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(54) Title: CELL LINE, SYSTEM AND METHOD FOR OPTICAL CONTROL OF SECONDARY MESSENGERS

(57) **Abstract**: A variety of methods, devices and compositions are implemented for light- activated molecules. One such method is implemented for generating secondary messengers in a cell. A nucleotide sequence for expressing a chimeric light responsive membrane protein (e.g., rhodopsin) is modified with one or more heterologous receptor subunits {e.g., an adrenergic receptor (alpha1, Beta2)}. The light responsive membrane protein is expressed in a cell for producing a secondary messenger in response to light.
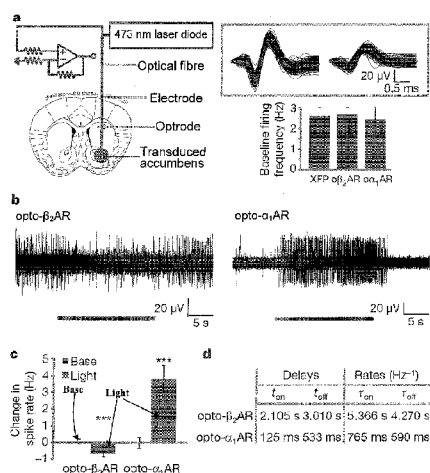
FIG. 4

CELL LINE, SYSTEM AND METHOD FOR
OPTICAL CONTROL OF SECONDARY MESSENGERS

Related Patent Document

5   This patent document claims the benefit, under 35 U.S.C. § 119(e), of

U.S. Provisional Patent Application Serial No. 61/057,108 filed on May 29, 2008 and

entitled "Cell Line, System and Method for Optical Control of Secondary Messengers;"

the underlying provisional application is fully incorporated herein by reference.

Incorporation-by-Reference of Material Submitted Electronically

10   Incorporated by reference in its entirety is a computer-readable nucleotide/

amino acid sequence listing submitted concurrently herewith, and identified as follows:

One 12,342 Byte ASCII (Text) file named "STFD195PCT_ST25.txt" created on

April 29, 2009.

Field of the Invention

15   The present invention relates generally to systems and approaches for generating

secondary messengers in response to optical stimulus and more particularly to a cell lines,

nucleotide sequences, chimeric proteins, and uses thereof, each relating to the production

of secondary messengers in response to light.

Background

20   Guanine nucleotide-binding proteins (G proteins) are believed to alternate

between an inactive guanosine diphosphate (GDP) state and an active guanosine

triphosphate (GTP) bound state. These two states have been linked to the release of a

secondary messenger within a cell. The released secondary messenger can function to

25   regulate downstream cell processes.

Secondary messengers include signaling molecules that are rapidly

generated/released. These molecules produce cellular responses by activating effector

proteins within the cell. Example cellular signaling systems include the phosphoinositol

system, the cyclic adenosine monophosphate (cAMP) system, and the arachidonic acid

30   system.

Changes between the different states of the G proteins can be triggered as a result

of proteins called G protein-coupled receptors (GPCRs), G protein-linked receptors

(GPLR), seven transmembrane domain receptors (7TM receptors) or heptahelical

receptors. This protein family includes a variety of transmembrane receptors. These receptors respond to external stimuli (*e.g.*, light, neurotransmitters, odors or hormones) by activating signal transduction pathways internal to the cell. Specifically, ligands bind and activate the transduction pathways thereby causing the G proteins to alternate states.

5   GPCR-related activity is associated with many diseases, and thus, GPCRs are the target of many pharmaceuticals and treatments.

It is believed that over 30% of all drugs on the market target G-protein coupled receptors (GPCRs) and that many of those drugs relate to the production or inhibition of the secondary messenger cAMP. There is an abundance of pathological processes that

10   directly involve cAMP, including neurophysiological, endocrinological, cardiac, metabolic, and immune diseases. In the study of complex mammalian behaviors, technological limitations have prevented spatiotemporally precise control over intracellular signaling processes. Current chemical-based methods for modulating secondary messenger levels, such as cAMP levels, operate relatively slowly and present

15   problems to study activity on the fast timescales that the body uses in connection with certain tissue, such as in nervous or cardiac tissue. These chemical-methods often lack the speed to probe these fast timescales (*e.g.*, while screening for novel therapeutics).

Summary

20   The present invention is directed to overcoming the above-mentioned challenges and others related to generation of secondary messengers and related imaging devices and their implementations. The present invention is exemplified in a number of implementations and applications, some of which are summarized below.

Consistent with an embodiment of the present invention, a method is implemented

25   for generating secondary messengers in a cell. A nucleotide sequence for expressing a chimeric light responsive membrane protein (*e.g.*, rhodopsin) is modified with one or more heterologous receptor subunits {*e.g.*, an adrenergic receptor (alpha1, Beta2)}. The light responsive membrane protein is expressed in a cell for producing a secondary messenger in response to light.

30   Consistent with an embodiment of the present invention, a method is implemented for assessing the efficacy of a putative treatment regimen (*e.g.*, a drug or electrical stimulus or anything that works via these secondary messengers) relating to intracellular messengers. A nucleotide sequence for expressing a chimeric light responsive membrane protein (rhodopsin) is modified with one or more heterologous receptor subunits {*e.g.*, an

adrenergic receptor (alpha1, Beta2)}. The light responsive membrane protein is expressed in a cell for producing a secondary messenger in response to light. The protein is exposed to light. The effects of the treatment are assessed.

An embodiment of the present invention is directed toward, a cell expressing a
5   chimeric light responsive membrane protein (rhodopsin) with one or more heterologous receptor subunits {e.g., an adrenergic receptor (alpha1, Beta2)}.

An embodiment of the present invention is directed toward, a nucleotide sequence for expressing a chimeric light responsive membrane protein (rhodopsin) with one or more heterologous receptor subunits {e.g., an adrenergic receptor (alpha1, Beta2)}.

10   The above summary of the present invention is not intended to describe each illustrated embodiment or every implementation of the present invention. The figures and detailed description that follow more particularly exemplify these embodiments.

Brief Description of the Drawings

The invention may be more completely understood in consideration of the detailed
15   description of various embodiments of the invention that follows in connection with the accompanying drawings, in which:

FIG. 1A shows a schematic showing optoGs and optoGq, consistent with example embodiments of the present invention;

FIG. 1B shows Enzyme-Linked Immunosorbent Assay (ELISA) of cAMP, cGMP,
20   and IP_1 of cells transfected with either nothing, optoGs, or optoGq, consistent with example embodiments of the present invention;

FIG. 1C shows Ca-imaging of cells transfected with mCherry fusion proteins of optoGs and optoGq, consistent with example embodiments of the present invention;

FIG. 2 shows Ca-imaging of cells transfected with mCherry fusion proteins of
25   optoGs and optoGq, consistent with example embodiments of the present invention;

FIG. 3A shows cAMP, IP_1 and IP_3 levels for HEK cells expressing various constructs, consistent with example embodiments of the present invention;

FIG. 3B shows a lentiviral express vector, GAD immunostaining of opto-α_1AR-expressing cells and observed pCREB activation in optoXR-expressing cells (mCherry+)
30   following 10 min optical stimulation, consistent with example embodiments of the present invention;

FIG. 4A shows optrode targeting of transduced accumbens, spike waveforms and baseline firing rates for indicated constructs, consistent with example embodiments of the present invention;

FIG. 4B shows *in vivo* optrode recordings with light stimulation, consistent with example embodiments of the present invention;

FIG. 4C shows change in spiking frequency with light versus baseline, consistent with example embodiments of the present invention;

FIG. 4D shows firing rate change kinetics, consistent with example embodiments of the present invention;

FIG. 5A shows stereotactic targeting of a transduced region, a freely moving mouse with implanted fiber optics, a schematic of place preference apparatus and test and a trace of a freely exploring mouse, consistent with example embodiments of the present invention;

FIG. 5B shows preferences for control and opto-α_1AR, consistent with example embodiments of the present invention; and

FIG. 5C shows results of total distance for various open field tests; consistent with example embodiments of the present invention.

