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Supplementary Information for

Reversible silencing of endogenous receptors in intact brain tissue using two-photon pharmacology

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Supplementary Materials and Methods

1. Diffusible photoswitches and other drugs.

Freely-diffusible photoswitchable allosteric modulators of $mGlu_5$ receptors were obtained as previously described for alloswitch **(1)** and compounds **1c-d**, **1j** and **6 (2)**. Other drugs used (quisqualate, DHPG, LY367385, TTX) were purchased from Tocris.

2. Microscopy, light sources, and general imaging conditions.

Imaging of cultured cells and brain slices, and photostimulation of alloswitch and its analogs were performed using an inverted laser-scanning confocal microscope (TCS SP5, Leica Microsystems) equipped with a HCX PL APO 40×/1.25-0.75-NA oil objective for imaging cultured cells, and a HC PL APO 20×/.0.7-NA CS air objective (Leica Microsystems) for imaging acute slices. The pulsed laser (PL) used for two-photon excitation (2PE) of alloswitch and Ca^{2+}-imaging was an IR laser MaiTai Wide Band (710–990 nm, Spectra Physics). Continuous-wave lasers (CWLs) used were: a LASOS 405-50 50 mW 405 nm laser diode for one-photon excitation (1PE) of compounds, and a LASOS LGK7872 65 mW Argon laser for visualization of OGB-1, GCaMP6s, or eYFP-tag on cells (488/514/561 nm lines, respectively). For all wavelengths, images were acquired by bidirectional raster scan at 400 Hz. For cultured cells, image size was 387.5×387.5 µm, pixel size 1.5 µm, and pixel dwell time 9.8 µs. For slices, image size was 775×775 µm and 512×512 pixels; pixel size and dwell time were 1.5 µm and 4.9 µs (or 3.0 µm and 9.8 µs for 256×256 pixels images). For experiments where a 2.5× optical zoom was applied, image and pixel size were 310 µm and 607 nm respectively, whereas dwell time was unchanged. Emitted fluorescence was collected using hybrid detectors. Frame rates and laser powers used for specific experiments are detailed in the corresponding sections.

3. Cell culture procedures and imaging.

3.1. Plasmids:

For "all-2P" experiments (2P-imaging of Fura-2 combined with 2P-photostimulation of alloswitches), we used a plasmid where the $mGlu_5$ receptor was in fusion with a fluorescent reporter ($mGlu_5$-eYFP; **(3)**) to visualize expressing cells and identify false-positives for photostimulation. For experiments combining 1P-imaging with 1P- or 2P-photostimulation, we co-transfected cells with a plasmid for GCaMP6s under a ubiquitous promoter for Ca^{2+}-imaging (Addgene, plasmid #40753) and a plasmid encoding for the $mGlu_5$ receptor lacking eYFP **(1)** to avoid bleed-through into the Ca^{2+}-reporter channel.

3.2. Cell culture and transfection:

HEK tsA201 cells were maintained at 37 ºC and 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco DMEM/F-12, Thermo Fisher Scientific 11320) with 10% Fetal Bovine Serum (Gibco heat-inactivated FBS, Thermo Fisher Scientific 10500-056-100ml) and 1% antibiotics (Penicillin/Streptomycin, Sigma-Aldrich P4333). We induced transient expression of the plasmids in 10^6 cells resuspended in 2 ml using the polymer X-tremeGENE 9 DNA Transfection Reagent (Roche Applied Sciences 06365787001) following manufacturer's instructions (1 µg total DNA and 3 µl reagent in 100 µl FBS-free culture medium). The Ca^{2+} reporter was co-transfected with the receptor in a proportion of 1:1, with other conditions unchanged. Cells were seeded onto 21-mm glass coverslips treated with Poly-L-Lysine (Sigma-Aldrich P4832) to allow cell adhesion. Pre-confluent cultures were used for experiments at 48 to 96 hours after transfection. Coverslips were mounted on an Attofluor cell chamber (Thermo Fisher scientific A7816) in bath solution containing (in mM): 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 HEPES, 10 D-glucose and 2 $CaCl_2$; pH was adjusted to 7.40 using NaOH.

3.3. "All-2P" experiments workflow (2P-imaging of Fura-2 in $mGlu_5$-eYFP cells):

3.3.1. Fura-2 loading:

Cells expressing $mGlu_5$-eYFP were loaded with the Ca^{2+}-indicator Fura-2 AM (10 µM, Thermo Fisher Scientific F1201) diluted in culture medium and 1% DMSO for 30 minutes in the cell incubator (37 °C and 5% CO_2), and then rinsed.

3.3.2. Identification of $mGlu_5$-eYFP-expressing HEK cells:

To double-check for $mGlu_5^+$ cells, fluorescence images of the eYFP-tag on the receptor were acquired using the 514-nm line of the Argon laser or the pulsed laser set at 900 nm.

3.3.3. 2P Ca^{2+}-imaging using Fura-2:

Fura-2 was excited with the pulsed laser at a frame rate of 0.2 Hz at NIR wavelengths (760, 780, 800, or 820 nm, see example traces) (**SI Appendix**, **Fig. S1**), approximately corresponding to twice the wavelength (380–410 nm) of 1P excitation with continuous-wave lasers. Fura-2 was used in the single-wavelength (non-ratiometric) modality, to accommodate fast imaging rates of the Ca^{2+} indicator. Using this setup, increases in intracellular Ca^{2+} are detected as decreases in the fluorescence emission of Fura-2 (consistent with its emission spectra following 380 nm excitation) (**SI Appendix, Fig. S1A**). Laser power was set to the minimal value allowing satisfactory visualization of Fura-2 (~3 mW on sample for all wavelengths used), to minimize potential 2PE-isomerization of alloswitch during imaging. Emitted fluorescence was collected using hybrid detectors with spectral range 420–515 nm and standard gain settings.

3.4. 1P-imaging of GCaMP6s/$mGlu_5$ co-transfected cells:

GCaMP6s fluorescence was monitored using the 488 nm line of the Argon laser, with light intensity adjusted to the lowest power allowed by the system (100 nW on sample) to avoid *cis*-to-*trans* photoisomerization of alloswitch. Images of GCaMP6s were acquired at a frame rate of 0.2 Hz. The pinhole of the confocal system was set at the maximum aperture (optical section thickness was 7.4 µm), and the emitted fluorescence was collected using hybrid detectors (490–550 nm). In a set of experiments, cell morphology and thickness was detected by imaging GCaMP6s at 900 nm using the pulsed laser.

4. Acute and organotypic rodent brain slices procedures and imaging.

4.1. Ethical statement:

Procedures were performed in accordance with the European guidelines for animal care and use in research, and were approved by the Animal Experimentation Ethics Committee at the University of Barcelona (OB-432/16).

4.2. Dissection of acute brain slices:

Acute brain slices were obtained from Sprague-Dawley rats (Envigo) of 6 to 15 postnatal days (P6–15). Animals were sacrificed by decapitation to remove the brain. Coronal slices (350-µm thick) were obtained in ice-cold cutting solution containing (in mM) 27 $NaHCO_3$, 1.5 NaH_2PO_4, 2.6 KCl, 222 sucrose, 2 $MgSO_4$ and 2 $CaCl_2$ and saturated with 95% O_2 and 5% CO_2, using a vibrating-knife microtome (1000S Vibratome, Leica). Hippocampal slices were collected in a holding chamber filled with artificial cerebrospinal fluid (aCSF) containing (in mM) 126 NaCl, 26 $NaHCO_3$, 1.145 NaH_2PO_4, 3 KCl, 10 glucose, 2 $MgSO_4$ and 2 $CaCl_2$, saturated with 95% O_2 and 5% CO_2. Slices were recovered in the holding chamber for at least 1 hour at RT before OGB-loading, and used not more than 7 hours after sacrifice.

4.3. Hippocampal organotypic slice cultures:

Hippocampal organotypic slices (400-µm thick) were obtained from P7 Sprague-Dawley rats (Janvier Labs) using a tissue chopper (McIlwain TC752) and cultured for 5-7 days-in-vitro (DIV) until biolistically transfected with plasmidic DNA for GCaMP6s (Addgene, plasmid #40753) and DsRed2 (Addgene, plasmid #15777) as described **(4)**. Slices were used for experiments at DIV 7-14.

4.4. Ca^{2+}-imaging of acute brain slices:

4.4.1. Bulk-loading of the Ca^{2+}-indicator Oregon Green BAPTA-1 AM:

A group of 4 slices was loaded with 20 µM OGB-1 (Life Technologies O6807) in oxygenated aCSF containing 0.04% (w/v) Pluronic F-127 (Sigma P2443) and 0.5% DMSO (Sigma 276855). Slices were loaded during 15 minutes at 30º, and for 10 more minutes at RT, and then returned to the holding chamber.

