(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 September 2016 (22.09.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/149599 A1

(51) International Patent Classification:
C07K 14/405 (2006.01) *A61N 5/06* (2006.01)
C12N 13/00 (2006.01)

(21) International Application Number:
PCT/US2016/023095

(22) International Filing Date:
18 March 2016 (18.03.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/135,470	19 March 2015 (19.03.2015)	US
62/149,812	20 April 2015 (20.04.2015)	US
62/261,821	1 December 2015 (01.12.2015)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2016/149599 A1

(54) Title: COMPOSITIONS AND METHODS FOR USE OF ANION CHANNEL RHODOPSINS

(57) Abstract: Methods and compositions used to identify and characterize a new class of rhodopsins derived from algae, which are highly sensitive and efficient anion- conducting channelrhodopsins. The rhodopsin domain of these anion-conducting channelrhodopsins have been cloned and expressed in mammalian systems and thus may be used in, among others, optogenetic applications and as therapeutic agents for electrically active cell mediated disorders.

DESCRIPTION

COMPOSITONS AND METHODS FOR USE OF ANION CHANNEL RHODOPSINS

[0001] This application claims benefit of priority to U.S. Provisional Applications Serial No. 62/135,470, filed March 19, 2015, No. 62/149,812, filed April 20, 2015, and No. 62/261,821, filed December 1, 2015, the entire contents of which are hereby incorporated by reference.

[0002] This invention was made with U.S. Government support under Grant Nos. R37GM027750, R21MH098288 and S10RR022531 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0003] This disclosure generally relates to methods and compositions that utilize channelrhodopsins derived from algae, and more particularly to anion-conducting channelrhodopsins which have novel characteristics, for optogenetic applications or for use as therapeutic agents.

2. Description of Related Art

[0004] Optogenetics (Deisseroth. *Nat Methods* 8 (1): 26-9, 2011), refers to using optical methods for probing and controlling genetically targeted neurons within intact neural circuits. Optogenetics involves the introduction of light-activated channels and enzymes that allow manipulation of neural activity with millisecond precision while maintaining cell-type resolution through the use of specific targeting mechanisms. Because the brain is a high-speed system, millisecond-scale temporal precision is central to the concept of optogenetics, which allows probing the causal role of specific action potential patterns in defined cells.

[0005] Light control of motility behavior (phototaxis and photophobic responses) in green flagellate algae is mediated by sensory rhodopsins homologous to phototaxis receptors and light-driven ion transporters in prokaryotic organisms. In the phototaxis process, excitation of the algal sensory rhodopsins leads to generation of transmembrane photoreceptor currents. When expressed in animal cells, the algal phototaxis receptors function as light-gated cation channels, which has earned them the name

"channelrhodopsins". Channelrhodopsins have become useful molecular tools for light control of cellular activity.

[0006] Originally, the source of these light-activated channels and enzymes were several microbial opsins, including Channelrhodopsin-2 (ChR2) a single-component light-activated cation channel from algae, which allowed millisecond-scale temporal control in mammals, required only one gene to be expressed in order to work, and responded to visible-spectrum light with a chromophore (retinal) that was already present and supplied to ChR2 by the mammalian brain tissue. The experimental utility of ChR2 was quickly proven in a variety of animal models ranging from behaving mammals to classical model organisms such as flies, worms, and zebrafish, and hundreds of groups have employed ChR2 and related microbial proteins to study neural circuits. Currently, several members of this family have been recruited as molecular tools for optogenetics, i.e. regulation of cellular activity with light. Phototaxis receptors from green (chlorophyte) flagellate algae (6), best known as channelrhodopsins (ChRs) owing to their function as light-gated cation channels are widely used to depolarize genetically targeted populations of excitable cells.

[0007] Hyperpolarizing rhodopsin ion pumps have been employed to suppress neuron firing, but they transport only a single charge per captured photon and therefore have limited capacity. Recently, ChRs were engineered to conduct Cl-, but these optogenetic tools still retain some cation conductance and could be made highly light- sensitive only at the expense of greatly slowing the channel kinetics with additional mutations (J. Wietek et al., Science 344, 409 (2014) and A. Berndt, S. Y. Lee, C. Ramakrishnan, K. Deisseroth, Science 344, 420 (2014)). Ideal for optogenetic hyperpolarization would be natural light-gated anion channels optimized by evolution to be highly conductive and anion-selective.

[0008] Described herein are modified and optimized rhodopsin domains derived from a newly identified class of channelrhodopsins, Anion Channel Rhodopsins (ACRs), light-gated anion channels that provide highly sensitive and efficient membrane hyperpolarization and neuronal silencing through light-gated chloride conduction.

SUMMARY OF THE INVENTION

[0009] The presently disclosed methods and compositions are based, in part, on the discovery and identification of a novel class of channelrhodopsins, Anion Channel Rhodopsins (ACRs). Light-gated rhodopsin cation channels from chlorophyte algae have transformed neuroscience research through their use as membrane-depolarizing optogenetic tools for targeted photoactivation of neuron firing. Photosuppression of neuronal action potentials has been limited by the lack of equally efficient tools for membrane hyperpolarization. Described herein are Anion Channel Rhodopsins (ACRs), a new family of light-gated anion channels that provide highly sensitive and efficient membrane hyperpolarization and neuronal silencing through light-gated chloride conduction. ACRs strictly conduct anions, completely excluding protons and larger cations, and hyperpolarize the membrane with 100-fold faster kinetics at 3000-fold lower light intensity than the most efficient currently available optogenetic proteins. Sequences encoding 7TM domains of *G. theta* opsins (295, 291 and 288 aa, corresponding to the JGI protein models 111593, 146828 and 161302, respectively) were optimized for human codon usage and were synthesized. The sequence information encoding the functional constructs 111593 (GtACR1: SEQ ID NO 1) and 146828 (GtACR2: SEQ ID NO: 3) will be represented in GenBank (accession numbers KP171708 and KP171709, respectively). Illumination of neurons expressing GtACR2 fully inhibited their action potential spikes. These ACRs provide new membrane-hyperpolarizing tools for use in establishing a high level of membrane potential for use as optogenetic tools for neuronal silencing of excited cells for among others, neuronal or neurologic disorders, such as but not limited to Parkinson's disease and epilepsy, as well as for cardiac disorders. Thus, ACRs provided herein can serve as new membrane-hyperpolarizing tools for use treatment of epilepsy, as well as for cardiac disorders.

[0010] In some embodiments herein is disclosed a recombinant nucleic acid operatively linked to a heterologous promoter sequence, said recombinant nucleic acid comprising: a sequence that encodes a peptide with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13; or a sequence that encodes a peptide comprising 225 contiguous amino acids selected from SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 13; or a sequence that hybridizes to the nucleotide sequence of SEQ ID NO:2, SEQ ID NO: 4, SEQ ID NOs: 14-15 or the complement thereof. In another embodiment the recombinant

nucleic acid comprises an expression vector. In another embodiment of the the recombinant nucleic acid the sequence that hybridized to the nucleotide sequence of SEQ ID NO:2, SEQ ID NO: 4, SEQ ID NOs: 14-15 or the complement thereof, further comprises hybridization to filter-bound DNA in 0.5 M NaHPO4, 7% sodium dodecyl sulfate, 1 mM EDTA at 65°C, and washing in 0.2x SSC/0.1% SDS at 42°C. In a further embodiment a recombinant host cell comprising a recombinant nucleic acid operatively linked to a heterologous promoter sequence, said recombinant nucleic acid comprising: a sequence that encodes a peptide with at least 70% homology to an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13; or a sequence that encodes a peptide comprising 225 contiguous amino acids selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13; or a sequence that hybridizes to the nucleotide sequence of SEQ ID NO:2, SEQ ID NO: 4, SEQ ID NOs:14-15 or the complement thereof is disclosed.

[0011] In further embodiments, ACRs can be used as membrane-depolarizing tools, with or without induction of action potentials, in compartments of neurons or cardiomyocytes. Thus, in some aspects, ACR coding sequences of the embodiments further comprise a signal sequence that targets the encoding mRNA or polypeptide a compartment of a host cell.

[0012] In some embodiments a host cell is an: isolated human cell; a non-human mammalian cell; a bacterial cell; a yeast cell; an insect cell; or a plant cell.

[0013] In alternative embodiments, the nucleic acid sequences described can be targeted to the genome of a cell using a CRISPR-associated protein-9 nuclease (Cas9) based system for genome-editing and genome targeting. In some embodiments, delivery to some cells may require delivery systems, such as, but not limited to those based on lentivirus (LVs), adenovirus (AdV) and adenoassociated (AAV).

[0014] In some embodiments a method of restoring photosensitivity to a retina of a subject suffering from vision loss or blindness is disclosed, said method comprising: delivering to the OFF-bipolar neurons of the retina of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 which encodes a rhodopsin domain of an ACR expressible in a retinal neuron; and expressing said vector in

said retinal neuron, wherein the expressed rhodopsin renders said retinal neuron photosensitive, thereby restoring photosensitivity to enable light-induced silencing of such neuron in said retina or a portion thereof. In a further embodiment the subject is mammalian, and in a still further embodiment the subject is human. In an embodiment of the method of restoring photosensitivity to a retina of a subject suffering from vision loss or blindness is disclosed, said method comprising: delivering to the retina of said subject an expression vector wherein the delivering comprises a pharmaceutically acceptable carrying agent.

[0015] In some further embodiments, there is provided an isolated nucleic acid molecule comprising a sequence encoding an anion-conducting channelrhodopsin having a sequence at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to a sequence according to SEQ ID NOs: 15-24. In certain aspects, the isolated nucleic acid molecule comprises a sequence that hybridized to the nucleotide sequence of one of SEQ ID NOs: 15-24 under stringent conditions comprising hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate, 1 mM EDTA at 65°C, and washing in 0.2x SSC/0.1% SDS at 42°C. and encodes an anion-conducting channelrhodopsin. In some aspects, the nucleic acid is a DNA. In other aspects, the nucleic acid is a RNA (*e.g.,* mRNA). In further embodiments, there is provided an expression vector comprising a nucleic acid molecule provided herein, such as a sequence at least about 90% identical to a sequence according to SEQ ID NOs: 15-24.

[0016] In even further embodiment, there is provided a recombinant host cell comprising a nucleic acid provided herein (e.g., a sequence at least about 90% identical to a sequence according to SEQ ID NOs: 15-24). In some aspects, the host cell is an isolated human cell. In other aspects, the host cell is a non-human mammalian cell. In some aspects, the host cell is a bacterial cell. In certain aspects, the host cell is a yeast cell. In other aspects, the host cell is an insect cell. In some aspects, the host cell is a plant cell. In certain aspects, host cell is an isolated neuronal cell. In particular, the host cell is an isolated electrically active cell.

[0017] In another embodiment, there is provided a method of treating a subject suffering from a disorder that involves electrically active cells comprising expressing in the subject an effective amount of an anion-conducting channelrhodopsin at the site of the electrically active cells. In some aspects, the subject is suffering from neuropathic pain the method comprising expressing in the subject an effective amount of an anion-conducting

channelrhodopsin at the site of the pain. In certain aspects, the subject has an amputated limb, diabetes, multiple sclerosis or has undergone a surgery.

[0018] In certain aspects, expressing comprises administering an anion-conducting channelrhodopsin to the subject. In some aspects, the anion-conducting channelrhodopsin comprises an amino acid sequence at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to a sequence of SEQ ID NOs: 1, 3 or 13. In certain aspects, the anion-conducting channelrhodopsin is encoded by a sequence at about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to sequence according to SEQ ID NOs: 2, 4 or 14-17. In some aspects, the anion-conducting channelrhodopsin further comprises a cell-penetrating peptide (CPP) sequence or a cellular receptor-binding sequence. As used herein the terms "cell penetrating peptide" refers to segments of polypeptide sequence that allow a polypeptide to cross the cell membrane (e.g., the plasma membrane in the case a eukaryotic cell). Examples of CPP segments include, but are not limited to, segments derived from HIV Tat (*e.g.,* GRKKRRQRRRPPQ; SEQ ID NO: 25), herpes virus VP22, the Drosophila Antennapedia homeobox gene product, protegrin I, Penetratin (RQIKIWFQNRRMKWKK; SEQ ID NO: 26) or melittin (GIGAVLKVLTTGLPALISWIKRKRQQ; SEQ ID NO: 27). In certain aspects the CPP comprises the T1 (TKIESLKEHG; SEQ ID NO: 28), T2 (TQIENLKEKG; SEQ ID NO: 29), 26 (AALEALAEALEALAEALEALAEAAAA; SEQ ID NO:30) or INF7 (GLFEAIEGFIENGWEGMIEGWYGCG; SEQ ID NO: 31) CPP sequence.

[0019] In some aspects, expressing comprises administering a vector encoding an anion-conducting channelrhodopsin to the subject. In certain aspects, the vector is a RNA vector. In other aspects, the vector is a DNA vector. In some aspects, the vector is a plasmid, a viral vector or an episomal vector. In certain aspects, the vector further comprises an inducible expression cassette for a suicide gene.

[0020] In certain aspects, the sequence encoding the anion-conducting channelrhodopsin is operably linked to a heterologous promoter. In some aspects, the promoter is an inducible or a repressible promoter. In certain aspects, the promoter is a tissue or cell type specific promoter. In particular, the promoter is neuronal cell specific promoter.

[0021] As used herein, "essentially free," in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of

the specified component resulting from any unintended contamination of a composition is therefore well below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

[0022] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one.

[0023] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

[0024] Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0025] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein. The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0027] FIG. 1A-F: Phylogeny and photoactivity of *G. theta* ACRs. (A) Phylogenetic tree of cation channelrhodopsins (CCRs) and anion-conducting

channelrhodopsins (ACRs). (**B** and **C**) ClustalW alignments of transmembrane helices 2 (**B**) and 3 (**C**). Abbreviated organism names are: *Gt*, *Guillardia theta*; *Cr*, *Chlamydomonas reinhardtii*; *Ca*, *Chlamydomonas augustae*; *Mv*, *Mesostigma viride*; *Hs*, *Halobacterium salinarum*; *Nm*, *Nonlabens marinus*. The last residue numbers are shown on the right. Conserved Glu residues in the helix 2 are highlighted yellow, and Glu residues in the position of bacteriorhodopsin Asp85 are highlighted red. (**D**) (Main figure) Photocurrents of *Gt*ACR1, *Gt*ACR2, and *Cr*ChR2 in HEK293 cells in response to a saturating light pulse at -60 mV. (Inset) Mean amplitudes of peak (solid bars) and stationary (hatched bars) currents (n = 18-20 cells). Based on noise analysis, the unitary conductance of *Gt*ACR2 was 25-fold greater than that reported for the most widely used cation-conducting channelrhodopsin *Cr*ChR2. (**E**) Dependence of the peak and stationary current amplitudes and rise rates on stimulus intensity. (**F**) Action spectra of photocurrents.

[0028] FIG. 2A-D: ACRs do not conduct cations. Photocurrents generated by *Gt*ACR1 (**A**) and *Gt*ACR2 (**B**) in HEK293 cells at E_h changed in 20-mV steps from -60 mV (bottom to top; for liquid junction potentials (LJP) values (Table 1). The pipette solution was standard, whereas the bath solution was as indicated. (**C**) Current-voltage relationships measured at different pH of the bath. The data (mean values ± SEM, *n* = 4-6 cells) were corrected for LJP and normalized to the value measured at -60 mV at pH 7.4. Representative data for *Cr*ChR2 are shown for comparison. (**D**) E_{rev} shifts measured upon variation of the cation composition of the bath. The data are mean values ± SEM (*n* = 3-6 cells).

[0029] FIG. 3A-D: Anion selectivity of ACRs. Photocurrents generated by *Gt*ACR1 (**A**) and *Gt*ACR2 (**B**) in HEK293 cells at E_h changed in 20-mV steps from -60 mV (bottom to top; for liquid junction potential (LJP) values (Table 1). The pipette solution was standard, whereas the bath solution was as indicated. (**C**) Current-voltage relationships measured at different Cl^- concentrations in the bath. The data (mean values ± SEM, *n* = 4-6 cells) were corrected for LJP and normalized to the value measured at -60 mV at 156 mM Cl^-. The dashed vertical lines show the Nernst equilibrium potential for Cl^- at the bath concentrations used. (**D**) E_{rev} shifts measured upon variation of the anion composition of the bath. The data are mean values ± SEM (*n* = 3-6 cells).

[0030] FIG. 4A-F: *Gt*ACR2 as a hyperpolarizing tool. (**A** and **C**) Photocurrents generated by *Gt*ACR2 in HEK293 cells (**A**) and in cultured pyramidal neurons (**C**) with low Cl^- concentration in the pipette and high Cl^-concentration in the bath at E_h changed in 20-mV

steps from -80 mV (bottom to top; for LJP values (Tables 1 and 2). **(B)** Light intensity dependence of photocurrents generated by GtACR2 and archaerhodopsin-3 (Arch) in HEK293 cells at 20 mV. The data for slow Cl^--conducting ChR mutants are from J. Wietek *et al.*, (2014) *ibid* and A. Berndt, *et al.*, (2014) *ibid.* The arrows show the difference in light sensitivity. **(D)** Current-voltage relationship measured in neurons as shown in **(C)**. The data (mean values ± SEM, n = 5 cells) were corrected for LJP (Table 2). The dashed vertical line shows the resting potential (E_{rest}). The data for Cl^-- conducting ChR mutants are from J. Wietek *et al.*, (2014) *ibid* and A. Berndt, *et al.*, (2014) *ibid.* **(E)** Photoinhibition of spiking induced by pulsed (10 ms, 10 kHz) current injection in a neuron expressing *Gt*ACR2. The light intensity was 8.5E-3 mW/mm^2. **(F)** Light intensity dependence of electrically evoked spikes. The difference in the amplitude between electrically evoked signals in the dark and light was calculated as shown.

[0031] FIG. 5: Phylogenetic tree of *G. theta* protein models. The models homologous to microbial rhodopsins were selected among those predicted by the Joint Genome Institute (JGI) sequencing project (see the world wide web at: genome.jgi.doe.gov/Guith1/Guith1.home.html) and aligned using ClustalW. The tree was constructed using the neighbor-joining method. GtR1, GtR2 and GtR3 are proteins identified previously. Eight models lack the conserved Lys residue in the seventh transmembrane helix that covalently links to retinal in known rhodopsins, and should therefore be considered opsin-related proteins.

[0032] FIG. 6A-B: Current-voltage relationships for GtACR1 measured at different Cl^- concentrations in the bath. (A) The data (mean values ± SEM, n = 4-6 cells) were for liquid junction potentials and normalized to the value measured at –60 mV at 156 mM Cl–. The dashed vertical lines show the Nernst equilibrium potential for Cl– at the bath concentrations used. (B) Photocurrents generated by GtACR1 (FIG. 6B) in HEK293 cells with low Cl concentration in the pipette and high Cl concentration in the bath The membrane potentials were changed in 20-mV steps from -80 mV at the amplifier output.

[0033] FIGs. 7A-7D: (A-C) Illustrate the photocurrents generated by *Psu*ACR1 in HEK293 cells at the membrane potentials changed in 20-mV steps from -60 mV at the amplifier output (bottom to top) in response to a 1-s light pulse (520 nm). The Cl^- concentrations of the pipette and bath solutions were as indicated. **(D)** The voltage dependencies of the peak current (IE curves) measured with solutions indicated in the legend.

The data (mean values ± SEM, n = 4-9 cells) were normalized to the value measured at -60 mV with standard solutions and corrected for liquid junction potentials.

[0034] FIG. 8: Illustrates shifts of the E_{rev} of *Psu*ACR1 photocurrents measured upon variation of the anion (left) or cation (right) composition of the bath from the value obtained with non-permeable Asp^- (for anions) or non-permeable NMG^+ (for cations). The data are mean values ± SEM (n = 3-9 cells).

[0035] FIG. 9: The action spectrum of *Psu*ACR1 photocurrents.

[0036] FIG. 10: (a), Typical photocurrents (black solid lines) generated by PsuACR1 in response to laser photoexcitation (6 ns, 532 nm) at indicated voltages at the amplifier output. The current decay was fit with three exponentials (dashed lines), from which the time constants shown on the plot were derived. (b) Channel closing rates of three known ACRs calculated as reciprocals of the time needed for the peak amplitude to decrease to 50%. (c) The voltage dependence of the amplitudes of current decay components. (d) The dark recovery of the peak current for PsuACR1, GtACR1 and GtACR2 measured in double-flash experiments with 5-ms light pulses of the saturating intensity at 520, 515 and 470 nm, respectively. The numbers on the plot show the dark interval required for 50% peak recovery for each protein. The data points in panels b-d are mean values ± SEM (n = 3-8 cells).

[0037] FIG. 11: (a) Fast negative current recorded from PsuACR1 at the reversal potential for channel current (solid line, left axis) and exponential fit of its rise and decay (dotted line), and channel current recorded from the same cell at -60 mV at the amplifier output (dashed line, right axis). (b) Voltage dependencies of fast negative current (squares and solid line, left axis) and channel current (circles and dashed line, right axis) in a typical cell expressing PsuACR1. (c) The dependence of fast negative current on the bath pH. The data points are mean values ± SEM (n = 3-5 cells).

[0038] FIG. 12: A ClustalW alignment of the rhodopsin domains of ACRs and C. *reinhardtii* CCRs. The numbers at left show the last residue numbers of each sequence fragment. The carboxylate residues conserved in helices 2 and 3 of CCRs are highlighted red; the corresponding polar and non-polar residues in ACRs are highlighted green and yellow, respectively.

[0039] FIG. 13: The voltage dependence of the time constants of PsuACR1 current decay components. The data points are mean values ± SEM (n = 8 cells).

DESCRIPTION OF THE SEQUENCE LISTING

[0040] The Sequence Listing shows the amino acid and nucleic acid sequences of anion channel rhodopsin domains that were derived from Sequences encoding 7TM domains of *G. theta* opsins (295, 291 and 288 aa, corresponding to the JGI protein models 111593, 146828 and 161302, respectively) were optimized for human codon usage and were synthesized.

[0041] SEQ ID NO: 1 is the amino acid sequence of *Gt*ACR1.

[0042] SEQ ID NO: 2 is a nucleic acid sequence that encodes *Gt*ACR1.

[0043] SEQ ID NO: 3 is the amino acid sequence of *Gt*ACR2.

[0044] SEQ ID NO: 4 is a nucleic acid sequence that encodes *Gt*ACR2.

[0045] SEQ ID NO: 5 is the amino acid sequence of *Gt*161302.

[0046] SEQ ID NO: 6 is a nucleic acid sequence that encodes *Gt*161302.

[0047] SEQ ID NO: 7 is the amino acid sequence of *Cr*ChR1 (*Chlamydomonas reinhardtii* channelrhodopsin 1, aka *Chlamydomonas* sensory rhodopsin A: GenBank accession number AF508965).

[0048] SEQ ID NO: 8 is the amino acid sequence of *Cr*ChR2 (*Chlamydomonas reinhardtii* channelrhodopsin 2, aka *Chlamydomonas* sensory rhodopsin B: GenBank accession number AF508966).

[0049] SEQ ID NO: 9 is the amino acid sequence of *Ca*ChR1 (*Chlamydomonas (Chloromonas) augustae* channelrhodopsin 1: GenBank accession number JN596951).

[0050] SEQ ID NO: 10 is the amino acid sequence of *Mv*ChR1 (*Mesostigma viride* channelrhodopsin 1: GenBank accession number JF922293).

[0051] SEQ ID NO: 11 is the amino acid sequence of *Hs*HR (*Halobacterium salinarum* halorhodopsin: GenBank accession number WP_010902090.1).

[0052] SEQ ID NO: 12 is the amino acid sequence of NmHR (Nonlabens marinus chloride pumping rhodopsin: GenBank accession number BAO55276.1).

[0053] SEQ ID NO: 13 is the amino acid sequence of PsuACR1.

[0054] SEQ ID NO: 14 is a synthetic nucleic acid sequence that encodes PsuACR1.

[0055] SEQ ID NO: 15 is a synthetic nucleic acid sequence that encodes PsuACR1.

[0056] SEQ ID NO: 16 is a synthetic nucleic acid sequence that encodes GtACR1.

[0057] SEQ ID NO: 17 is a synthetic nucleic acid sequence that encodes GtACR2.

[0058] SEQ ID NO: 18 is a synthetic nucleic acid sequence that encodes Gt161302.

[0059] SEQ ID NO: 19 is a synthetic nucleic acid sequence that encodes CrChR1.

[0060] SEQ ID NO: 20 is a synthetic nucleic acid sequence that encodes CrChR2.

[0061] SEQ ID NO: 21 is a synthetic nucleic acid sequence that encodes CaChR1.

[0062] SEQ ID NO: 22 is a synthetic nucleic acid sequence that encodes MChR1.

[0063] SEQ ID NO: 23 is a synthetic nucleic acid sequence that encodes HsHR.

[0064] SEQ ID NO: 24 is a synthetic nucleic acid sequence that encodes NmHR.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0065] Until recently channelrhodopsins have been phototaxis receptors that function as light-gated cation channels that when transfected into animal cells, are used for photoactivation of neuron firing. Described herein are a new class of light gated channels, anion channel rhodopsins (ACRs), that provide highly sensitive and efficient membrane hyperpolarization and neuronal silencing through light-gated chloride conduction. ACRs strictly conduct anions, completely excluding protons and larger cations, and hyperpolarize the membrane with 100-fold faster kinetics at 3000-fold lower light intensity than the most efficient currently available optogenetic proteins.

[0066] By screening phototaxis receptor currents among several algal species, highly efficient ACRs with rapid kinetics was identified and characterized. In some embodiments,

the disclosed methods provide a technology that facilities the identification and characterization of particularly useful channelrhodopsins from algae.

I. Definitions

[0067] In this disclosure, the use of the singular includes the plural, the word "a" or "an" means "at least one", and the use of "or" means "and/or", unless specifically stated otherwise. Furthermore, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both elements and components comprising one unit and elements or components that comprise more than one unit unless specifically stated otherwise.

[0068] As used herein, the term "about," when used in conjunction with a percentage or other numerical amount, means plus or minus 10% of that percentage or other numerical amount. For example, the term "about 80%," would encompass 80% plus or minus 8%.

[0069] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including, but not limited to, patents, patent applications, articles, books, and treatises, are hereby expressly incorporated herein by reference in their entirety for any purpose. In the event that one or more of the incorporated literature and similar materials defines a term in a manner that contradicts the definition of that term in this application, this application controls

[0070] As used herein, and unless otherwise indicated, the term a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder, for which the present methods and compositions may be used include, but are not limited to, neuronal dysfunctions, disorders of the brain, the central nervous system, the peripheral nervous system, neurological conditions, disorders of memory and leaning disorders, cardiac arrhythmias, Parkinson's disease, epilepsy, ocular disorders, spinal cord injury, nerve pain associated with, but not limited to autoimmune diseases (for example, multiple sclerosis, Guillain-Barré syndrome, myasthenia gravis, lupus, and inflammatory bowel disease); cancer and the chemotherapy and radiation used to treat it; compression/trauma (for example, pinched nerves in the neck, crush injuries, and carpal tunnel syndrome); diabetic neuropathy; medication side effects; and toxic substances; motor neuron diseases (for example amyotrophic lateral sclerosis, progressive

bulbar palsy, progressive muscular atrophy and primary lateral sclerosis); nutritional deficiencies (for example vitamins B6 and B12); Infectious disease; itch sensations associated with, but not limited to eczema, atopic dermatitis, dry skin and allergic itches; diseases and disorders that alter vagal nerve activity, among others.

[0071] As used herein, and unless otherwise indicated, the term ocular disorders for which the present methods and compositions 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, but are not limited to, glaucoma, cataracts, corneal dystrophy, keratoconus. Posterior segment disorders include, but are not limited to, 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.

[0072] As used herein, and unless otherwise indicated, the terms "treat," "treating," "treatment" and "therapy" contemplate an action that occurs while a patient is suffering from a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder, and which reduces the severity of one or more symptoms or effect of such a disorder. Where the context allows, the terms "treat," "treating," and "treatment" also refers to actions taken toward ensuring that individuals at increased risk of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder and which reduces the severity are able to receive appropriate surgical and/or other medical intervention prior to onset of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder and which reduces the severity. As used herein, and unless otherwise indicated, the terms "prevent," "preventing," and "prevention" contemplate an action that occurs before a patient begins to suffer from a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder, that delays the onset of, and/or inhibits or reduces the severity of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder.

[0073] As used herein, and unless otherwise indicated, the terms "manage," "managing," and "management" encompass preventing, delaying, or reducing the severity of a recurrence of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder in a patient who has already suffered from such a disease, disorder or condition. The terms encompass modulating the threshold, development, and/or duration of the disorder that involves electrically active cells or changing how a patient responds to the disorder that involves electrically active cells or the maintenance and/or establishment of a desirable membrane potential across the membrane of a cell.

[0074] As used herein, and unless otherwise specified, a "therapeutically effective amount" of a compound is an amount sufficient to provide any therapeutic benefit in the treatment or management of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder, or to delay or minimize one or more symptoms associated with a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder. A therapeutically effective amount of a compound means an amount of the compound, alone or in combination with one or more other therapies and/or therapeutic agents that provide any therapeutic benefit in the treatment or management of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder.

[0075] The term "therapeutically effective amount" can encompass an amount that alleviates a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder, improves or reduces a disorder that involves electrically active cells or improves overall therapy, or enhances the therapeutic efficacy of another therapeutic agent.

[0076] As used herein, and unless otherwise specified, a "prophylactically effective amount" of a compound is an amount sufficient to prevent or delay the onset of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder or one or more symptoms associated with a disorder that involves electrically active cells or prevent or delay its recurrence. A prophylactically effective amount of a compound means an amount of the compound, alone or in combination with one or more other treatment and/or prophylactic

agent that provides a prophylactic benefit in the prevention of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder. The term "prophylactically effective amount" can encompass an amount that prevents a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder, improves overall prophylaxis, or enhances the prophylactic efficacy of another prophylactic agent. The "prophylactically effective amount" can be prescribed prior to, for example, the development of a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder.

[0077] As used herein, "patient" or "subject" includes mammalian organisms which are capable of suffering from a disorder that involves electrically active cells, such as but not limited to neuronal dysfunction, a neuron mediated disorder, ocular disorder or cardiac disorder, as described herein, such as human and non-human mammals, for example, but not limited to, rodents, mice, rats, non-human primates, companion animals such as dogs and cats as well as livestock, e.g., sheep, cow, horse, etc.