While the invention is amenable to various modifications and alternative forms, specifics thereof have been shown by way of example in the drawings and will be described in detail. It should be understood, however, that the intention is not to limit the invention to the particular embodiments described. On the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention.

Detailed Description

The present invention is believed to be useful for enabling practical applications of a variety of optical-based systems and methods, and the invention has been found to be particularly suited for use in systems and methods dealing with optical control of secondary messenger levels within a cell. While the present invention is not necessarily limited to such applications, various aspects of the invention may be appreciated through a discussion of various examples using this context.

Embodiments of the present invention involve a chimeric membrane protein that responds to optical stimulus by causing the release of a secondary messenger within the cell. In a specific instance, the chimeric protein is a combination of a heterologous

receptor subunit and a protein that undergoes conformation in reaction to light via photoisomerization and thus is activated by light. Rhodopsins or retinylidene proteins provide an example group of light-responsive proteins that can be modified to include a heterologous receptor subunit.

5   According to an embodiment of the present invention, a protein believed to contain a seven transmembrane α-helical domain is modified to include a heterologous receptor subunit associated with a secondary messenger. When expressed in a cell membrane, the protein reacts to light by undergoing a conformal change. The conformal change triggers the release/production of the secondary messenger.

10   Embodiments of the present invention involve a nucleotide sequence for coding a chimeric membrane protein that responds to optical stimulus by causing the release of a secondary messenger within the cell.

Embodiments of the present invention involve a cell that expresses a heterologous and chimeric membrane protein. The chimeric membrane protein responds to optical

15   stimulus by triggering the release of a secondary messenger within the cell. In certain embodiments the expression of the chimeric membrane protein occurs *in vivo*. In other embodiments expression of the chimeric membrane protein occurs *in vitro*.

Embodiments of the present invention can implemented for production of any suitable secondary messenger by modifying a Guanine nucleotide-binding protein

20   coupled receptor protein (GPCR) to include the appropriate receptor subunit.

Embodiments of the present invention allow for the use of proteins that respond to a variety of wavelengths and intensities of light.

An embodiment of the present invention involves the use of a chimeric GPCR protein, as disclosed herein, to determine any downstream effect of the secondary

25   messenger activity of interest.

Embodiments of the present invention are directed to expression of a chimeric GPCR protein in a variety of cell types including, but not limited to, mammalian cells, stems cells, plant cells, and unicellular organisms like yeast and *E. coli*.

A specific embodiment of the present invention is related to an optimized

30   expression of a chimeric protein with attached fluorescent proteins for ease of visualization, and optimized use of the modality for studying downstream effects of the secondary messenger activity induced by light.

An embodiment of the present invention is directed to genetically targeting a chimeric GPCR protein, as disclosed herein, to specific cell populations for expression therein. Cell-type specific promoters exist that are selectively expressed in a target cell type (*e.g.*, Synapsin-1 for targeting neurons; Troponin variants for cardiac tissue).

5   Placing these promoters upstream of the chimeric GPCR protein in an expression vector can be used to target expression of the protein to a cell type of interest. This includes inducible, reversible, or otherwise controllable promoter systems such as Tet-response, ER-response, and Cre/Lox systems.

According to an example embodiment of the present invention, a genetically

10   encodeable protein is developed such that, when these are expressed in cell types of interest, cyclic adenosine monophosphate (cAMP) is produced in response to light. This can be useful, for example, to visualize downstream effects on cell physiology including, but not limited to, screening for pharmaceuticals. Other embodiments use a chimeric and heterologous GPCR that results in the release of secondary messengers in response to

15   light. Example secondary messengers include cAMP, cyclic guanosine monophosphate (cGMP), inositol trisphosphate/inositol 1,4,5-trisphosphate/triphosphoinositol (IP_3) and arachidonic acid.

Consistent with an embodiment of the present invention, a method is implemented for assessing the efficacy of a putative treatment regimen (*e.g.*, a drug or electrical

20   stimulus or anything that works via these secondary messengers) relating to intracellular messengers. A nucleotide sequence for expressing a chimeric light responsive membrane protein (*e.g.*, rhodopsin) is modified with one or more heterologous receptor subunits {*e.g.*, an adrenergic receptor (alpha1, Beta2)}. The light responsive membrane protein is expressed in a cell for producing a secondary messenger in response to light. The protein

25   is exposed to light. The effects of the treatment are assessed.

The light can be applied according to a desired stimulus profile. In one embodiment the expressed membrane protein responds to light within tens of milliseconds. Thus, the stimulus profile can include a series of light pulses in rapid succession and the resulting effects can be monitored using, for example, Ca^{2+} sensitive

30   dyes.

In one instance, the cell can first be stimulated without the treatment. Once the treatment is administered, the cell can then be stimulated again. The results of each test can be compared to assess the effectiveness of the treatment.

The treatment can include a wide variety of different implementations including, but not limited to, pharmaceuticals, modifications to the cell (genetic or otherwise), physical parameters of the cell (*e.g.*, temperature changes or electrical stimulus) or a treatment regimen applied to an organism.

In one embodiment, the treatment is the optical stimulus of the expressed membrane protein. In such an instance the effectiveness can be measured, for example, by monitoring the symptoms associated with a disorder to be treated.

In another embodiment, the treatment regimen is implemented as part of modeling a disease or disorder. For example, a disease model can be used (cells or animals) and the background/baseline state can be assessed before the protein is expressed and the treatment regimen evaluated.

Experimental results show that optically-evoked cAMP regulation of targeted ion channels can be visualized by transfecting cells with both the cAMP-inducer and a cAMP-targeted cation channel and visualizing resultant activity using Ca^{2+}-sensitive dyes. This suite of genetically-encodable, optically-activated modulators of secondary messenger activity can be useful in screening novel therapeutics as well as being a therapeutic modality itself, given the implication of cAMP in numerous diseases states, like ADHD and cardiac channelopathies. The protein can be engineered for use with various other secondary messengers (*e.g.*, IP_3), other colors for light activation by engineering the retinal binding site or choosing for the chimera a rhodopsin or cone opsin with a different absorbance/action spectrum, and other downstream effects of the secondary messenger, such as calcium signaling and/or kinase activity.

