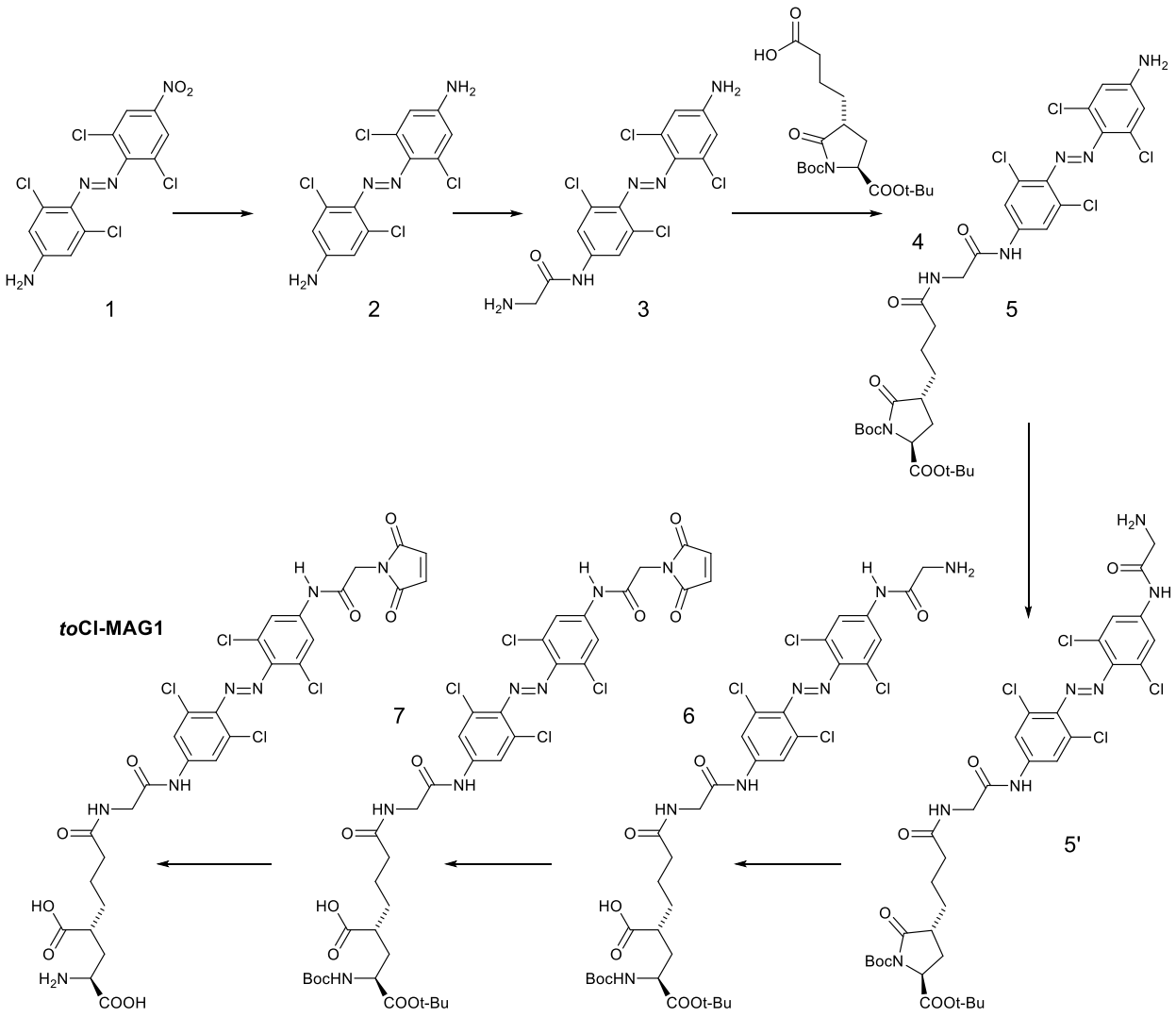
Supporting information for:

Long wavelength optical control of glutamate receptor ion channels using a tetra-*ortho*-substituted azobenzene derivative

Synthesis of *to*Cl-MAG1

Detailed synthetic scheme:



*Synthesis of 3,5-dichloro-4-((2,6-dichloro-4-nitrophenyl)diazenyl)aniline, **1 (NH_2-azo-NO_2)***

