SI Appendix

Transcriptional readout of neuronal activity via an engineered Ca^{2+} activated protease

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Cloning.

See Plasmid table for a list of genetic constructs used in this study. Each entry lists construct features including promoters, epitope tags, etc. For cloning, PCR fragments were amplified using Q5 polymerase (New England BioLabs (NEB)). The vectors were double-digested and ligated to gel-purified PCR products by T4 ligation or Gibson assembly. Ligated plasmid products were introduced by heat shock transformation into competent XL1-Blue bacteria.

HEK 293T cell culture and transfection.

HEK 293T cells from ATCC with fewer than 20 passages were cultured as monolayers in media composed of a 1:1 mixture of DMEM (Dulbecco's Modified Eagle medium, Gibco) and MEM (Minimum Essential Medium Eagle) supplemented with 10% (v/v) FBS (Fetal Bovine Serum, Sigma) and + 1% (v/v) pen-strep at 37 °C under 5% CO_2. For imaging at 10× magnification, we grew the cells in plastic 48-well plates that were pretreated with 50 µg/mL human fibronectin (Millipore) for at least 10 min at 37 °C before cell plating (to improve cell adherence). For imaging at 63× magnification, we grew cells on 7 × 7 mm glass cover slips placed inside 48-well plates. The coverslips were also pretreated with 50 µg/mL human fibronectin for at least 10 min at 37 °C before cell plating. Cells were transfected at 60–90% confluence with 1 mg/mL PEI max solution (polyethylenimine HCl Max pH 7.3). 96-well plates, used to quantify the luciferase activity, were treated in the same way.

For scFLARE experiments in HEK, cells were transfected in 48-well plates, and each well received a DNA mixture consisting of: 25 ng UAS:mCherry or UAS:Luciferase plasmid; and 100 ng of different scFLARE versions, along with 0.8 µL PEI max in 10 µL serum-free MEM media for 15 min at room temperature. DMEM/MEM with 10% FBS (100 µL) was then mixed with the DNA-PEI max solution and incubated with the HEK cells for 15-18 h before further processing. These quantities were scale up or down according the size of the well-plate used.

For FLARE experiments in HEK, (48-well plates), each well received a DNA mixture consisting of: 20 ng UAS:mCherry plasmid; 50 ng of uTEV1Δ or uTEV2Δ and 50 ng of membrane-anchored transcription factor in the same conditions described above.

Analysis of scFLARE performance in HEK 293T.

For fluorescence microscopy, cells were fixed with room temperature paraformaldehyde fixative solution (4% paraformaldehyde in PBS) for 10 min. Fixed cells were permeabilized by incubation with cold methanol at −20 °C for 5 min, washed with PBS once and then blocked with 2% PBS-B (w/v BSA in PBS) at room temperature for 1 h. HEK 293T cells were immunostained using mouse-anti-V5 antibody (1:2,000 dilution, Life Technologies, R96025) and/or rabbit-anti-HA antibody (1:2,000 dilution, Rockland, 600-401-384) , in a 2% PBS-B for 1 h at room temperature with gentle rocking, followed by washing twice with PBS-B. After washes, cells were incubated with anti-mouse-AlexaFlour405 antibody (1:2,000 dilution, Life Technologies, A-31553) and/or anti-rabbit-AlexaFlour647 antibody (1:2,000 dilution, Life Technologies, A-21245) for 1 h at room temperature with gentle rocking. After washing two times with PBS-B, cells were directly imaged with a 10× air objective on a Zeiss AxioObserver inverted confocal microscope.

Fluorescence microscopy of cultured cells.

Confocal imaging was performed on a Zeiss AxioObserver inverted confocal microscope with 10× air and 40× oil-immersion objectives, outfitted with a Yokogawa spinning disk confocal head,

a Quad-band notch dichroic mirror (405/488/568/647), and 405 (diode), 491 (DPSS), 561 (DPSS) and 640-nm (diode) lasers (all 50 mW). The following combinations of laser excitation and emission filters were used for the fluorophores: eGFP/citrine (491 laser excitation; 528/38 emission), mCherry (561 laser excitation; 617/73 emission), and differential interference contrast (DIC). All images were collected and processed using SlideBook (Intelligent Imaging Innovations)

Luciferase assays.

For the luciferase assays, we used the Bright-Glo luciferase assay system (Promega). The Bright-Glo reagent was thawed at room temperature in a water bath prior to usage. Media was aspirated from each well, and 50 µL of DPBS and 50 µL of Bright-Glo reagent were added to each well. Luminescence was immediately analyzed at 25°C using a plate reader (TECAN) with the Green-1 filter, and linear shaking.

FACS analysis of scFLARE and FLARE activation.

HEK 293T were trypsinized and transferred to a 5 mL polystyrene round-bottom tube with 1 mL of DPBS (0.2 mL yeast was diluted into 1 mL of DPBS).

For two-dimensional FACS analysis, we used a LSRII-UV flow cytometer (BD Biosciences) to analyze cells with 488 nm and 561 nm lasers and 525/50 (for citrine) and 610/20 (for mCherry) emission filters. To analyze and sort single cells, cells were plotted by forward-scatter area (FSC-A) and side-scatter area (SSC-A) and a gate was drawn around cells clustered between 10^4 – 10^5 FSC-A and 10^3 – 10^5 SSC-A to give population P1. Cells from population P1 were then plotted by side-scatter width (SSC-W) and side-scatter height (SSC-H) and a gate was drawn around cells clustered between 10 – 100 SSC-W and 10^3 – 10^5 SSC-H to give population P2. Cells from population P2 were then plotted by forward-scatter width (FSC-W) and forward-scatter height (FSC-H) and a gate was drawn around cells clustered between 10 – 100 FSC-W and 10^3 – 10^5 FSC-H to give population P3. Population P3 often represented >90% of the total population analyzed. From population P3, we then plotted Citrine (488 nm laser and 525/50 emission filter) on the x-axis (representing expression level of the protease or TEVcs) and mCherry (561 nm laser and 610/20 emission filter) on the y-axis (representing turn-on of the reporter gene). BD FACSDIVA software was used to analyze all data from FACS sorting and analysis.

Western Blot.

In a 6 well-plate, HEK 293T cells expressing the indicated constructs were plated, transfected, and stimulated as described above. After stimulation, we washed the cells once by gently pipetting a stream of DPBS into one side of the well. The washes were removed by aspiration and ~500 µL of DPBS were pipetted directly onto the cells. The supernatant was pipetted up and down until cells were detached. Pellets were collected by centrifuging the resulting cell suspension at 1,500 r.p.m. for 3 min. The supernatant was removed, and the pellet was lysed by resuspending in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1× protease inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF and 2 mM iodoacetamide, a TEV protease inhibitor) by gentle pipetting and incubating for 5 min at 4 °C. Lysates were clarified by centrifugation at 10,000 r.p.m. for 10 min at 4 °C. 20 µL of PLB (6x) Protein Loading buffer , 6X) were mixed with 100µL of the clarified lysate and boiled for 5 min prior PAGE gel separation.

Proteins were separated on 9% SDS-PAGE gels and then were transferred into a nitrocellulose membrane, and then stained by Ponceau S (5 min in 0.1% (w/v) Ponceau S in 5% acetic acid/water). The blots were then blocked in 3% BSA (w/v) in TBS-T (Tris-buffered saline,

0.1% Tween 20) for 45 min at room temperature. Blots were then stained with an anti-V5 mouse (Invitrogen 46-0705) in 3% BSA (w/v) in TBS-T for 1 at room temperature, washed two times with TBS-T for 10 min each, then stained with anti-mouse-HRP (Invitrogen 170-6516) in 3% BSA (w/v) in TBS-T for 45 min at room temperature. The blots were washed four times with TBS-T for 5 min each time before to development with Clarity Western ECL Blotting Substrates (Bio-Rad) and imaging on a Bio-Rad Gel Doc 2000. Quantitation of Western blots was performed using ImageJ on raw images under non-saturating conditions.

Production of AAV virus supernatant for neuron transduction.

AAV virus supernatant was used to transduce neuron cultures for FLARE experiments. To generate viruses, HEK 293T cells were transfected in a T25 flask, at 70–90% confluence. For each virus, we combined 0.875 µg of viral DNA (plasmid p36-p42, from the Plasmid Table), 0.725 µg AAV1 serotype plasmid (plasmid p44), 0.725 µg AAV2 serotype plasmid (plasmid p45), and 1.75 µg helper plasmid pDF6 (plasmid p46) with 20 µL PEI max and 200 µL serum-free DMEM [1]. The transfection mix was incubated for 15 min at room temperature and added to 5 mL of complete media. The media of the T25 flask was replaced with the mixture. After incubation for 48 h at 37 °C, the supernatant (containing secreted AAV virus) was collected and filtered through a 0.45-µm syringe filter (VWR). AAV virus was aliquoted into sterile Eppendorf tubes (0.5 mL/tube), flash frozen in liquid nitrogen and stored at −80 °C.

Viral transduction of cortical neuron cultures and stimulation.

A mixture of AAV viruses, prepared as above, encoding scFLARE2 and a suitable reporter, were added to DIV 11-12 cultured neurons in a 24 well-plate containing 1.5 mL of complete neurobasal media. Typical viral supernatant quantities used were: 200 µL of scFLARE2 virus and 100 µL of reporter virus (TRE:mCherry, TRE:mCherry-p2A-bReaChES, or TRE:mCherry-p2A-ePnHR3.0-TS). Lower levels of scFLARE2 expression generally give higher S/N in labeling. After incubation for 3 days at 37 °C, 30% of the media in each well was replaced with fresh complete neurobasal media.

After viral transduction, neurons were grown in the dark, wrapped in aluminum foil, and all subsequent manipulations were performed in a dark room with red light illumination to prevent unwanted activation of the LOV domain. Six days post-transduction (at DIV 17-18), neurons were stimulated in the presence or absence of blue light. To elevate cytosolic Ca^{2+} we used electrical stimulation generated by a Master 8 device (AMPI), which induces trains of electric stimuli. A stimulator isolator unit (Warner Instrument, SIU-102b) was used to provide constant current output ranging from 10–100 mA. Platinum iridium alloy (70:30) wire from Alfa-Aesar was folded into a pair of rectangles (0.7 cm × 1.5 cm) and placed right above the neurons on the edge of the well to act as electrodes. We used 5-second trains, each consisting of 32 1-ms 48 mA pulses at 20 Hz, lasting for a total of 15 min (see Supplementary Figure 8B for a schematic). Trains at different frequencies and for different stimulation times were used for different experiments. For blue-light irradiation, neuron plates were placed on top of the custom-built LED light box described above (“scFLARE experiments in HEK 293T”) and irradiated with 467-nm blue light at 60 mW/cm^2 and 33% duty cycle, 2 s of light every 6 s. For the dark condition, neurons were wrapped in aluminum foil. Prior to performing FLARE experiments, we checked each method of stimulation and the quality of our neuron cultures by performing GCaMP5f real-time calcium imaging. To

achieve high S/N labeling with scFLARE2, the basal activity of the cultures should be low, and neurons must to be sensitive to electric field stimulation.

Drug stimulation of neuron cultures.

