Non-invasive optical control of endogenous Ca\textsuperscript{2+} channels in awake mice

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Optogenetic approaches for controlling Ca\textsuperscript{2+} channels provide powerful means for modulating diverse Ca\textsuperscript{2+}-specific biological events in space and time. However, blue light-responsive photoreceptors are, in principle, considered inadequate for deep tissue stimulation unless accompanied by optic fiber insertion. Here, we present an ultra-light-sensitive optogenetic Ca\textsuperscript{2+} modulator, named monSTIM1 encompassing engineered cryptochrome2 for manipulating Ca\textsuperscript{2+} signaling in the brain of awake mice through non-invasive light delivery. Activation of monSTIM1 in either excitatory neurons or astrocytes of mice brain is able to induce Ca\textsuperscript{2+}-dependent gene expression without any mechanical damage in the brain. Furthermore, we demonstrate that non-invasive Ca\textsuperscript{2+} modulation in neurons can be sufficiently and effectively translated into changes in behavioral phenotypes of awake mice.
The brain utilizes a versatile \( \text{Ca}^{2+} \)-signaling toolkit to regulate manifold functions including memory, emotion, and locomotion \(^1\). Accumulating evidence suggests that abnormally modulated intracellular \( \text{Ca}^{2+} \) dynamics are correlated with brain dysfunctions such as neurodegenerative diseases \(^2\), but their causality and specific contribution of \( \text{Ca}^{2+} \) signaling per se on functional outcomes still remains elusive. Thus, to understand exact roles of neural \( \text{Ca}^{2+} \) signaling in brain functions, it is necessary to specifically control intracellular \( \text{Ca}^{2+} \) dynamics at designed time and space. The ubiquitously expressed \( \text{Ca}^{2+} \)-release-activated \( \text{Ca}^{2+} \) (CRAC) channel is selectively expressed in neurons \(^3\), which were typically constructed from cytosolic domains of STIM1 (Stromal interaction molecule 1) protein, a CRAC channel regulator \(^4\), and blue light-responsive plant photoreceptors, such as cryptochrome2 (CRY2) \(^5\) or the LOV2 (light-oxygen-voltage-sensing) domain \(^6\). These optogenetic tools are able to shape distinguished intracellular \( \text{Ca}^{2+} \) dynamics in response to various inputs of light and effectively evoke \( \text{Ca}^{2+} \)-responsive diverse biological events, including gene expression, cell migration, immune responses, and memory reinforcement. However, it has been found that excessive expression of designed elements alters basal \( \text{Ca}^{2+} \) concentration, even in the dark state \(^7\). In addition, it is presumed that these optogenetic modules inevitably necessitate the use of optic fibers for in vivo brain applications because of the poor tissue-penetration efficiency of blue light \(^8\), Long-term insertion of optic fiber in mice brain also introduces bio-compatibility issues, which induces astrocytic scar formation nearby implanted region \(^9\), thermal damage \(^10\) as well as compatibility issues, which induces astrocytic scar formation characteristics, including minimal alteration of basal \( \text{Ca}^{2+} \) concentration (Fig. 1a). But under excessive expression of the fusion protein, some cells exhibited slight increase of basal \( \text{Ca}^{2+} \) concentration \(^11\), possibly raising concerns of changed cellular contexts regardless of light illumination. We hypothesized that the dimeric nature of both CRY2 \(^12\) and STIM1 \(^13\), would contribute to the multimeric property of OptoSTIM1 without blue light, promoting constitutive \( \text{Ca}^{2+} \) influx through CRAC channels and leading to elevated basal \( \text{Ca}^{2+} \) level. On the basis of previous findings that CRY2 is the main determinant of the kinetic properties of OptoSTIM1 \(^13\), we reasoned that disrupting the dimeric interface of CRY2 would reduce light-independent self-association of OptoSTIM1 and prevent subsequent CRAC channel leakage. To investigate the putative dimerization region of \( A.\ thaliana \) CRY2 (ACRY2), we aligned the amino-acid sequence of ACRY2 with those of relatively well-characterized cryptochromes from other species and predicted its structure based on that of ACRY1 (Supplementary Figs. 1 and 2). Because the protrusion loop (Phe288–Ala306) in \( D.\ cryptochrome \) was reported to stabilize self-association through disulfide bond formation involving Cys296, we first located an equivalent protrusion loop (Lys268–Leu286) in ACRY2 model structure. Then, we selected the five amino acids, N27–SGE281, which potentially makes the close contact between two protrusion loops of ACRY2 (Fig. 1b). We then designed OptoSTIM1 variants containing E279A, E281A, in which the negatively charged glutamic acid residues Glu279 and Glu281 in the protrusion loop were replaced with the neutral amino-acid alanine (E279A and E281A) to reduce their potential electrostatic interaction, or in which the small residue Gly280 was converted into a bulky Tyr residue (G280W) to stabilize seryl dimerization during self-association. OptoSTIM1 variants containing G280W or E281A mutations in ACRY2 exhibited lower basal intracellular \( \text{Ca}^{2+} \) concentrations (\( [\text{Ca}^{2+}]_i \)) and similar maximal \( [\text{Ca}^{2+}]_i \), in response to activation, measured by the ratiometric \( \text{Ca}^{2+} \) indicator, Fura-2, compared with OptoSTIM1 bearing original CRY2 (Fig. 1c). This suggests that Gly280 and Glu281 might be involved in CRY2 self-association in the dark, but not in the light-driven activation of OptoSTIM1. Notably, the correlation between basal \( [\text{Ca}^{2+}]_i \), and protein expression level was lower for OptoSTIM1 carrying either CRY2G280W or CRY2E281A mutants compared with those bearing CRY2 or CRY2E279A (Supplementary Fig. 3c). Moreover, OptoSTIM1(CRY2E281A) produced a higher maximal \( [\text{Ca}^{2+}]_i \), following stimulation compared with other variants, but had activation (\( T_{\text{a}1/2} = 62 \) s) and deactivation (\( T_{\text{d}1/2} = 7 \) min) kinetics similar to those of the original OptoSTIM1. In contrast, OptoSTIM1(CRY2G280W) exhibited lower maximal \( [\text{Ca}^{2+}]_i \), slower activation, and faster deactivation compared with the original OptoSTIM1 (Fig. 1d and Supplementary Fig. 4).

