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(54) **Title**: METHODS FOR FUNCTIONAL BRAIN CIRCUIT ANALYSIS

(57) **Abstract**: Provided herein are methods for analyzing in vivo a brain circuit. A method of the present disclosure may include using optogenetics to stimulate a first region of a brain of an individual, in conjunction with functional magnetic resonance imaging (fMRI) of different regions of the brain to determine a dynamic functional connection between individual neurons of the first region and a second region of the brain. The method may further include identifying a third region of the brain, the neurons of which region mediate the dynamic functional connection between the first and second regions.

METHODS FOR FUNCTIONAL BRAIN CIRCUIT ANALYSIS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/265,291, filed December 9, 2015, which application is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under contract number NS087159 awarded by the National Institutes of Health. The government has certain rights in the invention.

INTRODUCTION

[0003] Functional studies of neuronal tissues involve stimulating one or more neurons with a defined stimulus and measuring the output response of the one or more neurons. Methods of stimulating neurons and measuring the output response include optogenetics, functional magnetic resonance imaging (fMRI), electrophysiology, electroencephalography (EEG) and behavioral monitoring.

[0004] Optogenetics uses light-activated polypeptides (channels and pumps) to modulate activity of neurons expressing the light-activated polypeptides in a light-dependent manner.

[0005] Blood oxygenation level-dependent (BOLD) fMRI is a widely used technology for non-invasive whole brain imaging. BOLD signals reflect complex changes in cerebral blood flow (CBF), cerebral blood volume (CBV), and cerebral metabolic rate of oxygen consumption ($CMRO_2$) following neuronal activity.

SUMMARY

[0006] Provided herein are methods for analyzing, *in vivo*, a brain circuit. A method of the present disclosure may include using optogenetics to stimulate a first region of a brain of an individual, in conjunction with functional magnetic resonance imaging (fMRI) of different regions of the brain to determine a dynamic functional connection between

individual neurons of the first region and a second region of the brain. The method may further include identifying a third region of the brain, the neurons of which region mediate the dynamic functional connection between the first and second regions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] **Figures 1A-1G** are a collection of images, graphs and diagrams showing that targeted stimulation of central thalamus evokes positive blood oxygenation level-dependent (BOLD) changes and increases in neuronal firing at the site of stimulation, according to embodiments of the present disclosure.

[0008] **Figures 2A-2H** are a collection of images and graphs showing the spatial characterization of evoked fMRI signals, according to embodiments of the present disclosure.

[0009] **Figures 3A-3E** are a collection of graphs and diagrams showing that the sign of evoked cortical activity depends on the frequency of central thalamic stimulation, according to embodiments of the present disclosure.

[0010] **Figures 4A-4H** are a collection of images, graphs and diagrams showing that Frequency-dependent spindle-like oscillations are evoked in zona incerta (ZI), according to embodiments of the present disclosure.

[0011] **Figures 5A-5I** are a collection of images, graphs and diagrams showing that cortical inhibition driven by 10 Hz central thalamus stimulation depends on normal incertal processing, according to embodiments of the present disclosure.

[0012] **Figures 6A-6D** are a collection of graphs showing that optogenetic stimulation of central thalamus in asleep animals modulates brain state in a frequency-dependent manner, according to embodiments of the present disclosure.

[0013] **Figure 7** is a collection of images showing specificity of ChR2 targeting for CaMKIIa-positive cells.

[0014] **Figure 8** is a collection of images showing representative fluorescence images of ChR2-EYFP at remote targets, illustrating the massive projections to forebrain from transfected relay neurons in the right central thalamus.

[0015] **Figures 9A-9B** are a collection of graphs showing that widespread and frequency-dependent recruitment of forebrain with optogenetics is distinct to stimulation of

intralaminar nuclei of central thalamus, according to embodiments of the present disclosure.

[0016] **Figures 10A-10C** are a collection of images and graphs showing that the frequency-dependent recruitment of forebrain by central thalamus and its control over cortical BOLD signal polarity are preserved when pulse width is held constant.

[0017] **Figure 11** is a collection of graphs and a table showing that cortical spikes that occur during periods of inhibition driven by 10 Hz central thalamus stimulation exhibit a non-uniform distribution over time, according to embodiments of the present disclosure.

[0018] **Figure 12** is an image showing a wide-field fluorescence image of eNpHR expression in zona incerta, overlaid with the estimated cone of activated eNpHR (i.e. inhibited neurons) shown to scale, according to embodiments of the present disclosure.

[0019] **Figure 13** is a graph showing that pre-stimulus activity is consistent across frequencies of stimulation in asleep rats, as quantified with EEG bandpower in delta, theta, alpha, and beta bands, according to embodiments of the present disclosure.

[0020] **Figure 14** shows the amino acid sequences of depolarizing light-activated polypeptides and derivatives thereof (SEQ ID NOs:1-20) that may find use in the present methods, according to embodiments of the present disclosure.

[0021] **Figure 15** shows the amino acid sequences of hyperpolarizing light-activated polypeptides and derivatives thereof (SEQ ID NOs:21-51) that may find use in the present methods, according to embodiments of the present disclosure.

DEFINITIONS

[0022] The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

[0023] The term "genetic modification" refers to a permanent or transient genetic change induced in a cell following introduction into the cell of a heterologous nucleic acid (e.g., a nucleic acid exogenous to the cell). Genetic change ("modification") can be accomplished by incorporation of the heterologous nucleic acid into the genome of the host cell, or by transient or stable maintenance of the heterologous nucleic acid as an extrachromosomal element. Where the cell is a eukaryotic cell, a permanent genetic change can be achieved by introduction of the nucleic acid into the genome of the cell. Suitable methods of genetic modification include viral infection, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate precipitation, direct microinjection, and the like.

[0024] A "plurality" contains at least 2 members. In certain cases, a plurality may have at least 10, at least 100, at least 1000, at least 10,000, at least 100,000, at least 10^6, at least 10^7, at least 10^8 or at least 10^9 or more members.

[0025] "Substantially" as used herein, may be applied to modify any quantitative representation that could permissibly vary without resulting in a change in the basic function to which it is related.

[0026] An "individual" as used herein, may be any suitable animal amenable to the methods and techniques described herein, where in some cases, the individual may be a vertebrate animal, including a mammal, bird, reptile, amphibian, etc. The individual may be any suitable mammal, e.g., human, mouse, rat, cat, dog, pig, horse, cow, monkey, non-human primate, etc.

[0027] A "set", as used herein, may include one or more elements.

[0028] "Functional", as used herein, may be used to describe a process that is physiologically relevant, i.e., relevant for carrying out a process that normally occurs in a living organism. The process may be a measured phenomenon that is representative of, or a direct or indirect read out of, an underlying, physiologically relevant process.

[0029] A "connection" as used herein, may refer to a structural and/or functional relationship between two distinct entities, e.g., cells (including neurons), regions of a tissue (such as regions of a brain), tissues, organs, etc. A functional connection between two regions of the brain may be achieved by direct and/or indirect structural connections (e.g., synaptic connections) between the two regions.

[0030] "Neural activity" as used herein, may refer to electrical activity of a neuron (e.g., changes in membrane potential of the neuron), as well as indirect measures of the electrical activity of one or more neurons. Thus, neural activity may refer to changes in field potential, changes in intracellular ion concentration (e.g., intracellular calcium concentration), and changes in magnetic resonance induced by electrical activity of neurons, as measured by, e.g., blood oxygenation level dependent (BOLD) signals in functional magnetic resonance imaging.

[0031] "Dynamic" as used herein, may be applied to describe a process that varies in the temporal dimension.

[0032] "Quantitative" as used herein, refers to a numerical property defined by or is related to magnitude, or to describe a system (e.g., brain circuit) whose output varies with different patterns of input.

[0033] "Qualitative" as used herein, may refer to a property that is not defined by the magnitude of a numerical quantity. For instance, a qualitative determination may include determinations in which a yes/no or on/off result is determined.

[0034] "Internal" as used herein, refers to any portion of a body that is not visually or optically accessible from outside the exterior surface of the body. In certain cases, the portion may be below the surface of the body at a depth of 0.1 mm or more.

[0035] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0036] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0037] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0038] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a neuron" includes a plurality of such neurons and reference to "the light-activated polypeptide" includes reference to one or more light-activated polypeptides and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0039] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the invention are specifically embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0040] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

[0041] Provided herein are methods for analyzing *in vivo* a brain circuit. A method of the present disclosure may include using optogenetics to stimulate a first region of a brain of an individual, in conjunction with functional magnetic resonance imaging (fMRI) scanning of different regions of the brain to determine a dynamic functional connection between individual neurons of the first region and a second region of the brain. The method may further include identifying a third region of the brain, the neurons of which region mediate the dynamic functional connection between the first and second regions, using optogenetic stimulation in combination with electrophysiological recording. The present methods can be applied to any suitable brain circuit to reveal functional connections between different regions of the brain at the cellular level, e.g., functional connections between specifically activated neurons of one region, neurons of a second region dynamically regulated by the activated neurons, and neurons of a third region that mediates the dynamic regulation.

METHODS

[0042] Methods of the present disclosure may use any number of combinations of suitable neuronal stimulation and neuronal activity measurement protocols, as necessary, to determine the functional connections between different brain regions. Suitable protocols include electrophysiology; light-induced modulation of neural activity; electroencephalography (EEG) recordings; functional imaging and behavioral analysis. Electrophysiology may include single electrode, multi electrode, and/or field potential recordings. Light-induced modulation of neural activity may include any suitable optogenetic method, as described further herein. Functional imaging may include fMRI, and any functional imaging protocols using genetically encoded indicators (e.g., calcium indicators, voltage indicators, etc.). Behavioral analysis may include any suitable behavioral assays, such as behavioral assays for arousal, memory (such as a water maze assay), conditioning (such as fear conditioning), sensory responses (responses to e.g., visual, somatosensory, auditory, gustatory, and/or olfactory cues).

[0043] Some protocols, such as fMRI, provide a non-invasive, brain-wide measure representative of neural activity. Some protocols, such as electrophysiology, provide cellular resolution and rapid measures of neural activity as well as cellular resolution and

rapid control of neural activity. Some protocols, such as optogenetics, provide spatially-targeted and temporally-defined control of action potential firing in defined groups of neurons. An appropriate combination of assays may be used to dissect a functional brain circuit. In some cases, the combination includes: optogenetics and fMRI; optogenetics and electrophysiology; optogenetics and EEG; optogenetics and behavioral analysis. Any other suitable combination, e.g., EEG and behavioral analysis; fMRI and electrophysiology; electrophysiology and behavioral analysis, etc., may also be used.

[0044] The methods disclosed herein are amenable to revealing causal links between different brain regions in a single living individual (e.g., a single mouse or rat, a single human, a single non-human mammal) by using one or more different combinations of neuronal stimulation and activity measurement protocols, as described above. Thus, in some embodiments, a brain functional circuit is assayed in a single animal using one or more combinations of optogenetics and fMRI; optogenetics and electrophysiology; optogenetics and EEG; and optogenetics and behavioral analysis. In some cases, a brain functional circuit is assayed in a single animal using all of optogenetics and fMRI; optogenetics and electrophysiology; optogenetics and EEG; and optogenetics and behavioral analysis. The order in which the different combinations of assays are performed on a single animal may be any suitable order. In some cases, the combinations of assays are performed in the order of: optogenetics and fMRI; optogenetics and EEG/optogenetics and behavioral analysis; and optogenetics and electrophysiology, where the pairs "optogenetics and EEG" and "optogenetics and behavioral" may be performed in any order. Other combinations of protocols may be performed at any suitable point before or after any of the combinations of protocols with optogenetics.

[0045] In general terms, an implementation of the present method may include using a combination of optogenetic stimulation of a defined set of neurons in a first region of the brain, and measuring the response at a whole-brain level by scanning the brain with fMRI, to determine a dynamic functional connection between the first region and a second region of the brain. Thus, the neurons in the first region may be modified to contain a light-activated polypeptide, e.g., a light-activated ion channel, where the light-activated polypeptide is configured to modulate the activity of, e.g., depolarize or hyperpolarize, the neuron upon stimulating the first region with a light stimulus of

appropriate wavelength, illumination volume and intensity. In some cases, the neurons of the first region express the light-activated polypeptide. In some cases, the neurons of the first region are genetically modified, e.g., by viral infection of a DNA construct containing nucleotide sequences encoding the light-activated polypeptide and any other appropriate regulatory elements, to express the light-activated polypeptide. Any suitable light-activated polypeptide may be used, as described further herein.

[0046] The light stimulus used to activate the light-activated polypeptide may include light pulses characterized by, e.g., frequency, pulse width, duty cycle, wavelength, intensity, etc. In some cases, the light stimulus includes two or more different sets of light pulses, where each set of light pulses is characterized by different temporal patterns of light pulses. The temporal pattern may be characterized by any suitable parameter, including, but not limited to, frequency, period (i.e., total duration of the light stimulus), pulse width, duty cycle, etc.

[0047] The variation in the property of the light pulses of a set may be reflected in a difference in the activity of the illuminated neurons. In some cases, where the neuron is depolarized by activation of the light-activated polypeptide, an increase in the frequency of the light pulses may cause an increase in the frequency of action potential firing in the illuminated neurons. In some embodiments, the frequency of action potential firing in the illuminated neurons scales quantitatively with the increase in the frequency of the light pulses. In some cases, a linear increase in the frequency of the light pulses may induce a linear, or non-linear but monotonic, increase in the frequency of action potential firing in the illuminated neurons.

[0048] The responses to the stimulation by different sets of light pulses may be measured by fMRI for different regions of the brain, and a comparison of the responses in each region may indicate a functional connection between neurons stimulated by the light stimulus to the first region and one or more other regions of the brain. In some cases, where the light stimulus contains sets of light pulses that differ quantitatively by a temporal parameter (e.g., varies by frequency or pulse width), a response to the light stimulus in a second region of the brain, as measured by fMRI, that is not linear (e.g., an increase in the action potential firing frequency of excitatory neurons in the first region does not lead to a corresponding increase in the response in the second region) may indicate that there is a functional connection between neurons of the first region and neurons of the second

region dependent on the temporal pattern of action potential firing of the neurons of the first region. The non-linear relationship between the variation in the light pulses and the variation in the fMRI response may be a non-monotonic or a qualitative relationship. In some cases, a quantitative change in the light pulse may cause a change in sign of the fMRI blood oxygenation level-dependent (BOLD) response (e.g., a positive or negative BOLD response is measured depending on the frequency of the light pulses).

[0049] For example, upon illuminating a first region of the brain with a first set of light pulses and a second set of light pulses that have a different temporal pattern, neurons in the first region may generate action potentials induced by the first set and second set of light pulses. fMRI can then be used to measure activity in a second region of the brain. In some cases, a first change in neural activity induced by the first set of light pulses in the second region can be measured by fMRI. In addition, a second change in neural activity induced by the second set of light pulses in the second region can also be measured by fMRI. In some instances, there may be a difference between the first measured change in neural activity and the second measured change in neural activity. Based on the difference between the first measured change in neural activity and the second measured change in neural activity, a dynamic functional connection between the neurons in the first region of the brain and the second region of the brain may be identified. For instance, the dynamic functional connection between the neurons in the first region of the brain and the second region of the brain may be identified by calculating a difference between the first measured change and the second measured change in neural activity. If the calculated difference between the first measured change and the second measured change in neural activity is qualitatively or quantitatively different, this may be an indication that there is a dynamic functional connection from the neurons in the first region of the brain to the neurons in the second region of the brain.

[0050] In some cases, the light stimulus contains two sets of light pulses, where the two sets are characterized by having different parameter values, such as different frequencies of light pulses. Where the two sets of light pulses have different frequencies, the duty cycle may be the same, or may be different. In some cases, the two sets of light pulses with different frequencies have the same pulse width. In other instances, the two sets of light pulses with different frequencies have different pulse widths.

[0051] The light pulses of a set may have any suitable frequency. In some cases, the set of light pulses contains a single pulse of light that is sustained throughout the duration of the light stimulus. In some cases, the light pulses of a set have a frequency of 0.1 Hz or more, e.g., 0.5 Hz or more, 1 Hz or more, 5 Hz or more, 10 Hz or more, 20 Hz or more, 30 Hz or more, 40 H or more, including 50 Hz or more, or 60 Hz or more, or 70 Hz or more, or 80 Hz or more, or 90 Hz or more, or 100 Hz or more, and have a frequency of 100,000 Hz or less, e.g., 10,000 Hz or less, 1,000 Hz or less, 500 Hz or less, 400 Hz or less, 300 Hz or less, 200 Hz or less, including 100 Hz or less. In some cases, the light pulses of a set have a frequency in the range of 0.1 to 100,000 Hz, e.g., 1 to 10,000 Hz, 1 to 1,000 Hz, including 5 to 500 Hz, or 10 to 100 Hz.

[0052] In some cases, the two sets of light pulses are characterized by having different parameter values, such as different pulse widths. Where the two sets of light pulses have different pulse widths, the duty cycles of the two sets of light pulses may be the same, or may be different. In some cases, the two sets of light pulses with different pulse widths have the same frequency. In other instances, the two sets of light pulses with different pulse widths have different frequencies.

[0053] The light pulses may have any suitable pulse width. In some cases, the pulse width is 0.1 ms or longer, e.g., 0.5 ms or longer, 1 ms or longer, 3 ms or longer, 5 ms or longer, 7.5 ms or longer, 10 ms or longer, including 15 ms or longer, or 20 ms or longer, or 25 ms or longer, or 30 ms or longer, or 35 ms or longer, or 40 ms or longer, or 45 ms or longer, or 50 ms or longer, and is 500 ms or shorter, e.g., 100 ms or shorter, 90 ms or shorter, 80 ms or shorter, 70 ms or shorter, 60 ms or shorter, 50 ms or shorter, 45 ms or shorter, 40 ms or shorter, 35 ms or shorter, 30 ms or shorter, 25 ms or shorter, including 20 ms or shorter. In some embodiments, the pulse width is in the range of 0.1 to 500 ms, e.g., 0.5 to 100 ms, 1 to 80 ms, including 1 to 60 ms, or 1 to 50 ms, or 1 to 30 ms.

[0054] In some cases, the two sets of light pulses are characterized by having different parameter values, such as different duty cycles. The duty cycle is a percentage (or ratio) related to the "on time" for a pulsed signal and can be calculated as follows:
duty cycle = (pulse width) / frequency.

[0055] Where the two sets of light pulses have different duty cycles, the pulse width and/or the frequency of the two sets of light pulses may be the same, or may be different. In some cases, the two sets of light pulses with different duty cycles have different pulse widths

and different frequencies. In other instances, the two sets of light pulses with different duty cycles have different pulse widths and the same frequency. In other instances, the two sets of light pulses with different duty cycles have the same pulse width and different frequencies.

[0056] In some cases, the two sets of light pulses are characterized by having the same duty cycle. Where the two sets of light pulses have the same duty cycle, the pulse width and/or the frequency of the two sets of light pulses may be different. In some cases, the two sets of light pulses with the same duty cycle have different pulse widths and different frequencies.

[0057] The duty cycle of the pulses may be any suitable duty cycle. In some cases, the duty cycle is 1% or more, e.g., 5% or more, 10% or more, 15% or more, 20% or more including 25% or more, or 30% or more, or 35% or more, or 40% or more, or 45% or more, or 50% or more, and may be 80% or less, e.g., 75% or less, 70% or less, 65% or less, 60% or less, 65% or less, 50% or less, 45% or less, including 40% or less, or 35% or less, or 30% or less. In certain embodiments, the duty cycle is in the range of 1 to 80%, e.g., 5 to 70%, 5 to 60%, including 10 to 50%, or 10 to 40%.

[0058] The average power of the light pulse, measured at the tip of an optical fiber delivering the light pulse to regions of the brain, may be any suitable power. In some cases, the power is 0.1 mW or more, e.g., 0.5 mW or more, 1 mW or more, 1.5 mW or more, including 2 mW or more, or 2.5 mW or more, or 3 mW or more, or 3.5 mW or more, or 4 mW or more, or 4.5 mW or more, or 5 mW or more, and may be 1,000 mW or less, e.g., 500 mW or less, 250 mW or less, 100 mW or less, 50 mW or less, 40 mW or less, 30 mW or less, 20 mW or less, 15 mW or less, including 10 mW or less, or 5 mW or less. In some embodiments, the power is in the range of 0.1 to 1,000 mW, e.g., 0.5 to 100 mW, 0.5 to 50 mW, 1 to 20 mW, including 1 to 10 mW, or 1 to 5 mW.

[0059] The wavelength and intensity of the light pulses may vary and may depend on the activation wavelength of the light-activated polypeptide, optical transparency of the region of the brain, the desired volume of the brain to be illuminated, etc.

[0060] The volume of a brain region illuminated by the light pulses may be any suitable volume. In some cases, the illuminated volume is 0.001 mm^3 or more, e.g., 0.005 mm^3 or more, 0.001 mm^3 or more, 0.005 mm^3 or more, 0.01 mm^3 or more, 0.05 mm^3 or more, including 0.1 mm^3 or more, and is 100 mm^3 or less, e.g., 50 mm^3 or less, 20 mm^3 or less,

10 mm^3 or less, 5 mm^3 or less, 1 mm^3 or less, including 0.1 mm^3 or less. In certain cases, the illuminated volume is in the range of 0.001 to 100 mm^3, e.g., 0.005 to 20 mm^3, 0.01 to 10 mm^3, 0.01 to 5 mm^3, including 0.05 to 1 mm^3.

[0061] Another aspect of the present methods includes identifying neurons in a third region of the brain that may mediate the dynamic functional connection between the first and second regions, as described above. Thus, the neurons of the third region may be said to represent a node, e.g., a modulatory node, of the dynamic functional connection between the first and second regions, where the neurons of the third region have functional connections to both the first and second regions. In some cases, identifying a modulatory node of the dynamic functional connection may include using one or more of electrophysiology; light-induced modulation of neural activity; EEG; functional imaging (e.g., fMRI) and behavioral analysis, as described above.

[0062] In some embodiments, neurons in a third region of the brain that may mediate the dynamic functional connection between the first and second regions may be identified by measuring the effect of disrupting the normal activity of the neurons of the third region on the dynamic properties of the functional connection between neurons of the first and second regions. If normal activity of the neurons of the third region is required for the dynamic functional connection, then the neurons of the third region may represent a modulatory node for the dynamic functional connection.

[0063] The normal activity of the neurons of the third region may be disrupted using any suitable method. In some cases, the activity is disrupted by activating a light-activated polypeptide, e.g., a light-activated ion pump for a normally activated neuron, in the neurons of the third region, via illumination of the third region with an appropriate light stimulus for activating the light-activated polypeptide. By comparing the change in response of neurons in the second region to a first set of light pulses that activates light-activated polypeptides in neurons of the first region, in the presence or absence of a third set of light pulses illuminating the third region, the role of the neurons of the third region in the dynamic functional connection between the first and second regions may be determined. Thus, if illumination of the third region to disrupt the normal activity of neurons of the third region reduces, abolishes or otherwise alters the response of the neurons in the second region to the first set of light pulses, the neurons of the third region may mediate the dynamic functional connection. In some cases, the response of

the neurons in the second region to the first set of light pulses may be reduced by 20% or more, e.g., 30% or more, 40% or more, 50% or more, 60% or more, 70% or more, 80% or more, 90% or more, and up to 100%. In some cases, the response of the neurons in the second region to the first set of light pulses may include a change in sign (e.g., a reduction in firing rate with normal activity of neurons of the third region is switched to an increase in firing rate with disruption of normal activity of neurons of the third region).

[0064] For example, the presence or absence of a modulatory node in the third region of the brain may be determined by illuminating the first region of the brain (which has a dynamic functional connection to the second region of the brain) with and without illumination of the third region of the brain with a set of light pulses. Upon illuminating the first region of the brain with a first set of light pulses, neurons in the first region may generate action potentials induced by the first set of light pulses. In addition, the third region of the brain may be concurrently illuminated with a third set of light pulses. Upon illumination of the third region, the activity of neurons in the third region may be disrupted, for example if neurons in the third region express a light-activated polypeptide as described herein. Neural activity may then be measured (e.g., fMRI) in the second region of the brain, which has a dynamic functional connection to the first region of the brain. For instance, fMRI can be used to measure a third change in neural activity induced by the first set of light pulses in the second region of the brain without illumination of the third region of the brain. In some cases, the third measured change in neural activity induced by the first set of light pulses in the second region of the brain without illumination of the third region of the brain provides a baseline measurement of the dynamic functional connection between the first region and the second region of the brain. In addition, a fourth change in neural activity induced by the first set of light pulses in the second region can also be measured while the third region of the brain is being illuminated by a third set of light pulses. In some instances, there may be a difference between the third measured change in neural activity and the fourth measured change in neural activity. Based on the difference between the third measured change in neural activity and the fourth measured change in neural activity, a modulatory node in the third region of the brain may be identified. The presence of a modulatory node in the third region of the brain may be an indication of a functional connection between the

third region of the brain and the first region of the brain and/or a functional connection between the third region of the brain and the second region of the brain. For instance, the modulatory node in the third region of the brain may be identified by calculating a difference between the third measured change and the fourth measured change in neural activity. If the calculated difference between the third measured change and the fourth measured change in neural activity is qualitatively or quantitatively different, this may be an indication that there is a modulatory node in the third region of the brain. For example, if the calculated difference between the third measured change and the fourth measured change in neural activity is qualitatively or quantitatively different, this may be an indication that there is a functional connection between the third region of the brain and the first region of the brain and/or a functional connection between the third region of the brain and the second region of the brain.

[0065] Optogenetic stimulation may be performed using any suitable method. Suitable methods are described in, e.g., U.S. Pat. No. 8,834,546, which is incorporated herein by reference. Neurons of a suitable region of the brain whose activity is to be modulated by light can be modified using a convenient method to express the light-activated polypeptide. In some cases, neurons of a brain region are genetically modified to express a light-activated polypeptide. In some cases, the neurons may be genetically modified using a viral vector, e.g., an adeno-associated viral vector, containing a nucleic acid having a nucleotide sequence that encodes the light-activated polypeptide. The viral vector may include any suitable control elements (e.g., promoters, enhancers, recombination sites, etc.) to control expression of the light-activated polypeptide according to cell type, timing, presence of an inducer, etc.

[0066] Suitable neuron-specific control sequences include, but are not limited to, a neuron-specific enolase (NSE) promoter (see, e.g., EMBL HSENO2, X51956; see also, e.g., U.S. Pat. No. 6,649,811, U.S. Pat. No. 5,387,742); an aromatic amino acid decarboxylase (AADC) promoter; a neurofilament promoter (see, e.g., GenBank HUMNFL, L04147); a synapsin promoter (see, e.g., GenBank HUMSYNIB, M55301); a thy-1 promoter (see, e.g., Chen et al. (1987) *Cell* 51:7-19; and Llewellyn et al. (2010) *Nat. Med.* 16:1161); a serotonin receptor promoter (see, e.g., GenBank S62283); a tyrosine hydroxylase promoter (TH) (see, e.g., *Nucl. Acids. Res.* 15:2363-2384 (1987) and *Neuron* 6:583-594 (1991)); a GnRH promoter (see, e.g., Radovick et al., *Proc. Natl.*

Acad. Sci. USA 88:3402-3406 (1991)); an L7 promoter (see, e.g., Oberdick et al., *Science* 248:223-226 (1990)); a DNMT promoter (see, e.g., Bartge et al., *Proc. Natl. Acad. Sci. USA* 85:3648-3652 (1988)); an enkephalin promoter (see, e.g., Comb et al., *EMBO J.* 17:3793-3805 (1988)); a myelin basic protein (MBP) promoter; a CMV enhancer/platelet-derived growth factor-β promoter (see, e.g., Liu et al. (2004) *Gene Therapy* 11:52-60); a motor neuron-specific gene Hb9 promoter (see, e.g., U.S. Pat. No. 7,632,679; and Lee et al. (2004) *Development* 131:3295-3306); and an alpha subunit of $Ca^{(2+)}$-calmodulin-dependent protein kinase II (CaMKIIα) promoter (see, e.g., Mayford et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:13250). Other suitable promoters include elongation factor (EF) 1α and dopamine transporter (DAT) promoters.

[0067] In some cases, cell type-specific expression of the light-activated polypeptide may be achieved by using recombination systems, e.g., Cre-Lox recombination, Flp-FRT recombination, etc. Cell type-specific expression of genes using recombination has been described in, e.g., Fenno et al., *Nat Methods*. 2014 Jul;11(7):763; and Gompf et al., *Front Behav Neurosci*. 2015 Jul 2;9:152, which are incorporated by reference herein.

[0068] In some cases the regions of the brain with neurons containing a light-activated peptide, is illuminated using one or more optical fibers. The optical fiber may be configured in any suitable manner to direct a light emitted from suitable source of light, e.g., a laser or light-emitting diode (LED) light source, to the region of the brain. The optical fiber may be any suitable optical fiber. In some cases, the optical fiber is a multimode optical fiber. The optical fiber may include a core defining a core diameter, where light from the light source passes through the core. The optical fiber may have any suitable core diameter. In some cases, the core diameter of the optical fiber is 10 μm or more, e.g., 20 μm or more, 30 μm or more, 40 μm or more, 50 μm or more, 60 μm or more, including 80 μm or more, and is 1,000 μm or less, e.g., 500 μm or less, 200 μm or less, 100 μm or less, including 70 μm or less. In some embodiments, the core diameter of the optical fiber is in the range of 10 to 1,000 μm, e.g., 20 to 500 μm, 30 to 200 μm, including 40 to 100 μm.

[0069] In certain embodiments, a cladding surrounds at least a portion of the core of the optical fiber. For instance, the cladding may surround substantially the entire outer circumferential surface of the optical fiber. In some cases, the cladding is not present on the ends of the optical fiber, such as at the end of the optical fiber that receives light

from the light source, and the opposite end of the optical fiber that transmits light to the neurons in the target region of the brain. The cladding may be any suitable type of cladding. In some cases, the cladding has a lower refractive index than the core of the optical fiber. Suitable materials for the cladding include, but are not limited to, plastic, resin, and the like, and combinations thereof.

[0070] In some cases, the optical fiber includes an outer coating. The outer coating may be disposed on the surface of the cladding. The coating may surround substantially the entire outer circumferential surface of the optical fiber. In some cases, the coating is not present on the ends of the optical fiber, such as at the end of the optical fiber that receives light from the light source, and the opposite end of the optical fiber that transmits light to the neurons in the target region of the brain. The coating may be a biologically compatible coating. A biologically compatible coating includes coatings that do not significantly react with tissues, fluids, or other substances present in the subject into which the optical fiber is inserted. In some cases, a biologically compatible coating is composed of a material that is inert (i.e., substantially non-reactive) with respect to the surrounding environment in which the optical fiber is used.

[0071] The optical fiber end that is implanted into the target region of the brain may have any suitable configuration suitable for illuminating a region of the brain with a light stimulus delivered through the optical fiber. In some cases, the optical fiber includes an attachment device at or near the distal end of the optical fiber, where the distal end of the optical fiber corresponds to the end inserted into the subject. In some cases, the attachment device is configured to connect to the optical fiber and facilitate attachment of the optical fiber to the subject, such as to the skull of the subject. Any suitable attachment device may be used. In some cases, the attachment device includes a ferrule, e.g., a metal, ceramic or plastic ferrule. The ferrule may have any suitable dimensions for holding and attaching the optical fiber. In some cases, the ferrule has a diameter in the range of 0.5 to 3 mm, e.g., 0.75 to 2.5 mm, or 1 to 2 mm.

[0072] In certain embodiments, methods of the present disclosure may be performed using any suitable electronic components to control and/or coordinate the various optical components used to illuminate the regions of the brain. The optical components (e.g., light source, optical fiber, lens, objective, mirror, and the like) may be controlled by a controller, e.g., to coordinate the light source illuminating the regions of the brain with

light pulses. The controller may include a driver for the light source that controls one or more parameters associated with the light pulses, such as, but not limited to the frequency, pulse width, duty cycle, wavelength, intensity, etc. of the light pulses. The controllers may be in communication with components of the light source (e.g., collimators, shutters, filter wheels, moveable mirrors, lenses, etc.).

[0073] A computational unit (e.g., a computer) may be used in the methods of the present disclosure to control and/or coordinate the light stimulus through the one or more controllers, and to analyze data from fMRI scanning of the regions of the brain. A computational unit may include any suitable components to analyze the measured fMRI images. Thus, the computational unit may include one or more of the following: a processor; a non-transient, computer-readable memory, such as a computer-readable medium; an input device, such as a keyboard, mouse, touchscreen, etc.; an output device, such as a monitor, screen, speaker, etc.; a network interface, such as a wired or wireless network interface; and the like.

[0074] fMRI may be performed using any suitable method. Suitable methods are described in, e.g., U.S. Pat. No. 8,834,546, which is incorporated herein by reference.

