Two-photon brightness of azobenzene photoswitches designed for glutamate receptor optogenetics

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Edited by Winfried Denk, Max Planck Institute for Medical Research, Heidelberg, Germany, and approved January 7, 2015 (received for review September 9, 2014)

Mammalian neurotransmitter-gated receptors can be conjugated to photoswitchable tethered ligands (PTLs) to enable photoactivation, or photoantagonism, while preserving normal function at neuronal synapses. \textsuperscript{1}MAG\textsuperscript{1} PTLs for ionotropic and metabotropic glutamate receptors (GlRs) are based on an azobenzene photoswitch that is optimally switched into the liganding state by blue or near-UV light, wavelengths that penetrate poorly into the brain. To facilitate deep-tissue photoactivation with near-infrared light, we measured the efficacy of two-photon (2P) excitation for two MAG molecules using nonlinear spectroscopy. Based on quantitative characterization, we find a recently designed second generation PTL, L-MAG\textsubscript{460}, to have a favorable 2P absorbance peak at 850 nm, enabling efficient 2P activation of the GluK2 kainate receptor, LiGluK. We also achieve 2P photoactivation of a metabotropic receptor, LimGluR, with a new mGluR-specific PTL, D-MAG\textsubscript{046}. 2P photoswitching is efficiently achieved using digital holography to shape illumination over single somata of cultured neurons. Simultaneous \textsuperscript{2}Ca\textsuperscript{2+}-imaging reports on 2P photoswitching in multiple cells with high temporal resolution. The combination of electrophysiology or \textsuperscript{2}Ca\textsuperscript{2+} imaging with 2P activation by optical wavefront shaping should make second generation PTL-controlled receptors suitable for studies of intact neural circuits.

Significance

MAGs (maleimide-azobenzene-glutamate) are photoswitches that covalently bind to genetically engineered glutamate receptors (GlRs) and, under the control of light, mimic or block the action of the excitatory neurotransmitter glutamate. However the blue and near-UV light that optimally photo-switch MAGs do not penetrate well into the brain. In this paper, we show how MAGs can instead be photoswitched by two-photon (2P) absorption of near-infrared light, which penetrates deeper into tissue. We demonstrate 2P control of MAG-dependent ionic currents in neurons, and synthesize a new MAG photoswitch to enable 2P activation of a G protein coupled receptor signaling cascade through a metabotropic GluR. These optogenetic tools bring exceptional spatiotemporal resolution and pharmacological specificity to the study of synaptic transmission and plasticity in intact neural circuits.


The authors declare no conflict of interest.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1416942112/-\textsuperscript{DC1}Supplemental.

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Modern neurobiology relies heavily on optical microscopy to observe, and, increasingly, to manipulate (1, 2), biological processes in live tissue. Among these methods, 2-photon-excited fluorescence microscopy (2PM) with near-infrared (NIR) light has emerged as an important technique for extending optical microscopy to highly scattering tissue (3, 4). Remarkably, barely 25 years after the first 2P-excited fluorescence image was published (5), 2PM is now performed in awake, behaving animals (4, 25). The rapid time to adoption of 2PM owes at least some credit to the availability of spectroscopic data on the 2P-excited efficacy, or brightness, of synthetic and genetically encoded fluorophores (15–17). Brightness, defined for fluorophores as the product of absorption cross-section and fluorescence quantum yield, gives the experimenter an objective metric to assess fluorescent reporters and identify appropriate optical parameters, such as the optimal excitation wavelength and range of light intensities (18). By comparison, relatively little information is available on the 2P properties of optogenetic and photopharmacological tools (12, 14). Wider adoption of 2P optical manipulations should follow from quantitative characterization of 2P brightness for optogenetic and photopharmacological reagents.

Here we quantify the 2P brightness of two types of photoswitchable tethered ligands (PTLs) based on azobenzene. PTLs comprise a modular class of genetically-targeted photochemical tools first conceived in the 1970s by Eranger and Lester for acetylcholine receptors (19, 20). PTLs have since been developed for a variety of other receptors and channels (2). Maleimide-azobenzene-glutamate (MAG) compounds are designed to be conjugated to the extracellular ligand binding domains of genetically engineered mammalian glutamate receptors (GlRs), enabling pharmacological manipulation of signaling pathways dependent on the excitatory neurotransmitter glutamate (21–25). MAGs have been used to study neurotransmission and neuroplasticity in a variety of preparations (26–29). Two families of MAGs have been synthesized to date: First generation MAGs have a symmetrically-substituted azobenzene core, e.g., L-MAG\textsubscript{0} (21) and D-MAG\textsubscript{0} (30). Second generation MAGs have an asymmetrically-substituted azobenzene core, e.g., L-MAG\textsubscript{460} (31) and MAG\textsubscript{2p} (32). The latter MAGs are single-color photoswitches incorporating an electron-donating amine and an electron-withdrawing amide substitution, creating a “push–pull” system that both red-shifts the trans isomer absorption spectrum and reduces the thermal stability of the cis isomer (31, 33).
It was recently reported that the asymmetric substitution in second generation PTLs enabled efficient 2P excitation in two antennae-bearing PTLs, MAG$_{2P}$, and MAGA$_{2P}$ (32). However, that study concluded that the bistable nature of a first generation MAG could ultimately generate a larger effect, albeit at a slower rate, by building up a population of cis isomers. We spectroscopically characterized trans-MAG0 and the original “push–pull” MAG, l-MAG$_{0a0}$, in solution, and determined the 1P and 2P absorption cross-sections and quantum yield of photoisomerization for these molecules. From experimental values, we determine that 2P-excited trans $\rightarrow$ cis photoswitching in l-MAG$_{0a0}$ occurs with efficiency comparable to 2P-excited fluorescence in EGFP (18). In other words, l-MAG$_{0a0}$ is as bright as EGFP. However, owing to a symmetry-disallowed 2P transition to the near-UV band, l-MAG0 and other first generation PTLs are ill-suited to the range of wavelengths most commonly used for 2PM (700–1,100 nm).

We validate these spectroscopic results with functional characterization of 2P photoactivation of MAGs in cultured cells expressing genetically engineered glutamate receptors, identifying sets of parameters suitable for 2P photoactivation of MAGs in several GluR–PTL combinations: We demonstrate that 2P excitation with 800–900-nm pulses efficiently activates LiGluR photocurrents in neurons labeled with l-MAG$_{0a0}$, but not with l-MAG0. Using digital holography (2P-DH) (13, 34), we demonstrate that activation of l-MAG$_{0a0}$ is compatible with functional imaging using the red genetically encoded calcium indicator R-GECOI.0 (35). This combination of optical tools facilitates fast, multicellular stimulation and recording, an important benchmark for the utility of PTLs for in vivo optogenetic studies. Finally, we synthesized a new compound with altered stereochemistry, and show that the resulting PTL, d-MAG$_{0a0}$, enables 2P control of an engineered G protein-coupled receptor (GPCR), LimGluR3 (30). We discuss why the 2P optical properties of MAGs generalize to a larger range of ligand substitutions, which could broaden the palette of PTLs for optogenetic neural circuit analysis in tissue.

**Results**

**One-Photon Absorption.** The 1P absorption spectra of the first and second generation MAGs, l-MAG0 and l-MAG$_{0a0}$, in DMSO are shown in Fig. 1 (blue solid lines). The absorption spectra of these PTLs can be described by inhomogenously broadened bands (Fig. 1, dashed lines) similar in relative strength and energies to the two lowest energy optical transitions in the core azobenzenes: trans-L-MAG0 (Fig. 1A) has a near-UV absorption peak at 376 nm, with a much weaker transition at 450 nm, consistent with $\pi$-$\pi^*$ and $\sigma$-$\pi^*$ electronic transitions in the substituted core, bis-para-diamidoazobenzene (36). The strength of these bands is altered in cis-L-MAG0 (Fig. 1B), which is sufficiently metastable that it can be measured with a conventional spectrophotometer by first generating a photostationary state with near-UV light. Second generation trans-L-MAG$_{0a0}$ has two overlapping visible absorption bands (Fig. 1C, dashed lines), which can be resolved by looking at the solvent dependence of the 1P absorption spectrum (SI Appendix, Fig. S1). As in the parent compound Disperse Red 1, these data support assignment of $\pi$-$\pi^*$ and $\sigma$-$\pi^*$ electronic transitions (37, 38). We determined an extinction coefficient, $e = 19,500 \pm 500 \, \text{M}^{-1} \cdot \text{cm}^{-1}$ at 425 nm in DMSO. The spectrum of cis-L-MAG$_{0a0}$ was determined by transient absorption (see below).

**Two-Photon Absorption.** MAGs are not fluorescent, so we measured the 2P absorption cross-section spectra ($\sigma_2$ spectra) directly using a nondegenerate white light continuum technique (39) (described in detail in SI Appendix, Figs. S2 and S3). The resulting spectra revealed strikingly different 1P and 2P absorption properties in the trans isomers of both MAGs (Fig. 1A and C, red symbols). The peak absorption ($\lambda_{\text{eff}} = 318 \pm 2$ nm) in first generation compound trans-L-MAG0 was blue-shifted 4,850 cm$^{-1}$ relative to the 1P absorption peak ($\lambda = 376$ nm in DMSO) (Fig. 1A), which is more consistent with a higher energy transition that does not appear in the 1P spectrum. When the 2P measurement was repeated in the background of a photostationary population of cis isomers, the 318-nm absorption markedly, and reversibly, decreased in intensity while absorption at the $n$-$\pi^*$ transition increased (Fig. 1B, red symbols). Based on 2P measurements on a related PTL (maleimide-azo-benzene-quaternary ammonium) in phosphate buffered solution (SI Appendix, Fig. S4), we saw no evidence that peak at 318-nm peak was grossly influenced either by solvent polarity or by resonance-enhanced 2P absorption due to overlap between the white light and 1P absorption spectra (SI Appendix). Trans-L-MAG$_{0a0}$ had peak $\sigma_2$ at the same transition energy as peak 1P absorption ($\lambda_{\text{eff}} = 427$ nm), but dropped off more steeply at low energies, resolving the $n$-$\pi^*$ absorption band (Fig. 1C, red symbols)
symbols). Taken together, the 2P absorption spectra of both MAGs are consistent with parity selection rules for dipolar transitions in centrosymmetric molecules. These rules predict that the lowest-energy 1P-allowed π-π* transition should be strictly forbidden for 2P absorption (9).

