gtctgctgtc gggagggaac tgctgtttgt gactaaccct
61 gtcgtcgtga acgggagtgt gctggtccct gaggaccagt gctactgtgc cggctggatc
121 gaatcacgcg gaaccaacgg ggcccagaca gctagcaatg tgctgcagtg gctggccgct
181 gggtttagta tcctgctgct gatgttctac gcctatcaga cttggaagtc aacctgcggc
241 tgggaggaaa tctacgtgtg cgctattgag atggtgaaag tgatcctgga gttcttcttc
301 gagttcaaga acccaagcat gctgtacctg gctactggac accgagtgca gtggctgaga
361 tatgcagaat ggctgctgac atgccccgtc atctgcattc acctgtccaa cctgacaggc
421 ctgagcaatg actactccag gagaactatg ggactgctgg tgtccgacat cggcagcatt
481 gtctggggag caacttctgc tatggcaacc ggatacgtga aggtcatctt tttctgcctg
541 gggctgtgct atggcgcaaa taccttttc cacgcagcca aggcctacat tgaggggtat
601 cataccgtgc caaaaggccg gtgccgacag gtggtcacag gaatggcttg gctgtttttc
661 gtctcttggg gaatgtttcc catcctgttc attctggggc ctgaagggtt cggcgtgctg
721 tctgtctacg gaagtacagt ggggcatact atcattgacc tgatgtccaa aaactgttgg
781 ggcctgctgg gacactatct gagagtgctg atccacgagc atatcctgat tcatggcga
841 attcggaaga ccacaaaact gaatatcggc ggaaccgaga ttgaagtgga aacactggtg
901 gaagacgagg ctgaggctgg ggctgtgaac aaggggactg gcaaa

[0063] A L132C/T159S double mutant Chop2 of the invention may be encoded by the following amino acid sequence (positions 132 and 159 underlined and bolded, SEQ ID NO: 19):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV ICIHLSNLTG LSNDYSRRTM GLLVSDIGSI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0064] A L132A single mutant Chop2 of the invention may be encoded by the following amino acid sequence (position 132 underlined and bolded, SEQ ID NO: 20):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV IAIHLSNLTG LSNDYSRRTM GLLVSDIGTI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0065] A L132A/T159C double mutant Chop2 of the invention may be encoded by the following nucleotide sequence (SEQ ID NO: 21):

1 ATGGACTACG GGGGGGCTCT GTCTGCTGTC GGGAGGGAAC TGCTGTTTGT GACTAACCCT
61 GTCGTCGTGA ACGGGAGTGT GCTGGTCCCT GAGGACCAGT GCTACTGTGC CGGCTGGATC
121 GAATCACGCG GAACCAACGG GGCCCAGACA GCTAGCAATG TGCTGCAGTG GCTGGCCGCT
181 GGGTTTAGTA TCCTGCTGCT GATGTTCTAC GCCTATCAGA CTTGGAAGTC AACCTGCGGC
241 TGGGAGGAAA TCTACGTGTG CGCTATTGAG ATGGTGAAAG TGATCCTGGA GTTCTTCTTC
301 GAGTTCAAGA ACCCAAGCAT GCTGTACCTG GCTACTGGAC ACCGAGTGCA GTGGCTGAGA
361 TATGCAGAAT GGCTGCTGAC ATGCCCCGTC ATCGCCATTC ACCTGTCCAA CCTGACAGGC
421 CTGAGCAATG ACTACTCCAG GAGAACTATG GGACTGCTGG TGTCCGACAT CGGCTGCATT
481 GTCTGGGGAG CAACTTCTGC TATGGCAACC GGATACGTGA AGGTCATCTT TTTCTGCCTG
541 GGGCTGTGCT ATGGCGCAAA TACCTTTTTC CACGCAGCCA AGGCCTACAT TGAGGGGTAT
601 CATACCGTGC CAAAAGGCCG GTGCCGACAG GTGGTCACAG GAATGGCTTG GCTGTTTTTC
661 GTCTCTTGGG GAATGTTTCC CATCCTGTTC ATTCTGGGGC CTGAAGGGTT CGGCGTGCTG

721 TCTGTCTACG GAAGTACAGT GGGGCATACT ATCATTGACC TGATGTCCAA AAACTGTTGG
781 GGCCTGCTGG GACACTATCT GAGAGTGCTG ATCCACGAGC ATATCCTGAT TCATGGCGAT
841 ATTCGGAAGA CCACAAAACT GAATATCGGC GGAACCGAGA TTGAAGTGGA AACACTGGTG
901 GAAGACGAGG CTGAGGCTGG GGCTGTGAAC AAGGGGACTG GCAAA

[0066] A L132A/T159C double mutant Chop2 of the invention may be encoded by the following amino acid sequence (positions 132 and 159 underlined and bolded, SEQ ID NO: 22):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV IAIHLSNLTG LSNDYSRRTM GLLVSDIGCI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWL1FF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0067] A T159A single mutant Chop2 of the invention may be encoded by the following amino acid sequence (position 159 underlined and bolded, SEQ ID NO: 23):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV ILIHLSNLTG LSNDYSRRTM GLLVSDIGAI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0068] A L132C/T159A double mutant Chop2 of the invention may be encoded by the following nucleotide sequence (SEQ ID NO: 24):

1 atggactacg ggggggctct gtctgctgtc gggagggaac tgctgtttgt gactaaccct
61 gtcgtcgtga acgggagtgt gctggtccct gaggaccagt gctactgtgc cggctggatc
121 gaatcacgcg gaaccaacgg ggcccagaca gctagcaatg tgctgcagtg gctggccgct
181 gggtttagta tcctgctgct gatgttctac gcctatcaga cttggaagtc aacctgcggc
241 tgggaggaaa tctacgtgtg cgctattgag atggtgaaag tgatcctgga gttcttcttc
301 gagttcaaga acccaagcat gctgtacctg gctactggac accgagtgca gtggctgag
361 tatgcagaat ggctgctgac atgccccgtc atctgcattc acctgtccaa cctgacagg
421 ctgagcaatg actactccag gagaactatg ggactgctgg tgtccgacat cggcgccatt
481 gtctggggag caacttctgc tatggcaacc ggatacgtga aggtcatctt tttctgcctg
541 gggctgtgct atggcgcaaa tacctttttc cacgcagcca aggcctacat tgaggggta
601 cataccgtgc caaaaggccg gtgccgacag gtggtcacag gaatggcttg gctgttttt
661 gtctcttggg gaatgtttcc catcctgttc attctggggc ctgaagggtt cggcgtgctg

721 tctgtctacg gaagtacagt ggggcatact atcattgacc tgatgtccaa aaactgttgg
781 ggcctgctgg gacactatct gagagtgctg atccacgagc atatcctgat tcatggcgat
841 attcggaaga ccacaaaact gaatatcggc ggaaccgaga ttgaagtgga aacactggtg
901 gaagacgagg ctgaggctgg ggctgtgaac aaggggactg gcaaa

[0069] A L132C/T159A double mutant Chop2 of the invention may be encoded by the following amino acid sequence (positions 132 and 159 underlined and bolded, SEQ ID NO: 25):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV ICIHLSNLTG LSNDYSRRTM GLLVSDIGAI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0070] A wild type (WT) Chop2 of the invention may be encoded by the following amino acid sequence (SEQ ID NO: 26):

1 MDYGGALSAV GRELLFVTNP VVVNGSVLVP EDQCYCAGWI ESRGTNGAQT ASNVLQWLAA
61 GFSILLLMFY AYQTWKSTCG WEEIYVCAIE MVKVILEFFF EFKNPSMLYL ATGHRVQWLR
121 YAEWLLTCPV ILIHLSNLTG LSNDYSRRTM GLLVSDIGTI VWGATSAMAT GYVKVIFFCL
181 GLCYGANTFF HAAKAYIEGY HTVPKGRCRQ VVTGMAWLFF VSWGMFPILF ILGPEGFGVL
241 SVYGSTVGHT IIDLMSKNCW GLLGHYLRVL IHEHILIHGD IRKTTKLNIG GTEIEVETLV
301 EDEAEAGAVN KGTGK

[0071] Mutant ChR2 proteins of the invention also demonstrate slower channel kinetics. Higher light sensitivity was found to correlate with slower channel kinetics, indicating a trade-off between light sensitivity and channel kinetics. Chop2 proteins that form the ChR2 proteins of the present invention may also comprise additional mutations or modifications that may improve channel kinetics, or increase the deactivation rate, of the ChR2. Particularly preferred ChR2 mutants balance the threshold of light sensitivity with channel kinetics.

Compositions and Kits

[0072] Compositions and kits of the invention comprise at least one nucleic acid molecule or polypeptide molecule that encodes a mutant Chop2 protein, and the resulting ChR2, of the invention. The at least one nucleic acid molecule or polypeptide molecule that encodes a mutant Chop2 protein of the invention may further include a pharmaceutically-acceptible carrier. Kits of the invention further include instructions for administering a composition of the invention to a subject.

Therapeutic Uses

[0073] Mutations were made on a codon optimized Chop2-GFP fusion protein to create single and double mutations at the L132 (Leucine 132) and T159 (threonine 159) sites. The functional properties of each mutant ChR2, or a combination thereof, were first examined in HEK cells. AAV2 virus vectors carrying mutant Chop2-GFP constructs driven by CAG promoter were made and injected intravitreally into the eyes of adult mice. Mutant Chop2-mediated light responses were examined by using multi-electrode array recordings from whole-mount retinas.

[0074] Single mutant ChR2, i.e., L132 and T159C, markedly lower the threshold light intensity that is required to evoke a ChR2-mediated photocurrent. Moreover, several double mutant ChR2 variants, including L132C/T159C, L132A/T159C, and L132C/T159S, were found to further increase the photocurrent above the results of any single mutant ChR2 at low light intensities. The double mutants exhibited a slower off-rate, which is likely to contribute to the increased photocurrent at the low light intensities. Spiking activity of retinal ganglion cells mediated by the L132C/T159C double mutant was observed at the light intensity of 10<13 >photon/cm<2>/s and at the wavelength of 473 nm. This light level is about 1.5 to 2 log units lower than the light level that is required to elicit the spiking activity with wild-type ChR2. The spike firing of retinal ganglion cells expressing L132C/T159C could follow a light flicker frequency of up to 15 Hz. Ongoing studies are evaluating the long-term expression and safety of mutant ChR2s of the invention in retinal neurons.

[0075] Furthermore, expression of the mutant Chop2 proteins, and the resulting ChR2 proteins, of the present invention was not found to cause neurotoxicity of up to two months after viral injection in mice, demonstrating the safety of the present invention for therapeutic use.

[0076] Vectors for use in the present invention can include various viral vectors, such as plasmids and recombinant viruses, i.e., recombinant adeno-associated virus (rAAV), recombinant adenoviruses, recombinant retroviruses, recombinant lentiviruses, and other viruses known in the art.

[0077] In some embodiments, the expression of the Chop2 proteins of the present invention is driven by a constitutive promoter, i.e., CAG promoter, CMV promoter, LTR. In other embodiments, the promoter is an inducible or a cell-specific promoter. Cell type-specific promoters that enable Chop2 protein expression in specific subpopulations of cells, i.e., retinal neuron cells or degenerating cells, may be preferred. These cells may include, but are not limited to, a retinal ganglion cell, a photoreceptor cell, a bipolar cell, a rod bipolar cell, an ON-type cone bipolar cell, a retinal ganglion cell, a photosensitive retinal ganglion cell, a horizontal cell, an amacrine cell, or an AII amacrine cell. Cell type-specific promoters are well known in the art. Particularly preferred cell type-specific promoters include, but are not limited to mGluR6, NK-3, and Pcp2(L7).

[0078] In some embodiments, use of different opsin genes in addition to the mutant Chop2 proteins of the present invention and targeted gene expression may further increase light sensitivity or improve vision. Visual information is processed through the retina through two pathways: an ON pathway which signals the light ON, and an OFF pathway which signals the light OFF. The existence of the ON and OFF pathway is important for the enhancement of contrast sensitivity. The visual signal in the ON pathway is relay from ON-cone bipolar cells to ON ganglion cells. Both ON-cone bipolar cells and ON-ganglion cells are depolarized in response to light. On the other hand, the visual signal in the OFF pathway is carried from OFF-cone bipolar cells to OFF ganglion cells. Both OFF-cone bipolar cells and OFF-ganglion cells are hypopolarized in response to light. Rod bipolar cells, which are responsible for the ability to see in dim light (scotopic vision), are ON bipolar cells (depolarized in response to light). Rod bipolar cells relay the vision signal through AII amacrine cells (an ON type retinal

cells) to ON an OFF cone bipolar cells.

[0079] Accordingly, a dual rhodopsin system can be used to recapitulate the ON and OFF pathways integral to visual processing and acuity. Briefly, a Chop2 protein of the present invention can be specifically targeted to ON type retinal neurons (i.e., ON type ganglion cells and/or ON type bipolar cells), while a hypopolarizing light sensor (i.e., halorhodopsin or other chloride pump known in the art) can be targeted to OFF type retinal neurons (i.e. OFF type ganglion cells and/or OFF type bipolar cells) to create ON and OFF pathways. The specific targeting to preferred cell subpopulations can be achieved through the use of different cell type-specific promoters. For example, Chop2 expression may be driven by the mGluR6 promoter for targeted expression in ON-type retinal neurons (i.e., ON type ganglion cells and/or ON type bipolar cells) while a hypopolarizing channel, such as halorhodopsin, expression is driven by the NK-3 promoter for targeted expression in OFF-type retinal neurons (i.e., OFF type ganglion cells and/or OFF type bipolar cells).

[0080] An alternative approach to restore ON and OFF pathways in the retina is achieved by, expressing a depolarizing light sensor, such as ChR2, to rod bipolar cells or AII amacrine. In this approach, the depolarization of rod bipolar cells or AII amacrine cells can lead to the ON and OFF responses at the levels of cone bipolar cells and the downstream retinal ganglion cells. Thus, the ON and OFF pathways that are inherent in the retina are maintained.

[0081] The present invention can be formulated to a pharmaceutical composition or medicament suitable for administration into a subject or patient. Suitable routes of administration include, for example, intravitreal, intraocular, or subretinal injection.

[0082] Such formulations comprise a pharmaceutically and/or physiologically acceptable vehicle, diluent, carrier or excipient, such as buffered saline or other buffers, e.g., HEPES, to maintain physiologic pH. For a discussion of such components and their formulation, see, generally, Gennaro, A E., Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins Publishers; 2003 or latest edition). See also, WO00/15822. If the preparation is to be stored for long periods, it may be frozen, for example, in the presence of glycerol.

[0083] The pharmaceutical composition described above is administered to a subject having a visual or blinding disease by any appropriate route, preferably by intravitreal or subretinal injection, depending on the retinal layer being targeted.

[0084] Disclosures from Bennett and colleagues (cited herein) concern targeting of retinal pigment epithelium—the most distal layer from the vitreal space. According to the present invention, the Chop2 construct or polypeptide is targeted to retinal cells, i.e., retinal ganglion cells or bipolar cells. Such cells are known to be reasonably well-accessible to intravitreal injection as disclosed herein. Intravitreal and/or subretinal injection can provide the necessary access to the bipolar cells, especially in circumstances in which the photoreceptor cell layer is absent due to degeneration—which is the case in certain forms of degeneration that the present invention is intended to overcome.

[0085] To test for the vector's ability to express the Chop2 mutants of the present invention, specifically in mammalian retinal neurons, by AAV-mediated delivery, a combination of a preferred promoter sequence linked to a reporter gene such as LacZ or GFP linked to a SV40 poly A sequence can be inserted into a plasmid and packaged into rAAV virus particles, concentrated, tested for contaminating adenovirus and titered for rAAV using an infectious center assay. The right eyes of a number of test subjects, preferably inbred mice, can be injected sub-retinally with about 1 μl of the rAAV preparation (e.g., greater than about 10<10 >infectious units ml). Two weeks later, the right (test) and left (control) eyes of half the

animals may be removed, fixed and stained with an appropriate substrate or antibody or other substance to reveal the presence of the reporter gene. A majority of the test retinas in injected eyes will exhibited a focal stained region, e.g., blue for LacZ/Xgal, or green for GFP consistent with a subretinal bleb of the injected virus creating a localized retinal detachment. All control eyes may be negative for the reporter gene product. Reporter gene expression examined in mice sacrificed at later periods is detected for at least 10 weeks post-injection, which suggests persistent expression of the reporter transgene.

[0086] In one embodiment, the Chop2 constructs are packaged in adenoviral vectors for transgene delivery. An effective amount of rAAV virions carrying a nucleic acid sequence encoding the Chop2 DNA under the control of the promoter of choice, preferably a constitutive CMV promoter or a cell-specific promoter such as mGluR6, is preferably in the range of between about 10<10 >to about 10<13 >rAAV infectious units in a volume of between about 150 and about 800 μl per injection. The rAAV infectious units can be measured according to McLaughlin, S K et al., 1988, J Virol 62:1963. More preferably, the effective amount is between about 10<10 >and about 10<12 >rAAV infectious units and the injection volume is preferably between about 250 and about 500 μl. Other dosages and volumes, preferably within these ranges but possibly outside them, may be selected by the treating professional, taking into account the physical state of the subject (preferably a human), who is being treated, including, age, weight, general health, and the nature and severity of the particular ocular disorder.

[0087] It may also be desirable to administer additional doses ("boosters") of the present nucleic acid(s) or rAAV compositions. For example, depending upon the duration of the transgene expression within the ocular target cell, a second treatment may be administered after 6 months or yearly, and may be similarly repeated. Neutralizing antibodies to AAV are not expected to be generated in view of the routes and doses used, thereby permitting repeat treatment rounds.

[0088] The need for such additional doses can be monitored by the treating professional using, for example, well-known electrophysiological and other retinal and visual function tests and visual behavior tests. The treating professional will be able to select the appropriate tests applying routine skill in the art. It may be desirable to inject larger volumes of the composition in either single or multiple doses to further improve the relevant outcome parameters.

Ocular Disorders

[0089] The ocular disorders for which the present Chop2 proteins, and the resulting ChR2 proteins, are intended and may be used to improve one or more parameters of vision include, but are not limited to, developmental abnormalities that affect both anterior and posterior segments of the eye. Anterior segment disorders include glaucoma, cataracts, corneal dystrophy, keratoconus. Posterior segment disorders include blinding disorders caused by photoreceptor malfunction and/or death caused by retinal dystrophies and degenerations. Retinal disorders include congenital stationary night blindness, age-related macular degeneration, congenital cone dystrophies, and a large group of retinitis-pigmentosa (RP)-related disorders. These disorders include genetically pre-disposed death of photoreceptor cells, rods and cones in the retina, occurring at various ages. Among those are severe retinopathies, such as subtypes of RP itself that progresses with age and causes blindness in childhood and early adulthood and RP-associated diseases, such as genetic subtypes of LCA, which frequently results in loss of vision during childhood, as early as the first year of life. The latter disorders are generally characterized by severe reduction, and often complete loss of photoreceptor cells, rods and cones. (Trabulsi, E I, ed., Genetic Diseases of the Eye, Oxford University Press, NY, 1998).

[0090] In particular, the Chop2 and ChR2 proteins of the present invention useful for the treatment and/or restoration of at least partial vision to subjects that have lost vision due to ocular disorders, such as RPE-associated retinopathies, which are characterized by a long-term preservation of ocular tissue structure despite loss of function and by the association between function loss and the defect or absence of a normal gene in the ocular cells of the subject. A variety of such ocular disorders are known, such as childhood onset blinding diseases, retinitis pigmentosa, macular degeneration, and diabetic retinopathy, as well as ocular blinding diseases known in the art. It is anticipated that these other disorders, as well as blinding disorders of presently unknown causation which later are characterized by the same description as above, may also be successfully treated by the Chop2 and ChR2 proteins of the present invention. Thus, the particular ocular disorder treated by the present invention may include the above-mentioned disorders and a number of diseases which have yet to be so characterized.

Optogenetics

[0091] The emerging field of optogenetics involves the combination of genetic and optical methods to control specific events in targeted cells of a living tissue. Optogeneics may be used within freely moving mammals and other animals. Moreover, the temporal precision (millisecond-timescale) of optogeneic methods are sufficient to function within intact biological systems.

[0092] The instant invention provides Chop2-gene therapy to retinal tissues of the eye, by introducing into retinal cells a nucleic acid or polypeptide encoding for at least one mutant form of Chop2. Mutant Chop2/ChR2 proteins of the invention are specifically adapted to be light-activated at lower thresholds of light intensities than their wild type counterparts. Accordingly, the mutant Chop2/ChR2 proteins of the invention can be used to activate cells of the retina and visual system using less damaging sources of illumination. The mutant Chop2/ChR2 proteins also conduct larger photocurrents upon activation, resulting in a more robust or efficacious response from the mutant Chop2/ChR2-expressing cells.

[0093] For example, mutant Chop2 proteins of the invention are administered to a subject through local, intravitreous or subretinal, injection of a nucleic acid molecule encoding a mutant Chop2, a mutant Chop2 polypeptide molecule, or a cell expressing a mutant Chop2/ChR2. Retinal cells of the subject express the mutant Chop2 proteins within the plasma membrane. When the transfected or transformed retinal cells encounter light radiation, the transfected or transformed retinal cells transduce an improved or restored signal.

[0094] These methods may be used in subjects of normal and/or impaired vision. Chop2/ChR2 mutants of the invention may preserve, improve, or restore vision. Moreover, Chop2/ChR2 mutants of the invention are used to preserve, improve, or restore the transduction of non-visual information from photosensitive retinal ganglion cells to the brain.

[0095] The term “vision” as used herein is defined as the ability of an organism to usefully detect light as a stimulus for differentiation or action. Vision is intended to encompass the following:
1. Light detection or perception—the ability to discern whether or not light is present;
2. Light projection—the ability to discern the direction from which a light stimulus is coming;
3. Resolution—the ability to detect differing brightness levels (i.e., contrast) in a grating or letter target; and
4. Recognition—the ability to recognize the shape of a visual target by reference to the differing contrast levels within the target.
Thus, “vision” includes the ability to simply detect the presence of light. The polypeptides and polynucleotides encoding mutant Chop2 of the present invention can be used to improve or

restore vision, wherein the improvement or restoration in vision includes, for example, increases in light detection or perception, increase in light sensitivity or photosensitivity in response to a light stimulus, increase in the ability to discern the direction from which a light stimulus is coming, increase in the ability to detect differing brightness levels, increase in the ability to recognize the shape of a visual target, and increases in visual evoked potential or transmission from the retina to the cortex. As such, improvement or restoration of vision may or may not include full restoration of sight, i.e., wherein the vision of the patient treated with the present invention is restored to the degree to the vision of a non-affected individual. The visual recovery described in the animal studies described below may, in human terms, place the person on the low end of vision function by increasing one aspect of vision (i.e., light sensitivity, or visual evoked potential) without restoring full sight. Nevertheless, placement at such a level would be a significant benefit because these individuals could be trained in mobility and potentially in low order resolution tasks which would provide them with a greatly improved level of visual independence compared to total blindness. Even basic light perception can be used by visually impaired individuals, whose vision is improved using the present compositions and methods, to accomplish specific daily tasks and improve general mobility, capability, and quality of life.

[0100] The degree of restoration of vision can be determined through the measurement of vision before, and preferably after, administering a vector comprising, for example, DNA encoding Chop2. Vision can be measured using any of a number of methods well-known in the art or methods not yet established. Vision, as improved or restored by the present invention, can be measured by any of the following visual responses:
1. a light detection response by the subject after exposure to a light stimulus—in which evidence is sought for a reliable response of an indication or movement in the general direction of the light by the subject individual when the light it is turned on;
2. a light projection response by the subject after exposure to a light stimulus in which evidence is sought for a reliable response of indication or movement in the specific direction of the light by the individual when the light is turned on;
3. light resolution by the subject of a light vs. dark patterned visual stimulus, which measures the subject's capability of resolving light vs dark patterned visual stimuli as evidenced by:
a. the presence of demonstrable reliable optokinetically produced nystagmoid eye movements and/or related head or body movements that demonstrate tracking of the target (see above) and/or
b. the presence of a reliable ability to discriminate a pattern visual stimulus and to indicate such discrimination by verbal or non-verbal means, including, for example pointing, or pressing a bar or a button; or
4. electrical recording of a visual cortex response to a light flash stimulus or a pattern visual stimulus, which is an endpoint of electrical transmission from a restored retina to the visual cortex, also referred to as the visual evoked potential (VEP). Measurement may be by electrical recording on the scalp surface at the region of the visual cortex, on the cortical surface, and/or recording within cells of the visual cortex.

[0107] Thus, improvement or restoration of vision, according to the present invention, can include, but is not limited to: increases in amplitude or kinetics of photocurents or electrical response in response to light stimulus in the retinal cells, increases in light sensitivity (i.e., lowering the threshold light intensity required for initiating a photocurrent or electrical response in response to light stimulus, thereby requiring less or lower light to evoke a photocurrent) of the retinal cells, increases in number or amplitude of light-evoked spiking or spike firings, increases in light responses to the visual cortex, which includes increasing in visual evoked potential transmitted from the retina or retinal cells to the visual cortex or the brain.

[0108] Both in vitro and in vivo studies to assess the various parameters of the present

invention may be used, including recognized animal models of blinding human ocular disorders. Large animal models of human retinopathy, e.g., childhood blindness, are useful. The examples provided herein allow one of skill in the art to readily anticipate that this method may be similarly used in treating a range of retinal diseases.

[0109] While earlier studies by others have demonstrated that retinal degeneration can be retarded by gene therapy techniques, the present invention demonstrates a definite physiological recovery of function, which is expected to generate or improve various parameters of vision, including behavioral parameters.

[0110] Behavioral measures can be obtained using known animal models and tests, for example performance in a water maze, wherein a subject in whom vision has been preserved or restored to varying extents will swim toward light (Hayes, J M et al., 1993, Behav Genet. 23:395-403).

[0111] In models in which blindness is induced during adult life or congenital blindness develops slowly enough that the individual experiences vision before losing it, training of the subject in various tests may be done. In this way, when these tests are re-administered after visual loss to test the efficacy of the present compositions and methods for their vision-restorative effects, animals do not have to learn the tasks de novo while in a blind state. Other behavioral tests do not require learning and rely on the instinctiveness of certain behaviors. An example is the optokinetic nystagmus test (Balkema G W et al., 1984, Invest Ophthalmol Vis Sci. 25:795-800; Mitchiner J C et al., 1976, Vision Res. 16:1169-71).

[0112] The present invention may also be used in combination with other forms of vision therapy known in the art to improve or restore vision. For example, the use of visual prostheses, which include retinal implants, cortical implants, lateral geniculate nucleus implants, or optic nerve implants. Thus, in addition to genetic modification of surviving retinal neurons using the present methods, the subject being treated may be provided with a visual prosthesis before, at the same time as, or after the molecular method is employed. The effectiveness of visual prosthetics can be improved with training of the individual, thus enhancing the potential impact of the Chop2 transformation of patient cells as contemplated herein. Training methods, such as habituation training characterized by training the subject to recognize recognize (i) varying levels of light and/or pattern stimulation, and/or (ii) environmental stimulation from a common light source or object as would be understood by one skilled in the art; and orientation and mobility training characterized by training the subject to detect visually local objects and move among said objects more effectively than without the training. In fact, any visual stimulation techniques that are typically used in the field of low vision rehabilitation are applicable here.

EXAMPLES

Example 1

Generation of Labeled Mutant Chop2 Constructs

[0113] Mutations were made on a codon optimized Chop2-GFP fusion protein to create single and double mutations at the L132 (Leucine 132) and T159 (Threonine 159) sites. Several mutants were generated, for example, single mutants such as L132A, L132C, T159A, T159C, and T 159S, and double mutants such as L132C/T159C, L132C/T159S, L132A/T159C, and L132C/T159A. Chop2-GFP transgenes were cloned into a rAAV vector under the control of a CAG promoter using methods known in the art.

Example 2

In Vitro Analysis of Mutant Chop2 Constructs

[0114] The functional properties of each mutant Chop2, or a combination thereof, were first examined in HEK cells. Chop2 constructs were delivered to HEK cells by adenoviral infection, for example. Upon expression of the WT or mutant Chop2, functional WT and mutant ChR2 channels were formed. Measurements of the light sensitivity and other properties of the ChR2 channels were assessed as described herein. The light stimuli (photons/cm<2>.s at 460 nm) were generated by a xenon arc lamp and attenuated by neutral density filters: ND4.0 (2.8×10<14>), ND3.0 (1.4×10<15>), ND2.5 (4.8×10<15>); ND2.0 (1.6×10<16>), ND1.0 (1.3×10<17>), ND0 (1.2×10<18>). Light evoked currents were measured from wild-type ChR2, T159C, L132C, L132C/T159C, and L132C/T159S. Patch clamp recordings were performed using methods known in the art.

[0115] Representative recordings from this experiment comparing light sensitivity between the Chop2 constructs demonstrated that mutations at L132 alone or in combination with mutation at T159 show increased photocurrent in comparison to WT (FIGS. 1A and 1B). FIG. 1B shows the same current traces at a different scale to illustrate the difference in amplitude of the photocurrents between WT ChR2 and ChR2 mutants more clearly. FIG. 1B specifically compares the current traces resulting from light stimulation using the neutral density filter (ND 2.5), equivalent to 4.8×10<15 >photos/cm<2>/s; the traces are designated by the arrows. The amplitude of the photocurrent of the L132C mutant is larger than that of WT; the amplitude of the photocurrent of double mutant L132C/T159C is larger than that of L132C; and the amplitude of the photocurrent of the L132C/T159S mutant larger than L132/T159C. The current traces of the ChR2 mutants, particularly double mutants L132C/T159C and L132C/T159S, also show slower deactivation kinetics when compared to WT and L132C.

[0116] FIG. 2 shows the representative recordings of light-evoked currents from WT ChR2, L132C, L132C/T159C, and L132C/T159S after stimulation by a 10 ms light pulse (1.2×10<18 >photons/cm<2>/s at 460 nm wavelength) to compare the deactivation time course, or decay time course after the light is off. Mutant ChR2 show longer deactivation time courses, with the double mutant L132C/T159S having the longest. Higher light sensitivity, as demonstrated by L132C/T159C and L132C/T159S, may be correlated with slower channel kinetics.

Example 3

In Vivo Ocular Administration and Analysis of Mutant Chop2 Constructs

[0117] AAV2 virus vectors carrying mutant Chop2-GFP constructs driven by CAG promoter were made and injected intravitreally into the eyes of C57BL/6J adult mice. Adult mice were anesthetized by IP injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Under a dissecting microscope, an incision was made by scissors through the eyelid to expose the sclera. A small perforation was made in the sclera region posterior to the lens with a needle and viral vector suspension of 0.8-1.5 μl at the concentration of approximately 10<11 >genomic particles/ml was injected into intravitreal space through the hole with a Hamilton syringe with a 32-gauge blunt-ended needle. For each animal, usually only one eye was injected with viral vectors carrying a Chop2 construct, and the other eye was uninjected or injected with control viral vectors carrying GFP alone. Upon expression of the WT or mutant Chop2 of the present invention, functional WT or mutant ChR2 channels were formed utilizing endogenous retinal, and the properties of these ChR2 proteins were assessed as described herein.

[0118] ChR2-mediated light responses were examined by using multi-electrode array recordings from whole-mount retinas. Light stimuli (photons/cm<2>/s) was generated by a

473 nm blue laser and attenuated by neutral density filters: ND0 (6.3×10<16>), ND1.0 (7.4×10<15>), ND1.5 (2.7×10<15>), ND2.0 (7.3×10<14>), ND2.5 (3.2×10<14>), ND3.0 (8.5×10<13>), ND3.5 (3.8×10<13>), and ND4.0 (9.5×10<12>).

[0119] The multielectrode array recordings were based on the procedures reported by Tian and Copenhagen (2003). Briefly, the retina was dissected and placed photoreceptor side down on a nitrocellulose filter paper strip (Millipore Corp., Bedford, Mass.). The mounted retina was placed in the MEA-60 multielectrode array recording chamber of 30 μm diameter electrodes spaced 200 μm apart (Multi Channel System MCS GmbH, Reutlingen, Germany), with the ganglion cell layer facing the recording electrodes. The retina was continuously perfused in oxygenated extracellular solution at 34° C. during all experiments. The extracellular solution contained (in mM): NaCl, 124; KCl, 2.5; CaCl2, 2; MgCl2, 2; NaH2PO4, 1.25; NaHCO3, 26; and glucose, 22 (pH 7.35 with 95% O2 and 5% CO2). Recordings were usually started 60 min after the retina was positioned in the recording chamber. The interval between onsets of each light stimulus was 10-15 s. The signals were filtered between 200 Hz (low cut off) and 20 kHz (high cut off). The responses from individual neurons were analyzed using Offline Sorter software (Plexon, Inc., Dallas, Tex.).

[0120] Single mutant Chop2/ChR2 mutants, i.e., L132 and T159C, markedly lower the threshold light intensity that is required to evoke a ChR2-mediated photocurrent. Moreover, several double mutants, including L132C/T159C, L132A/T159C, and L132C/T159S, were found to further increase the photocurrent at low light intensities. Different neutral density filters were used to attenuate the light stimuli to differentiate the light-evoked responses of the Chop2 constructs in low light. Spiking activity of retinal ganglion cells mediated by the mutants of the present invention was observed at the light intensities about 1.5 to 2 log units lower than the light level that is required to elicit the spiking activity with wild-type ChR2 (FIG. 3). Specifically, WT ChR2 exhibited did not exhibit any spiking activity in response to light stimuli with neutral density filter 2.5 (3.2×10<14 >photons/cm<2>/s) while ChR2 mutants (L132C, L132C/T159C, and L132C/T159S) demonstrate spiking activity. In fact, the ChR2 mutants still exhibited spiking activity in response to light with neutral density filters 3.0 and 3.5. Therefore, ChR2 mutants of the present invention possess higher light sensitivity and, thus, a markedly lower threshold light intensity that is required to elicit a ChR2-mediated photocurrent. Moreover, ChR2 double mutants possess a higher light sensitivity than single mutants, i.e. L132C. In addition, the spike firing of retinal ganglion cells expressing L132C/T159C and L132/T159S could follow a light flicker frequency of up to 15 Hz and 5 Hz, respectively (FIG. 4).

[0121] The L132C/T159A mutant shows high light sensitivity, probably the most light sensitive among these mutants, but it also shows extremely slow off-rate (the channel continue open for many sends after light off). Interestingly, it can be turned off more quickly using a light with long-wavelengths, such as yellow light. The L132C/T159A mutant (encoded by SEQ ID NOs: 24 and 25) demonstrates significant potential.

[0122] Given the trade-off between light sensitivity and channel kinetics, Chop2/ChR2 mutants that demonstrate a balance between light sensitivity and channel kinetics, such as L132C/T159C or L132C/T159S, may be suitable for the application of vision restoration.

Example 4

Analysis of Mutant Chop2 Constructs in Mouse Models of Disease

[0123] Mouse models of degenerative ocular diseases are known in the art. For example, homozygous rd1 (rd1/rd1) mice are a commonly used photoreceptor degeneration model. Rd1 mice carry a null mutation in a cyclic GMP phosphodiesterase, PDE6, similar to some forms

of retinitis pigmentosa in humans. Other well-established mouse models of ocular disease that may be of particular interest to demonstrate ChR2 mutant safety and efficacy include rds (also known as Prph<Rd2>), rd3, rd4, rd5, rd6, rd7, rd8, rd9, Pde6b<rd10>, or cpfl1 mice.

[0124] The Chop2-GFP constructs of the present invention can be injected intravitreally into the eyes of newborn (P1) or adult mice at 2-12 months of age. GFP signal can be observed in the Chop2-GFP-injected retinas, to determine the levels of ChR2 expression or expression in particular populations of cells, such as the retinal ganglion cells. Mutant Chop2-GFP expression can be monitored for a predetermined amount of time, i.e. 3-6 months, or 1 year after viral injection. Patch-clamp and multichannel array recordings can be performed using the methods known in the art and described herein to measure the light-evoked responses of mutant Chop2-GFP-expressing cells in vivo.

[0125] Additional techniques and tests are well-established in the art to test for the restoration of light sensitivity or vision. Visual evoked potentials from the Chop2-GFP expressing cells or visual cortex can be examined, as described in PCT publication WO 2007/131180. Other tests include behavioral assessments of the visual acuity in the mice, i.e., virtual optomotor test and visual water maze.

Example 5

Analysis of Long-Term Expression and Safety of Administration of Mutant Chop2 Constructs to Retinal Neurons

[0126] Neurotoxicity was assessed in C57BL/6J adult mice injected with Chop2 constructs of the present invention. The expression safety of Chop2 mutants in the retina was assessed by immunostaining and cell counting after exposure to strong blue light for two weeks. None of the mice were found to exhibit symptoms of neurotoxicity for up to two months after injection.

[0127] Additional ongoing studies are evaluating the long-term expression and safety of Chop2/ChR2 mutants of the invention in retinal neurons.

OTHER EMBODIMENTS

[0128] While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

[0129] The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

US8470790
Restoration of Visual Responses by In Vivo Delivery of Rhodopsin Nucleic Acids

Nucleic acid vectors encoding light-gated cation-selective membrane channels, in particular channelrhodopsin-2 (Chop2), converted inner retinal neurons to photosensitive cells in photoreceptor-degenerated retina in an animal model. Such treatment restored visual

perception and various aspects of vision. A method of restoring light sensitivity to a retina of a subject suffering from vision loss due to photoreceptor degeneration, as in retinitis pigmentosa or macular degeneration, is provided. The method comprises delivering to the subject by intravitreal or subretinal injection, the above nucleic acid vector which comprises an open reading frame encoding a rhodopsin, to which is operatively linked a promoter and transcriptional regulatory sequences, so that the nucleic acid is expressed in inner retinal neurons. These cells, normally light-insensitive, are converted to a light-sensitive state and transmit visual information to the brain, compensating for the loss, and leading to restoration of various visual capabilities.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention in the field of molecular biology and medicine relates to the use of microbial-type rhodopsins, such as the light-gated cation-selective membrane channel, channelrhodopsin-2 (Chop2) to convert inner retinal neurons to photosensitive cells in photoreceptor-degenerated retina, thereby restoring visual perception and various aspects of vision.

2. Description of the Background Art

Vision normally begins when rods and cones, also called photoreceptors, convert light signals to electrical signals that are then relayed through second- and third-order retinal neurons and the optic nerve to the lateral geniculate nucleus and, then to the visual cortex where visual images are formed (Baylor, D, 1996, Proc. Natl. Acad. Sci. USA 93:560-565; Wassle, H, 2004, Nat. Rev. Neurosci. 5:747-57). For a patient who is vision-impaired due to the loss of photoreceptors, visual perception may be induced by providing electrical stimulation at one of these downstream neuronal locations, depending on the nature of the particular impairment.

The severe loss of photoreceptor cells can be caused by congenital retinal degenerative diseases, such as retinitis pigmentosa (RP) (Sung, C H et al., 1991, Proc. Natl. Acad. Sci. USA 88 :6481-85; Humphries, P et al., 1992, Science 256:804-8; Weleber, R G et al., in: S J Ryan, Ed, Retina, Mosby, St. Louis (1994), pp. 335-466), and can result in complete blindness. Age-related macular degeneration (AMD) is also a result of the degeneration and death of photoreceptor cells, which can cause severe visual impairment within the centrally located best visual area of the visual field.

Both rodents and humans go progressively blind because, as rods and cones are lost, there is little or no signal sent to the brain. Inherited retinal degenerations that cause partial or total blindness affect one in 3000 people worldwide. Patients afflicted with Usher's Syndrome develop progressive deafness in addition to retinal degeneration. There are currently no effective treatments or cures for these conditions.

Basic research on approaches for retinal degeneration has long been classified into two approaches: (1) treatments to preserve remaining photoreceptors in patients with retinal degenerative disease, and (2) methods to replace photoreceptors lost to retinal degeneration. Patients afflicted with retinal disease often group themselves into those seeking ways to slow the loss of their diminishing vision and those who are already legally blind (“no light perception”), having lost their photoreceptors because of an inherited eye disease or trauma.

For the first approach, neuroprotection with neurotrophic factors (LaVail, M M et al., 1992, Proc. Natl. Acad. Sci. USA 89:11249-53) and virus-vector-based delivery of wild-type genes for recessive null mutations (Acland, G M et al., 2001, Nat. Genet. 28:92-95) have come the

furthest—to the point of a Phase I/II clinical trial (Hauswirth, W W, 2005, Retina 25, S60; Jacobson, S, Protocol #0410-677, World Wide Web URL: webconferences.com/nihoba/16_jun—2005.html) gaining approval in the U.S. for adeno-associated viral (AAV)-mediated gene replacement therapy for Leber's Congenital Amaurosis (LCA), a specific form of retinal degeneration. Unfortunately, for patients in advanced stages of retinal degeneration, this approach is not applicable, and the photoreceptor cells must be replaced.

For replacement, one approach involves transplantation (replacement) of normal tissues or cells to the diseased retina. Another involves electrical-stimulation of remaining non-visual neurons via retinal implants in lieu of the lost photoreceptive cells (prosthetic substitution). However, both methods face many fundamental obstacles. For example, for successful transplantation, the implanted tissue or cells must integrate functionally within the host retina. The electrical-stimulation approaches are burdened with mechanistic and technical difficulties as well as problems related to lack of long-term biocompatibility of the implanted bionic devices. In summary, there exist no effective vision-restoring therapies for inherited blinding disease.

The present inventors' strategy as disclosed herein, requires a suitable molecular “light-sensor.” Previous studies reported the heterologous expression of Drosophila rhodopsin (Zemelman, B V et al., 2002, Neuron 33:15-22) and, more recently, melanopsin, the putative photopigment of the intrinsic photosensitive retinal ganglion cells (Melyan, Z. et al., 2005, Nature 433:741-5; Panda, S. et al., 2005, Science 307:600-604; Qiu, X. et al., 2005, Nature 433:745-9). These photopigments, however, are coupled to membrane channels via a G protein signaling cascade and use cis-isoforms of retinaldehyde as their chromophore. As a result, expression of multiple genes would be required to render photosensitivity. In addition, their light response kinetics is rather slow. Recent studies aimed to improve the temporal resolution described the engineering of a light-sensitive K<-> channel (Banghart et al., 2004, Nat. Neurosci. 7:1381-6), though this required introduction of an exogenous “molecular tether” and use of UV light to unblock the channel. This engineered channel was proposed to be potentially useful for restoring light sensitivity in degenerate retinas, but its expression and function in retinal neurons remain unknown.