4.4.2. 1P Ca^{2+}-imaging in slices:

For imaging, a slice was transferred into a 35-mm dish chamber (MatTek, P35G-1.0-14-C) along with a dish insert (Warner Instruments, RC-33DL) to allow perfusion with oxygenated aCSF (**SI Appendix, Materials and Methods 4.2**) complemented with 100 µM LY367385 ($mGlu_1$ antagonist, Tocris 1237) and 1 µM TTX (Na_V channel blocker, Tocris 1069) to isolate Ca^{2+}-events solely induced by $mGlu_5$ activation (hereafter: *aCSF+LY/TTX*), and recirculated at 1.5–2 ml/min (Reglo peristaltic pump, Harvard Apparatus, 73-2449) at RT. Calcium events were recorded in *stratum orien*s to *stratum radiatum* of the CA1 region of the hippocampus by exciting OGB-1 with the 488 nm Argon laser set at a frame rate of 1–0.33 Hz and low

power (1.7–5.2 µW) to avoid unwanted *cis*-to-*trans* isomerization of alloswitch. The confocal pinhole was set to obtain optical sections of 6.182–17.5 µm.

4.4.3. Pressure-ejection of the $mGlu_5$ receptor agonist in slices:

Micropipettes with a tip resistance of 3–6 MΩ were prepared from 1.2/0.69-mm OD/ID borosilicate glass capillaries (World Precision Instruments, GC-120F-10) using a pipette puller (Sutter Instruments, P-97), and filled with *aCSF+LY/TTX* (**SI Appendix, Materials and Methods 4.3.2**) containing 1 mM (S)-3,5-DHPG, or 2 mM (*R,S*)-3,5-DHPG ($mGlu_{1/5}$ agonist, Tocris 0805 and 0342 respectively), and the astrocyte marker Sulforhodamine 101 (40 µM, SR101, Sigma S7635). Pipettes were held with a micromanipulator (Narishige MN-153) off the microscope stage, and placed in contact with the slice surface on top of the field of view in a shadow-guided manner using transmitted light. Agonist ejection was initiated manually using a pressure-ejection system (Eppendorf FemtoJet, VWR) set at 500–800 hPa during 1 s.

4.4.4. Identification of $SR101^+$-cells:

$SR101^+$-cells were visualized at 561 nm with the Argon laser to confirm ejection site and identify cell types.

4.5. Ca^{2+}-imaging in organotypic brain slices:

Organotypic slices (DIV 7-14) were perfused with oxygenated aCSF containing (in mM) 119 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 11 mM glucose. 100 µM LY367385 and 10 µM alloswitch were added to recirculating aCSF at least 10 minutes before imaging. 50 µM DHPG was then added to the bathed solution, and intracellular Ca^{2+} mobilization was monitored by exciting GCaMP6s with a 488 nm laser (<3 µW) at a frame rate of 0.5 Hz, using a HC PL APO CS2 63.0x1.40 OIL objective (Leica Microsystems) and 4x optical zoom. Image size was 512×512 pixels, pixel size 120 nm, and pixel dwell time 2.5 µs. To visualize the fine morphology of targeted neurons, z-stack images were acquired at 1 µm steps by exciting DsRed2 with a 561 nm laser (<2.5 µW).

5. Photostimulation of diffusible photoswitches.

5.1. Bath-application of compounds:

In cultured cells, agonist (3 µM quisqualate, Sigma-Aldrich Q2128) and photoswitches (1 µM from 1 mM stocks in DMSO; total DMSO was 0.1%) were supplemented as described previously **(1)**. In brief, compounds were added manually during image acquisition in the recording chamber by thoroughly pipetting a small volume to assure a good mixing of the solutions. In slices, recirculating *aCSF+LY/TTX* was replaced with oxygenated *aCSF+LY/TTX* containing 10 µM alloswitch (in 0.02% DMSO) to block responses of cells to puffing of the $mGlu_5$ agonist. Alloswitches were incubated for at least 7 minutes in both culture and slice experiments to allow silencing of $mGlu_5$ receptors before photostimulation.

5.2. Photostimulation:

Photostimulation at 1PE or 2PE was achieved by raster-scanning the field-of-view with the corresponding lasers, executed through the same light path used for Ca^{2+}-imaging. To do this, raster-scans for photostimulation were interleaved between two consecutive image acquisitions (= imaging cycle) of either Fura-2, GCaMP6s, or OGB-1. Photostimulation scans were repeated consecutively 13 and 10 times per imaging cycle in Fura-2-loaded and GCaMP6s-expressing cells, respectively, and 1 to 3 times per imaging cycle in slices. Image and pixel size, and pixel dwell time for photostimulation were the same as for imaging (**SI Appendix, Materials and Methods 2** and **Fig. S1, Fig. S4, S5 and S7**). Adjustments to the frame rate were introduced by the imaging software to accommodate for hardware changes occurring during video recordings (*i.e.* switching light wavelength and power, and stage or objective movements), and accounted for imaging frame rates reduced to ~0.15 Hz during 2PE in Fura-2-loaded cells, ~0.17 (1PE) and ~0.12 Hz (2PE) in GCaMP6s-cells, and average values of 0.3–0.4 Hz (1PE) and 0.3 Hz (2PE) in slices. All adjustments were recorded and considered at the time of data analysis and plotting, but are not represented in the movies shown (**SI Appendix**, **Movies 1–4**).

5.2.1. 1PE:

One-photon excitation (1PE) was accomplished using the 405 nm diode laser set at 2 µW in GCaMP6s-HEK cells, and 58–98 µW in slices (power measured after objective lens).

5.2.2. 2PE:

The light source for two-photon excitation (2PE) was the pulsed IR laser MaiTai Wide Band tuned at 780 nm, unless otherwise indicated. Laser power was adjusted to be 12 mW on sample in experiments with Fura-

2-loaded cells, 20 mW for GCaMP6s-expressing cells, and 25 mW for slices (powers measured after objective lens using a thermal-head power sensor; L30A-V1, Ophir Photonics). Note that in experiments where 2PE of compounds was combined with 2P-imaging of Fura-2, the software used for imaging and photostimulation arbitrarily increased the light intensity set for the imaging scans, which resulted in increased fluorescence of the sample, but did not correspond to a real change in intracellular Ca^{2+}, and neither masked the photostimulated Ca^{2+}-oscillations (**SI Appendix, Fig. S1D–E**). This increase in baseline fluorescence was not *post-hoc* corrected in any of the cell traces shown.

5.2.3. Photostimulation at axial distances other than the focal plane:

In experiments with cultured cells, photostimulation at varying axial distances was achieved by moving the motorized stage downwards, thus shifting the focal plane for 1PE or 2PE above the cells and inside the bath containing the photoswitches. For slice experiments, the presence of the ejection pipette prohibited any stage movement, thus photostimulation at axial distances above and below the imaging plane was achieved by moving the objective in either direction. Image settings and light powers were as already detailed above.

5.2.4. 2PE of cellular compartments:

For local 2PE in organotypic slices, a region covering the apical or basal dendritic trunk of a CA1 neuron (12×52 µm) was excited by pulsed illumination (780 nm, 12 mW at 1.5 Hz, 90 repetitions) by using the FRAP module of the Leica SP5 confocal system.

6. Image analysis and statistics.

6.1. Analysis of Fura-2 fluorescence and 2PE in cultured cells:

Cell profiles were defined in ImageJ, and the average fluorescence intensity for each time-point was extracted (F). For each identified cell, fluorescence changes (indicated as dF/F in the figures) relative to the first frame of each series (F_0) were calculated as $(F-F_0)/F_0$ and plotted in Excel with a custom-written VBA macro. Cells showing Ca^{2+}-events after bath-application of alloswitches and before photostimulation, if any, were excluded from further analysis. Cells displaying a single Ca^{2+}-event during 2PE were not considered as successfully excited. Only cells showing two or more Ca^{2+}-peaks were classified as responsive and considered for counting the number of peaks (# oscillations), identifying peak time of first, second and last oscillation observed (first time-point where the fluorescence signal clearly decreased relative to basal, indicating an increase in the intracellular Ca^{2+}, and before the minimum fluorescence was observed, corresponding to the peak amplitude of the Ca^{2+} response). These values were used to calculate the latency to the appearance of the first peak from the photostimulation onset (latency), the time between first and last peak (duration) and their average frequency (= # oscillations/duration). After all responding cells had been assigned a latency time and oscillatory frequency, we loaded the eYFP channel images to cross-check for transfected cells. No responding cells were found to lack $mGlu_5$-eYFP expression.

