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A Red-Shifted, Fast-Relaxing Azobenzene Photoswitch for Visible Light Control of an Ionotropic Glutamate Receptor

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Abstract

The use of azobenzene photoswitches has become a dependable method for rapid and exact modulation of biological processes and material science systems. The requirement of ultraviolet light for azobenzene isomerization is not ideal for biological systems due to poor tissue penetration and potentially damaging effects. While modified azobenzene cores with a red-shifted *cis*-to-*trans* isomerization have been previously described, they have not yet been incorporated into a powerful method to control protein function: the photoswitchable tethered ligand (PTL) approach. We report the synthesis and characterization of a red-shifted PTL, L-MAG0$_{460}$, for the light-gated ionotropic glutamate receptor LiGluR. In cultured mammalian cells, the LiGluR +L-MAG0$_{460}$ system is activated rapidly by illumination with 400–520 nm light to generate a large ionic current. The current rapidly turns off in the dark as the PTL relaxes thermally back to the *trans* configuration. The visible light excitation and single-wavelength behavior considerably simplify use and should improve utilization in tissue.

The development of synthetic photoswitches has been a boon to researchers in the material and biological sciences due to the precise spatiotemporal control that light allows.[1] Azobenzenes in particular have proved to be robust photoswitches that tolerate significant chemical modifications. For applications in neuroscience, we have developed a two-component approach that we refer to as chemical optogenetics.[2,3] Therein, a photoswitchable tethered ligand (PTL), usually derived from an azobenzene, is covalently attached to a protein to enable its reversible activation or block in response to flashes of light.[4,5] PTLs, for example when attached to neurotransmitter-gated ion channels and receptors, can be used to manipulate neuronal signaling in living cells and organisms. While

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Supporting Information
Experimental procedures, characterization for compounds, methods of receptor transfection, and illumination protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

Notes
The authors declare no competing financial interest.

they can be used like microbial opsins, which function as retinal-dependent pumps or ion channels,[6,7] to excite and inhibit neuronal firing with light, they offer the unique additional advantage of targeting native transmitter systems that control synaptic strength and plasticity, which are thought to be key for circuit function and memory formation.[8]

We focus here on a family of PTLs that use the excitatory neurotransmitter glutamate as a ligand to control ionotropic or metabotropic glutamate receptors (Figure 1a).[9,10] These PTLs are composed of three parts, maleimide–azobenzene–glutamate (MAG), such as L-MAG0 (Figure 1b, **1**), which bind covalently at their maleimide end to an engineered cysteine introduced into the ligand binding domain of a homotetrameric kainate receptor GluK2 (iGluR6) to generate the light-activated "LiGluR".[9,11] Irradiation with 380 nm light isomerizes the azobenzene core from the more stable *trans* state to the metastable *cis* state. This docks the glutamate into its binding pocket and opens the channel. Irradiation at ~500 nm reverses the processes and closes the channel.[11] Even in cases where labeling, photoswitching, or the ligand efficacy remain submaximal, LiGluR constitutes a powerful tool for *in vivo* studies, as low-affinity kainate receptors like GluK2 are not fully activated in many physiological conditions either (see SI). LiGluR has been used to evoke patterns of action potentials in neurons,[12] reproducibly inject calcium into glial cells and chromaffin cells to evoke transmitter release,[13,14] excite specific cells for neural circuit analysis *in vivo*,[15] and restore a retinal light response and visual behavior to mice blinded by photoreceptor cell degeneration.[16]