The present invention makes use of microbial-type rhodopsins similar to bacteriorhodopsin (Oesterhelt, D et al., 1973, Proc. Natl. Acad. Sci. USA 70:2853-7), whose conformation change is caused by reversible photoisomerization of their chromophore group, the all-trans isoform of retinaldehyde, and is directly coupled to ion movement through the membrane (Oesterhelt, D., 1998, Curr. Opin. Struct. Biol. 8:489-500). Two microbial-type opsins, channelopsin-1 and -2 (Chop1 and Chop2), have recently been cloned from Chlamydomonas reinhardtii (Nagel, G. et al., 2002, Science 296:2395-8; Sineshchekov, O A et al., 2002, Proc. Natl. Acad. Sci. USA 99:8689-94; Nagel, G. et al., 2003, Proc. Natl. Acad. Sci. USA 100, 13940-45) and shown to form directly light-gated membrane channels when expressed in Xenopus laevis oocytes or HEK293 cells in the presence of all-trans retinal. Chop2, a seven transmembrane domain protein, becomes photo-switchable when bound to the chromophore all-trans retinal. Chop2 is particularly attractive because its functional light-sensitive channel, channelrhodopsin-2 (Chop2 retinalidene abbreviated ChR2) with the attached chromophore is permeable to physiological cations. Unlike animal rhodopsins, which only bind the 11-cis conformation, Chop2 binds all-trans retinal isomers, obviating the need for the all-trans to 11-cis isomerization reaction supplied by the vertebrate visual cycle. However, the long-term compatibility of expressing ChR2 in native neurons in vivo in general and the properties of ChR2-mediated light responses in retinal neurons in particular remained unknown until the present invention.

The present strategy is feasible because histological studies, both in animal models of photoreceptor degeneration (Chang, B. et al., 2002, Vision Res. 42:517-25; Olshevskaya, E V

et al., 2004, J. Neurosci. 24:6078-85) and in postmortem patient eyes with almost complete photoreceptor loss due to RP (Santos, A H et al., 1997, Arch. Ophthalmol 115:511-15; Milam, A H et al., 1998, Prog. Retin. Eye Res. 1 7:175-205), reported the preservation of a significant number of inner retinal neurons.

Retinal gene therapy has been considered a possible therapeutic option for man. For example, U.S. Pat. No. 5,827,702 refers to methods for generating a genetically engineered ocular cell by contacting the cell with an exogenous nucleic acid under conditions in which the exogenous nucleic acid is taken up by the cell for expression. The exogenous nucleic acid is described as a retrovirus, an adenovirus, an adeno-associated virus or a plasmid. See, also, WO 00/15822 (Mar. 23, 2000) and WO 98/48097 (Oct. 29, 1998)

Efforts in such gene therapy have focused mainly on slowing down retinal degeneration in rodent models of primary photoreceptor diseases. Normal genes and mutation-specific ribozymes delivered to photoreceptors have prolonged the lifetime of these cells otherwise doomed for apoptotic cell death (Bennett, J., et al. 1996 Nat. Med. 2, 649-54; Bennett, J., et al. 1998, Gene Therapy 5, 1156-64; Kumar-Singh, R et al., 1998 Hum. Mol. Genet. 7, 1893-900; Lewin, A S et al. 1998, Nat. Med. 4, 967-71; Ali, R et al. 2000, Nat. Genet. 25, 306-10; Takahashi, M. et al., 1999, J Virol. 73, 7812-6; Lau, D., et al., 2000, Invest. Ophthalmol. Vis. Sci. 41, 3622-33; and LaVail, M M, et al. 2000, Proc Natl Acad Sci USA 97, 11488-93).

Retinal gene transfer of a reporter gene, green fluorescent protein (GFP), using a recombinant adeno-associated virus (rAAV) was demonstrated in normal primates (Bennett, J et al. 1999 Proc. Natl. Acad. Sci. USA 96, 9920-25). However, the restoration of vision in a blinding disease of animals, particularly in humans and other mammals, caused by genetic defects in retinal pigment epithelium (RPE) and/or photoreceptor cells has not been achieved. Jean Bennett and colleagues have described the rescue of photoreceptors using gene therapy in a model of rapid degeneration of photoreceptors using mutations of the RP65 gene and replacement therapy with the normal gene to replace or supplant the mutant gene. See, for example, US Patent Publication 2004/0022766 of Acland, Bennett and colleagues. This therapy showed some success in a naturally-occurring dog model of severe disease of retinal degenerations—the RPE65 mutant dog, which is analogous to human LCA.

Advantages of the present approach include the fact that it does not require introducing exogenous cells and tissues or physical devices, thus avoiding many obstacles encountered by existing approaches; the present invention is applicable for the reversal of vision loss or blindness caused by many retinal degenerative diseases. By expressing photosensitive membrane-channels or molecules in surviving retinal neurons of the diseased retina by viral based gene therapy method, the present invention can produce permanent treatment of the vision loss or blindness with high spatial and temporal resolution for the restored vision.

To the extent that any specific disclosure in the aforementioned publications or other publications may be considered to anticipate any generic aspect of the present invention, the disclosure of the present invention should be understood to include a proviso or provisos that exclude of disclaim any such species that were previously disclosed. The aspects of the present invention which are not anticipated by the disclosure of such publications are also unobvious from the disclosure of these publications, due at least in part to the unexpectedly superior results disclosed or alleged herein.

SUMMARY OF THE INVENTION

The present invention is directed to the genetic conversion of surviving light-insensitive inner retinal neurons in a retina in which photoreceptors are degenerating or have already died, into directly photosensitive neuronal cells, thereby imparting light sensitivity to such retinas and

restoring one or more aspects of visual responses and functional vision to a subject suffering from such degeneration. By restoring light sensitivity to a retina lacking this capacity, due to disease, the invention provides a mechanism for the most basic light-responses that are required for vision. Said another way, the present invention introduces a “light sensors” into retinal neurons that normally do not have them, to compensate for loss of retinal photoreceptor cells.

The present inventors and colleagues investigated the feasibility of using Chop2/ChR2 to restore light sensitivity to the retinas that have undergone rod and cone degeneration. The results presented herein show long-term expression of Chop2/ChR2 in rodent inner retinal neurons in vivo. The results also show that these inner retinal neurons can express a sufficient number of functional ChR2 channels to produce robust membrane depolarization or action potential firing without an exogenous supply of all-trans retinal. Furthermore, the present inventors demonstrated that the expression of ChR2 in a photoreceptor-deficient mouse model not only enables retinal ganglion cells to encode light signals but also restores visually evoked responses in the visual cortex.

The present invention is directed to the restoration of vision loss to individuals that have lost vision or are blind as a result of retinal photoreceptor degeneration. The invention enables retinal neurons in such a diseased retina to respond to light by expressing photosensitive membrane-channels or molecules in these retinal neurons. Preferred the light-sensitive channels or molecules are microbial type light-gate channel rhodopsins, such as ChR2, ChR1, light-driven ion pump, such as bacteriorhodopsins (Lanyi, J K, 2004, Annu Rev Physiol. 66:665-88), halorhodopsins (Lanyi, J K, 1990, Physiol Rev. 70:319-30), and their derivatives

As discovered by the present inventors, retinal neurons that are normally not light sensitive (directly) in the retinas of blind mice, such as retinal ganglion cells (RGCs) and bipolar cells, can respond to light when a green algae protein called channelrhodopsin-2 (ChR2), or a biologically active fragment or a conservative amino acid substitution variant thereof, is inserted into the neuronal cell membranes. The study was conducted with mice that had been genetically bred to lose rods and cones, the light-sensitive cells in the retina, a condition that models RP in humans. In addition to RP, there are many forms of retinal degenerative eye diseases that possibly could be treated by the present approach.

As disclosed herein, visual function can be restored by conveying light-sensitive properties to other surviving cells in the retina after the rods and cones have died. Using a DNA transfer approach, the present inventors introduced the light-absorbing protein ChR2 into the mouse retinal neurons that survived after the rods and cones had died. These cells became light sensitive and sent signals via the optic nerve and higher order visual pathways to the visual cortex where visual perception occurs. Using electrophysiologic means, it was shown that the signals reached the visual cortex in a majority of the ChR2-treated mice. The light sensitivity persisted for at least six months, suggesting that the subject might regain usable vision with additional maneuvers disclosed herein, such as expressing ChR2 in other types of retinal cells or modifying the light sensitivity and/or wavelength selectivity of ChR2, or using similar microbial proteins, to produce diverse light-sensitive channels to improve outcomes for the restoration of normal vision.

As noted by persons of skill in this art, this strategy represents a “paradigm shift in the field” referring to a “new field of re-engineering retinal interneurons as genetically modified ‘prosthetic’ cells,” The present invention “opened the possibility of genetically modifying the surviving retinal interneurons to function as a replacement light-sensing receptor,” (Flannery, J and Greenberg, K., 2006, Neuron. 50:1-3; written as a preview to a publication in the same issue of the present inventors and colleagues, Bi J. et al., Neuron 50, 23-33, 2006).

The present inventors capitalized upon advancements in the field by using viral vectors to transfer genes to retinal photoreceptor cells (Flannery J G et al., 1997, Proc. Natl. Acad. Sci. USA 94:6916-21). The conversion of light-insensitive retinal interneurons into photosensitive cells introduces an entirely new direction for treatments of blinding retinal degeneration.

In one embodiment of the present invention, retinal bipolar cells, certain amacrine cells and ganglion cells are targeted for transduction of the Chop2 DNA, to convert them functionally into photosensitive cells that subsume the function of rods and cones. The layering of cells in the retina is such that photoreceptor cells excite bipolar cells which excite ganglion cells to transmit signals to the visual cortex. It is preferred to express the channel opsin of the present invention in bipolar ON-type cells. Intravitreal and/or subretinal injections are used to deliver DNA molecules and virus vectors to reach the cells being targeted.

In one embodiment, the promoter is from a mGluR6 promoter-region of the Grm6 gene (GenBank accession number BC041684), a gene that controls expression of metabotropic glutamate receptor 6 ((Ueda Y et al., 1997, J Neurosc 17:3014-23). The genomic sequence is shown in GenBank accession number—AL627215. A preferred example of this promoter region sequence from the above GenBank record is SEQ ID NO:9 consisting of 11023 nucleotides—as shown in FIG. 8. The original Umeda et al., study employed a 10 kb promoter, but the actual length of the promoter and the sequence that comprises control elements of Grm6 can be adjusted by increasing or decreasing the fragment length. It is a matter of routine testing to select and verify the action of the optimally sized fragment from the Grm6 gene that drives transgenic expression of a selected coding sequence, preferably Chop2, in the desired target cells, preferably in bipolar cells which are rich in glutamate receptors, particularly the "on" type bipolar cells, which are the most bipolar cells in the retina (Nakajima, Y., et al., 1993, J Biol Chem 268:11868-73).

The present invention is directed to a method of restoring light sensitivity to a retina, comprising:
(a) delivering to retinal neurons a nucleic acid expression vector that encodes a light-gated channel rhodopsin or a light-driven ion pump rhodopsin expressible in the neurons, which vector comprises an open reading frame encoding the rhodopsin, and operatively linked thereto, a promoter sequence, and optionally, transcriptional regulatory sequences; and
(b) expressing the vector in the neurons, thereby restoring light sensitivity.

The rhodopsin is preferably channelrhodopsin-2 (Chop2) or a biologically active fragment or conservative amino acid substitution variant thereof.

The vector is preferably a rAAV viral vector.

The promoter may be a constitutive promoter such as a hybrid CMV enhancer/chicken ß-actin promoter (CAG) (as indicated below as part of SEQ ID NO:1), or a CMV promoter. The promoter may also be (i) an inducible or (ii) a cell type-specific promoter, preferred examples of the latter being the mGluR6 promoter (e.g., part of a promoter sequence SEQ ID NO:9), a Pcp2 (L7) promoter or a neurokinin-3 (NK-3) promoter.

A preferred vector in the above method comprises the CAG promoter, a woodchuck posttranscriptional regulatory element (WPRE), and a bovine or human growth hormone polyadenylation sequence.

In the present method, the retinal neurons are selected from ON- and OFF-type retinal ganglion cells, retinal rod bipolar cells, All amacrine cells and ON and OFF retinal cone bipolar cells. Preferably, the vector is targeted to and expressed in ON type ganglion cells and/or ON type bipolar cells If the vector comprises the NK-3 promoter, the vector is

preferably targeted to OFF cone bipolar cells.

The invention is also directed to method of restoring photosensitivity to retinal neurons of a subject suffering from vision loss or blindness in whom retinal photoreceptor cells are degenerating or have degenerated and died, which method comprises:
(a) delivering to the retina of the subject a nucleic acid vector that encodes a light-gated channel rhodopsin or a light-driven ion pump rhodopsin expressible in the neurons, which vector comprises an open reading frame encoding the rhodopsin, and operatively linked thereto, a promoter sequence, and optionally, transcriptional regulatory sequences;
(b) expressing the vector in the neurons, wherein the expression of the rhodopsin renders the neurons photosensitive, thereby restoring of photosensitivity to the retina.

In this method the rhodopsin is preferably Chop2 or a biologically active fragment or conservative amino acid substitution variant thereof. The vector is preferably a rAAV viral vector. Preferred promoters are as described above for the above-presented embodiment. Preferred target cells for the vector are as described above.

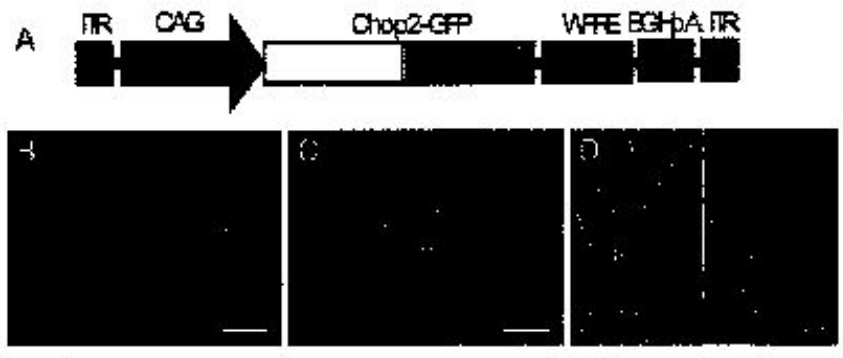
The restoration of photosensitivity using the above method preferably results in restoration of vision in the subject. The vision is preferably measured by one or more of the following methods:
(i) a light detection response by the subject after exposure to a light stimulus
(ii) a light projection response by the subject after exposure to a light stimulus;
(iii) light resolution by the subject of a light versus a dark patterned visual stimulus;
(iv) electrical recording of a response in the visual cortex to a light flash stimulus or a pattern visual stimulus

In this foregoing method, the vision loss or blindness may be a result of a degenerative disease, preferably, retinitis pigmentosa or age-related macular degeneration.

In another embodiment, the subject is also provided with a visual prosthesis before, at the same time as, or after delivery of the vector. Preferred visual prostheses comprise retinal implants, cortical implants, lateral geniculate nucleus implants, or optic nerve implants.

When employing the foregoing method, the subject's visual response may be subjected to training using one or more visual stimuli. The training is preferably achieved by one or more of the following methods:
(a) habituation training characterized by training the subject to recognize (i) varying levels of light and/or pattern stimulation, and/or (ii) environmental stimulation from a common light source or object; and
(b) orientation and mobility training characterized by training the subject to detect visually local objects and move among the objects more effectively than without the training.

BRIEF DESCRIPTION OF DRAWINGS



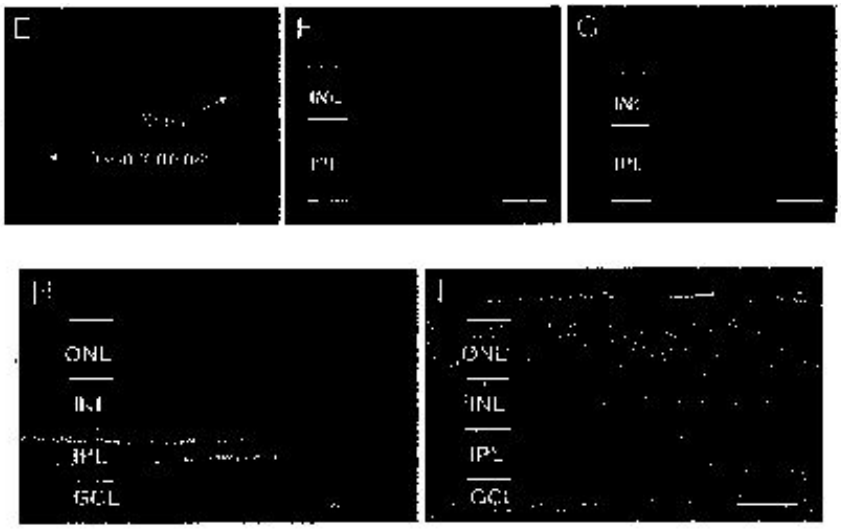


Fig. 1A – 1I

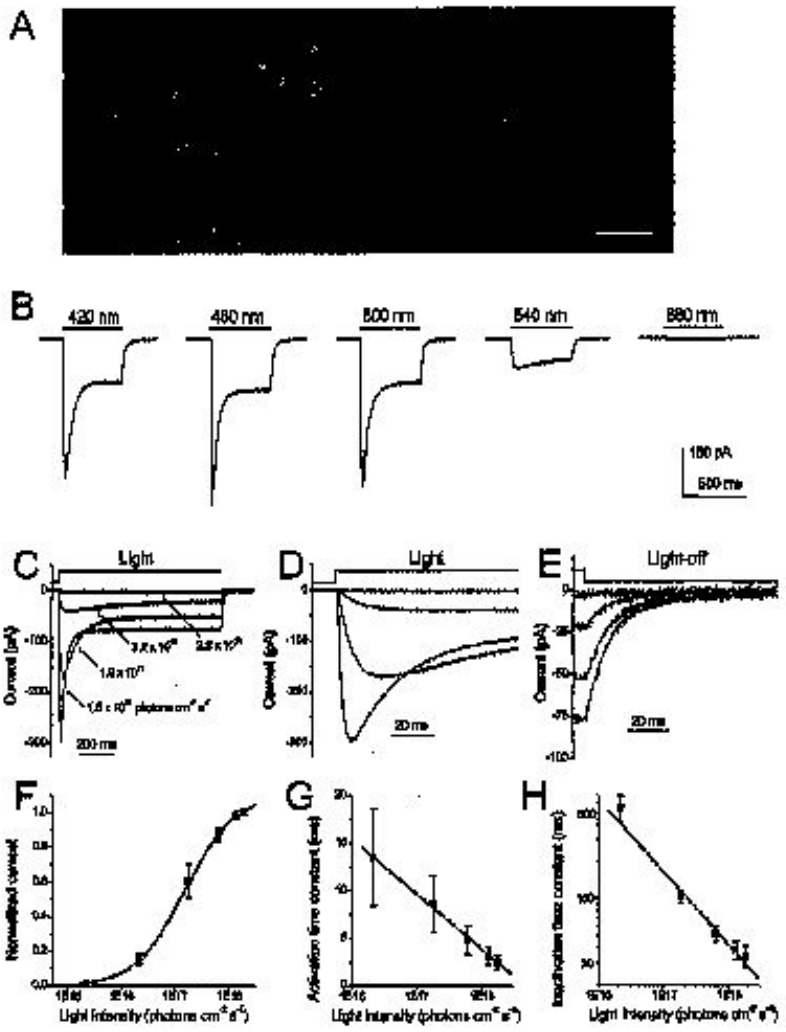
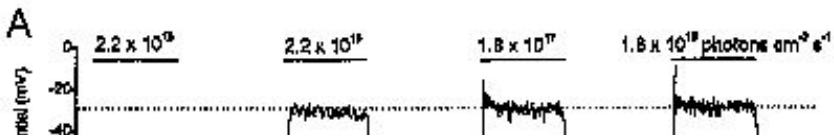


Fig. 2A – 2H



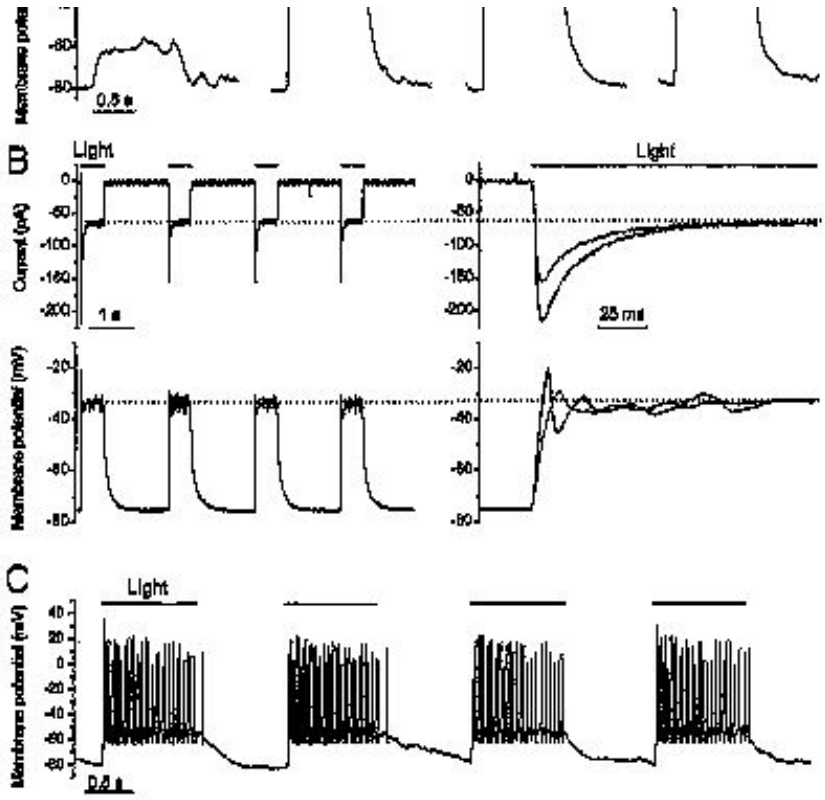


Fig. 3A – 3C

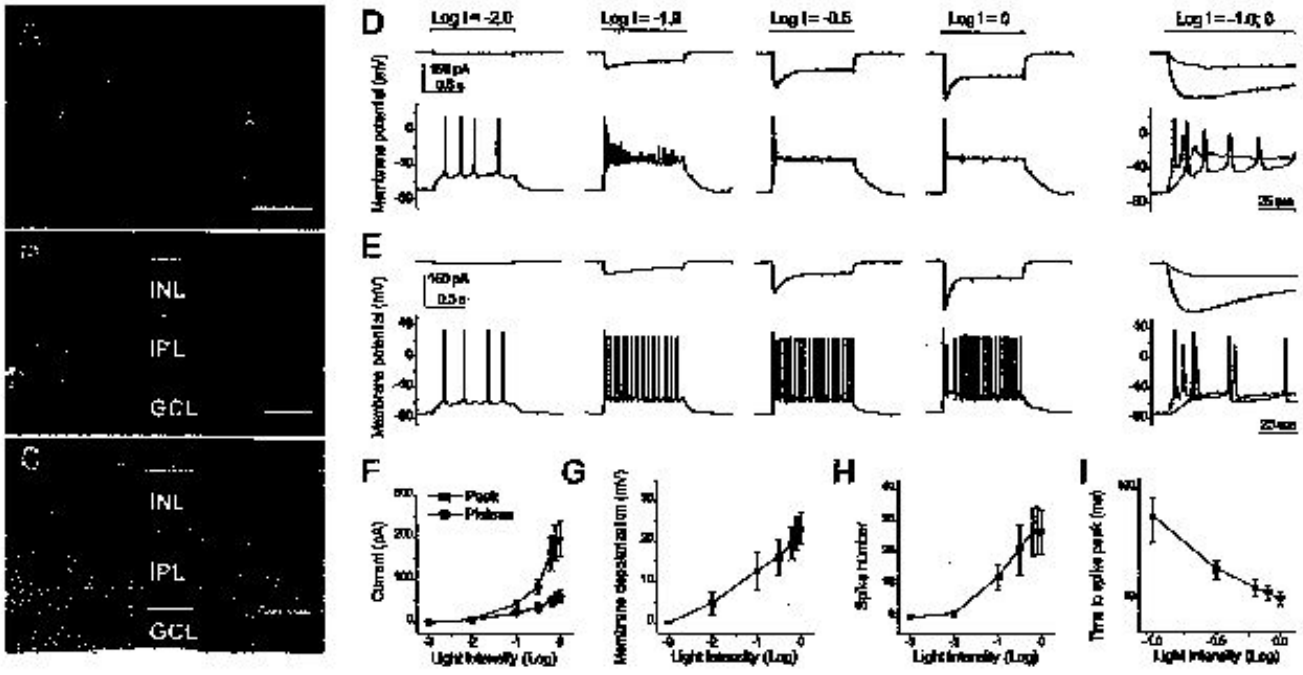
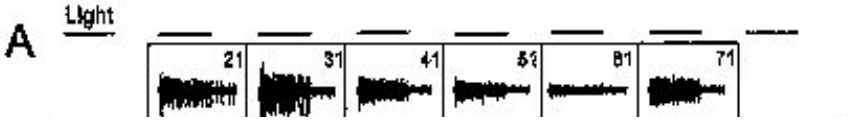


Fig. 4A – 4I



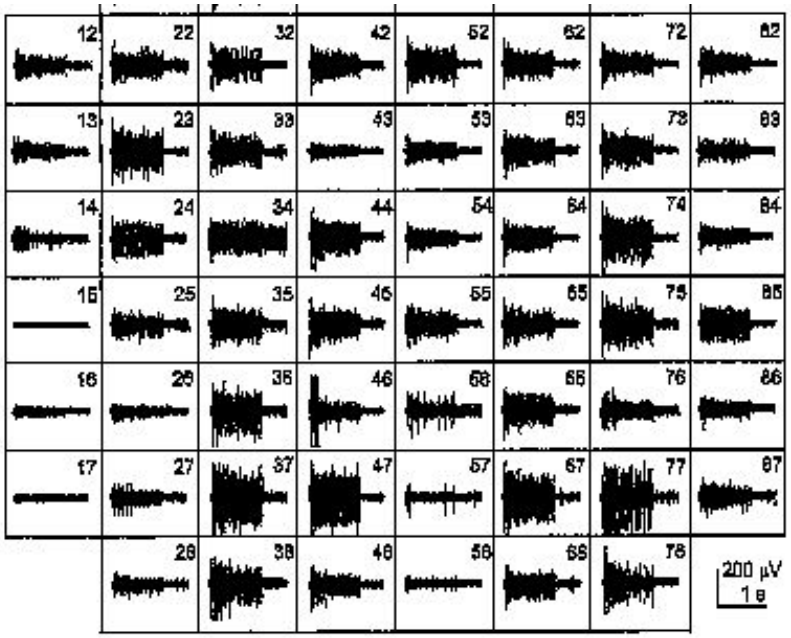


Fig. 5A

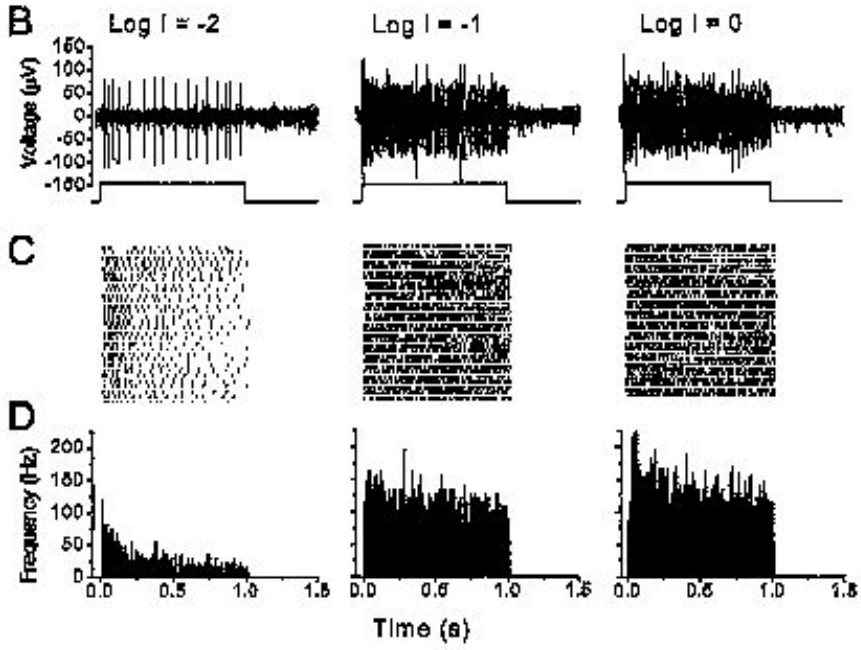
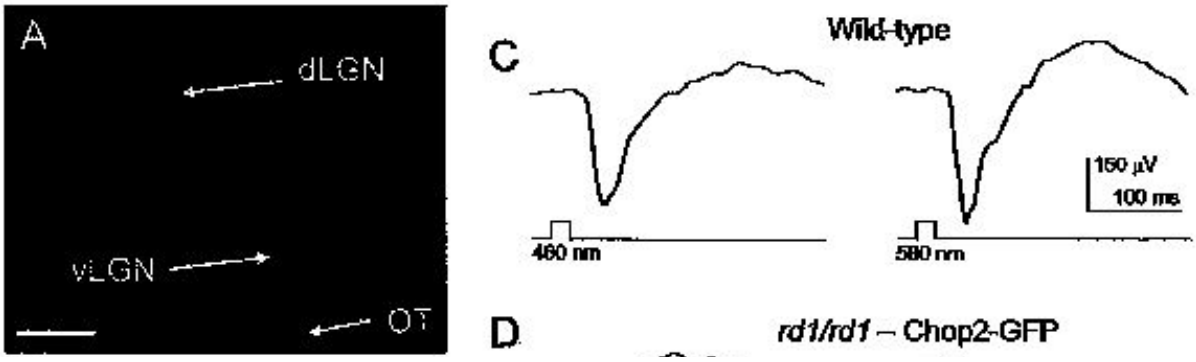


Fig. 5B – 5D



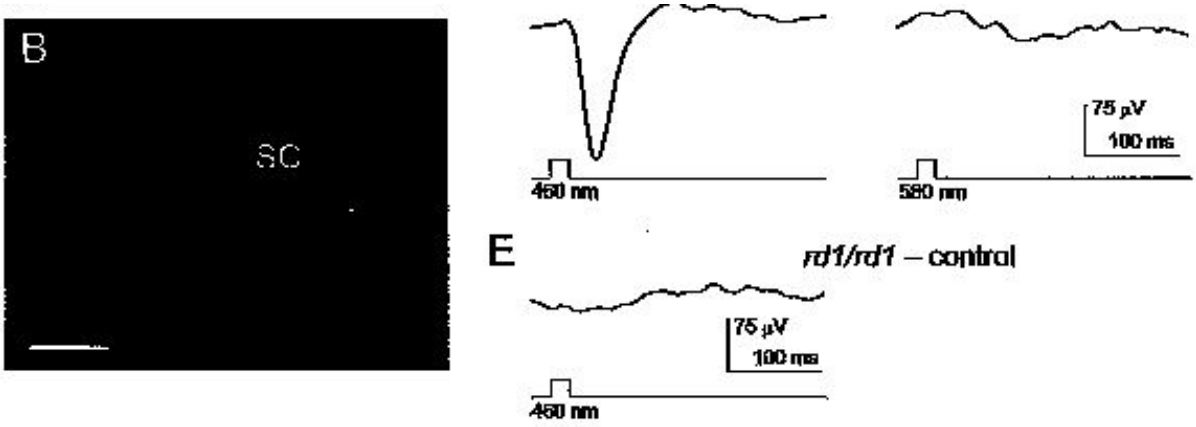


Fig. 6A – 6E

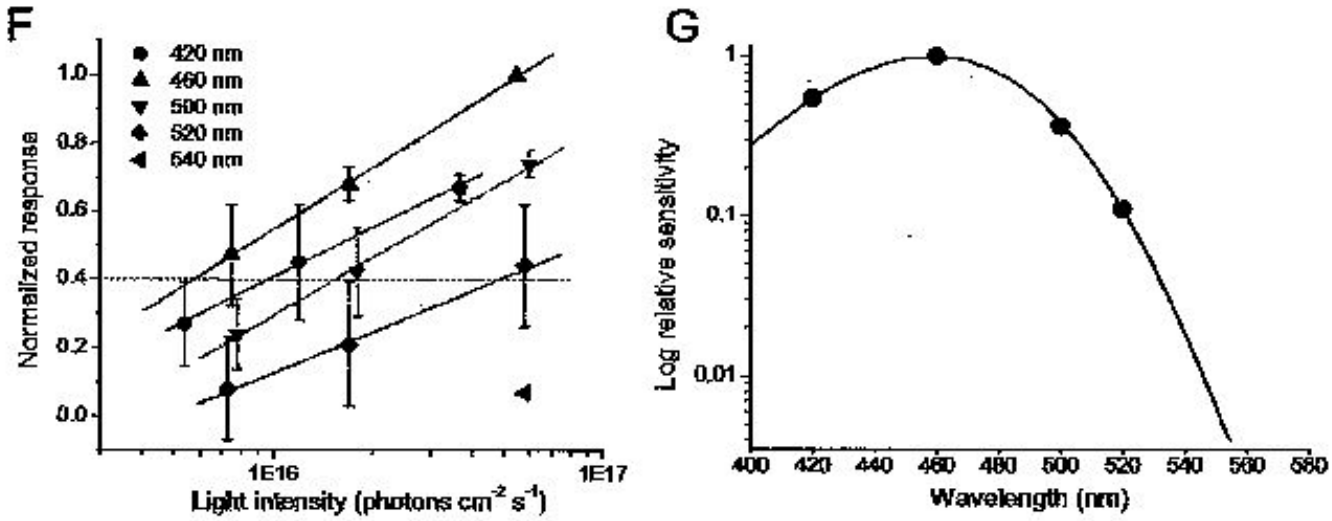


Fig. 6F – 6G

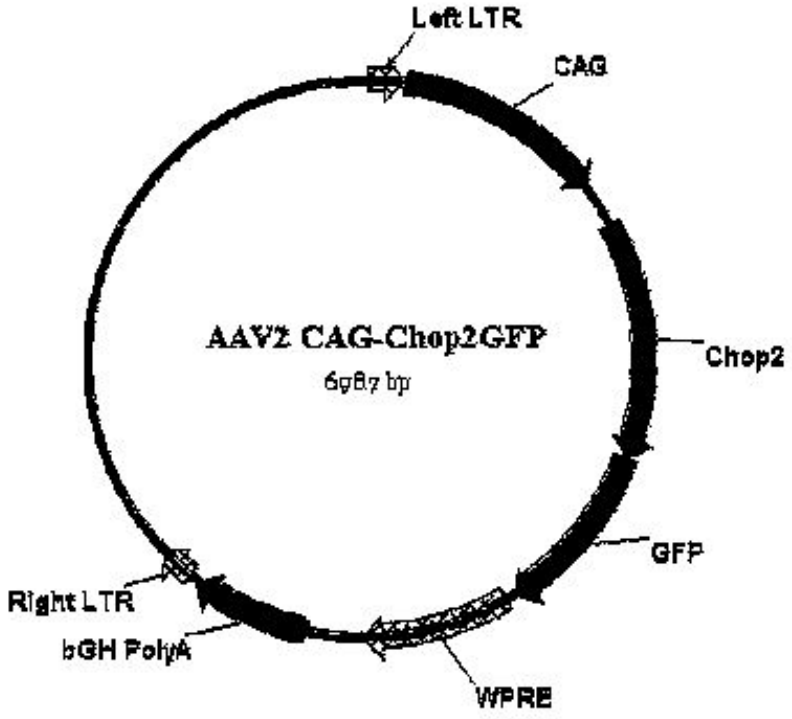


Fig. 7

FIG. 8/1

SEQ ID NO:9

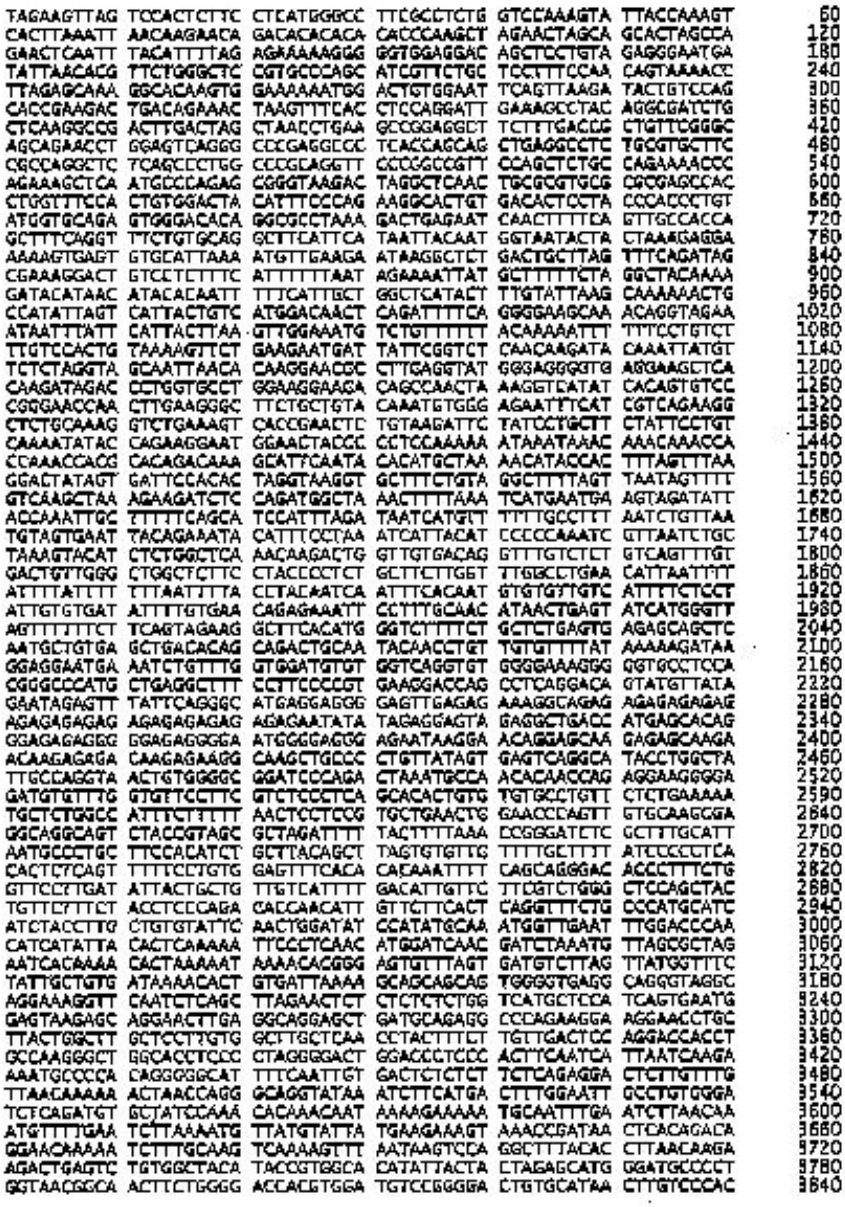


FIG. 8/2

SEQ ID NO:9

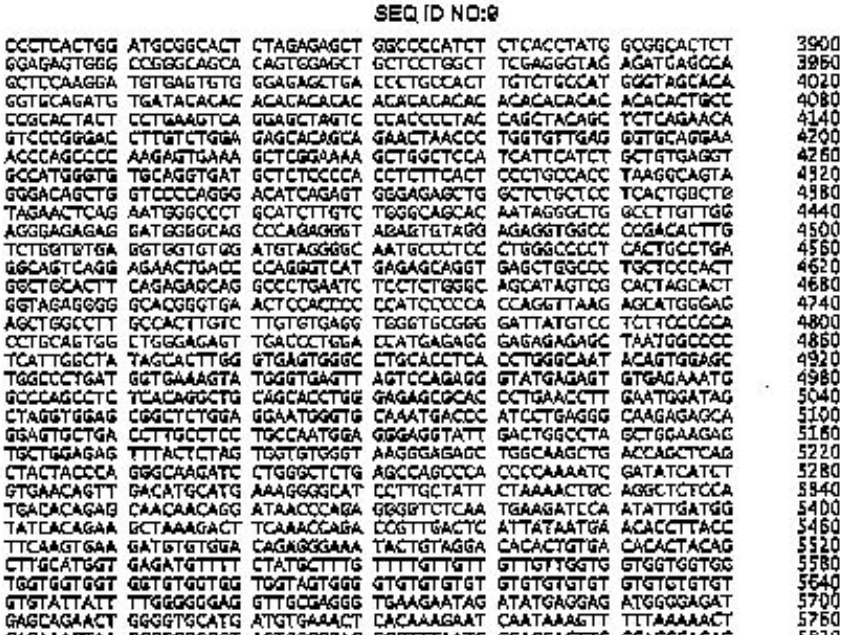


Fig. 8/3

SEQ ID NO:8

FIGS. 1A-1I. Expression of Chop2-GFP in Retinal Neurons In vivo. FIG. 1A shows the rAAV-CAG-Chop2-GFP-WPRE expression cassette. CAG: a hybrid CMV enhancer/chicken ß-actin promoter. WPRE: woodchuck posttranscriptional regulatory element. BGHpA: a bovine growth hormone polyadenylation sequence. (FIGS. 1B and 1C) Chop2-GFP fluorescence viewed in low (FIG. 1B) and high (FIG. 1C) magnifications from eyes two months after the viral vector injection. (FIG. 1D) Confocal images of a ganglion cell, which show a stacked image (left) and a single optical section image (right). (FIG. 1E) Chop2-GFP fluorescence in a horizontal cell, which shows GFP in soma, axon, and distal axon terminal. (FIGS. 1F and 1G) Chop2-GFP fluorescence in amacrine cells (FIG. 1F) and a retinal bipolar cell (FIGS. 1G). FIGS. 1H and 1I show fluorescence image (FIG. 1H) and phase contrast image (FIG. 1I) taken from a retina 12 months after the injection of Chop2-GFP viral vectors. Images in (FIGS. 1B-1E) were taken from flat whole-mounts of rat retinas. Images in (FIGS. 1F-1I) were taken from vertical slice sections of rat retinas. Scale bar: 200 μm in (FIG. 1B); 100 μm in (FIG.

1C); 15 μm in (FIG. 1D); 50 μm in (FIG. 1E), FIG. 1H), and (FIG. 1I); 25 μm in (FIG. 1F) and (FIG. 1G). ONL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL:

FIGS. 2A-2H. Properties of Light-Evoked Currents of the ChR2-expressing retinal neurons. (FIG. 2A) Phase contrast image (left) and fluorescence image (right) of a GFP-positive retinal neuron dissociated from the viral vector injected eye. Scale bar: 25 μm. (FIG. 2B) A recording of Chop2-GFP fluorescent retinal cell to light stimuli of wavelengths ranging from 420 to 580 nm. The light intensities were ranging from 1.0-1.6×10<18 >photons cm <2 >S 1. (FIG. 2C) A representative recording of the currents elicited by light stimuli at the wavelength of 460 nm with light intensities ranging from 2.2×10<15 >to 1.8×10<18 >photons cm<-2>s<-1>. (FIG. 2D) Current traces after the onset of the light stimulation from FIG. 2C shown in the expanded time scale. The line shows the fitting of one current trace by an exponential function: I(t)=a0+a1×(1-exp[-t/t1])+a2×(exp[-t/t2]), in which t1 and t2 represent the activation and inactivation time constant, respectively. (FIG. 2E) Current traces after the termination of the light stimulation from FIG. 2C shown in the expanded time scale. The line shows the fitting of one current trace by a single exponential function: I(t)=a0+a1×(exp[-t/t]), in which t represent the deactivation time constant. (FIG. 2F) Light-intensity response curve. The data points were fitted with a single logistic function curve. (FIGS. 2F and H) The relationships of light-intensity and activation time constant (FIG. 2G) and light-intensity and inactivation time constant (FIG. 2H) obtained from the fitting shown in FIG. 2D. All recordings were made at the holding potential of -70 mV. The data points in FIG. 2F-2H are shown as mean±SD (n=7).