Molecular 2P absorption cross-sections (σ₂) in Goepptert-Mayer units (Table 1: 1 GM = 10⁻¹⁹ cm² s · γ⁻¹) were determined from back-to-back measurements of MAGs and reference samples (SI Appendix, Figs. S3f and S5E). The resulting σ₂ values (Table 1) are remarkably consistent with those of the parent compounds and related compounds from both classes of azobenzenes (36, 38). Notably, the σ₂ of both MAGs are orders of magnitude larger than that of the most widely used caged neurotransmitter, MINI-glutamate (σ₂ = 0.04 ± 0.02 GM at 730 nm; ref. 40). The cross-section of trans-L-MAG0460 is at least as large as the most common fluorescent proteins (σ₂ = 40–100 GM; ref. 17) in the NIR.

Quantum Yield of MAG Photoisomerization. Brightness depends not only on the strength of absorption, but also on the quantum efficiency of the process of interest. To ascertain the quantum yield (QY) of trans → cis photoisomerization (denoted E→Z), we characterized the excited state reaction of the isolated MAGs in solution using broadband femtosecond transient absorption spectroscopy (SI Appendix, Fig. S2). Following excitation with 400-nm 100-fs laser pulses, time-resolved difference spectra were obtained with 150-fs resolution for the first 1 ns of the reaction (Fig. 2). In both MAGs, the kinetics near the peak 1P absorption show an instrument-limited transient bleach of the trans isomer (Fig. 2A, λprobe = 380 nm; Fig. 2C, λprobe = 420 nm) that partially recovers as a fraction of photoexcited molecules isomerize. The bleach decay occurs on two distinct timescales, mirrored in the kinetics of a red-shifted absorption (Fig. 2B, λprobe = 460 nm; Fig. 2D, λprobe = 540 nm). These time- and wavelength-resolved datasets were decomposed by global analysis (41) using a multi-state model based on the photodynamics of trans-azobenzene (42) (SI Appendix, Fig. S6). In L-MAG0, we found a net photo-product yield, ϕE→Z = 0.15 ± 0.05, which is within the range of reported values for trans→cis photoisomerization in azobenzene in DMSO via π-π* excitation (43). The difference spectrum associated with the photoproduct (Fig. 2B, Upper, green solid line) nicely decomposed into the cis and trans spectra derived from photostationary measurements (Fig. 2B, Lower, green circles).

Furthermore, from knowledge of the extinction coefficients of both isomers and the yield of the forward reaction, we can infer from photostationary spectra that the QY of cis-trans isomerization is 0.4, consistent with cis-azobenzene (42).

The L-MAG0460 data were fit to the same model to obtain ϕE→Z = 0.4 ± 0.1. A higher isomerization QY is consistent with excitation of the n-π* transition (42). The photoproduct spectrum had a red-shifted band (Fig. 2D, Lower, green circles), qualitatively consistent with a cis isomer (42). However, the 1-ns absorption spectrum extends to longer wavelengths than observed in photostationary measurements on related compounds (32), and may represent a vibrationally “hot” cis-L-MAG0460. Transient absorption following 2P excitation with the laser fundamental (800 nm, 100 fs, 16 mW) produced a similar photoproduct (Fig. 2D, Lower, red solid line). Thus, it appeared that 1P and 2P excitation in trans-L-MAG0460 are both effective at driving trans–cis photoisomerization.

From the experimental values for ε, σ₂, and ϕ, the brightness of each MAG isomer was defined as ϕεσ₂, for 1P and 2P excitation, respectively (Table 1). The trans isomer brightness represents the probability of photoswitching from the thermally equilibrated (dark) state. Compared with the 2P brightness of common fluorescent reports like EGFP (30 GM at 927 nm; ref. 17), these brightness values indicate that trans-L-MAG0460 is favored for the NIR light sources and intensities typical in 2PM. Trans-L-MAG0, on the other hand, is at least 20 times dimmer at NIR wavelengths. Whereas the large cross-section at 640 nm suggests that L-MAG0 might be a powerful tool if used in conjunction with visible-wavelength femtosecond light sources, the QY of this 1P-disallowed remains to be determined to properly quantify brightness.

Two-Photon Cis→Trans Photoswitching of L-MAG0. We next sought to validate 2P excitation of MAGs through functional measurements in live cells expressing engineered GluRs. For GluRs engineered to be agonized by the cis isomer, bistable L-MAG0 can mimic the chronic effect of soluble GluR agonists, with the unique feature of very fast and reversible action (23). The strength of MAG agonism is intrinsically limited by the largest population of cis isomers that can be attained, which is defined by the wavelength-dependent photostationary state (PSS). Based on the σ₂ spectra (Fig. 1), different PSS can be expected under 1P and 2P excitation. In fact, the cis isomer is significantly brighter than the trans isomer for wavelengths greater than 700 nm. Consequently, starting from a dark-equilibrated population of trans isomers, the rate of trans → cis 2P activation would quickly be exceeded by the rate of cis → trans 2P de-activation, tending to “turn off” the MAG effect. Therefore, we expected that L-MAG0 would inherently produce weaker cis-agonism under 2P excitation than under 1P excitation.

To test these predictions, we measured L-MAG0-activated photocurrents in the engineered GluK2 kainate receptor, LiGluR, using whole cell patch clamping on a laser-scanning microscope equipped with 405 nm and 488 nm lasers, as well as a Ti:sapphire laser (Fig. 3). Dissociated hippocampal neurons were transfected with an hSyn (human synapsin 1) gene promoter driven LiGluR (GluK2-L439C-K456A), where L439C is known to bind L-MAG0 as a cis-activated agonist (21–24) (Fig. 3A). The additional point mutation (K456A) reduces affinity for soluble glutamate and quickens receptor recovery to glutamate-dependent desensitization (44, 45). Photocurrents were induced by scanning the imaging laser over the patched cell somata and proximal processes (Fig. 3B, red dashed lines). The 1P excitation

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<td>‡Inferred from PSS of 96% cis isomers at 380 nm (22).</td>
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The rate of photocurrent reduction was consistent with the measured conditionally affect thermal reisomerization. Together, these results suggest ceased, suggesting that prolonged NIR exposure might adduced to thermal reisomerization. Kinetics at 2P PSS expected at 900 nm from the relative 2P f r o mt he s a m e c e l l( F i g .3

Again, a 405-nm laser was scanned over the soma to generate in photocurrent indicative of that do not undergo isomerization. Kinetics at 2P photocurrents were nearly identical (Fig. 4C), showing that LiGluR overexpressed in neurons is distributed throughout the plasma membrane (23, 46). Additional analysis showed that LiGluR overexpressed in neurons is distributed between cells (30–400 pA, mean = 8 ± 0.20 pA, n = 20). In many neurons, 1P excitation triggered action potentials in unclamped portions of the cell (seen as action currents, indicated by * in Fig. 4A).

For 2P excitation, light was targeted to the soma of each neuron (Fig. 4B, image, red region) by spatially patterning laser pulses from a Ti:sapphire laser using digital holography (13). Bouts of 50–100 ms of 840–850-nm robustly generated photocurrents (Fig. 4B, traces) that were similar to those evoked by 1P excitation. The decays of 1P and 2P photocurrents were nearly identical (Fig. 4C and D: τ1/2 = 200 ± 10 ms for 1P, n = 20; τ1/2 = 190 ± 20 ms for 2P, n = 11; p = 0.3, unpaired, two-way t test). These photocurrent kinetics are slightly faster than previous observations in HEK293 cells of 1P activation of GluK2-L439C and LiGluR channel dynamics contribute to photocurrent decay. Regardless, the effective decay kinetics do not depend on 1P vs. 2P excitation, nor on somatic vs. whole cell excitation, supporting our interpretation that the photocurrents arise from the same photopharmacological process.

The 2P-DH excitation with time-averaged intensity in the range of 1–2.3 mW/µm² stimulated photocurrents that were 38 ± 8% of the maximum current reached by 1P excitation of the same neuron (Fig. 4E, n = 11). The generation of smaller photocurrents by 2P-DH was consistent with the smaller fraction of the cell that was illuminated (compare blue region to red region in Fig. 4A and B, respectively) considering that studies showed that LiGluR overexpressed in neurons is distributed throughout the plasma membrane (23, 46). Additional analysis of photoswitching efficiency is presented in SI Appendix (SI Appendix, Fig. S7).

Simultaneous Ca²⁺-Imaging and 2P-DH Photoswitching. Another major challenge in optogenetics is how to stimulate and measure physiological function without interference between optical reporters and effectors. Thus, we next asked whether 2P-DH photoswitching of l-MAG0 was compatible with simultaneous functional imaging. Because LiGluR is Ca²⁺-permeable, we measured photoactivation of LiGluR + 1-MAG0 with the genetically encoded red fluorescent Ca²⁺ indicator, R-GECO1.0

Fig. 2. Photoswitching dynamics in MAGs. (A) Time-resolved changes in absorption for select wavelengths during trans-cis photosomerization reaction in l-MAG0. The time axis is linear until 10 ps, and logarithmic thereafter. Kinetics at ωprobe = 380 nm, near the peak 1P absorption of trans-l-MAG0, show the transient bleach and recovery of photoexcited molecules that do not undergo isomerization. Kinetics at ωprobe = 460 nm represent vibrationally excited isomerization photoproducts. Solid red lines represent fits obtained from global analysis (SI Appendix, Fig. S6). (B) The 1-ns difference spectrum (Upper, green solid line) was decomposed into trans (Upper, blue dashed; Lower, blue solid) and cis contributions. The putative cis spectrum (Lower, green circles) closely matches that of cis-l-MAG0, derived from photostationary measurements (Lower, green solid line). (C) Kinetics in the photosomerization of trans-l-MAG0 were similar, with trans isomer dynamics represented by the kinetics at ωprobe = 420 nm, and photoproduct dynamics seen at ωprobe = 540 nm. Solid red lines represent fits obtained from global analysis. (D) The 1-ns difference spectrum (Upper, green solid) was decomposed into trans (Upper, black dashed; Lower, solid blue) and a putative cis (green circles) absorption spectra with isomerization QY of 0.4 ± 0.1. The 2P excitation yielded a similar photoproduct (Lower, red solid).

with 405 nm generated a saturated photocurrent that was reversible with 488 nm (Fig. 3C, i, purple and green bars, respectively), consistent with previous studies on LiGluR (23). Scanning over the same region with a Ti:sapphire imaging laser (80-MHz repetition rate, time-averaged intensity < 20 mW/µm²) with wavelengths in the range 800-900 nm produced reversible inward currents with much slower onset (Fig. 3C, ii, yellow bars). The 2P photocurrents elicited in this manner never exceeded 15% of 1P photocurrents from the same cell (Fig. 3D, n = 8 with at least one 2P wavelength tested per cell), despite scan periods as long as 10 s. This value is consistent with the 2P PSS expected at 900 nm from the relative 2P brightness of trans and cis isomers of l-MAG0 (Table 1).