[0075] The brain regions of interest in the present methods (for optogenetically stimulating and/or measuring neural activity) may vary and may be any suitable region. In certain embodiments, the brain regions are anatomically and/or functionally defined regions of the brain. For example, the first region of the brain and the second region of the brain illuminated by light pulses as described herein may be anatomically distinct regions of the brain. Similarly, in some instances, the third region of the brain, which modulates the dynamic functional connection between the first and second regions, may be anatomically distinct from the first region and the second region of the brain. In some cases, where the brain is a mammalian brain, the brain region of interest is selected from at least a portion of the thalamus (including the central thalamus), sensory cortex (including the somatosensory cortex), zona incerta (ZI), ventral tegmental area (VTA), prefontal cortex (PFC), nucleus accumbens (NAc), amygdala (BLA), substantia nigra, ventral pallidum, globus pallidus, dorsal striatum, ventral striatum, subthalamic nucleus, hippocampus, dentate gyrus, cingulate gyrus, entorhinal cortex, olfactory cortex, primary motor cortex, and cerebellum. In some cases, different brain regions (e.g., the first and second brain regions) are separated at minimum by one or more, e.g., 2 or more, 3 or

more, 4 or more, 5 or more, including 7 or more synaptic connections, and are separated at minimum by 15 or fewer, e.g., 12 or fewer, 10 or fewer, 8 or fewer, including 6 or fewer synaptic connections. In some embodiments, the different brain regions are separated at minimum by 1 to 15 synaptic connections, e.g., 1 to 12 synaptic connections, 1 to 10 synaptic connections, 2 to 8 synaptic connections, including 3 to 6 synaptic connections.

[0076] Neurons of interest and that are present in the brain regions may be any suitable types of neurons. In some cases, the neurons are inhibitory neurons, or excitatory neurons. In some cases, the neurons are sensory neurons, interneurons, or motor neurons. In some cases, the neurons are, without limitation, dopaminergic, cholinergic, GABAergic, glutamatergic, or peptidergic neurons.

Light-activated polypeptides

[0077] As summarized above, aspects of the present disclosure include various brain regions containing neurons with, e.g., expressing, a light-activated polypeptide. The light-activated polypeptide may be a light-activated ion channel or a light-activated ion pump. The light-activated ion channel polypeptides are adapted to allow one or more ions to pass through the plasma membrane of a neuron when the polypeptide is illuminated with light of an activating wavelength. Light-activated proteins may be characterized as ion pump proteins, which facilitate the passage of a small number of ions through the plasma membrane per photon of light, or as ion channel proteins, which allow a stream of ions to freely flow through the plasma membrane when the channel is open. In some embodiments, the light-activated polypeptide depolarizes the neuron when activated by light of an activating wavelength. Suitable depolarizing light-activated polypeptides, without limitation, are shown in Figure 14. In some embodiments, the light-activated polypeptide hyperpolarizes the neuron when activated by light of an activating wavelength. Suitable hyperpolarizing light-activated polypeptides, without limitation, are shown in Figure 15.

[0078] In some embodiments, the light-activated polypeptides are activated by blue light. In some embodiments, the light-activated polypeptides are activated by green light. In some embodiments, the light-activated polypeptides are activated by yellow light. In some embodiments, the light-activated polypeptides are activated by orange light. In some embodiments, the light-activated polypeptides are activated by red light.

[0079] In some embodiments, the light-activated polypeptide expressed in a cell can be 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/or an N-terminal golgi export signal. The one or more amino acid sequence motifs which enhance light-activated 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 polypeptide. In some cases, the one or more amino acid sequence motifs which enhance light-activated polypeptide transport to the plasma membranes of mammalian cells is fused internally within a light-activated polypeptide. Optionally, the light-activated polypeptide and the one or more amino acid sequence motifs may be separated by a linker.

[0080] In some embodiments, the light-activated polypeptide can be 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 can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)).

[0081] A trafficking sequence can have a length of from about 10 amino acids to about 50 amino acids, e.g., from about 10 amino acids to about 20 amino acids, from about 20 amino acids to about 30 amino acids, from about 30 amino acids to about 40 amino acids, or from about 40 amino acids to about 50 amino acids.

[0082] ER export sequences that are suitable for use with a light-activated polypeptide include, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53); VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like. An ER export sequence can have a length of from about 5 amino acids to about 25 amino acids, e.g., from about 5 amino acids to about 10 amino acids,

from about 10 amino acids to about 15 amino acids, from about 15 amino acids to about 20 amino acids, or from about 20 amino acids to about 25 amino acids.

[0083] Signal sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such as one of the following: 1) the signal peptide of hChR2 (e.g., MDYGGALSAVGRELLFVTNPVVVNGS (SEQ ID NO:59)); 2) the β2 subunit signal peptide of the neuronal nicotinic acetylcholine receptor (e.g., MAGHSNSMALFSFSLLWLCSGVLGTEF (SEQ ID NO:60)); 3) a nicotinic acetylcholine receptor signal sequence (e.g., MGLRALMLWLLAAAGLVRESLQG (SEQ ID NO:64)); and 4) a nicotinic acetylcholine receptor signal sequence (e.g., MRGTPLLLVVSLFSLLQD (SEQ ID NO:61)).

[0084] A signal sequence can have a length of from about 10 amino acids to about 50 amino acids, e.g., from about 10 amino acids to about 20 amino acids, from about 20 amino acids to about 30 amino acids, from about 30 amino acids to about 40 amino acids, or from about 40 amino acids to about 50 amino acids.

[0085] In some embodiments, the signal peptide sequence in the protein can be deleted or substituted with a signal peptide sequence from a different protein.

[0086] Examples of light-activated polypeptides are described in, e.g., PCT App. No. PCT/US2011/028893, which is incorporated herein by reference. Representative light-activated polypeptides that find use in the present disclosure are further described below.

Depolarizing light-activated polypeptides

ChR

[0087] In some aspects, a depolarizing light-activated polypeptide is derived from *Chlamydomonas reinhardtii*, wherein the polypeptide is capable of transporting cations across a cell membrane when the cell is illuminated with light. In another embodiment, the light-activated polypeptide comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:1. The light used to activate the light-activated cation channel protein derived from *Chlamydomonas reinhardtii* can have a wavelength between about 460 and about 495 nm or can have a wavelength of about 480 nm. Additionally, light pulses having a temporal frequency of about 100 Hz can be used to activate the light-activated protein. In some embodiments, activation of the light-

activated cation channel derived from *Chlamydomonas reinhardtii* with light pulses having a temporal frequency of about 100 Hz can cause depolarization of the neurons expressing the light-activated cation channel. The light-activated cation channel protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the light-activated cation channel protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the light-activated cation channel protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The light-activated proton pump protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[0088] In some embodiments, the light-activated cation channel includes a T159C substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes a L132C substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes an E123T substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes an E123A substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes a T159C substitution and an E123T substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes a T159C substitution and an E123A substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes a T159C substitution, an L132C substitution, and an E123T substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes a T159C substitution, an L132C substitution, and an E123A substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes an L132C substitution and an E123T substitution of the amino acid sequence set forth in SEQ ID NO:1. In some embodiments, the light-activated cation channel includes an

L132C substitution and an E123A substitution of the amino acid sequence set forth in SEQ ID NO:1.

[0089] In some embodiments, a ChR2 protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs that enhance transport to the plasma membranes of neurons selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the ChR2 protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the ChR2 protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the ChR2 protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the ChR2 protein comprises a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can comprise any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[0090] In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[0091] In certain embodiments, the ChR2 protein can have an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:2.

[0092] In other embodiments, the light-activated polypeptide is a step function opsin (SFO) protein or a stabilized step function opsin (SSFO) protein that can have specific amino acid substitutions at key positions in the retinal binding pocket of the protein. In some embodiments, the SFO protein can have a mutation at amino acid residue C128 of SEQ ID NO:1. In other embodiments, the SFO protein has a C128A mutation in SEQ ID NO:1. In other embodiments, the SFO protein has a C128S mutation in SEQ ID NO:1. In another embodiment, the SFO protein has a C128T mutation in SEQ ID NO:1.

[0093] In some embodiments, the SSFO protein can have a mutation at amino acid residue D156 of SEQ ID NO:1. In other embodiments, the SSFO protein can have a mutation at both amino acid residues C128 and D156 of SEQ ID NO:1. In one embodiment, the SSFO protein has an C128S and a D156A mutation in SEQ ID NO:1. In another embodiment, the SSFO protein can comprise an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:1; and includes an alanine, serine, or threonine at amino acid 128; and includes a alanine at amino acid 156. In another embodiment, the SSFO protein can comprise a C128T mutation in SEQ ID NO:1. In some embodiments, the SSFO protein includes C128T and D156A mutations in SEQ ID NO:1.

[0094] In some embodiments the SFO or SSFO proteins provided herein can be capable of mediating a depolarizing current in the cell when the cell is illuminated with blue light. In other embodiments, the light can have a wavelength of about 445 nm. Additionally, in some embodiments the light can be delivered as a single pulse of light or as spaced pulses of light due to the prolonged stability of SFO and SSFO photocurrents. In some embodiments, activation of the SFO or SSFO protein with single pulses or spaced pulses of light can cause depolarization of a neuron expressing the SFO or SSFO protein. In some embodiments, each of the disclosed step function opsin and stabilized step function opsin proteins can have specific properties and characteristics for use in depolarizing the membrane of a neuronal cell in response to light.

[0095] Further disclosure related to SFO or SSFO proteins can be found in International Patent Application Publication No. WO 2010/056970, the disclosure of which is hereby incorporated by reference in its entirety.

[0096] In some cases, the ChR2-based SFO or SSFO comprises a membrane trafficking signal and/or an ER export signal. In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[0097] In certain embodiments, the SSFO protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:4.

***Volvox carteri* light-activated polypeptide**

[0098] In some embodiments, a suitable light-activated polypeptide is a cation channel derived from *Volvox carteri* (VChR1) and is activated by illumination with light of a wavelength of from about 500 nm to about 600 nm, e.g., from about 525 nm to about 550 nm, e.g., 545 nm. In some embodiments, the light-activated ion channel protein comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:5. The light-activated ion channel protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the light-activated ion channel protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the light-activated ion channel protein can comprise one or more conservative amino acid

substitutions and/or one or more non-conservative amino acid substitutions. The light-activated ion channel protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of a neuronal cell in response to light.

[0099] In some cases, a VChR1 light-activated cation channel protein comprises a core amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:5 and at least one (such as one, two, three, or more) amino acid sequence motifs which enhance transport to the plasma membranes of mammalian cells selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the light-activated proton ion channel comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the light-activated ion channel protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the light-activated ion channel protein comprises an N-terminal signal peptide, a C-terminal ER Export signal, and a C-terminal trafficking signal. In some embodiments, the light-activated ion channel protein comprises a C-terminal ER Export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER Export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER Export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00100] In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g.,

KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00101] In certain embodiments, the VChR1protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:6.

Step function opsins and stabilized step function opsins based on VChR1

[00102] In other embodiments, the light-activated polypeptide is a SFO or an SSFO based on VChR1. In some embodiments, the SFO protein can have a mutation at amino acid residue C123 of SEQ ID NO:5. In other embodiments, the SFO protein has a C123A mutation in SEQ ID NO:5. In other embodiments, the SFO protein has a C123S mutation in SEQ ID NO:5. In another embodiment, the SFO protein has a C123T mutation in SEQ ID NO:5.

[00103] In some embodiments, the SFO protein can have a mutation at amino acid residue D151 of SEQ ID NO:5. In other embodiments, the SFO protein can have a mutation at both amino acid residues C123 and D151 of SEQ ID NO:5. In one embodiment, the SFO protein has an C123S and a D151A mutation in SEQ ID NO:5.

[00104] In some embodiments an SFO or SSFO protein is capable of mediating a depolarizing current in the cell when the cell is illuminated with blue light. In some embodiments, the light has a wavelength of about 560 nm. Additionally, in some embodiments the light is delivered as a single pulse of light or as spaced pulses of light due to the prolonged stability of SFO and SSFO photocurrents. In some embodiments, activation of the SFO or SSFO protein with single pulses or spaced pulses of light can cause depolarization of a neuron expressing the SFO or SSFO protein. In some embodiments, each of the disclosed step function opsin and stabilized step function opsin proteins can have specific properties and characteristics for use in depolarizing the membrane of a neuronal cell in response to light.

[00105] In some cases, the VChR1-based SFO or SSFO comprises a membrane trafficking signal and/or an ER export signal. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier

potassium channel Kir2.1. Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

C1V1 chimeric cation channels

[00106] In other embodiments, the light-activated cation channel protein is a C1V1 chimeric protein derived from the VChR1 protein of *Volvox carteri* and the ChR1 protein 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. In some embodiments, the C1V1 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 ChR1. In another embodiment, the portion of the intracellular loop domain of the C1V1 chimeric protein can be replaced with the corresponding portion from ChR1 extending to amino acid residue A145 of the ChR1. In other embodiments, the C1V1 chimeric 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. In yet another embodiment, the portion of the intracellular loop domain of the C1V1 chimeric protein can be replaced with the corresponding portion from ChR1 extending to amino acid residue W163 of the ChR1. In other embodiments, the C1V1 chimeric protein comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:7.

[00107] In some embodiments, the C1V1 protein mediates a depolarizing current in the cell when the cell is illuminated with green light. In some embodiments, the light has 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 C1V1 chimeric protein is not capable of mediating a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein is not capable of mediating a depolarizing current in the cell when the cell is illuminated with light having a wavelength of about 405 nm. Additionally, in some embodiments, light pulses having a temporal frequency of about 100 Hz can be used to activate the C1V1 protein.

[00108] In some cases, the C1V1 polypeptide comprises a membrane trafficking signal and/or an ER export signal. In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00109] In certain embodiments, the C1V1 protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:8.

C1V1 variants

[00110] In some aspects, a suitable light-activated polypeptide comprises 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 C1V1 chimeric proteins described herein can 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.

[00111] Accordingly, suitable light-activated proteins include C1V1 chimeric light-activated proteins that can 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 comprises an amino acid substitution at amino acid residue E122 of SEQ ID NO:7. In some embodiments, the C1V1 protein comprises a substitution at amino acid residue E162 of SEQ ID NO:7. In other embodiments, the C1V1 protein comprises a substitution at both amino acid residues E162 and E122 of SEQ ID NO:7.

[00112] In some aspects, the C1V1-E122 mutant chimeric protein is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In some embodiments the light is green light. In other embodiments, the light has a wavelength of between about 540 nm to about 560 nm. In some embodiments, the light has a wavelength of about 546 nm. In other embodiments, the C1V1-E122 mutant chimeric protein mediates a depolarizing current in the cell when the cell is illuminated with red light. In some embodiments, the red light has a wavelength of about 630 nm. In some embodiments, the C1V1-E122 mutant chimeric protein does not mediate a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein does not mediate a depolarizing current in the cell when the cell is illuminated with light having a wavelength of about 405 nm. Additionally, in some embodiments, light pulses having a temporal frequency of about 100 Hz can be used to activate the C1V1-E122 mutant chimeric protein. In some embodiments, activation of the C1V1-E122 mutant chimeric protein with light pulses having a frequency of 100 Hz can cause depolarization of the neurons expressing the C1V1-E122 mutant chimeric protein.

[00113] In other aspects, the C1V1-E162 mutant 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 535 nm to about 540 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 mutant chimeric protein does not mediate a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein does not mediate a depolarizing current in the cell when the cell is illuminated with light having a wavelength of about 405 nm. Additionally, in some embodiments, light pulses having a temporal frequency of about 100 Hz can be used to activate the C1V1-E162 mutant chimeric protein. In some embodiments, activation of the C1V1-E162 mutant chimeric protein with light pulses having a frequency of 100 Hz can cause depolarization-induced synaptic depletion of the neurons expressing the C1V1-E162 mutant chimeric protein.

[00114] In yet other aspects, the C1V1-E122/E162 mutant 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 mutant chimeric protein does not mediate a depolarizing current in the cell when the cell is illuminated with violet light. In some embodiments, the chimeric protein does not mediate a depolarizing current in the cell when the cell is illuminated with light having a wavelength of about 405 nm. In some embodiments, the C1V1-E122/E162 mutant chimeric protein can exhibit less activation when exposed to violet light relative to C1V1 chimeric proteins lacking mutations at E122/E162 or relative to other light-activated cation channel proteins. Additionally, in some embodiments, light pulses having a temporal frequency of about 100 Hz can be used to activate the C1V1-E122/E162 mutant chimeric protein. In some embodiments, activation of the C1V1- E122/E162 mutant chimeric protein with light pulses having a frequency of 100 Hz can cause depolarization-induced synaptic depletion of the neurons expressing the C1V1-E122/E162 mutant chimeric protein.

[00115] In some cases, the C1V1 variant polypeptide comprises a membrane trafficking signal and/or an ER export signal. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence

KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

C1C2 chimeric cation channels

[00116] In other embodiments, the light-activated cation channel protein is a C1C2 chimeric protein derived from the ChR1 and the ChR2 proteins from *Chlamydomonas reinhardti,* wherein the protein is responsive to light and is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In another embodiment, the light-activated polypeptide comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:9. The light-activated cation channel protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the light-activated cation channel protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the light-activated cation channel protein comprises one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The light-activated proton pump protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00117] In some embodiments, a C1C2 protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs that enhance transport to the plasma membranes of neurons selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the C1C2 protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the C1C2 protein comprises an N-terminal signal peptide and a C-

terminal trafficking signal. In some embodiments, the C1C2 protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the C1C2 protein comprises a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00118] In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00119] In certain embodiments, the C1C2 protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:10.

ReaChR

[00120] In some aspects, a depolarizing light-activated polypeptide is a red shifted variant of a depolarizing light-activated polypeptide derived from *Chlamydomonas reinhardtii*; such light-activated polypeptides are referred to herein as a "ReaChR polypeptide" or "ReaChR protein" or "ReaChR." In another embodiment, the light-activated polypeptide

comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:11. The light used to activate the ReaChR polypeptide can have a wavelength between about 590 and about 630 nm or can have a wavelength of about 610 nm. The ReaChR protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the light-activated cation channel protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the ReaChR protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The ReaChR containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00121] In some embodiments, a ReaChR protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs that enhance transport to the plasma membranes of neurons selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the ReaChR protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the ReaChR protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the ReaChR protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the ReaChR protein comprises a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00122] In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00123] In certain embodiments, the ReaChR protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:12.

SdChR

[00124] In some aspects, a depolarizing light-activated polypeptide is a SdChR polypeptide derived from *Scherffelia dubia,* wherein the SdChR polypeptide is capable of transporting cations across a cell membrane when the cell is illuminated with light. In some cases, the SdChR polypeptide comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:13. The light used to activate the SdChR polypeptide can have a wavelength between about 440 and about 490 nm or can have a wavelength of about 460 nm. The SdChR protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the SdChR protein to regulate the polarization state of the plasma membrane of the cell. In some instances, the SdChR protein comprises one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The SdChR protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00125] In some embodiments, a SdChR protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs that enhance transport to the plasma membranes of neurons selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the SdChR protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the SdChR protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the SdChR protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the SdChR protein comprises a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00126] In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00127] In certain embodiments, the SdChR protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:14.

CnChR1

[00128] In some aspects, a depolarizing light-activated polypeptide can be, e.g. CnChR1, derived from *Chlamydomonas noctigama,* wherein the CnChR1 polypeptide is capable of transporting cations across a cell membrane when the cell is illuminated with light. In some cases, the CnChR1 polypeptide comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:15. The light used to activate the CnChR1 polypeptide can have a wavelength between about 560 and about 630 nm or can have a wavelength of about 600 nm. The CnChR1 protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the CnChR1 protein to regulate the polarization state of the plasma membrane of the cell. In some cases, the CnChR1 protein comprises one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The CnChR1 protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00129] In some embodiments, a CnChR1protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs that enhance transport to the plasma membranes of neurons selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the CnChR1protein includes an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the CnChR1protein includes an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the CnChR1protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the CnChR1protein comprises a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00130] In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00131] In certain embodiments, the CnChR1protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:16.

CsChrimson

[00132] In other embodiments, the light-activated cation channel protein is a CsChrimson chimeric protein derived from a CsChR protein of *Chloromonas subdivisa* and CnChR1 protein from *Chlamydomonas noctigama*, wherein the N terminus of the protein comprises the amino acid sequence of residues 1-73 of CsChR followed by residues 79-350 of the amino acid sequence of CnChR1; is responsive to light; and is capable of mediating a depolarizing current in the cell when the cell is illuminated with light. In another embodiment, the CsChrimson polypeptide comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:17. The CsChrimson protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native

amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the CsChrimson protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the CsChrimson protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. A CsChrimson protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00133] In some embodiments, a CsChrimson protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs that enhance transport to the plasma membranes of neurons selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the CsChrimson protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the CsChrimson protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the CsChrimson protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the CsChrimson protein comprises a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00134] In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a

trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00135] In certain embodiments, the CsChrimson protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:18.

ShChR1

[00136] In some aspects, a depolarizing light-activated polypeptide can be, e.g. ShChR1, derived from *Stigeoclonium helveticum,* wherein the ShChR1 polypeptide is capable of transporting cations across a cell membrane when the cell is illuminated with light. In some cases, the ShChR1 polypeptide comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:19. The light used to activate the ShChR1 protein derived from *Stigeoclonium helveticum* can have a wavelength between about 480 and about 510 nm or can have a wavelength of about 500 nm. The ShChR1 protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the ShChR1 protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the ShChR1 protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. A ShChR1 protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport cations across a cell membrane.

[00137] In some embodiments, a ShChR1 protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs that enhance transport to the plasma membranes of neurons selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the ShChR1 protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In

some embodiments, the ShChR1 protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the ShChR1 protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the ShChR1protein comprises a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00138] In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00139] In certain embodiments, the ShChR1 protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:20.

[00140] Other suitable depolarizing light-activated polypeptides are described in, e.g., Klapoetke et al. Nat Methods 2014 11:338.

Hyperpolarizing light-activated polypeptides

Arch

[00141] In some embodiments, a suitable light-activated polypeptide is an Archaerhodopsin (Arch) proton pump (e.g., a proton pump derived from *Halorubrum sodomense*) that can transport one or more protons across the plasma membrane of a cell when the cell is illuminated with light. The light can have a wavelength between about 530 and about 595 nm or can have a wavelength of about 560 nm. In some embodiments, the Arch protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:21. The Arch protein can additionally have substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the Arch protein to transport ions across the plasma membrane of a neuron. Additionally, the Arch protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. An Arch protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of a neuron in response to light.

[00142] In some embodiments, the Arch protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs selected from a signal peptide, an ER export signal, and a membrane trafficking signal, that enhance transport to the plasma membranes of neurons. In some embodiments, the Arch protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the Arch protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the Arch protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the Arch protein includes a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 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 include a fluorescent protein, for example, but not limited to, a yellow

fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00143] In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal can include the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can include an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00144] In certain embodiments, the Arch protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:22.

ArchT

[00145] In some embodiments, a suitable light-activated protein is an Archaerhodopsin (ArchT) proton pump (e.g., a proton pump derived from *Halorubrum sp.* TP009) that can transport one or more protons across the plasma membrane of a cell when the cell is illuminated with light. The light can have a wavelength between about 530 and about 595 nm or can have a wavelength of about 560 nm. In some embodiments, the ArchT protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:23 (ArchT). The ArchT protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the ArchT protein to transport ions across the plasma membrane of a neuron. Additionally, the ArchT protein can comprise one or

more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The ArchT protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of a neuron in response to light.

[00146] In some cases, the ArchT polypeptide comprises a membrane trafficking signal and/or an ER export signal. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00147] In certain embodiments, the ArchT protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:24.

GtR3

[00148] In some embodiments, the light-activated polypeptide is responsive to blue light and is a proton pump protein derived from *Guillardia theta*, wherein the proton pump protein is capable of mediating a hyperpolarizing current in the cell when the cell is illuminated with blue light; such a protein is referred to herein as a "GtR3 protein" or a "GtR3 polypeptide". The light can have a wavelength between about 450 and about 495 nm or can have a wavelength of about 490 nm. In some embodiment, a GtR3 protein comprises an amino acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:25 (GtR3). The GtR3 protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or

decrease the ability of the GtR3 protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the GtR3 protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The GtR3 protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to hyperpolarize the plasma membrane of a neuronal cell in response to light.

[00149] In some cases, a GtR3 protein comprises a core amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:25 and at least one (such as one, two, three, or more) amino acid sequence motifs which enhance transport to the plasma membranes of mammalian cells selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, GtR3 protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the GtR3 protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the light-activated proton pump protein comprises an N-terminal signal peptide, a C-terminal ER Export signal, and a C-terminal trafficking signal. In some embodiments, the GtR3 protein comprises a C-terminal ER Export signal and a C-terminal trafficking signal. In some embodiments, the signal peptide comprises the amino acid sequence MDYGGALSAVGRELLFVTNPVVVNGS (SEQ ID NO:59). In some embodiments, the first 19 amino acids are replaced with MDYGGALSAVGRELLFVTNPVVVNGS (SEQ ID NO:59). In some embodiments, the C-terminal ER Export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The GtR3 protein may further comprise a fluorescent protein, for example, but not limited to, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER Export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00150] In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV

(SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00151] In certain embodiments, a GtR3 protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:26.

Oxy

[00152] In some embodiments, a light-activated protein is an *Oxyrrhis marina* (Oxy) proton pump that can transport one or more protons across the plasma membrane of a cell when the cell is illuminated with light. The light can have a wavelength between about 500 and about 560 nm or can have a wavelength of about 530 nm. In some embodiments, the Oxy protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:27. The Oxy protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the Oxy protein to transport ions across the plasma membrane of a neuron. Additionally, the Oxy protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. The Oxy protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of a neuron in response to light.

[00153] In some embodiments, an Oxy protein comprises at least one (such as one, two, three, or more) amino acid sequence motifs that enhance transport to the plasma membranes of neurons selected from the group consisting of a signal peptide, an ER

export signal, and a membrane trafficking signal. In some embodiments, the Oxy protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the Oxy protein includes an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the Oxy protein comprises an N-terminal signal peptide, a C-terminal ER export signal, and a C-terminal trafficking signal. In some embodiments, the Oxy protein comprises a C-terminal ER export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The Oxy protein may further comprise a fluorescent protein, for example, but not limited to, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00154] In some cases, the Oxy polypeptide comprises a membrane trafficking signal and/or an ER export signal. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00155] In certain embodiments, the Oxy protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:28.

Mac

[00156] In some embodiments, the light-activated proton pump protein (referred to herein as "Mac protein") is responsive to light and is derived from *Leptosphaeria maculans*, wherein the Mac proton pump protein is capable of pumping protons across the membrane of a cell when the cell is illuminated with 520 nm to 560 nm light. The light can have a wavelength between about 520 nm to about 560 nm. In some cases, a Mac protein comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:29 or SEQ ID NO:30 (Mac; Mac 3.0). The Mac protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the Mac protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the Mac protein can comprise one or more conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. A Mac protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to pump protons across the plasma membrane of a neuronal cell in response to light.

[00157] In other aspects, a Mac protein comprises a core amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:29 and at least one (such as one, two, three, or more) amino acid sequence motifs which enhance transport to the plasma membranes of mammalian cells selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the Mac protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the Mac protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the Mac protein comprises an N-terminal signal peptide, a C-terminal ER Export signal, and a C-terminal trafficking signal. In some embodiments, the Mac protein comprises a C-terminal ER Export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER Export signal and the C-terminal trafficking signal are linked by a linker. The linker can comprise any of about 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500

amino acids in length. The Mac protein may further comprise a fluorescent protein, for example, but not limited to, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER Export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00158] In some cases, the Mac polypeptide includes a membrane trafficking signal and/or an ER export signal. In some embodiments, the trafficking signal can be derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00159] Further disclosure related to light-activated proton pump proteins can be found in International Patent Application No. PCT/US2011/028893, the disclosure of which is hereby incorporated by reference in its entirety.

NpHR

[00160] In some cases, a suitable light-activated chloride pump protein is derived from *Natronomonas pharaonis*; such a protein is referred to herein as an "NpHR protein" or an "NpHR polypeptide." In some embodiments, the NpHR protein can be responsive to amber light as well as red light and can mediate a hyperpolarizing current in the neuron when the NpHR protein is illuminated with amber or red light. The wavelength of light that can activate the NpHR protein can be between about 580 and 630 nm. In some embodiments, the light can be at a wavelength of about 589 nm or the light can have a wavelength greater than about 630 nm (*e.g.* less than about 740 nm). In another embodiment, the light has a wavelength of around 630 nm. In some embodiments, the

NpHR protein can hyperpolarize a neural membrane for at least about 90 minutes when exposed to a continuous pulse of light. In some embodiments, the NpHR protein comprises an amino acid sequence at least about 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:31. Additionally, the NpHR protein can comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the NpHR protein to regulate the polarization state of the plasma membrane of the cell. In some embodiments, the NpHR protein comprises one or more conservative amino acid substitutions. In some embodiments, the NpHR protein comprises one or more non-conservative amino acid substitutions. A NpHR protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to hyperpolarize the plasma membrane of a neuronal cell in response to light.

[00161] In some cases, an NpHR protein comprises a core amino acid sequence at least about 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:31; and an endoplasmic reticulum (ER) export signal. This ER export signal can be fused to the C-terminus of the core amino acid sequence or can be fused to the N-terminus of the core amino acid sequence. In some embodiments, the ER export signal is linked to the core amino acid sequence by a linker. The linker can be any of about 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments, the ER export signal comprises the amino acid sequence FXYENE (SEQ ID NO:57), where X can be any amino acid. In another embodiment, the ER export signal comprises the amino acid sequence VXXSL, where X can be any amino acid. In some embodiments, the ER export signal comprises the amino acid sequence FCYENEV (SEQ ID NO:58).

[00162] Endoplasmic reticulum (ER) export sequences that are suitable for use include, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52)) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55);

FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like. An ER export sequence can have a length of from about 5 amino acids to about 25 amino acids, e.g., from about 5 amino acids to about 10 amino acids, from about 10 amino acids to about 15 amino acids, from about 15 amino acids to about 20 amino acids, or from about 20 amino acids to about 25 amino acids.

[00163] In other aspects, an NpHR protein comprises core amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:31 and a trafficking signal (*e.g.,* which can enhance transport of the NpHR protein 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, which can be any of about 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The NpHR protein may further comprise a fluorescent protein, for example, but not limited to, a 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 other embodiments, the trafficking signal can comprise the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56).

[00164] In some aspects, an NpHR protein comprises a core amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:31; and at least one (such as one, two, three, or more) amino acid sequence motifs which enhance transport to the plasma membranes of mammalian cells selected from the group consisting of an ER export signal, a signal peptide, and a membrane trafficking signal. In some embodiments, the NpHR protein includes an N-terminal signal peptide, a C-terminal ER Export signal, and a C-terminal trafficking signal. In some embodiments, the C-terminal ER Export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The NpHR protein can also further comprise a fluorescent protein, for example, but not limited to, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER

Export signal can be more C-terminally located than the trafficking signal. In other embodiments the trafficking signal is more C-terminally located than the ER Export signal. In some embodiments, the signal peptide includes the amino acid sequence MTETLPPVTESAVALQAE (SEQ ID NO:62). In another embodiment, the NpHR protein includes an amino acid sequence at least 95% identical to SEQ ID NO:31. In another embodiment, the NpHR protein includes an amino acid sequence at least 95% identical to SEQ ID NO:31.

[00165] Moreover, in other aspects, an NpHR protein comprises a core amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:31, wherein the N-terminal signal peptide of SEQ ID NO:31 is deleted or substituted. In some embodiments, other signal peptides (such as signal peptides from other opsins) can be used. The light-activated protein can further comprise an ER transport signal and/or a membrane trafficking signal described herein.

[00166] In some embodiments, the light-activated protein is an NpHR protein that comprises an amino acid sequence at least 75%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the sequence shown in SEQ ID NO:31. In some embodiments, the NpHR protein further comprises an endoplasmic reticulum (ER) export signal and/or a membrane trafficking signal. For example, the NpHR protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:31 and an endoplasmic reticulum (ER) export signal. In some embodiments, the amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:31 is linked to the ER export signal through a linker. In some embodiments, the ER export signal comprises the amino acid sequence FXYENE (SEQ ID NO:57), where X can be any amino acid. In another embodiment, the ER export signal comprises the amino acid sequence VXXSL, where X can be any amino acid. In some embodiments, the ER export signal comprises the amino acid sequence FCYENEV (SEQ ID NO:58). In some embodiments, the NpHR protein comprises an amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:31, an ER export signal, and a membrane trafficking signal. In other embodiments, the NpHR protein comprises, from the N-terminus to the C-terminus, the amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:31, the ER export signal, and the

membrane trafficking signal. In other embodiments, the NpHR protein comprises, from the N-terminus to the C-terminus, the amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:31, the membrane trafficking signal, and the ER export signal. In some embodiments, the membrane trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In some embodiments, the membrane trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). In some embodiments, the membrane trafficking signal is linked to the amino acid sequence at least 95% identical to the sequence shown in SEQ ID NO:31 by a linker. In some embodiments, the membrane trafficking signal is linked to the ER export signal through a linker. The linker may be 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, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments, the light-activated protein further comprises an N-terminal signal peptide.

[00167] Further disclosure related to light-activated chloride pump proteins can be found in U.S. Patent Application Publication Nos: 2009/0093403 and 2010/0145418 as well as in International Patent Application NO: PCT/US2011/028893, the disclosures of each of which are hereby incorporated by reference in their entireties.