FIGS. 3A-3C. Properties of Light-Evoked Voltage Responses of ChR2-Expressing Retinal Neurons. (FIG. 3A) A representative recordings from GFP-positive nonspiking neurons. The voltage responses were elicited by four incremental light stimuli at the wavelength of 460 nm with intensities ranging from 2.2×10<15 >to 1.8×10<18 >photons cm<-2>s<-1 >in current clamp. The dotted line indicates the saturated potential level. (FIG. 3B) A representative recording from GFP-positive nonspiking neurons to repeat light stimulations. The light-evoked currents (top traces) and voltage responses (bottom traces) from a same cells were shown. Left panel shows the superimposition of the first (red) and second (black) traces in an expanded time scale. The dotted line indicates the sustained component of the currents (top) and plateau membrane potential (bottom). (FIG. 3C) A representative recording of GFP-positive spiking neurons to repeated light stimulations. The responses in FIGS. 3B and 3C were evoked by light at the wavelength of 460 nm with the intensity of 1.8×10<18 >photons cm<-2>s<-1>.

FIGS. 4A-4I. Expression and Light-Response Properties of ChR2 in Retinal Neurons of rd1/rd1 Mice. (FIG. 4A) Chop2-GFP fluorescence viewed in flat retinal whole-mount of a 15 month old mouse with the Chop2-GFP viral vector injection at 9 months of age. (FIG. 4B) Chop2-GFP fluorescence viewed in vertical section from the retina of a 6 month old mouse with the injection of Chop2-GFP viral vectors at 3 months of age. (FIG. 4C) Light microscope image of a semithin vertical retinal section from a 5 month old mouse (Chop2-GFP viral vectors injected at postnatal day 1). Scale bar: 50 μm in (FIG. 4A) and 30 μm in (FIGS. 4B and 4C). (FIGS. 4D-4E) show representative recordings of transient spiking (FIG. 4D) and sustained spiking (FIG. 4E) GFP-positive neurons. The responses were elicited by light of four incremental intensities at the wavelength of 460 nm. The light intensity without neutral density (Log I=0) was 3.6×10<17 >photons cm<-2 >s<-1>. The currents were recorded at the holding potential of -70 mV. The superimposed second (solid black) and fourth (dashed or red) current and voltage traces are shown in the right panel in the expanded time scale. (FIGS. 4F-4I) show the relationships of the amplitude of current (FIG. 4F), membrane depolarization (FIG. 4G), the number of spikes (FIG. 4H), and the time to the first spike peak (FIG. 4I) to light intensity. Recordings were made from rd1/rd1 mice at ?4 months of age. The data points are the mean ±SE (n=6 in FIG. 4F-4H and n=4 in FIG. 4I).

FIG. 5A-5D. Multielectrode Array Recordings of the ChR2-Expressing Retinas of rd1/rd1 Mice. (FIG. 5A) A sample recording of light-evoked spike activities from the retinas of rd1/rd1 mice (ages?4 months). The recording was made in the present of CNQX (25 μM) and AP5 (25 μM). Prominent light-evoked spike activity was observed in 49 out of 58 electrodes (electrode 15 was for grounding and electrode 34 was defective). (FIG. 5B) Sample light-evoked spikes recorded from a single electrode to three incremental light intensities. (FIG. 5C) The raster plots of 30 consecutive light-elicited spikes originated from a single neuron. (FIG. 5D) The averaged spike rate histograms. The light intensity without neutral density filters (Log I=0) was 8.5×10<17 >photons cm<-2>s<-1>. The responses shown in FIG. 5A were elicited by a single light pulse without neutral density filters.

FIG. 6A-6E. Central Projections of Chop2-GFP-Expressing Retinal Ganglion Cells and Visual-Evoked Potentials in rd1/rd1 Mice. (FIG. 6A) GFP labeled terminal arbors of retinal ganglion cells in ventral lateral geniculate nucleus and dorsal lateral geniculate nucleus. (FIG. 6B) GFP-labeled terminal arbors of retinal ganglion cells in superior colliculus. OT: optical track; vLGN: ventral lateral geniculate nucleus; dLGN: dorsal lateral geniculate nucleus; SC: superior colliculus. Scale bar: 200 μm in FIG. 6A), 100 μm in FIG. 6B). (FIG. 6C) VEPs recorded from a wild-type mouse. The responses were observed both to the wavelengths of 460 and 580 nm. (FIG. 6D) VEPs recorded from an rd1/rd1 mouse injected with Chop2-GFP viral vectors. The responses were elicited only by light at the wavelength of 460 nm but not at the wavelength of 580 nm. (FIG. 6E) No detectable VEPs were observed from rd1/rd1 mice injected with viral vectors carrying GFP alone. The light intensities measured at the corneal surface at the wavelengths of 460 and 580 nm were 5.5×10<16 >and 5.2×10<16 >photons cm<-2>s<-1>, respectively. (FIG. 6F) Plot of the amplitude of VEPs from rd1/rd1 mice injected with Chop2-GFP viral vectors to various light intensities at the wavelengths of 420, 460, 500, 520, and 540 nm. For each eye, the responses are normalized to the peak response obtained at 460 nm. The data are the mean±SD (n=3 eyes). Spectral sensitivity at each wavelength was defined as the inverse of the interpolated light intensity to produce 40% of the normalized peak response, as indicated by the dot line. (FIG. 6G) The sensitivity data points were fitted by a vitamin-A1-based visual pigment template with a peak wavelength of 461 nm.

FIG. 7 shows a map of the viral expression construct rAAV2-CAG-Chop2-GFP-WPRE (SEQ ID NO: 1), which comprises a Chop2-GFP fragment, an operatively linked a hybrid CMV enhancer/chicken ß-actin promoter (CAG), a woodchuck posttranscriptional regulatory element (WPRE), and a bovine growth hormone (BGH) polyadenylation sequence.

FIG. 8 (sheets 1-3) presents the sequence (SEQ ID NO:9)—11023 nt's—of the mGluR6 promoter region of the Grm6 gene (GenBank No. BC041684). The genomic sequence is provided in GenBank No. AL627215.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method for treating an ocular disorder in a human, other mammalian or other animal subject. In particular, the ocular disorder is one which involves a mutated or absent gene in a retinal pigment epithelial cell or a photoreceptor cell. The method of this invention comprises the step of administering to the subject by intravitreal or subretinal injection of an effective amount of a recombinant virus carrying a nucleic acid sequence encoding an ocular cell-specific normal gene operably linked to, or under the control of, a promoter sequence which directs the expression of the product of the gene in the ocular cells and replaces the lack of expression or incorrect expression of the mutated or absent gene.

Ocular Disorders

The ocular disorders for which the present methods are intended and may be used to improve

one or more parameters of vision include, but are not limited to, developmental abnormalities that affect both anterior and posterior segments of the eye. Anterior segment disorders include glaucoma, cataracts, corneal dystrophy, keratoconus. Posterior segment disorders include blinding disorders caused by photoreceptor malfunction and/or death caused by retinal dystrophies and degenerations. Retinal disorders include congenital stationary night blindness, age-related macular degeneration, congenital cone dystrophies, and a large group of retinitis-pigmentosa (RP)-related disorders. These disorders include genetically pre-disposed death of photoreceptor cells, rods and cones in the retina, occurring at various ages. Among those are severe retinopathies, such as subtypes of RP itself that progresses with age and causes blindness in childhood and early adulthood and RP-associated diseases, such as genetic subtypes of LCA, which frequently results in loss of vision during childhood, as early as the first year of life. The latter disorders are generally characterized by severe reduction, and often complete loss of photoreceptor cells, rods and cones. (Trabulsi, E I, ed., Genetic Diseases of the Eye, Oxford University Press, NY, 1998).

In particular, this method is useful for the treatment and/or restoration of at least partial vision to subjects that have lost vision due to ocular disorders, such as RPE-associated retinopathies, which are characterized by a long-term preservation of ocular tissue structure despite loss of function and by the association between function loss and the defect or absence of a normal gene in the ocular cells of the subject. A variety of such ocular disorders are known, such as childhood onset blinding diseases, retinitis pigmentosa, macular degeneration, and diabetic retinopathy, as well as ocular blinding diseases known in the art. It is anticipated that these other disorders, as well as blinding disorders of presently unknown causation which later are characterized by the same description as above, may also be successfully treated by this method. Thus, the particular ocular disorder treated by this method may include the above-mentioned disorders and a number of diseases which have yet to be so characterized.

Visual information is processed through the retina through two pathways: an ON pathway which signals the light ON, and an OFF pathway which signals the light OFF (Wassle, supra). It is generally believed that the existence of the ON and OFF pathway is important for the enhancement of contrast sensitivity. The visual signal in the ON pathway is relay from ON-cone bipolar cells to ON ganglion cells. Both ON-cone bipolar cells and ON-ganglion cells are depolarized in response to light. On the other hand, the visual signal in the OFF pathway is carried from OFF-cone bipolar cells to OFF ganglion cells. Both OFF-cone bipolar cells and OFF-ganglion cells are hypopolarized in response to light. Rod bipolar cells, which are responsible for the ability to see in dim light (scotopic vision), are ON bipolar cells (depolarized in response to light). Rod bipolar cells relay the vision signal through AII amacrine cells (an ON type retinal cells) to ON an OFF cone bipolar cells.

The present Examples show functional consequence of expressing ubiquitously expressing light sensitive channels, namely ChR2, in retinal ganglion cells by CAG promoter, and suggest that this sufficient for restoring useful vision. However, targeting of depolarizing membrane channels, such as ChR2, to the ON-type retinal neurons might result in better useful vision. In addition, expression of light sensors in more distal retinal neurons, such as bipolar cells, would utilize the remaining signal processing functions of the degenerate retina. Furthermore, by expressing a depolarizing light sensor, such as ChR2, in ON type retinal neurons (ON type ganglion cells and/or ON type bipolar cells) and expressing a hypopolarizing light sensor, such as halorhodopsin (a chloride pump) (Han, X et al., 2007, PLoS ONE, March 21;2:e299; Zhang, F et al., 2007; Nature 446:633-9; present inventors' results) in OFF type retinal neurons (OFF type ganglion cells and/or OFF type bipolar cells) could create ON and OFF pathways in photoreceptor degenerated retinas.

An alternative approach to restore ON and OFF pathways in the retina is achieved by, expressing a depolarizing light sensor, such as ChR2, to rod bipolar cells or AII amacrine. This

is because the depolarization of rod bipolar cells or AII amacrine cells can lead to the ON and OFF responses at the levels of cone bipolar cells and the downstream retinal ganglion cells and, thus, the ON and OFF pathways that are inherent in the retina could be maintained (Wässle, 2004).

According to the present invention, the followings approaches are used to restore the light sensitivity of inner retinal neurons:

(1) Ubiquitously expressing light sensitive channels, such as ChR2, are employed to produced membrane depolarization in all types of ganglion cells (both ON and OFF ganglion cells), or all types of bipolar cells (rod bipolar cells, and ON and OFF cone bipolar cells). The AAV vector with CAG promoter has already partially achieved this approach in rodent retinas, as exemplified herein.

(2) A depolarizing light sensor, such as ChR2, is targeted to ON type retinal neurons such as ON type ganglion cells or ON type bipolar cells. A study from Dr. J. G. Flannery's group has identified the fragments of a human gap junctional protein (connexin-36) promoter to target GFP in ON-type retinal ganglion cells by using AAV-2 virus vector (Greenberg K P et al., 2007, In vivo Transgene Expression in ON-Type Retinal Ganglion Cells: Applications to Retinal Disease. ARVO abstract, 2007). A readily packable shorter version of mGluR6 promoter of (<2.5 kb) would allow targeting of ChR2 to ON type bipolar cells (both rod bipolar cells and ON type cone bipolar cells).

(3) Cell specific promoters are used to target the specific types of retinal neurons. A promoter that could target rod bipolar cells is Pep2 (L7) promoter (Tomomura, M et al., 2001, Eur J Neurosci. 14:57-63). The length of the active promoter is preferably less that 2.5 Kb so it can be packaged into the AAV viral cassette.

(4) A depolarizing light sensor, such as ChR2, is targeted to ON type ganglion cells or ON type cone bipolar cells and a hypopolarizing light sensor, such as halorhodopsin, to OFF type ganglion cells or OFF type cone bipolar cells to create ON and OFF pathways. As described above, an adequately short (packable) version of mGluR6 promoter (<2.5 kb) would allow targeting of ChR2 to ON type bipolar cells. The Neurokinin-3 (NK-3) promoter would be used to target halorhodopsin to OFF cone bipolar cells (Haverkamp, S et al., 2002, J Comparative Neurology, 455:463-76.

Vectors

According to the various embodiments of the present invention, a variety of known nucleic acid vectors may be used in these methods, e.g., recombinant viruses, such as recombinant adeno-associated virus (rAAV), recombinant adenoviruses, recombinant retroviruses, recombinant poxviruses, and other known viruses in the art, as well as plasmids, cosmids and phages, etc. Many publications well-known in the art discuss the use of a variety of such vectors for delivery of genes. See, e.g., Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, latest edition; Kay, M A. et al., 2001, Nat. Med., 7:33-40; and Walther W et al., 2000, Drugs 60:249-71).

Methods for assembly of the recombinant vectors are well-known. See, for example, WO 00/15822 and other references cited therein, all of which are incorporated by reference.

There are advantages and disadvantages to the various viral vector systems. The limits of how much DNA can be packaged is one determinant in choosing which system to employ. rAAV tend to be limited to about 4.5 kb of DNA, whereas lentivirus (e.g., retrovirus) system can accommodate 4-5 kb.

AAV Vectors

Adeno-associated viruses are small, single-stranded DNA viruses which require a helper virus for efficient replication (Berns, K I, Parvoviridae: the viruses and their replication, p. 1007-1041 (vol. 2), in Fields, B N et al., Fundamental Virology, 3rd Ed., (Lippincott-Raven Publishers, Philadelphia (1995)). The 4.7 kb genome of AAV has two inverted terminal repeats (ITR) and two open reading frames (ORFs) which encode the Rep proteins and Cap proteins, respectively. The Rep reading frame encodes four proteins of molecular weights 78, 68, 52 and 40 kDa. These proteins primarily function in regulating AAV replication and rescue and integration of the AAV into the host cell chromosomes. The Cap reading frame encodes three structural proteins of molecular weights 85 (VP 1), 72 (VP2) and 61 (VP3) kDa which form the virion capsid (Berns, supra). VP3 comprises >80% of total AAV virion proteins.

Flanking the rep and cap ORFs at the 5' and 3' ends are 145 bp ITRs, the first 125 bp's of which can form Y- or T-shaped duplex structures. The two ITRs are the only cis elements essential for AAV replication, rescue, packaging and integration of the genome. Two conformations of AAV ITRs called “flip” and “flop” exist (Snyder, R O et al., 1993, J Virol., 67:6096-6104; Berns, K I, 1990 Microbiol Rev, 54:316-29). The entire rep and cap domains can be excised and replaced with a transgene such as a reporter or therapeutic transgene (Carter, B J, in Handbook of Parvoviruses, P. Tijsser, ed., CRC Press, pp. 155-168 (1990)).

AAVs have been found in many animal species, including primates, canine, fowl and human (Murphy, F A et al., The Classification and Nomenclature of Viruses: Sixth Rept of the Int'l Comme on Taxonomy of Viruses, Arch Virol, Springer-Verlag, 1995). Six primate serotypes are known (AAV1, AAV2, AAV3, AAV4, AAV5 and AAV6).

The AAV ITR sequences and other AAV sequences employed in generating the minigenes, vectors, and capsids, and other constructs used in the present invention may be obtained from a variety of sources. For example, the sequences may be provided by any of the above 6 AAV serotypes or other AAV serotypes or other densoviruses, including both presently known human AAV and yet to yet-to-be-identified serotypes. Similarly, AAVs known to infect other animal species may be the source of ITRs used in the present molecules and constructs. Capsids from a variety of serotypes of AAV may be combined in various mixtures with the other vector components (e.g., WO01/83692 (Nov. 8, 2001) incorporated by reference). Many of these viral strains or serotypes are available from the American Type Culture Collection (ATCC), Manassas, Va., or are available from a variety of other sources (academic or commercial).

It may be desirable to synthesize sequences used in preparing the vectors and viruses of the invention using known techniques, based on published AAV sequences, e.g. available from a variety of databases. The source of the sequences utilized to prepare the present constructs is not considered to be limiting. Similarly, the selection of the AAV serotype and species (of origin) is within the skill of the art and is not considered limiting

The Minigene

As used herein, the AAV sequences are typically in the form of a rAAV construct (e.g., a minigene or cassette) which is packaged into a rAAV virion. At minimum, the rAAV minigene is formed by AAV ITRs and a heterologous nucleic acid molecule for delivery to a host cell. Most suitably, the minigene comprises ITRs located 5' and 3' to the heterologous sequence. However, minigene comprising 5' ITR and 3' ITR sequences arranged in tandem, e.g. 5' to 3' or a head-to-tail, or in another configuration may also be desirable. Other embodiments include a minigene with multiple copies of the ITRs, or one in which 5' ITRs (or conversely, 3' ITRs) are

located both 5' and 3' to the heterologous sequence. The ITRs sequences may be located immediately upstream and/or downstream of the heterologous sequence; intervening sequences may be present. The ITRs may be from AAV5, or from any other AAV serotype. A minigene may include 5' ITRs from one serotype and 3' ITRs from another.

The AAV sequences used are preferably the 145 bp cis-acting 5' and 3' ITR sequences (e.g., Carter, B J, supra). Preferably, the entire ITR sequence is used, although minor modifications are permissible. Methods for modifying these ITR sequences are well-known (e.g., Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 3<rd >Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 2001; Brent, R et al., eds., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 2003; Ausubel, F M et al., eds., Short Protocols in Molecular Biology, 5thedition, Current Protocols, 2002; Carter et al., supra; and Fisher, K et al., 1996 J Virol. 70:520-32). It is conventional to engineer the rAAV virus using known methods (e.g., Bennett, J et al. 1999, supra). An example of such a molecule employed in the present invention is a “cis-acting” plasmid containing the heterologous sequence, preferably the Chop2 sequence, flanked by the 5' and 3' AAV ITR sequences.

The heterologous sequence encodes a protein or polypeptide which is desired to be delivered to and expressed in a cell. The present invention is directed to Chop2 sequences under the control of a selected promoter and other conventional vector regulatory components.

The Transgene Being Targeted and Expressed

In a most preferred embodiment, the heterologous sequence is a nucleic acid molecule that functions as a transgene. The term “transgene” as used herein refers to a nucleic acid sequence heterologous to the AAV sequence, and encoding a desired product, preferably Chop2 and the regulatory sequences which direct or modulate transcription and/or translation of this nucleic acid in a host cell, enabling expression in such cells of the encoded product. Preferred polypeptide products are those that can be delivered to the eye, particularly to retinal neurons.

The transgene is delivered and expressed in order to treat or otherwise improve the vision status of a subject with an ocular disorder that may result from any of a number of causes, including mutations in a normal photoreceptor-specific gene. The targeted ocular cells may be photoreceptor cells (if not totally degenerated) or, more preferably, other retinal neurons, namely, bipolar cells and retinal ganglion cells.

Using an mGluR6 promoter operatively linked to a Chop2 opsin coding sequence and a reporter gene, e.g. GFP or another fluorescent protein, an insert of about 4.5 kb is preferred—1 kb for the opsin, 0.7 kb for the reporter, 10 kb—for the mGluR6 promoter region and about 0.4 kb for conventional transcriptional regulatory factors.

Use of different opsin genes allows selection of desired wavelengths as the absorption maxima of different channel proteins differ. In one embodiment, the reported gene is moved to the red part of the visual spectrum.

Similarly, based on the studies reported herein, the brightness of the light needed to stimulate evoked potential in transduced mouse retinas, indicates that a channel opsin with increased light sensitivity may be more desirable. This can be achieved by selection of a suitable naturally occurring opsin, for example other microbial-type rhodopsins, or by modifying the light sensitivity of Chop2 as well as its other properties, such as ion selectivity and spectral sensitivity, to produce diversified light-sensitive channels to better fit the need for vision restoration.

Different transgenes may be used to encode separate subunits of a protein being delivered, or

to encode different polypeptides the co-expression of which is desired. If a single transgene includes DNA encoding each of several subunits, the DNA encoding each subunit may be separated by an internal ribozyme entry site (IRES), which is preferred for short subunit-encoding DNA sequences (e.g., total DNA, including IRES is <5 kB). Other methods which do not employ an IRES may be used for co-expression, e.g. the use of a second internal promoter, an alternative splice signal, a co- or post-translational proteolytic cleavage strategy, etc., all of which are known in the art.

The coding sequence or non-coding sequence of the nucleic acids useful herein preferably are codon-optimized for the species in which they are to be expressed. Such codon-optimization is routine in the art.

While a preferred transgene encodes a full length polypeptide, preferably Chop2 (SEQ ID NO:6, the present invention is also directed to vectors that encode a biologically active fragment or a conservative amino acid substitution variant of Chop2 (or of any aother polypeptide of the invention to be expressed in retinal neurons). Non-limiting examples of useful fragments are the polypeptide with the sequence SEQ ID NO:3 and SEQ ID NO:8. The fragment or variant is expressed by the targets cells being transformed and is able to endow such cells with light sensitivity that is functionally equivalent to that of the full length or substantially full length polypeptide having a native, rather than variant, amino acid sequence. A biologically active fragment or variant is a "functional equivalent"—a term that is well understood in the art and is further defined in detail herein. The requisite biological activity of the fragment or variant, using any method disclosed herein or known in the art to establish activity of a channel opsin, has the following activity relative to the wild-type native polypeptide: about 50%, about 55%, about 60 %, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, and any range derivable therein, such as, for example, from about 70% to about 80%, and more preferably from about 81% to about 90%; or even more preferably, from about 91% to about 99%.

It should be appreciated that any variations in the coding sequences of the present nucleic acids and vectors that, as a result of the degeneracy of the genetic code, express a polypeptide of the same sequence, are included within the scope of this invention.

The amino acid sequence identity of the variants of the present invention are determined using standard methods, typically based on certain mathematical algorithms. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of Meyers and Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The nucleotide and amino acid sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (Altschul et al. (1990) J. Mol. Biol. 215:403-10). BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to, e.g. DAN encoding Chop2 of C. reinhardtii. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to

obtain amino acid sequences homologous to the appropriate reference protein such as Chop2. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized (Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See World Wide Web URL ncbi.nlm.nih.gov.

The preferred amino acid sequence variant has the following degrees of sequence identity with the native, full length channel opsin polypeptide, preferably Chop2 from C. reinhardtii (SEQ ID NO:6) or with a fragment thereof (e.g., SEQ ID NO:3 or 8): about 50%, about 55%, abou 60 %, about 65%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%, and any range derivable therein, such as, for example, from about 70% to about 80%, and more preferably from about 81% to about 90%; or even more preferably, from about 91% to about 99% identity. A preferred biologically active fragment comprises or consists of SEQ ID NO:3, which corresponds to residues 1-315 of SEQ ID NO:6, or comprises or consists of SEQ ID NO:8.

Any of a number of known recombinant methods are used to produce a DNA molecule encoding the fragment or variant. For production of a variant, it is routine to introduce mutations into the coding sequence to generate desired amino acid sequence variants of the invention. Site-directed mutagenesis is a well-known technique for which protocols and reagents are commercially available (e.g., Zoller, M J et al., 1982, Nucl Acids Res 10:6487-6500; Adelman, J P et al., 1983, DNA 2:183-93). These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases.

In terms of functional equivalents, it is well understood by those skilled in the art that, inherent in the definition of a “biologically functional equivalent” protein, polypeptide, gene or nucleic acid, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted.

In particular, the shorter the length of the polypeptide, the fewer amino acids changes should be made. Longer fragments may have an intermediate number of changes. The full length polypeptide protein will have the most tolerance for a larger number of changes. It is also well understood that where certain residues are shown to be particularly important to the biological or structural properties of a polypeptide residues in a binding regions or an active site, such residues may not generally be exchanged. In this manner, functional equivalents are defined herein as those poly peptides which maintain a substantial amount of their native biological activity.

For a detailed description of protein chemistry and structure, see Schulz, G E et al., Principles of Protein Structure, Springer-Verlag, New York, 1978, and Creighton, T. E., Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions that may be made in the protein molecule may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz et al. (supra) and FIG. 3-9 of Creighton (supra). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1	Small aliphatic, nonpolar or slightly polar residues	Ala, Ser, Thr (Pro, Gly);
2	Polar, negatively charged residues and	Asp, Asn, Glu, Gln; their amides
3	Polar, positively charged residues	His, Arg, Lys;
4	Large aliphatic, nonpolar residues	Met, Leu, Ile, Val (Cys)
5	Large aromatic residues	Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking a side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation, which is important in protein folding.

The hydropathy index of amino acids may also be considered in selecting variants. Each amino acid has been assigned a hydropathy index on the basis of their hydrophobicity and charge characteristics, these are: Ile (+4.5); Val (+4.2); Leu (+3.8); Phe (+2.8); Cys (+2.5); Met (+1.9); Ala (+1.8); Glycine (-0.4); Thr (-0.7); Ser (-0.8); Trp (-0.9); Tyr (-1.3); Pro (-1.6); His (-3.2); Glu (-3.5); Gln (-3.5); Asp (-3.5); Asn (-3.5); Lys (-3.9); and Arg (-4.5). The importance of the hydropathy index in conferring interactive biological function on a proteinaceous molecule is generally understood in the art (Kyte and Doolittle, 1982, J. Mol. Biol. 157:105-32). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathy index or score and still retain a similar biological activity. In making changes based upon the hydropathy index, the substitution of amino acids whose hydropathy indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biological functional equivalent polypeptide thereby created is intended for use in certain of the present embodiments. U.S. Pat. No. 4,554,101, discloses that the greatest local average hydrophilicity of a proteinaceous molecule, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the molecule. See U.S. Pat. No. 4,554,101 for a hydrophilicity values. In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Regulatory Sequences

The minigene or transgene of the present invention includes appropriate sequences operably linked to the coding sequence or ORF to promote its expression in a targeted host cell. “Operably linked” sequences include both expression control sequences such as. promoters that are contiguous with the coding sequences and expression control sequences that act in trans or distally to control the expression of the polypeptide product.

Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (e.g., Kozak consensus sequence); sequences that enhance nucleic acid or protein stability; and when desired, sequences that enhance protein processing and/or secretion. Many varied expression control sequences, including native and non-native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized herein. depending upon the type of expression desired.

Expression control sequences for eukaryotic cells typically include a promoter, an enhancer, such as one derived from an immunoglobulin gene, SV40, CMV, etc., and a polyadenylation sequence which may include splice donor and acceptor sites. The polyadenylation sequence

generally is inserted 3' to the coding sequence and 5' to the 3' ITR sequence. PolyA from bovine growth hormone is a suitable sequence.

The regulatory sequences useful herein may also contain an intron, such as one located between the promoter/enhancer sequence and the coding sequence. One useful intron sequence is derived from SV40, and is referred to as the SV40 T intron sequence. Another includes the woodchuck hepatitis virus post-transcriptional element. (See, for example, Wang L and Verma, I, 1999, Proc Nat'l Acad Sci USA, 96:3906-10).

An IRES sequence, or other suitable system as discussed above, may be used to produce more than one polypeptide from a single transcript. n exemplary IRES is the poliovirus IRES which supports transgene expression in photoreceptors, RPE and ganglion cells. Preferably, the IRES is located 3' to the coding sequence in the rAAV vector.

The promoter may be selected from a number of constitutive or inducible promoters that can drive expression of the selected transgene in an ocular setting, preferably in retinal neurons. A preferred promoter is “cell-specific”, meaning that it is selected to direct expression of the selected transgene in a particular ocular cell type, such as photoreceptor cells.

Examples of useful constitutive promoters include the exemplified??? CMV immediate early enhancer/chicken ß-actin (CßA) promoter-exon 1-intron 1 element, the RSV LTR promoter/enhancer, the SV40 promoter, the CMV promoter, the dihydrofolate reductase (DHFR) promoter, and the phosphoglycerol kinase (PGK) promoter.

Additional useful promoters are disclosed in W. W. Hauswirth et al., 1998, W098/48027 and A. M. Timmers et al., 2000, WO00/15822. Promoters that were found to drive RPE cell-specific gene expression in vivo include (1) a 528-bp promoter region (bases 1-528 of a murine 11-cis retinol dehydrogenase (RDH) gene (Driessen, C A et al., 1995, Invest. Ophthalmo!. Vis. Sci. 36:1988-96; Simon, A. et al., 1995, J. Biol. Chem 270:1107-12, 1995; Simon, A. et al., 1996, Genomics 36:424-3) Genbank Accession Number X97752); (2) a 2274-bp promoter region) from a human cellular retinaldehyde-binding protein (CRALBP) gene (Intres, R et al., 1994, J. Bio!. Chem. 269:25411-18; Kennedy, B N et al., 1998, J. Bio!. Chem. 273:5591-8, 1998), Genbank Accession Number L34219); and (3) a 1485-bp promoter region from human RPE65 (Nicoletti, A et al., 1998, Invest. Ophthalmol. Vis. Sci. 39:637-44, Genbank Accession Number U20510). These three promoters (labeled with the following SEQ ID numbers in WO00/15822? 2.3 amd 3) promoted RPE-cell specific expression of GFP. It is envisioned that minor sequence variations in the various promoters and promoter regions discussed herein—whether additions, deletions or mutations, whether naturally occurring or introduced in vitro, will not affect their ability to drive expression in the cellular targets of the present invention. Furthermore, the use of other promoters, even if not yet discovered, that are characterized by abundant and/or specific expression in retinal cells, particularly in bipolar or ganglion cells, is specifically included within the scope of this invention.

An inducible promoter is used to control the amount and timing of production of the transgene product in an ocular cell. Such promoters can be useful if the gene product has some undesired, e.g. toxic, effects in the cell if it accumulates excessively. Inducible promoters include those known in the art, such as the Zn-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (M MTV) promoter; the T7 promoter; the ecdysone insect promoter; the tetracycline-repressible system; the tetracycline-inducible system; the RU486-inducible system; and the rapamycin-inducible system. Any inducible promoter the action of which is tightly regulated and is specific for the particular target ocular cell type, may be used. Other useful types of inducible promoters are ones regulated by a specific physiological state, e.g. temperature, acute phase, a cell's replicating or differentiation state.

Selection of the various vector and regulatory elements for use herein are conventional, well-described, and readily available. See, e.g. Sambrook et al., supra; and Ausubel et al., supra. It will be readily appreciated that not all vectors and expression control sequences will function equally well to express the present transgene, preferably Chop2. Clearly, the skilled artisan may apply routine selection among the known expression control sequences without departing from the scope of this invention and based upon general knowledge as well as the guidance provided herein. One skilled in the art can select one or more expression control sequences, operably link them to the coding sequence being expressed to make a minigene, insert the minigene or vector into an AAV vector, and cause packaging of the vector into infectious particles or virions following one of the known packaging methods for rAAV.

Production of the rAAV

The rAAV used in the present invention may be constructed and produced using the materials and methods described herein and those well-known in the art. The methods that are preferred for producing any construct of this invention are conventional and include genetic engineering, recombinant engineering, and synthetic techniques, such as those set forth in reference cited above.

Briefly, to package an rAAV construct into an rAAV virion, a sequences necessary to express AAV rep and AAV cap or functional fragments thereof as well as helper genes essential for AAV production must be present in the host cells. See, for example U.S. Patent Pub. 2007/0015238, which describes production of pseudotyped rAAV virion vectors encoding AAV Rep and Cap proteins of different serotypes and AdV transcription products that provide helper functions For example, AAV rep and cap sequences may be introduced into the host cell in any known manner including, without limitation, transfection, electroporation, liposome delivery, membrane fusion, biolistic deliver of DNA-coated pellets, viral infection and protoplast fusion. Devices specifically adapted for delivering DNA to specific regions within and around the eye for the purpose of gene therapy have been described recently (for example, U.S. Patent Pub. 2005/0277868, incorporated by reference) are used within the scope of this invention. Such devices utilize electroporation and electromigration, providing, e.g. two electrodes on a flexible support that can be placed behind the retina. A third electrode is part of a hollow support, which can also be used to inject the molecule to the desired area. The electrodes can be positioned around the eye, including behind the retina or within the vitreous.

These sequences may exist stably in the cell as an episome or be stably integrated into the cell's genome. They may also be expressed more transiently in the host cell. As an example, a useful nucleic acid molecule comprises, from 5' to 3', a promoter, an optional spacer between the promoter and the start site of the rep sequence, an AAV rep sequence, and an AAV cap sequence.

The rep and cap sequences, along with their expression control sequences, are preferably provided in a single vector, though they may be provided separately in individual vectors. The promoter may be any suitable constitutive, inducible or native promoter. The delivery molecule that provides the Rep and Cap proteins may be in any form., preferably a plasmid which may contain other non-viral sequences, such as those to be employed as markers. This molecule typically excludes the AAV ITRs and packaging sequences. To avoid the occurrence of homologous recombination, other viral sequences, particularly adenoviral sequences, are avoided. This plasmid is preferably one that is stably expressed.

Conventional genetic engineering or recombinant DNA techniques described in the cited references are used. The rAAV may be produced using a triple transfection method with either the calcium phosphate (Clontech) or Effectene™ reagent (Qiagen) according to manufacturer's

instructions. See, also, Herzog et al., 1999, Nat. Med. 5:56-63.

The rAAV virions are produced by culturing host cells comprising a rAAV as described herein which includes a rAAV construct to be packaged into a rAAV virion, an AAV rep sequence and an AAV cap sequence, all under control of regulatory sequences directing expression.

Suitable viral helper genes, such as adenovirus E2A, E4Orf6 and VA, may be added to the culture preferably on separate plasmids. Thereafter, the rAAV virion which directs expression of the transgene is isolated in the absence of contaminating helper virus or wildtype AAV.

It is conventional to assess whether a particular expression control sequence is suitable for a given transgene, and choose the one most appropriate for expressing the transgene. For example, a target cell may be infected in vitro, and the number of copies of the transgene in the cell monitored by Southern blots or quantitative PCR. The level of RNA expression may be monitored by Northern blots quantitative RT-PCR. The level of protein expression may be monitored by Western blot, immunohistochemistry, immunoassay including enzyme immunoassay (EIA) such as enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA) or by other methods. Specific embodiments are described in the Examples below.

Pharmaceutical Compositions and Methods of the Invention

The rAAV that comprises the Chop2 transgene and cell-specific promoter for use in the target ocular cell as described above should be assessed for contamination using conventional methods and formulated into a sterile or aseptic pharmaceutical composition for administration by, for example, subretinal injection.

Such formulations comprise a pharmaceutically and/or physiologically acceptable vehicle, diluent, carrier or excipient, such as buffered saline or other buffers, e.g. HEPES, to maintain physiologic pH. For a discussion of such components and their formulation, see, generally, Gennaro, A E., Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins Publishers; 2003 or latest edition). See also, WO00/15822. If the preparation is to be stored for long periods, it may be frozen, for example, in the presence of glycerol.

The pharmaceutical composition described above is administered to a subject having a visual or blinding disease by any appropriate route, preferably by intravitreal or subretinal injection, depending on the retinal layer being targeted.

Disclosures from Bennett and colleagues (cited herein) concern targeting of retinal pigment epithelium—the most distal layer from the vitreal space. According to the present invention, the DNA construct is targeted to either retinal ganglion cells or bipolar cells. The ganglion cells are reasonably well-accessible to intravitreal injection as disclosed herein. Intravitreal and/or subretinal injection can provide the necessary access to the bipolar cells, especially in circumstances in which the photoreceptor cell layer is absent due to degeneration—which is the case in certain forms of degeneration that the present invention is intended to overcome.

To test for the vector's ability to express the transgene, specifically in mammalian retinal neurons, by AAV-mediated delivery, a combination of a preferred promoter sequence linked to a reporter gene such as LacZ or GFP linked to a SV40 poly A sequence can be inserted into a plasmid and packaged into rAAV virus particles, concentrated, tested for contaminating adenovirus and titered for rAAV using an infectious center assay. The right eyes of a number of test subjects, preferably inbred mice, are injected sub-retinally with about 1 μl of the rAAV preparation (e.g., greater than about 10<10 >infectious units ml). Two weeks later, the right (test) and left (control) eyes of half the animals are removed, fixed and stained with an

appropriate substrate or antibody or other substance to reveal the presence of the reporter gene. A majority of the test retinas in injected eyes will exhibited a focal stained region, e.g. blue for LacZ/Xgal, or green for GFP consistent with a subretinal bleb of the injected virus creating a localized retinal detachment. All control eyes are negative for the reporter gene product. Reporter gene expression examined in mice sacrificed at later periods is detected for at least 10 weeks post-injection, which suggests persistent expression of the reporter transgene.

An effective amount of rAAV virions carrying a nucleic acid sequence encoding the Chop2 DNA under the control of the promoter of choice, preferably a constitutive CMV promoter or a cell-specific promoter such as mGluR6, is preferably in the range of between about 10<10 >to about 10<13 >rAAV infectious units in a volume of between about 150 and about 800 μl per injection. The rAAV infectious units can be measured according to McLaughlin, S K et al., 1988, J Virol 62:1963. More preferably, the effective amount is between about 10<10 >and about 10<12 >rAAV infectious units and the injection volume is preferably between about 250 and about 500 μl. Other dosages and volumes, preferably within these ranges but possibly outside them, may be selected by the treating professional, taking into account the physical state of the subject (preferably a human), who is being treated, including, age, weight, general health, and the nature and severity of the particular ocular disorder.

It may also be desirable to administer additional doses ("boosters") of the present nucleic acid or rAAV compositions. For example, depending upon the duration of the transgene expression within the ocular target cell, a second treatment may be administered after 6 months or yearly, and may be similarly repeated. Neutralizing antibodies to AAV are not expected to be generated in view of the routes and doses used, thereby permitting repeat treatment rounds.

The need for such additional doses can be monitored by the treating professional using, for example, well-known electrophysiological and other retinal and visual function tests and visual behavior tests. The treating professional will be able to select the appropriate tests applying routine skill in the art. It may be desirable to inject larger volumes of the composition in either single or multiple doses to further improve the relevant outcome parameters.

Restoration or Improvement of Light Sensitivity and Vision

Both in vitro and in vivo studies to assess the various parameters of the present invention may be used, including recognized animal models of blinding human ocular disorders. Large animal models of human retinopathy, e.g. childhood blindness, are useful. The examples provided herein allow one of skill in the art to readily anticipate that this method may be similarly used in treating a range of retinal diseases.

While earlier studies by others have demonstrated that retinal degeneration can be retarded by gene therapy techniques, the present invention demonstrates a definite physiological recovery of function, which is expected to generate or improve various parameters of vision, including behavioral parameters.

Behavioral measures can be obtained using known animal models and tests, for example performance in a water maze, wherein a subject in whom vision has been preserved or restored to varying extents will swim toward light (Hayes, J M et al., 1993, Behav Genet 23:395-403).

In models in which blindness is induced during adult life or congenital blindness develops slowly enough that the individual experiences vision before losing it, training of the subject in various tests may be done. In this way, when these tests are re-administered after visual loss to test the efficacy of the present compositions and methods for their vision-restorative effects, animals do not have to learn the tasks de novo while in a blind state. Other behavioral tests do not require learning and rely on the instinctiveness of certain behaviors. An example is the

optokinetic nystagmus test (Balkema G W et al., 1984, Invest Ophthalmol Vis Sci. 25:795-800; Mitchiner J C et al., 1976, Vision Res. 16:1169-71).

As is exemplified herein, the transfection of retinal neurons with DNA encoding Chop2 provides residual retinal neurons, principally bipolar cells and ganglion cells, with photosensitive membrane channels. Thus, it was possible to measure, with a strong light stimulus, the transmission of a visual stimulus to the animal's visual cortex, the area of the brain responsible for processing visual signals; this therefore constitutes a form of vision, as intended herein. Such vision may differ from forms of normal human vision and may be referred to as a sensation of light, also termed “light detection” or “light perception.”

Thus, the term “vision” as used herein is defined as the ability of an organism to usefully detect light as a stimulus for differentiation or action. Vision is intended to encompass the following:
1. Light detection or perception—the ability to discern whether or not light is present
2. Light projection—the ability to discern the direction from which a light stimulus is coming;
3. Resolution—the ability to detect differing brightness levels (i.e., contrast) in a grating or letter target; and
4. Recognition—the ability to recognize the shape of a visual target by reference to the differing contrast levels within the target.

Thus, “vision” includes the ability to simply detect the presence of light. This opens the possibility to train an affected subject who has been treated according to this invention to detect light, enabling the individual to respond remotely to his environment however crude that interaction might be. In one example, a signal array is produced to which a low vision person can respond to that would enhance the person's ability to communicate by electronic means remotely or to perform everyday tasks. In addition such a person's mobility would be dramatically enhanced if trained to use such a renewed sense of light resulting from “light detection.” The complete absence of light perception leaves a person with no means (aside from hearing and smell) to discern anything about objects remote to himself.

The methods of the present invention that result in light perception, even without full normal vision, also improve or permit normally regulated circadian rhythms which control many physiological processes including sleep-wake cycles and associated hormones. Although some blind individuals with residual retinal ganglion cells (RGCs) can mediate their rhythms using RGC melanopsin, it is rare for them to do so. Thus, most blind persons have free-running circadian rhythms. Even when such individuals do utilize the melanopsin pathway, the effect is very weak effect. The methods of the present invention are thus expected to improve health status of blind individuals by enabling absent light entrainment or improving weakened (melanopsin-mediated) light entrainment of their circadian rhythms. This leads to better health and well-being of these subjects.

In addition to circadian rhythms, the present invention provides a basis to improve deficits in other light-induced physiological phenomena. Photoreceptor degeneration may result in varying degrees of negative masking, or suppression, of locomotor activity during the intervals in the circadian cycle in which the individual should be sleeping. Another result is suppression of pineal melatonin. Both of these contribute to the entrainment process. Thus, improvement in these responses or activities in a subject in whom photoreceptors are degenerating or have degenerated contributes, independently of vision per se, to appropriate sleep/wake cycles that correspond with the subject's environment in the real world.

Yet another benefit of the present invention is normalization of pupillary light reflexes because regulation of pupil size helps modulate the effectiveness of light stimuli in a natural feed back loop. Thus, the present invention promotes re-establishment of this natural feedback loop,

making vision more effective in subject treated as described herein.

In certain embodiments, the present methods include the measurement of vision before, and preferably after, administering a vector comprising, for example, DNA encoding Chop2. Vision is measured using any of a number of methods well-known in the art or ones not yet establshed. Most preferred herein are the following visual responses:
(1) A light detection response by the subject after exposure to a light stimulus—in which evdence is sought for a reliable response of an indication or movement in the general direction of the light by the subject individual when the light it is turned on is .
(2) a light projection response by the subject after exposure to a light stimulus in which evidence is sought for a reliable response of indication or movement in the specific direction of the light by the individual when the light is turned on.
(3) light resolution by the subject of a light vs. dark patterned visual stimulus, which measures the subject's capability of resolving light vs dark patterned visual stimuli as evidenced by:
(a) the presence of demonstrable reliable optokinetically produced nystagmoid eye movements and/or related head or body movements that demonstrate tracking of the target (see above) and/or
(b). the presence of a reliable ability to discriminate a pattern visual stimulus and to indicate such discrimination by verbal or non-verbal means, including, for example pointing, or pressing a bar or a button; or
(4) electrical recording of a visual cortex response to a light flash stimulus or a pattern visual stimulus, which is an endpoint of electrical transmission from a restored retina to the visual cortex. Measurement may be by electrical recording on the scalp surface at the region of the visual cortex, on the cortical surface, and/or recording within cells of the visual cortex.