This synthesis was modified slightly from Samanta et al [1]. Sodium nitrate (0.20g, 2.88 mmol) was added to a stirred solution of cold concentrated sulfuric acid (2 mL) on an ice bath. The mixture was heated to 70^{o}C for 5 min and cooled to 0^{o}C. To this ice cold mixture was added dropwise a cold 0^{o}C solution of 2,6-dichloro-4-nitroaniline (0.60g, 2.88 mmol) in a mixture of

acetic acid/DMF (3.6 mL/ 9 mL). The reaction was stirred at 0°C for 2h. To this diazotized intermediate was added dropwise a cold 0°C solution of sodium 3,5-dichloro anilinomethanesulfonate (0.4 g, 1.44 mmol) in 6 mL of DMF synthesized according to literature procedures [1]. The reaction mixture was stirred at 0°C for 1h and then brought to room temperature where it was stirred for an additional 72h. The mixture was then poured onto ice and the dark red tar-like precipitate obtained was filtered. The wet precipitate was basified with 15 mL of an aqueous sodium hydroxide solution (3g NaOH/50 mL deionized water) and heated to 70°C for 30 min. Following basification, the reaction mixture was extracted with ethylacetate, concentrated, and purified using silica gel column chromatography (75:15:10 hexane/$CHCl_3$/EtOAc) to yield **1** (136 g, 26 %) as a brownish solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (s, 2H), 6.76 (s, 2H), 4.56 (br, 2H).

Synthesis of 4,4'-(diazene-1,2-diyl)bis(3,5-dichloroaniline), ***2 (NH_2-azo-NH_2)***

Compound **1** (0.120g**,** 0.31 mmol), was dissolved in 6.5 mL dioxane and diluted with 4.5 mL of deionized water. Fresh anhydrous sodium sulfide (stored at 4°C and protected from light) (0.076g, 0.976 mmol) was dissolved in 2 mL of deionized water and added to the solution containing compound **1**. The reaction mixture was then refluxed at 90°C for 3h at which time 50 mL of ethyl acetate was added and the solution extracted against brine (2x). The ethylacetate layer was concentrated and the resulting bright red solid was purified by silica gel chromatography (50:25:25 hexane/$CHCl_3$/EtOAc to 50:50 hexane/EtOAc carried out in the dark) to yield **2** (0.07g, 65 %) as a bright red solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.71 (s, 2H), 6.54 (s, 2H), 4.07 (br, 2H), 3.93 (br, 2H).

Synthesis of 2-amino-N-(4-((4-amino-2,6-dichlorophenyl)diazenyl)-3,5 dichlorophenyl)acetamide, ***3 (Gly-azo-NH_2)***

The diamino compound **3** (0.05g, 0.14 mmol) was dissolved in 5.2 mL of anhydrous THF to which DMAP (0.0017g, 0.014 mmol) and dry DIPEA (63 μL, 0.35 mmol) were added. The solution was stirred at 0°C for 15 min prior to the dropwise addition of Fmoc-Glycine pre-activated as an acid chloride. The reaction was left at 0°C for one hour and then brought to room temperature for two hours. The Fmoc-Glycine acid chloride solution was prepared as follows: Fmoc-Glycine (0.062g, 0.21 mmol) was suspended in 600 μL of dry DCM. To this suspension was added 105 μL of an oxalyl chloride solution in anhydrous THF (105 μL of a 2.0M solution in THF, 0.21 mmol). Upon the subsequent addition of anhydrous DMF (5 μL), violent bubbling was observed and the reaction solution was left for 1h at room temperature. After 1h, the yellow Fmoc-glycine acid chloride solution was concentrated and resuspended in 2 mL of anhydrous THF. This Fmoc-Glycine acid chloride solution was then added dropwise as described above via oven dried pasteur pipette at 0°C. Following reaction completion, the solution was diluted with 20 mL of ethylacetate and extracted against sodium bicarbonate (2x), and brine (2x). Following concentration of the ethylacetate layer, the reddish solid was purified by silica column chromatography (50:50 hexane/ethylacetate carried out in the dark) to yield the Fmoc-protected version of compound **3**. The Fmoc group was subsequently removed by dissolving the compound in anhydrous DMF (2.28 mL) to which 23 μL of piperidine was added (0.23 mmol) with the solution left stirring for 6h at room temperature. Following the completion of the deprotection

reaction, 20 mL of ethyl acetate was added and extracted against brine (2x). The ethyl acetate layer was concentrated and the red compound purified by silica gel chromatography (95:5 to 90:10 DCM/MeOH) to yield compound **3** (0.027g, 48% yield over 2 steps) as a red solid. ^{1}H NMR (400 MHz, MeOD) δ 7.78 (s, 2H), 7.67 (s, 2H), 6.75 (s, 2H), 6.56 (s, 2H), 3.50 (s, 2H), 3.46 (s, 2H).^{13}C NMR (101 MHz, DMSO) δ 172.77, 152.11, 150.23, 143.47, 142.43, 139.37, 139.20,135.5, 133.80, 131.17, 126.58, 126.37, 125.10, 119.09, 118.79, 113.69, 112.98, 48.55, 45.46 (both cis and trans chemical shifts are apparent in most cases). ESI-HRMS: m/z calculated for $C_{14}H_{11}Cl_4N_5O$: 405.9790 $[M+H]^+$; found: 405.9791