FIGs. 1A, 1B and 1C show experimental data from optoGs and optoGq, two examples of light-activated inducers of secondary messenger signaling ('optoXRs') that have been developed. These light-activated inducers are a rhodopsin/GPCR chimerism. OptoGq provides light-responsive control of Gq signaling, whereas, OptoGs, provides light-responsive control of Gs signaling.

In both optoGs and optoGq it has been shown that there is negligible difference in baseline cAMP and IP_3 levels in darkness and that there is no crossover to other secondary messenger pathways such as cGMP. The increased cAMP levels seen with light stimulation of optoGq is an expected downstream effect of IP_3 production.

FIG. 1A shows a schematic of optoGs and optoGq, consistent with example embodiments of the present invention. For each protein, the intracellular loops of

rhodopsin are replaced with those of adrenergic proteins normally coupled to either Gs (beta2) or Gq (alpha1). The genetic coding sequences are optimized for expression in human and murine cells. Examples of the resulting sequences include optoGs: Seq. Id. No. 1 and Seq. Id. No. 2; and optoGq: Seq. Id No. 3 and Seq. Id. No 4.

5   As is appreciated by the skilled artisan, the amino acid sequences of the proteins are presented as non-limiting examples in support of embodiments which extend to variations (e.g., point mutations) in the genetic sequence that otherwise provide consistent, interchangeable or equivalent results.

           FIG. 1B shows Enzyme-Linked Immunosorbent Assay (ELISA) of cAMP (top),

10   cGMP (middle), and IP$_1$ (bottom; a degradation product of IP$_3$) of cells transfected with either nothing, optoGs, or optoGq, consistent with an example embodiment of the present invention. The results of FIG. 1B were obtained from cells that were stimulated with 504 nm light (20 nm bandwidth) for one minute per spot or kept in the dark, as indicated.

           Stimulation was implemented using an environment-controlled inverted culture

15   microscope (Leica DMI6000B). In the cAMP assay, some cells were treated with 10uM forskolin for 30 minutes as a saturating, positive control of the assay. OptoGs significantly increased cAMP levels in response to light. No significant baseline increase of cAMP, or deviations of cGMP or IP$_3$ levels with optoGs were found. OptoGq significantly increased IP3 levels in response to light without significantly altering cGMP

20   levels. An increase in cAMP levels with IP$_3$ production is believed to be a consequence of intracellular Ca^{2+} release.

           FIG. 1C shows Ca-imaging of cells transfected with mCherry fusion proteins of optoGs and optoGq, consistent with example embodiments of the present invention. To detect cAMP, a cAMP-selective mutant of the cyclic nucleotide gated Ca^{2+} channel

25   CNGA2 was transfected in excess of optoGs. IP$_3$ activates release of intracellular Ca^{2+} stores, thereby providing a reliable signal of Gq activation. A control population was also transfected with mCherry alone with the mutant CNGA2 in excess. Cells were loaded with fura-2 (20-25 minute incubation) and 2 ms exposures of 340 nm and 380 nm were acquired every two seconds. In each of optoGs and optoGq the acquisitions alone were

30   sufficient to yield a Ca signal, while no significant signal was detected in the control population.

           FIG. 1 shows data obtained from a specific experimental setup, however, the invention is not so limited. For example, various deliver techniques other than

transfecting are contemplated including, but not limited to, viral transduction, ballistic gene delivery (gene gun), and spontaneous nucleic acid uptake.

The base-rhodopsin can be modified for use with any suitable heterologous receptor subunits, such as Gi- coupled receptors like the alpha2-adrenergic receptor or the dopamine D2 receptor or the serotonin 5HT2A receptor; or other Gs- or Gq-coupled receptors like the dopamine D1A receptor or the metabotropic glutamate receptors.

According to one example embodiment, the base-rhodopsin is a protein derived from the bovine *Bos taurus*.

According to one embodiment the base-protein other than the base-rhodopsin mentioned above can also be used and includes various 7-transmembrane proteins, such as the cone opsins (red, green, or blue), rhodopsins of other species, and ligand-gated receptors like the dopamine or serotonin receptors.

Various implementations relate to *in vivo* applications in mammals. These implementations include, but are not limited to, testing and confirming neural circuit and disease models.

FIGs. 3A and 3B show experimental data from an *in vivo* application of optoGs (opto-β_2AR) and optoGq (opto-α_1AR), which are two examples of light-activated inducers of secondary messenger signaling. Aspects of the present invention relate to the use and development of a versatile family of genetically encoded optical tools ('optoXRs') that leverage common structure–function relationships among G-protein-coupled receptors (GPCRs) to recruit and control, with high spatiotemporal precision, receptor-initiated biochemical signaling pathways.

The results shown in FIGs. 3A and 3B relate to two specific optoXRs that selectively recruit distinct, targeted signaling pathways in response to light. The two optoXRs exerted opposing effects on spike firing in nucleus accumbens in vivo, and precisely timed optoXR photostimulation in nucleus accumbens by itself sufficed to drive conditioned place preference in freely moving mice. The optoXR approach allows testing of hypotheses regarding the causal impact of biochemical signaling in behaving mammals, in a targetable and temporally precise manner.

Optical control over intracellular signaling was implemented in mammals, using shared structure–function relationships among GPCRs to develop and express in vivo multiple distinct opsin/GPCR2 chimeras with novel transduction logic that couples signal to effector. Consistent with various implementations, one or more chimeric opsin-

receptor proteins are engineered to be functional within mammals *in vivo*, targetable to specific cells, and responsive to precisely timed light pulses. Such approaches allow for the use of high-speed optical stimulus (and protein response) to test for and characterize intracellular biochemical events at precisely-defined and behaviorally-relevant times. A few non-limiting example implementations include, pulsatile versus tonic modulation, synchrony between different modulatory systems, and other fundamental physiological and pathological processes in defined cell types over a range of timescales.

Mammalian implementations have been successfully implemented. In one example implementation, the intracellular loops of rhodopsin were replaced with those of specific adrenergic receptors by first aligning conserved residues of the Gq-coupled human α_{1a} adrenergic receptor (α_1AR) and the Gs-coupled hamster β_2-adrenergic receptor (β_2AR) with the Gt-coupled bovine rhodopsin (FIG. 1A). Exchanges of intracellular regions (including carboxy-terminal domains) were engineered for each receptor based on structural models to transfer G-protein coupling from Gt, and optimized each receptor for *in vivo* expression in mammals. Upon activation by varied ligands, the native receptors can explore multiple ensemble states to recruit canonical and non-canonical pathways in a ligand-biased signaling phenomenon. The optoXRs are likely to select a single active ensemble state upon sensing light in a manner dependent on biological context.