SR95531 (Gabazine, CAS 104104-50-9), and (S)-3,5-DHPG (162870-29-3) were acquired from TOCRIS. AMPA (74341-63-2) was purchased from Sigma-Aldrich. 100x stock solutions were prepared in DMSO. Neurons were plated in 6-well plates and processed as outlined in the previous section. Six days post-transduction (at DIV 17-18), neurons expressing scFLARE2 and TRE:mCherry were treated with various drugs. Around 1 mL of the 2 mL media from each well was transferred into an eppendorf tube. The drug was diluted in 200 µL of the media and added gently to the top of the media in each well, to avoid mechanical stimulation of the neurons. The final concentrations used were: 10 µM of SR95531, 10 µM of AMPA, and 50 µM of (S)-3,5-DHPG. Stimulation time was 5 min. For blue light illumination, the 6-well plate was placed on top of a custom-built LED light box that delivers 467-nm blue light at 60 mW/cm2 intensity and 33% duty cycle (2 s of light every 6 s). To terminate the drug stimulation, the solution in each well was removed by gentle pipetting and the neurons were washed twice with the media that was previously saved in Eppendorf tubes. 18 hours later, neurons were fixed and analyzed by microscopy.

Immunostaining of fixed neurons, imaging and data analysis.

After stimulation, neurons were incubated in dark conditions at 37 °C before fixation with prewarm paraformaldehyde fixative solution (4% paraformaldehyde, 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 0.12 M sucrose, pH 7.3) for 10 min.

Fixed neurons were permeabilized by incubation with cold methanol at −20 °C for 5 min, washed with PBS once and then blocked with 2% PBS-B (w/v BSA in PBS) at room temperature for 1 h. Neurons were immunostained using rabbit-anti-VP16 antibody (1:2,000 dilution, Abcam ab4808), in a 2% PBS-B for 1 h at room temperature with gentle rocking, followed by washing twice with PBS-B. The neurons were then incubated with anti-rabbit-AlexaFlour647 antibody (1:2,000 dilution, Life Technologies, A21245) for 1 h at room temperature with gentle rocking. After washing two times with PBS-B, the neurons were directly imaged in their 24-well plates using a 10× air objective on a Zeiss AxioObserver inverted confocal microscope.

For imaging, ten fields of view were collected per condition. For each field of view, a mask was created to encompass regions with VP16 expression (reflecting scFLARE expression). In these masked regions, the mean mCherry fluorescence intensity was calculated, and background was subtracted. These mean mCherry intensity values were calculated individually for 10 fields of view per condition and plotted in a dot plot.

Virus infusion in mice.

Adult wild-type male C57BL/6 mice 12-20 weeks old (Jackson Laboratory, Bar Harbor, ME) were used for all experiments. All procedures were carried out in accordance with the National Institutes of Health guidelines for animal care and use and were approved by the Administrative Panel on Laboratory Animal Care of Stanford University. All surgeries were conducted under aseptic conditions using a small-animal stereotaxic instrument (Leica Biosystems Inc., Buffalo Grove, IL). Mice were anaesthetized with isoflurane (5% for induction, 1.5–2.0% after) in the

stereotaxic frame for the entire surgery and their body temperature was maintained using a heating pad. The hippocampus was targeted using the following coordinates from bregma: -2.3 mm AP, ±1.5 mm ML, and −1.35 mm DV. A 2:1 mixture of scFLARE2:TRE-mCherry virus was injected either bilaterally (direct labeling experiments) or unilaterally (left hemisphere, indirect labeling experiments) using a 10-µL microsyringe with a beveled 33 gauge microinjection needle (nanofil; WPI, Sarasota, FL). A total volume of 1.5 µL of virus was injected in each hemisphere (750nL at -1.35 DV and 750nL at -1.55 DV) at a rate of 10 nL/s using a microsyringe pump (UMP3; WPI, Sarasota, FL) and its controller (Micro4; WPI, Sarasota, FL). After each injection the needle was raised 100 µm for an additional 10 min to allow for viral diffusion at the injection site and then slowly withdrawn.

scFLARE2 labeling in mice.

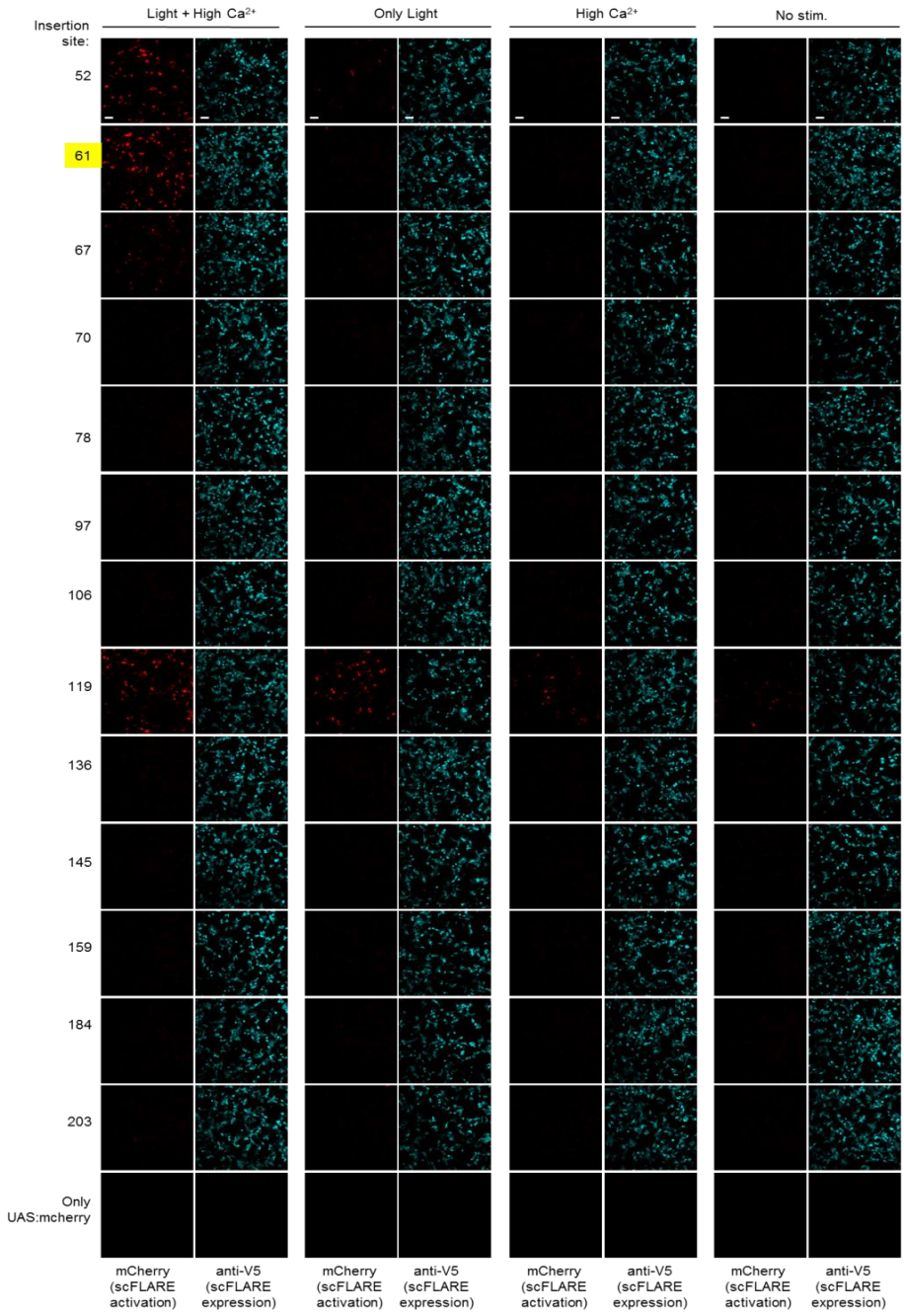
Light was delivered 6-7 d following viral injection. For light delivery, the optical fiber implant from one hemisphere was connected to a 473-nm diode-pumped solid state (DPSS) laser (Shanghai Laser & Optics Century Co., Ltd, China). In direct labeling experiments the optical fiber for the other hemisphere, or for indirect labeling experiments the optical fiber for the animals in the dark group, was capped to prevent light penetrance. For anesthetized experiments, mice received light following implant surgery and remained under anesthesia for an additional 30 min following light administration. For awake and kainate experiments, mice were allowed 2 h to recover from implant surgery before light delivery. For kainate experiments, closed-loop seizure detection and light delivery were carried out as previously described [2]. Briefly, EEG recording electrodes for kainate injected animals were connected to an electrical commutator (PlasticsOne; Roanoke, VA) routed to an amplifier (BrownLee 410; Automate Scientific, Inc., Berkeley, CA), and in turn connected to a digitizer (National Instruments USB-6221; National Instruments, Austin, TX) and a computer running custom MATLAB recorder and seizure detection software. When a seizure was detected, the software enabled light delivery. Animals in all groups receiving light had one single session of 10 mW 473-nm light delivered at 2 s pulses every 4 s (50% duty cycle), for 10 min total. The light delivery was triggered either by the closed-loop system in kainate injected animals or manually by the experimenter for animals in the awake or anesthetized group.

Brain slice preparation and immunohistochemistry.