Two properties of L-MAG0 (**1**) and other "regular" azobenzene switches impose limitations for neuroscience applications. First, the most efficient isomerization of **1** from *trans* to *cis* is triggered by illumination at 380 nm, i.e., in the UV-A range. Ultraviolet light is problematic in biological systems for several reasons: prolonged exposure can be damaging, it penetrates mammalian tissue poorly, and it does not transmit through the lens of the human eye, for which visual restoration depends on detection in the visible range (~400–680 nm). The second property is the bistability of the "regular" azobenzene core of L-MAG0 (**1**). Although the *cis* isomer thermally relaxes to the lower energy *trans* form over time, the half-life is on the order of minutes to hours. For neuroscience applications, this requires a second aligned light source at the longer wavelength to rapidly pump the switch back into the *trans* state. For application to vision restoration, this comparatively slow thermal relaxation prevents the system from mimicking the spontaneous turn-off of the native rhodopsin of the photoreceptor cell. The requirement for a second wavelength to return from the metastable *cis* state also means that a substantial part of the spectrum has an impact on the PTL, reducing room for optical reporters with which one would want to combine the light-gated system. These problems would all be overcome with a modified azobenzene core that is "activated" at one wavelength in the visible part of the spectrum and then rapidly relaxes back to the "inactive" *trans* state in the dark.

The literature on azobenzenes suggests increasing electron density as a strategy to red-shift the absorption and lower the energy barrier for the *cis*-to-*trans* isomerization.[17,18] Both effects are achieved in the "amino" and "push–pull" azobenzenes. Push–pull azobenzenes are so-called because one benzene ring is *ortho* or *para* substituted with an electron-donating group, while the other ring is substituted with an electron withdrawing group. This

both red-shifts the absorbance of the *trans* isomer by ~100 nm and greatly decreases the thermal stability of the *cis* isomer. Initially, these concepts were applied to azobenzene cross-linkers of peptide helices.[19-22] Additionally, diffusible photochromic ligands modified to be push–pull have been made as reversibly caged potassium channel blockers[23-25] and AMPA receptor agonists,[26] but substituted azobenzenes have not yet been tested as PTLs. Potential problems include steric hindrance to operation of the tethered ligand and slowing of thermal relaxation due to binding interaction of the PTL ligand headgroup. With this in mind, we designed the compound L-MAG0$_{460}$ (Figure 1c, **2**) to obtain the advantage of the donor–acceptor red-shift, but with minimal variation to the structure so that it would resemble L-MAG0 (**1**) as closely as possible. Modification of the acetamido-group in the 4′-position to an electron-donating tertiary amine, in conjunction with the 4-acetamido group, was expected to yield a sufficiently strong push–pull system.

The readily available industrial dye “disperse red 1” (**3**, Scheme 1) was chosen as our azobenzene precursor. Using an Appel reaction, we converted the hydroxyl group on **3** into a bromide and in a subsequent two-step Gabriel synthesis to a free amine. The selective reduction of the aromatic nitro group with sodium sulfide yielded azoaniline **4**. Protection of the more nucleophilic primary amine in **4** with a fluorenylmethyloxycarbonyl (Fmoc) group, followed by amide coupling with known pyroglutamate derivative **5**,[9] yielded the advanced intermediate **6**. Deprotection of the primary amine was followed by hydrolysis of the pyroglutamate with concomitant saponification of the ethyl ester, and then installation of the maleimide. Finally, acidic removal of the *tert*-butyloxycarbonyl (Boc) protecting group provided L-MAG0$_{460}$ (**2**) as its hydrochloride salt.

As expected, changing the 4-acetamido group to a dialkylamine in **2** resulted in a strong (~100 nm) red-shift of the absorption, with a maximum at 462 nm in aqueous solution (Figure 2b). To characterize the photoswitching properties of **2** and to test its applicability as a PTL, we labeled the ionotropic glutamate receptor GluK2. For attachment we chose the position L439C where regular L-MAG0 (**1**) acts as a highly efficient *cis* agonist.[9,11,27,28] GluK2(439C) receptors expressed on the surface of HEK293 cells were covalently labeled by incubation with the red-shifted L-MAG0$_{460}$ (**2**) and excess reagent was removed by washing. Patch clamp electrophysiology was used to assess the photoinduced activation of LiGluR with covalently tethered **2**.