It is known in the art that it is often difficult to make children who have only light perception appreciate that they have this vision. Training is required to get such children to react to their visual sensations. Such a situation is mimicked in the animal studies exemplified below. Promoting or enhancing light perception, which the compositions and methods of the present invention will accomplish, is valuable because patients with light perception not only are trainable to see light, but they can usually be trained to detect the visual direction of the light, thus enabling them to be trained in mobility in their environment. In addition, even basic light perception can be used by visually impaired individuals, including those whose vision is improved using the present compositions and methods, along with specially engineered electronic and mechanical devices to enable these individuals to accomplish specific daily tasks. Beyond this and depending on their condition, they may even be able to be trained in resolution tasks such as character recognition and even reading if their impairment permits. Thus it is expected that the present invention enhances the vision of impaired subjects to such a level that by applying additional training methods, these individuals will achieve the above objectives.

Low sensitivity vision may emulate the condition of a person with a night blinding disorder, an example of which is Retinitis Pigmentosa (RP), who has difficulty adapting to light levels in his environment and who might use light amplification devices such as supplemental lighting and/or night vision devices.

Thus, the visual recovery that has been described in the animal studies described below would, in human terms, place the person on the low end of vision function. Nevertheless, placement at such a level would be a significant benefit because these individuals could be trained in mobility and potentially in low order resolution tasks which would provide them with a greatly improved level of visual independence compared to total blindness.

The mice studied in the present Examples were rendered completely devoid of photoreceptors; this is quite rare, even in the worst human diseases. The most similar human state is RP. In

most cases of RP, central vision is retained till the very end. In contrast, in the studied mouse model, the mouse becomes completely blind shortly after birth.

Common disorders encountered in low vision are described by J. Tasca and E. A. Deglin in Chap. 6 of Essentials of Low Vision Practice, R. L. Brilliant, ed., Butterworth Heinemann Publ., 1999, which is incorporated by reference in its entirety. There is reference to similar degenerative conditions, but these references show form vision that is measurable as visual acuity. Ganglion cell layers are not retained in all forms of RP, so the present approach will not work for such a disorder.

When applying the present methods to humans with severe cases of RP, it is expected that central vision would be maintained for a time at some low level while the peripheral retina degenerated first. It is this degenerating retina that is the target for re-activation using the present invention. In essence, these individuals would be able to retain mobility vision as they approached blindness gradually.

Subjects with macular degeneration, characterized by photoreceptor loss within the central "sweet spot" of vision (Macula Lutea), are expected to benefit by treatment in accordance with the present invention, in which case the resolution capability of the recovered vision would be expected to be higher due to the much higher neuronal density within the human macula.

While it is expected that bright illumination of daylight and artificial lighting that may be used by a visually impaired individual will suffice for many visual activities that are performed with vision that has recovered as a result of the present treatments. It is also possible that light amplification devices may be used, as needed, to further enhance the affected person's visual sensitivity. The human vision system can operate over a 10 log unit range of luminance. On the other hand, microbial type rhodopsins, such as ChR2, operate over up to a 3 log unit range of luminance. In addition, the light conditions the patient encounters could fall outside of the operating range of the light sensor. To compensate for the various light conditions, a light pre-amplification or attenuation device could be used to expand the operation range of the light conditions. Such device would contain a camera, imaging processing system, and microdisplays, which can ne assembled from currently available technologies, such as night vision goggles and/or 3D adventure and entertainment system. (See, for example the following URL on the Worldwide web—emagin.com/.)

The present invention may be used in combination with other forms of vision therapy known in the art. Chief among these is the use of visual prostheses, which include retinal implants, cortical implants, lateral geniculate nucleus implants, or optic nerve implants. Thus, in addition to genetic modification of surviving retinal neurons using the present methods, the subject being treated may be provided with a visual prosthesis before, at the same time as, or after the molecular method is employed.

The effectiveness of visual prosthetics can be improved with training of the individual, thus enhancing the potential impact of the Chop2 transformation of patient cells as contemplated herein. An example of an approach to training is found in US 2004/0236389 (Fink et al.), incorporated by reference. The training method may include providing a non-visual reference stimulus to a patient having a visual prosthesis based on a reference image. The non-visual reference stimulus is intended to provide the patient with an expectation of the visual image that the prosthesis will induce. Examples of non-visual reference stimuli are a pinboard, Braille text, or a verbal communication. The visual prosthesis stimulates the patient's nerve cells, including those cells whose responsiveness has been improved by expressing Chop2 as disclosed herein, with a series of stimulus patterns attempting to induce a visual perception that matches the patient's expected perception derived from the non-visual reference stimulus. The patient provides feedback to indicate which of the series of stimulus patterns induces a

perception that most closely resembles the expected perception. The patient feedback is used as a "fitness function" (also referred to as a cost function or an energy function). Subsequent stimuli provided to the patient through the visual prosthesis are based, at least in part, on the previous feedback of the patient as to which stimulus pattern(s) induce the perception that best matches the expected perception. The subsequent stimulus patterns may also be based, at least in part, on a fitness function optimization algorithm, such as a simulated annealing algorithm or a genetic algorithm.

Thus, in certain embodiments of this invention, the method of improving or restoring vision in a subject further comprises training of that subject, as discussed above. Preferred examples of training methods are:

(a) habituation training characterized by training the subject to recognize (i) varying levels of light and/or pattern stimulation, and/or (ii) environmental stimulation from a common light source or object as would be understood by one skilled in the art; and
(b) orientation and mobility training characterized by training the subject to detect visually local objects and move among said objects more effectively than without the training.
In fact, any visual stimulation techniques that are typically used in the field of low vision rehabilitation are applicable here.

The remodeling of inner retinal neurons triggered by photoreceptor degeneration has raised a concerns about retinal-based rescue strategies after the death of photoreceptors (Strettoi and Pignatelli 2000, Proc Natl Acad Sci USA. 97:11020-5; Jones, B W et al., 2003, J Comp Neurol 464:1-16; Jones, B W and Marc, R E, 2005, Exp Eye Res. 81:123-37; Jones, B W et al., 2005, Clin Exp Optom. 88:282-91). Retinal remodeling is believed to result from deafferentation, the loss of afferent inputs from photoreceptors—in other words, the loss of light induced activities So after death of rods and coned, there is no light evoked input to retinal bipolar cells and ganglion cells, and through them to higher visual centers. In response to the loss of such input, the retina and higher visual network are triggered to undergo remodeling, in a way seeking other forms of inputs. Said otherwise, the retina needs to be used to sense light in order to maintain its normal network, and with the loss of light sensing, the network will deteriorate via a remodeling process. This process is not an immediate consequence of photoreceptor death; rather it is a slow process, providing a reasonably long window for intervention.

Thus, an additional utility of restoring light sensitivity to inner retinal neurons in accordance with the present invention is the prevention or delay in the remodeling processes in the retina, and, possibly, in the higher centers. Such retinal remodeling may have undesired consequences such as corruption of inner retinal network, primarily the connection between bipolar and retinal ganglion cells. By introducing the light-evoked activities in bipolar cells or ganglion cells, the present methods would prevent or diminish the remodeling due to the lack of input; the present methods introduce this missing input (either starting from bipolar cells or ganglion cells), and thereby stabilize the retinal and higher visual center network. Thus, independently of its direct effects on vision, the present invention would benefit other therapeutic approaches such as photoreceptor transplantation or device implants,.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

SYNOPSIS OF EXAMPLES

(references cited in the following sections may appear in a list at the end).

Methods

A Chop2-GFP chimera was made by linking a nucleic acid encoding green fluorescent protein (GFP) (part of SEQ ID NO: 1 as shown below) to a nucleic acid (SEQ ID NO:2) encoding an active fragment (SEQ ID NO:3) of channelopsin-2 (Chop2) such that an expressed protein has the GFP linked to the C-terminus of the Chop2 region. Both these sequences constitute the "transgene" as discussed above. The Chop2-GFP DNA was transfected into HEK293 cells under control of a CMV promoter.

A viral construct (SEQ ID NO: 1) was made by subcloning the Chop2-GFP into an AAV-2 viral cassette containing a CAG promoter. A map of this construct is shown in FIG. 7. The viral vectors were injected into the eye of newborn rats. The expression of Chop2-GFP was examined by GFP fluorescence in retinal whole-mounts or slice sections. The function of the Chop2-GFP was assessed by whole-cell patch clamp recordings.

Results

Bright GFP fluorescence was detected within 18-24 hrs in HEK cells after the transfection. The fluorescence was localized predominantly to the plasma membrane. The preserve of the function of the Chop2-GFP chimera was confirmed by patch-clamp recordings. Substantial light-gated currents were also observed in the Chop2-GFP-expressing HEK cells without adding the exogenous all-trans retinal, indicating that a significant number of functional Chop2-GFP channels were formed in HEK cells using only endogenous precursor for the chromophore group. Three to four weeks after the injection, GFP fluorescence was observed in the retinal neurons of the injected eyes. Bright GFP-fluorescence was observed in many ganglion cells and horizontal cells, some amacrine cells, and, occasionally, bipolar cells for at least 10 weeks following injection. The Chop2-GFP-expressing retinal neurons exhibited robust membrane depolarization in response to light stimulation and did not require an exogenous source of all-trans retinal.

Thus, the inventors demonstrated that the selected AAV vector construct efficiently targeted retinal ganglion cells and effectively delivered the Chop2-GFP cDNA and expressed protein at high levels after intravitreal injection in both normal and diseased retinas. When endogenous retinal was bound to the Chop2, it could be photoswitched, and neural activity could be evoked in retinas and at cortical levels. This was shown by several techniques-initially by in vitro patch-clamp recordings of individual dissociated RGCs, followed by multielectrode array recordings of whole-mount retina preparations representative of a large population of RGCs. Finally, in vivo cortical recordings from live blind mice demonstrated that critical connections were functionally maintained to higher visual centers.

Conclusion

The progressive in vitro and in vivo results show that ectopic expression of Chop2 is a therapeutic strategy for restoring light sensitivity to a "blind" retina. Functional expression of a directly light-gated membrane channel, Chop2, was demonstrated in rat retinal neurons in vivo. Thus, expression of light-gated membrane channels in second- or third-order retinal neurons is a useful strategy for restoration of light perception after photoreceptor degeneration.

Example I

Materials and Methods

DNA and Viral Vector Constructions

The DNA fragment encoding the N-terminal fragment (Met<1>-Lys<315>) of Chop2 (Nagel

et al., 2003) was cloned into pBluescript vector (Stratagene) containing the last exon of a mouse protamine 1 gene containing polyadenylation signal (mP1) and GFP cDNA inserted in frame at the 3' end of the Chop2 coding fragment to produce a Chop2-GFP fusion protein. The function of Chop2-GFP chimera was verified in transfected HEK293 cells.

The viral expression construct rAAV2-C AG-Chop2-GFP-WPR E was made by subcloning the Chop2-GFP fragment into an adeno-associated (serotype-2) viral expression cassette. The viral cassette comprised a hybrid CMV enhancer/chicken ß-actin promoter (CAG), a woodchuck posttranscriptional regulatory element (WPRE), and a bovine growth hormone (BGH) polyadenylation sequence. Viral vectors were packaged and affinity purified (GeneDetect).

The vector map is shown in FIG. 7.

The nucleic acid sequence of this vector (SEQ ID NO: 1) is shown below in annotated form (with the annotations as described):

ITR's (at both ends) (UPPER CASE underscore)
CAG promoter sequence (Lower case, bold, italic)
Kozak sequence (lower case double underscore)
Chop2 coding sequence (lower case, bold)
Green fluorescent protein coding sequence (lower case, bold underscored)
WPRE (regulatory element): (UPPER CASE)
The BGH Poly A sequence is not marked.

The remaining sequence (all lower case), including between Chop2 and GFP, is vector sequence

The Chop2 coding sequence from the above vector is shown below as SEQ ID NO:2. Numbering indicates both nucleotide number and codon number. The encoded polypeptide (SEQ ID NO:3) is also shown. Again, this is the N-terminal 315 residues of Chop2 polypeptide (SEQ ID NO:6).

atg gat tat gga ggc gcc ctg agt gcc gtt ggg cgc gag ctg cta ttt 48
M D Y G G A L S A V G R E L L F 16
gta acg aac cca gta gtc gtc aat ggc tct gta ctt gtg cct gag gac 96
V T N P V V V N G S V L V P E D 32
cag tgt tac tgc gcg ggc tgg att gag tcg cgt ggc aca aac ggt gcc 144
Q C Y C A G W I E S R G T N G A 48
caa acg gcg tcg aac gtg ctg caa tgg ctt gct gct ggc ttc tcc atc 192
Q T A S N V L Q W L A A G F S I 64
cta ctg ctt atg ttt tac gcc tac caa aca tgg aag tca acc tgc ggc 240
L L L M F Y A Y Q T W K S T C G 80
tgg gag gag atc tat gtg tgc gct atc gag atg gtc aag gtg att ctt 288
W E E I Y V C A I E M V K V I L 96
gag ttc ttc ttc gag ttt aag aac ccg tcc atg ctg tat cta gcc aca 336
E F F F E F K N P S M L Y L A T 112
ggc cac cgc gtc cag tgg ttg cgt tac gcc gag tgg ctt ctc acc tgc 384
G H R V Q W L R Y A E W L L T C 128
ccg gtc att ctc att cac ctg tca aac ctg acg ggc ttg tcc aac gac 432
P V I L I H L S N L T G L S N D 144
tac agc agg cgc act atg ggt ctg ctt gtg tct gat att ggc aca att 480
Y S R R T M G L L V S D I G T I 160
gtg tgg ggc gcc act tcc gct atg gcc acc gga tac gtc aag gtc atc 528
V W G A T S A M A T G Y V K V I 176

ttc ttc tgc ctg ggt ctg tgt tat ggt gct aac acg ttc ttt cac gct 576
F F C L G L C Y G A N T F F H A 192
gcc aag gcc tac atc gag ggt tac cat acc gtg ccg aag ggc cgg tgt 624
A K A Y I E G Y H T V P K G R C 208
cgc cag gtg gtg act ggc atg gct tgg ctc ttc ttc gta tca tgg ggt 672
R Q V V T G M A W L F F V S W G 224
atg ttc ccc atc ctg ttc atc ctc ggc ccc gag ggc ttc ggc gtc ctg 720
M F P I L F I L G P E G F G V L 240
agc gtg tac ggc tcc acc gtc ggc cac acc atc att gac ctg atg tcg 768
S V Y G S T V G H T I I D L M S 256
aag aac tgc tgg ggt ctg ctc ggc cac tac ctg cgc gtg ctg atc cac 816
K N C W G L L G H Y L R V L I H 272
gag cat atc ctc atc cac ggc gac att cgc aag acc acc aaa ttg aac 864
E H I L I H G D I R K T T K L N 288
att ggt ggc act gag att gag gtc gag acg ctg gtg gag gac gag gcc 912
I G G T E I E V E T L V E D E A 30
gag gct ggc gcg gtc aac aag ggc acc ggc aag 945
E A G A V N K G T G K 315

A native nucleic acid sequence that encodes the full length Chop2 protein of C. reinhardtii (GenBank Accession #AF461397) has the following nucleotide sequence (SEQ ID NO:4). Note that the coding sequence begins at the ATG codon beginning at nt 28.

1 gcatctgtcg ccaagcaagc attaaacATG gattatggag gcgccctgag tgccgttggg
61 cgcgagctgc tatttgtaac gaacccagta gtcgtcaatg gctctgtact tgtgcctgag
121 gaccagtgtt actgcgcggg ctggattgag tcgcgtggca caaacggtgc ccaaacggcg
181 tcgaacgtgc tgcaatggct tgctgctggc ttctccatcc tactgcttat gttttacgcc
241 taccaaacat ggaagtcaac ctgcggctgg gaggagatct atgtgtgcgc tatcgagatg
301 gtcaaggtga ttctcgagtt cttcttcgag tttaagaacc cgtccatgct gtatctagcc
361 acaggccacc gcgtccagtg gttgcgttac gccgagtggc ttctcacctg cccggtcatt
421 ctcattcacc tgtcaaacct gacgggcttg tccaacgact acagcaggcg caccatgggt
481 ctgcttgtgt ctgatattgg cacaattgtg tggggcgcca cttccgccat ggccaccgga
541 tacgtcaagg tcatcttctt ctgcctgggt ctgtgttatg gtgctaacac gttctttcac
601 gctgccaagg cctacatcga gggttaccac accgtgccga agggccggtg tcgccaggtg
661 gtgactggca tggcttggct cttcttcgta tcatggggta tgttccccat cctgttcatc
721 ctcggccccg agggcttcgg cgtcctgagc gtgtacggct ccaccgtcgg ccacaccatc
781 attgacctga tgtcgaagaa ctgctggggt ctgctcggcc actacctgcg cgtgctgatc
841 cacgagcata tcctcatcca cggcgacatt cgcaagacca ccaaattgaa cattggtggc
901 actgagattg aggtcgagac gctggtggag gacgaggccg aggctggcgc ggtcaacaag
961 ggcaccggca agtacgcctc ccgcgagtcc ttcctggtca tgcgcgacaa gatgaaggag
1021 aagggcattg acgtgcgcgc ctctctggac aacagcaagg aggtggagca ggagcaggcc
1081 gccagggctg ccatgatgat gatgaacggc aatggcatgg gtatgggaat gggaatgaac
1141 ggcatgaacg gaatgggcgg tatgaacggg atggctggcg gcgccaagcc cggcctggag
1201 ctcactccgc agctacagcc cggccgcgtc atcctggcgg tgccggacat cagcatggtt
1261 gacttcttcc gcgagcagtt tgctcagcta tcggtgacgt acgagctggt gccggccctg
1321 ggcgctgaca acacactggc gctggttacg caggcgcaga acctgggcgg cgtggacttt
1381 gtgttgattc accccgagtt cctgcgcgac cgctctagca ccagcatcct gagccgcctg
1441 cgcggcgcgg gccagcgtgt ggctgcgttc ggctgggcgc agctggggcc catgcgtgac
1501 ctgatcgagt ccgcaaacct ggacggctgg ctggagggcc cctcgttcgg acagggcatc
1561 ctgccggccc acatcgttgc cctggtggcc aagatgcagc agatgcgcaa gatgcagcag
1621 atgcagcaga ttggcatgat gaccggcggc atgaacggca tgggcggcgg tatgggcggc
1681 ggcatgaacg gcatgggcgg cggcaacggc atgaacaaca tgggcaacgg catgggcggc
1741 ggcatgggca acggcatggg cggcaatggc atgaacggaa tgggtggcgg caacggcatg
1801 aacaacatgg gcggcaacgg aatggccggc aacggaatgg gcggcggcat gggcggcaac

1861 ggtatgggtg gctccatgaa cggcatgagc tccggcgtgg tggccaacgt gacgccctcc
1921 gccgccggcg gcatgggcgg catgatgaac ggcggcatgg ctgcgcccca gtcgcccggc
1981 atgaacggcg gccgcctggg taccaacccg ctcttcaacg ccgcgccctc accgctcagc
2041 tcgcagctcg gtgccgaggc aggcatgggc agcatgggag gcatgggcgg aatgagcgga
2101 atgggaggca tgggtggaat ggggggcatg ggcggcgccg gcgccgccac gacgcaggct
2161 gcgggcggca acgcggaggc ggagatgctg cagaatctca tgaacgagat caatcgcctg
aagcgcgagc ttggcgag

2221

The coding portion of SEQ ID NO:4 is shown below as SEQ ID NO:5, organized as 737 triplet codons (plus a stop codon) that encode a 737 amino acid polypeptide. The ATG start codon and the TAA stop codon are highlighted.

ATG gat tat gga ggc gcc ctg agt gcc gtt ggg cgc gag ctg cta ttt
gta acg aac cca gta gtc gtc aat ggc tct gta ctt gtg cct gag gac
cag tgt tac tgc gcg ggc tgg att gag tcg cgt ggc aca aac ggt gcc
caa acg gcg tcg aac gtg ctg caa tgg ctt gct gct ggc ttc tcc atc
cta ctg ctt atg ttt tac gcc tac caa aca tgg aag tca acc tgc ggc
tgg gag gag atc tat gtg tgc gct atc gag atg gtc aag gtg att ctc
gag ttc ttc ttc gag ttt aag aac ccg tcc atg ctg tat cta gcc aca
ggc cac cgc gtc cag tgg ttg cgt tac gcc gag tgg ctt ctc acc tgc
ccg gtc att ctc att cac ctg tca aac ctg acg ggc ttg tcc aac gac
tac agc agg cgc acc atg ggt ctg ctt gtg tct gat att ggc aca att
gtg tgg ggc gcc act tcc gcc atg gcc acc gga tac gtc aag gtc atc
ttc ttc tgc ctg ggt ctg tgt tat ggt gct aac acg ttc ttt cac gct
gcc aag gcc tac atc gag ggt tac cac acc gtg ccg aag ggc cgg tgt
cgc cag gtg gtg act ggc atg gct tgg ctc ttc ttc gta tca tgg ggt
atg ttc ccc atc ctg ttc atc ctc ggc ccc gag ggc ttc ggc gtc ctg
agc gtg tac ggc tcc acc gtc ggc cac acc atc att gac ctg atg tcg
aag aac tgc tgg ggt ctg ctc ggc cac tac ctg cgc gtg ctg atc cac
gag cat atc ctc atc cac ggc gac att cgc aag acc acc aaa ttg aac
att ggt ggc act gag att gag gtc gag acg ctg gtg gag gac gag gcc
gag gct ggc gcg gtc aac aag ggc acc ggc aag tac gcc tcc cgc gag
tcc ttc ctg gtc atg cgc gac aag atg aag gag aag ggc att gac gtg
cgc gcc tct ctg gac aac agc aag gag gtg gag cag gag cag gcc gcc
agg gct gcc atg atg atg atg aac ggc aat ggc atg ggt atg gga atg
gga atg aac ggc atg aac gga atg ggc ggt atg aac ggg atg gct ggc
ggc gcc aag ccc ggc ctg gag ctc act ccg cag cta cag ccc ggc cgc
gtc atc ctg gcg gtg ccg gac atc agc atg gtt gac ttc ttc cgc gag
cag ttt gct cag cta tcg gtg acg tac gag ctg gtg ccg gcc ctg ggc
gct gac aac aca ctg gcg ctg gtt acg cag gcg cag aac ctg ggc ggc
gtg gac ttt gtg ttg att cac ccc gag ttc ctg cgc gac cgc tct agc
acc agc atc ctg agc cgc ctg cgc ggc gcg ggc cag cgt gtg gct gcg
ttc ggc tgg gcg cag ctg ggg ccc atg cgt gac ctg atc gag tcc gca
aac ctg gac ggc tgg ctg gag ggc ccc tcg ttc gga cag ggc atc ctg
ccg gcc cac atc gtt gcc ctg gtg gcc aag atg cag cag atg cgc aag
atg cag cag atg cag cag att ggc atg atg acc ggc ggc atg aac ggc
atg ggc ggc ggt atg ggc ggc ggc atg aac ggc atg ggc ggc ggc aac
ggc atg aac aac atg ggc aac ggc atg ggc ggc ggc atg ggc aac ggc
atg ggc ggc aat ggc atg aac gga atg ggt ggc ggc aac ggc atg aac
aac atg ggc ggc aac gga atg gcc ggc aac gga atg ggc ggc ggc atg
ggc ggc aac ggt atg ggt ggc tcc atg aac ggc atg agc tcc ggc gtg
gtg gcc aac gtg acg ccc tcc gcc gcc ggc ggc atg ggc ggc atg atg

aac ggc ggc atg gct gcg ccc cag tcg ccc ggc atg aac ggc ggc cgc
ctg ggt acc aac ccg ctc ttc aac gcc gcg ccc tca ccg ctc agc tcg
cag ctc ggt gcc gag gca ggc atg ggc agc atg gga ggc atg ggc gga
atg agc gga atg gga ggc atg ggt gga atg ggg ggc atg ggc ggc gcc
ggc gcc gcc acg acg cag gct gcg ggc ggc aac gcg gag gcg gag atg
ctg cag aat ctc atg aac gag atc aat cgc ctg aag cgc gag ctt ggc
gag taa 2214 nt's

The full length Chop2 protein of C. reinhardtii (GenBank Accession #AF461397) encoded by SEQ ID NO's 3 and 4, has the following amino acid sequence, SEQ ID NO:6:

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNG 50
AQT
ASNVLQWLAAGFSILLLMFYAYQTWKSTCGWEEIYVCAIEMVKVILE 100
FFF
EFKNPSMLYLATGHRVQWLRYAEWLLTCPVILIHLSNLTGLSNDYSR 150
RTM
GLLVSDIGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFHAAKAYI 200
EGY
HTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLSVYGSTV 250
GHT
IIDLMSKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVE 300
TLV
EDEAEAGAVNKGTGKYASRESFLVMRDKMKEKGIDVRASLDNSKEVE 350
QEQ
AARAAMMMMNGNGMGMGMGMNGMNGMGGMNGMAGGAKPGLELTPQLQ 400
PGR
VILAVPDISMVDFFREQFAQLSVTYELVPALGADNTLALVTQAQNLG 450
GVD
FVLIHPEFLRDRSSTSILSRLRGAGQRVAAFGWAQLGPMRDLIESAN 500
LDG
WLEGPSFGQGILPAHIVALVAKMQQMRKMQQMQQIGMMTGGMNGMGG 550
GMG
GGMNGMGGGNGMNNMGNGMGGGMGNGMGGNGMNGMGGGNGMNNMGGN 600
GMA
GNGMGGGMGGNGMGGSMNGMSSGVVANVTPSAAGGMGGMMNGGMAAP 650
QSP
GMNGGRLGTNPLFNAAPSPLSSQLGAEAGMGSMGGMGGMSGMGGMGG 700
MGG
MGGAGAATTQAAGGNAEAEMLQNLMNEINRLKRELGE 737

Another useful Chop2 sequence useful for the present invention is a nucleic acid of 933 nt's (including the stop codon) encoding a 310 aa polypeptide (a biologically active fragment of the full length native Chop2) is a synthetic construct derived from Chlamydomonas reinhardtii? (See EF474017 and Zhang et al., 2007, Nature in press). This sequence is codon-optimized for human expression. The nt sequence shown below is SEQ ID NO:7, and the encoded a.a. sequence shown is SEQ ID NO:8. The polypeptide with the a.a. sequence SEQ ID NO:8 is a fragment of SEQ ID NO:6 truncated at the C-terminus and with Pro replacing Asn at 310.

atg gac tat ggc ggc gct ttg tct gcc gtc gga cgc gaa ctt ttg ttc 48
M D Y G G A L S A V G R E L L F 16
gtt act aat cct gtg gtg gtg aac ggg tcc gtc ctg gtc cct gag gat 96
V T N P V V V N G S V L V P E D 32

caa tgt tac tgt gcc gga tgg att gaa tct cgc ggc acg aac ggc gct 144
Q C Y C A G W I E S R G T N G A 48
cag acc gcg tca aat gtc ctg cag tgg ctt gca gca gga ttc agc att 192
Q T A S N V L Q W L A A G F S I 64
ttg ctg ctg atg ttc tat gcc tac caa acc tgg aaa tct aca tgc ggc 240
L L L M F Y A Y Q T W K S T C G 80
tgg gag gag atc tat gtg tgc gcc att gaa atg gtt aag gtg att ctc 288
W E E I Y V C A I E M V K V I L 96
gag ttc ttt ttt gag ttt aag aat ccc tct atg ctc tac ctt gcc aca 336
E F F F E F K N P S M L Y L A T 112
gga cac cgg gtg cag tgg ctg cgc tat gca gag tgg ctg ctc act tgt 384
G H R V Q W L R Y A E W L L T C 128
cct gtc atc ctt atc cac ctg agc aac ctc acc ggc ctg agc aac gac 432
P V I L I H L S N L T G L S N D 144
tac agc agg aga acc atg gga ctc ctt gtc tca gac atc ggg act atc 480
Y S R R T M G L L V S D I G T I 160
gtg tgg ggg gct acc agc gcc atg gca acc ggc tat gtt aaa gtc atc 528
V W G A T S A M A T G Y V K V I 176
ttc ttt tgt ctt gga ttg tgc tat ggc gcg aac aca ttt ttt cac gcc 576
F F C L G L C Y G A N T F F H A 192
gcc aaa gca tat atc gag ggt tat cat act gtg cca aag ggt cgg tgc 624
A K A Y I E G Y H T V P K G R C 208
cgc cag gtc gtg acc ggc atg gca tgg ctg ttt ttc gtg agc tgg ggt 672
R Q V V T G M A W L F F V S W G 224
atg ttc cca att ctc ttc att ttg ggg ccc gaa ggt ttt ggc gtc ctg 720
M F P I L F I L G P E G F G V L 240
agc gtc tat ggc tcc acc gta ggt cac acg att att gat ctg atg agt 768
S V Y G S T V G H T I I D L M S 256
aaa aat tgt tgg ggg ttg ttg gga cac tac ctg cgc gtc ctg atc cac 816
E H I L I H G D I R K T T K L N 272
gag cac ata ttg att cac gga gat atc cgc aaa acc acc aaa ctg aac 864
I G G T E I E V E T L V E D E A 288
atc ggc gga acg gag atc gag gtc gag act ctc gtc gaa gac gaa gcc 912
I G G T E I E V E T L V E D E A 304
gag gcc gga gcc gtg cca taa 933
E A G A V P stop 310

AAV Vector Injection

All of the animal experiments were at the institutional level and were in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Newborn (PI) rat pups (Sprague-Dawley and Long-Evans) and mouse pups (C57BL/6J and C3H/HeJ or rd1/rd1) were anesthetized by chilling on ice. Adult mice (rd1/rd1) were anesthetized by IP injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Under a dissecting microscope, an incision was made by scissors through the eyelid to expose the sclera. A small perforation was made in the sclera region posterior to the lens with a needle and viral vector suspension of 0.8-1.5 µl at the concentration of approximately 10<11 >genomic particles/ml was injected into intravitreal space through the hole with a Hamilton syringe with a 32-gauge blunt-ended needle. For each animal, usually only one eye was injected with viral vectors carrying Chop2-GFP and the other eye was uninjected or injected with control viral vectors carrying GFP alone. After the injection, animals were kept on a 12/12 hr light/dark cycle. The light illumination of the room housing the animals measured at the wavelength of 500 nm was 6.0×10<14 >photons cm<-2>s<-1>.

Histology

Animals were sacrificed at various time points after the vector injection. The expression of Chop2-GFP fluorescence was examined in flat whole-mount retinas, vertical retinal, and coronal brain sections. The dissected retinas and brains were fixed with 4% paraformaldehyde in PBS for 0.5-2 hr at room temperature and 24 hr at 4° C., respectively. The fixed retinas (embedded in 3% agarose) and brains were cut by using a vibratome. The retinal and brain sections or the retinal whole mounts were mounted on slides and covered with Vectashield medium (Vector Laboratories). GFP fluorescence was visualized under a fluorescence microscope equipped with exciter, dichroic, and emission filters of 465-495 nm, 505 nm, and 515-555 nm, respectively, and most images were obtained with a digital camera (Axiocam, Zeiss). Some images were obtained with a confocal microscope (TCS SP2, Leica). For light microscopy of semithin vertical retinal section, eyes were enucleated, rinsed in PBS, and fixed in 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorenson's phosphate buffer (pH 7.4) at 4° C. for 3 hr. The eyes were then dehydrated in graded ethanols and embedded in plastic and cut into 1 μm sections and stained with a methylene blue/azure mixture.

Patch-Clamp Recordings

Dissociated retinal cells and retinal slice were prepared as previously described (Pan, 2000 and Cui et al., 2003). Recordings with patch electrodes in the whole-cell configuration were made by an EPC-9 amplifier and PULSE software (Heka Electronik, Lambrecht, Germany). Recordings were made in Hanks' solution containing (in mM): NaCl, 138; NaHCO3, 1; Na2HPO4, 0.3; KCl, 5; KH2PO4, 0.3; CaCl2, 1.25; MgSO4, 0.5; MgCl2, 0.5; HEPES-NaOH, 5; glucose, 22.2; with phenol red, 0.001% v/v; adjusted to pH 7.2 with 0.3 N NaOH.

The electrode solution contained (in mM): K-gluconate, 133; KCl, 7; MgCl2, 4; EGTA, 0.1; HEPES, 10; Na-GTP, 0.5; and Na-ATP, 2; pH adjusted with KOH to 7.4. The resistance of the electrode was 13 to 15 MO. The recordings were performed at room temperature (~22° C.).

Multielectrode Array Recordings

The multielectrode array recordings were based on the procedures reported by Tian and Copenhagen (2003). Briefly, the retina was dissected and placed photoreceptor side down on a nitrocellulose filter paper strip (Millipore Corp., Bedford, Mass.). The mounted retina was placed in the MEA-60 multielectrode array recording chamber of 30 μm diameter electrodes spaced 200 μm apart (Multi Channel System MCS GmbH, Reutlingen, Germany), with the ganglion cell layer facing the recording electrodes. The retina was continuously perfused in oxygenated extracellular solution at 34° C. during all experiments. The extracellular solution contained (in mM): NaCl, 124; KCl, 2.5; CaCl2, 2; MgCl2, 2; NaH2PO4, 1.25; NaHCO3, 26; and glucose, 22 (pH 7.35 with 95% O2 and 5% CO2). Recordings were usually started 60 min after the retina was positioned in the recording chamber. The interval between onsets of each light stimulus was 10-15 s. The signals were filtered between 200 Hz (low cut off) and 20 kHz (high cut off). The responses from individual neurons were analyzed using Offline Sorter software (Plexon, Inc., Dallas, Tex.).

Visual-Evoked Potential Recordings

Visual-evoked potential recordings were carried out in wild-type mice of the C57BL/6 and 129/Sv strains aged 4-6 months and in the rd1/rd1 mice aged 6-11 months. Recordings were performed 2-6 months after viral vector injection.

After general anesthesia (i.p. injection of ketamine (100 mg/kg) and acepromazine (0.8 mg/kg), animals were mounted in a stereotaxic apparatus. Body temperature was either

unregulated or maintained at 34° C. with a heating pad and a rectal probe. Pupils were dilated with 1% atropine and 2.5% accu-phenylephrine. A small portion of the skull (~1.5×1.5 mm) centered about 2.5 mm from the midline and 1 mm rostral to the lambdoid suture was drilled and removed. Recordings were made from visual cortex (area V1) by a glass micropipette (resistance ~0.5 M after filling with 4 M NaCl) advanced 0.4 mm beneath the surface of the cortex at the contralateral side of the stimulated eye. The stimuli were 20 ms pluses at 0.5 Hz. Responses were amplified (1,000 to 10,000), band-pass filtered (0.3-100 Hz), digitized (1 kHz), and averaged over 30-250 trials.

Light Stimulation

For dissociated cell and retinal slice recordings, light stimuli were generated by a 150 W xenon lamp-based scanning monochromator with bandwidth of 10 nm (TILL Photonics, Germany) and coupled to the microscope with an optical fiber. For multielectrode array recordings, light responses were evoked by the monochromator or a 175 W xenon lamp-based illuminator (Lambda LS, Sutter Instrument) with a band-pass filter of 400-580 nm and projected to the bottom of the recording chamber through a liquid light guider. For visual evoked potential, light stimuli were generated by the monochromator and projected to the eyes through the optical fiber. The light intensity was attenuated by neutral density filters. The light energy was measured by a thin-type sensor (TQ82017) and an optical power meter (Model: TQ8210) (Advantest, Tokyo, Japan).

Example 2

Expression of Chop2 in Retinal Neurons In Vivo

To directly visualize the expression and localization of Chop2 proteins, the C-terminal portion of the Chop2 channel was replaced with GFP, to make a Chop2-GFP chimera. The adeno-associated virus (AAV) vectors was selected to target the expression of Chop2-GFP fusion protein into retinal neurons because the capability of AAV vectors to deliver transgenes into nondividing cells, including inner retinal neurons (Harvey et al., 2002 and Martin et al., 2003), and to integrate the transgenes into the host genome (Flotte, 2004).

A viral expression cassette, rAAV2-C AG-Chop2-GFP-WPRE, was made by subcloning the Chop2-GFP chimera into an AAV serotype-2 expression cassette containing a hybrid CMV enhancer/chicken ß-actin (CAG) promoter (FIG. 1A). To establish the expression and function of Chop2 channels in retinal neurons in general, we first examined the expression of Chop2 in nondystrophic retinas. The viral vector was injected into the intravitreal space in the eyes of postnatal day 1 rats and mice. Three to four weeks after the injection, bright GFP fluorescence was observed in retinal neurons of all injected eyes (FIGS. 1B-1H), confirming that Chop2-GFP was expressed. The expression was usually confluent throughout the retina (FIG. 1B).

The Chop2-GFP-fluorescence was predominantly observed in retinal ganglion cells (FIGS. 1C and 1D; also see FIG. 1H). The fluorescence signal was observed throughout the inner plexiform layer (IPL) (FIG. 1H), indicating that the viral vector targeted the expression of Chop2-GFP both in ON and OFF ganglion cells. The expressing of Chop2-GFP was also frequently observed in horizontal cells (FIG. 1E), amacrine cells (FIG. 1F), and, occasionally, in bipolar cells (FIG. 1G).

The GFP signal was predominantly localized to the plasma membrane (FIG. 1D), consistent with the GFP tag being anchored to the membrane by a seven-transmembrane portion of the Chop2 channel. Once expressed in a cell, the GFP signal was extended over the entire cell including distal processes and axon terminals (see FIGS. 1C and 1E). Bright GFP fluorescence was found to be stable for 12 months or more after the injection (FIG. 1H), whereas no gross

changes in retinal morphology were noticed (FIG. 1I). These results indicated that long-term stable expression of Chop2-GFP was achieved in inner retinal neurons in vivo.

Example 3

Properties of Light-Evoked Currents of ChR2-Expressing Inner Retinal Neurons

Functional properties of the Chop2 channels were examined in inner retinal neurons by using whole-cell patch-clamp recordings. The recordings were performed in acutely dissociated cells so that photoreceptor-mediated light responses were confidently excluded. Chop2-GFP-positive cells were identified by their GFP fluorescence (FIG. 2A). The precursor for the Chop2 chromophore group, all-trans retinal, was not added because it might be ubiquitously present in cells (Kim et al, 1992 and Thompson and Gal, 2003). Light-evoked responses were observed in all recorded GFP fluorescent cells (n=34), indicating that functional ChR2 (Chop2 with the chromophore attached) can be formed in retinal neurons with the retinal chromophore groups already present in the cells. Consistently, the expression of functional ChR2 channels has also been recently reported in cultured hippocampal neurons without the supply of exogenous retinal chromophore groups (Boyden et al, 2005; but see Li et al, 2005).

The properties of the ChR2-mediated light responses were first examined in voltage clamp. Light-evoked currents were observed in Chop2-GFP-expressing inner retinal neurons by light stimuli up to the wavelength of 580 nm with the most sensitive wavelength around 460 nm (FIG. 2B), consistent with the reported peak spectrum sensitivity of ChR2 (Nagel et al, 2003). The amplitude and the kinetics of the currents were dependent on the light intensity (FIG. 2C). FIGS. 2D and 2E show in the expanded time scale the current traces right after the onset and the termination of the light stimulation, respectively. Detectable currents were observed in most recorded cells at a light intensity of 2.2×10<15 >photons cm<-2>s<-1>. In some cells, currents were observed at a light intensity of 2×10<14 >photons cm<-2>s<-1 >(not shown). At higher light intensities, the currents displayed both transient and sustained components, similar to the properties of the nonfusion ChR2 (Nagel et al., 2003). The relationship between the light intensity and peak current is shown in FIG. 2F (n=7). The activation and inactivation kinetics of the currents were also dependent on the light intensity (FIG. 2D). The initial phase of the current could be well fitted by an exponential function with a single activation and inactivation constant, as illustrated in FIG. 2D (red trace). The activation and inactivation time constants versus light intensity are plotted in FIGS. 2G and 2H, respectively. On the other hand, the deactivation kinetics of the currents after the light off was not light-intensity dependent. The current decay trace could be well fitted by a single exponential function as shown in FIG. 2E (red trace). The time constant was 17.1±6.5 ms (mean±SD, n=7).

The next experiment examined whether the ChR2-mediated currents were sufficient to drive membrane depolarization. FIG. 3A shows the representative responses from a nonspiking neuron in response to four incremental light intensities at the wavelength of 460 nm. Detectable responses were observed in most recorded cells at a light intensity of 2.2×10<15 >photons cm<-2>s<-1>. At higher light intensities, the membrane depolarization approached a saturated level. The ChR2-mediated light responses to repeated light stimulations were further examined. The transient component of the currents diminished to repeated stimulations whereas the sustained component of the currents was stable (top traces in FIG. 3B). This was clearly seen in the expanded time scale in the right panel of FIG. 3B by comparing the superimposed first (red trace) and the second (black trace) light-evoked currents. For the same cell, in current clamp, the stimulations evoked robust membrane depolarizations (bottom traces in FIG. 3B). The membrane depolarizations reached an almost identical level, except for the initial portion of the response. This was also shown in the expanded time scale (right panel), which superimposed the first (red trace) and the second (black trace) light-evoked responses. FIG. 3C shows a representative recording of spiking neurons to repeated light

stimulations. Again, the stimulations elicited almost identical membrane depolarizations accompanied by multiple spikes. Taken together, these results demonstrated that the ChR2-mediated currents in second- and third-order retinal neurons are sufficient to drive membrane depolarization and/or spike firing.

Example 4

Expression of Chop2 in Photoreceptor-Deficient rd1/rd1 Mice

Having established the expression and function of ChR2 in wild-type retinas, we went on to address whether the expression of ChR2 could restore light responses in retinas after photoreceptor degeneration. To this end, the experiments were carried out in homozygous rd1 (rd1/rd1) mice (Bowes et al., 1990), a photoreceptor degeneration model with a null mutation in a cyclic GMP phosphodiesterase, PDE6, similar to some forms of retinitis pigmentosa in humans (McLaughlin et al., 1993). The Chop2-GFP viral vector was injected intravitreally into the eyes of newborn (P1) or adult mice at 2-12 months of age. Similar to the results observed in wild-type animals, bright GFP signal was observed in Chop2-GFP-injected retinas, predominately in retinal ganglion cells (FIGS. 4A and 4B). At the time of the recording experiments (?4 months of age unless otherwise indicated), photoreceptor cells were absent (FIG. 4C). The expression of Chop2-GFP was observed in the rd1/rd1 mice up to 16 months of age (3-6 months after the viral injection) as the case shown in FIG. 4A from a 15 month old rd1/rd1 mouse. These results indicate that inner retinal neurons in this photoreceptor-deficient model not only survive long after the complete death of photoreceptors but also retain the capability of stable expression of Chop2-GFP.