*Synthesis of di-tert-butyl (2S,4R)-4-(4-((2-((4-((E)-(4-amino-2,6-dichlorophenyl)diazenyl)-3,5-dichlorophenyl)amino)-2-oxoethyl)amino)-4-oxobutyl)-5-oxopyrrolidine-1,2-dicarboxylate (**5**) **(PyroGlu-Gly-azo-NH$_2$)***

Compound **3** (0.027g, 0.067 mmol) was dissolved in 250 µL anhydrous DMF and diluted with 3.42 mL anhydrous DCM. To this solution was added dry DIPEA (41.3 µL, 0.23 mmol), EDCI (0.013g, 0.067 mmol), and HOBt (0.015g, 0.09 mmol). After stirring for 5 min at room temperature, 4-((3R,5S)-1,5-bis(tert-butoxycarbonyl)-2-oxopyrrolidin-3-yl)butanoic acid (**4**), prepared as described previously [2] (0.023g,0.06 mmol) was added to the solution and the reaction was allowed to proceed for three hours at room temperature. Following reaction completion, the solution was diluted with ethylacetate (20 mL) and extracted against sodium bicarbonate (2x) and brine (2x). The ethylacetate layer was concentrated and the red solid dissolved in DCM where it was again extracted against sodium bicarbonate (2x) and brine (2x). The DCM layer was then collected and concentrated with the obtained red solid purified by silica gel column chromatography (97:3 to 95:5 DCM/MeOH) yielding compound **5** (0.041g**,** 80 % yield) as a red solid. ^{1}H NMR (400 MHz, MeOD) δ 7.78 (s, 2H), 7.64 (s, 2H), 6.74 (s, 2H), 6.57 (s, 2H), 4.51 (m, 1H), 4.02 (s, 2H), 3.97 (s, 2H), 2.61 (m, 1H), 2.32 (m, 2H), 2.22 (m, 1H), 2.06 (m, 1H), 1.87 (m,1H) 1.73 (m, 2H), 1.65 (m, 1H), 1.50 (s, 9H), 1.49 (s, 9H).^{13}C NMR (101 MHz, DMSO) δ 174.73,174.07, 172.51, 170.47, 170.2, 168.66,151.91, 150.56, 148.72, 143.16, 142.51, 139.24, 139.04, 136.41, 133.85, 131.20, 126.65, 126.35, 125.11, 118.96, 118.67, 113.59, 112.93, 82.01, 81.51, 59.67, 57.34, 42.63, 40.74, 34.81, 33.70, 29.58, 27.39, 22.32, 21.68, 20.55, 13.92. ESI-HRMS: m/z calculated for $C_{32}H_{38}Cl_4N_6O_7Na$: 781.1432 $[M+Na]^+$; found: 781. 1433

*Synthesis of di-tert-butyl (2S,4R)-4-(4-((2-((4-((E)-(4-(2-aminoacetamido)-2,6-dichlorophenyl)diazenyl)-3,5-dichlorophenyl)amino)-2-oxoethyl)amino)-4-oxobutyl)-5-oxopyrrolidine-1,2-dicarboxylate (**5'**) **(PyroGlu-Gly-azo-Gly)***

Compound **5** (0.041g, 0.054 mmol) was dissolved in 3 mL of anhydrous THF to which dry DIPEA (96 µL, 0.50 mmol) and DMAP (2.4mg, 0.02 mmol) were added followed by stirring at 0^{o}C. Fmoc-Gly (0.090g, 0.30 mmol) was dissolved in 912 µL of anhydrous DCM to which oxalyl chloride (165 µL of a 2M oxalyl chloride solution in anhydrous THF, 0.33 mmol) and 8 µL anhydrous DMF was added. After stirring for 1h at room temperature, the acid chloride was concentrated, redissolved in 1.8 mL anhydrous THF, and added dropwise via a pasteur pipette to the solution containing compound **5** at 0^{o}C. The reaction was stirred at 0^{o}C for 1h and then stirred at room temperature for an additional 3h. Following the completion of the reaction, the