[0078] As used herein, the term "conservative substitution" generally refers to amino acid replacements that preserve the structure and functional properties of a protein or polypeptide. Such functionally equivalent (conservative substitution) peptide amino acid sequences include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequences encoded by a nucleotide sequence that result in a silent change, thus producing a functionally equivalent gene product. Conservative amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example: nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

[0079] As used herein, a "redshift" is a shift to longer wavelength. In contrast a "blueshift" would be a shift to shorter wavelength. These terms apply to both light-emitting and light-absorbing objects.

[0080] As used herein the phrase “rhodopsin domain” refers to the "rhodopsin fold", a 7-transmembrane-helix (7TM) structure characteristic of rhodopsins. As used herein, the channelopsin is the apoprotein, while channelrhodopsin is the protein and retinal. As used herein the term “channelrhodopsin” describes retinylidene proteins (rhodopsins) that function as light-gated ion channels.

[0081] The percent identity or homology is determined with regard to the length of the relevant amino acid sequence. Therefore, if a polypeptide of the present invention is comprised within a larger polypeptide, the percent homology is determined with regard only to the portion of the polypeptide that corresponds to the polypeptide of the present invention and not the percent homology of the entirety of the larger polypeptide. “Percent identity” or “% identity,” with reference to nucleic acid sequences, refers to the percentage of identical nucleotides between at least two polynucleotide sequences aligned using the Basic Local Alignment Search Tool (BLAST) engine. See Tatusova *et al.* (1999) FEMS Microbiol Lett. 174:247-250. The BLAST engine is provided to the public by the National Center for Biotechnology Information (NCBI), Bethesda, Md. To align two polynucleotide sequences, the BLAST which employs the “blastn” program is used.

[0082] “Percent identity” or “% identity,” with reference to polypeptide sequences, refers to the percentage of identical amino acids between at least two polypeptide sequences aligned using the Basic Local Alignment Search Tool (BLAST) engine. See Tatusova et al. (1999) ibid. The BLAST engine is provided to the public by the National Center for Biotechnology Information (NCBI), Bethesda, Md. To align two polypeptide sequences, the BLAST which employs the “blastp” program is used.

II. Channelrhodopsins

[0083] Sequences encoding 7TM domains of *G. theta* opsins (295, 291 and 288 aa, corresponding to the Joint Genome Institute (JGI) sequencing project (http://genome.jgi.doe.gov/Guith1/Guith1.home.html) protein models 111593, 146828 and 161302, respectively) were optimized for human codon usage and were synthesized and two have been identified and characterized as highly efficient ACRs with rapid kinetics. Some embodiments provided herein are amino acid and nucleic acid sequences of functional domains of novel ACRs that are also functionally characterized. Several such ACRs have been determined to have highly sensitive and efficient a ACRs domains of the

channelrhodopsins were cloned and identified as *Gt*ACR1 (SEQ ID NO: 1 and 2), *Gt*ACR2 (SEQ ID NO: 3 and 4) or *Gt*161302 (SEQ ID NO: 5 and 6) which were derived from anion-conducting channelrhodopsins of *Guillardia theta*. The functional construct from 111593 is identified as GtACR1 (SEQ ID NO: 1 and 2) and the functional construct from 146828 is identified as GtACR2 (SEQ ID NO: 3 and 4) and were deposited in GenBank (accession numbers KP171708 and KP171709). Also provided in some embodiments is the use and composition of these novel ACR domains, identified as *Gt*ACR1, *Gt*ACR2 or *Gt*161302 (SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5).

[0084] In some embodiments, are conserved variants of GtACR (for example, GtACR1 or GtACR2) or a peptide fragment thereof. A "conservative" amino acid substitution refers to the substitution of an amino acid in a polypeptide with another amino acid having similar properties, such as size or charge. In certain embodiments, a polypeptide comprising a conservative amino acid substitution maintains at least one activity of the unsubstituted polypeptide. A conservative amino acid substitution may encompass non-naturally occurring amino acid residues which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include, but are not limited to, peptidomimetics and other reversed or inverted forms of amino acid moieties.

[0085] In some embodiments, are any of the disclosed methods, wherein the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin having the amino acid sequence of all or part of SEQ ID NOS: 1 or 3, or a biologically active fragment thereof that retains the biological activity of the encoded rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin or a biologically active conservative amino acid substitution variant of SEQ ID NOS: 1 or 3 or of said fragment.

A. Channelrhodopsin Polypeptides

[0086] In some embodiments, are isolated polypeptides that encode a rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin. In some embodiments, an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the isolated polypeptide has at least 85% homology to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the isolated polypeptide has between 85% - 95%- 100% homology to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[0087] In some embodiments, is a protein composition comprises a polypeptide having the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[0088] The peptide amino acid sequences that can be used in various embodiments including the anion-conducting channelrhodopsin amino acid sequences described herein (SEQ ID NOS: 1 or 3), as well as analogues and derivatives thereof and functional fragments such as but not limited to the rhodopsin/7TM domain. In fact, in some embodiments the any desired peptide amino acid sequences encoded by particular nucleotide sequences can be used, as is the use of any polynucleotide sequences encoding all, or any portion, of desired peptide amino acid sequences. The degenerate nature of the genetic code is well-known, and, accordingly, each anion-conducting channelrhodopsin peptide amino acid-encoding nucleotide sequence is generically representative of the well-known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the anion-conducting channelrhodopsin peptide amino acid sequences described herein, when taken together with the genetic code (see, e.g., "Molecular Cell Biology", Table 4-1 at page 109 (Darnell et al., eds., W. H. Freeman & Company, New York, NY, 1986)), are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

[0089] Such functionally equivalent peptide amino acid sequences (conservative substitutions) include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequences encoded by a nucleotide sequence, but that result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example: nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

[0090] Naturally occurring residues may be divided into classes based on common side chain properties: hydrophobic (Met, Ala, Val, Leu, Ile); neutral hydrophilic (Cys, Ser, Thr, Asn, Gln); acidic (Asp, Glu); basic (His, Lys, Arg); residues that influence chain orientation (Gly, Pro); and aromatic (Trp,Tyr, Phe). For example, non-conservative

substitutions may involve the exchange of a member of one of these classes for a member from another class.

[0091] In making substitutions, according to certain embodiments, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. They are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine (−1.3); proline (−1.6); histidine (−3.2); glutamate (−3.5); glutamine (−3.5); aspartate (−3.5); asparagine (−3.5); lysine (−3.9); and arginine (−4.5).

[0092] The importance of the hydropathic amino acid index in conferring interactive biological function on a protein, in certain instances, is understood in the art (Kyte *et al.*, J. Mol. Biol., 157:105-131 (1982)). It is known that in certain instances, certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, in certain embodiments, the substitution of amino acids whose hydropathic indices are within ±2 is included. In certain embodiments, those which are within ±1 are included, and in certain embodiments, those within ±0.5 are included.

[0093] Substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biologically functional protein or peptide thereby created is intended for use in immunological embodiments, as in the present case. In certain embodiments, the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e., with a biological property of the protein.

[0094] The following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0±1); glutamate (+3.0±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−0.5±1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3); valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5) and tryptophan (−3.4). In making changes based upon similar hydrophilicity values, in certain embodiments, the substitution of amino acids whose hydrophilicity values are within ±2 is included, in certain embodiments,

those which are within ±1 are included, and in certain embodiments, those within ±0.5 are included.

[0095] A skilled artisan will be able to determine suitable variants of a polypeptide as set forth herein using well-known techniques. In certain embodiments, one skilled in the art may identify suitable areas of the molecule that may be changed without destroying activity by targeting regions not believed to be important for activity. In certain embodiments, one can identify residues and portions of the molecules that are conserved among similar polypeptides.

[0096] In certain embodiments, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

[0097] Additionally, in certain embodiments, one skilled in the art can review structure-function studies identifying residues in similar polypeptides that are important for activity or structure. In view of such a comparison, in certain embodiments, one can predict the importance of amino acid residues in a protein that correspond to amino acid residues which are important for activity or structure in similar proteins. In certain embodiments, one skilled in the art may opt for chemically similar amino acid substitutions for such predicted important amino acid residues.

[0098] In certain embodiments, one skilled in the art can also analyze the three-dimensional structure and amino acid sequence in relation to that structure in similar polypeptides. In certain embodiments, in view of such information, one skilled in the art may predict the alignment of amino acid residues of a polypeptide with respect to its three dimensional structure. In certain embodiments, one skilled in the art may choose not to make radical changes to amino acid residues predicted to be on the surface of the protein, since such residues may be involved in important interactions with other molecules.

[0099] Moreover, in certain embodiments, one skilled in the art may generate test variants containing a single amino acid substitution at each desired amino acid residue. In certain embodiments, the variants can then be screened using activity assays known to those skilled in the art. In certain embodiments, such variants could be used to gather information about suitable variants. For example, in certain embodiments, if one discovered that a change to a particular amino acid residue resulted in destroyed, undesirably reduced, or unsuitable

activity, variants with such a change may be avoided. In other words, in certain embodiments, based on information gathered from such routine experiments, one skilled in the art can readily determine the amino acids where further substitutions should be avoided either alone or in combination with other mutations.

[00100] A number of scientific publications have been devoted to the prediction of secondary structure. See, e.g., Moult J., Curr. Op. in Biotech., 7(4):422-427 (1996), Chou *et al.*, Biochemistry, 13(2):222-245 (1974); Chou *et a*l., Biochemistry, 113(2):211-222 (1974); Chou *et al.*, Adv. Enzymol. Relat. Areas Mol. Biol., 47:45-148 (1978); Chou *et al.*, Ann. Rev. Biochem., 47:251-276 and Chou *et al.*, Biophys. J., 26:367-384 (1979). Moreover, computer programs are currently available to assist with predicting secondary structure. One method of predicting secondary structure is based upon homology modeling. For example, two polypeptides or proteins which have a sequence identity of greater than 30%, or similarity greater than 40% often have similar structural topologies. The growth of the protein structural database (PDB) has provided enhanced predictability of secondary structure, including the potential number of folds within a polypeptide's structure. See, e.g., Holm *et al.*, Nucl. Acid. Res., 27(1):244-247 (1999). It has been suggested (Brenner et al., Curr. Op. Struct. Biol., 7(3):369-376 (1997)) that there are a limited number of folds in a given polypeptide or protein and that once a critical number of structures have been resolved, structural prediction will become dramatically more accurate.

[00101] Additional methods of predicting secondary structure include "threading" (see, e.g., Jones, D., Curr. Opin. Struct. Biol., 7(3):377-87 (1997); Sippl *et al.*, Structure, 4(1):15-19 (1996)), "profile analysis" (see, e.g., Bowie *et al.*, Science, 253:164-170 (1991); Gribskov *et al.*, Meth. Enzym., 183:146-159 (1990); Gribskov *et al.*, Proc. Nat. Acad. Sci., 84(13):4355-4358 (1987)), and "evolutionary linkage" (see, e.g., Holm *et al.*, Nucl. Acid. Res., 27(1):244-247 (1999), and Brenner *et al.*, Curr. Op. Struct. Biol., 7(3):369-376 (1997)).

[00102] In certain embodiments, a variant of the reference channelrhodopsin or rhodopsin domain (*Gt*ACR) includes a glycosylation variant wherein the number and/or type of glycosylation sites have been altered relative to the amino acid sequence of the reference anion-conducting channelrhodopsin or rhodopsin domain (*Gt*ACR). In certain embodiments, a variant of a polypeptide comprises a greater or a lesser number of N-linked glycosylation sites relative to a native polypeptide. An N-linked glycosylation site is characterized by the

sequence: Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X may be any amino acid residue except proline. The substitution of amino acid residues to create this sequence provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions which eliminate this sequence will remove an existing N-linked carbohydrate chain. In certain embodiments, a rearrangement of N-linked carbohydrate chains is provided, wherein one or more N-linked glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked sites are created. Exemplary variants include cysteine variants wherein one or more cysteine residues are deleted from or substituted for another amino acid (e.g., serine) relative to the amino acid sequence of the reference channelrhodopsin or rhodopsin domain (*Gt*ACR). In certain embodiments, cysteine variants may be useful when polypeptides and proteins must be refolded into a biologically active conformation such as after the isolation of insoluble inclusion bodies. In certain embodiments, cysteine variants have fewer cysteine residues than the native polypeptide. In certain embodiments, cysteine variants have an even number of cysteine residues to minimize interactions resulting from unpaired cysteines.

[00103] According to certain embodiments, amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and/or (4) confer or modify other physiochemical or functional properties on such polypeptides. According to certain embodiments, single or multiple amino acid substitutions (in certain embodiments, conservative amino acid substitutions) may be made in a naturally-occurring sequence (in certain embodiments, in the portion of the polypeptide outside the domain(s) forming intermolecular contacts). In certain embodiments, a conservative amino acid substitution typically may not substantially change the structural characteristics of the reference sequence (e.g., in certain embodiments, a replacement amino acid should not tend to break a helix that occurs in the reference sequence, or disrupt other types of secondary structure that characterizes the reference sequence).

[00104] Examples of certain art-recognized polypeptide secondary and tertiary structures are described, for example, in Proteins, Structures and Molecular Principles (Creighton, Ed., W. H. Freeman and Company, New York (1984)); Introduction to Protein Structure (C. Branden and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton et at. Nature 354:105 (1991).

[00105] In other embodiments, are methods and compositions that provide an ACR with improved properties and characteristics that enhance the application of the compositions in, among other things, optogenetic techniques. Improved properties include, but are not limited to adaptation to human codon usage and synthesis. These embodiments provide greater sensitivity and efficient membrane hyperpolarization and neuronal silencing through light-gated chloride conduction.

B. Fusion Proteins

[00106] The use of fusion proteins in which a polypeptide or peptide, or a truncated or mutant version of peptide is fused to an unrelated or homologous protein, polypeptide, or peptide, and can be designed on the basis of the desired peptide encoding nucleic acid and/or amino acid sequences described herein. Such fusion proteins include, but are not limited to: IgFc fusions, which stabilize proteins or peptides and prolong half-life *in vivo*; fusions to any amino acid sequence that allows the fusion protein to be anchored to the cell membrane; or fusions to an enzyme, fluorescent protein, or luminescent protein that provides a marker function. Fusion proteins to homologous proteins include, but are not limited to, those that are produced from genes that are engineered to encode a portion of the anion-conducting channelrhodopsin fused to a portion of a homologous (orthologous or paralogous) protein of the same of related function. For example, chimeras between different channelrhodopsins may be made to combine beneficial properties uniquely present in each. In some aspects, a chimeric channelrhodopsin of the embodiments comprises about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of its sequence from a first channelrhodopsin and the remaining sequence from a second channelrhodopsin. In some aspects, a chimeric channelrhodopsin comprises the rhodopsin domain of a first channelrhodopsin and the remaining sequence from a second channelrhodopsin. In yet further aspects, a chimeric channelrhodopsin can comprise 1, 2, 3, 4, 5 or 6 of its transmembrane domains from a first channelrhodopsin and the remaining transmembrane domains from a second channelrhodopsin.

[00107] In certain embodiments, a fusion protein may be readily purified by utilizing an antibody that selectively binds to the fusion protein being expressed. In alternate embodiments, a fusion protein may be purified by subcloning peptide encoding nucleic acid sequence into a recombination plasmid, or a portion thereof, is translationally fused to an amino-terminal (N-terminal) or carboxy-terminal (C-terminal) tag consisting of six histidine

residues (a "His-tag"; *see, e.g.*, Janknecht *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8972-8976, 1991). Extracts from cells expressing such a construct are loaded onto Ni^{2+} nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

C. Nucleic Acids Encoding Channelrhodopsins

[00108] In some embodiments, a recombinant nucleic acid operatively linked to a heterologous promoter sequence, said recombinant nucleic acid comprising: a sequence that encodes a peptide with at least 85% homology to an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13; or a sequence that encodes a peptide comprising 225 contiguous amino acids selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13; or a sequence that hybridizes to the nucleotide sequence of SEQ ID NO:2, SEQ ID NO:4 or the complement thereof.

[00109] In some embodiments, are isolated nucleic acid molecules comprising a nucleotide sequence that encode highly efficient and sensitive anion-conducting channelrhodopsins derived from algae. In some embodiments, the rhodopsin domain encodes the peptides whose sequence is described in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, are isolated nucleic acid molecules comprising a nucleotide sequence that encodes the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 3. In some embodiments, are expression vectors comprising a nucleic acid sequence that encodes the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, are host cells comprising an expression vector comprising a nucleic acid sequence that encodes the amino acid sequences of SEQ ID NO: 1 or SEQ ID NO: 3 or a nucleic acid sequence, fragment of portion thereof of the nucleic acid sequences of SEQ ID NO:2 or SEQ ID NO:4.

[00110] In some embodiments, are isolated nucleic acid molecules comprising a nucleotide sequence that was derived from cDNA and encode the rhodopsin domain of an ACR. In some embodiments, the rhodopsin domain encodes the peptides whose sequence is described in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, are isolated nucleic acid molecules that were derived from cDNA that comprise a nucleotide sequence that encodes the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, are expression vectors comprising a nucleic acid sequence that encodes the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID

NO: 13. In some embodiments, are host cells comprising a recombinant expression vector comprising a nucleic acid sequence that was derived from cDNA and encode the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[00111] In some embodiments are isolated peptides comprising an amino acid sequence encoded by at least a portion of the cDNA derived nucleic acid sequences that encode the 7TM or rhodopsin domain of a highly efficient and sensitive ACR. In some embodiments, are isolated peptides comprising an amino acid sequence encoded by a cDNA derived nucleic acid sequence that encodes an amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3.

[00112] In some embodiments are isolated peptides comprising a contiguous sequence encoded by a nucleic acid sequence that encodes the anion rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin derived. In some embodiments, are isolated peptides comprising an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3, or fragment thereof. In some embodiments, are isolated peptides comprising an amino acid sequence encoded by at least a portion of a nucleic acid sequence of a group consisting of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO: 6 and which functions as a anion rhodopsin or anion-conducting channelrhodopsin.

[00113] In some embodiments, are isolated peptides comprising an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 or a 7 TM domain /rhodopsin domain encoded by a cDNA derived nucleic acid sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO: 6 and which functions as an anion-conducting channelrhodopsin.

[00114] In some embodiments, isolated nucleic acid molecules are provided comprising a nucleotide sequence that encodes the rhodopsin of a highly efficient and sensitive anion-conducting channelrhodopsin. In some embodiments, the rhodopsin encodes a peptide whose sequence is shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, are isolated nucleic acid molecules comprising a nucleotide sequence that encodes the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, are expression vectors comprising a nucleic acid sequence that encodes the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, are host cells comprising a expression vector comprising a nucleic acid sequence that encodes the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 3 or

SEQ ID NO: 13. In some embodiments, are peptides comprising a sequence that encodes the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin. In some embodiments, are isolated peptides comprising an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the isolated peptides comprise an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13, or fragments thereof.

[00115] In some embodiments, are isolated nucleic acid molecules wherein said nucleic acid molecule has a sequence of SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NOs: 14-15. In other embodiments, are expression vectors comprising a nucleic acid sequence selected from that shown in SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NOs: 14-15 and those that encode the amino acid sequences shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, are host cells comprising a expression vector comprising a nucleic acid sequence shown in SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NOs: 14-15 and those that encode the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, an isolated nucleic acid comprises a nucleotide sequence that encodes the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin. In some embodiments, the nucleotide sequence encodes at least 16 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes at least 20 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes at least 35 contiguous amino acids of SEQ ID NO: 1, or SEQ ID NO: 3. In some embodiments, the nucleotide sequence encodes at least 50 contiguous amino acids of SEQ ID NO:1 or SEQ ID NO: 3. In some embodiments, the nucleotide sequence encodes at least 75 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes at least 33 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes a peptide comprising any contiguous portion of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[00116] In some embodiments, are isolated nucleic acids that comprise a nucleotide sequence that encodes the rhodopsin domain of a novel anion-conducting channelrhodopsins derived from *Guillardia theta*. In some embodiments, the nucleotide sequence encodes at least 16 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes at least 20

contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes at least 35 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes at least 50 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes at least 75 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes at least 33 contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments, the nucleotide sequence encodes a peptide comprising SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[00117] In some embodiments, an isolated nucleic acid comprising a nucleotide sequence that encodes a functional domain of an anion-conducting channelrhodopsin of *Guillardia theta*. In some embodiments are isolated nucleic acid that encodes at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 75, 100, 125, 150, 175, 200, 205, 210, 215, 220, 225, 228, 229, 230, 235, 240 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 296 or more contiguous amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 or fragments thereof. Further, in some embodiments, any range derivable between any of the above-described integers.

[00118] In other embodiments, the present invention provides for an isolated polypeptide or an isolated nucleic acid encoding a polypeptide having in some embodiments between about 70% and about 75%; in further embodiments between about 75% and about 80%; in further still embodiments between about 80% and 90%; or even more further between about 90% and about 99% of amino acids (for example 95%) that are identical to (or homologous to) the amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 or fragments thereof.

[00119] In other embodiments, the present invention provides for an isolated nucleic acid encoding a polypeptide having between about 70% and about 75%; or more preferably between about 75% and about 80%; or more preferably between about 80% and 90%; or even more preferably between about 90% and about 99% of amino acids that are identical to the amino acids of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 or fragments thereof.

[00120] In some embodiments, the nucleic acid segments, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, enhancers, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like. In some embodiments, for example, are recombinant nucleic acids comprising a nucleotide sequence that encodes amino acids of SEQ ID NO: 1, SEQ ID NO:3 or fragments thereof, operably linked to a heterologous promoter.

[00121] In certain embodiments the invention provides an isolated nucleic acid obtained by amplification from a template nucleic acid using a primer selected from appropriate primer that can be used with SEQ ID NO:2 or SEQ D NO:4.

[00122] In some embodiments, are any of the disclosed methods wherein the expression vectors include, but are not limited to, AAV viral vector. In some embodiments, are any of the disclosed methods wherein the promoter is a constitutive promoter. In some embodiments, are any of the disclosed methods wherein the constitutive promoter includes, but is not limited to, a CMV promoter or a hybrid CMV enhancer/chicken β-actin (CAG) promoter. In some embodiments, are any of the disclosed methods wherein the promoter includes, but is not limited to, an inducible and/or a cell type-specific promoter.

[00123] In some embodiments is a cDNA-derived nucleic acid comprising a nucleic acid sequence that encodes an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13. In some embodiments is a cDNA-derived nucleic acid comprising a nucleic acid sequence that encodes an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13, wherein the cDNA-derived nucleic acid comprises the nucleic acid sequence of SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NOs: 14-15. In other embodiments is an expression vector comprising the cDNA-derived nucleic acid comprising a nucleic acid sequence that encodes an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[00124] Channelrhodopsin nucleic acid sequences for use in the disclosed methods and compositions include, but are not limited to, the active portion of the presently disclosed algal derived anion-conducting channelrhodopsins GtACR1 (amino acid SEQ ID NO: 1, and nucleic acid sequence SEQ ID NO: 2) and GtACR2 (amino acid SEQ ID NO: 3, and nucleic acid sequence SEQ ID NO: 4) , including but not limited to those described, such

as but not limited to the nucleic acid sequences that encode the rhodopsin domain, an active portion of the presently disclosed algal derived anion-conducting channelrhodopsins, such as but not limited to the rhodopsin domains disclosed (SEQ ID NO: 1).

[00125] In some embodiments, the use of an active portion of a presently disclosed anion-conducting channelrhodopsin, such as but not limited to the rhodopsin domain, includes all or portions of the sequences described herein (and expression vectors comprising the same), and additionally contemplates the use of any nucleotide sequence encoding a contiguous an active portion of the presently disclosed anion-conducting channelrhodopsins, such as but not limited to the rhodopsin domain, open reading frame (ORF) that hybridizes to a complement of an anion-conducting channelrhodopsin or channelopsin sequence described herein under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1x SSC/0.1% SDS at 68°C ("Current Protocols in Molecular Biology", Vol. 1 and 2 (Ausubel et al., eds., Green Publishing Associates, Incorporated, and John Wiley & Sons, Incorporated, New York, NY, 1989)), and encodes a functionally equivalent anion-conducting channelrhodopsin (or active portion thereof, such as but not limited to the rhodopsin domain) gene product or the active portion thereof. Additionally contemplated is the use of any nucleotide sequence that hybridizes to the complement of a DNA sequence that encodes an anion-conducting channelrhodopsin amino acid sequence under moderately stringent conditions, e.g., washing in 0.2x SSC/0.1% SDS at 42°C ("Current Protocols in Molecular Biology", supra), yet still encodes a functionally equivalent anion-conducting channelrhodopsin product. Functional equivalents of anion-conducting channelrhodopsin include, but are not limited to, naturally occurring versions of anion-conducting channelrhodopsin present in other or the same species (orthologs, paralogs and more generally homologs), and mutant versions of anion-conducting channelrhodopsin, whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, or directed evolution, as described in, for example, U.S. Patent No. 5,837,458) or active portion thereof, such as but not limited to the rhodopsin domain. The disclosure also includes the use of degenerate nucleic acid variants (due to the redundancy of the genetic code) of the identified channelrhodopsin polynucleotide sequences.

[00126] Additionally contemplated is the use of polynucleotides encoding anion-conducting channelrhodopsin ORFs, or their functional equivalents, encoded by

polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar to the corresponding regions of the anion-conducting channelrhodopsin sequences described herein (as measured by BLAST sequence comparison analysis using, for example, the University of Wisconsin GCG sequence analysis package (SEQUENCHER 3.0, Gene Codes Corporation, Ann Arbor, MI) using default parameters).

[00127] In certain embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors which encode a protein or peptide that includes within its amino acid sequence an amino acid sequence of a channelrhodopsin or a functional portions or variant thereof, such as those identified and cloned: *GtACR1* and *GtACR2* (SEQ ID NOS: 1 and 3) and *PsuACR1* (SEQ ID NO: 13). In some embodiments, a portion of a channelrhodopsin and has relatively few amino acids which are not identical to, or a biologically functional equivalent of, the amino acids of the full-length channelrhodopsin. The term "functional equivalent" is well understood in the art. Accordingly, sequences which have between about 70% and about 80%; or more preferably, between about 85% and about 90%; or even more preferably, between about 90 and 95% and about 99%; of amino acids which are identical or functionally equivalent to the amino acids of the identified and cloned: GtACR1 (SEQ ID NO: 1), GtACR2 (SEQ ID NO: 3) or PsuACR1 (SEQ ID NO: 13).

[00128] The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared which include a short stretch complementary to nucleic acids that encode the polypeptides of SEQ ID NOS: 1 and 3, such as about 10 to 15 or 20, 30, or 40 or so nucleotides, and which are up to 2000 or so base pairs in length. DNA segments with total lengths of about 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 500, 200, 100 and about 50 base pairs in length are also contemplated to be useful.

[00129] In some embodiments, isolated nucleic acids that encode the amino acids of a channelrhodopsin or fragment thereof and recombinant vectors incorporating nucleic acid sequences which encode a channelrhodopsin protein or peptide and that includes

within its amino acid sequence an amino acid sequence in accordance with SEQ ID NOS: 1 and 3. In some embodiments, a purified nucleic acid segment that encodes a protein that encodes a channelrhodopsin or fragment thereof, the recombinant vector may be further defined as an expression vector comprising a promoter operatively linked to said channelrhodopsin-encoding nucleic acid segment.

[00130] In additional embodiments, is a host cell, made recombinant with a recombinant vector comprising channelrhodopsin-encoding nucleic acid segments. The recombinant host cell may be a prokaryotic cell or a eukaryotic cell. As used herein, the term "engineered" or "recombinant" cell is intended to refer to a cell into which a recombinant gene, such as a gene encoding a channelrhodopsin, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced gene. Engineered cells are thus cells having a gene or genes introduced through the hand of man. Recombinantly introduced genes will either be in the form of a copy of a genomic gene or a cDNA gene, or will include genes positioned adjacent to a promoter not naturally associated with the particular introduced gene. In some embodiments, nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of about 14, 15-20, 30, 40, 50, or even of about 100 to about 200 nucleotides or so, identical or complementary to the channelrhodopsin-encoding nucleic acid sequences.

[00131] In some embodiments, the channelrhodopsin-encoding nucleic acid sequences described herein can be targeted to the genome of a host cell using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins. See Sander and Joung, *Nature Biotechnology*, 32(4): 347- 355, incorporated herein by reference. In some embodiments, the CRISPR/Cas nuclease or CRISPR/Cas nuclease system includes a non-coding RNA molecule (guide) RNA, which sequence-specifically binds to DNA, and a Cas protein (*e.g.*, Cas9), with nuclease functionality (*e.g.*, two nuclease domains). In some embodiments, a Cas nuclease and gRNA (including a fusion of crRNA specific for the target sequence and fixed tracrRNA) are introduced into the cell. In general, target sites at the 5' end of the gRNA target the Cas nuclease to the target site, *e.g.*, the gene, using complementary base pairing. In some embodiments, the target site is selected based on its location immediately 5' of a protospacer adjacent motif (PAM) sequence, such as typically

NGG, or NAG. In this respect, the gRNA is targeted to the desired sequence by modifying the first 20 nucleotides of the guide RNA to correspond to the target DNA sequence.

[00132] In some embodiments, the CRISPR system induces DSBs at the target site, followed by disruptions as discussed herein. In other embodiments, Cas9 variants, deemed "nickases" are used to nick a single strand at the target site. In some aspects, paired nickases are used, *e.g.*, to improve specificity, each directed by a pair of different gRNAs targeting sequences such that upon introduction of the nicks simultaneously, a 5' overhang is introduced.

[00133] In some aspects, one or more guide RNAs (ribonucleic acids) direct an enzyme having nuclease activity expressed by the cell, such as a DNA binding protein having nuclease activity, to a target location on the DNA (deoxyribonucleic acid) wherein the enzyme cuts the DNA and an exogenous donor nucleic acid described herein is inserted into the DNA, such as by homologous recombination. Exemplary methods are described, for example, in US Patent Publication No. 20140357530 and International Publication No. WO2015006290, both incorporated herein by reference.

[00134] Accordingly, certain embodiments of the present disclosure are based on the use of exogenous DNA (*e.g.*, channelrhodopsin-encoding nucleic acid sequences), nuclease enzymes such as DNA binding proteins and guide RNAs to co-localize to DNA and digest or cut the DNA with insertion of the exogenous DNA, such as by homologous recombination. Such DNA binding proteins are readily known to those of skill in the art to bind to DNA for various purposes. Such DNA binding proteins may be naturally occurring. DNA binding proteins included within the scope of the present disclosure include those which may be guided by RNA, referred to herein as guide RNA. According to this aspect, the guide RNA and the RNA guided DNA binding protein form a co-localization complex at the DNA. Such DNA binding proteins having nuclease activity are known to those of skill in the art, and include naturally occurring DNA binding proteins having nuclease activity, such as Cas9 proteins present, for example, in Type II CRISPR systems. Such Cas9 proteins and Type II CRISPR systems are well documented in the art. See Makarova et al., Nature Reviews, Microbiology, Vol. 9, June 2011, pp. 467-477 including all supplementary information hereby incorporated by reference in its entirety.