To further enhance the photosensitivity of OptoSTIM1 (CRY2E281A), we employed the previously reported superior CRY2-clustering systems, CRY2oloig (E490G) \(^26\) and CRY2clust (A9) \(^27\), where C-terminal amino-acid sequence of CRY2 is extended with nine residues (ARDPDDLGN). Combining CRY2E281A with either CRY2E490G or A9 minimally affected \( [\text{Ca}^{2+}]_i \), in both dark and light conditions beyond that displayed by OptoSTIM1(CRY2E281A) (Fig. 1c and Supplementary Figs. 3a, b). Interestingly, compared with other variants, OptoSTIM1(CRY2E281A–A9) exhibited much higher sensitivity to light in that ~47% of OptoSTIM1(CRY2E281A–A9)-expressing cells remained effectively responsive to a light intensity of 1 \( \mu \text{W mm}^{-2} \) and showed ~55-fold higher sensitivity than original OptoSTIM1 (Fig. 1e–g and Supplementary Figs. 6, 7). In contrast, OptoSTIM1(CRY2E281A–E490G) showed lower responsiveness to light and slower deactivation kinetics. OptoSTIM1 variants showed differential activation and deactivation kinetics in terms of \( [\text{Ca}^{2+}]_i \), dynamics, probably owing to each mutation rendering distinguished clustering characteristic of CRY2. Interestingly, they consistently exhibited faster activation and slower deactivation kinetics upon elevated light density (Supplementary Fig. 8). In these characterizations, we utilized R-GECO1 as an

**Results**

**Engineering ACRY2 to improve properties of OptoSTIM1.** Previously we demonstrated that oligomerization of OptoSTIM1 \(^16\), optogenetic \( \text{Ca}^{2+} \) modulator, through blue light-driven CRY2 homo-association was able to efficiently activate endogenous CRAC channels, in turn elevating intracellular \( \text{Ca}^{2+} \) concentration (Fig. 1a). But under excessive expression of the fusion protein, some cells exhibited slight increase of basal \( \text{Ca}^{2+} \) concentration \(^6\), possibly raising concerns of changed cellular contexts regardless of light illumination. We hypothesized that the dimeric nature of both CRY2 \(^22\) and STIM1\(^23,24\) would
Fig. 1 Development of ultra-light-sensitive OptoSTIM1. 

a Schematic representation of working mechanism of the OptoSTIM1. PM, plasma membrane; STIM1ct, C-terminal fragment of STIM1 (a.a 238–685).

b Model structure of AtCRY2 predicted by SWISS-MODEL. Expanded view on the right represents loop structure in the potential dimeric interface highlighted in black box on the crystal structure; amino acids for the target region for mutagenesis are aligned. Reds indicate specific residues mutagenized.

c Graph showing correlation between [Ca\(^{2+}\)]\(_i\) in dark (x axis) and [Ca\(^{2+}\)]\(_i\) upon light stimulation (y axis) measured using Fura-2.

d Plot representing correlation between half-maximal time point for reaching saturated R-GECO1 level upon light stimulation (x axis) and basal R-GECO1 level in dark (y axis) for each indicated variant (n ≥ 100 for each variant).

e Graph showing maximal R-GECO1 fluorescence intensity based on varying light density (1–50 µW mm\(^{-2}\)). Blue light was delivered at each indicated power density at 5-second intervals for 1 minute using a 488 nm laser (n ≥ 100 for each variant at each light density).