Next, we tested 2P deactivation of LiGluR photocurrents. Again, a 405-nm laser was scanned over the soma to generate a saturated photocurrent, where ~90% of the MAG molecules are in cis conformation. When the 2P laser was subsequently scanned over the same area, we observed a reversible reduction in photocurrent indicative of cis → trans photoswitching (Fig. 3C, iii, yellow, red, and brown bars and summary in Fig. 3E). The rate of photocurrent reduction was consistent with the measured σg spectrum of the cis isomer (Fig. 1B), although we found that often photocurrents continued to decrease even after 2P exposure ceased, suggesting that prolonged NIR exposure might additionally affect thermal reisomerization. Together, these results demonstrate that, although it is possible to weakly activate LiGluR + l-MAG0 by 2P excitation, 2P deactivation is the more efficient process, which intrinsically limits the efficacy of l-MAG0 when used at Ti:sapphire laser wavelengths.

Fast 2P Photoswitching of l-MAG0 in Cultured Neurons with 2P Digital Holography. Many optogenetic applications require manipulating select cells within a population with high spatiotemporal precision. Two-photon digital holography (2P-DH) is an effective method to flexibly target light to many regions simultaneously, or to produce an extended image to excite large patches of membrane for summation of single-channel ionic currents (13). It was recently shown that 2P scanning excitation of MAG2p could depolarize neurons, but scans of nearly 1 s were necessary (32). We asked whether faster photoactivation might be attained with 2P-DH.

To test activation of trans-l-MAG0 by 2P-DH, we again transfected dissociated hippocampal neurons with LiGluR. It was shown previously that l-MAG0 covalently tethered to position L439C activates the channel under blue light, while photocurrent decreases in the dark by thermal isomerization of l-MAG0 (22). Here, 1P stimulation was achieved with a 470-nm LED used as a wide-field light source that illuminated the entire neuron (Fig. 4A, image, blue shadow). The 50–100-ms pulses of blue light elicited LiGluR photocurrents of a stereotypical shape (Fig. 4A, traces): Photocurrent switched on rapidly, reached a peak current (SMAX) that was approximately sustained through the duration of the stimulation pulse and decayed slowly (relative to onset) following the end of the light pulse. SMAX varied between cells (30–400 pA, mean = 8 ± 0.20 pA, n = 20). In many neurons, 1P excitation triggered action potentials in unclamped portions of the cell (seen as action currents, indicated by * in Fig. 4A).

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Previous work demonstrated that 1P activation of L-MAG0 at 560 nm does not generate significant LiGluR photocurrent (31); consequently, we reasoned that imaging R-GECO1.0 at this wavelength would produce little background MAG activation. Initial tests in HeLa cells expressing LiGluR showed that following whole-cell 2P-DH stimulation with 840 nm (100 ms exposure at 0.3–1.1 mW/μm²), R-GECO reports Ca²⁺ transients that depend on L-MAG0 (Fig. 5A, Upper, red trace). In cells not treated with L-MAG0, 2P-DH stimulation resulted in modest bleaching of R-GECO1.0 basal fluorescence (Fig. 5A, Lower, gray trace) represent fluorescence recovery after photobleaching. Because 2P bleaching of R-GECO1.0 should be independent of the PTL, L-MAG0-dependent increases in R-GECO1.0 fluorescence (Fig. 5B) likely underestimate the actual size of the Ca²⁺ transient. Nonetheless, 2P photoswitching of L-MAG0 appears compatible with imaging with a red genetically encoded Ca²⁺ indicator with a reasonable signal-to-noise ratio.

To further explore the potential of all-optical, ligand-specific optogenetics, we used the combination of 2P-DH photoswitching and functional imaging to report on Ca²⁺ activity in a population of cells in response to sequential targeted 2P photostimulation. Holograms for three regions of interest (ROIs) were generated using a laser-scanning microscope. ROIs were patch clamped and photoactivated on a laser-scanning microscope. (C) Representative whole cell voltage-clamp recording showing the effects of 1P and 2P laser scanning excitation. Each colored bar represents a stimulation period during which laser light at the designated wavelength was rastered bidirectionally over the cell somata: (i) 405-nm light reproducibly elicited saturated, bistable photocurrents reversible with 488 nm. (ii, iii) Starting from a 1P-saturated LiGluR photocurrent, 2P stimulation reversibly decreased the amplitude of photocurrent, indicating closure of LiGluR channels by cis–trans photoswitching. (D) At the highest intensities tolerated by the cells (20 mW/μm² time-averaged intensity), 2P laser scanning generated photocurrents no greater than 15% of the photocurrents stimulated by 405-nm laser scanning of the same cell. Bars were calculated as the mean current photoactivated from a dark resting state for 2–6 photoswitching cycles, normalized to the cell’s 1P response, and then averaged across cells. The number of independent cells measured at each wavelength is denoted in parentheses. Error bars are SEM. (E) Bars represent the mean relative change in current from two to four photoswitching cycles, averaged across cells. Error bars are SEM.

Two-Photon Photoactivation of an Engineered mGluR with D-MAG0460

Finally, we sought to extend 2P MAG activation to optically manipulate intracellular signaling cascades using a photoactivatable G protein-coupled receptor (GPCR). We turned to the recently described LimGluR2 and LimGluR3, which are based on the group II mGluRs, mGluR2 and mGluR3 (30). LimGluRs allow for photoactivation of the G₁ₒ signaling cascade (Fig. 6A) using the first generation PTL, d-MAG0, a variant of L-MAG0 with altered stereoreactivity but similar optical properties. To achieve 2P stimulation, we synthesized a new red-shifted compound, d-MAG0460 (Fig. 6B, SI Appendix). Following labeling with d-MAG0460, activation of LimGluR2 (mGluR2-L300C) or LimGluR3 (mGluR3-Q306C) was assessed in HEK293T cells using the G protein activated inward rectifying potassium channel, GIRK, as a readout of activation of the G₁ₒ signaling pathway (47). LimGluR3 showed robust photoactivation in response to blue light, while LimGluR2 was poorly activated by d-MAG0460 (Fig. 6C). LimGluR3 + d-MAG0460-dependent GIRK currents reversed in the dark on the time scale of 5–10 s (Fig. 6D), reflecting the slow off kinetics of the G protein signaling cascade rather than the fast (subsecond) thermal isomerization kinetics of the PTL. In contrast to LiGluR + L-MAG0460 (Fig. 4), the current rise time did not depend on 1P intensity and 5–8-s blue light exposure
thermally with the same rate, $\tau$.

The wavelength dependence of LimGluR3 $\mu$ either light source elicited long-lived ionic currents. Action currents are indicated by *. Scale bar is 20 μm.

**Discussion**

**Comparison of MAGs with Other Optogenetic Tools.** Because MAGs can be used for controlling neuron depolarization when tethered to an engineered ion channel (e.g., LiGluR), we note that the 2P absorption cross-sections are smaller than that of Channelrhodopsin2 (230–260 GM) (12, 39). Although it can be difficult to compare $\sigma_2$ values measured by different methods (16, 17), Yamaguchi and Tahara reported a comparable $\sigma_2$ of 230 GM for all-trans retinal in solution determined by the same 2P spectrophotometry technique used here (39). Because most opsins have very high QY (>0.5) for retinal isomerization, it is reasonable to expect that ChR2 is brighter than either MAG. However, L-MAG0460 offer some flexibility in 2P excitation compared with opsins because of their relatively slow off-kinetics (52, 53). Two-photon stimulation of opsins can be used for controlling neuron depolarization when tethered to an engineered ion channel (e.g., LiGluR). We next used 2P-DH to activate LimGluR3 $\Delta$-MAG0460. Photocurrents evoked in this way by 850-nm excitation had kinetics similar to those evoked by 1P photoactivation (Fig. 6D).

The wavelength dependence of LimGluR3 $\Delta$-MAG0460 currents was measured by testing multiple excitation wavelengths within individual cells (Fig. 6F, $n \geq 3$ cells for all wavelengths). A peak at 850–860 nm is apparent, but longer wavelength produced larger signals than we expected from the L-MAG0460 absorption spectrum (Fig. 1A). Upon further investigation, we observed that, in the absence of $\Delta$-MAG0460, 2P-DH illumination of cultured cells occasionally evoked inward currents that were distinguishable from LimGluR3 photocurrents by their relatively small amplitude and rapid kinetics (Fig. 6G, Left). The wavelength dependence of MAG-independent photocurrents showed that the nonspecific current increased in amplitude with longer wavelength (Fig. 6G, Right). By comparing these signals with the NIR absorption of water (48) (Fig. 6G, Right, dashed line), we conjecture that these nonspecific currents arise from heat, as has been described before in cells exposed to intense NIR laser pulses (49–51). By comparing the relative currents, normalized in each cell to the 950-nm signal, we see that the largest MAG-dependent currents occur at a wavelength of 850 nm, functionally corroborating the measured 2P absorption spectrum of $\Delta$-MAG0460 (Fig. 1C). Post hoc, we estimated the amplitude of MAG-dependent currents by scaling the amplitude of photocurrents by the dark relaxation kinetics, and found 2P-DH at 850 nm produced on average 85 ± 13% of the currents generated by 1P photoactivation in the same cell (Fig. 6E, red open squares). Because the entire cell was illuminated for both 1P and 2P excitation, the similar size of evoked currents supports comparable efficacy for 1P and 2P photoswitching of $\Delta$-MAG0460. Overall, these experiments show that $\Delta$-MAG0460 may be used to evoke mGluR3-dependent G protein signaling by 2P photoswitching, thus expanding the applications of these tools to contexts where spatial precision and deep tissue penetration are required to activate a GPCR.
on glutamate receptors. In Table 1, we compare the brightness of MAGs to other photopharmacological reagents that act tamate receptor desensitization blockers, so these optical conditions should be relevant to in vivo preparations. Notably, in contrast to the study evoked by 2P laser scanning (32). Notably, in contrast to the study of efficient 2P photoswitching of L-MAG0460 by 2P-DH (32). Continued 2P exposure will not generate a larger LiGluR-specific photocurrent, and may even result in nonspecific effects as we observed in Fig. 6. On the other hand, the reasonably high 2P brightness of the cis isomer provides an advantage for 2P control of trans-activated MAGs and related azobenzene PTLs (2, 20, 25).