[00135] Exemplary DNA binding proteins having nuclease activity function to nick or cut double stranded DNA. Such nuclease activity may result from the DNA binding protein having one or more polypeptide sequences exhibiting nuclease activity. Such exemplary DNA binding proteins may have two separate nuclease domains with each domain responsible for cutting or nicking a particular strand of the double stranded DNA. Exemplary polypeptide sequences having nuclease activity known to those of skill in the art include the McrA-HNH nuclease related domain and the RuvC-like nuclease domain. Accordingly, exemplary DNA binding proteins are those that in nature contain one or more of the McrA-HNH nuclease related domain and the RuvC-like nuclease domain.

D. Recombinant Expression

[00136] While the desired peptide amino acid sequences described can be chemically synthesized (see, e.g., "Proteins: Structures and Molecular Principles" (Creighton, ed., W. H. Freeman & Company, New York, NY, 1984)), large polypeptides sequences may advantageously be produced by recombinant DNA technology using techniques well-known in the art for expressing nucleic acids containing a nucleic acid sequence that encodes the desired peptide. Such methods can be used to construct expression vectors containing peptide encoding nucleotide sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination (*see, e.g.*, "Molecular Cloning, A Laboratory Manual", *supra*, and "Current Protocols in Molecular Biology", *supra*). Alternatively, RNA and/or DNA encoding desired peptide encoding nucleotide sequences may be chemically synthesized using, for example, synthesizers (*see, e.g.*, "Oligonucleotide Synthesis: A Practical Approach" (Gait, ed., IRL Press, Oxford, United Kingdom, 1984)).

[00137] A variety of host-expression vector systems may be utilized to express peptide encoding nucleotide sequences. When the desired peptide or polypeptide is soluble or a soluble derivative, the peptide or polypeptide can be recovered from the host cell culture, *i.e.*, from the host cell in cases where the peptide or polypeptide is not secreted, and from the culture media in cases where the peptide or polypeptide is secreted by the host cell. However, suitable expression systems also encompass engineered host cells that express the desired polypeptide or functional equivalents anchored in the cell membrane. Purification or enrichment of the desired peptide from such expression systems can be accomplished using appropriate detergents and lipid micelles, and methods well-known to those skilled in the art.

Furthermore, such engineered host cells themselves may be used in situations where it is desired not only to retain the structural and functional characteristics of the peptide, but to assess biological activity, *e.g.*, in certain drug screening assays.

[00138] In certain applications, transient expression systems are desired. However, for long-term, high-yield production of recombinant proteins or peptides, stable expression is generally preferred. For example, cell lines that stably express the desired protein, polypeptide, peptide, or fusion protein may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. Following the introduction of the foreign DNA, engineered cells are allowed to grow for about 1-2 days in an enriched media, and then switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection, and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci, which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express the desired gene products or portions thereof. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of a desired protein, polypeptide or peptide.

[00139] A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, *Cell* 11:223-232, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski, *Proc. Natl. Acad. Sci. USA* 48:2026-2034, 1962), and adenine phosphoribosyltransferase (Lowy *et al.*, *Cell* 22:817-823, 1980) genes, which can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Anti-metabolite resistance can also be used as the basis of selection for the following genes: dihydrofolate reductase (dhfr), which confers resistance to methotrexate (Wigler *et al.*, *Proc. Natl. Acad. Sci. USA* 77:3567-3570, 1980, and O'Hare *et al.*, *Proc. Natl. Acad. Sci. USA* 78:1527-1531, 1981); guanine phosphoribosyl transferase (gpt), which confers resistance to mycophenolic acid (Mulligan and Berg, *Proc. Natl. Acad. Sci. USA* 78:2072-2076, 1981); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colbere-Garapin *et al.*, *J. Mol. Biol.* 150:1-14, 1981); and hygromycin B phosphotransferase (hpt), which confers resistance to hygromycin (Santerre *et al.*, *Gene* 30:147-156, 1984).

[00140] Host cells/expression systems that may be used for purpose of providing compositions to be used in the disclosed methods include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with a recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vector containing a desired peptide encoding nucleotide sequence; yeast (*e.g.*, *Saccharomyces cerevisiae*, *Pichia pastoris*) transformed with a recombinant yeast expression vector containing a desired peptide encoding nucleotide sequence; insect cell systems infected with a recombinant virus expression vector (*e.g.*, baculovirus) containing a desired peptide encoding nucleotide sequence; plant cell systems infected with a recombinant virus expression vector (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV), or transformed with a recombinant plasmid expression vector (*e.g.*, Ti plasmid), containing a desired peptide encoding nucleotide sequence; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring a recombinant expression construct containing a desired peptide encoding nucleotide sequence and a promoter derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter, the vaccinia virus 7.5K promoter).

[00141] In bacterial systems, a number of different expression vectors may be advantageously selected depending upon the use intended for the desired gene product being expressed. For example, when a large quantity of such a protein is to be produced, such as for the generation of pharmaceutical compositions comprising a desired peptide, or for raising antibodies to the protein, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to: the *E. coli* expression vector pUR278 (Ruther and Müller-Hill, *EMBO J.* 2:1791-1794, 1983), in which a desired peptide encoding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye and Inouye, *Nucleic Acids Res.* 13:3101-3110, 1985, and Van Heeke and Schuster, *J. Biol. Chem.* 264:5503-5509, 1989); and the like. pGEX vectors (GE Healthcare, Piscataway, NJ) may also be used to express a desired peptide moiety as a fusion protein with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads, followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned desired peptide encoding gene product can be released from the GST moiety.

[00142] In an exemplary insect system, *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) is used as a vector to express a desired peptide encoding sequence. The virus grows in *Spodoptera frugiperda* cells. A desired peptide encoding sequence may be cloned individually into a non-essential region (for example the polyhedrin gene) of the virus, and placed under control of an *Ac*NPV promoter (for example the polyhedrin promoter). Successful insertion of a desired peptide encoding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). The recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted polynucleotide is expressed (*see*, *e.g.*, Smith *et al.*, *J. Virol.* 46:584-593, 1983, and U.S. Patent No. 4,215,051).

[00143] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a desired peptide encoding nucleotide sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric sequence may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing desired peptide products in infected hosts (*see*, *e.g.*, Logan and Shenk, *Proc. Natl. Acad. Sci. USA* 81:3655-3659, 1984). Specific initiation signals may also be required for efficient translation of inserted desired peptide encoding nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In some cases exogenous translational control signals, including, perhaps, the ATG initiation codon, may be provided. Furthermore, the initiation codon should be in phase with the reading frame of the desired peptide encoding coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, *etc.* (*see*, *e.g.*, Nevins, *CRC Crit. Rev. Biochem.* 19:307-322, 1986).

[00144] In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review, *see*, *e.g.*, "Current Protocols in Molecular Biology", *supra*, Ch. 13, Bitter *et al.*, *Meth. Enzymol.* 153:516-544, 1987, "DNA Cloning", Vol. II, Ch.

3 (Glover, ed., IRL Press, Washington, D.C., 1986); Bitter, *Meth. Enzymol.* 152:673-684, 1987, "The Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance" (Strathern *et al.*, eds., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1981), and "The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression" (Strathern *et al.*, eds., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1982).

[00145] In plants, a variety of different plant expression vectors can be used, and expression of a desired peptide encoding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA or 19S RNA promoters of CaMV (Brisson *et al.*, *Nature* 310:511-514, 1984), or the coat protein promoter of TMV (Takamatsu *et al.*, *EMBO J.* 6:307-311, 1987) may be used. Alternatively, plant promoters such as the promoter of the small subunit of RUBISCO (Coruzzi *et al.*, *EMBO J.* 3:1671-1679, 1984, and Broglie *et al.*, *Science* 224:838-843, 1984), or heat shock promoters, *e.g.*, soybean hsp17.5-E or hsp17.3-B (Gurley *et al.*, *Mol. Cell. Biol.* 6:559-565, 1986) may be used. These constructs can be introduced into plant cells using, for example, Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, or electroporation. For reviews of such techniques, *see*, *e.g.*, Weissbach and Weissbach, in "Methods in Plant Molecular Biology", Section VIII (Schuler and Zielinski, eds., Academic Press, Inc., New York, NY, 1988), and "Plant Molecular Biology", 2nd Ed., Ch. 7-9 (Grierson and Covey, eds., Blackie & Son, Ltd., Glasgow, Scotland, United Kingdom, 1988).

[00146] In addition, a host cell strain may be chosen that modulates the expression of the inserted desired peptide encoding sequence, or modifies and processes the desired peptide encoding nucleic acid sequence in a desired fashion. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may affect certain functions of the protein. Different host cells have characteristic and specific mechanisms for post-translational processing and modification of proteins and peptides. Appropriate cell lines or host systems can be chosen to ensure the correct or desired modification and processing of the desired protein, polypeptide, or peptide expressed. To this end, eukaryotic host cells that possess the cellular machinery for desired processing of the primary transcript, and glycosylation and/or phosphorylation of desired peptide encoding nucleic acid sequence be used. Such mammalian host cells include, but are not limited to, Chinese hamster ovary (CHO), VERO, baby hamster kidney (BHK), HeLa, monkey kidney (COS), MDCK, 293, 3T3, WI38, human hepatocellular carcinoma (*e.g.*, Hep G2),and U937 cells.

[00147] In some embodiments, a recombinant host cell comprising one of the nucleic acid sequences described. In some embodiments, a protein composition comprising one of the polypeptides described.

III. Methods of Use

[00148] In some embodiments, molecular engineered variants (some with improved activity) of the described a highly efficient and sensitive anion-conducting channelrhodopsin by site-specific mutagenesis and chimera construction. In some embodiments, the channelrhodopsins serve as receptors for phototaxis and the photophobic response. Their photoexcitation initiates depolarization of the cell membrane.

[00149] In some embodiments, the rhodopsin domains of several anion-conducting channelrhodopsins were cloned and determined to have channel activity when they were expressed in mammalian HEK293 cells. Using these methods new anion-conducting channelrhodopsin variants, were determined to have improved properties with regards to, among other applications, optogenetics.

[00150] One of the major challenges for optogenetic applications, especially in living animals, are scattering of the stimulating light by biological tissues and its absorption by hemoglobin. Optogenetic tools with long-wavelength absorption would exhibit minimal light attenuation from these effects, but most microbial rhodopsins do not fall into this category. For instance, the absorption maximum of ChR2, which possesses several other useful properties and is thereby most frequently used as a depolarizing tool in optogenetics, is 470 nm.

[00151] Long-wavelength absorption by optogenetic tools is generally considered desirable to increase the penetration depth of the stimulus light by minimizing tissue scattering and absorption by hemoglobin. In some embodiments, the long-wavelength sensitivity of optogenetic microbial rhodopsins is enhanced using 3,4-Dehydroretinal (A2 retinal). A2 retinal (3,4-dehydroretinal) is a natural retinoid, its 11-*cis* form being found in photoreceptor cells of certain invertebrates, fish and amphibians, where it may constitute the only retinal, or an additional chromophore to A1 retinal. The presence of an additional double bond in the ß-ionone ring of the chromophore results in pigments that absorb light at longer wavelengths, as compared to those formed with A1 (regular) retinal. Variations in A1/A2 ratio cause natural adaptive tuning of spectral sensitivity of vision in the organisms during

adaptation to external conditions. Reconstitution of bleached microbial rhodopsins (bacteriorhodopsin, halorhodopsin, sensory rhodopsins I and II) *in vitro* with all-*trans* 3,4-dehydroretinal (A2 retinal) also shifts their absorption spectra to longer wavelengths. In some embodiments, spectral properties of optogenetic tools were modified by incorporation of all-*trans* A2 retinal. The addition of A2 retinal, both ion pumps and channelrhodopsins form functional pigments with significantly red-shifted absorption.

[00152] In some embodiments, the long-wavelength sensitivity of optogenetic microbial rhodopsins is enhanced using A2 retinal. In some embodiments, chromophore substitution provides a complementary strategy to improve the efficiency of optogenetic tools. Substitution of A1 retinal by A2 retinal significantly shifts the spectral sensitivity of tested rhodopsins to longer wavelengths typically without altering other aspects of their function.

[00153] Optogenetic techniques involve the introduction of light-activated channels and enzymes that allow manipulation of neural activity and control of neuronal function. Thus, in some embodiments, the disclosed methods and compositions can be introduced into cells and facilitate the manipulation of the cells activity and function. See, for example, US publication 20130090454 of US application serial number 13/622,809, as well as, Mattis, J., Tye, K. M., Ferenczi, E. A., Ramakrishnan, C., O'Shea, D. J., Prakash, R., Gunaydin, L. A., Hyun, M., Fenno, L. E., Gradinaru, V., Yizhar, O., and Deisseroth, K. (2012) Principles for applying optogenetic tools derived from direct comparative analysis of microbial opsins. Nat. Methods 9, 159 –172; and Zhang, F., Vierock, J., Yizhar, O., Fenno, L. E., Tsunoda, S., Kianianmo- meni, A., Prigge, M., Berndt, A., Cushman, J., Polle, J., Magnuson, J., Hege- mann, P., and Deisseroth, K. (2011) The microbial opsin family of optogenetic tools. Cell 147, 1446 –1457).

[00154] Optogenetic techniques, and thus the disclosed methods and compositions, can be used to characterize the functions of complex neural circuits and information processing in the normal brain and during various neurological conditions; functionally map the cerebral cortex; characterize and manipulate the process of learning and memory; characterize and manipulate the process of synaptic transmission and plasticity; provide light-controlled induction of gene expression; provide optical control of cell motility and other activities.

[00155] Clinical applications of the disclosed methods and compositions include (but are not limited to) optogenetic approaches to therapy such as: restoration of vision by introduction of channelrhodopsins in post-receptor neurons in the retina for ocular disorder gene-therapy treatment of age-dependent macular degeneration, diabetic retinopathy, and retinitis pigmentosa, as well as other conditions which result in loss of photoreceptor cells; control of cardiac function by using channelrhodopsins incorporated into excitable cardiac muscle cells in the atrioventricular bundle (bundle of His) to control heart beat rhythm rather than an electrical pacemaker device; restoration of dopamine-related movement dysfunction in Parkinsonian patients; amelioration of depression; recovery of breathing after spinal cord injury; provide noninvasive control of stem cell differentiation and assess specific contributions of transplanted cells to tissue and network function. Any group of electrically active cells may be amenable to ACR suppression, including, but not limited to those listed above and cardiomyocytes. Such ACRs are also potentially useful for efficient photoinhibition of cardiomyocyte action potentials thereby enabling treatment of cardiac dysfunctions including, but not limited to, tachycardia. In some embodiments, the presently described compositions and methods can be used to facilitate optical stimulation of cardiac cells and tissues, without negative electrophysiological effects of current cardiac anti-arrhythmia therapies and alleviate symptoms by stimulating or silencing specific regions with abnormal excitation in the heart or the brain. In some embodiments, such optogenetic-based techniques could be used to silence, restore or reset irregular heartbeat in patients some of which now receive implantable devices.

[00156] In some embodiments, the presently described compositions and methods can be used to influence cardiac cells or regions by either direct viral gene delivery (such as but not limited to AAV) or by delivery of ACR-carrying donor cells (such as but not limited to cardiomyocytes, Purkinje and His bundle cells, etc.) generated or transformed in culture.

[00157] In some embodiments, a method of membrane hyperpolarization of a cell in a subject suffering from a neuron mediated disorder, said method comprising: delivering to the cell of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13, which encodes a rhodopsin domain of an anion-conducting

channelrhodopsin expressible in said cell; and expressing said vector in said cell, wherein the expression of the rhodopsin results in membrane hyperpolarization.

[00158] In some embodiments, a method of neuronal silencing in a subject suffering from a neuron mediated disorder, said method comprising: delivering to a target neuron of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13, which encodes a rhodopsin domain of an anion-conducting channelrhodopsin expressible in said target neuron; and expressing said vector in said target neuron, wherein the expression of the rhodopsin results in silencing of the signal from the target neuron.

[00159] In some embodiments, a method of restoring photosensitivity to a retina of a subject suffering from vision loss or blindness, said method comprising: delivering to the retina of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 which encodes a rhodopsin domain of an anion-conducting channelrhodopsin expressible in a retinal neuron; and expressing said vector in said retinal neuron, wherein the expressed rhodopsin renders a high level of membrane potential in said retinal neuron.

[00160] Therefore, in some embodiments an anion-conducting channelrhodopsin, light-gated anion channels that provide highly sensitive and efficient membrane hyperpolarization is provided as GtACR1 (SEQ ID NO: 1) and GtACR2 (SEQ ID NO: 3) which were derived from anion-conducting channelrhodopsin of *Guillardia theta* and may be used to enhance optogenetic techniques and optogenetic approaches to therapy.

[00161] Anion-conducting channelrhodopsins, functional or active portions thereof, such as but not limited to the rhodopsin domain, and functional equivalents include, but are not limited to, naturally occurring versions of ACR and those that are orthologs and homologs, and mutant versions of ACR, whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, or directed evolution, as described in, for example, U.S. Patent No. 5,837,458). Also included are the use of degenerate nucleic acid variants (due to the redundancy of the genetic code) of the disclosed algae ACR derived polynucleotide sequences.

[00162] In some embodiments, are methods of treating a neuronal disorder, comprising: (a) delivering to a target neuron a nucleic acid expression vector that encodes a rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin, expressible in said target neuron, said vector comprising an open reading frame encoding the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin, operatively linked to a promoter sequence, and optionally, a transcriptional regulatory sequence; and (b) expressing said vector in said target neuron, wherein the expressed rhodopsin that results in highly sensitive and efficient membrane hyperpolarization and neuronal silencing of said target neuron upon exposure to light. In some embodiments, the rhodopsin domain is encoded by SEQ ID NO: 1 or SEQ ID NO:3.

[00163] In some embodiments, are methods of treating a neuronal disorder, comprising: (a) delivering to a target neuron a nucleic acid expression vector that encodes a rhodopsin domain of an anion-conducting channelrhodopsin derived from algae, expressible in said target neuron, said vector comprising an open reading frame encoding the rhodopsin domain of an anion-conducting channelrhodopsin, operatively linked to a promoter sequence, and optionally, a transcriptional regulatory sequence; and (b) expressing said vector in said target neuron, wherein the expressed rhodopsin silences said target neuron upon exposure to light. In some embodiments, the rhodopsin domain is encoded by SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[00164] In some embodiments, are methods of restoring light sensitivity to a retina, comprising: (a) delivering to a retinal neuron a nucleic acid expression vector that encodes a rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin, expressible in the retinal neuron; said vector comprising an open reading frame encoding the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin operatively linked to a promoter sequence, and optionally, a transcriptional regulatory sequence; and (b) expressing said vector in said retinal neuron, wherein the expressed rhodopsin renders said retinal neuron photosensitive, thereby restoring light sensitivity to said retina or a portion thereof. In some embodiments, the rhodopsin domain is encoded by SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[00165] In some embodiments, are methods of restoring light sensitivity to a retina, comprising: (a) delivering to a retinal neuron a nucleic acid expression vector that encodes a rhodopsin domain of a highly efficient and sensitive anion-conducting

channelrhodopsin, expressible in the retinal neuron; said vector comprising an open reading frame encoding the rhodopsin domain of a a highly efficient and sensitive anion-conducting channelrhodopsin operatively linked to a promoter sequence, and optionally, a transcriptional regulatory sequence; and (b) expressing said vector in said retinal neuron, wherein the expressed rhodopsin renders said retinal neuron photosensitive, thereby restoring light sensitivity to said retina or a portion thereof. In some embodiments, the rhodopsin domain is encoded by SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[00166] In some embodiments, are methods of restoring photosensitivity to a retina of a subject suffering from vision loss or blindness in whom retinal photoreceptor cells are degenerating or have degenerated and died, said method comprising: (a) delivering to the retina of said subject a nucleic acid vector that encodes a rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin expressible in a retinal neuron; said vector comprising an open reading frame encoding the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin operatively linked to a promoter sequence, and optionally, a transcriptional regulatory sequence; and (b) expressing said vector in said retinal neuron, wherein the expressed rhodopsin renders said retinal neuron photosensitive, thereby restoring photosensitivity to said retina or a portion thereof. In some embodiments, the rhodopsin domain is encoded by SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13.

[00167] In some embodiments, the presently described compositions and methods can be used to facilitate optical stimulation of cardiac cells and tissues, without negative electrophysiological effects of current cardiac anti-arrhythmia therapies and alleviate symptoms by stimulating or silencing specific regions with abnormal excitation in the heart or the brain. In some embodiments, such optogenetic based techniques could be used to silence, restore or reset irregular heartbeat in patients some of which now receive implantable devices.

[00168] In some embodiments, the presently described compositions and methods can be used to influence cardiac cells or regions by either direct viral gene delivery (such as but not limited to AAV) or by delivery of ACR-carrying donor cells (such as but not limited to cardiomyocytes, Purkinje and His bundle cells, etc.) generated or transformed in culture.

[00169] In some embodiments is a method of treating a disorder in an electrically active cell in a subject suffering from a disorder that involves electrically active cells, said method comprising: (a) delivering to the cell of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 expressible in said cell; and (b) expressing said vector in said electrically active cell, wherein the expressed rhodopsin silences the signal from said electrically active cell.

[00170] In some embodiments is a method of treating a disorder in an electrically active cell in a subject suffering from a disorder that involves electrically active cells, said method comprising: (a) delivering to said subject a transgenic cell comprising an expression vector comprising a polynucleotide that encodes an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 expressible in said transgenic cell; and (b) expressing said vector in said transgenic cell, wherein the expression silences the signal from a electrically active cell.

[00171] In some embodiments is a method of silencing an electrically active cell in a subject suffering from an electrically active cell mediated disorder, said method comprising: (a) delivering to a target neuron of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 which encodes a rhodopsin domain of an anion-conducting channelrhodopsin expressible in said target neuron; and (b) expressing said vector in said target electrically active cell, wherein the expressed rhodopsin results in silencing of the signal from the electrically active cell. In some embodiments is a recombinant host cell, wherein said host cell is an isolated an electrically active cell.

[00172] In some embodiments, a method of treating a neuronal disorder comprises:(a) delivering to a target neuron a nucleic acid expression vector that encodes a rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin, expressible in said target neuron; said vector comprising an open reading frame encoding the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin operatively linked to a promoter sequence, and optionally, transcriptional regulatory sequences; and (b) expressing the expression vector in the target neuron, wherein the expressed anion-conducting channelrhodopsin silences the target neuron upon exposure to light. In some embodiments an above-described expression vector also comprises one or

more transcriptional regulatory sequences operably linked to the promoter and rhodopsin domain sequences. In some embodiments, the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin has the amino acid sequence of all or part of SEQ ID NOS: 1 or 3 and the rhodopsin domain sequences of SEQ ID NO: 1 or 3, or a biologically active fragment thereof that retains the biological activity of the encoded rhodopsin domain of a channelrhodopsin or is a biologically active conservative amino acid substitution variant of SEQ ID NOS: 1 or 3 or of said fragment. In some embodiments, the expression vector comprises an AAV viral vector. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the constitutive promoter is a CMV promoter or a hybrid CMV enhancer/chicken β-actin (CAG) promoter. In some embodiments, the promoter is an inducible and/or a cell type-specific promoter.

[00173] In some embodiments, a method of restoring light sensitivity to a retina comprises (a) delivering to a retinal neuron in a subject a nucleic acid expression vector that encodes a rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin, expressible in the retinal neuron; said expression vector comprising an open reading frame encoding the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin operatively linked to a promoter sequence, and optionally, one or more transcriptional regulatory sequences; and (b) expressing the expression vector in the retinal neuron, wherein the expressed rhodopsin renders the retinal neuron photosensitive, thereby restoring light sensitivity to the retina or a portion thereof. In some embodiments, the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin has the amino acid sequence of all or part of SEQ ID NOS: 1 or 3 and the rhodopsin domain sequences of SEQ ID NO: 1 or 3, or a biologically active fragment thereof that retains the biological activity of the encoded rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin or is a biologically active conservative amino acid substitution variant of SEQ ID NOS: 1, 3 or 13 and the rhodopsin domain sequences of SEQ ID NO: 1, 3 or 13, or of said fragment. In some embodiments, the expression vector comprises an AAV (*e.g.,* AAV2) viral vector. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the constitutive promoter is a CMV promoter or a hybrid CMV enhancer/chicken β-actin (CAG) promoter. In some embodiments, the promoter is an inducible and/or a cell type-specific promoter.

[00174] In some embodiments, a method of restoring photosensitivity to a retina of a subject suffering from vision loss or blindness in whom retinal photoreceptor cells are degenerating or have degenerated and died comprises: (a) delivering to the retina of the subject a nucleic acid expression vector that encodes a rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin expressible in retinal neurons; said expression vector comprising an open reading frame encoding the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin operatively linked to a promoter sequence, and optionally, transcriptional regulatory sequences; and (b) expressing the expression vector in the retinal neuron, wherein the expression of the rhodopsin renders the retinal neuron photosensitive, thereby restoring photosensitivity to said retina or a portion thereof. In some embodiments, the rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin has the amino acid sequence of all or part of SEQ ID NOS: 1, 3 or 13 and the rhodopsin domain sequences of SEQ ID NO: 1, 3 or 13, or a biologically active fragment thereof that retains the biological activity of the encoded rhodopsin domain of a highly efficient and sensitive anion-conducting channelrhodopsin or is a biologically active conservative amino acid substitution variant of SEQ ID NOS: 1, 3 or 13 and the rhodopsin domain sequences of SEQ ID NO: 1, 3 or 13, or of said fragment. In some embodiments, the expression vector comprises an AAV (*e.g.*, AAV2) viral vector. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the constitutive promoter is a CMV promoter or a hybrid CMV enhancer/chicken β-actin (CAG) promoter. In other embodiments, the promoter is an inducible and/or a cell type-specific promoter.

A. Compositions as Therapeutics

[00175] The use of channelrhodopsins, or active fragments thereof such as but not limited to the rhodopsin domain as therapeutics. In certain embodiments the presently disclosed compositions and are used to improve optogenetic techniques and applications as well as can be used to aid in diagnosis, prevention, and/or treatment of among other things neuron mediated disorders, neurologic disorders (such as Parkinson's disease) and as therapy for ocular disorders.

[00176] In certain embodiments the presently disclosed compositions can be administered in combination with one or more additional compounds or agents ("additional active agents") for the treatment, management, and/or prevention of among other things

neuron mediated disorders, neurologic disorders (such as Parkinson's disease) and as therapy for ocular disorders. Such therapies can be administered to a patient at therapeutically effective doses to treat or ameliorate, among other things, neuron mediated disorders, neurologic disorders (such as Parkinson's disease) and as therapy for ocular disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in any delay in onset, amelioration, or retardation of disease symptoms.

[00177] Toxicity and therapeutic efficacy of such compositions can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, expressed as the ratio LD_{50}/ED_{50}. Compositions that exhibit large therapeutic indices are preferred. Compounds that exhibit toxic side effects may be used in certain embodiments, however, care should usually be taken to design delivery systems that target such compositions preferentially to the site of affected tissue, in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00178] Data obtained from cell culture assays and animal studies can be used in formulating a range of dosages for use in humans. The dosages of such compositions lie preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending on the dosage form employed and the route of administration utilized. For any composition, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test composition that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Plasma levels may be measured, for example, by high performance liquid chromatography.

[00179] When the therapeutic treatment of among other things neurologic disorders (such as Parkinson's disease) and as therapy for ocular disorders is contemplated, the appropriate dosage may also be determined using animal studies to determine the maximal tolerable dose, or MTD, of a bioactive agent per kilogram weight of the test subject. In general, at least one animal species tested is mammalian. Those skilled in the art regularly extrapolate doses for efficacy and avoiding toxicity to other species, including human.

Before human studies of efficacy are undertaken, Phase I clinical studies help establish safe doses.

[00180] Additionally, the bioactive agent may be coupled or complexed with a variety of well-established compositions or structures that, for instance, enhance the stability of the bioactive agent, or otherwise enhance its pharmacological properties (*e.g.*, increase *in vivo* half-life, reduce toxicity, *etc.*).

[00181] Such therapeutic agents can be administered by any number of methods known to those of ordinary skill in the art including, but not limited to, inhalation, subcutaneous (sub-q), intravenous (I.V.), intraperitoneal (I.P.), intramuscular (I.M.), or intrathecal injection, or topically applied (transderm, ointments, creams, salves, eye drops, and the like), as described in greater detail below.

B. Pharmaceutical Compositions

[00182] Pharmaceutical compositions for use in accordance with the presently described compositions may be formulated in conventional manners using one or more physiologically acceptable carriers or excipients.

[00183] The pharmaceutical compositions can comprise formulation materials for modifying, maintaining, or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. Suitable formulation materials include, but are not limited to: amino acids (for example, glycine, glutamine, asparagine, arginine and lysine); antimicrobials; antioxidants (for example, ascorbic acid, sodium sulfite and sodium hydrogen-sulfite); buffers (for example, borate, bicarbonate, Tris-HCl, citrates, phosphates and other organic acids); bulking agents (for example, mannitol and glycine); chelating agents (for example, ethylenediamine tetraacetic acid (EDTA)); complexing agents (for example, caffeine, polyvinylpyrrolidone, beta-cyclodextrin, and hydroxypropyl-beta-cyclodextrin); fillers; monosaccharides, disaccharides, and other carbohydrates (for example, glucose, mannose and dextrins); proteins (for example, serum albumin, gelatin and immunoglobulins); coloring, flavoring, and diluting agents; emulsifying agents; hydrophilic polymers (for example, polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (for example, sodium); preservatives (for example, benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben,

propylparaben, chlorhexidine, sorbic acid and hydrogen peroxide); solvents (for example, glycerin, propylene glycol and polyethylene glycol); sugar alcohols (for example, mannitol and sorbitol); suspending agents; surfactants or wetting agents (for example, pluronics, PEG, sorbitan esters, polysorbates (for example, polysorbate 20 and polysorbate 80), triton, tromethamine, lecithin, cholesterol, and tyloxapal); stability enhancing agents (for example, sucrose and sorbitol); tonicity enhancing agents (for example, alkali metal halides (for example, sodium or potassium chloride), mannitol, and sorbitol); delivery vehicles; diluents; excipients; and pharmaceutical adjuvants ("Remington's Pharmaceutical Sciences", 18th Ed. (Gennaro, ed., Mack Publishing Company, Easton, PA, 1990)).

[00184] Additionally, the described therapeutic peptides can be linked to a half-life extending vehicle. Certain exemplary half-life extending vehicles are known in the art, and include, but are not limited to, the Fc domain, polyethylene glycol, and dextran (*see*, *e.g.*, PCT Patent Application Publication No. WO 99/25044).