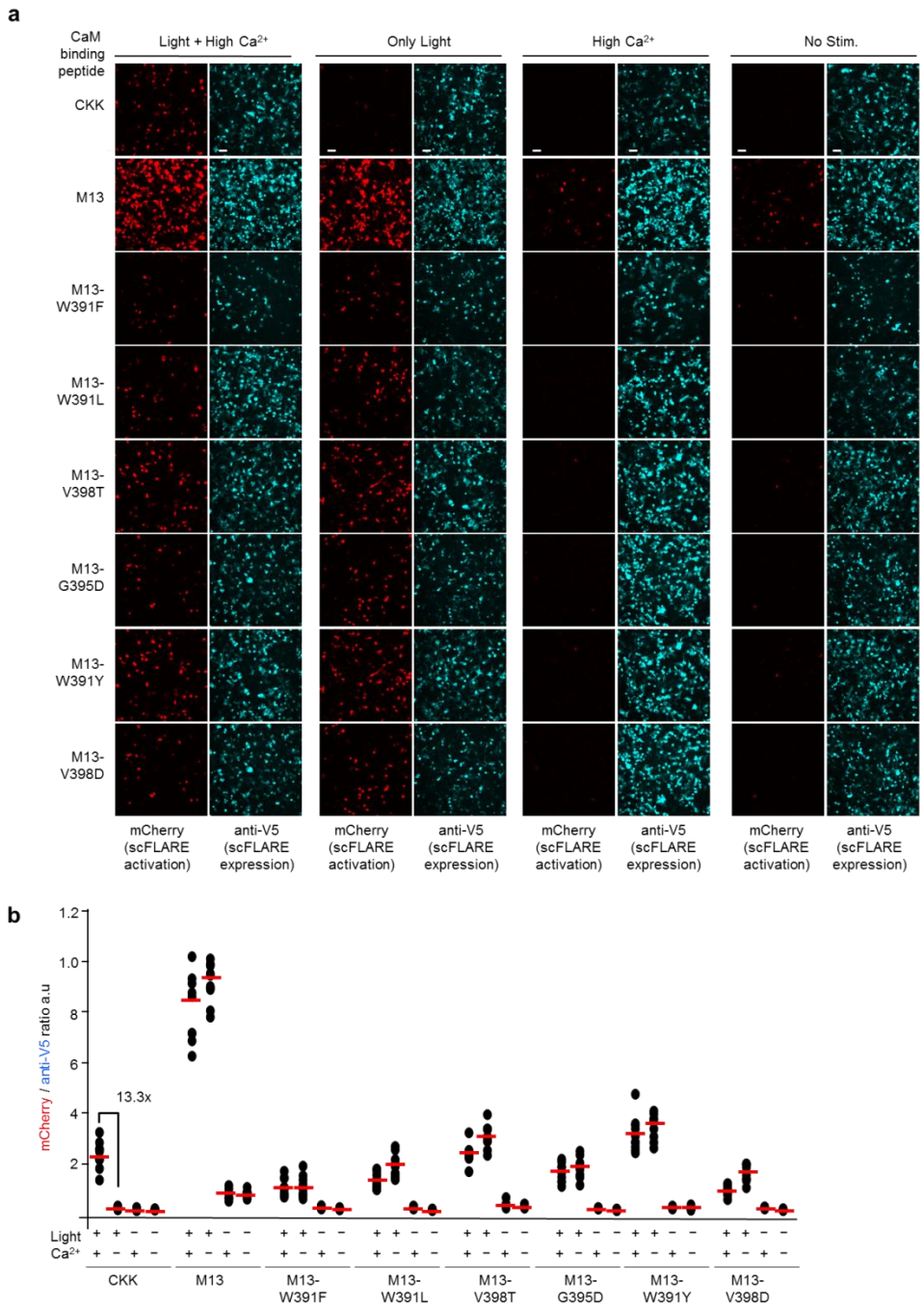
Animals were euthanized 18-24 h after light administration by being deeply anesthetized with a mixture of ketamine and xylazine (80-100 mg/kg ketamine, 5-10mg/kg xylazine; intraperitoneal) and transcardially perfused with 10 mL of 0.9% sodium chloride solution followed by 10 mL of cold 4% PFA dissolved in phosphate buffer solution. The excised brains were held in a 4% PFA solution for at least 24 h before being sectioned into 60 µm slices using a vibratome (Leica VT1200S; Leica Biosystems Inc., Buffalo Grove, IL). For immunohistochemistry, slices were incubated in blocking buffer containing 1% bovine serum albumin (BSA) and 0.5% Triton-X in TBS (tris buffered saline) for 1 h at room temperature, then incubated with rabbit anti-VP16 antibody (1:2,000; abcam, Cambridge, MA) in TBS containing 1% BSA and 0.5% Triton-X overnight at 4ºC. Slices were subsequently washed in TBS (4 × 10 min) before incubating in anti-rabbit-AlexaFluor647 secondary (1:1000; Thermo Fisher Scientific, Waltham, MA) for 2 h at room

temperature. Afterwards slices were washed in TBS (4 x 10 min) before mounting on glass slides and cover-slipped using Vectashield Antifade Mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

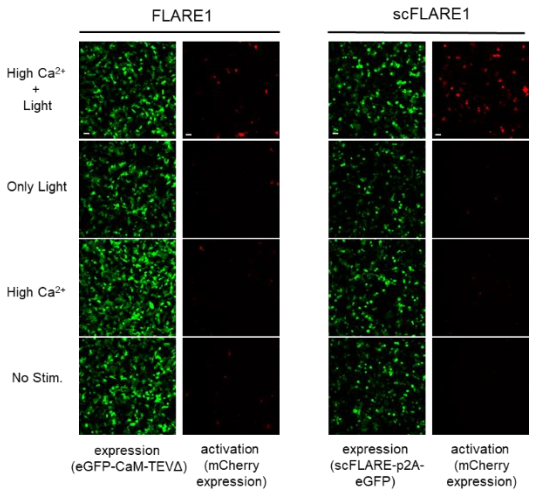
SI Figures



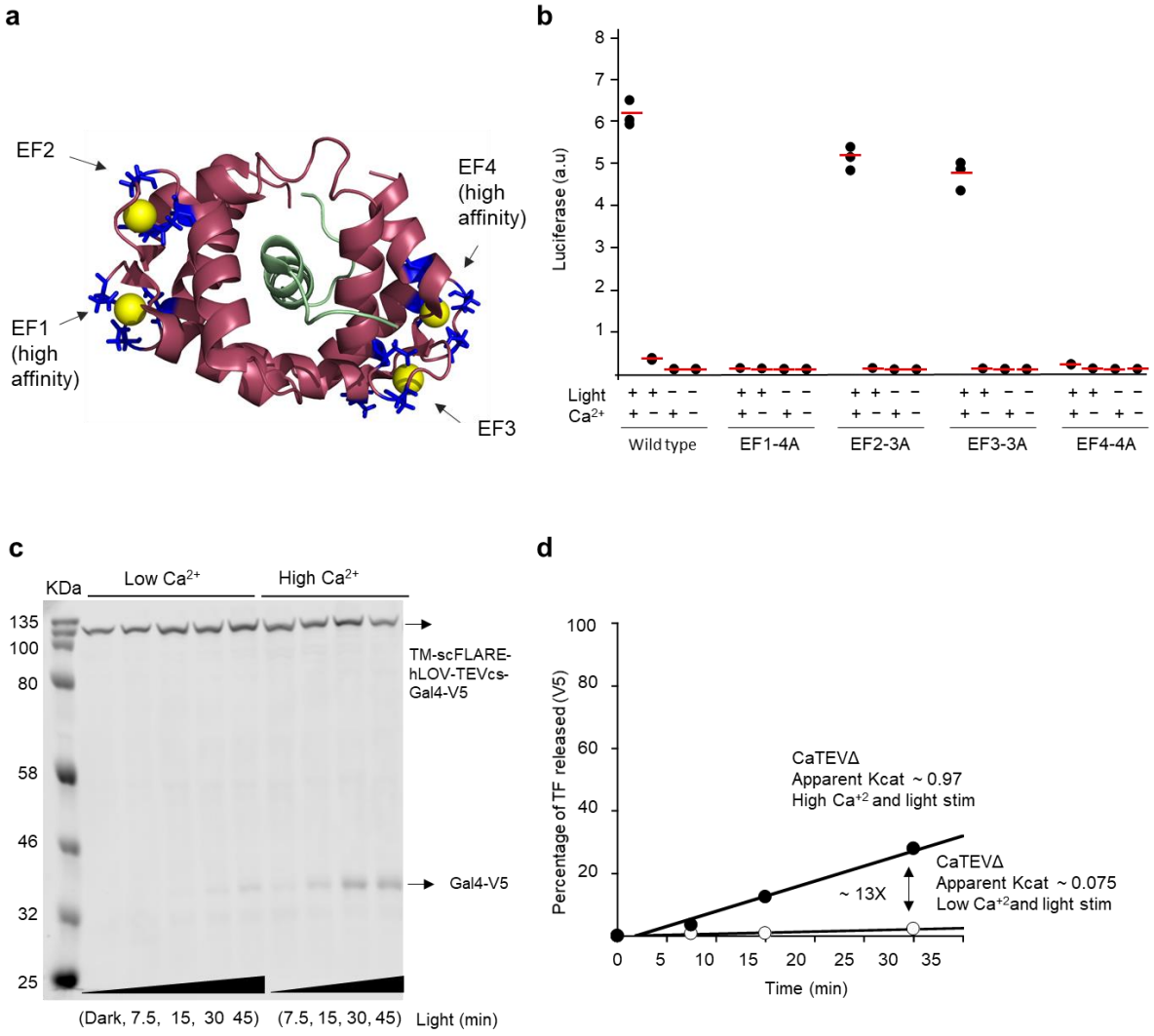
Supplementary Figure 1. Screening of calmodulin insertion sites in TEV. Related to Figure 1F, but with microscopy readout of scFLARE activation rather than luciferase/platereader readout. HEK 293T cells were transiently transfected with UAS:mCherry and an scFLARE variant containing a Ca-TEV with the Ca^{2+} responsive module inserted into TEV at the indicated insertion site. Stimulation was performed using 6 mM $CaCl_2$ and 2 µM Ionomycin for 10 min in the absence or presence of blue light (467 nm, 60 mW/cm^2, 33% duty cycle (2s light every 6s)). Eight hours later, cells were fixed and permeabilized, stained with anti-V5 antibody, and imaged. This experiment was performed once. Scale bars, 10 µm.



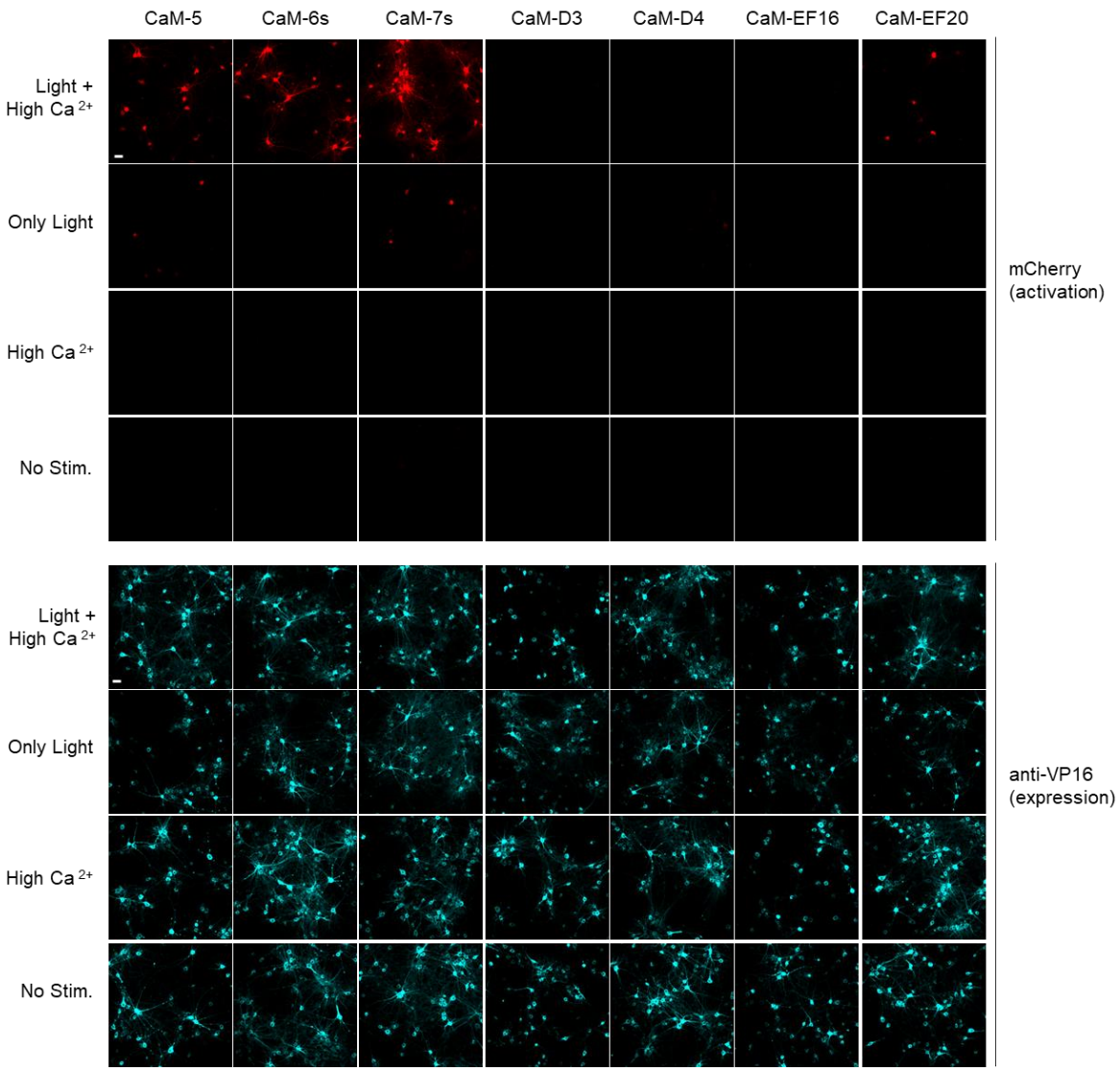
Supplementary Figure 2. Screening of calmodulin-binding peptides in scFLARE. a) HEK 293T cells were transiently transfected with UAS:mCherry and scFLARE variants containing the indicated calmodulin-binding peptide. CKK is a 26-residue peptide, corresponding to the CaM-binding domain of rat Ca^{2+}/CaM-dependent protein kinase kinase (CaMKK). M13 is a synthetic peptide and its sequence is the same as the calmodulin-binding domain of skeletal muscle myosin light chain kinase (skMLCK) (residues 577-602). It was used as a CaM binding peptide in the GCaMP series. Stimulation, staining, and imaging was performed as in Supplementary Figure 1. This experiment was performed once. Scale bars, 10 μm. **b**) Quantitation of data from (a). mCherry/anti-V5 signal ratios were calculated from mean mCherry and V5 intensities across 10 fields of view per condition (>100 cells per field of view; red line, mean).



