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Orthogonal Optical Control of a G Protein-Coupled Receptor with a SNAP-Tethered Photochromic Ligand

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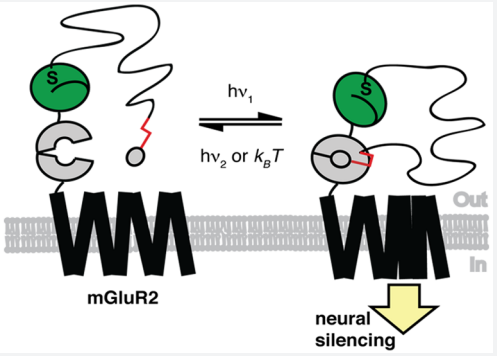
Supporting Information

ABSTRACT: The covalent attachment of synthetic photoswitches is a general approach to impart light sensitivity onto native receptors. It mimics the logic of natural photoreceptors and significantly expands the reach of optogenetics. Here we describe a novel photoswitch design—the photoswitchable orthogonal remotely tethered ligand (PORTL)—that combines the genetically encoded SNAP-tag with photochromic ligands connected to a benzylguanine via a long flexible linker. We use the method to convert the G protein-coupled receptor mGluR2, a metabotropic glutamate receptor, into a photoreceptor (SNAG-mGluR2) that provides efficient optical control over the neuronal functions of mGluR2: presynaptic inhibition and control of excitability. The PORTL approach enables multiplexed optical control of different native receptors using distinct bioconjugation methods. It should be broadly applicable since SNAP-tags have proven to be reliable, many SNAP-tagged receptors are already available, and photochromic ligands on a long leash are readily designed and synthesized.



INTRODUCTION

The ability to covalently link synthetic molecules with proteins has significantly increased the power of molecular biology and has provided new therapeutic approaches via antibody drug conjugates. In recent years, chemical biologists have developed methods that not only can be used *in vitro* and in cell cultures but also can be applied *in vivo*, even in large animals and, potentially, in humans.[1]

Important issues in bioconjugation are the speed, selectivity, and orthogonality of the reaction and the extent to which the target protein needs to be modified to enable covalent attachment. Engineered cysteines have proved popular since they represent a minimal change in the protein structure and reliably react with certain electrophiles, such as maleimides.[1] More selective methods depend on the expansion of the genetic code[2] and otherwise inert molecules that specifically react with protein motifs.[3] These include self-labeling "tags", such as the SNAP-tag,[4] the CLIP-tag,[5] or the Halo-tag,[6] and amino acid sequences that can be modified using external enzymes.[7]

Bioconjugation has also played an important role in photopharmacology, which is an effort to control biological activity with synthetic photoswitches.[8] While soluble photochromic ligands (PCLs) are diffusion limited, photoswitchable tethered ligands (PTLs) covalently attach to an engineered site in the target protein. This places the ligand in the vicinity of its binding pocket, so that light maneuvers it in and out of a position where it can bind.[9] The PTL approach allows for precise targeting since the bioconjugation motif, which is usually an engineered cysteine for maleimide conjugation, can be genetically encoded and selectively expressed in cells of interest. By contrast, PCLs act on native receptors, making for easier use, especially in therapeutic settings, albeit with less precision.

Although PTLs have proven to be powerful for controlling neural signaling and animal behavior,[10] they have faced the disadvantages of cysteine/maleimide chemistry. Maleimides hydrolyze under physiological conditions and conjugate to glutathione, making them incompatible with the intracellular environment. Moreover, both in the cell and on the cell surface, they are likely to react with accessible native cysteines that are not at the designed PTL anchoring site. Although attachment to the introduced cysteine can be enhanced by affinity labeling due to increased times of proximity when the ligand binds in the binding pocket,[11] the susceptibility of maleimides to unwanted nucleophiles, including water, makes them less than ideal for applications in photopharmacology.

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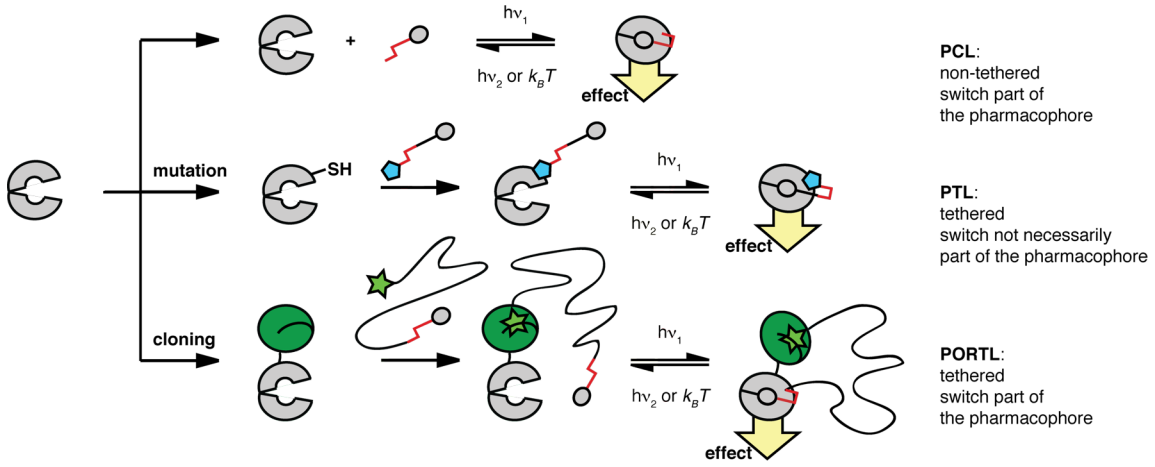

Figure 1. PORTL concept. A photochromic ligand (PCL) is freely diffusible, and the switch is part of the pharmacophore (top). This is not necessarily the case in a photoswitchable tethered ligand (PTL) (middle). The photoswitchable orthogonal remotely tethered ligand approach (PORTL, bottom) combines the switch as part of the pharmacophore with a long, flexible tether that allows for anchoring on a remote site.

