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(54) Title: LIGHT-ACTIVATED CHIMERIC OPSINS AND METHODS OF USING THE SAME

(57) Abstract: Provided herein are compositions comprising light-activated chimeric proteins expressed on plasma membranes and methods of using the same to selectively depolarize excitatory or inhibitory neurons.

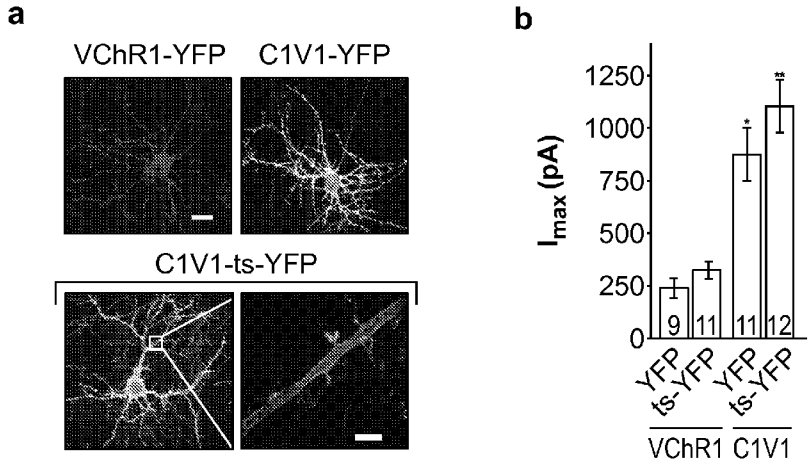

FIG. 1

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LIGHT-ACTIVATED CHIMERIC OPSINS AND METHODS OF USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application Nos. 61/410,736 filed on November 5, 2010; 61/410,744 filed on November 5, 2010; and 61/511,912 filed on July 26, 2011, the disclosures of each of which are incorporated herein by reference in their entireties.

TECHNICAL FIELD

This application pertains to compositions comprising animal cells expressing light-activated chimeric proteins on their plasma membranes and methods of using the same to selectively depolarize excitatory or inhibitory neurons residing in the same microcircuit in the pre-frontal cortex.

BACKGROUND

The neurophysiological substrates of most psychiatric disorders are poorly understood, despite rapidly emerging information on genetic factors that are associated with complex behavioral phenotypes such as those observed in autism and schizophrenia (Cichonet al., *The American Journal of Psychiatry* 166(5):540 (2009); O'Donovan et al., *Human Genetics* 126(1): 3 (2009)). One remarkable emerging principle is that a very broad range of seemingly unrelated genetic abnormalities can give rise to the same class of psychiatric phenotype (such as social behavior dysfunction; Folstein & Rosen-Sheidley, *Nature Reviews* 2(12):943 (2001)). This surprising pattern has pointed to the need to identify simplifying circuit-level insights that could unify diverse genetic factors under a common pathophysiological principle.

One such circuit-level hypothesis is that elevation in the ratio of cortical cellular excitation and inhibition (cellular E/I balance) could give rise to the social and cognitive deficits of autism (Rubenstein, *Current Opinion in Neurology* 23(2):118; Rubenstein & Merzenich, *Genes, Brain, and Behavior* 2(5):255 (2003)). This hypothesis could potentially unify diverse streams of pathophysiological evidence, including the observation that many autism-related genes are linked to gain-of-function phenotypes in ion channels and synaptic proteins (Bourgeron, *Current Opinion in Neurobiology* 19 (2), 231 (2009)) and that ~30% of autistic patients also show clinically apparent seizures (Gillberg & Billstedt, *Acta Psychiatrica Scandinavica*, 102(5):321 (2000)). However, it

has not been clear if such an imbalance (to be relevant to disease symptoms) would be operative on the chronic (e.g. during development) or the acute timescale. Furthermore, this hypothesis is by no means universally accepted, in part because it has not yet been susceptible to direct testing. Pharmacological and electrical interventions lack the necessary specificity to selectively favor activity (in a manner fundamentally distinct from receptor modulation) of neocortical excitatory cells over inhibitory cells, whether in the clinical setting or in freely behaving experimental mammals during social and cognitive tasks. It is perhaps related to challenges such as this that the social and cognitive deficits of autism and schizophrenia have proven largely unresponsive to conventional psychopharmacology treatments in the clinic.

Optogenetics is the combination of genetic and optical methods used to control specific events in targeted cells of living tissue, even within freely moving mammals and other animals, with the temporal precision (millisecond-timescale) needed to keep pace with functioning intact biological systems. The hallmark of optogenetics is the introduction of fast light-activated channel proteins to the plasma membranes of target neuronal cells that allow temporally precise manipulation of neuronal membrane potential while maintaining cell-type resolution through the use of specific targeting mechanisms. Among the microbial opsins which can be used to investigate the function of neural systems are the channelrhodopsins (ChR2, ChR1, VChR1, and SFOs) used to promote depolarization in response to light. In just a few short years, the field of optogenetics has furthered the fundamental scientific understanding of how specific cell types contribute to the function of biological tissues such as neural circuits *in vivo*. Moreover, on the clinical side, optogenetics-driven research has led to insights into Parkinson's disease and other neurological and psychiatric disorders.

However, there are limitations to existing optogenetic tools for exploring the hypothesis that elevation in the ratio of cortical E/I balance might be associated with the social and cognitive deficits of autism and other disorders such as schizophrenia. Conventional channelrhodopsin photocurrents display significant desensitization which precludes the generation of step-like changes in E/I balance (instead requiring ramping or pulsing, which would not be suitable for investigation of stable changes in cellular E/I balance); moreover, both SFOs and conventional ChRs are driven by blue light, which precludes within-preparation comparison of the effects of driving different populations of circuit elements (such as excitatory and inhibitory neurons). Therefore, what is needed is a tool that would allow the manipulation of cortical E/I balances and the monitoring of

gamma oscillations in cortical slices to permit the investigation of how these manipulations affect downstream neurons residing in the same microcircuit in the pre-frontal cortex.

BRIEF SUMMARY OF THE INVENTION

Provided herein are compositions comprising chimeric light-activated protein cation channels which are capable of mediating a depolarizing current in the cell when the cell is illuminated with light.

Provided herein are animal cells comprising a light-activated protein expressed on the cell membrane, wherein the protein is (a) a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1; (b) is responsive to light; and (c) is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In some embodiments, the cells are isolated or in a cell culture medium.

Also provided herein is a population of cells comprising the cell expressing the chimeric protein described herein on the cell membrane. Also provided herein are non-human animals and brain tissue slices comprising a cell expressing the chimeric protein described herein on the cell membrane.

Provided herein are polynucleotide comprising a nucleotide sequence encoding a light activated protein expressed on the cell membrane, wherein the protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1; is responsive to light; and is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. Vectors (such as expressing vectors) comprising the polynucleotides are also provided. In some embodiments, the expression vector is a viral vector (e.g., an AAV vector, a retroviral vector, an adenoviral vector, a HSV vector, or a lentiviral vector).

Also provided herein are methods of using the animal cells expressing the chimeric protein described herein on the cell membrane, the methods comprise activating the chimeric protein with light.

Also provided herein are methods of selectively depolarizing excitatory or inhibitory neurons residing in the same microcircuit, the methods comprising: selectively depolarizing an excitatory neuron comprising a first light-activated protein, wherein the first light activated protein is depolarized when exposed to light having a first wavelength; or selectively depolarizing an inhibitory neuron comprising a second light-activated protein, wherein the second light activated protein is depolarized when exposed to light having a second wavelength.

In some embodiments, the first or the second light activated protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1. In some embodiments, wherein the first light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO: 1, 3, 5, or 7, and wherein the second light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:11, 12, 13, or 14.

A method of selectively depolarizing excitatory or inhibitory neurons residing in the same microcircuit, the method comprising: expressing a first light-activated protein in an excitatory neuron; and expressing a second light activated protein in an inhibitory neuron, wherein the first light activated protein is independently depolarized when exposed to light having a first wavelength and wherein the second light activated protein is independently depolarized when exposed to light having a second wavelength. In some embodiments, the first or the second light activated protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1. In some embodiments, the first light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO: 1, 3, 5, or 7, and wherein the second light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:11, 12, 13, or 14.

Also provided herein are methods for identifying a chemical compound that selectively inhibits the depolarization of excitatory or inhibitory neurons residing in the same microcircuit, the method comprising:(a) selectively depolarizing an excitatory neuron comprising a first light-activated protein with light having a first wavelength or selectively depolarizing an inhibitory neuron comprising a second light-activated protein with light having a second wavelength; (b) measuring an excitatory post synaptic potential (EPSP) in response to selectively depolarizing the excitatory neuron comprising a first light-activated protein or measuring an inhibitory post synaptic current (IPSC) in response to selectively depolarizing an inhibitory neuron comprising a second light-activated protein; (c) contacting the excitatory neuron or the inhibitory neuron with a chemical compound; (d) measuring the excitatory post synaptic potential (EPSP) or measuring the inhibitory post synaptic current (IPSC) to determine if contacting either the excitatory neuron or the inhibitory neuron with the chemical compound selectively inhibits the depolarization of either neuron. In some embodiments, the first or the second light activated protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from

Chlamydomonas reinhardti, wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1. In some embodiments, the first light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO: 1, 3, 5, or 7, and wherein the second light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:11, 12, 13, or 14.

It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention. These and other aspects of the invention will become apparent to one of skill in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts engineering of an improved red-shifted channelrhodopsin for combinatorial optogenetics. **(a)** Confocal images of cultured hippocampal neurons transfected with VChR 1-eYFP or C1V1-eYFP under the control of the CaMKIIa promoter. Box denotes region expanded in the last panel, showing dendritic membrane localization of C1V1-tsYFP. Scale bars: 20 μm *(left),* 4 μm *(right).* **(b)** Peak photocurrents from whole-cell patch clamp recordings in-cultured hippocampal neurons expressing indicated opsins. **(c)** Sequence alignment of ChR1, ChR2 and VChR1. Splice sites for two C1V1 variants are indicated. Putative transmembrane helices 1-7 are indicated with bars (TM1-7); mutated amino acids indicated in grey. **(d)** Photocurrent amplitudes recorded in HEK cells expressing C1V1 splice variants 1 and 2. **(e)** Single confocal plane images of cultured hippocampal neurons transfected with indicated opsins, fused to EYFP. DNA concentration was matched across constructs. **(f)** Action spectra of ChR2, VChR1, ClV1 wt, C1V (E122T), C1V1 (E162T), and C1V1 (E122T/E162T). Photocurrents were collected with 2ms light pulses in HEK293 cells. **(g)** Ion permeance of C1V1 splice variant 1 as measured by photocurrent magnitude at -40 mV in HEK cells by whole cell patch clamp using cation-isolating external solutions. Data were normalized to the maximum peak Na current. **(h)** Schematic of the C1V1 chimera with point mutation positions indicated in white. ChR1 sequence indicated with black; VChR1 sequence with grey.

FIG. 2 depicts testing an improved red-shifted channelrhodopsin for combinatorial optogenetics. **(a)** Representative traces and summary plot of channel closure time constant (to_{ff}) in cultured neurons expressing the indicated opsins; traces are normalized to peak current. **(b)** C1V1-E122T inactivation compared to deactivation of ChR2. **(c)** Inactivation of current in C1V1 double mutant E122T/E162T versus other C1V1 variants. **(d)** Mean peak photocurrents recorded in cultured neurons expressing the indicated opsins in response to a 2ms 542nm light pulse.

FIG. 3 depicts photocurrents from acute slice recordings in prefrontal pyramidal neurons. **(a)** Peak photocurrents show consistent correlation with integrated fluorescence intensity. **(b)** Fluorescence-photocurrent relationship in ChR2(H134R) and C1V1(E122T/E162T). Black lines are linear fits to the data. **(c)** Acute slice recordings in prefrontal pyramidal neurons stimulated with 560 nm light pulse trains or current injections at the indicated frequencies. Summary graph shows population data (n = 6). **(d)** Fraction of successful spikes to current injections (200 pA, 10 ms pulses; top left) or 2 ms light pulses at the indicated wavelengths and light power densities. All pulse trains consisted of 20x2 ms pulses delivered through the microscope objective using a Sutter DG-4 light source, filtered using 20 nm bandpass filters and additional neutral density filters to attenuate light power (n = 6 cells in 2 slices). **(e)** Voltage-clamp responses to 542 nm and 630 nm light pulses in cells expressing C1V1-E122T or C1V1-E 122T/E 162T (top). Current-clamp recording in a C1V1-E 122T expressing cell shows spiking in response to a 5 Hz train of 50 ms 630 nm light at 3.2 mW mm^{-2} (bottom). **(f)** Kinetics of red light response in C1V1(E122T). Activation time constants (τ_{on}) of photocurrents recorded from cultured neurons expressing C1V1(E122T) at 540 nm and 630 nm. Note that light powers were 3.2 mW mm-2 at 630 nm and 7.7 mW mm^{-2} at 540 nm (n = 5 cells, p = 0.0006 paired *t*-test). **(g)** Voltage clamp traces show responses in a neuron expressing C1V1(E122T) to 630 nm light pulses. Pulse lengths are indicated above traces. τ_{on} calculated from the 150 ms trace is 67ms. **(h)** Current clamp recording from a neuron expressing C1V1(E122T) showing spikes elicited by 50ms pulses at 630 nm (power density 3.2 mW mm^{-2}).

FIG. 4 depicts independent activation of excitatory pyramidal neurons and inhibitory parvalbumin-expressing cells. **(a)** Current clamp recordings from cultured hippocampal neurons expressing C1V1(E1 22T/E162T) or ChR2(H134R) in response to 2ms light pulses at 560nm or 405nm (5 Hz; 7.6 mW/mm^2 at both wavelengths). **(b)** Recording configuration in double-injected animals expressing C1V1 in cortical pyramidal neurons and ChR2 (H134R) in inhibitory parvalbumin-positive interneurons. To independently express opsins, PV::Cre mice were injected with a two-virus mix containing Lenti-CaMKIIα-C1V1(E122T/E162T) and AAV5-EF la-DIO-Ch R2 (H 134R). **(c)** Voltage clamp recordings from a non-expressing PYR neuron receiving synaptic input from C1V1-expressing PYR-cells and ChR2-expressing PV-cells. Clamped at OmV, 405nm light pulses trigger short-latency IPSCs while 560nm pulses evoke only small, long-latency inhibitory synaptic responses. **(d)** Voltage clamp recording from the same cell shown in **c**. Clamped at -65mV, 560nm light pulses trigger EPSCs but 405nm pulses do not evoke

detectable synaptic currents. Gray lines show individual events; black lines show light pulse-triggered averages. **(e)** mPFC optrode recording in an anesthetized PV::Cre mouse injected with CaMKIIa::C1V1(E162T)-ts-eYFP and Ef1a-DIO::ChR2-eYFP (diagram illustrates experimental setup). Violet (405 nm) light pulses are presented with variable delay (At) relative to green light pulses (example traces). **(f)** Summary graph shows probability of green light-evoked spikes with violet pulses preceding the green light pulses by the indicated delays. Individual points are from single recordings. Black line shows average for all recordings (> 3 recording sites per bin). **(g)** Optrode recording from a mouse injected with viruses showing one presumed pyramidal unit and one presumed PV unit, firing in response to 561 nm stimulation (*right*, upper waveform) and 405nm stimulation (*right*, lower waveform), respectively.

FIG. 5 depicts combinatorial optogenetic excitation in distinct intact-system preparations. **(a)** Combinatorial projection control with C1V1-E122T/E 162T and ChR2-H134R *in vitro*. Experimental paradigm showing expression of C1V1 and ChR2 in cortico-thalamic (CT) and ventrobasal (VB) thalamo-cortical cells (TC), respectively. **(b)** Voltage-clamp recording from an nRT cell receiving projections both from CT and TC cells. Simultaneous stimulation (At = O ms) leads to a linear summation of evoked EPSCs from both projections. **(c)** Individual subthreshold inputs from TC and CT fibers lead to spiking in an nRT neuron only when inputs are precisely co-incident. **(d)** Delays between CT and TC inputs are indicated on the left. Horizontal dashed lines indicate truncated spikes. Normalized number of action potentials (from 6 nRT cells) evoked by CT and TC fibers activated with variable latencies (At) indicates that CT and TC inputs lead to effective integration only if coincident within 5 ms. Summary data represent mean ± SEM.

FIG. 6 depicts spectrotemporal separation and combinatorial control: circuit modulation and emergent patterns in altered E/I states under ongoing synaptic activity. **(a)** Experimental paradigm for SSFO activation of PV neurons and C1V1 activation in pyramidal neurons. **(b)** Voltage clamp recording at 0 mV from a pyramidal neuron in an acute slice preparation from a PV::Cre mouse expressing CaMKIIa::C1V1(E162T) and DIO-SSFO. SSFO and C1V1 are activated by a blue light pulse (2) and IPSC frequency is increased by sustained SSFO activity (3; compare upper and lower traces in inset for pre- and post-activation IPSC activity). A sustained yellow light pulse deactivates SSFO and activates C1V1 and transiently increases IPSC frequency (4). Population power spectra (right) show gamma frequency activity during optical excitatory neuron activation (590 nm pulse) that is increased during coactivation of excitatory and PV neurons (470 nm pulse).

Diagrams below traces show predicted activity of C1V1 and SSFO during the experiment. **(c)** The observed gamma frequency peak was not dependent on prior PV neuron stimulation via SSFO. **(d)** Summary IPSC frequencies from **(b)** and **(c)** at baseline and after the initial blue or orange pulse. Diagrams below traces show predicted activity of C1V1 and SSFO during the experiment.

DETAILED DESCRIPTION

This invention provides, *inter alia,* compositions comprising animal cells expressing light-activated chimeric proteins on their plasma membranes and methods of using the same to selectively depolarize excitatory or inhibitory neurons residing in the same microcircuit in the pre-frontal cortex. The inventors have developed chimeric proteins possessing unique physiochemical properties which for the first time permit experimental manipulation of cortical E/I elevations and the ability to monitor gamma oscillations in cortical slices. These unique light-sensitive chimeric proteins can be expressed in either excitatory or inhibitory neural circuits in the prefrontal cortex of nonhuman animals which can then be depolarized in response to light having particular wavelengths. Furthermore, brain slices from non-human animals containing cortical excitatory or inhibitory neurons expressing the chimeric light-sensitive proteins disclosed herein can be used to search for chemical compounds which can selectively inhibit the depolarization of either excitatory or inhibitory neurons residing within the same neural circuit.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are well known to those skilled in the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989) and *Molecular Cloning: A Laboratory Manual*, third edition (Sambrook and Russel, 2001), (jointly referred to herein as "Sambrook"); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987, including supplements through 2001); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York; Harlow and Lane (1999) *Using Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (jointly referred to herein as "Harlow and Lane"), Beaucage et al. eds., *Current Protocols in Nucleic Acid Chemistry* John Wiley & Sons, Inc., New York, 2000), *Handbook of Experimental Immunology*, 4th edition (D. M. Weir & C. C. Blackwell, eds., Blackwell Science Inc.,

1987); and *Gene Transfer Vectors for Mammalian Cells* (J. M. Miller & M. P. Calos, eds., 1987).

Definitions

As used herein, the singular form "a", "an", and "the" includes plural references unless indicated otherwise.

An "animal" can be a vertebrate, such as any common laboratory model organism, or a mammal. Mammals include, but are not limited to, humans, farm animals, sport animals, pets, primates, mice, rats, and other rodents.

An "amino acid substitution" or "mutation" as used herein means that at least one amino acid component of a defined amino acid sequence is altered or substituted with another amino acid leading to the protein encoded by that amino acid sequence having altered activity or expression levels within a cell.

A "chimeric protein" is a protein comprising one or more portions derived from one or more different proteins. Chimeric proteins may be produced by culturing a recombinant cell transfected with a nucleic acid that encodes the chimeric protein.

It is intended that every maximum numerical limitation given throughout this specification includes every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

V1C1 Chimeric Proteins and Cells Expressing the Same

In some aspects, the animal cells disclosed herein comprise a chimeric light-sensitive protein, known as "C1V1," which is derived from the VChR1 cation channel from *Volvox carteri* and the ChR1 cation channel from *Chlamydomonas Reinhardti*. The protein may be comprised of the amino acid sequence of VChR1, but additionally can have at least the first and second transmembrane helices of the VChR1 polypeptide replaced by the corresponding first and second transmembrane helices of ChR1. C1V1 chimeric opsin proteins are assembled from pieces of other opsin proteins that do not express well alone in neurons and which are potent, redshifted, and stable channelrhodopsins. In some embodiments, the animal cell may express a second light-activated protein on the plasma membrane of the cell. The second light-activated protein can be capable of mediating a

hyperpolarization of the cell plasma membrane in response to activation by light. Examples of light-activated proteins capable of mediating a hyperpolarization of the cell plasma membrane can be found, for example, in International Patent Application No: PCT/US2011/028893, the disclosure of which is incorporated by reference herein in its entirety.