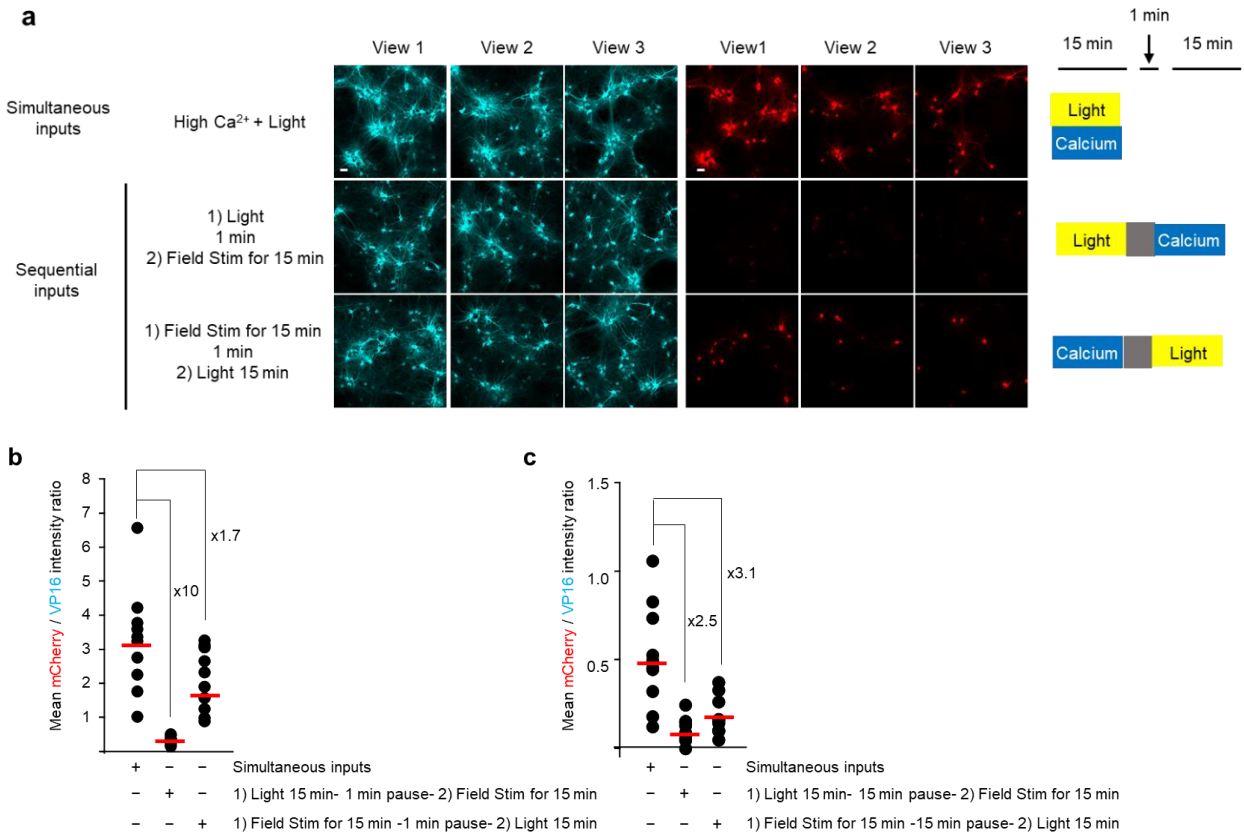
Supplementary Figure 3. Comparison of FLARE1 and scFLARE1 in HEK. HEK 293T cells were transiently transfected with UAS:mCherry and either FLARE1 constructs or scFLARE1-p2A-eGFP. After 15 h of expression, cells were stimulated and imaged as in Supplementary Figure 1. Scale bars 10 μm.



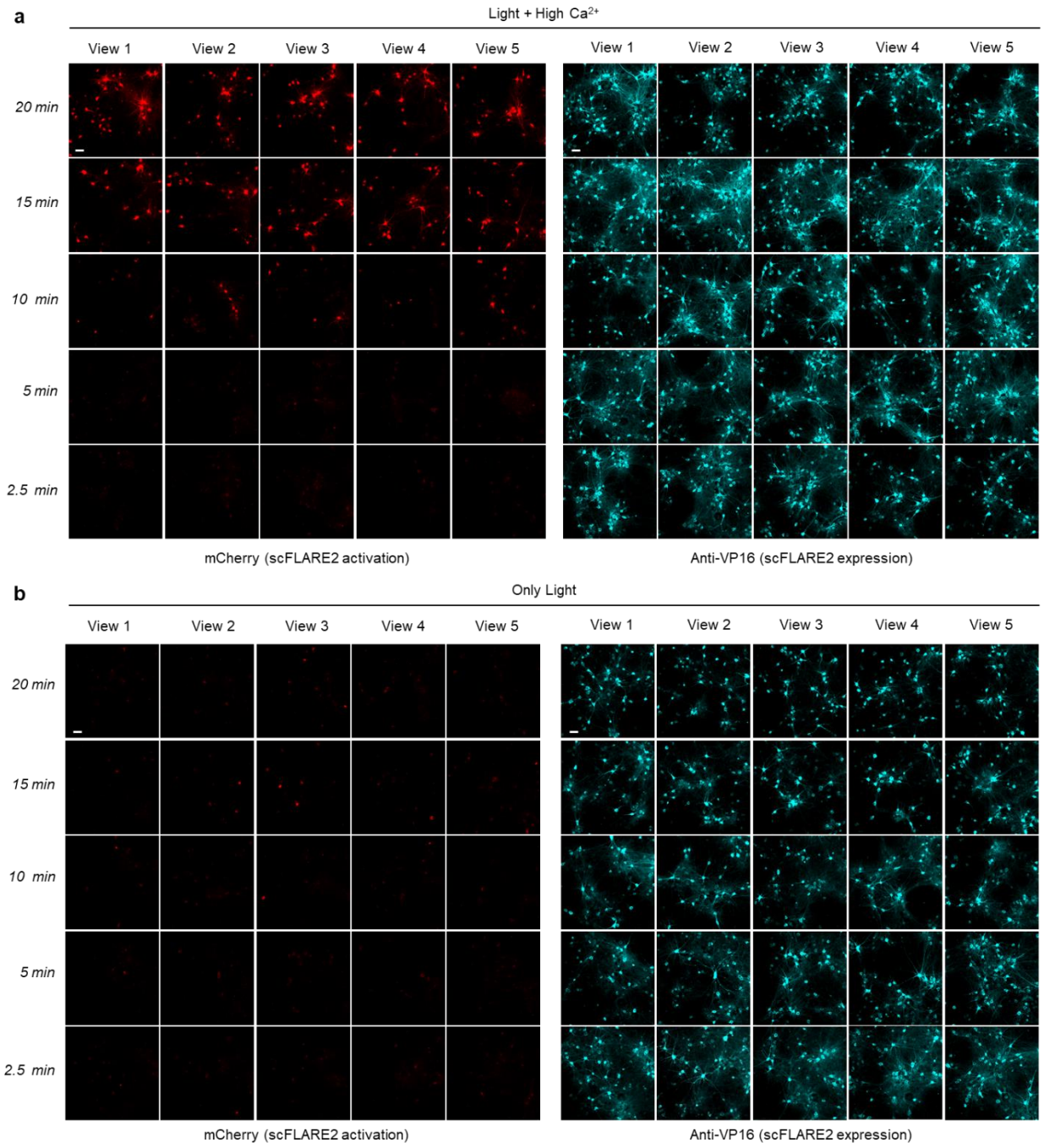
Supplementary Figure 4. Mechanistic analysis of scFLARE1. a) Crystal structure of calmodulin-CKK complex in the Ca^{2+}-bound state (PDB: 1CKK [3]). Four EF hands are shown in blue, and Ca^{2+} ions are yellow. **b**) Mutagenesis of Ca^{2+} binding residues within Ca-TEV of scFLARE. HEK 293T cells were prepared, stimulated, and analyzed as in Figure 1F (10 minutes of stimulation). EF1 and EF4 are the high Ca^{2+} affinity lobes of calmodulin while EF2 and EF3 are low Ca^{2+} affinity [4, 5]. EF1 comprises the following combined mutations (D22A, D24A, D24A and D31A), EF2 (D56A, D58A, D60A and E67A), EF3 (D93A, D95A and E104A) and EF4 (D128A, D130A, A132A and E140A). This experiment was performed once with three technical replicates per condition (red lines, mean). **c**) Western blot analysis of scFLARE cleavage at different time points at high and low Ca^{2+} under blue light illumination. **d**) Quantification of CaTEV reaction rates based on Gal4-V5 signal. This experiment was replicated three times with similar results.