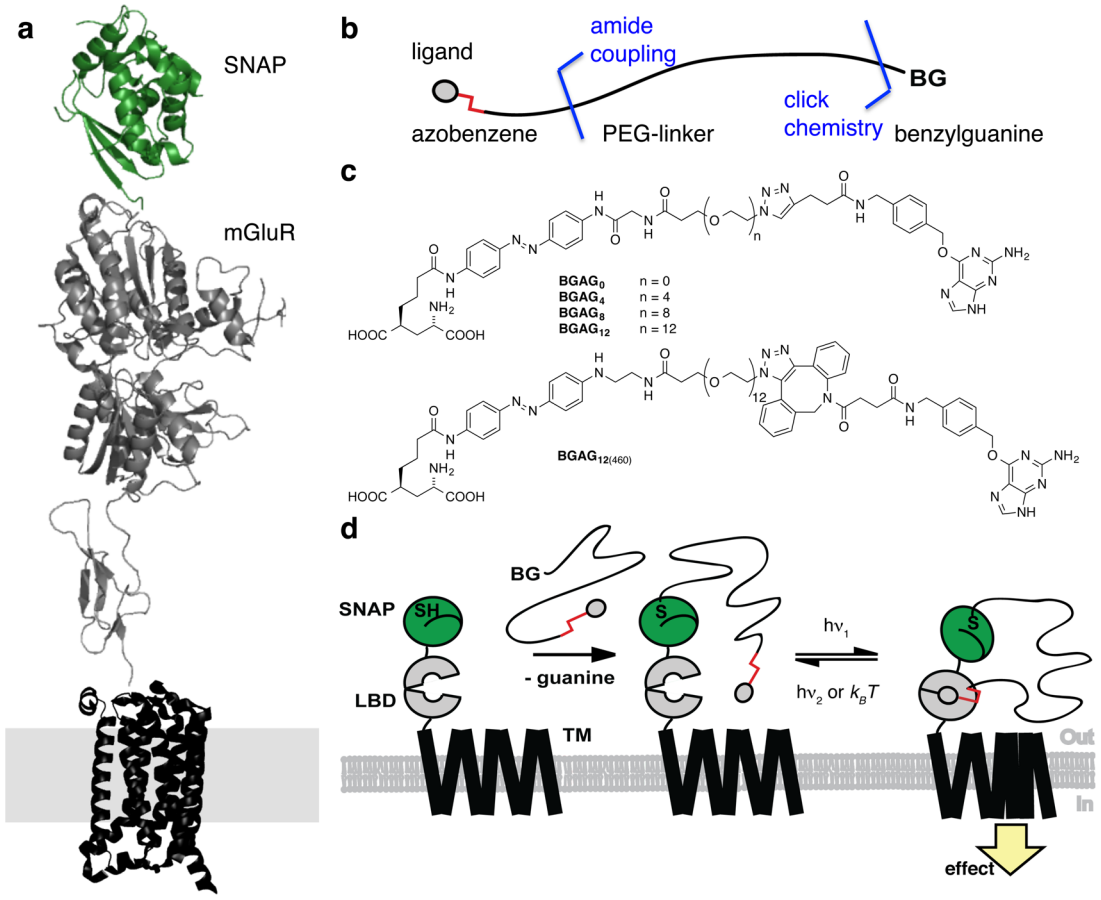

Figure 2. Concept and Design of PORTL compounds for SNAP-tag conjugation. (a) Model of a SNAP-mGluR subunit showing the relative dimensions of the domains (SNAP pdb, 3kzy; mGluR3-LBD pdb, 2e4u; mGluR5-7TM pdb, 4oo9). The mGluR extracellular domains are shown in gray, and the transmembrane domains are shown in black, while the SNAP-tag is shown in green. (b) Schematic design of a photoswitchable orthogonal remotely tethered ligand (PORTL) using amide coupling and click chemistry to ensure synthetic modularity. (c) PORTL consisting of a ligand connected to an azobenzene a flexible linker (PEG-chain) of various length and a benzylguanine (BG). Only one regioisomer is shown in $BGAG_{12(460)}$. Depending on the substitution pattern on the azobenzene the switching wavelength can be tuned. (d) Schematic showing the logic of PORTL-mediated reversible activation and deactivation of a target receptor with two orthogonal wavelengths of light.

A solution to these challenges could be the introduction of electrophiles that react with very high selectivity and yet are stable toward water. Under normal circumstances, this requires a larger protein tag, moving the site of attachment far away from the ligand-binding site, typically in the range of several nanometers. Although tethers with photoswitches placed in series could be designed, multiple isomerization states of the tether and the spread of conformations of the long entropic

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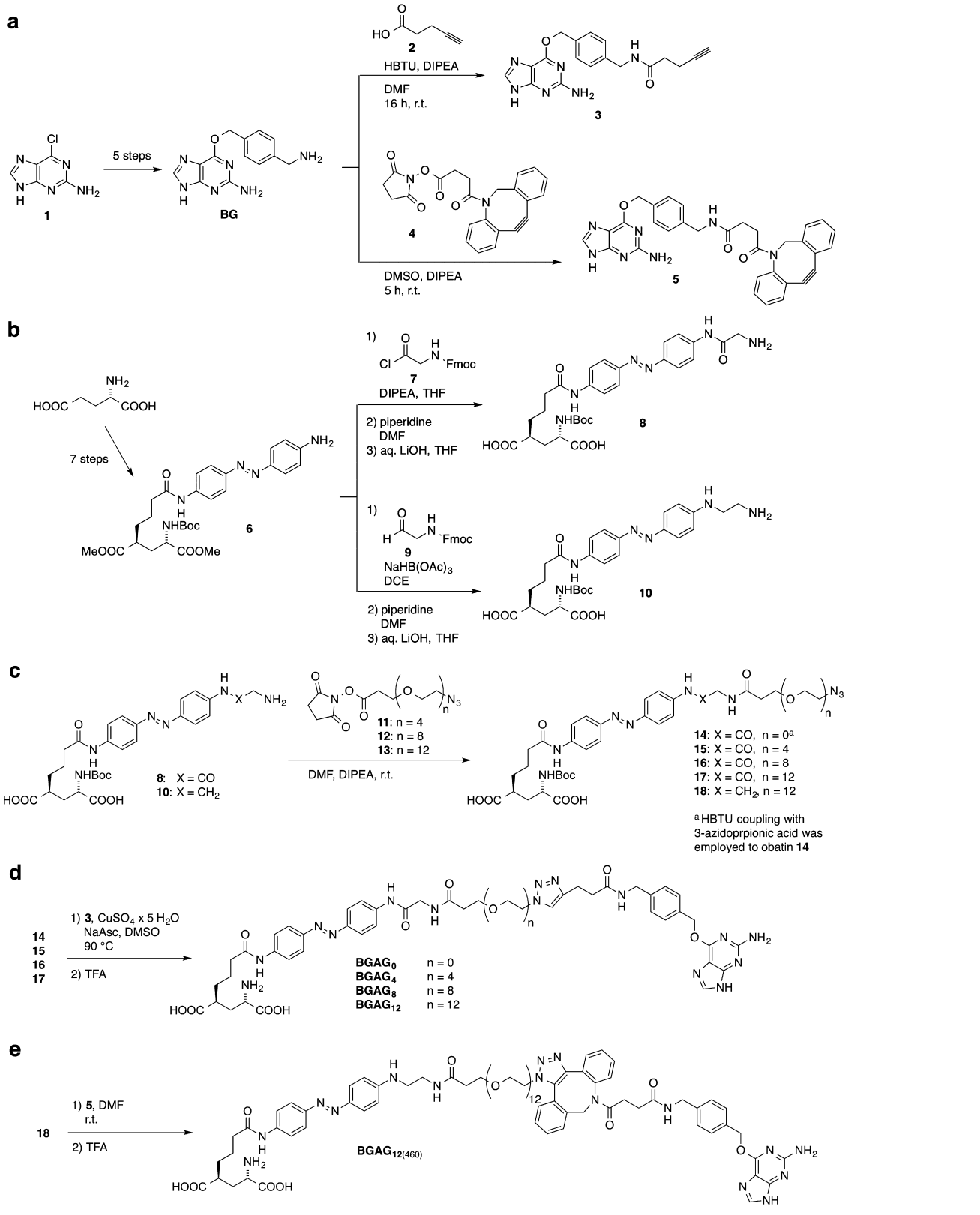