Embodiments of the present disclosure may also be directed toward modified or mutated versions of C1V1. These proteins can be used alone or in combination with a variety of other opsins to assert optical control over neurons. In particular, the use of modified C1V1, in connection with other opsins, is believed to be useful for optical control over nervous system disorders. Specific uses of C1V1 relate to optogenetic systems or methods that correlate temporal, spatial and/or cell type-specific control over a neural circuit with measurable metrics.

V1C1 chimeric proteins

Provided herein are light-activated chimeric proteins expressed on an animal cell plasma membrane. In some aspects the light-activated protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti.* In some embodiments, the chimeric protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the corresponding first and second transmembrane helices of ChR1. In other embodiments, the chimeric protein comprises the amino acid sequence of VChR1 having the first and second transmembrane helices replaced by the corresponding first and second transmembrane helices of ChR1 and further comprises at least a portion of the intracellular loop domain located between the second and third transmembrane helices replaced by the corresponding portion from ChR1. In some embodiments, the entire intracellular loop domain between the second and third transmembrane helices of the chimeric light-activated protein can be replaced with the corresponding intracellular loop domain from ChR1. In other embodiments, the portion of the intercellular loop domain located between the second and third transmembrane helices that is replaced with the corresponding portion of ChR1 can extend to A145 of SEQ ID NO:1. In other embodiments, the chimeric protein comprises the amino acid sequence of VChR1 having the first and second transmembrane helices and the intracellular loop domain replaced by the corresponding first and second transmembrane helices and intracellular loop domain of ChR1 and further comprises at least a portion of the third transmembrane helix replaced by the corresponding portion of ChR1. In another embodiment, the portion of the third transmembrane helix replaced by the corresponding

portion from ChR1 can extend to W163 of SEQ ID NO:1. In some embodiments, the light-activated chimeric protein comprises the amino acids 1-145 of ChR1 and amino acids 102-316 of VChR1. In some embodiments, the light-activated chimeric protein comprises the amino acids 1-162 of ChR1 and amino acids 119-316 of VChR1. In some embodiments, the light-activated chimeric protein can comprise an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 1 without the signal peptide sequence. In some embodiments, the light-activated chimeric protein can comprise an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 1

In other embodiments, the light activated chimeric protein is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In some embodiments the light can be green light. In other embodiments, the light can have a wavelength of between about 540 nm to about 560 nm. In some embodiments, the light can have a wavelength of about 542 nm. In some embodiments, the chimeric protein may not be capable of mediating a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein may not be capable of mediating a depolarizing current in the cell when the cell is illuminated with light having a wavelength of 405 nm.

In some embodiments, the protein can further comprise a C-terminal fluorescent protein. In some specific embodiments, the C-terminal fluorescent protein can be enhanced yellow fluorescent protein (EYFP), green fluorescent protein (GFP), cyan fluorescent protein (CFP), or red fluorescent protein (RFP). In some embodiments, the light-activated chimeric protein is modified by the addition of a trafficking signal (ts) which enhances transport of the protein to the cell plasma membrane. In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In some embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV. In some embodiments, the signal peptide sequence in the protein may be replaced with a different signal peptide sequence.

In some embodiments, the animal cell can be a neuronal cell, a muscle cell, or a stem cell. In one embodiment, the animal cell is a neuronal cell. In some embodiments the neuronal cell can be an excitatory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In still other embodiments, the inhibitory neuron can be a parvalbumin neuron. In some

embodiments the neuronal cell can be an inhibitory neuron located in the pre-frontal cortex of a non-human animal. In some embodiments the neuronal cell can be an inhibitory neuron located in the pre-frontal cortex of a non-human animal.

In some embodiments, the animal cells can further comprise a second light-activated protein expressed on the cells' plasma membrane. In some embodiments, the second light-activated protein can be capable of mediating a hyperpolarizing current in the cell when the cell is illuminated with light. In some embodiments the second light-activated protein can be NpHr, eNpHr2.0, eNpHr3.0, eNpHr3.1 or GtR3. Additional information regarding other light-activated cation channels, anion pumps, and proton pumps can be found in U.S. Patent Application Publication Nos: 2009/0093403; and International Patent Application No: PCT/US2011/028893, the disclosures of which are hereby incorporated by reference herein in their entirety. In some embodiments, the light-activated chimeric protein can have enhanced photocurrents in neural cells exposed to light relative to cells expressing other light-activated cation channel proteins. In some embodiments, the enhancement in photocurrent provided by the light-activated chimeric protein can be any of 1 fold, 2 fold, 3 fold, 4 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, 10 fold, 11 fold, 12 fold, 13 fold, 14 fold, or 15 fold, greater than cells expressing other light-activated cation channel proteins, inclusive.

Also provided herein is one or more light-activated proteins expressed on an animal cell plasma membrane, wherein said one or more light activated proteins comprises a core amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 12, 13, or 14 and further comprising a trafficking signal (e.g., which enhances transport to the plasma membrane). The trafficking signal may be fused to the C-terminus of the core amino acid sequence or may be fused to the N-terminus of the core amino acid sequence. In some embodiments, the trafficking signal can be linked to the core amino acid sequence by a linker. The linker can comprise any of 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The linker may further comprise a fluorescent protein, for example, but not limited to, an enhanced yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In some embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV.

V1C1 chimeric mutant variants

In some aspects, the invention includes polypeptides comprising substituted or mutated amino acid sequences, wherein the mutant polypeptide retains the characteristic light-activatable nature of the precursor C1V1 chimeric polypeptide but may also possess altered properties in some specific aspects. For example the mutant light-activated chimeric proteins described herein may exhibit an increased level of expression both within an animal cell or on the animal cell plasma membrane; an altered responsiveness when exposed to different wavelengths of light, particularly red light; and/or a combination of traits whereby the chimeric C1V1 polypeptide possess the properties of low desensitization, fast deactivation, low violet-light activation for minimal cross-activation with other light-activated cation channels, and/or strong expression in animal cells.

Light-activated chimeric proteins comprising amino acid substitutions or mutations include those in which one or more amino acid residues have undergone an amino acid substitution while retaining the ability to respond to light and the ability to control the polarization state of a plasma membrane. For example, light-activated proteins comprising amino acid substitutions or mutations can be made by substituting one or more amino acids into the amino acid sequence corresponding to SEQ ID NO:1. In some embodiments, the invention includes proteins comprising altered amino acid sequences in comparison with the amino acid sequence in SEQ ID NO:1, wherein the altered light-activated chimeric protein retains the characteristic light-activated nature and/or the ability to regulate ion flow across plasma membranes of the protein with the amino acid sequence represented in SEQ ID NO:1 but may have altered properties in some specific aspects.

Amino acid substitutions in a native protein sequence may be conservative or non-conservative and such substituted amino acid residues may or may not be one encoded by the genetic code. The standard twenty amino acid "alphabet" is divided into chemical families based on chemical properties of their side chains. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and side chains having aromatic groups (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a chemically similar side chain (*i.e.*, replacing an amino acid possessing a basic side chain

with another amino acid with a basic side chain). A "non-conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a chemically different side chain (*i.e.*, replacing an amino acid having a basic side chain with an amino acid having an aromatic side chain). The amino acid substitutions may be conservative or non-conservative. Additionally, the amino acid substitutions may be located in the C1V1 retinal binding pocket, in one or more of the C1V1 intracellular loop domains, and/or in both the retinal binding pocket or the intracellular loop domains.

Accordingly, provided herein are C1V1 chimeric light-activated proteins that may have specific amino acid substitutions at key positions throughout the retinal binding pocket of the VChR1 portion of the chimeric polypeptide. In some embodiments, the C1V1 protein can have a mutation at amino acid residue E122 of SEQ ID NO:1. In some embodiments, the C1V1 protein can have a mutation at amino acid residue E162 of SEQ ID NO:1. In other embodiments, the C1V1 protein can have a mutation at both amino acid residues E162 and E122 of SEQ ID NO:1. In some embodiments, each of the disclosed mutant C1V1 chimeric proteins can have specific properties and characteristics for use in depolarizing the membrane of an animal cell in response to light.

C1V1-E122 mutant polypeptides

Provided herein are the light-activated C1V1 chimeric proteins disclosed herein expressed on an animal cell plasma membrane, wherein one or more amino acid residues have undergone an amino acid substitution while retaining C1V1 activity (i.e., the ability to catalyze the depolarization of an animal cell in response to light activation), and wherein the mutation can be at a glutamic acid residue corresponding to E122 of SEQ ID NO:1 (C1V1-E122). In some embodiments, the C1V1-E122 mutant chimeric light-activated protein comprises substitutions introduced into the amino acid sequence shown in SEQ ID NO:1 at amino acid E122 that can result in the chimeric protein having increased sensitivity to light, increased sensitivity to particular wavelengths of light, and/or increased ability to regulate the polarization state of the plasma membrane of the cell relative to C1V1 chimeric light-activated proteins that do not have a mutation at E122. In some embodiments, the mutation can be a conservative amino acid substitution. In some embodiments, the mutation can be a non-conservative amino acid substitution. In some embodiments, the mutation at amino acid residue E122 can be to threonine (C1V1-E122T). In other embodiments, the light-activated chimeric protein can comprise an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 3 without the signal peptide sequence. In other embodiments, the light-activated

chimeric protein can comprise an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 3. In other embodiments, the C1V1-E122 mutant chimeric light-activated protein may be fused to a C-terminal trafficking signal. In some embodiments, the trafficking signal can be linked to the C1V1-E122 mutant chimeric light-activated protein by a linker. The linker can comprise any of 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The linker may further comprise a fluorescent protein, for example, but not limited to, an enhanced yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In some embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV.

In other embodiments, the C1V1-E122 chimeric protein is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In some embodiments the light can be green light. In other embodiments, the light can have a wavelength of between about 540 nm to about 560 nm. In some embodiments, the light can have a wavelength of about 546 nm. In other embodiments, the C1V1-E122 chimeric protein can mediate a depolarizing current in the cell when the cell is illuminated with red light. In some embodiments, the red light can have a wavelength of about 630 nm. In some embodiments, the C1V1-E122 chimeric protein may not be capable of mediating a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein may not be capable of mediating a depolarizing current in the cell when the cell is illuminated with light having a wavelength of 405 nm. In some embodiments, the animal cell can be a neuronal cell, a muscle cell, or a stem cell. In one embodiment, the animal cell can be a neuronal cell. In some embodiments the neuronal cell can be an excitatory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In some embodiments the neuronal cell can be an inhibitory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In still other embodiments, the inhibitory neuron can be a parvalbumin neuron. In some embodiments, the animal cells can further comprise a second light-activated protein expressed on the cells' plasma membrane. In some embodiments, the second light-activated protein can be capable of mediating a hyperpolarizing current in the cell when the cell is

illuminated with light. In some embodiments the second light-activated protein can be NpHr, eNpHr2.0, eNpHr3.0, eNpHr3.1 or GtR3.

C1V1-E162 mutant polypeptides

Provided herein are the light-activated C1V1 chimeric proteins disclosed herein expressed on an animal cell plasma membrane, wherein one or more amino acid residues have undergone an amino acid substitution while retaining C1V1 activity (i.e., the ability to catalyze the depolarization of an animal cell in response to light activation), wherein the mutation can be at a glutamic acid residue corresponding to E162 of SEQ ID NO:1 (C1V1-E162). In some embodiments, the C1V1-E162 mutant chimeric light-activated protein comprises substitutions introduced into the amino acid sequence shown in SEQ ID NO:1 at amino acid E162 that can result in the chimeric protein having increased sensitivity to light, increased sensitivity to particular wavelengths of light, and/or increased ability to regulate the polarization state of the plasma membrane of the cell relative to C1V1 chimeric light-activated proteins that do not have a mutation at E162. In some embodiments, the mutation can be a conservative amino acid substitution. In some embodiments, the mutation can be a non-conservative amino acid substitution. In some embodiments, the mutation at amino acid residue E162 can be to threonine (C1V1-E162T). In other embodiments, the light-activated chimeric protein can comprise an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 5 without the signal peptide sequence. In other embodiments, the light-activated chimeric protein can comprise an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 5. In other embodiments, the C1V1-E162 mutant chimeric light-activated protein may be fused to a C-terminal trafficking signal. In some embodiments, the trafficking signal can be linked to the C1V1-E162 mutant chimeric light-activated protein by a linker. The linker can comprise any of 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The linker may further comprise a fluorescent protein, for example, but not limited to, an enhanced yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In some embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV.

In other embodiments, the C1V1-E162 chimeric protein is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In some

embodiments the light can be green light. In other embodiments, the light can have a wavelength of between about 540 nm to about 535 nm. In some embodiments, the light can have a wavelength of about 542 nm. In other embodiments, the light can have a wavelength of about 530 nm. In some embodiments, the C1V1-E162 chimeric protein may not be capable of mediating a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein may not be capable of mediating a depolarizing current in the cell when the cell is illuminated with light having a wavelength of 405 nm. In some embodiments, the C1V1-E162 chimeric protein can further comprise a C-terminal fluorescent protein. In some embodiments, the animal cell can be a neuronal cell, a muscle cell, or a stem cell. In one embodiment, the animal cell can be a neuronal cell. In some embodiments the neuronal cell can be an excitatory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In some embodiments the neuronal cell can be an inhibitory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In still other embodiments, the inhibitory neuron can be a parvalbumin neuron. In some embodiments, the animal cells can further comprise a second light-activated protein expressed on the cells' plasma membrane. In some embodiments, the second light-activated protein can be capable of mediating a hyperpolarizing current in the cell when the cell is illuminated with light. In some embodiments the second light-activated protein can be NpHr, eNpHr2.0, eNpHr3.0, eNpHr3.1 or GtR3. In some embodiments, the C1V1-E162 light-activated chimeric protein can have an accelerated photocycle relative C1V1 proteins lacking mutations at E162 or relative to other light-activated cation channel proteins. In some embodiments, the C1V1-E162 light-activated chimeric protein can have a photocycle more than 1 fold, 1.5 fold, 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold, or 5 fold faster than C1V1 proteins lacking mutations at E162 or relative to other light-activated cation channel proteins, inclusive.

C1V1-E122/E162 double mutant polypeptides

Provided herein are the light-activated C1V1 chimeric proteins disclosed herein expressed on an animal cell plasma membrane, wherein one or more amino acid residues have undergone an amino acid substitution while retaining C1V1 activity (i.e., the ability to catalyze the depolarization of an animal cell in response to light activation), wherein the mutations can be at glutamic acid residues corresponding to E122 and E162 of SEQ ID NO:1 (C1V1-E122/E162). In some embodiments, the C1V1-E122/E162 mutant chimeric light-activated protein can comprise substitutions introduced into the amino acid sequence

shown in SEQ ID NO:1 at amino acid E122 and E162 that can result in the chimeric protein having increased sensitivity to light, increased sensitivity to particular wavelengths of light, and/or increased ability to regulate the polarization state of the plasma membrane of the cell relative to C1V1 chimeric light-activated proteins that do not have a mutation at E122 and E162. In some embodiments, the mutations can be conservative amino acid substitutions. In some embodiments, the mutations can be non-conservative amino acid substitutions. In some embodiments, the mutations can be both conservative and non-conservative amino acid substitutions. In some embodiments, the mutation at amino acid residue E122 and at E162 can both be to threonine (C1V1-E122T/E162T). In other embodiments, the light-activated chimeric protein can comprise an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 7 without the signal peptide sequence. In other embodiments, the light-activated chimeric protein can comprise an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO: 7. In other embodiments, the C1V1-E122/E162 mutant chimeric light-activated protein may be fused to a C-terminal trafficking signal. In some embodiments, the trafficking signal can be linked to the C1V1-E122/E162 mutant chimeric light-activated protein by a linker. The linker can comprise any of 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The linker may further comprise a fluorescent protein, for example, but not limited to, an enhanced yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In some embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV.

In other embodiments, the C1V1-E122/E162 chimeric protein is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In some embodiments the light can be green light. In other embodiments, the light can have a wavelength of between about 540 nm to about 560 nm. In some embodiments, the light can have a wavelength of about 546 nm. In some embodiments, the C1V1-E122/E162 chimeric protein may not be capable of mediating a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein may not be capable of mediating a depolarizing current in the cell when the cell is illuminated with light having a wavelength of 405 nm. In some embodiments, the C1V1-E122/E162 chimeric protein can exhibit less activation when exposed to violet light relative to C1V1 proteins

lacking mutations at E122/E162 or relative to other light-activated cation channel proteins. In some embodiments, the animal cell can be a neuronal cell, a muscle cell, or a stem cell. In one embodiment, the animal cell can be a neuronal cell. In some embodiments the neuronal cell can be an excitatory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In some embodiments the neuronal cell can be an inhibitory neuron located in the pre-frontal cortex of a non-human animal. In still other embodiments, the inhibitory neuron can be a parvalbumin neuron. In some embodiments, the animal cells can further comprise a second light-activated protein expressed on the cells' plasma membrane. In some embodiments, the second light-activated protein can be capable of mediating a hyperpolarizing current in the cell when the cell is illuminated with light. In some embodiments the second light-activated protein can be NpHr, eNpHr2.0, eNpHr3.0, eNpHr3.1 or GtR3. In some embodiments, the C1V1-E122/E162 mutant light-activated chimeric protein can have decreased inactivation relative to C1V1 proteins lacking mutations at E122/E162 or relative to other light-activated cation channel proteins. In some embodiments, the C1V1-E122/E162 mutant light-activated chimeric protein can inactivate by any of about 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26v, 27%, 28%, 29%, or 30% compared to C1V1 proteins lacking mutations at E122/E162 or relative to other light-activated cation channel proteins, inclusive. In some embodiments, the C1V1- E122/E162 light-activated chimeric protein can have a photocycle more than 1 fold, 1.5 fold, 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold, 5 fold, 5.5 fold, 6 fold, 6.5 fold, 7 fold, 7.5 fold, 8 fold, 8.5 fold, 9 fold, 9.5 fold, or 10 fold faster than C1V1 proteins lacking mutations at E122/E162 or relative to other light-activated cation channel proteins, inclusive.

Enhanced intracellular transport amino acid motifs

The present disclosure provides for the modification of light-activated chimeric proteins expressed in a cell by the addition of one or more amino acid sequence motifs which enhance transport to the plasma membranes of mammalian cells. Light-activated chimeric proteins having components derived from evolutionarily simpler organisms may not be expressed or tolerated by mammalian cells or may exhibit impaired subcellular localization when expressed at high levels in mammalian cells. Consequently, in some embodiments, the chimeric light-activated protein expressed in a cell is fused to one or more amino acid sequence motifs selected from the group consisting of a signal peptide, an endoplasmic reticulum (ER) export signal, a membrane trafficking signal, and an N-terminal golgi export signal. The one or more amino acid sequence motifs which enhance

light-activated chimeric protein transport to the plasma membranes of mammalian cells can be fused to the N-terminus, the C-terminus, or to both the N- and C-terminal ends of the light-activated protein. Optionally, the light-activated protein and the one or more amino acid sequence motifs may be separated by a linker. In some embodiments, the light-activated chimeric protein is modified by the addition of a trafficking signal (ts) which enhances transport of the protein to the cell plasma membrane. In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In some embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV. Additional protein motifs which can enhance light-activated protein transport to the plasma membrane of a cell are described in U.S. Patent Application No. 12/041,628, which is incorporated herein by reference in its entirety. In some embodiments, the signal peptide sequence in the chimeric protein is deleted or substituted with a signal peptide sequence from a different protein.

Animal cells, non-human animals, and brain slices

Provided herein are cells comprising the light activated chimeric proteins disclosed herein. In some embodiments, the cells are animal cells. In some embodiments, the animal cells comprise the C1V1 protein corresponding to SEQ ID NO:1. In other embodiments, the animal cells comprise the mutant C1V1-E122T protein corresponding to SEQ ID NO:3. In other embodiments, the animal cells comprise the mutant C1V1-E162T protein corresponding to SEQ ID NO:5. In other embodiments, the animal cells comprise the mutant C1V1-E122T/E162T protein corresponding to SEQ ID NO7. In some embodiments, the animal cell can be a neuronal cell, a muscle cell, or a stem cell. In one embodiment, the animal cell can be a neuronal cell. In some embodiments the neuronal cell can be an excitatory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In some embodiments the neuronal cell can be an inhibitory neuron located in the pre-frontal cortex of a non-human animal. In still other embodiments, the inhibitory neuron can be a parvalbumin neuron.