f Representative R-GECO1 images of cells expressing either OptoSTIM1(CRY2) or OptoSTIM1(CRY2E281A-A9) (488 nm, 1–100 µW mm\(^{-2}\), 5-second intervals for 1 minute). Graphs on the right indicate fluorescence change of R-GECO1 upon light stimulation (Black, CRY2; Blue, CRY2E281A-A9). Scale bar, 10 µm.

g Heat plot showing extent of reactive cell population upon light stimulation with each indicated light density. Images and quantified data are representative of multiple experiments (n > 3). Data represent means ± s.e.m.
intensiometric Ca\(^{2+}\) biosensor. As the previous study showed that R-GECO1 itself can be activated by blue light and elicit change of fluorescence\(^{28}\), we examined if there is any unintended additive effect by R-GECO1 on our quantified results. Notably, HeLa cells solely expressing R-GECO1, showed subtle change (<3\%) of fluorescence intensity upon continuous or transient exposure to blue light, which is much smaller than typical dynamic range (>600\%) of fluorescence change we observe throughout our optogenetic Ca\(^{2+}\) modulators (Supplementary Fig. 10d). Therefore, this result indicates that photoactivation of R-GECO1 had a negligible effect on our experimental condition. From all the results above, we conclude that OptoSTIM1(CRY2\(^{E281A}\)-A9) showed performance and characteristics closest to the goal of our CRY2 engineering effort, exhibiting lower basal [Ca\(^{2+}\)], regardless of expression level with superior light responsiveness for effective induction of Ca\(^{2+}\)- influx. We termed this ultra-photosensitive variant OptoSTIM1(CRY2\(^{E281A}\)-A9) as monster-OptoSTIM1, abbreviated as monSTIM1.

### Characteristics of monSTIM1

As previously described, fundamental properties of OptoSTIM1 include plasma membrane translocation upon light stimulation and possessing a specific window of photoactivatable light spectrum\(^{6}\) (400–500 nm) for compatible use with red-shifted biosensors. To verify whether monSTIM1 retains these properties, first, we closely examined the change of subcellular distribution of monSTIM1 during the process of light irradiation. By utilizing plasma membrane marker protein, iRFP670-PM(KRas4B tail), we clearly show translocation of light-activated monSTIM1 to the plasma membrane (Supplementary Fig. 10a–c). Second, to investigate light spectrum for monSTIM1 activation, we stimulated monSTIM1-expressing cells with different wavelengths of light. We observed that monSTIM1 efficiently responds to 457 or 488 nm light, but weakly or does not respond to the 405 nm and wavelength of light longer than 514 nm (Supplementary Fig. 10d) equivalent to what OptoSTIM1 has previously shown. Among distinguished features between OptoSTIM1 and monSTIM1, next we particularly focused on to address what drives elevated basal [Ca\(^{2+}\)] by OptoSTIM1 compared with that of monSTIM1, which might change the physiological context of cells under overexpressed condition. We hypothesized that dimeric feature of both SOAR (a.a 336–485) domain in the C-terminus of STIM1 and CRY2 would contribute to induction of higher oligomeric state of OptoSTIM1 in the absence of blue light and, in turn, basal [Ca\(^{2+}\)] increment, and CRY2\(^{E281A}\) mutation on monSTIM1 would reduce the degree of oligomeric state in the dark. To address whether CRY2\(^{E281A}\) mutation indeed results in the change of basal oligomeric state, we compared homo-association property of OptoSTIM1 and monSTIM1 by utilizing InCell SMART-i (Intracellular supra-molecular assembly readout trap for interactions)\(^{29}\), which readily assesses protein interactions in the form of cluster formation (Supplementary Fig. 11a). We monitored cluster formation upon rapamycin treatment in HeLa cells co-expressing FKBP-V14H (GFP), FRB-mScarlet-Ft (Ferritin) with EGFP-STIM1ct (a.a 238–685), OptoSTIM1 (EGFP-CRY2-STIM1ct), or monSTIM1 (EGFP-CRY2\(^{E281A}\)-A9-STIM1ct) both in the dark and light. Relatively smaller cell population with cluster formation was visualized with cells expressing either EGFP-STIM1ct (14.2\%) or monSTIM1 (11\%), whereas 39.2\% of OptoSTIM1-expressing cell population showed cluster formation (Supplementary Fig. 11b, c). Light-stimulated cells expressing either OptoSTIM1 or monSTIM1 showed robust and comparable level of cluster formation inside the cells, owing to homo-oligomerization of CRY2. This result suggests that CRY2\(^{E281A}\) would have less-oligomeric property than CRY2 in the absence of blue light, thereby significantly attenuating elevated basal [Ca\(^{2+}\)] even under over-expressed condition.