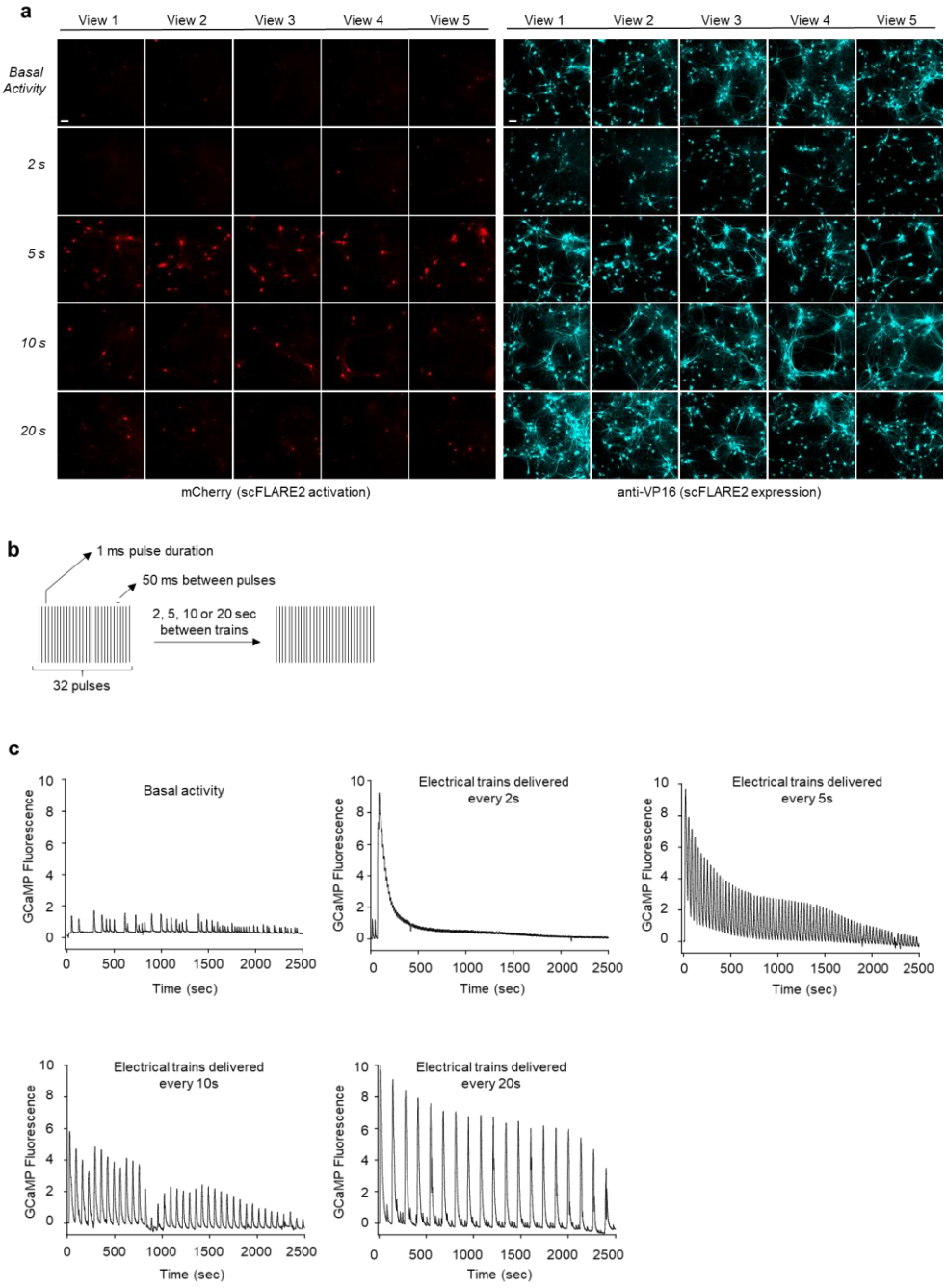
Supplementary Figure 5. Comparison of calmodulin domains in scFLARE in neurons. Fluorescence images representative of the data used to generate the graph in Figure 3C. Rat cortical neuron cultures were transduced with scFLARE and TRE:mCherry AAV1/2s. At DIV18, neurons were stimulated with light (467 nm, 60 mW/cm^2, 33% duty cycle (2 s every 6 s)) and high Ca^{+2} (5-s electrical trains consisting of 32 1-ms 50 mA pulses at 20 Hz) for 15 minutes. 18 hours later, neurons were fixed, immunostained with anti-VP16 antibody and imaged by confocal microscopy. Scale bars, 10 µm.



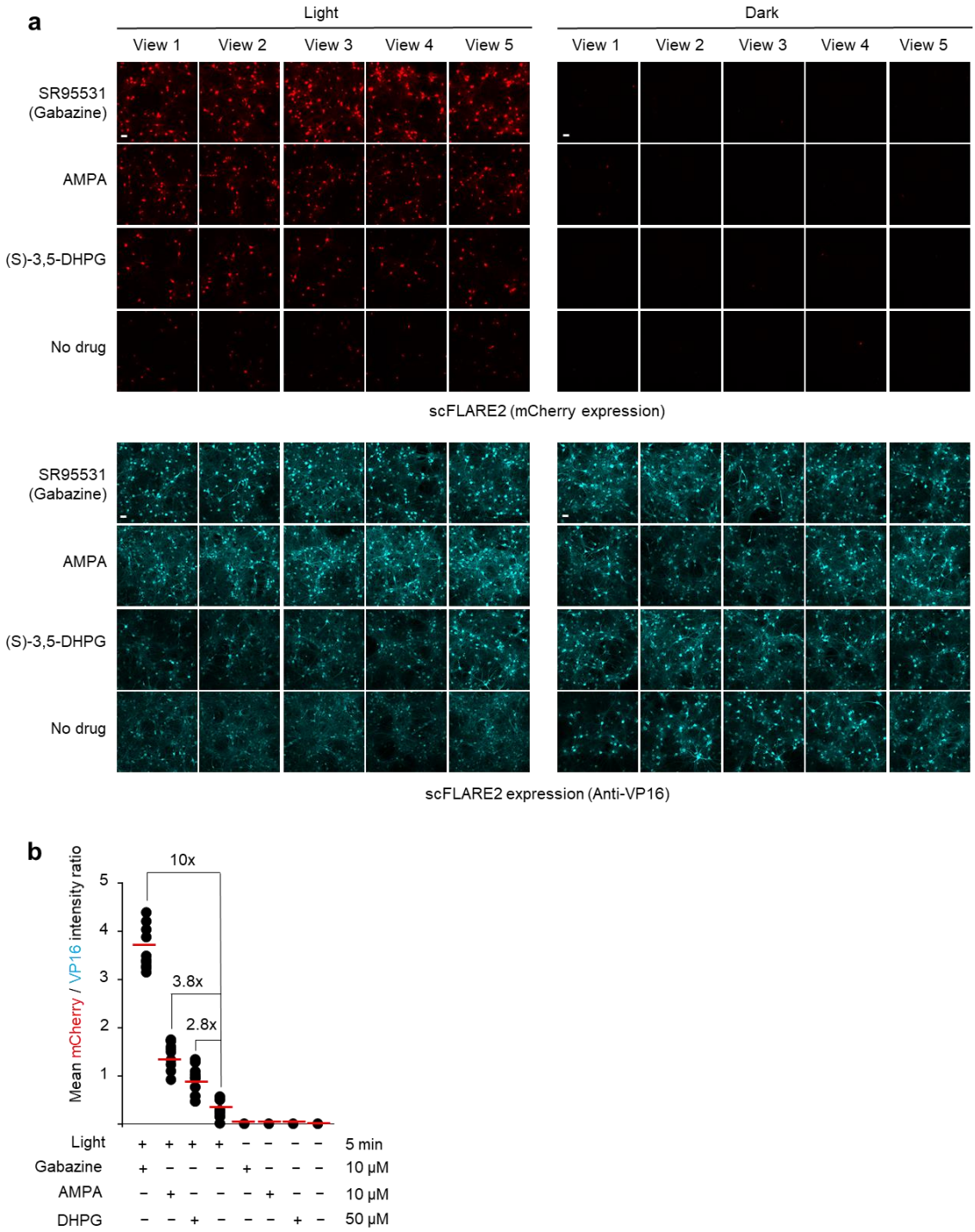
Supplementary Figure 6. Reversibility of CaTEV protease in scFLARE2. a) Rat cortical neurons were transduced with scFLARE2 and TRE:mCherry AAV1/2s. At day 18 in vitro (DIV18), neurons were stimulated electrically (6-s trains consisting of 32 1-ms 50 mA pulses at 20 Hz) in the presence of blue light (467 nm, 60 mW/cm^2, 33% duty cycle (2s light every 6s)) for a total of 15 min. In the case of sequential inputs, a 1min pause separated the two inputs 18 hours later, cells were fixed, permeabilized, stained with anti-VP16 antibody, and imaged. This experiment was repeated 2 times. Scale bars, 10 µm. **b**) Quantification of experiment in (a). **c**) Quantification of experiment in Figure 3E, 10 fields of view per condition.



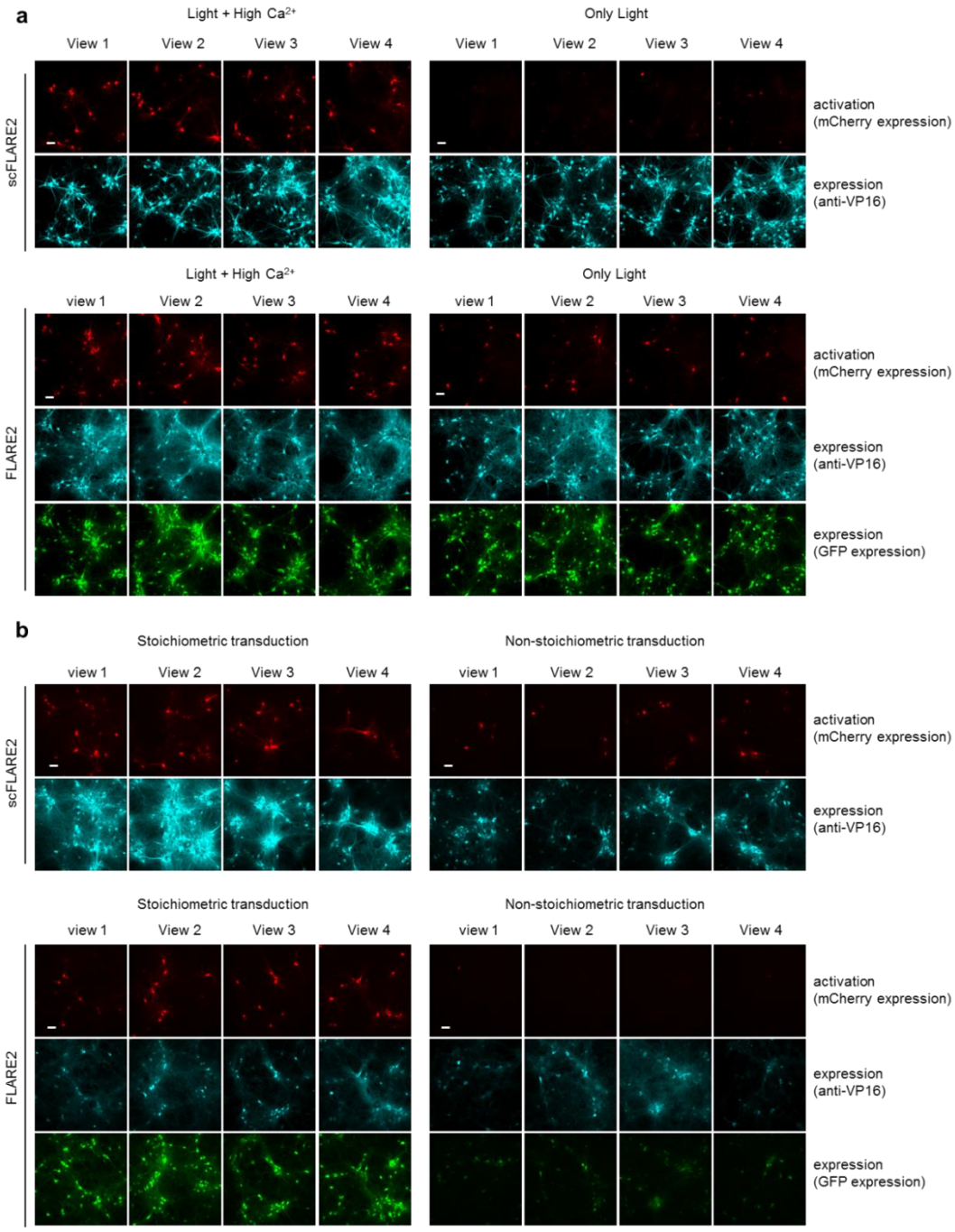
Supplementary Figure 7. scFLARE2 time course. Fluorescence images used to generate plot in Figure 3F. (5 fields of view shown). Rat cortical neuron cultures were transduced with scFLARE2 and TRE:mCherry AAV1/2s. At DIV18, neurons were stimulated with light (467 nm, 60 mW/cm^2, 33% duty cycle (2 s every 6 s)) and high Ca^{2+} (5-s electrical trains consisting of 32 1-ms 50 mA pulses at 20 Hz) for the indicated times. 18 hours later, neurons were fixed, immunostained with anti-VP16 antibody and imaged by confocal microscopy. This experiment was performed two times. Scale bars, 10 μm.