Two-Photon Optical Transitions in PTLs. The absorption spectra in Fig. 1 compel some consideration of parity selection rules for optical absorption. Although the early theory of Goeppert-Mayer detailed the formal extension of quantum selection rules to multiphoton transitions, these predictions could not be experimentally validated until the development of the laser (9). Now, it is widely validated that 1P and 2P absorption can be substantially different, especially in small molecules with high degree of molecular symmetry (56). The influence of parity is apparent in the absorption spectrum of azobenzene, where the centrosymmetric trans isomer has a dipolar electronic transition between the symmetric highest occupied molecular orbital (HOMO) and the anti-symmetric lowest unoccupied molecular orbital (LUMO). PTLs appear to retain the azobenzene electronic symmetry for a variety of ligand substituents, as seen experimentally by the similarity of 1P absorption of first generation PTLs (2). Two-photon absorption in these simple molecules can be approximated as the product of two dipolar transitions, requiring that the electronic wavefunctions in the initial and final states have the same symmetry. The reversal of parity selection rules leads to the prediction that the 1P-allowed π-π* transition should become 2P forbidden. This prediction appears upheld by both of the first generation PTLs, as seen by their blue-shifted 2P absorption bands (Fig. 1 and SI Appendix, Fig. S3). Furthermore, the observation that the bent cis isomer of L-MAG0 had common 1P and 2P transitions is consistent with the parity formalism applied to non-centrosymmetric molecules.

It is interesting to note that the asymmetrically substituted L-MAG0460 also showed blue-shifted 2P absorption consistent with a 2P-forbidden transition at 460 nm. Although the reduced symmetry of this photoswitch could relax the parity selection rules for π-π* transitions, our measurements are collectively more consistent with a highly blue-shifted n-π* band. The ordering of electronic states in pseudostilbenes is still debated (38), but electronic structure calculations on related molecules Disperse Red 1 and Disperse Orange 3 show significant population of an n-like HOMO-1 state (38). The character of these electronic transitions is significant for at least two reasons. First, a higher photoisomerization yield is often associated with n-π* transition because it directly accesses the reactive excited state (42). Second, it suggests that solvent stabilization of a nonplanar trans conformation underlies the favorable 2P absorption in push–pull PTLs. This insight may be important in designing new 2P azobenzenes with both thermal bistability and favorable π-π* interactions. For instance, an alternative chemistry for red-shifting azobenzene absorption is to break the symmetry of the trans isomer by steric specificity at high concentrations (2). Several other caged glutamate compounds have been developed with higher QY of photolytic cleavage or more favorable absorption at visible wave-lengths (36, 37), but the 2P efficacy of most of these compounds have not been objectively quantified. Caging groups with good nonlinear absorption, such as the diethylamino coumarin chromophore recently described by Olson et al. (55), may significantly improve the 2P efficacy of such compounds.

In general, a lack of quantitative data about these compounds limits the ability to compare optogenetic and optopharmacological tools. Characterization of 2P properties should not only help researchers select the most appropriate reagents, but also devise the most effective optical stimulation protocols. For instance, by characterizing the brightness of both isomers of L-MAG0, we showed that the maximum current one can expect from LiGluR + L-MAG0 is limited by the 2P PSS. Continued 2P exposure will not generate a larger LiGluR-specific photocurrent, and may even result in nonspecific effects as we observed in Fig. 6. On the other hand, the reasonably high 2P brightness of the cis isomer provides an advantage for 2P control of trans-activated MAGs and related azobenzene PTLs (2, 20, 25).

**Fig. 5.** The 2P-DH photoswitching of L-MAG0460 is compatible with red Ca2+ imaging on a spinning disk confocal microscope. (A) Representative fluorescence traces from 2P-DH targeted HeLa cells coexpressing LiGluR and R-GECO1.0. (B) Cells not treated with MAG are bleached by 840-nm pulses (n = 6), and cells treated with L-MAG0460 have stereotypical Ca2+ transients (n = 4). (C) Representative image of basal fluorescence of R-GECO1.0. Optical recordings were made from many cells with 50–100-ms time resolution, and 2P-DH stimulation of individual cells was targeted by encoding ROIs as separate holograms. Scale bar is 50 μm. (D) The fidelity of each hologram was visualized by imaging 2P-excited fluorescence from a fluorescein film. Speckles in the 2P image are common in 2P-DH. (E) Ca2+ transients recorded from three cells stimulated in rapid sequence by 840-nm projections of the holograms corresponding to D.

relatively insensitive to the method of 2P stimulation. Indeed, our findings of efficient 2P photoswitching of L-MAG0460 by 2P-DH complement the recent report of two antenna-bearing MAGs with similar thermal relaxation, where LiGluR photocurrents were evoked by 2P laser scanning (32). Notably, in contrast to the study in ref. 32, our characterization in neurons was done without glutamate receptor desensitization blockers, so these optical conditions should be relevant to in vivo preparations.

Considering their ligand-specific nature, it is worthwhile to compare MAGs to other photopharmacological reagents that act on glutamate receptors. In Table 1, we compare the brightness of L-MAG0 and L-MAG0460 with two popular types of caged glutamate (14). MINI-glutamate is still the most widely used compound in 2P applications, despite low brightness, due to its high
crowding with ortho substitutions (57). It was recently demonstrated that tetra-ortho-chloro MAG (toCl-MAG1) has both enhanced n-π* absorption in the trans isomer and thermal bistability in agonizing LiGluR (52). Our results suggest that such molecules may also have favorable 2P absorption. Although these ideas remain to be experimentally validated, identifying general strategies for designing 2P azobenzenes would accelerate the development of new PTLs for optical manipulation of neurophysiology. These measurements may also possibly find wider impact in industrial and research applications where azobenzenes are used as molecular photoswitches, such as optical storage (58) and optomechanical manipulation of biomolecules (59).

Summary

In summary, we have quantitatively characterized the 2P brightness of MAG PTLs with either a symmetrically or asymmetrically-substituted azobenzene core. We found that both types of MAGs have 2P absorption cross-sections that are relatively large compared with other optical tools widely used in neuroscience, and we identified functional conditions for 2P photoactivation of each MAG in conjugation with engineered ionotropic and metabotropic GluRs. We demonstrated that digital holographic 2P stimulation of MAGs can effectively be used to depolarize cells, or activate GPCR signaling cascades, with either electrophysiological or imaging readout with red-shifted genetically

Fig. 6. Photoactivation of mGluR signaling with o-MAG0460. (A) Group II metabotropic GluRs couple glutamate to a G_i/o signaling cascade. Engineered mGluRs (LimGluRs) require MAGs with altered stereochemistry. (B) o-MAG0460 is a second generation PTL with similar optical properties to l-MAG0460. (C) The efficacy of o-MAG0460 photoactivation (445 nm) in LimGluR2 and LimGluR3 was assessed as the percentage of GIRK current observed relative to saturating glutamate (1 mM). (D) Whole cell voltage-clamp recordings from HEK293T cells expressing LimGluR3 and labeled with o-MAG0460 show that blue light activates a G protein signaling cascade, measurable by GIRK currents. The kinetics of the photocurrent are considerably slower than photoswitching. (E) Whole cell excitation by 2P-DH with 850 nm produced on average 85 ± 13% of the MAG-dependent current of wide-field 470-nm excitation (n = 7). (F) The wavelength dependence of LimGluR3 + o-MAG0460 currents, n > 3 cells for all wavelengths. Each symbol represents an individual cell. Data points were first scaled by light intensity (relative to 850 nm), and then normalized to the each cell’s response to 850 nm. (G) Additionally 2P-DH produced a relatively small inward current in some cells, independent of MAG, which had easily distinguishable kinetics and different wavelength dependence (n > 2 cells for all wavelengths). Each symbol represents an individual cell. Black represents mean and SEM. The action spectrum of nonspecific current increase diminishes with longer wavelength. Accounting for the MAG-independent current, the largest MAG-dependent currents occur at a wavelength of 850 nm, functionally corroborating the measured 2P absorption spectrum of o-MAG0460 (Fig. 1A).
encoded fluorescent indicators. These results highlight the possibility of optically mimicking elements of excitatory neurotransmission using a combination of 2P optical tools and genetically specified pharmacology that can add novel dimensions to studies of intact neural circuits.

Materials and Methods
Femtosecond Absorption Spectroscopy. Femtosecond transient absorption spectra were recorded using a home-built nonlinear spectrophotometer (SI Appendix, Fig. S2A) based on an amplified Ti:sapphire laser system (Newport; SpitfirePro), as described elsewhere (1). In brief, short pulsed light at 800 nm (2.1 W time-averaged power, 1-kHz repetition rate, 800 nm, 40-fs pulse FWHM) was split into independent optical paths to generate pump and probe pulses. Pump pulses resonant with the 1P absorption of trans-L-MAG0 and trans-L-MAG0D; 90°, ∼2.5 mW, 100 fs) were obtained from the fundamental pulse by second-harmonic generation in a 880 crystal (29.2°, 1 mm thick). For 2P excitation of L-MAG0D, the pump pulse was the laser fundamental (16 mW). The probe pulse was a coherent white light generated by self-phase modulation of ∼1 μW nm focused into a 2-mm-thick CaF2 window. Pump-induced changes in absorption (ΔA) spectra were recorded for a time series of pump-probe delays with 10-fs resolution by controlling the optical path length of one pulse with a computer-controlled translation stage. At each time point, ΔA(λ, t) was calculated from pairs of probe-transmission spectra obtained at 1 kHz while optically chopping pump exposure at 500 Hz. Self-referenced probe transmission allowed detection changes in optical density <10−6. Pump and probe pulses were linearly polarized at magic angle (54.8°) to obtain isotropic response. MAGs in DMSO were measured in 1-mm path at 0.5 μM to get reasonable signal to noise. Consequently, cross-phase modulation was considerable. We took care in the experiments to check that all optical powers were measured after the objective with a λ-calibrated piezolectric meter (Thorlabs). Radiant flux (photons−s−1) was calculated from measured power P for center wavelength λ as:

\[ I_{\text{w}} = \frac{P}{\lambda \cdot \text{area}} \]

The factor g accounted for the time distribution of photons for different light sources: LED, g = 1; 2P-DH: \( g = \left( \frac{g_0}{g_f} \right) \), where we used value \( g_f \) for Gaussian pulse shape (6), repetition rate \( r = 80 \text{ MHz} \), and a pulse duration \( \tau = 400 \text{ fs} \), measured by autocorrelation of NIR pulses at the objective (Intelligent Imaging Innovations, Inc.). The ROI areas used to calculate light intensity for 2P-DH were determined from spatial calibration of the image within Slidebook 5.5 imaging software. Time-averaged powers at the objective for 2P-DH experiments ranged from 75 to 300 mW. Scanning excitation used NIR time-averaged power less than 20 mW (80 MHz, ca. 300 fs).