Dunaliella salina light-activated polypeptide

[00168] In some embodiments, a suitable light-activated ion channel protein is, e.g., a DsChR protein derived from *Dunaliella salina*, wherein the ion channel protein is capable of mediating a hyperpolarizing current in the cell when the cell is illuminated with light. The light can have a wavelength between about 470 nm and about 510 nm or can have a wavelength of about 490 nm. In some embodiments, a DsChR protein comprises an amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:34. The DsChR protein can additionally comprise substitutions, deletions, and/or insertions introduced into a native amino acid sequence to increase or decrease sensitivity to light, increase or decrease sensitivity to particular wavelengths of light, and/or increase or decrease the ability of the DsChR protein to regulate the polarization state of the plasma membrane of the cell. Additionally, the DsChR protein can comprise one or more

conservative amino acid substitutions and/or one or more non-conservative amino acid substitutions. A DsChR protein containing substitutions, deletions, and/or insertions introduced into the native amino acid sequence suitably retains the ability to transport ions across the plasma membrane of a neuronal cell in response to light.

[00169] In some case, a DsChR protein comprises a core amino acid sequence at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:34; and at least one (such as one, two, three, or more) amino acid sequence motifs which enhance transport to the plasma membranes of mammalian cells selected from the group consisting of a signal peptide, an ER export signal, and a membrane trafficking signal. In some embodiments, the DsChR protein comprises an N-terminal signal peptide and a C-terminal ER export signal. In some embodiments, the DsChR protein comprises an N-terminal signal peptide and a C-terminal trafficking signal. In some embodiments, the DsChR protein comprises an N-terminal signal peptide, a C-terminal ER Export signal, and a C-terminal trafficking signal. In some embodiments, the DsChR protein comprises a C-terminal ER Export signal and a C-terminal trafficking signal. In some embodiments, the C-terminal ER Export signal and the C-terminal trafficking signal are linked by a linker. The linker can be any of about 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, or 500 amino acids in length. The DsChR protein may further comprise a fluorescent protein, for example, but not limited to, a yellow fluorescent protein, a red fluorescent protein, a green fluorescent protein, or a cyan fluorescent protein. In some embodiments the ER Export signal is more C-terminally located than the trafficking signal. In some embodiments the trafficking signal is more C-terminally located than the ER Export signal.

[00170] In some cases, the DsChR polypeptide comprises a membrane trafficking signal and/or an ER export signal. In some embodiments, the trafficking signal is derived from the amino acid sequence of the human inward rectifier potassium channel Kir2.1. In other embodiments, the trafficking signal comprises the amino acid sequence KSRITSEGEYIPLDQIDINV (SEQ ID NO:56). Trafficking sequences that are suitable for use can comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, amino acid sequence identity to an amino acid sequence such a trafficking sequence of human inward rectifier potassium

channel Kir2.1 (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In some cases, the ER export signal is, e.g., VXXSL (where X is any amino acid; SEQ ID NO:52) (e.g., VKESL (SEQ ID NO:53), VLGSL (SEQ ID NO:54); etc.); NANSFCYENEVALTSK (SEQ ID NO:55); FXYENE (SEQ ID NO:57) (where X is any amino acid), e.g., FCYENEV (SEQ ID NO:58); and the like.

[00171] In certain embodiments, the DsChR protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:35.

Anion Channel Polypeptides based on C1C2

[00172] In some embodiments, a light-activated anion channel polypeptide is a C1C2 protein. In some embodiments, a C1C2 polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:36. In some embodiments, the amino acid sequence of the C1C2 protein is modified by introducing one or more of the following mutations into the amino acid sequence: T98S, E129S, E140S, E162S, V156K, H173R, T285N, V281K and/or N297Q. In some embodiments, a C1C2 protein comprises the amino acid sequence of the protein C1C2 with all 9 of the above-listed amino acid substitutions, such that the amino acid sequence of the C1C2 polypeptide is that set forth in SEQ ID NO:36.

[00173] In some embodiments, a C1C2 polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:36; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 amino acid substitutions selected from T98S, E129S, E140S, E162S, V156K, H173R, T285N, V281K and/or N297Q, relative to the amino acid sequence of C1C2 (SEQ ID NO:36). In some embodiments, a C1C2 polypeptide includes an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:36; and includes T98S, E129S, E140S, E162S, and T285N substitutions relative to the amino acid sequence of C1C2. In some embodiments, a C1C2 polypeptide

includes an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:36; and includes V156K, H173R, V281K, and N297Q substitutions relative to the amino acid sequence of C1C2.

[00174] In some embodiments, a C1C2 polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:36; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S98, S129, S140, S162, K156, R173, N285, K281, and Q297, where the amino acid numbering is as set forth in SEQ ID NO:36. In some embodiments, a C1C2 polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:36; and includes S98, S129, S140, S162, K156, R173, N285, K281, and Q297, where the amino acid numbering is as set forth in SEQ ID NO:36. In any one of these embodiments, a C1C2 polypeptide can comprise a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a C1C2 polypeptide can comprise an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a C1C2 polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). Thus, in certain embodiments, the C1C2 protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:36.

[00175] In some embodiments, a C1C2 polypeptide is based on the amino acid sequence of the protein C1C2 (SEQ ID NO:36), wherein the amino acid sequence has been modified by replacing the first 50 N-terminal amino acids of C1C2 with amino acids 1-11 from the protein ChR2 (MDYGGALSAVG) (SEQ ID NO:63). In some embodiments, a suitable light-activated anion channel polypeptide is referred to as "ibC1C2" and comprises an amino acid sequence having at least 58%, at least 60%, at

least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:40; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258, where the amino acid numbering is as set forth in SEQ ID NO:40. In some embodiments, a suitable light-activated anion channel polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:40; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258, where the amino acid numbering is as set forth in SEQ ID NO:40. In some embodiments, a suitable light-activated anion channel polypeptide comprises the amino acid sequence set forth in SEQ ID NO:40. In any one of these embodiments, a suitable anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a suitable anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a suitable anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). Thus, in certain embodiments, the ibC1C2 protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:40.

[00176] In some embodiments, a suitable light-activated anion channel polypeptide is based on the amino acid sequence of the protein C1C2 (SEQ ID NO:36), wherein the cysteine amino acid residue at position 167 has been replaced by a threonine residue. In some embodiments, a suitable light-activated anion channel polypeptide, e.g., $SwiChR_{CT}$, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:38; and comprises 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S98, S129, S140, S162, K156, R173, N285, K281, and Q297; and includes T167. In some embodiments, a suitable light-activated anion channel polypeptide comprises an amino

acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:38; and includes S98, S129, S140, S162, K156, R173, N285, K281, and Q297; and includes T167, where the amino acid numbering is as set forth in SEQ ID NO:38. In some embodiments, a light-activated anion channel polypeptide comprises the amino acid sequence provided in SEQ ID NO:38. In some of these embodiments, the light-activated polypeptide exhibits prolonged stability of photocurrents. In some embodiments, the first 50 amino acids are replaced with MDYGGALSAVG (SEQ ID NO:63). In any one of these embodiments, a suitable anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a suitable anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a suitable anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00177] In some embodiments, a suitable light-activated anion channel polypeptide is based on the amino acid sequence of the protein C1C2, wherein the cysteine amino acid residue at position 167 has been replaced by an alanine residue. In some embodiments, a suitable light-activated anion channel polypeptide, $SwiChR_{CA}$, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:38; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S98, S129, S140, S162, K156, R173, N285, K281, and Q297; and includes A167, where the amino acid numbering is as set forth in SEQ ID NO:38. In some embodiments, a suitable light-activated anion channel polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:38; and includes S98, S129, S140, S162, K156, R173, N285, K281, and Q297; and includes A167, where the amino acid numbering is as set forth in SEQ ID NO:38. In some embodiments, the first 50 amino acids are replaced with

MDYGGALSAVG (SEQ ID NO:63). In any one of these embodiments, a suitable anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide includes an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00178] In some embodiments, a suitable light-activated anion channel polypeptide is based on the amino acid sequence of the protein C1C2, wherein the cysteine amino acid residue at position 167 has been replaced by a serine residue. In some embodiments, a suitable light-activated anion channel polypeptide, $SwiChR_{CS}$, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:38; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S98, S129, S140, S162, K156, R173, N285, K281, and Q297; and includes S167, where the amino acid numbering is as set forth in SEQ ID NO:38. In some embodiments, a suitable light-activated anion channel polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:38; and includes S98, S129, S140, S162, K156, R173, N285, K281, and Q297; and includes S167, where the amino acid numbering is as set forth in SEQ ID NO:38. In some embodiments, the first 50 amino acids are replaced with MDYGGALSAVG (SEQ ID NO:63). In any one of these embodiments, a suitable anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide includes an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00179] In certain embodiments, the SwiChR protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:39.

[00180] In some embodiments, a suitable light-activated anion channel polypeptide, SwiChR, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:38; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S98, S129, S140, S162, K156, R173, N285, K281, and Q297; includes N195, or A195; and includes A167, where the amino acid numbering is as set forth in SEQ ID NO:38. In some embodiments, a suitable light-activated anion channel polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:38; and includes S98, S129, S140, S162, K156, R173, N285, K281, and Q297; includes A167; and includes N195, or A195, where the amino acid numbering is as set forth in SEQ ID NO:38. In some embodiments, the first 50 amino acids are replaced with MDYGGALSAVG (SEQ ID NO:63). In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00181] In some embodiments, a suitable light-activated anion channel polypeptide is based on the amino acid sequence of the protein C1C2 with one or more of the modifications described above, wherein the aspartate amino acid residue at original position 195 has been replaced by an alanine residue. In certain embodiments wherein the first 50 N-terminal amino acids of the protein are replaced by amino acids 1-11 from the protein ChR2, the aspartate amino acid residue at position 156 (which corresponds to

original position 195 of the C1C2 amino acid sequence set forth in SEQ ID NO:36) is replaced by an alanine residue.

[00182] In some embodiments, a suitable hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein C1C2 with one or more of the modifications described above, wherein the aspartate amino acid residue at original position 195 has been replaced by an asparagine residue. In certain embodiments wherein the first 50 N-terminal amino acids of the protein are replaced by amino acids 1-11 from the protein ChR2, the aspartate amino acid residue at position 156 (which corresponds to original position 195 of the C1C2 amino acid sequence set forth in SEQ ID NO:36) is replaced by an asparagine residue.

[00183] In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:40; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258; and includes A128, T128 or S128, where the amino acid numbering is as set forth in SEQ ID NO:40. In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:40; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258; and includes A128, T128 or S128, where the amino acid numbering is as set forth in SEQ ID NO:40. In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a suitable anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a suitable anion channel polypeptide includes both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

Anion Channel Proteins based on ChR2

[00184] In some embodiments, a suitable hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein ChR2. The amino acid sequence of ChR2 is set forth in SEQ ID NO:42. In some embodiments, the amino acid sequence of the ChR2 protein has been modified by introducing one or more of the following mutations into the amino acid sequence: A59S, E90S, E101S, E123S, Q117K, H134R, V242K, T246N and/or N258Q. In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises the amino acid sequence of the protein ChR2 with all 9 of the above-listed amino acid substitutions, such that the amino acid sequence of the polypeptide is provided in SEQ ID NO:42 (iChR2).

[00185] In some embodiments, a suitable light-activated anion channel polypeptide iChR2 comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:42; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 amino acid substitutions selected from A59S, E90S, E101S, E123S, Q117K, H134R, V242K, T246N and/or N258Q, relative to the amino acid sequence of ChR2 (SEQ ID NO:1).

[00186] In some embodiments, a suitable light-activated polypeptide ("iChR2") comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:42; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, K242, N246 and Q258, where the amino acid numbering is as set forth in SEQ ID NO:42. In some embodiments, an iChR2 polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:42; and includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of: S59, S90, S101, S123, K117, R134, K242, N246, Q258, and either N156 or A156, and either T128, A128, or S128, where the amino acid numbering is as set forth in SEQ ID NO:42. In some embodiments, an iChR2 polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at

least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:42; and includes S59, S90, S101, S123, K117, R134, K242, N246 and Q258, where the amino acid numbering is as set forth in SEQ ID NO:42. In any one of these embodiments, an iChR2 polypeptide can comprise a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, an iChR2 polypeptide can comprise an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, an iChR2 polypeptide can comprise both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). Thus in certain embodiments, the iChR2 protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:43.

Anion Channel Polypeptides based on C1V1

[00187] In some embodiments, a suitable hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein C1V1. The amino acid sequence of C1V1 is set forth in SEQ ID NO:44. In some embodiments, the amino acid sequence of the C1V1 protein has been modified by introducing one or more of the following mutations into the amino acid sequence: T98S, E129S, E140S, E162S, V156K, H173R, A285N, P281K and/or N297Q. In some embodiments, a hyperpolarizing light-activated polypeptide comprises the amino acid sequence of the protein C1V1 with all 9 of the above-listed amino acid substitutions, such that the amino acid sequence of the polypeptide is provided in SEQ ID NO:44.

[00188] In some embodiments, a suitable light-activated anion channel polypeptide, iC1V1, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:44; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 amino acid substitutions selected from T98S, E129S, E140S, E162S, V156K, H173R, A285N, P281K and/or N297Q, relative to the amino acid sequence of C1V1 (SEQ ID NO:7).

[00189] In some embodiments, a suitable light-activated anion channel polypeptide, iC1V1, comprises an amino acid sequence having at least 58%, at least 60%, at least

65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:44; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S98, S129, S140, S162, K156, R173, N285, K281, and Q297, where the amino acid numbering is as set forth in SEQ ID NO:44. In some embodiments, a suitable light-activated anion channel polypeptide (referred to as "iC1V1"), comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:44; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S98, S129, S140, S162, K156, R173, N285, K281, and Q297, and includes N195, where the amino acid numbering is as set forth in SEQ ID NO:44. In some embodiments, a suitable light-activated anion channel polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:44; and includes S98, S129, S140, S162, K156, R173, N285, K281, and Q297, where the amino acid numbering is as set forth in SEQ ID NO:44. In any one of these embodiments, a suitable anion channel polypeptide includes a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide includes an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a suitable anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). Thus in certain embodiments, the iC1V1protein can have an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:45.

[00190] In some embodiments, a suitable hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein C1V1 (SEQ ID NO:7), wherein the amino acid sequence has been modified by replacing the first 50 N-terminal amino acids of C1V1 with amino acids 1-11 from the protein ChR2 (MDYGGALSAVG) (SEQ ID NO:63). In some embodiments, a suitable hyperpolarizing light-activated polypeptide, ibC1V1, comprises an amino acid sequence having at least 58%, at least 60%, at least

65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:46; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258, where the amino acid numbering is as set forth in SEQ ID NO:46. In some embodiments, a suitable hyperpolarizing light-activated polypeptide (referred to as "ibC1V1"), comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:46; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258, and includes N156, where the amino acid numbering is as set forth in SEQ ID NO:46. In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:46; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258, where the amino acid numbering is as set forth in SEQ ID NO:46. In some embodiments, a suitable light-activated anion channel polypeptide comprises the amino acid sequence set forth in SEQ ID NO:46. In any one of these embodiments, a suitable anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a suitable anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). Thus in certain embodiments, an ibC1V1 protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:47.

[00191] In some embodiments, a suitable hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein C1V1 (SEQ ID NO:7), wherein the cysteine amino acid residue at position 167 has been replaced by a threonine residue. In

some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:7; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S98, S129, S140, S162, K156, R173, N285, K281, and Q297; and includes T167. In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:44; and includes S98, S129, S140, S162, K156, R173, N285, K281, and Q297; and includes T167, S167 or A167, where the amino acid numbering is as set forth in SEQ ID NO:44. In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:46; and includes S98, S129, S140, S162, K156, R173, N285, K281, and Q297; includes T167, S167 or A167; and includes A195 or N195, where the amino acid numbering is as set forth in SEQ ID NO:46. In some embodiments, the first 50 amino acids are replaced with MDYGGALSAVG (SEQ ID NO:63). In any one of these embodiments, a suitable hyperpolarizing light-activated polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a suitable hyperpolarizing light-activated polypeptidecomprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a suitable hyperpolarizing light-activated polypeptide includes both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00192] In some embodiments, a suitable hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein C1V1 with one or more of the modifications described above, wherein the aspartate amino acid residue at original position 195 has been replaced by an alanine residue. In certain embodiments wherein the first 50 N-terminal amino acids of the protein are replaced by amino acids 1-11 from

the protein ChR2, the aspartate amino acid residue at position 156 (which corresponds to original position 195 of the C1V1 amino acid sequence set forth in SEQ ID NO:7) is replaced by an alanine residue.

[00193] In some embodiments, a suitable hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein C1V1 with one or more of the modifications described above, wherein the aspartate amino acid residue at original position 195 has been replaced by an asparagine residue. In certain embodiments wherein the first 50 N-terminal amino acids of the protein are replaced by amino acids 1-11 from the protein ChR2, the aspartate amino acid residue at position 156 (which corresponds to original position 195 of the C1V1 amino acid sequence set forth in SEQ ID NO:7) is replaced by an asparagine residue.

[00194] In some embodiments, a suitable hyperpolarizing light-activated polypeptide, ibC1V1, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:46; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258; and includes T128, A128, or S128, where the amino acid numbering is as set forth in SEQ ID NO:46. In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:46; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258; and includes T128, A128, or S128, where the amino acid numbering is as set forth in SEQ ID NO:46. In any one of these embodiments, a suitable anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a suitable anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a suitable anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00195] In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:46; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258; and includes T128, A128, or S128; and includes A156 or N156, where the amino acid numbering is as set forth in SEQ ID NO:46. In some embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:46; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258; and includes T128, A128, or S128; and includes A156 or N156, where the amino acid numbering is as set forth in SEQ ID NO:46. In any one of these embodiments, a suitable hyperpolarizing light-activated polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a suitable hyperpolarizing light-activated polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide includes both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

Anion Channel Polypeptides based on ReaChR

[00196] In some embodiments, a subject hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein ReaChR. The amino acid sequence of ReaChR is set forth in SEQ ID NO:11. In some embodiments, the amino acid sequence of the ReaChR protein has been modified by introducing one or more of the following mutations into the amino acid sequence: T99S, E130S, E141S, E163S, V157K, H174R, A286N, P282K and/or N298Q. In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises the amino acid sequence of the protein ReaChR with all 9 of the above-listed amino acid substitutions, such that the amino acid sequence of the polypeptide is provided in SEQ ID NO:48.

[00197] In some embodiments, a subject light-activated anion channel polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:48; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 amino acid substitutions selected from T99S, E130S, E141S, E163S, V157K, H174R, A286N, P282K and/or N298Q, relative to the amino acid sequence of ReaChR (SEQ ID NO:11).

[00198] In some embodiments, a subject light-activated anion channel polypeptide, iReaChR, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:48; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S99, S130, S141, S163, K157, R174, N286, K281, and Q298, where the amino acid numbering is as set forth in SEQ ID NO:48. In some embodiments, a subject light-activated anion channel polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:48; and includes S99, S130, S141, S163, K157, R174, N286, K281, and Q298, where the amino acid numbering is as set forth in SEQ ID NO:48. In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a

subject anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide includes both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). Thus in certain embodiments, the iReaChR protein comprises an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:49.

[00199] In some embodiments, a subject light-activated anion channel polypeptide, iReaChR, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:48; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S99, S130, S141, S163, K157, R174, N286, K281, and Q298, and includes N196, where the amino acid numbering is as set forth in SEQ ID NO:48. In some embodiments, a subject light-activated anion channel polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:48; and includes S99, S130, S141, S163, K157, R174, N286, K281, and Q298, and includes N196, where the amino acid numbering is as set forth in SEQ ID NO:48. In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00200] In some embodiments, a subject hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein ReaChR (SEQ ID NO:11), wherein the amino acid sequence has been modified by replacing the first 51 N-terminal amino acids of ReaChR with amino acids 1-11 from the protein ChR2 (MDYGGALSAVG) (SEQ ID

NO:63). In some embodiments, a subject hyperpolarizing light-activated polypeptide, ibReaChR, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:50; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258, where the amino acid numbering is as set forth in SEQ ID NO:50. In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:50; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258, where the amino acid numbering is as set forth in SEQ ID NO:50. In some embodiments, a subject light-activated anion channel polypeptide comprises the amino acid sequence set forth in SEQ ID NO:50. In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). Thus in certain embodiments, the ibReaChR protein can have an amino acid sequence that is at least 75%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence shown in SEQ ID NO:51.

[00201] In some embodiments, a subject hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein ReaChR (SEQ ID NO:11), wherein the amino acid sequence has been modified by replacing the first 51 N-terminal amino acids of ReaChR with amino acids 1-11 from the protein ChR2 (MDYGGALSAVG) (SEQ ID NO:63). In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence

set forth in SEQ ID NO:11; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258, and includes N156, where the amino acid numbering is as set forth in SEQ ID NO:11. In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:11; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258, and includes N156, where the amino acid numbering is as set forth in SEQ ID NO:11. In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00202] In some embodiments, a subject hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein ReaChR (SEQ ID NO:11), wherein the cysteine amino acid residue at position 168 has been replaced by a threonine residue. In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:11; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S99, S130, S141, S163, K157, R174, N286, K281, and Q298; and includes T168, S168 or A168. In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:11; and includes S99, S130, S141, S163, K157, R174, N286, K281, and Q298; and includes T168, S168 or A168, where the amino acid numbering is as set forth in SEQ ID NO:11. In some embodiments, the first 51 amino acids are replaced with MDYGGALSAVG (SEQ ID

NO:63). In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00203] In some embodiments, a subject hyperpolarizing light-activated polypeptide, iReaChR, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:48; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S99, S130, S141, S163, K157, R174, N286, K281, and Q298; includes A196 or N196; and includes T168, S168, or A168, where the amino acid numbering is as set forth in SEQ ID NO:48. In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth SEQ ID NO:48; and includes S99, S130, S141, S163, K157, R174, N286, K281, and Q298; includes A196 or N196; and includes T168, S168, or A168, where the amino acid numbering is as set forth in SEQ ID NO:48. In some embodiments, the first 51 amino acids are replaced with MDYGGALSAVG (SEQ ID NO:63). In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide includes both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00204] In some embodiments, a subject hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein ReaChR with one or more of the modifications described above, wherein the aspartate amino acid residue at original

position 196 has been replaced by an alanine residue. In certain embodiments wherein the first 51 N-terminal amino acids of the protein are replaced by amino acids 1-11 from the protein ChR2, the aspartate amino acid residue at position 156 (which corresponds to original position 196 of the ReaChR amino acid sequence set forth in SEQ ID NO:11) is replaced by an alanine residue.

[00205] In some embodiments, a subject hyperpolarizing light-activated polypeptide is based on the amino acid sequence of the protein ReaChR with one or more of the modifications described above, wherein the aspartate amino acid residue at original position 196 has been replaced by an asparagine residue. In certain embodiments wherein the first 51 N-terminal amino acids of the protein are replaced by amino acids 1-11 from the protein ChR2, the aspartate amino acid residue at position 156 (which corresponds to original position 196 of the ReaChR amino acid sequence set forth in SEQ ID NO:11) is replaced by an asparagine residue.

[00206] In some embodiments, a subject hyperpolarizing light-activated polypeptide, ibReaChR, comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:50; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258; and includes T128, S128 or A128, where the amino acid numbering is as set forth in SEQ ID NO:50. In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:50; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258; and includes T128, where the amino acid numbering is as set forth in SEQ ID NO:50. In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide comprises an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g.,

KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

[00207] In some embodiments, a subject hyperpolarizing light-activated polypeptide, ibReaChR, includes an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:50; and includes 1, 2, 3, 4, 5, 6, 7, 8, or 9 of: S59, S90, S101, S123, K117, R134, N246, K242, and Q258; includes T128, S128 or A128; and includes A156 or N156, where the amino acid numbering is as set forth in SEQ ID NO:50. In some embodiments, a subject hyperpolarizing light-activated polypeptide comprises an amino acid sequence having at least 58%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO:50; and includes S59, S90, S101, S123, K117, R134, N246, K242, and Q258; includes T128, S128 or A128; and includes A156 or N156, where the amino acid numbering is as set forth in SEQ ID NO:50. In any one of these embodiments, a subject anion channel polypeptide comprises a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)). In any one of these embodiments, a subject anion channel polypeptide includes an ER export signal (e.g., FCYENEV (SEQ ID NO:58)). In any one of these embodiments, a subject anion channel polypeptide comprises both a membrane trafficking signal (e.g., KSRITSEGEYIPLDQIDINV (SEQ ID NO:56)) and an ER export signal (e.g., FCYENEV (SEQ ID NO:58)).

UTILITY

[00208] The methods of the present disclosure find a variety of uses. As described above, the methods of the present disclosure find use in dissecting and analyzing functional brain circuits. In some cases, the present method may provide a way to identify new roles for anatomically and/or functionally defined neurons in functional circuits. In some cases, the anatomical or structural connections between regions of the brain may not be sufficient to provide insight into the functional role of the connections, and a method of the present disclosure may reveal the functional connection.

[00209] In some cases, the present methods may provide a way to establish a causative link at a cellular level for functional connections between regions of the brain for which only correlative data is available. Thus, in some cases, the present methods can demonstrate the presence or absence of a causative relationship underlying a correlative observation in a brain circuit.

[00210] In certain embodiments, the present methods provide for selectively activating a specific population of neurons, via a combination of selective expression of light-activated polypeptides and selective illumination of brain regions, at different temporal frequencies, wherein the number of neurons activated at each frequency remains substantially the same. Thus, an effect of increased frequency of light pulses activating a first region on the response at a functionally connected second region of the brain may be attributed mainly to the change in frequency, and not on other factors, e.g., recruitment of more neurons in a frequency-dependent manner.

[00211] The present methods also find use in probing the effect of deep brain stimulation (DBS) of brain regions, e.g., the central thalamus, insula, cingulate, subthalamic nucleus (STN), globus pallidus interna (GPI), zona incerta (ZI), etc., that may find use in the treatment of various neurological disorders, such as pain, depression, addiction, Alzheimer's disease, attention deficit disorder, autism, anorgasmia, cerebral palsy, bipolar depression, unipolar depression, epilepsy, generalized anxiety disorder, acute head trauma, hedonism, obesity, obsessive-compulsive disorder (OCD), acute pain, chronic pain, Parkinson's disease, persistent vegetative state, phobia, post-traumatic stress disorder, rehabilitation/regenesis for post-stroke, post- head trauma, social anxiety disorder, Tourette's Syndrome, hemorrhagic stroke, and ischemic stroke. The present methods, in some cases, may provide a way to probe the effect of a single parameter of stimulation, such as light pulse frequency or pulse width, of a defined population of neurons, on global brain dynamics, as well as cellular level functional circuits.

EXAMPLES

[00212] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the

only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1: Materials and Methods

[00213] **Animals.** Female Sprague-Dawley rats (>11 weeks old, 250-350g) were used as subjects for all thalamic injections. Animals were individually housed under a 12 hour light-dark cycle and provided with food and water *ad libitum*. Animal husbandry and experimental manipulation were in strict accordance with National Institute of Health, Univeristy of California, Los Angeles (UCLA) Institutional Animal Care and Use Committee (IACUC), and Stanford University IACUC guidelines.

[00214] **Viral injections and fiber placement.** pAAV5-CaMKIIa-hChR2(H134R)-EYFP-WPRE plasmid was obtained from the Deisseroth lab at Stanford University. Concentrated virus was produced at the vector core of the University of North Carolina at Chapel Hill. Rats were anesthetized with isoflurane (induction 5%, maintenance 2-3%; Sigma-Aldrich, St. Louis, Missouri, USA) and secured in a stereotactic frame. Standard procedures for sterile surgery were followed. Buprenorphine was administered to minimize pain. Artificial tears were applied to the eyes. The head was shaved, and 70% ethanol and betadine were applied to the bare scalp following a midline incision. A small craniotomy was performed with a dental drill above the targeted coordinate. Two microliters of virus were injected through a 34 gauge needle (World Precision Instruments Inc., Sarasota, FL) at 150 nl/min with a micro-syringe pump controller at the desired coordinates in central thalamus or other subcortical targets for control experiments: **I)** CL and PC nuclei of central thalamus (-3.2 mm AP, +1.5 mm ML, -5.6 mm DV; $n = 47$ animals for imaging); **II)** ventral posteromedial nucleus (-2.5 mm AP, +2.6 mm ML, -6.0 mm DV); **III)** anterior thalamic nuclei (-3.1 mm AP, +1.8 mm ML, -5.3 mm DV); **IV)** posterior thalamic nuclei (-4.6 mm AP, +1.8 mm ML, -5.0 mm DV);

V) intermediate hippocampus (-5.8 mm AP, +5.2 mm ML, -3.4 mm DV, n = 8 animals). All injections were made in the right hemisphere. The syringe needle was left in place for an additional 10 minutes before being slowly withdrawn. Custom-designed guide cannulas (Plastics One) or fiber-optic cannulas (Doric Lenses Inc.) were mounted on the skull and secured using metabond (Parkell). Incisions were sutured, and animals were kept on a heating pad until recovery from anesthesia. Buprenorphine was injected subcutaneously twice daily for 48 hours post-operatively to minimize discomfort. The original cohort of 47 central thalamus animals was further refined to a group of 18 after screening for implant locations less than 0.85 mm away from the target coordinate (estimated with T2 magnetic resonance imaging (MRI) scans; Figure 1B). Two additional animals were excluded due to lack of thalamic activation, leaving 16 animals for analysis.

[00215] In a second cohort of rats, concentrated AAV5-hSyn-eNpHR3.0-mCherry virus produced at the University of North Carolina at Chapel Hill vector core was injected into the right zona incerta (-3.96 mm AP, +2.8 mm ML, +7.4 mm DV, n = 4 animals) after completion of the ChR2 injection into the central thalamus as described above. Both injections were performed during the same surgery. 0.5 microliters of eNpHR virus were injected through a 34 gauge needle at 100 nl/min. Following the injection, the syringe needle was left in place for approximately 10 minutes before being slowly withdrawn. Recovery details were the same as described above.

[00216] **Optogenetic functional MRI (ofMRI) data acquisition**. Functional MRI (fMRI) scanning was performed using a 7T Bruker Biospec small animal MRI system at UCLA. Animals were initially anesthetized with 5% isoflurane and intubated before placement onto custom-made MRI-compatible cradles with ear and tooth securement. A 39 mm outer diameter, 25 mm inner diameter custom-designed transmit/receive single-loop surface coil was centered over the region of interest on the skull to maximize signal-to-noise ratio. An optical fiber of 62.5 μm core diameter was connected to a 473 nm laser source (Laserglow Technologies, Toronto, Canada) and coupled with the implanted fiber-optic cannula. A single ofMRI scan consisted of a block design with six 20 s pulse trains of light (10, 40, or 100 Hz in randomized order) delivered once per minute over 6 minutes. Five to six consecutive scans were collected during each session. For all experiments, the optical fiber output power was calibrated to 2.5 mW. A duty

cycle of 30% was used across frequencies to maintain the total amount of light delivery, resulting in unique pulse widths of 30, 7.5, and 3 ms for 10, 40, and 100 Hz, respectively. In a series of control experiments using a second cohort of animals with validated probe locations ($n = 3$), the duty cycle was varied while the pulse width was held constant at 3 ms (Figure 10).

[00217] During fMRI scanning, animals were placed into the iso-center of the magnet while artificially ventilated (45~60 strokes/min) under light anesthesia using a ventilator and calibrated vaporizer with a mixture of O_2 (35 %), N_2O (63.5 %), and isoflurane (1.3-1.5 %). To ensure stable (blood oxygenation level dependent) BOLD signals, expiratory CO_2 was kept at 3-4 % and body temperature was maintained at 36.5-37.5 °C using heated airflow. T2-weighted high-resolution anatomical images were acquired with a fast spin echo sequence prior to fMRI scanning to check for brain damage and validate the optical fiber's location (137 μm resolution in-plane resolution with 35×35 mm^2 field of view (FOV), 0.5 mm slice thickness, 32 coronal slices). Gradient recalled echo (GRE) BOLD methods were used to acquire fMRI images during photostimulation. The fMRI image acquisition was designed to have 35×35 mm^2 in-plane field of view (FOV) and 0.5×0.5×0.5 mm^3 spatial resolution with a sliding window reconstruction to update the image every repetition time (TR). The two-dimensional, multi-slice gradient-echo sequence used a four-interleave spiral readout (96, 97), 30° flip angle, 750 ms TR, and 12 ms echo time, resulting in 23 coronal slices (128 × 128 matrix size). The spiral k-space samples were reconstructed through a 2-dimensional gridding reconstruction method. Finally, real-time motion correction was performed using a previously described graphical processing unit (GPU)-based system. Scans with significant motion, identified by careful visual inspection for spiral artifacts and activations at the boundary of the brain, which indicates large motion, were excluded from analysis. This condition for exclusion was established prior to data collection.

[00218] **fMRI data analysis.** All fMRI data processing was performed using the Matlab® software environment (MathWorks, Inc., Natick, MA) and mrVista (Stanford Vision and Imaging Science and Technology Laboratory, Stanford, CA; web(dot)stanford(dot)edu/group/vista/cgi-bin/wiki/index(dot)php/MrVista). Motion-corrected images belonging to consecutive scans of the same stimulation paradigm and scanning session were first averaged together. The average four-dimensional (4D)

images were then aligned to a common coordinate frame, using a six degree-of-freedom rigid body transformation. If multiple scanning sessions were performed on the same animal at the same frequency (typically 1, at most 4), the resulting images from each session were first averaged together before any inter-subject analysis to achieve maximum signal-to-noise ratio (SNR) while weighting the images from all animal subjects equally.