reaction solution was diluted with 20 mL ethylacetate and extracted against sodium bicarbonate and brine (2x each). The ethylacetate layer was concentrated, and the orange solid redissolved in DCM (20ml) and again extracted as described above. The DCM layer was concentrated and the orange solid obtained was dissolved in 2.3 mL anhydrous DMF to which piperidine was added (46ul, 0.46 mmol). The deprotection reaction was carried out for 6h at room temperature following which time the reaction solution was diluted with 20ml ethylacetate and extracted against brine (2x). The orange solid was dissolved in DMSO and purified via RP-HPLC (10% to 60% ACN/30min, 0.1% formic acid in both water and ACN). The collected fractions were pooled, neutralized to pH 6-7 and extracted with ethylacetate to yield **5'**, (0.030g, 67%) as an orange solid. (**5'** can also be sufficiently purified using silica column chromatography with 95:5 to 90:10 DCM/MeOH) ^{1}H NMR (400 MHz, CD_3OD) δ 7.87 (s, 2H), 7.85 (s, 2H), 7.68 (s, 2H), 7.66 (s, 2H), 4.52 (m, , 1H), 4.03 (s, 2H), 3.97 (s, 2H), 3.75 (s, 2H), 3.70 (s, 2H), 2.63 (m, 1H), 2.34 (m, 2H), 2.25 (m, 1H), 2.08 (m, 1H), 1.87 (m, 1H), 1.74 (m, 2H), 1.50 (s, 9H), 1.49 (s, 9H), 1.44 (m, 1H). ^{13}C NMR (400 MHz, DMSO) δ 175.20, 173.41, 170.94, 165.87, 149.18, 142.70, 141.56, 141.28, 140.79, 127.99, 125.66, 119.69, 119.28, 82.50, 82.01, 57.80, 45.79, 43.38(cis/trans), 41.21, 35.31 (cis/trans), 30.08, 27.94,27.92, 22.84 (cis/trans). ESI-HRMS: m/z calculated for $C_{34}H_{41}Cl_4N_7O_8Na$: 838.1680 $[M+Na]^+$; found: 838. 1687

Synthesis of (R)-6-((2-((4-((E)-(4-(2-aminoacetamido)-2,6-dichlorophenyl)diazenyl)-3,5-dichlorophenyl)amino)-2-oxoethyl)amino)-2-((S)-3-(tert-butoxy)-2-((tert-butoxycarbonyl)amino)-3-oxopropyl)-6-oxohexanoic acid ***6) (glutamate-gly-azo-gly)***

Compound **5'** (0.02g, 0.024mmol) was dissolved in 1 mL of THF at 0°C. This was followed by the addition of 1 mL of 1M LiOH precooled to 0°C with the reaction carried out for 1h at 0°C. Following the completion of the reaction, the solution was neutralized with 1 mL of 1M HCl precooled to 0°C and concentrated *in vacuo* to remove the THF layer. To this suspension was added DMSO followed by centrifugation. The coloured supernatant was then injected into the HPLC using the following gradient: 10%-60% ACN/30min, (0.1% formic acid in both water and ACN). The desired fraction was collected, neutralized with sodium bicarbonate and concentrated yielding **6** as an orange solid (0.015g, 75%). ^{1}H NMR (400 MHz, MeOD) δ 7.90 (s, 2H), 7.88 (s, 2H), 7.72 (s, 2H), 7.69 (s, 2H), 4.05 (s, 2H), 3.99 (s, 2H), 3.96 (m,1H) 3.95 (s, 2H), 3.90 (s, 2H), 2.53 (s, 1H), 2.34 (m, 2H), 2.11 (s, 1H), 1.67 (s, 4H), 1.58 (s, 1H), 1.46 (s,9H),1.44 (s, 9H). ^{13}C NMR (500 MHz, MeOD) δ 182.48 , 175.9, 173, 168.87, 159.50,156.6, 150.9, 142.4, 139.9, 128.09, 128.01, 125.97, 125.91, 119.68, 119.46, 119.37, 119.16 – 119.12, 81.06, 78.78, 53.57, 45.00, 44.35, 42.8, 35.2, 34.25, 32.43, 31.26, 27.36, 26.88, 23.55, 22.32.
ESI-HRMS: m/z calculated for $C_{34}H_{44}Cl_4N_7O_9$: 834.1954 $[M+H]^+$; found: 834. 1949

Synthesis of (R)-2-((S)-3-(tert-butoxy)-2-((tert-butoxycarbonyl)amino)-3-oxopropyl)-6-((2-((3,5-dichloro-4-((E)-(2,6-dichloro-4-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetamido)phenyl)diazenyl)phenyl)amino)-2-oxoethyl)amino)-6-oxohexanoic acid ***7 (glutamate-gly-azo-gly-malemide)***