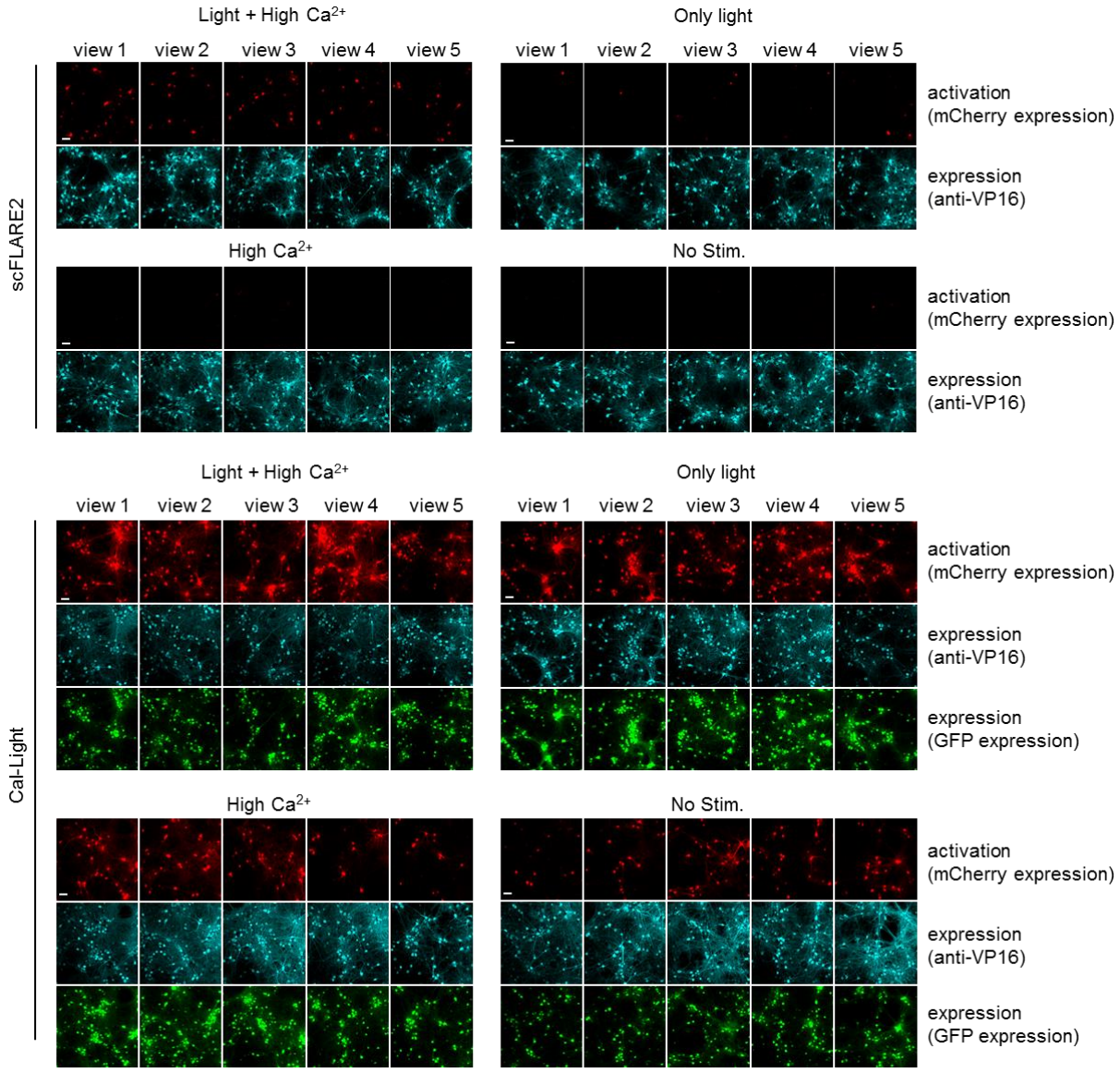
Supplementary Figure 8. scFLARE2 activation is dependent on stimulation frequency. a) Fluorescence images used to generate plot in Figure 3H. Rat cortical neuron cultures were transduced with scFLARE2 and TRE:mCherry AAV1/2s. At DIV18, neurons were stimulated for a total of 15 minutes with light (467 nm, 60 mW/cm^2, 33% duty cycle (2 s every 6 s)) and electrical trains (32 1-ms 50 mA pulses at 20 Hz) where the interval between trains varied by the indicated times (2 sec between trains, 5 sec between trains, etc.). 18 hours later, neurons were fixed, immunostained with anti-VP16 antibody and imaged by confocal microscopy. This experiment was performed two times. Scale bars, 10 μm. **b**) Schematic showing the pattern of electrical trains used to stimulate cultured neurons. Each train consists of 32 1-msec 48 mA pulses at 20 Hz (50 msec between each pulse). Our standard conditions space the trains 6 seconds apart. For high-frequency stimulation, the trains are spaced only 2 seconds apart. **c**) GCaMP fluorescence time courses for rat cortical neurons expressing GCaMP5 [6] and stimulated as in (b).



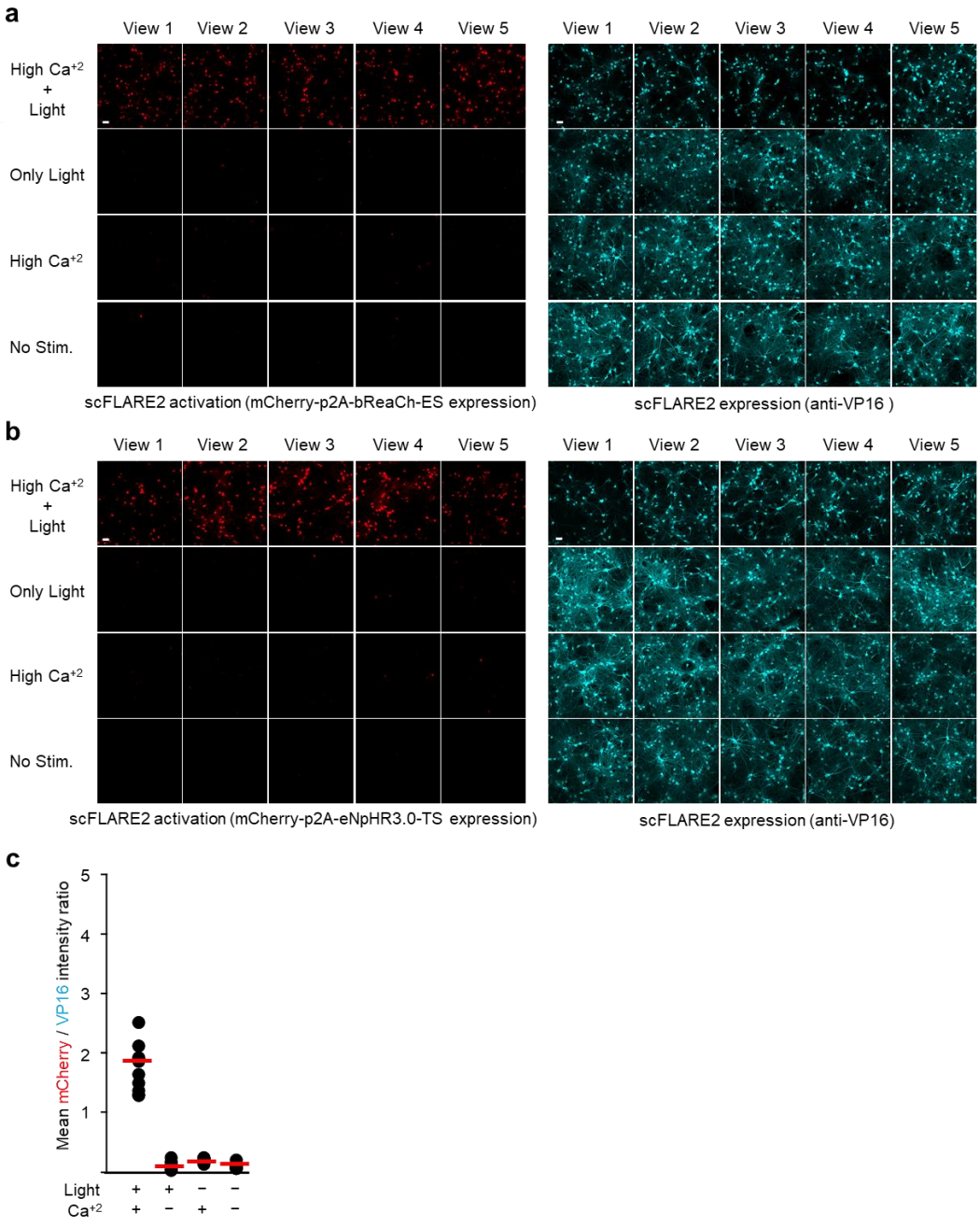
Supplementary Figure 9. scFLARE2 labeling of neurons in response to drug stimulation. a) Rat cortical neuron cultures were transduced with AAV1/2s encoding scFLARE2 and TRE:mCherry. At DIV18, neurons were illuminated with blue light (467 nm, 60 mW/cm^2, 33% duty cycle (2 s every 6 s)) in the presence of SR95531 (10 μM), AMPA (10 μM), or (S)-3,5-DHPG (50 μM) for 5 min. 18 hours later, neurons were fixed, immunostained with anti-VP16 antibody and imaged by confocal microscopy. Scale bars, 10 μm. **b**) Quantification of experiment in (a).