### Utilizing CRY2\(^{E281A}\)-A9 to regulate receptor tyrosine kinase

In order to examine applicability of the light-sensitive CRY2\(^{E281A}\), A9 module to other CRY2-based optogenetic tools, we employed OptoFGFR1 (optical activation of fibroblast growth factor receptor 1)\(^{30}\) and replaced original CRY2 with CRY2\(^{E281A}\)-A9 (Supplementary Fig. 12a). In this case, we barely detected differences in both basal [Ca\(^{2+}\)], and light-induced maximal [Ca\(^{2+}\)], between cells expressing either OptoFGFR1 or OptoFGFR1(CRY2\(^{E281A}\), A9). We suspect that this phenomenon is due to inherent working mechanism of FGFR1 in elevating [Ca\(^{2+}\)], which triggers transient IP\(_3\)-induced Ca\(^{2+}\) ion release from intracellular stores, such as ER (Supplementary Fig. 12b), where we might have saturated activity of OptoFGFR1 in this particular light-illuminating condition. However, upon titrating exposure time of light, we found that 76\% of cells expressing OptoFGFR1(CRY2\(^{E281A}\), A9) was reactive to 1.5 s exposure of light, whereas only 15\% of cells expressing OptoFGFR1 responded (Supplementary Fig. 12c, d), further demonstrating the versatility of CRY2\(^{E281A}\)-A9 to generate light-sensitive optogenetic module.

### Non-invasive monSTIM1 activation in vivo

This ultra-photosensitivity led us to anticipate that monSTIM1 might be able to drive Ca\(^{2+}\)-dependent molecular activity in neurons of the intact brain in response to non-invasive light illumination in a similar way that we reported previously\(^{31}\). To test this conjecture, we designed a light-illuminating cage in which an LED solid-state array capable of delivering blue light (473 nm) of ~1 mW cm\(^{-2}\) to the head of mice was attached to the cage lid (Fig. 2b and Supplementary Fig. 13). In this condition, we could not find any noticeable change in mice behavior by light illumination per se (Supplementary Movie 1). Then, excitatory neurons of the somatosensory cortex (S1) were transduced with lentiviral constructs of OptoSTIM1, monSTIM1, or light-insensitive OptoSTIM1(CRY2\(^{D387A}\)). After 4 weeks, mice were exposed to blue light for 30 minutes at homecage without removal of hairs and skin, and then killed 60 minutes later to assess for the expression level of c-Fos, a Ca\(^{2+}\)-dependent immediate-early gene (Fig. 2a). Strikingly, mice expressing monSTIM1 showed significant induction of c-Fos expression compared with control groups including mice in ambient room light condition without blue light illumination and mice expressing light-insensitive OptoSTIM1(CRY2\(^{D387A}\)) with blue light exposure for 30 minutes. We found the c-Fos signal was predominantly localized to cells expressing monSTIM1 (Fig. 2c, d). We next sought to validate the functionality of monSTIM1 in astrocytes, another major cell type in the brain. Activation of monSTIM1, expressed under the control of the GfaABC1D promoter, efficiently induced expression of c-Fos in astrocytes. Specifically, c-Fos was detected in 74\% of the monSTIM1-positive astrocyte population in the S1 region, whereas control groups showed no noticeable c-Fos induction. To evaluate the suitability of monSTIM1 for deep-brain modulation, we examined c-Fos induction in monSTIM1-expressing astrocytes in the dentate gyrus (DG) and thalamic (TH) regions of the brain under the same light-stimulation condition we used for cortical stimulation. We found that 57\% (DG) and 44\% (TH) of cell population expressing monSTIM1 showed c-Fos expression upon light stimulation (Fig. 2e, f). The decreased percentages of c-Fos-positive astrocytes in DG and TH compared with that of S1 reflect that penetration efficiency of blue light was gradually reduced as a function of depth in the brain. We also observed that 21.5\% of monSTIM1-positive excitatory neurons in the
hippocampus CA1 region showed c-Fos expression. Therefore, these results demonstrate that monSTIM1 is able to effectively induce intracellular Ca\textsuperscript{2+} signaling in deep-brain regions through non-invasive light activation.