Genes encoding chimeras (opto-α_1AR and optoβ_2AR) were fused to a fluorescent protein. Validation of functional optoXR expression, was accomplished through imaged $[Ca^{2+}]_i$ (intracellular calcium concentration) in HEK cells transfected with opto-α_1AR alone (expected to recruit$[Ca^{2+}]_i$ via Gq), or with both opto-β_2AR (expected to recruit cyclic AMP via Gs) and the cAMP-gated Ca^{2+} channel CNGA2-C460W/E583M. Ratiometric $[Ca^{2+}]_i$ imaging demonstrated that 60 s of green light stimulation (504 +/- 6 nm, 7 mW mm^{-2}) was sufficient to drive prominent $[Ca^{2+}]_i$ signals downstream of either optoXR but not in control conditions (FIG. 2), revealing functional expression. To test specificity of the signaling controlled by each optoXR, transduced HEK cells were illuminated with 3 mW mm^{-2} 504 +/- 6 nm light for 60 s and then lysed and analyzed for levels of cGMP, cAMP and IP_1 (a degradation product of IP_3) via immunoassays. The canonical pattern was as expected for opto-β_2AR corresponding to its molecular design, as optical stimulation yielded significant production of cAMP in opto-β_2AR-expressing cells (FIG. 3A, top), comparable to that achieved with pharmacological stimulation of the wild-type β_2AR and without recruitment of IP_3 (FIG. 3A, middle), $[Ca^{2+}]_i$ (FIG. 2), or

substantial dark activity. In contrast, optical stimulation yielded significant upregulation of IP_3 signaling in opto-α_1AR-expressing cells (FIG. 3A, middle), comparable to levels induced by pharmacological stimulation of the wild-type α_1AR. Together with the $[Ca^{2+}]_i$ elevations (FIG. 2), these data reveal the pattern expected for Gq recruitment, a pattern

5   not seen in opto-β_2AR-expressing cells (FIG. 3A, top). Optical stimulation of cells expressing either construct was unable to modulate cGMP levels (FIG. 3A, bottom), further indicating the signaling specificity of the chimeric proteins. Similar assays revealed that the optoXRs retain an action spectrum close to that of native rhodopsin, are able to integrate signals over a range of biologically suitable light fluxes, and can activate

10   non-canonical pathways to a similar extent as wild-type receptors, as for p42/p44-MAPK signaling.

        OptoXR performance in intact neural tissue has been tested, including whether or not supplementation of retinal cofactors was necessary. In one such test, lentiviral vectors carrying the optoXR fusion genes under control of the synapsin-I promoter (to

15   target biochemical modulation to local neurons rather than other potentially Gs/Gq-responsive cellular tissue elements such as glia and endothelial cells; FIG. 3B, top left) were stereotactically injected into the nucleus accumbens of adult mice. This strategy targets biochemical modulation to neurons with somatodendritic compartments in accumbens (~95%GABAergic medium spiny neurons, without further subtype

20   specificity; FIG. 3B, left) and excludes fibers of passage or afferent presynaptic terminals as these lentiviruses do not transduce cells via axons. Two weeks after transduction, acute coronal slices of accumbens were prepared in artificial cerebrospinal fluid, optically stimulated for 10 min, and immediately fixed and stained for Ser 133-phosphorylated CREB (pCREB), a biochemical integrator of both cAMP and Ca^{2+}-coupled signaling

25   cascades. Without supplementation of exogenous retinoids, significantly elevated pCREB was observed in the optoXR-expressing populations (FIG. 3B, right) and not in non-illuminated tissue.

        The functional consequences of optoXR activation on accumbens local electrical activity was determined by recording multi-unit *in vivo* neuronal firing with an optrode

30   targeted to transduced accumbens (FIG. 4A). No significant differences in baseline firing rates were observed in the dark with either construct (FIG. 4A, bottom right). Optical stimulation resulted in decreased network firing in opto-β_2AR-expressing accumbens (left trace in FIG. 4B illustrates effect kinetics; summary data shown in FIG. 4C and 4D

respectively), in agreement with previous pharmacological studies targeting Gs. Optical

stimulation increased firing in opto-α_1AR-expressing accumbens (FIG. 4B right; FIG. 4C,

4D). Spike frequency histograms showed that the kinetics of optoXR effects on firing

rates was consistent with biochemical rather than electrical initiation of the signal

5   (FIG. 4D). These electrophysiological data, in combination with the earlier biochemical

validations, support that optoXRs can be functionally expressed *in vivo*, to permit

differential photoactivatable control of intracellular cascades and to modulate network

physiology.

In one implementation, optogenetics were used to assess the ability of precisely

10   timed optoXR stimulation to modulate behavior in freely moving mice. Portable solid-

state light delivery was combined with transgenic expression of optoXRs to optically

control intracellular signaling within accumbens neurons in the temporally precise

manner used for operant behavior (FIG. 5A). Confocal analysis revealed expression to be

limited to local accumbens neurons; in particular no labeling was observed in afferent

15   fibers, in distant regions projecting to accumbens, in glia, or in surrounding regions.

Optical stimulation was targeted to transduced accumbens as part of a three-day operant

conditioned place preference assay (FIG. 5A). On each day of the test, animals were

allowed to freely explore the place preference apparatus (FIG. 5A, bottom). On day 1,

animals freely explored the apparatus without optical stimulation. On day 2, whenever

20   the animal freely entered the designated conditioned chamber, a laser-diode-coupled

optical fiber registered to the transduced region delivered light pulses at 10 Hz to

approximate the likely intensity of monoaminergic input during strong reward. Path

tracing revealed that the flexible optical fiber approach allowed full and unimpeded

exploration of all chambers (FIG. 5A, bottom). On day 3, animals again freely explored

25   the apparatus without optical stimulation, and the time spent in the conditioned chamber

was quantified by two independent, blinded scorers. Notably, animals expressing opto-

α_1AR showed a robust increase in preference for the conditioned side of the apparatus

following optical stimulation (FIG. 5B). This effect of temporally precise biochemical

modulation was reproducible across two separate cohorts of opto-α_1AR animals ($n=5-6$,

30   $P<0.05$, Student's t-test for each cohort for time in conditioned chamber; $n=11$, $P<0.01$

for the total population), whereas the other opsin genes, opto-β_2AR and ChR2, appeared

less effective in driving preference. The effect of opto-α_1AR stimulation in accumbens

neurons was specific to reward-related behavior and did not extend to direct modulation

of anxiety-related behaviors or locomotor activity, as identical optical stimulation delivered to a cohort of the same animals in an open field test revealed no significant effect on distance travelled or preference for wall proximity (FIG. 5C).

A specific and non-limiting implementation that is consistent with the above experiments is now described. *In vivo* recording and analysis was performed using optrodes consisting of a multi-mode optical fiber 200 mm in diameter (Thorlabs) coupled to a recording electrode (1MV tungsten, A-M Systems) with an electrode/fiber tip-to-tip distance of 200–400 mm were lowered into the transduced accumbens (electrode tip 4.8–5.2mm below bregma) of mice placed in a stereotactic frame (David Kopf Instruments) and anaesthetized under isoflurane. Light from a 473nm diode laser (CrystaLaser) was delivered through the fiber. Electrical signals were bandpass filtered and amplified (0.3–1 kHz, 1800 Microelectrode AC Amplifier, A-M Systems) and analyzed with pClamp 10.0 (Molecular Devices). Spikes were detected by threshold and individually confirmed by inspection.