6.2. Analysis of GCaMP6s-fluorecence and 1PE/2PE in cultured cells:

Cell profile and fluorescence intensities were obtained as described in the previous section (**SI Appendix, Materials and Methods 6.1**) for all cells displaying fluorescence changes at any time-point. Cells with unstable fluorescence in the absence of stimulation were discarded. In experiments where both 1PE and 2PE were done sequentially on the same cell, cells were further sorted if showing inconsistent oscillatory responses to 1PE, defined as either having ≤2 peaks/5 minutes, or when 1PE responses were not successfully reproduced in the second 1PE photostimulation (1PE at the focal plane). The remaining cells were classified as '*1P-oscillating*' or '*1P-CaEvents*' and pooled to count total number of cells analyzed. Cells were further classified in '*2P-oscillating-0µm*', '*2P-CaEvents-0µm*', '*2P-CaEvents-10µm*' or '*2P-NOresponse*', to determine % of photoactivated ('*oscillating*' + '*CaEvents*') and oscillating cells ('*oscillating*' only) over the total number of cells considered for analysis. No cells were found active at 2PE and inactive at 1PE. No cells were found to display Ca^{2+}-events when 2PE was done 30 µm above the cell plane, or events in the form of oscillations 10 or 30 µm away from the cells, and the corresponding categories were not created. For both experiment types, traces of cells showing oscillatory behaviors were analyzed using Igor Pro (WaveMetrics) to determine: first time-point above basal Ca^{2+} (t_{ON}), number of oscillatory events observed (# oscillations), and last time-point before return to baseline or stop of oscillatory behavior (t_{OFF}). These data were used to calculate the time delay between the beginning of the photostimulation and the onset of the oscillatory behavior (latency), overall duration of the oscillatory behavior (duration = t_{ON} - t_{OFF}), and number (#

oscillations) and average frequency of calcium oscillations (freq. of oscillations = # oscillations / duration). Graphs and statistics were generated with Prism (GraphPad).

6.3. Analysis of responses to 1PE/2PE in slices:

Acute slices. To assist OGB fluorescence image analysis for 2PE experiments in slices, an ImageJ macro was written at ADMCF (Advanced Digital Microscopy Core Facility, IRBB) which detects cell somata after noise reduction and registration (as required), extracts fluorescence changes relative to baseline (dF/F) over time, and classifies cells as responsive versus non-responsive based on the presence or absence of significant fluorescence peaks above noise following stimulation. Code and detection pipeline, together with a sample time-lapse are available online (https://sites.google.com/a/irbbarcelona.org/adm/image-j-fiji#TOC-Somata-segmentation-and-time-response-analysis-in-acute-rodent-brain-slices-). For all detected regions-of-interest (ROIs), exported dF/F traces were graphed using a custom-written macro in Excel. Responding and non-responding cells were validated manually for each condition, and then counted as percentages relative to the total number of cells detected in the field of view. First, ROIs displaying spontaneous activity (fluorescence spikes before stimulation) or poor signal-to-noise ratios were manually excluded from further analysis; second, when detectable changes in fluorescence where observed over 20 to 30 frames after agonist ejection, cells were counted as "responding", and otherwise classified as "silent"; finally, the peak amplitude of responding cells was extracted as the maximum dF/F value observed within this frame range. Detection of ROIs with ImageJ, dF/F calculation relative to first frame fluorescence, and cell classification were done manually for a set of lower magnification 1PE experiments (**Fig. 5**) using the same criteria. For experiments where efficacy (number of responding cells) and efficiency (peak amplitude of the cell response) of photoactivation at the focal plane were measured along with those of photostimulation at 4 other axial distances (**Fig. 7** and **SI Appendix, Fig. S7C**), cells that were insensitive to either DHPG or alloswitch-1 (**SI Appendix, Fig. S7B**) were excluded from further analysis.

Organotypic slices. Average intensity of GCaMP6s fluorescence was extracted from regions-of-interest located at three subcellular compartments (cell soma, and initial portion of the apical or basal dendritic trunk) as dF/F. Responses were quantified as dF/F peak amplitude during the 2PE period, and shown as % over baseline.

6.4. Data and statistics:

Data are represented as mean ± s.e.m. unless otherwise indicated. Number of cells, slices and animals used are indicated in figure captions. Graphs were generated with Igor Pro 6 (WaveMetrics) or Prism 5 (GraphPad) and figures with Illustrator CC (Adobe). Statistics performed using Prism software are indicated in figure captions. Gaussian fittings were done using Origin software using data points with amplitudes significantly different from the peak. The full width at half maximum (FWHM) was calculated by multiplying the standard deviation of the fitted curve by a factor of 2.355.

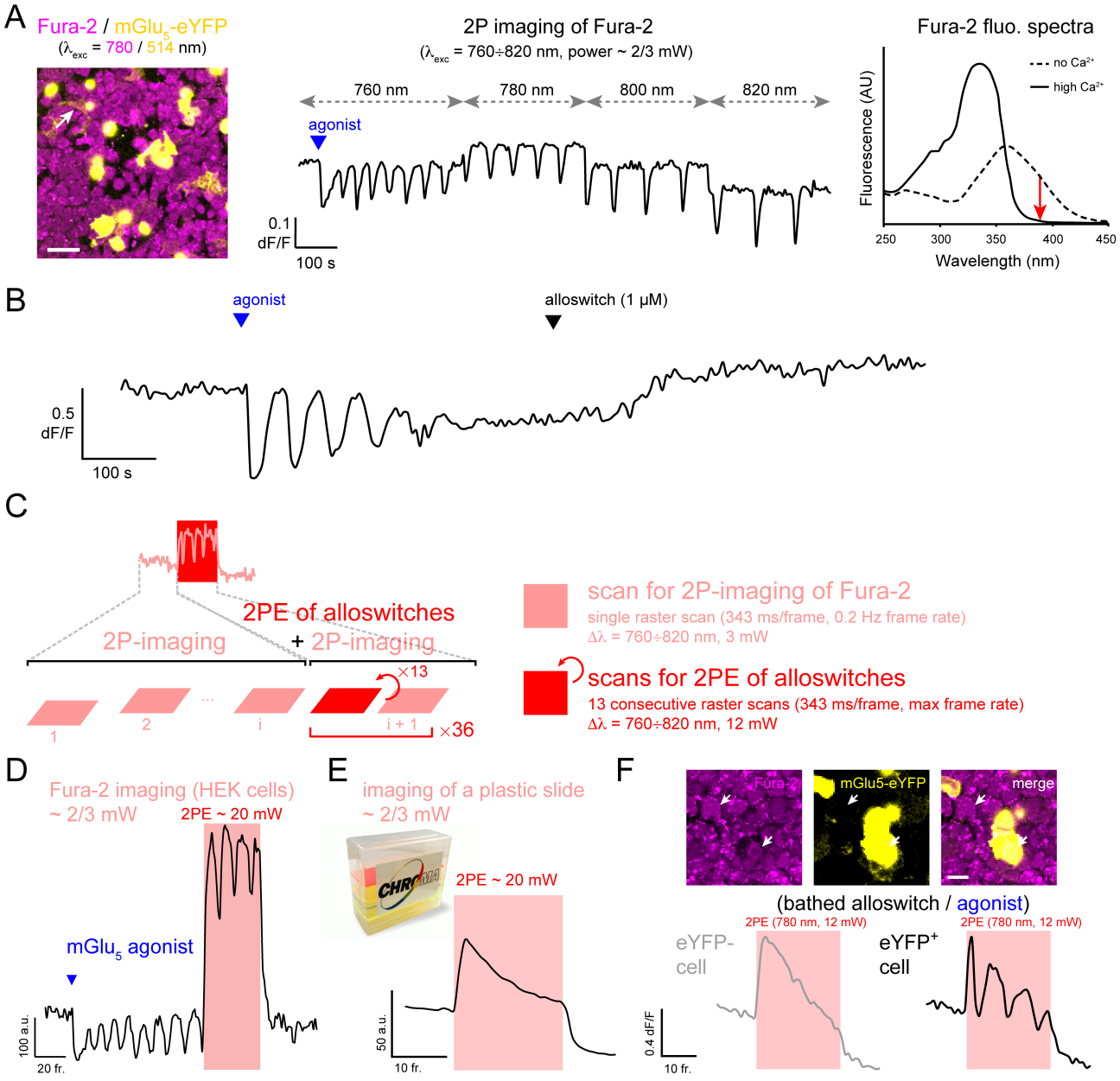

Fig. S1. (related to Fig. 1)