Modulating mice behaviors through monSTIM1. At last, we explored whether induction of Ca\textsuperscript{2+} signaling through non-invasive light delivery impacted specific behaviors of awake mice. We have previously demonstrated that the activity of voltage-dependent L-type Ca\textsuperscript{2+} channels (Ca\textsubscript{a,1.2}, Cav1.2) in the anterior cingulate cortex (ACC) is involved in social fear learning in mice (e.g., observational fear response\textsuperscript{32}). Mice with an ACC-limited deletion of the Ca\textsubscript{a,1.2} gene showed reduced observational fear, likely owing to impaired synaptic transmission or neuronal excitability. In addition, previous studies demonstrated that Ca\textsuperscript{2+} signaling is involved in electrophysiological properties of excitatory neurons of the ACC. Ca\textsuperscript{2+}-stimulated proteins such as Cav1.2, CaM, CaMKIV and AC133 are known to contribute to the expression of immediate-early genes, thereby promoting long-term potentiation in ACC\textsuperscript{34}. However, the causal relationship between direct activation of Ca\textsuperscript{2+} signaling and observational fear has not been tested. Accordingly, we targeted excitatory pyramidal neurons in the ACC with EGFP, monSTIM1 or light-insensitive OptoSTIM1 and examined socially transmitted fear responses (Fig. 3a). In these experiments, only the observer mouse was illuminated for 30 minutes in the homecage and then both observer and demonstrator mice were moved into observational fear behavioral chambers separated by a transparent Plexiglas partition. Mice were habituated to the apparatus for 5 minutes, and repetitive foot shocks were applied only to the demonstrator at 10-second intervals for 4 minutes to evoke vicarious freezing response in the observer (Fig. 3b). Notably,
mice in which monSTIM1 was activated displayed significantly higher freezing levels during the training period (4 minutes). Twenty-four hours later, observers re-exposed to the same conditioning chamber also showed increased contextual fear memory compared with mice in the control groups (Fig. 3c, d), indicating that Ca²⁺ signaling in the ACC contributed to both short- and long-term social fear responses. In accordance with our previous study demonstrating functional lateralization in modulation of observational fear, we also observed lateralized brain functions of Ca²⁺ signals in observational fear, demonstrating that activation of Ca²⁺ signals by monSTIM1 in the right, and not in the left, hemisphere resulted in observational fear response (Fig. 3e, f). Given that activation of monSTIM1 in the ACC did not cause any alterations in locomotor activity or anxiety-like behavior during open-field tests (Supplementary Fig. 14), these results collectively demonstrate that monSTIM1-mediated Ca²⁺ induction in the ACC selectively enhances socially transmitted fear response in mice.

Next we explored whether non-invasive activation of monSTIM1 in the CA1 hippocampus, deeper brain area than the ACC, could still exhibit the functionality to modulate brain functions such as reinforcement of contextual memory as we previously described. Mice expressing monSTIM1 were illuminated with blue light at the homecage and moved to conditioning
chamber to conduct Pavlovian fear conditioning administering an auditory cue (90 dB, 3 kHz for 30 s) with pairing of an electric foot shock (2 s, 0.7 mA) (Fig. 3g). To test memory formation, mice were re-exposed to either the same context (day 2) or the tone (day 3) sequentially. Consistent with our previous finding, monSTIM1-stimulated mice exhibited enhanced context-specific memory compared with non-stimulated mice but no differences in tone memory, demonstrating that monSTIM1 activation through non-invasive light delivery sufficiently modulate specific brain function and corresponding behavioral outcome (Fig. 3h–j).

Discussion

In this study, we present an ultra-light-sensitive optogenetic Ca²⁺ modulator achieved with CRY2 engineering, which includes mutated single amino acid on predicted CRY2 dimeric interface (CRY2E281A) and superior clustering module (CRY2clust (A9)). We demonstrate that high propensity of OptoSTIM1 to achieve advanced oligomeric state in the basal condition through homo-interaction of both CRY2 and STIM1ct drives elevation of resting Ca²⁺ level. We also show that CRY2E281A mutant, when incorporated into OptoSTIM1 in place of CRY2, attenuates increased in a significant manner over the course of 30 min post ligand stimulus in hippocampal excitatory neurons [15], indicating that Ca²⁺-responsive molecular machineries may take timescale of minutes to impose their influences on higher level phenotypes.

Combined with approaches for expressing genes in the brain through systemic delivery of engineered viruses [16] and various transgenic lines for gene recombination, the approach demonstrated here should allow bona fide non-invasive expression and activation of Ca²⁺ signaling in a broad range of cell types, allowing fundamental roles of Ca²⁺ in manifold brain functions to be elucidated.

Methods

Sequence alignment and model building. Sequences of cryptochrome proteins from various species were aligned using the web-based software MultiAlin [17]. The crystal structure of ArCRY2 was constructed by homology modeling using SWISS-MODEL software. ArCRY1 structure (PDB code: 1U3D) was selected as templates for model building based on the calculated Global Model Quality Estimation (GMQE) score (>0.5), and the model with highest Qualitative Model Energy ANalysis-Z (QMEAN-Z, −1.63) was selected as the final ArCRY2 model. This final ArCRY2 model was structurally aligned to Orthosiphon cryptochrome dimer (PDB code: 4K03) to predict the potential dimeric interface of ArCRY2.