Illumination with blue light readily induced channel opening, resulting in large inward currents (Figure 2a). This demonstrates that excitation of L-MAG0$_{460}$ (**2**) significantly populates the *cis* configuration and that the structural modification at the 4′-position of the azobenzene does not impede ligand-binding or the closure of the ligand binding domain that is required for the channel to activate and open. Varying the irradiation wavelength allowed us to record an action spectrum, which shows a broad plateau between 440 and 480 nm, along with some activation by green light. The degree of LiGluR+2 activation across the spectrum closely follows the absorption spectrum of the *trans* form (Figure 2b). The exception to this agreement was at the peak at 445 nm—a difference that may reflect the polarity of the local environment surrounding L-MAG0$_{460}$ (**2**) once it has covalently bound to LiGluR. Polar solvents often red-shift the absorbance of azobenzenes,[17] as we indeed observed with L-MAG0$_{460}$ (Figure S1).

In stark contrast to regular L-MAG0 (**1**), which in the dark remains in its *cis* configuration for extended times (τ = 25.5 min[11]), red-shifted L-MAG0$_{460}$ (**2**) relaxes back to *trans* to close the channel on a much shorter time scale when the light is turned off (τ_{mean} = 0.71 s (*n* = 3), Figure 3a). This shows that the thermal relaxation of **2** bound to the glutamate receptor is >2200 times faster compared to **1**.

We wondered whether the broad action spectrum of **2** in the blue/green region, in combination with the minimal absorption at higher wavelengths (Figure 2b) would enable activation by white light. Indeed, we found that the unfiltered polychromatic output of our Xe-lamp light source gave a high level of activation, close to the maximal activation observed with monochromatic 445 nm light (Figure 3b). A final important feature is that LigluR+**2** maintains fidelity over hundreds of switching cycles (Figure S2).

Thus, LiGluR+**2** acts as a single-wavelength photoswitch that supports repeated activation with pulses of light and can be activated by either narrow bands of light or light sources with broad emission spectra. It needs no illumination to be deactivated and deactivates rapidly enough to match the time course of many signaling events. These properties make **2** a versatile PTL, which maintains the same molecular specificity as **1** for genetically engineered glutamate receptors.

In summary, we report what is to our knowledge the synthesis of the first PTL with a red-shifted azobenzene core. L-MAG0$_{460}$ (**2**) operates as a potent photoswitch for LiGluR that is made from the calcium permeant, excitatory kainate receptor GluK2. It generates large and reproducible currents when irradiated with blue-green light and spontaneously turns off in the dark. This fast, spontaneous deactivation is an important functional feature that could be generally applicable to PTLs with low-affinity ligands. The red-shift and single-wavelength dependence should enable deeper penetration in brain tissue while minimizing phototoxicity and achieving control with only one light source. An exciting future application will be to build on the demonstration that LiGluR+**1** restores light sensitivity to the retina in a mouse disease model that causes photoreceptor degeneration.[16] The broad excitation at visible wavelengths, coupled to the spontaneous turning off in the dark, suggests that L-MAG0$_{460}$ (**2**), which has an activation spectrum similar to blue cone photoreceptors (S-cones), is the better photoswitch for this application. Finally, due to the inherent modularity in the synthesis of MAG-type PTLs, other variants with this red-shifted azobenzene core should be straightforward to synthesize. Indeed, since PTLs attach to the surface of the signaling protein, it makes sense that they would be tolerant to substitutions that can tune their spectral and kinetic properties, offering a direct and rational approach to modifying protein function. This will provide a valuable expansion of the chemical optogenetic toolset for the remote control of ion channels and cellular signaling cascades.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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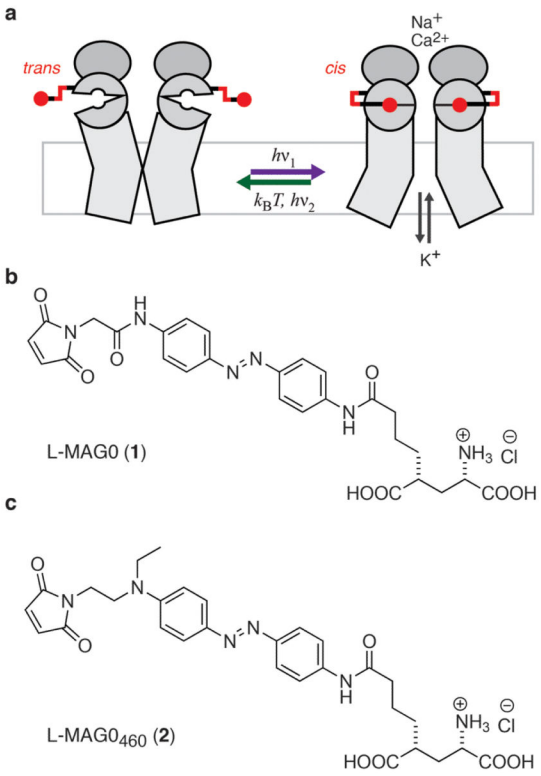