Also provided herein, are non-human animals comprising the light activated chimeric proteins disclosed herein expressed on the cell membrane of the cells in the animals. In some embodiments, the animal cells comprise the C1V1 protein corresponding to SEQ ID NO:1. In other embodiments, the animal cells comprise the mutant C1V1-E122T protein corresponding to SEQ ID NO:3. In other embodiments, the animal cells comprise the mutant C1V1-E162T protein corresponding to SEQ ID NO:5. In other

embodiments, the animal cells comprise the mutant C1V1-E122T/E162T protein corresponding to SEQ ID NO7. In some embodiments, the animals comprising the light-activated chimeric proteins described herein are transgenically expressing said light-activated chimeric proteins. In other embodiments, the animals comprising the light-activated chimeric proteins described herein have been virally transfected with a vector carrying the light-activated protein such as, but not limited to, an adenoviral vector.

Provided herein are living brain slices from a non-human animal comprising the light-activated chimeric proteins described herein expressed on the cell membrane of the cells in the slices. In some embodiments, the brain slices are from non-human animals transgenically expressing the light-activated chimeric proteins described herein. In other embodiments, the brain slices are from non-human animals that have been virally transfected with a vector carrying said light-activated protein such as, but not limited to, an adenoviral vector. In some embodiments, the brain slices are coronal brain slices. In some embodiments, the brain slices are any of about 100 μm, about 150 μm, about 200 μm, about 250 μm, about 300 μm, about 350 μm, about 400 μm, about 450 μm, or about 500 μm thick, inclusive, including any thicknesses in between these numbers.

Isolated polynucleotides

Provided herein are isolated C1V1 polynucleotides that encode any chimeric polypeptides described herein that, for example, have at least one activity of a C1V1 polypeptide. The disclosure provides isolated, synthetic, or recombinant polynucleotides comprising a nucleic acid sequence having at least about 70%, e.g., at least about 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%; 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or complete (100%) sequence identity to the nucleic acid of SEQ ID NO:2, 4, 6 or 8 over a region of at least about 10, e.g., at least about 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 nucleotides.

The disclosure specifically provides a nucleic acid encoding C1V1 and/or a mutant variant thereof. For example, the disclosure provides an isolated nucleic acid molecule, wherein the nucleic acid molecule encodes: (1) a polypeptide comprising an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:1; (2) a polypeptide comprising an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:3, (3) a polypeptide comprising an amino acid sequence with at least 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:5; or (4) a polypeptide comprising an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence represented by SEQ ID NO:7.

Promoters and vectors

The disclosure also provides expression cassettes and/or vectors comprising the above-described nucleic acids. Suitably, the nucleic acid encoding a chimeric protein of the disclosure is operably linked to a promoter. Promoters are well known in the art. Any promoter that functions in the host cell can be used for expression of C1V1 and/or any variant thereof of the present disclosure. Initiation control regions or promoters, which are useful to drive expression of a C1V1 chimeric protein or variant thereof in a specific animal cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these nucleic acids can be used.

Specifically, where recombinant expression of C1V1 chimeric proteins in an excitatory neural cell is desired, a human calmodulin-dependent protein kinase II alpha (CaMKIIα) promoter may be used. In other embodiments, an elongation factor 1a (EF-1a) promoter in conjunction with a Cre-inducible recombinant AAV vector can be used with parvalbumin-Cre transgenic mice to target expression C1V1 chimeric proteins to inhibitory neurons.

Also provided herein are vectors comprising the polynucleotides disclosed herein encoding a C1V1 chimeric polypeptide or any variant thereof. The vectors that can be administered according to the present invention also include vectors comprising a polynucleotide which encodes an RNA (e.g., RNAi, ribozymes, miRNA, siRNA) that when transcribed from the polynucleotides of the vector will result in the accumulation of light-activated chimeric proteins on the plasma membranes of target animal cells. Vectors which may be used, include, without limitation, lentiviral, HSV, and adenoviral vectors. Lentiviruses include, but are not limited to HIV-1, HIV-2, SIV, FIV and EIAV. Lentiviruses may be pseudotyped with the envelope proteins of other viruses, including, but not limited to VSV, rabies, Mo-MLV, baculovirus and Ebola. Such vectors may be prepared using standard methods in the art.

In some embodiments, the vector is a recombinant AAV vector. AAV vectors are DNA viruses of relatively small size that can integrate, in a stable and sitespecific manner, into the genome of the cells that they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do

not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions that carry the encapsidation functions: the left-hand part of the genome, that contains the rep gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome, that contains the cap gene encoding the capsid proteins of the virus.

The application of AAV as a vector for gene therapy has been rapidly developed in recent years. Wild-type AAV could infect, with a comparatively high titer, dividing or non-dividing cells, or tissues of mammal, including human, and also can integrate into in human cells at specific site (on the long arm of chromosome 19) (Kotin, R. M., et al, *Proc. Natl. Acad. Sci. USA* 87: 2211-2215, 1990) (Samulski, R. J, et al, *EMBO J.* 10: 3941-3950, 1991 the disclosures of which are hereby incorporated by reference herein in their entireties). AAV vector without the *rep* and *cap* genes loses specificity of site-specific integration, but may still mediate long-term stable expression of exogenous genes. AAV vector exists in cells in two forms, wherein one is episomic outside of the chromosome; another is integrated into the chromosome, with the former as the major form. Moreover, AAV has not hitherto been found to be associated with any human disease, nor any change of biological characteristics arising from the integration has been observed. There are sixteen serotypes of AAV reported in literature, respectively named AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV13, AAV14, AAV15, and AAV16, wherein AAV5 is originally isolated from humans (Bantel-Schaal, and H. zur Hausen. 1984. *Virology* 134: 52-63), while AAV1-4 and AAV6 are all found in the study of adenovirus (Ursula Bantel-Schaal, Hajo Delius and Harald zur Hausen. *J. Virol.* 1999, 73: 939-947).

AAV vectors may be prepared using standard methods in the art. Adeno-associated viruses of any serotype are suitable (*See*, e.g., Blacklow, pp. 165-174 of "*Parvoviruses and Human Disease*" J. R. Pattison, ed. (1988); Rose, *Comprehensive Virology* 3:1, 1974; P. Tattersall "The Evolution of Parvovirus Taxonomy" In *Parvoviruses* (JR Kerr, SF Cotmore. ME Bloom, RM Linden, CR Parrish, Eds.) p5-14, Hudder Arnold, London, UK (2006); and DE Bowles, JE Rabinowitz, RJ Samulski "*The Genus Dependovirus*" (JR Kerr, SF Cotmore. ME Bloom, RM Linden, CR Parrish, Eds.) p15-23, Hudder Arnold, London, UK (2006), the disclosures of which are hereby incorporated by reference herein in their

entireties). Methods for purifying for vectors may be found in, for example, U.S. Pat. Nos. 6566118, 6989264, and 6995006 and WO/1999/011764 titled "Methods for Generating High Titer Helper-free Preparation of Recombinant AAV Vectors", the disclosures of which are herein incorporated by reference in their entirety. Preparation of hybrid vectors is described in, for example, PCT Application No. PCT/US2005/027091, the disclosure of which is herein incorporated by reference in its entirety. The use of vectors derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (*See* e.g., International Patent Application Publication Nos: 91/18088 and WO 93/09239; U.S. Patent Nos: 4,797,368, 6,596,535, and 5,139,941; and European Patent No: 0488528, all of which are herein incorporated by reference in their entirety). These publications describe various AAV-derived constructs in which the rep and/or cap genes are deleted and replaced by a gene of interest, and the use of these constructs for transferring the gene of interest *in vitro* (into cultured cells) or *in vivo* (directly into an organism). The replication defective recombinant AAVs according to the invention can be prepared by co-transfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (*rep* and *cap* genes), into a cell line that is infected with a human helper virus (for example an adenovirus). The AAV recombinants that are produced are then purified by standard techniques.

In some embodiments, the vector(s) for use in the methods of the invention are encapsidated into a virus particle (e.g. AAV virus particle including, but not limited to, AAV1, AAV2, AAV3, AAV4, AAV5,AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV13, AAV14, AAV15, and AAV16). Accordingly, the invention includes a recombinant virus particle (recombinant because it contains a recombinant polynucleotide) comprising any of the vectors described herein. Methods of producing such particles are known in the art and are described in US Patent No. 6,596,535.

For the animal cells described herein, it is understood that one or more vectors may be administered to neural cells, heart cells, or stem cells. If more than one vector is used, it is understood that they may be administered at the same or at different times to the animal cell.

Methods of the Invention

Provided herein are methods for selectively depolarizing excitatory or inhibitory neurons residing in the same microcircuit by expressing in those neurons the light-activated chimeric proteins described herein. In some embodiments, a first light-activated protein,

such as those disclosed herein, can be expressed in an excitatory neuron while a second light-activated protein can be expressed in an inhibitory neuron. In some embodiments, the first light-activated protein expressed in the excitatory neuron can be activated by a different wavelength of light than the second light-activated protein expressed in the inhibitory neuron. In some embodiments, the first and second light-activated proteins can be expressed in a living non-human animal or in a living brain slice from a non-human animal.

In other embodiments, a method is provided for identifying a chemical compound that selectively inhibits the depolarization of excitatory or inhibitory neurons residing in the same neural circuit by expressing in those neurons the light-activated chimeric proteins described herein. In some embodiments, a first light-activated protein can be expressed in an excitatory neuron while a second light-activated protein can be expressed in an inhibitory neuron. In some embodiments, the first light-activated protein expressed in the excitatory neuron can be activated by a different wavelength of light than the second light-activated protein expressed in the inhibitory neuron. In some embodiments, the first and second light-activated proteins can be expressed in a living non-human animal or in a living brain slice from a non-human animal.

Methods for selectively altering the E/I balance in neurons residing in the same microcircuit

In some aspects, there is provided a method for selectively depolarizing excitatory or inhibitory neurons residing in the same microcircuit, the method comprising: selectively depolarizing an excitatory neuron comprising a first light-activated protein, wherein the first light-activated protein is depolarized when exposed to light having a first wavelength or selectively depolarizing an inhibitory neuron comprising a second light-activated protein, wherein the second light-activated protein is depolarized when exposed to light having a second wavelength. In some embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 1. In other embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 3. In some embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 5. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:11. In some

embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:12. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 13. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 14. More information regarding the disclosure of other light-activated cation channels can be found in U.S. Patent Application Publication No: 2007/0054319; U.S. Patent Application No: 61/410,704; and International Patent Application Publication No: WO 2010/056970, the disclosures of each of which are hereby incorporated by reference in their entireties.

In other aspects, there is provided a method for selectively depolarizing excitatory or inhibitory neurons residing in the same microcircuit, the method comprising: expressing a first light-activated protein in an excitatory neuron; and expressing a second light-activated protein in an inhibitory neuron, wherein the first light-activated protein is independently depolarized when exposed to light having a first wavelength and wherein the second light-activated protein is independently depolarized when exposed to light having a second wavelength. In some embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 1. In other embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 3. In some embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 5. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:11. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:12. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:13. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:14.

In some embodiments, the first light-activated protein can be activated by green light. In one embodiment, the first light-activated protein can be activated by light having a wavelength of about 560 nm. In one embodiment, the first light-activated protein can be activated by red light. In another embodiment, the first light-activated protein can be activated by light having a wavelength of about 630 nm. In other embodiments, the second light-activated protein can be activated by violet light. In one embodiment, the second light-activated protein can be activated by light having a wavelength of about 405 nm. In other embodiments, the second light activated protein can be activated by green light. In some embodiments, the light-activated proteins are activated by light pulses that can have a duration for any of about 1 millisecond (ms), about 2 ms, about 3, ms, about 4, ms, about 5 ms, about 6 ms, about 7 ms, about 8 ms, about 9 ms, about 10 ms, about 15 ms, about 20 ms, about 25 ms, about 30 ms, about 35 ms, about 40 ms, about 45 ms, about 50 ms, about 60 ms, about 70 ms, about 80 ms, about 90 ms, about 100 ms, about 200 ms, about 300 ms, about 400 ms, about 500 ms, about 600 ms, about 700 ms, about 800 ms, about 900 ms, about 1 sec, about 1.25 sec, about 1.5 sec, or about 2 sec, inclusive, including any times in between these numbers. In some embodiments, the light-activated proteins are activated by light pulses that can have a light power density of any of about 0.05 mW mm^{-2}, about 0.1 mW mm^{-2}, about 0.25 mW mm^{-2}, about 0.5 mW mm^{-2}, about 0.75 mW mm^{-2}, about 1 mW mm^{-2}, about 2 mW mm^{-2}, about 3 mW mm^{-2}, about 4 mW mm^{-2}, about 5 mW mm^{-2}, about 6 mW mm^{-2}, about 7 mW mm^{-2}, about 8 mW mm^{-2}, about 9 mW mm^{-2}, about 10 mW mm^{-2}, about 11 mW mm^{-2}, about 12 mW mm^{-2}, about 13 mW mm^{-2}, about 14 mW mm^{-2}, about mW mm^{-2}, about 16 mW mm^{-2}, about 17 mW mm^{-2}, about 18 mW mm^{-2}, about 19 mW mm^{-2}, about 20 mW mm^{-2}, about 21 mW mm^{-2}, about 22 mW mm^{-2}, about 23 mW mm^{-2}, about 24 mW mm^{-2}, or about 25 mW mm^{-2}, inclusive, including any values between these numbers. In some embodiments the neuronal cell can be an excitatory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In some embodiments the neuronal cell can be an inhibitory neuron located in the pre-frontal cortex of a non-human animal. In still other embodiments, the inhibitory neuron can be a parvalbumin neuron. In some embodiments, the inhibitory and excitatory neurons can be in a living non-human animal. In other embodiments, the inhibitory and excitatory neurons can be in a brain slice from a non-human animal.

Methods for identifying a chemical compound that selectively alters the E/I balance in neurons residing in the same microcircuit

In some aspects, there is provided a method for identifying a chemical compound that selectively inhibits the depolarization of excitatory or inhibitory neurons residing in the same microcircuit, the method comprising: (a) selectively depolarizing an excitatory neuron comprising a first light-activated protein with light having a first wavelength or selectively depolarizing an inhibitory neuron comprising a second light-activated protein with light having a second wavelength; (b) measuring an excitatory post synaptic potential (EPSP) in response to selectively depolarizing the excitatory neuron comprising a first light-activated protein or measuring an inhibitory post synaptic current (IPSC) in response to selectively depolarizing an inhibitory neuron comprising a second light-activated protein; (c) contacting the excitatory neuron or the inhibitory neuron with a chemical compound; (d) measuring the excitatory post synaptic potential (EPSP) or measuring the inhibitory post synaptic current (IPSC) to determine if contacting either the excitatory neuron or the inhibitory neuron with the chemical compound selectively inhibits the depolarization of either neuron. In some embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 1. In other embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 3. In some embodiments, the first light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO: 5. In some aspects, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:11. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:12. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:13. In some embodiments, the second light-activated protein can comprise a protein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence shown in SEQ ID NO:14. In some embodiments, the chemical compound can be a member of a combinatorial chemical library. In other embodiments, the method further comprises

assaying the chemical compound to determine if it adversely affects the function of cardiac tissue or the cardiac action potential in mammals.

In some embodiments, the first light-activated protein can be activated by green light. In one embodiment, the first light-activated protein can be activated by light having a wavelength of about 560 nm. In one embodiment, the first light-activated protein can be activated by red light. In another embodiment, the first light-activated protein can be activated by light having a wavelength of about 630 nm. In other embodiments, the second light-activated protein can be activated by violet light. In one embodiment, the second light-activated protein can be activated by light having a wavelength of about 405 nm. In some embodiments, the light-activated proteins can be activated by light pulses that can have a duration for any of about 1 millisecond (ms), about 2 ms, about 3, ms, about 4, ms, about 5 ms, about 6 ms, about 7 ms, about 8 ms, about 9 ms, about 10 ms, about 15 ms, about 20 ms, about 25 ms, about 30 ms, about 35 ms, about 40 ms, about 45 ms, about 50 ms, about 60 ms, about 70 ms, about 80 ms, about 90 ms, about 100 ms, about 200 ms, about 300 ms, about 400 ms, about 500 ms, about 600 ms, about 700 ms, about 800 ms, about 900 ms, about 1 sec, about 1.25 sec, about 1.5 sec, or about 2 sec, inclusive, including any times in between these numbers. In some embodiments, the light-activated proteins can be activated by light pulses that can have a light power density of any of about 0.05 mW mm^{-2}, about 0.1 mW mm^{-2}, about 0.25 mW mm^{-2}, about 0.5 mW mm^{-2}, about 0.75 mW mm^{-2}, about 1 mW mm^{-2}, about 2 mW mm^{-2}, about 3 mW mm^{-2}, about 4 mW mm^{-2}, about 5 mW mm^{-2}, about 6 mW mm^{-2}, about 7 mW mm^{-2}, about 8 mW mm^{-2}, about 9 mW mm^{-2}, about 10 mW mm^{-2}, about 11 mW mm^{-2}, about 12 mW mm^{-2}, about 13 mW mm^{-2}, about 14 mW mm^{-2}, about mW mm^{-2}, about 16 mW mm^{-2}, about 17 mW mm^{-2}, about 18 mW mm^{-2}, about 19 mW mm^{-2}, about 20 mW mm^{-2}, about 21 mW mm^{-2}, about 22 mW mm^{-2}, about 23 mW mm^{-2}, about 24 mW mm^{-2}, or about 25 mW mm^{-2}, inclusive, including any values between these numbers. In some embodiments the neuronal cell can be an excitatory neuron located in the pre-frontal cortex of a non-human animal. In other embodiments, the excitatory neuron can be a pyramidal neuron. In some embodiments the neuronal cell can be an inhibitory neuron located in the pre-frontal cortex of a non-human animal. In still other embodiments, the inhibitory neuron can be a parvalbumin neuron. In some embodiments, the inhibitory and excitatory neurons can be in a living non-human animal. In other embodiments, the inhibitory and excitatory neurons can be in a brain slice from a non-human animal.

EXEMPLARY EMBODIMENTS

The present disclosure relates to a light-activated chimera opsin that modifies a membrane voltage when expressed therein. While the present disclosure is not necessarily limited in these contexts, various aspects of the disclosure may be appreciated through a discussion of examples using these and other contexts.

Various embodiments of the present disclosure relate to a light-activated opsin modified for expression in cell membranes including mammalian cells. The opsin is derived from a combination of two different opsins, *Volvox* channelrhodopsin (VChR1) and *Chlamydomonas reinhardtii* channelrhodopsin (ChR1). The opsin can be useful for expressing at levels of a higher rate than either of the individual opsins from which it is derived.

In certain more specific embodiments, the genetic sequence of ChR1/VChR1 chimera (C1V1) is primarily VChRl. Portions of the VChRl sequence associated with trafficking are replaced with homologous sequences from ChRl.

Various embodiments relate to modification directed toward the addition of a trafficking signal to improve expression in mammalian cells.

Certain aspects of the present disclosure are directed to further modified versions of C1V1. For example, certain embodiments include a mutation E162T to C1V1, which experiments suggest provides an accelerated photocycle (*e.g.,* almost 3-fold).

Various embodiments of the present disclosure relate to an optogenetic system or method that correlates temporal, spatial and/or cell-type-specific control over a neural circuit with measurable metrics. The optogenetic system uses a variety of opsins, including C1V1 and/or C1V1 variants, to assert control over portions of neural circuits. For instance, various metrics or symptoms might be associated with a neurological disorder. The optogenetic system targets a neural circuit within a patient for selective control thereof. The optogenetic system involves monitoring the patient for the metrics or symptoms associated with the neurological disorder. In this manner, the optogenetic system can provide detailed information about the neural circuit, its function and/or the neurological disorder.

Consistent with the embodiments discussed herein, particular embodiments relate to studying and probing disorders using a variety of opsins. Other embodiments relate to the identification and/or study of phenotypes and endophenotypes. Still other embodiments relate to the identification of treatment targets.

Aspects of the present disclosure are directed toward the artificial inducement of disorder/disease states on a fast-temporal time scale. The use of an opsin such as C1V1 can be particularly useful based on characteristics regarding an accelerated photocycle. Moreover, certain embodiments allow for reversible disease states, which can be particularly useful for establishing baseline/control points for testing and/or for testing the effects of a treatment on the same animal when exhibiting the disease state and when not exhibiting the disease state. The use of opsins such as C1V1 allows for the control of a cell using a light source. The C1V1 reacts to light, causing a change in the membrane potential of the cell. The removal of the light and the subsequent cessation of the activation of C1V1 allows for the cell to return to its baseline state. Various other possibilities exist, some of which are discussed in more detail herein.