Panel A: Single-wavelength imaging of the Ca^{2+} indicator Fura-2 using a pulsed laser. 2P-imaging of Fura-2 in **Figs. 1–2** of main text was performed in the single-wavelength (non-ratiometric) modality, to accommodate faster rates for imaging of the Ca^{2+} indicator and coincident photostimulation of the compounds at 2P. **Left**: Cultured HEK cells expressing $mGlu_5$-eYFP receptors (yellow) and loaded with the ratiometric Ca^{2+} indicator Fura-2 AM (magenta). Numbers in parenthesis indicate wavelengths used for Fura-2 and eYFP excitation with pulsed and continuous-wave lasers, respectively. Arrowhead points at cell whose Ca^{2+} trace is shown in **B**. Scale bar is 50 μm. **Center**: Fura-2 was excited with the pulsed laser at different NIR wavelengths (760 to 820, in 20 nm steps) and a frame rate of 0.2 Hz. 2PE imaging wavelengths approximately correspond to 380–410 nm of 1PE imaging of Fura-2 with continuous-wave lasers. Therefore, increases in intracellular Ca^{2+} induced by bath-application of agonist (quisqualate, 3 μM) in cultured cells expressing $mGlu_5$ are detected as decreases in Fura-2 fluorescence, consistent with its fluorescence emission at 380 nm (red arrow in right panel). **Right**: UV-vis spectra of Fura-2 excited at

wavelengths indicated by horizontal axis, and in presence of increasing concentrations of free Ca^{2+} (modified from *leica-microsystems.com*). Red arrow illustrates direction of changes in Fura-2 fluorescence upon increases in free Ca^{2+} when the indicator is excited at 380 nm.

Panel B: Silencing of $mGlu_5$ receptors using alloswitch in "all-2P experiments". Ca^{2+} trace in a cultured cell expressing $mGlu_5$ and loaded with Fura-2 (excited with the pulsed laser at 780 nm, frame rate 0.2 Hz). Bath-application of agonist (quisqualate, 3 μM) at time indicated by blue arrowhead induces intracellular Ca^{2+} signaling (downward shift in 780 nm excited fluorescence of Fura-2), which is reverted to baseline after addition of alloswitch (1 μM, black arrowhead).

Panels C–E: "all-2P experiments" schematics (Figs. 1-2) and increased baseline fluorescence of samples during photostimulation. Panel C: Schematics of the imaging-photostimulation setup for "all-2P experiments" (shown in **Figs. 1–2** of main text). Imaging of Fura-2 at 2PE is interleaved with photostimulations of alloswitches at 2PE, and performed at the same wavelength but different power values of the pulsed laser, by raster-scanning with an inverted confocal microscope. For 2PE imaging of Fura-2, low-power (3 mW) and slow frame rates (0.2 Hz) are used, whereas photostimulation of alloswitches is achieved by consecutive raster scans at the same wavelength ($\Delta\lambda$ = 760, 780, 800, or 820 nm) but high power (12 mW) and maximum frame rate. The top trace is an example of a Ca^{2+} imaging session in cultured cells, and zoom-in of the first part of the trace shows sequential imaging scans for visualization of Ca^{2+} dynamics through 2P-imaging of Fura-2. Zoom-in of the photostimulation period (red box) indicates that photostimulation was accomplished by 13 sequential scans at maximum speed and was followed by one imaging scan at the same wavelength but low power; this cycle of "photostimulation+imaging" was repeated 36 times for calculation of oscillatory frequencies during photostimulation. Laser power during imaging scans was set to minimal values allowing satisfactory visualization of Fura-2 (~3 mW on sample for all wavelengths used), with the rationale of minimizing unwanted 2PE of alloswitch molecules due to imaging of Fura-2.

Panels D–E: In "all-2P experiments", steady increases in the basal fluorescence of all samples were observed during photostimulation periods (light-red boxes). These increases reflected increased basal fluorescence of the sample rather than real decreases in intracellular Ca^{2+}, and were due to an arbitrarily increased light power of imaging scans during photostimulation done by the microscope software (see (**SI Appendix, Materials and Methods 5.2.2** for details). To verify this, low-power (3 mW) scans for 2P-imaging of Fura-2 in cultured HEK cells (**D**) or for visualization of a fluorescent plastic slide by Chroma (**E**) were combined with mock high-power (20 mW) 2PE scans during photostimulation periods. Importantly, the increased fluorescence during photostimulation was compatible with the detection of Ca^{2+} oscillations, as illustrated in **panel D**. These increases in baseline fluorescence were not *post-hoc* corrected in any of the cell traces shown throughout the paper. Agonist for $mGlu_5$ activation is quisqualate (3 μM, blue triangle); fluorescence intensity is shown in arbitrary units over frame number.

Panel F: Control photostimulation in $mGlu5^-$ cells. Oscillatory responses characteristic of $mGlu_5$ receptors (**bottom right**) were absent in $mGlu_5$-$eYFP^-$ cells (**bottom left**). Traces correspond to cells indicated by arrows in photomicrographs (**top**; $eYFP^-$ and $eYFP^+$ cell, leftmost and rightmost arrow, respectively). Scale bar, 20 μm. Note that the increase in basal fluorescence during light stimulation is observed in both $eYFP^+$ and $eYFP^-$ cells, and is comparable to that in plastic fluorescent slides (**SI Appendix**, **Fig. S1E**). Experiment was done as in **Fig. 1C** of the main text: cells were bathed with 10 μM alloswitch and 3 μM quisqualate, and photorelease of the $mGlu_5$-bock was achieved by 2PE of alloswitch at 780 nm and 12 mW.

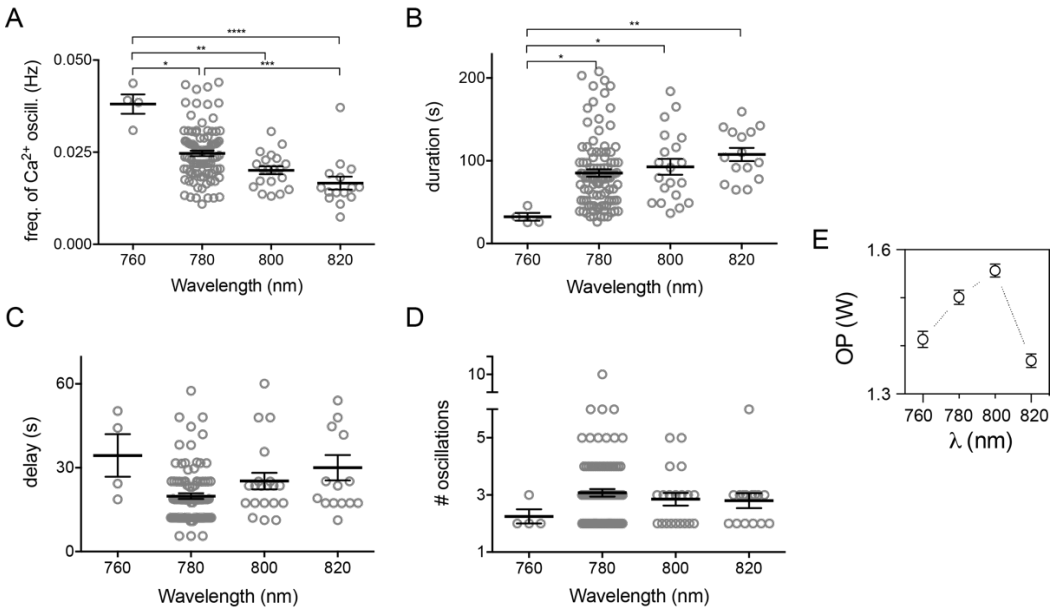

Fig. S2. (related to Fig. 2)

Optimization of 2PE conditions for Fura-2 fluorescence imaging and alloswitch isomerization.

Panels A–D: Full dataset and statistics for functional efficiency of 2PE of alloswitch at different infrared wavelengths (**Fig. 2** of main text). In brief, Fura-2 fluorescence was used to monitor Ca^{2+} oscillations in cultured HEK cells expressing $mGlu_5$-eYFP receptors. Oscillations were photoinduced by 2PE (13 scans, 12 mW laser power) of alloswitch (1 μM) in bath along with agonist (quisqualate, 3 μM) at wavelengths indicated by horizontal axis. Graphs illustrate functional efficiency of alloswitch isomerization at 2PE, expressed as the frequency of photoinduced Ca^{2+} oscillations (**A**), duration of the oscillatory behavior (**B**), time for onset of first Ca^{2+} peak after photostimulation start (delay [aka latency], **C**), and average number of oscillations counted during 2PE (# oscillations, **D**). Data represented as mean ± s.e.m., n = 4–95 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, using Dunn's multiple comparison test after the Kruskal–Wallis test.