Functional Imaging. R-GECO was imaged on an upright spinning disk confocal microscope (Intelligent Imaging Innovations, Inc.) with a 561-nm diode laser and 20×/0.75NA water dipping objective (Zeiss). Images were obtained by an EM-CCD camera at frame rates of 10–20 Hz. The 2P-DH light path was coupled to the microscope through a sideport and projected onto the sample through the imaging objective. The 2P bouts were controlled by a Pockels’ cell and timed with camera acquisition through Slidebook imaging software. Ca2+ transients represent mean ΔF/ΔF0 over pixels within ROIs.

Data Analysis. All spectroscopy and physiology data analysis was done with custom scripts in Matlab. All values represent mean ± SEM. Errors on calculated values were determined using SE propagation, unless otherwise stated.

Details of tissue culture, electrophysiology, and MAG synthesis are provided in SI Appendix.

ACKNOWLEDGMENTS. We thank Matthew Banghart for the generous gift of MAQ: Olivier Thoumine, Cherie Stanley, and Grant Kauwe for the low-affinity GluK2 construct (La-LiGlur); Andreas Reiner for guidance on working with D-MAG0460; Hildeg Adesnik for electrophysiology data acquisition software; and Karl Kilborn and Brian Bodenmeister for customizing Slidebook software. Funding was provided by the NIH Nanomedicine Development Center for the Optical Control of Biological Function (PN2EY018241) (to E.Y.L.), NIH Ruth L. Kirschstein National Research Service Award (F32EY022860) (to M.A.K.), and the National Science Foundation (CHE-1413739) (to D.S.L.).

Supporting Information

Two-photon brightness of azobenzene photoswitches designed for glutamate receptor optogenetics

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Supporting Methods

Tissue culture and transfection. Dissociated postnatal hippocampal neurons (P0-P5) were prepared from Sprague Dawley rats (Charles River) at high density (80K cells/cover slip) and transfected using the Calcium-Phosphate transfection method as described previously (1), with 1 µg of cDNA encoding LA-LiGluR (GluK2-L439C-K456A) and 0.3 µg of EGFP. The additional point mutation (K456A) reduces glutamate affinity and quickens receptor recovery to glutamate desensitization (2). HeLa cells were maintained in DMEM with 5% FBS and transiently transfected with (in µg): 0.8 CMV-LiGluR6 and 1 CMV-R-GECO1.0 (red-calcium indicator (3)), using Lipofectamine 2000 (Invitrogen). Functional imaging was performed 2-3 days after transfection. Prior to imaging, cells were labeled with 100 µM L-MAG0460 and treated with 0.3 mg/mL concavalin A (Sigma) to block desensitization of LiGluR. HEK293 and 293T cells were maintained in DMEM with 5% FBS and transiently transfected 24-36 hrs prior to experiments with (in µg): 0.7 LimGluR2 (mGluR2-L300C) or LimGluR3 (mGluR3-Q306C), 0.7 GIRK1-F137S and 0.1 tdTomato using Lipofectamine 2000. All cells were grown at 37°C.

Electrophysiology. Patch clamp recordings used a Multichannel 700B amplifier in the whole cell mode. Recordings were carried out after 15 DIV for hippocampal neurons. For LiGluR experiments, cells were incubated with 100 µM of either L-MAG0 or L-MAG0460 for 45 min at 37°C, 5% CO2. The labeling solution contained (in mM): 150 NMDG-HCl, 3 KCl, 0.5 CaCl2, 5 MgCl2, 10 HEPES, 5 D-glucose, pH 7.4. Cells were voltage-clamped at -60 mV. Pipettes had resistances of 8-12 MΩ and were filled with a solution containing (in mM): 140 K-gluconate, 10 NaCl, 2 MgATP, 1 CaCl2, 2 MgCl2 10 HEPES, 5 EGTA, pH 7.4. The extracellular recording solution contained (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl2, 10 D-glucose, 2.5 CaCl2, 10 HEPES, pH 7.3. For LimGluR experiments HEK293 or 293T cells were incubated in 50 µM D-MAG-0460 for 45-60 minutes at room temperature in a labeling solution containing (in mM): 135 NaCl, 5.4 KCl, 10 Heps, 2.5 CaCl2, 1 MgCl2, pH 7.4. Extracellular high potassium recording solution contained (in mM): 120 KCl, 25 NaCl, 10 Heps, 2 CaCl2, 1 MgCl2, pH 7.4. Pipettes had resistances of 6-10 MΩ and were filled with a solution containing (in mM): 140 KCl, 10 HEPES, 3 Na2ATP, 0.2 Na2GTP, 5 EGTA 3 MgCl2, pH 7.4. Cells were voltage clamped at -60 mV. Electrophysiological recordings were digitized using Matlab (Mathworks), which was also used to synchronize illumination by means of analog signals controlling...
either an LED driver (Thorlabs), or Pockel’s cell (ConOptics). The 470 nm LED focal spot size was measured by knife edge to have a Gaussian xy-profile with $1/e^2$ diameter $200 \pm 10 \mu m$.

**General synthetic methods.** Unless otherwise noted: all reactions were monitored with Merck silica gel 60 F254 plates and visualized with 254 nm light, iodine on silica, or a charring solution of ceric ammonium molybdate (CAM). Flash chromatography was carried out using Agela Technologies 32-63 D 60 Å silica gel (normal phase) or 50 μm FLASH C18 silica gel (reverse phase). All chemicals were used as obtained from commercial sources. Non-aqueous reactions were carried out under nitrogen atmosphere and magnetically stirred in oven-dried glassware. Unless dry-loading the flash column, all organic extracts were washed with brine, dried over sodium sulfate and filtered through filter paper; solvents were then removed with a rotary evaporator at aspirator pressure. All NMR spectra were measured in deuterated chloroform (CDCl$_3$) or methanol (CD$_3$OD) with Bruker AV, AM, or DRX spectrometers at 400 MHz, 500 MHz, and 600 MHz for $^1$H spectra and 151 MHz for $^{13}$C spectra. Spectra were calibrated to residual solvent peaks as reported by Gottlieb and Nudelman (4) and processed using MestReNova v. 7.0 software. Optical rotations were measured using a Perkin-Elmer 241 Polarimeter at 25 °C and 589 nm, sample concentrations are reported in g/100 ml. High resolution mass spectra (HRMS) were obtained using the Micro-Mass Facility operated by the College of Chemistry, University of California at Berkeley using electrospray ionization (ESI).

**Scheme S1. Synthetic route used for D-MAGO$_{460}$.”**
Synthetic procedures

Azobenzene S1. Previously described glutamate derivative (5) (251 mg, 0.692 mmol) was dried by reconcentration from benzene and placed under high vacuum for 1 h. It was then combined in a flask with azobenzene (6) (368 mg, 0.725 mmol), DIPEA (0.45 mL, 2.75 mmol), HBTU (275 mg, 0.725 mmol), and dissolved in DMF (20 mL). After stirring for 3 days, the reaction was diluted with EtOAc and then extracted 3 times with brine; the organic phase was dried and concentrated. Column chromatography on silica gel (dry load, 50% EtOAc in CH₂Cl₂) gave 355 mg (60%) of azobenzene S1 as a red solid.

Rf 0.36 (20% EtOAc in CH₂Cl₂); ¹H NMR (600 MHz, MeOD) δ 7.72 (m, 6H), 7.64 (d, J = 7.6 Hz, 2H), 7.50 (d, J = 7.4 Hz, 2H), 7.26 (t, J = 7.5 Hz, 2H), 7.18 (t, J = 7.4 Hz, 2H), 6.70 (d, J = 8.9 Hz, 2H), 4.25 (d, J = 7.0 Hz, 2H), 4.18 (m, 1H), 4.04 (t, J = 6.9 Hz, 1H), 3.64 (s, 3H), 3.62 (s, 3H), 3.31 (m, 4H), 3.19 (s, 2H), 2.49 (t, J = 6.7 Hz, 1H), 2.33 (s, 2H), 1.95 (m, 2H), 1.62 (m, 4H), 1.39 (s, 9H), 1.03 (t, J = 7.0 Hz, 3H); ¹³C NMR (151 MHz, MeOD) δ 177.2, 174.1, 173.6, 158.7, 157.7, 151.5, 150.5, 145.1, 144.4, 142.4, 141.1, 128.7, 128.1, 126.10, 126.07, 123.8, 121.1, 120.9, 112.3, 80.7, 67.6, 53.4, 52.7, 52.4, 50.5, 48.3, 46.2, 43.4, 39.3, 37.6, 34.7, 32.7, 28.7, 24.1, 12.7; HRMS (ESI+) m/z calcd for C₄₇H₅₇O₉N₆ [M+H]+: 849.4182, found 849.4173; [α]D = -10 (c = 0.9 in MeOH).

Azobenzene S2. Azobenzene S1 (214 mg, 0.252 mmol) was dissolved in THF (30 mL) and piperidine (0.25 mL, 2.52 mmol) was added. The reaction ran overnight but was not complete (as judged by TLC) so an additional volume of piperidine was added (0.5 mL, 5.04 mmol). After a further 5 h, the reaction was complete (by TLC). The reaction mixture was diluted with EtOAc and then extracted with a saturated
NaHCO₃ solution and brine; the organic phase was dried and concentrated. Column chromatography on silica gel (95:5:1 of CH₂Cl₂:MeOH:NEt₃) gave 149 mg (94%) of azobenzene S₂ as a red solid.