Figure 3. Synthesis of BGAGs. (a) Synthesis of BG-alkynes **3** and **5** for click chemistry. (b) Diversification of previously described **6** to give blue azobenzene glutamate **8** and red-shifted azobenzene glutamate **10**. (c) PEG-chain installation. (d) Cu(I)-catalyzed alkyne azide click to obtain BGAGs. (e) Strain promoted alkyne azide click to obtain $BGAG_{12(460)}$.

spring[12] could complicate control and prevent clean changes in biological activity upon irradiation.

Here we introduce a new concept, termed PORTL (photoswitchable orthogonal remotely tethered ligand), that overcomes the limitations of maleimide chemistry and lays to rest concerns about off-target attachment (Figure 1). Like a PTL, a PORTL consists of a bioconjugation handle, a photoswitchable group, and a ligand. In this case, however,

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the switch does not primarily impact the overall length, pointing angle, and flexibility of the tether, but rather the pharmacology of the tethered ligand. As such, the switch becomes an integral part of the pharmacophore and the change in biological activity is designed to result not from a change in the relative position of the ligand with respect to its binding site but, rather, from a change in the efficacy of the ligand, as it does in a PCL. Therefore, the tether can be long and flexible, allowing for the use of larger bioconjugation motifs, such as a SNAP-tag, which provide an anchoring site at a more remote location with respect to the ligand-binding site. The SNAP-tag is a modified DNA repair enzyme that functions as a self-labeling domain by selectively and quickly reacting with benzylguanine (BG) electrophiles.[4] It enables specific and efficient labeling with BG fluorophores in cultured cells and in brain slice.[13] Importantly, unlike maleimides, BGs are essentially inert toward water, regular cysteines, and glutathione, making them ideal for labeling in physiological systems that include extracellular and intracellular targets.[4b,14]

We demonstrate the validity of the PORTL concept by fusing the class C G protein-coupled receptor (GPCR), mGluR2, with a SNAP-tag and endowing it with a synthetic azobenzene photoswitch through benzylguanine chemistry. The resulting photoreceptor, termed SNAG-mGluR2 (SNAP-tagged-azobenzene-glutamate receptor), permits rapid, repeatable, high-efficacy photoagonism of mGluR2 with thermally bistable and fast relaxing photoswitches. SNAG-mGluR2 allows for optical manipulation of neuronal excitability and synaptic transmission in hippocampal neurons. We also show that the SNAG approach may be combined with the cysteine attachment of a conventional PTL to allow for orthogonal optical control of two glutamate receptors within the same cell, paving the way for other multiplexing strategies.

RESULTS

Design of PORTL Photoswitches for Metabotropic Glutamate Receptors. mGluRs are class C GPCRs that mediate many aspects of glutamatergic signaling in the brain and serve as drug targets for a number of neurological disorders.[15] The defining structural feature of class C GPCRs is a large, bilobed extracellular ligand-binding domain (LBD) that assembles as a dimer and mediates receptor activation. We previously developed photoswitchable versions of mGluR2, -3, and -6, termed "LimGluRs", via cysteine conjugation of D-maleimide-azobenzene glutamate ("D-MAG") molecules to the LBD near the glutamate binding site.[10g,16] This work indicated that mGluRs are amenable to agonism by azobenzene-conjugated glutamate compounds. In addition, previous work has shown that *N*-terminal SNAP-tagged mGluRs retain normal function and may be efficiently labeled in living cells.[17] In order to take advantage of the many attractive properties of the SNAP-tag linkage relative to that of cysteine-maleimide, we sought to optically control the LBD of mGluR2 via PORTL conjugation to a genetically encoded SNAP-tag fused to the LBD.

To design a new family of photoswitches we first analyzed SNAP and mGluR crystal structures to determine the dimensions required to permit a compound conjugated to an *N*-terminal SNAP-tag via a BG group at one end to reach the orthosteric binding site within the mGluR LBD via a glutamate at the other end (Figure 2a). We decided to place the central photoswitchable azobenzene unit close to the glutamate ligand based on the logic that the ability of the glutamate moiety to dock in the binding pocket and activate mGluR2 would be altered by photoisomerization of the azobenzene, as achieved earlier for soluble photochromic ligands[18] rather than a length-dependent change in the ability to reach the binding site. Furthermore, we hypothesized that a long, flexible polyethylene glycol linker between the BG and azobenzene units would span the necessary distance between the SNAP domain and the mGluR2 LBD and permit the glutamate moiety to reach the binding site (Figure 2b).

Based on our previous work, which indicated that agonism of mGluR2 via glutamate-azobenzene molecules requires 4′ D stereochemistry, which we refer to as "D-MAG",[10g] we decided to maintain this feature in our new SNAP-tag photoswitches. We opted to construct the linker between BG and azobenzene out of monodisperse PEG-polymers of different sizes. PEG polymers do not strongly adhere to protein surfaces and are known to be conformationally very flexible.[1] To allow this system to be adopted for other pharmacophores in the future, we designed the synthetic chemistry to be flexible as well, using amide bond formation and click chemistry for rapid assembly. Alkyne−azide click chemistry has been extensively used for bioorthogonal reactions and can be employed in the presence of benzylguanines.[19] Both the Cu(I)-catalyzed click chemistry or the cyclooctyne strain promoted version, which can be used *in vivo,* are available.

Together, these considerations led to the design of two families of benzylguanine-azoglutamates with either a diacyl azodianiline switch ($BGAG_n$), as used in the original set of D-MAGs for a 2-wavelength on/off bistable optical control of mGluRs,[10g] or a red-shifted azobenzene switch ($BGAG_{n(460)}$), as used more recently for single wavelength single or two-photon control of an mGluR[16,20] (Figure 2c). In these molecules, the first index denotes the number of ethylene glycol repeat units and the tether length, whereas the number in parentheses indicates the wavelength that results in maximum *cis*-azobenzene content upon irradiation.