Panel E: Output power of the pulsed laser at the wavelengths used for photostimulation of alloswitch in A–D.

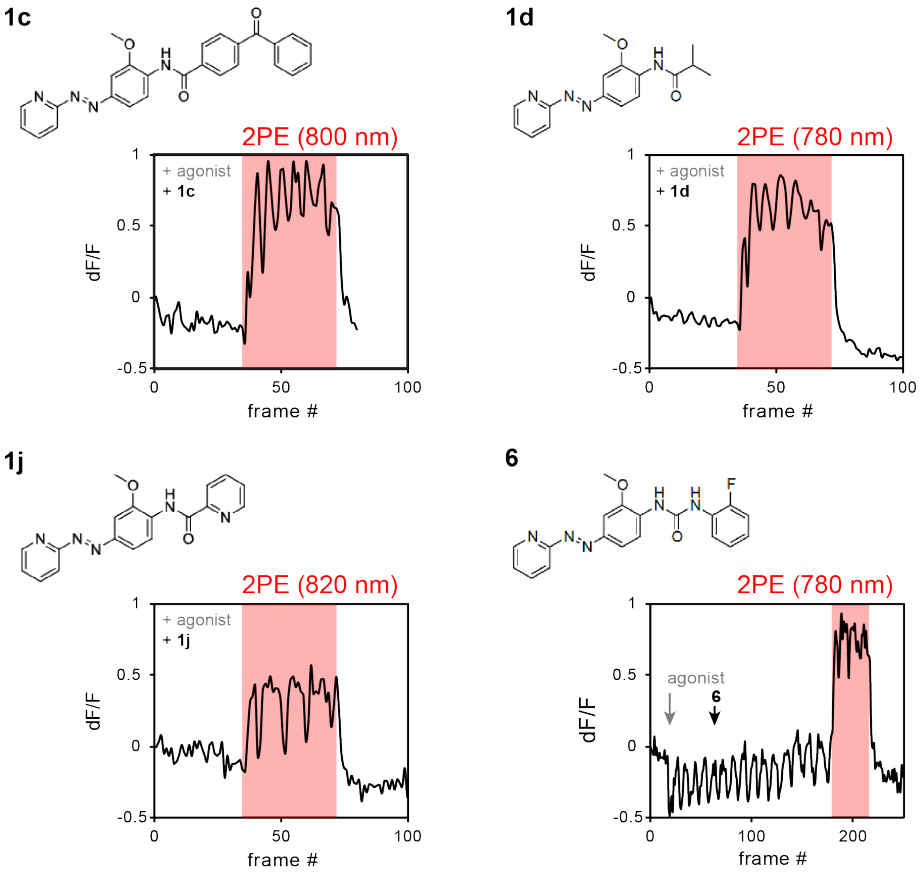

Fig. S3. (related to Fig. 1)

"All-2P experiments", structures of alloswitches used, and example traces of their 2PE.

Schematics of imaging-photostimulation setup for "all-2P experiments" can be found in **Fig. S1C**.

Compounds **1c-d, 1j, 6** are alloswitch analogs used for "all-2P experiments" other than alloswitch (names correspond to compounds published in Gómez-Santacana *et al.,* 2017; parent alloswitch structure is shown in **Fig. 1** of main text). Compound structures are shown, along with example traces of 2PE at wavelengths indicated above trace boxes. Traces show changes in Fura-2 fluorescence (dF/F) in $mGlu_5$-eYFP expressing HEK cells, supplemented with agonist (quisqualate, 3 µM) and alloswitch analogs (1 µM) prior to experiment (except for compound **6** where additions are indicated by grey and black arrows, respectively).

Compounds were selected based on structural variations aimed at improving the performance of alloswitch at 2PE. In particular, we focused on replacing the 2-chlorophenyl group of alloswitch with an aliphatic amide (**1d**) or aromatic amides with stronger electron acceptors (benzophenone in **1c**, pyridine in **1j**) or donors (2-flurophenyl urea in **6**), thus changing internal charge transfer upon electron excitation (push-pull character) of the phenylazopyridine, and, as such, two-photon absorption **(5)**. Consistent with an increased push-pull character, compounds **1d** and **6** display, respectively, improved potency and red-shifted spectrum compared to alloswitch **(2)**. Effective photoisomerization during 2PE at indicated wavelengths was observed for all alloswitch analogs tested except compound **6**, that failed to silence Ca^{2+} oscillations induced by agonist at the concentration used (1 µM) and no inferences about his 2PE character were possible. Extensive wavelength-dependence characterization (as performed for the parent molecule in **Fig. 2** of main text) is required to determine relative performance of alloswitch analogs at 2PE.

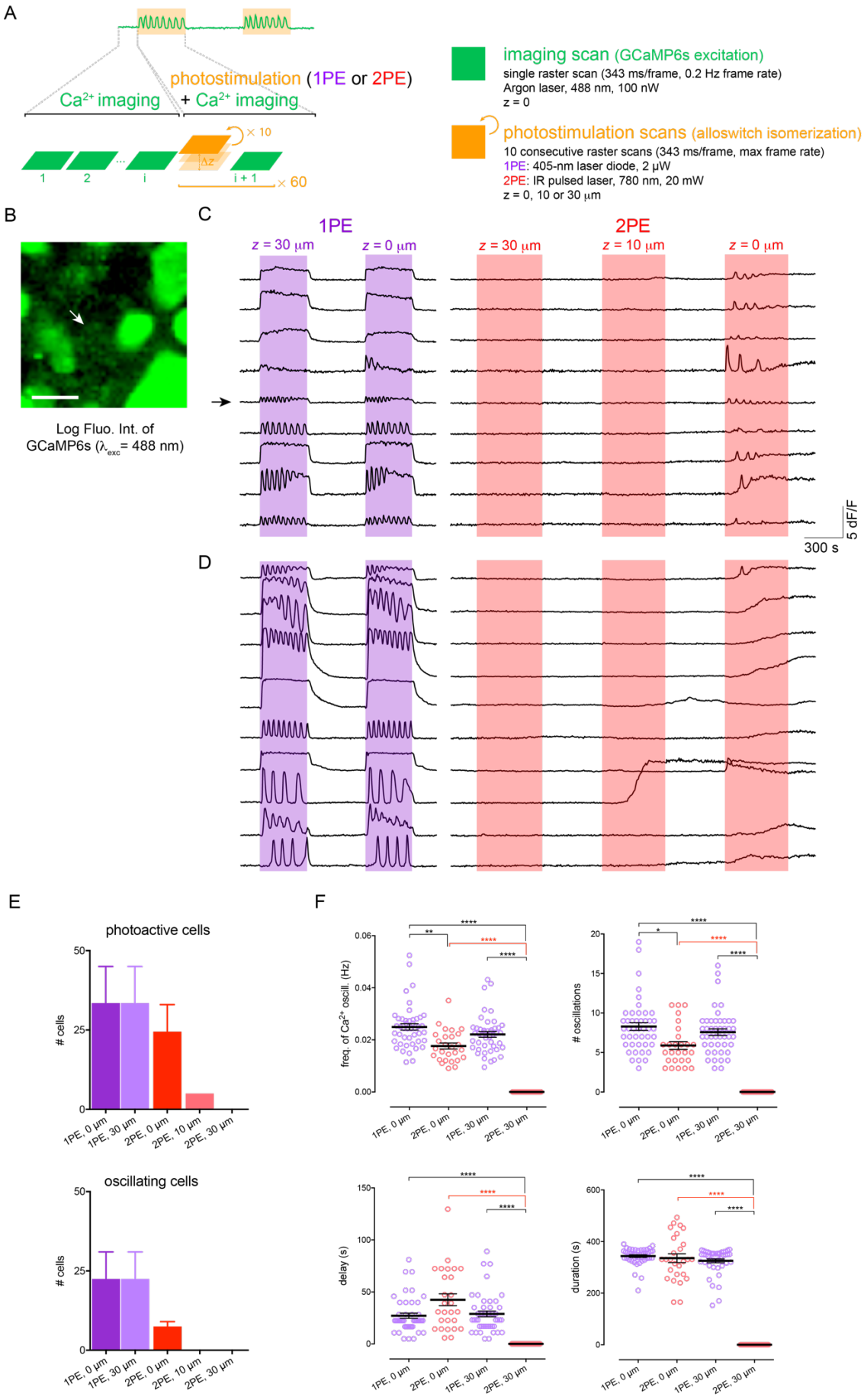
A
photostimulation (1PE or 2PE)
Ca2+ imaging
+ Ca2+ imaging
× 10
Δz
1
2
i
i + 1
× 60
imaging scan (GCaMP6s excitation)
single raster scan (343 ms/frame, 0.2 Hz frame rate)
Argon laser, 488 nm, 100 nW
z = 0
photostimulation scans (alloswitch isomerization)
10 consecutive raster scans (343 ms/frame, max frame rate)
1PE: 405-nm laser diode, 2 µW
2PE: IR pulsed laser, 780 nm, 20 mW
z = 0, 10 or 30 µm
B
Log Fluo. Int. of
GCaMP6s (λexc = 488 nm)
C
1PE
2PE
z = 30 µm
z = 0 µm
z = 30 µm
z = 10 µm
z = 0 µm
5 dF/F
300 s
D
E
photoactive cells
cells
1PE, 0 µm
1PE, 30 µm
2PE, 0 µm
2PE, 10 µm
2PE, 30 µm
oscillating cells
F
freq. of Ca2+ oscill. (Hz)
oscillations
delay (s)
duration (s)