Example 5

Light-Evoked Responses of ChR2-Expressing Surviving Inner Retinal Neurons of rd1/rd1 Mice

The light response properties of the ChR2-expressing retinal neurons in rd1/rd1 mice were examined by whole-cell patch-clamp recording in retinal slices. The recordings were made from the GFP-positive cells located in the ganglion cell layer. Light-evoked currents were observed in GFP-positive cells. The magnitude of the current was again dependent on the light intensity (top traces in FIGS. 4D and 4E; also see light intensity and current relationships shown in FIG. 4F). Two groups of ChR2-expressing retinal neurons were observed based on their response properties: a group of transient spiking neurons (FIG. 4D) and a group of sustained spiking neurons (FIG. 4E). The membrane depolarization and/or spike rates were also dependent on the light intensity (bottom traces in FIGS. 4D and 4E). Furthermore, light at higher intensities markedly accelerated the kinetics of the voltage responses as illustrated in the right panels of FIGS. 4D and 4E by superimposing the second traces (black) and the fourth traces (red) in an expanded time scale. The relationships of light intensity to the membrane depolarization, the spike firing rate, and the time to the first spike peak are shown in FIGS. 4G, 4H, and 4I, respectively. These results demonstrate that the surviving retinal third-order neurons with the expression of ChR2 are capable of encoding light intensity with membrane depolarization and/or action potential firing and response kinetics.

Example 6

Multielectrode Array Recordings of ChR2-Mediated Retinal Activities

The spike coding capability of the photoreceptor-deficient retina of rd1/rd1 mice were examined after the expression of ChR2 by use of multielectrode array recordings from whole-mount retinas. As shown from a sample recording in FIG. 5A, spike firings with fast kinetics

in response to light on and off were observed in Chop2-GFP-expressing retinas (n=11 retinas). The light-evoked spike firings were not affected by the application of CNQX (25-50 μM) plus APV (25-50 μM) (n=3), indicating that the responses are originated from the ChR2 of the recorded cells. No such light-evoked spike firings were observed in retinas that were either injected with viral vectors carrying GFP alone (n=2 retinas) or left uninjected (n=3). The latter confirmed the absence of photoreceptor-originated light responses. The light-evoked spike firings were not affected by suramine (100 μM) (n=2), which has been reported to be able to block melanopsin receptor-mediated photocurrent (Melyan et al., 2005 and Qiu et al., 2005).

In addition, the response kinetics to both light on and off (see FIG. 5B) were much faster than those generated by the intrinsically photosensitive retinal ganglion cells (Tu et al., 2005). These results indicated that a significant contribution to the observed light responses from the intrinsically photosensitive ganglion cells under our recording conditions is unlikely. The light-evoked responses were often found to be picked up by the majority of the electrodes (see FIG. 5A), consistent with the observation that Chop2-GFP was extensively expressed in the retinas. The vast majority of the responses were sustained during light stimulation. FIG. 5B illustrates the raw traces recorded by a single electrode in response to three incremental light stimuli. The raster plots of the spike activity sorted from a single neuron of the recording were shown in FIG. 5C. The firing frequency was remarkably stable during the course of the recording. The averaged spike rate histograms are shown in FIG. 5D. Again, the spike frequency was increased to the higher light intensity. The light responses could be recorded for up to 5 hr. These results demonstrate further that the ChR2-expressing retinal ganglion cells can reliably encode light intensity with spike firing rate.

Example 7

Visual-Evoked Potentials

A study was conducted to test whether the ChR2-mediated light responses in the retinas of rd1/rd1 mice were transmitted to the visual cortex. The expression of transgenes, such as GFP, in retinal ganglion cells as achieved by AAV infection was reported to be able to extend to their terminations in higher visual centers in the brain (Harvey et al., 2002). Therefore the anatomical projections of the axon terminals of Chop2-GFP-expressing retinal ganglion cells were first examined. Consistently, Chop2-GFP labeled axon terminals of retinal ganglion cells were observed in several regions of the brain, including ventral lateral geniculate nucleus and dorsal lateral geniculate nucleus (FIG. 6A), as well as superior colliculus (FIG. 6B). These results indicate that the central projections of retinal ganglion cells in the degenerate retinas are maintained.

Visual evoked potentials (VEPs) from visual cortex were then examined. First, as illustrated in FIG. 6C, VEPs were observed in all tested wild-type mice (4-6 months of age) in response to light stimuli at the wavelengths of both 460 and 580 nm (n=6 eyes). When tested in Chop2-GFP-injected eyes of rd1/rd1 mice (6-11 months of age), VEPs were observed in the majority of the eyes (nine out of 13) in response to light stimulus at the wavelength of 460 nm but not to light stimulus at the wavelength of 580 nm (FIG. 6D), consistent with the light sensitivity of ChR2 channels (see FIG. 2B). The average amplitude of the VEPs in the Chop2-GFP-injected eyes in response to the light stimulus at the wavelength of 460 nm was 110±34 μV (mean±SE; n=10), which is smaller than that observed in wild-type mice (274±113 μV; n=6), although these two values are not significantly different (one-way ANOVA test, $p<0.1$). The lower amplitudes of the VEPs in the Chop2-transfected mice compared to the wild-type mice are not surprising because the expression of ChR2 was probably only achieved in a small portion of the retinal ganglion cells. The average latency to the peak of the VEPs in the Chop2-GFP-injected eyes was 45±1.7 ms (n=10), which is shorter than that observed in wild-type mice (62±2.8 ms; n=6). These two values were significantly different ($p<0.01$). The latter would be

predicted because the light response mediated by ChR2 in retinal ganglion cells originates two synapses downstream of the photoreceptors. As a control, no detectable VEPs were observed to light stimulus at the wavelength of 460 nm in the eyes of the age-matched rd1/rd1 mice that were injected with viral vectors carrying GFP alone (n=5) (FIG. 6E). In addition, no detectable VEPs were observed in uninjected rd1/rd1 mice (n=3; 5 months of age) to the wavelengths ranging from 420 to 620 nm (not shown), confirming that rd1/rd1 mice at ?5 months of age are completely blind based on VEPs.

To further ensure that the VEPs in the blind rd1/rd1 mice originate from ChR2 expressed in their retinas, the action spectrum of the VEP were measured by plotting their normalized amplitudes in response to varying light wavelengths and intensities to obtain the relative sensitivity of the response (FIG. 6F) (n=3). The data points were well fitted by a vitamin-A1-based visual pigment template (Partridge and De Grip, 1991) with a peak wavelength at 461 nm (FIG. 6G), a good match to the reported peak action spectrum of ChR2 at ~460 nm (Nagel et al., 2003). Taken together, these results demonstrated that expression of ChR2 in the photoreceptor-deficient retinas can restore visually evoked responses in the brain.

Example 8

Discussion of Examples 1-7

The results presented herein demonstrated that the strategy of restoration of light responses in photoreceptor-deficient rodent retinas based on the expression of ChR2 is mechanistically and technically feasible. Most importantly, the results showed that ChR2 satisfies several major criteria for its use as a light sensor in retinal neurons. First, by delivery of an AAV vector carrying fused Chop2-GFP, the inventors showed the ability of retinal neurons to tolerate the prolonged expression of Chop2. To date, the expression of Chop2-GFP proteins had been achieved in nondystrophic rat retinal neurons for 12 months and in photoreceptor deficient rd1/rd1 mice for 6 months in vivo after the viral injection. The present results therefore indicate that the expression of ChR2 in retinal neurons is biocompatible under normal light cycle conditions.

Second, these results showed that a sufficient number of ChR2 can be formed in retinal neurons, with only endogenous chromophore groups as supplied by regular diet, to produce robust membrane depolarizations and/or action potential firings in the retina and VEPs in visual cortex. It is worth emphasizing here that, unlike animal visual pigments that rapidly lose their chromophore after its photoisomerization from 11-cis to all-trans retinal (Wald, 1968), for microbial-type rhodopsins, photoisomerization from all-trans to 11-cis retinal is reversible and both isomers remain attached to the protein (Oesterhelt, 1998). Once the ChR2 complex is formed, the light-sensitive channel can sustain multiple cycles of photoisomerization with the same chromophore moiety. Although the efficacy of the de novo ChR2 formation might be expected to depend on the availability of the chromophore group, the need for constant resupply of the chromophore to form new ChR2 does not appear to impose a limitation on overall ChR2 function. As observed in the multielectrode array recordings, ChR2 respond repeatedly to light stimulation for several hours in vitro without loss of activity. These results thus indicate that the turn-over rate for ChR2 is fairly slow, an additional advantage for use as an artificially produced light sensor.

Furthermore, as reported originally in cell expression systems (Nagel et al., 2003), later in hippocampal neurons (Boyden et al., 2005, Ishizuka et al., 2006 and Li et al., 2005), and now shown in retinal neurons, a number of properties of the ChR2 channel are highly advantageous for its use as a light sensor.

First, the ChR2 channel is permeable to the cations that underlie neuronal membrane

excitability. Thus, activation of ChR2 channels by light can directly produce membrane depolarizations to mimic the ON-responses of inner retinal neurons. Indeed, as shown herein, the light-evoked responses mediated by ChR2 in nonspiking and spiking retinal neurons remarkably resemble the light responses of ON-bipolar cells and sustained ON-ganglion cells (Werblin and Dowling, 1969 and Kaneko, 1970).

Second, the activation kinetics of the current in response to light are extremely fast, whereas the sustained components of the currents do not show apparent inactivation to continuous or repeated light illuminations. Thus, the ChR2-expressing neurons can signal with rapid kinetics but without pigment inactivation. Consistently, the expression of ChR2 has been shown to allow optical control of neural excitability with high temporal resolution (Boyden et al., 2005, Ishizuka et al., 2006 and Li et al., 2005). Furthermore, it is shown here that the magnitude and activation kinetics of the light-evoked current depend upon light irradiance over a 3-log-unit range. As demonstrated in the whole-cell and multielectrode array recordings, this would allow the encoding of various light intensities with graded membrane depolarizations and/or spike rates.

Also of importance for the feasibility of the strategy of restoring light sensitivity in retinas after photoreceptor degeneration, results of this study show that many inner retinal neurons survive in aged rd1/rd1 mice (up to 16 months of age) and are capable of expressing ChR2 long after the death of all photoreceptors. This is consistent with histological studies showing that many inner retinal neurons survive, despite some remodeling, in this mouse model (Jimenez et al., 1996, Strettoi and Pignatelli, 2000 and Chang et al., 2002). Moreover, the present studies using ChR2 showed that the surviving inner retinal neurons retained their physiological capability to encode light signals with membrane depolarizations and/or action potential firings and to transmit visual signals to the visual cortex. Thus, the strategy based on the expression of ChR2 is suitable at least for certain retinal degenerative diseases at certain stages.

The remodeling of inner retinal neurons triggered by photoreceptor degeneration raised some concerns for the retinal-based rescue strategy after the death of photoreceptors (Strettoi and Pignatelli, 2000, Jones et al., 2003 and Jones and Marc, 2005). However, retinal degenerative diseases are heterogeneous as to the time course of the degeneration, survival an d functional state of different cell types (Chang et al., 2002). The use of ChR2 is a powerful tool for undertaking such studies.

Retinal remodeling is believed to be caused by deafferentation (Jones and Marc, 2005). Therefore, the restoration of the light sensitivity in inner retinal neurons may be able to prevent or delay the remodeling processes.

Finally, according to the present invention, viral-based gene delivery systems, such as AAV vectors (Flannery et al., 1997, Bennett et al., 1999, Ali et al., 2000 and Acland et al., 2001), are tools for introducing Chop2 into retinal neurons as demonstrated herein.

The present results showed that that viral construct with AAV serotype-2 and CAG promoter achieved robust expression of Chop2 in ganglion cells. However, because the expression of Chop2 with this construct appears to target both ON- and OFF-type ganglion cells, it remains to be determined how the conversion of both ON- and OFF-ganglion cells into ON-type affects the visual perception.

Behavior studies in primates reported that pharmacological blockade of the ON channel in the retina did not severely impair such vision functions as the detection of light decrement and the perception of shape (Schiller et al., 1986). Therefore, targeting of ChR2 to the ON channel, for example to ON-type ganglion cells, is expected to result in useful vision.

It is also contemplated herein to express ChR2 in the more distal retinal neurons, such as bipolar cells; this approach would utilize the remaining signal processing functions of the degenerate retina. Targeting ChR2 to rod bipolar cells is particularly attractive because the depolarization of rod bipolar cells can lead to the ON and OFF responses at the levels of cone bipolar cells and retinal ganglion cells (Wassle, 2004), thereby maintaining the ON and OFF channels that are inherent in the retina.

The threshold light intensity required for producing responses in ChR2-expressing retinas appeared to be near 10<14>-10<15 >photons cm<-2>s<-1>. For comparison, the thresholds for normal rod and cone photoreceptors are about 10<6 >and 10<10 >photons cm<-2>s<-1>, respectively (Dacey et al., 2005). Therefore, the ChR2-expressing retinas would operate in substantially higher photonic range. The relatively low light sensitivity of the ChR2-expressing retinas compared to the normal retinas could be due to a number of factors. First, there may be a low cross-sectional density of ChR2 molecules in the transfected retinal neurons compared with the visual pigments in rods and cones. Second, the ChR2-expressing inner retinal neurons lack the unique multilayer photoreceptor membrane organization, typical for the outer segments of rods and cones, which developed to achieve higher pigment density and thus increase the probability of catching photons (Steinberg, et al., 1980). Third, unlike visual pigments that propagate their signal through amplification cascade (Stryer, 1991), the directly light-gated ChR2 channels lack such amplification capabilities. Finally, in normal retinas, amplification of visual signals occurs as the signals converge from multiple photoreceptors to ganglion cells (Barlow et al., 1971). This process was not yet achieved in the ChR2-transfected retinas. It is not yet evident which of these factors contributes the most to the decreased light sensitivity of the ChR2-expressing retinas remains. Interestingly, ChR2 mediated phototaxis to low-intensity light in green algae (Sineshchekov et al., 2002; but see Kateriya et al. [2004]). Therefore, the light sensitivity of ChR2 in retinal neurons may have been altered by modifications introduced in the Chop2 molecule for the heterologous expression. Such a difference may also reflect different structural and functional organization of algae and mammalian cells.

Nevertheless, for clinical usage, light intensifying devices can be used to expand the light operation range.

At present, no treatment is available for restoring vision once the photoreceptor cells have been lost. As noted above, transplantation of normal photoreceptor cells or progenitor cells (Bok, 1993 and Lund et al., 2001) or direct electrical stimulation of the surviving second- and third-order retinal neurons via retinal implants (Zrenner, 2002) have been proposed as possible strategies for restoration of light responses in the retina after rod and cone degeneration. An important advantage of the present invention is that it does not involve the introduction of tissues or devices into the retina and, therefore, may largely avoid the complications of immune reactions and bioincompatibilities. In addition, the present approach is expected to achieve high spatial resolution for the restored “vision” because the approach targets the cellular level. Thus, the expression of microbial-type channel rhodopsins, such as ChR2, in surviving retinal neurons is a strategy for the treatment of complete blindness caused by rod and cone degeneration.

References Cited in Examples Sections

Acland et al., 2001—G. M. Acland, G. D. Aguirre, J. Ray, Q. Zhang, T. S. Aleman, A. V. Cideciyan, S. E. Pearce-Kelling, V. Anand, Y. Zeng and A. M. Maguire et al., Gene therapy restores vision in a canine model of childhood blindness, Nat. Genet. 28 (2001), pp. 92-95
Ali et al., 2000—R. R. Ali, G. M. Sarra, C. Stephens, M. D. Alwis, J. W. Bainbridge, P. M.

Munro, S. Fauser, M. B. Reichel, C. Kinnon and D. M. Hunt et al., Restoration of photoreceptor ultrastructure and function in retinal degeneration slow mice by gene therapy, Nat. Genet. 25 (2000), pp. 306 310
Auricchio et al., 2001—A. Auricchio, G. Kobinger, V. Anand, M. Hildinger, E. O'Connor, A. M. Maguire, J. M. Wilson and J. Bennett, Exchange of surface proteins impacts on viral vector cellular specificity and transduction characteristics: the retina as a model, Hum. Mol. Genet. 10 (2001), pp. 3075-3081
Banghart et al., 2004—M. Banghart, K. Borges, E. Isacoff, D. Trauner and R. H. Kramer, Light-activated ion channels for remote control of neuronal firing, Nat. Neurosci. 7 (2004), pp. 1381-1386
Barlow et al., 1971—H. B. Barlow, W. R. Levick and M. Yoon, Responses to single quanta of light in retinal ganglion cells of the cat, Vision Res. 3 (1971), pp. 87-101
Baylor, 1996—D. Baylor, How photons start vision, Proc. Natl. Acad. Sci. USA 93 (1996), pp. 560 565
Bennett et al., 1999—J. Bennett, A. M. Maguire, A. V. Cideciyan, M. Schnell, E. Glover, V. Anand, T. S. Aleman, N. Chirmule, A. R. Gupta and Y. Huang et al., Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina, Proc. Natl. Acad. Sci. USA 96 (1999), pp. 9920-9925
Bok, 1993—D. Bok, Retinal transplantation and gene therapy. Present realities and future possibilities, Invest. Ophthalmol. Vis. Sci. 34 (1993), pp. 473-476
Bowes et al., 1990—C. Bowes, T. Li, M. Danciger, L. C. Baxter, M. L. Applebury and D. B. Farber, Retinal degeneration in the rd mouse is caused by a defect in the beta subunit of rod cGMP-phosphodiesterase, Nature 347 (1990), pp. 677-680
Boyden et al., 2005—E. S. Boyden, F. Zhang, E. Bamberg, G. Nagel and K. Deisseroth, Millisecond-timescale, genetically targeted optical control of neural activity, Nat. Neurosci. 8 (2005), pp. 1263-1268
Chang et al., 2002—B. Chang, N. L. Hawes, R. E. Hurd, M. T. Davisson, S. Nusinowitz and J. R. Heckenlively, Retinal degeneration mutants in the mouse, Vision Res. 42 (2002), pp. 517-525
Cui et al., 2003—J. Cui, Y. P. Ma, S. A. Lipton and Z.-H. Pan, Glycine receptors and glycinergic synaptic input at the axon terminals of mammalian retinal rod bipolar cells, J. Physiol. 553 (2003), pp. 895-909
Dacey et al., 2005—D. M. Dacey, H. W. Liao, B. B. Peterson, F. R. Robinson, V. C. Smith, J. Pokorny, K. W. Yau and P. D. Gamlin, Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN, Nature 433 (2005), pp. 749-754
Fitzsimons et al., 2002—H. L. Fitzsimons, R. J. Bland and M. J. During, Promoters and regulatory elements that improve adeno-associated virus transgene expression in the brain, Methods 28 (2002), pp. 227-236
Flannery et al., 1997—J. G. Flannery, S. Zolotukhin, M. I. Vaquero, M. M. LaVail, N. Muzyczka and W. W. Hauswirth, Efficient photoreceptor-targeted gene expression in vivo by recombinant adeno-associated virus, Proc. Natl. Acad. Sci. USA 94 (1997), pp. 6916-6921
Flotte, 2004—T. R. Flotte, Gene therapy progress and prospects: recombinant adeno-associated virus (rAAV) vectors, Gene Ther. 11 (2004), pp. 805-810
Harvey et al., 2002—A. R. Harvey, W. Kamphuis, R. Eggers, N. A. Symons, B. Blits, S. Niclou, G. J. Boer and J. Verhaagen, Intravitreal injection of adeno-associated viral vectors results in the transduction of different types of retinal neurons in neonatal and adult rats: a comparison with lentiviral vectors, Mol. Cell. Neurosci. 21 (2002), pp. 141 157
Humphries et al., 1992—P. Humphries, P. Kenna and G. J. Farrar, On the molecular genetics of retinitis pigmentosa, Science 256 (1992), pp. 804-808
Ishizuka et al., 2006—T. Ishizuka, M. Kakuda, R. Araki and H. Yawo, Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels, Neurosci. Res. 54 (2006), pp. 85-94
Jimenez et al., 1996—A. J. Jimenez, J. M. Garcia-Fernandez, B. Gonzalez and R. G. Foster, The spatio-temporal pattern of photoreceptor degeneration in the aged rd/rd mouse retina, Cell

Tissue Res. 284 (1996), pp. 193 202
Jones and Marc, 2005—B. W. Jones and R. E. Marc, Retinal remodeling during retinal degeneration, Exp. Eye Res. 81 (2005), pp. 123-137
Jones et al., 2003—B. W. Jones, C. B. Watt, J. M. Frederick, W. Baehr, C. K. Chen, E. M. Levine, A. H. Milam, M. M. Lavail and R. E. Marc, Retinal remodeling triggered by photoreceptor degenerations, J. Comp. Neurol. 464 (2003), pp. 1-16
Kaneko, 1970—A. Kaneko, Physiological and morphological identification of horizontal, bipolar, and amacrine cells in the goldfish retina, J. Physiol. 207 (1970), pp. 623-633
Kateriya et al., 2004—S. Kateriya, G. Nagel, E. Bamberg and P. Hegemann, “Vision” in single-celled algae, News Physiol. Sci. 19 (2004), pp. 133-137
Kim et al., 1992—C. I. Kim, M. A. Leo and C. S. Lieber, Retinol forms retinoic acid via retinal, Arch. Biochem. Biophys. 294 (1992), pp. 388-393
Li et al., 2005—X. Li, D. V. Gutierrez, M. G. Hanson, J. Han, M.D. Mark, H. Chiel, P. Hegemann, L. T. Landmesser and S. Herlitze, Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin, Proc. Natl. Acad. Sci. USA 102 (2005), pp. 17816-17821
Lund et al., 2001—R. D. Lund, A. S. Kwan, D. J. Keegan, Y. Sauve, P. J. Coffey and J. M. Lawrence, Cell transplantation as a treatment for retinal disease, Prog. Retin. Eye Res. 20 (2001), pp. 415-449
Martin et al., 2003—K. R. Martin, H. A. Quigley, D. J. Zack, H. Levkovitch-Verbin, J. Kielczewski, D. Valenta, L. Baumrind, M. E. Pease, R. L. Klein and W. W. Hauswirth, Gene therapy with brain-derived neurotrophic factor as a protection: retinal ganglion cells in a rat glaucoma model, Invest. Ophthalmol. Vis. Sci. 44 (2003), pp. 4357-4365
McLaughlin et al., 1993—M. E. McLaughlin, M. A. Sandberg, E. L. Berson and T. P. Dryja, Recessive mutations in the gene encoding the beta-subunit of rod phosphodiesterase in patients with retinitis pigmentosa, Nat. Genet. 4 (1993), pp. 130 134
Melyan et al., 2005—Z. Melyan, E. E. Tarttelin, J. Bellingham, R. J. Lucas and M. W. Hankins, Addition of human melanopsin renders mammalian cells photoresponsive, Nature 433 (2005), pp. 741 745
Milam et al., 1998—A. H. Milam, Z. Y. Li and R. N. Fariss, Histopathology of the human retina in retinitis pigmentosa, Prog. Retin. Eye Res. 17 (1998), pp. 175-205
Nagel et al., 2002—G. Nagel, D. Ollig, M. Fuhrmann, S. Kateriya, A. M. Musti, E. Bamberg and P. Hegemann, Channelrhodopsin-1: a light-gated proton channel in green algae, Science 296 (2002), pp. 2395 2398
Nagel et al., 2003—G. Nagel, T. Szellas, W. Huhn, S. Kateriya, N. Adeishvili, P. Berthold, D. Ollig, P. Hegemann and E. Bamberg, Channelrhodopsin-2, a directly light-gated cation-selective membrane channel, Proc. Natl. Acad. Sci. USA 100 (2003), pp. 13940-13945
Oesterhelt, 1998—D. Oesterhelt, The structure and mechanism of the family of retinal proteins from halophilic archaea, Curr. Opin. Struct. Biol. 8 (1998), pp. 489-500
Oesterhelt and Stoeckenius, 1973—D. Oesterhelt and W. Stoeckenius, Functions of a new photoreceptor membrane, Proc. Natl. Acad. Sci. USA 70 (1973), pp. 2853-2857
Olshevskaya et al., 2004—E. V. Olshevskaya, P. D. Calvert, M. L. Woodruff, I. V. Peshenko, A. B. Savchenko, C. L. Makino, Y. S. Ho, G. L. Fain and A. M. Dizhoor, The Y99C mutation in guanylyl cyclase-activating protein 1 increases intracellular Ca<2+> and causes photoreceptor degeneration in transgenic mice, J. Neurosci. 24 (2004), pp. 6078-6085
Pan, 2000—Z.-H. Pan, Differential expression of high- and two types of low-voltage-activated calcium currents in rod and cone bipolar cells of the rat retina, J. Neurophysiol. 83 (2000), pp. 513-527
Panda et al., 2005—S. Panda, S. K. Nayak, B. Campo, J. R. Walker, J. B. Hogenesch and T. Jegla, Illumination of the melanopsin signaling pathway, Science 307 (2005), pp. 600-604
Partridge and De Grip, 1991—J. C. Partridge and W. J. De Grip, A new template for rhodopsin (vitamin A1 based) visual pigments, Vision Res. 31 (1991), pp. 619-630
Qiu et al., 2005—X. Qiu, T. Kumbalasiri, S. M. Carlson, K. Y. Wong, V. Krishna, I. Provencio and D. M. Berson, Induction of photosensitivity by heterologous expression of melanopsin,

Nature 433 (2005), pp. 745-749
Santos et al., 1997—A. Santos, M. S. Humayun, E. de Juan Jr., R. J. Greenburg, M. J. Marsh, I. B. Klock and A. H. Milam, Preservation of the inner retina in retinitis pigmentosa. A morphometric analysis, Arch. Ophthalmol. 115 (1997), pp. 511-515
Schiller et al., 1986—P. H. Schiller, J. H. Sandell and J. H. Maunsell, Functions of the ON and OFF channels of the visual system, Nature 322 (1986), pp. 824-825
Sineshchekov et al., 2002—O. A. Sineshchekov, K. H. Jung and J. L. Spudich, Two rhodopsins mediate phototaxis to low- and high-intensity light in Chlamydomonas reinhardtii, Proc. Natl. Acad. Sci. USA 99 (2002), pp. 8689-8694
Steinberg et al., 1980—R. H. Steinberg, S. K. Fisher and D. H. Anderson, Disc morphogenesis in vertebrate photoreceptors, J. Comp. Neurol. 190 (1980), pp. 501-518
Strettoi and Pignatelli, 2000—E. Strettoi and V. Pignatelli, Modifications of retinal neurons in a mouse model of retinitis pigmentosa, Proc. Natl. Acad. Sci. USA 97 (2000), pp. 11020-11025
Stryer, 1991—L. Stryer, Visual excitation and recovery, J. Biol. Chem. 266 (1991), pp. 10711-10724
Sung et al., 1991—C. H. Sung, C. M. Davenport, J. C. Hennessey, I. H. Maumenee, S. G. Jacobson, J. R. Heckenlively, R. Nowakowski, G. Fishman, P. Gouras and J. Nathans, Rhodopsin mutations in autosomal dominant retinitis pigmentosa, Proc. Natl. Acad. Sci. USA 88 (1991), pp. 6481-6485
Suzuki et al., 2003—T. Suzuki, K. Yamasaki, S. Fujita, K. Oda, M. Iseki, K. Yoshida, M. Watanabe, H. Daiyasu, H. Toh and E. Asamizu et al., Archaeal-type rhodopsins in Chlamydomonas: model structure and intracellular localization, Biochem. Biophys. Res. Commun. 301 (2003), pp. 711-717
Tian and Copenhagen, 2003—N. Tian and D. R. Copenhagen, Visual stimulation is required for refinement of ON and OFF pathways in postnatal retina, Neuron 39 (2003), pp. 85-96
Thompson and Gal, 2003—D. A. Thompson and A. Gal, Vitamin A metabolism in the retinal pigment epithelium: genes, mutations, and diseases, Prog. Retin. Eye Res. 22 (2003), pp. 683-703
Tu et al., 2005—D. C. Tu, D. Zhang, J. Demas, E. B. Slutsky, I. Provencio, T. E. Holy and R. N. Van Gelder, Physiologic diversity and development of intrinsically photosensitive retinal ganglion cells, Neuron 48 (2005), pp. 987-999
Wald, 1968—G. Wald, The molecular basis of visual excitation, Nature 219 (1968), pp. 800-807
Wassle, 2004—H. Wassle, Parallel processing in the mammalian retina, Nat. Rev. Neurosci. 5 (2004), pp. 747-757
Weleber, 1994—R. G. Weleber, Retinitis pigmentosa and allied disorders. In: S. J. Ryan, Editor, Retina, Mosby, St. Louis, Mo. (1994), pp. 335-466
Werblin and Dowling, 1969—F. S. Werblin and J. E. Dowling, Organization of the retina of the mudpuppy, Necturus maculosus. II. Intracellular recording, J. Neurophysiol. 32 (1969), pp. 339-355
Zemelman et al., 2002—B. V. Zemelman, G. A. Lee, M. Ng and G. Miesenbock, Selective photostimulation of genetically chARGed neurons, Neuron 33 (2002), pp. 15-22
Zrenner, 2002—E. Zrenner, Will retinal implants restore vision?, Science 295 (2002), pp. 1022 1025

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AAV-Mediated Subcellular Targeting of Heterologous Rhodopsins in Retinal Ganglion Cells

Microbial type rhodopsins, such as the light-gated cation-selective membrane channel, channelrhodopsin-2 (Chop2/ChR2) or the ion pump halorhodopsin (HaloR) are expressed in

retinal ganglion cells upon transduction using recombinant AAV vectors. Selective targeting of these transgenes for expression in discrete subcellular regions or sites is achieved by including a sorting motif in the vector that can target either the central area or surround (off-center) area of these cells. Nucleic acid molecules comprising nucleotide sequences encoding such rhodopsins and sorting motifs and their use in methods of differential expression of the transgene are disclosed. These compositions and methods provide significant improvements for restoring visual perception and various aspects of vision, particular in patients with retinal disease.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The present invention in the field of molecular biology and medicine relates to the targeting of microbial-type rhodopsins, such as the light-gated cation-selective membrane channel, channelrhodopsin-2 (Chop2 or ChR2) or the ion pump halorhodopsin (HaloR) in retinal ganglion cells as a basis for restoring visual perception and various aspects of vision.

[0004] 2. Description of the Background Art

[0005] Vision normally begins when rods and cones (photoreceptors) convert light signals to electrical signals that are then relayed through second- and third-order retinal neurons and the optic nerve to the lateral geniculate nucleus and, then to the visual cortex where visual images are formed (Baylor, D, 1996, Proc. Natl. Acad. Sci. USA 93:560-565; Wassle, H, 2004, Nat. Rev. Neurosci. 5:747-57). The severe loss of photoreceptor cells can be caused by congenital retinal degenerative diseases, such as retinitis pigmentosa (RP) (Sung, C H et al., 1991, Proc. Natl. Acad. Sci. USA 88: 6481-85; Humphries, P et al., 1992, Science 256:804-8; Weleber, R G et al., in: S J Ryan, Ed, Retina, Mosby, St. Louis (1994), pp. 335-466), and can result in complete blindness. Age-related macular degeneration (AMD) also results from degeneration and death of photoreceptor cells, which can cause severe visual impairment within the centrally located best visual area of the visual field.

[0006] As rods and cones are lost in humans as well as rodents and other animals, little or no signal is sent to the brain. There are currently no effective treatments or cures for inherited retinal degenerations that cause partial or total blindness.

[0007] Approaches to treatment of retinal degeneration include (1) preservation of remaining photoreceptors in patients with retinal degenerative disease, and (2) replacement of photoreceptors lost to retinal degeneration. For the first approach, neuroprotection with neurotrophic factors (LaVail, M M et al., 1992, Proc. Natl. Acad. Sci. USA 89:11249-53) and virus-vector-based delivery of wild-type genes for recessive null mutations (Acland, G M et al., 2001, Nat. Genet. 28:92-95) have come the furthest—to the point of clinical trials (Hauswirth, W W, 2005, Retina 25, S60; Jacobson, S. Protocol #0410-677, for adeno-associated viral (AAV)-mediated gene replacement therapy in Leber's Congenital Amaurosis (LCA), a specific form of retinal degeneration. This approach is not applicable in patients in advanced stages of retinal degeneration where photoreceptor cells must be replaced. One replacement approach involves transplantation of normal tissue or cells to the diseased retina. Another involves electrical-stimulation of remaining light-insensitive neurons via retinal implants in lieu of the lost cells (prosthetic substitution). Both methods face many obstacles. Hence, there is a continuing need for vision-restoring therapies for inherited blinding disease.

[0008] Histological studies in animal models of photoreceptor degeneration and in postmortem human eyes from patients with almost complete photoreceptor loss due to RP showed preservation of a significant number of inner retinal neurons, making retinal gene therapy a

possible therapeutic option (e.g., U.S. Pat. No. 5,827,702; WO 00/15822 (2000) and WO 98/48097 (1998)).

[0009] Retinal gene transfer of a reporter gene, green fluorescent protein (GFP), using a recombinant AAV (rAAV) was demonstrated in normal primates (Bennett, J et al. 1999 Proc. Natl. Acad. Sci. USA 96, 9920-25). However, the restoration of vision in a blinding disease of animals, particularly in humans and other mammals, caused by genetic defects in retinal pigment epithelium (RPE) and/or photoreceptor cells has not been achieved. Bennett and colleagues have described rescue of photoreceptors by gene therapy in a mutant RPE65 gene model of rapid degeneration of photoreceptors and replacement therapy with the normal gene to replace/supplant the mutant gene. (US Pat Publ 2004/0022766, Acland et al.). This therapy showed some success in a naturally-occurring dog model of human LCA—the RPE65 mutant dog.

[0010] Heterologous expression of Drosophila rhodopsin (Zemelman, B V et al., 2002, Neuron 33:15-22) and melanopsin, the putative photopigment of the intrinsic photosensitive retinal ganglion cells ("ROC") has been reported (Melyan, Z. et al., 2005, Nature 433:741-5; Panda, S. et al., 2005, Science 307:600-604; Qiu, X. et al., 2005, Nature 433:745-9). These photopigments, however, are coupled to membrane channels via a G protein signaling cascade and use cis-isoforms of retinaldehyde as their chromophore. Expression of multiple genes would be required to render photosensitivity and their light response kinetics is rather slow.

[0011] The present inventor's work, including the present invention, utilizes microbial-type rhodopsins that are similar to bacteriorhodopsin (Oesterhelt, D et al., 1973, Proc. Natl. Acad. Sci. USA 70:2853-7), whose conformation change is caused by reversible photoisomerization of their chromophore group, all-trans retinaldehyde, and is directly coupled to ion movement through the membrane (Oesterhelt, D., 1998, Curr. Opin. Struct. Biol. 8:489-500). Two microbial-type opsins, channelopsin-1 and -2 (Chop1 and Chop2), have been cloned from Chlamydomonas reinhardtii (Nagel, G. et al., 2002, Science 296:2395-8; Sineshchekov, O A et al., 2002, Proc. Natl. Acad. Sci. USA 99:8689-94; Nagel, G. et al., 2003, Proc. Natl. Acad. Sci. USA 100, 13940-45) and shown to form directly light-gated membrane channels when expressed in Xenopus laevis oocytes or HEK293 cells in the presence of all-trans retinal. Chop2, a seven transmembrane domain protein, becomes photo-switchable when bound to the chromophore all-trans retinal. Chop2 is particularly attractive because its functional light-sensitive channel, channelrhodopsin-2 (Chop2 retinalidene abbreviated ChR2) with the attached chromophore is permeable to physiological cations. Unlike animal rhodopsins, which only bind the 11-cis conformation, Chop2/ChR2 binds all-trans retinal isomers, obviating the need for all-trans to 13-cis isomerization supplied by the vertebrate visual cycle.

[0012] However, the long-term compatibility of expressing ChR2 in native neurons in vivo in general and the properties of ChR2-mediated light responses in retinal neurons in particular remained unknown until the work of the present inventor and colleagues. Indeed their work (and that of others) represent the pioneering demonstration of the (a) feasibility of restoring light sensitivity to a degenerate retina, (b) transmission of light-driven information to higher visual centers, and mediation of visually guided behaviors through such prosthetic interventions. This work proved that the insertion of such "optical neuromodulators" or "light sensors" as ChR2 into normally photo-insensitive retinal neurons is a promising approach for restoring sight to profoundly blind individuals. These strategies included the delivery of the directly photosensitive cation channel ChR2 and the photosensitive chloride pump halorhodopsin (abbreviated herein "HaloR" and elsewhere "NpHR" or "eNpHR" because of its origin from Natronobacterium pharaonis (Lanyi, J K et al. J. Biol. Chem. 265:1253-1260 (1990). Such work has been reported by the present inventor's group (Bi, A. et al., Neuron 50:23-33 (2006), Ivanova, E et al., Mol. Vis. 15:1680-9 (2009), Zhang, Y. et al., J Neurosci. 29:9186-96 (2009), primarily with ChR2. Others have delivered and expressed ChR2 (Lagali

et al., Nat. Neurosci. 11:667-675 (2008); NpHR by (Busskamp V. et al., Science 329, 413-417 (2010); synthetically engineered potassium (SPARK) and glutamate (LiOluR) channels (Greenberg, K P et al., Invest. Ophthalmol. Vis. Sci. 47, 4750 (2006; abstract); Kolstad et al., Invest. Ophthalmol. Vis. Sci 49:3897 (2009; Abstract) and the G protein-coupled receptor melanopsin (Lin, B. et al., Proc. Natl. Acad. Sci. USA 105:16009-16014 (2008)) in normally nonphotosensitive bipolar, amacrine, and ganglion cells or nonfunctional photoreceptors.

[0013] The present inventor and colleagues (Bi, A. et al., Neuron 50:23-33 (2006); WO2007/131180) disclosed adeno-associated virus (AAV2)-mediated expression of exogenously delivered light-gated membrane cation channel, ChR2, or light-driven chloride ion pump, HaloR, in inner retinal neurons and demonstrated that expression of ChR2 in surviving inner retinal neurons of a mouse with photoreceptor degeneration can restore the ability of the retina to encode light signals and transmit the light signals to the visual cortex.

[0014] The present inventor and colleagues (Zhang, Y. et al., J Neurosci. 29:9186-96 (2009 Jul. 22). reported that the expression HaloR can effectively restore OFF responses in inner retinal neurons of mice with retinal degeneration. HaloR-expressing RGCs respond to light with rapid hypopolarization and suppression of spike activity. After termination of the light stimulus, their membrane potential exhibited a rapid rebound overshoot with robust sustained or transient spike firing. Coexpression of ChR2/HaloR in RGCs produced ON, OFF, and even ON-OFF responses, depending on the wavelength of the light stimulus. Suggesting that the expression of multiple microbial rhodopsins such as ChR2 and HaloR is a possible strategy to restore both ON and OFF light responses in the retina after the death of rod and cone photoreceptors.

[0015] The present invention is a refinement and significant step forward of the inventor's prior work, being directed to differential, subcellular “site-selective expression” of these light-sensor-encoding nucleic acids by adding sorting or targeting motifs to the vectors that confer such selectivity. This adds to the “spatial resolution” of vision restoration achieved in this manner in those suffering vision loss or blindness caused, for example, by any of a number of retinal degenerative diseases. The present inventor's approach does not require, introducing exogenous cells and tissues or physical devices, thus avoiding obstacles encountered by existing approaches, though the combined use of the present approach with visual prostheses or devices is also envisioned.

SUMMARY OF THE INVENTION

[0016] The present inventor has discovered that differentially targeted expression of ChR2 and HaloR to different subcellular regions in RGCs recreates the antagonistic center-surround receptive field in these cells that further permits improvement of the visual spatial processing for restored vision. The primary spatial distinction of expression is in center vs. peripheral regions of the cells. Peripheral is also referred to in the art as the “surround” or as “off center,” terms that are well understood.

[0017] RGCs are rendered light sensitive by expression of ChR2 and/or HaloR selectively in somatodendritic region while being kept to a minimum in the axonal region. This enables maintenance of visual spatial processing. This is based on the discovery that a number of “sorting motifs” also referred to here as “targeting motifs, “sorting sequences” or “targeting sequences” present in a vector that comprises the light sensor encoding nucleic acid. Such a motif mediates site- or region-selective expression of the ChR2 or HaloR in subcellular regions of a retinal neuron, preferably an RGC. This targeting serves as a basis for enhanced spatial control and specificity, and results in transmission of appropriate signals, providing better contrast, which more closely resembling signals from a healthy, intact retina, to higher centers of the visual cortex to compensate for damage and degeneration in retinal

photoreceptors.

[0018] The present invention is directed to a nucleic acid molecule encoding a rhodopsin for differential expression in subcellular regions of a retinal neuron, preferably an RGC, which molecule comprises:
(a) a first nucleotide sequence encoding a light-gated channel rhodopsin or a light-driven ion pump rhodopsin;
(b) linked in frame to (a), a second nucleotide sequence encoding a peptide or polypeptide sorting motif; and
(c) operatively linked to (a) and (b), a promoter sequence, and optionally, transcriptional regulatory sequences; and
(d) a polyadenylation sequence preferably from bovine growth hormone (bGHpolyA).

[0023] Preferably the nucleic promoter and regulator sequence comprise a cytomegalovirus enhancer/chicken ß-actin promoter (CAG), preferably SEQ ID NO:26, and woodchuck hepatitis virus posttranscriptional regulatory element, preferably SEQ ID NO:27, and (d) is preferably SEQ ID NO:28.

[0024] The nucleic acid molecule may further comprise, linked in frame with (a) and (b), a third nucleotide sequence encoding a reporter polypeptide, preferably GFP; a preferred sequence is SEQ ID NO:25.

[0025] In the above nucleic acid molecule, the light-gated channel rhodopsin is preferably ChR2, such as SEQ ID:22-, or a biologically active fragment, most preferably SEQ ID NO: 22. The light driven ion pump rhodopsin is preferably HaloR, most preferably SEQ ID NO:24.

[0026] In one embodiment of the above nucleic acid molecule, the sorting motif is one that targets the center of the neuron's receptive field, for example, to one or more of the following subcellular regions: the soma, the proximal dendritic region, or the axon initial segment. Preferred sorting motif-encoding sequences are a nucleotide sequence encoding (a) voltage-gated potassium channel 2.1 (Kv2.1), which is or comprises SEQ ID NO:1; or (b) the ankyrin binding domain of voltage-gated sodium channel 1.6 (Nav1.6), which is or comprises SEQ ID NO:3. The encoded amino acid sequence of the motif is preferably (a) the sequence of Kv2.1, which is or comprises SEQ ID NO:2; or (b) the sequence of the ankyrin-binding domain of Nav1.6, which is or comprises SEQ ID NO:4.

[0027] In another preferred embodiment of the above nucleic acid molecule, the motif is one that targets the rhodopsin (±the reporter gene) to the surround or off-center part of the neuron's receptive field, for example, to the somatodendritic region of the neurons. Preferred sorting motif-encoding sequences are a nucleotide sequence encoding (a) the cytoplasmic C-terminal segment of neuroligin-1 (NLG-1), which is or comprises SEQ ID NO:5; or (b) the myosin binding domain of melanophilin (MLPH), which is or comprises SEQ ID NO:7. The encoded amino acid sequence of the motif is preferably (a) the sequence of the cytoplasmic C-terminal segment of NLG-1 which is or comprises, SEQ ID NO:6; or (b) the sequence of the myosin-binding domain of MLPH, which is or comprises SEQ ID NO:8.