Behavioral analysis was performed using optical stimulation that was applied through an optical fiber (200 mm diameter, Thor Labs) coupled to a 473 nm blue diode laser (CrystaLaser) and registered with a cannula targeting accumbens (0–100 mm from tip). Light was delivered with 50 ms pulse width for optoXRs via a function generator (Agilent 33220A). Place preference was conducted in a standard apparatus (SD Instruments) with walls between chambers removed to permit free exploration. Data were analyzed from video for amount of time spent in each chamber by two independent, blinded observers using a custom tallying script run in MATLAB (Mathworks). For open field tests, animals were placed in a square open field measuring 40340 cm; light stimulation was delivered with the same parameters as for place preference experiments. Videos were analyzed using automated software (Viewpoint), for total time and distance in the central 15315 cm square versus the outer annulus (remainder of the field).

Statistical analysis, where indicated, was performed using two-tailed Student's t-tests (calculated in Microsoft Excel) or one-way ANOVA with Tukey post-hoc tests (GraphPad Prism) were used. All summary bar graphs are presented as mean +/- s.e.m., with significance denoted as follows: $*P<0.05$, $**P<0.01$, $***P<0.001$.

Further details supporting the surprising results and effectiveness of various embodiments of the present invention can be found in *Temporally precise in vivo control*

of intracellular signalling, Raag D. Airan, *et al.*, *Nature* 458, 1025-1029 (23 April 2009), which is fully incorporated herein by reference.

The following description provides details for specific and non-limiting method that is consistent with an embodiment of the present invention. Numerous variations of this methodology are envisioned and within the scope of the present invention.

Vector construction

Mammalian codon optimized sequences of opto-α_1AR and opto-β_2AR (amino acid sequences in FIG. 1A) were synthesized and cloned into pcDNA3.1, and fused to the N-terminus of mCherry or YFP (with its start codon deleted) using the NotI site. The linker between the optoXR and mCherry/YFP is 5' GCGGCCGCC 3'. Lentiviral vectors containing Synapsin I optoXR mCherry were constructed by cloning the transgene for each optoXR mCherry into the AgeI and EcoRI sites of the pLenti SynapsinI hChR2 mCherry WPRE vector.

Lentiviral Production

High titer lentivirus was produced. Briefly, HEK 293FT cells were plated to 90% confluence in a 4-layer cell factory (Nunc) cultured with DMEM containing 10% FBS. Cells were co-transfected with 690 μg of the lentiviral vector described above and two helper plasmids (690 μg of pΔCMVR8.74 and 460 μg of pMD2.G). Media was changed at 15 h post transfection. At 24 h post transfection, media was changed with 200-220 mL of serum free UltraCULTURE (Cambrex) containing 5 mM sodium butyrate. At 40 h post transfection, the culture supernatant, now containing viruses, was spun at 1000 rpm for 5 min to remove cellular debris and then filtered using a 0.45 μm low-protein-binding filter flask. The clarified supernatant was then ultra centrifuged for 2 h at 55,000g using an SW 28 rotor (Beckman) to precipitate the virus. After centrifugation, supernatant was discarded and the resultant viral pellet was dissolved in a total of 100 μL of cold (4°C) PBS. The resuspended virus was centrifuged for 5 min at 7000 rpm to remove remaining cellular and viral debris. Aliquots were frozen at -80°C until further use.

Animal surgery and behavior

Female C57BL/6 mice, 10-12 weeks old, were housed and handled according to the Laboratory Vertebrate Animals protocol of Stanford University. Virus solution was delivered to the right nucleus accumbens as follows. Animals were anaesthetized under isoflurane and fur was sheared from the top of the head. While under isoflurane anesthesia, the head of the animal was placed in a stereotactic frame (David Kopf

Instruments). A midline scalp incision was made and a ~1 mm diameter craniotomy was

drilled 1.10mm anterior, and 1.45 mm lateral to bregma. A beveled 33 gauge needle

(NanoFil, World Precision Instruments) pre-loaded with virus was then lowered into the

accumbens (needle tip at 4.70-4.80 mm ventral to bregma) and 1.0 μL of virus was

5   injected at 100 nL/min using an automated syringe pump (NanoFil, World Precision

Instruments). Following injection, 3-5 min was allowed for tissue relaxation and fluid

diffusion before retraction of the needle. For animals targeted for acute slice or in vivo

recording experiments, the craniotomy was filled with dental cement (Lang Dental) and

the incision was closed using VetBond (3M). For animals targeted for behavioral

10   analysis, cannulas (C316G, cut 4.5 mm below the pedestal; PlasticsOne) were placed with

the pedestal flush to the skull. Cannulae were secured using Metabond (Parkell) and

dental cement (Lang Dental). Following drying of VetBond or cement, animals were

removed from the frame and allowed to recover for at least one week before further

manipulation. Control animals for behavioral experiments underwent the same

15   manipulations (surgery, cannula implantation, light stimulation) as experimental animals,

and were injected with vehicle (PBS) alone instead of virus. For place preference

experiments, animals that did not show a baseline preference for either side chamber

(>70% or <10%) or for the central chamber (>40%) were admitted into the study; >90%

of all animals met these criteria for an unbiased, balanced place preference design.

20   *Acute slice preparation*

Animals were anaesthetized under isoflurane and decapitated using surgical shears

(Fine Science Tools). Coronal, 275 μm-thick slices containing accumbens were cut and

stored in a cutting solution containing 64mM NaCl, 2.5mM KCl, 1.25mM NaH_2PO_4,

25mM $NaHCO_3$, 10mM glucose, 120mM sucrose, 0.5mM $CaCl_2$ and 7mM $MgCl_2$

25   (equilibrated with 95% O_2/5% CO_2). Following slicing, slices were incubated in the

cutting solution at 32-35°C for 30 min and then at room temperature until

experimentation. For *ex vivo* optoXR stimulation, slices were loaded on the stage of an

upright microscope (BX51W, Olympus) and perfused with an artificial cerebrospinal

fluid containing 124mM NaCl, 3mM KCl, 1.25mM NaH_2PO_4, 26mM $NaHCO_3$, 10mM

30   glucose, 2.4mM $CaCl_2$, and 1.3mM $MgCl_2$ (equilibrated with 95% O_2/5% CO_2). Light

from a 300W Lambda DG-4 (Sutter) was passed through a 473 nm ± 20 nm bandpass

filter (Semrock) and applied to the slices using a 4X objective (0.28 NA) for 10 min

followed immediately by fixation for later analysis.

Signaling validation assays

HEK293FT cells (Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) in 24 well plates and changed to serum-free medium 4-6 hrs post-transfection. For Ca^{2+} imaging, cells plated on matrigel-coated coverslips were loaded

5   with 5μg/ml fura-2 AM in F-127 Pluronic/DMSO (Probes) in Tyrode containing 1μM ATR, at 37°C and 5% atmospheric CO_2 for 20-25 min. Following loading, coverslips were imaged at 340nm/380nm on an Olympus BX51W using Metafluor (Axon Instruments) controlling a 300W Lambda DG-4 (Sutter). For immunoassays, 18-24 hrs after transfection, 1μM ATR and 50mM LiCl (to prevent IP_1 degradation) were added and

10   plates transferred to an environmentally-controlled microscope (Leica DMI6000; 37°C, 5% atmospheric CO_2). 5 regions/well were optically stimulated for 1 min each (Sutter 300W Lambda DG-4; Semrock 504/12nm bandpass filter; 10X 0.30 NA objective); 3 wells/condition. Following incubation (cAMP/cGMP: 20 min; IP_1: 1 hr), cells were lysed and analyze by HTRF (CisBio) and a Biotek Synergy4 reader.