Compound **6** (0.015g, 0.018mmol) was dissolved in 500 µl of anhydrous DMF to which dry DIPEA (100µl, 0.58mmol) was added at 0°C. *N*-methoxycarbonylmaleimide (0.030g, 0.19mmol) was dissolved in 500µl of anhydrous DMF at 0°C, and added to the solution

containing compound **6**. The reaction was allowed to proceed for 30 min at 0°C and then brought to room temperature for 1h. Following the completion of the reaction, the solution was diluted with ethylacetate and extracted against brine (2x). The ethylacetate layer was dried with sodium sulfate and concentrated resulting in the isolation of a yellowish-orange solid. The solid was purified first by silica column chromatography (90:10:0.6:0.6 DCM/MeOH/H_2O/Acetic acid) followed by HPLC using the following gradient: 35-100% ACN/30min (neutral deionized water and ACN used) with the desired fraction collected and extracted with ethylacetate. The ethylacetate layer was dried using sodium sulfate and concentrated to yield **7** (0.010g, 60%) as a yellowish-orange solid. ^{1}H NMR (400 MHz, DMSO) δ 10.87 (s, 1H), 10.76 (s, 1H), 10.55 (s, 1H), 10.42 (s, 1H), 8.23 (m, 1H), 7.90 (s, 2H), 7.85 (s, 2H), 7.71 (s, 2H), 7.66 (s, 2H), 7.18 (s, 2H), 7.15 (s, 2H), 4.34 (s, 2H), 4.28 (s, 2H), 4.14 (m, 1H), 3.91 (d, *J* = 5.6Hz, 2H), 3.85 (d, *J* = 5.8Hz,2H), 3.76 (m, 1H), 2.33 (m, 1H), 2.16 (m, 2H), 1.89 (m, 1H), 1.59 (m, 1H), 1.49 (m, 3H), 1.40 (m, 1H), 1.38 (s, 18H). ^{13}C NMR (500 MHz, DMSO) δ 195.8, 171.16, 170.95, 170.78, 166.8, 155.9, 155.7, 144.85, 141.35, 135.45, 127.99, 119.81, 119.22, 80.35, 60.20, 45.87, 43.44, 30.52, 28.66, 28.10, 21.85, 21.21. ESI-HRMS: m/z calculated for $C_{38}H_{43}Cl_4N_7O_{11}$: 912.1714 $[M-H]^-$; found: 912. 1724

*Synthesis of **toCl-MAG1***

Anhydrous ethylacetate (5 mL) under nitrogen, was added to a sealed, dry, round bottom flask connected on one end to a bubbler. One end of a 12 inch cannula was inserted into the flask and was submerged in the ethylacetate. The other end was inserted into a large round bottom flask containing NaCl (14g, 0.241 moles) equipped with a sealed dropper containing concentrated sulfuric acid (13ml, 0.241 moles). The sulfuric acid was allowed to drip slowly into the flask containing NaCl which resulted in the bubbling of HCl gas into ethyl acetate. After 1h, 3 mL of the HCl saturated ethyl acetate solution was added to **7** (0.010g, 0.011mmol), and stirred at room temperature in the dark for 2hr. To this mixture containing visible precipitate was added 6 mL of diethyl ether. Following trituration of the precipitate with diethylether, *to*Cl-MAG1 was isolated (0.008g, 96%). ^{1}H NMR (500 MHz, DMSO/phosphate buffer pD 7 in D_2O) δ 7.80 (s, 2H), 7.77 (s, 2H), 7.61 (s, 2H), 7.59 (s, 2H), 6.96 (s, 2H), 6.93 (s, 2H), (methylene protons corresponding to the maleimide coupled glycine are obscured by phosphate, 4.34(s,2H)) 3.88 (s, 2H), 3.82 (s, 2H), 3.78 (m, 1H), 2.16 (m, 3H),1.93 (m,1H) 1.85 (m, 1H), 1.67 (m, 1H), 1.48 (m, 3H).ESI-HRMS: m/z calculated for $C_{29}H_{26}Cl_4N_7O_9$: 756.0552 $[M-H]^-$; found: 756. 0552

NMR of *to*Cl-MAG1 in methanol:

^{1}H NMR (500 MHz, MeOD) δ 7.85 (s, 2H), 7.83 (s, 2H), 7.67 (s, 2H), 7.63 (s, 2H), 6.95 (s, 2H), 6.93 (s, 2H), 4.42-4.34 (dd,2H), 4.04 (m, 3H), 3.53 – 3.44 (q, 2H, diethylether), 3.03 – 2.98 (m, 1H), 2.54 – 2.29 (m, 4H), 1.87 – 1.59 (m, 4H), 1.20 (t, 3H, diethylether).