Plasmid construction. Construction of expression plasmids for R-GECO1 and OptoSTIM1 were previously described in detail [18]. The OptoSTIM1 used in the current study encompassed the photolyase homology region of CRY2 (amino acids 1–498) and C-terminal of STIM1 (amino acids 238–685), both of which were subjected to mutagenesis through polymerase chain reaction (PCR)-driven overlap extension using forward and reverse mutagenic oligonucleotides used for mutagenesis of CRY2 were as follows: Flanking primers, 5′-GTCGCCGCCCCTCAAGGATACCTGTCG-3′ (forward) and 5′-CTCCGCGCCTCCCCATTGGTGGCAGCAGATCATAATC-3′ (reverse); E279A, 5′-AAACGGCGCGCGCAAGAAGGGCCGCGATGTTCCTG-3′ (forward) and 5′-GCTTTCTCGCTGTTTTTATCGCGAGCCCA-3′ (reverse). The resulting PCR-amplified sequences encoding CRY2 mutants were cloned into the OptoSTIM1(CRY2) vector at AgeI and EcoRI sites or BopEI and BamHI sites. OptoSTIM1 containing oligomeric mutants (CRY2olig (E490G) and CRY2clust (A9)) were constructed as previously described [19]. For construction of OptoSTIM1 bearing both CRY2olig (E490G) and oligomeric mutants (CRY2olig (E490G) and CRY2clust (A9)), a sequence encoding the CRY2olig (E490G) (5′-GGCGAAGAAAGCGCCGATCTGTTCCTG-3′) and CRY2clust (A9) (5′-GCTTTCTCCATCTTTCTGTTTTTATCGCGAGCCA-3′) (reverse) was flanked by AgeI and BspEI restriction enzymes to generate pIQ670-C1 vector. The pIQ670-C1 vector was constructed by cloning CRY2olig (E490G) into AgeI and BspEI sites of pIQ670-C1 vector. The pIQ670-N1 vector was created by exchanging CRY2olig (E490G) fragment with AgeI and BspEI restriction enzymes to generate pIQ670-C1 vector. Next, the tail sequence of KRas4B tail (20 amino acids; KMSKDGKKKKKKSKTKCVIM) was fused to the pmScarlet-Ferritin (FT) using BspEI and KpnI sites to generate pIQ670-PM (KRas4B tail). sequence encoding.

Cell culture and transfection. HeLa cells and HEK293T (ATCC) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; PAABio Laboratories GmbH) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a humidified 10% CO₂ atmosphere. Cells were transfected using either a Microcarrier (Neon Transfection System; Invitrogen) or Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions.

Live cell imaging. Prepared cells were plated on 96-well polystyrene coverslip bottom plates (μ-Plate 96-Well bts; ibidi). R-GECO1 fluorescence imaging with blue light illumination for OptoSTIM1 activation was performed using a Nikon A1R confocal microscope (Nikon Instruments), mounted onto an inverted Eclipse Ti body (Nikon), equipped with a CFI Plan Apochromat VC objective (×60 /1.4-numerical aperture (NA)) and digital zooming Nikon imaging software (NIS). Images were collected in FRB-EGFP FT using an A1R and B2BFT with an A1R and B2BFT with a 100× objective ( org. N.A. 1.4; 37°C) by calculating in a Chamäleon TC system (Cell Live Instruments, Inc., Korea). Immediately before imaging, the medium was replaced with OPTI-MEM (Invitrogen). Blue light photo-excitation (power density,
500 µW mm⁻²) was delivered with a 488-nm laser at 5-second intervals for 1 minute, unless stated otherwise.

**Fura-2 imaging and calibration.** HeLa cells were loaded by incubating at room temperature for 30 minutes with Fura-2 AM (Invitrogen), dissolved in dimethyl sulfoxide and diluted to 2 μM in DMEM, and washed three times for 5 minutes at each step. Fura-2 imaging was performed by intermittent excitation with 340 nm and 380 nm filtered fluorescent light using a LAMBDA DG-4 lamp (Sutter Instrument Company), equipped with an XCIPlan Fluor objective. The emission signal passing through a 510-nm emission filter was collected with a Nikon DS-Qi monochrome digital camera. Free [Ca²⁺]ᵢ was calculated according to the formula, [Ca²⁺]ᵢ = K_dissociation free Ca²⁺ + R_{free}×R_{max}[Ca^{2+}] + R_{free}×R_{max}[Ca^{2+}]max where K_dissociation is the dissociation constant of Fura-2 for Ca²⁺, R_{free} and R_{max} are ratios to zero free Ca²⁺ and saturating Ca²⁺, respectively, and [Ca^{2+}]max and [Ca^{2+}]max are fluorescence intensities for zero free Ca²⁺ and saturating free Ca²⁺, respectively, at an excitation wavelength of 380 nm. According to manufacturer's instruction (Fura-2 CA1D Imaging Calibration Kit (Invitrogen)), the K_dissociation in our experimental condition is set as 261 × 10⁻² M.

**InCell SMART-i assay.** HeLa cells were transfected using a Microcarrier (Neon Transfection System; Invitrogen) in a condition of two pulses of electric shock at 980 V for 35 milliseconds. 12 hours post transfection, cells were treated for 2 hours with dimethyl sulfoxide (DMSO) or Rapamycin (Calbiochem), dissolved in DMSO as 2 μM stock solution and diluted to 500 nM in DMEM before use. To induce oligomerization of CRY2-fused proteins, cells were exposed to pulsed illumination of light (5-s irradiation every 10-s, 470 nm, 100 µm² mm⁻²) administered with a blue LED array for 30 minutes before fixation. Cells were then fixed with 4% paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS) for 20 minutes and washed with PBS for three times. Cells were imaged using a confocal microscope and analyzed with NIS-element AR 64-bit version 3.2i; Laboratory Imaging software provided from Nikon.