Various aspects of the present disclosure are directed to an E122T mutation of a C1V1 opsin. In certain embodiments of the present disclosure, the E122T mutation shifts maximum absorption of C1V1 or its variants toward the red light spectrum with respect to the un-mutated opsin.

Various embodiments of the present disclosure relate to an opsin modified for expression in mammalian cells and shifted, with respect to ChR2, for maximum absorption in the green light spectrum. The C1V1 opsin is derived from a combination of opsins and expresses at a higher rate than either of the opsins from which it is derived. The opsin, C1V1, is derived from *Volvox* channelrhodopsin (VChR1) and *Chlamydomonas reinhardtii* channelrhodopsin (ChR1). The resulting opsin, C1V1 and its variants, have a maximum absorption at wavelengths between 530 nm and 546 nm.

Certain aspects of the present disclosure are directed to further modified versions of C1V1. For example, certain embodiments include a mutation E122T, which shifts the maximum absorption of C1V1 towards the red light spectrum. Other modifications can include an additional mutation E162T, which experiments suggest provides an accelerated photocycle in addition to the red shift provided by the E122T mutation.

In some embodiments, there is provided a transmembrane molecule derived from VChR1 and having the traffic sequences replaced with homologous sequences from ChRl. In some embodiments, the molecule further includes a mutation E122T. In other embodiments, the molecule further includes mutations at E162T and E122T. In certain embodiments, the molecule activates an ion channel in response to green light. In one embodiment, the molecule has a maximum light absorption of approximately 546nm. In

another embodiment, the molecule has a maximum light absorption of approximately 535nm.

In some embodiments, there is provided an animal cell comprising: an integrated exogenous molecule which expresses an ion channel that is responsive to red light; the exogenous molecule derived from VChR1 and including transmembrane traffic sequences thereof replaced by homologous sequences from ChR1. In some embodiments, the exogenous molecule further includes E122T. In other embodiments, the cell has a neural firing ratio of about 14% to 94% in response to light having wavelengths of 405nm and 560 nm, respectively. In other embodiments, the cell has a neural firing ratio of about 11% to 72% in response to light having wavelengths of 405nm and 560 nm, respectively.

Additional example embodiments of the present disclosure relate to the use of a hybrid ChR1/VChR1 chimera that contains no ChR2 sequence at all, is derived from two opsins genes that do not express well individually, and is herein referred to as C1V1. Embodiments of the present disclosure also relate to improvements of the membrane targeting of VChR1 through the addition of a membrane trafficking signal derived from the $K_{ir}2.1$ channel. Confocal images from cultured neurons expressing VChRl-EYFP revealed a large proportion of intracellular protein compared with ChR2; therefore, membrane trafficking signal (ts) derived from the $K_{ir}2.1$ channel was used to improve the membrane targeting of VChRl. Membrane targeting of this VChRl-ts-EYFP was slightly enhanced compared with VChRl-EYFP; however, mean photocurrents recorded from cultured hippocampal neurons expressing VChRl-ts-EYFP were only slightly larger than those of VChRl-EYFP. Accordingly, embodiments of the present disclosure relate to VChR1, which has been modified by exchanging helices with corresponding helices from other ChRs. For example, robust improvement has been discovered in two chimeras where helices 1 and 2 were replaced with the homologous segments from ChRl. It was discovered that whether splice sites were in the intracellular loop between helices 2 and 3 (at ChR1 residue Ala145) or within helix 3 (at ChR1 residue Trp163), the resulting chimeras were both robustly expressed and showed similarly enhanced photocurrent and spectral properties. This result was unexpected as ChR1 is only weakly expressed and poorly integrated into membranes of most mammalian host cells.

Specific aspects of the present disclosure relate to microbial opsin genes adapted for neuroscience, allowing transduction of light pulse trains into millisecond-timescale membrane potential changes in specific cell types within the intact mammalian brain (e.g., channelrhodopsin (ChR2), *Volvox* channelrhodopsin (VChR1) and halorhodopsin

(NpHR)). ChR2 is a rhodopsin derived from the unicellular green algae *Chlamydomonas reinhardtii*. The term "rhodopsin" as used herein is a protein that comprises at least two building blocks, an opsin protein, and a covalently bound cofactor, usually retinal (retinaldehyde). The rhodopsin ChR2 is derived from the opsin Channelopsin-2 (Chop2), originally named Chlamyopsin-4 (Cop4) in the *Chlamydomonas* genome. The temporal properties of one depolarizing channelrhodopsin, ChR2, include fast kinetics of activation and deactivation, affording generation of precisely timed action potential trains. For applications seeking long timescale activation, it has been discovered that the normally fast off-kinetics of the channelrhodopsins can be slowed. For example, certain implementations of channelrhodopsins apply 1mW/mm^2 light for virtually the entire time in which depolarization is desired, which can be less than desirable.

Much of the discussion herein is directed to ChR2. Unless otherwise stated, the disclosure includes a number of similar variants. Examples include, but are not limited to, Chop2, ChR2-310, Chop2-310, and *Volvox* channelrhodopsin (VChR1). For further details on VChRl, reference can be made to "Red-shifted optogenetic excitation: a tool for fast neural control derived from *Volvox carteri,*" *Nat Neurosci.* June 2008, 11(6):631-3. Epub 2008 Apr 23, the disclosure of which is fully incorporated herein by reference in its entirety. In other implementations, similar modifications can be made to other opsin molecules. For instance, modifications/mutations can be made to ChR2 or VChR1 variants. Moreover the modified variants can be used in combination with light-activated ion pumps.

Embodiments of the present disclosure include relatively minor amino acid variants of the naturally occurring sequences. In one instance, the variants are greater than about 75% homologous to the protein sequence of the naturally occurring sequences. In other variants, the homology is greater than about 80%. Yet other variants have homology greater than about 85%, greater than 90%, or even as high as about 93% to about 95% or about 98%. Homology in this context means sequence similarity or identity, with identity being preferred. This homology can be determined using standard techniques known in sequence analysis. The compositions of embodiments of the present disclosure include the protein and nucleic acid sequences provided herein, including variants which are more than about 50% homologous to the provided sequence, more than about 55% homologous to the provided sequence, more than about 60% homologous to the provided sequence, more than about 65% homologous to the provided sequence, more than about 70% homologous to the provided sequence, more than about 75% homologous to the provided sequence, more than about 80% homologous to the provided sequence, more than about 85% homologous to the

provided sequence, more than about 90% homologous to the provided sequence, or more than about 95% homologous to the provided sequence.

As used herein, "stimulation of a target cell" is generally used to describe modification of the properties of the cell. For instance, the stimulus of a target cell may result in a change in the properties of the cell membrane that can lead to the depolarization or polarization of the target cell. In a particular instance, the target cell is a neuron and the stimulus affects the transmission of impulses by facilitating or inhibiting the generation of impulses (action potentials) by the neuron.

For further details on light-activated opsins, reference can be made to PCT publication No. WO 2010/056970, entitled "Optically-Based Stimulation of Target Cells and Modifications Thereto," to Deisseroth et al., which is fully incorporated herein by reference in its entirety.

EXAMPLES

Example 1: Development of chimeric channelrhodopsin variant C1V1

In this example, a tool that would permit the driving of cortical E/I elevations and the monitoring of gamma oscillations in cortical slices, as well as *in vivo* in live animal experiments, was sought, with three key properties: 1) much higher potency to enable dose-response investigation; 2) low desensitization to allow for step-like changes in E/I balance; and 3) redshifted excitation to allow comparative drive of different populations within the same preparation.

These experiments were initially attempted with VChR1, which displays both a redshift and reduced desensitization[14], but previous investigation suggested that photocurrents in cells expressing VChR1 were small (~100-150 pA[14]), and did not elicit robust synaptic activity in downstream cells (not shown). Indeed, when first attempting to express VChR1 in cells, only small photocurrents were observed, (**FIG. 1A**) consistent with previous findings. Adding a membrane trafficking signal derived from the Kir2.1 channel to generate VChR1-is-EYFP delivered only a modest trend toward enhanced photocurrents compared with VChR1-EYFP (**FIG 1B**). However, noting that in ChR2, replacing transmembrane segments with the homologous region from ChR1 increased membrane targeting and enhanced photocurrents, it was hypothesized that a similar systematic exchange between the helices of VChR1 with the corresponding helices from other ChRs, might similarly result in enhanced membrane expression in HEK cells.

Materials and Methods

Chimeric channelrhodopsin variant C1V1 was generated by fusing either a wild-type or human codon-optimized channelrhodopsin-1 with a human codon-adapted VChR1 (GenBankTM accession number ACD70142.1) by overlap extension PCR. C1V1 splice variants were generated by overlap PCR. Variant one contained the first 145 amino acids of ChR1 and amino acids 102 to 316 of VChR1. Variant two contained the first 162 amino acids of ChR1 and amino acids 119 to 316 of VChRl. The resultant chimeric PCR fragments were cloned into pECFP-N1 (Clonetech, Mountain View, CA) and into lentiviral expression vectors under the CaMKIIα promoter. The membrane trafficking signal was derived from the Kir2.1 channe1. Mutations were confirmed by sequencing the coding sequence and splice sites. For AAV-mediated gene delivery, opsin-EYFP fusions along with the CaMKIIα promoter were subcloned into a modified version of the pAAV2-MCS vector. Cre-dependent opsin expression was achieved by cloning the opsin-EYFP cassette in the reverse orientation between pairs of incompatible lox sites (loxP and 1ox2722) to generate a double floxed inverted open reading frame (D10) under the control of the elongation factor la (EF- 1α) promoter. All constructs are available from the Deisseroth Lab (www.optogenetics.org).

HEK293 cells were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 2mM glutamine (Biochrome, Berlin, Germany), and 1% (w/w) penicillin/streptomycin. Cells were seeded onto coverslips at a concentration of 0.175 x 106 cells/ml and supplemented with 1 μM all-trans retinal. Transient transfection was performed with Fugene 6 (Roche, Mannheim, Germany) and recordings were done 20-28 hours later. Photocurrents in transiently transfected HEK293 cells were recorded by conventional whole-cell patch-clamp. The external solution contained [mM]: 140 NaC1, 2 $CaC1_2$, 2 $MgC1_2$, 2 KCl, 10 HEPES (pH 7.2). The internal solution contained [mM]: 110 NaCl, 10 EGTA, 2 $MgC1_2$, 1 $CaC1_2$, 5 KC1, 10 HEPES (pH was adjusted to 7.2 either using CsOH or HC1). Patch pipettes were pulled with micropipette puller model P-97 (Sutter Instrument Co., Novato, CA) from microhaematocrit-tubes (Hecht-Assistant, Sondheim, Germany) with 1.5-2 MΩ resistance. HEK cell whole-cell patch-clamping was performed with an EPC 7 (HEKA, Elektronik GmbH, Lambrecht, Germany) amplifier. Analog data was sampled at 20kHz, digitized with Digidata1440 (Molecular Devices, Foster City, CA) and displayed using pClamp10.1 Software (Molecular Devices, Foster City, CA). For recording wavelength dependence, a light guide from a Polychrome V unit (TILL Photonics, Planegg, Germany) was mounted on the epiluminescence port of an Olympus

IX70 microscope. For reflecting light into the objective a beam splitter (70% R / 30% T) was used resulting in a final photon density of ~ 1 x 10_{22} photons m^{-2} s^{-1} at 470 nm on the coverslip. For recording the action spectra only 50% of the light intensity was used. The polychrome V Unit was controlled with Tillvision Software (TILL Photonics, Planegg, Germany) synchronized with the pClamp Software.

Results

Interestingly, the most robust improvement in chimeras was found where helix 1 and 2 were replaced with the homologs from ChR1 (**FIG. 1C-D**). Two chimeric ChR1-VChR1 channels for membrane targeting and photocurrent size were tested in cultured HEK293 cells. The first was joined in the second intracellular loop after A1a145 of ChR1, and the second was joined within helix three after Trp163 of ChR1 (**FIG. 1C**). Whereas both variants were nearly equally well expressed in HEK293 cells (**FIG. 1D**), in cultured neurons the second variant expressed more robustly (**FIG. 1E**) and showed greatly enhanced peak photocurrents (888 ± 128 pA, n = 11 cells; $p < 0.0005$) compared with VChR1-EYFP (**FIG. 1B**). The action spectrum peak remained robustly redshifted relative to ChR2 (**Table 1; FIG. 1F**), and the ionic selectivity of the chimera was similar to that previously reported for ChR2 and VChR1 (**FIG. 1G**) Adding the Kir2.1 trafficking sequence to this hybrid trended to further increased photocurrents (1104 ± 123 pA, n = 12 cells; $p < 0.0005$ compared with VChR1-EYFP, p = 0.23 compared with C1V1-EYFP; **FIG. 1B; Tables 1- 2**). The resulting hybrid ChR1/VChR1 chimera contains no ChR2 sequence at all, is remarkably derived from two opsin genes that do not express well alone, and is here referred to as ClV1 (**FIG. 1 A, H**).

Example 2: Optimization of photocurrent kinetics of C1V1

Fast deactivation properties[28] of this redshifted opsin would be required for maximal temporal as well as spectral separation from other opsins that are activated by wavelengths located towards the blue end of the visible spectrum. However, it was found that the photocurrents displayed by C 1V1-ts-EYFP exhibited >10-fold slower decay than ChR2, and even slower decay than the original VChR1 (**FIG. 2A;** To_{ff} 156 ± 12 ms and 132 ± 12 ms for C1V1-ts-EYFP (n = 4) and VChR1-EYFP (n = 5), respectively; **Table 1**), potentially precluding the use of ClV1 for applications requiring rapid firing rates. To correct the photocurrent kinetics of C1V1, the chromophore region was searched using known structural models [22,28] (**FIG. 1H**) for mutations with faster photocycle kinetics, reduced inactivation and reduced blue absorption. Next, glutamate-122 was mutated to threonine, based on

studies of the glutamate-rich motif in helix 2 showing that this mutation reduces inactivation.[3]

Materials and Methods

All point mutations in C1V1 vectors were generated in the plasmids by site-directed mutagenesis (Agilent Technologies, Palo Alto, CA). The membrane trafficking signal was derived from the $K_{ir}2.1$ channel. Mutations were confirmed by sequencing the coding sequence and splice sites.

Results

The ChETA-homologous mutation E162T[28] markedly accelerated the photocycle almost 3-fold To_{ff} 58 ± 4.7 ms, n = 7 cells; **FIG. 2A, Table 1).** Surprisingly, whereas analogous mutations in ChR2 or other microbial opsins have caused a red-shift[28,29], in C1V1 this mutation shifted the action spectrum in the undesired direction, hypsochromic to 530 nm (**FIG. 1F**; **Table 1**). C1V1-E122T inactivated only by 26% compared to 46% deactivation of ChR2 (**FIG. 2B, Table 1**); in addition, the spectrum was further red-shifted to 546 nm (**FIG. 1F, Table 1**) and showed a striking loss of the problematic blue shoulder of the C1V1 action spectrum. Finally, in the double mutant E122T/E162T, inactivation of the current was even lower than in the E122T mutant and the photocycle was still faster compared to E162T To_{ff} 34 ± 4.9 ms, n = 7 cells; **FIG. 2A, FIG. 2C, Table 1**), while preserving the redshift and absence of the blue shoulder of the action spectrum. Moreover, while the E122 mutant severely reduced photocurrent amplitude (**FIG. 2D**, the double mutant restored the very high currents characteristic of the original C1V1-ts. Thus, multiple surprising and useful properties of the individual mutations were conserved in the double mutant, trafficking-enhanced ClV1 chimera.

Table 1: Spectral/kinetic properties of ChR2, VChR1 and C1V1 variants.

Peak activation wavelength was recorded in HEK cells using 2 ms light pulses at the peak activation wavelength. Deactivation kinetics (τ_{off}) and peak photocurrents were recorded in cultured hippocampal neurons using 2 ms light pulses at the maximal activation wavelength. To identify the optimal variants for combinatorial activation with ChR2, the percent response at 405 nm and 560 nm was recorded in HEK cells. Desensitization of the photocurrent was recorded using 300 ms light pulses, quantifying the decay of the peak photocurrent (I_{max}) to the steady state.

	Absorption maximum (nm)	Toff Kinetics pH7.2 (ms)	Peak current (pA) at -60 Mv*	Ratio 405/560	Desensitation %
ChR2	460± 6 (N=5)	10 ± 1(N=5)	816± 181 (N=5)	60% : 8% (N = 7)	65±8 (N=5)
VChR1	543±7 (N= 7)	85 ± 11(N= 6)	284±54(N= 5)	9% : 82% (N = 7)	53±10 (N=18)
C1V1	539 ±4(N= 10)	60 ±6(N= 6)	1035± 158 (N =6)	28% : 86% (N = 10)	46±12 (N=14)
C1V1(E162T)	530 ±4(N= 6)	23 ±5(N= 4)	1183±53(N= 6)	20% : 71% (N = 6)	41±12 (N=7)
C1V1(E122T)	546 ±5(N= 4)	55 ±8(N= 5)	572±21 (N= 5)	14% : 94% (N = 4)	26±6 (N 4)
C1V1(E122T, E162T)	535 ±5(N= 7)	12 ±1(N= 5)	1072±89(N= 9)	11% : 72% (N = 7)	11±9 (N=9)

Table 2: Summary of p-values from unpaired *t*-test comparisons for peak photocurrent amplitude across all opsins shown in Table 1. Photocurrents were recorded in cultured neurons using a 2 ms light pulse at 540 nm (VChR1 and C1V1 variants) or 470 nm (ChR2(H134R)).

VchR1-YFP	VChR1-ts-YFP	C1V1-YFP	C1V1-ts-YFP	C1V1 (E162T) – ts-Y	C1V1 (E162T/E122T) -ts-YFP	ChR2(H134R)-YFP	
1.0000	0.5770	0.0188	0.0029	6.5E-06	1.1E-05	0.0448	**VChr1-YFP**
	1.0000	0.266	0.0039	1.1E-06	0.0015	0.0579	**VChR1-ts-YFP**
		1.0000	0.3372	0.0399	0.0788	0.8175	**C1V1-YFP**
			1.0000	0.4099	0.8442	0.4222	**C1V1-ts-YFP**
				1.0000	0.3254	0.1490	**C1V1(E162T)-ts-Y**
					1.0000	0.3001	**C1V1(E162T/E122T)-ts-YFP**
						1.0000	**ChR2(H134R)-YFP**

Thus, multiple useful properties of the individual mutations were conserved together in the double mutant.

Example 3: Use of novel C1V1 chimeras in prefrontal cortex neurons

To test these novel C1V1 opsin genes in neurons, lentiviral vectors encoding C1V1-ts-EYFP and the point mutation combinations above were generated. These opsins were then expressed in cultured hippocampal neurons and recorded whole-cell photocurrents under identical stimulation conditions (2ms pulses, 542nm light, 5.5 mW mm^{-2}) to determine whether the improvement in photocurrent amplitude resulted directly from the increased expression of ClV1 compared to VChR1.

Materials and Methods

Animals

Wild-type or transgenic Parvalbumin::Cre C57/BL6J male mice were group housed three to five to a cage and kept on a reverse 12 hour light/dark cycle with *ad libitum* food and water. Experimental protocols were approved by Stanford University IACUC and meet guidelines of the National Institutes of Health guide for the Care and Use of Laboratory Animals.

Whole cell patch-clamp electrophysiology in hippocampal and cortical neurons

Primary hippocampal cultures were isolated from PO Sprague-Dawley rats, plated on Matrigel (Invitrogen)-coated glass coverslips and treated with FUDR to inhibit glia overgrowth. Endotoxin-free plasmid DNA was transfected in cultured neurons using a HEPES buffered Saline/CaPO4 mix. Electrophysiological recordings from individual neurons identified by fluorescent protein expression were obtained in Tyrode media ([mM] 150 NaC1, 4 KC1, 2 MgC12, 2 MgC12, 10 D-glucose, 10 HEPES, pH 7.35 with NaOH) using a standard internal solution ([mM] 130 KGluconate, 10 KC1, 10 HEPES, 10 EGTA, 2 MgC12, pH 7.3 with KOH) in 3-5 MΩ glass pipettes. For cortical slice physiology, acute 300 gm coronal slices from 8-9 week old wild-type C57BL/6J or PV::Cre mice previously injected with virus were obtained in ice-cold sucrose cutting solution ([mM] 11 D-glucose, 234 sucrose, 2.5 KC1, 1.25 NaH2PO4, 10 MgSO4, 0.5 CaC12, 26 NaHCO3) using a Vibratome (Leica). Slices were recovered in oxygenated Artificial Cerebrospinal Fluid (ACSF; [mM] 124 NaC1, 3 KC1, 1.3 MgC12, 2.4 CaC12, 1.25 NaH2PO4, 26 NaHCO3, 10 D-glucose) at 32°C for one hour. Individual neuron patches were obtained after identifying fluorescent protein expression from indicated prefrontal cortical layer under constant ACSF perfusion. Filtered light from a broad-wavelength xenon lamp source (Sutter Instruments

DG-4) was coupled to the fluorescence port of the microscope (Leica DM-LFSA). Band pass filters (Semrock) had 20 nm bandwidth, and were adjusted with additional neutral density filters (ThorLabs) to equalize light power output across the spectrum.