NMR for t-butylester protected *to*Cl-MAG1 (from incomplete deprotection) ^{1}H NMR (500 MHz, DMSO) δ 11.12,11.06 cis/trans (s, 1H), 10.76 ,10.64 cis/trans (s, 1H), 8.30 (m, 1H), 7.94 (s, 2H), 7.88 (s, 2H), 7.75 (s, 2H), 7.70 (s, 2H), 7.17 (s, 2H), 7.15 (s, 2H), 4.35 (d, *J* = 7.2 Hz, 2H), 4.30 (d, *J* = 6.6 Hz, 2H), 3.93 (d, *J* = 5.5 Hz, 2H), 3.87 (d, *J* = 5.6 Hz, 2H), 3.80 (m, 1H), 3.66

(m,1H), 2.99 (m, 1H), 2.19 (m, 2H), 2.11 (m, 1H), 1.78 (m, 1H), 1.54 (m, 4H), 1.46(s, 9H). ESI-HRMS: m/z
$[M+H]^+$: 814.13.

Electrophysiological characterization of LiGluR toCl-MAG1

Experiments were performed as described previously. [3] The homomeric ionotropic glutamate receptor GluK2(Q) L439C (LiGluR) was expressed in HEK 293T cells using a previously described pcDNA-expression vector [2, 4, 5]and Lipofectamine 2000 (Invitrogen) as transfection agent. YFP was co-transfected as fluorescent marker protein to identify transfected cells. Cell-surface receptors were labeled after 24 - 48 h expression at 37°C. [6]

The PTL *to*Cl-MAG1 was dissolved in dry DMSO to yield a ~50 mM stock solution, which was stored at -20°C. Prior to labeling, cells were washed with external solution (see below) and incubated for 2 min with 0.3 mg/ml concanavalin A (ConA) to suppress ligand-induced desensitization. [7] Labeling was performed for 40 min at room temperature with ~50 µM *to*Cl-MAG1 in external solution, after which the cells were thoroughly washed with external solution to remove any unreacted PTL.

Whole-cell recordings were performed at room temperature (22 - 24°C) in voltage-clamp mode, typically at -75 mV using an Axopatch 200B headstage/amplifier (Molecular Devices) on an inverted microscope (Olympus IX series). The extracellular solution contained 138 mM NaCl, 1.5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.3. Patch pipettes were pulled from borosilicate glass to give 3 - 7 MΩ resistance, when filled with internal solution (135 mM K-gluconate, 10 mM NaCl, 10 mM HEPES, 2 mM $MgCl_2$, 2 mM MgATP, 1 mM EGTA, pH 7.4).

Regular photoswitching was performed using Xe-lamp light sources, either a DG4 light source (Sutter) in combination with excitation filters (379/34 nm and 445/20 nm; 'center'/'full width >90%') (Fig. 2d, Fig. S2) or a Polychrome V light source (Till Photonics; 15 nm FWHM bandwidth). The latter allowed continuously testing wavelengths between 340 - 680 nm in 20 nm increments (Fig. 2b, Figs. S1, S3-5, S6). The light sources were coupled via liquid light guides to the back-port of the inverted microscope to give homogeneous epi-illumination through a 40x LUCPlanFLN NA 0.60 FN 22 objective (Olympus). High intensity illumination with 625 nm light (Fig. 2c) was achieved by coupling a collimated LED light source (Thorlabs, M625L3-C1) into the back-port using a double lamp adapter (Olympus, U-DULHA) equipped with a 560 nm dichroic mirror. The light intensity at the sample stage was measured using a photodiode. The intensity dependence (Fig. S2) was measured using a set of ND filters (Omegafilters).

Data were analyzed using Clampfit 10.4 (Molecular Devices) and Profit 6.1 (Quantumsoft). The photoswitching kinetics were well described by a single exponential function: $A(t) = A_0 \cdot e^{-k \cdot t}$. The rate constants of PSS equilibration (Fig. S5) were corrected for the spectral intensity output of the Polychrome light source to an uniform irradiance of 1 mW/mm^2 assuming a linear relation between rate constant and light intensity (Fig. S2).

Supplementary Figures

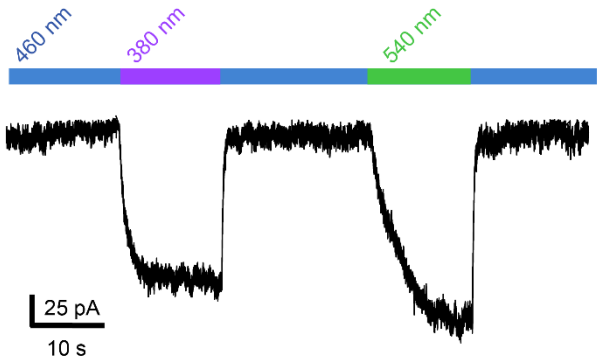


Figure S1. Direct comparison of photo-activation with 380 nm and 540 nm light.