[00185] These agents may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

[00186] The agents may also be formulated as compositions for rectal administration such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

[00187] In addition to the formulations described previously, the agents may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. For example, agents may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil), ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack may for example

comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

[00188] Active compositions can be administered by controlled release means or by delivery devices that are well-known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos. 3,845,770, 3,916,899, 3,536,809, 3,598,123, 4,008,719, 5,674,533, 5,059,595, 5,591,767, 5,120,548, 5,073,543, 5,639,476, 5,354,556, and 5,733,566. Such dosage forms can be used to provide slow or controlled-release of one or more active ingredients using, for example, hydropropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination thereof, to provide the desired release profile in varying proportions. Exemplary sustained release matrices include, but are not limited to, polyesters, hydrogels, polylactides (*see*, *e.g.*, U.S. Patent No. 3,773,919 and European Patent Application Publication No. EP 058,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (*see*, *e.g.*, Sidman *et al.*, *Biopolymers* 22:547-556, 1983), poly (2-hydroxyethyl-methacrylate) (*see*, *e.g.*, Langer *et al.*, *J. Biomed. Mater. Res.* 15:167-277, 1981, and Langer, *Chemtech* 12:98-105, 1982), ethylene vinyl acetate (Langer *et al.*, *supra*), and poly-D(-)-3-hydroxybutyric acid (European Patent Application Publication No. EP 133,988). Sustained release compositions may include liposomes, which can be prepared by any of several methods known in the art (*see*, *e.g.*, Eppstein *et al.*, *Proc. Natl. Acad. Sci. USA* 82:3688-3692, 1985, and European Patent Application Publication Nos. EP 036,676, EP 088,046, and EP 143,949). Suitable controlled-release formulations known to those of ordinary skill in the art, including those described herein, can be readily selected for use with the presently disclosed compositions. Certain embodiments encompass single unit dosage forms suitable for oral administration such as, but not limited to, tablets, capsules, gelcaps, and caplets that are adapted for controlled-release.

[00189] All controlled-release pharmaceutical products have a common goal of improving therapy over that achieved by their non-controlled counterparts. Ideally, use of an optimally designed controlled-release preparation in medical treatment is characterized by a minimum of drug substance being employed to cure or control the condition in a minimum amount of time. Advantages of controlled-release formulations include extended activity of the drug, reduced dosage frequency, and increased patient compliance. In addition, controlled-release formulations can be used to affect the time of onset of action or other

characteristics, such as blood levels of the drug, and can thus affect the occurrence of side (*e.g.*, adverse) effects.

[00190] Most controlled-release formulations are designed to initially release an amount of active ingredient that promptly produces the desired therapeutic effect, and gradually and continually release other amounts of active ingredient to maintain this level of therapeutic or prophylactic effect over an extended period of time. In order to maintain this relatively constant level of active ingredient in the body, the drug must be released from the dosage form at a rate that will replace the amount of active ingredient being metabolized and excreted from the body. Controlled-release of an active ingredient can be stimulated by various conditions including, but not limited to, pH, temperature, enzymes, water, or other physiological conditions or compositions.

[00191] In some cases, active ingredients of the disclosed methods and compositions are preferably not administered to a patient at the same time or by the same route of administration. Therefore, in some embodiments are kits that, when used by the medical practitioner, can simplify the administration of appropriate amounts of active ingredients to a patient.

[00192] A typical kit comprises a single unit dosage form of one or more of the therapeutic agents disclosed, alone or in combination with a single unit dosage form of another agent that may be used in combination with the disclosed compositions. Disclosed kits can further comprise devices that are used to administer the active ingredients. Examples of such devices include, but are not limited to, syringes, drip bags, patches, and inhalers.

[00193] Disclosed kits can further comprise pharmaceutically acceptable vehicles that can be used to administer one or more active ingredients. For example, if an active ingredient is provided in a solid form that must be reconstituted for parenteral administration, the kit can comprise a sealed container of a suitable vehicle in which the active ingredient can be dissolved to form a particulate-free sterile solution that is suitable for parenteral administration. Examples of pharmaceutically acceptable vehicles include, but are not limited to: Water for Injection USP; aqueous vehicles such as, but not limited to, Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and polypropylene glycol; and non-aqueous vehicles such

as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate. However, in specific embodiments, the disclosed formulations do not contain any alcohols or other co-solvents, oils or proteins.

C. Transgenic Animals

[00194] The present disclosure provides methods and compositions for the creation and use of both human and non-human transgenic animals that carry an algae derived anion-conducting channelrhodopsin transgene in all their cells, as well as non-human transgenic animals that carry an algae derived anion-conducting channelrhodopsin transgene in some, but not all their cells, for example in certain electrically active cells. Human and non-human mammals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees, can be used to generate transgenic animals carrying an algae derived anion-conducting channelrhodopsin polynucleotide (and/or expressing an algae derived polypeptide) may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head or head-to-tail tandems. An algae derived anion-conducting channelrhodopsin transgene may also be selectively introduced into and activated in a particular cell-type (*see*, *e.g.*, Lakso *et al.*, *Proc. Natl. Acad. Sci. USA* 89:6232-6236, 1992). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell-type of interest, and will be apparent to those of skill in the art.

[00195] Should it be desired that an algae-derived anion-conducting channelrhodopsin, or fragment thereof, transgene be integrated into the chromosomal site of the endogenous copy of the mammalian anion-conducting channelrhodopsin gene, gene targeting is generally preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous anion-conducting channelrhodopsin gene are designed for the purpose of integrating, *via* homologous recombination with chromosomal sequences, into and disrupting the function of the endogenous channelrhodopsin gene (*i.e.*, "knock-out" animals). In this way, the expression of the endogenous channelrhodopsin gene may also be eliminated by inserting non-functional sequences into the endogenous channelrhodopsin gene. The transgene may also be selectively introduced into a particular cell-type, thus inactivating the endogenous channelrhodopsin gene in only that cell-type (*see*, *e.g.*, Gu *et al.*, *Science* 265:103-106, 1994).

The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell-type of interest, and will be apparent to those of skill in the art.

[00196] Any technique known in the art may be used to introduce a channelrhodopsin, or fragment thereof, transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to: pronuclear microinjection (U.S. Patent No. 4,873,191); retrovirus-mediated gene transfer into germ lines (van der Putten *et al.*, *Proc. Natl. Acad. Sci. USA* 82:6148-6152, 1985); gene targeting in embryonic stem cells (Thompson *et al.*, *Cell* 56:313-321, 1989); electroporation of embryos (Lo, *Mol. Cell. Biol.* 3:1803-1814, 1983); sperm-mediated gene transfer (Lavitrano *et al.*, *Cell* 57:717-723, 1989); and positive-negative selection, as described in U.S. Patent No. 5,464,764. For a review of such techniques, *see*, *e.g.*, Gordon, *Int. Rev. Cytol.* 115:171-229, 1989.

[00197] Once transgenic animals have been generated, the expression of the recombinant channelrhodopsin gene, or fragment thereof, may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the channelrhodopsin transgene has taken place. The level of mRNA expression of the channelrhodopsin transgene in the tissues of the transgenic animals may also be assessed using techniques that include, but are not limited to, Northern blot analysis of cell-type samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of an algae derived channelrhodopsin-expressing tissue can also be evaluated immunocytochemically using antibodies selective for the channelrhodopsin transgene product.

D. Transgene Based Therapies

[00198] In certain embodiments the presently disclosed compositions and are used to improve optogenetic techniques and applications as well as can be used to aid in diagnosis, prevention, and/or treatment of neurologic disorders, such as but not limited to Parkinson's disease, as well as for ocular disorders.

[00199] In some embodiments, methods and compositions are used to identify and characterize multiple channelrhodopsins derived from algae. The cloning and expression of the rhodopsin domain of the channelrhodopsins and expression in mammalian cells

demonstrates that these channelrhodopsins have improved characteristics that can be used for optogenetic applications as well as therapeutic agents.

[00200] For example, a disclosed method and composition may be used in, among other things, retinal gene therapy for mammals (as described in, among others, U.S. Patent Nos. *5,827,702, 7824869 and US Patent Publication Number 20100015095 as well as in WIPO publications WO 2000*/15822 and WO 1998/48097). A genetically engineered ocular cell is produced 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. 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). 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) has been used to treat a naturally-occurring dog model of severe disease of retinal degenerations—the RPE65 mutant dog, which is analogous to human LCA. By expressing photosensitive membrane-channels or molecules in surviving retinal neurons of the diseased retina by viral based gene therapy method, the present invention may produce permanent treatment of the vision loss or blindness with high spatial and temporal resolution for the restored vision.

[00201] The nucleic acids sequences that encode an active portion of the presently disclosed anion-conducting channelrhodopsins, include but are not limited to the nucleic acid sequences that encode the rhodopsin domains identified in SEQ ID NOS: 1, 3 and 13 or the rhodopsin domain sequences of SEQ ID NO: 2.

[00202] In some embodiments, there is provided a method of modifying a target polynucleotide in a eukaryotic cell, such as a neuron. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, said cleavage comprises cleaving one or two strands at the location of

the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in an insertion of one or more channelrhodopsin-encoding nucleotides in said target polynucleotide.

[00203] An exemplary DNA binding protein is an RNA guided DNA binding protein of a Type II CRISPR System. An exemplary DNA binding protein is a Cas9 protein. According to one aspect, an engineered Cas9-gRNA system is provided which enables RNA-guided genome cutting in a site specific manner, if desired, and modification of the genome by insertion of exogenous channelrhodopsin-encoding nucleic acids provided herein. The guide RNAs are complementary to target sites or target loci on the DNA. The guide RNAs can be crRNA-tracrRNA chimeras. The Cas9 binds at or near target genomic DNA. The one or more guide RNAs bind at or near target genomic DNA. The Cas9 cuts the target genomic DNA and exogenous donor DNA is inserted into the DNA at the cut site.

[00204] Accordingly, methods are directed to the use of a guide RNA with a Cas9 protein and an exogenous channelrhodopsin-encoding nucleic acid to multiplex insertions of exogenous channelrhodopsin-encoding nucleic acids into DNA within a cell expressing Cas9 by cycling the insertion of nucleic acid encoding the RNA and exogenous donor nucleic acid, expressing the RNA, colocalizing the RNA, Cas9 and DNA in a manner to cut the DNA, and insertion of the exogenous donor nucleic acid. The method steps can be cycled in any desired number to result in any desired number of DNA modifications.

[00205] In some embodiments, introduction and expression of channelrhodopsins, such as those described herein, in ocular neuronal cells, for example, impart 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, a mechanism for the most basic light-responses that are required for vision is provided. In some embodiments, the functional domains of anion-conducting channelrhodopsins, such as GtACR1 and GtACR2 may be used to restore light sensitivity to the retinas that have undergone rod and cone degeneration by expressing the channelrhodopsin in inner retinal neurons in vivo. In some embodiments these channelrhodopsins may be introduced using techniques that include, but are not limited to, retinal implants, cortical implants, lateral geniculate nucleus implants, or optic nerve implants

[00206] In some embodiments, the anion-conducting channelrhodopsins are inserted into the retinal neurons that survived after the rods and cones have died in an area or portion of the retina of a subject, using the transfer of nucleic acids, alone or within an expression vector. Such expression vectors may be constructed, for example, by introduction of the desired nucleic acid sequence into a virus system known to be of use for gene therapy applications, such as, but not limited to, AAV (*e.g.,* AAV2), retroviruses and alike.

[00207] In some embodiments the anion-conducting channelrhodopsins may be inserted into retinal interneurons. These cells then can become light sensitive and send signals via the optic nerve and higher order visual pathways to the visual cortex where visual perception occurs, as has been demonstrated electrophysiologicly in mice. In some embodiments, among other routes, intravitreal and/or subretinal injections may be used to deliver channelrhodopsin molecules or virus vectors expressing the same.

[00208] In some embodiments, the active portion of the presently disclosed algal derived anion-conducting channelrhodopsins, such as but not limited to the rhodopsin domain of these anion-conducting channelrhodopsins, can be used to restore light sensitivity to a retina, by delivering to retinal neurons a nucleic acid expression vector that encodes algal derived anion-conducting channelrhodopsins (such as but not limited to the rhodopsin domain of these anion-conducting channelrhodopsins) that is 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 expressing the vector in the neurons, thereby restoring light sensitivity.

[00209] In certain embodiments the channel rhodopsin can be algal derived anion-conducting channelrhodopsins such as, but not limited to functional domains of anion-conducting channelrhodopsins, such as, but not limited to, *Gt*ACR1, *Gt*ACR2 or *Gt*161302 or a biologically active fragment or conservative amino acid substitution variant thereof, such as but not limited to the rhodopsin domain. The vector system may be recombinant AAV (*e.g.,* AAV2), the promoter may be a constitutive promoter such as, but not limited to, a CMV promoter or a hybrid CMV enhancer/chicken β-actin promoter (CAG).

IV. Examples

[00210] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the

techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – Materials and Methods

[00211] Molecular Biology Methods: Sequences encoding 7TM domains of *G. theta* opsins (295, 291 and 288 aa, corresponding to the JGI protein models 111593, 146828 and 161302, respectively) were optimized for human codon usage and were synthesized (Genewiz, South Plainfield, NJ, USA) and cloned into the mammalian expression vector pcDNA3.1 (Life Technologies, Grand Island, NY, USA) in frame with an EYFP tag. The sequence information encoding the functional constructs 111593 (*Gt*ACR1) and 146828 (*Gt*ACR2) were deposited in GenBank (accession numbers KP171708 and KP171709, respectively). The gene encoding archaerhodopsin-3 (Arch) was kindly provided by Dr. Edward S. Boyden (Massachusetts Institute of Technology, Boston, MA, USA). Mutations were introduced using a QuikChange XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) and verified by DNA sequencing.

[00212] HEK293 Recording Methods: HEK293 (human embryonic kidney) cells were transfected using the ScreenFectA transfection reagent (Waco Chemicals USA, Richmond, VA, USA). All-trans-retinal (Sigma, St Louis, MO, USA) was added as a stock solution in ethanol at the final concentration of 5 μM. Measurements were performed 48-72 h after transfection with an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA). The signals were digitized with a Digidata 1440A using pClamp 10 software (both from Molecular Devices). Patch pipettes with resistances of 2-5 MΩ were fabricated from borosilicate glass. The composition of solutions is shown in table S1. A 4 M salt bridge was used in all experiments; liquid junction potentials (LJP) were calculated using the ClampEx built-in LJP calculator. Continuous light pulses were provided by a Polychrome IV light source (T.I.L.L. Photonics GMBH, Grafelfing, Germany) in combination with a mechanical shutter (Uniblitz Model LS6, Vincent Associates, Rochester, NY, USA; half-opening time 0.5 ms). The light intensity was attenuated with the built-in Polychrome system or with

neutral density filters. Maximal quantum density at the focal plane of the 40x objective lens was 8.5 mW/mm^2.

[00213] In experiments aimed to test cation permeability Erev shifts were calculated by subtraction of the reference value measured at 150 mM NMG^+ in the bath from the values measured at 150 mM Na^+ (pH 5.4), 150 mM Na^+ (pH 7.4), 150 mM K^+ (pH 7.4) or 75 mM Ca^{2+} (pH 7.4). The Cl^- concentration in the bath was 155.6 mM with all cations. In tests of anion permeability E_{rev} shifts were calculated by subtraction of the Reference value measured at 150 mM Asp^- from the value measured at 75 mM SO_4^{2-}or 150 mM of F^-, Br^-, I^- or $NO3^-$. The Na^+ concentration in the bath was 150 mM with all anions except F- (Table 1).

Table 1: Composition of pipette and bath solutions and liquid junction potentials in experiments with HEK293 cells.

	NaCl	KCl	$CaCl_2$	$MgCl_2$	Na_2EGTA	HEPES	NMG	Glucose	NaAsp	NaF	NaBr	NaI	$NaNO_3$	Na_2SO_4	HCl	LJP pip. stand	LJP pip. Asp
Pipette standard	—	126	0.5	2	5	25	12.2	—	—	—	—	—	—	—	—	—	—
Pipette Asp	—	—	0.5	2	5	25	12.2	—	126	—	—	—	—	—	—	—	—
Bath standard	150	—	1.8	1	—	10	4.6	5	—	—	—	—	—	—	—	4.7	12.9
Bath Asp	—	—	1.8	1	—	10	4.6	5	150	—	—	—	—	—	—	-7	—
Bath pH 5.4	150	—	1.8	1	—	10	—	5	—	—	—	—	—	—	—	4.7	—
Bath K	—	150	1.8	1	—	10	4.6	5	—	—	—	—	—	—	—	0.3	—
Bath Ca	—	—	75	1	—	10	4.6	5	—	—	—	—	—	—	—	8.4	—
Bath NMG	1.5	—	1.8	1	—	10	148.5	5	—	—	—	—	—	—	148.5	10.7	—
Bath F	5.6	—	—	—	—	10	4.6	5	—	150	—	—	—	—	—	13.8	—
Bath Br	—	—	1.8	1	—	10	4.6	5	—	—	150	—	—	—	—	4.5	—
Bath I	—	—	1.8	1	—	10	4.6	5	—	—	—	150	—	—	—	4.3	—
Bath NO_3	—	—	1.8	1	—	10	4.6	5	—	—	—	—	150	—	—	3.3	—
Bath SO_4	—	—	1.8	1	—	10	4.6	5	—	—	—	—	—	75	—	-2.6	—

Abbreviations used: Asp, aspartate; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LJP, liquid junction potential; NMG, N-Methyl-D-glucamine. All concentrations are in mM.

[00214] **Neuronal recording**: For neuronal expression the *Gt*ACR2-EYFP construct was transferred to the pFUGW lentivirus vector provided by Dr. Carlos Lois (Massachusetts Institute of Technology, Boston, MA, USA). The lentivirus was produced by triple transfection of HEK293FT cells (Invitrogen Grand Island, NY, USA) with the envelope plasmid pCMV-VSVG, the packaging plasmid pΔ8.9 (both from Dr. Lois) and the pFUGW-*Gt*ACR2-EYFP plasmid using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). Hippocampi of E18 Sprague Dawley rats were obtained as part of a kit from BrainBits (Springfield, IL, USA), and primary neuronal cultures were prepared using the protocol provided by the company. Cells were cultured in NbActiv4 medium on poly-lysine coated coverslips and supplemented with 0.4 μM all-*trans* retinal (final concentration, in addition to retinyl acetate present in the medium). Patch-clamp measurements were carried out 10 to 19 days after transfection. The same photoexcitation source and measuring setup was used as described above for HEK cells. Spiking was measured in the current clamp mode. The composition of solutions is shown in Table 2.

Table 2: Composition of pipette and bath solutions and liquid junction potentials in experiments with neurons.

	K_2SO_4	KCl	NaCl	$CaCl_2$	$MgCl_2$	HEPES	Glucose	LJP
Pipette	67.5	—	—	—	2	—	—	—
Bath Tyrode	—	2	125	3	1	25	30	11.3

Abbreviations used: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LJP, liquid junction potential; NMDG, N-Methyl-D-glucamine. All concentrations are in mM.

Example 2 – Characterization of rhodopsins

[00215] Of the approximately 50 known ChRs from chlorophytes, all that have been tested are exclusively cation channels. However, several rhodopsin sequences that have been cloned from these organisms did not exhibit channel activity. The nuclear genome sequence of the cryptophyte Guillardia theta has been completely sequenced (B. A. Curtis *et al.*, Nature 492, 59 (2012)). A BLAST search of model proteins returned 53 hits with homology to microbial (type I) rhodopsins. None were highly homologous to ChRs, but the models of one particular cluster (FIG. 1A) do contain some key residues characteristic of chlorophyte ChRs (FIG. 1B).

[00216] The sequences encoding the 7TM domains of *G. theta* proteins 111593, 146828 and 161302 were well expressed in HEK293 cells. The first two constructs generated photocurrents, whereas the third did not. As shown below, the first two function as light-gated anion channels; therefore we named them *Gt*ACR1 and *Gt*ACR2 (*Guillardia theta* Anion Channel Rhodopsins 1 and 2).

[00217] Using standard solutions (126 mM KCl in the pipette and 150 mM NaCl in the bath, pH 7.4; for other components Table 1) the currents generated by *Gt*ACR1 and *Gt*ACR2 were inward at the holding potential (E_h) -60 mV (FIG. 1D, main figure). The mean stationary currents from *Gt*ACR1 and *Gt*ACR2 were, respectively, 8- and 6-fold larger than those from *Cr*ChR2, the most frequently used optogenetic tool, with significantly smaller inactivation (FIG. 1, inset). Half-decay times of photocurrents were 200 and 90 ms for *Gt*ACR1 and *Gt*ACR2, respectively. The dependence of the current rise rate on the stimulus intensity exhibited a higher saturation level than the amplitude (FIG. 1E) and therefore was used for construction of the action spectra. *Gt*ACR1 showed maximal sensitivity to 515 nm light with a shoulder on the short-wavelength slope of the spectrum (FIG. 1F). The sensitivity of *Gt*ACR2 peaked at 470 nm with additional bands at 445 and 415 nm (FIG. 1F).

[00218] Using standard solutions (see above) the sign of *Gt*ACR1 and *Gt*ACR2 photocurrents reversed upon membrane depolarization (FIGs. 2A and B, respectively). In the tested range from - 60 to 60 mV the current voltage relationships (*IE* curves) were linear (FIG. 2C), unlike those for chlorophyte ChRs (described in D. Gradmann, A. Berndt, F. Schneider, P. Hegemann, *Biophys. J.* 101, 1057 (2011). To characterize the ion permeability of *G. theta* rhodopsins *IE* curves were measured and determined E_{rev} upon variation of the ionic composition of the bath solution. In contrast to chlorophyte ChRs, for which protons are the most highly permeant ions, E_{rev} of the currents generated by *Gt*ACR1 and *Gt*ACR2 were not affected by pH (FIG. 2C). Moreover, no E_{rev} shifts were observed when the large non-permeable organic cation N-methyl-glucamine (NMG^+) was replaced with Na^+, K^+, or Ca^{2+} (FIG. 2D). Indicating that *Gt*ACR1 and *Gt*ACR2 are not permeable to cations conducted by chlorophyte ChRs.

[00219] When most of the Cl^- in the bath was replaced with the large organic anion aspartate yielding a Nernst equilibrium potential for Cl^- (E_{Cl}) of 81 mV, E_{rev} shifted to 80 mV (FIGs. 3A-C), as would be expected only if the currents generated by the *Gt*ACR1

and *Gt*ACR2 were exclusively due to passive Cl^- transport. Next, we compared permeabilities of different anions by substituting them for non-permeable Asp^- in the bath. For both *G. theta* ACRs, I^-, $NO3^-$, or Br^- caused even greater E_{rev} shifts than Cl^-. F^- caused a smaller shift, whereas SO_4^{2-}- was non-permeable. The permeability sequence $NO_3^- > I^- > Br^- > Cl^- > F^- > SO_4^{2-} = Asp^-$ determined for ACRs is in accord with the lyotropic series characteristic of many classic Cl^- channels from animal cells.

[00220] A conspicuous feature of ACRs is a non-carboxylic residue in the position of the proton acceptor Asp85 in bacteriorhodopsin (BR), where nearly all cation-selective ChRs contain a Glu residue (column 5 of helix 3, highlighted red in FIG. 1C). Its replacement with Glu reduced the currents > 1000-fold without inhibiting expression. The kinetics of these very small remaining currents changed dramatically. But most importantly, this mutation eliminated anion selectivity of ACRs, as was evident from the practically unchanged E_{rev} after replacement of chloride with aspartate in the bath (FIG. 6A), in a dramatic contrast to the wild type. Therefore, the absence of a carboxylate residue in the proton acceptor position appears to be required for anion selectivity of ACRs. A non-ionizable residue at the corresponding position is also typical of chloride-pumping rhodopsins from haloarchaea and marine flavobacteria (FIG. 1C), where the residue forms part of the chloride binding site in the unphotolyzed state as shown for haloarchaeal halorhodopsin.

[00221] The cytoplasmic Cl^- concentration in most animal cells including neurons is low. Under such conditions (6 mM of Cl^- in the pipette and 156 mM in the bath) *G. theta* ACRs generated hyperpolarizing currents in HEK293 cells at E_h above E_{Cl} (FIG. 4A for *Gt*ACR2, FIG. 6B for *Gt*ACR1). The amplitude of *Gt*ACR2 photocurrents was similar, but the kinetics was faster than of *Gt*ACR1 currents, which is advantageous for control of neuronal activity. The high amplitude of the photocurrents makes ACRs very promising as optogenetic tools for light- induced hyperpolarization. *Gt*ACR2 generated hyperpolarizing photocurrents of the same amplitude as the proton pump *archaerhodopsin-3* (*Arch*), a popular tool for optogenetic spike suppression, at 10,000-fold lower light intensity (FIG. 4B). The maximal amplitude of hyperpolarizing photocurrents of the most efficient Cl^--conducting ChR mutants was <0.6 nA at the expense of dramatically slower kinetics. With *Gt*ACR2 the currents of such amplitude and 100-fold faster kinetics were observed at 3000-fold lower light intensity (FIG. 4B). These properties make *G. theta* ACRs more efficient hyperpolarizing optogenetic tools than previously available.

[00222] In cultured rat pyramidal neurons *Gt*ACR2-generated hyperpolarizing currents at E_h above -88 mV (FIG. 4C and D). This value corresponds exactly to E_{Cl} under our conditions (Table 2), confirming that GtACR2 exclusively conducts anions in neurons. This strict selectivity is a second advantage of ACRs over the previously reported Cl^--conducting ChR mutants for which E_{rev} is 25-30 mV more positive than E_{Cl}, due to residual cation permeability. The range of potentials at which GtACR2 hyperpolarizes the membrane is therefore significantly wider and extends through the values typical for resting potentials of neurons (FIG. 4D). In current clamp experiments *Gt*ACR2 completely inhibited electrically evoked spikes at light intensities above 3x10-3 mW/mm2 with high temporal precision (FIGs. 4E and F).

[00223] Though not wishing to be limited by a particular mechanism, it appears that the mechanism of anion conductance in ACRs is different from that of the Cl^--conducting ChR mutants, as might be expected from their large difference in sequence. ACRs contain a Glu residue corresponding to Glu90 of the cation selectivity filter described for *Cr*ChR2. To confer Cl^- permeability to this cation-conducting channel, Glu90 required replacement with an uncharged (Ser (15)) or cationic (Lys or Arg (14)) residue. However, the presence of the Glu90 homolog in ACRs shows it is not a barrier to anion permeation in the anion channels unlike in the cation channels. Phylogenetically and functionally, ACRs comprise a distinct class of rhodopsins fundamentally different from cation channelrhodopsins (CCRs). As natural anion channels, ACRs provide hyperpolarizing optogenetic tools optimized by evolution for extremely high light-sensitivity, absolute anion selectivity, and rapid kinetics.

Example 3 – PsuACR1 is a light-gated anion channel (ACR)

[00224] An additional ACR has been identified in the marine cryptophyte *Proteomonas sulcata which* shows a close similarity to those ACRs described above that were derived from *G. theta*. This sequence, has been identified as *Ps*ChR1 (see GenBank: KF992074, incorporated herein by reference) and has been shown to generate photocurrents when expressed in cultured neurons (Klapoetke NC, Murata Y, Kim SS, Pulver SR, Birdsey-Benson A, Cho YK, Morimoto TK, Chuong AS, Carpenter EJ, Tian Z, Wang J, Xie Y, Yan Z, Zhang Y, Chow BY, Surek B, Melkonian M, Jayaraman V, Constantine-Paton M, Wong GK, Boyden ES. Nat Methods. 2014 Mar; 11(3):338-46)), but these photocurrents were not characterized in detail and the measurements did not distinguish between cationic and anionic conductance. In the example below it is demonstrated that the corresponding *P. sulcata*

protein exhibits light-gated anion conductance similar to that of the ACRs from *G. theta*, although the amplitude of its photocurrents in cultured mammalian cells is smaller. Therefore, it will be referred to herein as *Psu*ACR1 (SEQ ID NO: 13 and SEQ ID NO: 14), the third letter from the species name was added because the abbreviation *Ps* was used earlier for a CCR from the green alga *Platymonas subcordiformis*.

[00225] In addition to, *Psu*ACR1 primary protein sequence exhibits a high overall similarity and several characteristic features found in the *G. theta* derived ACRs (SEQ ID NO: 1 and 3) described above. It contains a Glu residue (Glu-64) corresponding to Glu-90 of *Cr*ChR2 that had to be replaced with a neutral or positively charged residue to convert *Cr*ChR2 to a Cl^-conducting channel. The position of the proton acceptor in bacteriorhodopsin (Asp-85) is occupied by a non-carboxylic residue, although in *Psu*ACR1 it is Ala (Ala-93) rather than Ser, as in *G. theta* derived ACRs (SEQ ID NO: 1 and 3). Finally, neither of the three residues predicted to form an inner cation channel gate according to the crystal structure of C1C2 chimera (Tyr-109, His-173 and His-304) are conserved in either *Psu*ACR1 ACRs (SEQ ID NO: 13 and 14) or *G. theta* derived ACRs (SEQ ID NO: 1 and 3): Tyr-109 is replaced with Met in all three proteins; His-173 (the position, corresponding to Asp-96 in bacteriorhodopsin), with Leu in *Gt*ACR1 and Gln (as in rhodopsin sodium pumps) in *Gt*ACR2 (SEQ ID NO: 3) and *Psu*ACR1 (SEQ ID NO: 13); His-304 is replaced with Ala in *Gt*ACR1, Ser in *Gt*ACR2, and Arg in *Psu*ACR1 (SEQ ID NO: 13).

[00226] To establish the electrophysiological properties of *Psu*ACR1 (SEQ ID NO: 13 and 14) it was expressed it in human embryonic kidney (HEK293) cells and measured photocurrents under voltage clamp conditions and the findings appear in FIG. 7. A series of current traces recorded with our standard solutions (126 mM KCl in the pipette and 150 mM NaCl in the bath, pH 7.4) in response to a light pulse of the saturating intensity are shown in FIG. 7A. The mean peak amplitude at -60 mV at the amplifier output was 707 ± 184 pA (mean ± SEM, n = 18 cells), which was roughly ten times less than that of the currents generated by ACRs derived from *G. theta* (SEQ ID NO: 1 and 3) under the same conditions. Another difference was that *Psu*ACR1 (SEQ ID NO: 13 and 14) currents displayed rapid inactivation under continuous illumination to a plateau level of 56 ± 2% (mean ± SEM, n = 18 cells), whereas inactivation of photocurrents generated by the *G. theta* derived ACRs (SEQ ID NO: 1 and 3) was much smaller and slower. The sign of *Psu*ACR1 (SEQ ID NO: 13 and 14) photocurrents reversed when the membrane potential was shifted to

more positive values. The dependence of its peak amplitude on the holding potential (E_h) (the IE curve) was linear (FIG. 7D, black line), as that for *G. theta* ACRs (SEQ ID NO: 1 and 3), in contrast to the outward rectification typical of CCRs. The reversal potential (E_{rev}) of photocurrents recorded in standard solutions was close to zero (FIG. 7D, black symbols and line).