15   *Immunohistochemistry and confocal analysis*

Following *in vivo* stimulation, mice were transcardially perfused with ice-cold 4% paraformaldehyde (PFA) in PBS (pH 7.4) 90 min after termination of stimulation. Brains were removed and fixed overnight in 4% PFA and then equilibrated in 30% sucrose in PBS. Coronal, 40 μm-thick sections were cut on a freezing microtome and stored in

20   cryoprotectant at 4°C until processed for immunohistochemistry. Free-floating sections were washed in PBS and then incubated for 30 min in 0.3% Tx100 and 3% normal donkey serum (NDS). For acute slice experiments, immediately following stimulation the 275 μm-thick slices were fixed for 1 hr in ice-cold 4% PFA and incubated with 0.5% Tx100 and 3% NDS. For MAPK assays, immediately following HEK293 cell

25   stimulation, coverslips were fixed for 15 min, incubated with 0.6% H2O2 and then permeabilized with 0.1% Tx100 in 3% NDS. Primary antibody incubations were conducted overnight in 0.01% Tx100 and 3% NDS for mouse anti-GAD67 1:500, Millipore, Billerica, MA; rabbit anti-cfos 1:500, Calbiochem, San Diego, CA; rabbit anti-phospho-CREB Ser133 1:500, Millipore. Sections were washed and incubated with

30   secondary antibodies (1:1000) conjugated to either FITC or Cy5 (Jackson Laboratories, West Grove, PA) for 3 hrs at room temperature. Following 20 min incubation with DAPI (1:50,000) sections were washed and mounted on microscope slides with PVD-DABCO. The remaining overnight primary antibody incubations (rabbit anti-phosphoErk1/2; anti-

phospho-MAPK p38 1:500, Promega, Madison, WI; mouse monoclonal anti-dopamine

D1 receptor 1:50, Chemicon; rabbit polyclonal anti-dopamine D2 receptor 1:50,

Millipore; goat polyclonal anti-choline acetyltransferase 1:200, Millipore) were followed

by incubation with biotinylated secondary antibody (1:500, Jackson Laboratories), avidin-

biotin-horseradish peroxidase treatment (ABC kit, Vector Labs, Burlingame, CA), and

TSA detection (Perkin Elmer, Shelton, CT) according to manufacturer's instructions.

Confocal fluorescence images were acquired on a Leica TCS SP5 scanning laser

microscope using a 20X/0.70NA or a 40X/1.25NA oil immersion objective. Four serial

stack images per condition were acquired within a 500 μm region beneath the cannula

tract. DAPI staining was used to delineate nuclei for determination of the mean pixel

intensity of cfos or pCREB immunoreactivity using Volocity (Improvision) software.

Positive or pCREB-active cells were identified by intensity threshold, and image

acquisition and analysis were performed blind to the experimental conditions.

Table S1

Raw numerical pCREB intensities (au) for data represented in FIG. 3B. Mean and

SEM in bold for each subgroup; p-values for two-tailed t-test of subgroup versus control

in italics.

	opto-α_1AR		opto-β_2AR	
mCherry	-	+	-	+
Mean	65.326	97.95309	63.6385	82.83284
SEM	3.758281	7.199024	3.847409	6.907057
p-value vs. mCherry-		*0.000272*		*0.019559*

Table S2

Raw numerical baseline firing rates (Hz) for data presented in FIG. 4A. Mean and

SEM in bold for each subgroup; p-values for t-test of subgroup versus control in italics.

	XFP	oα_1AR	oβ_2AR
Mean	2.596154	2.439357	2.687798
SEM	0.436406	0.603845	0.346556
p-value vs XFP		*0.834496*	*0.869791*

Table S3

Raw numerical changes in firing rate (Hz) for data presented in FIG. 4C

calculated within the baseline itself ('Base') and between the baseline and the light

stimulation periods ('Light').

	opto-β_2AR		Opto-α_1AR	
	Base	Light	Base	Light
Mean	0.061788	-0.68113	-0.01287	3.816198
SEM	0.134665	0.162402	0.336387	0.812251
p-value vs Base		*0.000861*		*0.000239*

5

Accordingly, embodiments of the present invention relate to optogenetic control

of intracellular signaling and are useful for temporally precision while operating *in vivo*

within behaving mammals, while displaying extremely low dark activity, and recruiting

the complex fabric of multiple signaling molecules downstream of native receptors,

10   thereby unifying in a single technology many of the individual positive aspects of other

approaches. Similar embodiments directly probe the causal significance of seven-

transmembrane-dependent signaling pathways triggered by other modulators, including

myriad neurotransmitters and endocrine hormones. Other embodiments use an optoXR

approach in ways that extend beyond excitable cells to capitalize upon the versatile

15   integration of fiber-optic depth targeting with optogenetically targeted photosensitivity.

One such embodiment relates to probing causal significance of temporally precise

biochemical signaling in diverse non-excitable tissues.

Embodiments of the present invention relate to considerations of the phenomenon

of ligand-biased signaling, wherein varied ligands can stabilize ensemble receptor

20   conformational states and thereby bias the intracellular action of the receptor in coupling

to alternative transduction cascades. The optoXRs are used to induce these alternative

cascades to similar levels as with pharmacological manipulation (for example, opto-β_2AR

can induce similar changes in MAPK activation compared with native ligand acting on

the wild-type β_2AR); however, individual optoXRs may not always be found to permit

25   control of all of the conformational states that contribute to ligand biased signaling.

Retinal-based tools can be particularly useful due to the presence of the endogenous

chromophore in mammalian tissues, and the extremely low activity in the dark.

Optogenetics can take the form of diverse effectors linked to fast, single-component retinal-binding modules, capitalizing on the temporal precision of optics.

Embodiments of the present invention use optoXR methods to complement microbial opsin strategies, providing another dimension of fast, targetable cellular control operative in behaving mammals.

Consistent with another embodiment of the present invention, wavelength-shifted versions of the optoXRs, based on known opsin genes with different action spectra, are used. Such optoXRs can be particularly useful for providing separable channels of biochemical and electrical control.

Variants of the specific protein sequences discussed herein are consistent with embodiments of the present invention. Some variants are greater than about 75% homologous to these protein sequences, while others are greater than about 80%, 85% or 90%. In some embodiments the homology will be as high as about 93 to about 95 or about 98%. The compositions of the present invention include the protein and nucleic acid sequences provided herein including variants which are more than about 50% homologous to the provided sequence up to and including 100% homologous.

The various embodiments discussed herein could be integrated with fast circuit readout technologies for increasingly sophisticated interrogation and reverse engineering of neural circuitry, both in normal operation and in disease states.

The various embodiments described above are provided by way of illustration only and should not be construed to limit the invention. Based on the above discussion and illustrations, those skilled in the art will readily recognize that various modifications and changes may be made to the present invention without strictly following the exemplary embodiments and applications illustrated and described herein. For instance, such changes may include variations of the secondary messenger produced. Such modifications and changes do not depart from the true spirit and scope of the present invention, which is set forth in the following claims.