Activation with 540 nm light yields more photocurrent amplitude, but is slower than with 380 nm light (see also Fig. S3-5).

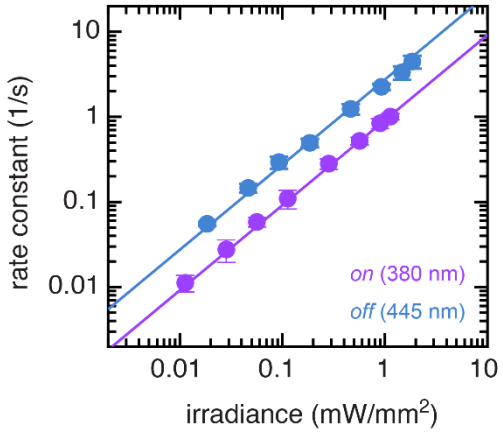


Figure S2. The photoswitching kinetics depends on the light intensity.

Rate constants from single exponential fits of the activation (380 nm) and deactivation (445 nm) kinetics of LiGluR *to*Cl-MAG1. The points represent means ± s.d. (n = 4 cells). In both directions the speed of photoswitching is directly proportional to the irradiance with sensitivities of 0.96 ± 0.02 mm^2/(s mW) and 2.74 ± 0.1 mm^2/(s mW) for 380 nm and 445 nm, respectively (solid lines).

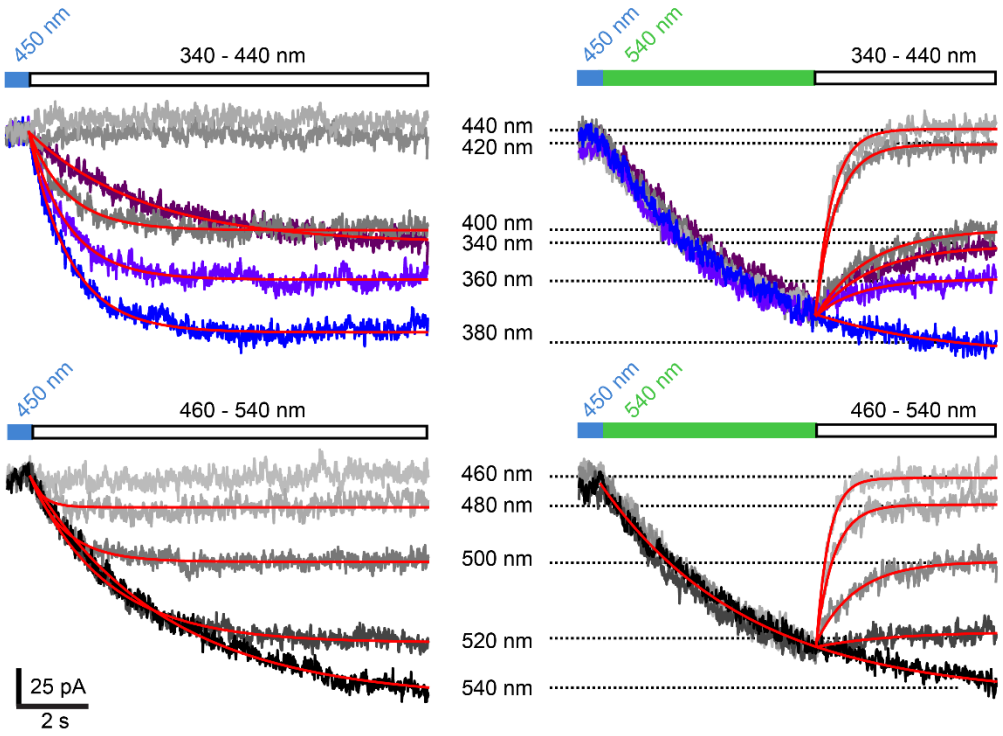


Figure S3. Photo-stationary state (PSS) equilibration of LiGluR *to*Cl-MAG1.

To test for full PSS equilibration, photoswitching in the *trans*-to-*cis* direction (left panels) was compared to photoswitching in the *cis*-to-*trans* direction (right panels): LiGluR *to*Cl-MAG1 was either pre-equilibrated to a predominantly *trans* PSS (450 nm) or *cis* PSS (540 nm) and then illuminated with light of the indicated wavelengths (open bars). In the wavelength range of 340 - 540 nm full PSS equilibration was achieved within 30 s (~1 mW/mm^2), whereas >540 nm equilibration remained incomplete on this timescale (see Fig. S4). The activation and deactivation kinetics are well described by single exponential functions (red lines; for rate constants see Fig. S5).