[00227] When most of Cl^- in the bath was replaced with aspartate, the E_{rev} shifted to 72 ± 2 mV (mean ± SEM, n = 5 cells). A typical series of photocurrents measured under these conditions is shown in FIG. 7B, and an IE curve, in FIG. 7D (red symbols and line). When Cl^- was replaced with Asp^- in the pipette, but the bath solution was standard, the E_{rev} shifted to -70 ± 2 mV (mean ± SEM, n = 4 cells). Photocurrents and an IE curve for these conditions are shown in FIGs. 8C and D (blue symbols and line), respectively. These results indicated that *Psu*ACR1 photocurrents were due to passive Cl^- transport.

[00228] When Cl^- in the bath was replaced with other anions and measured the IE curves. The shifts of the E_{rev} value measured with each anion from that with non-permeable Asp^- are shown in FIG. 8. As for *G. theta* ACRs, I^- was the most, and F^-, the least permeable halide, whereas divalent sulphate was practically impermeable. Similarly, practically no shift of the E_{rev} was measured with Na^+, K^+ or Ca^{2+} in the bath instead of non-permeable N-methyl-glucamine (NMG^+), or when the bath pH was decreased from 7.4 to 5.4 (FIG. 8). These results confirmed that *Psu*ACR1 is a light-gated anion channel.

[00229] The action spectrum of *Psu*ACR1 photocurrents measured at pH 7.4 has the maximum at 520 nm and a shoulder at 550 nm (FIG. 9). A decrease to pH 5.4 did not shift the position of the spectral maximum.

[00230] To characterize the kinetics of PsuACR1 photocurrent under single-turnover conditions, laser flashes (6 ns) were used for photoexcitation (FIG. 10a). Compared to the previously characterized ACRs from G. theta, channel closing in PsuACR1 at pH 7.4 was ~8 times faster (FIG. 10b). The current decay was fit with 3 exponentials. The third, slowest component made the least contribution to the overall amplitude. The voltage dependencies (IE curves) of the amplitudes of the three decay components of PsuACR1 photocurrents are shown in FIG. 10c. The reversal potentials of all components were the same within experimental error. The time constants (τ) of all components were only weakly voltage-dependent (FIG. 13), in contrast to previous observations in GtACR1. The rate of the

peak current recovery was measured in double-flash experiments. For PsuACR1 it was ~ 5 times faster than that for GtACR2, and ~8 times faster than that for GtACR1 (FIG. 10d).

[00231] At the reversal potential for channel currents a fast negative signal was recorded, the time course of which was limited by the time resolution of the recording system (FIG. 11a). The inward direction of the fast current recorded from PsuACR1 corresponded to that of the initial charge transfer associated with retinal isomerization. The amplitude of this current in PsuACR1 did not depend on the Vh or pH of the bath in the tested range (FIG. 11b and c), which further indicated the origin of this current as retinal isomerization.

* * *

[00232] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent they provide exemplary procedural or other details supplementary to those set forth herein, are incorporated herein by reference.

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WHAT IS CLAIMED IS:

1. A recombinant nucleic acid operatively linked to a heterologous promoter sequence, said recombinant nucleic acid comprising:

(a) a sequence that encodes a peptide with at least 85% homology to an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13; or

(b) a sequence that encodes a peptide comprising 225 contiguous amino acids selected from SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13; or

(c) a sequence that hybridizes to the nucleotide sequence of SEQ ID NO:2, SEQ ID NO:4 or the complement thereof.

2. The recombinant nucleic acid of claim 1, wherein it comprises an expression vector.

3. A recombinant host cell comprising a recombinant nucleic acid of claim 1.

4. The recombinant host cell of claim 3, wherein said host cell is an isolated human cell.

5. The recombinant host cell of claim 3, wherein said host cell is a non-human mammalian cell.

6. The recombinant host cell of claim 3, wherein said host cell is a bacterial cell.

7. The recombinant host cell of claim 3, wherein said host cell is a yeast cell.

8. The recombinant host cell of claim 3, wherein said host cell is an insect cell.

9. The recombinant host cell of claim 3, wherein said host cell is a plant cell.

10. The recombinant host cell of claim 3, wherein said host cell is an isolated neuronal cell.

11. The recombinant host cell of claim 3, wherein said host cell is an isolated electrically active cell.

12. A method of membrane hyperpolarization of a cell in a subject suffering from a neuron mediated disorder, said method comprising:

(a) delivering to the cell of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence at least 90% identical to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 13, which encodes a rhodopsin domain of an anion-conducting channelrhodopsin expressible in said cell; and

(b) expressing said vector in said cell, wherein the expressed rhodopsin silences the signal from said neuron.

13. A method of neuronal silencing in a subject suffering from a neuron mediated disorder, said method comprising:

(a) delivering to a target neuron of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence at least 90% identical to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 13 which encodes a rhodopsin domain of an anion-conducting channelrhodopsin expressible in said target neuron; and

(b) expressing said vector in said target neuron, wherein the expressed rhodopsin results in silencing of the signal from the target neuron.

14. A method of restoring photosensitivity to a retina of a subject suffering from vision loss or blindness, said method comprising:

(a) delivering to the retina of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence at least 90% identical to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 13 which encodes a rhodopsin domain of an anion-conducting channelrhodopsin expressible in a retinal neuron; and

(b) expressing said vector in said retinal neuron, wherein the expressed rhodopsin renders a high level of membrane potential in said retinal neuron.

15. The method of claim 11 wherein, said method comprises:

(a) delivering to the cell of said subject an expression vector that encodes a rhodopsin domain; said vector comprising an open reading frame encoding the

rhodopsin domain of an anion-conducting channelrhodopsin selected from SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 13, operatively linked to a promoter sequence; and

(b) expressing said vector in said cell, wherein the expressed rhodopsin renders a high level of membrane potential in said cell.

16. The recombinant nucleic acid of claim 1, wherein in step c) said sequence that hybridized to the nucleotide sequence of SEQ ID NO:2, or the complement thereof, further comprises hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate, 1 mM EDTA at 65°C, and washing in 0.2x SSC/0.1% SDS at 42°C.

17. The method of claim 13, wherein said subject is mammalian.

18. The method of claim 13, wherein said subject is human.

19. The method of claim 13, wherein said delivering comprises a pharmaceutically acceptable carrying agent.

20. A cDNA-derived nucleic acid comprising a nucleic acid sequence that encodes an amino acid sequence comprising SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 13 .

21. The cDNA-derived nucleic acid of claim 19, wherein the cDNA-derived nucleic acid comprises the nucleic acid sequence of SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NOs: 14-15.

22. An expression vector comprising the cDNA-derived nucleic acid of claim 19.

23. A method of treating a disorder in an electrically active cell in a subject suffering from a disorder that involves electrically active cells, said method comprising:

(a) delivering to the cell of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence at least 90% identical to a sequence selected from the group consisting of SEQ ID NOs: 1, SEQ ID NO: 3 or SEQ ID NO: 13, expressible in said cell; and

(b) expressing said vector in said electrically active cell, wherein the expressed rhodopsin silences the signal from said electrically active cell.

24. A method of treating a disorder in an electrically active cell in a subject suffering from a disorder that involves electrically active cells, said method comprising:

(a) delivering to said subject a transgenic cell comprising an expression vector comprising a polynucleotide that encodes an amino acid sequence at least 90% identical to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 13 expressible in said transgenic cell; and

(b) expressing said vector in said transgenic cell, wherein the expression silences the signal from a electrically active cell.

25. A method of silencing an electrically active cell in a subject suffering from an electrically active cell mediated disorder, said method comprising:

(a) delivering to a target neuron of said subject an expression vector comprising a polynucleotide that encodes an amino acid sequence at least 90% identical to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 13, which encodes a rhodopsin domain of an anion-conducting channelrhodopsin expressible in said target neuron; and

(b) expressing said vector in said target electrically active cell, wherein the expressed rhodopsin results in silencing of the signal from the electrically active cell.

26. An isolated nucleic acid molecule comprising a sequence encoding an anion-conducting channelrhodopsin having a sequence at least about 90% identical to a sequence according to SEQ ID NOs: 15-24.

27. The isolated nucleic acid molecule of claim 26, comprising a sequence having a sequence at least about 95% identical to a sequence according to SEQ ID NOs: 15-24.

28. The isolated nucleic acid molecule of claim 26, where the nucleic acid is a DNA.

29. The isolated nucleic acid molecule of claim 26, where the nucleic acid is a RNA.

30. The isolated nucleic acid molecule of claim 29, where the nucleic acid is a mRNA.

31. An expression vector comprising a nucleic acid molecule according to claim 26.

32. A recombinant host cell comprising a nucleic acid of claim 26.

33. The recombinant host cell of claim 32, wherein said host cell is an isolated human cell.

34. The recombinant host cell of claim 32, wherein said host cell is a non-human mammalian cell.

35. The recombinant host cell of claim 32, wherein said host cell is a bacterial cell.

36. The recombinant host cell of claim 32, wherein said host cell is a yeast cell.

37. The recombinant host cell of claim 32, wherein said host cell is an insect cell.

38. The recombinant host cell of claim 32, wherein said host cell is a plant cell.

39. The recombinant host cell of claim 32, wherein said host cell is an isolated neuronal cell.

40. The recombinant host cell of claim 32, wherein said host cell is an isolated electrically active cell.

41. A method of treating a subject suffering from a disorder that involves electrically active cells comprising expressing in the subject an effective amount of an anion-conducting channelrhodopsin at the site of the electrically active cells.

42. The method of claim 41, wherein the subject is suffering from neuropathic pain the method comprising expressing in the subject an effective amount of an anion-conducting channelrhodopsin at the site of the pain.

43. The method of claim 41, wherein said expressing comprises administering an anion-conducting channelrhodopsin to the subject.

44. The method of claim 43, wherein the anion-conducting channelrhodopsin further comprises a cell-penetrating peptide (CPP) sequence or a cellular receptor-binding sequence.

45. The method of claim 41, wherein said expressing comprises administering a vector encoding an anion-conducting channelrhodopsin to the subject.

46. The method of claim 45, wherein the vector is a RNA vector.

47. The method of claim 45, wherein the vector is a DNA vector.

48. The method of claim 47, wherein the sequence encoding the anion-conducting channelrhodopsin is operably linked to a heterologous promoter.

49. The method of claim 48, wherein the promoter is an inducible or a repressible promoter.

50. The method of claim 48, wherein the promoter is a tissue or cell type specific promoter.

51. The method of claim 50, wherein the promoter is neuronal cell specific promoter.

52. The method of claim 47, wherein the vector is a plasmid, a viral vector or an episomal vector.

53. The method of claim 47, wherein the vector further comprises an inducible expression cassette for a suicide gene.

54. The method of claim 41, wherein the subject has an amputated limb, diabetes, multiple sclerosis or has undergone a surgery.

55. The method of claim 41, wherein the anion-conducting channelrhodopsin comprise an amino acid sequence at least about 90% identical to a sequence of SEQ ID NOs: 1, 3 or 13.

56. The method of claim 45, wherein the anion-conducting channelrhodopsin is encoded by a sequence at about 90% identical to sequence according to SEQ ID NOs: 2, 4 or 14-17.

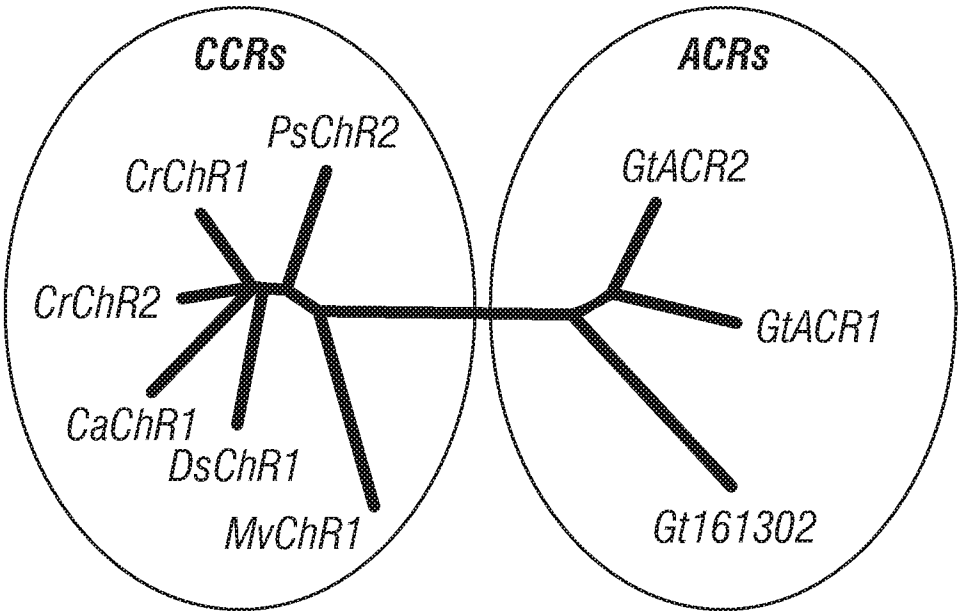

FIG. 1A

helix 2

*Gt*ACR1	WEAIYLPTTE----MITYSL	74	SEQ ID NO: 32
*Gt*ACR2	WESVYLPFVE----SITYAL	70	SEQ ID NO: 33
*Gt*161302	WEFVLVPLTE----CFVYGL	67	SEQ ID NO: 34
*Cr*ChR1	WEEIYVATIEMIKFIIEYFH	139	SEQ ID NO: 35
*Cr*ChR2	WEEIYVCAIEMVKVILEFFF	100	SEQ ID NO: 36
*Ca*ChR1	WEEVYVCCIELVFICFELYH	146	SEQ ID NO: 37
*Mv*ChR1	WEVWFVACIETSIYIIAITS	119	SEQ ID NO: 38
*Hs*HR	RPRLIWGATLMIPLVSISSY	77	SEQ ID NO: 39
*Nm*HR	ALSCIVMVSAGLILNSQAVM	71	SEQ ID NO: 40

FIG. 1B

helix 3

ARMASWLCTCPIMLGLVSNMA	113	SEQ ID NO: 41
SRMASWLCTCPIMLGQISNMA	109	SEQ ID NO: 42
TRTVLWLATVPIILNQINGMA	106	SEQ ID NO: 43
LRYAEWLLTCPVILIHLSNLT	178	SEQ ID NO: 44
LRYAEWLLTCPVILIHLSNLT	139	SEQ ID NO: 45
LRYSEWLLCCPVILIHLSNVT	185	SEQ ID NO: 46
LRYMEWLMTCPVILIALSNIT	158	SEQ ID NO: 47
GRYLTWALSTPMILLALGLLA	127	SEQ ID NO: 48
YRYVNWMATIPCLLLQLLIVL	114	SEQ ID NO: 49

FIG. 1C

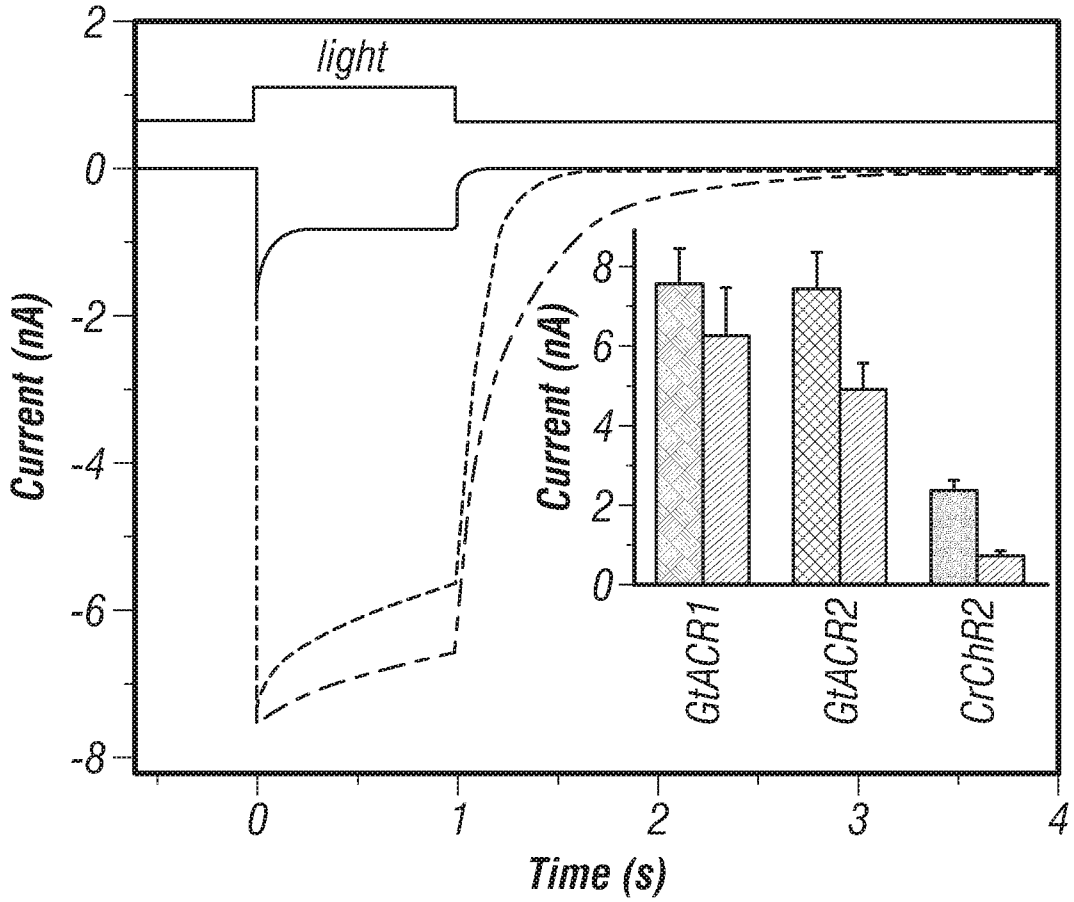

FIG. 1D

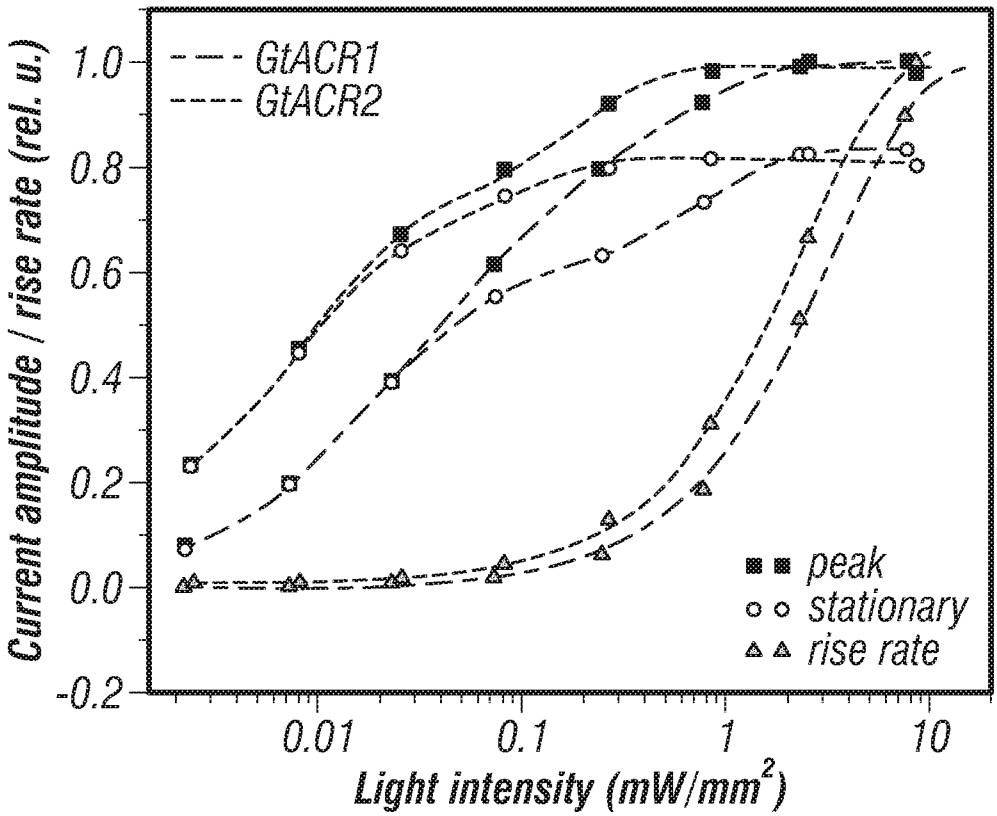
GtACR1
GtACR2
peak
stationary
rise rate
Current amplitude / rise rate (rel. u.)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0.01
0.1
1
10
Light intensity (mW/mm²)

FIG. 1E

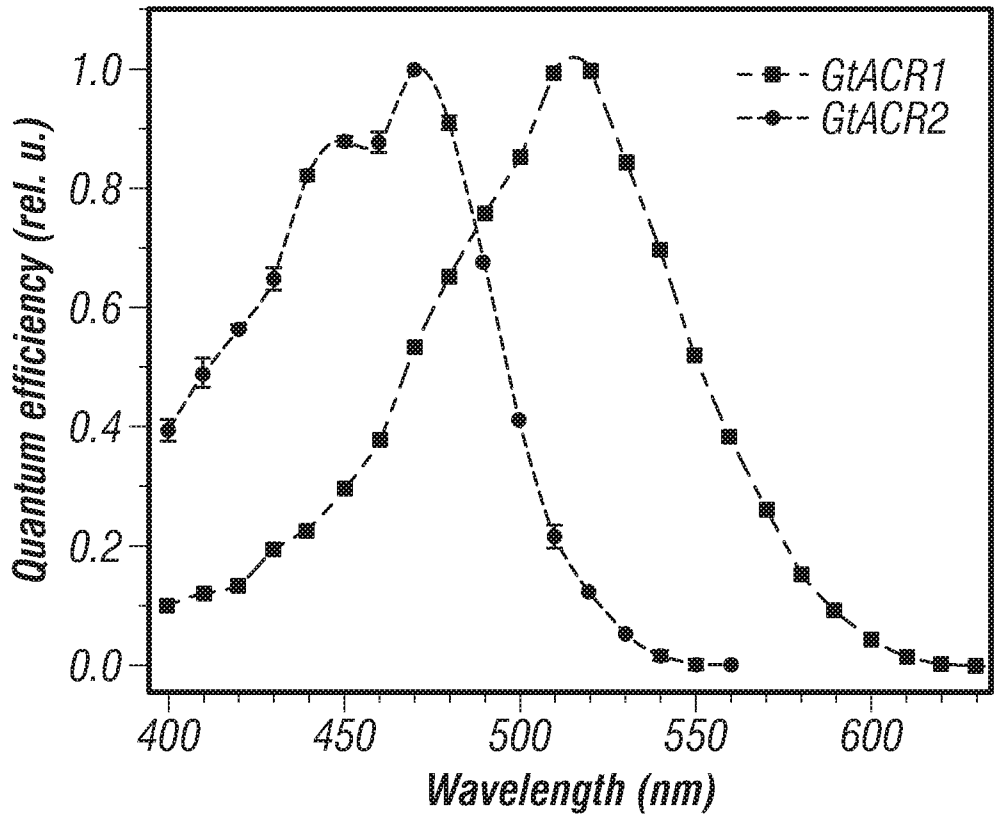
GtACR1
GtACR2
Quantum efficiency (rel. u.)
1.0
0.8
0.6
0.4
0.2
0.0
400
450
500
550
600
Wavelength (nm)

FIG. 1F

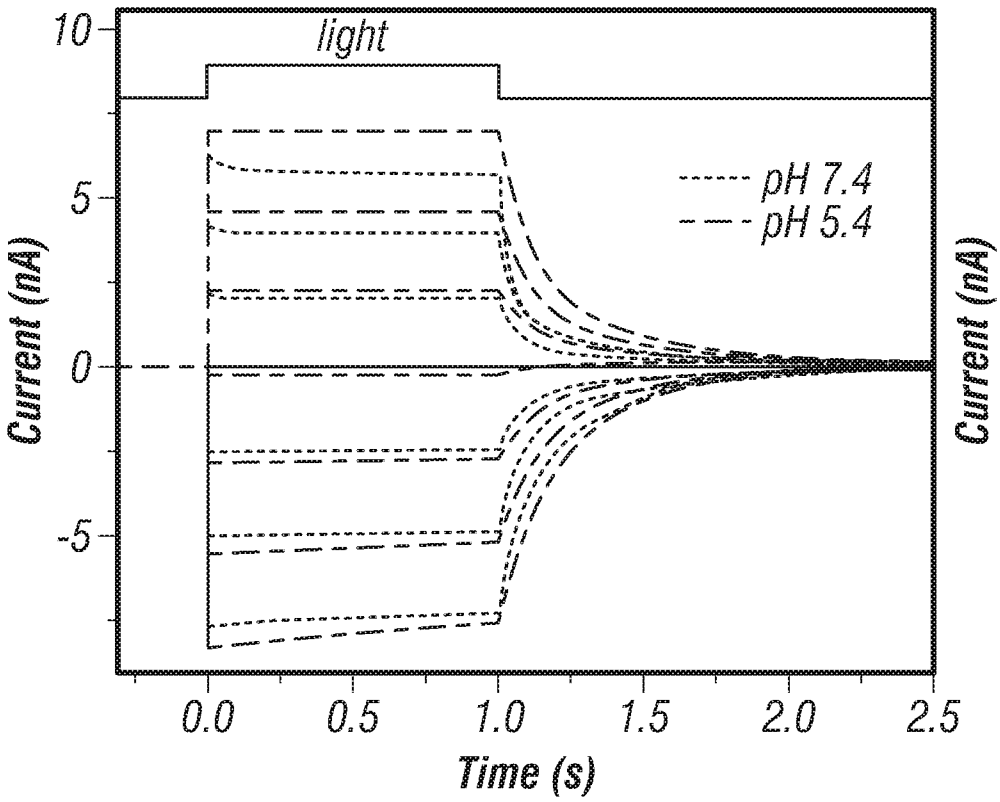
light
10
5
0
-5
pH 7.4
pH 5.4
Current (nA)
Current (nA)
0.0
0.5
1.0
1.5
2.0
2.5
Time (s)

FIG. 2A

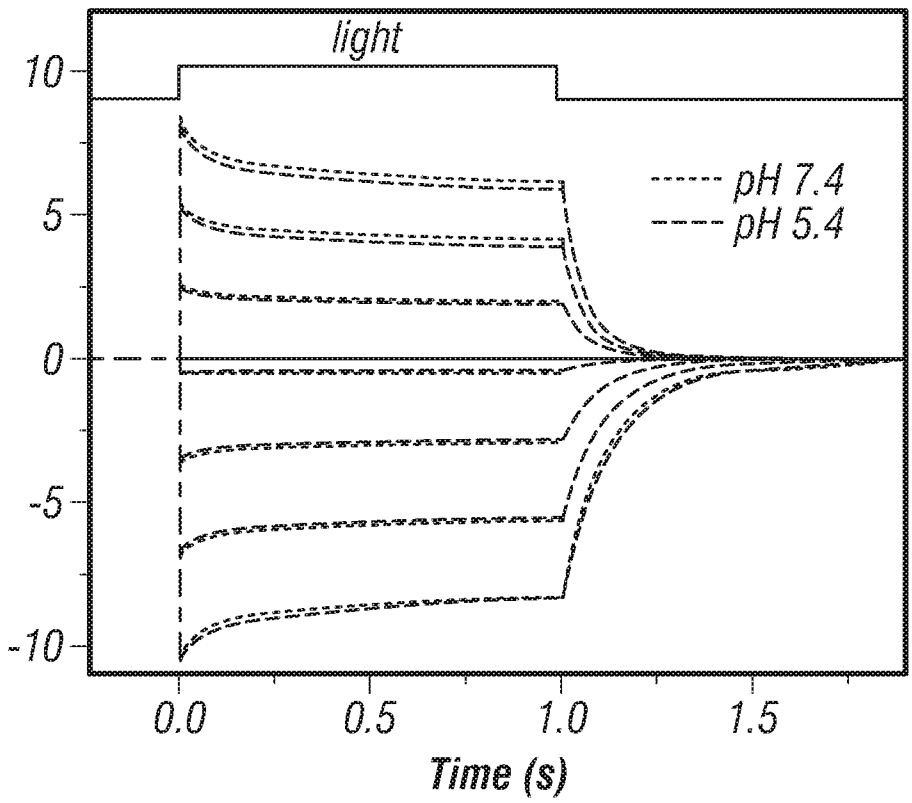
light
10
5
0
-5
-10
pH 7.4
pH 5.4
0.0
0.5
1.0
1.5
Time (s)

FIG. 2B

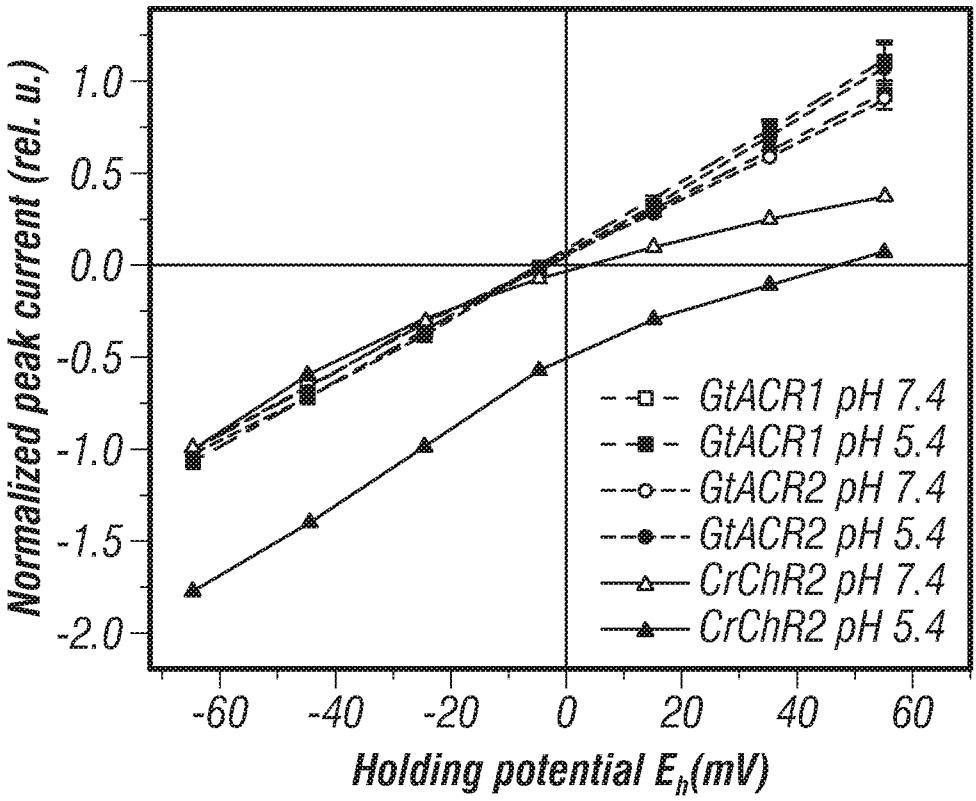
Normalized peak current (rel. u.)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-60
-40
-20
0
20
40
60
Holding potential E_h(mV)
GtACR1 pH 7.4
GtACR1 pH 5.4
GtACR2 pH 7.4
GtACR2 pH 5.4
CrChR2 pH 7.4
CrChR2 pH 5.4