[0028] Also provided is a recombinant adeno-associated virus expression vector, preferably an AAV2 vector, comprising any of the above nucleic acid molecules. In the vector, the sequence of the nucleic acid molecule is flanked at its 5' end by a 5' inverted terminal repeat (ITR) and at its 3' end by a 3' ITR of the AAV, preferably AAV2. The sequence of these ITR is preferably SEQ ID NO:17 and SEQ ID NO:18, respectively.

[0029] As above, in one embodiment of the expression vector, the sorting motif is one that targets the center of the neuron's receptive field. A preferred nucleotide sequence encoding the

motif is (a) the sequence encoding Kv2.1, which is or comprises SEQ ID NO:1; or (b) the sequence encoding the ankyrin binding domain of Nav1.6, which is or comprises SEQ ID NO:3. Preferably, in the expression vector, the amino acid sequence of the encoded motif is (a) the acid sequence of Kv2.1, which is or comprises SEQ ID NO:3; or (b) the sequence of the ankyrin binding domain of Nav1.6, which is or comprises SEQ ID NO:4.

[0030] In another embodiment of the expression vector, the sorting motif is one that targets the surround or off-center of the neuron's receptive field. Here, the motif is selected from the group consisting of nucleotide sequence encoding (a) the cytoplasmic C-terminal segment of NLG-1, which is or comprises SEQ ID NO:5; or (b) myosin binding domain of MLPH, which is or comprises SEQ ID NO:7. Preferably, in the expression vector, the amino acid sequence of the encoded motif is (a) the sequence of the cytoplasmic C-terminal segment NLG-1, which is or comprises SEQ ID NO:6; or (b) the sequence of the myosin-binding domain of MLPH, which is or comprises SEQ ID NO:8.

[0031] The above expression vector can have one of the following schematic structures:

[0000]
(a) 5'-ITR-CAG-ChR2-GFP-{Motif}-WPRE-bGHpolyA-ITR-3'
(b) 5'-ITR-CAG-ChR2-{Motif}-WPRE-bGHpolyA-ITR-3'
(c) 5'-ITR-CAG-HaloR-GFP-{Motif}-WPRE-bGHpolyA-ITR-3'
(d) 5'-ITR-CAG-HaloR-{Motif}-WPRE-bGHpolyA-ITR-3'
wherein {Motif} is nucleotide sequence encoding the sorting motif, and wherein, any two or more of ChR2, GFP and Motif or HaloR, GFP and Motif, are linked in-frame. In the foregoing, vector, the Motif is preferably selected from the group consisting of

(i) the nucleotide sequence encoding Kv2.1, which is or comprises SEQ ID NO:1; or
(ii) the nucleotide sequence encoding the ankyrin binding domain of Nav1.6, which is or comprises SEQ ID NO:3.
(iii) the nucleotide sequence encoding cytoplasmic C-terminal segment of NLG-1, which is or comprises SEQ ID NO:5; or
(iv) the nucleotide sequence encoding myosin binding domain of MLPH, which is or comprises SEQ ID NO:7.

[0036] A preferred expression vector for targeting ChR2 to the center of the neuron's receptive field has the schematic structure and nucleotide sequence selected from the following group

[0000]
SEQ ID NO: 32
(a) 5'-ITR-CAG-ChR2-GFP-{Kv2.1 Motif)-WPRE-bGHpolyA-ITR-3',;
SEQ ID NO: 33
(b) 5'-ITR-CAG-ChR2-{Kv2.1 Motif)-WPRE-bGHpolyA-ITR-3',;
SEQ ID NO: 34
(c) 5'-ITR-CAG-ChR2-GFP-{Nav2.6 Motif)-WPRE-bGHpolyA-ITR-3',
and;
SEQ ID NO: 35
(d) 5'-ITR-CAG-ChR2-{Nav2.6 Motif)-WPRE-bGHpolyA-ITR-3',.

[0037] A preferred expression vector for targeting ChR2 to the surround or off-center of the neuron's receptive field has the schematic structure and nucleotide sequence selected from the following group

[0000]
SEQ ID NO: 36
(a) 5'-ITR-CAG-ChR2-GFP-{NLG-1 Motif)-WPRE-bGHpolyA-ITR-3',;
SEQ ID NO: 37
(b) 5'-ITR-CAG-ChR2-{NLG-1 Motif)-WPRE-bGHpolyA-ITR-3',;
SEQ ID NO: 38
(c) 5'-ITR-CAG-ChR2-GFP-{MLPH Motif)-WPRE-bGHpolyA-ITR-3',
and;
SEQ ID NO: 39
(d) 5'-ITR-CAG-ChR2-{MLPH Motif)-WPRE-bGHpolyA-ITR-3',.

[0038] A preferred expression vector targeting HaloR to the center of the neuron's receptive field has the schematic structure and nucleotide sequence selected from the following group:

[0000]
SEQ ID NO: 40
(a) 5'-ITR-CAG-HaloR-GFP-{Kv2.1 Motif)-WPRE-bGHpolyA-ITR-3',;
SEQ ID NO: 41
(b) 5'-ITR-CAG-HaloR-{Kv2.1 Motif)-WPRE-bGHpolyA-ITR-3',;
SEQ ID NO: 42
(c) 5'-ITR-CAG-HaloR-{Nav2.6 Motif)-WPRE-bGHpolyA-ITR-3',
and;
SEQ ID NO: 43
(d) 5'-ITR-CAG-HaloR-GFP-{Nav2.6 Motif)-WPRE-bGHpolyA-ITR-3',;
[0039] A preferred expression vector for targeting HaloR to the surround or off-center of the neuron's receptive field has the schematic structure and nucleotide sequence selected from the following group
[0000]
SEQ ID NO: 44
(a) 5'-ITR-CAG-HaloR-GFP-{NLG-1 Motif)-WPRE-bGHpolyA-ITR-3',;
SEQ ID NO: 45
(b) 5'-ITR-CAG-HaloR-{NLG-1 Motif)-WPRE-bGHpolyA-ITR-3',;
SEQ ID NO: 46
(c) 5'-ITR-CAG-HaloR-GFP-{MLPH Motif)-WPRE-bGHpolyA-ITR-3',
and;
SEQ ID NO: 47
(c) 5'-ITR-CAG-HaloR-{MLPH Motif)-WPRE-bGHpolyA-ITR-3',.

[0040] Preferably the above expression vector further comprises AAV vector backbone nucleotide sequence SEQ ID NO:29 linked to the 3' end of the AAV 3'ITR sequence.

[0041] The present invention is directed to a method of restoring light sensitivity to a retina, comprising:

(a) delivering to retinal neuron, preferably an RGC, a nucleic acid expression vector that encodes
(i) a light-gated channel rhodopsin or a light-driven ion pump rhodopsin;
(ii) a sorting motif that targets (i) to be expressed in selected subcellular regions of the neurons;
(iii) optionally, a reporter polypeptide; and
(iv) operatively linked to (i), (ii) and (iii) a promoter sequence, and optionally, transcriptional regulatory sequences; and
(b) expressing the vector in the neurons,
wherein the expression of the sorting motif with the rhodopsin results in selected expression of the rhodopsin and, when present, the reporter polypeptide, in subcellular regions of the RGC for which the motifs are selective, thereby restoring the light sensitivity.

[0048] Also provided is a method of selectively expressing a light-gated channel rhodopsin or a light-driven ion pump rhodopsin in a desired subcellular site or sites of a retinal neuron, preferably an RGC, comprising

a) delivering to the RGC a nucleic acid molecule or expression vector that encodes
(i) a light-gated channel rhodopsin, preferably ChR2, or a light-driven ion pump rhodopsin, preferably HaloR;
(ii) a sorting motif that targets the rhodopsin to be expressed in the desired site or sites;
(iii) operatively linked to (i) and (ii) a promoter sequence, and optionally, transcriptional regulatory sequences; and
(b) expressing the vector in the desired sites of the RGC.

[0054] In one embodiment of the method, the desired subcellular site is soma, proximal dendritic region, or axon initial segment, where preferably the motif is one that targets the rhodopsin to the center of the RGCs receptive field.

[0055] In another embodiment of the method, the desired subcellular site is the somatodendritic region, where preferably the motif is one that targets the surround or off-center of the RGCs receptive field.

[0056] In all the above methods, the nucleic acid molecule comprises any of the molecules above and the vector is the any of expression vectors above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0057] FIG. 1 is a group of photomicrographs comparing fluorescence intensity (originally green, converted to white, on black background) from green fluorescent protein (GFP) encoded in frame with ChR2 with or without (control) a sorting motif. The sorting motifs tested, as indicated in abbreviated form in the panels (described in more detail elsewhere in this document), were: Kv2.1, Nav1.6, AMPAR, Kv4.2, MLPH, nAchR, NGL-1 AND TLCN. The arrow-heads in each panel point to the axon of the ChR2-GFP expressing RGCs. The results appear in tabular form in Table 2, below.

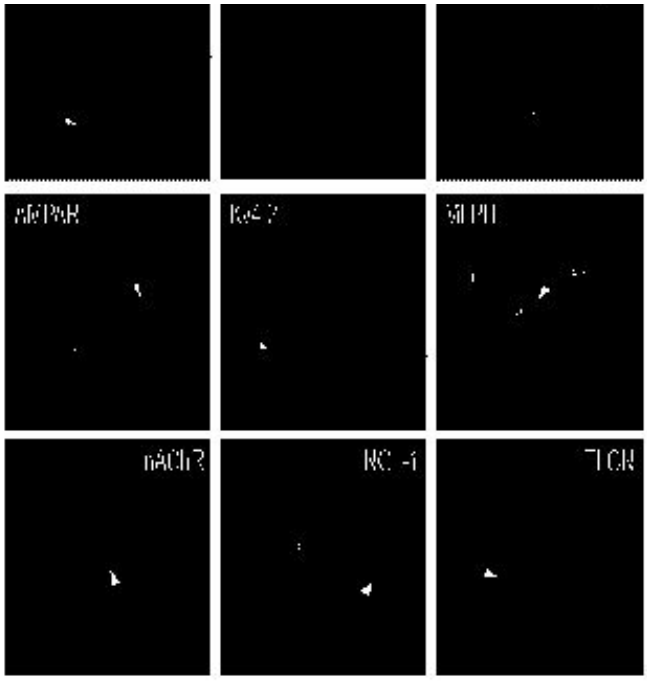

FIG. 1

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0058] The present inventors discovered that certain protein sorting motifs used in AAV-mediated transduction direct targeted expression of Chop2 or HaloR or, for visualization, a test reporter gene (Green fluorescent protein, GFP) to RGCs results in differential expression of the targeted reporter gene in different compartments or subcellular sites of the RGCs.

[0059] The present Examples show differential expression of ubiquitously expressing light sensitive channels, namely ChR2 driven by the CAG promoter and under the influence of various targeting motifs in distinct subcellular regions or sites of retinal ganglion cells.

[0060] However, targeting of depolarizing membrane channels, such as ChR2, to the ON-type retinal neurons might result in better useful vision.

[0061] In addition, expression of light sensors in more distal retinal neurons, such as bipolar cells, would utilize the remaining signal processing functions of the degenerate retina.

[0062] By expressing a depolarizing light sensor, such as ChR2, in ON type retinal neurons (ON type ganglion cells and/or ON type bipolar cells) and expressing a hypopolarizing light sensor, such as HaloR (a chloride pump) (Han, X et al., 2007, PLoS ONE, March 21; 2:e299; Mang, F et al., 2007; Nature 446:633-9; present inventors' results) in OFF type retinal neurons (OFF type ganglion cells and/or OFF type bipolar cells) could create ON and OFF pathways in photoreceptor degenerated retinas.

[0063] According to the present invention, the followings approaches used to restore the light sensitivity of inner retinal neurons are enhanced by the use, disclosed herein, of peptide/polypeptide sorting motifs expressed using recombinant vectors in selected subcellular sites/regions of retinal neurons, particularly RGC.

[0064] (1) Ubiquitously expressing light sensitive channels, such as ChR2, are employed to produced membrane depolarization in all types of ganglion cells (both ON and OFF ganglion cells), or all types of bipolar cells (rod bipolar cells, and ON and OFF cone bipolar cells). The

AAV vector with CAG promoter has already partially achieved this approach in rodent retinas, as exemplified herein.

[0065] (2) A depolarizing light sensor, such as ChR2, is targeted to ON type retinal neurons such as ON type ganglion cells or ON type bipolar cells. Fragments of a human gap junctional protein (connexin-36) promoter were found to target GFP in ON-type retinal ganglion cells by using AAV-2 virus vector (Greenberg K P et al., 2007, ARVO abstract, 2007). A readily packable shorter version of mGluR6 promoter of (<2.5 kb) would allow targeting of ChR2 to ON type bipolar cells (both rod bipolar cells and ON type cone bipolar cells).

[0066] (3) Cell specific promoters are used to target the specific types of retinal neurons. A promoter that could target rod bipolar cells is Pcp2 (L7) promoter (Tomomura, M et al., 2001, Eur J. Neurosci. 14:57-63). The length of the active promoter is preferably less that 2.5 Kb so it can be packaged into the AAV viral cassette.

[0067] (4) A depolarizing light sensor, such as ChR2, is targeted to ON type ganglion cells or ON type cone bipolar cells and a hypopolarizing light sensor, such as halorhodopsin, to OFF type ganglion cells or OFF type cone bipolar cells to create ON and OFF pathways. As described above, an adequately short (packable) version of mGluR6 promoter (<2.5 kb) would allow targeting of ChR2 to ON type bipolar cells. The Neurokinin-3 (NK-3) promoter would be used to target halorhodopsin to OFF cone bipolar cells (Haverkamp, S et al., 2002, J. Compar. Neurol. 455.463-76.

[0068] (5) A depolarizing light sensor, such as ChR2, is targeted to rod bipolar cells and their target AII amacrine cells, an ON type retinal cell (which communicate with ON and OFF cone bipolar cells).

[0069] Sorting Motifs

[0070] Table 1 describes the sorting peptide/polypeptide motifs examined by the present inventors presenting both the nucleotide and amino acid sequences, and a conclusion about their effects on sorting or targeting of the linked encoded proteins to different subcellular sites.

[0000]

TABLE 1

Description of Sorting Motifs.

Name	Source Protein (ref)	Sorting Motif	Subcellular Targeted Site (Receptive Field)
Kv2.1	Voltage-gated potassium channel 2.1<1>	Cytoplasmic C-terminus	Proximal dendrites, somata (center)
aa sequence: (SEQ ID NO: 2)		nt sequence: (SEQ ID NO: 1)	
QSQPILNTKEMAPQSKPPEELEMSSM		CAG TCT CAG CCC ATC CTG AAC ACT AAG GAG ATG GCC	
PSPVAPLPARTEGVIDMRSMSSIDSF		CCT CAG AGT AAA CCC CCT GAG GAA CTG GAA ATG AGC	
ISCATDFPEATRF (65)		TCC ATG CCA TCT CCA GTG GCT CCT CTG CCA GCT AGG ACC GAG GGC GTG ATT GAC ATG AGA GAC ATG TCT AGT ATC GAT AGC TTC ATT TCC TGC GCC ACC GAC TTC CCC GAA GCT ACA AGG TTT	
Nav1.6	Voltage-gated sodium channel 1.6<2,3>	Ankyrin binding domain	Axon initial segment, somata (center)
aa sequence: (SEQ ID NO: 4)		nt sequence: (SEQ ID NO: 3)	
TVRVPIAVGE SDFENLNTED		ACC GTG AGG GTG CCC ATC GCC GTG GGC GAG	

AGC GAC
VSSESDP (27) TTC GAG AAC CTG AAC ACC GAG GAC GTG AGC AGC GAG
AGC GAC CCC
Neuroligin-1<4>
NLG-1 Cytoplasmic Somatodendrtic
C-terminal (surround = off center)
aa sequence: (SEQ ID NO: 6) nt sequence: (SEQ ID NO: 5)
VVLRTACPPDYTLAMRRSPDDVPLMT GTG GTG CTG AGG ACT GCC TGC CCC CCT GAC TAC ACC
PNTITM (31) CTG GCT ATG AGG AGA AGC CCA GAC GAT GTG CCC CTG ATG ACC CCC AAC ACC ATC ACA ATG
Melanophilin<5>
MLPH Myosin binding Somatodendritic
domain (surround = off center)
aa sequence: (SEQ ID NO: 8) nt sequence: (SEQ ID NO: 7)
RDQPLNSKKKKRLLSFRDVDFEEDSD AGG GAC CAG CCT CTG AAC AGC AAA AAG AAA AAG AGG
(26) CTC CTG AGC TTC AGG GAC GTG GAC TTC GAG GAG GAC AGC GAC
nAchR Nicotinic acetylcholine Tyrosine-Dileucine Somatodendriticreceptor a7 subunit<6>
(surround = off center)
aa sequence: (SEQ ID NO: 10) nt sequence: (SEQ ID NO: 9)
GEDKVRPACQHKPRRCALASVELSAG GGC GAG GAC AAG GTG CGG CCC GCC TGT CAG CAC AAG
AGPPTSNGNLLYIGFRGLEGM (47) CCT CGG CGG TGC AGC CTG GCC AGC GTG GAG CTG AGC
GCC GGC GCC GGC CCA CCC ACC AGC AAC GGC AAC CTG
CTG TAC ATC GGC TTC AGA GGC CTG GAG GGC ATG
Kv4.2 Voltage-gated potassium Dileucine Somatodendriticchannel 4.2<7>
(surround = off center)
aa sequence: (SEQ ID NO: 12) nt sequence: (SEQ ID NO: 11)
FEQQHHHLLH CLEKTT (16) TTC GAG CAG CAG CAC CAC CAC CTG CTG CAC TGC CTG
GAG AAG ACC ACC
Telencephalin<8>
TLCN Phenylalanine-based Somatodendritic
(surround = off-center)
aa sequence: (SEQ ID NO: 14) Nucleotide sequence: (SEQ ID NO: 13)
QSTACKKGEYNVQEAESSGEAVCLNG CAG AGC ACA GCC TGC AAA AAG GGC GAG TAC AAC GTG
AGGGAGGAAGAEGGPEAAGGAAESPA CAG GAA GCT GAG AGC TCT GGC GAA GCC GTG TGT CTG
EGEVFAIQLTSA (65) AAC GGC GCC GGA GGC GGT GCC GGC GGA GCT GCC GGC
GCT GAG GGT GGC CCT GAG GGC GCT GGA GGT GCC GCT
GAG AGC CCC GCT GAG GGC GAA GTC TTT GCC ATC CAG
CTG ACA TCT GCT
AMPAR AMPA receptor GluR1 Cytoplasmic Somatodendriticsubunit<9>
C-terminal (surround = off-center)
aa sequence: (SEQ ID NO: 16) Nucleotide sequence: (SEQ ID NO: 15)
EFCYKSRSESKRMKGFCLIPQQSINE GAG TTC TGC TAC AAG AGC AGG TCC GAA TCT AAG AGA
AIRTSTLPRNSGA (39) ATG AAA GGC TTT TGT CTG ATC CCC CAG CAG AGC ATC AAC GAG GCC ATT CGG ACC AGT ACA CTG CCT CGC AAT

AGC GGA GCT
(Legend to Table 1)
Name: Each sorting motif was named based on the “source protein” from which it was derived.
Motif: the functional name or location of each motif.
Subcellular targeted site: the reported site of preferential subcellular targeting.
Receptive Field: the central vs. surround (off-center or peripheral) region of the cell
Superscripted numbers refer to the following references:<1>
Lim ST, et al. . Neuron. 25: 385-97 (2000).<2>

Garrido, J. et al. Science 300: 2091 (2003).<3>

Bioko, T. et al., J. Neurosci. 232306-2313 (2003).<4>

Rosales, C. et al. Eur. J. Neurosci. 22, 2381-2386 (2005).<5>

Lewis, T. et al. Nat. Neurosci. 12, 568-576 (2009).<6>

Xu, J. et al. J. Neurosci. 26: 9780-9793 (2006).<7>

Rivera, J. et al. Nat. Neurosci. 6: 243-250 (2003).<8>

Mitsui, S. et al., J. Neurosci. 25: 1122-1131 (2005).<9>

Dotti, F. et al., J. Neurosci. 20: 1-5 (2000).

Name: Each sorting motif was named based on the protein from which it was derived.

[0071] The functional consequence of expressing ubiquitously expressing light sensitive channels, namely ChR2, in RGC by CAG promoter, coupled with the targeting to selected subcellular sites suggest that this will contribute to restoring useful vision. However, targeting of depolarizing membrane channels, such as ChR2, to ON-type retinal neurons might result in better useful vision. By expressing a depolarizing light sensor, such as ChR2, in the desired subcellular regions of ON type retinal neurons (ON type RGC and/or ON type bipolar cells) and expressing a hypopolarizing light sensor, such as HaloR in selected subcellular sites of OFF type retinal neurons (OFF type RGC and/or OFF type bipolar cells) could create even more useful ON and OFF pathways in photoreceptor degenerated retinas that is possible without the selective targeting mediated by the sorting motifs described here. A preferred embodiment would be:

[0072] (1) By employing a “center-targeting” motif, such as Kv2.1 or Nav1.6, target ChR2 to the center receptive field of ON RGC, while targeting HaloR to the surround (-Off-center) of such cells using motifs such as NLG-1 or MLPH. Activation by light of such cells would result in depolarization (stimulation) of the center and hypopolarization (inhibition) of the surround.

[0073] (2) By employing a “center-targeting” motif, such as Kv2.1 or Nav1.6, target HaloR to the center receptive field of OFF RGC, while targeting ChR2 to the surround of such cells using motifs such as NLG-1 or MLPH. Activation by light of such cells would result in inhibition of the center and stimulation of the surround.

[0074] Such combined treatment would enhance not only signal transmission but contrast and hence visual resolution in such molecularly enhanced or modified cells. This more closely resembles the physiological effects of signals transmitted to these cells by retinal

photoreceptors in a normal vision state. Such specificity and selectivity would be aided by the use of ON cell-specific promoters and OFF cell-specific promoters compared to the ubiquitous promoters exemplified here. Once such promoters are identified, they would be inserted into the various vectors described here in place of CAG. Use of the present composition and methods

Vectors

[0075] According to the various embodiments of the present invention, a variety of known nucleic acid vectors may be used in these methods, e.g., recombinant viruses, such as recombinant adeno-associated virus (rAAV), recombinant adenoviruses, recombinant retroviruses, recombinant poxviruses, and other known viruses in the art, as well as plasmids, cosmids and phages, etc. Many publications well-known in the art discuss the use of a variety of such vectors for delivery of genes. See, e.g., Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, latest edition; Kay, M A. et al., 2001, Nat. Med., 7:33-40; and Walther W et al., 2000, Drugs 60:249-71). Methods for assembly of the recombinant vectors are well-known. See, for example, WO00/15822 and other references cited therein, all of which are incorporated by reference. There are advantages and disadvantages to the various viral vector systems. The limits of how much DNA can be packaged is one determinant in choosing which system to employ. rAAV tend to be limited to about 4.5 kb of DNA, whereas lentivirus (e.g., retrovirus) system can accommodate 4-5 kb.

[0076] AAV Vectors

[0077] Adeno-associated viruses are small, single-stranded DNA viruses which require a helper virus for efficient replication (Berns, K I, Parvoviridae: the viruses and their replication, p. 1007-1041 (vol. 2), in Fields, B N et al., Fundamental Virology, 3rd Ed., (Lippincott-Raven Publishers, Philadelphia (1995)). The 4.7 kb genome of AAV has two inverted terminal repeats (ITR) and two open reading frames (ORFs) which encode the Rep proteins and Cap proteins, respectively. The Rep reading frame encodes four proteins of molecular weights 78, 68, 52 and 40 kDa. These proteins primarily function in regulating AAV replication and rescue and integration of the AAV into the host cell chromosomes. The Cap reading frame encodes three structural proteins of molecular weights 85 (VP 1), 72 (VP2) and 61 (VP3) kDa which form the virion capsid (Berns, supra). VP3 comprises >80% of total AAV virion proteins.

[0078] Flanking the rep and cap ORFs at the 5' and 3' ends are 145 bp ITRs, the first 125 bp's of which can form Y- or T-shaped duplex structures. The two ITRs are the only cis elements essential for AAV replication, rescue, packaging and integration of the genome. Two conformations of AAV ITRs called “flip” and “flop” exist (Snyder, R O et al., 1993, J. Virol., 67:6096-6104; Berns, K I, 1990 Microbiol Rev, 54:316-29). The entire rep and cap domains can be excised and replaced with a transgene such as a reporter or therapeutic transgene (Carter, B J, in Handbook of Parvoviruses, P. Tijsser, ed., CRC Press, pp. 155-68 (1990)).

[0079] AAVs have been found in many animal species, including primates, canine, fowl and human (Murphy, F A et al., The Classification and Nomenclature of Viruses: Sixth Rept of the Int'l Comm on Taxonomy of Viruses, Arch Virol, Springer-Verlag, 1995). Six primate serotypes are known (AAV1, AAV2, AAV3, AAV4, AAV5 and AAV6) (and more are known that infect other classes of mammals)

[0080] The AAV ITR sequences and other AAV sequences employed in generating the minigenes, vectors, and capsids, and other constructs used in the present invention may be obtained from a variety of sources. For example, the sequences may be provided by any of the above 6 AAV serotypes or other AAV serotypes or other densoviruses, including both presently known human AAV and yet to yet-to-be-identified serotypes. Similarly, AAVs

known to infect other animal species may be the source of ITRs used in the present molecules and constructs. Capsids from a variety of serotypes of AAV may be combined in various mixtures with the other vector components (e.g., WO01/83692 (Hildiger et al.; U.S. Pat. No. 7,056,502; US Pat Pub. 2003/0013189 (Wilson et al.,), Indeed there are advantages to various virion types related to their vulnerability to pre-existing immunity in humans, the efficiency of transduction, and/or duration of expression. Thus it may be preferable to use pseudotyped, rAAV virions wherein the rAAV2 ITRs described herein are combined with AAV5 capsid proteins. Such constructs may be advantageous because humans are less likely to have been pre-exposed to AAV5 vs. AAV2, and therefore are less likely to have immunological memory (e.g., circulating antibodies or capsid-specific T lymphocytes). For other descriptions of the use of various of these rAAV virions, see, for example, WO2005/021768 (Tak et al.); Adriaansen, J et al., Ann Rheum Dis 2005, 64:1677-1684; US Pat. Pub. 2004-072351 (Womer et al.); U.S Pat. Pub. 2005/0255089 (Chiorini et al.), Adriaansen, J et al., Ann Rheum Dis 2005, 64:1677-1684, all of these references concerning rAAV are incorporated by reference in their entirety. In general, while rAAV vectors have been exemplified herein, the present invention includes AAV2 ITR's combined with capsid proteins of any of 6 known primate AAV serotypes. It is also known in the art that certain mutations in capsid proteins can enhance transfection efficiency, and it would within the ordinary skill of the art to test and select appropriate mutations for use in the present invention. Many of these viral strains or serotypes are available from the American Type Culture Collection (ATCC), Manassas, Va., or are available from a variety of other sources (academic or commercial).

[0081] It may be desirable to synthesize sequences used in preparing the vectors and viruses of the invention using known techniques, based on published AAV sequences, e.g., available from a variety of databases. The source of the sequences utilized to prepare the present constructs is not considered to be limiting. Similarly, the selection of the AAV serotype and species (of origin) is within the skill of the art and is not considered limiting

[0082] The rAAV Minigene or Cassette

[0083] As used herein, the rAAV construct (e.g., a minigene or cassette) is packaged into a rAAV virion. At minimum, the rAAV minigene is formed by AAV ITRs and a heterologous nucleic acid molecule for delivery to a host cell. Most suitably, the minigene comprises ITRs, most preferably AAV2 ITRs, located 5' and 3' to the heterologous sequence (rhodopsin protein and targeting sequence) being expressed. Vectors comprising 5' ITR and 3' ITR sequences arranged in tandem, e.g., 5' to 3' or a head-to-tail, or in another configuration may also be useful. Other embodiments include a minigene with multiple copies of the ITRs, or one in which 5' ITRs (or conversely, 3' ITRs) are located both 5' and 3' to the heterologous sequence. The ITRs sequences may be located immediately upstream and/or downstream of the heterologous sequence; intervening sequences may be present. As noted, the preferred ITRs are from AAV2, but they may also originate from AAV5 or from any other AAV serotype. Moreover, the present construct or minigene may include 5' ITRs from one serotype and 3' ITRs from another.

[0084] The AAV sequences used are preferably the 140145 bp cis-acting 5' and 3' ITR sequences (e.g., Carter, B J, supra). Preferably, the entire ITR sequence is used, although minor modifications are permissible. The most ITR's used in the present examples are

[0000]

5' ITR:
(SEQ ID NO: 17)
cctgcaggca gctgcgcgct cgctcgctca ctgaggccgc ccgggcaaag cccgggcgtc
gggcgacctt tggtcgcccg gcctcagtga gcgagcgagc gcgcagagag ggagtggcca

actccatcac tagggttcc t 141

3' ITR:
(SEQ ID NO: 18)
aggaacccct agtgatggag ttggccactc cctctctgcg cgctcgctcg ctcactgagg
ccgggcgacc aaaggtcgcc cgacgcccgg gctttgcccg ggcggcctca gtgagcgagc
gagcgcgcag ctgcctgcag g 141

[0085] Methods for modifying these ITR sequences are well-known (e.g., Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 3<rd >Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 2001; Brent, R et al., eds., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 2003; Ausubel, F M et al., eds., Short Protocols in Molecular Biology, 5thedition, Current Protocols, 2002; Carter et al., supra; and Fisher, K et al., 1996 J. Virol. 70:520-32). It is conventional to engineer the rAAV virus using known methods (e.g., Bennett, J et al. 1999, supra).

[0086] An example of such a molecule employed in the present invention is a “cis-acting” plasmid containing the heterologous sequence, preferably the ChR2 (any of SEQ ID NO:30-39) or HaloR sequence (any of SEQ ID NO:40-47, with or without an in-frame GFP sequence, with an in-frame sorting motif, promoter/regulatory sequences, all flanked by the 5' and 3' AAV ITR sequences.

[0087] The heterologous sequence encodes a protein or polypeptide which is desired to be delivered to and expressed in a cell and a targeting motif that differentially targets the polypeptide to particular subcellular regions of the cell, preferably an RGC.

[0088] The Transgene(s) Being Targeted and Expressed

[0089] In a most preferred embodiment, the heterologous sequence is a nucleic acid molecule that functions as a transgene. The term “transgene” as used herein refers to a nucleic acid sequence heterologous to the AAV sequence, and encoding a desired product, preferably ChR2 or HaloR plus the sorting motif, and the regulatory sequences which direct or modulate transcription and/or translation of this nucleic acid in a host cell, enabling expression in such cells of the encoded product. Preferred polypeptide products are those that can be delivered to the eye, particularly to retinal neurons, most preferably to RGC.

[0090] The transgene/targeting sequence is delivered and differentially expressed in selected subcellular sites as directed by the sorting motif, in order to treat or otherwise improve the vision status of a subject with an ocular disorder. The targeted ocular cells are preferably retinal neurons, namely, bipolar cells and most preferably, RGC.

[0091] Based on the studies reported in WO2007/131180, the brightness of the light needed to stimulate evoked potential in transduced mouse retinas, indicates that a channel opsin with increased light sensitivity may be more desirable. This can be achieved by selection of a suitable naturally occurring opsin, for example other microbial-type rhodopsins, or by modifying the light sensitivity of ChR2 as well as its other properties, such as ion selectivity and spectral sensitivity, to produce diversified light-sensitive channels to better fit the need for vision restoration.

[0092] Different transgenes may be used to encode separate subunits of a protein being delivered, or to encode different polypeptides the co-expression of which is desired. If a single transgene includes DNA encoding each of several subunits, the DNA encoding each subunit may be separated by an internal ribozyme entry site (IRES), which is preferred for short subunit-encoding DNA sequences (e.g., total DNA, including IRES is <5 kB). Other methods

which do not employ an IRES may be used for co-expression, e.g., the use of a second internal promoter, an alternative splice signal, a co- or post-translational proteolytic cleavage strategy, etc., all of which are known in the art.

[0093] The coding sequence or non-coding sequence of the present nucleic acids, including all domains to be expressed preferably are codon-optimized for the species in which they are to be expressed, particularly mammals and humans. Such codon-optimization is routine in the art.

[0094] While a preferred transgene encodes a full length polypeptide, preferably ChR2, the present invention is also directed to vectors that encode a biologically active fragment of ChR2 (nucleotides: SEQ ID NO:19; amino acids: SEQ ID NO:20) or a (preferably conservative) amino acid substitution variant or mutant of ChR2, or a full length HaloR (nucleotide SEQ ID NO:23; amino acid SEQ ID NO:24) or a biologically active fragment, variant, mutant, or fusion/chimeric nucleic acid encoding a fusion protein. A preferred point mutation named CatCh (calcium translocating channelrhodopsin (mutation at L132C) mediates an accelerated response time and a voltage response that is ~70-fold more light sensitive than that of wild-type ChR2; these properties stem from enhanced Ca2+ permeability. (Kleinlogel, S et al., Nature Neuroscience 14:513-518 (2011)). Such variants, mutants and fragments of any other polypeptide of the invention to be expressed in retinal neurons are within the scope of this invention. When a fragment or variant of the full length and native coding sequence is expressed by the targets cells being transformed and is able to endow such cells with light sensitivity that is functionally equivalent to that of the full length or substantially full length polypeptide having a native, rather than variant, amino acid sequence. A biologically active fragment or variant is a "functional equivalent"—a term that is well understood in the art and is further defiled in detail herein. The requisite biological activity of the encoded fragment or variant, using any method disclosed herein or known in the art to establish activity of a channel opsin, has the following activity relative to the wild-type native polypeptide: about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, and any range derivable therein, such as, for example, from about 70% to about 80%, and more preferably from about 81% to about 90%; or even more preferably, from about 91% to about 99%.

[0095] It should be appreciated that any variations in the coding sequences of the present nucleic acids and vectors that, as a result of the degeneracy of the genetic code, express a polypeptide of the same sequence, are included within the scope of this invention.

[0096] The amino acid sequence identity of the encoded polypeptide variants of the present invention are determined using standard methods, typically based on certain mathematical algorithms. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of Meyers and Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The nucleotide and amino acid sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and

XBLAST programs (Altschul et al. (1990) J. Mol. Biol. 215:403-10). BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to, e.g., DAN encoding Chop2 of C. reinhardtii. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the appropriate reference protein such as Chop2. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized (Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See World Wide Web URL ncbi.nlm.nih.gov.

[0097] The preferred amino acid sequence variant has the following degrees of sequence identity with the native, full length channel opsin polypeptide, preferably Chop2 from C. reinhardtii (SEQ ID NO:_) or with a fragment thereof (e.g., SEQ ID NO:_): about 50%, about 55%, about 60%, about 65%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%, and any range derivable therein, such as, for example, from about 70% to about 80%, and more preferably from about 81% to about 90%; or even more preferably, from about 91% to about 99% identity. A preferred biologically active fragment comprises or consists of SEQ ID NO:3, which corresponds to residues 1-315 of the full length SEQ ID NO:6, or comprises or consists of SEQ ID NO:8.

[0098] Any of a number of known recombinant methods are used to produce a DNA molecule encoding the fragment or variant. For production of a variant, it is routine to introduce mutations into the coding sequence to generate desired amino acid sequence variants of the invention. Site-directed mutagenesis is a well-known technique for which protocols and reagents are commercially available (e.g., Zoller, M J et al., 1982, Nucl Acids Res 10:6487-6500; Adelman, J P et al., 1983, DNA 2:183-93). These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases.

[0099] In terms of functional equivalents, it is well understood by those skilled in the art that, inherent in the definition of a “biologically functional equivalent” protein, polypeptide, gene or nucleic acid, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted.

[0100] In particular, the shorter the length of the polypeptide, the fewer amino acids changes should be made. Longer fragments may have an intermediate number of changes. The full length polypeptide protein will have the most tolerance for a larger number of changes. It is also well understood that where certain residues are shown to be particularly important to the biological or structural properties of a polypeptide residues in a binding regions or an active site, such residues may not generally be exchanged. In this manner, functional equivalents are defined herein as those poly peptides which maintain a substantial amount of their native biological activity.

[0101] For a detailed description of protein chemistry and structure, see Schulz, G E et al., Principles of Protein Structure, Springer-Verlag, New York, 1978, and Creighton, T. E., Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions that may be made in the protein molecule may be based on analysis of the frequencies of amino acid changes between

a homologous protein of different species, such as those presented in Table 1-2 of Schulz et al. (supra) and FIG. 3-9 of Creighton (supra). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

[0000]
1 Small aliphatic, nonpolar or slightly polar residues Ala, Ser, Thr (Pro, Gly);
2 Polar, negatively charged residues and their amides Asp, Asn, Glu, Gln;
3 Polar, positively charged residues His, Arg, Lys;
4 Large aliphatic, nonpolar residues Met, Leu, Ile, Val (Cys)
5 Large aromatic residues Phe, Tyr, Trp.

[0102] The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking a side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation, which is important in protein folding.

[0103] The hydropathy index of amino acids may also be considered in selecting variants. Each amino acid has been assigned a hydropathy index on the basis of their hydrophobicity and charge characteristics, these are: Ile (+4.5); Val (+4.2); Leu (+3.8); Phe (+2.8); Cys (+2.5); Met (+1.9); Ala (+1.8); Glycine (-0.4); Thr (-0.7); Ser (-0.8); Trp (-0.9); Tyr (-1.3); Pro (-1.6); His (-3.2); Glu (-3.5); Gln (-3.5); Asp (-3.5); Asn (-3.5); Lys (-3.9); and Arg (-4.5). The importance of the hydropathy index in conferring interactive biological function on a proteinaceous molecule is generally understood in the art (Kyte and Doolittle, 1982, J. Mol. Biol. 157:105-32). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathy index or score and still retain a similar biological activity. In making changes based upon the hydropathy index, the substitution of amino acids whose hydropathy indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biological functional equivalent polypeptide thereby created is intended for use in certain of the present embodiments. U.S. Pat. No. 4,554,101, discloses that the greatest local average hydrophilicity of a proteinaceous molecule, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the molecule. See U.S. Pat. No. 4,554,101 for a hydrophilicity values. In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

[0000] Vector Components and their Sequences.

[0104] Promoters/Regulatory Sequences

[0105] The expression vector of the present invention includes appropriate sequences operably linked to the coding sequence(s) or ORF(s) to promote its expression in a targeted host cell. “Operably linked” sequences include both expression control sequences such as. promoters that are contiguous with the coding sequences and expression control sequences that act in trans or distally to control the expression of the polypeptide product.

[0106] Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (e.g., Kozak consensus sequence); sequences that enhance nucleic acid

or protein stability; and when desired, sequences that enhance protein processing and/or secretion. Many varied expression control sequences, including native and non-native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized herein. depending upon the type of expression desired.

[0107] Expression control sequences for eukaryotic cells typically include a promoter, an enhancer, such as one derived from an immunoglobulin gene, SV40, CMV, etc., and a polyadenylation sequence which may include splice donor and acceptor sites. The polyadenylation (polyA) sequence generally is inserted 3' to the coding sequence and 5' to the 3' ITR sequence. The polyA from bovine growth hormone (bGH) is a suitable sequence and is abbreviated “bGHpolyA” (SEQ ID NO:28).

[0108] The regulatory sequences useful herein may also contain an intron, such as one located between the promoter/enhancer sequence and the coding sequence. One useful intron sequence is derived from SV40, and is referred to as the SV40 T intron sequence. Another includes the woodchuck hepatitis virus post-transcriptional element. (See, for example, Wang L and Verma, I, 1999, Proc Nat'l Acad Sci USA, 96:3906-10).

[0109] An IRES sequence, or other suitable system as discussed above, may be used to produce more than one polypeptide from a single transcript. An exemplary IRES is the poliovirus IRES which supports transgene expression in photoreceptors, RPE and ganglion cells. Preferably, the IRES is located 3' to the coding sequence in the present vector, preferably an rAAV vector.

[0110] The promoter may be selected from a number of constitutive or inducible promoters that can drive expression of the selected transgene in an ocular setting, preferably in retinal neurons. A preferred promoter is “cell-specific”, meaning that it is selected to direct expression of the selected transgene in a particular ocular cell type, such as photoreceptor cells.

[0111] A preferred constitutive promoters include the exemplified hybrid cytomegalovirus (CMV) immediate early enhancer/chicken ß-actin promoter-exon 1-intron 1 element (together abbreviated as “CAG”; SEQ ID NO:26, herein) used along with woodchuck hepatitis virus posttranscriptional regulatory element (abbreviated herein as “WPRE”; SEQ ID NO:27 herein). However, for human safety, other posttranscriptional regulatory elements known in the art can readily be substituted for WPRE.

[0112] Other useful promoters include RSV LTR promoter/enhancer, the SV40 promoter, the CMV promoter, the dihydrofolate reductase (DHFR) promoter, and the phosphoglycerol kinase (PGK) promoter. Additional useful promoters are disclosed in W. W. Hauswirth et al., 1998, WO98/48027 and A. M. Timmers et al., 2000, WO00/15822. Promoters that were found to drive RPE cell-specific gene expression in vivo include (1) a 528-bp promoter region (bases 1-528 of a murine 11-cis retinol dehydrogenase (RDH) gene (Driessen, C A et al., 1995, Invest. Ophthalmol. Vis. Sci. 36:1988-96; Simon, A. et al., 1995, J. Biol. Chem. 270:1107-12, 1995; Simon, A. et al., 1996, Genomics 36:424-3) Genbank Accession Number X97752); (2) a 2274-bp promoter region) from a human cellular retinaldehyde-binding protein (CRALBP) gene (Intres, R et al., 1994, J. Bio!. Chem. 269:25411-18; Kennedy, B N et al., 1998, J. Bio!. Chem. 273:5591-8, 1998), Genbank Accession Number L34219); and (3) a 1485-bp promoter region from human RPE65 (Nicoletti, A et al., 1998, Invest. Ophthalmol. Vis. Sci. 39:637-44, Genbank Accession Number U20510). These three promoters in WO00/15822 promoted RPE-cell-specific expression of GFP. It is envisioned that minor sequence variations in the various promoters and promoter regions discussed herein—whether additions, deletions or mutations, whether naturally occurring or introduced in vitro, will not affect their ability to drive expression in the cellular targets of the coding sequences of the present invention.

Furthermore, the use of other promoters, even if not yet discovered, that are characterized by abundant and/or specific expression in retinal cells, particularly in bipolar or ganglion cells, is specifically included within the scope of this invention.