What is claimed is:

1.   A method for generating secondary messengers in a cell, the method comprising:

        modifying a nucleotide sequence for expressing a chimeric light responsive rhodopsin-based membrane protein with one or more heterologous receptor subunits; and

        expressing the light responsive membrane protein in a cell for producing a secondary messenger in response to light.

2.   The method of claim 1, wherein the one or more heterologous receptor subunits include an adrenergic receptor.

3.   The method of claim 1, wherein the chimeric light responsive rhodopsin-based membrane protein is a seven transmembrane protein.

4.   The method of claim 1, wherein the secondary messenger is one of cAMP, cyclic guanosine monophosphate (cGMP), inositol trisphosphate/inositol 1,4,5-trisphosphate/triphosphoinositol (IP$_3$) and arachidonic acid.

5.   The method of claim 1, wherein the step of expressing is accomplished *in vivo*.

6.   The method of claim 1, wherein the step of expressing is accomplished *in vitro*.

7.   The method of claim 1, further including the step of optically stimulating the expressed light responsive membrane protein.

8.   A method for assessing the efficacy of a putative treatment regimen relating to intracellular messengers, the method comprising:

        modifying a nucleotide sequence for expressing a chimeric light responsive rhodopsin-based membrane protein with one or more heterologous receptor subunits;

        expressing the light responsive membrane protein in a cell for producing a secondary messenger in response to light;

        exposing the protein to light; and

        assessing the effects of the treatment.

9.   A cell expressing a chimeric light responsive rhodopsin-based membrane protein with one or more heterologous receptor subunits

10.   The cell of claim 9, wherein the one or more heterologous receptor subunits are adrenergic receptors of at least one of alpha1 and Beta2.

10.   A nucleotide sequence for expressing a chimeric light responsive membrane rhodopsin-based protein with one or more heterologous receptor subunits.