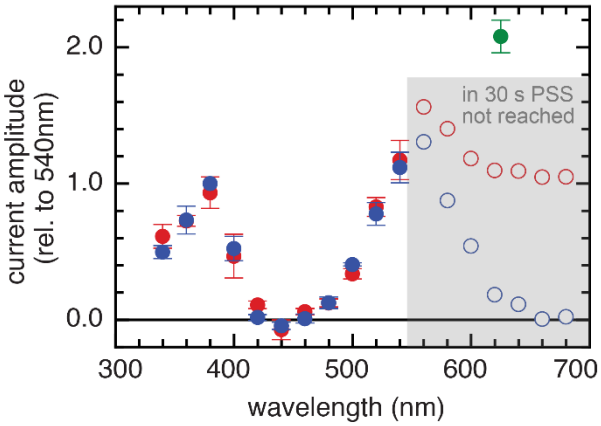


Figure S4. Activation spectrum and PSS equilibration of LiGluR *to*Cl-MAG1.

The photocurrent reaches a local maximum at 380 nm and a global maximum ≥560 nm, indicating PSSs with high *cis* content. Minimal photoactivation is observed at 440 nm, indicating a PSS with maximal *trans* population. Blue and red symbols denote switching after 450 nm and 540 nm pre-equilibration, respectively (see Fig. S3). Between 340 - 540 nm (~1 mW/mm^2) full PSS equilibration was achieved within 30 s (closed symbols), whereas >540 nm equilibration remained incomplete (open symbols) on this timescale. Nevertheless, *to*Cl-MAG1 can be efficiently switched to the *cis* form using high intensity red light (see Fig. 2c; green data point above). Data points with error bars represent means ± s.d. (n = 4 - 5 cells).

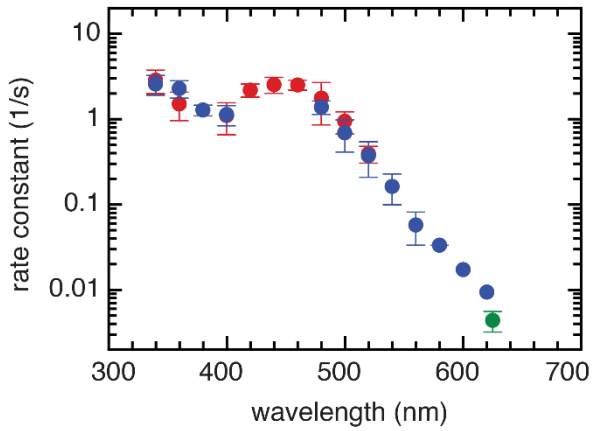


Figure S5. Analysis of the PSS equilibration kinetics.

Photoswitching <400 nm (near UV) and in the 460 nm region is fast, reflecting the absorbance maxima of *to*Cl-MAG1 in aqueous solution (see Fig. 1). Photo-

equilibration slows down >500 nm, where the *to*Cl-MAG1 absorbance drops off. The rate constants were obtained from single exponential fits (see Fig. S3) and corrected for the differences in light intensity to represent a uniform irradiance of 1 mW/mm^2 across all wavelengths (see Materials and Methods). As expected, equilibration in the *trans*-to-*cis* direction (blue symbols) is as fast as in the *cis*-to-*trans* direction (red symbols). Above 600 nm high light intensities are necessary to achieve isomerization on the minutes timescale (see Fig. 2c; green data point above). Data points with error bars represent means ± s.d. (*n* = 4 - 5 cells).

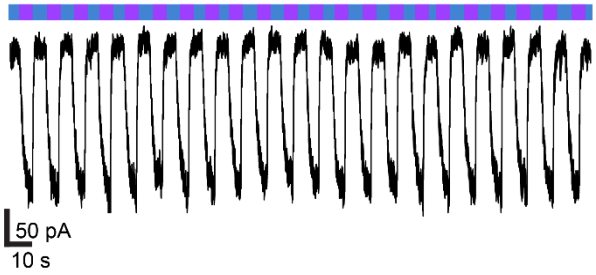


Figure S6. Reversibility and photostability of *to*Cl-MAG1 photoswitching.

Repetitive photoswitching with 380 nm/460 nm illumination (5 s *on*/*off* cycle) demonstrates excellent photostability of *to*Cl-MAG1.

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