FIG. 2C

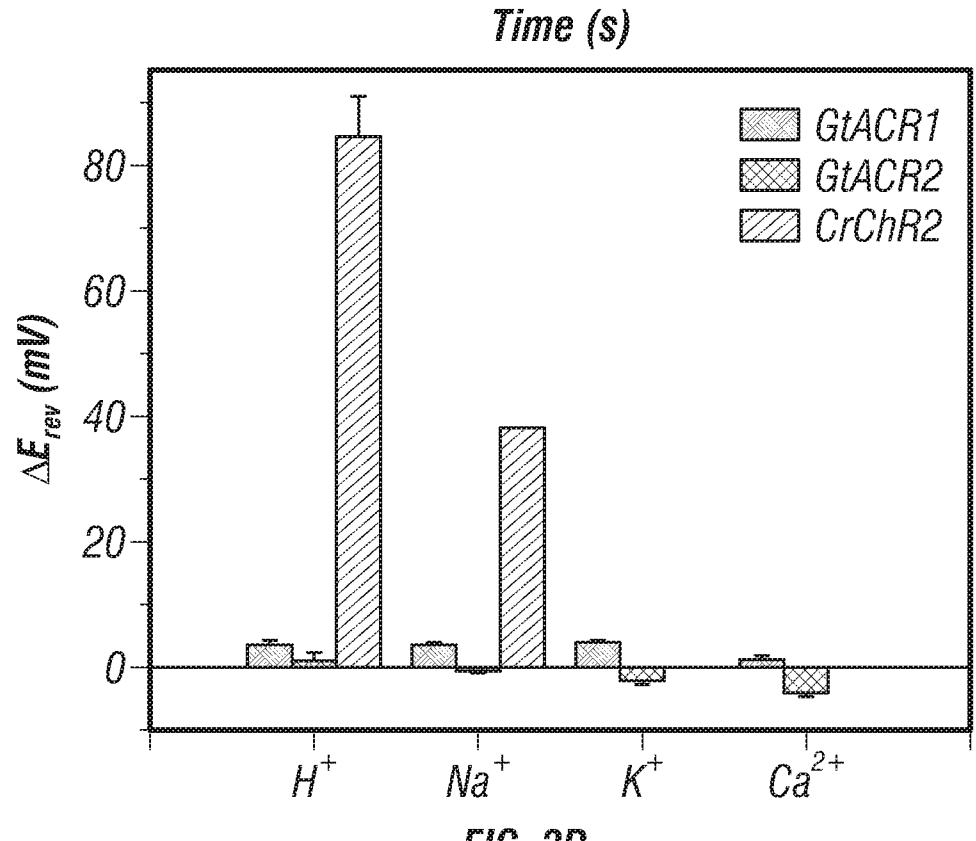
Time (s)
ΔE_{rev} (mV)
80
60
40
20
0
GtACR1
GtACR2
CrChR2
H^+
Na^+
K^+
Ca^{2+}

FIG. 2D

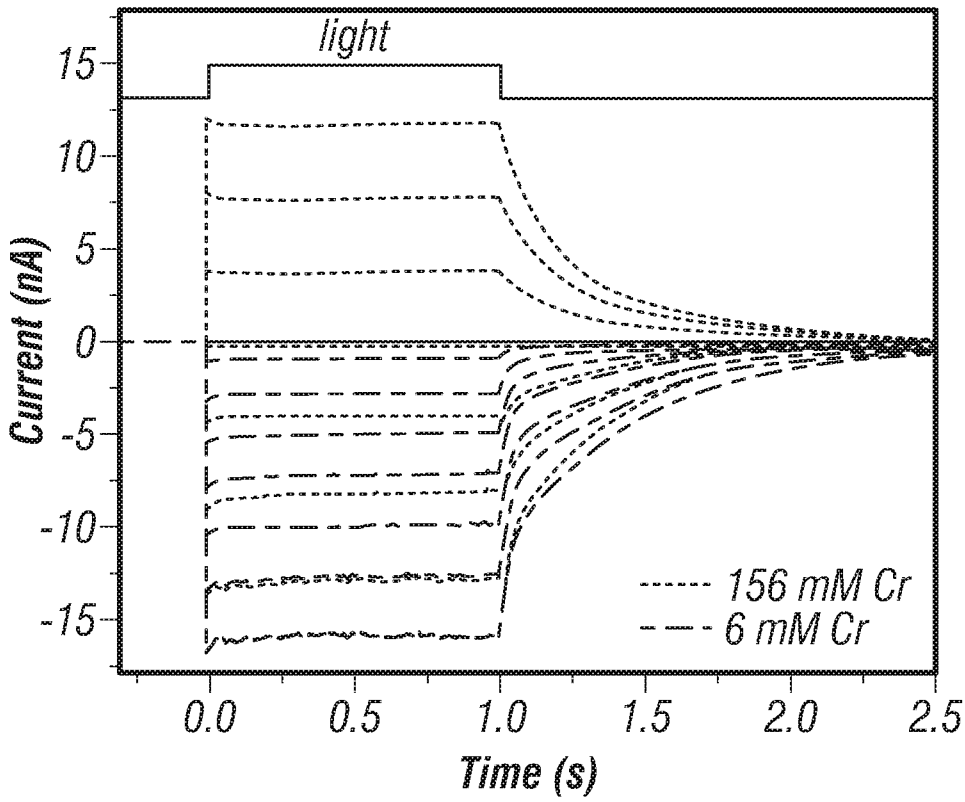
light
15
10
5
0
-5
-10
-15
Current (nA)
156 mM Cr
6 mM Cr
0.0
0.5
1.0
1.5
2.0
2.5
Time (s)

FIG. 3A

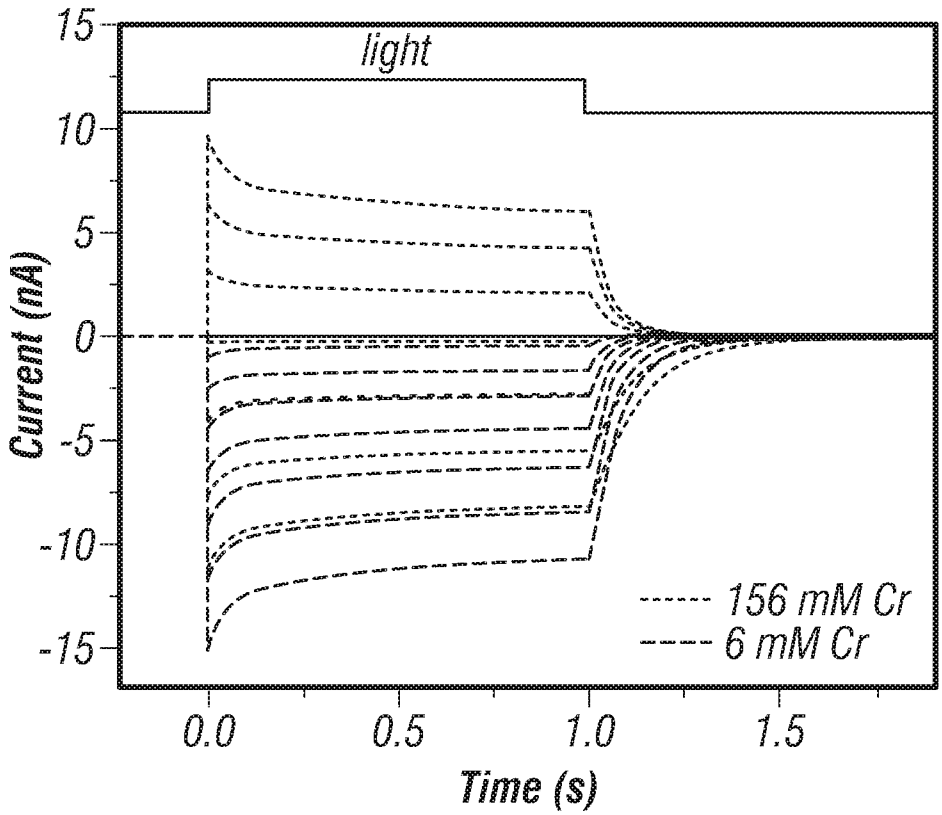
light
15
10
5
0
-5
-10
-15
Current (nA)
156 mM Cr
6 mM Cr
0.0
0.5
1.0
1.5
Time (s)

FIG. 3B

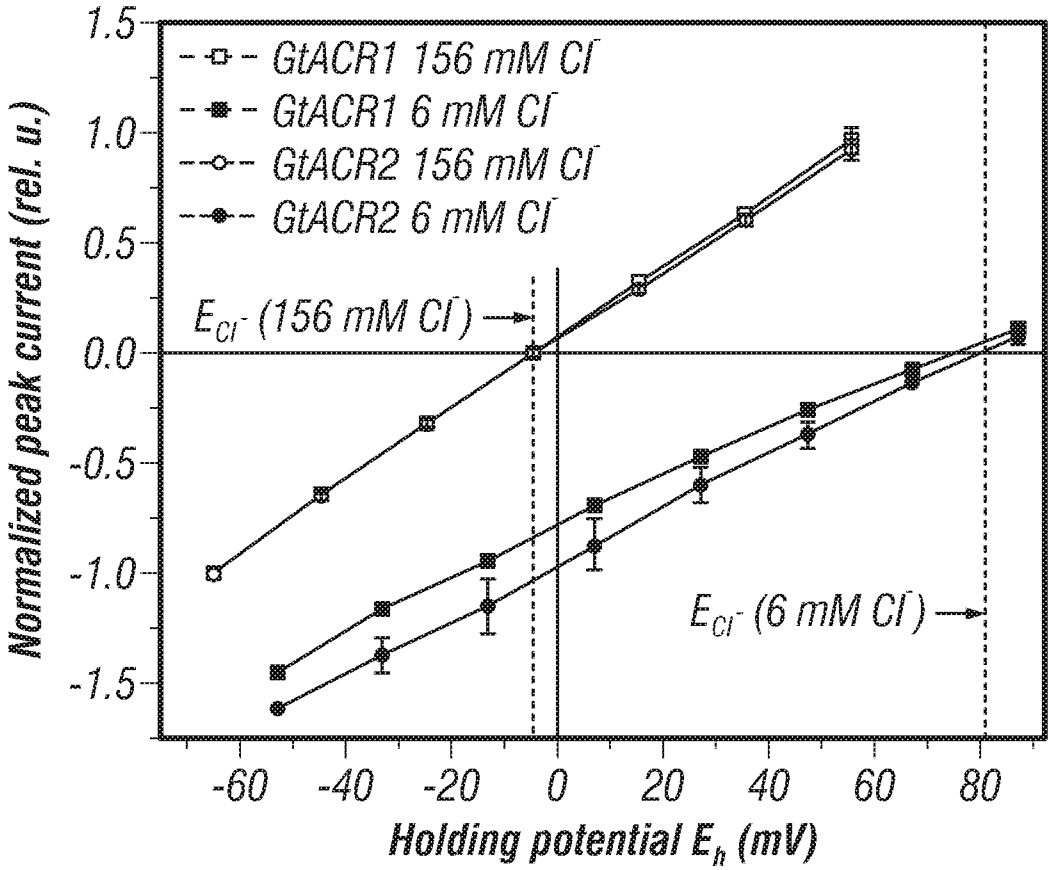
GtACR1 156 mM Cl⁻
GtACR1 6 mM Cl⁻
GtACR2 156 mM Cl⁻
GtACR2 6 mM Cl⁻
E_{Cl^-} (156 mM Cl⁻)
E_{Cl^-} (6 mM Cl⁻)
Normalized peak current (rel. u.)
Holding potential E_h (mV)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-60
-40
-20
0
20
40
60
80

FIG. 3C

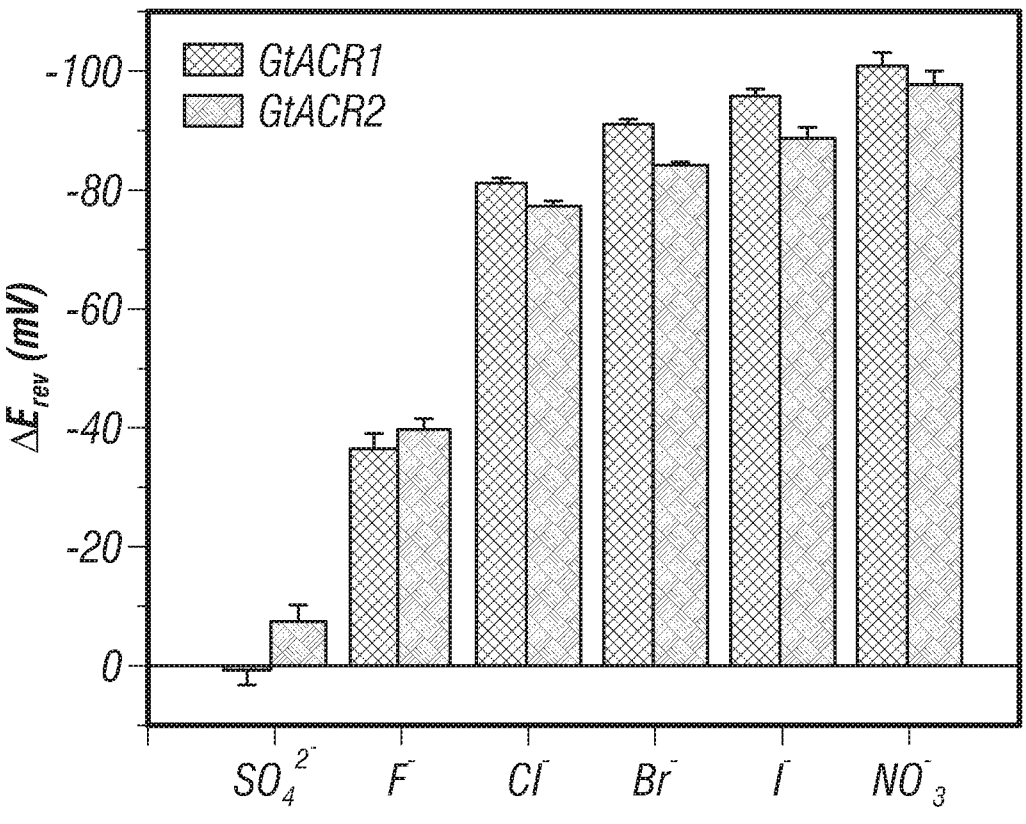
GtACR1
GtACR2
ΔE_{rev} (mV)
-100
-80
-60
-40
-20
0
SO_4^{2-}
F^-
Cl^-
Br^-
I^-
NO_3^-

FIG. 3D

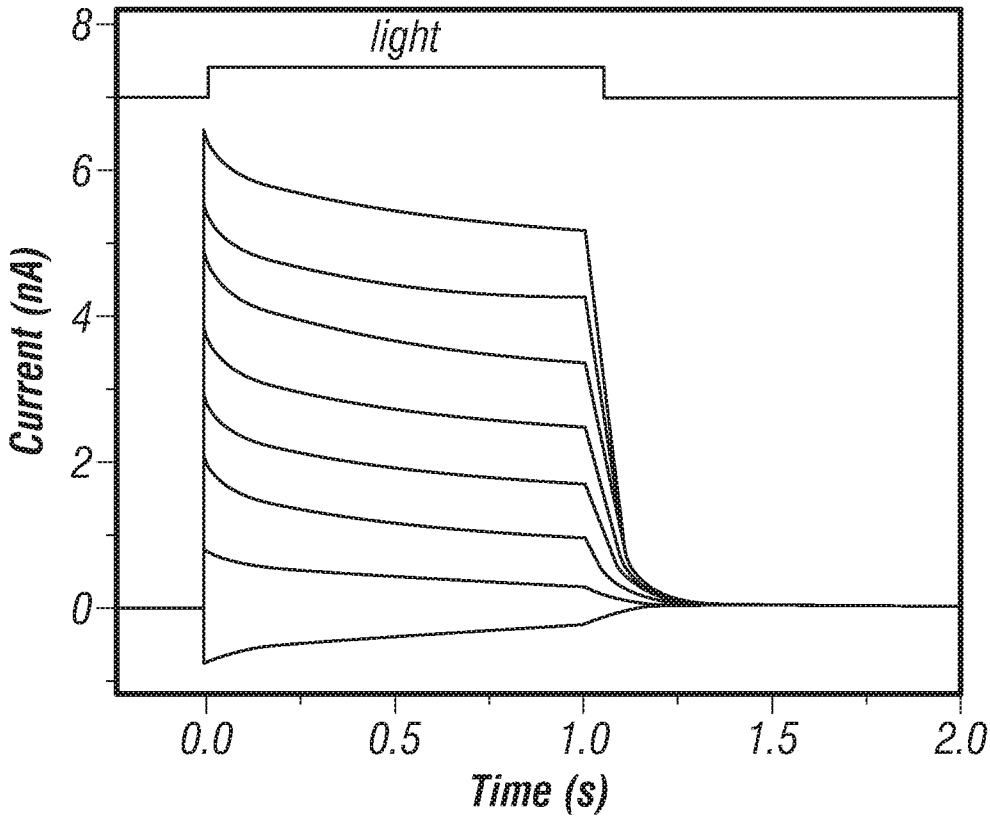
light
8
6
4
2
0
Current (nA)
0.0
0.5
1.0
1.5
2.0
Time (s)

FIG. 4A

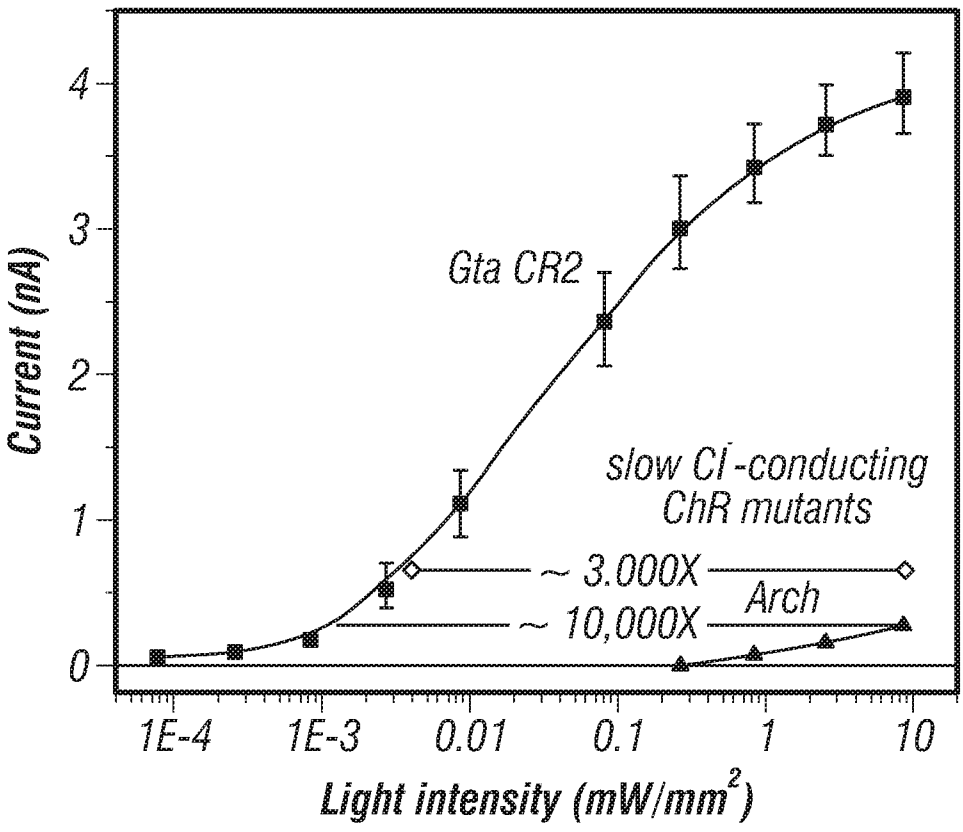
4
3
2
1
0
Current (nA)
Gta CR2
slow Cl-conducting
ChR mutants
~ 3,000X
~ 10,000X
Arch
1E-4
1E-3
0.01
0.1
1
10
Light intensity (mW/mm2)

FIG. 4B

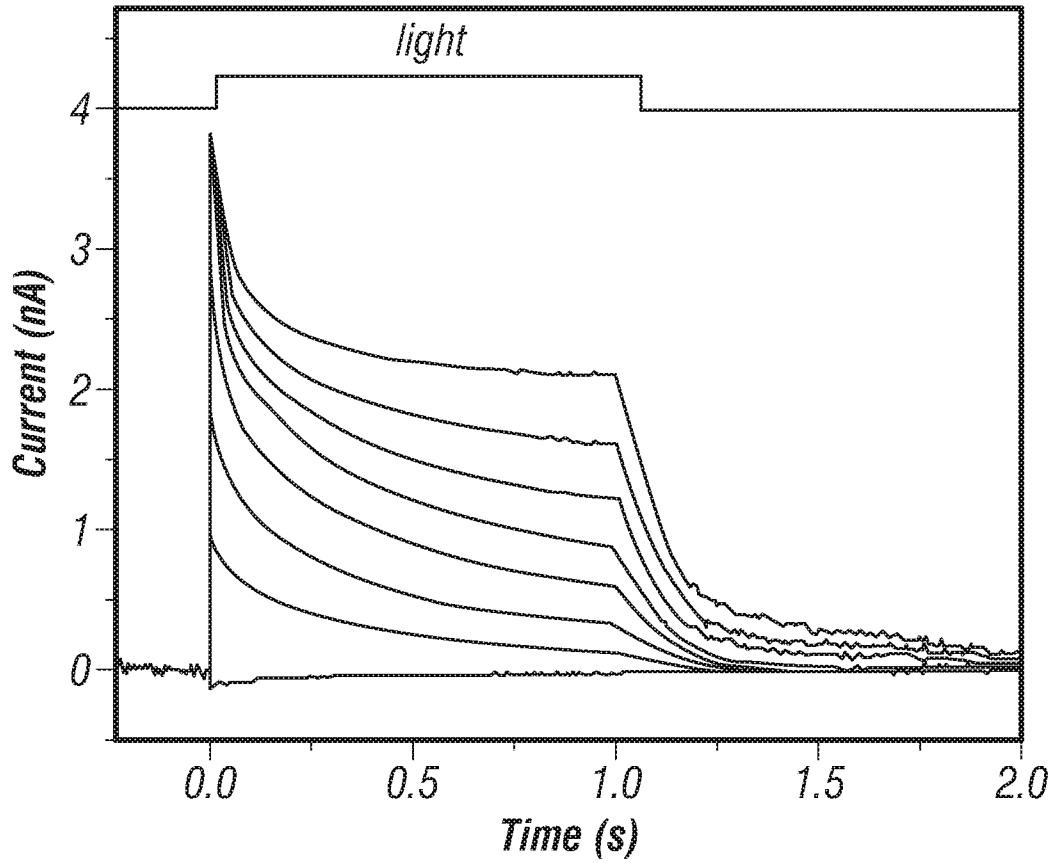
light
Current (nA)
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Time (s)

FIG. 4C

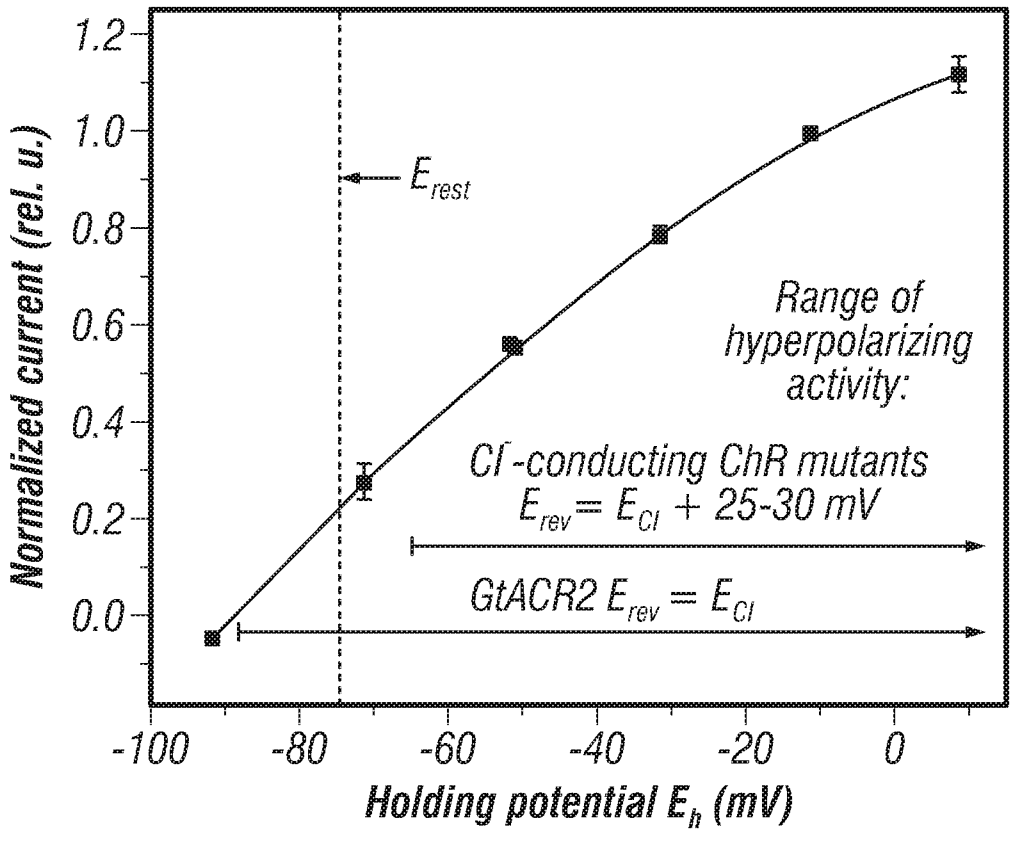
Normalized current (rel. u.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
E_{rest}
Range of hyperpolarizing activity:
Cl^--conducting ChR mutants
$E_{rev} = E_{Cl}$ + 25-30 mV
GtACR2 $E_{rev} = E_{Cl}$
-100
-80
-60
-40
-20
0
Holding potential E_h (mV)

FIG. 4D

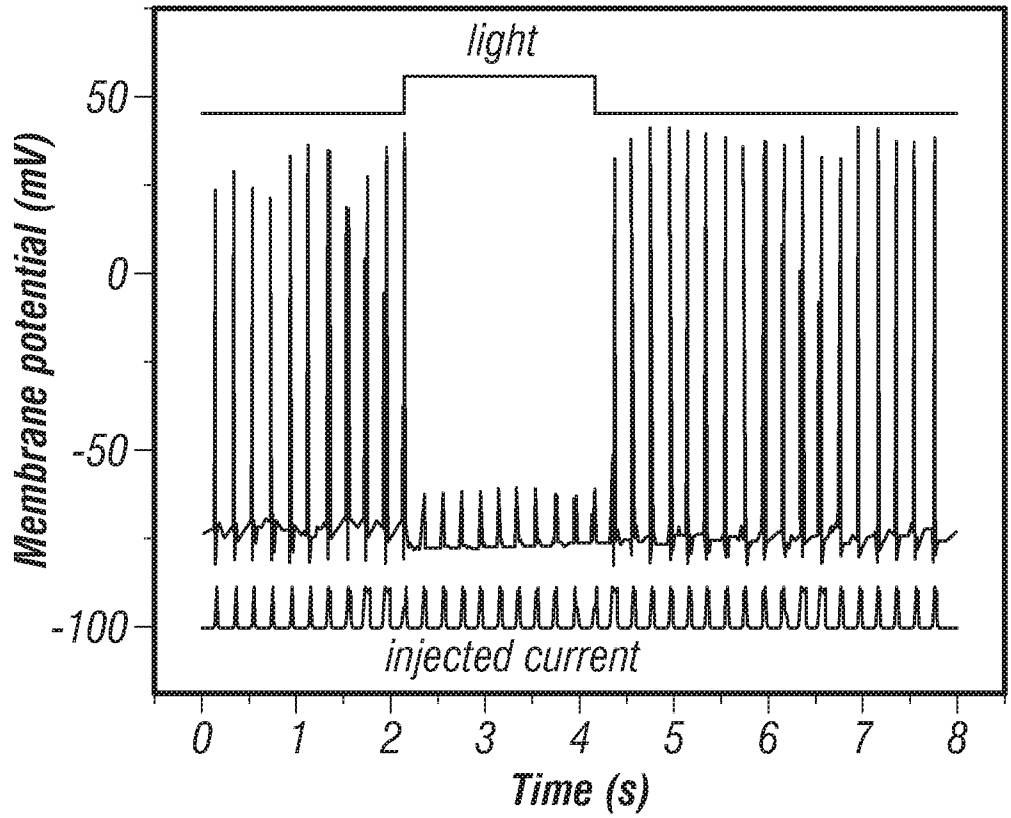
light
50
0
-50
-100
Membrane potential (mV)
injected current
0
1
2
3
4
5
6
7
8
Time (s)