Rᶠ 0.41 (10% MeOH in CH₂Cl₂, plus 1% NEt₃); ¹H NMR (600 MHz, MeOD) δ 7.78 (m, 4H), 7.70 (d, J = 8.6 Hz, 2H), 6.83 (d, J = 8.9 Hz, 2H), 4.18 (m, 1H), 3.69 (s, 3H), 3.67 (s, 3H), 3.56 (m, 1H), 3.48 (m, 2H), 3.35 (s, 1H), 2.99 (m, 2H), 2.52 (s, 1H), 2.39 (s, 2H), 1.97 (m, 2H), 1.65 (m, 4H), 1.42 (s, 9H), 1.17 (m, 3H); ¹³C NMR (151 MHz, MeOD) δ 177.2, 174.2, 173.8, 157.8, 151.4, 150.5, 144.9, 141.3, 126.0, 123.8, 121.2, 112.8, 80.7, 54.7, 53.4, 52.7, 52.3, 50.9, 46.4, 43.5, 39.1, 37.6, 34.7, 32.7, 28.7, 24.1, 12.4; HRMS (ESI+) m/z calcd for C₃₂H₄₇O₇N₆ [M+H]+: 627.3501, found 627.3500; [α]₀ = -17 (c = 0.84 in MeOH).

Azobenzene S₃. Azobenzene S₂ (149 mg, 0.237 mmol) was dissolved in THF (20 mL) and cooled in an ice bath to 0 °C, at which point an aqueous solution of LiOH (7 mL, 1 M) was added. The reaction was stirred at 0 °C for 1 h and then allowed to warm to rt while stirring for another 2 h. The reaction mixture was then acidified at 0 °C with formic acid and concentrated to remove the THF. The remaining aqueous solution was loaded onto a reverse phase column (gradient from 20% MeOH in 0.1% formic acid to 100% MeOH), giving 81 mg (57%) of azobenzene S₃ as a brown/yellow solid.

¹H NMR (600 MHz, MeOD) δ 8.32 (s, 1H), 7.82 (d, J = 8.6 Hz, 2H), 7.77 (d, J = 8.5 Hz, 2H), 7.70 (d, J = 8.4 Hz, 2H), 6.90 (d, J = 8.6 Hz, 2H), 4.14 (t, J = 7.3 Hz, 2H), 3.70 (t, J = 7.3 Hz, 2H), 3.55 (q, J = 7.1 Hz, 2H), 3.18 (t, J = 7.0 Hz, 2H), 2.53 (m, 1H), 2.42 (m, 2H), 1.96 (t, J = 7.4 Hz, 2H), 1.78 (m, 1H), 1.69 (m, 3H), 1.44 (s, 9H), 1.21 (t, J = 7.0 Hz, 3H); ¹³C NMR (151 MHz, DMF) δ 177.0, 174.9, 163.6, 155.7, 150.2, 148.6, 143.7, 141.7, 124.9, 122.9, 119.5, 112.1, 78.2, 52.8, 47.2, 45.1, 42.3, 37.0, 35.2, 31.6, 28.0, 23.5, 11.8; HRMS (ESI+) m/z calcd for C₃₀H₄₃O₇N₆ [M+H]+: 599.3188, found 599.3185; [α]₀ = +40 (c = 0.3 in MeOH).
**D-MAG0<sub>460</sub>.** Azobenzene S3 (26.1 mg, 0.0436 mmol) was dissolved in a saturated aqueous solution of NaHCO<sub>3</sub> (2 mL) and cooled to 0 °C. N-methoxycarbonyl maleimide (33.8 mg, 0.218 mmol) was added, followed by THF (2 mL) 15 minutes later. The reaction was allowed to warm to rt and stirred another 1 h, at which point the reaction mixture was then acidified at 0 °C with formic acid and extracted three times with EtOAc, dried, and concentrated. Column chromatography on silica gel (5:95:1:1 MeOH:CH<sub>2</sub>Cl<sub>2</sub>:AcOH:H<sub>2</sub>O, then 10:90:1:1 MeOH:CH<sub>2</sub>Cl<sub>2</sub>:AcOH:H<sub>2</sub>O) gave 14.5 mg of a dark brown solid. This sensitive intermediate was then stirred in a saturated solution of HCl in dry EtOAc for 3 hours. Removal of solvent in vacuo, followed by three triturations with Et<sub>2</sub>O and one with hexanes, gave 13.7 mg (51% over 2 steps) of **D-MAG0<sub>460</sub>** as a dark purple, glassy solid.

<sup>1</sup>H NMR (600 MHz, MeOD) δ 8.01 (d, <i>J</i> = 8.9 Hz, 2H), 7.80 (m, 4H), 7.19 (m, 2H), 6.82 (s, 2H), 3.99 (t, <i>J</i> = 7.1 Hz, 1H), 3.87 (m, 4H), 3.75 (m, 2H), 2.80 (m, 1H), 2.47 (m, 2H), 2.17 (m, 1H), 2.05 (m, 1H), 1.77 (m, 4H), 1.31 (m, 3H); <sup>13</sup>C NMR (151 MHz, DMF) δ 176.8, 172.7, 172.2, 171.9, 151.2, 149.4, 144.4, 142.7, 135.8, 125.9, 123.7, 120.4, 112.7, 52.3, 49.9, 48.7, 46.0, 41.8, 37.6, 33.5, 32.4, 23.9, 12.9; HRMS (ESI+) m/z calcd for C<sub>29</sub>H<sub>35</sub>O<sub>7</sub>N<sub>6</sub> [M+H]<sup>+</sup>: 579.2562, found 579.2561; [α]<sub>D</sub> = -6 (c = 0.7 in MeOH).
Supporting Results

S1. Measurement and analysis of 2P absorption spectra. 2P absorption is a third-order nonlinear process that depends on both the 2-photon absorption cross-section (σ₂) and the squared concentration of incident photons. As noted by Xu and Webb (7), accurate measurement of 2P absorption spectra depends strongly on spatial and temporal properties of the laser field, E(z,ω,t). Differences in laser conditions can lead to very different reported values for σ₂, making it difficult to compare materials, or even the relative strength of transitions within a material. We sought to avoid many of these complications by making simultaneous measurement of 2P absorption over a broad range of transition energies using a non-degenerate (2-color) white light spectrophotometry method (8–10). This nonlinear spectrophotometry technique uses the same experimental apparatus as femtosecond transient absorption (Fig. S2a), where the pump-dependent change in absorption for a weak probe pulse, E(ω), has been described theoretically in terms of the third-order polarization response (11, 12):

$$\Delta A(\omega) \sim \omega \text{Im} \frac{P^{(3)}(\omega)}{E(\omega)}$$  \hspace{1cm} (1)

In contrast to transient absorption, for the 2-color-2P absorption measurement, pump and probe pulses are detuned from the 1-photon absorption spectrum of the photoswitch (Fig. S2b). Under these conditions, changes in probe transmission are observed only when the pulses are temporally overlapped. Neither beam has sufficient energy or intensity to excite a photoisomerization independently, as confirmed by the lack of a persistent bleach of the initial isomer (Fig. S2c). The third-order polarization response seen by the probe pulse can be written:

$$P^{(3)}(\omega) \equiv \chi^{(3)}(\omega = \omega_{\text{NIR}} + \omega - \omega_{\text{NIR}}) E(\omega)E_{\text{NIR}}(\omega_{\text{NIR}})E_{\text{NIR}}(\omega_{\text{NIR}})$$  \hspace{1cm} (2)

Two types of phenomena contribute to Eq. 2: (1) absorption that occur when photons from both laser pulses interact with the same molecule, including both 2P absorption and stimulated Raman scattering; (2) cross-phase modulation of E(ω,t) by the pump |E_{\text{NIR}}(\omega_{\text{NIR}}, t)|². The latter signal predominantly arises from the χ(3) properties of the solvent and cuvette windows. It can be seen from measurements in the absence of a 2P absorber (either in transparent solvent or in empty cuvette), that the cross-phase modulation signal has a time-integral equal to zero and does not contribute to Eq. 1, and therefore can be subtracted from the signal measured in the PTL sample if conditions are identical. Comparing the sample and solvent measurements yields a spectrally-resolved measure of the χ(3) contribution of the sample:

$$\Delta A(\omega) = \Delta A_{\text{sample}} - \Delta A_{\text{solvent}} = \left[ \omega \text{Im} \frac{P^{(3)}(\omega)}{E(\omega)} \right]_{\text{sample}} - \left[ \omega \text{Im} \frac{P^{(3)}(\omega)}{E(\omega)} \right]_{\text{solvent}}$$  \hspace{1cm} (3a)

$$\Delta A(\omega) = \chi^{(3)}(\omega)E_{\text{NIR}}(\omega_{\text{NIR}})E_{\text{NIR}}(\omega_{\text{NIR}}) \propto c \sigma_2(\lambda) I_{\text{NIR}}$$  \hspace{1cm} (3b)
Note that Eq. 3b is linear in NIR intensity. From Eq. 3b, two samples measured under identical conditions, a ratio of $\Delta A(\lambda_{WL}, \lambda_{NIR})$ measurements is sufficient to determine a relative 2P cross-section without knowledge of the exact spatial and temporal properties of $I_{WL}$ and $I_{NIR}$. The unknown cross-section $\sigma_2(\lambda)$ is given by:

$$\sigma_2(\lambda) = \frac{\Delta A}{\Delta A_{ref}} \frac{c_{ref}}{c} \sigma_2(\lambda)_{ref}$$  \hspace{1cm} (4)

For measurement of photoswitches, back-to-back measurements were made in neat solvent in the same sample cuvette to verify cancellation of cross-phase modulation and identify contributions to $\Delta A_{WL}$ not due to 2P absorption (Figs. S3, S4, S5). Laser parameters for 2P absorption measurements are summarized in Table S1. For measurements with $\lambda_{NIR} = 1360$ nm, we used coumarin 153 in methanol as a reference sample, with a reported $\sigma_2 = 45$ GM (7); for measurements where $\lambda_{NIR} = 800$ nm, we used MNI-glutamate in water, with a reported $\sigma_2 = 0.04 \pm 0.02$ GM at 730 nm (13). Spectroscopic parameters for reference samples are summarized in Table S2. In the measured spectrum of L-MAGO, we observe an absorption feature at $\lambda = 360$ nm (Fig. S3g). Upon analysis, this is seen to correspond to Raman scattering of the 800 nm pulse by the 2994, 2913 cm$^{-1}$ CH stretching modes of DMSO (14) (Fig. S3h). The peaks remaining after solvent corrections can be attributed to Raman scattering from the azobenzene moiety (Fig. S3h).