Cultured cell images were acquired on the same microscope using a Retiga Exi CCD camera (Qimaging, Inc.) at 100 ms exposure with 30 gain. Illumination power density was 12 mW mm^{-2} at 500 nm with a standard EYFP filter set. Quantification of fluorescence was performed with ImageJ software by marking a region containing the soma and proximal neurites and calculating for each cell the total integrated pixel intensity in that region, rather than average fluorescence, since photocurrents are likely to be related to the total number of membrane-bound channels rather than average channel expression per area.

Virus preparation and injection

Both Lentiviral- and AAV-mediated gene delivery were used for heterologous expression of opsins in mice. Indicated opsins were driven by either Human calmodulin-dependent protein kinase II alpha (CaMKIIα) promoter to target cortical excitatory neurons or Elongation Factor 1a (EF-1a) in conjunction with a Cre-inducible cassette and followed by the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). Cre-inducible recombinant AAV vector was produced by the University of North Carolina Vector Core (Chapel Hill, NC, USA) and used in conjunction with parvalbumin::Cre transgenic mice to target parvalbumin positive nterneurons. Briefly, AAV constructs were subcloned into a modified version of the pAAV2-MCS, serotyped with AAV5 coat proteins and packaged by the viral vector core at the University of North Carolina. The final viral concentration of AAV vectors as 1x 10^{12} genome copies (gc)/mL. Lentiviral constructs were generated as reported. All constructs are available from the Deisseroth Lab (www.optogenetics.org). Stereotactic viral injections were carried out under protocols approved by Stanford University. Juvenile (4-6 weeks) mice kept under isoflurane anesthesia were arranged in a stereotactic frame (Kopf Instruments) and leveled using bregma and lambda skull landmarks. Craniotomies were preformed so as to cause minimal damage to cortical tissue. Infralimbic prefrontal cortex (IL; from bregma: 1.8mm anterior, 0.35mm lateral, -2.85mm ventral) was targeted using a 10uL syringe and 35g beveled needle (Word Precision Instruments). Virus was infused at a rate of 0.1μL/min. Subjects injected with virus for behavioral studies were additionally implanted with a chronic fiber optic coupling device to facilitate light delivery either with or without an attached penetrating cerebral fiber for local delivery to target cortical region as noted (Doric Lenses, Canada). Penetrating fibers were stereotactically inserted to a depth of -2.5mm from the

same anterior and lateral coordinates and affixed using adhesive luting cement (C&B MetaBond) prior to adhesive closure of the scalp (Vetbond, 3M). Animals were administered analgesic relief following recovery from surgery.

Results

Recordings from cultured hippocampal neurons expressing individual constructs and an integrated fluorescence reading were obtained from each individual cell. In individual cells, fluorescence levels closely correlated with the measured photocurrent amplitudes across constructs (**FIG. 3A**). It was therefore concluded that the potently increased photocurrent of C1V1 resulted chiefly from improved expression in neurons. Since the double mutant C1V1-E122T/E162T showed superior performance along all dimensions tested (photocurrent size, inactivation kinetics, and action spectrum), performance to ChR2(H134R) was also directly compared by measuring integrated somatic YFP fluorescence and peak photocurrents. Surprisingly, C1V1-E122T/E162T cells showed stronger photocurrents than ChR2-H134R cells at equivalent fluorescence levels (**FIG. 3B**), potentially suggestive of increased unitary conductance.

To examine whether C1V1-E122T/E162T would be suitable for optically driving spiking in pyramidal neurons, adeno-associated viral vectors harboring the C1V1-E122T/E162T-ts-EYFP gene under the CaMKIIa promoter (AAV5-CaMKIIa-C1V1-E122T/E162T-ts-EYFP) were generated and injected the virus into the prefrontal cortex of mice. Responses were recorded from expressing neurons in acute slices with 2 ms light pulse trains and compared with responses to trains of current injection (10 ms, 200 pA) at identical frequencies. It was found that the frequency response of neurons to 560 nm pulse trains was indistinguishable from the response to current injections at the same frequencies (**FIG. 3C**; n = 6 cells in 2 slices), suggesting that intrinsic properties of the cell and not ClV1 kinetics limit spiking performance at high rates. Similar performance properties were seen across a range of green, yellow, and amber illumination conditions, with strong performance at the moderate light intensities (<10 mW/mm^2) suitable for *in vivo* applications in mammals (**FIG. 3D**). Indeed, responses at 540 nm and 590 nm were similarly effective at evoking precisely timed action potentials, with lower fidelity at lower light powers as expected (**FIG. 3D**).

With the prominently red-shifted action spectrum, the possibility that ClV1 might even be used to drive spiking with red light, not previously reported with any opsin and potentially important for allowing improved spectral separation as well as control of neurons in deeper tissue, was considered. Whether any C1V1 variants could be used to

drive spikes using far-red light was therefore examined. Although the kinetics of C1V1-E122T were slower than those of C1V1-El22T/E162T, its action spectrum was the most red-shifted of all variants (**FIG. 1F**), and indeed it was found that cells expressing C1V1-E122T responded more strongly to red light (630nm) than cells expressing the double mutant (**FIG. 3E**, *top*). Although on-kinetics of the E122T mutant at 630nm were slower than at 545nm (**FIG. 3F**), photocurrents were recruited using longer pulses of 630 nm light at moderate intensity (**FIG. 3G**) that sufficed to elicit defined spike trains (**FIG. 3H**; **FIG. 3E**, *bottom*).

Example 4: Use of novel C1V1 chimeras in living brain slices from the prefrontal cortex neurons of mice

The study sought to determine whether inhibitory and excitatory neurons residing within the same microcircuit could be targeted with the introduction of C1V1 variants. Independent activation of two neuronal populations within living brain slices was explored; in this case CaMKIIα-C1V1-E122T/E162Tts eYFP and EF1a-DIO-ChR2-H134R-EYFP were expressed in mPFC of PV::Cre mice.

Materials and Methods

Acute 300 μm coronal slices isolated from 8-9 week old wild-type C57BL/6J or PV::Cre mice previously injected with virus were obtained in ice-cold sucrose cutting solution ([mM] 11 D-glucose, 234 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgSO_4$, 0.5 $CaCl_2$, 26 $NaHCO_3$) using a Vibratome (Leica). Slices were recovered in oxygenated Artificial Cerebrospinal Fluid (ACSF; [mM] 124 NaCl, 3 KCl, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 D-glucose) at 32°C for one hour. Individual neuron patches were obtained after identifying fluorescent protein expression from indicated prefrontal cortical layer under constant ACSF perfusion. Filtered light from a broad-wavelength xenon lamp source (Sutter Instruments DG-4) was coupled to the fluorescence port of the microscope (Leica DM-LFSA). Slice physiology data were imported into Matlab and analyzed using custom-written software. Power spectra were calculated using the wavelet method as described by Sohal et al.[55] Briefly, for each frequency f, the recorded traces were first filtered with a bandpass filter between $f \pm 5$ Hz. The filtered traces were then convolved with the wavelet function:

$$W(f,t) = s(t) * g(f,t)$$

$$G(f,t) = e^{(-t^2)(2\sigma^2 s^{-2}ft)^{-1}}$$

where * denotes convolution, $\sigma = 5/(6f)$. The squared amplitude of $W(f,t)$ over a 500 msec window was then used to measure the power at various frequencies. All power spectra from slice recordings were normalized to $1/f$.

Cultured cell images were acquired on the same microscope using a Retiga Exi CCD camera (Qimaging inc.) at 100 ms exposure with the 30 gain. Illumination power density was 12 mW mm^{-2} at 500 nm with a standard EYFP filter set. Quantification of fluorescence was done with ImageJ software by marking a region containing the soma and proximal neuritis and calculating for each cell the total integrated pixel intensity in that region, rather than average fluorescence, since photocurrents are likely to be related to the total number of membrane-bound channels rather than average channel expression per area.

Using current clamp, a single pyramidal cell was stimulated with a train of simulated EPSC waveforms. Individual sEPSC events had peak current magnitudes of 200 pA and decayed with a time constant of 2 ms. Each experiment was divided into 10 sweeps, each 10 seconds long and separated by 5 seconds to minimize rundown. Each sweep was divided into 500 ms segments. The total number of sEPSCs in each 500 ms segment was randomly chosen from a uniform distribution between 0 and 250. Then, the times of the sEPSCs within the 500 ms segment were randomly selected from a uniform distribution extending across the entire segment, simulating excitatory input from a population of unsynchronized neurons. Empirically, these stimulation parameters reliably drove pyramidal neurons at firing rates from 0 - 30 Hz. In conditions marked as baseline, a 10 sec pulse of 590 nm light was delivered to completely inactivate the opsin before running the sEPSC protocol. In conditions where the opsin was activated, a 1 sec pulse of 470 nm light preceded the sEPSC protocol.

To understand the net effect of altered E/I balance on information processing, the mutual information between each neuron's input sEPSC rate and output spike rate, which captures relevant changes in the shape of the I-O curve and in the response variability was computed. First, the joint distribution of sEPSC rate and spike rate by binning in time, sEPSC rate, and spike rate were estimated and the building of a joint histogram. Time bins were 125 ms wide, and sEPSC rate was divided into 10 equally spaced bins from 0 to 500 Hz, although the mutual information results were consistent across a wide range of binning parameters. Spike rate was binned using the smallest meaningful bin width given the time bin width (e.g. 8 Hz bin width for 125 ms time bins). From this joint histogram, compute

mutual information, as previously described was computed equaling the difference between response entropy and noise entropy.

Response entropy quantifies the total amount of uncertainty in the output spike rate of the neuron. Noise entropy quantifies the uncertainty that remains in the output spike rate given the input rate. Note that the maximum information that neural responses can transmit about the input stimulus is the entropy of the stimulus set. For 10 equally spaced input sEPSC rate bins and a uniform distribution of input rate over these bins, the entropy of the input rate is $\log_2(10) = 3.322$ bits.

Mutual information calculated from undersampled probability distributions can be biased upwards. Consequently, all reported values of mutual information, response entropy and noise entropy were corrected for bias due to undersampling. This correction is done by computing values from smaller fractions (from one-half to one-eighth) of the full data and extrapolating to the limit of infinite data. Using 125 ms time windows, the correction factors were always less than 0.07 bits.

Vectors were created and injections were performed as above.

Results

Using this array of multiply engineered opsin genes, the possibilities for combinatorial control of cells and projections within intact mammalian systems was explored. First, it was asked whether excitatory and inhibitory neurons residing within the same microcircuit could be separably targeted by the respective introduction of C1V1 variants and conventional ChRs into these two cell populations. It was found that cultured hippocampal neurons expressing C1V1-E122T/E162T spiked in response to 2 ms green light pulses (560nm) but not violet light pulses. In contrast, cells expressing ChR2-H134R spiked in response to 2 ms 405nm light pulses, but not in response to 2ms 561 nm light pulses (**FIG. 4A**). This principle was therefore tested within living brain slices; in this case AAV5-CaMKIIa::C1V1-E122T/E 162T-ts-mCherry along with AAV5-EFla-DIO::ChR2-H134R-EYFP in was expressed in mPFC of PV::Cre mice (**FIG. 4B**). In pyramidal neurons not expressing any opsin, 405 nm light pulses triggered robust and fast inhibitory postsynaptic currents due to direct activation of PV cells (**FIG. 4C**), while 561 nm light pulses triggered both short-latency EPSCs (**FIG. 4D**) and the expected long-latency polysynaptic IPSCs arising from C1V1-expressing pyramidal cell drive of local inhibitory neurons (**FIG. 4C**).

Excitation of these independent cellular elements *in vivo* with optrode recordings was then explored (**FIG. 4E**, *left*). To examine the inhibitory effect of PV cell activity on

pyramidal neuron spiking, an experimental protocol in which 5Hz violet light pulses (to activate ChR2 in PV cells) preceded 5 Hz green light pulses (to activate C1V1 in excitatory pyramidal neurons) with varying inter-pulse intervals was designed. When violet and green light pulses were separated by 100 ms (**FIG. 4E**, *top trace*), responses to green light pulses were not affected by the violet pulses. However, as delays between violet and green pulses were reduced, green light-induced events became more readily inhibited and were completely abolished when light pulses were presented with sufficient synchrony (**FIG. 4E**, *bottom trace*; summary data in **FIG. 4F**). These data demonstrate combinatorial optogenetic activation within an intact mammal (driving one population alone or in precise temporal combination with another), capitalizing on the speed of the opsins and the properties of the delivered light pulses.

Example 5: Effect of independent activation of corticothalamic (CT) and thalamocortical (TC) glutamatergic axons impinging upon neurons of the reticular thalamic nucleus

To validate the combinatorial control property for axonal projections instead of direct cellular somata stimulation, the effect of independent activation of corticothalamic (CT) and thalamocortical (TC) glutamatergic axons impinging upon neurons of the reticular thalamic nucleus (nRT) (**FIG. 5A**) was examined in thalamic slices.

Materials and Methods

C57BL/6J wild-type (postnatal days 90-120) were anesthetized with pentobarbital (100 mg/kg, i.p.) and decapitated. The thalamic slice preparation and whole-cell patch-clamp recordings were performed. Recordings were obtained from nRT (reticular thalamic) and TC (relay thalamocortical) neurons visually identified using differential contrast optics with a Zeiss (Oberkochen, Germany), Axioskop microscope, and an infrared video camera. For EPSCs and current-clamp recordings, the internal solution contained (in mM): 120 K-gluconate, 11 KC1, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 1 EGTA. pH was adjusted to 7.4 with KOH (290 mOsm). E_{Cl}^- was estimated ~ -60 mV based on the Nernst equation. Potentials were corrected for -15 mV liquid junction potential. For voltage-clamp experiments neurons were clamped at -80 mV and EPSCs were pharmacologically isolated by bath application of the GABAA receptor antagonist picrotoxin (50 μM, Tocris). In all recording conditions, access resistance was monitored and cells were included for analysis only if the access resistance was <18 MΩ and the change of resistance was <25% over the course of the experiment.

600 nL rAAV5/CamKIIα-hChR2(H134R)-EYFP or 900 nL rAAV5-CaMKIIαC1V1(E122T/E162T)-TS-mCherry virus was injected stereotaxically into ventrobasal thalamus (VB) or barrel cortex, respectively, of C57BL/6J wild-type mice *in vivo*, between post-natal days 30-35. Intra-cortical and intra-thalamic (VB) injections were performed in the same mice (n=6). Intra-cortical injections were preformed (from bregma) 1.3 mm posterior, 3 mm lateral, 1.15 mm below the cortical surface. Intra-thalamic injections were 1.7 mm posterior, 1.5 mm lateral, 3.5 mm below the cortical surface. Mice were sacrificed ~2-3 months following injections and horizontal brain thalamic slices were made for optical stimulation and *in vitro* recordings as described above. VB thalamus was removed to avoid disynaptic activation of nRT neurons via the CT-TC-nRT pathway. Cutting VB thalamus from slices removed all photosensitive cell bodies from the preparation, enabled direct examination of CTnRT and TC-nRT projections, and did not affect the electrical membrane properties of nRT neurons (not shown). Optical activation of ChR2-expressing TC and C1V1-expressing CT axons were performed with 405 nm and 560 nm laser stimuli, respectively (5 ms duration light pulses, 2-3 mW) (OEM Laser Systems, MI) delivered with optic fiber (BFL 37-300, ThorLabs) upstream along the CT and TC pathways projecting to nRT. Minimal stimulation intensity was used, defined as the light power that resulted in 50 to 70% failures (30 – 50% successes), fixed response kinetics and low response amplitude variability. Consequent minimal evoked EPSCs presumably resulted from selective optical activation of single CT or TC axons presynaptic to the recorded cell. The stimulation light power was slightly increased (~5% above minimal stimulation) until the number of failures became 0. CT and TC inputs were (simultaneously) stimulated and minimal evoked EPSCs and EPSPs (each individually subthreshold for action potential firing) were recorded in nRT cells.

Statistical significance was calculated using paired or unpaired two-tailed *t*-tests, as applicable. Data were analyzed using Matlab Statistics toolbox or Microsoft Excel

Results

Minimal stimulation of TC axons evoked large and fast excitatory post-synaptic currents (EPSCs) in nRT neurons, whereas minimal stimulation of CT axons evoked small and slow EPSCs in nRT neurons (**FIG. 5B**), both typical for these pathways.

Next the synaptic integration of CT and TC inputs under variable delay conditions between these two inputs was examined. Subthreshold EPSPs from each pathway became suprathreshold for action potential firing only when coincident within 5 ms (**FIG. 5C-D**). The temporal precision of C1V1 and ChR2 activation allowed a reliable control of the delay

between the TC and CT inputs and thus allowed determination of a narrow window (~5ms) of effective synaptic integration in nRT cells, not previously observable with existing electrical, pharmacological, or optogenetic techniques due to the reciprocal connectivity of cortex and thalamus as well as the close approximation of CT and TC axons. These results demonstrate for the first time, in the same intact preparation, independent activation of distinct axonal projections to examine their combinatorial effects on the same target cell.

Example 6: Use of C1V1 and SSFO to achieve spectrotemporal separation of neural activation within the same circuit

In both of the above two preparations, visible-spectrum violet (405 nm) and green (560 nm) lasers were used to achieve separable activation of the two opsins. While 405 nm lasers deliver safe non-UV light, for many applications it may be preferable to use 470 nm laser light for the blue-responsive opsin, since 470 nm light will penetrate tissue more deeply, scatter less, and be more easily and economically delivered from common blue light sources. While this may seem impossible since 470 nm light will partially activate C1V1 (**FIG. 1G**) as well as ChR2, combinatorial control could be achievable even with 470 nm light, capitalizing on both the temporal properties of SSFO and the redshifted nature of C1V1 to achieve "spectrotemporal separation" within intact mammalian tissue. To test this possibility, it was decided to directly compare, within the same preparation, the effects on rhythmic activity of stably potentiating either excitatory or inhibitory cells (**FIG. 6A**)

Materials and Methods

ChR2-D156A and SSFO were generated by inserting point mutations into the pLenti-CaMKIIα¬ChR2-EYFP-WPRE vector using site-directed mutagenesis (Quikchange II XL; Stratagene). Viral gene delivery, coronal brain sectioning, and patch clamp recording were performed as above. Double virus injections to express CaMKIIa::C1V1 and DIO-SSFO in the mPFC of PV::Cre mice were performed.

While handling cells or tissues expressing SSFO, care was taken to minimize light exposure to prevent activation by ambient light. Before each experiment, a 20s pulse of 590 nm light was applied to convert all of the SSFO channels to the dark state and prevent run-down of photocurrents. For acquisition of SSFO activation and deactivation spectra, recordings from cultured neurons were made in voltage clamp mode. For recording activation spectra, a 1 s pulse of varying wavelength was applied, followed by a 10 s 590 nm pulse. Deactivation spectra were acquired by first applying a 1 s 470 nm pulse to activate SSFO, followed by a 10 s pulse of varying wavelength. Net activation or deactivation was calculated by dividing the photocurrent change after the first or second

pulse, respectively, by the maximum photocurrent change induced by the peak wavelength for that cell. Negative values in deactivation spectra resulted from traces in which, for example, a 10 s 470nm pulse led to a slight increase in photocurrent rather than deactivate the channels. This could be the result of the relatively wide (20 nm) band-pass filter width used for these recordings with the Sutter DG-4. Intermediate wavelengths (between 470nm and 520nm) are expected to have a mixed effect on the channel population for the same reasons.

Photon flux calculations for SSFO integration properties were conducted by calculating the photon flux through the microscope objective at each light power, and then dividing to reach the photon flux across the cell surface, based on the diameter of the recorded cells and approximating cell shape as a spheroid.