Synthesis of Benzylguanines-Azobenzenes-Glutamates (BGAGs). Our synthesis of BGAGs started with guanine derivative **1**, which was converted into the known benzylguanine (BG) in 5 steps[4a] (Figure 3a). Coupling with 4-pentynoic acid (**2**) then yielded BG-alkyne **3**. Alternatively, cyclooctyne **4** was linked to BG by amidation to obtain BG-DBCO **5**. On the ligand side, we utilized several steps from the reported synthesis of D-MAGs starting from L-glutamate[10g] to synthesize glutamate azobenzene **6** via Fratér−Seebach allylation.[21] Acylation with glycine derivative **7**, followed by deprotection, gave primary amine **8**, whereas reductive amination with aldehyde **9**[22] and deprotection yielded diamine **10** (Figure 3b). Coupling of both **8** and **10** with bifunctional PEG-O-Su esters of varying length yielded azides **15**−**18** (whereas **14** was obtained by HBTU-mediated coupling) that were ready for click chemistry (Figure 3c).

BGAGs with a "regular" azobenzene switch were synthesized by Cu(I) catalyzed azide alkyne click chemistry, followed by deprotection, which yielded $BGAG_{0,4,8,12}$ (Figure 3d). It should be noted that high temperatures and high catalyst loadings were needed to drive the click-reaction to completion and that the red-shifted version could not be obtained from **18** and **3** under these conditions. Therefore, strain promoted reaction of **18** with **5**, followed by deprotection of the amino acid moiety, was used instead, which gave the red-shifted photoswitch $BGAG_{12(460)}$. The purity of all BGAGs was assessed by ^1H

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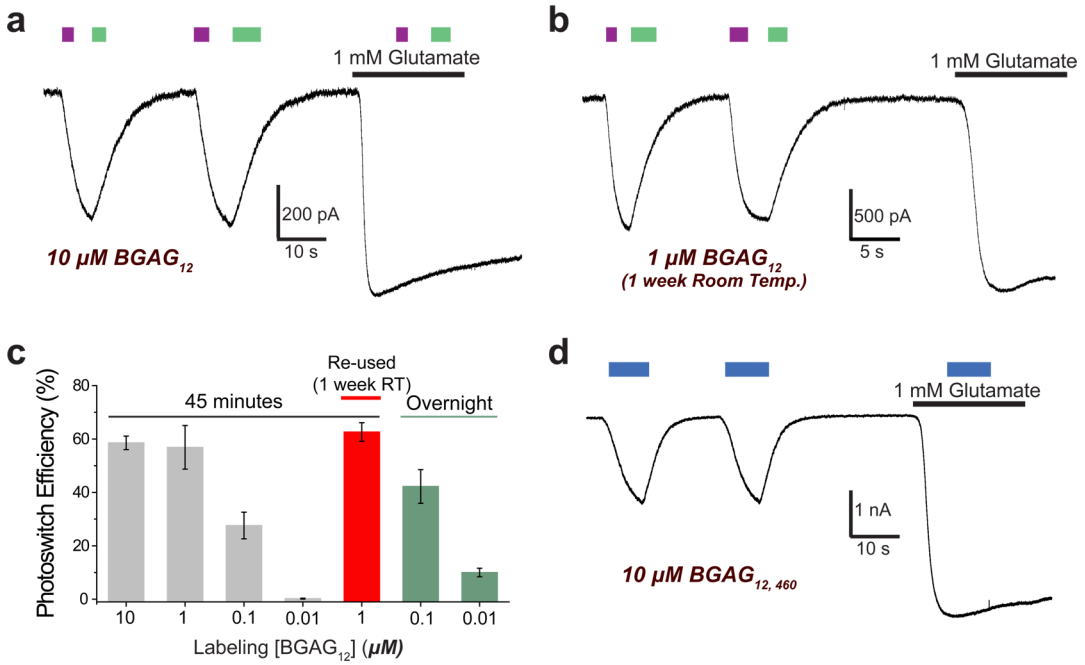

Figure 4. Optical control of SNAG-mGluR2 in HEK293T cells coexpressing SNAP-mGluR2 and GIRK. (a) Representative patch-clamp trace demonstrating the reversible optical control of SNAG-mGluR2 (SNAP-mGluR2 + $BGAG_{12(460)}$). Photoactivation is achieved with a brief pulse of UV light (λ = 380 nm, purple) and reversed by a brief pulse of green light (λ = 500 nm, green). Application of saturating 1 mM glutamate gives full activation and prevents further photoswitching. (b) Representative trace showing photoactivation of SNAG-mGluR2 using 1 μM $BGAG_{12}$ after it was incubated for 1 week in aqueous buffer. (c) Summary of the efficiency of photoactivation of SNAG-mGluR2 (compared to 1 mM glutamate) following different $BGAG_{12}$ labeling conditions. Error bars represent SEM. (d) Representative trace showing photoactivation of $SNAG_{460}$-mGluR2 (SNAP-mGluR2 + $BGAG_{12(460)}$) with blue light (λ = 445 nm). Relaxation occurs spontaneously in the dark.

NMR, HR-MS, and HPLC (see Supporting Information and Figure S1).

Optical Control of SNAG-mGluR2 in HEK293T Cells. After synthesizing the set of BGAG molecules, we next sought to test whether they could be efficiently conjugated to SNAP-mGluR2 and used to optically manipulate mGluR2 function (Figure 2d). We first expressed a GFP-fusion construct (SNAP-mGluR2-GFP) in HEK293T cells and saw efficient labeling with a BG-conjugated Alexa dye that was limited to the cell surface (Figure S2), as previously reported.[17b,17c] This indicated that charged, BG-conjugated compounds are unlikely to cross the membrane and will, thus, primarily target receptors on the cell surface. Furthermore, *in vitro* studies showed, unlike maleimides, no dependence of SNAP labeling on the presence of a reducing or oxidizing environment (Figure S3).