FIG. 4E

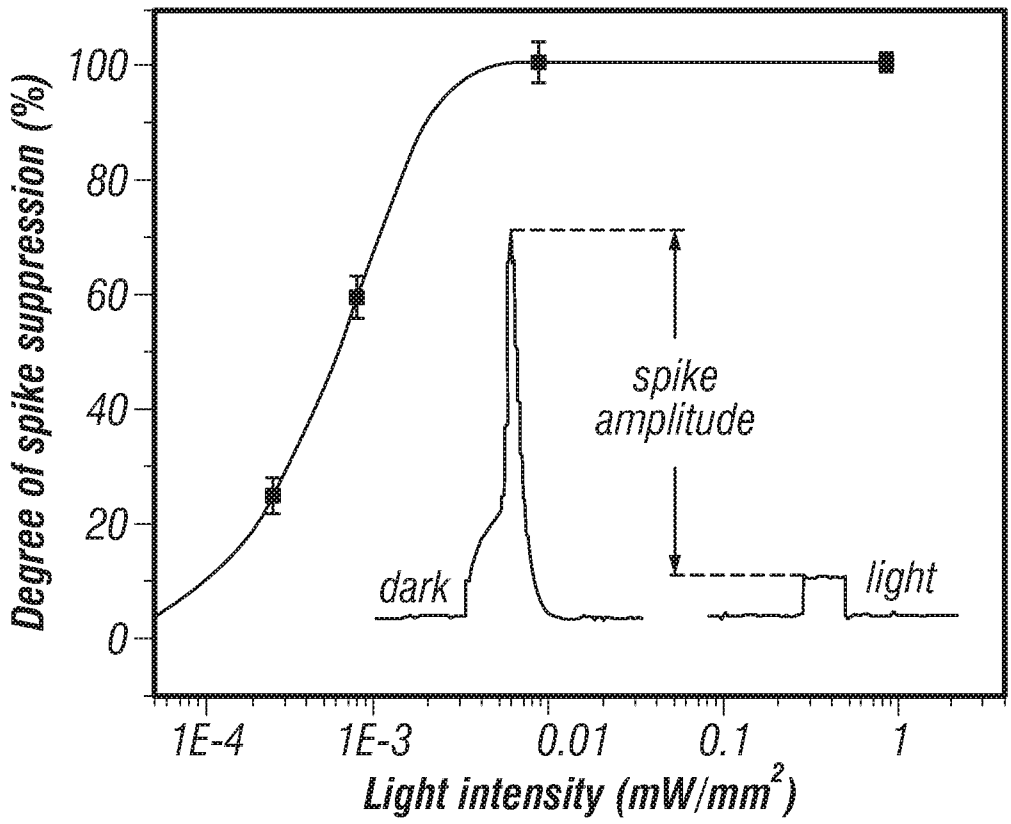
100
80
60
40
20
0
Degree of spike suppression (%)
spike
amplitude
dark
light
1E-4
1E-3
0.01
0.1
1
Light intensity (mW/mm²)

FIG. 4F

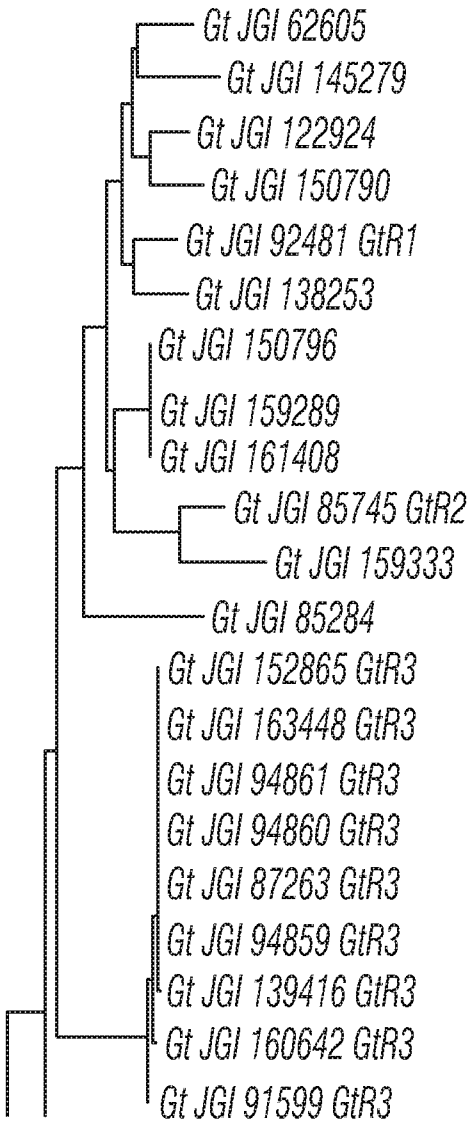
Gt JGI 62605
Gt JGI 145279
Gt JGI 122924
Gt JGI 150790
Gt JGI 92481 GtR1
Gt JGI 138253
Gt JGI 150796
Gt JGI 159289
Gt JGI 161408
Gt JGI 85745 GtR2
Gt JGI 159333
Gt JGI 85284
Gt JGI 152865 GtR3
Gt JGI 163448 GtR3
Gt JGI 94861 GtR3
Gt JGI 94860 GtR3
Gt JGI 87263 GtR3
Gt JGI 94859 GtR3
Gt JGI 139416 GtR3
Gt JGI 160642 GtR3
Gt JGI 91599 GtR3

FIG. 5

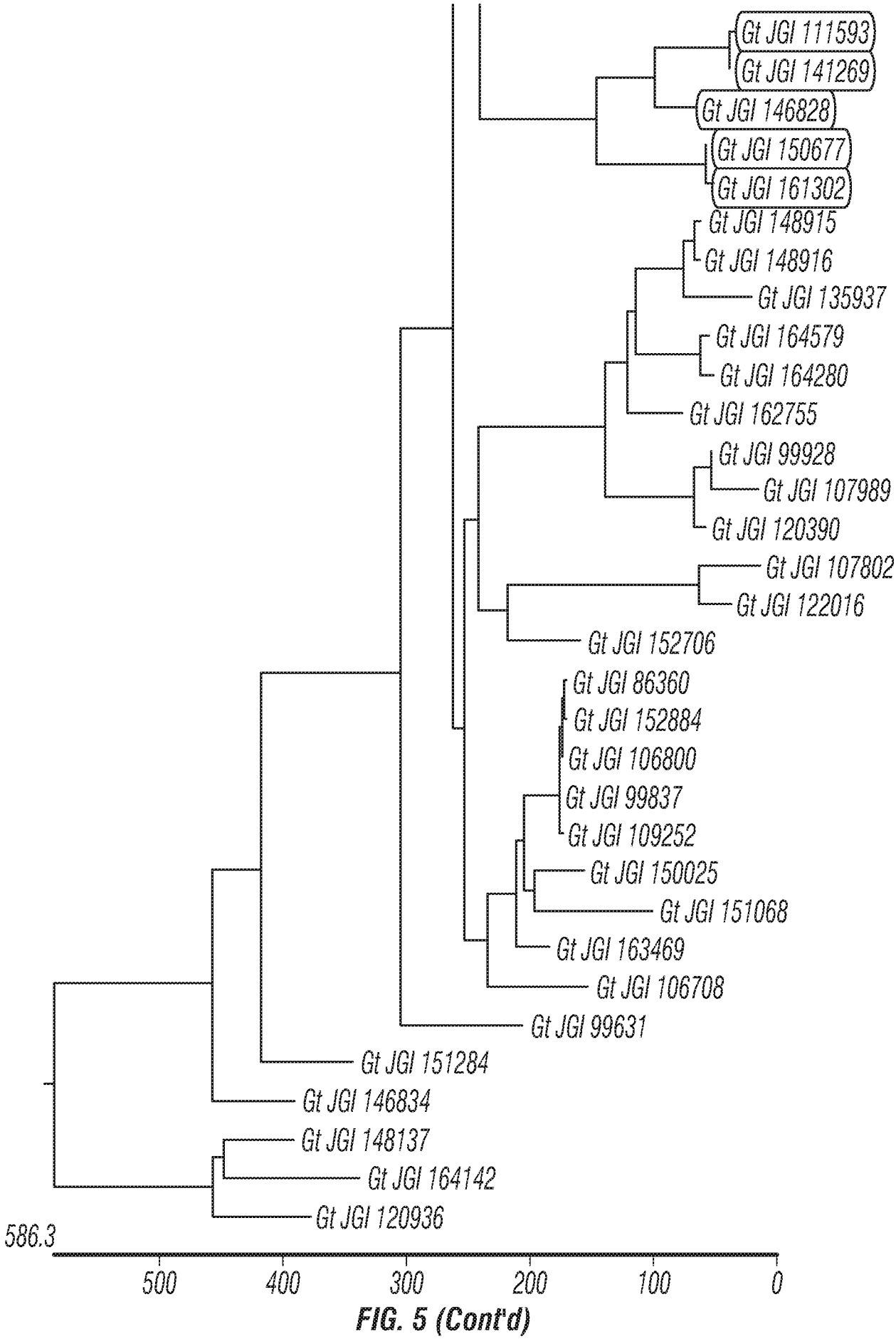
Gt JGI 111593
Gt JGI 141269
Gt JGI 146828
Gt JGI 150677
Gt JGI 161302
Gt JGI 148915
Gt JGI 148916
Gt JGI 135937
Gt JGI 164579
Gt JGI 164280
Gt JGI 162755
Gt JGI 99928
Gt JGI 107989
Gt JGI 120390
Gt JGI 107802
Gt JGI 122016
Gt JGI 152706
Gt JGI 86360
Gt JGI 152884
Gt JGI 106800
Gt JGI 99837
Gt JGI 109252
Gt JGI 150025
Gt JGI 151068
Gt JGI 163469
Gt JGI 106708
Gt JGI 99631
Gt JGI 151284
Gt JGI 146834
Gt JGI 148137
Gt JGI 164142
Gt JGI 120936
586.3
500
400
300
200
100
0

FIG. 5 (Cont'd)

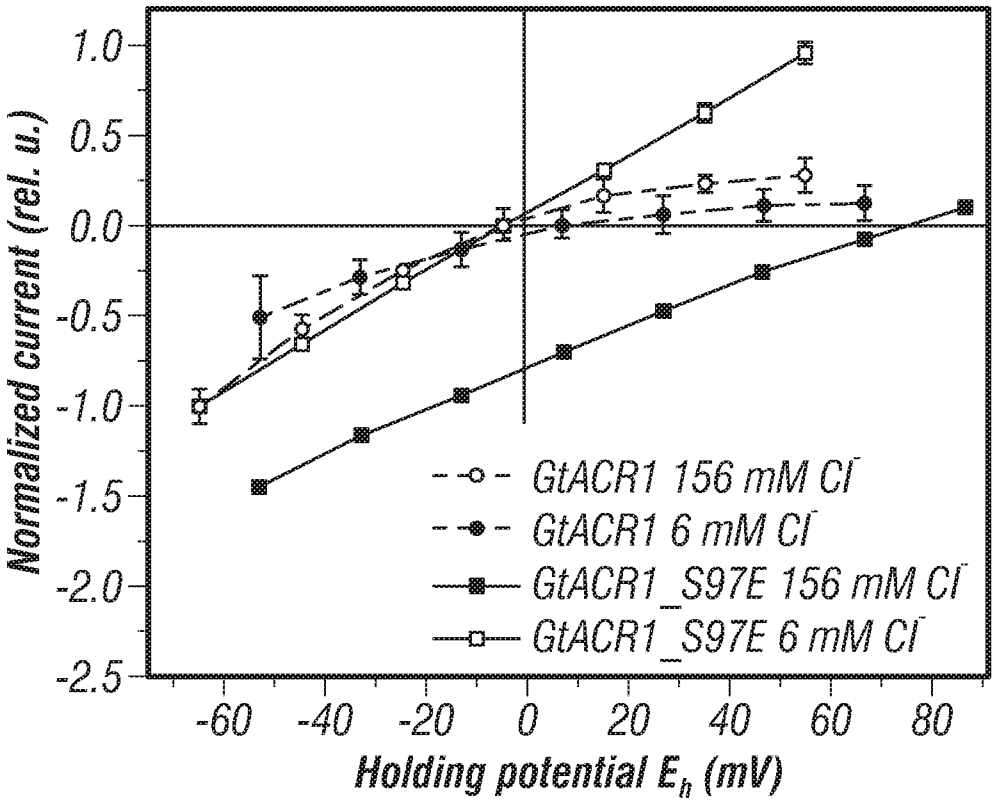
Normalized current (rel. u.)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-60
-40
-20
0
20
40
60
80
Holding potential E_h (mV)
GtACR1 156 mM Cl⁻
GtACR1 6 mM Cl⁻
GtACR1_S97E 156 mM Cl⁻
GtACR1_S97E 6 mM Cl⁻

FIG. 6A

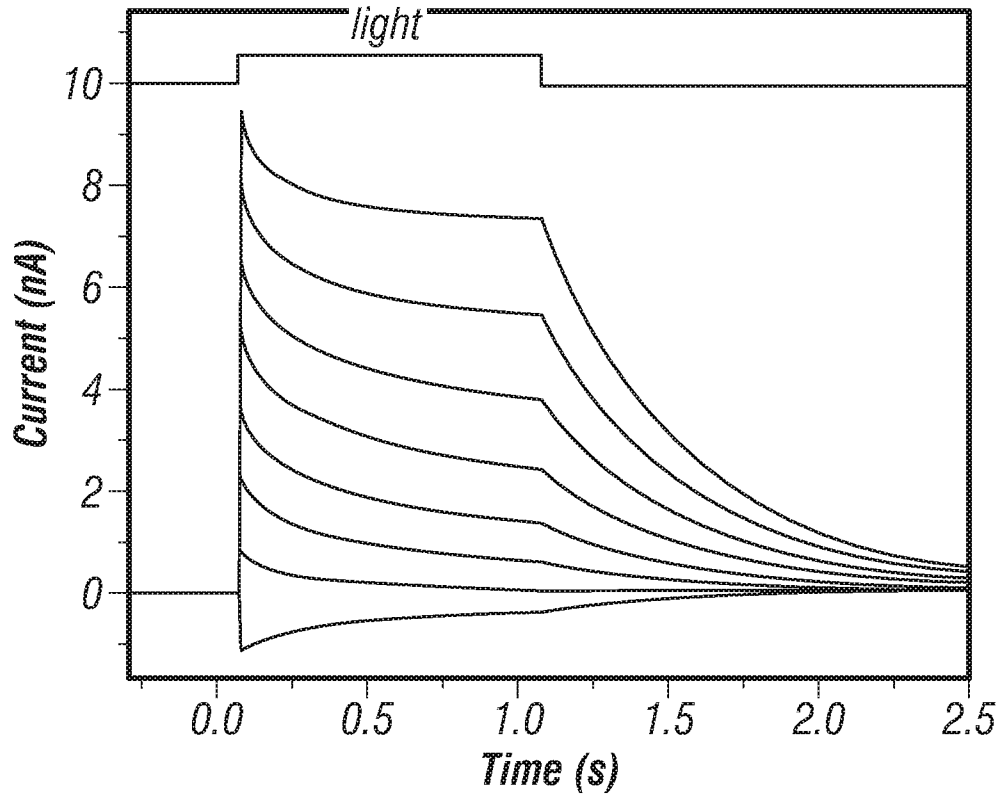
light
Current (nA)
10
8
6
4
2
0
0.0
0.5
1.0
1.5
2.0
2.5
Time (s)

FIG. 6B

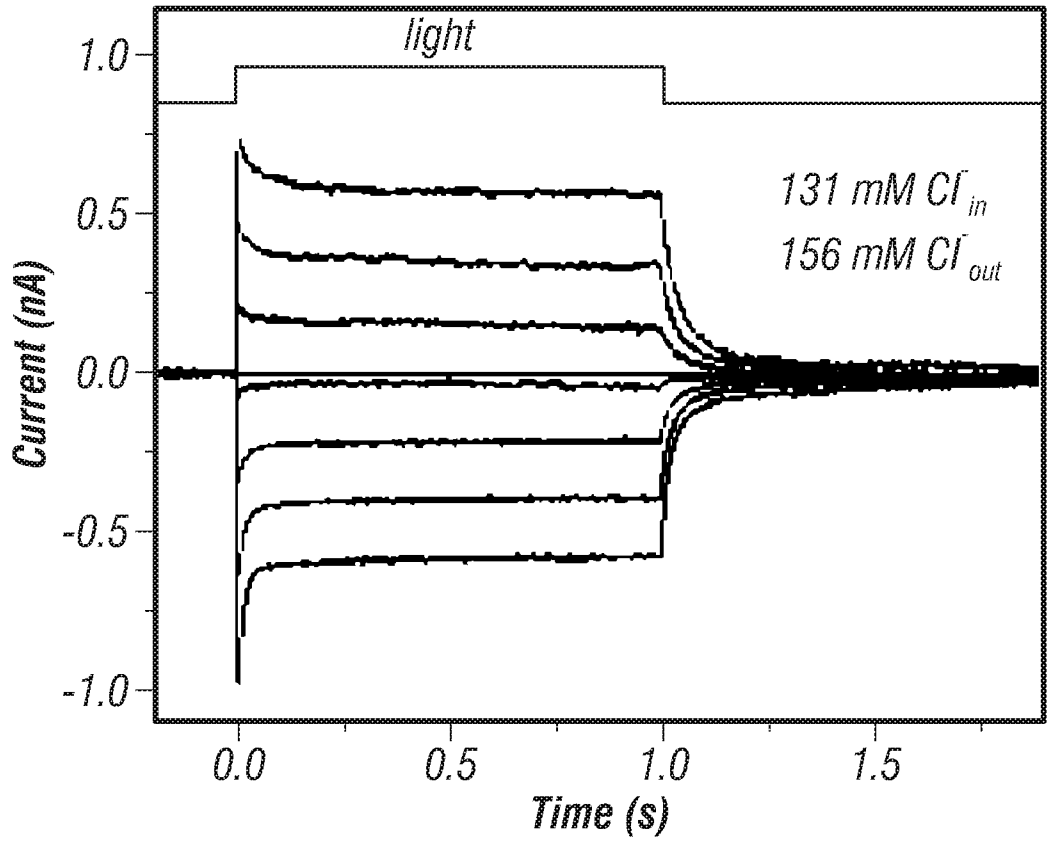
light
1.0
0.5
0.0
-0.5
-1.0
Current (nA)
131 mM Cl⁻ in
156 mM Cl⁻ out
0.0
0.5
1.0
1.5
Time (s)

FIG. 7A

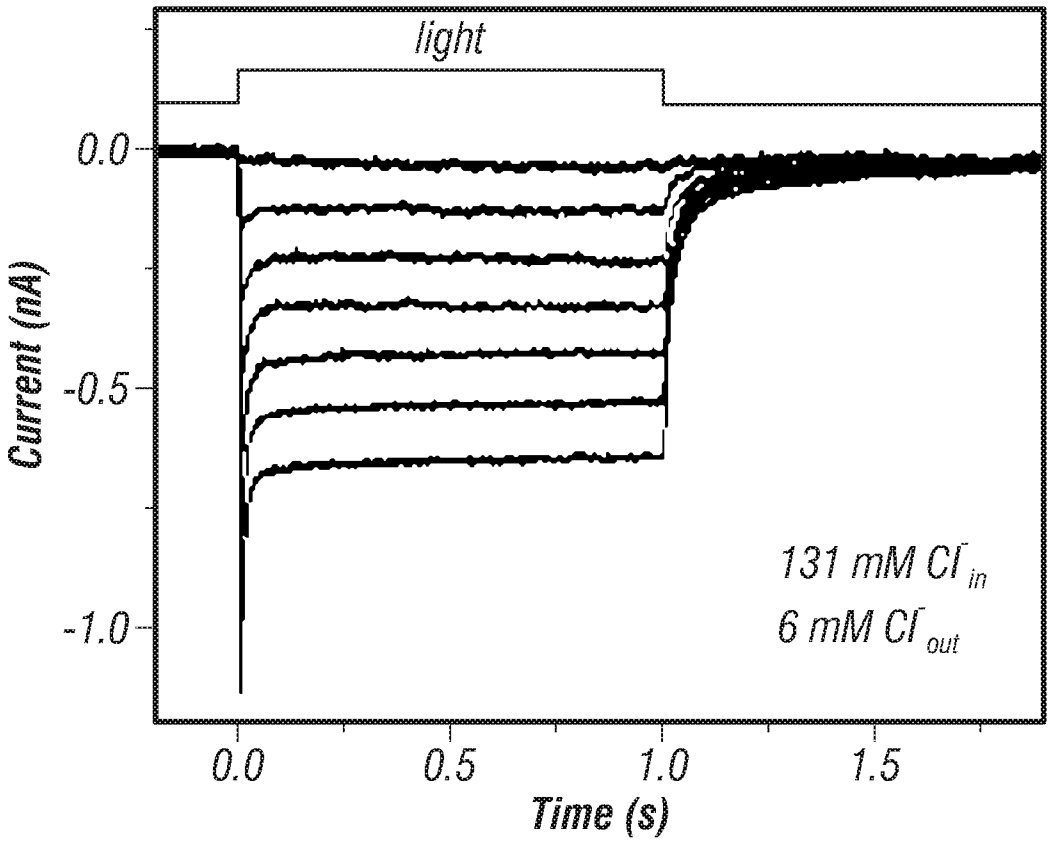
light
0.0
-0.5
-1.0
Current (nA)
131 mM Cl⁻ in
6 mM Cl⁻ out
0.0
0.5
1.0
1.5
Time (s)

FIG. 7B

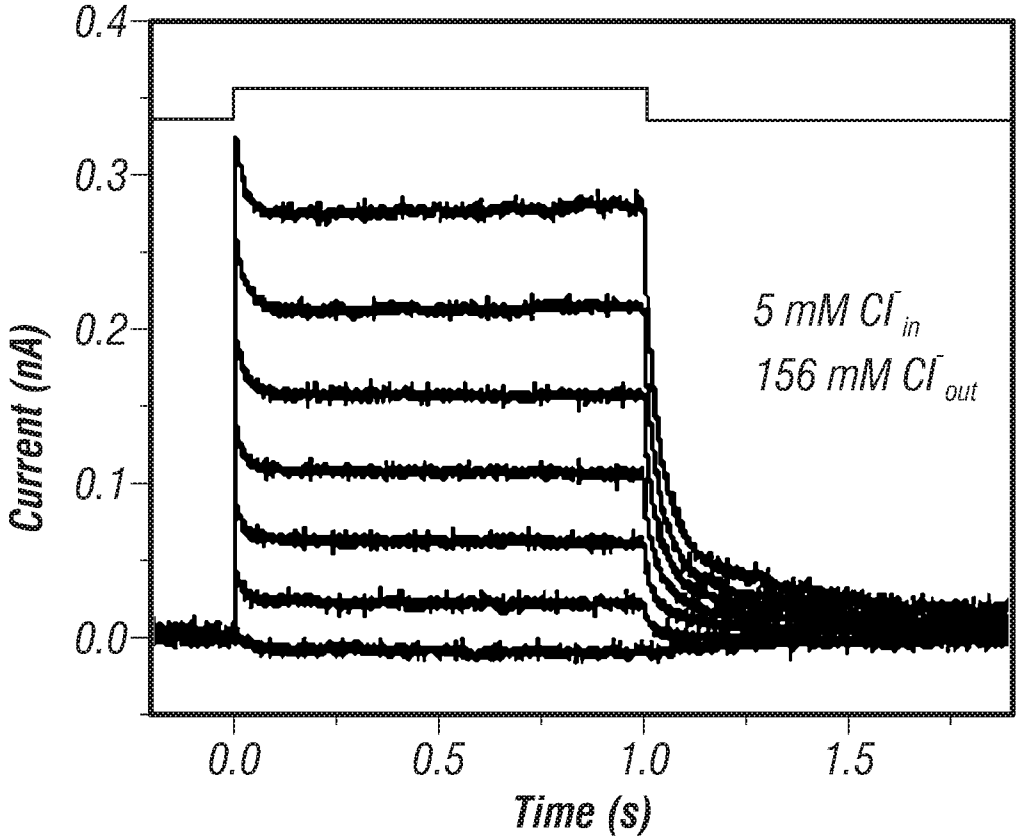
0.4
0.3
0.2
0.1
0.0
Current (nA)
5 mM Cl⁻ in
156 mM Cl⁻ out
0.0
0.5
1.0
1.5
Time (s)

FIG. 7C

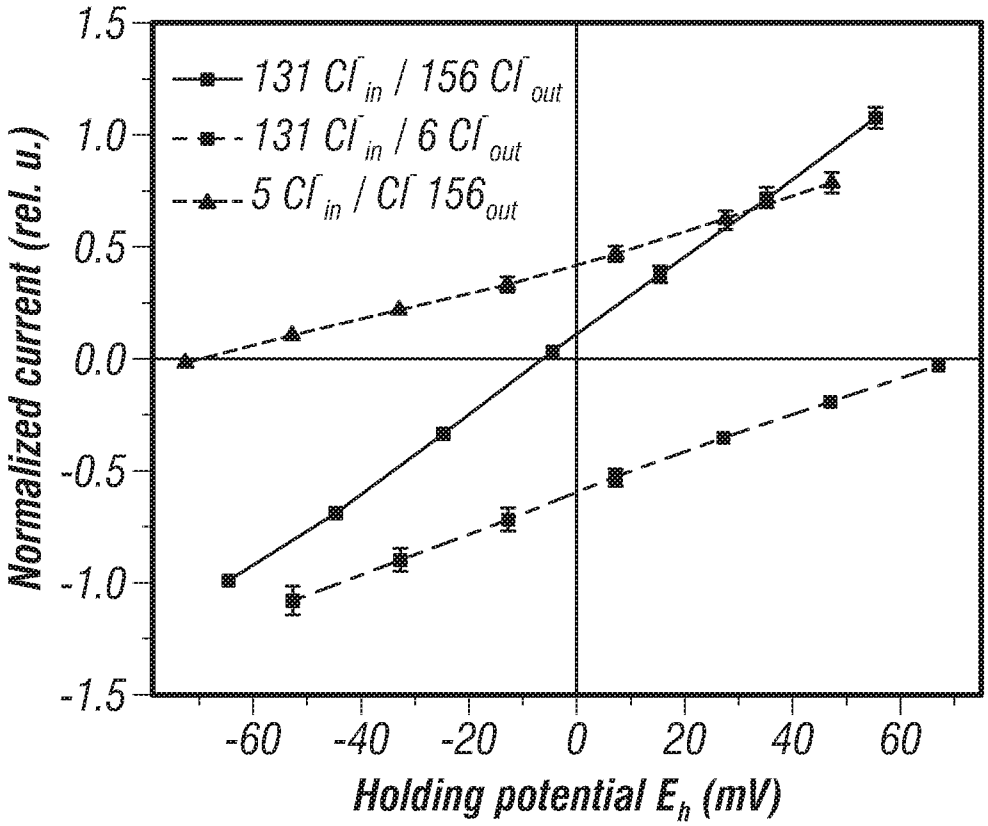
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Normalized current (rel. u.)
131 Cl⁻ in / 156 Cl⁻ out
131 Cl⁻ in / 6 Cl⁻ out
5 Cl⁻ in / Cl⁻ 156 out
-60
-40
-20
0
20
40
60
Holding potential Eh (mV)

FIG. 7D

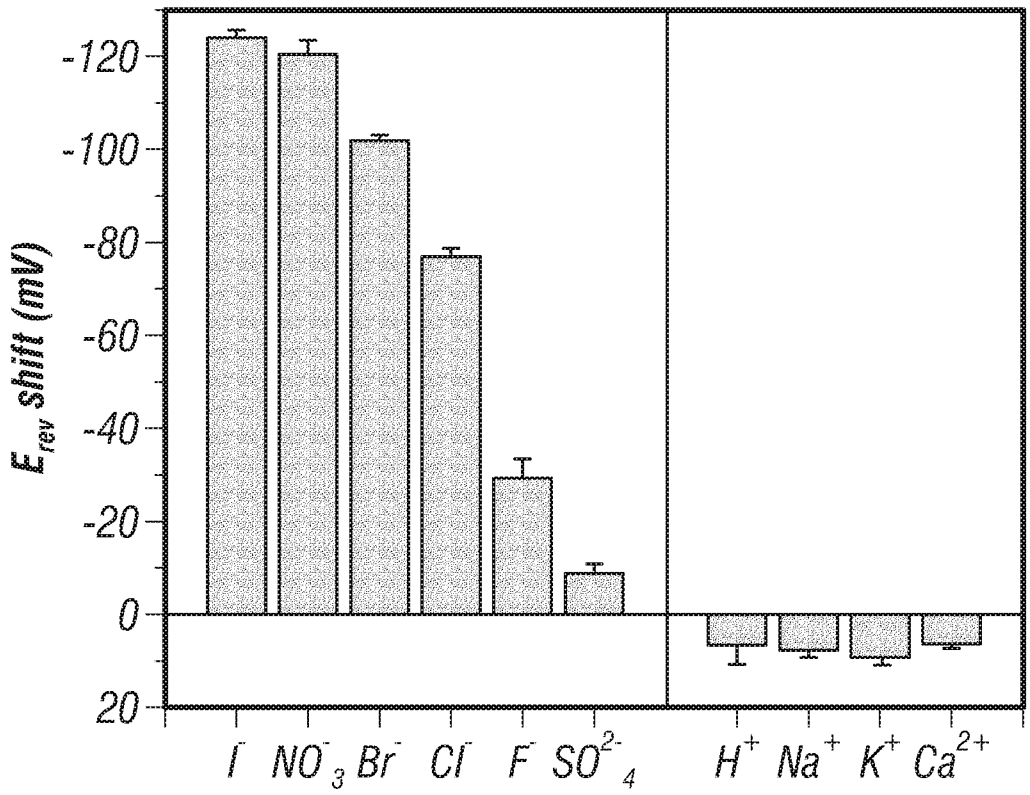
-120
-100
-80
-60
-40
-20
0
20
E_{rev} shift (mV)
I^- NO_3^- Br^- Cl^- F^- SO_4^{2-}
H^+ Na^+ K^+ Ca^{2+}