Figure 1.

Photoswitchable tethered ligands enable ligand-gated ion channels to be controlled by light. (a) Cartoon showing light-gating, with a shorter wavelength inducing isomerization to *cis*, followed by ligand binding and channel opening, while a longer wavelength reverses the process. This can also occur thermally (k_BT). (b) Modular design of maleimide–azobenzene–glutamate (MAG) photoswitches exemplified by regular L-MAG0 (**1**). (c) Structure of the red-shifted PTL L-MAG0$_{460}$ (**2**).

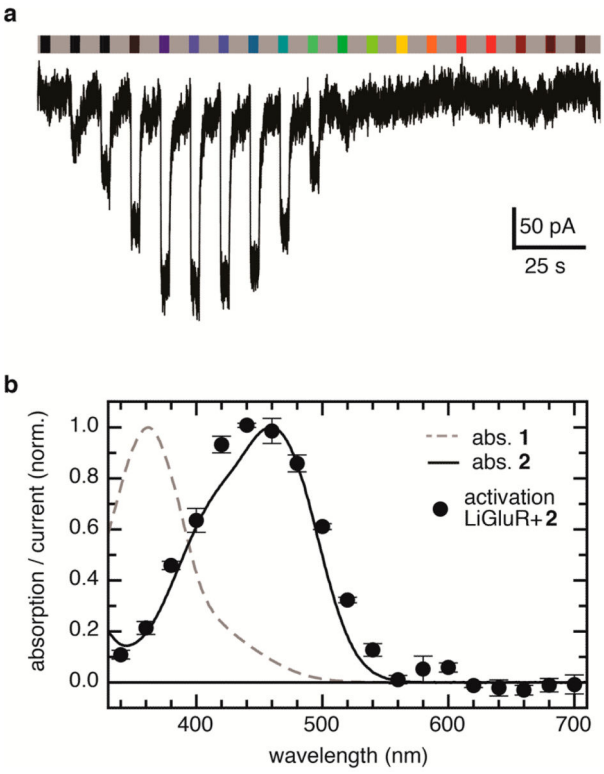


Figure 2.

(a) Representative action spectrum showing the activation of LiGluR+**2** with visible light. (b) Comparison of the absorption spectra of **1** and **2** (in phosphate-buffered saline, pH 7.4) and the LiGluR+**2** action spectrum (as in (a)) obtained from voltage-clamp recordings (mean ± SEM, $n = 4$).