[00219] Time series were calculated for each voxel in these individual-animal images as the percent modulation of the BOLD signal relative to a 30 s baseline period collected prior to stimulation. Boxcar detrending with a window size of 1 minute was also performed to correct for possible scanner drift. Next, a coherence value was calculated for each voxel's time series as the magnitude of its Fourier transform at the frequency of repeated stimulation blocks (i.e. 1/60 Hz) divided by the sum-of-squares of all frequency components. Voxels with a coherence value greater than 0.35 were considered to be significantly synchronized to stimulation. Assuming Gaussian noise and ~470 degrees of freedom (computed using the statistical parametric mapping (SPM) software environment), the Bonferroni-corrected *P* value for this threshold can be estimated to be less than 10^{-9}. Activation volume (Figure 2) was defined as the number of significant voxels that exhibited a positive response within a predefined region of interest, multiplied by the volume per voxel. Positive responses were identified as having a phase in the interval $[0, \pi/2] \cup [3\pi/2, 2\pi]$. Phase represents the temporal shift of the response when it is modeled as a sinusoid, and was calculated as the argument of each voxel's Fourier transform at the frequency of repeated stimulation blocks (i.e. 1/60 Hz).

[00220] Hemodynamic response functions (HRFs) were calculated as the average 60 s response of a voxel's six-cycle, 6-minute time series. Time series and HRFs displayed for figures were generated by averaging the mean time series or mean HRF of voxels with a coherence value greater than 0.35 in the corresponding ROI across animals. In some cases, the first data point's value was subtracted from each voxel's HRF to define its relative percent modulation from the onset of stimulation.

[00221] To generate average activation maps (Figure 2), the 4D fMRI images from experiments at the same stimulation location and frequency were normalized and averaged together across animals. The averaged images were then processed according to the above Fourier domain analyses. Coherence values were overlaid onto all voxels

having a coherence above the 0.35 threshold. Warm and cool colormaps generated using Matlab®'s 'hot' and 'winter' functions were used for positive and negative responses, respectively, to illustrate the localization of negative BOLD to the somatosensory cortex. These activation maps were overlaid onto corresponding T2-weighted anatomical images with a digital standard rat brain atlas. The same atlas was used to segment ROIs. An identical analysis pipeline was used for activation maps in Figure 10 with a representative animal.

[00222] **Electroencephalogram (EEG) electrode implantation**. EEG electrodes were implanted upon completion of ofMRI experiments in a subset of animals. Surgical preparation and recovery details were the same as those used for virus injection. Stainless steel screws (0-80, 1.5 mm diameter, Plastics One) attached to 2 cm of insulated wire (30 gauge, R30Y0100, Wire Wrapping Wire, O.K. Industries) were used as EEG electrodes and secured on the skull using dental cement. The recording electrode was placed approximately 2 mm anterior of bregma and 2 mm to the right of midline. The reference electrode was located approximately 5 mm anterior of bregma and 3 mm to the left of midline.

[00223] **Video-EEG acquisition and analysis**. Prior to video-EEG recording, animals were anesthetized under 5% isoflurane for approximately 5 min for optical fiber coupling and EEG wire connection. Animals were then transferred to a light- and sound-controlled experimental chamber where they were allowed to move freely. Behavioral experiments began after animals recovered from anesthesia and subsequently fell asleep for 15 min (as indicated by lack of motion and real-time EEG output readings). For each experiment, the animal was video-recorded during 5 min of sleep, followed by 20 s of optical stimulation (10, 40, or 100 Hz, 473 nm laser, 2.5 mW laser power, 30% duty cycle), and then an additional 5 min post-stimulation period. EEG data was acquired throughout the experiment at 1 kHz with an MP150 data acquisition unit and EEG100C amplifier (Biopac Systems Inc., Santa Barbara, CA), using EL254S Ag-AgCl electrodes and Gel102 conductive EEG paste. A digital camera was used to video-record the experiment. All behavioral experiments were performed during the animals' light cycle.

[00224] EEG recordings were classified using the Biopac Acqknowledge® software by an experienced electroencephalographer blind to treatment into a single best category: normal, low-voltage fast, spikes, spike-waves, or evolving electrographic seizure. Video

clips paired to each EEG recording were classified into one of the following categories to further assess the animal's brain state: sleep (i.e. no change), awakening (animal is alert and exploring), absence seizure (animal is immobile and appears frozen for the duration of stimulation, but returns to a sleeping state once stimulation ends), or convulsive seizure. All observed behavioral responses could be classified into one of these categories. Band power in Figure 13 was quantified using Matlab®'s 'bandpower' function and normalized by the signal's total power from 0 Hz to one half the sampling rate (500 Hz).

[00225] ***In vivo* electrophysiology**. Upon completion of ofMRI and EEG behavioral experiments, *in vivo* electrophysiology experiments were performed in a subset of animals. Animals were anesthetized with 5% isoflurane for induction and maintained at 2-3% until any craniotomies were complete. Isoflurane was kept at 0.8-1.2% during the recording session, and artificial tears were applied to the eyes. Recordings in Figures 4 and 5 were performed under ventilation conditions identical to fMRI experiments. After securing the animal within a stereotactic frame, small craniotomies were performed using a dental drill above the region of interest. For stimulation, the cannula implanted at central thalamus was connected to a 473 nm laser source (Laserglow Technologies) with an output power level of 2.5 mW via an optical fiber. The cannula implanted at zona incerta was connected to a 593 nm laser source (Laserglow Technologies) calibrated to 2.5-3.0 mW. An acute 16-channel microelectrode array was targeted to the recording site using stereotactic instruments (NeuroNexus Technologies; A1x16 standard model linear electrode array for local and cortical recordings; V1-16-Poly2 polytrode array for ZI recordings; 0.35±0.5 MOhm impedance). A stainless steel reference screw was placed above the cerebellum. Continuous field potential and single unit spiking events were recorded using the Plexon omniplex system with plexcontrol software (Plexon Inc., TX, USA). When only ChR2 was activated, recordings were performed for 20 seconds without stimulation, followed by repeated stimulation cycles (20 s on, 40 s off) at 10, 40, or 100 Hz with 30% duty cycle. When ChR2 and eNpHR were activated together, the same stimulation paradigm was followed, except that a 30 s period of continuous 593 nm light delivery began 5 s before the onset of ChR2 excitation. When only eNpHR was activated (Figure 5D), a 20 or 30 s period of continuous 593 nm light delivery was used, with 40 or 30 s periods of no light delivery between repeated cycles, respectively. For

single unit responses, the Plexon multichannel acquisition processor was used to amplify and band-pass filter the neuronal signals (150 Hz – 8 kHz). Signals were digitized at 40 kHz and processed to extract action potentials in real-time. To separate the field potential, a low-pass filter (200 Hz cutoff frequency, 4-pole Bessel filter) was used and signals were downsampled to 1 kHz. Simultaneous EEG data was collected at 1 kHz during zona incerta recordings in Figure 4 using the MP150 data acquisition unit and EEG100C amplifier (Biopac).

[00226] **Zona incerta electrophysiology analysis.** For the analysis in Figure 4, field potential recordings were high pass filtered with a cutoff frequency of 2 Hz to eliminate respiratory artifacts. Spindle-like oscillations (SLOs) occurring during the stimulus were then quantified on a per trial basis using a *post-hoc* custom algorithm. Briefly, an SLO was identified when the recording's magnitude reached at least 6 standard deviations above its mean absolute value. If the recording did not exceed this value for the preceding 500 ms, and was above this value for at least 2% of samples over the next 500 ms, an SLO was counted. This method of quantification accurately captured the large-amplitude oscillations that could be visually discerned (see Figure 4D).

[00227] **Fluorescence imaging and immunohistochemistry**. Upon completion of *in vivo* ofMRI, behavioral, and electrophysiology experiments, rats were deeply anesthetized with isoflurane in a knockdown box and transcardially perfused with 0.1M phosphate buffered saline (PBS) and ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were extracted and fixed in 4% PFA overnight at 4 °C. The brains were equilibrated in 10%, 20%, and then 30% sucrose in PBS at 4 °C. Coronal sections (50 µm) were prepared on a freezing microtome (HM 430 Sliding Microtome, Thermo Scientific Inc.). Consecutive sections (500 µm apart) were mounted and examined with a fluorescence microscope (Leica EL6000). For quantitative immunohistochemistry (Figure 7), free-floating sections were processed with 5% normal donkey serum, and 0.4% Triton X-100 for 60 min. Sections were then exposed at 4 °C for 48 hr to primary antibodies against mouse monoclonal CaMKIIα (CaMKIIα, 1:500, 05-532, Millipore®, Billerica, MA). After washing with PBS, sections incubated for 2 hr at room temperature with Alexa Fluor® 647-conjugated AffiniPure donkey anti-mouse IgG (1:250, Jackson Laboratories, West Grove, PA). Slices were then washed and mounted (DAPI-Fluoromount G,

SouthernBiotech, Birmingham, AL). Immuno-fluorescence was assessed with a laser confocal microscope (Leica CTR 6500).

[00228] For high-resolution, whole-brain fluorescence imaging (Figures 1A, 4H, and Figure 8), frozen brains were embedded using stainless steel Tissue-Tek® base molds and Neg-50 embedding medium (Richard-Allan Scientific (Thermo); $n = 2$ animals) (103). Post-freezing, the Neg-50 embedded brain was sectioned on a Microm™ HM550 cryostat using the tape-transfer method with all sections mounted directly onto slides. Alternating sections, cut at 20 μm, were separated to form two distinct series per brain. One slide series of the sectioned material was processed for Nissl cell body staining, using a thionin-based protocol and coverslipped with DPX mounting medium. The alternate series was dehydrated and directly coverslipped with DPX for fluorescence imaging. Whole-slide digital imaging was performed using a Hamamatsu NanoZoomer® 2.0-HT system at 0.46 μm/pixel, with fluorescence scans at 12-bit depth using a tri-pass filter cube. Following data conversion to lossless jp2 (JPEG2000), individual brain sections were aligned and registered using rigid two-dimensional (2-D) image transformation.

[00229] **Statistics.** All statistical tests were performed in Matlab®. Non-parametric tests were used throughout the analysis. For *in vivo* electrophysiology measurements at thalamus and zona incerta, one-tailed Wilcoxon signed-rank tests were used to evaluate changes in firing rate between the pre-stimulation and stimulation periods. For measurements at sensory cortex in Figure 3G, a two-tailed version of the test was used to evaluate either increases or decreases in firing rate. For results in Table 1, the average pre-stimulus firing rate (20 s bin) was compared to the average firing rate of four 5 s bins over the 20 s period of stimulation using a one-tailed Wilcoxon signed rank test, uncorrected for multiple comparisons. One-sided Wilcoxon rank sum tests were used to evaluate differences in SLO occurrence (Figure 4E), as well as changes in cortical or incertal firing when eNpHR activation was coupled with central thalamus stimulation (Figures 5F and 5H). For electrophysiology results, independence was assumed between repeated trials. All other assumptions for these tests were satisfied. For volumetric comparisons in Figure 2E-G, one-sided Wilcoxon signed-rank tests were used to identify increases in the volume of BOLD activation between 10 and 40 Hz and 10 and 100 Hz (corrected for multiple comparisons). Note that variance was generally similar across

groups being compared. Significance was determined at the $\alpha = 0.05$ cutoff level. No statistical methods were used to estimate sample size. All statistical tests used to compare changes with frequency (Figures 2E-2G, and Figure 9B) were performed pairwise, with an equal number of animals used for each frequency.

Example 2: High-Frequency Stimulation of Central Thalamus Relay Neurons Drives Widespread Forebrain Activation In Vivo

[00230] To investigate the specific role of central thalamus, optogenetic techniques were applied to control relay cells in a spatially and temporally precise manner. A stereotactic injection in the right CL and PC intralaminar nuclei of central thalamus was performed with adeno-associated virus carrying channelrhodopsin-2 (ChR2) and the fluorescent reporter protein EYFP under control of the CaMKIIa promoter. This promoter is expressed primarily in excitatory neurons, the vast majority of which in thalamus are relay cells. 35% of cells identified within the bulk injection area were EYFP-positive, and 97% of EYFP-positive cells co-expressed CaMKIIa, indicating high sensitivity for stimulation of excitatory neurons ($n = 2$ rats, 831 cells; Figure 7). While ChR2-EYFP expression extended beyond these two nuclei (Figure 1A), targeted stimulation of the intralaminar nuclei was achieved by (a) stereotactic placement of the implanted optical fiber, as confirmed with high-resolution T2-weighted structural MR images (Figure 1B,C), and (b) spatially restricted illumination (Figure 1A,B). 47 rats were initially injected and cannulated using the central thalamus as the stereotactic target (-3.2 mm AP, +1.5 mm ML, -5.5 mm DV). However, the intralaminar nuclei are relatively small and difficult to accurately target. Therefore only a subset of these animals were used based on the empirically observed distribution of optical fiber tip locations using T2-weighted MRI scans (Figure 1B; <0.85 mm distance from target coordinate). Of the 18 rats that had an accurately localized implant location, two exhibited a general absence of fMRI activity – most notably at the site of stimulation – and were excluded, leaving 16 animals for further analysis.

[00231] **Figure 1. Targeted stimulation of central thalamus evokes positive BOLD changes and increases in neuronal firing at the site of stimulation.** (*Figure 1A*) Representative wide-field fluorescence image shows robust ChR2-EYFP expression throughout central thalamus, overlaid with the estimated cone of excited tissue shown to scale. (*Figure 1B*) Empirically observed locations of fiber optic implants in initial cohort

of 47 rats, estimated using high-resolution structural MRI scans. 18 of these animals had implant locations that were accurately localized to the central thalamus (<0.85 mm from target site, shown as dashed circle and cross). Two were excluded based on lack of thalamic activation, leaving $n = 16$ rats for further analysis. Black dots indicate implant coordinates of 16 animals used for analysis. Gray dots indicate implant coordinates of 31 rejected animals. (*Figure 1C*) Representative T2-weighted anatomical MRI scan used to estimate implant location, marked with arrow. (*Figure 1D*) Schematic of 23 coronal slices acquired during ofMRI experiments. Slice numbers correspond to activation maps in Figure 2. (*Figure 1E*) Average time series of significantly modulated voxels within the ipsilateral thalamus ROI (see Figure 2D) exhibit robust positive BOLD responses during repeated 20 s periods of stimulation at 10, 40, and 100 Hz, indicated by blue bars. Values are mean ± s.e.m. across animals ($n = 16$, 10, and 16 for each frequency, respectively). (*Figure 1F*) Diagram of local *in vivo* optrode recordings during optical stimulation of central thalamus. Inset shows spike waveforms of recorded neurons. (*Figure 1G*) Representative peri-event time histogram of a recorded neuron showing the increase in firing rate within central thalamus during optical stimulation at each of the three frequencies tested. See also Figure 7.

[00232] **Figure 7. Specificity of ChR2 targeting for CaMKIIa-positive cells.** Immunohistochemistry confirms the specific targeting of ChR2-EYFP to CaMKIIa-positive neurons in central thalamus. 35% of cells identified within the bulk injection area were EYFP-positive, and 97% of EYFP-positive cells co-expressed CaMKIIa ($n = 2$ rats, 831 cells). Scale bar, 10 μm.

[00233] In order to achieve a small volume of directly excited tissue limited to the intralaminar nuclei, a 62.5 μm diameter optical fiber was used. Assuming that an intensity of 1 mW/mm^2 is required for ChR2 activation, the specific power exiting from the fiber optic's tip in these experiments (2.5 mW) corresponds to a penetration depth of 1.08 mm and a volume of 0.08 mm^3 over which ChR2+ neurons can be excited. Figure 1A illustrates this penetration depth and activation cone (11.7° half-angle of divergence) to scale with the targeted nuclei, showing that stimulation is well restricted to the central thalamus. These two factors (MR-validated stereotactic fiber placement and a small volume of excited tissue) suggest that the effects reported here primarily derive from stimulation of excitatory relay neurons within the central thalamus.

[00234] To explore the anatomical connectivity of transfected neurons in central thalamus, *ex vivo* fluorescence microscopy images of ChR2-EYFP expression were collected. Due to the spread of viral transfection (Figure 1A), it is possible that the reported fluorescence reflects projections from adjacent thalamic nuclei as well. Nevertheless, in agreement with known projection systems of central thalamus, EYFP-expressing axons were observed throughout forebrain, including frontal cortex and striatum (Figure 8). In particular, the medial prefrontal, lateral prefrontal, cingulate, motor, and sensory cortices all received strong projections. This input was highly convergent at the superficial layers, with moderate but weaker projections present in middle layers as well. Furthermore, projections were significantly restricted to the hemisphere ipsilateral to virus injection for both cortex and striatum. While these anatomical connections provide a strong foundation for understanding how central thalamus can influence brain state, they do little to explain the dynamic nature of these circuits – for example, how stimulation of central thalamus at different frequencies can lead to distinct behavioral responses. Therefore, to dissect the functional significance of these massive forebrain projections and visualize the large-scale spatial and temporal dynamics evoked by central thalamus stimulation, optical stimulation with simultaneous *in vivo* whole-brain functional imaging was combined.

[00235] **Figure 8. Representative fluorescence images of ChR2-EYFP at remote targets illustrate the massive projections to forebrain from transfected relay neurons in the right central thalamus.** The bottom two rows provide magnified images of cortical and striatal regions used for quantitative ofMRI analysis. The top row provides the whole-brain slices from which these magnified images come. EYFP-expressing axonal projections are primarily localized to the ipsilateral hemisphere and to superficial layers in cortex.

[00236] During optogenetic fMRI experiments, twenty-three coronal slices with 0.5×0.5 mm^2 in-plane resolution and 0.5 mm thickness were acquired at a frame rate of 750 ms using spiral k-space trajectories and a sliding window reconstruction algorithm to achieve high spatiotemporal resolutions with whole-brain coverage (bregma +5.2 to -5.3 mm; Figure 1D). Novel inverse Gauss-Newton methods were also used to correct for possible motion artifacts and optimize the robustness of detecting optogenetically-evoked responses. For each experiment, 20 s periods of stimulation were delivered every

minute for 6 minutes at 10, 40, or 100 Hz. This form of continuous steady-state stimulation mimics the approach used in clinical DBS and has been showed to evoke robust fMRI responses with optogenetic stimuli. Indeed, stimulation at all three frequencies resulted in a robust positive blood-oxygen-level-dependent (BOLD) signal at the site of stimulation that was highly synchronized to light delivery, increased upon optical activation, and gradually returned to baseline following the end of stimulation (Figure 1E). To confirm that this BOLD signal reflected underlying neuronal firing patterns, simultaneous single-unit recordings was performed with stimulation using an optrode at the central thalamus (Figure 1F). In agreement with the fMRI signal, stimulations at 10, 40, and 100 Hz all resulted in robust increases in the local neuronal firing rate (Figure 1G; $n = 5$ neurons, $P < 0.05$, Wilcoxon signed-rank test between the 20 s pre-stimulation and stimulation periods, 12 trials for each neuron).

[00237] Both locally in the thalamus and at downstream, synaptically connected brain regions, the frequency of stimulation had an effect in determining the extent of ipsilateral and contralateral BOLD activation – defined here as positive BOLD signals significantly synchronized to the block stimulation paradigm (see Example 1). In general, a much larger volume of brain tissue was activated by stimulation at 40 and 100 Hz compared to 10 Hz, with frontocortical areas and striatum being strongly activated at high frequencies (Figure 2A-2C). To quantify these spatial differences in recruitment patterns, the total volume of positive and statistically significant BOLD signals evoked by stimulation in select ROIs was calculated (Figure 2D). This difference in activation volume between low (10 Hz) and high (40 or 100 Hz) stimulation frequencies was significant at the thalamus, striatum, and medial prefrontal, lateral prefrontal, cingulate, motor, and sensory cortices (Figure 2E-G). Striatal activity was primarily localized to the dorsal sector, with negligible activity occurring in the ventral region (Figure 2B,C). Furthermore, BOLD activation was generally restricted to the ipsilateral hemisphere, although activation volumes in the contralateral striatum, lateral prefrontal cortex, motor cortex, and sensory cortex were all significantly greater during 100 Hz stimulation compared to 10 Hz stimulation (Figure 2F-H).

[00238] **Figure 2. Spatial characterization of evoked fMRI signals.** (*Figures 2A-2C*) Average coherence maps of brain-wide activity during stimulation of excitatory central thalamus relay neurons at 10, 40, and 100 Hz. Warm colors indicate positive BOLD

responses, while cool colors indicate negative BOLD responses (see Methods). (*Figure 2D*) Regions of interest (ROIs) used for quantitative analysis of spatial ofMRI activation patterns. (*Figure 2E*) The amount of active volume (positive signal with coherence > 0.35) in the ipsilateral thalamus is significantly greater during 40 and 100 Hz stimulations than 10 Hz stimulation. Thalamic recruitment is relatively limited on the contralateral side. (*Figure 2F*) Activation of the ipsilateral striatum is significantly greater during 40 and 100 Hz stimulations than 10 Hz stimulation. Activation of the contralateral striatum is limited across frequencies, although there is an increase from 10 to 100 Hz. (*Figure 2G*) Medial and lateral prefrontal cortex exhibit a significantly greater volume of activation during 40 and/or 100 Hz stimulation than 10 Hz stimulation. Activity in the contralateral hemisphere is limited across all tested frequencies, although there is an increase from 10 to 100 Hz. (*Figure 2H*) Activation of cingulate, motor, and somatosensory cortex is each greater during 40 and 100 Hz stimulations than 10 Hz stimulation. The contralateral motor and sensory cortices are also activated to a greater extent during 40 and/or 100 Hz stimulation. Scale bars in *Figure 2A* through *Figure 2C* represent 2 mm. Asterisks in *Figure 2E* through *Figure 2H* indicate significant differences for 10 versus 40 Hz and 10 versus 100 Hz stimulations. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, one-sided Wilcoxon signed-rank tests, corrected for multiple comparisons. Error bars represent mean ± s.e.m. across animals. $n = 16$, 10, and 16 animals for 10, 40, and 100 Hz, respectively. Abbreviations are as follows: i- (ipsilateral), c- (contralateral), Cg (cingulate cortex), MC (motor cortex), MPFC (medial prefrontal cortex), LPFC (lateral prefrontal cortex), SC (sensory cortex), Str (striatum), Th (thalamus). See also Figures 8, 9A-9B, and 10A-10C.

[00239] These results provide a direct, region-specific visualization of the widespread driving effect that central thalamus has been shown to exert over forebrain, and link prior anatomical and physiological studies on arousal regulation to spatially precise and quantitative measures of cortical and striatal activation. For example, the evoked responses are consistent with the unilateral nature of thalamocortical projections (Figure 8), but reveal that the contralateral cortex can still be modulated by unilateral stimulation of central thalamus, particularly at high frequencies. Importantly, stimulation of other thalamic nuclei failed to evoke similarly widespread activity in the striatum and cortex (Figure 9A). Furthermore, large differences in forebrain activation between 10 and 40

Hz stimulations were not observed for other forms of subcortical stimulation (Figure 9B), suggesting this is a distinct property of central thalamus.

[00240] **Figures 9A-9B. Widespread and frequency-dependent recruitment of forebrain with optogenetics is distinct to stimulation of intralaminar nuclei of central thalamus.** (*Figure 9A*) Volumes of striatal and cortical activation (i.e. positive BOLD signals with coherence greater than 0.35) during 40 Hz stimulation of central thalamus, presented with activation volumes during stimulation of other thalamic nuclei. Central thalamus is the only target to result in significant recruitment of striatum and prefrontal and frontal cortical regions. (*Figure 9B*) Comparison of frequency dependent effects of central thalamus stimulation with those of intermediate hippocampus (IH) stimulation. Unlike central thalamus stimulation, which recruits significantly more volume in striatum, motor cortex, and sensory cortex at 40 Hz than at 10 Hz (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$; one-sided Wilcoxon signed-rank test), recruitment of these regions during hippocampal stimulation does not exhibit a significant dependence on frequency. Note that activation data was not available (N/A) at MPFC or LPFC for hippocampus stimulation due to differences in field of view. Abbreviations are as follows: i- (ipsilateral), c- (contralateral), Cing. ctx (cingulate cortex), MPFC (medial prefrontal cortex), LPFC (lateral prefrontal cortex), Sens. ctx (sensory cortex).

[00241] Throughout these experiments, a constant duty cycle of 30% was used to maintain the total amount of light delivery across frequencies and control for possible heating artifacts. Because 20 s pulse train was kept for all stimulation frequencies and avoid possible differences introduced by neuronal adaptation, maintaining a constant duty cycle required unique pulse widths for each frequency (i.e. 30, 7.5, and 3 ms for 10, 40, and 100 Hz, respectively). To rule out the possibility that these changes in pulse width were the primary cause of the above differences in forebrain recruitment, stimulations were repeated while maintaining a 3 ms pulse width. Visualization and quantification of evoked fMRI responses show that the increase in cortical and striatal activation with frequency was preserved (Figures 10A and 10B). These data suggest that stimulation frequency was the primary factor in modulating forebrain fMRI activation.\

[00242] **Figures 10A-10C. The frequency-dependent recruitment of forebrain by central thalamus and its control over cortical BOLD signal polarity are preserved when pulse width is held constant.** (*Figure 10A*) Representative coherence map of

brain-wide activity during stimulation of excitatory central thalamus relay neurons at 10, 40, and 100 Hz using a constant pulse width of 3 ms. Warm colors indicate positive BOLD responses, while cool colors indicate negative BOLD responses (see Example 1). (*Figure 10B*) Quantification of positive BOLD responses in cortex and striatum ($n = 3$ animals). Gray lines indicate animal-specific results, normalized to 100 Hz stimulation. Black lines indicate the average across animals. All six regions exhibit an increase in recruitment with frequency, consistent with the study's main results when pulse width was varied to keep the duty cycle and total amount of light delivery constant. Regions of interest (ROIs) are the same as those used in Figure 2. (*Figure 10C*) Hemodynamic response functions evoked in somatosensory cortex during 10, 40, and 100 Hz stimulation of central thalamus using a constant pulse width of 3 ms. Consistent with the study's main results, a negative BOLD signal is evoked at 10 Hz, while slow and fast positive BOLD responses are evoked at 40 and 100 Hz, respectively.

Example 3: Central Thalamus Stimulation Frequency Controls Cortical Excitation/Inhibition Balance

[00243] The temporal dynamics of cortical responses evoked during low- and high-frequency central thalamus stimulation were next examined. Despite targeted activation of excitatory neurons, the somatosensory cortex exhibited a strong negative BOLD signal during 10 Hz stimulation, suggesting a suppression of baseline activity (Figures 2A and 3A, 3B). In stark contrast, central thalamus stimulations at 40 and 100 Hz led to positive changes in the BOLD signal at the somatosensory cortex (Figures 2B, 2C and 3A, 3B). Thus, stimulation of the same excitatory population at different frequencies resulted in completely opposite responses at a downstream target. Importantly, these responses were preserved when pulse width was held constant in control experiments, indicating that stimulation frequency was the primary factor controlling this effect (Figures 10A and 10C).

[00244] **Figure 3. The sign of evoked cortical activity depends on the frequency of central thalamic stimulation.** (*Figures 3A, 3B*) 10 Hz stimulation of central thalamus evokes a strong negative BOLD signal throughout ipsilateral somatosensory cortex, while 40 and 100 Hz stimulations evoke positive responses. Time series come from the sensory cortex ROI defined in Figure 2D. Hemodynamic response function (HRF) shows the average response to a single 20 s period of stimulation, indicated by blue bar.

Error bars represent mean ± s.e.m. across animals. n = 16, 10, and 16 for 10, 40, and 100 Hz, respectively. (*Figure 3C*) Diagram of *in vivo* recordings at somatosensory cortex during stimulation of excitatory central thalamus relay neurons. Inset shows spike waveforms of recorded neurons. (*Figures 3D, 3E*) Representative peri-event time histogram of a recorded neuron, and corresponding quantification of firing rate during the 20 s periods before, during, and after stimulation. Neural firing rate decreased within the somatosensory cortex during 10 Hz central thalamus stimulation, but increased during 40 and 100 Hz stimulations (n = 17 trials each, * $P < 0.05$, *** $P < 0.001$ pre vs. ON, two-tailed Wilcoxon signed-rank test; see Table 1 for further analysis). Values are mean ± s.e.m. See also Figure 10.

[00245] While similar findings of frequency-dependent polarity changes have been hinted at in previous studies, downstream positive and negative BOLD signals that result from selective stimulation of excitatory neurons at distinct frequencies have not yet been visualized and validated with electrophysiology. To define the neuronal underpinnings of these signals, single-unit extracellular recordings were performed in the somatosensory cortex during central thalamus stimulation (Figure 3C). In agreement with the BOLD activity observed during ofMRI experiments, 10 Hz stimulation resulted in a decrease in neuronal firing rate between pre-stimulation and stimulation periods (Figures 3D, 3E; n = 10 of 11 recorded neurons). Conversely, stimulations at 40 and 100 Hz both led to increases in neuronal firing (Figures 3D, 3E; n = 11 of 11 recorded neurons). Because the evoked firing rates appeared to change over the course of stimulation, the pre-stimulation firing rate to the average firing rates were specifically compared during consecutive 5 s periods of the 20 s stimulus (i.e. 0-5 s, 5-10 s, 10-15 s, and 15-20 s; uncorrected $P < 0.05$, Wilcoxon signed rank test; 17 trials for each neuron). Interestingly, the decrease in firing rate during 10 Hz stimulation occurred primarily over the interval from 5 to 15 s after stimulation began, while the increase in firing rate during 40 Hz stimulation occurred primarily over the first 10 s (Table 1). On the other hand, the increase in neuronal firing rate during 100 Hz stimulation was generally maintained throughout the 20 s stimulation period (Table 1). Such differences may reflect short-term plasticity of the thalamocortical pathway, which has previously been shown to exhibit frequency-dependent properties. Peri-stimulus time histograms also revealed that spike events occurring during inhibition had a non-uniform distribution

over time, which peaked between 6 and 34 ms after light onset (Figure 11). These data suggest that the glutamatergic thalamocortical input at 10 Hz sometimes generated action potentials. Notably, however, not every light pulse resulted in an immediate action potential.

[00246] **Table 1.** Electrophysiology results from sensory cortex single-unit recordings. See also Table 1-source data 1.

Stimulation Frequency	Effect on Sensory Cortex Firing Rate	Percentage of neurons with significant change in firing rate (n = 11)			
		0-5 s after stim. onset	5-10 s after stim. onset	10-15 s after stim. onset	15-20 s after stim. onset
10 Hz	Increase	0%	0%	0%	0%
	Decrease	0%	91%	82%	9%
40 Hz	Increase	100%	91%	36%	55%
	Decrease	0%	0%	0%	0%
100 Hz	Increase	100%	82%	82%	82%
	Decrease	0%	0%	0%	0%

[00247] **Figure 11. Cortical spikes that occur during periods of inhibition driven by 10 Hz central thalamus stimulation exhibit a non-uniform distribution over time.** (*Top panels*) Average peristimulus time histograms (PSTHs) of spike events in somatosensory cortex during 10 Hz central thalamus stimulation for six animals. Analysis was restricted to the 5 s time bin with the greatest number of neurons inhibited for each animal. PSTHs were calculated by aligning spike counts to the onset of individual 30 ms light pulses, summing over the 50 pulses delivered during the 5 s stimulation period, averaging across trials, and binning at 5 ms intervals for each inhibited neuron. Histograms were normalized by the corresponding spike count value during the 20 s pre-stimulation baseline period (represented by the dashed line), and averaged across neurons for each animal. Shaded rectangles represent the 30 ms light pulse. Note that spike events are reduced relative to baseline for the majority of the 100 ms inter-stimulus period, but spike events that do occur have a non-uniform distribution that peaks 6-34 ms after stimulus onset. These patterns suggest that some thalamic stimuli induce spike events in cortex, despite the net suppression of activity relative to pre-stimulation levels. Animals presented include two used for ChR2-electrophysiology experiments in Table 1 and four used for combined ChR2-eNpHR electrophysiology

experiments in Figure 5. (*Bottom table*) Summary of PSTH peak latencies and spike fidelity for inhibited neurons in somatosensory cortex. Peak latency was defined as the 5 ms bin with highest spike count for each neuron's PSTH. Spike fidelity represents the percentage of light pulses in the given 5 s bin of inhibition that evoke at least one spike during the 30 ms pulse. Values represent mean +/- s.t.e. across cells in the figure and table.

[00248] Together, these ofMRI and electrophysiological data indicate that neuronal activity throughout somatosensory cortex is suppressed at low frequencies of central thalamus stimulation and increased at high frequencies of stimulation. Because the stimulations were restricted to excitatory neurons with cell bodies located in central thalamus, the causal relationship between stimulation frequency and cortical excitation/inhibition can be attributed to the neurons' initial firing pattern. These results add to a growing body of literature in systems neuroscience suggesting that a neuronal population's firing pattern can have vastly different – even opposite – effects on downstream regions depending on its specific temporal code.