We next tested the ability of BGAGs to photoactivate SNAP-mGluR2 using whole cell patch-clamp electrophysiology in HEK293T cells cotransfected with the G protein-activated inward rectifier potassium (GIRK) channel. Cells expressing SNAP-mGluR2 were initially incubated with 10 μM $BGAG_{12}$ for 45 min at 37 °C. Following extensive washing to remove excess, nonattached photoswitches, photoisomerization to the *cis* configuration with a brief (<1 s) bout of illumination at 380 nm produced robust photoactivation that persisted in the dark and was reversed by a brief (~1 s) bout of illumination at 500 nm to isomerize the azobenzene back to the *trans* state (Figures 4a, S2a). mGluR2 photoactivation via $BGAG_{12}$ was highly reproducible. In the photoswitch "off" state (i.e., in the dark or following illumination at 500 nm), responses to the native neurotransmitter ligand glutamate were intact. Photocurrents were abolished at high glutamate concentrations, suggesting that $BGAG_{12}$ does not function as a partial agonist (Figure 4a). Light responses were ~60% of the responses to saturating glutamate (59.3 ± 2.8%, n = 10 cells), consistent with both efficient conjugation and receptor activation. Importantly, cells expressing wild type mGluR2 (i.e., with no SNAP-tag) and incubated with $BGAG_{12}$ showed no light responses (Figure S4b), confirming that there is no BGAG conjugation in the absence of a SNAP-tag by performing the wash-in and wash-out protocol in the same manner as for SNAP-mGluR2. Given the successful optical control of mGluR2, we termed the tool that combines SNAP-mGluR2 and BGAG "SNAG-mGluR2". SNAG-mGluR2 showed similar photocurrent efficacy and kinetics to the previously reported LimGluR2.[10g] SNAG-mGluR2 photoactivation was fully blocked by the competitive mGluR2 antagonist LY341495, without altering the baseline current, supporting the interpretation that $BGAG_{12}$ activates mGluR2 via its native, orthosteric binding site and does not significantly activate in the *trans* configuration of the azobenzene (Figure S4c). The apparent affinity for glutamate of SNAG-mGluR2 was comparable to that of SNAP-mGluR2 not labeled by $BGAG_{12}$ and, indeed, of wild type mGluR2 (Figure S4d), indicating that normal mGluR2 function is maintained.

We next tested different labeling conditions of $BGAG_{12}$ and found that 45 min incubation with ≥1 μM $BGAG_{12}$ showed optimal labeling (Figure S5a,b). However, photocurrents were still observed with 100 nM labeling for 45 min (Figure S5c) and could even be observed with concentrations as low as 10 nM with overnight labeling (Figure S5d–f). Remarkably, the labeling solution could be reused for multiple experiments for 1 week following dilution in aqueous buffer at room temperature, without a decline in efficacy of optical activation (Figure 4b,c). This result is in stark contrast to maleimide-based MAG

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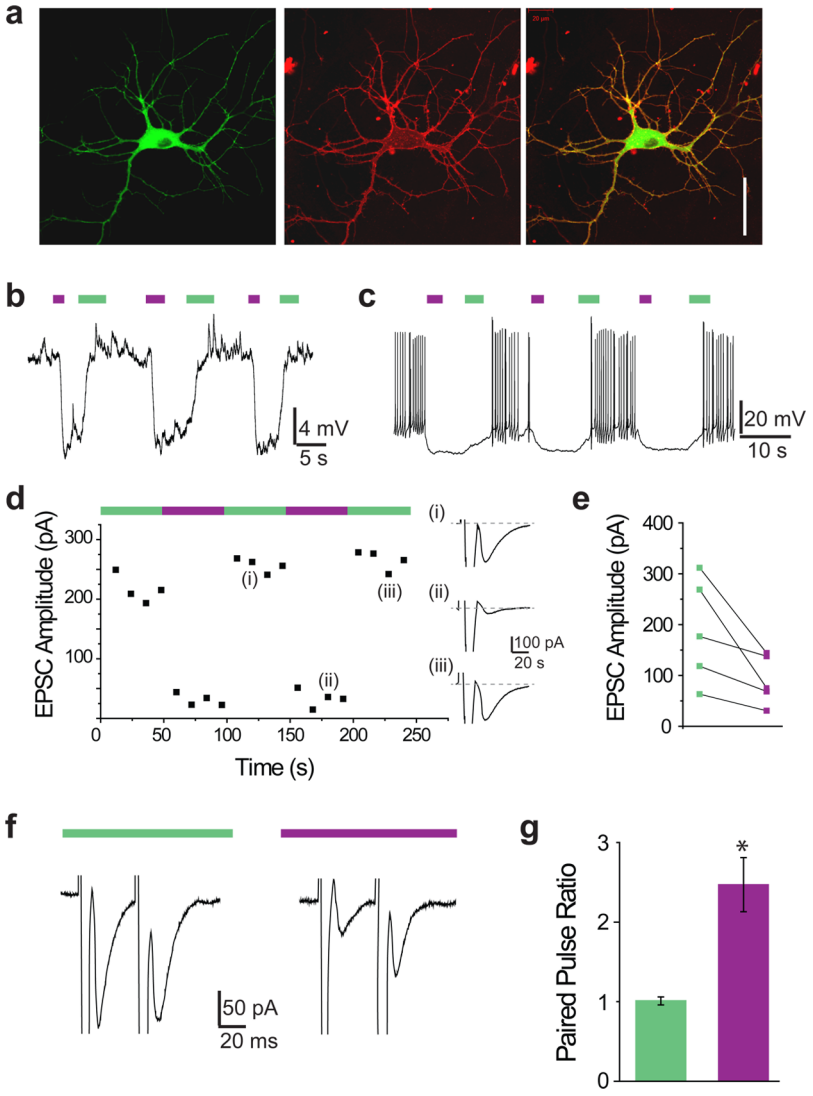

Figure 5. Optical control of SNAG-mGluR2 in hippocampal neurons. (a) Representative confocal images showing the expression of SNAP-mGluR2-GFP (left) and its labeling with BG-Alexa-647 (middle) in hippocampal neurons. In the merge (right) of the two images it is clear that dye labeling occurs on the surface of the neuron only. (b, c) Representative recording showing SNAG-mGluR2 mediated hyperpolarization (b) and silencing (c) of hippocampal neurons in whole cell patch-clamp recordings. Violet bars indicate 380 nm illumination, and green bars indicate 500 nm illumination. (d) Time course of autaptic EPSC amplitude for a representative neuron showing rapid, reversible inhibition of synaptic transmission by SNAG-mGluR2. (i), (ii), and (iii) show individual traces associated with data points. (e) Summary of SNAG-mGluR2 mediated optical synaptic inhibition by 380 nm light in all cells tested. (f) Representative recording showing an increase in paired pulse ratio in response to SNAG-mGluR2 activation using an interstimulus interval of 50 ms. (g) Summary of paired pulse ratio in 500 nm (green) or 380 nm (violet) for the same cell as in panel f.

photoswitches, which typically need to be applied at concentrations up to 100−200 μM[10g,11] and are hydrolyzed in water with a half-life in the range of minutes to hours.[1]

To further explore the mechanism of photoswitching in SNAG-mGluR2, we synthesized a PCL version of $BGAG_{12}$ where the BG group was omitted ("AG_{12}"; Figure S6a). AG_{12} photoagonized SNAP-mGluR2 with the same directionality as $BGAG_{12}$ (Figure S6b), supporting the hypothesis that photoswitching is based on the relative efficacy of the azobenzene-glutamate moiety in *cis* versus *trans*, rather than a length or geometry-dependent change in the ability to reach the binding site. We also tested BGAG variants ranging in length from 0 to 8 PEG repeats and found comparable photoactivation of SNAG-mGluR2 to $BGAG_{12}$ for all versions (Figure S7), suggesting similar effective concentrations of the ligand near the binding pocket.