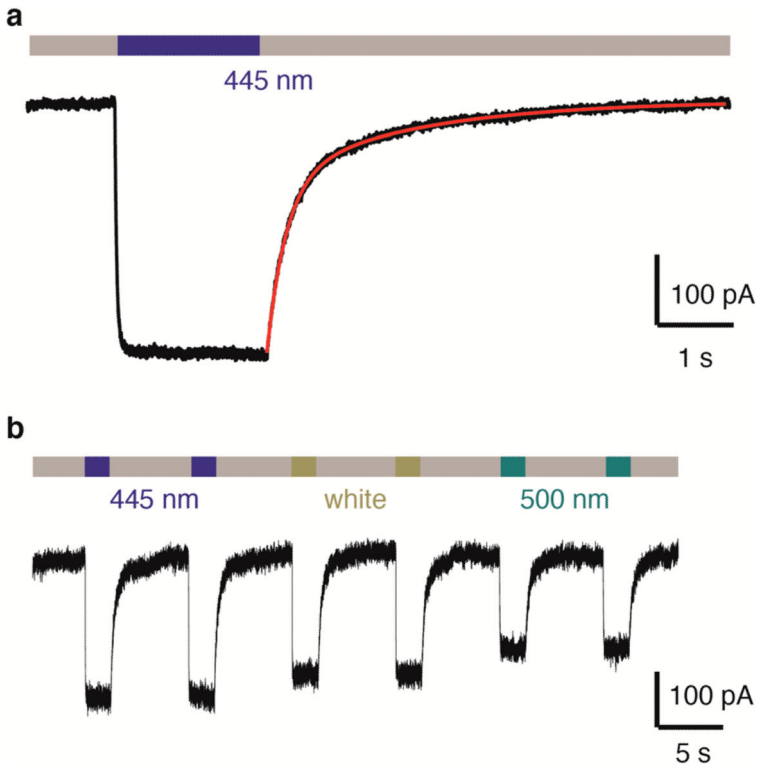
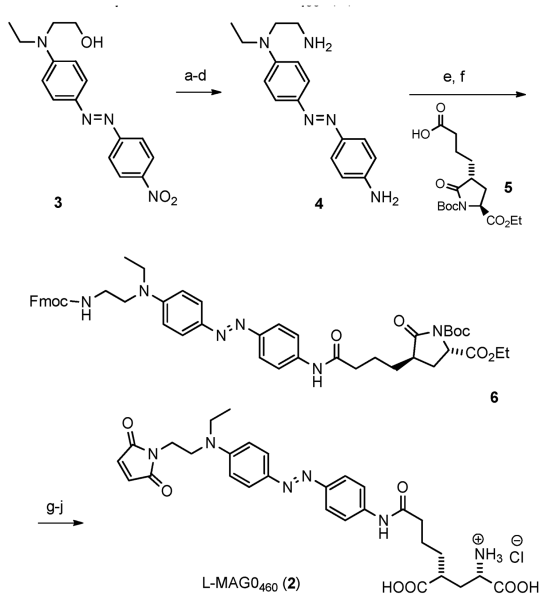


Figure 3.

(a) Activation by 445 nm light and fast thermal relaxation in the dark (τ_{mean} = 0.71 s (*n* = 3)). (b) Activation by 445 nm light, unfiltered white light and 500 nm light.



[a]Reagents and conditions: (a) CBr_4, PPh_3, CH_2Cl_2, 0 °C (90%); (b) potassium phthalimide, DMF, 90 °C; (c) H_2NNH_2, EtOH, 60 °C (68%, two steps); (d) Na_2S, 1,4-dioxane, H_2O, 90 °C (82%); (e) FmocCl, DIPEA, CH_2Cl_2 (46%); (f) **5**, HOBt, EDCl, DIPEA, MeCN (53%); (g) piperidine, THF; (h) 1 M LiOH(aq), (40%, two steps); (i) *N*-(methoxycarbonyl) maleimide, $NaHCO_3$ H_2O, THF, 0 °C; (j) HCl, EtOAc (90%, two steps).

Scheme 1.
Synthesis of L-MAG0$_{460}$ (2)[a]