Fig. S4. (related to Fig. 3 and Fig. 4)

Panels A–D: Schematics of photostimulation experiments at different axial distances in cultured cells (Figs. 3 and 4). Panel A: Schematics of setup used to combine imaging of intracellular Ca^{2+} (green) with 1PE or 2PE photostimulation (orange) of alloswitches, by raster-scanning with an inverted confocal microscope. The top trace corresponds to a Ca^{2+} imaging session in cultured cells, and zoom-in of the first part of the trace shows sequential imaging scans for visualization of Ca^{2+} through GCaMP6s fluorescence. Zoom-in of the photostimulation period (light orange box) indicates that photostimulation was accomplished by 10 scans at maximum speed followed by one imaging scan, and this cycle photostimulation-imaging was repeated 60 times. The 10 photostimulation scans were done at one axial position (Δz = 30, 10 or 0 μm) above the imaging plane (z = 0). Details of scanning and laser settings, as well as axial positions are listed on the right. **Panel B**: Cultured HEK cells co-expressing $mGlu_5$ receptors and GCaMP6s (excited at 488 nm, 100 nW; shown as logarithm of fluorescence intensity for better visualization) for Ca^{2+} imaging at 1PE. Arrow indicates cell shown by arrow in **C**, scale bar is 20 μm.

Panels C-D: Example traces of cultured HEK cells where changes in intracellular Ca^{2+} were monitored by imaging GCaMP6s in the presence of $mGlu_5$ agonist (quisqualate, 3 μM) and alloswitch (1 μM), and in the absence or presence of photostimulation of alloswitch at 1PE (violet boxes; 405 nm, 2 μW, repetition rate 0.17 Hz) or 2PE (red boxes; 780 nm, 20 mW, repetition rate 0.12 Hz) performed at axial distances indicated above colored boxes. Cells oscillating when photostimulated at 2PE at the imaging plane (z = 0) are shown in **C**; examples of cells showing Ca^{2+} events other than oscillations under 2PE (used to compute % of photostimulated cells in **Fig. 4, panel D**) are shown in **D**. Ca^{2+} traces are shown as fluorescence changes relative to first frame (dF/F) of GCaMP6s (excited at 488 nm, 100 nW).

Panel E: Average number of cells photoactive or oscillating at 1PE and 2PE, and different axial positions of photostimulation (related to cell percentages presented in Fig. 4D). In brief, changes in intracellular Ca^{2+} were monitored by imaging GCaMP6s in the presence of $mGlu_5$ agonist (quisqualate, 3 μM) and alloswitch (1 μM), and in the absence or presence of photostimulation of alloswitch at 1PE (violet boxes; 405 nm, 2 μW, repetition rate 0.17 Hz) or 2PE (red boxes; 780 nm, 20 mW, repetition rate 0.12 Hz) performed at axial distances indicated by horizontal axes. Photoactive cells (**top**) comprise cells responding to photostimulation with Ca^{2+} oscillations (oscillating cells, **bottom**) or with other types of intracellular Ca^{2+} events (examples of cells photoactive under 2PE but not oscillating can be viewed in **panel D**). Ca^{2+} events observed were either steady Ca^{2+} increases or peak-and-plateau responses. Cell classification was conducted as detailed in **SI Appendix, Materials and Methods, 6.2**. The relative abundance of Ca^{2+} responses other than oscillations in cultured cells is not surprising, given the different amounts of receptor and G-protein subunit expression have been previously demonstrate to alter the phenotype of intracellular Ca^{2+} events in response to activation of $mGlu_5$ receptors **(6, 7)**. The total number of cells responsive to 1PE at the imaging plane ('1PE, 0 μm', **top**) was used to calculate the percentage of photoactive and oscillating cells shown in **Fig. 4D** of main text. Data are average number of cells for each condition; n = 67 cells from 2 independent experiments.

Panel F: Full datasets and statistics for photoactivation experiments at 1PE or 2PE and different axial distances in cultured cells (shown in Fig. 4E of main text). Briefly, cultured HEK cells co-expressing $mGlu_5$ receptors and GCaMP6s for Ca^{2+} recordings were imaged as detailed in **Fig. 4**. The oscillatory behavior in response to agonist (quisqualate, 3 μM) was monitored during silencing of receptors with alloswitch (1 μM) and coincident photoactivation at 1PE (violet) or 2PE (red) done at different axial distances (indicated in horizontal axes) from the cell imaging plane (0 μm). Photoactivation is quantified as frequency and total number of calcium oscillations, time for the peak of the first oscillation observed after light onset (delay [aka latency]), and overall duration of the oscillatory behavior (duration). Lines and error bars represent mean ± s.e.m. plotted above individual cells (empty circles). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, using Dunn's correction for multiple comparisons after the Kruskal-Wallis test. D'Agostino and Pearson omnibus normality test was significant only for the dataset '2PE, 0 μm' in all variables measured except for the delay [aka latency]. For 1PE conditions, n = 45 from 2 independent experiments; for 2PE, n = 27 from 3 independent experiments.

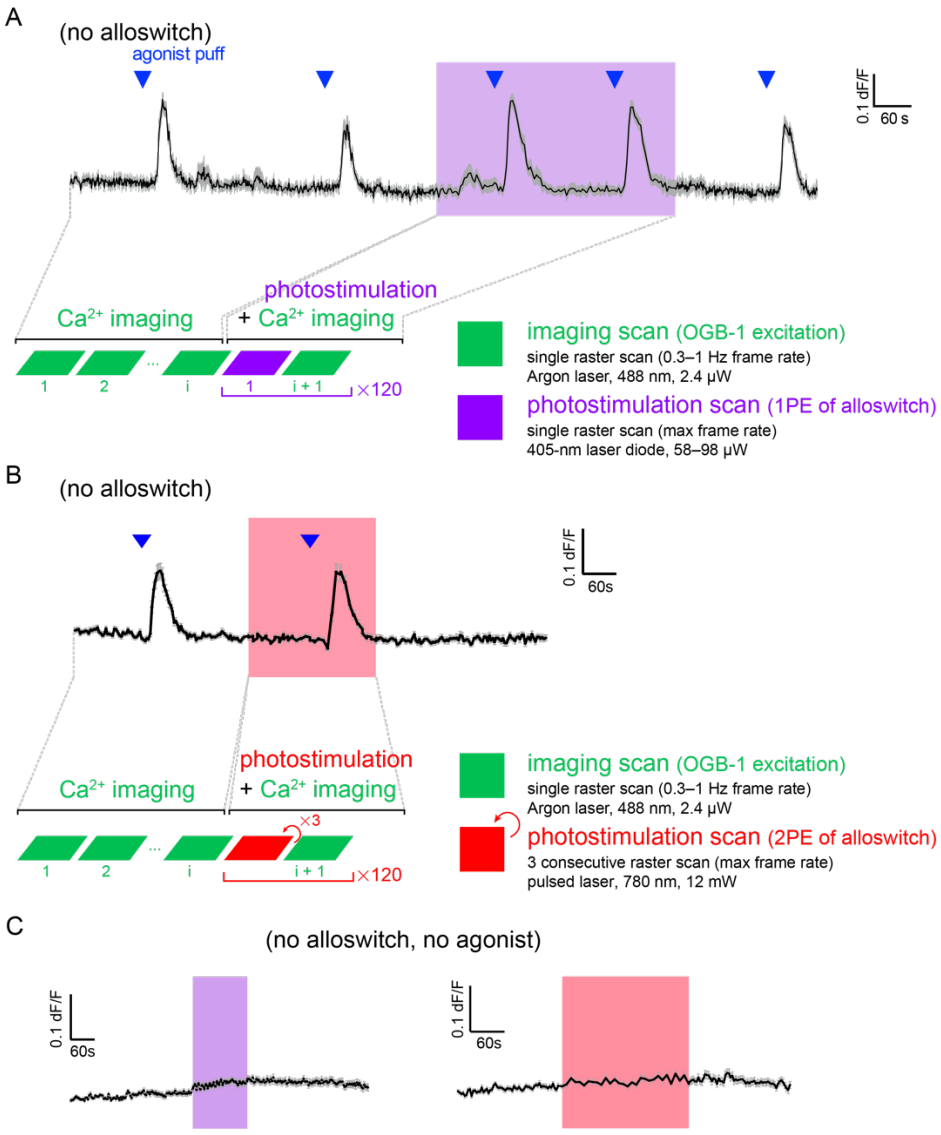

Fig. S5. (related to Fig. 5 and Fig. 6)

Panels A–B: Schematics of imaging-photostimulation cycles in rat acute brain slices and control experiments.