We next tested the red-shifted version of $BGAG_{12}$, $BGAG_{12(460)}$, to see if we could develop a SNAG-mGluR2 variant that is controlled with a single wavelength of visible light. Following labeling with 10 μM $BGAG_{12(460)}$, photoactivation of SNAP-mGluR2 was achieved reproducibly in response to illumination with blue light (420−470 nm bandpass) (Figure 4d). Relaxation occurred rapidly in the dark following illumination, as expected, and the photoactivation was ∼35% relative to saturating glutamate (34.9 ± 4.2%, n = 18 cells). We termed the combination of SNAP-mGluR2 and $BGAG_{12(460)}$ "$SNAG_{460}$-mGluR2".

Having developed multiple versions of SNAG-mGluR2 that were able to efficiently photoactivate mGluR2, we next wondered if this toolset could be used to optically manipulate mGluR2 in its native neuronal setting.

Optical Manipulation of Excitability and Synaptic Transmission via SNAG-mGluR2 in Hippocampal Neu-

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rons. mGluR2, like other neuronal $G_{i/o\text{-coupled}}$ GPCRs, primarily signals either somatodendritically, to hyperpolarize membranes through the activation of GIRK channels, or presynaptically, to inhibit neurotransmitter release by a number of mechanisms, including inhibition of voltage-gated calcium channels.[15a] We hypothesized that SNAG-mGluR2 would efficiently gate both of those canonical functions in neurons.

We first expressed SNAP-mGluR2-GFP in dissociated hippocampal neurons and labeled with BG-Alexa-647 to determine if SNAP-BG conjugation could occur efficiently in neuronal cultures, which are considerably denser than HEK 293T cell cultures. We observed strong SNAP-mGluR2-GFP expression and surface labeling with Alexa-647 (Figure 5a), indicating that the SNAP tethering approach is suitable to neurons. Importantly, untransfected cells did not show BG-Alexa-647 fluorescence (Figure S8), confirming the specificity of the labeling chemistry. Next, instead of labeling with BG-Alexa-647, we labeled with $BGAG_{12}$ and observed rapid membrane hyperpolarization (~2−8 mV) in response to illumination at 380 nm, which was reversed by illumination at 500 nm (Figure 5b). When the neurons were at depolarized potentials that induced firing, the light-induced hyperpolarization was sufficient to inhibit the action potentials (Figure 5c).

To test for presynaptic inhibition, we cultured hippocampal neurons at low density to promote the formation of autapses, i.e., synapses between the axon and dendrites of the same neuron. In autaptic neurons, photoactivation of SNAG-mGluR2 reversibly decreased excitatory postsynaptic current (EPSC) amplitude by up to 70% (average = 48.3 ± 7.3%, n = 5 cells) (Figure 5d,e). Optical inhibition of EPSC amplitude was accompanied by an increase in paired pulse ratio (Figure 5f,g) and a decrease in synaptic depression during high frequency trains (Figure S9), consistent with a presynaptic reduction in the probability of transmitter release. Together, these observations demonstrate that the SNAG system is well suited for neuronal cells and that SNAG-mGluR2 itself is a powerful tool for optical manipulation of neuronal inhibition via native mGluR2-mediated mechanisms that control neural firing and transmitter release.

Dual Optical Control of SNAG-mGluR2 and LiGluR via Orthogonal Photoswitch Labeling. A major goal in physiology is to be able to independently manipulate different receptors within the same preparation using different wavelengths of light. This type of experiment could be extremely powerful for deciphering the different roles, and potential crosstalk, of different signaling pathways within a cell or neural circuit. With this goal in mind, we wondered if SNAG-mGluR2 could be used in conjunction with a previous generation photoswitchable receptor to provide individual optical control of two receptors within the same cell. We turned to LiGluR, a GluK2 ionotropic glutamate receptor that is photoactivated by molecules of the maleimide-azobenzene-glutamate (MAG) family through cysteine-maleimide linkage.[18a,23] To test this, we coexpressed SNAP-mGluR2 along with its GIRK channel effector and LiGluR (GluK2-L439C) in HEK293T cells. We labeled the cells with $BGAG_{12}$ for 30 min, and then with L-$MAG0_{460}$, a blue light-activated, spontaneously relaxing version of MAG with similar spectral properties to $BGAG_{12(460)}$.[20] Due to the spectral and light sensitivity differences between the two photoswitches, we were able to independently and sequentially activate SNAG-mGluR2 and LiGluR (Figure 6a, see Methods). Photoactivation of SNAG-mGluR2 with dim illumination at 380 nm induced slow inward photocurrents, which were deactivated by illumination at 590 nm, as shown above. 590 nm yellow light was used to ensure orthogonality to L-$MAG0_{460}$. In contrast, photoactivation of LiGluR-L-$MAG0_{460}$ by illumination at 500 nm induced rapid, spontaneously relaxing photocurrents, as shown earlier.[20] When only one of the receptors was expressed, only its characteristic photoresponse was seen. In the case of SNAG-mGluR2 this was a slow ON, slow OFF photocurrent induced by illumination at 380 and 500 nm, respectively, whereas in the case of LiGluR-L-$MAG0_{460}$ this was a rapid, spontaneously relaxing photocurrent, which was triggered by illumination at 500 nm, which turned off spontaneously in the dark (Figure 6b,c). Although a SNAP-tag does react slowly with maleimides (Figure S3), this can be circumvented by first labeling with BGAG to saturation before applying MAG compounds. Together these experiments show that the PORTL approach based on conjugation of BGAGs to SNAP-tagged receptors allows for independent, dual optical control within the same preparation, a major step forward for chemical optogenetics.