Example 4: Low-Frequency Central Thalamus Stimulation Drives Incertal Oscillations

[00249] Given that stimulation was restricted to excitatory neurons, it was hypothesized that the suppression of cortex during 10 Hz stimulation might be related to the frequency-dependent modulation of a GABAergic population. The response properties of the zona incerta (ZI), which has been implicated in providing a powerful GABAergic modulation of 10 Hz spike-wave activity in spontaneous absence seizures in the rat, was investigated. Anatomically, ZI sends direct GABAergic projections to somatosensory thalamic nuclei and sensory cortex. Functionally, ZI has also been shown to selectively gate sensory information processing in higher-order thalamic nuclei through GABAergic inhibition. To investigate the involvement of zona incerta, single-unit and field potential electrophysiology recordings were performed in this region during simultaneous optogenetic stimulation of central thalamus at 10 or 40 Hz (Figure 4A). EEG recordings were simultaneously collected in frontal cortex to directly evaluate the relationship between ZI activity and whole-brain arousal state, which is typically measured with forebrain EEG. The zona incerta was targeted using stereotactic localization and the well-defined somatotopic representation of this region. The electrode was targeted to -3.96 mm AP, +2.2-2.6 mm ML, +6.7-7.2 mm DV from dura. The zona incerta was

identified according to a compatible depth reading, spike latencies consistent with a polysynaptic response (on the order of 10 ms; Figure 4B), and a receptive field that responds to contralateral whisker stimulation, which zona incerta is known to possess (64). The electrode was initially lowered through the dorsal part of the VP thalamus (approximately 1.5 mm above zona incerta), which also responds to whisker stimulation, until the recorded neurons did not respond to such a stimulus. The electrode was then lowered for another ~1.5 mm until the recorded neurons fired in response to whisker stimulation, indicating the zona incerta had been reached.

[00250] Figure 4. Frequency-dependent spindle-like oscillations are evoked in zona incerta (ZI). (*Figure 4A*) Diagram of *in vivo* recordings at zona incerta and simultaneous EEG recordings in frontal cortex during optical stimulation of central thalamus in anesthetized animals. (*Figure 4B*) Representative peri-event time histograms of spiking activity from recorded ZI neurons aligned to the onset of individual light pulses, summed over all pulses and trials. Peak spike latencies are approximately 10 and 8 ms for 10 Hz (left) and 40 Hz (right) stimulations, suggesting that recordings are performed at least one synapse away from the stimulated population in central thalamus. Schematics at top illustrate the 30% duty cycle pulse trains which lasted 20 s for each frequency. (*Figure 4C*) Representative peri-event time histograms over the 20 s period of stimulation show increases in ZI firing during 10 and 40 Hz stimulations. Among the 28 isolated single-units in zona incerta ($n = 2$ animals), most exhibited a significant increase in firing rate during stimulation ($n = 26$ and 22 out of 28 neurons, respectively; $P < 0.05$, one-tailed Wilcoxon signed-rank test with 20 trials for each cell). (*Figure 4D*) Representative field potential recordings from the same channel and trial number during 10 Hz (top) and 40 Hz (bottom) stimulation of central thalamus. Four amplitude-modulated, spindle-like oscillations (SLOs) are evoked during 10 Hz stimulation (marked by black triangles), while none are evoked during 40 Hz stimulation. Inset shows a zoomed-in SLO. (*Figure 4E*) The number of SLOs was greater during 10 Hz stimulation than 40 Hz stimulation across 11 of 12 available channels ($n = 2$ animals, 20 trials each, $P < 0.01$, one-tailed Wilcoxon rank sum test). (*F*) When more than one SLO was evoked within the same 20 s period of 10 Hz stimulation, the distribution of inter-event intervals was centered at 6.6 ± 0.2 s (s.e.m.). (*Figure 4G*) Representative EEG recordings collected in frontal cortex during central thalamus stimulation and

simultaneous ZI recordings. 10 Hz stimulation evokes a spike-wave response, which is associated with loss of consciousness and perceptual awareness, while 40 Hz stimulation evokes a low-voltage fast response indicative of arousal. (*Figure 4H*) ChR2-positive processes were observed in zona incerta, providing a basis for its recruitment during stimulation of central thalamus. i.c.: internal capsule.

[00251] Out of 28 isolated ZI neurons, the majority exhibited increases in their firing rate during central thalamus stimulation at both 10 and 40 Hz (Figure 4C; $n = 26$ and 22, respectively; $P < 0.05$, Wilcoxon signed rank test between the 20 s pre-stimulation and stimulation periods, 20 trials for each neuron). However, a key difference was that large, amplitude-modulated spindle-like oscillations (SLOs) in the field potential occurred significantly more often during 10 Hz stimulation than 40 Hz stimulation (Figures 4D, 4E). These oscillations exhibited an inter-event interval centered around 6.6 ± 0.2 s (s.e.m.), similar to those observed in thalamus during sleep onset (Figure 4F). Consistent with this, simultaneous EEG recordings in frontal cortex revealed strong spike-wave modulation during 10 Hz stimulation and lower amplitude, fast oscillations during 40 Hz stimulation, which are associated with loss of consciousness and aroused brain states, respectively (Figure 4G). EYFP-expressing axons were also observed in zona incerta (Figure 4H), indicating that central thalamus relay neurons may have direct connections to zona incerta and providing a possible anatomical substrate for these responses

Example 5: Cortical Inhibition Driven by Central Thalamus Stimulation Depends on Evoked Incertal Activity

[00252] The observation of spindle-like oscillations in zona incerta during 10, but not 40, Hz central thalamus stimulation indicates that this region can be uniquely engaged by central thalamus-driven networks. However, it remains unknown whether the evoked activity in zona incerta plays a causal role in driving the frequency-dependent inhibition of somatosensory cortex. To address this question, the inhibitory opsin halorhodopsin (eNpHR) fused to the mCherry fluorescent marker and controlled by the pan-neuronal hSyn promoter was injected into zona incerta of four animals expressing ChR2-EYFP in central thalamus (Figures 5A and 5B, Figure 12). Two new stimulation paradigms were explored: (1) 20 or 30 s continuous eNpHR activation, and (2) 20 s, 10 Hz central thalamus stimulation performed within a 30 s period of continuous eNpHR activation.

Single-unit recordings were performed simultaneously at the zona incerta and sensory cortex during concurrent activation of these two opsins (Figure 5C).

[00253] Figure 5. Cortical inhibition driven by 10 Hz central thalamus stimulation depends on normal incertal processing. (*Figure 5A*) Wide-field fluorescence image shows robust eNpHR-mCherry expression spatially localized to the right zona incerta. Scale bar, 1 mm. (*Figure 5B*) Confocal images show eNpHR-mCherry localized to somatic membrane of neurons in zona incerta. Scale bar, 10 μm. 209 out of 882 DAPI-stained cells co-expressed mCherry in ZI (24%, $n = 2$ animals). (*Figure 5C*) Schematic of cortical electrophysiology recordings during 10 Hz stimulation of central thalamus and continuous (cont.) inhibition of zona incerta using ChR2 and eNpHR, respectively. (*Figure 5D*) Peri-event time histogram of a representative neuron in zona incerta whose firing rate is suppressed during activation of eNpHR with 593 nm light. (*Figure 5E*) Peri-event time histogram of a representative neuron in zona incerta whose firing rate remains suppressed throughout the period of 10 Hz central thalamus stimulation during eNpHR activation (compare to Figure 4C). (*Figure 5F*) Activation of eNpHR in zona incerta significantly reduces the change in incertal firing rate evoked by 10 Hz central thalamus stimulation in 60 of 70 neurons ($P < 0.05$, one-sided Wilcoxon rank sum test). Changes in firing rate are normalized to pre-stimulation levels. (*Figure 5G*) Peri-event time histograms from a representative cortical neuron show that the inhibitory response evoked by 10 Hz central thalamus stimulation is reversed by simultaneously suppressing activity in zona incerta. Firing rates are normalized to the average pre-stimulation values. (*Figure 5H*) Quantification of evoked changes in cortical firing rate during 10 Hz central thalamus stimulation with and without concurrent eNpHR activation. 50 out of 76 cells exhibit reduced inhibition when central thalamus stimulation is paired with eNpHR activation ($P < 0.05$, Wilcoxon rank sum test over 1 s bins). Changes in firing rate are normalized to pre-stimulation levels. (*Figure 5I*) Confocal images show mCherry-positive axonal projections from zona incerta in somatosensory cortex. Scale bar, 20 μm.

[00254] Figure 12. Wide-field fluorescence image of eNpHR expression in zona incerta, overlaid with the estimated cone of activated eNpHR (i.e. inhibited neurons) shown to scale. Penetration depth and volume were calculated to be 0.64 mm and 0.024 mm^3, respectively, using the methods described in and a threshold light

intensity of 5 mW/mm^2. The optical fiber had a diameter of 105 μm, NA of 0.22, and half-angle of divergence of 9.3°. Penetration depth and activation volume correspond to an optical power of 3 mW exiting the fiber optic's tip. Stimulation coordinate corresponds to -3.96 mm AP, +2.4 mm ML, and -6.7 mm DV. The thalamic reticular nucleus, another region of dense GABAergic neurons, is shown for reference.

[00255] Among the 70 neurons recorded in zona incerta, delivery of 593 nm light resulted in a decrease in firing for 62 cells ($P < 0.05$, Wilcoxon signed rank test between 20 s pre-stimulation period and 20 or 30 s stimulation period, 15-20 trials for each neuron), indicating that illumination of halorhodopsin was successful in suppressing incertal activity. The evoked decrease in neuronal firing rate typically lasted throughout the duration of 593 nm light delivery (Figure 5D). When halorhodopsin activation in ZI was paired with 10 Hz stimulation of central thalamus, the previously described increase in incertal firing (Figure 4C) was disrupted. In 60 out of 70 neurons, the difference in incertal firing rate between the 20 s 10 Hz central thalamus stimulation period and the pre-stimulation period was significantly reduced with concurrent eNpHR activation (Figure 5F; $P < 0.05$, one-sided Wilcoxon rank sum test, $n = 10$-20 trials). Figure 5E illustrates the suppression of zona incerta activity throughout the 20 s period of 10 Hz central thalamus stimulation in a representative neuron. These data indicate that activation of halorhodopsin significantly suppressed the incertal firing evoked by 10 Hz central thalamus stimulation, and successfully disrupted incertal processing.

[00256] To determine whether this suppression of zona incerta affected the cortical activity driven by central thalamus stimulation, the changes in somatosensory cortex firing rate evoked by ChR2 activation with and without illumination of eNpHR was quantified. 76 somatosensory cortex neurons were recorded, and the 20 s period of central thalamus stimulation was divided into four 5 s bins as before. Consistent with the data presented in Figure 3, 68 cells (89%) exhibited a decrease in firing during 10 Hz stimulation of central thalamus (uncorrected $P < 0.05$, Wilcoxon signed rank test; 10-15 trials for each neuron). Strikingly, however, suppression of zona incerta activity with eNpHR reversed this effect. Across animals, 50 out of 76 neurons (66%) exhibited reduced inhibition when central thalamus stimulation was paired with eNpHR activation (Figure 5H; $P < 0.05$, Wilcoxon rank sum test over 1 s bins; 10-20 trials for each neuron). Indeed, a fraction of cells switched from inhibitory responses to excitatory

ones. Figure 5G illustrates the firing patterns of one cell that exhibited an inhibitory response during 10 Hz central thalamus stimulation that was eliminated when zona incerta was simultaneously suppressed with eNpHR. Collectively, these data suggest that incertal activity during 10 Hz central thalamus stimulation has a net inhibitory effect on somatosensory cortex. In support of this influence being through direct anatomical connections, mCherry-positive axons were observed in the sensory cortex (Figure 5I), consistent with previous reports of incerto-cortical projections. These findings present a conceptually novel role of zona incerta in central thalamus arousal circuits.

Example 6: Central Thalamus Stimulation Modulates Brain State in a Frequency-Dependent Manner

[00257] Finally, to relate these findings more directly to behavior associated with central thalamus arousal circuits and previous electrical stimulation studies, 10, 40, and 100 Hz stimulations in asleep, unanaesthetized animals were performed with simultaneous video and EEG recordings (see Example 1). Control (pre-stimulus) activity was consistent across frequencies of stimulation, as quantified with EEG band power in delta, theta, alpha, and beta bands (Figure 13). During 10 Hz stimulation, the majority of animals exhibited behavior indicative of an absence seizure, including freezing and behavioral arrest throughout stimulation followed by a return to sleep (Figure 6A; $n = 4/7$). In addition, the most common EEG response was a transition to slow spike-wave discharges (Figure 6B,C; $n = 5/7$), which are typically associated with loss of consciousness. In stark contrast, stimulations at 40 and 100 Hz resulted in behavioral transitions to an awake state, reflected by exploration and goal-directed movement (Figure 6A; $n = 4/7$ and 4/6, respectively). Similarly, the most common EEG pattern evoked by these high-frequency stimulations was a low-voltage-fast response (Figure 6B; $n = 3/7$ and 6/6, respectively), indicative of cortical activation and desynchronization. Collectively, these phenomena are consistent with the patterns of cortical and striatal recruitment observed with ofMRI. Moreover, the slow spike-wave and low-voltage-fast EEG responses evoked during behavioral experiments (Figure 6C,D) match those recorded under anesthetized conditions (Figure 4G), further linking the network activation patterns revealed by ofMRI to the arousal responses reported here, as well as those reported in early stimulation studies.

[00258] **Figure 6. Optogenetic stimulation of central thalamus in asleep animals modulates brain state in a frequency-dependent manner.** (*Figure 6A*) Low-frequency stimulation (10 Hz) in a majority of animals (n = 4/7) evokes behavioral absence seizures, while high-frequency stimulations (40 and 100 Hz) cause a majority of animals to awaken (n = 4/7 and 4/6, respectively). Dashed boxes indicate the most common response for each frequency, with arrows indicating the corresponding transition from sleep. (*Figure 6B*) Low-frequency stimulation typically evokes spike-wave responses in EEG (n = 5/7), consistent with the behavioral reading of absence seizures. The most frequent EEG response during high-frequency stimulations is low voltage fast (n = 3/7 and 6/6), indicative of arousal. N, normal. SW, spike-wave. lvf, low voltage fast. s, spiking. e, evolving seizure. (*Figures 6C, 6D*) Representative traces of EEG responses classified as spike-wave and low voltage fast. Insets show 4 s magnification. Importantly, these EEG patterns match those recorded under anesthetized conditions (Figure 4G), further linking the responses visualized with ofMRI to the reported behavioral responses. See also Figure 13.

[00259] **Figure 13. Pre-stimulus activity is consistent across frequencies of stimulation in asleep rats, as quantified with EEG bandpower in delta, theta, alpha, and beta bands.**

[00260] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. A method for *in vivo* circuit analysis of a brain of an individual, comprising:

i) illuminating a first region of a brain with:

a first set of light pulses; and

a second set of light pulses, wherein the first set has a different temporal pattern of light pulses from the second set,

wherein the first region comprises neurons that generate action potentials upon activation, using light pulses of the first and second sets, of a first light-activated polypeptide expressed by the neurons of the first region, wherein the action potentials are generated at a frequency that scales quantitatively with the temporal pattern of the light pulses of the first and second sets;

ii) measuring, in a second region of the brain using functional magnetic resonance imaging (fMRI) scanning of a plurality of brain regions:

a first change in neural activity induced by the first set of light pulses; and

a second change in neural activity induced by the second set of light pulses; and

iii) identifying a dynamic functional connection from the neurons of the first region to neurons of the second region based on a first difference between the first measured change and the second measured change.

2. The method of claim 1, wherein the identifying (iii) comprises:

calculating the first difference between the first measured change and the second measured change;

determining that a first functional connection from the neurons of the first region to the neurons of the second region is a dynamic functional connection when the first measured change and the second measured change are qualitatively or quantitatively different.

3. The method of claim 2, wherein the first difference is non-linear relative to the difference in the temporal pattern of the first set and second set of light pulses.

4. The method of any one of claims 1 to 3, wherein the first difference is a change in sign.

5. The method of any one of claims 1 to 4, wherein the quantitatively different temporal pattern comprises different frequency and/or pulse width of the light pulses.

6. The method of any one of claims 1 to 5, wherein the first brain region is a first internal region of the brain.

7. The method of claim 6, wherein the first internal region of the brain comprises at least a part of a region selected from the group consisting of thalamus, sensory cortex, zona incerta (ZI), ventral tegmental area (VTA), prefontal cortex (PFC), nucleus accumbens (NAc), amygdala (BLA), substantia nigra, ventral pallidum, globus pallidus, dorsal striatum, ventral striatum, subthalamic nucleus, hippocampus, dentate gyrus, cingulate gyrus, entorhinal cortex, olfactory cortex, primary motor cortex, and cerebellum.

8. The method of any one of claims 1 to 7, wherein the first region is illuminated with one or more optical fibers configured to transmit the first set and second set of light pulses.

9. The method of claim 8, wherein a volume of the first region illuminated by the light pulses is 100 mm^3 or less.

10. The method of any one of claims 1 to 9, wherein the first and second regions are anatomically distinct regions of the brain.

11. The method of any one of claims 1 to 10, wherein the neurons of the first region are excitatory neurons.

12. The method of any one of claims 1 to 11, wherein the brain comprises a third region comprising neurons whose activity is modulated by activity of the neurons of the first region, and wherein the method further comprises:

iv) illuminating the first region by the first set of light pulses, with and without illumination of the third region with a third set of light pulses, wherein modulation of the

activity of neurons of the third region is disrupted by activation, upon illumination of the third region, of a second light-activated polypeptide expressed by the neurons of the third region;

v) measuring, in the second region:

a third change in neural activity induced by the first set of light pulses without illumination of the third region; and

a fourth change in neural activity induced by the first set of light pulses with illumination of the third region by the third set of light pulses; and

vi) identifying, based on a second difference between the third measured change and the fourth measured change, a modulatory node of the dynamic functional connection, the modulatory node comprising neurons of the third region, wherein the neurons of the third region have a functional connection between neurons of the first region and the second region.

13. The method of claim 12, wherein the identifying (vi) comprises:

calculating the second difference between the third measured change and the fourth measured change; and

determining that the neurons of the third region mediate the first measured change when the second difference is a qualitative or quantitative difference.

14. The method of claim 13, wherein the second difference is smaller in magnitude than the first difference.

15. The method of any one of claims 12 to 14, wherein the measuring (v) comprises measuring changes in neural activity using electrophysiology.

16. The method of any one of claims 12 to 15, wherein the third brain region is a second internal region of the brain.

17. The method of claim 16, wherein the second internal region of the brain comprises at least a part of a region selected from the group consisting of thalamus, sensory cortex, zona incerta (ZI), ventral tegmental area (VTA), prefontal cortex (PFC), nucleus accumbens (NAc), amygdala (BLA), substantia nigra, ventral pallidum, globus pallidus, dorsal striatum, ventral

striatum, subthalamic nucleus, hippocampus, dentate gyrus, cingulate gyrus, entorhinal cortex, olfactory cortex, primary motor cortex, and cerebellum.

18. The method of any one of claims 12 to 17, wherein the third region is illuminated with one or more optical fibers configured to transmit the third set of light pulses.

19. The method of any one of claims 12 to 18, wherein the first and second regions are anatomically distinct regions of the brain.

20. The method of any one of claims 1 to 19, wherein the first light-activated polypeptide is a light-activated ion channel.

21. The method of any one of claims 1 to 20, wherein the second light-activated polypeptide is a light-activated ion pump.

22. The method of any one of claims 1 to 21, wherein the individual is a mammal.

23. The method of any one of claims 1 to 22, wherein the individual is anesthetized.

24. A method for *in vivo* circuit analysis of a brain of an individual, comprising:

i) determining a dynamic functional connection between:

a first region of the brain comprising neurons configured to depolarize or hyperpolarize upon light-induced activation of a light-activated polypeptide expressed in the neurons of the first region; and

a second region of the brain,

using light-induced modulation of neural activity of the first population of neurons, in conjunction with fMRI scanning of a plurality of brain regions that comprises the second region; and

ii) identifying a modulatory node of the dynamic functional connection, the modulatory node comprising neurons of a third region, wherein the neurons of the third region have a functional connection between neurons of the first region and the second region, using light-

induced modulation of neural activity in combination with electrophysiology and/or functional imaging.

25. The method of claim 24, wherein the neurons of the third region are configured to depolarize or hyperpolarize in response to a light stimulus.

26. The method of any one of claims 24 and 25, wherein the first and second regions are anatomically distinct regions of the brain.

27. The method of any one of claims 24 to 26, wherein the neurons of the first region are excitatory neurons.

28. The method of any one of claims 24 to 27, wherein the determining (i) comprises performing the method according to any one of claims 1 to 11.

Figure 1A

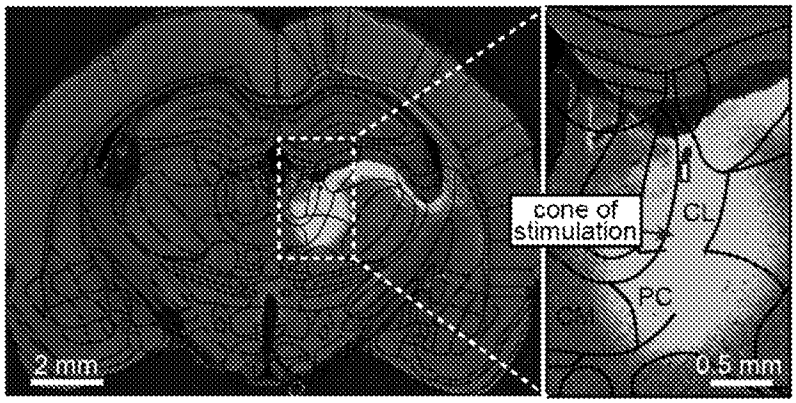


Figure 1B

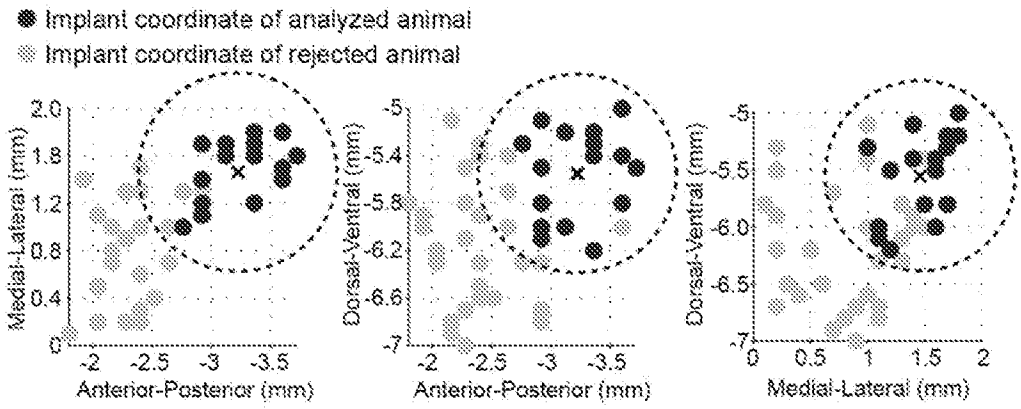


Figure 1C

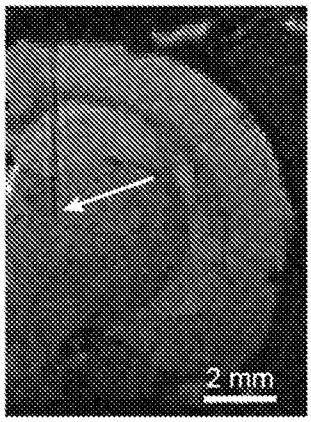


Figure 1D

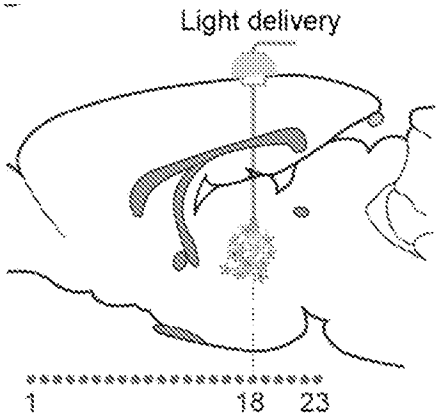


Figure 1E

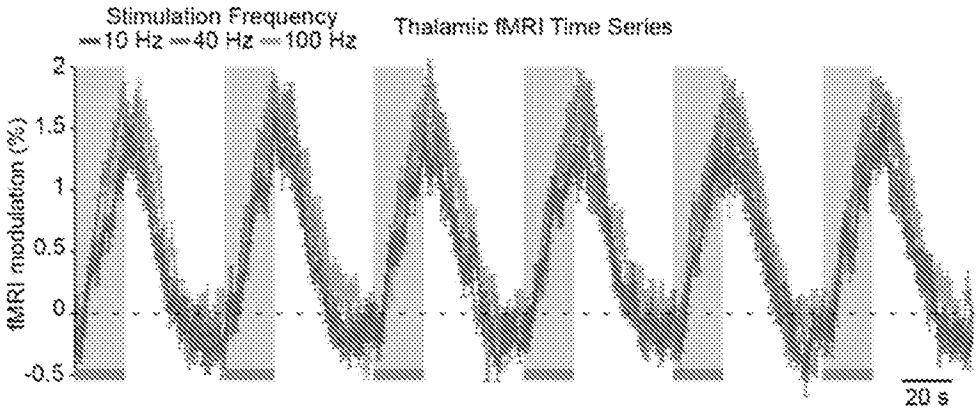


Figure 1F

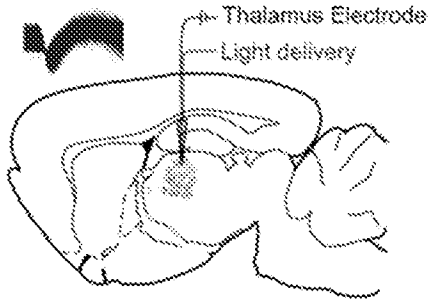


Figure 1G

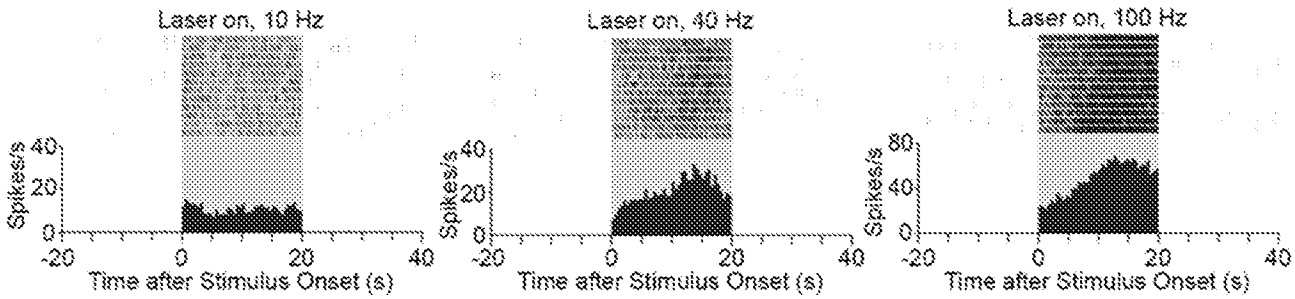


Figure 2A

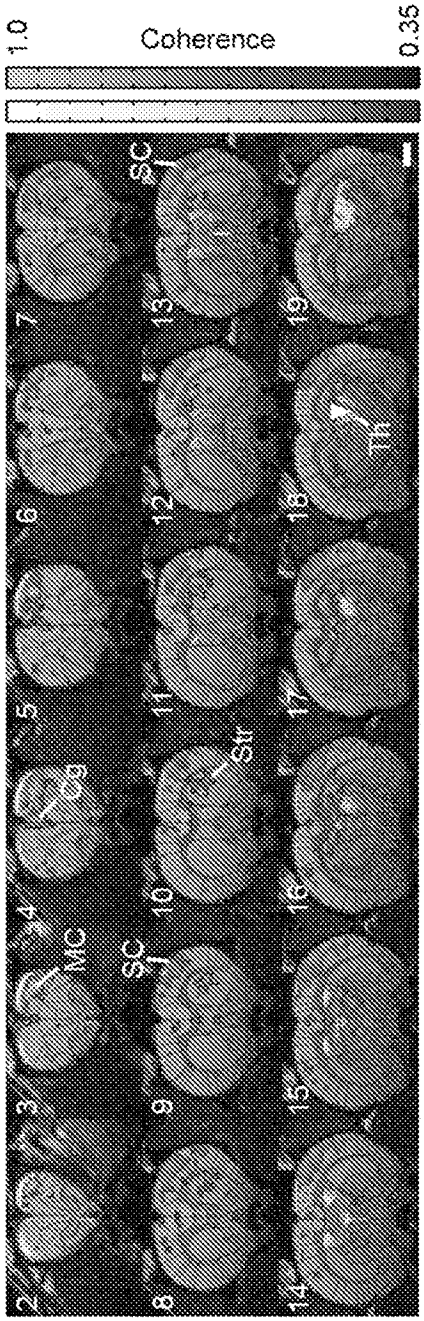


Figure 2B

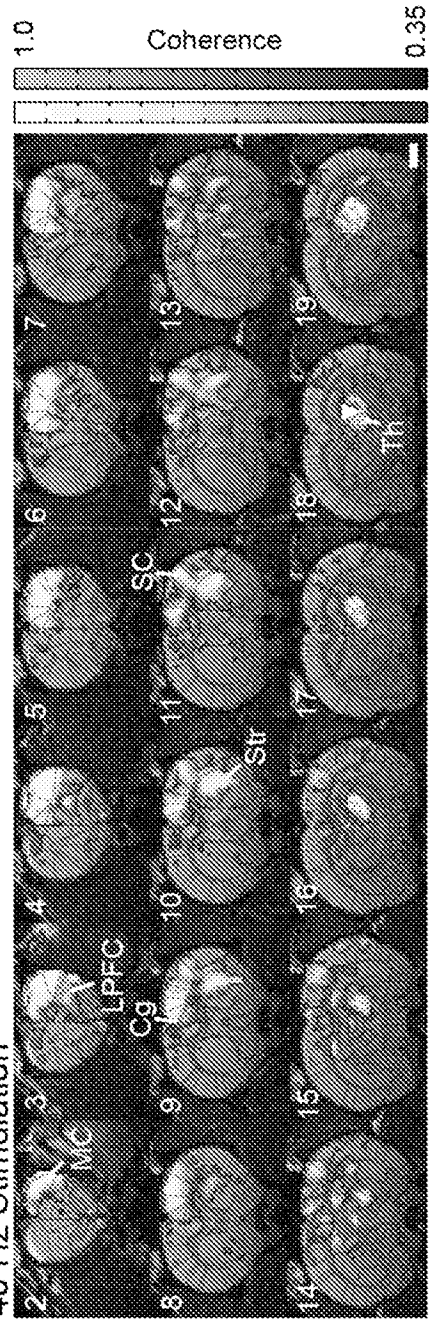


Figure 2C

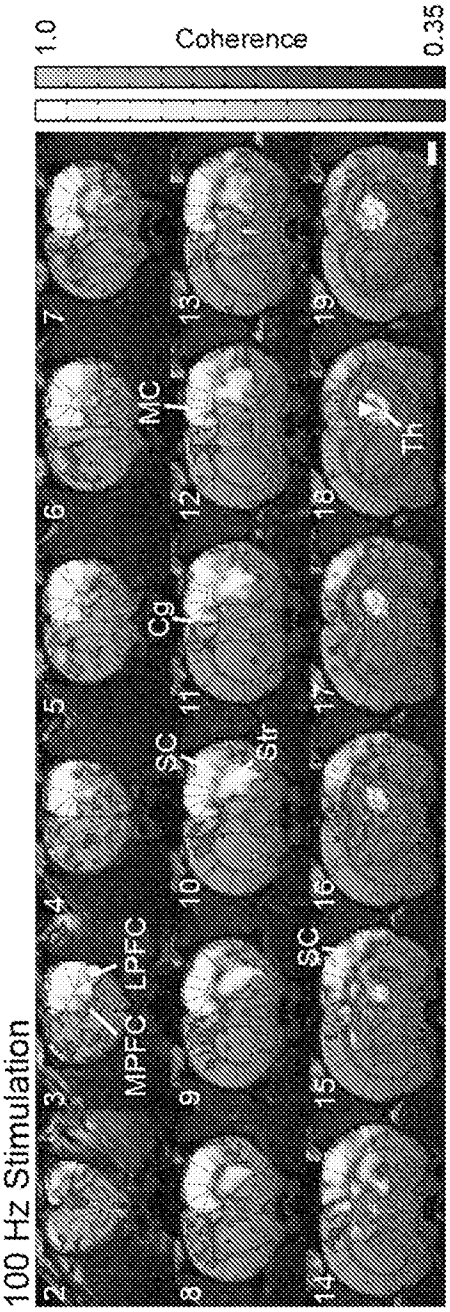


Figure 2D

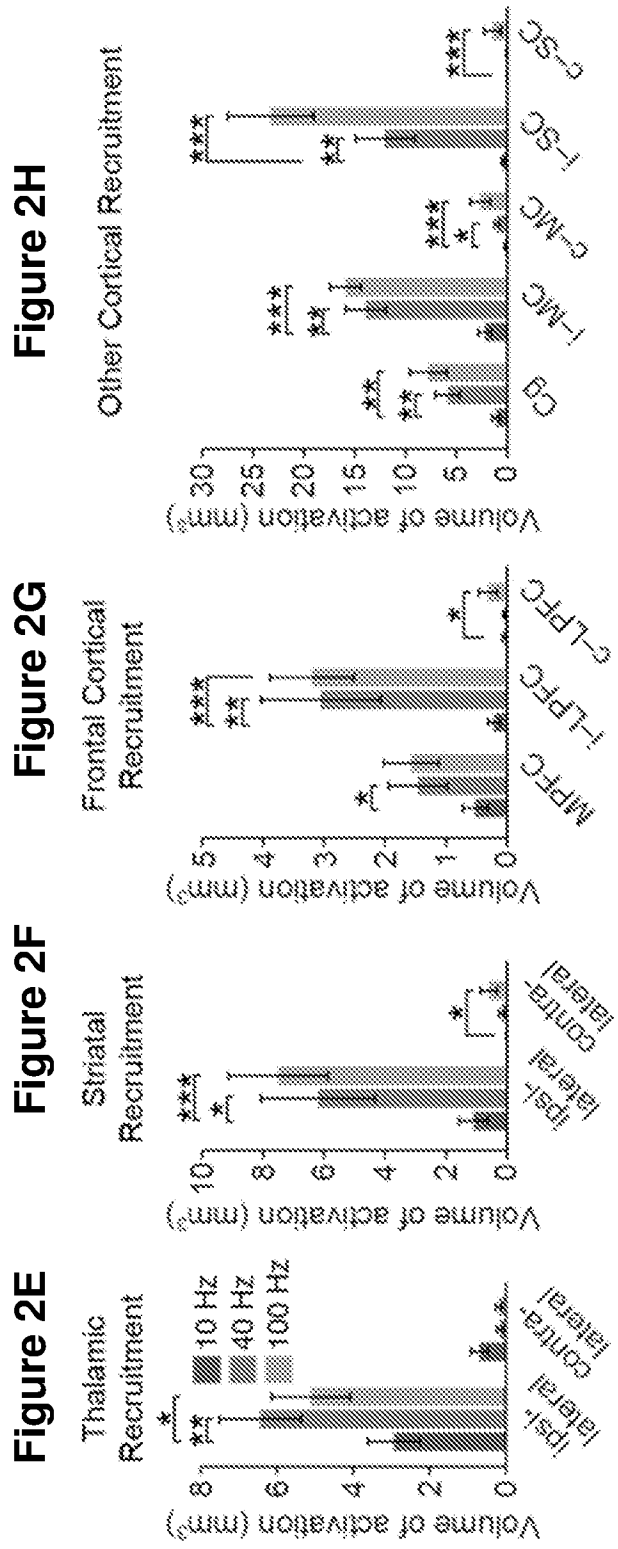


Figure 2E
Thalamic Recruitment
10 Hz
40 Hz
100 Hz
Volume of activation (mm³)
Ipsi-lateral
Contra-lateral
Figure 2F
Striatal Recruitment
Volume of activation (mm³)
Ipsi-lateral
Contra-lateral
Figure 2G
Frontal Cortical Recruitment
Volume of activation (mm³)
MPFC
i_LPFC
c_LPFC
Figure 2H
Other Cortical Recruitment
Volume of activation (mm³)
Cg
i_MC
c_MC
i_SC
c_SC