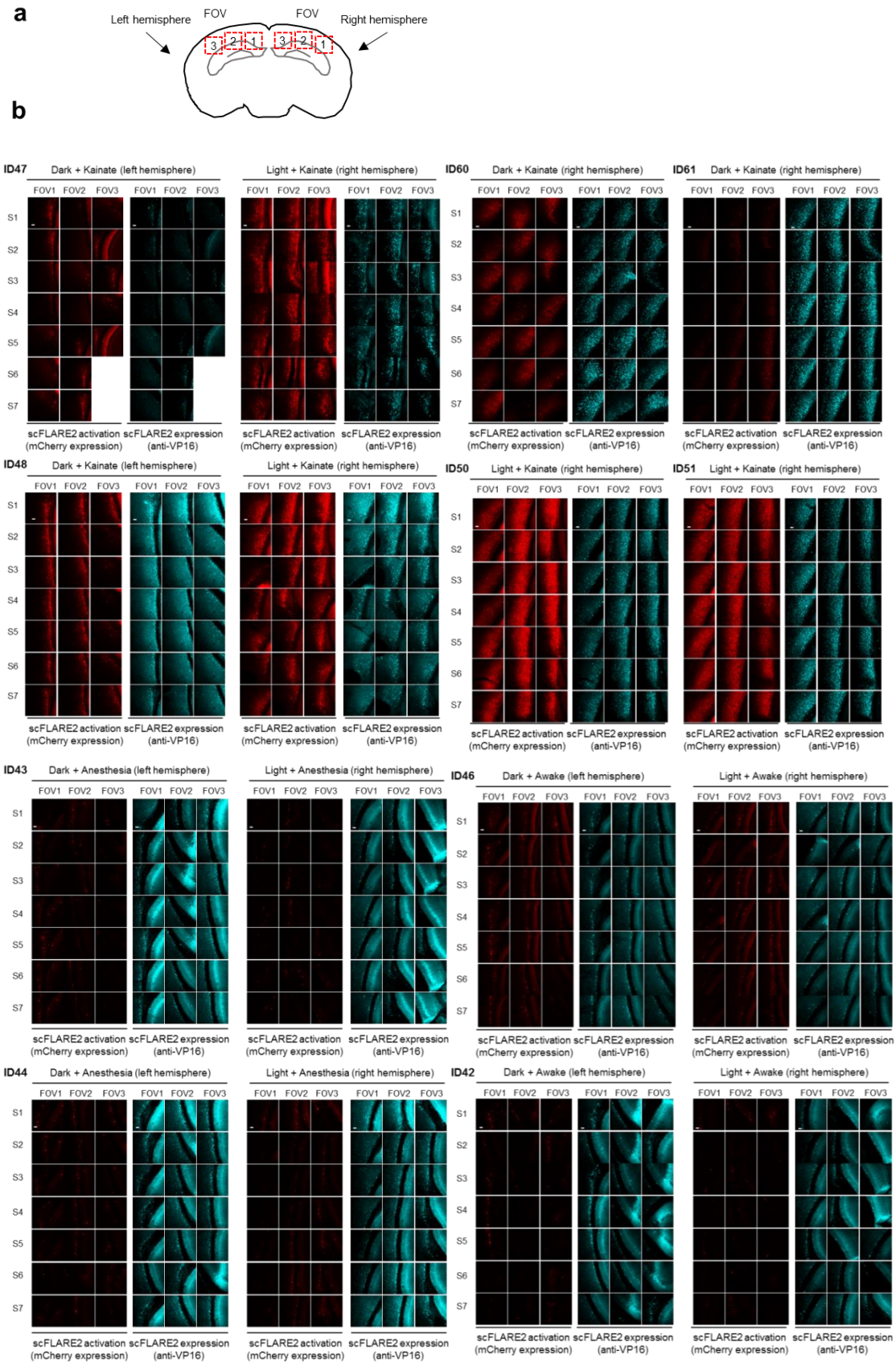
Supplementary Figure 10. FLARE2 and scFLARE2 performance at (a) high and (b) non-stoichiometric expression levels. Related to Figure 4C. **a**) Rat cortical neuron cultures were transduced with TRE:mCherry and scFLARE2 or FLARE2 concentrated AAV1/2 viruses to achieve higher expression levels. At DIV18, neurons were stimulated with light (467 nm, 60 mW/cm2, 33% duty cycle (2 s every 6 s)) and high Ca^{2+} (5-s electrical trains consisting of 32 1-ms 50 mA pulses at 20 Hz) for 15 minutes. 18 hours later, neurons were fixed, immunostained with anti-VP16 antibody and imaged by confocal microscopy. This experiment was repeated 2 times. Scale bars, 10 μm. **b**) Same as (a) but we used non-stoichiometric amounts of FLARE2 viruses (0.25:1 ratio of protease:TF rather than 1:1 ratio of protease:TF). This experiment was performed once. Scale bars, 10 μm.

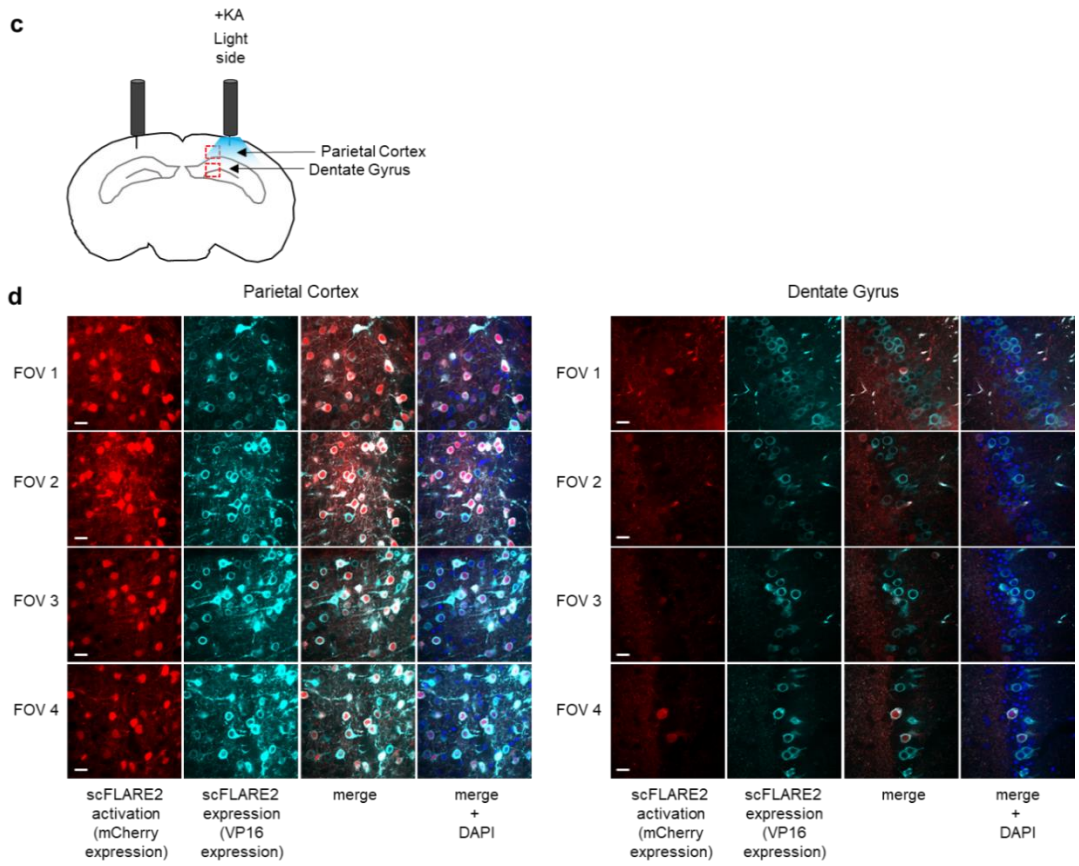


Supplementary Figure 11. Comparison of scFLARE2 to Cal-Light. Fluorescence images used to generate plot in Figure 4F. (5 fields of view shown) Rat cortical neurons were transduced with AAV1/2s encoding scFLARE2 and Cal-Light. At day 18 in vitro (DIV18), neurons were stimulated electrically (6-s trains consisting of 32 1-ms 50 mA pulses at 20 Hz) in the presence of blue light (467 nm, 60 mW/cm^2, 33% duty cycle (2s light every 6s)) for a total of 15 min. 18 hours later, cells were fixed, permeabilized, stained with anti-VP16 antibody, and imaged. Scale bars, 10 µm.

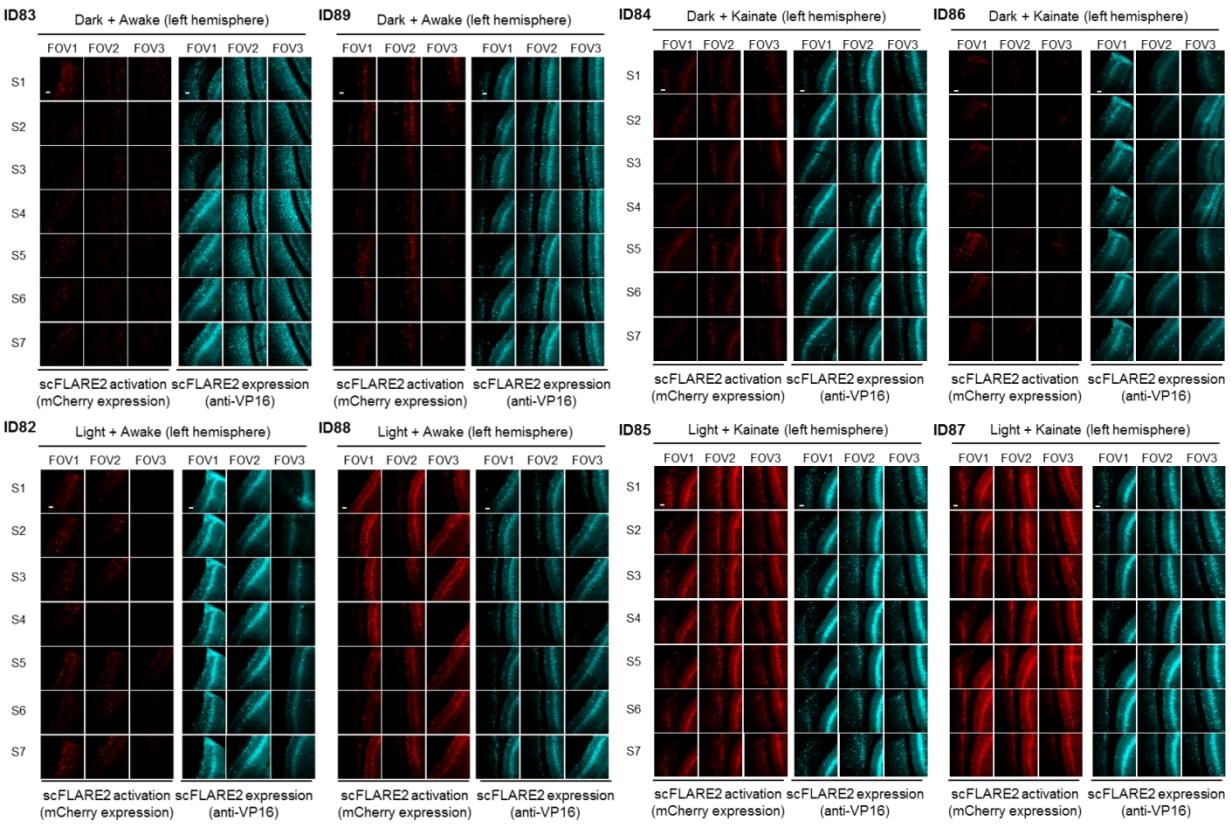


Supplementary Figure 12. scFLARE2 drives the activity-dependent expression of channelrhodopsin. Related to Figure 5. Rat cortical neurons were transduced with AAV1/2s encoding scFLARE2 and TRE:mCherry-p2A-bReaChES (**a**) or TRE:mCherry-p2A-eNpHR3.0-TS (**b**). At day 18 in vitro (DIV18), neurons were stimulated electrically (6-s trains consisting of 32 1-ms 50 mA pulses at 20 Hz) in the presence of blue light (467 nm, 60 mW/cm^2, 33% duty cycle (2s light every 6s)) for a total of 15 min. 18 hours later, cells were fixed, permeabilized, stained with anti-VP16 antibody, and imaged. Scale bars, 10 µm. **c**) Quantification of experiment in (b).