**Subjects.** All mice were handled and cared for according to the directives of the Animal Care and Use Committee of KAIST (Daejeon, Korea). All in vivo mice experiments were carried out on 8–13-week-old male C57BL/6 J mice purchased from Jackson Laboratory. Mice were housed in cages with free access to food pellets and water, and were kept on a 12-hour light–dark cycle (8 am to 8 pm) at 22 °C and 40% humidity. All behavior experiments were performed during the light phase of the light–dark cycle at the same time of day.

**Preparation of lentivirus.** The plasmid for lentiviral vectors containing CaMKIIα promoter was produced as previously described. Plasmids for other variants were generated by cloning exchange PCR-amplified CRY2 variants (CRY2E281A-A9, CRY2E281A-A9, CRY2E281A-A9, CRY2E281A-A9, CRY2E281A-A9) into pLenti-CaMKIIα-EYFP-CRY2-STIM1 with AgeI and EcoRI sites. Construct pLenti-CaMKIIα-EYFP was constructed by replacing ChETA-EYFP of pLenti-CaMKIIα-ChETA-EYFP (addgene #26967, RRID:Addgene_26967) vector to EYFP of pEGFP-C1 using BamHI and BsrGI sites. Plasmid containing GsaABC1D promoter was generated by cloning exchange PCR-amplified GsaABC1D promoter into pLenti-CaMKIIα-EYFP-CRY2-STIM1 using PscI and BamHI sites. The lentivirus vector was co-transfected with VSVG- and A589 required for the production of the lentiviral particles into the HEK293T cell line by using the jetPEI transfection reagent. After 72 hours transfection, the supernatant was collected and centrifuged 626 × g for 5 minutes and then filtered through 0.45 μm filtration unit (Millipore). For purifying lentivirus, we carried out by ultracentrifugation (107,000 × g) for 2 hours at 4 °C. After ultracentrifugation, supernatant was removed and the pellet was resuspended in PBS, aliquoted and stored at −80 °C. Titration of lentivirus was measured using Lenti-X² qRT-PCR titration kit (Takara) according to the manufacturer’s instructions. The viral titers were 7.88 × 10¹² and 2.23 × 10¹² genome copies ml⁻¹ for CaMKIIα promoter-bearing OptoSTIM1 and monSTIM1 viruses, respectively, and 6.81 × 10¹² and 8.42 × 10¹¹ genome copies ml⁻¹ for GsaABC1D promoter-bearing monSTIM1 and Opto-STIM1(CRY2E281A-A9) viruses, respectively.

**Stereotaxic surgery and in vivo light-stimulation condition.** Stereotaxic viral injection was performed using 8-week-old male C57BL/6J mice. Surgical procedures were performed under stereotaxic guidance. Before surgery, surgical tools were sterilized at 240 °C in a hot bead sterilizer. All mice, maintained at 37 °C using a heating plate, were anesthetized with 0.022 ml/g Avertin 1 hours after light illumination (except for experiments performed under dark conditions) and perfused transcardially, first with PBS and then with 10 ml of 4% PFA in PBS. Brains were extracted and incubated in 4% PFA at 4 °C overnight. Brains were transferred to PBS, and 60-μm coronal slices were prepared using a vibratome (Leica). For immunostaining, slices were placed in PBS containing 0.2% Triton X-100 and 5% normal goat serum for 1 hour, after which the solution was replaced with primary antibody diluted in PBS containing 0.1% Triton X-100 and 2% normal goat serum. After incubating overnight at 4 °C, slices were rinsed five times with PBS containing 0.2% Tween-20 (10 minutes each), followed by a 1-hour incubation with secondary antibody. Tissue sections were then washed in PBS containing 0.2% Tween-20 (10 minutes each) and mounted on microscope slides with VECTASHIELD antifade mounting medium containing DAPI(4',6-diamidino-2-phenylindole) (H-1200, Vector Laboratories). Fluorescence images were captured with ×10 and ×60 objectives using a Nikon A1R confocal microscope (Nikon Instruments). All ×60 images were acquired as z-stacks by binning 3-μm-depths per image plane. Antibodies used for immunohistochemistry were as follows: (cry2E281A-A9) (1:2000, A10162, Thermo Fisher Scientific) and Alexa 488-conjugated anti-chicken secondary antibody (1:2000, A-11039, Thermo Fisher Scientific), to stain for EGFP-CRY2(variants)-STIM1, and rabbit polyclonal anti-c-Fos primary antibody (1:1000, ab190289, Abcam) and Alexa 594-conjugated anti-secondary antibody (1:2000, A-11102, Thermo Fisher Scientific). To analyze c-Fos⁺ cells, more than five coronal brain sections in each mice samples were used to count the number of c-Fos⁺ cells with containing DAPI⁺ and surrounded EGFP signal. Statistical significance was evaluated using a Sidak’s multiple comparisons test.