We also measured the 2P absorption of another first-generation PTL, maleimide-azobenzene-quaternary ammonium (MAQ) (15, 16) in phosphate buffered solution (Figure S4). Despite a bathochromic shift in the near-UV $\pi-\pi^*$ absorption band ($\lambda_{sp} = 360$ nm in phosphate buffered solution, pH 7.4), the 2P absorption of trans-MAQ was very similar to trans-L-MAGO (Fig. 1a). The lack of a solvent shift supports the interpretation that the absorption peak at 318 nm is not due to vibronic enhancement within the lowest energy $\pi-\pi^*$ transition, but rather corresponds to a different electronic transition. Considering that the trans-MAQ measurement used a different combination of white light and NIR wavelengths (Figure S4b), we also saw no evidence that these $\sigma_2$ spectra were grossly influenced by resonance-enhanced 2P absorption due to overlap between the white light and 1P absorption spectra.

**S2. Photoisomerization dynamics in MAGs.** The photoisomerization model for 400 nm excitation of L-MAGO and L-MAGO$\text{460}$ was based on trans-azobenzene, as shown in Figure S6. Photoexcitation promotes molecules from the dark-equilibrated trans (E) isomer ground state to the first 1P-allowed (S$_2$) excited state, where relaxation occurs through internal conversion with an effective time constant $\tau_{1c} = 130$ fs. Approximately 45% of photoexcited molecules relax to an S$_1$ excited state where they structurally evolve away from the Frank-Condon region with $\tau_{FC}$. Isomerization occurs from S$_1$, resulting in a net quantum yield for the cis isomer of 0.15 in L-MAGO. Failed isomerization attempts result in a vibrationally excited trans population that thermally equilibrates with $\tau_{vib}$. In L-MAGO$\text{460}$, the n-\pi and \pi-\pi* transitions are inverted and the entire photoexcited population goes through S$_1$, resulting in a higher yield of cis photoprodut ($\phi_{\text{cis}} = 0.4$). Time constants are summarized in Table S3, where the effective rate constants ($\tau_1$, $\tau_2$) are related to Figure S6 by ($\tau_1$)$^{-1} = k_20 + k_{21}$ and ($\tau_2$)$^{-1} = k_{10} + k_{E-\text{cis}}$, and $\tau_{FC} = (k_{21})^{-1}$. All values have an error of ± 10%.
S3. Relationship of L-MAG0\textsubscript{460} photocurrent kinetics to photoswitching. Since the photoactive effects of PTLs (e.g., LiGluR photocurrents controlled by MAGs) are reversibly triggered by \textit{trans} \rightarrow \textit{cis} isomerization (denoted as E-Z) and turned off by \textit{cis} \rightarrow \textit{trans} isomerization (Z-E). If we assume that instantaneous photocurrent can be approximated by the concentration of \textit{cis} isomer [Z], modulo an efficiency factor \(\eta_\gamma\), then the current can modeled by rate equations for a unimolecular reaction, where \(k_{\text{ir}}\) is the rate constant for thermal isomerization and the rate constants \(k_\gamma\) represent photoisomerization for \(\gamma\)-photon absorption (\(\gamma = 1,2\)):

\[
\frac{ds}{dt} \approx \eta_\gamma \frac{d[Z]}{dt}
\]

\[
\frac{d[Z]}{dt} = k_\gamma^{E\rightarrow Z}[E] - k_\gamma^{Z\rightarrow E}[Z] - k_{\text{KT}}[Z]
\]

The LiGluR + L-MAG0\textsubscript{460} photocurrent dark relaxation kinetics were presumably rate-limited by thermal relaxation of the \textit{cis} isomer, as described previously (6). We can readily observe from the recordings in Figure 4 that, at the light intensities of the experiment (1P: 0.02 – 0.26 \(\mu\text{W}/\mu\text{m}^2 = 0.6 – 8.7 \times 10^{13} \gamma \text{ s}^{-1}\text{cm}^{-2}\); 2P: 0.3 – 2.3 \(\mu\text{W}/\mu\text{m}^2 = 1.2 – 9.5 \times 10^{23} \gamma \text{ s}^{-1}\text{cm}^{-2}\)), photoisomerization rates \(k_\gamma\) were collectively much faster than \(k_{\text{KT}}\) for both 1P and 2P LiGluR photocurrents. Therefore, we estimate that LiGluR currents saturate as a \textit{photostationary} state (PSS) is reached:

\[
S_{\text{max}} \approx \eta_\gamma[Z]_{\text{PSS}} = \eta_\gamma k_\gamma^{E\rightarrow Z}[E]_{\text{PSS}}
\]

Eq. 6 predicts that \(S_{\text{max}}\) should be independent of light intensity for a given cell. Although \(S_{\text{max}}\) varied widely between cells, we can still say something about relative photocurrent, \(s(t) = S(t)/S_{\text{max}}\), in terms of fractional population [Z]:

\[
\frac{ds}{dt} = k_\gamma^{E\rightarrow Z}(1 - [Z]) - k_\gamma^{Z\rightarrow E}[Z]
\]

The rate constants for photoisomerization depend on the light intensity-dependent rate of absorption and reaction quantum yield:

\[
k(\lambda)_\gamma^{E\rightarrow Z} = \frac{1}{\gamma} \Phi_{\gamma}^{E\rightarrow Z} \sigma(\lambda)_\gamma^E I(\lambda)_\gamma
\]

\[
k(\lambda)_\gamma^{Z\rightarrow E} = \frac{1}{\gamma} \Phi_{\gamma}^{Z\rightarrow E} \sigma(\lambda)_\gamma^Z I(\lambda)_\gamma
\]

where \(\sigma(\lambda)_\gamma\) is the \(\gamma\)-photon absorption cross-section, and \(I(\lambda)\) is the light intensity in units \(\gamma \text{ s}^{-1}\text{cm}^{-2}\). Combining Eqs. 7 and 8, the rate at which \(S_{\text{max}}\) is approached is:

\[
\frac{ds}{dt} = -\frac{\eta_\gamma}{\gamma} \left[ \Phi_{\gamma}^{E\rightarrow Z} \sigma(\lambda)_\gamma^E + \Phi_{\gamma}^{Z\rightarrow E} \sigma(\lambda)_\gamma^Z \right] I(\lambda)_\gamma [Z] + \eta_\gamma k_\gamma^{E\rightarrow Z}
\]
The solution to Eq. 9 is a decaying exponential with rate constant:

\[ k_{on} = \eta \gamma \left[ \phi_{\gamma}^{E \rightarrow Z} \sigma(\lambda) + \phi_{\gamma}^{Z \rightarrow E} \sigma(\lambda) \right] I(\lambda) \gamma \]  

Eq. 10 predicts that the rate of photocurrent onset under saturation conditions should depend on light intensity linearly for 1P photoswitching, and as \( I^2 \) for 2P photoswitching.

Experimentally, the photocurrent rise for 1P excitation was well fit by a single exponential. By measuring the rise time for several light intensities in the same cell, we found that the rates constant showed a linear relationship with light intensity (Fig. S7c). A minimum intensity of 0.1 \( \mu \text{W}/\mu \text{m}^2 \) produced a steady state photocurrent indicative of equilibrium between \( \text{trans} \rightarrow \text{cis} \) and \( \text{cis} \rightarrow \text{trans} \) isomerization rates. Across cells, 470 nm light of intensity 0.36 \( \mu \text{W}/\mu \text{m}^2 \) yielded an average \( \tau_{on} = 4.6 \pm 0.4 \text{ ms} \), while 2P-DH photocurrents had an average \( \tau_{on} = 11.5 \pm 2.4 \text{ ms} \) (Fig. S7b). For most cells, the on-rates for 2P photoactivation was sufficient to reach a steady state current (Fig. S7b) with 2P intensities tolerated by healthy neurons (290 ± 10 mW time-averaged power distributed into 130-300 \( \mu \text{m}^2 \) regions). Cell “tolerance” was judged by stability over repeated photoswitching cycles (representative examples after 30 cycles shown in Fig. 4a, b). We conclude that 2P photoactivation of LiGluR + L-MAG0460 is efficient enough to generate maximum current density without cell damage.

As noted by Rickgauer and Tank for Channelrhodopsin2 (17), photocurrent kinetics can provide complementary quantitative measures of spectroscopic values given a model relating photophysics to physiological mechanism. They developed a framework relating the ChR2 photocycle to photocurrents measured at saturation, and based on fitting experimental functional data determined a value of \( \sigma^2 \) for ChR2 (260 ± 20 GM at 920 nm). Although the origin of photocurrent saturation in ChR2 is a thermodynamic photocycle rather than photochromism, a similar type of framework can be extended to photochromic PTLs through the photostationary model described above. In fact, using the experimental values for brightness in Eq. 10, we obtain intensity-dependence rate constants that agree well with the photocurrent rise times measured in neurons with LiGluR (Fig. S7f, dashed = predicted, symbols = experimental), assuming an efficiency factor \( \eta_1 = \eta_2 = 0.1 \), and that the 2P absorption of \text{cis}-L-MAG0460 follows the 1P absorption (as in \text{cis}-L-MAG0).

**S4. Interpretation of LimGluR-dependent photocurrents.** LimGluR3 + D-MAG0460-dependent GIRK current had considerably different kinetics than LiGluR photocurrents, despite the chemical similarity of L-MAG0460 and D-MAG0460. These differences can be attributed to the nature of LimGluR and the indirect readout of LimGluR activity (Figure S8). Considering that similar excitation intensities produced a photostationary \text{cis} population in LiGluR + L-MAG0460 (Figure 4), the slower onset LimGluR3 + D-MAG0460 photoactivation suggests that G protein signaling is rate-limiting, or possibly that second messenger recruitment to LimGluR competes with thermal relaxation of D-MAG0460.
References


Supporting Figure Legends

**Figure S1.** Visible absorption of L-MAG0\textsubscript{460} is composed of two overlapping bands. The trans-L-MAG0\textsubscript{460} 1P absorption spectrum in DMSO and water can be decomposed in two absorption bands. The larger solvent shift of the lower energy band is consistent with a π-π* transition, suggesting an inversion of the transition energies.