a
Left hemisphere
FOV
FOV
Right hemisphere
3
2
1
3
2
1
b
ID47
Dark + Kainate (left hemisphere)
Light + Kainate (right hemisphere)
ID60
Dark + Kainate (right hemisphere)
ID61
Dark + Kainate (right hemisphere)
ID48
Dark + Kainate (left hemisphere)
Light + Kainate (right hemisphere)
ID50
Light + Kainate (right hemisphere)
ID51
Light + Kainate (right hemisphere)
ID43
Dark + Anesthesia (left hemisphere)
Light + Anesthesia (right hemisphere)
ID46
Dark + Awake (left hemisphere)
Light + Awake (right hemisphere)
ID44
Dark + Anesthesia (left hemisphere)
Light + Anesthesia (right hemisphere)
ID42
Dark + Awake (left hemisphere)
Light + Awake (right hemisphere)
FOV1 FOV2 FOV3
S1
S2
S3
S4
S5
S6
S7
scFLARE2 activation (mCherry expression)
scFLARE2 expression (anti-VP16)



Supplementary Figure 13. Confocal fluorescence images showing scFLARE2 direct activation in the mouse brain. a) Schematic of the region where viruses were injected, and pictures taken. Each row shows 3 consecutive fields of view. Scale bars, 10 μm**. b**) These micrographs were used to generate the graphs and quantitation shown in Figures 6D. **c**) Schematic of the regions where high-magnification images were taken. **d**) Confocal images of parietal cortex and dentate gyrus. Scale bars, 10 μm.



Supplementary Figure 14. Confocal fluorescence images showing scFLARE2 indirect activation in the mouse brain. Related to Figure 6F. mCherry expression and corresponding VP16 (FLARE expression marker) images in brain sections from mice treated as in Figure 6E. Each row shows 3 consecutive fields of view. Scale bars, 10 μm.

Movie legends

- S. Video 1-Cortical rat neurons expressing GCaMP5 Basal activity.
- S. Video 2-Cortical rat neurons expressing GCaMP5 Electrical Stim Interval 2 sec.
- S. Video 3-Cortical rat neurons expressing GCaMP5 Electrical Stim Interval 5 sec.
- S. Video 4-Cortical rat neurons expressing GCaMP5 Electrical Stim Interval 10 sec.
- S. Video 5-Cortical rat neurons expressing GCaMP5 Electrical Stim Interval 20 sec.
- S. Video 6-Cortical rat neurons expressing GCaMP5 treated with SR95331.
- S. Video 7-Cortical rat neurons expressing GCaMP5 treated with AMPA.
- S. Video 8-Cortical rat neurons expressing GCaMP5 treated with (S)-3,5-DHPG.

Rat cortical neurons were transduced with GCaMP5 AAV1/2s. At day 18 in vitro (DIV18), neurons were stimulated electrically (32 1-ms 50 mA pulses at 20 Hz) at different trains intervals (2, 5, 10 and 20 sec between trains) or treated with indicated drugs. The GCaMP fluorescence signal was recorded. Basal activity (no electrical stimulation or drug) was also recorded.

Plasmid legends

ID	Plasmid name	Plasmid Vector	Promoter	Terminator	Expression in	Tags	Ab. R	Used in figures	Comments
p1	CD4-CIBN-CaTEVΔ52-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p2	CD4-CIBN-CaTEVΔ61-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, 1G, S. Figure 1	
p3	CD4-CIBN-CaTEVΔ67-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p4	CD4-CIBN-CaTEVΔ70-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p5	CD4-CIBN-CaTEVΔ78-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p6	CD4-CIBN-CaTEVΔ97-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p7	CD4-CIBN-CaTEVΔ106-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p8	CD4-CIBN-CaTEVΔ119-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p9	CD4-CIBN-CaTEVΔ136-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p10	CD4-CIBN-CaTEVΔ145-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p11	CD4-CIBN-CaTEVΔ159-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p12	CD4-CIBN-CaTEVΔ184-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p13	CD4-CIBN-CaTEVΔ203-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, S. Figure 1	
p14	CD4-CIBN-CaTEVΔ-S153N-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, 1G, 1H, 2D	scFLARE1 (Addgene #158738)
								S. Figure 3	
p15	CD4-CIBN-CaTEVΔ-T30A-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, 1G	
p16	CD4-CIBN-CaTEVΔ-T30A/S153N-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 1F, 1G	
p17	CD4-CIBN-CaTEVΔ-S153N-M13-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 2	
p18	CD4-CIBN-CaTEVΔ-S153N-M13-W391F-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 2	
p19	CD4-CIBN-CaTEVΔ-S153N-M13-W391L-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 2	
p20	CD4-CIBN-CaTEVΔ-S153N-M13-V398T-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 2	
p21	CD4-CIBN-CaTEVΔ-S153N-M13-G395D-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 2	
p22	CD4-CIBN-CaTEVΔ-S153N-M13-W391Y-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 2	
p23	CD4-CIBN-CaTEVΔ-S153N-M13-V398D-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 2	
p24	CD4-V5-CIBN-CaTEVΔ-S153N-hLOV-(No TEVcs)-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 2D	
p25	CD4-HA-CIBN- Dead CaTEVΔ-S153N-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	Figure 2D	
p26	CD4-CIBN-CaTEV-S153N-hLOV-GAL4-p2A-eGFP	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5 / eGFP	Amp	Figure 2A	scFLARE1-p2A-eGFP (Addgene #158699)
p27	eGFP-CaM-uTEV1Δ (S153N)	AAV	CMV	PolyA	Mammalian / HEK	eGFP/ V5	Amp	Figure 2A	
p28	eGFP-CaM-uTEV2Δ (T30A/S153N)	AAV	CMV	PolyA	Mammalian / HEK	eGFP/ V5	Amp	Figure 2A. S. Figure 3D	
p29	CD4-CIBN-MKII-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / HA	Amp	Figure 2A	
p30	UAS-mCherry	AAV	CMV	WPRE/PolyA	Mammalian / HEK		Amp	Figure 1G. S. Figure 1, 2, 3	Plasmid #135457
p31	UAS-Luciferase	AAV	CMV	WPRE/PolyA	Mammalian / HEK		Amp	Figure F, G. S. Figure 2B, 3	Plasmid #104840
p32	CD4-CIBN-CaTEVΔ-S153N-EF1-A4-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 3	
p33	CD4-CIBN-CaTEVΔ-S153N-EF2-A4-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 3	
p34	CD4-CIBN-CaTEVΔ-S153N-EF3-A3-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 3	
p35	CD4-CIBN-CaTEVΔ-S153N-EF3-A4-hLOV-GAL4	AAV	CMV	PolyA	Mammalian / HEK	Flag / V5	Amp	S. Figure 3	
p36	NRX-TM-Nav1.6-CIBN-CaTEVΔ (CaM5)-S153N-tTA	AAV	Synapsin	PolyA	Neuronal	HA/VP16	Amp	S. Figure 4, 5	
p37	NRX-TM-Nav1.6-CIBN-CaTEVΔ (CaM6s)-S153N-tTA	AAV	Synapsin	PolyA	Neuronal	HA/VP16	Amp	S. Figure 5	
p38	NRX-TM-Nav1.6-CIBN-CaTEVΔ (CaM7s)-S153N-tTA	AAV	Synapsin	PolyA	Neuronal	HA/VP16	Amp	Figure 3D, 3E, H.	scFLARE2 (Addgene #158700)
								s. Figure 5-14	
p39	NRX-TM-Nav1.6-CIBN-CaTEVΔ (D3)-S153N-tTA	AAV	Synapsin	PolyA	Neuronal	HA/VP16	Amp	S. Figure 5	
p40	NRX-TM-Nav1.6-CIBN-CaTEVΔ (D4)-S153N-tTA	AAV	Synapsin	PolyA	Neuronal	HA/VP16	Amp	S. Figure 5	
p41	NRX-TM-Nav1.6-CIBN-CaTEVΔ (EF16)-S153N-tTA	AAV	Synapsin	PolyA	Neuronal	HA/VP16	Amp	S. Figure 5	
p42	NRX-TM-Nav1.6-CIBN-CaTEVΔ (EF20)-S153N-tTA	AAV	Synapsin	PolyA	Neuronal	HA/VP16	Amp	S. Figure 5	
p43	TRE:mCherry	AAV	TRE/CMVm	WPRE/PolyA	Mammalian / neuronal		Amp	S. Figure 5, 6, 7, 8, 9	Plasmid #92202
p44	NRX-TM-Nav1.6-CIBN-hLOV-TEVcs-tTA	AAV	Synapsin	PolyA	Neuronal	HA/VP16	Amp	Figure 3H. S. Figure 4, 8	
p45	eGFP-CaM-uTEV1Δ (S153N)	AAV	Synapsin	PolyA	Neuronal	eGFP/ V5	Amp	Figure 3H. S. Figure 4, 8	
p46	TRE-mcherry-p2A-bReaches-TS	AAV	TRE/CMVm	WPRE/PolyA	Neuronal	mCherry/ HA	Amp	Figure 5	Reporter gene (Addgene #158701)
p47	TRE-mcherry-p2A-Chrimson-TS	AAV	TRE/CMVm	WPRE/PolyA	Neuronal	mCherry/ HA	Amp		Reporter gene (Addgene #158702)
p48	TRE-mcherry-p2A-eNpHR3.0-TS	AAV	TRE/CMVm	WPRE/PolyA	Neuronal	mCherry/ HA	Amp	S. Figure 9	Reporter gene (Addgene #158703)
p49	AAV1						Amp		
p50	AAV2						Amp		
p51	DF6						Amp		

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