Results

SSFO is a novel multiply-engineered channelrhodopsin with a decay constant of 29 minutes that can be effectively deactivated at the same wavelengths that activate C1V1 and permits bistable excitation of neurons over many minutes with enhanced light sensitivity. Information regarding SSFOs can be found in International Patent Application Publication No: WO 2010/056970 and United States Patent Application Nos: 61/410,704 and 61/410,711, the contents of which are hereby incorporated by reference herein in their entireties. Double virus injections to express CaMKIIa::C1V1 and DIO::SSFO in acute slices from the mPFC of PV::Cre mice were performed. Under these conditions, excitatory pyramidal cells should respond to redshifted light, and inhibitory PV cells to blue light. Indeed, in response to a 1 s 470 nm light pulse to activate SSFO in the PV cells, the rate of ongoing IPSCs was stably increased from 8.5 ± 1.2 Hz at baseline (period 3, **FIG. 6B**) to 16.8 ± 2.1 Hz after the blue light pulse (period 2; n = 4 recordings, $p < 0.005$, paired t-test; **FIG. 6C**), showing persistent activation of the SSFO-expressing inhibitory cells. Even though 470 nm light will also transiently activate C 1V1, this activation can only occur during the light pulse itself due to the very fast deactivation of C1V1 after light-off; the prolonged post-light period is characterized by SSFO activity only (**FIG. 6B**), illustrating temporal separation of optogenetic control modes. Interestingly, during this prolonged period of elevated PV neuron activity, no significantly elevated peak in the IPSC power spectrum was elicited, suggesting that direct activation of PV neurons alone in this reduced preparation is insufficient to elicit gamma synchrony in the network. However, in marked contrast, during the 470 nm light pulse itself when the same level of PV neuron activation but also partial activation of C 1V 1-expressing pyramidal cells is also expected, a

pronounced gamma peak was consistently observed (peak frequency 39.2 ± 3.5 Hz; $n = 4$ recordings;) that extended into the high-gamma range (>60Hz).

Moreover, in the same experiments (indeed, later in the same recorded sweeps), direct activation in this case of C1V1-pyramidal cells alone with 590 nm light(which simultaneously activates C1V1 in PY cells and deactivates the SSFO in PV cells) led to robust gamma synchrony, with a lower frequency peak (26.6 ± 1 Hz, $n = 6$ recordings). Demonstrating that any residual PV neuron activity linked to the prior history of SSFO activation in PV cells was not necessary for this effect, otherwise-identical sweeps with only a history of C1V1 activation in the pyramidal cells and no prior history of elevated IPSC rate elicited the same result). These results illustrate the integrated principle of spectrotemporal combinatorial control, and also suggest that elevating activity in pyramidal neurons can give rise through network properties to gamma oscillations[31]. Interestingly, during the 470 nm light pulse, when activation of both PV and pyramidal cells was expected, gamma synchrony was consistently observed at higher frequencies than when only excitatory neurons were activated, supporting and extending information on the coordinated importance of both PV and pyramidal cells in eliciting gamma oscillations.[31-33]

Conclusion

In the course of this work, a family of new tools was generated that are referred to as C1V1 variants. C1V1 is a red-shifted opsin gene assembled, remarkably, from pieces of other opsin genes that do not express well alone in neurons, but which were identified in earlier genomic searches (VChR1 and ChR1). C1V1 contains no ChR2 sequence at all, yet its multiply- engineered variants reported here now represent the most potent, most redshifted, and most stable channelrhodopsins known. Mutagenesis in key amino acid positions throughout the retinal binding pocket led to the generation of (1) C1V1(E162T), a high-expressing redshifted opsin gene generated as a fast homolog of the ChETA mutation; (2) C1V1(E122T) which displays the reddest action spectrum shoulder and can even be used to fire action potentials with red light (3) C1V1(E122T/E162T) – a combination mutant with the lowest desensitization, fastest deactivation, least violet-light activation for minimal cross-activation with ChR2, and strong expression. Indeed, C1V1 variants may be selected for different applications based on considerations of current size, deactivation kinetics, and action spectrum (**Table 1**)-- for example, in two-photon work, since 2P activation of ChR2 has been difficult due to current size and rapid kinetics of channel closure, C1V1(E162T) is likely to be of interest. The C1V1 variants enabled

direct testing of the hypothesis that increasing levels of elevated cellular E/I balance would give rise to increasing intensities of gamma rhythmicity, a phenomenon previously linked to both schizophrenia and autism. Of course, the different tools are also synergistic; using C1V1 variants together with ChR2 permitted reliable and separate driving of spiking in the two distinct neuronal populations addressed in this study – the excitatory pyramidal neurons and the fast-spiking, parvalbumin-expressing inhibitory interneurons, and confirm that steady elevated cellular E/I balance was effective at generating gamma-band circuit activity, capitalizing on both kinetic and spectral differences in the optogenetic tools. This type of combinatorial activation can be extended beyond multiple cell types to multiple neural pathway types—for example, the separable activation of spiking, within a single brain region, in two converging axonal afferent pathways arising from distinct locations-- a long-sought goal of systems neuroscience.

The examples, which are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way, also describe and detail aspects and embodiments of the invention discussed above. The foregoing examples and detailed description are offered by way of illustration and not by way of limitation. All publications, patent applications, and patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or patent were specifically and individually indicated to be incorporated by reference. Various embodiments described above, and discussed in the attached Appendices may be implemented together and/or in other manners. One or more of the aspects in the present disclosure and in the Appendices can also be implemented in a more separated or integrated manner, as should be apparent and is useful in accordance with particular target applications. In particular, all publications and appendices cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

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SEQUENCES

SEQ ID NO:1 (Humanized C1V1 amino acid sequence)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVI
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IVWGATSAMCTGWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGICRELVRVMAWTFFVAWG
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SEQ ID NO:2 (Humanized C1V1 nucleotide sequence)

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WLRYAEWLLTCPVLLIHLSNLTGLKDDYSKRTMGLLVSDVGCIVWGATSAMCTGWTKILFFLISLSYGMYTYFHAAK
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SEQ ID NO:10 (Alternative Humanized C1V1 nucleotide sequence (C1V1_25))

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SEQ ID NO:11 ChR2 amino acid sequence

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SVYGSTVGHTIIDLMSKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLV
EDEAEAGAVP

SEQ ID NO:12 ChR2(H134R)

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLAA
GFSILLLMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHRVQWLR
YAEWLLTCPVILIRLSNLTGLSNDYSRRTMGLLVSDIGTIVWGATSAMATGYVKVIFFCL
GLCYGANTFFHAAKAYIEGYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVL
SVYGSTVGHTIIDLMSKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLV
EDEAEAGAVP

SEQ ID NO:13 SFO

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLAAGFSILL
LMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHRVQWLRYAEWLLTSPVILI
HLSNLTGLSNDYSRRTMGLLVSDIGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFHAAKAYIEGY
HTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLSVYGSTVGHTIIDLMSKNCWGLLGHYL
RVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAVP

SEQ ID NO:14 (SSFO)

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLAAGFSILL
LMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHRVQWLRYAEWLLTSPVILI
HLSNLTGLSNDYSRRTMGLLVSAIGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFHAAKAYIEGY
HTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLSVYGSTVGHTIIDLMSKNCWGLLGHYL
RVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAVP

What is claimed is:

1. An animal cell comprising a light-activated protein expressed on the cell membrane, wherein the protein

is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1;

is responsive to light; and

is capable of mediating a depolarizing current in the cell when the cell is illuminated with light.

2. The animal cell of claim 1, wherein the protein further comprises a replacement within the intracellular loop domain located between the second and third transmembrane helices of the chimeric light responsive protein, wherein at least a portion of the intracellular loop domain is replaced by the corresponding portion from the ChR1.

3. The animal cell of claim 2, wherein the portion of the intracellular loop domain is replaced with the corresponding portion from the ChR1 extending to amino acid residue A145 of the ChR1.

4. The animal cell of claim 2, wherein the protein further comprises a replacement within the third transmembrane helix of the chimeric light responsive protein, wherein at least a portion of the third transmembrane helix is replaced by the corresponding sequence of ChR1.

5. The animal cell of claim 4, wherein the portion of the intracellular loop domain is replaced with the corresponding portion from the ChR1 extending to amino acid residue W163 of the ChR1.

6. The animal cell of claim 1, wherein the chimeric protein comprises an amino acid sequence at least 95% identical to the amino acid sequence shown in SEQ ID NO:1.

7. The animal cell of claim 6, further comprising a mutation at amino acid residue(s) E122 and/or E162 of SEQ ID NO:1.

8. The animal cell of claim 7, wherein the mutation at amino acid E122 is to threonine.

9. The animal cell of claim 7, wherein the mutation at amino acid E162 is to threonine.

10. The animal cell of claim 1, wherein the chimeric protein comprises the amino acid sequence of SEQ ID NO:3.

11. The animal cell of claim 1, wherein the chimeric protein comprises the amino acid sequence of SEQ ID NO:1.

12. The animal cell of claim 1, wherein the chimeric protein comprises the amino acid sequence of SEQ ID NO:7.

13. The animal cell of any one of claims 1-12, wherein the protein further comprises a C-terminal membrane trafficking signal.

14. The animal cell of claim 13, wherein the C-terminal membrane trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV.

15. The animal cell of claim 13 or 14, wherein the membrane trafficking signal is linked to the C-terminus of the protein by a linker.

16. The animal cell of any one of claims 1-15, wherein the protein further comprises a C-terminal fluorescent protein selected from the group consisting of EYFP, GFP, CFP, and RFP.

17. The animal cell of any one of claims 1-16, wherein the animal cell is selected from the group consisting of: a neuronal cell, a muscle cell, and a stem cell.

18. The animal cell of any one of claims 1-17, further comprising a second light-activated protein expressed on the cell membrane.

19. The animal cell of claim 18, wherein the second light-activated protein is a protein capable of mediating a hyperpolarizing current in the cell when the cell is illuminated with light.

20. The animal cell of claim 19, wherein the second light-activated protein is selected from the group consisting of: an NpHr, an eNpHr2.0, an eNpHr3.0, an eNpHr3.1 and a GtR3.

21. A population of cells comprising the cell of any one of claims 1-20.

22. A non-human animal comprising a cell of any one of claims 1-20.

23. A brain tissue slice comprising a cell of any one of claims 1-20.

24. An isolated polynucleotide comprising a nucleotide sequence encoding a light activated protein expressed on the cell membrane, wherein the protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1; is responsive to light; and is capable of mediating a depolarizing current in the cell when the cell is illuminated with light.

25. The isolated polynucleotide of claim 24, wherein the polynucleotide comprises a sequence at least 95% identical to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:1.

26. The isolated polynucleotide of claim 24, wherein the polynucleotide comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:3.

27. The isolated polynucleotide of claim 24, wherein the polynucleotide comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:5.

28. The isolated polynucleotide of claim 24, wherein the polynucleotide comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:7.

29. An expression vector comprising any of the polynucleotide of claims 25-28.

30. The expression vector of claim 29, wherein the expression vector is a viral vector.

31. The expression vector of claim 30, wherein the viral vector is selected from the group consisting of: an AAV vector, a retroviral vector, an adenoviral vector, a HSV vector, and a lentiviral vector.

32. A method of using the cell of any one of claims 1-20, comprising activating the light-activated protein with light.

33. A method of using the cell of claim 32, comprising activating the light-activated protein with red light.

34. A method of using the cell of claim 32, comprising activating the light-activated protein with green light.

35. A method of selectively depolarizing excitatory or inhibitory neurons residing in the same microcircuit, the method comprising: selectively depolarizing an excitatory neuron comprising a first light-activated protein, wherein the first light activated protein is depolarized when exposed to light having a first wavelength; or selectively depolarizing an inhibitory neuron comprising a second light-activated protein, wherein the second light activated protein is depolarized when exposed to light having a second wavelength; wherein the first or the second light activated protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1.

36. A method of selectively depolarizing excitatory or inhibitory neurons residing in the same microcircuit, the method comprising: expressing a first light-activated protein in an excitatory neuron; and expressing a second light activated protein in an inhibitory neuron, wherein the first light activated protein is independently depolarized when exposed to light having a first wavelength and wherein the second light activated protein is independently depolarized when exposed to light having a second wavelength; wherein the first or the second light activated protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1.

37. The method of claim 35 or 36, wherein the first light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO: 1, 3, 5, or 7, and wherein the second light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:11, 12, 13, or 14.

38. The method of claim 37, wherein the first light-activated protein is activated by green light and the second light-activated protein is activated by violet light.

39. The method of any one of claims 35-38, wherein the inhibitory and excitatory neurons are in a living non-human animal.

40. The method of any one of claims 35-39, wherein the inhibitory and excitatory neurons are in living brain slices from a non-human animal.

41. The method of any one of claims 35-40, wherein the inhibitory and excitatory neurons are from the prefrontal cortex.

42. The method of any one of claims 35-41, wherein the excitatory neuron comprises a pyramidal neuron and the inhibitory neuron comprises a parvalbumin neuron.

43. A method for identifying a chemical compound that selectively inhibits the depolarization of excitatory or inhibitory neurons residing in the same microcircuit, the method comprising:

(a) selectively depolarizing an excitatory neuron comprising a first light-activated protein with light having a first wavelength or selectively depolarizing an inhibitory neuron comprising a second light-activated protein with light having a second wavelength; wherein the first or the second light activated protein is a chimeric protein derived from VChR1 from *Volvox carteri* and ChR1 from *Chlamydomonas reinhardti,* wherein the protein comprises the amino acid sequence of VChR1 having at least the first and second transmembrane helices replaced by the first and second transmembrane helices of ChR1;

(b) measuring an excitatory post synaptic potential (EPSP) in response to selectively depolarizing the excitatory neuron comprising a first light-activated protein or measuring an inhibitory post synaptic current (IPSC) in response to selectively depolarizing an inhibitory neuron comprising a second light-activated protein;

(c) contacting the excitatory neuron or the inhibitory neuron with a chemical compound; and

(d) measuring the excitatory post synaptic potential (EPSP) or measuring the inhibitory post synaptic current (IPSC) to determine if contacting either the excitatory neuron or the inhibitory neuron with the chemical compound selectively inhibits the depolarization of either neuron.

44. The method of claim 43, wherein the first light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO: 1, 3, 5, or 7, and wherein the second light-activated protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:11, 12, 13, or 14.

45. The method of claim 44, wherein the first light-activated protein is activated by green light and the second light-activated protein is activated by violet light.

46. The method of any one of claims 43-45, wherein the inhibitory and excitatory neurons are in a living non-human animal.

47. The method of any one of claims 43-46, wherein the inhibitory and excitatory neurons are in living brain slices from a non-human animal.

48. The method of any one of claims 43-47, wherein the inhibitory and excitatory neurons are from the prefrontal cortex.

49. The method of any one of claims 43-48, wherein the excitatory neuron comprises a pyramidal neuron and the inhibitory neuron comprises a parvalbumin neuron.

50. The method of any one of claims 43-49, wherein the chemical compound is a member of a combinatorial chemical library.

51. The method any one of claims 43-50, further comprising assaying the chemical compound on cardiac tissue to determine if the compound adversely affects cardiac action potential.

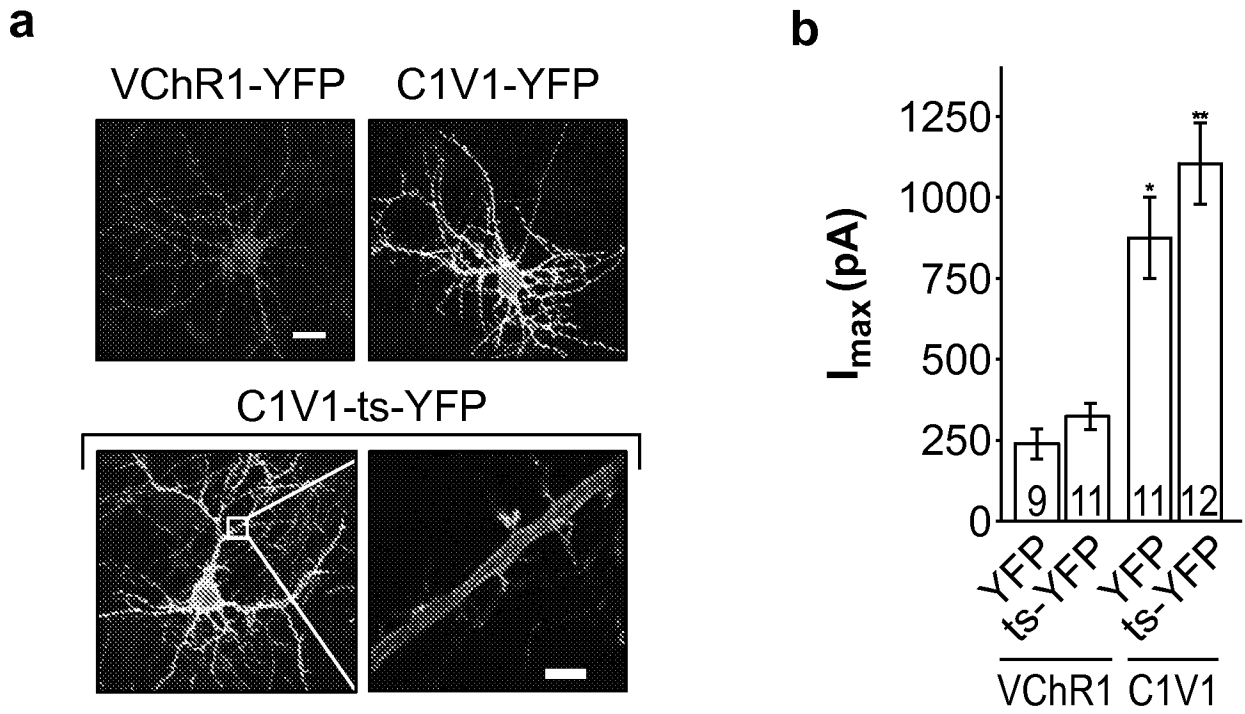

a
VChR1-YFP
C1V1-YFP
C1V1-ts-YFP
b
I_{max} (pA)
1250
1000
750
500
250
0
9
11
11
12
*
**
YFP
ts-YFP
YFP
ts-YFP
VChR1
C1V1

FIG. 1

c

```
ChR1    MSRRPWLLAL ALAVALAAGS AGASTGSDAT VPVATQDGPD YVFHRAHERM 60
ChR2    .......... .....MDYGG ALSAVG.... .......... .......REL 14
VChR1   .......... .......... .......... .......... ..MDYPVARS 8

ChR1    LFQTSYTLEN NGSVICIPNN GQCFCLAWLK SNGTNAEKLA ANILQWITFA 100
ChR2    LFVTNPVVVN .GSVLVP..E DQCYCAGWIE SRGTNGAQTA SNVLQWLAAG 61
VChR1   LIVRYPTDLG NGTVCMP..R GQCYCEGWLR SRGTSIEKTI AITLQWVVFA 56
                                                    TM1

ChR1    LSALCLMFYG YQTWKSTCGW EEIYVATIEM IKFIIEYFHE FDEPAVIYSS 150
ChR2    FSILLLMFYA YQTWKSTCGW EEIYVCAIEM VKVILEFFFE FKNPSMLYLA 111
VChR1   LSVACLGWYA YQAWRATCGW EEVYVALIEM MKSIIEAFHE FDSPATLWLS 106
                                                    Splice 1
        TM2

        Splice 2
ChR1    NGNKTVWLRY AEWLLTCRVI LIHLSNLTGL ANDYNKRTMG LLVSDIGTIV 200
ChR2    TGHRVQWLRY AEWLLTCPVI LIHLSNLTGL SNDYSRRTMG LLVSDIGTIV 161
VChR1   SGNGVVWMRY GEWLLTCPVL LIHLSNLTGL KDDYSKRTMG LLVSDVGCIV 156
          TM3                                TM4

ChR1    WGTTAALSKG YVRVIFFLMG LCYGIYTFFN AAKVYIEAYH TVPKGICRDL 250
ChR2    WGATSAMATG YVKVIFFCLG LCYGANTFFH AAKAYIEGYH TVPKGRCRQV 211
VChR1   WGATSAMCTG WTKILFFLIS LSYGMYTYFH AAKVYIEAFH TVPKGICREL 206
                    TM5

ChR1    VRYLAWLYFC SWAMFPVLFL LGPEGFGHIN QFNSAIAHAI LDLASKNAWS 300
ChR2    VTGMAWLFFV SWGMFPILFI LGPEGFGVLS VYGSTVGHTI IDLMSKNCWG 261
VChR1   VRVMAWTFFV AWGMFPVLFL LGTEGFGHIS PYGSAIGHSI LDLIAKNMWG 256
          TM6                                TM7

ChR1    MMGHFLRVKI HEHILLYGDI RKKQKVNVAG QEMEVETMVH EEDD       344
ChR2    LLGHYLRVLI HEHILIHGDI RKTTKLNIGG TEIEVETLVE DEAEAGAVP  310
VChR1   VLGNYLRVKI HEHILLYGDI RKKQKITIAG QEMEVETLVA EEED       300
```