Figure 3A

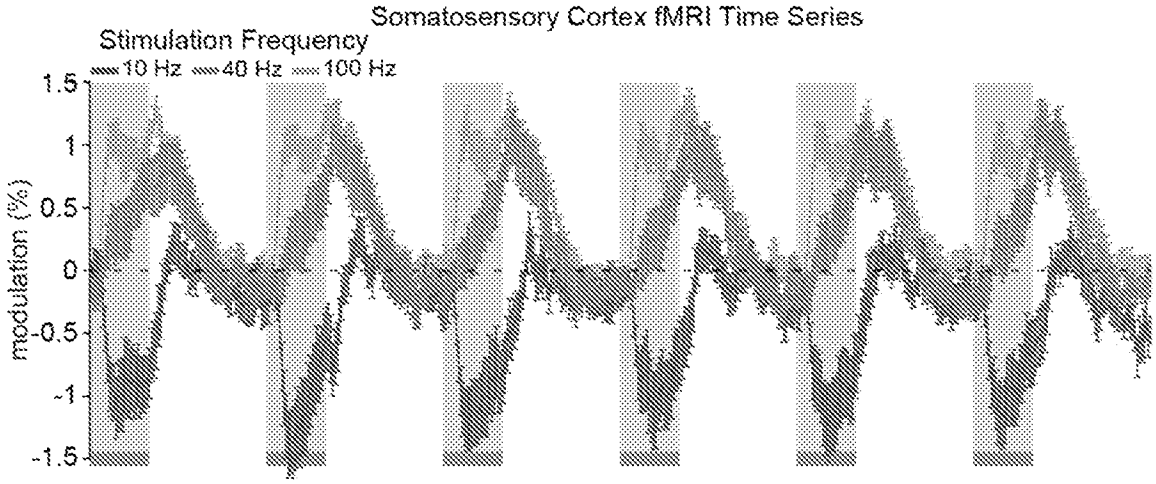


Figure 3B

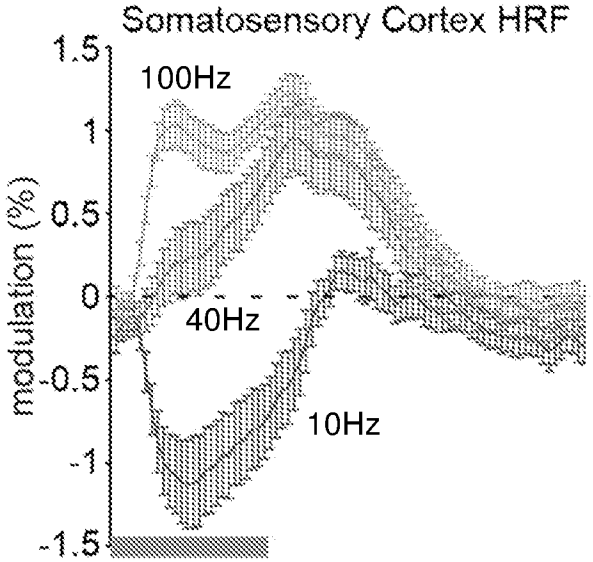


Figure 3C

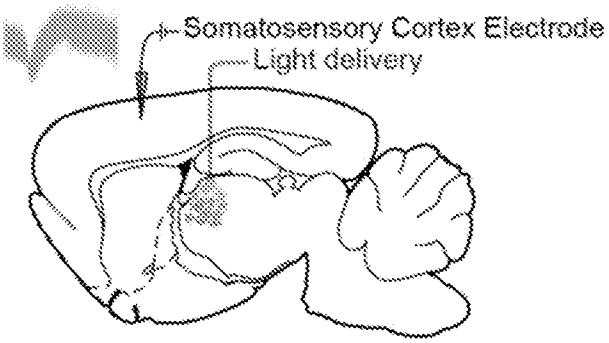


Figure 3D

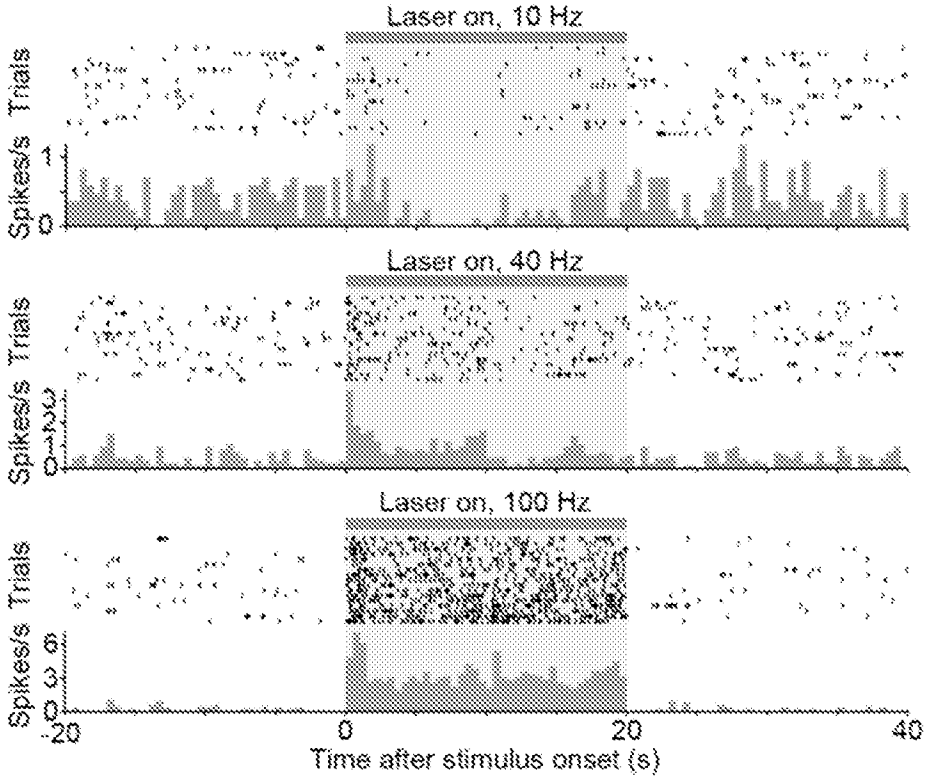


Figure 3E

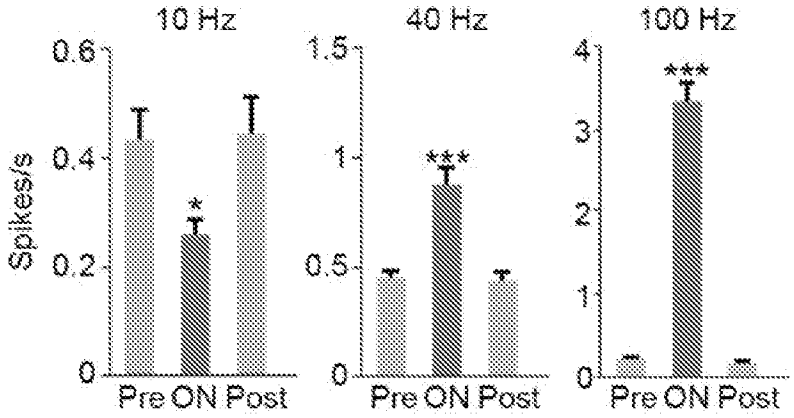


Figure 4A

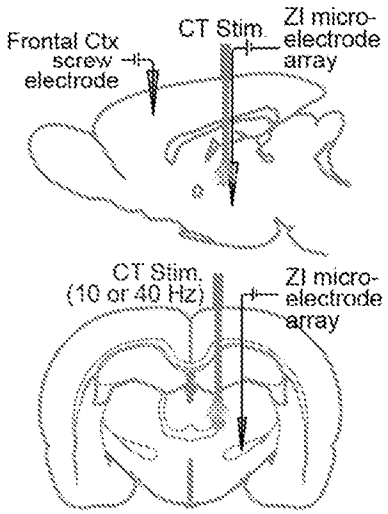


Figure 4B

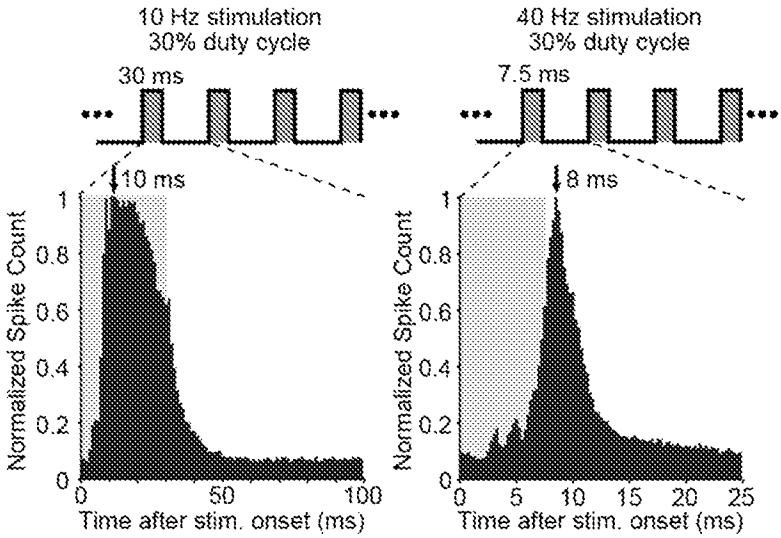


Figure 4C

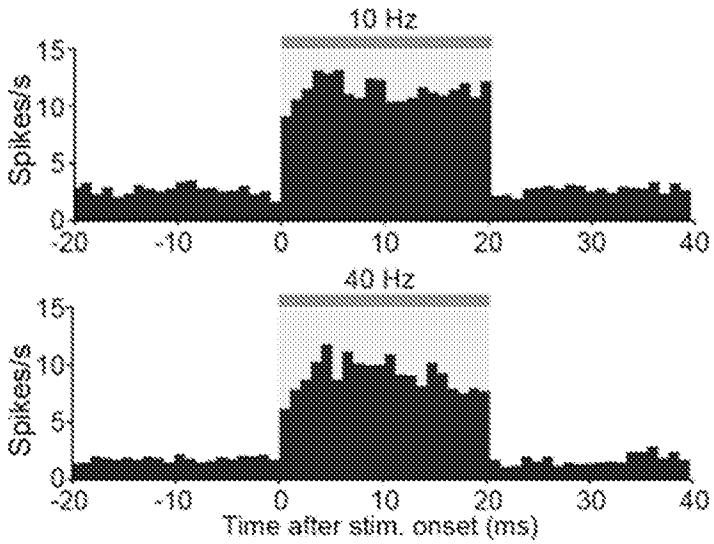


Figure 4D

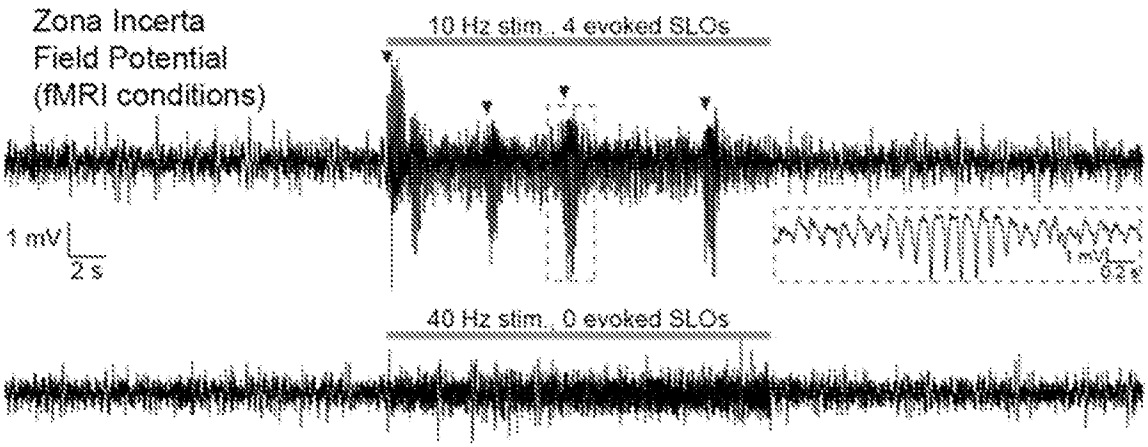


Figure 4E

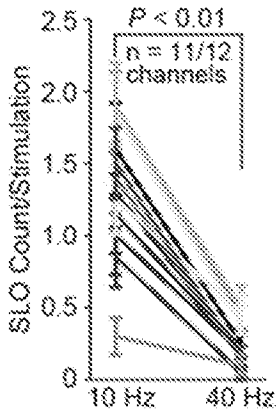


Figure 4F

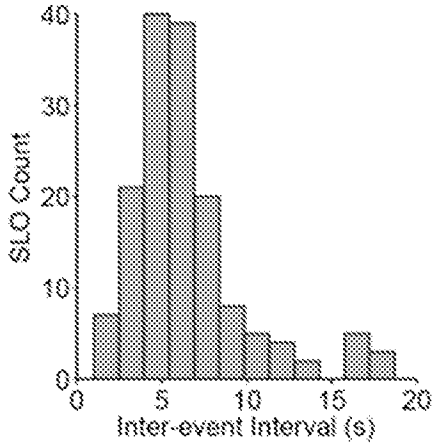


Figure 4G

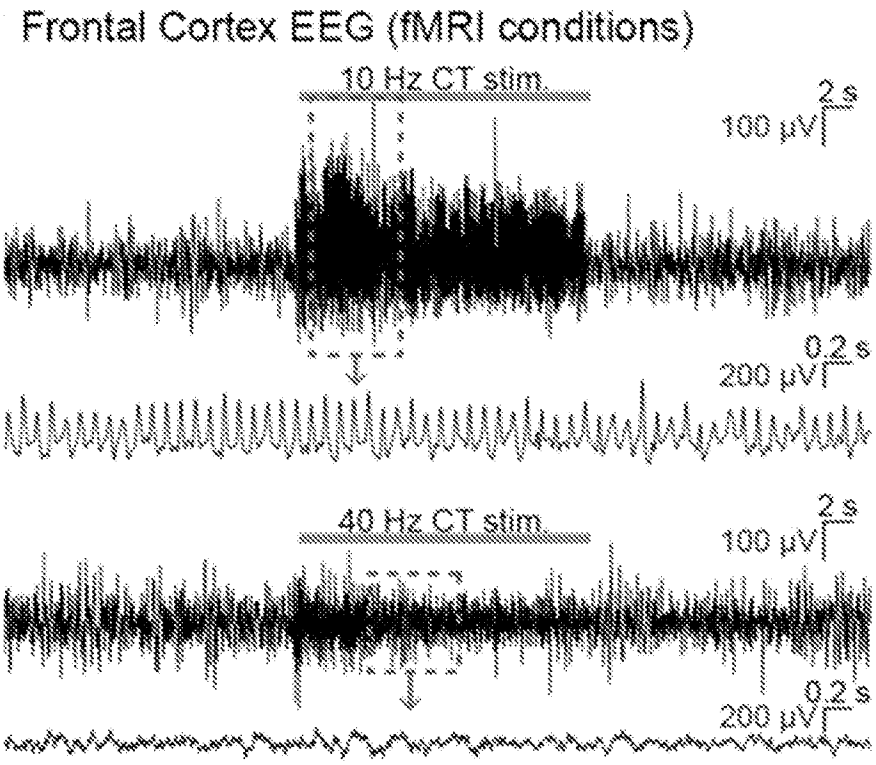


Figure 4H

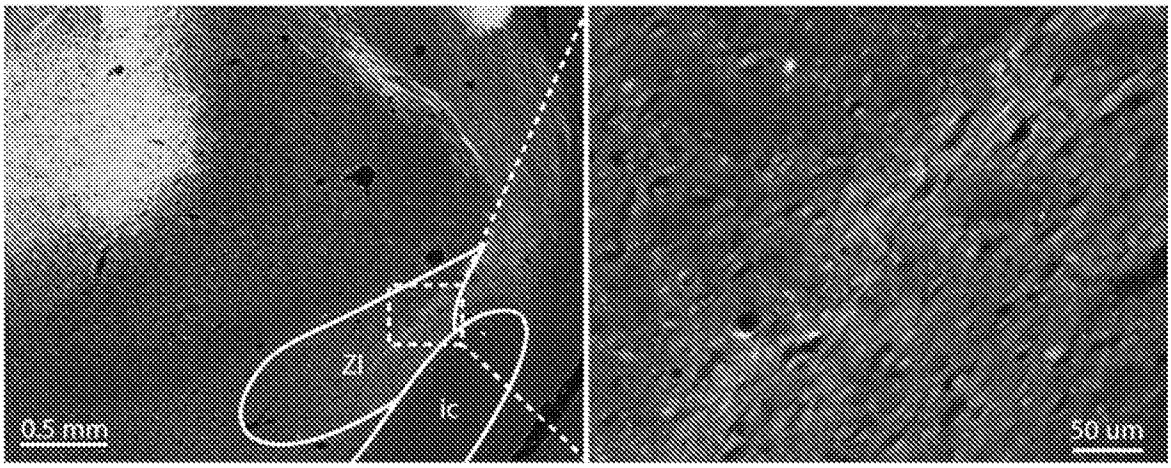


Figure 5A

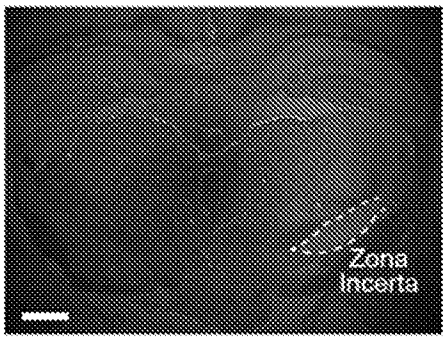


Figure 5B

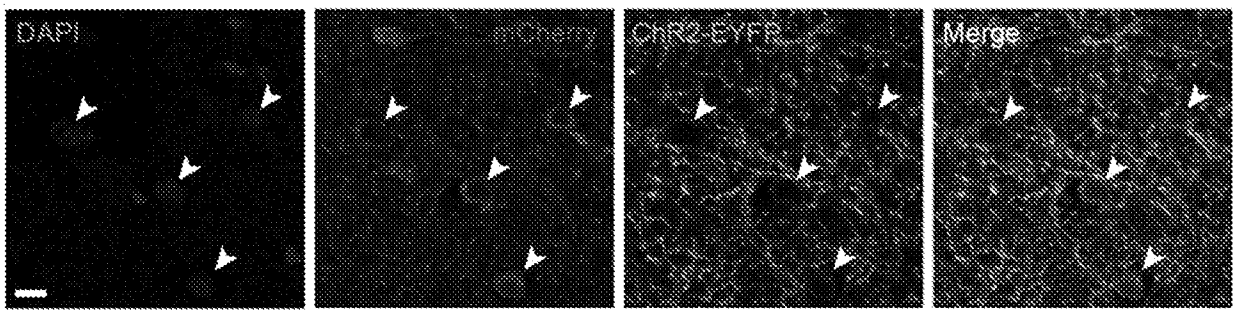


Figure 5C

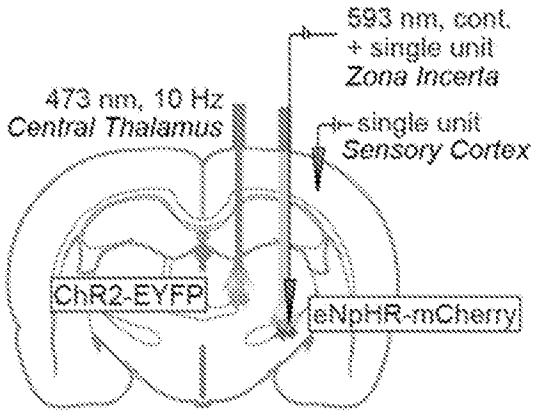


Figure 5D

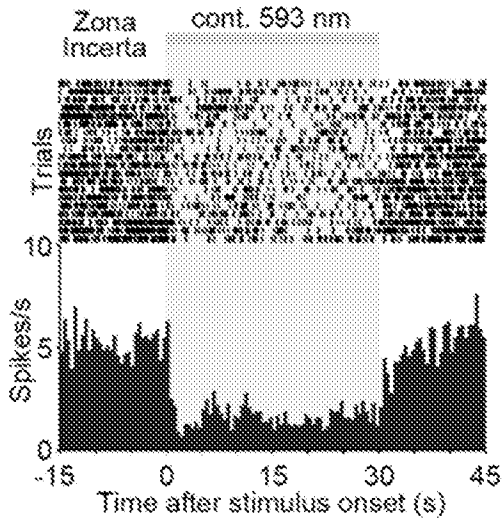


Figure 5E

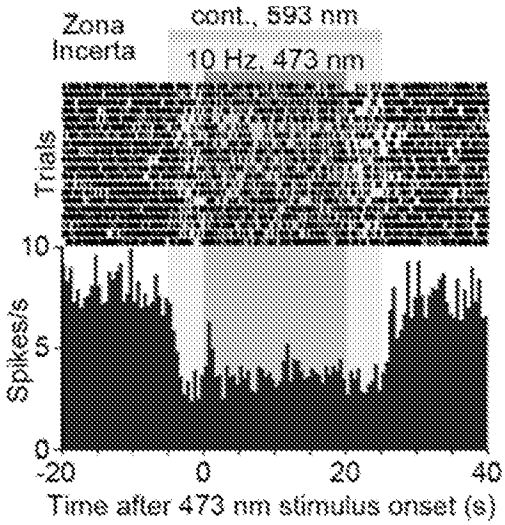


Figure 5F

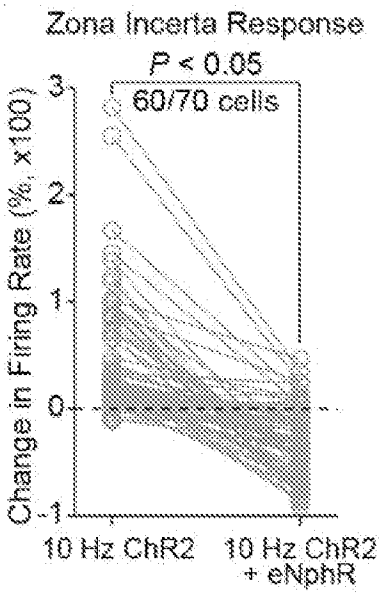


Figure 5G

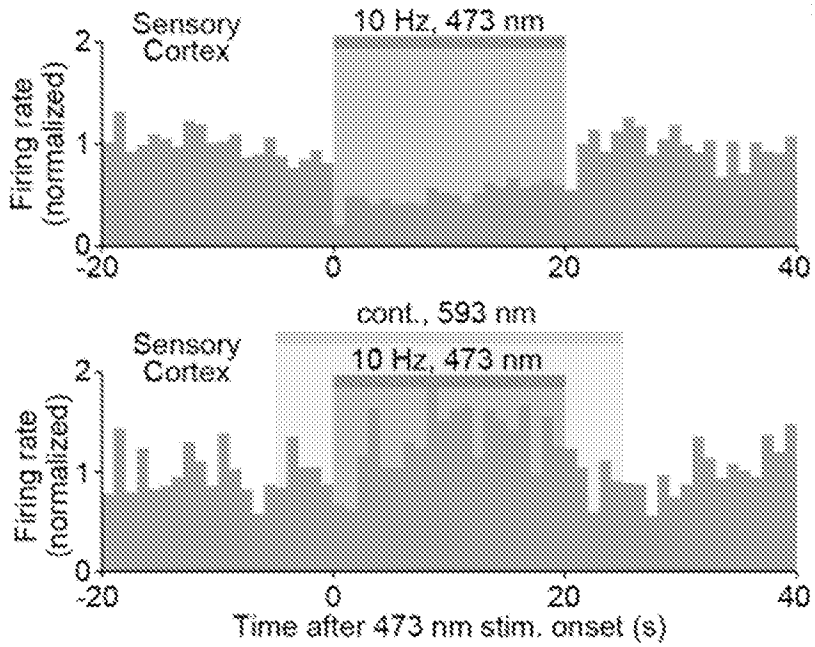


Figure 5H

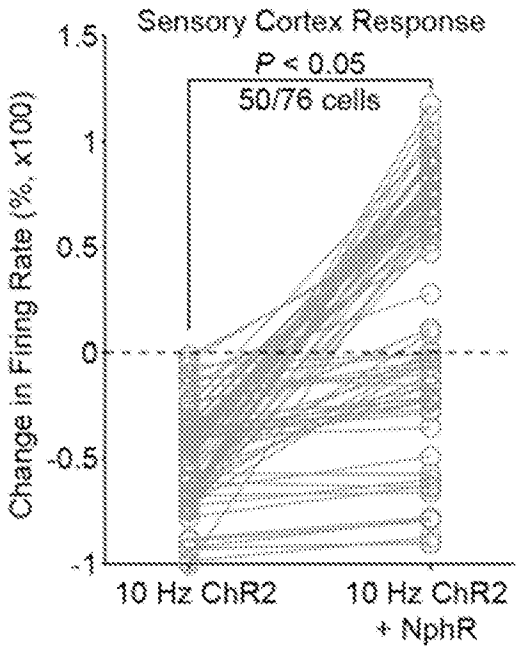


Figure 5I

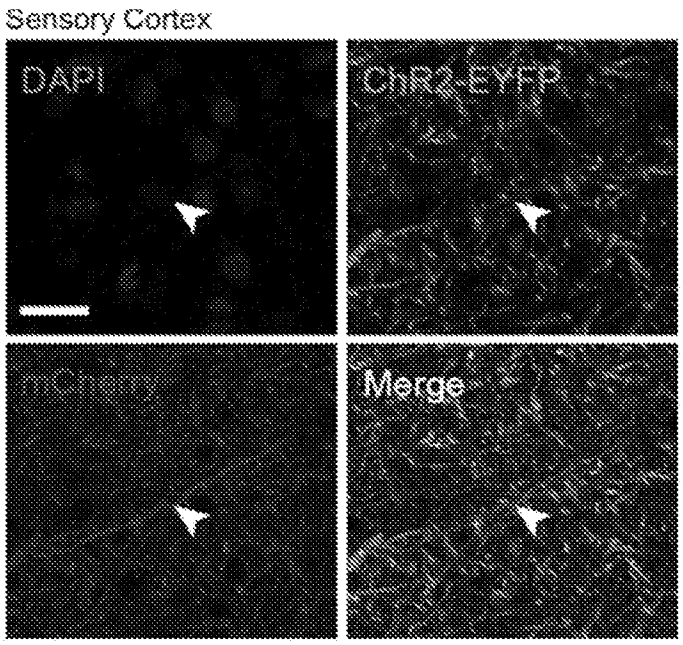


Figure 6A

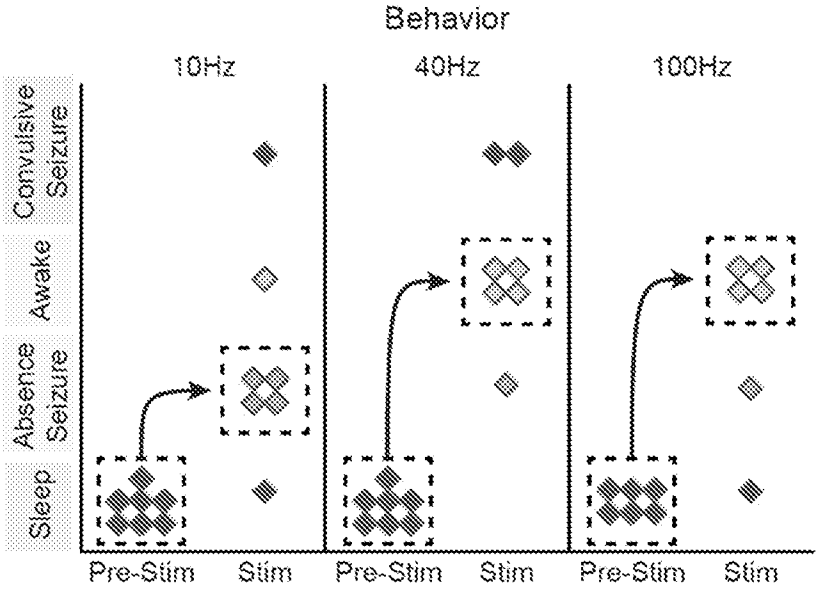


Figure 6B

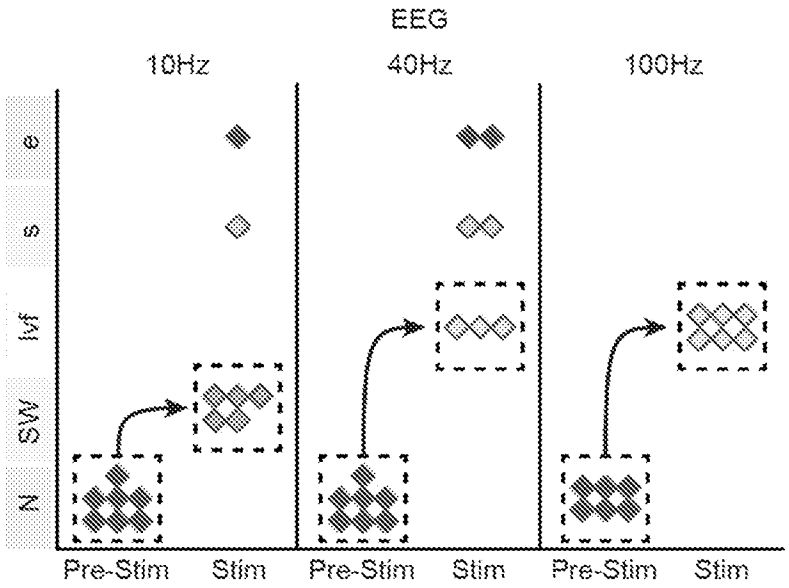


Figure 6C

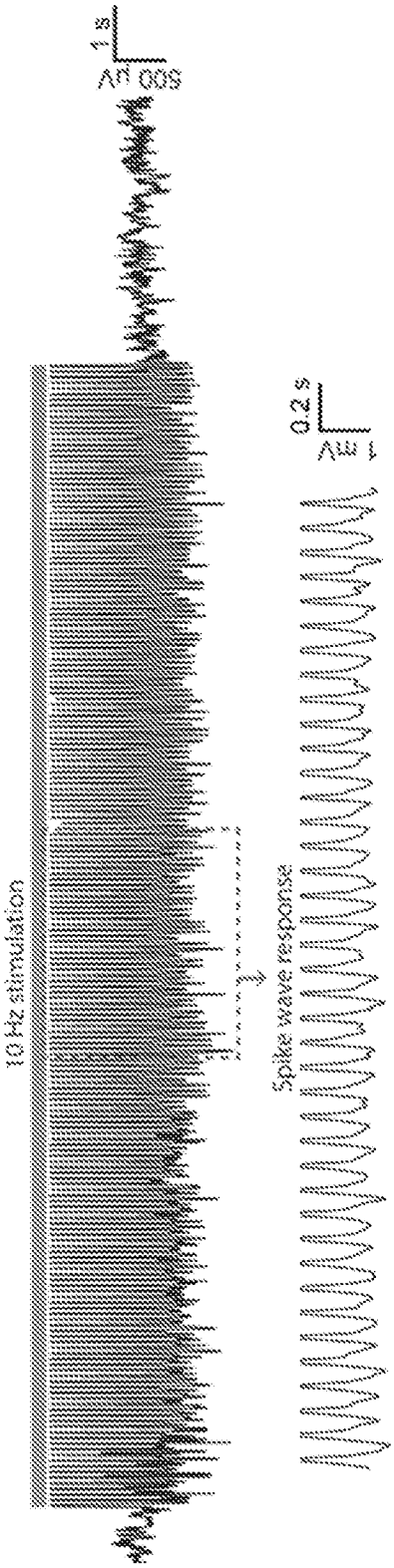


Figure 6D

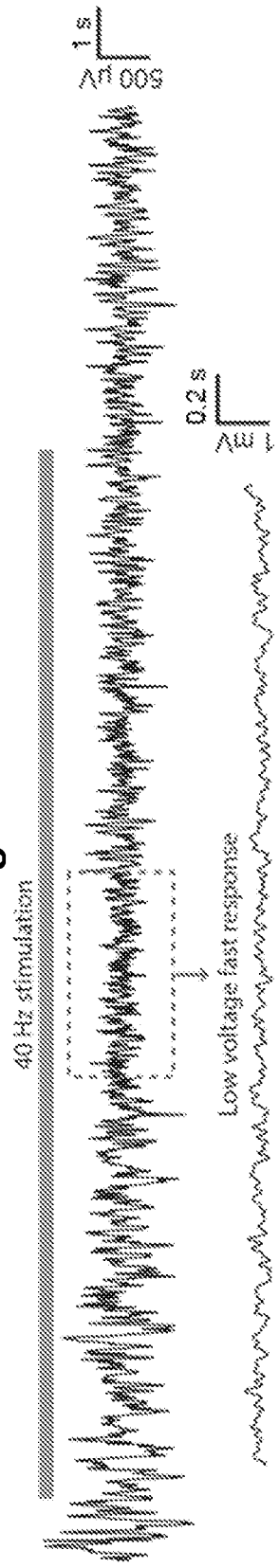


Figure 7

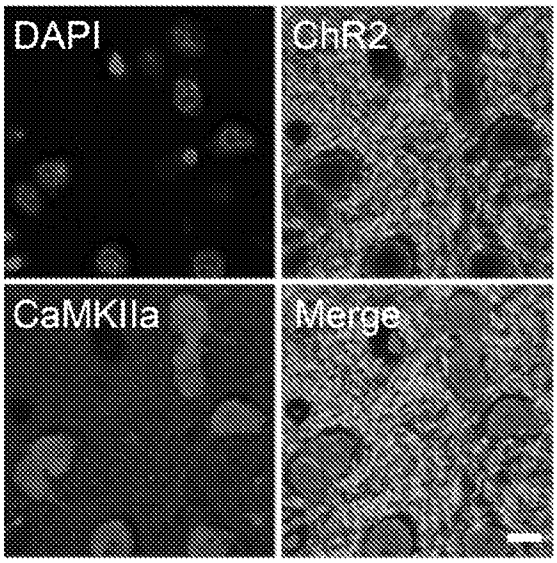


Figure 8

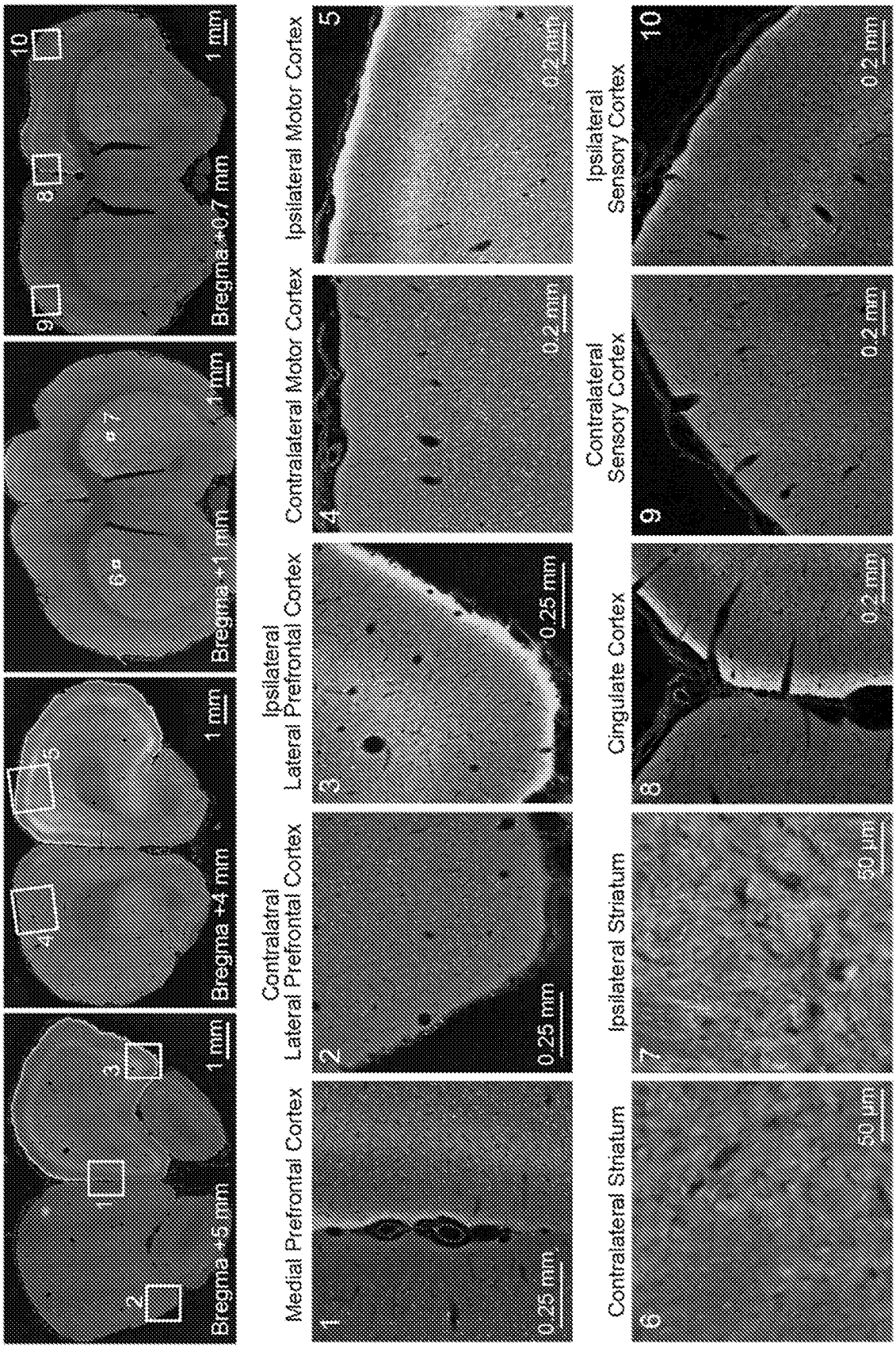

Bregma +5 mm
Bregma +4 mm
Bregma +1 mm
Bregma +0.7 mm
1 mm
Medial Prefrontal Cortex
Contralatral Lateral Prefrontal Cortex
Ipsilateral Lateral Prefrontal Cortex
Contralateral Motor Cortex
Ipsilateral Motor Cortex
Contralateral Striatum
Ipsilateral Striatum
Cingulate Cortex
Contralateral Sensory Cortex
Ipsilateral Sensory Cortex
0.25 mm
0.2 mm
50 µm

Figure 9A

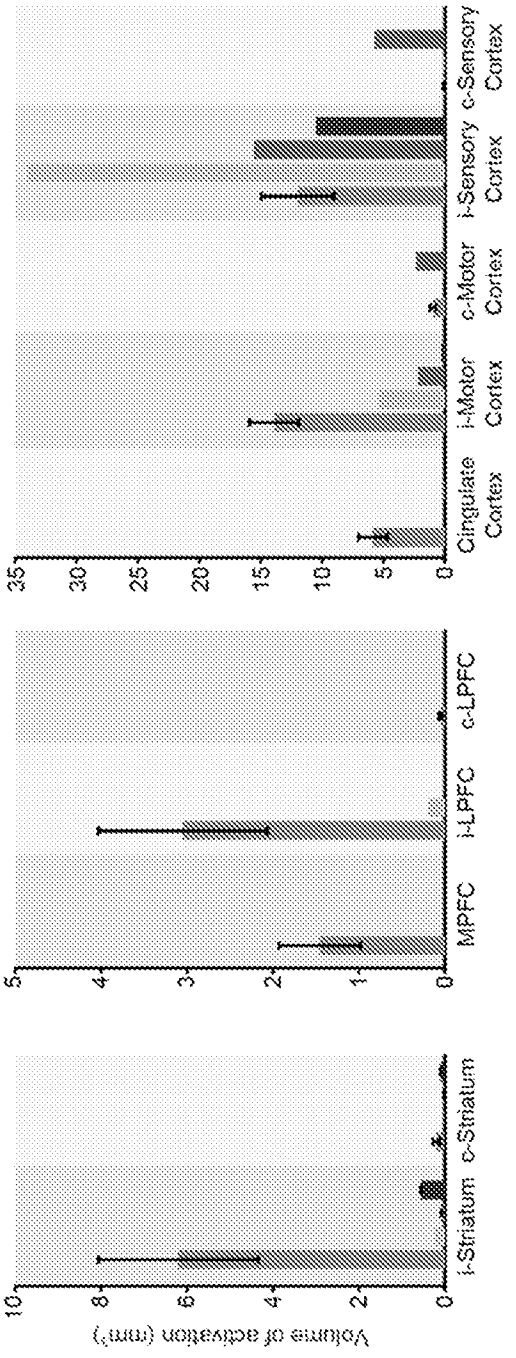


Figure 9B

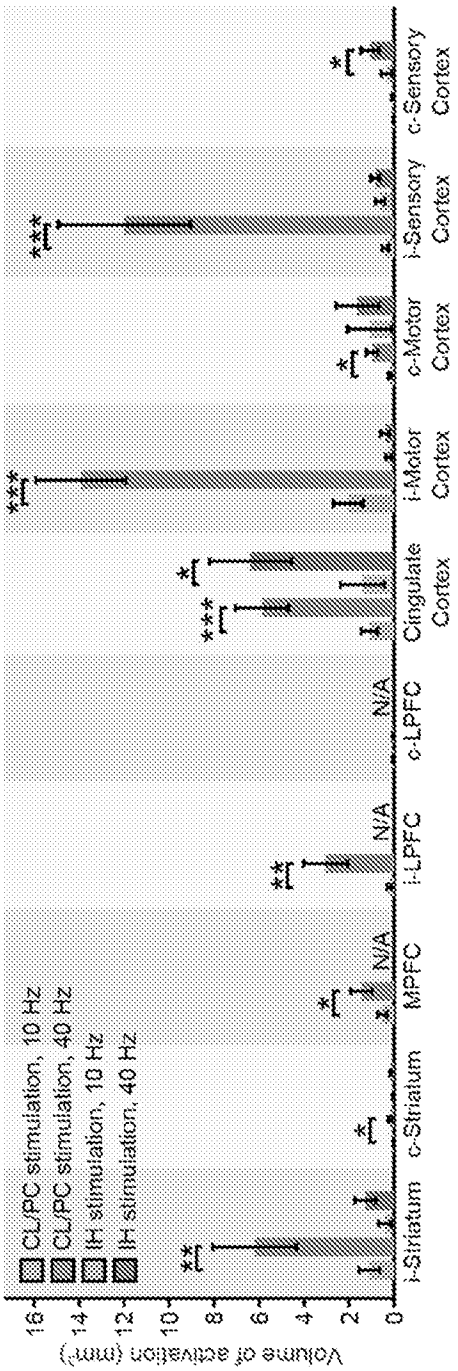


Figure 10A

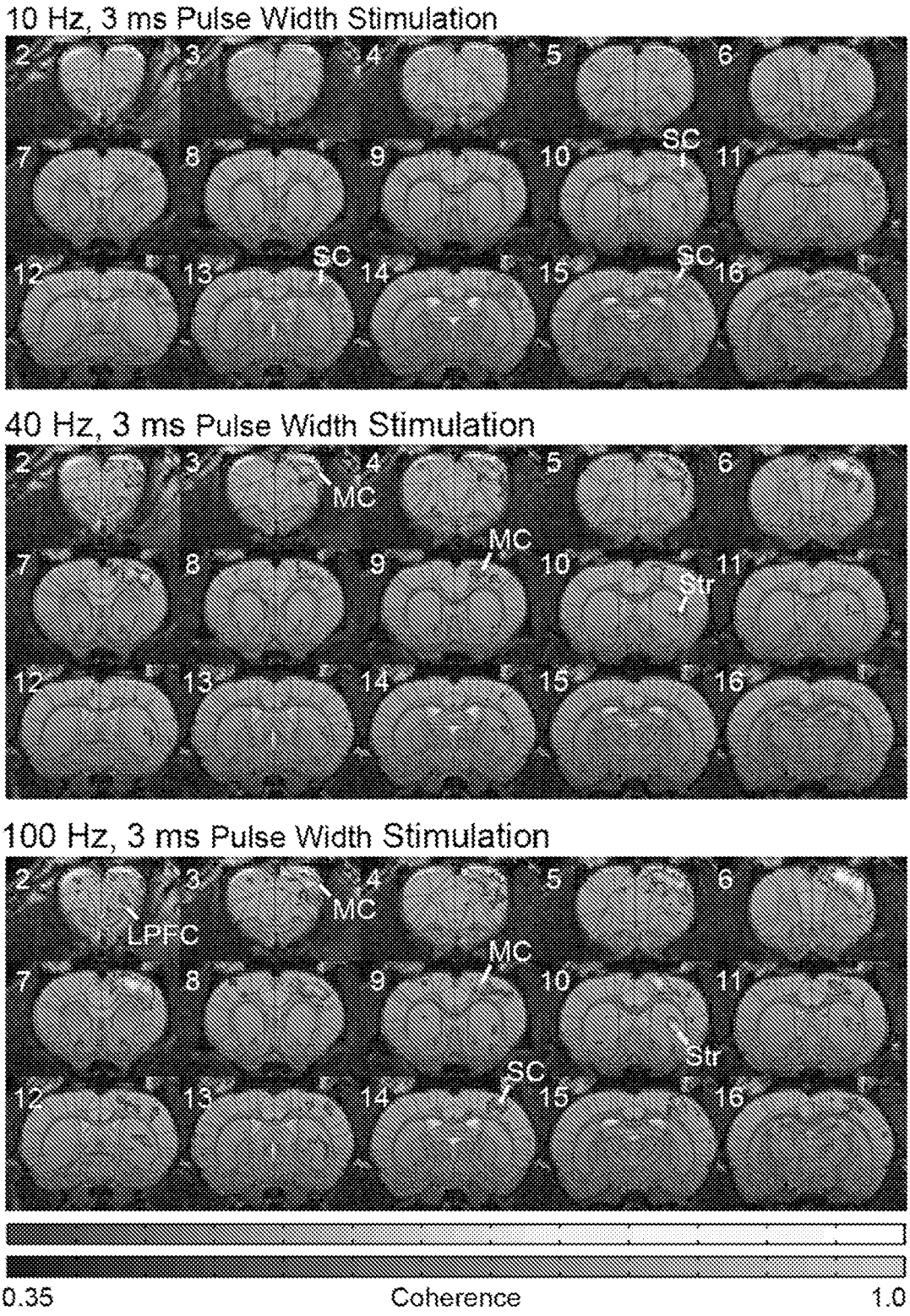

10 Hz, 3 ms Pulse Width Stimulation
2
3
4
5
6
7
8
9
10
SC
11
12
13
SC
14
15
SC
16
40 Hz, 3 ms Pulse Width Stimulation
2
3
4
MC
5
6
MC
7
8
9
10
11
Str
12
13
14
15
16
100 Hz, 3 ms Pulse Width Stimulation
2
3
4
MC
5
6
LPFC
MC
7
8
9
10
11
Str
SC
12
13
14
15
16
0.35
Coherence
1.0

Figure 10B

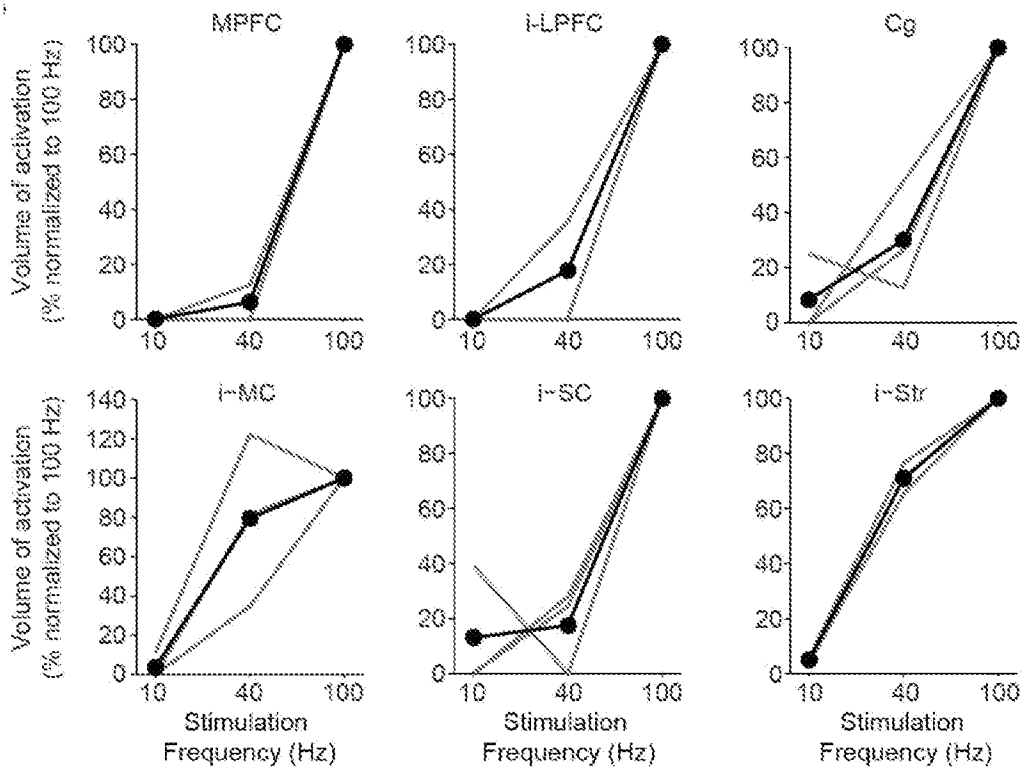


Figure 10C

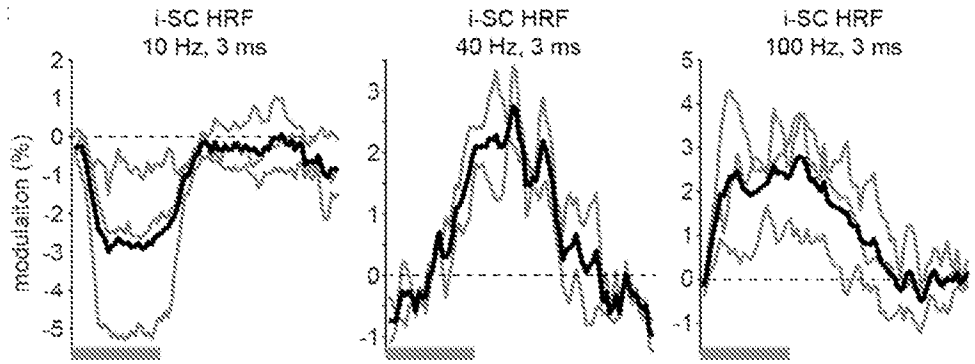
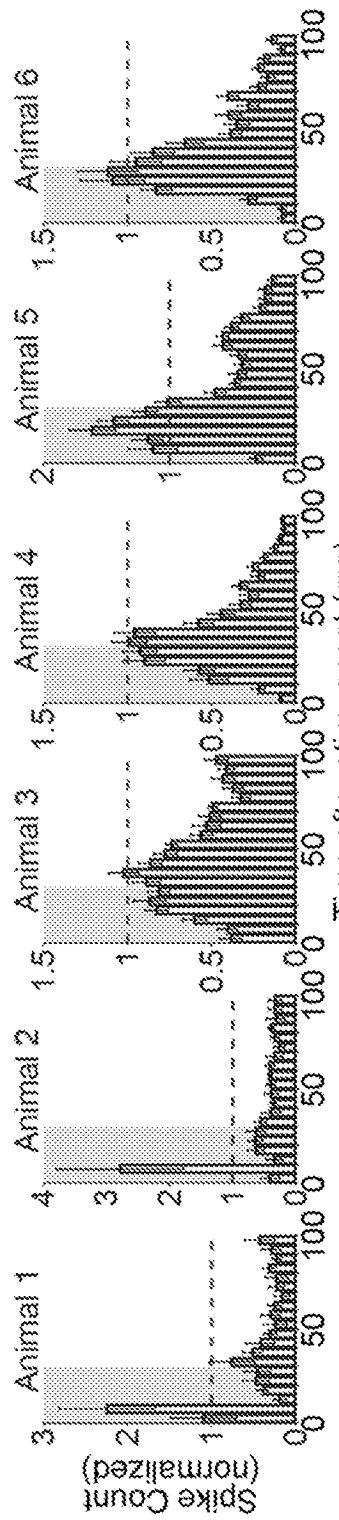


Figure 11



	Pre-stimulation firing rate (Hz)	Suppressed firing rate (Hz)	Peak latency (ms)	Spike fidelity (%)	Number of cells
Animal 1	2.2 ± 0.6	1.2 ± 0.4	5.8 ± 1.1	5.0 ± 1.4	6
Animal 2	5.6 ± 1.5	3.3 ± 1.3	7.5 ± 0.0	14.6 ± 6.3	4
Animal 3	10.6 ± 1.9	6.6 ± 1.2	33.5 ± 3.2	14.1 ± 2.1	10
Animal 4	14.0 ± 2.3	6.3 ± 1.3	32.5 ± 2.6	17.3 ± 3.3	23
Animal 5	12.4 ± 2.1	8.4 ± 1.6	21.9 ± 3.3	31.3 ± 4.7	18
Animal 6	8.4 ± 2.1	4.2 ± 1.2	26.9 ± 2.3	11.2 ± 2.5	17

Figure 12

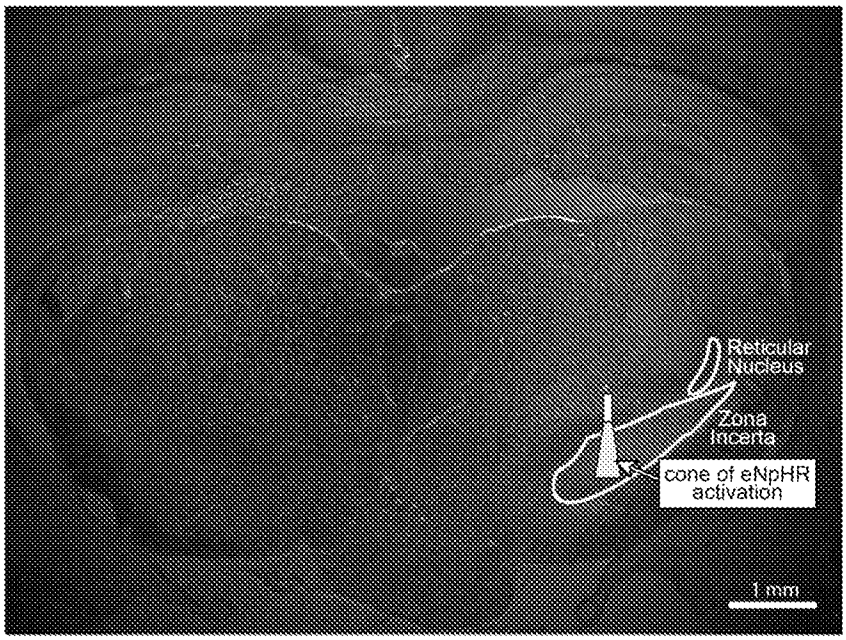


Figure 13

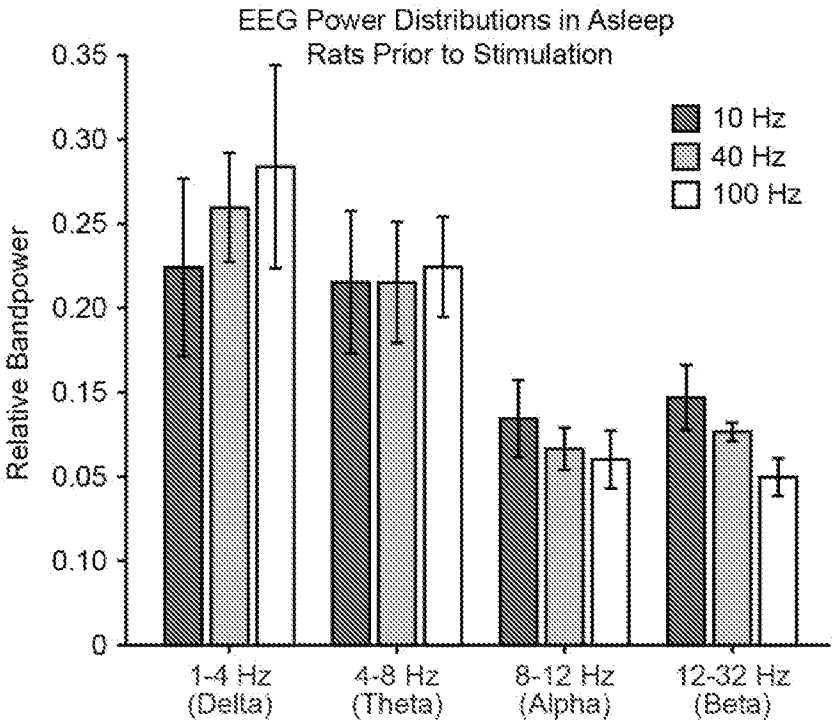


Figure 14

(Depolarizing opsins)

Amino acid sequence of ChR2 (SEQ ID NO:1)

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLAAGF
SILLLMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHRVQWLRYAEWLL
TCPVILIHLSNLTGLSNDYSRRTMGLLVSDIGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFH
AAKAYIEGYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLSVYGSTVGHTIIDLM
SKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAVP

Amino acid sequence of ChR2 with ER export and trafficking signal sequences (SEQ ID NO:2)

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLAAGF
SILLLMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHRVQWLRYAEWLL
TCPVILIHLSNLTGLSNDYSRRTMGLLVSDIGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFH
AAKAYIEGYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLSVYGSTVGHTIIDLM
SKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAVP*AAA***KSRITSEGE**
YIPLDQIDINVFCYENEV

amino acid sequence of a ChR2 SSFO (SEQ ID NO:3)

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLAAGF
SILLLMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHRVQWLRYAEWLL
T**S**PVILIHLSNLTGLSNDYSRRTMGLLVS**A**IGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFH
AAKAYIEGYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLSVYGSTVGHTIIDLM
SKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAVP

amino acid sequence of a ChR2 SSFO with ER export and trafficking signal sequences (SEQ ID NO:4)

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLAAGF
SILLLMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHRVQWLRYAEWLL
T**S**PVILIHLSNLTGLSNDYSRRTMGLLVS**A**IGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFH
AAKAYIEGYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLSVYGSTVGHTIIDLM
SKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAVP*AAA***KSRITSEGE**
YIPLDQIDINVFCYENEV

Figure 14 (Cont.)

Amino acid sequence of a VChR1 (SEQ ID NO:5)

Mdypvarslivryptdlgngtvcmprgqcycegwlrsrgtsiektiaitlqwvvfalsvaclgw
yayqawratcgweevyvaliemmksiieafhefdspatlwlssgngvvwmrygewlltcpvlli
hlsnltglkddyskrtmgllvsdvgcivwgatsamctgwtkilfflislsygmytyfhaakvyi
eafhtvpkgicrelvrvmawtffvawgmfpvlfllgtegfghispygsaighsildliaknmwgvl
gnylrvkihehillygdirkkqkitiagqemevetlvaeeed

Amino acid sequence of a VChR1 with ER export and trafficking signal sequences (SEQ ID NO:6)

Mdypvarslivryptdlgngtvcmprgqcycegwlrsrgtsiektiaitlqwvvfalsvaclgw
yayqawratcgweevyvaliemmksiieafhefdspatlwlssgngvvwmrygewlltcpvlli
hlsnltglkddyskrtmgllvsdvgcivwgatsamctgwtkilfflislsygmytyfhaakvyi
eafhtvpkgicrelvrvmawtffvawgmfpvlfllgtegfghispygsaighsildliaknmwgvl
gnylrvkihehillygdirkkqkitiagqemevetlvaeeed*AAA***KSRITSEGEYIPLDQIDINV**FCY
ENEV

amino acid sequence of C1V1 (SEQ ID NO:7)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENN
GSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWITFALSALCLMFYGYQTWKSTCGWEEIY
VATIEMIKFIIEYFHEFDEPAVIYSSNGNKTVWLRYAEWLLTCPVLLIHLSNLTGLKDDYSKRT
MGLLVSDVGCIVWGATSAMCTGWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGICRELV
RVMAWTFFVAWGMFPVLFLLGTEGFGHISPYGSAIGHSILDLIAKNMWGVLGNYLRVKIHEHI
LLYGDIRKKQKITIAGQEMEVETLVAEEED

amino acid sequence of C1V1 with ER export and trafficking signal sequences (SEQ ID NO:8)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENN
GSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWITFALSALCLMFYGYQTWKSTCGWEEIY
VATIEMIKFIIEYFHEFDEPAVIYSSNGNKTVWLRYAEWLLTCPVLLIHLSNLTGLKDDYSKRT
MGLLVSDVGCIVWGATSAMCTGWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGICRELV
RVMAWTFFVAWGMFPVLFLLGTEGFGHISPYGSAIGHSILDLIAKNMWGVLGNYLRVKIHEHI
LLYGDIRKKQKITIAGQEMEVETLVAEEED*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of a C1C2 (SEQ ID NO:9)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPNN
GQCFCLAWLKSNGTNAEKLAANILQWITFALSALCLMFYGYQTWKSTCGWEEIYVATIEMIKFIIEYFHE
FDEPAVIYSSNGNKTVWLRYAEWLLTCPVILIHLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALSKG
YVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLS
VYGSTVGHTIIDLMSKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV

Figure 14 (Cont.)

Amino acid sequence of a C1C2 with ER export and trafficking signal sequences (SEQ ID NO:10)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPNN
GQCFCLAWLKSNGTNAEKLAANILQWITFALSALCLMFYGYQTWKSTCGWEEIYVATIEMIKFIIEYFHE
FDEPAVIYSSNGNKTVWLRYAEWLLTCPVILIHLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALSKG
YVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLS
VYGSTVGHTIIDLMSKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV*AA*
*A***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of ReaChR (red shifted ChR) (SEQ ID NO:11)

MVSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPN
NGQCFCLAWLKSNGTNAEKLAANILQWVTFALSVACLGWYAYQAWRATCGWEEVYVALIEMMKSIIEAFH
EFDSPATLWLSSGNGVVWMRYGEWLLTCPVILIHLSNLTGLKDDYSKRTMGLLVSDVGCIVWGATSAMCT
GWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGLCRQLVRAMAWLFFVSWGMFPVLFLLGPEGFGHI
SPYGSAIGHSILDLIAKNMWGVLGNYLRVKIHEHILLYGDIRKKQKITIAGQEMEVETLVAEEEDKYESS

Amino acid sequence of ReaChR (red shifted ChR) with ER export and trafficking signal sequences (SEQ ID NO:12)

MVSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPN
NGQCFCLAWLKSNGTNAEKLAANILQWVTFALSVACLGWYAYQAWRATCGWEEVYVALIEMMKSIIEAFH
EFDSPATLWLSSGNGVVWMRYGEWLLTCPVILIHLSNLTGLKDDYSKRTMGLLVSDVGCIVWGATSAMCT
GWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGLCRQLVRAMAWLFFVSWGMFPVLFLLGPEGFGHI
SPYGSAIGHSILDLIAKNMWGVLGNYLRVKIHEHILLYGDIRKKQKITIAGQEMEVETLVAEEEDKYESS
*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of SdChR (CheRiff) (SEQ ID NO:13)

Mggapapdahsappgndsaggseyhapagyqvnppyhpvhgyeeqcssiyiyygalweqetargfqwfavflsalfl
afygwhaykasvgweevyvcsvelikvileiyfeftspamlflyggnitpwlryaewlltcpvilihlsnitglsee
ynkrtmallvsdlgticmgvtaalatgwvkwlfyciglvygtqtfynagiiyvesyyimpaggckklvlamtavyys
swlmfpglfifgpegmhtlsvagstightiadllskniwgllghflrikihehiimygdirrpvssqflgrkvdvla
fvteedkv

Amino acid sequence of SdChR (CheRiff) with ER export and trafficking signal sequences (SEQ ID NO:14)

Mggapapdahsappgndsaggseyhapagyqvnppyhpvhgyeeqcssiyiyygalweqetargfqwfavflsalfl
afygwhaykasvgweevyvcsvelikvileiyfeftspamlflyggnitpwlryaewlltcpvilihlsnitglsee
ynkrtmallvsdlgticmgvtaalatgwvkwlfyciglvygtqtfynagiiyvesyyimpaggckklvlamtavyys
swlmfpglfifgpegmhtlsvagstightiadllskniwgllghflrikihehiimygdirrpvssqflgrkvdvla
fvteedkv*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Figure 14 (Cont.)