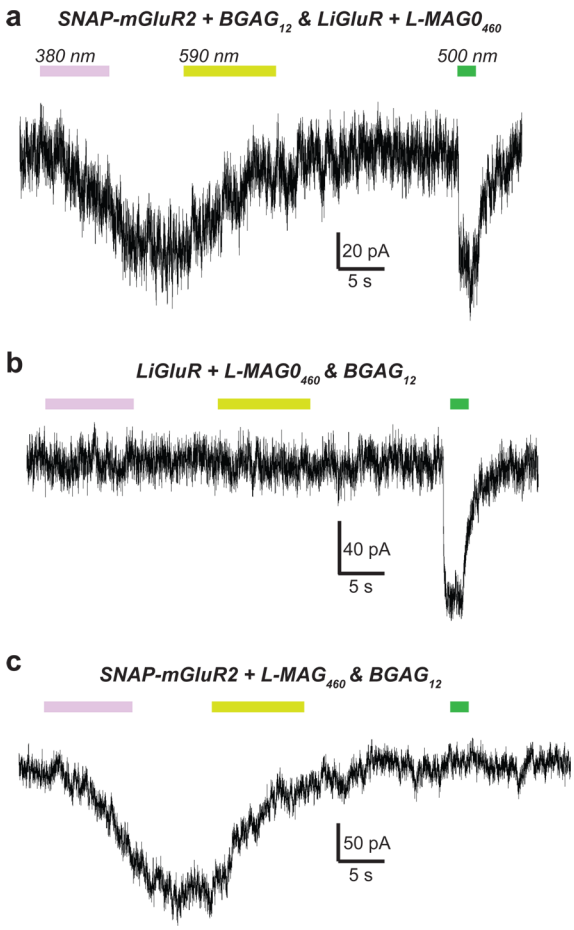

Figure 6. Dual optical control of SNAG-mGluR2 and LiGluR in HEK 293T cells via orthogonal labeling of $BGAG_{12}$ and MAG_{460}. (a−c) Representative traces showing the responses to dim 380 nm light (<0.005 mW/mm^2; purple bars), 590 nm light (~1 mW/mm^2; yellow bars), and 500 nm light (~1 mW/mm^2; green bars) in cells treated with $BGAG_{12}$ and L-$MAG0_{460}$. Cells expressing both SNAP-mGluR2 and LiGluR show a slow SNAG-mGluR2-mediated response to 380 nm light that is reversed by 590 nm light and a fast LiGluR-mediated response to 500 nm light (a). In the absence of SNAP-mGluR2, the slow response to 380 nm is not seen (b), and in the absence of LiGluR, the fast response to 500 nm is not seen (c), confirming the origins of each current.

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DISCUSSION

Photoswitchable tethered ligands (PTLs) provide a powerful component of the optogenetic arsenal for biophysical, synaptic, neural circuit, behavioral, and disease treatment applications.[8,23] Unlike opsin-based approaches, which rely on the exogenous expression of non-native light-gated membrane proteins, PTLs offer target-specific control of native signaling proteins through the bioconjugation of synthetic light-controlled compounds. They allow one to study the physiological roles of individual proteins with a high subtype specificity and spatiotemporal and genetic precision compared to classical pharmacological or genetic techniques. Until the present, PTL anchoring to the signaling protein of interest has been almost exclusively based on the covalent attachment of a maleimide group on the PTL to an engineered cysteine positioned near the pore or ligand binding pocket of the protein.[24] Even on extracellular parts of proteins, where most native cysteines are disulfide bonded and not subject to attack by a maleimide, there are many free cysteines where PTLs will attach. As a result, the specificity of action of cysteine-reactive PTLs relies not on unique targeting but on the insensitivity of other proteins to the minor repositioning of tethered ligands.[10a,18a,25] Still, there would be a major advantage if protein attachment could be bio-orthogonal and so highly specific. Maleimide-cysteine attachment has proven viable in small animals, such as zebrafish, and easily accessible tissues, such as the outer retina of mouse. However, maleimides may be inefficient in larger systems due to slow diffusion and competition with hydrolysis, and is restricted to the extracellular environment, since inside the cell competition for the target cysteine by glutathione at millimolar concentrations would be forbidding. In addition, attachment to a native accessible cysteine, such as in enzyme active sites, could be deadly. Our goal was to create a new orthogonal and efficient strategy for specific PTL attachment that is easy to generalize. We present a solution to these challenges in the form of a second generation PTL, termed PORTL, an approach built around the conjugation of BG-labeled photoswitches to genetically encoded SNAP-tags.

The PORTL approach takes advantage of the fact that the SNAP-tag reacts with BGs in a very efficient and selective way that is fully orthogonal to native chemical reactions.[4] Unlike first-generation PTLs, which need to be tethered near the site of ligand binding,[10a,g,18a,25,26] PORTL tethers the photoswitch farther away, on a separate domain, providing a useful separation between the attachment point and functional headgroup of the compound by a long linker. In principle, the photoswitch could also be attached to a separate transmembrane protein, an antibody, or a membrane anchor. This physical separation is expected to place the ligand headgroup of a PORTL at a relatively lower local concentration than a conventional PTL. The headgroup would then be photoswitched between active and inactive states like a photochromic ligand, and should be inactive ideally in the dark. Aspects of this logic were previously applied to a photoswitchable ligand attached via a long flexible tether to a $GABA_A$ receptor, although in that case the ligand was a potentiator, not an agonist, the ligand was active in the dark, and the attachment was to an introduced cysteine.[27] A further feature to our design is that the predicted relatively low local concentration of PORTL headgroups may help ensure the lack of basal modulation of receptor activity by the relaxed state of the photoswitch.

With these considerations in mind, we designed and synthesized benzylguanine-azoglutamate (BGAG) PORTL compounds that may be attached to a SNAP-tagged version of the class C GPCR mGluR2 to produce the chemical optogenetic tool termed "SNAG-mGluR2". SNAG-mGluR2 permits the high-efficacy, rapid, repeatable photoactivation of mGluR2 with a 2-color, bistable BGAG (SNAG-mGluR2) or a 1-color, spontaneously relaxing $BGAG_{12(460)}$ ($SNAG_{460}$-mGluR2). In both cases, SNAG-mGluR2 remains inactive in the dark and is activated in the high-energy state in response to either near UV (~380 nm, $BGAG_n$) or visible light (~460 nm, $BGAG_{12(460)}$). Consistent with our predictions about the mechanism of PORTL photoactivation, untethered photoswitches that mimic the azobenzene-glutamate part of BGAG showed the same directionality of photoswitching on mGluR2, suggesting that the efficacy of the photoswitchable ligand is higher in *cis* than *trans* and independent of the tether. Importantly, since it maintains the entire full-length sequence of mGluR2, SNAG-mGluR2 should also retain all native signaling properties ranging from ligand binding to G protein coupling to downstream regulation. Consistent with this, SNAG-mGluR2 permitted efficient optical manipulation of two distinct native downstream targets of mGluR2 in neurons: a somato-dendritic control of excitability and a presynaptic control of synaptic transmission.