11.   A nucleotide sequence according to Seq. Id. No. 1 or Seq. Id. No. 3 and point mutations thereof.

12.   A cell expressing the amino acid according to Seq. Id. No. 2 or Seq. Id No. 4 and point mutations thereof.
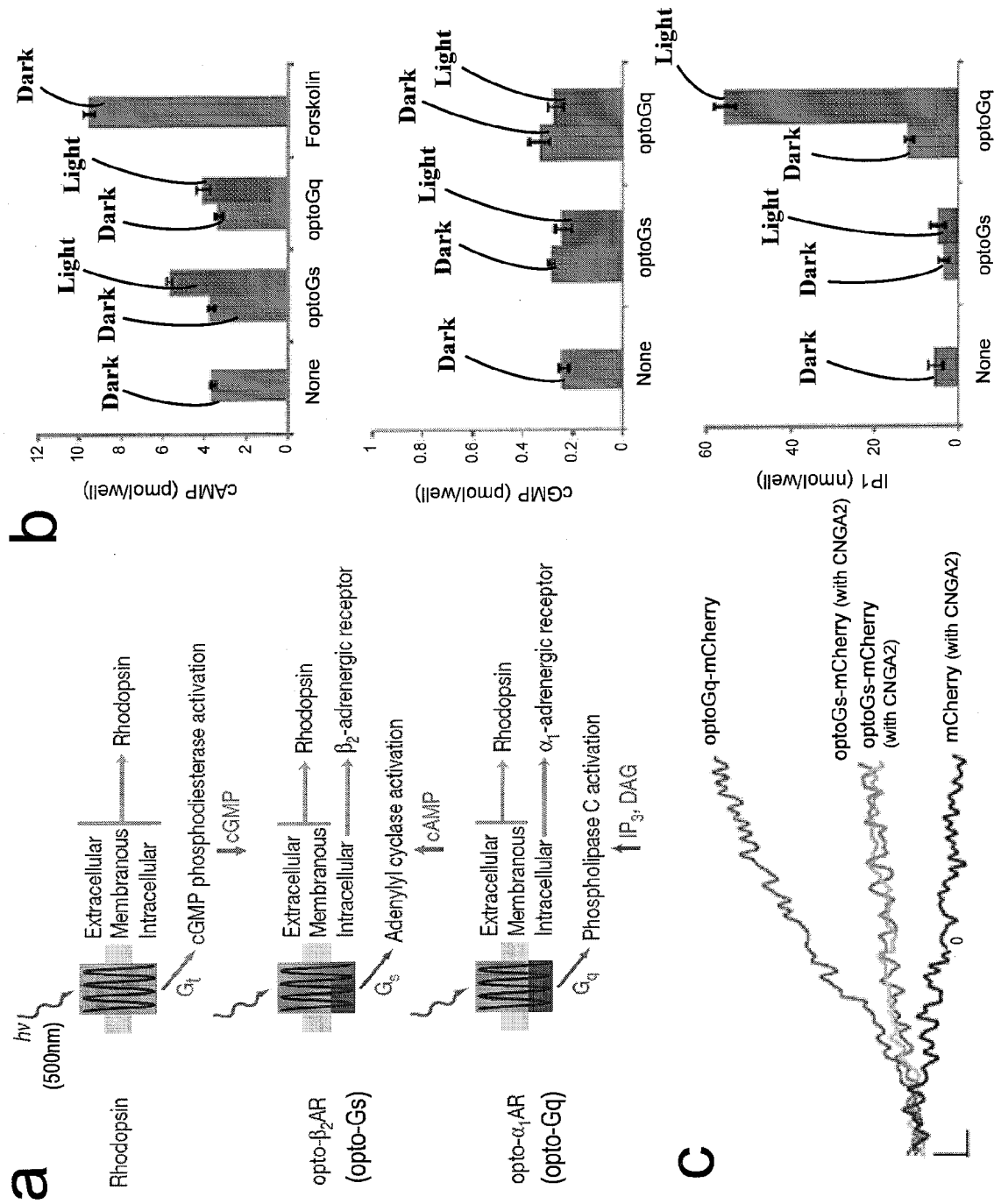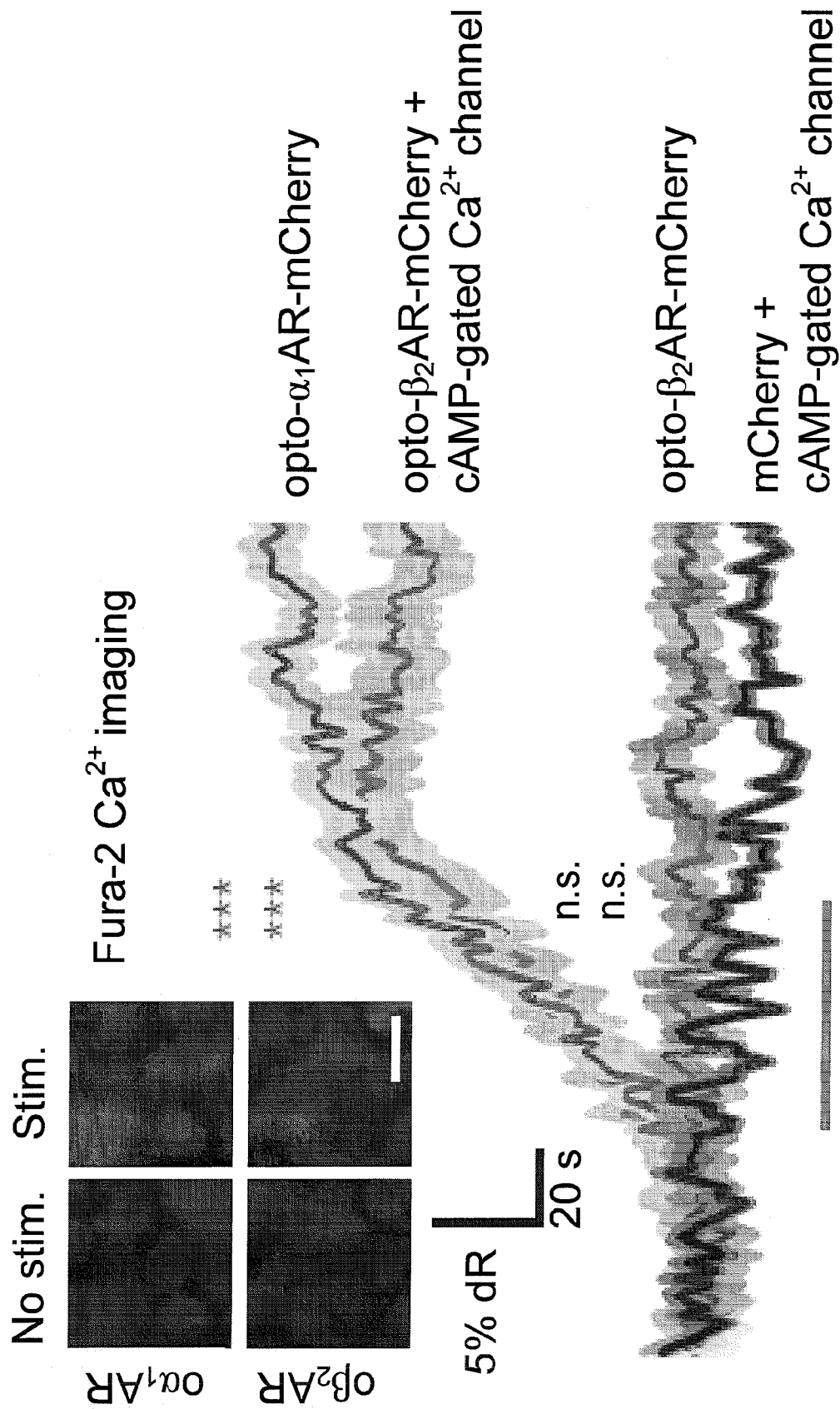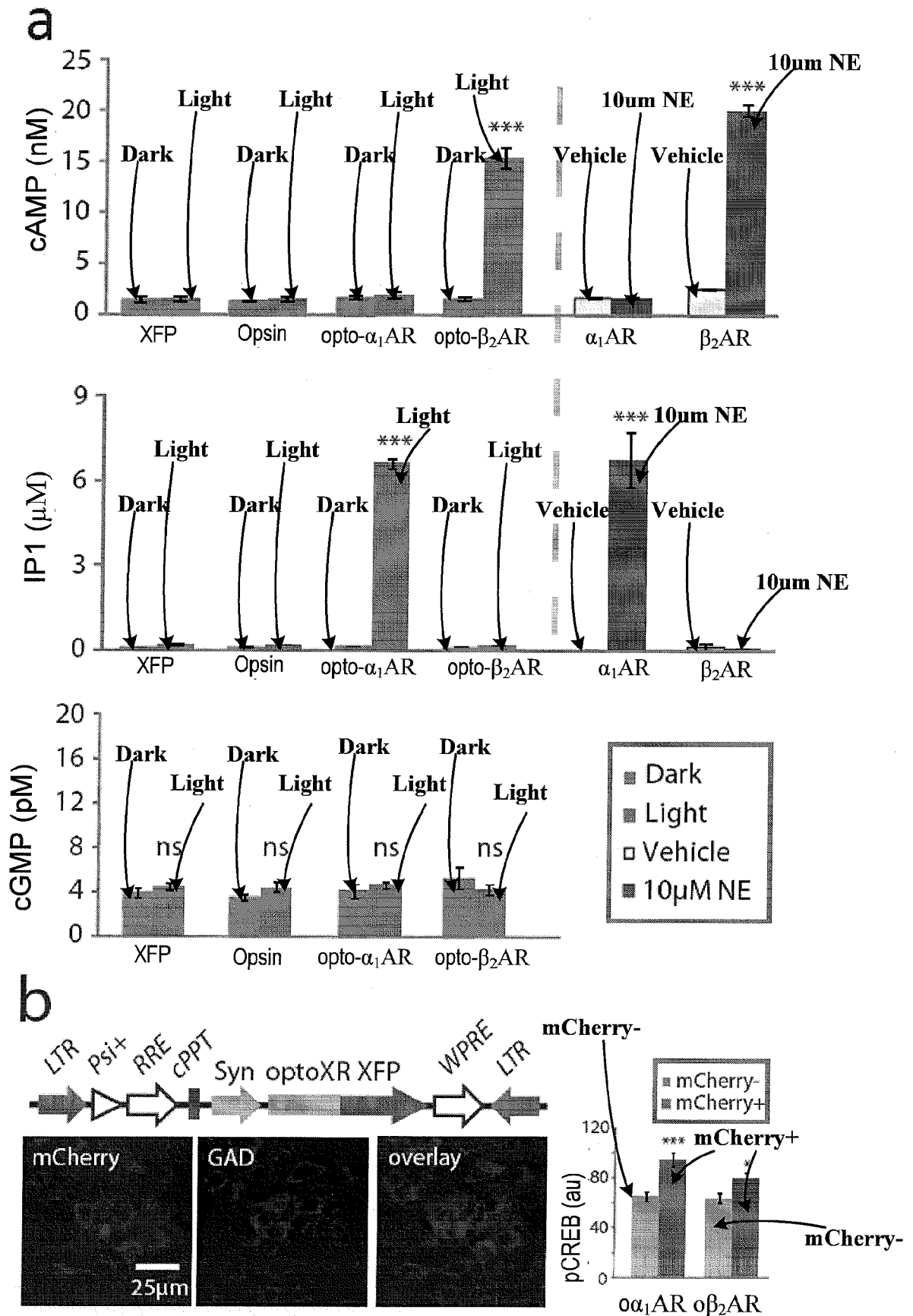
FIG. 1

FIG. 2

FIG. 3

a

473 nm laser diode

Optical fibre

Electrode

Optrode

Transduced accumbens

20 µV | 0.5 ms

XFP oβ$_2$AR oα$_1$AR

b

opto-β$_2$AR

opto-α$_1$AR

20 µV | 5 s

20 µV | 5 s

c

Base
Light

Base

Light

***

***

opto-β$_2$AR opto-α$_1$AR

d

	Delays		Rates (Hz^{-1})	
	t_{on}	t_{off}	τ_{on}	τ_{off}
opto-β$_2$AR	2.105 s	3.010 s	5.366 s	4.270 s
opto-α$_1$AR	125 ms	533 ms	765 ms	590 ms

FIG. 4

FIG. 5