**Figure S2.** Femtosecond spectrophotometry. (a) Block diagram of optical setup for broadband femtosecond pump-probe spectroscopy: WLG, white light generation; SHG, second-harmonic generation in BBO. The optical path length between pump and probe pulses is varied with a computer-controlled translation stage. At each delay point, the pump beam is blocked with an optical chopper and the transmission spectrum of the white light pulse is collected with and without pump exposure. Difference spectra are calculated as $\Delta A(\lambda, t) = -\log \left( \frac{I(\lambda,t)_{\text{pump}}}{I(\lambda)_{\text{no pump}}} \right)$. (b) Transient absorption, the pulse wavelengths are selected to be sensitive to photo-excited changes in electronic states. Nonlinear optical effects are frequently observed in pump-probe measurements in the brief period when the pump and probe pulses are coincident (TIME-ZERO). Though sometimes called a “coherent artifact” in transient absorption spectroscopy, this two color interaction can be exploited for measurement of two-photon absorption (right). (c) Example of time- and wavelength-resolved ΔA data. Note the x-axis is logarithmic for values past 1 ps. Following the pump pulse, ΔA dynamics result from the relaxation of non-equilibrium populations; the formation of stable photoproducts causes a persistent ΔA. (d) Example of time- and wavelength-resolved ΔA data for 2-color 2P absorption measurement. Note that no signal persists past “TIME-ZERO”.

**Figure S3.** Anatomy of the 2P absorption measurement. (a) 2P absorption can involve two photons with degenerate properties (necessarily half the electronic transition energy), or non-degenerate photon pairs that summate to the transition energy. Non-resonant photons must interact with the same molecule within the lifetime of virtual transition states (dashed lines) for 2P absorption. (b) Non-degenerate 2P absorption spectra was measured using the nonlinear spectrophotometer described in Figure S2, where pump and probe wavelengths were detuned from the PTL 1P absorption. The white light (WL) probe was tuned to longer wavelengths, while the resonant pump pulse was replaced with a non-resonant NIR pulse so that WL and NIR energies summate to the transition energy. (c) Changes in white light are induced by the NIR pulse, but unlike a transient absorption measurement, no measurable excited state population is generated and $\Delta A(\lambda_{\text{WL}})$ returns to baseline. (d) The ΔA signal has contributions from cross-phase modulation (XPM) and 2P absorption. Viewed as time-resolved spectra (shown at 150 fs intervals), XPM is seen as loss ($\Delta A(\lambda_{\text{WL}}) > 0$) in the center wavelength and gain ($\Delta A(\lambda_{\text{WL}}) < 0$) at new wavelengths generated as the nonlinear field of the NIR pulse changes the instantaneous frequency of the white light. XPM is nearly identical in back-to-back measurements of PTL (solid lines) and neat solvent (dashed lines). The difference represents the contribution due to 2P absorption of the PTL. (e) The measurement has better than 400 cm\textsuperscript{-1} spectral resolution. (f) Viewed in the time domain, the cross-correlation of WL and NIR pulses is $\approx$150 fs. (g) The time-integrated $\Delta A(\lambda_{\text{WL}})$ signal is directly proportional to 2P absorption. The signal in solvent confirms that XPM contributes no net loss of probe light. (h) Anti-Stokes Stimulated Raman scattering of $\lambda_{\text{NIR}} = 800$ nm by the 2994, 2913 cm\textsuperscript{-1} CH stretching modes of DMSO was stimulated.
by the white light probe and appears as a positive signal in the time-integrated ΔA signal for neat DMSO (black) and L-MAG0 in DMSO (red = trans, green = cis). (h) The peaks remaining after solvent corrections can be attributed to Raman scattering from the azobenzene moiety. (i) For the wavelength combination in panel b, we used MNI-glutamate as the reference sample with known 2P cross-section. Red symbols are the 2P absorption signals scaled to reported σ2 values. The 1P absorption spectrum is shown for reference (blue lines).

**Figure S4. 2P absorption of MAQ.** (a) 2P absorption spectrum of a related first generation PTL, maleimide-azobenzene-quaternary ammonium (MAQ) (15, 16) was very similar to that of trans-L-MAG0 (Fig. 1a). The lack of a solvent shift supports the interpretation that the absorption peak at 318 nm is not a vibronically-enhanced π-π* transition, but rather corresponds to a different electronic transition. (b) The trans-MAQ measurement used a different combination of white light and NIR wavelengths. (c) Dispersion of WL probe. (d) Time-resolved difference spectra at 100-fs intervals give an estimate of spectral resolution. Cross-correlation signals were measured in neat solvent (dashed) to identify contributions from cross-phase modulation and Raman scattering.

**Figure S5. 2P absorption of L-MAG0_{460} and reference spectra.** (a) Overlap of WL probe spectrum with L-MAG0_{460} and L-MAG0 was minimized. (b) Evaluation of 2P spectra was limited to use only the spectral region where the WL probe was linearly chirped (λ < 650 nm). (c) Time-resolved difference spectra at 100 fs intervals in DMSO give an estimate of spectral resolution. (d) The time-integrated ΔA(λ_{WL}) signal is directly proportional to 2P absorption. The signal in solvent confirms that XPM contributes no net loss of probe light. Some resonance-enhanced 2P absorption (denoted with **) was observed on the high-energy side of L-MAG0_{460} spectrum where there is greatest overlap between the WL and MAG 1P absorption. (e) The reference spectrum for this combination of wavelengths was coumarin 153 in methanol. Red symbols are the 2P absorption signals scaled to reported σ2 values. The 1P absorption spectrum is shown for reference (blue lines).

**Figure S6. Photoisomerization dynamics in MAGs.** (a) Heat map representation of time-resolved changes in absorption during trans-cis photoisomerization reaction in L-MAG0. The x-axis is linear until 1 ps, and logarithmic thereafter. (b) Photoisomerization model for 400 nm excitation of L-MAG0: The π-π* transition in dark-equilibrated trans (E) isomer promotes molecules to the S2 excited state, where they relax through internal conversion with an effective time constant τ1 = 130 fs. Approximately 45% of photoexcited molecules relax to an S1 excited state where they structurally evolve away from the Frank-Condon region with τ_{FC}. Isomerization occurs from S1, resulting in a net quantum yield for the cis isomer. Failed isomerization attempts result in a vibrationally excited trans population that thermally equilibrates with τ_{vib}. (c) Kinetics at various probe wavelengths show the simplicity of the photodynamics. (d) Time-resolved spectra at select probe delays. (e) Concentrations of state populations obtained global analysis with the target model depicted in panel b. (f) Species Associated Difference Spectra (SADS) show the net wavelength-dependence of the exponential time constants (Table S3). (g) Heat map representation of transient absorption during trans-L-MAG0_{460} photoreaction. (h) 400 nm directly excites molecules to the photoisomerization intermediate state because of the inversion of transition energies. (i) Kinetics at various probe wavelengths show slightly more complex photodynamics than in L-MAG0. (j) Time-resolved
spectra at select probe delays. (k) Concentrations of state populations obtained global analysis with the target model depicted in panel h. (l) Species Associated Difference Spectra (SADS) show the net wavelength-dependence of the exponential time constants. The vibrationally excited population (blue, green lines) equilibrate within ~15 ps to a bleach spectrum that matches the ground state absorption spectrum.

**Figure S7. Stationary state photocurrents in LiGluR + L-MAG0460.** (a) Neuronal LiGluR photocurrents showed no correlation between maximum photocurrent amplitude and rate constant of photocurrent onset ($k_{on} = \tau_{on}^{-1}$). (b) Comparison of $\tau_{on}$ for 1P and 2P photocurrents from Figure 4 with 1P power series measured within individual cells (1**). (c) Intensity-dependence of 1P photocurrents recorded from single cells. An intensity of ≈ 0.1 $\mu$W/µm$^2$ produced a steady state photocurrent that could be sustained for the duration of light pulse. (d) $k_{on}$ showed a linear dependence on light intensity. (e) Example of 2P photocurrent elicited by 2P-DH time-averaged intensity of 1.8 ± 0.3 mW/µm$^2$ at 850 nm. (f) The measured rate constants (symbols) are consistent with predicted values predicted by the PSS model (Eq. 10) using the experimental values for 1P and 2P brightness and an efficiency factor, $\eta = 0.1$.

**Figure S8. GPCR signaling is rate-limiting step in photoagonism of LimGluR3 by D-MAG0460.** (a) The LimGluR3 + D-MAG0460-dependent GIRK current increased steadily with light exposure, approaching a maximum steady state after 5-8 s at 0.4 $\mu$W/µm$^2$. Well below this intensity, LiGluR + L-MAG0460 photocurrents reached stationary state within 100 ms (Fig. S7). (b) Intensity dependence of LimGluR3 + D-MAG0460 signal recorded from a single cell. (c) The rate constant of GIRK current rise, measured here as the time to reach 90% of the current at 5 s, did not depend on intensity (n=3 cells, at least 3 intensities tested per cell).
Proton NMR spectra of new compounds.
Table S1. Summary of laser conditions used in 2P absorption measurements.

<table>
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<tr>
<th>PTL</th>
<th>λ_{NIR} (nm)</th>
<th>WL medium</th>
<th>λ_{eff} (nm)</th>
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<tr>
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<td>1420</td>
<td>CaF$_2$</td>
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<tr>
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<td>Sapphire</td>
<td>400-450</td>
<td>Coumarin 153</td>
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<td>YAG</td>
<td>300-375</td>
<td>MNI-glutamate</td>
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Table S2. Summary of reference absorption values.

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</thead>
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<td>solvent</td>
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<td>λ$_{max}$ (nm)</td>
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<td>423$^b$</td>
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<td>E (M$^{-1}$cm$^{-1}$)</td>
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<td>14700$^f$</td>
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<tr>
<td>2λ (nm)</td>
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<tr>
<td>σ$_2$ (GM)</td>
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<td>45 ± 7</td>
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</tbody>
</table>

$^a$ Reference (13).

$^b$ Reported extinction coefficients at 423 nm: ε = 14700 M$^{-1}$cm$^{-1}$ in MeOH (18), ε = 18900 M$^{-1}$cm$^{-1}$ in EtOH (19).

$^c$ Reference (20).

Table S3. Fitted parameters for MAG photoisomerization in DMSO.

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<th>PTL</th>
<th>λ$_{pump}$ (nm)</th>
<th>τ$_1$ (ps)</th>
<th>τ$_{FC}$ (ps)</th>
<th>τ$_2$ (ps)</th>
<th>(k$^{e\rightarrow z}$)$^{-1}$ (ps)</th>
<th>τ$_{vib}^e$ (ps)</th>
<th>τ$_{vib}^z$</th>
<th>Φ$^{e\rightarrow z}$</th>
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<td>--</td>
<td>0.11</td>
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<td>--</td>
<td>0.2</td>
<td>0.6</td>
<td>2</td>
<td>15</td>
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<tr>
<td>L-MAGO$_{460}$</td>
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<td>n.d.</td>
<td>n.d.</td>
<td>2</td>
<td>15</td>
<td>3</td>
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</table>

n.d., not determined.
Carroll_Figure S4