In line with the attractive properties of SNAP-tag conjugation, BGAG photosensitizes SNAP-mGluR2 at concentrations 100−1000× lower than typically used for maleimide-based PTLs, minimizing potential activation of glutamate receptors during photoswitch incubation. Furthermore, owing to its insensitivity to hydrolysis by water, BGAG remains reactive over not minutes but days, and stocks diluted in aqueous buffer may be reused without a loss of labeling efficiency. Taken together, these properties should make the PORTL approach ideally suited for labeling in intact tissue or *in vivo*, as was recently shown for fluorophore conjugation to a SNAP-tag in the nervous system of mouse.[13b]

Another major advantage of the PORTL approach is its modularity, which will allow it to be widely applicable to many protein targets with a variety of photoswitches. The SNAP-tag is well characterized and has been used extensively to label fusion proteins with fluorophores or to create semisynthetic probes for the sensing of small molecules.[13a] Like GFP, the SNAP-tag can be fused to proteins of interest without significantly altering their activity. Indeed, several SNAP-tagged transmembrane class A, class B, and class C GPCRs, including all of the mGluRs,[17a,28] have been described, and many of these are commercially available.

To facilitate the application of this approach to a wide range of target proteins, we designed our synthetic strategy to be as modular and efficient as possible, taking advantage of the power of click chemistry. Building on existing pharmacology and the growing repertoire of PCLs, PORTL compounds may be synthesized with different headgroups for many other target proteins of interest. These compounds may include photoswitchable agonists, antagonists, or allosteric modulators. Relative to the challenge of finding optimal cysteine residues for maleimide-based photoswitch conjugation with first-generation PTLs, the PORTL system will greatly facilitate the design and implementation of new photosensitive proteins. For instance, voltage-gated potassium channels[10a] and nicotinic acetylcholine receptors,[29] which had been previously out under optical control using maleimides, could be amenable to the

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PORTL approach. In addition, the PORTL system with the SNAP-tag will enable the optical control of intracellular targets because, unlike maleimide, the benzylguanine-labeling motif is unaffected by the reducing environment of the cell.

Finally, a major breakthrough in this study that is made possible by the PORTL system is the demonstration of the ability to orthogonally optically manipulate SNAG-mGluR2 and the maleimide-based LiGluR in the same cell. The ability to separately label and manipulate multiple receptor populations may be especially useful for probing crosstalk between proteins at the molecular, cellular, or circuit level. In the future, combination of SNAP-tethered photoswitches with PORTL compounds targeting the orthogonal SNAP-variant CLIP[5] or the unrelated Halo tag[6] may greatly expand the ability to optically control multiple receptor populations independently in the same preparation. Tuning of the spectral properties of the azobenzene photoswitch will further facilitate the ability to complex multiple tools within the same preparation. Overall, the PORTL approach brings us closer toward the overarching goal of obtaining the ability to individually and precisely photoactivate or inhibit the fundamental signaling molecules of the brain in concert in behaving animals. Even in the absence of optical control, tethered pharmacology (which PORTL represents) holds great promise as a means to precisely control biological function.

METHODS

Chemical Synthesis and Availability of Photoswitches. Details on the chemical synthesis of BGAGs and their precursors and characterization data can be found in the Supporting Information. BGAGs are available for academic use from the Trauner laboratory upon request.

HEK293T and Hippocampal Neuron Electrophysiology. HEK293T cell recordings were performed as described previously.[10g] Cells were seeded on 18 mm glass coverslips and transfected with 0.7 μg/well SNAP-mGluR2 (and/or LiGluR: GluK2-L439C) and GIRK1-F137S DNA, along with 0.1 μg/well tdTomato as a transfection marker, using Lipofectamine 2000 (Invitrogen). Whole-cell HEK cell recordings were performed 24−48 h later at room temperature (22−24 °C) using an Axopatch 200B headstage/amplifier (Molecular Devices) on an inverted microscope (Olympus IX series) or an EPC10 USB patch clamp amplifier (HEKA) and PatchMaster software (HEKA) on a Leica DM IL LED. Recordings were performed in high potassium (HK) extracellular solution containing (in mM) 120 KCl, 29 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, pH 7.4. Glass pipettes of resistance between 4 and 8 MΩ were filled with intracellular solution containing (in mM) 140 KCl, 10 Hepes, 3 Na_2ATP, 0.2 Na_2GTP, 5 EGTA, 3 $MgCl_2$, pH 7.4. Voltage-clamp recordings were typically performed at −60 mV. Drugs were purchased from Tocris, diluted in HK solution, and applied using a gravity-driven perfusion system. Data were analyzed with Clampfit (Molecular Devices) or IgorPro (v6.22, wavemetrics).

Prior to recording, cells were washed with extracellular labeling solution and labeled with BGAG variants at the reported concentrations for 45−50 min in an incubator at 37 °C. The extracellular labeling solution contained (in mM) 138 NaCl, 1.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, pH 7.4. For overnight labeling experiments, BGAG was diluted in HEK cell culture media (DMEM + 5% FBS). For experiments involving LiGluR, following BGAG incubation cells were incubated for 5 min at room temperature with 0.3 mg/mL concanavalin A to prevent receptor desensitization followed by 50 μM L-$MAG0_{460}$ for 30 min at room temperature. Illumination was mediated by Xe-lamp (DG4, Sutter) in combination with excitation filters. Neutral density filters (Omegafilters) were used to vary the light intensity.

Dissociated hippocampal neuron cultures were prepared from postnatal P0 or P1 mice on 12 mm glass coverslips as previously described.[10g] Neurons were transfected with SNAP-mGluR2 (1.5 μg/well) and tdTomato (0.25 μg/well as a transfection marker) using the calcium phosphate method at DIV9. Whole cell patch clamp experiments were performed 3−6 days after transfection (DIV 12−15). Labeling was performed using the same protocol as HEK cells except BGAG was diluted in extracellular recording solution containing (in mM) 138 NaCl, 1.5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose, 5 HEPES, pH 7.4. Glass pipettes of resistance 4−8 MΩ were filled with an intracellular solution containing (in mM) 140 K-gluconate, 10 NaCl, 5 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 2 MgATP, and 0.3 Na_2GTP, pH 7.2. Autaptic neurons were voltage clamped at −60 mV, and a 2−3 ms voltage step to +20 mV was used to evoke a spike followed (∼3−5 ms later) by an EPSC. Stimulation was performed once every 12 s to prevent rundown.

Confocal imaging of SNAP-mGluR2-GFP and Alexa dye-labeled constructs was performed on a Zeiss LSM780 AxioExaminer. Dye labeling was performed in appropriate extracellular solutions for 45 min at 1 μM in an incubator at 37 °C, followed by extensive washing before imaging.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.5b00260.

Preparation of BGAGs, characterization by NMR, HRMS, and HPLC, and supporting figures (PDF)

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Notes

The authors declare no competing financial interest.

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