FIG. 8

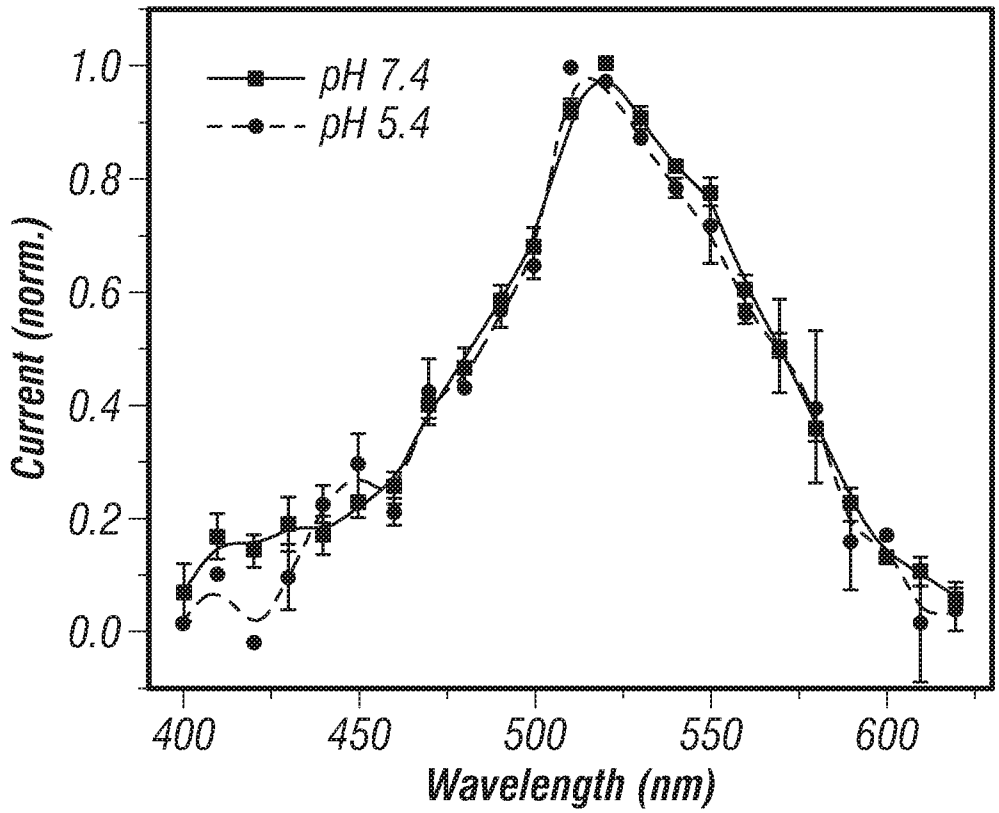
pH 7.4
pH 5.4
1.0
0.8
0.6
0.4
0.2
0.0
Current (norm.)
400
450
500
550
600
Wavelength (nm)

FIG. 9

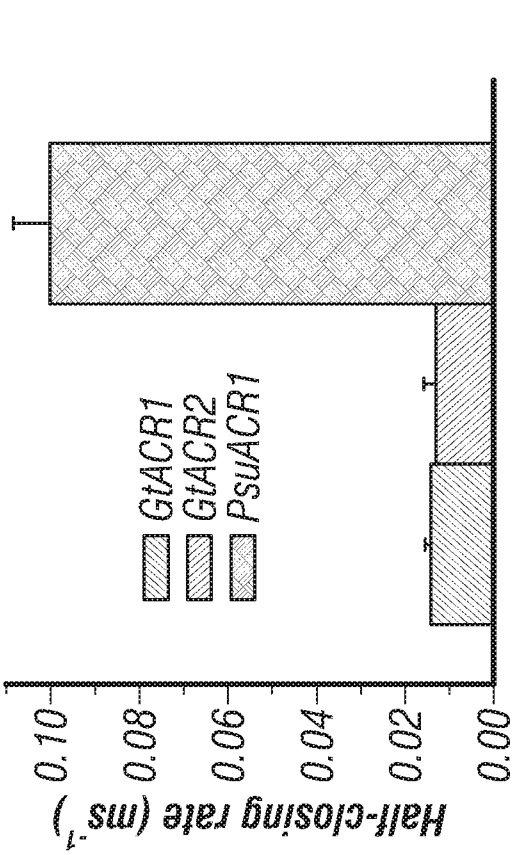

FIG. 10B

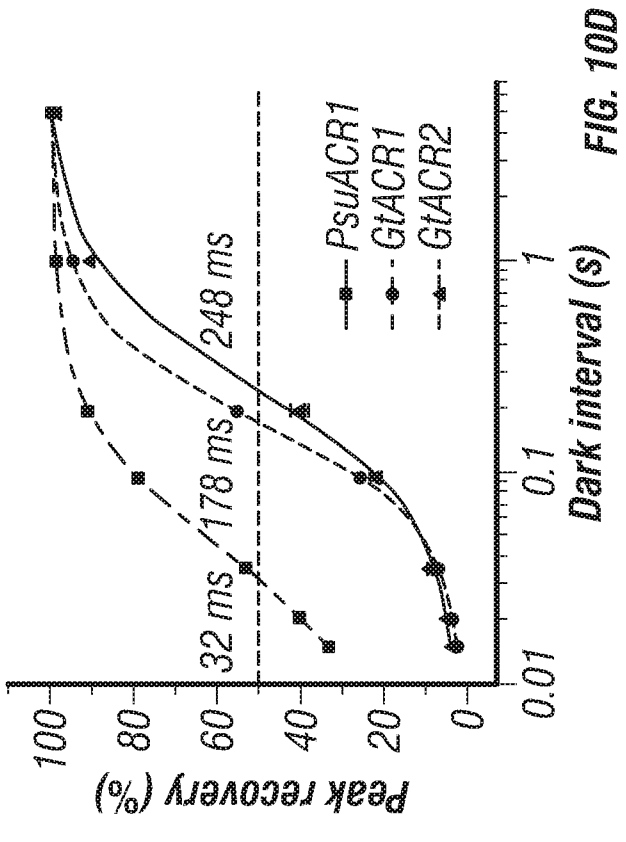

FIG. 10D

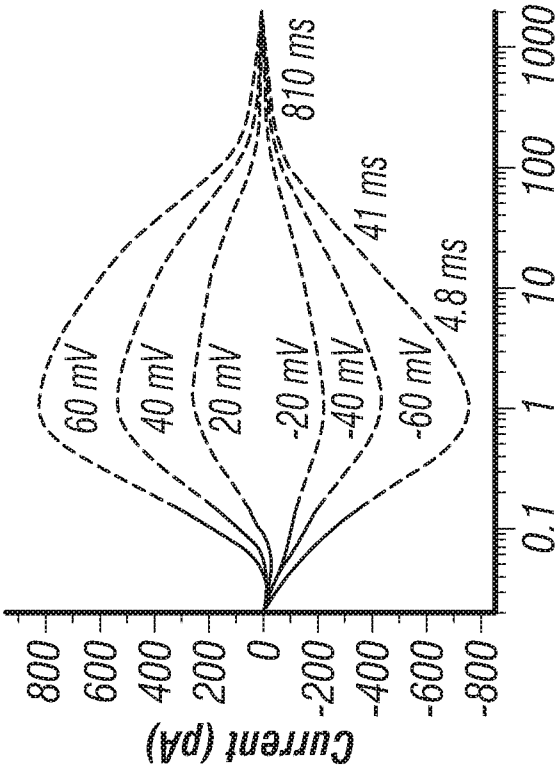

FIG. 10A

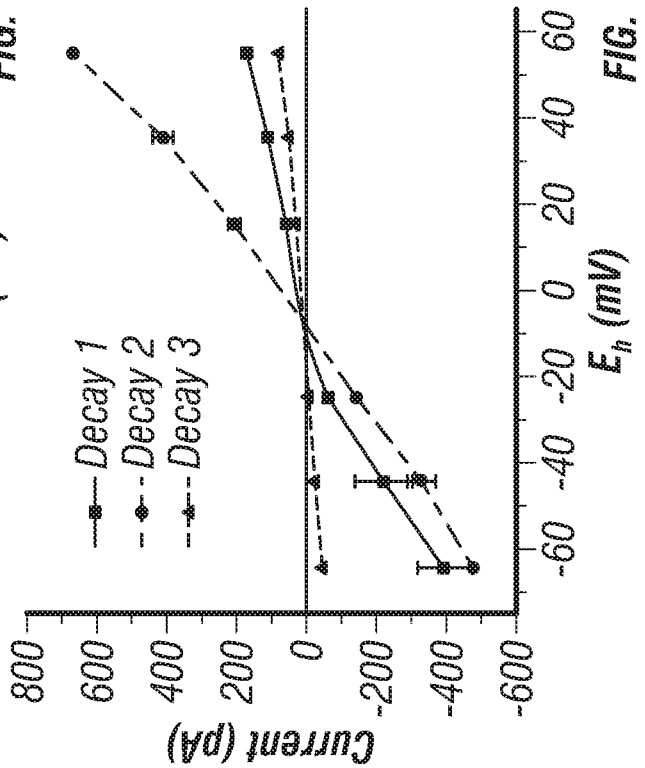

FIG. 10C

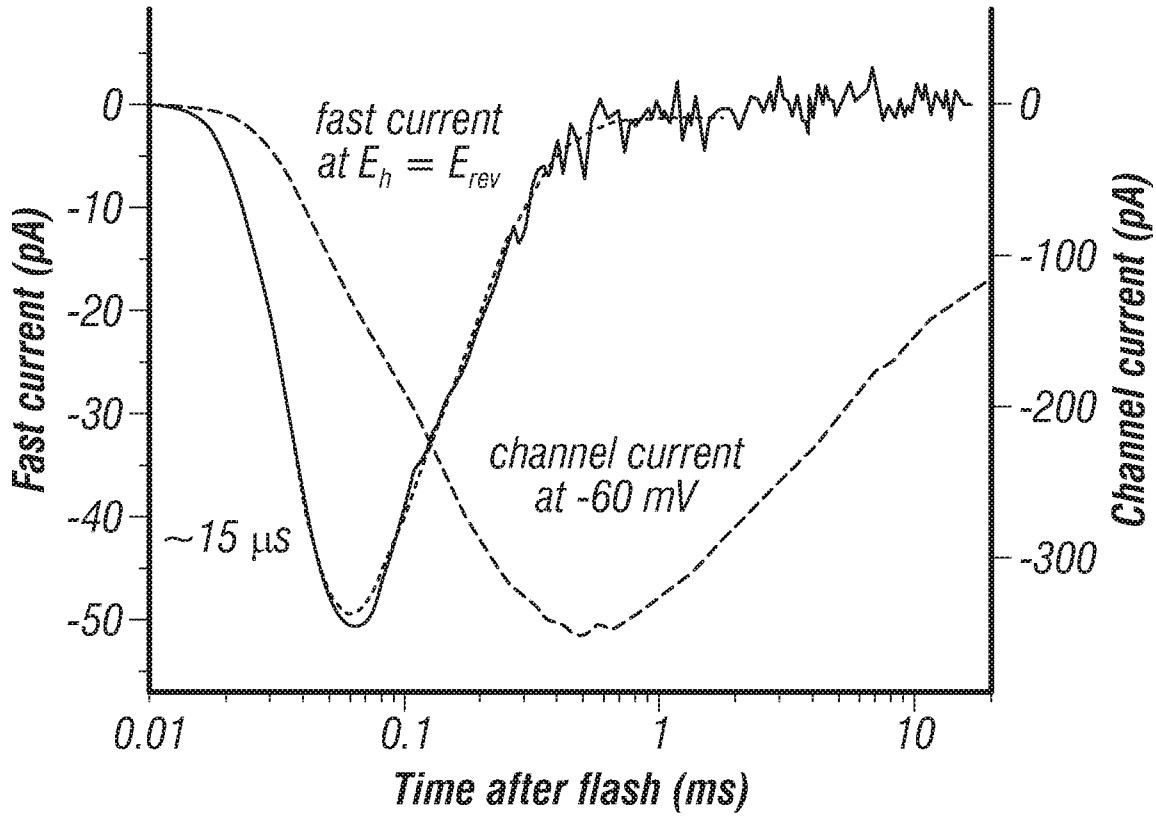

FIG. 11A

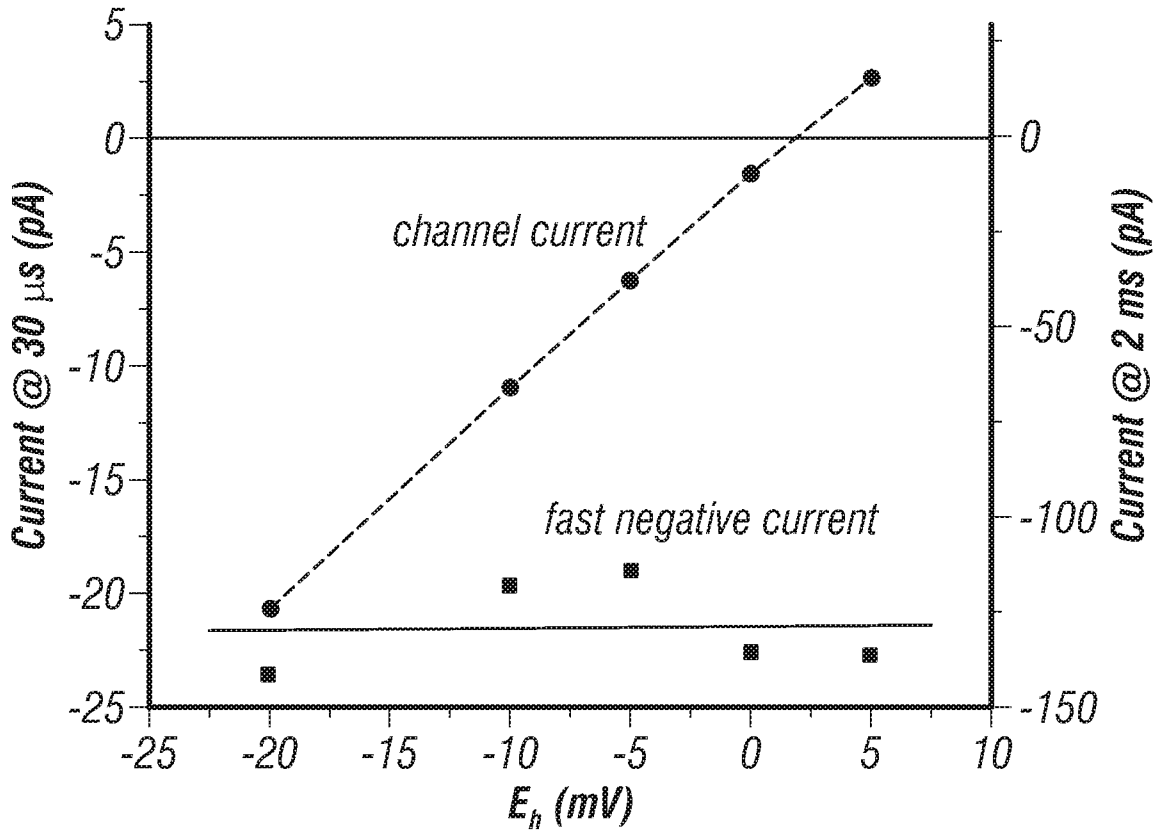

FIG. 11B

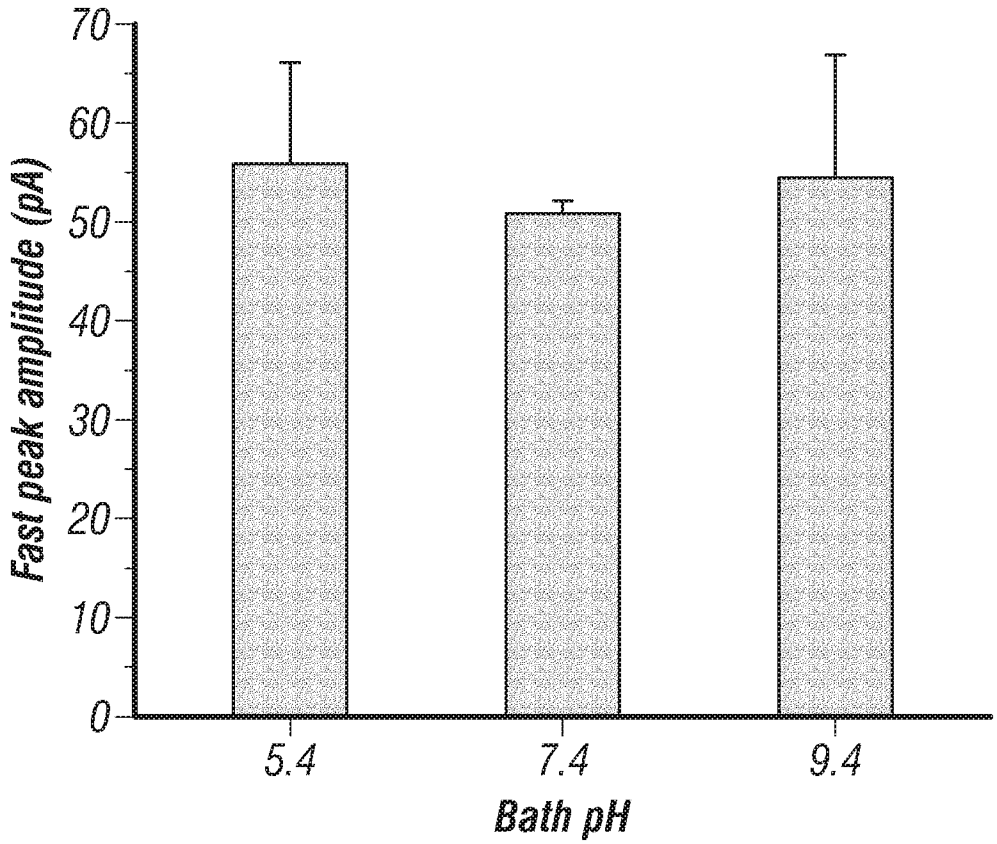

FIG. 11C

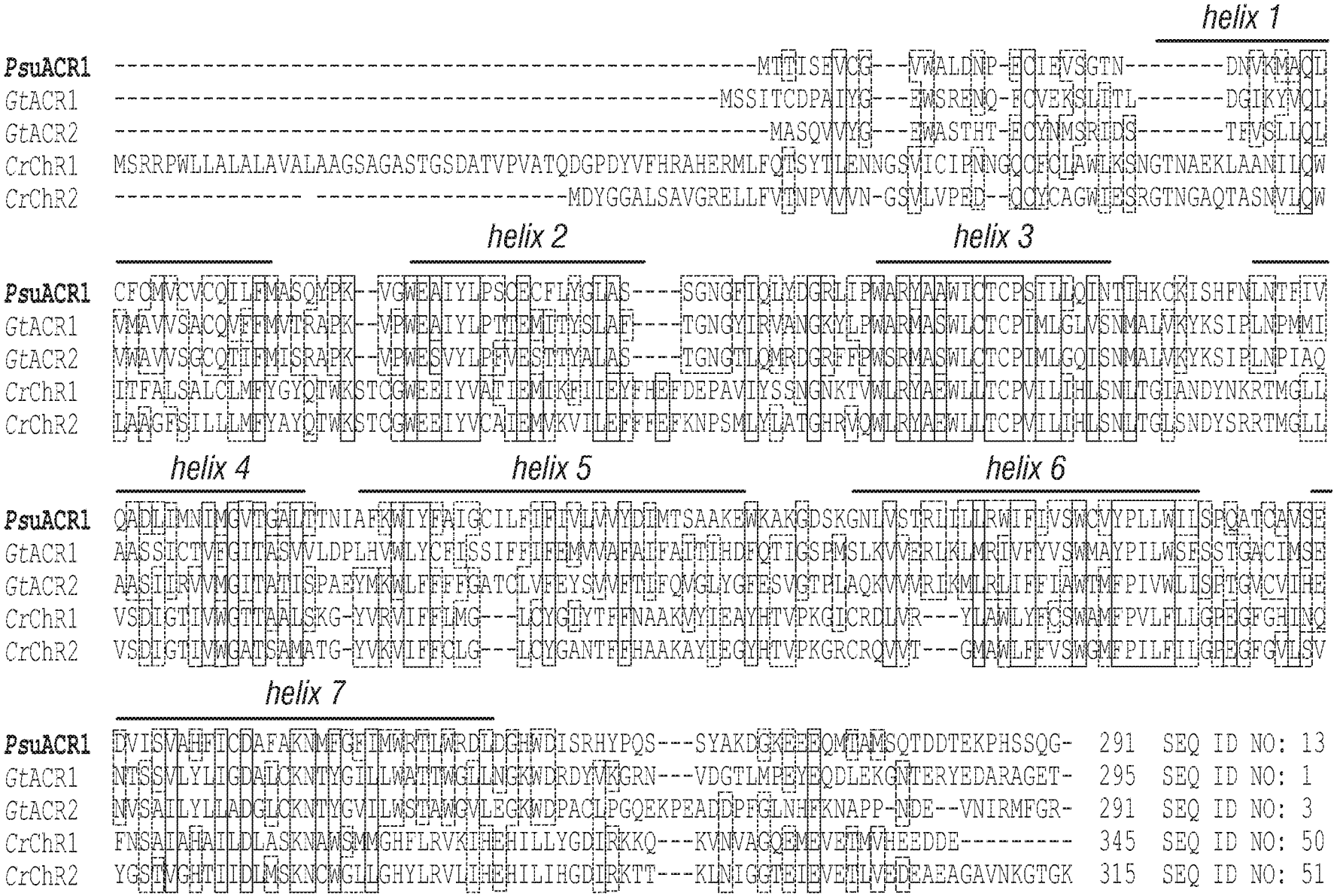
helix 1
helix 2
helix 3
helix 4
helix 5
helix 6
helix 7
PsuACR1
GtACR1
GtACR2
CrChR1
CrChR2
SEQ ID NO: 13
SEQ ID NO: 1
SEQ ID NO: 3
SEQ ID NO: 50
SEQ ID NO: 51

FIG. 12

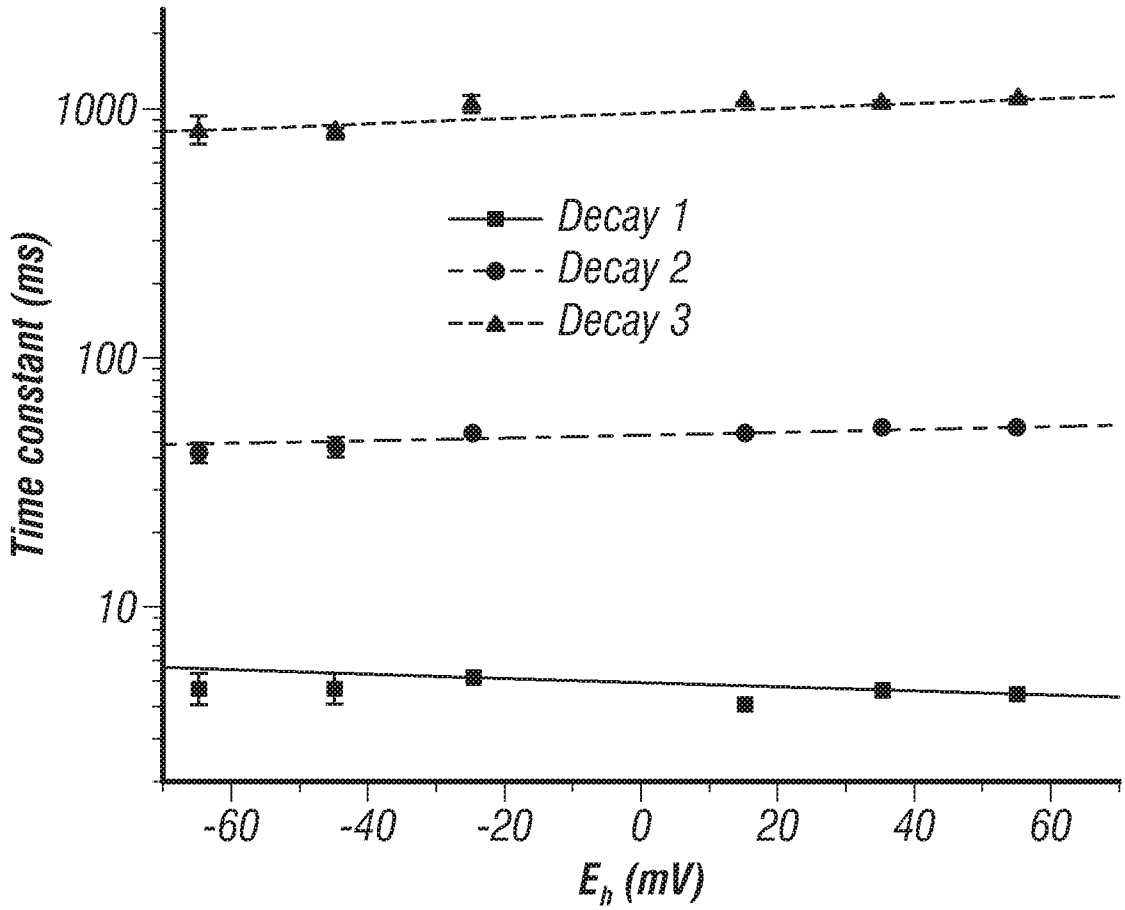
1000
100
10
Time constant (ms)
Decay 1
Decay 2
Decay 3
-60
-40
-20
0
20
40
60
E_h (mV)

FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/23095

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/405; C12N 13/00; A61N 5/06 (2016.01)
CPC - C07K 14/405; C12N 15/86, 5/87; A61N 5/0622
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C07K 14/405; C12N 13/00; A61N 5/06 (2016.01)
CPC: C07K 14/405; C12N 15/86, 5/87; A61N 5/0622

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C07K 14/405; C12N 15/86, 5/87; A61N 5/0622 (text search)
USPC: 435/455, 325, 320.1, 173.4; 536/23.7; 607/88 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Patents; Google Scholar
Search terms: Channelrhodopsin (ChR), microbial rhodopsin, anion channel, light gated channel, hyperpolarization, cation channel, algae (e.g. Guillardia), optogenetic%, vector, expression, polynucleotide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y -- A	BERNDT et al. Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. Science 25 April 2014 Vol 344 No 6182 Pages 420-424 and online Supplemental Material [Available on the internet: <URL: http: www.sciencemag.org/content/344/6182/420/suppl/DC1>]. Especially abstract, online Supplemental Materials.	1-4, 10, 11, 20, 22 ---------- 16, 21
Y -- A	Uniprot Accession L1J207. Uncharacterized Protein (online) 06 March 2013 retrieved 30 May 2015] [Available on the internet: http://www.uniprot.org/uniprot/L1J207]. Especially pg 1-3.	1-4, 10, 11, 20, 22 ---------- 16, 21
A	GenBank Accession KF992074. Synthetic construct PsChR1 gene (online) 29 January 2014 [retrieved 4 June 2016]. Available on the Internet: <URL: http://www.ncbi.nlm.nih.gov/nuccore/KF992074>. Especially pg 1-2.	16, 21
A,P	Govorunova et al. Natural light-gated anion channels: A family of microbial rhodopsins for advanced optogenetics. Science 07 August 2015 Vol 349 No 6248 Pages 647-650. entire article.	1-4, 10, 11, 20, 22
A, P	GenBank Accession KP171708.1. Synthetic construct ACR1 gene, partial cds. (online) 01 July 2015 [retrieved 04 June 2016] Available on the internet: <URL: http://www.ncbi.nlm.nih.gov/nuccore/KP171708>. Especially pg 1-2.	16, 21

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 August 2016 (01.08.2016)	22 AUG 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/23095

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
GenCore ver 6.4.1 SEQ ID NOs: 1, 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/23095

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----Go to Extra Sheet for continuation-------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 10,11, 16, 20-22, limited to SEQ ID NOs: 1-2 and human host cell

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/23095

-------continued from Box III (Lack of Unity of Invention)---------

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-11, 16, 20-22, 26-40, drawn to a composition comprising an isolated nucleic acid molecule comprising a sequence encoding an anion conducting channelrhodopsin that may be linked to a heterologous promoter sequence.
The nucleic acid composition or peptide encoded by the nucleic acid will be searched to the extent that the peptide encodes an amino acid sequence with at least 85% sequence identity to the first named sequence SEQ ID NO: 1 (claim 1) or the nucleotide encoding said peptide sequence, SEQ ID NO: 2 (claim 1), and the first named recombinant host cell, an isolated human cell (claim 3). It is believed that claims 1-4, 10, 11, 16, 20-22 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NOs: 1-2; and an isolated human cell. Additional nucleic acids and amino acid sequences of a channelrhodopsin expressed in a selected host cell will be searched upon payment of additional fees [see instant Specification para [0041-0064] for the description of sequences.]. Applicant must specify the claims that encompass any additional elected nucleic acids, peptides, and host cells . Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: nucleic acid sequence SEQ ID NO: 22 (encodes Mesostigma viride channelrhodopsin 1 ((MvChR1)) in an isolated human host cell (claims 26-33, 39, 40).

Group II: Claims 12-15, 17-19, 23-25, 41-56, drawn to a method of membrane hyperpolarization or neuronal silencing.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ has the special technical feature of a composition comprising a nucleic acid or peptide encoded by said nucleic acid encoding an anionic channelrhodopsin, not required by Group II.

Group II has the special technical feature of specific method steps to cause membrane hyperpolarization or neuronal silencing in a cell or a cell of a subject, not required by Group I+.

Among the inventions listed as Groups I+ is the specific nucleic acid and amino acid sequences, and recombinant host cell, recited therein. Each of the inventions of Group I+ requires a unique nucleic acid and amino acid sequences and host cell, not required by the other inventions.

Common Technical Feature:

Groups I+ and II share the common technical feature of a polynucleotide that encodes an amino acid sequence of a rhodopsin domain of an anion-conducting channelrhodopsin linked to a heterologous promoter sequence and expressed in a host cell.

However, said common technical feature does not represent a contribution over the prior art, and is obvious the publication titled "Structure-guided transformation of channelrhodopsin into a light-activated chloride channel" by BERNDT et al. (hereinafter "Berndt") [published 25 April 2014 in Science Vol 344 No 6182 Pages 420-424, with online supplemental material available at www.sciencemag.org/content/344/6182/420/suppl/DC1], in view of Accession L1J207 by Uniprot (hereinafter "Uniprot") (online) 6 March 2013 [retrieved 30 May 2015] [Available on the internet: http://www.uniprot.org/uniprot/L1J207].

As to the common technical feature and claim 1, Berndt teaches a polynucleotide that encodes an amino acid sequence at least 90% identical to a rhodopsin domain of an anion-conducting channelrhodopsin expressible in said cell using a heterologous promoter sequence (abstract; we designed and characterized a class of channelrhodopsins (originally cation-conducting) converted into chloride-conducting anion channels. These tools enable fast optical inhibition of action potentials and can be engineered to display step-function kinetics for stable inhibition, outlasting light pulses and for orders-of-magnitude-greater light sensitivity of inhibited cells; Supplementary Material and Methods, pg 2, para 2, Point mutagenesis of C1C2.....We used AAV vectors bearing the CaMKII alpha promoter for protein expression in neurons, and a pcDNA3.1 vector bearing the CMV promoter for expression in HEK cells"). In addition, Uniprot entry L1J207 (hereinafter 'L1J207) (06 March 2013) [retrieved on 30 May 2016 from http://www.uniprot.org/uniprot/L1J207] teaches a microbial rhodopsin peptide sequence (pg 1; "the ion-translocating microbial rhodopsin (mr) family") having 100% sequence identity with instant SEQ ID NO: 1 (pg 2-3; AA 1-295 100% sequence identity).

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+ and II lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note concerning claim 15: Claim 15 is interpreted to depend from 12, not clam 11 as written, because claim 11 is a dependent composition claim.

Note concerning claim 21: Claim 21, a composition claim, is written to depend from method claim 19, and is more appropriately dependent on composition claim 20, and is interpreted as such.

Note concerning claim 22: Claim 22, a composition claim, is written to depend from method claim 19, and is more appropriately dependent on composition claim 20, and is interpreted as such.