**Observational fear conditioning**. Observational fear conditioning tests were performed in a chamber consisting of two attached identical chambers (18 × 17.5 × 38 cm) separated by the middle of the two chamber by a transparent Plexiglas divider. The floor of the cage consisted of stainless steel 5-mm rods, 1 cm apart, similar to passive-avoidance cages (Coulbourn Instruments). The space beneath the rods allowed sounds and smells to be shared during experiments. Before commencing observational fear learning experiments, mice were handled for 10 minutes over 3 days. On test day, mice were habituated to the behavior chamber for 1 hour immediately before the test. For transcranial light stimulation, the observer mouse was illuminated with blue light (1 μm² cm⁻²) for 30 minutes, and then was moved into the apparatus chamber containing a different mouse (demonstrator) in the next chamber. As demonstrators, 10–12-week-old mice of the same strain (C57BL/6 J) were used. In all experiments, observer and demonstrator mice were non-siblings and were housed in separate cages. Both observer and demonstrator mice were habituated to the apparatus for 5 minutes, after which a 2-second foot shock (1 mA) was delivered every 10 s for 4 minutes only to the demonstrator mouse using a programmed animal shocker (Coulbourn Instruments). On day 2 (24 hours after) the observer mouse was placed in the same chamber as the demonstrator mouse and observed for observational fear learning to access contextual memory test, and were observed for 4 minutes. Fear responses were video-recorded and quantified. Data were not obtained in a blinded manner, but two other blinded investigators verified the reliability of the results. Statistical significance was evaluated using a Tukey’s test.

**Pavlovian fear conditioning**. Pavlovian fear conditioning experiment was performed as same as what we described in previous study. In brief, mice were housed in a homecage with covering LED illuminating cage lid and delivered blue light for 30 minutes prior to conditioning. Afterwards, mice were moved into conditioning chamber (Context A) with shock floor (Coulbourn Instruments) to deliver electric foot shock at the bottom of chamber. Mice were habituated for 2 minutes and then gave three paired tone (30 s, 60 dB, 3 kHz)-shock (2 s, 0.7 mA) for every 2 minutes intervals. After last conditioning session, mice were left in conditioned chamber for another 90 s before moving in the homecage. The chamber was wiped sequentially with 70% ethanol and distilled water inter-training period. On day 2, mice were re-exposed into same conditioning chamber without tone presentation and recorded for 5 minutes to access the percentage of freezing before the test context. On day 2, mice were tested in the test context which composed cylindrical shape chamber with white floor and scented with 40% diluted mouthwash (Context B). During 6 minutes recording, tone stimulation only presented at 3 minutes later after exposing the context B. Freezing behavior of mice was recorded and quantified with FreezeFrame software (Actimetrics). Two other blinded investigators verified the reliability of the results. Statistical significance was evaluated using a Tukey’s test.

**Open-field test.** All mice were habituated to the testing room for 30 minutes immediately before the testing session. Mice were illuminated with blue light (473 nm) at a power density of 1 mW cm⁻² for 1 hour to activate OptoSTIM1 transcranially and sequentially transferred into the open-field box for 30 minutes for 10 minutes velocity and time spent in center were quantifying using an automated IR detection system (Optimouse). Statistical significance was evaluated using a Tukey’s multiple comparisons test.
Quantification and statistical analysis. Images were taken and analyzed using NIH-element AR 64-bit version 3.21; Laboratory Imaging software provided from Nikon. Time-measurement tool was used to quantify change of R-GECO1 intensity. Initial intensity of R-GECO1 was set as 100 and 500 (A.U.) what we previously described. Statistical significance was assessed by a two-tailed Student’s t test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability The data that support the findings of this study are available within the paper and its Supplementary Information files. Extra data are available from the corresponding author upon reasonable request.

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Author contributions
W.D.H., H.-S.S., S.L., T.K., and S.K. conceived the idea and directed the work; S.K., T.K., J.-H.C., N.K., H.P., Seoohn.K, H.M.K., S.L., H.-S.S., and W.D.H. designed experiments; S.K., T.K., N.K., and J.L. performed experiments; and S.K., T.K., S.L., N.K., J.-H.C., Seoohn.K, and W.D.H. wrote the manuscript.

Competing interests
South Korean patent no. 10-2018-0139283 has been awarded to Institute for Basic Science (to S.K., T.K., S.L., and W.D.H. being the inventors) for the m-stim1 technology described in this paper. The technology has been sold to Hulux and W.D.H. is a shareholder. All other authors declare no competing interests.
Additional information
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