[0113] Another useful promoter is from a mGluR6 promoter-region of the Grm6 gene (GenBank accession number BC041684), a gene that controls expression of metabotropic glutamate receptor 6 ((Ueda Y et al., 1997, J Neurosc17:3014-23). The genomic sequence is shown in GenBank accession number—AL627215. A preferred example of this promoter region sequence from the above GenBank record consists of 11023 nucleotides. The original Umeda et al., study employed a 10 kb promoter, but the actual length of the promoter and the sequence that comprises control elements of Grm6 can be adjusted by increasing or decreasing the fragment length. It is a matter of routine testing to select and verify the action of the optimally sized fragment from the Grm6 gene that drives transgenic expression of a selected coding sequence, preferably ChR2 or HaloR, in the desired target cells, preferably in bipolar cells which are rich in glutamate receptors, particularly the “on” type bipolar cells, which are the most bipolar cells in the retina (Nakajima, Y., et al., 1993, J Biol Chem 268:11868-73). Use of such a large promoter is not compatible with the packaging capabilities of rAAV virions, so would require a different delivery vector system known in the art, or identification of a shorter sequence (<2.5 kb) that could e packaged in a rAAV vector of the present invention.

[0114] Another promoter is the Pcp2 (L7) promoter (Tomomura, M et al., 2001, Eur J. Neurosci. 14:57-63). Again, the length of the active promoter is preferably less that 2.5 Kb so it can be packaged into the rAAV viral cassette.

[0115] The neurokinin-3 (NK-3) promoter. could be used to target Halor R to OFF cells (Haverkamp, S et al., 2002, J Comparative Neurology, 455:463-76.)

[0116] An inducible promoter is used to control the amount and timing of production of the transgene product in an ocular cell. Such promoters can be useful if the gene product has some undesired, e.g., toxic, effects in the cell if it accumulates excessively. Inducible promoters include those known in the art, such as the Zn-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter; the T7 promoter; the ecdysone insect promoter; the tetracycline-repressible system; the tetracycline-inducible system; the RU486-inducible system; and the rapamycin-inducible system. Any inducible promoter the action of which is tightly regulated and is specific for the particular target ocular cell type, may be used. Other useful types of inducible promoters are ones regulated by a specific physiological state, e.g., temperature, acute phase, a cell's replicating or differentiation state.

[0117] Selection of the various vector and regulatory elements for use herein are conventional, well-described, and readily available. See, e.g., Sambrook et al., supra; and Ausubel et al., supra. It will be readily appreciated that not all vectors and expression control sequences will function equally well to express the present transgenes Chop2 or HaloR. Clearly, the skilled artisan may apply routine selection among the known expression control sequences without departing from the scope of this invention and based upon general knowledge as well as the guidance provided herein. One skilled in the art can select one or more expression control sequences, operably link them to the coding sequence being expressed to make a minigene, insert the minigene or vector into an AAV vector, preferably rAAV2, and cause packaging of the vector into infectious particles or virions following one of the known packaging methods for rAAV.

[0118] Production of the rAAV

[0119] The rAAV2 used in the present invention may be constructed and produced using the materials and methods described herein and those well-known in the art. The methods that are preferred for producing any construct of this invention are conventional and include genetic engineering, recombinant engineering, and synthetic techniques, such as those set forth in reference cited above.

[0120] Briefly, to package an rAAV construct into an rAAV virion, a sequences necessary to express AAV rep and AAV cap or functional fragments thereof as well as helper genes essential for AAV production must be present in the host cells. See, for example U.S. Pat. Pub. 2007/0015238, which describes production of pseudotyped rAAV virion vectors encoding AAV Rep and Cap proteins of different serotypes and AdV transcription products that provide helper functions For example, AAV rep and cap sequences may be introduced into the host cell in any known manner including, without limitation, transfection, electroporation, liposome delivery, membrane fusion, biolistic deliver of DNA-coated pellets, viral infection and protoplast fusion. Devices specifically adapted for delivering DNA to specific regions within and around the eye for the purpose of gene therapy have been described (for example, U.S. Pat. Pub. 2005/0277868, incorporated by reference) are used within the scope of this invention. Such devices utilize electroporation and electromigration, providing, e.g., two electrodes on a flexible support that can be placed behind the retina. A third electrode is part of a hollow support, which can also be used to inject the molecule to the desired area. The electrodes can be positioned around the eye, including behind the retina or within the vitreous.

[0121] These sequences may exist stably in the cell as an episome or be stably integrated into the cell's genome. They may also be expressed more transiently in the host cell. As an example, a useful nucleic acid molecule comprises, from 5' to 3', a promoter, an optional spacer between the promoter and the start site of the rep sequence, an AAV rep sequence, and an AAV cap sequence.

[0122] The rep and cap sequences, along with their expression control sequences, are preferably provided in a single vector, though they may be provided separately in individual vectors. The promoter may be any suitable constitutive, inducible or native promoter. The delivery molecule that provides the Rep and Cap proteins may be in any form, preferably a plasmid which may contain other non-viral sequences, such as those to be employed as markers. This molecule typically excludes the AAV ITRs and packaging sequences. To avoid the occurrence of homologous recombination, other viral sequences, particularly adenoviral sequences, are avoided. This plasmid is preferably one that is stably expressed.

[0123] Conventional genetic engineering or recombinant DNA techniques described in the cited references are used. The rAAV may be produced using a triple transfection method with either the calcium phosphate (Clontech) or Effectene™ reagent (Qiagen) according to manufacturer's instructions. See, also, Herzog et al., Nat. Med. 5:56-63 (1999).

[0124] The rAAV virions are produced by culturing host cells comprising a rAAV as described in Bi et al., supra, and WO2007/131180, which includes a rAAV construct to be packaged into a rAAV virion, an AAV rep sequence and an AAV cap sequence, all under control of regulatory sequences directing expression.

[0125] Suitable viral helper genes, such as adenovirus E2A, E40rf6 and VA, may be added to the culture preferably on separate plasmids. Thereafter, the rAAV virion which directs expression of the transgene is isolated in the absence of contaminating helper virus or wildtype AAV.

[0126] It is conventional to assess whether a particular expression control sequence is suitable for a given transgene, and choose the one most appropriate for expressing the transgene. For

example, a target cell may be infected in vitro, and the number of copies of the transgene in the cell monitored by Southern blots or quantitative PCR. The level of RNA expression may be monitored by Northern blots quantitative RT-PCR. The level of protein expression may be monitored by Western blot, immunohistochemistry, immunoassay including enzyme immunoassay (EIA) such as enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA) or by other methods. Specific embodiments are described below.

Preferred Vectors of the Invention

[0127] This section lists a number of vectors useful in the present invention that comprise the following nucleotide sequences encoding
(a) Light Sensor: ChR2 coding sequence (preferably SEQ ID NO:21) or HaloR coding sequence (SEQ ID NO:23)
(b) Optionally, a reporter “gene” preferably GFP (SEQ ID NO:25)
(c) 5' and 3' ITRs from AAV2, SEQ ID NO:17 and 18, respectively.
(d) CAG Promoter/Regulatory sequence (SEQ ID NO:26)
(e) Posttranscriptional Regulatory element WPRE (SEQ ID NO:27)
(f) Polyadenylation sequence (SEQ ID NO:28)
In addition to the foregoing, the vector preferably contains
(g) the rAAV2 backbone sequences (SEQ ID NO:29) located 3' from the 3' ITR.
These vectors, their “schematic representation” several linear vector diagrams and annotated sequences are shown below. The following annotation is used in all the sequences:
ITR's: lower case, bold, italic, underscore
CAG: UPPERCASE (underscore)
Chop2/ChR2 (used interchangeably here): UPPERCASE, ITALIC
GFP: UPPERCASE (nonbold, non-italic)
Sorting Motif: UPPERCASE, (double underscore)
WPRE UPPERCASE (underscore)
bGHpolyA: UPPERCASE, (italic)
intervening vector nucleotides/cloning carryover: lower case (not italic)
(1) Two examples of vectors that do not have the Sorting Motif present but are “poised” for insertion of the motif (with the insertion point shown in the sequence)

[0000]

SEQ ID NO: 30
ITR—CAG—ChR2—GFP—{insertion site for Sorting Motif}—WPRE—bGHpolyA—ITR'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC

ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc
end ChR2??
??start GFP
AAA GGA GAA GAA CTC TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA
GAT GGT GAT GTT AAC GGC CAC AAG TTC TCT GTC AGT GGA GAG GGT GAA
GGT GAT GCA ACA TAC GGA AAA CTT ACC CTG AAG TTC ATC TGC ACT ACT
GGC AAA CTG CCT GTT CCA TGG CCA ACA CTA GTC ACT ACT CTG TGC TAT
GGT GTT CAA TGC TTT TCA AGA TAC CCG GAT CAT ATG AAA CGG CAT GAC
TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGG ACC ATC
TTC TTC AAA GAT GAC GGC AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT
GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT GAC TTC
AAG GAA GAT GGC AAC ATT CTG GGA CAC AAA TTG GAA TAC AAC TAT AAC
TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA ATC AAA
GTG AAC TTC AAG ACC CGC CAC AAC ATT GAA GAT GGA AGC GTT CAA CTA
GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT
TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG AAA GAT
CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA ACA GCT

CCCTGTGACC CCTCCCCAGT GCCTCTCCTG GCCCTGGAAG TTGCCACTCC
AGTGCCCACC
AGCCTTGTCC TAATAAAATT AAGTTGCATC ATTTTGTCTG ACTAGGTGTC
CTTCTATAAT
ATTATGGGGT GGAGGGGGGT GGTATGGAGC AAGGGGCAAG TTGGGAAGAC
AACCTGTAGG
GCCTGCGGGG TCTATTGGGA ACCAAGCTGG AGTGCAGTGG CACAATCTTG
GCTCACTGCA
ATCTCCGCCT CCTGGGTTCA AGCGATTCTC CTGCCTCAGC CTCCCGAGTT
GTTGGGATTC
CAGGCATGCA TGACCAGGCT CAGCTAATTT TTGTTTTTTT GGTAGAGACG
GGGTTTCACC
ATATTGGCCA GGCTGGTCTC CAACTCCTAA TCTCAGGTGA TCTACCCACC
TTGGCCTCCC
AAATTGCTGG GATTACAGGC GTGAACCACT GCTCCCTTCC CTGTCCTTct gattttgtag
end bGH-polyA??

(B) SEQ ID NO: 31 (Same as above but without GFP)
ITR—CAG—ChR2—(insertion site for Sorting Motif)—WPRE—bGHpolyA—ITR'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA

GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc
end ChR2?

ctctagagtc gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG
??start bGH-polyA
GCCCTGGAAG TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT
AAGTTGCATC
ATTTTGTCTG ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT
GGTATGGAGC
AAGGGGCAAG TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA
ACCAAGCTGG
AGTGCAGTGG CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA
AGCGATTCTC
CTGCCTCAGC CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT
CAGCTAATTT
TTGTTTTTTT GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC
CAACTCCTAA
TCTCAGGTGA TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC
GTGAACCACT

SEQ ID NO: 32 5'-ITR—CAG—ChR2—GFP—(Kv2.1 Motif)—WPRE—bGHpolyA—ITR-3

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC

GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc

end ChR2??

??start GFP

AAA GGA GAA GAA CTC TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA
GAT GGT GAT GTT AAC GGC CAC AAG TTC TCT GTC AGT GGA GAG GGT GAA
GGT GAT GCA ACA TAC GGA AAA CTT ACC CTG AAG TTC ATC TGC ACT ACT
GGC AAA CTG CCT GTT CCA TGG CCA ACA CTA GTC ACT ACT CTG TGC TAT
GGT GTT CAA TGC TTT TCA AGA TAC CCG GAT CAT ATG AAA CGG CAT GAC
TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGG ACC ATC
TTC TTC AAA GAT GAC GGC AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT
GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT GAC TTC
AAG GAA GAT GGC AAC ATT CTG GGA CAC AAA TTG GAA TAC AAC TAT AAC
TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA ATC AAA
GTG AAC TTC AAG ACC CGC CAC AAC ATT GAA GAT GGA AGC GTT CAA CTA
GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT
TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG AAA GAT
CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA ACA GCT

CCTCCCCAGT GCCTCTCCTG GCCCTGGAAG TTGCCACTCC AGTGCCCACC
AGCCTTGTCC
TAATAAAATT AAGTTGCATC ATTTTGTCTG ACTAGGTGTC CTTCTATAAT
ATTATGGGGT
GGAGGGGGGT GGTATGGAGC AAGGGGCAAG TTGGGAAGAC AACCTGTAGG
GCCTGCGGGG
TCTATTGGGA ACCAAGCTGG AGTGCAGTGG CACAATCTTG GCTCACTGCA
ATCTCCGCCT
CCTGGGTTCA AGCGATTCTC CTGCCTCAGC CTCCCGAGTT GTTGGGATTC
CAGGCATGCA
TGACCAGGCT CAGCTAATTT TTGTTTTTTT GGTAGAGACG GGGTTTCACC
ATATTGGCCA
GGCTGGTCTC CAACTCCTAA TCTCAGGTGA TCTACCCACC TTGGCCTCCC
AAATTGCTGG
GATTACAGGC GTGAACCACT GCTCCCTTCC CTGTCCTTct gattttgtag gtaaccacgt

end bGH-polyA??

SEQ ID NO: 33: (same as above but without GFP)
5'-ITR—CAG—ChR2—(Kv2.1 Motif)—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc
end ChR2??

CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG GCCCTGGAAG
TTGCCACTCC
AGTGCCCACC AGCCTTGTCC TAATAAAATT AAGTTGCATC ATTTTGTCTG
ACTAGGTGTC
CTTCTATAAT ATTATGGGGT GGAGGGGGGT GGTATGGAGC AAGGGGCAAG
TTGGGAAGAC
AACCTGTAGG GCCTGCGGGG TCTATTGGGA ACCAAGCTGG AGTGCAGTGG
CACAATCTTG
GCTCACTGCA ATCTCCGCCT CCTGGGTTCA AGCGATTCTC CTGCCTCAGC
CTCCCGAGTT
GTTGGGATTC CAGGCATGCA TGACCAGGCT CAGCTAATTT TTGTTTTTTT
GGTAGAGACG
GGGTTTCACC ATATTGGCCA GGCTGGTCTC CAACTCCTAA TCTCAGGTGA
TCTACCCACC
TTGGCCTCCC AAATTGCTGG GATTACAGGC GTGAACCACT GCTCCCTTCC
CTGTCCTTct
end bGH-polyA??

(E) SEQ ID NO: 34:
5'-ITR—CAG—ChR2—GFP—{Nav1.6 Motif}—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2

atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc
end ChR2??
??start GFP
AAA GGA GAA GAA CTC TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA
GAT GGT GAT GTT AAC GGC CAC AAG TTC TCT GTC AGT GGA GAG GGT GAA
GGT GAT GCA ACA TAC GGA AAA CTT ACC CTG AAG TTC ATC TGC ACT ACT
GGC AAA CTG CCT GTT CCA TGG CCA ACA CTA GTC ACT ACT CTG TGC TAT
GGT GTT CAA TGC TTT TCA AGA TAC CCG GAT CAT ATG AAA CGG CAT GAC
TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGG ACC ATC
TTC TTC AAA GAT GAC GGC AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT
GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT GAC TTC
AAG GAA GAT GGC AAC ATT CTG GGA CAC AAA TTG GAA TAC AAC TAT AAC
TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA ATC AAA
GTG AAC TTC AAG ACC CGC CAC AAC ATT GAA GAT GGA AGC GTT CAA CTA
GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT
TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG AAA GA
CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA ACA GCT

ctctagagtc gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG
??start bGH-polyA
GCCCTGGAAG TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT
AAGTTGCATC
ATTTTGTCTG ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT
GGTATGGAGC
AAGGGGCAAG TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA
ACCAAGCTGG
AGTGCAGTGG CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA
AGCGATTCTC
CTGCCTCAGC CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT
CAGCTAATTT
TTGTTTTTTT GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC
CAACTCCTAA
TCTCAGGTGA TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC

GTGAACCACT
GCTCCCTTCC CTGTCCTTct gattttgtag gtaaccacgt gcggaccgag
end bGH-polyA??

(F) SEQ ID NO: 35 (same as above without GFP)
5'-ITR—CAG—ChR2—{Nav1.6Motif}—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc
end ChR2??

GCCTCTCCTG GCCCTGGAAG TTGCCACTCC AGTGCCCACC AGCCTTGTCC
TAATAAAATT
AAGTTGCATC ATTTTGTCTG ACTAGGTGTC CTTCTATAAT ATTATGGGGT
GGAGGGGGGT
GGTATGGAGC AAGGGGCAAG TTGGGAAGAC AACCTGTAGG GCCTGCGGGG
TCTATTGGGA
ACCAAGCTGG AGTGCAGTGG CACAATCTTG GCTCACTGCA ATCTCCGCCT
CCTGGGTTCA
AGCGATTCTC CTGCCTCAGC CTCCCGAGTT GTTGGGATTC CAGGCATGCA
TGACCAGGCT
CAGCTAATTT TTGTTTTTTT GGTAGAGACG GGGTTTCACC ATATTGGCCA
GGCTGGTCTC
CAACTCCTAA TCTCAGGTGA TCTACCCACC TTGGCCTCCC AAATTGCTGG
GATTACAGGC
GTGAACCACT GCTCCCTTCC CTGTCCTTct gattttgtag gtaaccacgt gcggaccgag
end bGH-polyA??

(G) SEQ ID NO: 36:

5'-ITR—CAG—ChR2—GFP—{NLG1 Motif}—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc\ end
ChR2??
??start GFP
AAA GGA GAA GAA CTC TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA
GAT GGT GAT GTT AAC GGC CAC AAG TTC TCT GTC AGT GGA GAG GGT GAA
GGT GAT GCA ACA TAC GGA AAA CTT ACC CTG AAG TTC ATC TGC ACT ACT
GGC AAA CTG CCT GTT CCA TGG CCA ACA CTA GTC ACT ACT CTG TGC TAT
GGT GTT CAA TGC TTT TCA AGA TAC CCG GAT CAT ATG AAA CGG CAT GAC
TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGG ACC ATC
TTC TTC AAA GAT GAC GGC AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT
GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT GAC TTC
AAG GAA GAT GGC AAC ATT CTG GGA CAC AAA TTG GAA TAC AAC TAT AAC
TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA ATC AAA
GTG AAC TTC AAG ACC CGC CAC AAC ATT GAA GAT GGA AGC GTT CAA CTA
GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT
TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG AAA GAT
CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA ACA GCT

CCTCCCCAGT GCCTCTCCTG GCCCTGGAAG TTGCCACTCC AGTGCCCACC
AGCCTTGTCC
TAATAAAATT AAGTTGCATC ATTTTGTCTG ACTAGGTGTC CTTCTATAAT
ATTATGGGGT
GGAGGGGGGT GGTATGGAGC AAGGGGCAAG TTGGGAAGAC AACCTGTAGG
GCCTGCGGGG
TCTATTGGGA ACCAAGCTGG AGTGCAGTGG CACAATCTTG GCTCACTGCA
ATCTCCGCCT

CCTGGGTTCA AGCGATTCTC CTGCCTCAGC CTCCCGAGTT GTTGGGATTC CAGGCATGCA
TGACCAGGCT CAGCTAATTT TTGTTTTTTT GGTAGAGACG GGGTTTCACC ATATTGGCCA
GGCTGGTCTC CAACTCCTAA TCTCAGGTGA TCTACCCACC TTGGCCTCCC AAATTGCTGG
GATTACAGGC GTGAACCACT GCTCCCTTCC CTGTCCTTct gattttgtag gtaaccacgt
end bGH-polyA??

(H) SEQ ID NO: 37 (same as above but without GFP)
5'-ITR—CAG—ChR2—(NLG-1Motif)—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc
end ChR2??

CCCTGTGACC CCTCCCCAGT GCCTCTCCTG GCCCTGGAAG TTGCCACTCC AGTGCCCACC
AGCCTTGTCC TAATAAAATT AAGTTGCATC ATTTTGTCTG ACTAGGTGTC CTTCTATAAT
ATTATGGGGT GGAGGGGGGT GGTATGGAGC AAGGGGCAAG TTGGGAAGAC AACCTGTAGG
GCCTGCGGGG TCTATTGGGA ACCAAGCTGG AGTGCAGTGG CACAATCTTG GCTCACTGCA
ATCTCCGCCT CCTGGGTTCA AGCGATTCTC CTGCCTCAGC CTCCCGAGTT GTTGGGATTC
CAGGCATGCA TGACCAGGCT CAGCTAATTT TTGTTTTTTT GGTAGAGACG GGGTTTCACC
ATATTGGCCA GGCTGGTCTC CAACTCCTAA TCTCAGGTGA TCTACCCACC

TTGGCCTCCC
AAATTGCTGG GATTACAGGC GTGAACCACT GCTCCCTTCC CTGTCCTTct gattttgtag
end bGH-polyA??

(I) SEQ ID NO: 38:
5'-ITR—CAG—ChR2—GFP—{MLPH Motif}—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc
end ChR2??
??start GFP
AAA GGA GAA GAA CTC TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA
GAT GGT GAT GTT AAC GGC CAC AAG TTC TCT GTC AGT GGA GAG GGT GAA
GGT GAT GCA ACA TAC GGA AAA CTT ACC CTG AAG TTC ATC TGC ACT ACT
GGC AAA CTG CCT GTT CCA TGG CCA ACA CTA GTC ACT ACT CTG TGC TAT
GGT GTT CAA TGC TTT TCA AGA TAC CCG GAT CAT ATG AAA CGG CAT GAC
TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGG ACC ATC
TTC TTC AAA GAT GAC GGC AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT
GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT GAC TTC
AAG GAA GAT GGC AAC ATT CTG GGA CAC AAA TTG GAA TAC AAC TAT AAC
TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA ATC AAA
GTG AAC TTC AAG ACC CGC CAC AAC ATT GAA GAT GGA AGC GTT CAA CTA
GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT
TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG AAA GAT
CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA ACA GCT

gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG
GCCCTGGAAG
??start bGH-polyA

TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT AAGTTGCATC ATTTTGTCTG
ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT GGTATGGAGC AAGGGGCAAG
TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA ACCAAGCTGG AGTGCAGTGG
CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA AGCGATTCTC CTGCCTCAGC
CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT CAGCTAATTT TTGTTTTTTT
GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC CAACTCCTAA TCTCAGGTGA
TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC GTGAACCACT GCTCCCTTC

(J) SEQ ID NO: 39 (same as above without GFP)
5'-ITR—CAG—ChR2—{MLPH-Motif}—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start ChR2
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG GAT TAT GGA
GGC GCC CTG AGT GCC GTT GGG CGC GAG CTG CTA TTT GTA ACG AAC CCA
GTA GTC GTC AAT GGC TCT GTA CTT GTG CCT GAG GAC CAG TGT TAC TGC
GCG GGC TGG ATT GAG TCG CGT GGC ACA AAC GGT GCC CAA ACG GCG TCG
AAC GTG CTG CAA TGG CTT GCT GCT GGC TTC TCC ATC CTA CTG CTT ATG
TTT TAC GCC TAC CAA ACA TGG AAG TCA ACC TGC GGC TGG GAG GAG ATC
TAT GTG TGC GCT ATC GAG ATG GTC AAG GTG ATT CTT GAG TTC TTC TTC
GAG TTT AAG AAC CCG TCC ATG CTG TAT CTA GCC ACA GGC CAC CGC GTC
CAG TGG TTG CGT TAC GCC GAG TGG CTT CTC ACC TGC CCG GTC ATT CTC
ATT CAC CTG TCA AAC CTG ACG GGC TTG TCC AAC GAC TAC AGC AGG CGC
ACT ATG GGT CTG CTT GTG TCT GAT ATT GGC ACA ATT GTG TGG GGC GCC
ACT TCC GCT ATG GCC ACC GGA TAC GTC AAG GTC ATC TTC TTC TGC CTG
GGT CTG TGT TAT GGT GCT AAC ACG TTC TTT CAC GCT GCC AAG GCC TAC
ATC GAG GGT TAC CAT ACC GTG CCG AAG GGC CGG TGT CGC CAG GTG GTG
ACT GGC ATG GCT TGG CTC TTC TTC GTA TCA TGG GGT ATG TTC CCC ATC
CTG TTC ATC CTC GGC CCC GAG GGC TTC GGC GTC CTG AGC GTG TAC GGC
TCC ACC GTC GGC CAC ACC ATC ATT GAC CTG ATG TCG AAG AAC TGC TGG
GGT CTG CTC GGC CAC TAC CTG CGC GTG CTG ATC CAC GAG CAT ATC CTC
ATC CAC GGC GAC ATT CGC AAG ACC ACC AAA TTG AAC ATT GGT GGC ACT
GAG ATT GAG GTC GAG ACG CTG GTG GAG GAC GAG GCC GAG GCT GGC GCG
GTC AAC AAG GGC ACC GGC AAG gaattcggag gcggaggtgg agctagc
end ChR2??

GCCTCTCCTG GCCCTGGAAG TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT
AAGTTGCATC ATTTTGTCTG ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT
GGTATGGAGC AAGGGGCAAG TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA
ACCAAGCTGG AGTGCAGTGG CACAATCTTG GCTCACTGCA ATCTCCGCCT

CCTGGGTTCA
AGCGATTCTC CTGCCTCAGC CTCCCGAGTT GTTGGGATTC CAGGCATGCA
TGACCAGGCT
CAGCTAATTT TTGTTTTTTT GGTAGAGACG GGGTTTCACC ATATTGGCCA
GGCTGGTCTC
CAACTCCTAA TCTCAGGTGA TCTACCCACC TTGGCCTCCC AAATTGCTGG
GATTACAGGC
GTGAACCACT GCTCCCTTCC CTGTCCTTct gattttgtag gtaaccacgt gcggaccgag
end bGH-polyA??

SEQ ID NO: 40:
5'-ITR—CAG—HaloR—GFP—(Kv2.1Motif)—WPRE—bGHpolyA—ITR-3

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start HaloR
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG ACT GAG ACA
TTG CCA CCG GTA ACG GAA TCG GCT GTT GCG CTA CAG GCG GAG GTG ACC
CAG AGG GAG CTG TTC GAG TTC GTT CTC AAC GAC CCC CTC CTC GCC AGT
TCG CTG TAT ATT AAT ATC GCA CTG GCA GGG CTG TCG ATA CTG CTT TTC
GTG TTC ATG ACG CGC GGA CTC GAC GAC CCA CGG GCG AAA CTC ATC GCC
GTT TCG ACG ATT TTG GTG CCG GTG GTC TCT ATC GCG AGC TAC ACC GGC
CTT GCA TCG GGG CTC ACC ATC AGC GTC CTC GAG ATG CCA GCC GGC CAC
TTC GCC GAG GGG TCC TCG GTG ATG CTC GGC GGC GAA GAG GTA GAC GGC
GTC GTG ACG ATG TGG GGC CGC TAT CTG ACG TGG GCC CTT TCG ACA CCG
ATG ATA CTG CTG GCG CTT GGG CTG CTT GCT GGC TCT AAC GCC ACG AAG
CTC TTT ACC GCC ATC ACC TTC GAC ATC GCG ATG TGT GTC ACC GGC CTC
GCA GCC GCG CTG ACG ACC TCT TCG CAC CTG ATG CGG TGG TTC TGG TAC
GCC ATC AGT TGT GCG TGT TTC CTC GTC GTC CTC TAC ATC CTG CTC GTC
GAG TGG GCA CAG GAC GCC AAG GCT GCC GGT ACT GCG GAT ATG TTC AAT
ACG CTG AAG CTG CTG ACC GTT GTC ATG TGG CTC GGC TAC CCC ATC GTG
TGG GCA CTC GGC GTT GAG GGC ATC GCC GTT CTT CCG GTC GGA GTC ACG
TCG TGG GGA TAC AGC TTC CTC GAC ATC GTC GCG AAG TAC ATC TTC GCG
TTC CTG CTG CTC AAC TAC CTC ACG TCG AAC GAG AGC GTC GTC TCC GGC
TCG ATA CTC GAC GTG CCG TCC GCG TCG GGC ACT CCC GCT GAC GAC
end HaloR??
gaattcggag gcggaggtgg agctagc AAA GGA GAA GAA CTC TTC ACT GGA GTT
??start GFP
GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT GGT GAT GTT AAC GGC CAC
AAG TTC TCT GTC AGT GGA GAG GGT GAA GGT GAT GCA ACA TAC GGA AAA
CTT ACC CTG AAG TTC ATC TGC ACT ACT GGC AAA CTG CCT GTT CCA TGG
CCA ACA CTA GTC ACT ACT CTG TGC TAT GGT GTT CAA TGC TTT TCA AGA
TAC CCG GAT CAT ATG AAA CGG CAT GAC TTT TTC AAG AGT GCC ATG CCC
GAA GGT TAT GTA CAG GAA AGG ACC ATC TTC TTC AAA GAT GAC GGC AAC
TAC AAG ACA CGT GCT GAA GTC AAG TTT GAA GGT GAT ACC CTT GTT AAT
AGA ATC GAG TTA AAA GGT ATT GAC TTC AAG GAA GAT GGC AAC ATT CTG
GGA CAC AAA TTG GAA TAC AAC TAT AAC TCA CAC AAT GTA TAC ATC ATG
GCA GAC AAA CAA AAG AAT GGA ATC AAA GTG AAC TTC AAG ACC CGC CAC
AAC ATT GAA GAT GGA AGC GTT CAA CTA GCA GAC CAT TAT CAA CAA AAT
ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA CCA GAC AAC CAT TAC CTG
TCC ACA CAA TCT GCC CTT TCG AAA GAT CCC AAC GAA AAG AGA GAC CAC
ATG GTC CTT CTT GAG TTT GTA ACA GCT GCT GGG ATT ACA CAT GGC ATG

gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG
GCCCTGGAAG
??start bGH-polyA
TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT AAGTTGCATC
ATTTTGTCTG
ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT GGTATGGAGC
AAGGGGCAAG
TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA ACCAAGCTGG
AGTGCAGTGG
CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA AGCGATTCTC
CTGCCTCAGC
CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT CAGCTAATTT
TTGTTTTTTT
GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC CAACTCCTAA
TCTCAGGTGA
TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC GTGAACCACT
GCTCCCTTCC

SEQ ID NO: 41 (same as above without the GFP)
5'-ITR—CAG—HaloR—(Kv2.1Motif)—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start HaloR
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG ACT GAG ACA
TTG CCA CCG GTA ACG GAA TCG GCT GTT GCG CTA CAG GCG GAG GTG ACC
CAG AGG GAG CTG TTC GAG TTC GTT CTC AAC GAC CCC CTC CTC GCC AGT
TCG CTG TAT ATT AAT ATC GCA CTG GCA GGG CTG TCG ATA CTG CTT TTC
GTG TTC ATG ACG CGC GGA CTC GAC GAC CCA CGG GCG AAA CTC ATC GCC
GTT TCG ACG ATT TTG GTG CCG GTG GTC TCT ATC GCG AGC TAC ACC GGC
CTT GCA TCG GGG CTC ACC ATC AGC GTC CTC GAG ATG CCA GCC GGC CAC
TTC GCC GAG GGG TCC TCG GTG ATG CTC GGC GGC GAA GAG GTA GAC GGC
GTC GTG ACG ATG TGG GGC CGC TAT CTG ACG TGG GCC CTT TCG ACA CCG
ATG ATA CTG CTG GCG CTT GGG CTG CTT GCT GGC TCT AAC GCC ACG AAG
CTC TTT ACC GCC ATC ACC TTC GAC ATC GCG ATG TGT GTC ACC GGC CTC
GCA GCC GCG CTG ACG ACC TCT TCG CAC CTG ATG CGG TGG TTC TGG TAC
GCC ATC AGT TGT GCG TGT TTC CTC GTC GTC CTC TAC ATC CTG CTC GTC
GAG TGG GCA CAG GAC GCC AAG GCT GCC GGT ACT GCG GAT ATG TTC AAT
ACG CTG AAG CTG CTG ACC GTT GTC ATG TGG CTC GGC TAC CCC ATC GTG
TGG GCA CTC GGC GTT GAG GGC ATC GCC GTT CTT CCG GTC GGA GTC ACG
TCG TGG GGA TAC AGC TTC CTC GAC ATC GTC GCG AAG TAC ATC TTC GCG
TTC CTG CTG CTC AAC TAC CTC ACG TCG AAC GAG AGC GTC GTC TCC GGC
TCG ATA CTC GAC GTG CCG TCC GCG TCG GGC ACT CCC GCT GAC GAC
end HaloR??

ctctagagtc gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG
??start bGH-polyA
GCCCTGGAAG TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT

AAGTTGCATC
ATTTTGTCTG ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT
GGTATGGAGC
AAGGGGCAAG TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA
ACCAAGCTGG
AGTGCAGTGG CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA
AGCGATTCTC
CTGCCTCAGC CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT
CAGCTAATTT
TTGTTTTTTT GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC
CAACTCCTAA
TCTCAGGTGA TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC
GTGAACCACT
GCTCCCTTCC CTGTCCTTct gattttgtag gtaaccacgt gcggaccgag
end bGH-polyA??

(M) SEQ ID NO: 42
5'-ITR—CAG—HaloR—GFP—(Nav1.6 Motif)—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start HaloR
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG ACT GAG ACA
TTG CCA CCG GTA ACG GAA TCG GCT GTT GCG CTA CAG GCG GAG GTG ACC
CAG AGG GAG CTG TTC GAG TTC GTT CTC AAC GAC CCC CTC CTC GCC AGT
TCG CTG TAT ATT AAT ATC GCA CTG GCA GGG CTG TCG ATA CTG CTT TTC
GTG TTC ATG ACG CGC GGA CTC GAC GAC CCA CGG GCG AAA CTC ATC GCC
GTT TCG ACG ATT TTG GTG CCG GTG GTC TCT ATC GCG AGC TAC ACC GGC
CTT GCA TCG GGG CTC ACC ATC AGC GTC CTC GAG ATG CCA GCC GGC CAC
TTC GCC GAG GGG TCC TCG GTG ATG CTC GGC GGC GAA GAG GTA GAC GGC
GTC GTG ACG ATG TGG GGC CGC TAT CTG ACG TGG GCC CTT TCG ACA CCG
ATG ATA CTG CTG GCG CTT GGG CTG CTT GCT GGC TCT AAC GCC ACG AAG
CTC TTT ACC GCC ATC ACC TTC GAC ATC GCG ATG TGT GTC ACC GGC CTC
GCA GCC GCG CTG ACG ACC TCT TCG CAC CTG ATG CGG TGG TTC TGG TAC
GCC ATC AGT TGT GCG TGT TTC CTC GTC GTC CTC TAC ATC CTG CTC GTC
GAG TGG GCA CAG GAC GCC AAG GCT GCC GGT ACT GCG GAT ATG TTC AAT
ACG CTG AAG CTG CTG ACC GTT GTC ATG TGG CTC GGC TAC CCC ATC GTG
TGG GCA CTC GGC GTT GAG GGC ATC GCC GTT CTT CCG GTC GGA GTC ACG
TCG TGG GGA TAC AGC TTC CTC GAC ATC GTC GCG AAG TAC ATC TTC GCG
TTC CTG CTG CTC AAC TAC CTC ACG TCG AAC GAG AGC GTC GTC TCC GGC
TCG ATA CTC GAC GTG CCG TCC GCG TCG GGC ACT CCC GCT GAC GAC
end HaloR??
gaattcggag gcggaggtgg agctagc AAA GGA GAA GAA CTC TTC ACT GGA GTT
??start GFP
GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT GGT GAT GTT AAC GGC CAC
AAG TTC TCT GTC AGT GGA GAG GGT GAA GGT GAT GCA ACA TAC GGA AAA
CTT ACC CTG AAG TTC ATC TGC ACT ACT GGC AAA CTG CCT GTT CCA TGG
CCA ACA CTA GTC ACT ACT CTG TGC TAT GGT GTT CAA TGC TTT TCA AGA
TAC CCG GAT CAT ATG AAA CGG CAT GAC TTT TTC AAG AGT GCC ATG CCC
GAA GGT TAT GTA CAG GAA AGG ACC ATC TTC TTC AAA GAT GAC GGC AAC
TAC AAG ACA CGT GCT GAA GTC AAG TTT GAA GGT GAT ACC CTT GTT AAT
AGA ATC GAG TTA AAA GGT ATT GAC TTC AAG GAA GAT GGC AAC ATT CTG
GGA CAC AAA TTG GAA TAC AAC TAT AAC TCA CAC AAT GTA TAC ATC ATG

GCA GAC AAA CAA AAG AAT GGA ATC AAA GTG AAC TTC AAG ACC CGC CAC
AAC ATT GAA GAT GGA AGC GTT CAA CTA GCA GAC CAT TAT CAA CAA AAT
ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA CCA GAC AAC CAT TAC CTG
TCC ACA CAA TCT GCC CTT TCG AAA GAT CCC AAC GAA AAG AGA GAC CAC
ATG GTC CTT CTT GAG TTT GTA ACA GCT GCT GGG ATT ACA CAT GGC ATG

CCCTGTGACC CCTCCCCAGT GCCTCTCCTG GCCCTGGAAG TTGCCACTCC
AGTGCCCACC
AGCCTTGTCC TAATAAAATT AAGTTGCATC ATTTTGTCTG ACTAGGTGTC
CTTCTATAAT
ATTATGGGGT GGAGGGGGGT GGTATGGAGC AAGGGGCAAG TTGGGAAGAC
AACCTGTAGG
GCCTGCGGGG TCTATTGGGA ACCAAGCTGG AGTGCAGTGG CACAATCTTG
GCTCACTGCA
ATCTCCGCCT CCTGGGTTCA AGCGATTCTC CTGCCTCAGC CTCCCGAGTT
GTTGGGATTC
CAGGCATGCA TGACCAGGCT CAGCTAATTT TTGTTTTTTT GGTAGAGACG
GGGTTTCACC
ATATTGGCCA GGCTGGTCTC CAACTCCTAA TCTCAGGTGA TCTACCCACC
TTGGCCTCCC
AAATTGCTGG GATTACAGGC GTGAACCACT GCTCCCTTCC CTGTCCTTct gattttgtag
end bGH-polyA??