Panel A: Schematics at the **bottom** of the figure illustrate the setup used to monitor intracellular Ca^{2+} in acute brain slices experiments where OGB-1 imaging (green) was combined with photostimulation at 1PE (violet) by raster-scanning with an inverted confocal microscope. The photostimulation period (light violet box in top trace) is composed of 120 repetitions of: one raster scan of the 405 nm laser diode, followed by one 488 nm imaging scan for visualization of OGB-1. Details of scanning and laser settings used across experiments are listed on the right. **Top** of figure shows the average Ca^{2+} trace (mean ± s.e.m., n = 16 cells; 1 P9 rat brain slice) of a control experiment for photostimulation at 1PE in hippocampal slices, performed in the absence of alloswitch. 1PE (violet box) is done at a laser power (98 µW, pixel dwell time = 2.5 µs) almost twice the power used for successful photostimulation of alloswitch in **Fig. 5D** (58 µW). Intracellular Ca^{2+} is shown as changes in OGB-1 fluorescence (dF/F) over time. Activation of endogenous $mGlu_5$ receptors is done by pressure-ejection of agonist as in experiments in **Fig. 5** (DHPG, 1 mM; 800 hPa, 1 s), before, during and after photostimulation.

Panel B: Average trace (n=112 cells, 1 animal) of DHPG (1mM; 500 hPa, 1 s)-mediated Ca^{2+} response at both dark and 2PE (25 mW, 0.3 Hz) in the absence of alloswitch and corresponding schematics of how imaging and photostimulation have been performed.

Panel C: Control experiments to determine the effect of photostimulation alone on intracellular Ca^{2+} in slices. Left: Average trace (n=238 cells, 3 animals) of 1P light control experiments. Acute brain slices were perfused with normal ACSF and illuminated with the 405 nm laser diode (violet box) at 58 µW light power and 0.4 Hz illumination rate. **Right:** Average trace (n = 171 cells, 2 animals) of 2PE control experiments on acute brain slices. 780 nm (red box) at 25 mW, 0.3 Hz.

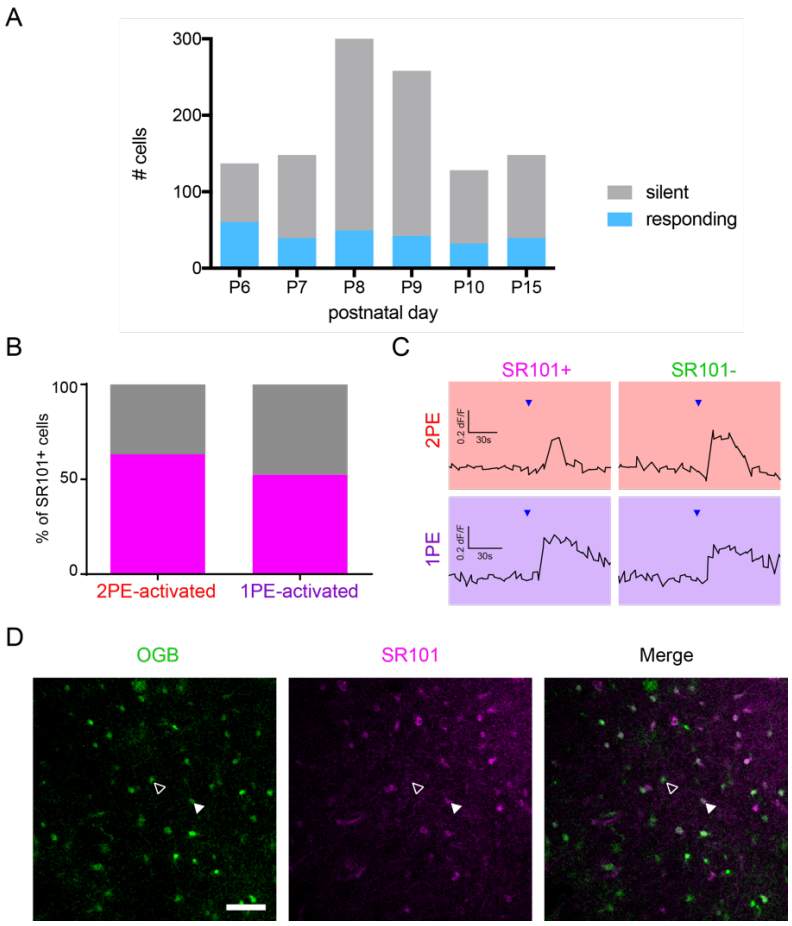

Fig. S6. (related to Figs. 5–7)

Panel A: Outline of experiments performed at different postnatal ages in acute rodent brain slices. Percentage of cells in *naïve* slices obtained from animals of different ages (P6–P15) responding to local puffing of $mGlu_5$ agonist (DHPG, 1 mM; 500–800 hPa, 1 s). Exploratory experiments were performed at indicated ages to identify optimal experimental conditions; P8–P9 rats were selected for further experiments because of optimal OGB-1 labeling (in our hands, labeling was poorer after P10). The increased number in labeled cells could also be due to better survival of cells after sectioning at these ages, which might account for the relatively constant number of responding cells across ages.

Panels B–D: Proportion of $SR101^+$ cells photoactivated in hippocampal CA1 *stratum radiatum* as in Figs. 6 and 7 of main text. Panel B: Percentage of $SR101^+$ cells among all cells analyzed in **Fig. 7** of the main text (n=114 cells from 3 animals). For 2PE, 63 ± 15% of the responding cells were $SR101^+$ and for 1PE, 53 ± 14% (Unpaired t-test, p=0.633). **Panel C:** Example traces of $SR101^+$ cell and $SR101^-$ cell during 2PE (red boxes) and 1PE (violet boxes) are presented as changes in OGB fluorescence (dF/F) over time. Agonist (blue arrowheads, 1 mM DHPG) was locally ejected at 500 hPa during 1 second. The average amplitude of calcium responses (n = 114 cells, 3 animals) was slightly higher (p = 0.035, unpaired t-test) in astrocytes ($SR101^+$) compared to neurons ($SR101^-$) during photoactivation of alloswitch at 1PE but not 2PE (p = 0.091, unpaired t-test), probably reflecting a more extended volumetric spread of astrocytes compared to neurons. **Panel D:** Cells in *stratum radiatum* labeled with OGB-1 (left), astrocytic marker SR101(middle), and the two channels merged (right). One photoactivated $SR101^+$ (white arrowhead) and one $SR101^-$ (empty arrowhead) cell are indicated. Scale bar is 50 µm.