FIG. 1 (Cont. 1)

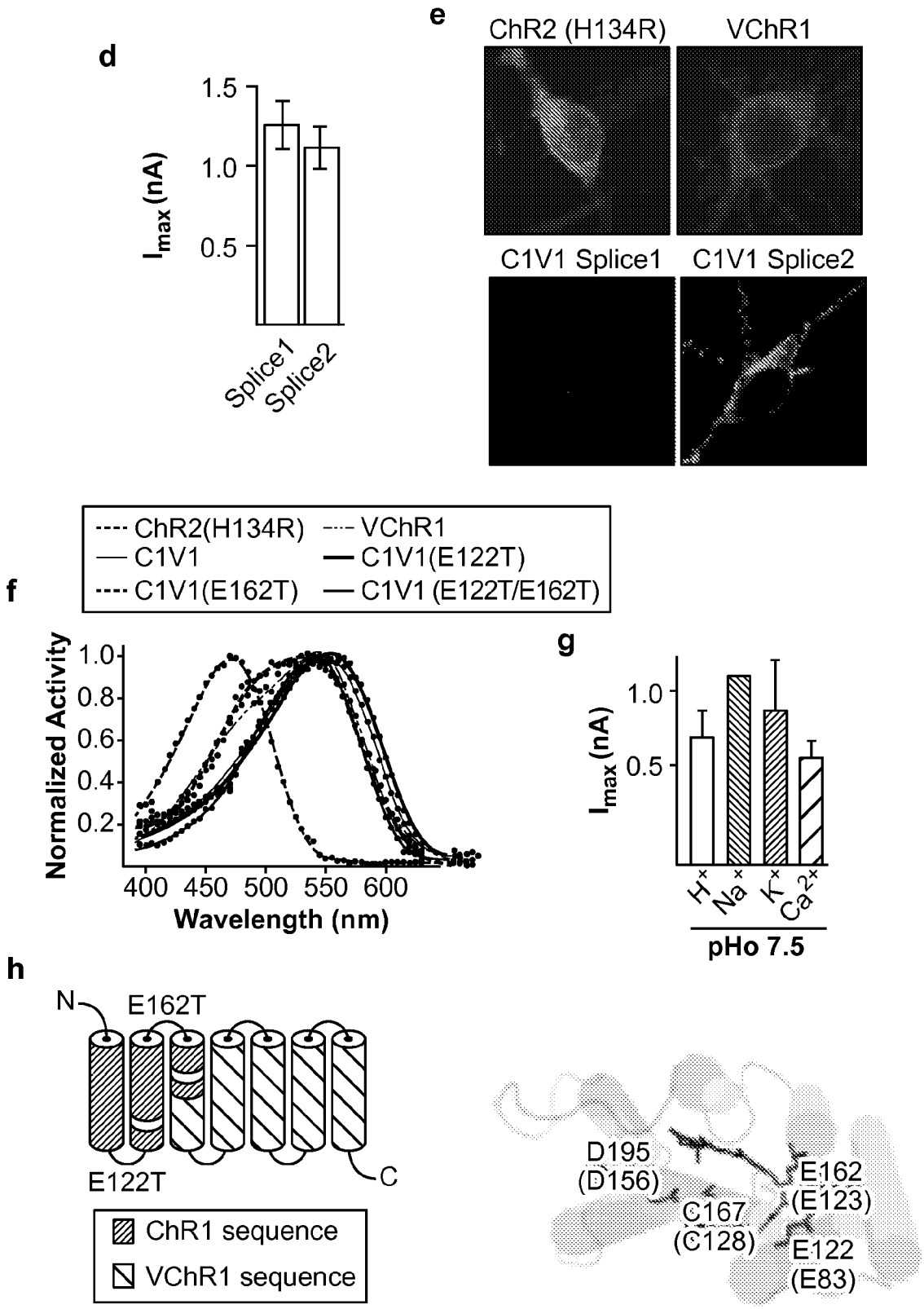

d
1.5
1.0
0.5
I_{max} (nA)
Splice1
Splice2
e
ChR2 (H134R)
VChR1
C1V1 Splice1
C1V1 Splice2
f
ChR2(H134R)
VChR1
C1V1
C1V1(E122T)
C1V1(E162T)
C1V1 (E122T/E162T)
Normalized Activity
1.0
0.8
0.6
0.4
0.2
400 450 500 550 600
Wavelength (nm)
g
1.0
0.5
I_{max} (nA)
H^+
Na^+
K^+
Ca^{2+}
pHo 7.5
h
N
E162T
E122T
C
ChR1 sequence
VChR1 sequence
D195
(D156)
E162
(E123)
C167
(C128)
E122
(E83)

FIG. 1 (Cont. 2)

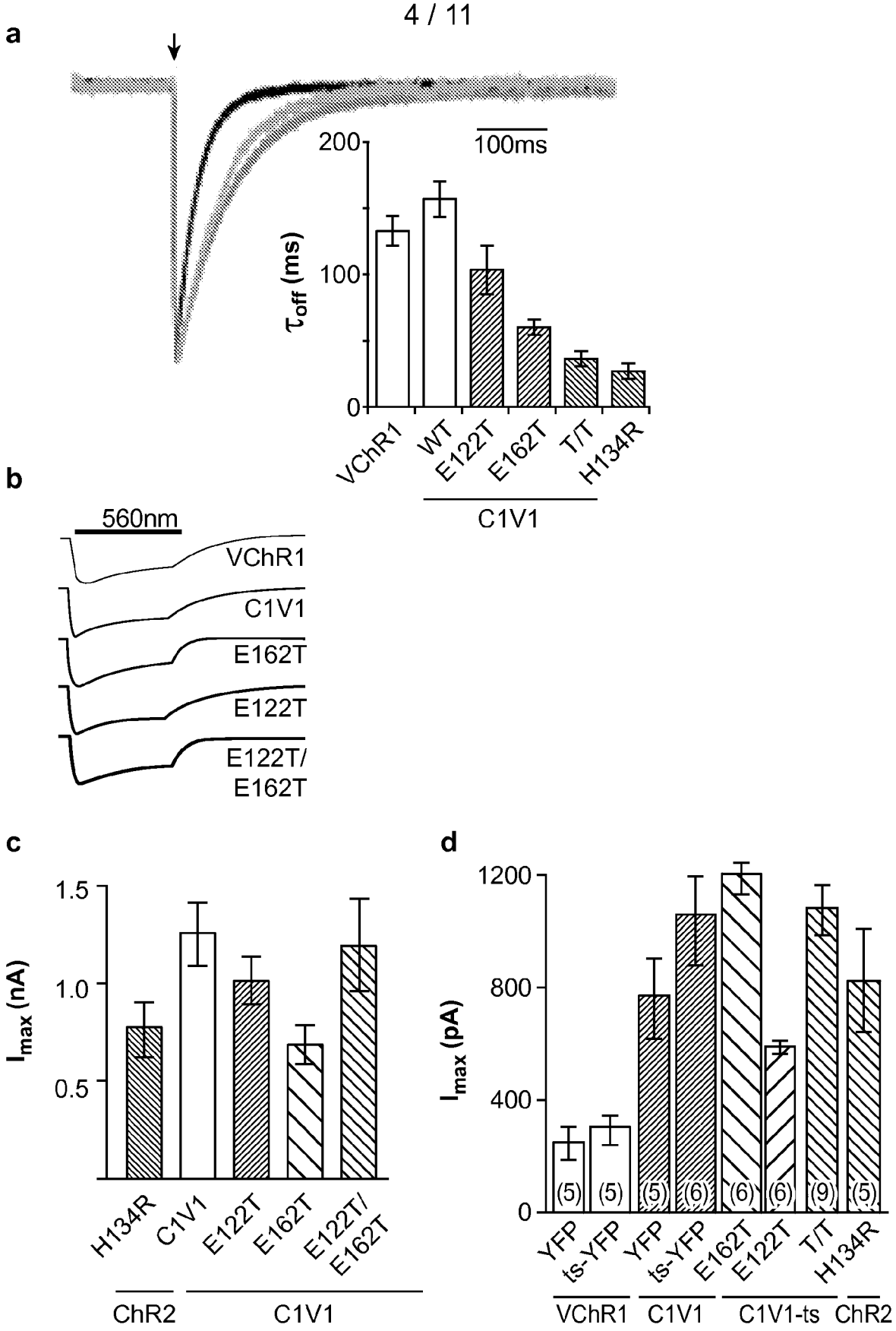

a
100ms
200
100
0
τ_{off} (ms)
VChR1
WT
E122T
E162T
T/T
H134R
C1V1
b
560nm
VChR1
C1V1
E162T
E122T
E122T/
E162T
c
1.5
1.0
0.5
I_{max} (nA)
H134R
C1V1
E122T
E162T
E122T/
E162T
ChR2
C1V1
d
1200
800
400
0
I_{max} (pA)
(5)
(5)
(5)
(6)
(6)
(6)
(9)
(5)
YFP
ts-YFP
YFP
ts-YFP
E162T
E122T
T/T
H134R
VChR1
C1V1
C1V1-ts
ChR2

FIG. 2

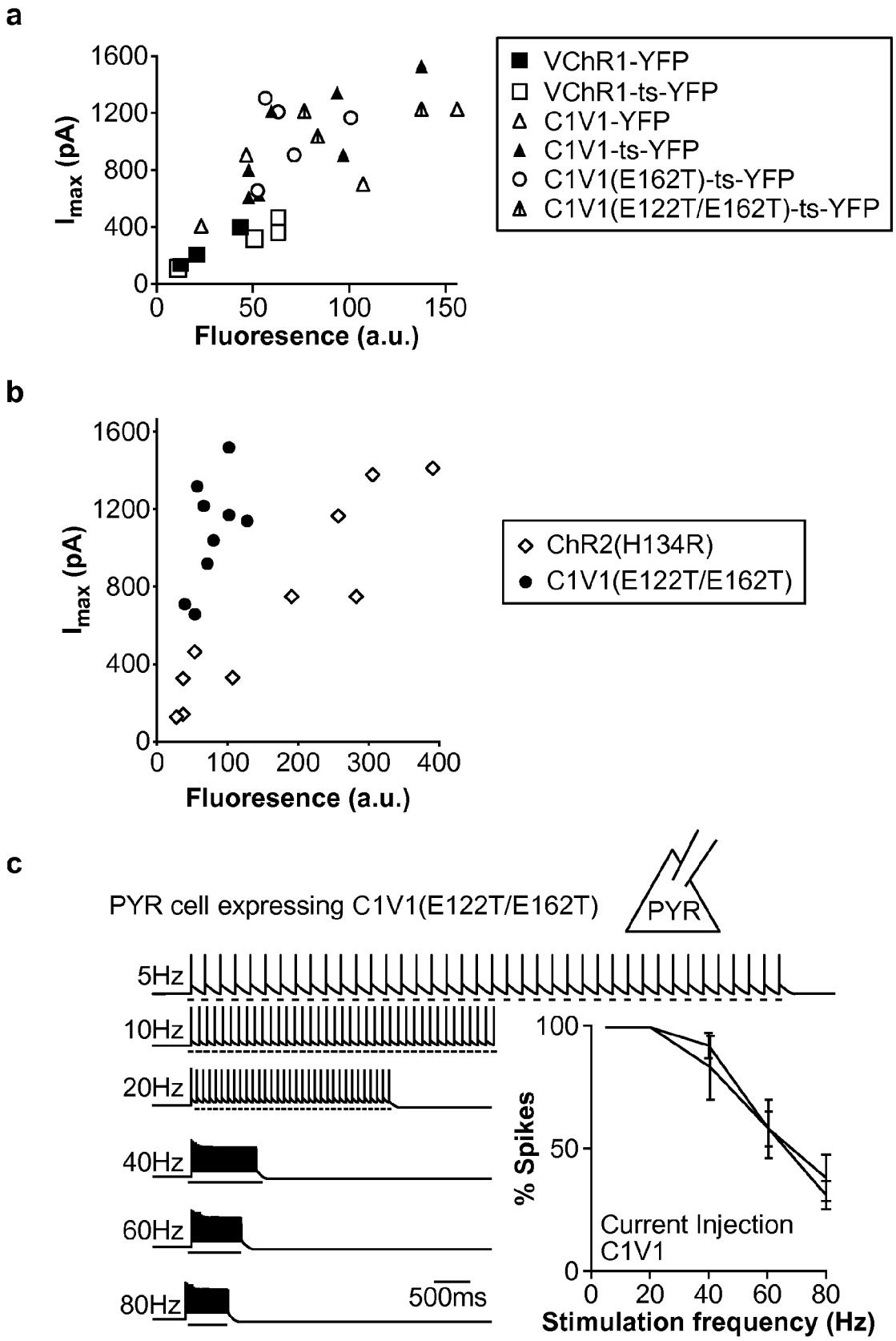

a
1600
1200
800
400
0
I_{max} (pA)
0
50
100
150
Fluoresence (a.u.)
VChR1-YFP
VChR1-ts-YFP
C1V1-YFP
C1V1-ts-YFP
C1V1(E162T)-ts-YFP
C1V1(E122T/E162T)-ts-YFP
b
1600
1200
800
400
0
I_{max} (pA)
0
100
200
300
400
Fluoresence (a.u.)
ChR2(H134R)
C1V1(E122T/E162T)
c
PYR cell expressing C1V1(E122T/E162T)
PYR
5Hz
10Hz
20Hz
40Hz
60Hz
80Hz
500ms
100
50
0
% Spikes
Current Injection
C1V1
0
20
40
60
80
Stimulation frequency (Hz)

FIG. 3

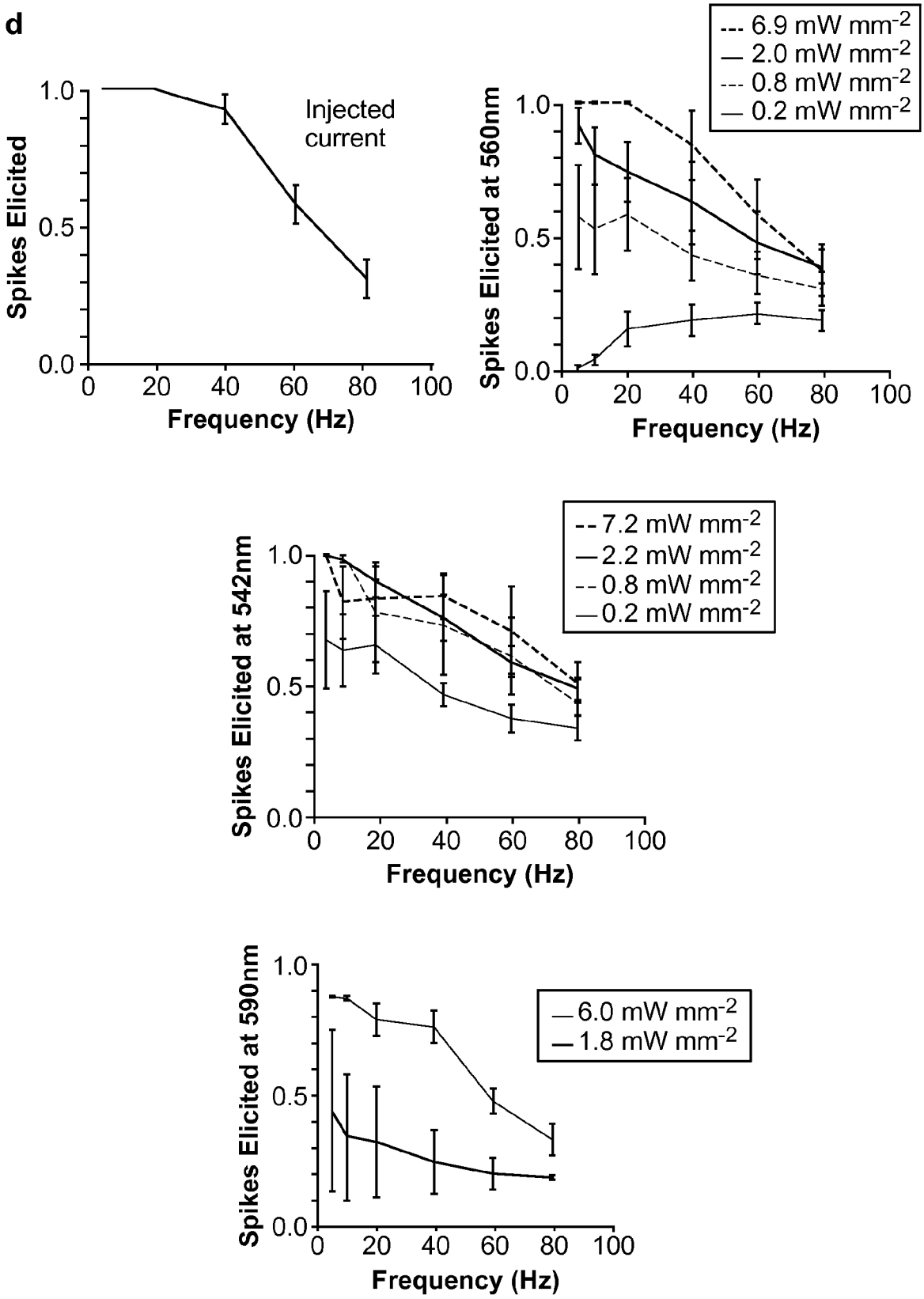
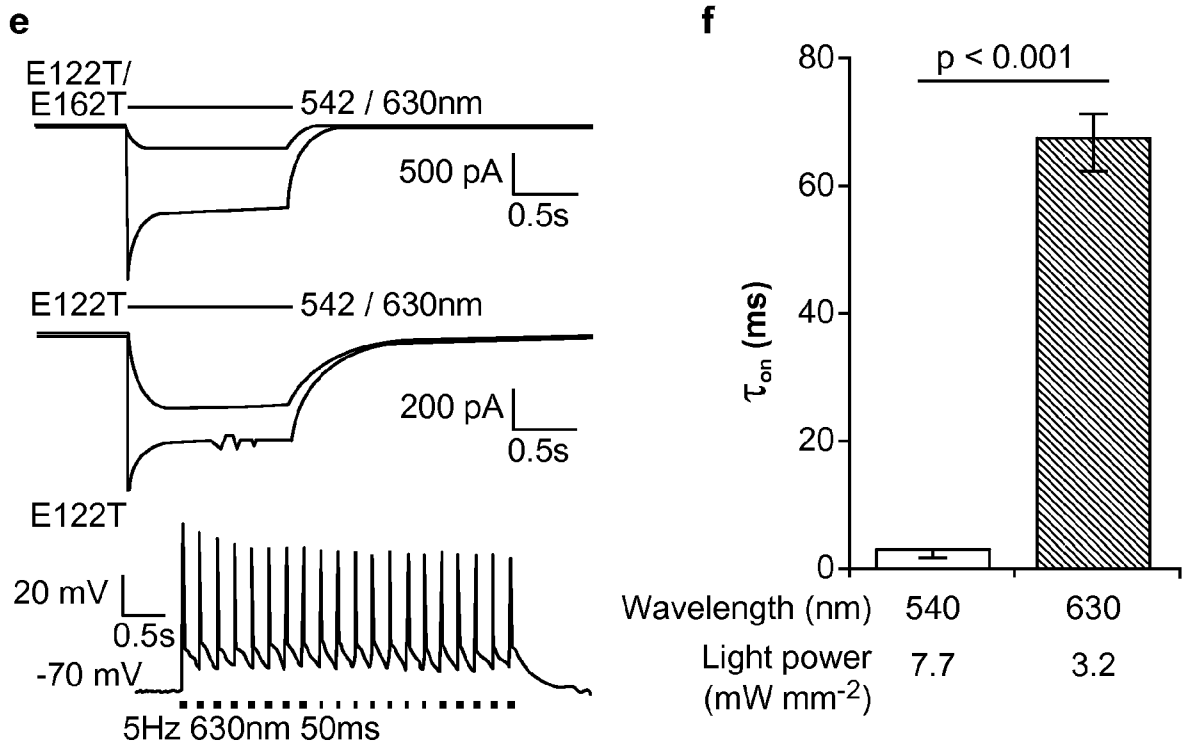
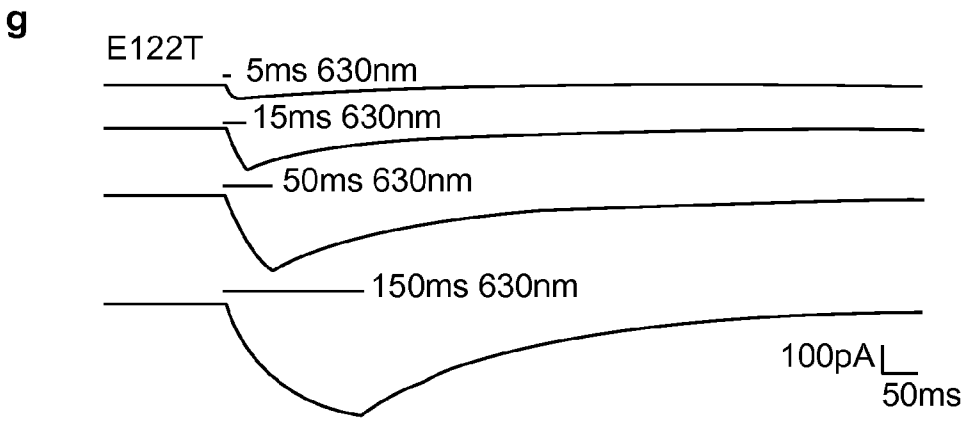

d
Spikes Elicited
1.0
0.5
0.0
0
20
40
60
80
100
Frequency (Hz)
Injected current
Spikes Elicited at 560nm
--- 6.9 mW mm-2
— 2.0 mW mm-2
--- 0.8 mW mm-2
— 0.2 mW mm-2
Spikes Elicited at 542nm
-- 7.2 mW mm-2
— 2.2 mW mm-2
-- 0.8 mW mm-2
— 0.2 mW mm-2
Spikes Elicited at 590nm
— 6.0 mW mm-2
— 1.8 mW mm-2