(N) SEQ ID NO: 43 (same as above without GFP)
5'-ITR—CAG—HaloR—(Nav1.6 Motif)—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start HaloR
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG ACT GAG ACA
TTG CCA CCG GTA ACG GAA TCG GCT GTT GCG CTA CAG GCG GAG GTG ACC
CAG AGG GAG CTG TTC GAG TTC GTT CTC AAC GAC CCC CTC CTC GCC AGT
TCG CTG TAT ATT AAT ATC GCA CTG GCA GGG CTG TCG ATA CTG CTT TTC
GTG TTC ATG ACG CGC GGA CTC GAC GAC CCA CGG GCG AAA CTC ATC GCC
GTT TCG ACG ATT TTG GTG CCG GTG GTC TCT ATC GCG AGC TAC ACC GGC
CTT GCA TCG GGG CTC ACC ATC AGC GTC CTC GAG ATG CCA GCC GGC CAC
TTC GCC GAG GGG TCC TCG GTG ATG CTC GGC GGC GAA GAG GTA GAC GGC
GTC GTG ACG ATG TGG GGC CGC TAT CTG ACG TGG GCC CTT TCG ACA CCG
ATG ATA CTG CTG GCG CTT GGG CTG CTT GCT GGC TCT AAC GCC ACG AAG
CTC TTT ACC GCC ATC ACC TTC GAC ATC GCG ATG TGT GTC ACC GGC CTC
GCA GCC GCG CTG ACG ACC TCT TCG CAC CTG ATG CGG TGG TTC TGG TAC
GCC ATC AGT TGT GCG TGT TTC CTC GTC GTC CTC TAC ATC CTG CTC GTC
GAG TGG GCA CAG GAC GCC AAG GCT GCC GGT ACT GCG GAT ATG TTC AAT
ACG CTG AAG CTG CTG ACC GTT GTC ATG TGG CTC GGC TAC CCC ATC GTG
TGG GCA CTC GGC GTT GAG GGC ATC GCC GTT CTT CCG GTC GGA GTC ACG
TCG TGG GGA TAC AGC TTC CTC GAC ATC GTC GCG AAG TAC ATC TTC GCG
TTC CTG CTG CTC AAC TAC CTC ACG TCG AAC GAG AGC GTC GTC TCC GGC
TCG ATA CTC GAC GTG CCG TCC GCG TCG GGC ACT CCC GCT GAC GAC
end HaloR??

gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG GCCCTGGAAG

??start bGH-polyA

TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT AAGTTGCATC ATTTTGTCTG
ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT GGTATGGAGC AAGGGGCAAG
TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA ACCAAGCTGG AGTGCAGTGG
CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA AGCGATTCTC CTGCCTCAGC
CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT CAGCTAATTT TTGTTTTTTT
GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC CAACTCCTAA TCTCAGGTGA
TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC GTGAACCACT GCTCCCTTCC

(O) SEQ ID NO: 44

5'-ITR—CAG—HaloR—GFP—(NLG-1 Motif)—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc

??start HaloR

atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG ACT GAG ACA
TTG CCA CCG GTA ACG GAA TCG GCT GTT GCG CTA CAG GCG GAG GTG ACC
CAG AGG GAG CTG TTC GAG TTC GTT CTC AAC GAC CCC CTC CTC GCC AGT
TCG CTG TAT ATT AAT ATC GCA CTG GCA GGG CTG TCG ATA CTG CTT TTC
GTG TTC ATG ACG CGC GGA CTC GAC GAC CCA CGG GCG AAA CTC ATC GCC
GTT TCG ACG ATT TTG GTG CCG GTG GTC TCT ATC GCG AGC TAC ACC GGC
CTT GCA TCG GGG CTC ACC ATC AGC GTC CTC GAG ATG CCA GCC GGC CAC
TTC GCC GAG GGG TCC TCG GTG ATG CTC GGC GGC GAA GAG GTA GAC GGC
GTC GTG ACG ATG TGG GGC CGC TAT CTG ACG TGG GCC CTT TCG ACA CCG
ATG ATA CTG CTG GCG CTT GGG CTG CTT GCT GGC TCT AAC GCC ACG AAG
CTC TTT ACC GCC ATC ACC TTC GAC ATC GCG ATG TGT GTC ACC GGC CTC
GCA GCC GCG CTG ACG ACC TCT TCG CAC CTG ATG CGG TGG TTC TGG TAC
GCC ATC AGT TGT GCG TGT TTC CTC GTC GTC CTC TAC ATC CTG CTC GTC
GAG TGG GCA CAG GAC GCC AAG GCT GCC GGT ACT GCG GAT ATG TTC AAT
ACG CTG AAG CTG CTG ACC GTT GTC ATG TGG CTC GGC TAC CCC ATC GTG
TGG GCA CTC GGC GTT GAG GGC ATC GCC GTT CTT CCG GTC GGA GTC ACG
TCG TGG GGA TAC AGC TTC CTC GAC ATC GTC GCG AAG TAC ATC TTC GCG
TTC CTG CTG CTC AAC TAC CTC ACG TCG AAC GAG AGC GTC GTC TCC GGC
TCG ATA CTC GAC GTG CCG TCC GCG TCG GGC ACT CCC GCT GAC GAC

end HaloR?

gaattcggag gcggaggtgg agctagc AAA GGA GAA GAA CTC TTC ACT GGA GTT

??start GFP

GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT GGT GAT GTT AAC GGC CAC
AAG TTC TCT GTC AGT GGA GAG GGT GAA GGT GAT GCA ACA TAC GGA AAA
CTT ACC CTG AAG TTC ATC TGC ACT ACT GGC AAA CTG CCT GTT CCA TGG
CCA ACA CTA GTC ACT ACT CTG TGC TAT GGT GTT CAA TGC TTT TCA AGA
TAC CCG GAT CAT ATG AAA CGG CAT GAC TTT TTC AAG AGT GCC ATG CCC
GAA GGT TAT GTA CAG GAA AGG ACC ATC TTC TTC AAA GAT GAC GGC AAC
TAC AAG ACA CGT GCT GAA GTC AAG TTT GAA GGT GAT ACC CTT GTT AAT

AGA ATC GAG TTA AAA GGT ATT GAC TTC AAG GAA GAT GGC AAC ATT CTG
GGA CAC AAA TTG GAA TAC AAC TAT AAC TCA CAC AAT GTA TAC ATC ATG
GCA GAC AAA CAA AAG AAT GGA ATC AAA GTG AAC TTC AAG ACC CGC CAC
AAC ATT GAA GAT GGA AGC GTT CAA CTA GCA GAC CAT TAT CAA CAA AAT
ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA CCA GAC AAC CAT TAC CTG
TCC ACA CAA TCT GCC CTT TCG AAA GAT CCC AAC GAA AAG AGA GAC CAC
ATG GTC CTT CTT GAG TTT GTA ACA GCT GCT GGG ATT ACA CAT GGC ATG

gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG
GCCCTGGAAG
??start bGH-polyA
TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT AAGTTGCATC
ATTTTGTCTG
ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT GGTATGGAGC
AAGGGGCAAG
TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA ACCAAGCTGG
AGTGCAGTGG
CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA AGCGATTCTC
CTGCCTCAGC
CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT CAGCTAATTT
TTGTTTTTTT
GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC CAACTCCTAA
TCTCAGGTGA
TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC GTGAACCACT
GCTCCCTTCC

P. SEQ ID NO: 45 (same as above but without GFP)
5'-ITR—CAG—HaloR—(NLG-1 Motif)—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start HaloR
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG ACT GAG ACA
TTG CCA CCG GTA ACG GAA TCG GCT GTT GCG CTA CAG GCG GAG GTG ACC
CAG AGG GAG CTG TTC GAG TTC GTT CTC AAC GAC CCC CTC CTC GCC AGT
TCG CTG TAT ATT AAT ATC GCA CTG GCA GGG CTG TCG ATA CTG CTT TTC
GTG TTC ATG ACG CGC GGA CTC GAC GAC CCA CGG GCG AAA CTC ATC GCC
GTT TCG ACG ATT TTG GTG CCG GTG GTC TCT ATC GCG AGC TAC ACC GGC
CTT GCA TCG GGG CTC ACC ATC AGC GTC CTC GAG ATG CCA GCC GGC CAC
TTC GCC GAG GGG TCC TCG GTG ATG CTC GGC GGC GAA GAG GTA GAC GGC
GTC GTG ACG ATG TGG GGC CGC TAT CTG ACG TGG GCC CTT TCG ACA CCG
ATG ATA CTG CTG GCG CTT GGG CTG CTT GCT GGC TCT AAC GCC ACG AAG
CTC TTT ACC GCC ATC ACC TTC GAC ATC GCG ATG TGT GTC ACC GGC CTC
GCA GCC GCG CTG ACG ACC TCT TCG CAC CTG ATG CGG TGG TTC TGG TAC
GCC ATC AGT TGT GCG TGT TTC CTC GTC GTC CTC TAC ATC CTG CTC GTC
GAG TGG GCA CAG GAC GCC AAG GCT GCC GGT ACT GCG GAT ATG TTC AAT
ACG CTG AAG CTG CTG ACC GTT GTC ATG TGG CTC GGC TAC CCC ATC GTG
TGG GCA CTC GGC GTT GAG GGC ATC GCC GTT CTT CCG GTC GGA GTC ACG
TCG TGG GGA TAC AGC TTC CTC GAC ATC GTC GCG AAG TAC ATC TTC GCG
TTC CTG CTG CTC AAC TAC CTC ACG TCG AAC GAG AGC GTC GTC TCC GGC
TCG ATA CTC GAC GTG CCG TCC GCG TCG GGC ACT CCC GCT GAC GAC

end HaloR??

ctctagagtc gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG
??start bGH-poly
GCCCTGGAAG TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT
AAGTTGCATC
ATTTTGTCTG ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT
GGTATGGAGC
AAGGGGCAAG TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA
ACCAAGCTGG
AGTGCAGTGG CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA
AGCGATTCTC
CTGCCTCAGC CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT
CAGCTAATTT
TTGTTTTTTT GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC
CAACTCCTAA
TCTCAGGTGA TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC
GTGAACCACT\
(Q) SEQ ID NO: 46
5'-ITR—CAG—HaloR—GFP—(MLPH Motif)—WPRE—bGHpolyA—ITR-3'

ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start HaloR
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG ACT GAG ACA
TTG CCA CCG GTA ACG GAA TCG GCT GTT GCG CTA CAG GCG GAG GTG ACC
CAG AGG GAG CTG TTC GAG TTC GTT CTC AAC GAC CCC CTC CTC GCC AGT
TCG CTG TAT ATT AAT ATC GCA CTG GCA GGG CTG TCG ATA CTG CTT TTC
GTG TTC ATG ACG CGC GGA CTC GAC GAC CCA CGG GCG AAA CTC ATC GCC
GTT TCG ACG ATT TTG GTG CCG GTG GTC TCT ATC GCG AGC TAC ACC GGC
CTT GCA TCG GGG CTC ACC ATC AGC GTC CTC GAG ATG CCA GCC GGC CAC
TTC GCC GAG GGG TCC TCG GTG ATG CTC GGC GGC GAA GAG GTA GAC GGC
GTC GTG ACG ATG TGG GGC CGC TAT CTG ACG TGG GCC CTT TCG ACA CCG
ATG ATA CTG CTG GCG CTT GGG CTG CTT GCT GGC TCT AAC GCC ACG AAG
CTC TTT ACC GCC ATC ACC TTC GAC ATC GCG ATG TGT GTC ACC GGC CTC
GCA GCC GCG CTG ACG ACC TCT TCG CAC CTG ATG CGG TGG TTC TGG TAC
GCC ATC AGT TGT GCG TGT TTC CTC GTC GTC CTC TAC ATC CTG CTC GTC
GAG TGG GCA CAG GAC GCC AAG GCT GCC GGT ACT GCG GAT ATG TTC AAT
ACG CTG AAG CTG CTG ACC GTT GTC ATG TGG CTC GGC TAC CCC ATC GTG
TGG GCA CTC GGC GTT GAG GGC ATC GCC GTT CTT CCG GTC GGA GTC ACG
TCG TGG GGA TAC AGC TTC CTC GAC ATC GTC GCG AAG TAC ATC TTC GCG
TTC CTG CTG CTC AAC TAC CTC ACG TCG AAC GAG AGC GTC GTC TCC GGC
TCG ATA CTC GAC GTG CCG TCC GCG TCG GGC ACT CCC GCT GAC GAC
end HaloR??
gaattcggag gcggaggtgg agctagc AAA GGA GAA GAA CTC TTC ACT GGA GTT
??start GFP
GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT GGT GAT GTT AAC GGC CAC
AAG TTC TCT GTC AGT GGA GAG GGT GAA GGT GAT GCA ACA TAC GGA AAA
CTT ACC CTG AAG TTC ATC TGC ACT ACT GGC AAA CTG CCT GTT CCA TGG
CCA ACA CTA GTC ACT ACT CTG TGC TAT GGT GTT CAA TGC TTT TCA AGA
TAC CCG GAT CAT ATG AAA CGG CAT GAC TTT TTC AAG AGT GCC ATG CCC
GAA GGT TAT GTA CAG GAA AGG ACC ATC TTC TTC AAA GAT GAC GGC AAC

TAC AAG ACA CGT GCT GAA GTC AAG TTT GAA GGT GAT ACC CTT GTT AAT
AGA ATC GAG TTA AAA GGT ATT GAC TTC AAG GAA GAT GGC AAC ATT CTG
GGA CAC AAA TTG GAA TAC AAC TAT AAC TCA CAC AAT GTA TAC ATC ATG
GCA GAC AAA CAA AAG AAT GGA ATC AAA GTG AAC TTC AAG ACC CGC CAC
AAC ATT GAA GAT GGA AGC GTT CAA CTA GCA GAC CAT TAT CAA CAA AAT
ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA CCA GAC AAC CAT TAC CTG
TCC ACA CAA TCT GCC CTT TCG AAA GAT CCC AAC GAA AAG AGA GAC CAC
ATG GTC CTT CTT GAG TTT GTA ACA GCT GCT GGG ATT ACA CAT GGC ATG

CCCTGTGACC CCTCCCCAGT GCCTCTCCTG GCCCTGGAAG TTGCCACTCC
AGTGCCCACC
AGCCTTGTCC TAATAAAATT AAGTTGCATC ATTTTGTCTG ACTAGGTGTC
CTTCTATAAT
ATTATGGGGT GGAGGGGGGT GGTATGGAGC AAGGGGCAAG TTGGGAAGAC
AACCTGTAGG
GCCTGCGGGG TCTATTGGGA ACCAAGCTGG AGTGCAGTGG CACAATCTTG
GCTCACTGCA
ATCTCCGCCT CCTGGGTTCA AGCGATTCTC CTGCCTCAGC CTCCCGAGTT
GTTGGGATTC
CAGGCATGCA TGACCAGGCT CAGCTAATTT TTGTTTTTTT GGTAGAGACG
GGGTTTCACC
ATATTGGCCA GGCTGGTCTC CAACTCCTAA TCTCAGGTGA TCTACCCACC
TTGGCCTCCC
AAATTGCTGG GATTACAGGC GTGAACCACT GCTCCCTTCC CTGTCCTTct gattttgtag
end bGH-polyA??
(R) SEQ ID NO: 47 (same as above without GFP)
5'-ITR—CAG—HaloR—(MLPH Motif)—WPRE—bGHpolyA—ITR-3'
ttcatgcctt cttctttttc ctacagctcc tgggcaacgt gctggttatt gtgctgtctc
??start HaloR
atcattttgg caaagaatta agcttgagct cgcgatccgc agcc ATG ACT GAG ACA
TTG CCA CCG GTA ACG GAA TCG GCT GTT GCG CTA CAG GCG GAG GTG ACC
CAG AGG GAG CTG TTC GAG TTC GTT CTC AAC GAC CCC CTC CTC GCC AGT
TCG CTG TAT ATT AAT ATC GCA CTG GCA GGG CTG TCG ATA CTG CTT TTC
GTG TTC ATG ACG CGC GGA CTC GAC GAC CCA CGG GCG AAA CTC ATC GCC
GTT TCG ACG ATT TTG GTG CCG GTG GTC TCT ATC GCG AGC TAC ACC GGC
CTT GCA TCG GGG CTC ACC ATC AGC GTC CTC GAG ATG CCA GCC GGC CAC
TTC GCC GAG GGG TCC TCG GTG ATG CTC GGC GGC GAA GAG GTA GAC GGC
GTC GTG ACG ATG TGG GGC CGC TAT CTG ACG TGG GCC CTT TCG ACA CCG
ATG ATA CTG CTG GCG CTT GGG CTG CTT GCT GGC TCT AAC GCC ACG AAG
CTC TTT ACC GCC ATC ACC TTC GAC ATC GCG ATG TGT GTC ACC GGC CTC
GCA GCC GCG CTG ACG ACC TCT TCG CAC CTG ATG CGG TGG TTC TGG TAC
GCC ATC AGT TGT GCG TGT TTC CTC GTC GTC CTC TAC ATC CTG CTC GTC
GAG TGG GCA CAG GAC GCC AAG GCT GCC GGT ACT GCG GAT ATG TTC AAT
ACG CTG AAG CTG CTG ACC GTT GTC ATG TGG CTC GGC TAC CCC ATC GTG
TGG GCA CTC GGC GTT GAG GGC ATC GCC GTT CTT CCG GTC GGA GTC ACG
TCG TGG GGA TAC AGC TTC CTC GAC ATC GTC GCG AAG TAC ATC TTC GCG
TTC CTG CTG CTC AAC TAC CTC ACG TCG AAC GAG AGC GTC GTC TCC GGC
TCG ATA CTC GAC GTG CCG TCC GCG TCG GGC ACT CCC GCT GAC GAC
gagagatctA CGGGTGGCAT CCCTGTGACC CCTCCCCAGT GCCTCTCCTG
GCCCTGGAAG
??start bGH-polyA
TTGCCACTCC AGTGCCCACC AGCCTTGTCC TAATAAAATT AAGTTGCATC
ATTTTGTCTG

ACTAGGTGTC CTTCTATAAT ATTATGGGGT GGAGGGGGGT GGTATGGAGC AAGGGGCAAG
TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTGGGA ACCAAGCTGG AGTGCAGTGG
CACAATCTTG GCTCACTGCA ATCTCCGCCT CCTGGGTTCA AGCGATTCTC CTGCCTCAGC
CTCCCGAGTT GTTGGGATTC CAGGCATGCA TGACCAGGCT CAGCTAATTT TTGTTTTTTT
GGTAGAGACG GGGTTTCACC ATATTGGCCA GGCTGGTCTC CAACTCCTAA TCTCAGGTGA
TCTACCCACC TTGGCCTCCC AAATTGCTGG GATTACAGGC GTGAACCACT GCTCCCTTCC

Pharmaceutical Compositions and Methods of the Invention

[0136] The vectors that comprises the ChR2 or HaloR transgene and the targeting motifs disclosed herein for use to target retinal neurons as described above should be assessed for contamination using conventional methods and formulated into a sterile or aseptic pharmaceutical composition for administration by, for example, subretinal injection.

[0137] Such formulations comprise a pharmaceutically and/or physiologically acceptable vehicle, diluent, carrier or excipient, such as buffered saline or other buffers, e.g., HEPES, to maintain physiologic pH. For a discussion of such components and their formulation, see, generally, Gennaro, A E., Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins Publishers; 2003 or latest edition). See also, WO00/15822. For prolonged storage, the preparation may be frozen, for example, in glycerol.

[0138] The pharmaceutical composition described above is administered to a subject having a visual or blinding disease by any appropriate route, preferably by intravitreal or subretinal injection, depending on the retinal layer being targeted.

[0139] Disclosures from Bennett and colleagues (cited herein) concern targeting of retinal pigment epithelium—the most distal layer from the vitreal space. According to the present invention, the DNA construct is targeted to either retinal ganglion cells or bipolar cells. The ganglion cells are reasonably well-accessible to intravitreal injection. Intravitreal and/or subretinal injection can provide the necessary access to the bipolar cells, especially in circumstances in which the photoreceptor cell layer is absent due to degeneration—which is the case in certain forms of degeneration that the present invention is intended to overcome.

[0140] To test for the vector's ability to express the transgene, specifically in mammalian retinal neurons, preferably RGC, by AAV-mediated delivery, a combination of a preferred promoter sequence linked to a reporter gene such as GFP or LacZ can be packaged into rAAV virus particles, concentrated, tested for contaminating adenovirus and titered for rAAV. The right eyes of a number of test subjects, preferably inbred mice, are injected sub-retinally with about 1 μl of the rAAV preparation (e.g., greater than about 10<10 >infectious units ml). Two weeks later, the right (test) and left (control) eyes of half the animals are removed, fixed and stained with an appropriate substrate or antibody or other substance to reveal the presence of the reporter gene. A majority of the test retinas in injected eyes will exhibited a focal stained region, e.g., blue for LacZ/Xgal, or green for GFP consistent with a subretinal bleb of the injected virus creating a localized retinal detachment. All control eyes are negative for the reporter gene product. Reporter gene expression examined in mice sacrificed at later periods is detected for at least 10 weeks post-injection, which suggests persistent expression of the reporter transgene.

[0141] An effective amount of rAAV virions carrying a nucleic acid sequence according to this invention encoding the ChR2 or HaloR and targeting motif under the control of the promoter of choice, preferably CAG or a cell-specific promoter such as mGluR6, is preferably in the range of between about 10<10 >to about 10<13 >rAAV infectious units in a volume of between about 150 and about 800 μl per injection. The rAAV infectious units can be measured according to McLaughlin, S K et al., 1988, J Virol 62:1963. More preferably, the effective amount is between about 10<10 >and about 10<12 >rAAV infectious units and the injection volume is preferably between about 250 and about 500 μl. Other dosages and volumes, preferably within these ranges but possibly outside them, may be selected by the treating professional, taking into account the physical state of the subject (preferably a human), who is being treated, including, age, weight, general health, and the nature and severity of the particular ocular disorder.

[0142] It may also be desirable to administer additional doses ("boosters") of the present nucleic acid or rAAV compositions. For example, depending upon the duration of the transgene expression within the ocular target cell, a second treatment may be administered after 6 months or yearly, and may be similarly repeated. Neutralizing antibodies to AAV are not expected to be generated in view of the routes and doses used, thereby permitting repeat treatment rounds.

[0143] The need for such additional doses can be monitored by the treating professional using, for example, well-known electrophysiological and other retinal and visual function tests and visual behavior tests. The treating professional will be able to select the appropriate tests applying routine skill in the art. It may be desirable to inject larger volumes of the composition in either single or multiple doses to further improve the relevant outcome parameters.

[0144] Ocular Disorders

[0145] The ocular disorders for which the present methods are intended and may be used to improve one or more parameters of vision include, but are not limited to, developmental abnormalities that affect both anterior and posterior segments of the eye. Anterior segment disorders include glaucoma, cataracts, corneal dystrophy, keratoconus. Posterior segment disorders include blinding disorders caused by photoreceptor malfunction and/or death caused by retinal dystrophies and degenerations. Retinal disorders include congenital stationary night blindness, age-related macular degeneration, congenital cone dystrophies, and a large group of retinitis-pigmentosa (RP)-related disorders. These disorders include genetically pre-disposed death of photoreceptor cells, rods and cones in the retina, occurring at various ages. Among those are severe retinopathies, such as subtypes of RP itself that progresses with age and causes blindness in childhood and early adulthood and RP-associated diseases, such as genetic subtypes of LCA, which frequently results in loss of vision during childhood, as early as the first year of life. The latter disorders are generally characterized by severe reduction, and often complete loss of photoreceptor cells, rods and cones. (Trabulsi, E I, ed., Genetic Diseases of the Eye, Oxford University Press, NY, 1998).

[0146] In particular, this method is useful for the treatment and/or restoration of at least partial vision to subjects that have lost vision due to ocular disorders, such as RPE-associated retinopathies, which are characterized by a long-term preservation of ocular tissue structure despite loss of function and by the association between function loss and the defect or absence of a normal gene in the ocular cells of the subject. A variety of such ocular disorders are known, such as childhood onset blinding diseases, retinitis pigmentosa, macular degeneration, and diabetic retinopathy, as well as ocular blinding diseases known in the art. It is anticipated that these other disorders, as well as blinding disorders of presently unknown causation which later are characterized by the same description as above, may also be successfully treated by

this method. Thus, the particular ocular disorder treated by this method may include the above-mentioned disorders and a number of diseases which have yet to be so characterized.

[0147] Visual information is processed through the retina through two pathways: an ON pathway which signals the light ON, and an OFF pathway which signals the light OFF (Wassle, supra). It is generally believed that the existence of the ON and OFF pathway is important for the enhancement of contrast sensitivity. The visual signal in the ON pathway is relay from ON-cone bipolar cells to ON ganglion cells. Both ON-cone bipolar cells and ON-ganglion cells are depolarized in response to light. On the other hand, the visual signal in the OFF pathway is carried from OFF-cone bipolar cells to OFF ganglion cells. Both OFF-cone bipolar cells and OFF-ganglion cells are hypopolarized in response to light. Rod bipolar cells, which are responsible for the ability to see in dim light (scotopic vision), are ON bipolar cells (depolarized in response to light). Rod bipolar cells relay the vision signal through All amacrine cells (an ON type retinal cell) to ON an OFF cone bipolar cell.

[0000] Electrical/Visual activity Recording and Measurement

[0148] Patch-Clamp Recordings

[0149] Dissociated retinal cells and retinal slice are prepared, e.g., as described by Pan, Z.-H. J. Neurophysiol. 83 513-527 (2000); J. Cui, Y P et al., J. Physiol. 553:895-909 (2003)). Recordings with patch electrodes in the whole-cell configuration can be made by an EPC-9 amplifier and PULSE software (Heka Electronik, Lambrecht, Germany) Recordings are preferably made in Hanks' solution containing (in mM): NaCl, 138; NaHCO3, 1; Na2HPO4, 0.3; KCl, 5; KH2PO4, 0.3; CaCl2, 1.25; MgSO4, 0.5; MgCl2, 0.5; HEPES-NaOH, 5; glucose, 22.2; with phenol red, 0.001% v/v; adjusted to pH 7.2 with 0.3 N NaOH. The electrode solution contains (in mM): K-gluconate, 133; KCl, 7; MgCl2, 4; EGTA, 0.1; HEPES, 10; Na-GTP, 0.5; and Na-ATP, 2; pH adjusted with KOH to 7.4. The resistance of the electrode is about 13 to 15 MO. The recordings are performed at room temperature.

[0150] Multielectrode Array Recordings

[0151] The multielectrode array recordings were are on the procedures reported by Tian, N. et al., Neuron 39:85-96 (2003). Briefly, retinas are dissected and placed photoreceptor side down on a nitrocellulose filter paper strip. The mounted retina is placed in the MEA-60 multielectrode array recording chamber of 30 μm diameter electrodes spaced 200 μm apart (Multi Channel System MCS GmbH, Reutlingen, Germany), with the ganglion cell layer facing the recording electrodes. The retina is continuously perfused in oxygenated extracellular solution at 34° C. The extracellular solution preferably contains (in mM): NaCl, 124; KCl, 2.5; CaCl2, 2; MgCl2, 2; NaH2PO4, 1.25; NaHCO3, 26; and glucose, 22 (pH 7.35 with 95% O2 and 5% CO2). Recordings are usually started 60 min after the retina is positioned in the recording chamber. The interval between onsets of each light stimulus is generally 10-15 s. The signals are filtered between 200 Hz (low cut off) and 20 kHz (high cut off). The responses from individual neurons are analyzed using, e.g., Offline Sorter software (Plexon, Inc., Dallas, Tex.).

[0152] Visual-Evoked Potential Recordings

[0153] Visual-evoked potential recordings are carried out, for example, in wild-type mice of the C57BL/6 and 129/Sv strains aged 4-6 months and in rdl/rdl mice aged 6-11 months. Recordings are performed 2-6 months after viral vector injection. After general anesthesia, animals are mounted in a stereotaxic apparatus. Body temperature may be unregulated or maintained at 34° C. with a heating pad and a rectal probe. Pupils are dilated with 1% atropine and 2.5% accu-phenylephrine. A small portion of the skull (~1.5×1.5 mm) centered about 2.5

mm from the midline and 1 mm rostral to the lambdoid suture is drilled and removed. Recordings are made from visual cortex (area V1) by a glass micropipette (resistance ~0.5 M after filling with 4 M NaCl) advanced 0.4 mm beneath the surface of the cortex at the contralateral side of the stimulated eye. The stimuli are 20 ms pluses at 0.5 Hz. Responses are amplified (1,000 to 10,000), band-pass filtered (0.3-100 Hz), digitized (1 kHz), and averaged over 30-250 trials.

Light Stimulation

[0154] For dissociated cell and retinal slice recordings, light stimuli are generated by a 150 W xenon lamp-based scanning monochromator with bandwidth of 10 nm (TILL Photonics, Germany) and coupled to the microscope with an optical fiber. For multielectrode array recordings, light responses are evoked by the monochromator or a 175 W xenon lamp-based illuminator (Lambda L S, Sutter Instrument) with a band-pass filter of 400-580 nm and projected to the bottom of the recording chamber through a liquid light guider. For visual evoked potential, light stimuli are generated by the monochromator and projected to the eyes through the optical fiber. The light intensity is attenuated by neutral density filters. The light energy is measured by a thin-type sensor (TQ82017) and an optical power meter (e.g., Model: TQ8210, Advantest, Tokyo, Japan).

Restoration or Improvement of Light Sensitivity and Vision

[0155] Both in vitro and in vivo studies to assess the various parameters of the present invention may be used, along with any recognized animal model of a blinding human ocular disorder. Large animal models of human retinopathy, e.g., childhood blindness, are useful. The examples provided herein allow one of skill in the art to readily appreciate that this method may be used similarly to treat a range of retinal diseases.

[0156] While earlier studies by others have demonstrated that retinal degeneration can be retarded by gene therapy techniques, the present invention demonstrates a definite physiological recovery of function, which is expected to generate or improve various parameters of vision, including behavioral parameters. Behavioral measures can be obtained using known animal models and tests, for example performance in a water maze, wherein a subject in whom vision has been preserved or restored to varying extents will swim toward light (Hayes, J M et al., 1993, Behav Genet 23:395-403).

[0157] In models in which blindness is induced during adult life or in congenital blindness that develops slowly enough for the individual to experience vision before its loss, training in various tests may be done. When these tests are re-administered after visual loss to test the efficacy of the present compositions and methods for their vision-restorative effects, animals do not have to learn the tasks de novo while in a blind state. Other behavioral tests do not require learning and rely on instinctiveness of certain behaviors. An example is the optokinetic nystagmus test (Balkema G W et al., 1984, Invest Ophthal V is Sci. 25:795-800; Mitchiner J C et al., 1976, Vision Res. 16:1169-71).

[0158] As is exemplified herein, the transfection of retinal neurons with DNA encoding Chop2 provides residual retinal neurons, principally bipolar cells and ganglion cells, with photosensitive membrane channels. Thus, it was possible to measure, with a strong light stimulus, the transmission of a visual stimulus to the animal's visual cortex, the area of the brain responsible for processing visual signals; this therefore constitutes a form of vision, as intended herein. Such vision may differ from forms of normal human vision and may be referred to as a sensation of light, also termed “light detection” or “light perception.”

[0159] Thus, the term “vision” as used herein is defined as the ability of an organism to

usefully detect light as a stimulus for differentiation or action. Vision is intended to encompass:

1. Light detection or perception—the ability to discern whether or not light is present
2. Light projection—the ability to discern the direction from which a light stimulus is coming;
3. Resolution—the ability to detect differing brightness levels (i.e., contrast) in a grating or letter target;
4. Recognition—the ability to recognize the shape of a visual target by reference to the differing contrast levels within the target.

Thus, “vision” includes the ability to simply detect the presence of light. This opens the possibility to train an affected subject who has been treated according to this invention to detect light, enabling the individual to respond remotely to his environment however crude that interaction might be. In one example, a signal array is produced to which a low vision person can respond to that would enhance the person's ability to communicate by electronic means remotely or to perform everyday tasks. In addition such a person's mobility would be dramatically enhanced if trained to use such a renewed sense of light resulting from “light detection.” The complete absence of light perception leaves a person with no means (aside from hearing and smell) to discern anything about objects remote to himself.

[0164] The methods of the present invention that result in light perception, even without full normal vision, also improve or support normally regulated circadian rhythms which control many physiological processes including sleep-wake cycles and associated hormones. Although some blind individuals with residual RGCs can mediate their rhythms using RGC melanopsin, it is rare for them to do so. Thus, most blind persons have free-running circadian rhythms. Even when they do utilize the melanopsin pathway, the effect is very weak. The methods of the present invention are thus expected to improve health status of blind individuals by enabling absent light entrainment or improving weakened (melanopsin-mediated) light entrainment of circadian rhythms which leads to better overall health and well-being.

[0165] In addition to rhythms, the present invention provides a basis to improve deficits in other light-induced physiological phenomena. Photoreceptor degeneration may result in varying degrees of negative masking, or suppression, of locomotor activity during the intervals in the circadian cycle in which the individual should be sleeping. Suppression of pineal melatonin may occur. Both contribute to the entrainment process. Thus, improvement in these responses/activities in a subject in whom photoreceptors are or have degenerated contributes, independently of vision per se, to appropriate sleep/wake cycles that correspond with the subject's environment in the real world.

[0166] Yet another benefit of the present invention is normalization of pupillary light reflexes because regulation of pupil size helps modulate the effectivenees of light stimuli in a natural feed back loop. Thus, the present invention promotes re-establishment of this natural feedback loop, making vision more effective in subject treated as described herein.

[0167] In certain embodiments, the present methods include the measurement of vision before, and preferably after, administering the present vector. Vision is measured using any of a number of methods well-known in the art or ones not yet established. Most preferred are:
(1) A light detection response by the subject after exposure to a light stimulus—in which evidence is sought for a reliable response of an indication or movement in the general direction of the light by the subject individual when the light it is turned on is.
(2) a light projection response by the subject after exposure to a light stimulus in which evidence is sought for a reliable response of indication or movement in the specific direction of the light by the individual when the light is turned on.
(3) light resolution by the subject of a light vs. dark patterned visual stimulus, which measures the subject's capability of resolving light vs dark patterned visual stimuli as evidenced by:

(a) the presence of demonstrable reliable optokinetically produced nystagmoid eye movements and/or related head or body movements that demonstrate tracking of the target (see above) and/or
(b). the presence of a reliable ability to discriminate a pattern visual stimulus and to indicate such discrimination by verbal or non-verbal means, including, for example pointing, or pressing a bar or a button; or
(4) electrical recording of a visual cortex response to a light flash stimulus or a pattern visual stimulus, which is an endpoint of electrical transmission from a restored retina to the visual cortex. Measurement may be by electrical recording on the scalp surface at the region of the visual cortex, on the cortical surface, and/or recording within cells of the visual cortex.

[0174] It is known in the art that it is often difficult to make children who have only light perception appreciate that they have this vision. Training is required to get such children to react to their visual sensations. Such a situation is mimicked in the animal studies exemplified below. Promoting or enhancing light perception, which the compositions and methods of the present invention will accomplish, is valuable because patients with light perception not only are trainable to see light, but they can usually be trained to detect the visual direction of the light, thus enabling them to be trained in mobility in their environment. In addition, even basic light perception can be used by visually impaired individuals, including those whose vision is improved using the present compositions and methods, along with specially engineered electronic and mechanical devices to enable these individuals to accomplish specific daily tasks. Beyond this and depending on their condition, they may even be able to be trained in resolution tasks such as character recognition and even reading if their impairment permits. Thus it is expected that the present invention enhances the vision of impaired subjects to such a level that by applying additional training methods, these individuals will achieve the above objectives.

[0175] Low sensitivity vision may emulate the condition of a person with a night blinding disorder, an example of which is Retinitis Pigmentosa (RP), who has difficulty adapting to light levels in his environment and who might use light amplification devices such as supplemental lighting and/or night vision devices.

[0176] Thus, the visual recovery that has been described in the animal studies described below would, in human terms, place the person on the low end of vision function. Nevertheless, placement at such a level would be a significant benefit because these individuals could be trained in mobility and potentially in low order resolution tasks which would provide them with a greatly improved level of visual independence compared to total blindness.

[0177] The mice studied in the present Examples were rendered completely devoid of photoreceptors; this is quite rare, even in the worst human diseases. The most similar human state is RP. In most cases of RP, central vision is retained till the very end. In contrast, in the studied mouse model, the mouse becomes completely blind shortly after birth.

[0178] Common disorders encountered in low vision are described by J. Tasca and E. A. Deglin in Chap. 6 of Essentials of Low Vision Practice, R. L. Brilliant, ed., Butterworth Heinemann Publ., 1999, which is incorporated by reference in its entirety. There is reference to similar degenerative conditions, but these references show form vision that is measurable as visual acuity. Ganglion cell layers are not retained in all forms of RP, so the present approach will not work for such a disorder.

[0179] When applying the present methods to humans with severe cases of RP, it is expected that central vision would be maintained for a time at some low level while the peripheral retina degenerated first. It is this degenerating retina that is the target for re-activation using the present invention. In essence, these individuals would be able to retain mobility vision as they

approached blindness gradually.

[0180] Subjects with macular degeneration, characterized by photoreceptor loss within the central “sweet spot” of vision (Macula Lutea), are expected to benefit by treatment in accordance with the present invention, in which case the resolution capability of the recovered vision would be expected to be higher due to the much higher neuronal density within the human macula.

[0181] While it is expected that bright illumination of daylight and artificial lighting that may be used by a visually impaired individual will suffice for many visual activities that are performed with vision that has recovered as a result of the present treatments. It is also possible that light amplification devices may be used, as needed, to further enhance the affected person's visual sensitivity. The human vision system can operate over a 10 log unit range of luminance. On the other hand, microbial type rhodopsins, such as ChR2, operate over up to a 3 log unit range of luminance. In addition, the light conditions the patient encounters could fall outside of the operating range of the light sensor. To compensate for the various light conditions, a light pre-amplification or attenuation device could be used to expand the operation range of the light conditions. Such device would contain a camera, imaging processing system, and microdisplays, which can ne assembled from currently available technologies, such as night vision goggles and/or 3D adventure and entertainment system. (See, for example the following URL on the Worldwide web—emagin.com/.)

[0182] The present invention may be used in combination with other forms of vision therapy known in the art. Chief among these is the use of visual prostheses, which include retinal implants, cortical implants, lateral geniculate nucleus implants, or optic nerve implants. Thus, in addition to genetic modification of surviving retinal neurons using the present methods, the subject being treated may be provided with a visual prosthesis before, at the same time as, or after the molecular method is employed.

[0183] The effectiveness of visual prosthetics can be improved with training of the individual, thus enhancing the potential impact of the ChR2 or HaloR transformation of patient cells as discussed herein. An example of an approach to training is found in US 2004/0236389 (Fink et al.), incorporated by reference. The training method may include providing a non-visual reference stimulus to a patient having a visual prosthesis based on a reference image. The non-visual reference stimulus is intended to provide the patient with an expectation of the visual image that the prosthesis will induce. Examples of non-visual reference stimuli are a pinboard, Braille text, or a verbal communication. The visual prosthesis stimulates the patient's nerve cells, including those cells whose responsiveness has been improved by expressing ChR2 and/or HaloR as disclosed herein, with a series of stimulus patterns attempting to induce a visual perception that matches the patient's expected perception derived from the non-visual reference stimulus. The patient provides feedback to indicate which of the series of stimulus patterns induces a perception that most closely resembles the expected perception. The patient feedback is used as a “fitness function” (also referred to as a cost function or an energy function). Subsequent stimuli provided to the patient through the visual prosthesis are based, at least in part, on the previous feedback of the patient as to which stimulus pattern(s) induce the perception that best matches the expected perception. The subsequent stimulus patterns may also be based, at least in part, on a fitness function optimization algorithm, such as a simulated annealing algorithm or a genetic algorithm.

[0184] Thus, in certain embodiments of this invention, the method of improving or restoring vision in a subject further comprises training of that subject, as discussed above. Preferred examples of training methods are:

(a) habituation training characterized by training the subject to recognize (i) varying levels of

light and/or pattern stimulation, and/or (ii) environmental stimulation from a common light source or object as would be understood by one skilled in the art; and
(b) orientation and mobility training characterized by training the subject to detect visually local objects and move among said objects more effectively than without the training.
In fact, any visual stimulation techniques that are typically used in the field of low vision rehabilitation are applicable here.

[0187] The remodeling of inner retinal neurons triggered by photoreceptor degeneration has raised a concerns about retinal-based rescue strategies after the death of photoreceptors (Strettoi and Pignatelli 2000, Proc Natl Acad Sci USA. 97:11020-5; Jones, B W et al., 2003, J Comp Neurol 464:1-16; Jones, B W and Marc, R E, 2005, Exp Eye Res. 81:123-37; Jones, B W et al., 2005, Clin Exp Optom. 88:282-91). Retinal remodeling is believed to result from deafferentation, the loss of afferent inputs from photoreceptors—in other words, the loss of light induced activities So after death of rods and coned, there is no light evoked input to retinal bipolar cells and ganglion cells, and through them to higher visual centers. In response to the loss of such input, the retina and higher visual network are triggered to undergo remodeling, in a way seeking other forms of inputs. Said otherwise, the retina needs to be used to sense light in order to maintain its normal network, and with the loss of light sensing, the network will deteriorate via a remodeling process. This process is not an immediate consequence of photoreceptor death; rather it is a slow process, providing a reasonably long window for intervention.

[0188] Thus, an additional utility of restoring light sensitivity to inner retinal neurons in accordance with the present invention is the prevention or delay in the remodeling processes in the retina, and, possibly, in the higher centers. Such retinal remodeling may have undesired consequences such as corruption of inner retinal network, primarily the connection between bipolar and RGCs. By introducing the light-evoked activities in bipolar cells or RGCs, the present methods would prevent or diminish the remodeling due to the lack of input; the present methods introduce this missing input (either starting from bipolar cells or ganglion cells), and thereby stabilize the retinal and higher visual center network. Thus, independently of its direct effects on vision, the present invention would benefit other therapeutic approaches such as photoreceptor transplantation or device implants.

[0189] Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Example I

Transgene Expression in Different Cellular Sites or Compartments

A. Materials and Methods

Viral Vectors:

[0190] Adeno-associated virus serotype 2 (rAAV2) cassette carrying a channelopsin-2 and GFP (Chop2-GFP) fusion construct (Bi, A. et al. Neuron 50:23-33 (2006); WO2007/131180. were modified by inserting subcellular sorting motifs at the 3' end of GFP (or, if no reporter is present, at the 3' end of ChR2 or HaloR. As described above. viral vectors carrying the transgene of ChR2-GFP-(motif) with a hybrid CMV early enhancer/chicken ß-actin) promoter (CAG) were packaged and affinity purified at the Gene Transfer Vector Core of the University of Iowa. Design of the vectors was is described above.

Animal and Viral Vector Injection:

[0191] 3-4 adult C57BL/6J mice aged 1-2 months per construct were used for the study. The mice were anesthetized by intraperitoneal injection of ketamine (120 mg/kg) and xylazine (15 mg/kg). Under a dissecting microscope, a small perforation was made with a needle in the sclera region posterior to the limbus, and 1.0 μl of viral vector suspension at a concentration of >1×10<12 >gv/ml was injected into the intravitreal space of each eye. Four weeks after viral vector injection, animals were sacrificed by CO2 asphyxiation followed by decapitation and enucleation.

Histology:

[0192] Enucleated eyes were fixed in 4% paraformaldehyde in phosphate buffer (PB) for 20 minutes and the dissected retina flat mounted onto a microscope slide for histological studies. The flat mounts were examined under a Zeiss Apotome microscope and Zstack images were taken at ~562 ms exposure time at optical sections of 1 μm apart in order to capture the axon, soma, and entire depth of the dendritic tree of each RGC.

Image Analysis and Fluorescence Intensity Ratio Calculations:

[0193] Intensity profiles of axon, soma, and dendrites for each RGC were measured in ImageJ (obtained from NIH) by applying lines of width of 5 pixels. For each RGC, axon intensity profile was obtained by averaging 3 measurements, somatic intensity profile was obtained by averaging 3 measurements, and dendritic intensity profile was obtained by averaging 9 measurements (3 proximal, 3 intermediate, and 3 distal). Dendrite/axon (D/A) and soma/axon (S/A) intensity ratios were then calculated from the average values for each RGC.

Statistical Analysis of Fluorescence Intensity Ratios:

[0194] A one-way analysis of variance (ANOVA) was conducted with Bonferroni correction. P<0.05 is considered significantly different for somatic fluorescence intensity (Soma F.I.) measurements, dendrite to axon (D/A) ratios and soma to axon (S/A) ratios between groups.

B. Results

[0195] Results are shown in FIG. 1 and in Table 2 below.

[0000]

TABLE 2
Comparison of Transduced GFP Expression in Different Cellular Sites or Compartments Mediated by Different Motifs:

Sorting Motif	n*	Fluorescence Intensity at subcellular site Mean ± SE Soma	Dendrite	Axon	Conclusion: targeted site (receptivce field)
Control	29	146.0 ± 8.3	65.2 ± 4.2	36.6 ± 1.9	2.31 ± 0.88<†>18.8 ± 1.4<†>
Kv2.1	24	117.7 ± 6.0			Soma, proximal dendritic (center) 74.7 ± 8.2<†>10.6 ± 3.3<†>25.3 ± 1.6<†>
Nav1.6	24				Axon initial segment, soma (center)20.8 ± 1.9<†>
MLPH	25	128.7 ± 9.3	73.5 ± 4.6		Somatodendritic (surroung = off center)23.2 ± 1.9<†>
NLG-1	25	133.2 ± 7.2	76.2 ± 3.1		Somatodendritic (surroung = off center)47.9 ± 3.0<†>
AMPAR	23	143.2 ± 8.8	81.5 ± 3.8		No selective targeting in this

Kv4.2 26 142.0 ± 8.9 76.6 ± 4.8 41.1 ± 2.9 experiment
nAChR 29 120.0 ± 4.8 67.3 ± 3.3 31.8 ± 1.8
TLCN 19 157.3 ± 15.9 53.4 ± 5.5 31.2 ± 3.4
*n = number of cells analyzed<†>

Difference from control significant at $p < 0.05$

[0196] Use of the Kv2.1 motif and targeted ChR2, and would similarly target HaloR, to soma and proximal dendritic regions (the center of receptive field) of RGCs. Use of Nav1.6 motif targets to soma and axon initial segments (the center of the receptive field). Kv2.1 appears to achieve such targeting more effectively than does Nav1.6.

[0197] Use of NLG and MLPH sorting motifs targeted ChR2 (and would target HaloR) to distal dendritic regions (the surround of the receptive field) because, compared to control, they are more biased to distal dendritic regions. NLG appears to do this better.

[0198] Use of Kv2.1, Nav1.6, NLG and MLPH reduces expression of the ChR2 or HaloR in the axons of retinal ganglion cells. Although not shown directly in FIG. 1 or Table 2, The ankyrin binding domain of Nav1.6 preferentially targeted Chop2-GFP to the axon initial segments as well as decreased expression in the dendrites of RGCs with D/A ratio 4.5 fold less than control. However the overall fluorescence intensity was lower for Nav1.6 compared to the control which contributed to the lack of significant difference in the S/A ratio compared to control. A previous (preliminary) study reported use of Anbthe ankyrin binding domain to target Chop2 to the somata of rabbit retinal ganglion cells via biolistic gene transfer (Greenberg, K. P. et al. Invest. Ophthal. V is Sci 2009 (abstract) 2009)

[0199] Motifs from nAchR, KV4.2, TLCN, and AMPAR did not show statistically significant differences from the control group in somatic fluorescence, D/A ratio, and S/A ratio in this study. However, it is believed that with varying conditions, further modified vectors, etc., these too are useful as sorting motifs for targeting of, and spatially selective expression of tranduced ChR2 or HaloR in RGC.

Example II

Physiological Responses of Cells Expressing ChR2 and

[0200] Studies were conducted (data not shown) in which the RGCs transduced by vectors comprising ChR2 and the Kv2.1 motif (center-targeting) which indeed showed enhanced expression in the center (Soma, proximal dendritic, were tested for electrical responses to light stimuli. A light slit was used to move a light along the cell, and recordings were made where the cell responded by depolarization. The responsiveness of such cells were enhanced compared to those of controls (transduced with vector not containing the sorting motif) indicating a close correlation between the histological evidence for site-specific expression of a transgene (GFP) and spatial organization of a transgene similarly introduced (ChR2). These results confirm the utility of this approach to evoking improved light responsiveness with organization reflective of normal retinal function (spatial specificity) in cells treated using the present methods.