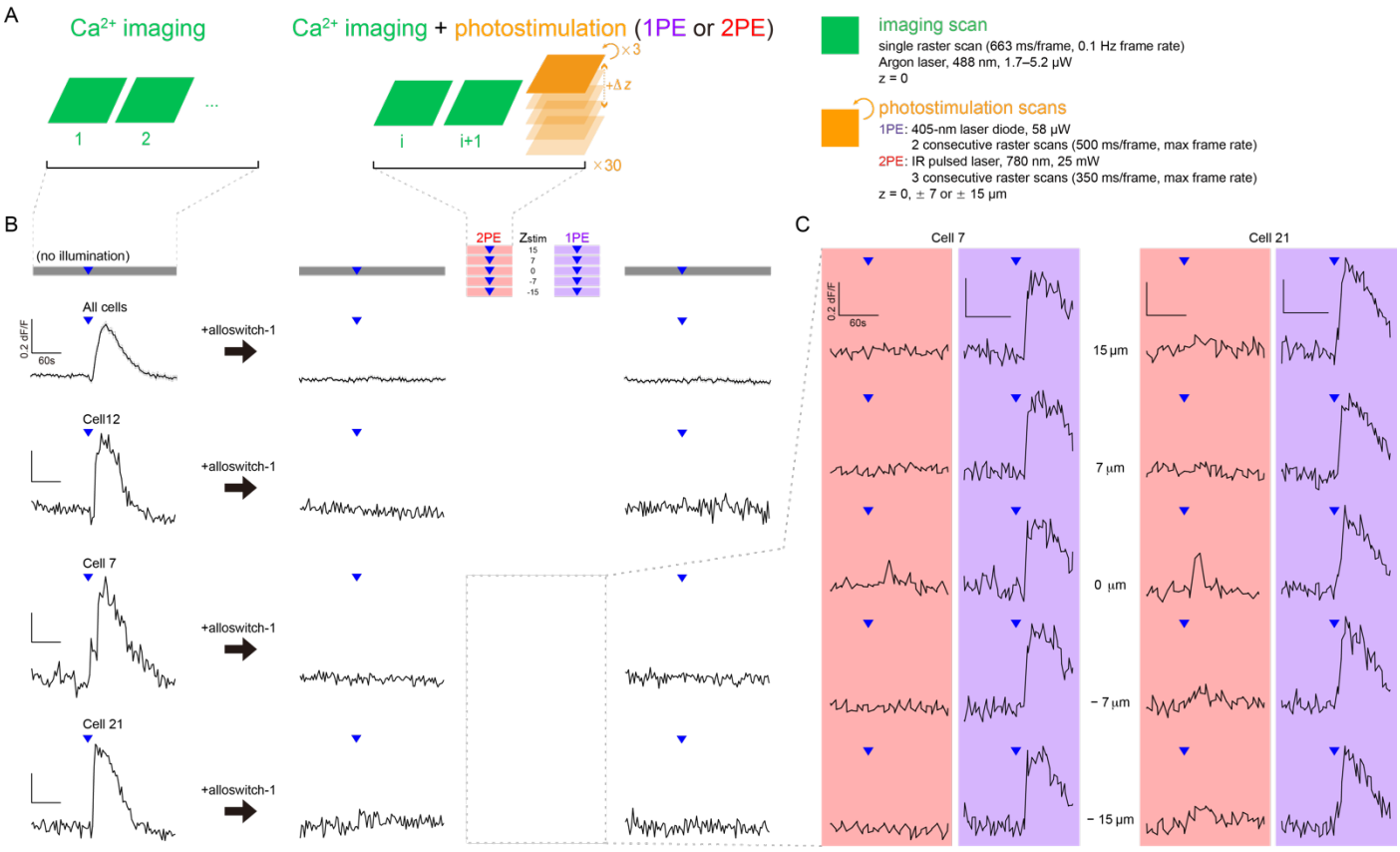

Fig. S7. (related to Fig. 7)

Inhibition of mGlu$_5$ activity by alloswitch was assessed throughout experiments shown in Fig. 7 of the main text.

Panel A: Schematics for experiments in slices as in **Fig. 7**, illustrating how Ca^{2+} imaging and photostimulation of alloswitch were performed along with changes in axial position for photostimulation (Δz).

Panel B: DHPG-mediated (blue triangles) Ca^{2+} responses were monitored in the absence of illumination (grey bars) before and after applying alloswitch, and during photostimulation (colored bars). After consecutive stimulations with 2PE (red) and 1PE (violet) at 5 different axial distances (z_{stim}) from the cell imaging plane, the inhibition exerted by *trans*-alloswitch was maintained in the absence of illumination. Top trace corresponds to averaged recordings from all cells analyzed in **Fig. 7C–D** of the main text; n=114 cells from 3 animals. Cell 12 is the same cell shown in **Fig. 7B**. Cell 7 and Cell 21 are additional examples of cells responding with Ca^{2+} peaks to agonist ejection (blue triangles) before addition of alloswitch (left traces), and demonstrating complete silencing by *trans*-alloswitch before (middle traces) and after (right traces) light stimulation protocols at 5 different axial distances (z_{stim}) and two light conditions (red/violet).

Panel C: Ca^{2+} responses of Cell 7 and Cell 21 from **A** during 2PE (red) and 1PE (violet) at different axial distances (from the top, z_{stim}= +15, +7, 0 ,-7, -15 µm) show a reversible control of mGlu$_5$-mediated response by photoswitching, which is axially restricted for 2PE but not for 1PE.

Movie S1. (related to Fig. 3)

Photorescue of $mGlu_5$ receptors by 1PE and 2PE in HEK cells.

Video shows GCaMP6s fluorescence in cultured HEK cells co-transfected with $mGlu_5$ receptors as in **Fig. 3**. Cell in the middle corresponds to cell indicated by arrow in **Fig. 3B** and Ca^{2+} trace in **Fig. 3C**, and responds with Ca^{2+} oscillations in all conditions. Intracellular events are recorded at conditions indicated by labels ("agonist" = 3 μM quisqualate; '+ alloswitch/no light' = 1 μM alloswitch supplemented in bath in addition to agonist; '1PE' = 405 nm laser diode, 2 μW; '2PE' = NIR pulsed laser, 780 nm, 20 mW) (**SI Appendix**, **Materials and Methods 5.2**). Photostimulation periods start on frame 6 after label, and last 60 frames each. GCaMP6s fluorescence is excited by raster scanning with a 488 nm Argon laser (100 nW, 343 ms/frame). Frame rate is 0.2 Hz during imaging alone, 0.17 Hz during 1P photostimulation, and 0.12 Hz during 2P photostimulation. Picture size is 62×62 μm.

Movie S2. (related to Fig. 4)

Axial-plane specific photorescue of $mGlu_5$ receptors by 2PE but not 1PE in HEK cells.

Video of GCaMP6s fluorescence in cultured HEK cells co-transfected with $mGlu_5$ receptors. Photocontrol of cell Ca^{2+} occurs regardless of the axial distance for 1PE of alloswitch, and is axial-plane selective for 2PE. The field of view corresponds to image and cell Ca^{2+} trace in **Fig. 4B–C** in main text. Ca^{2+} events are recorded in different conditions indicated by labels ('agonist' = 3 μM quisqualate; '+ alloswitch/no light' = 1 μM alloswitch supplemented in bath in addition to agonist; '1PE' = 405 nm laser diode, 2 μW; '2PE' = NIR pulsed laser, 780 nm, 20 mW; 'light OFF' = end of photostimulation) (**SI Appendix**, **Materials and Methods 5.2**). Axial position of photostimulation (*z* in main figures) is indicated between asterisks, and is 30 or 10 μm above cells, or corresponds to the cell imaging plane (0 μm). GCaMP6s fluorescence is excited by raster scanning with a 488 nm Argon laser (100 nW, 343 ms/frame). Frame rate is 0.2 Hz during imaging alone, 0.17 Hz during 1P photostimulation, and 0.12 Hz during 2P photostimulation. Picture size is 129×129 μm.

Movie S3. (related to Fig. 5)

Silencing and photorescue of $mGlu_5$ receptors by alloswitch and 1PE in acute brain slices.

Video of OGB-1 fluorescence in a P9 rat acute brain slice, corresponding to images and Ca^{2+} traces in **Fig. 5B–D** in main text. Intracellular Ca^{2+} events are recorded at baseline and different drug and photostimulation conditions indicated by labels ('baseline' = aCSF containing TTX/LY367385; '+ alloswitch/no light' = 10 μM alloswitch supplemented in recirculating aCSF; white dot indicates site and time of agonist pressure-ejection: DHPG, 1 mM and 800 hPa, 1 s; '1PE' = 405 nm laser diode, 58 μW, 120 raster scans at 0.3 Hz) (**SI Appendix**, **Materials and Methods 5.2**). OGB-1 fluorescence is excited by raster scanning with a 488 nm Argon laser (2.4 μW, 1 Hz frame rate). Frame rate for OGB-1 is 0.3 Hz during photostimulation. Picture size is 350×350 μm.

Movie S4. (related to Fig. 6)

Photorescue of $mGlu_5$ receptors by 2PE in acute brain slices.

Video of OGB-1 fluorescence in a P10 rat acute brain slice, corresponding to Ca^{2+} traces in **Fig. 6B**. Intracellular Ca^{2+} events are recorded in the dark and under photostimulation conditions indicated by labels ('+ alloswitch/no light' = 10 μM alloswitch supplemented in bubbling reservoir; '2PE' = 780 nm pulsed laser, 25 mW, 120 raster scans at 0.3 Hz) (**SI Appendix**, **Materials and Methods 5.2**). The white dot indicates the site and time of agonist pressure-ejection (DHPG, 1 mM; 500 hPa, 1 s). OGB-1 fluorescence is excited by raster scanning with a 488 nm Argon laser (2.4 μW, 1 Hz frame rate). Frame rate for OGB-1 is 0.2 Hz during photostimulation. Picture size is 310×310 μm.

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