FIG. 3 (Cont. 1)


e
E122T/
E162T
542 / 630nm
500 pA
0.5s
E122T
542 / 630nm
200 pA
0.5s
E122T
20 mV
0.5s
-70 mV
5Hz 630nm 50ms
f
p < 0.001
80
60
40
20
0
τon (ms)
Wavelength (nm) 540 630
Light power (mW mm-2) 7.7 3.2


g
E122T
5ms 630nm
15ms 630nm
50ms 630nm
150ms 630nm
100pA
50ms

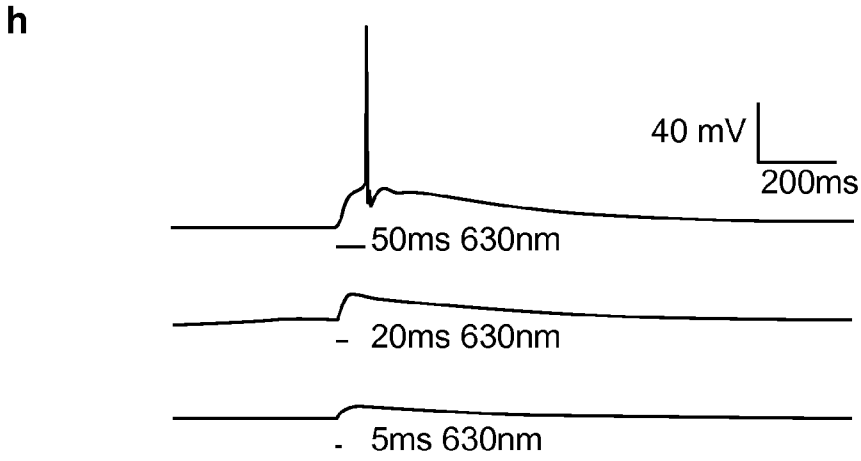

h
40 mV
200ms
50ms 630nm
20ms 630nm
5ms 630nm

FIG. 3 (Cont. 2)

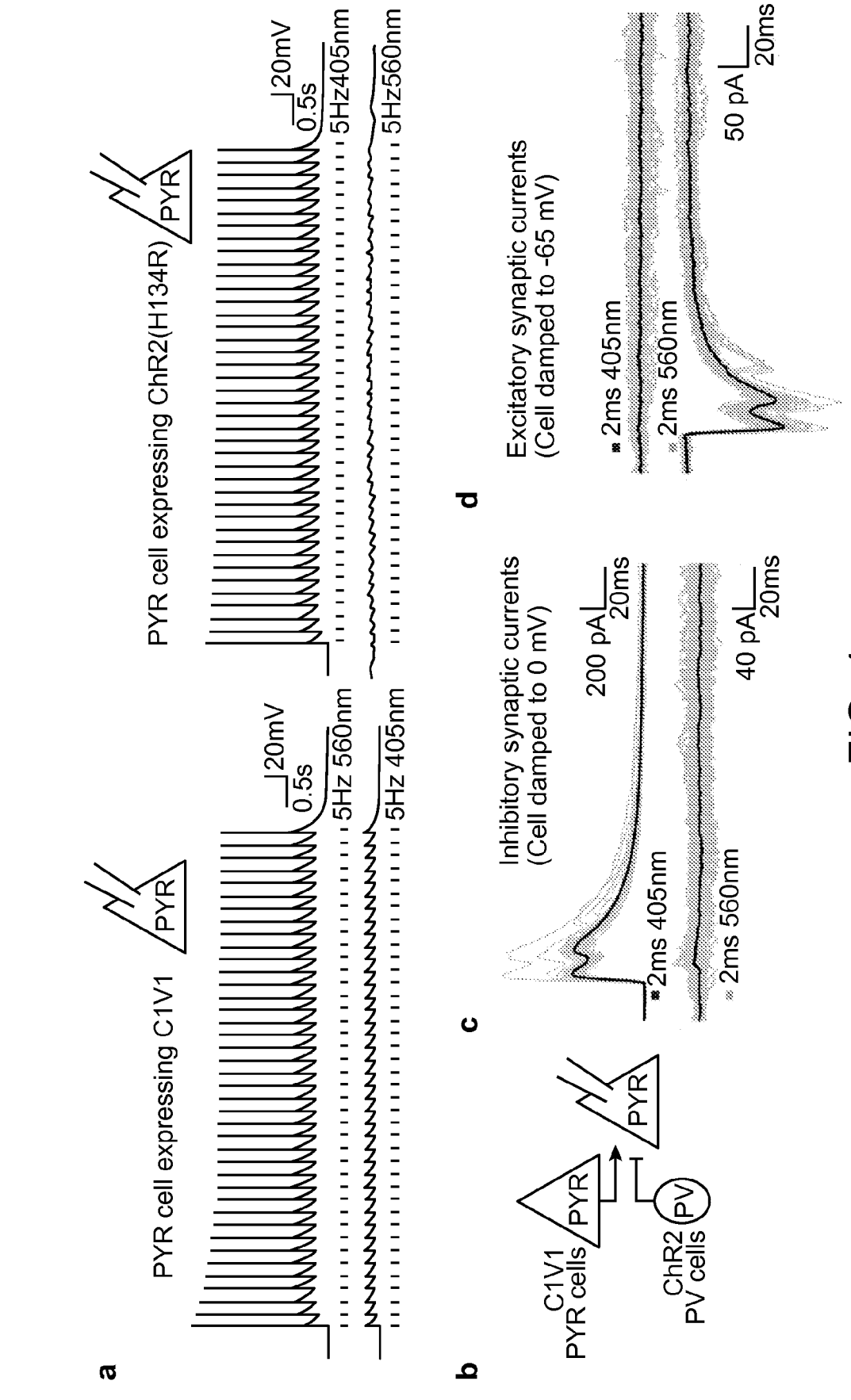

a
PYR cell expressing C1V1
PYR
20mV
0.5s
5Hz 560nm
5Hz 405nm
PYR cell expressing ChR2(H134R)
PYR
20mV
0.5s
5Hz405nm
5Hz560nm
b
C1V1
PYR cells
PYR
ChR2
PV cells
PV
c
Inhibitory synaptic currents
(Cell damped to 0 mV)
200 pA
20ms
2ms 405nm
40 pA
20ms
2ms 560nm
d
Excitatory synaptic currents
(Cell damped to -65 mV)
2ms 405nm
2ms 560nm
50 pA
20ms

FIG. 4

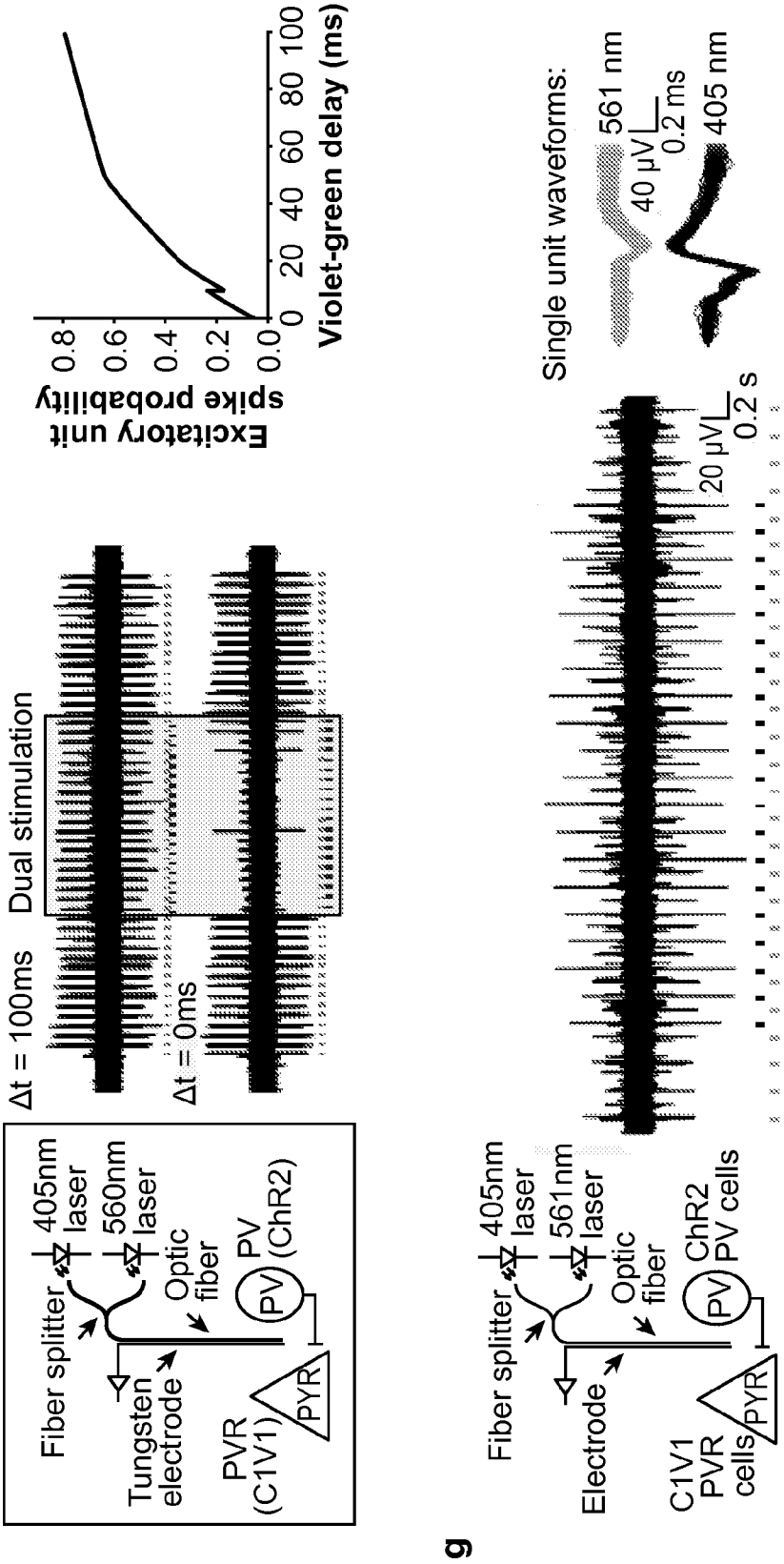

e
Fiber splitter
405nm laser
560nm laser
Optic fiber
PV (ChR2)
Tungsten electrode
PVR (C1V1)
PYR
Δt = 100ms
Dual stimulation
Δt = 0ms
f
Excitatory unit spike probability
0.0
0.2
0.4
0.6
0.8
0 20 40 60 80 100
Violet-green delay (ms)
g
Fiber splitter
405nm laser
561nm laser
Optic fiber
ChR2 PV cells
Electrode
C1V1 PVR cells
PYR
20 μV
0.2 s
Single unit waveforms:
561 nm
40 μV
0.2 ms
405 nm

FIG. 4 (Cont.)

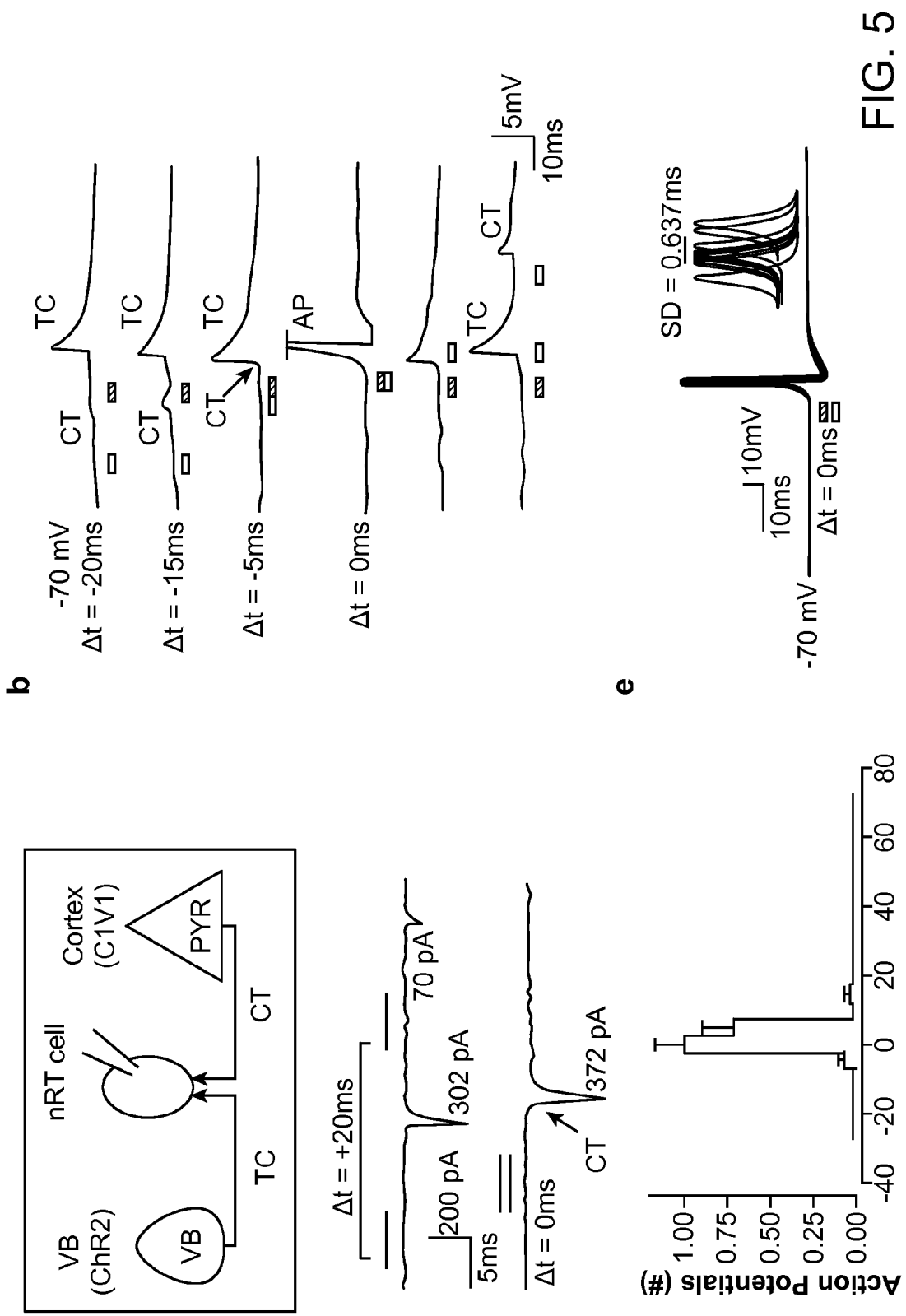

a
VB (ChR2)
nRT cell
Cortex (C1V1)
VB
PYR
TC
CT
b
-70 mV
Δt = -20ms
Δt = -15ms
Δt = -5ms
Δt = 0ms
TC
CT
AP
5mV
10ms
c
Δt = +20ms
200 pA
5ms
302 pA
70 pA
Δt = 0ms
372 pA
d
Action Potentials (#)
1.00
0.75
0.50
0.25
0.00
-40
-20
0
20
40
60
80
Δt (ms)
e
SD = 0.637ms
10mV
10ms
-70 mV
Δt = 0ms

FIG. 5

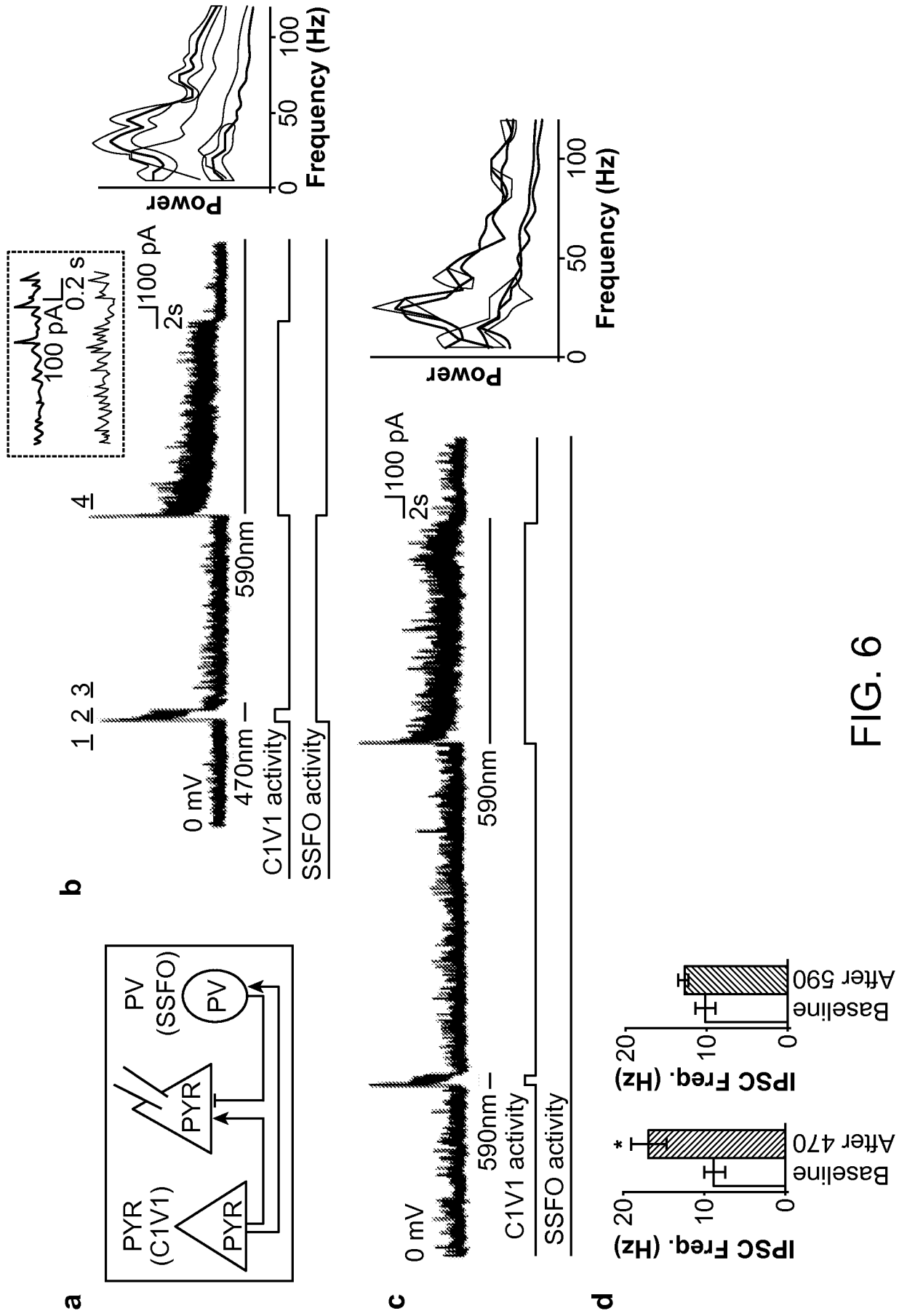

a
PYR (C1V1)
PYR
PV (SSFO)
PV
b
1 2 3
4
0 mV
470nm
590nm
C1V1 activity
SSFO activity
100 pA
2s
100 pA
0.2 s
Power
Frequency (Hz)
0
50
100
c
0 mV
590nm
C1V1 activity
SSFO activity
100 pA
2s
Power
Frequency (Hz)
d
IPSC Freq. (Hz)
Baseline
After 470
After 590
*

FIG. 6