Amino acid sequence of CnChR1 (Chrimson) (SEQ ID NO:15)

Maelissatrslfaagginpwpnpyhhedmgcggmtptgecfstewwcdpsyglsdagygycfveatggylvvgvek
kqawlhsrgtpgekigaqvcqwiafsiaialltfygfsawkatcgweevyvccvevlfvtleifkefsspatvylst
gnhayclryfewllscpviliklsnlsglkndyskrtmglivscvgmivfgmaaglatdwlkwllyivsciyggymy
fqaakcyveanhsvpkghcrmvvklmayayfaswgsypilwavgpegllklspyansighsicdiiakefwtflahh
lrikihehilihgdirkttkmeiggeeveveefveeededtv

Amino acid sequence of CnChR1 (Chrimson) with ER export and trafficking signal sequences (SEQ ID NO:16)

Maelissatrslfaagginpwpnpyhhedmgcggmtptgecfstewwcdpsyglsdagygycfveatggylvvgvek
kqawlhsrgtpgekigaqvcqwiafsiaialltfygfsawkatcgweevyvccvevlfvtleifkefsspatvylst
gnhayclryfewllscpviliklsnlsglkndyskrtmglivscvgmivfgmaaglatdwlkwllyivsciyggymy
fqaakcyveanhsvpkghcrmvvklmayayfaswgsypilwavgpegllklspyansighsicdiiakefwtflahh
lrikihehilihgdirkttkmeiggeeveveefveeededtv*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of CsChrimson (SEQ ID NO:17)

Msrlvaaswllalllcgitstttassapaasstdgtaaaavshyamngfdelakgavvpedhfvcgpadkcycsawl
hsrgtpgekigaqvcqwiafsiaialltfygfsawkatcgweevyvccvevlfvtleifkefsspatvylstgnhay
clryfewllscpviliklsnlsglkndyskrtmglivscvgmivfgmaaglatdwlkwllyivsciyggymyfqaak
cyveanhsvpkghcrmvvklmayayfaswgsypilwavgpegllklspyansighsicdiiakefwtflahhlriki
hehilihgdirkttkmeiggeeveveefveeededtv

Amino acid sequence of CsChrimson with ER export and trafficking signal sequences (SEQ ID NO:18)

Msrlvaaswllalllcgitstttassapaasstdgtaaaavshyamngfdelakgavvpedhfvcgpadkcycsawl
hsrgtpgekigaqvcqwiafsiaialltfygfsawkatcgweevyvccvevlfvtleifkefsspatvylstgnhay
clryfewllscpviliklsnlsglkndyskrtmglivscvgmivfgmaaglatdwlkwllyivsciyggymyfqaak
cyveanhsvpkghcrmvvklmayayfaswgsypilwavgpegllklspyansighsicdiiakefwtflahhlriki
hehilihgdirkttkmeiggeeveveefveeededtv*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of ShChR1 (Chronos) (SEQ ID NO:19)

metaatmthafisavpsaeatirgllsaaavvtpaadahgetsnattagadhgcfphinhgtelqhkiavglqwftv
ivaivqlifygwhsfkattgweevyvcvielvkcfielfhevdspatvyqtnggaviwlrysmwlltcpvilihlsn
ltglheeyskrtmtilvtdignivwgitaaftkgplkilffmiglfygvtcffqiakvyiesyhtlpkgvcrkicki
mayvffcswlmfpvmfiagheglglitpytsgighlildliskntwgflghhlrvkihehilihgdirktttinvag
enmeietfvdeeeeggv

Figure 14 (Cont.)

Amino acid sequence of ShChR1 (Chronos) with ER export and trafficking signal sequences (SEQ ID NO:20)

metaatmthafisavpsaeatirgllsaaavvtpaadahgetsnattagadhgcfphinhgtelqhkiavglqwftv
ivaivqlifygwhsfkattgweevyvcvielvkcfielfhevdspatvyqtnggaviwlrysmwlltcpvilihlsn
ltglheeyskrtmtilvtdignivwgitaaftkgplkilffmiglfygvtcffqiakvyiesyhtlpkgvcrkicki
mayvffcswlmfpvmfiagheglglitpytsgighlildliskntwgflghhlrvkihehilihgdirktttinvag
enmeietfvdeeeeggv*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Figure 15

(hyperpolarizing opsins)
amino acid sequence of Archaerhodopsin-3 (SEQ ID NO:21)
MDPIALQAGYDLLGDGRPETLWLGIGTLLMLIGTFYFLVRGWGVTDKDAREYYAVTILVPGIASAAYLSM
FFGIGLTEVTVGGEMLDIYYARYADWLFTTPLLLLDLALLAKVDRVTIGTLVGVDALMIVTGLIGALSHT
AIARYSWWLFSTICMIVVLYFLATSLRSAAKERGPEVASTFNTLTALVLVLWTAYPILWIIGTEGAGVVG
LGIETLLFMVLDVTAKVGFGFILLRSRAILGDTEAPEPSAGADVSAAD

amino acid sequence of eArch3.0 (SEQ ID NO:22)
MDPIALQAGYDLLGDGRPETLWLGIGTLLMLIGTFYFLVRGWGVTDKDAREYYAVTILVPGIASAAYLSM
FFGIGLTEVTVGGEMLDIYYARYADWLFTTPLLLLDLALLAKVDRVTIGTLVGVDALMIVTGLIGALSHT
AIARYSWWLFSTICMIVVLYFLATSLRSAAKERGPEVASTFNTLTALVLVLWTAYPILWIIGTEGAGVVG
LGIETLLFMVLDVTAKVGFGFILLRSRAILGDTEAPEPSAGADVSAAD*RPVVAAAA***KSRITSEGEYIPLD**
QIDINVFCYENEV

Amino acid sequence of ArchT (SEQ ID NO:23)

MDPIALQAGYDLLGDGRPETLWLGIGTLLMLIGTFYFIVKGWGVTDKEAREYYSITILVP
GIASAAYLSMFFGIGLTEVTVAGEVLDIYYARYADWLFTTPLLLLDLALLAKVDRVSIGT
LVGVDALMIVTGLIGALSHTPLARYSWWLFSTICMIVVLYFLATSLRAAAKERGPEVAST
FNTLTALVLVLWTAYPILWIIGTEGAGVVGLGIETLLFMVLDVTAKVGFGFILLRSRAIL
GDTEAPEP

Amino acid sequence of ArchT with ER export and trafficking signal sequences (SEQ ID NO:24)

MDPIALQAGYDLLGDGRPETLWLGIGTLLMLIGTFYFIVKGWGVTDKEAREYYSITILVP
GIASAAYLSMFFGIGLTEVTVAGEVLDIYYARYADWLFTTPLLLLDLALLAKVDRVSIGT
LVGVDALMIVTGLIGALSHTPLARYSWWLFSTICMIVVLYFLATSLRAAAKERGPEVAST
FNTLTALVLVLWTAYPILWIIGTEGAGVVGLGIETLLFMVLDVTAKVGFGFILLRSRAIL
GDTEAPEP*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Figure 15 (Cont.)

amino acid sequence of GtR3 (SEQ ID NO:25)
MLVGEGAKLDVHGCKTVDMASSFGKALLEFVFIVFACITLLLGINAAKSKAASRVLFPATFVTGIASIAY
FSMASGGGWVIAPDCRQLFVARYLDWLITTPLLLIDLGLVAGVSRWDIMALCLSDVLMIATGAFGSLTVG
NVKWVWWFFGMCWFLHIIFALGKSWAEAAKAKGGDSASVYSKIAGITVITWFCYPVVWVFAEGFGNFSVT
FEVLIYGVLDVISKAVFGLILMSGAATGYESI

amino acid sequence of GtR3 with ER export and trafficking signal sequences (SEQ ID NO:26)
MLVGEGAKLDVHGCKTVDMASSFGKALLEFVFIVFACITLLLGINAAKSKAASRVLFPATFVTGIASIAY
FSMASGGGWVIAPDCRQLFVARYLDWLITTPLLLIDLGLVAGVSRWDIMALCLSDVLMIATGAFGSLTVG
NVKWVWWFFGMCWFLHIIFALGKSWAEAAKAKGGDSASVYSKIAGITVITWFCYPVVWVFAEGFGNFSVT
FEVLIYGVLDVISKAVFGLILMSGAATGYESI*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of rhodopsin type II proton pump (Oxy) (SEQ ID NO:27)
MAPLAQDWTYAEWSAVYNALSFGIAGMGSATIFFWLQLPNVTKNYRTALTITGIVTLIATYHYFRIFNSW
VAAFNVGLGVNGAYEVTVSGTPFNDAYRYVDWLLTVPLLLVELILVMKLPAKETVCLAWTLGIASAVMVA
LGYPGEIQDDLSVRWFWWACAMVPFVYVVGTLVVGLGAATAKQPEGVVDLVSAARYLTVVSWLTYPFVYI
VKNIGLAGSTATMYEQIGYSAADVTAKAVFGVLIWAIANAKSRLEEEGKLRA

Amino acid sequence of rhodopsin type II proton pump with ER export and trafficking signal sequences(SEQ ID NO:28)
MAPLAQDWTYAEWSAVYNALSFGIAGMGSATIFFWLQLPNVTKNYRTALTITGIVTLIATYHYFRIFNSW
VAAFNVGLGVNGAYEVTVSGTPFNDAYRYVDWLLTVPLLLVELILVMKLPAKETVCLAWTLGIASAVMVA
LGYPGEIQDDLSVRWFWWACAMVPFVYVVGTLVVGLGAATAKQPEGVVDLVSAARYLTVVSWLTYPFVYI
VKNIGLAGSTATMYEQIGYSAADVTAKAVFGVLIWAIANAKSRLEEEGKLRA*AAA***KSRITSEGEYIPLDQ**
IDINVFCYENEV

Figure 15 (Cont.)

Amino acid sequence of L. maculans rhodopsin (Mac) (SEQ ID NO:29)
MIVDQFEEVLMKTSQLFPLPTATQSAQPTHVAPVPTVLPDTPIYETVGDSGSKTLWVVFVLMLIASAAFT
ALSWKIPVNRRLYHVITTIITLTAALSYFAMATGHGVALNKIVIRTQHDHVPDTYETVYRQVYYARYIDW
AITTPLLLLDLGLLAGMSGAHIFMAIVADLIMVLTGLFAAFGSEGTPQKWGWYTIACIAYIFVVWHLVLN
GGANARVKGEKLRSFFVAIGAYTLILWTAYPIVWGLADGARKIGVDGEIIAYAVLDVLAKGVFGAWLLVT
HANLRESDVELNGFWANGLNREGAIRIGEDDGA

Amino acid sequence of Mac 3.0 (SEQ ID NO:30)
MIVDQFEEVLMKTSQLFPLPTATQSAQPTHVAPVPTVLPDTPIYETVGDSGSKTLWVVFVLMLIASAAFT
ALSWKIPVNRRLYHVITTIITLTAALSYFAMATGHGVALNKIVIRTQHDHVPDTYETVYRQVYYARYIDW
AITTPLLLLDLGLLAGMSGAHIFMAIVADLIMVLTGLFAAFGSEGTPQKWGWYTIACIAYIFVVWHLVLN
GGANARVKGEKLRSFFVAIGAYTLILWTAYPIVWGLADGARKIGVDGEIIAYAVLDVLAKGVFGAWLLVT
HANLRESDVELNGFWANGLNREGAIRIGEDDGARPVVAVSKAAA**KSRITSEGEYIPLDQIDINV***FCYENE*
V

amino acid sequence of NpHR (SEQ ID NO:31)

MTETLPPVTESAVALQAEVTQRELFEFVLNDPLLASSLYINIALAGLSILLFVFMTRGLDDPRAKLI
AVSTILVPVVSIASYTGLASGLTISVLEMPAGHFAEGSSVMLGGEEVDGVVTMWGRYLTWALST
PMILLALGLLAGSNATKLFTAITFDIAMCVTGLAAALTTSSHLMRWFWYAISCACFLVVLYILLV
EWAQDAKAAGTADMFNTLKLLTVVMWLGYPIVWALGVEGIAVLPVGVTSWGYSFLDIVAKYI
FAFLLLNYLTSNESVVSGSILDVPSASGTPADD

amino acid sequence of NpHR3.0 (SEQ ID NO:32)

MTETLPPVTESAVALQAEVTQRELFEFVLNDPLLASSLYINIALAGLSILLFVFMTRGLDDPRAKLI
AVSTILVPVVSIASYTGLASGLTISVLEMPAGHFAEGSSVMLGGEEVDGVVTMWGRYLTWALST
PMILLALGLLAGSNATKLFTAITFDIAMCVTGLAAALTTSSHLMRWFWYAISCACFLVVLYILLV
EWAQDAKAAGTADMFNTLKLLTVVMWLGYPIVWALGVEGIAVLPVGVTSWGYSFLDIVAKYI
FAFLLLNYLTSNESVVSGSILDVPSASGTPADD**AAA**KSRITSEGEYIPLDQIDINFCYENEV

amino acid sequence of NpHR3.1 (SEQ ID NO:33)

MVTQRELFEFVLNDPLLASSLYINIALAGLSILLFVFMTRGLDDPRAKLIAVSTILVPVVSIASYTG
LASGLTISVLEMPAGHFAEGSSVMLGGEEVDGVVTMWGRYLTWALSTPMILLALGLLAGSNAT
KLFTAITFDIAMCVTGLAAALTTSSHLMRWFWYAISCACFLVVLYILLVEWAQDAKAAGTADM
FNTLKLLTVVMWLGYPIVWALGVEGIAVLPVGVTSWGYSFLDIVAKYIFAFLLLNYLTSNESVVS
GSILDVPSASGTPADD**AAA**KSRITSEGEYIPLDQIDINFCYENEV

Figure 15 (Cont.)

Amino acid sequence of *Dunaliella salina* channelrhodopsin (SEQ ID NO:34)

Mrrresqlaylclfvliagwaprltesapdlaerrppserntpyanikkvpnitepnanvqldg
walyqdfyylagsdkewvvgpsdqcycrawskshgtdregeaavvwayivfaicivqlvyfmfa
awkatvgweevyvniielvhialviwvefdkpamlylndgqmvpwlrysawllscpvilihlsn
ltglkgdyskrtmgllvsdigtivfgtsaalappnhvkvilftigllyglftfftaakvyieay
htvpkgqcrnlvramawtyfvswamfpilfilgregfghityfgssighfileifsknlwslg
hglryrirqhiiihgnltkknkiniagdnveveeyvdsndkdsdv

Amino acid sequence of *Dunaliella salina* channelrhodopsin with ER export and trafficking signal sequences (SEQ ID NO:35)

mrrresqlaylclfvliagwaprltesapdlaerrppserntpyanikkvpnitepnanvqldg
walyqdfyylagsdkewvvgpsdqcycrawskshgtdregeaavvwayivfaicivqlvyfmfa
awkatvgweevyvniielvhialviwvefdkpamlylndgqmvpwlrysawllscpvilihlsn
ltglkgdyskrtmgllvsdigtivfgtsaalappnhvkvilftigllyglftfftaakvyieay
htvpkgqcrnlvramawtyfvswamfpilfilgregfghityfgssighfileifsknlwslg
hglryrirqhiiihgnltkknkiniagdnveveeyvdsndkdsdv*AAA***KSRITSEGEYIPLDQID**
INVFCYENEV

Amino acid sequence of a iC1C2 (SEQ ID NO:36)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPNN
GQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFYGYQTWKSTCGWEEIYVATISMIKFIIEYFHS
FDEPAVIYSSNGNKTKWLRYASWLLTCPVILIRLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALSKG
YVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLS
KYGSNVGHTIIDLMSKQCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV

Amino acid sequence of a iC1C2 with ER export and trafficking signal sequences (SEQ ID NO:37)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPNN
GQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFYGYQTWKSTCGWEEIYVATISMIKFIIEYFHS
FDEPAVIYSSNGNKTKWLRYASWLLTCPVILIRLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALSKG
YVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLS
KYGSNVGHTIIDLMSKQCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV*AA*
*A***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of a SwiChR (iC1C2-C167A or T or S) (SEQ ID NO:38)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPNN
GQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFYGYQTWKSTCGWEEIYVATISMIKFIIEYFHS
FDEPAVIYSSNGNKTKWLRYASWLLT**X**PVILIRLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALSKG
YVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLS
KYGSNVGHTIIDLMSKQCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV

Figure 15 (Cont.)

Amino acid sequence of a SwiChR (iC1C2-C167A or T or S) with ER export and trafficking signal sequences (SEQ ID NO:39)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPNN
GQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFYGYQTWKSTCGWEEIYVATISMIKFIIEYFHS
FDEPAVIYSSNGNKTKWLRYASWLLT**X**PVILIRLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALSKG
YVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLS
KYGSNVGHTIIDLMSKQCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV*AA*
*A***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of ibC1C2 (SEQ ID NO:40)

MDYGGALSAVGLFQTSYTLENNGSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFY
GYQTWKSTCGWEEIYVATISMIKFIIEYFHSFDEPAVIYSSNGNKTKWLRYASWLLTCPVILIRLSNLTG
LANDYNKRTMGLLVSDIGTIVWGTTAALSKGYVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGRCRQ
VVTGMAWLFFVSWGMFPILFILGPEGFGVLSKYGSNVGHTIIDLMSKQCWGLLGHYLRVLIHEHILIHGD
IRKTTKLNIGGTEIEVETLVEDEAEAGAV

Amino acid sequence of ibC1C2 with ER export and trafficking signal sequences (SEQ ID NO:41)

MDYGGALSAVGLFQTSYTLENNGSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFY
GYQTWKSTCGWEEIYVATISMIKFIIEYFHSFDEPAVIYSSNGNKTKWLRYASWLLTCPVILIRLSNLTG
LANDYNKRTMGLLVSDIGTIVWGTTAALSKGYVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGRCRQ
VVTGMAWLFFVSWGMFPILFILGPEGFGVLSKYGSNVGHTIIDLMSKQCWGLLGHYLRVLIHEHILIHGD
IRKTTKLNIGGTEIEVETLVEDEAEAGAV*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of iChR2 (SEQ ID NO:42)

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLSAGFSILLLMFY
AYQTWKSTCGWEEIYVCAISMVKVILEFFFSFKNPSMLYLATGHRVKWLRYASWLLTCPVILIRLSNLTG
LSNDYSRRTMGLLVSDIGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFHAAKAYIEGYHTVPKGRCRQ
VVTGMAWLFFVSWGMFPILFILGPEGFGVLSKYGSNVGHTIIDLMSKQCWGLLGHYLRVLIHEHILIHGD
IRKTTKLNIGGTEIEVETLVEDEAEAGAVP

Amino acid sequence of iChR2 with ER export and trafficking signal sequences (SEQ ID NO:43)

MDYGGALSAVGRELLFVTNPVVVNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLSAGFSILLLMFY
AYQTWKSTCGWEEIYVCAISMVKVILEFFFSFKNPSMLYLATGHRVKWLRYASWLLTCPVILIRLSNLTG
LSNDYSRRTMGLLVSDIGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFHAAKAYIEGYHTVPKGRCRQ
VVTGMAWLFFVSWGMFPILFILGPEGFGVLSKYGSNVGHTIIDLMSKQCWGLLGHYLRVLIHEHILIHGD
IRKTTKLNIGGTEIEVETLVEDEAEAGAVP*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Figure 15 (Cont.)

Amino acid sequence of iC1V1 (SEQ ID NO:44)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPNN
GQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFYGYQTWKSTCGWEEIYVATISMIKFIIEYFHS
FDEPAVIYSSNGNKTKWLRYASWLLTCPVLLIRLSNLTGLKDDYSKRTMGLLVSDVGCIVWGATSAMCTG
WTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGICRELVRVMAWTFFVAWGMFPVLFLLGTEGFGHIS
KYGSNIGHSILDLIAKQMWGVLGNYLRVKIHEHILLYGDIRKKQKITIAGQEMEVETLVAEEED

Amino acid sequence of iC1V1 with ER export and trafficking signal sequences (SEQ ID NO:45)

MSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPNN
GQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFYGYQTWKSTCGWEEIYVATISMIKFIIEYFHS
FDEPAVIYSSNGNKTKWLRYASWLLTCPVLLIRLSNLTGLKDDYSKRTMGLLVSDVGCIVWGATSAMCTG
WTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGICRELVRVMAWTFFVAWGMFPVLFLLGTEGFGHIS
KYGSNIGHSILDLIAKQMWGVLGNYLRVKIHEHILLYGDIRKKQKITIAGQEMEVETLVAEEEDAAA**KSR**
ITSEGEYIPLDQIDINVFCYENEV

Amino acid sequence of ibC1V1 (SEQ ID NO:46)

MDYGGALSAVGLFQTSYTLENNGSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFY
GYQTWKSTCGWEEIYVATISMIKFIIEYFHSFDEPAVIYSSNGNKTKWLRYASWLLTCPVLLIRLSNLTG
LKDDYSKRTMGLLVSDVGCIVWGATSAMCTGWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGICRE
LVRVMAWTFFVAWGMFPVLFLLGTEGFGHISKYGSNIGHSILDLIAKQMWGVLGNYLRVKIHEHILLYGD
IRKKQKITIAGQEMEVETLVAEEED

Amino acid sequence of ibC1V1 with ER export and trafficking signal sequences (SEQ ID NO:47)

MDYGGALSAVGLFQTSYTLENNGSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWISFALSALCLMFY
GYQTWKSTCGWEEIYVATISMIKFIIEYFHSFDEPAVIYSSNGNKTKWLRYASWLLTCPVLLIRLSNLTG
LKDDYSKRTMGLLVSDVGCIVWGATSAMCTGWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGICRE
LVRVMAWTFFVAWGMFPVLFLLGTEGFGHISKYGSNIGHSILDLIAKQMWGVLGNYLRVKIHEHILLYGD
IRKKQKITIAGQEMEVETLVAEEEDAAA**KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of iReaChR (SEQ ID NO:48)

MVSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPN
NGQCFCLAWLKSNGTNAEKLAANILQWVSFALSVACLGWYAYQAWRATCGWEEVYVALISMMKSIIEAFH
SFDSPATLWLSSGNGVKWMRYGSWLLTCPVILIRLSNLTGLKDDYSKRTMGLLVSDVGCIVWGATSAMCT
GWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGLCRQLVRAMAWLFFVSWGMFPVLFLLGPEGFGHI
SKYGSNIGHSILDLIAKQMWGVLGNYLRVKIHEHILLYGDIRKKQKITIAGQEMEVETLVAEEEDKYESS

Figure 15 (Cont.)

Amino acid sequence of iReaChR with ER export and trafficking signal sequences (SEQ ID NO:49)

MVSRRPWLLALALAVALAAGSAGASTGSDATVPVATQDGPDYVFHRAHERMLFQTSYTLENNGSVICIPN
NGQCFCLAWLKSNGTNAEKLAANILQWVSFALSVACLGWYAYQAWRATCGWEEVYVALISMMKSIIEAFH
SFDSPATLWLSSGNGVKWMRYGSWLLTCPVILIRLSNLTGLKDDYSKRTMGLLVSDVGCIVWGATSAMCT
GWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGLCRQLVRAMAWLFFVSWGMFPVLFLLGPEGFGHI
SKYGSNIGHSILDLIAKQMWGVLGNYLRVKIHEHILLYGDIRKKQKITIAGQEMEVETLVAEEEDKYESS
*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

Amino acid sequence of ibReaChR (SEQ ID NO:50)

MDYGGALSAVGLFQTSYTLENNGSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWVSFALSVACLGWY
AYQAWRATCGWEEVYVALISMMKSIIEAFHSFDSPATLWLSSGNGVKWMRYGSWLLTCPVILIRLSNLTG
LKDDYSKRTMGLLVSDVGCIVWGATSAMCTGWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGLCRQ
LVRAMAWLFFVSWGMFPVLFLLGPEGFGHISKYGSNIGHSILDLIAKQMWGVLGNYLRVKIHEHILLYGD
IRKKQKITIAGQEMEVETLVAEEEDKYESS

Amino acid sequence of ibReaChR with ER export and trafficking signal sequences (SEQ ID NO:51)

MDYGGALSAVGLFQTSYTLENNGSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWVSFALSVACLGWY
AYQAWRATCGWEEVYVALISMMKSIIEAFHSFDSPATLWLSSGNGVKWMRYGSWLLTCPVILIRLSNLTG
LKDDYSKRTMGLLVSDVGCIVWGATSAMCTGWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGLCRQ
LVRAMAWLFFVSWGMFPVLFLLGPEGFGHISKYGSNIGHSILDLIAKQMWGVLGNYLRVKIHEHILLYGD
IRKKQKITIAGQEMEVETLVAEEEDKYESS*AAA***KSRITSEGEYIPLDQIDINV**FCYENEV

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/64250

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61B 3/16; A61N 5/06 (2017.01)
CPC - A61B 5/055; A61N 5/062; A61N 5/06
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61B 3/16; A61N 5/06 (2017.01); USPC - 600/411; 600/410; 607/88
CPC - A61B 5/055; A61N 5/062; A61N 5/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8) - A61B 3/16; A61N 5/06 (2017.01); USPC - 600/411; 600/410; 607/88
CPC - A61B 5/055; A61N 5/062; A61N 5/06 - see keyword below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline, Google: imaging, circuit, neural, in vivo, brain, circuit, analysis, individual, illuminating, first region, light, pulse, second, different, temporal, pattern, region, neurons, action potential, spike, activation, fMRI, scan, light-activated polypeptide, measuring, functional magnetic resonance imag

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/0289386 A1 (JUNIOR UNIVERSITY; THE BOARD OF TRUSTEES OF THE LELAND STANFORD) 31 October 2013 (31.10.2013), Abstract, para [0003], [0004], [0006], [0007], [0009], [0011], [0022], [0023], [0031], [0034], [0045], [0048], [0051], [0055], [0055], [0058], [0072], [0076], [0082], Fig 5C, Fig 6f-i, and SEQ ID NO: 1	1-4
Y	GROSSMAN et al., Modeling Study of the Light Stimulation of a Neuron Cell With Channelrhodopsin-2 Mutants. IEEE Trans Biomed Eng. 2011, Vol. 58(6), p. 1742-51. Abstract; pg 1742, Fig 1; pg 1743, col 1, top para, para 2, and Fig 2; pg 1745, col 2, up para, and Fig 4; pg 1746, col 1, last para, col 2, top para and last para, Fig 5, and Fig 6; and pg 1747, col 2, last para	1-4
A	BERNDT et al., High-efficiency channelrhodopsins for fast neuronal stimulation at low light levels. Proc Natl Acad Sci U S A. 2011, Vol. 108(18), p. 7595-600. Entire documentation, especially Abstract; pg 7595, col 1, up para, and col 2, top para; and pg 7598, Fig 5C	1-4
A	ENTCHEVA et al, Channelrhodopsin2 Current During the Action Potential: "Optical AP Clamp" and Approximation. Sci Rep. 2014, Vol. 4:5838. PDF File: pg 1-7. Entire documentation, especially Abstract; pg 1, para 1; and pg 2, Fig 1	1-4
A	SHMUEL et al., Sustained Negative BOLD, Blood Flow and Oxygen Consumption Response and Its Coupling to the Positive Response in the Human Brain. Neuron. 2002, Vol. 36(6), p. 1195-210. Entire documentation, especially Abstract; and pg 1198, Fig 3C; pg 1199, Fig 4; pg 1200, Fig 5; pg 1202, Fig 6; and pg 1204, col 2, para 3	1-4

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
27 January 2017	23 MAR 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/64250

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

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

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

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

3. ☒ Claims Nos.: 5-23 and 27-28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

Group I, Claims 1-4, directed to a method for in vivo circuit analysis of a brain of an individual, comprising: i) illuminating a first region of a brain with: a first set of light pulses; and a second set of light pulses, wherein the first set has a different temporal pattern of light pulses from the second set.

Group II, claims 24-26, directed to a method for in vivo circuit analysis of a brain of an individual, comprising: i) determining a dynamic functional connection between: a first region of the brain comprising neurons configured to depolarize or hyperpolarize upon light-induced activation of a light-activated polypeptide expressed in the neurons of the first region.

**********Continued in the extra sheet**********

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/64250

Continuation of:
Box III (unity of invention is lacking)

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

Special Technical Feature
Group I includes the special technical feature of a first set of light pulses; and a second set of light pulses, wherein the first set has a different temporal pattern of light pulses from the second set, not required by Group II.

Group II includes the special technical feature of a first region of the brain comprising neurons configured to depolarize or hyperpolarize upon light-induced activation, and ii) identifying a modulatory node of the dynamic functional connection, the modulatory node comprising neurons of a third region, not required by Group I.

Common Technical Features
The inventions of Groups I-II share the technical feature of a method for in vivo circuit analysis of a brain of an individual, comprising:
i) illuminating a first region of a brain with: a first set of light pulses; wherein the first region comprises neurons upon activation, of a first light-activated polypeptide expressed by the neurons of the first region (or Group II: a first region of the brain comprising neurons upon light-induced activation of a light-activated polypeptide expressed in the neurons of the first region);
ii) measuring, in a second region of the brain using functional magnetic resonance imaging (fMRI) scanning of a plurality of brain regions: a first change in neural activity induced by the first set of light pulses (or Group II: using light-induced modulation of neural activity of the first population of neurons, in conjunction with fMRI scanning of a plurality of brain regions that comprises the second region); and
iii) identifying a dynamic functional connection from the neurons of the first region to neurons of the second region (part of claim 1).

However, these shared technical features do not represent a contribution over prior art as being anticipated by US 2013/0289386 A1 to JUNIOR UNIVERSITY; THE BOARD OF TRUSTEES OF THE LELAND STANFORD to (hereinafter 'LELAND_STANFORD') as follows:

LELAND_STANFORD discloses a method for in vivo circuit analysis of a brain of an individual (para [0022] - 'a neuronal population for optogenetic stimulation and fMRI analysis'; para [0003] - 'Blood oxygenation level-dependent functional magnetic resonance imaging (BOLD fMRI) ... non-invasive whole brain imaging...the neural circuits'; para [0058] - 'subjects used for fMRI'; para [0006] - 'integrating high-field fMRI output with optogenetic stimulation of cells. A light-activated, light-responsive molecule... allow millisecond scale targeted. activity modulation in vivo. ... in the animal'; para [0076] - 'In Vivo Recording and Analysis'), comprising:
i) illuminating a first region of a brain with: a first set of light pulses (para [0004] - 'a target neural cell population in a first region of a brain to express light-responsive molecules. Using a light pulse, the light-responsive molecules in the target neural cell population are stimulated'; para [0064] - 'Optical stimulation ...with steady illumination');
--wherein the first region comprises neurons upon activation, of a first light-activated polypeptide expressed by the neurons of the first region (para [0004] - 'modifying a target neural cell population in a first region of a brain to express light-responsive molecules. Using a light pulse, the light-responsive molecules in the target neural cell population are stimulated', wherein 'to express light-responsive molecules' comprising 'a first light-activated polypeptide expressed', and wherein 'a first region of a brain to express light-responsive molecules. Using a light pulse, the light-responsive molecules ... are stimulated' is 'the first region comprises neurons upon activation, of a first light-activated polypeptide expressed by the neurons of the first region'; para [0009] - 'delivering a light-responsive molecule (e.g., ChR2) to neural cells of a first brain region. The neural cells of the first brain region may be stimulated ...applying light pulses to, the first brain region', wherein 'a light-responsive molecule (e.g., ChR2)' is 'a first light-activated polypeptide expressed'; para [0055] 'channelrhodopsin (ChR2)'; Sequence List - SEQ ID NO: 1, which is 100% identical to the SEQ ID NO: 1 of the Application; Specification: [00184] - 'light-activated polypeptide is based on the amino acid sequence of the protein ChR2'; [00185] - 'amino acid sequence of ChR2 (SEQ ID NO: 1)'; para [00230] - 'channelrhodopsin-2 (ChR2)');
ii) measuring, in a second region of the brain using functional magnetic resonance imaging (fMRI) scanning of a plurality of brain regions: a first change in neural activity induced by the first set of light pulses (Abstract - 'a target neural cell population in a first region of a brain to express light-responsive molecules. Using a light pulse, the light-responsive molecules in the target neural cell population are stimulated. Multiple regions of the brain are scanned via magnetic resonance imaging'; para [0007] - 'a target neural cell population in a first region of a brain to express light-responsive molecules. ... the light-responsive molecules are stimulated in the target neural cell population by using a light pulse. While the target neural cell population is being stimulated, multiple regions of the brain are scanned using an fMRI machine. The fMRI scans are used to observe neural reaction in response to the stimulation in at least one of the multiple regions of the brain and to determine therefrom the network communication'; para [0003] - 'Blood oxygenation level-dependent functional magnetic resonance imaging (BOLD fMRI)... non-invasive whole brain imaging'; para [0004]; para [0009]); and
iii) identifying a dynamic functional connection from the neurons of the first region to neurons of the second region (para [0004] - 'modifying a target neural cell population in a first region of a brain to express light-responsive molecules. Using a light pulse, the light-responsive molecules in the target neural cell population are stimulated. Multiple regions of the brain are scanned via magnetic resonance imaging. The scans allow for observation of a neural reaction in response to the stimulation in at least one of the multiple regions of the brain. ... a determination is made whether neural projection in a second region of the brain are connected to at least some of the cells in the modified target cell population in the first region of the brain'; [0009] - 'delivering a light-responsive molecule (e.g., ChR2) to neural cells of a first brain region. The neural cells of the first brain region may be stimulated by ... applying light pulses to, the first brain region. Multiple regions of the brain may be scanned by acquiring magnetic resonance images of first and second brain regions to identify the neural cells of the second brain region that are connected to the neural cells of the first brain region'; Abstract).

Without a shared special technical feature, the inventions lack unity with one another.

Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 5-23 and 27-28 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.