Published in final edited form as:

Methods Mol Biol. 2019 ; 1947: 103–136. doi:10.1007/978-1-4939-9121-1_6.

Optical Regulation of Class C GPCRs by Photoswitchable Orthogonal Remotely Tethered Ligands

Amanda Acosta-Ruiz¹, Johannes Broichhagen², and Joshua Levitz¹

¹Department of Biochemistry, Weill Cornell Medicine, New York, NY, USA.

²Department of Chemical Biology, Max Planck Institute for Medical Research, Heidelberg, Germany.

Abstract

G protein-coupled receptors (GPCRs) respond to a wide range of extracellular cues to initiate complex downstream signaling cascades that control myriad aspects of cell function. Despite a long-standing appreciation of their importance to both basic physiology and disease treatment, it remains a major challenge to understand the dynamic activation patterns of GPCRs and the mechanisms by which they modulate biological processes at the molecular, cellular, and tissue levels. Unfortunately, classical methods of pharmacology and genetic knockout are often unable to provide the requisite precision needed to probe such questions. This is an especially pressing challenge for the class C GPCR family which includes receptors for the major excitatory and inhibitory neurotransmitters, glutamate and GABA, which signal in a rapid, spatially-delimited manner and contain many different subtypes whose roles are difficult to disentangle. The desire to manipulate class C GPCRs with spatiotemporal precision, genetic targeting, and subtype specificity has led to the development of a variety of photopharmacological tools. Of particular promise are the photoswitchable orthogonal remotely tethered ligands (“PORTLs”) which attach to self-labeling tags that are genetically encoded into full length, wild-type metabotropic glutamate receptors (mGluRs) and allow the receptor to be liganded and un-liganded in response to different wavelengths of illumination. While powerful for studying class C GPCRs, a number of detailed considerations must be made when working with these tools. The protocol included here should provide a basis for the development, characterization, optimization, and application of PORTLs for a wide range of GPCRs.

Keywords

Optogenetics; Photopharmacology; Tethered ligands; G protein-coupled receptors; Metabotropic glutamate receptors; PORTLs; Neuromodulation; SNAP tag

1 Introduction

G protein-coupled receptors (GPCRs) provide one of the primary molecular mechanisms for the intracellular response to extracellular signals in a wide range of physiological systems and serve as the largest family of drug targets in biology [1]. The major conserved structural feature of GPCRs is a seven helix transmembrane domain (TMD) which undergoes conformational changes following ligand binding that permits coupling with intracellular

heterotrimeric G proteins to initiate diverse signaling cascades [2]. Of particular importance in the nervous system is the class C GPCR family which contains receptors for neurotransmitters [3, 4], ions [5], and hormones [6, 7]. Class C GPCRs are characterized structurally by the presence of large, bi-lobed extracellular ligand binding domains (LBD) which mediate constitutive dimerization. The largest subfamily of class C GPCRs are the eight metabotropic glutamate receptors (mGluR1–8) which show distinct, yet overlapping localization and physiological roles [3, 4]. Despite great progress over the last two decades of research, mechanistic understanding of class C GPCRs at the biophysical, cellular, and neurophysiological levels remains incomplete. A deeper understanding of the activation and signaling mechanisms of class C GPCRs could provide key insight into the fundamental roles of G protein signaling in neurobiology, as well as lead to the development of improved therapeutics for many conditions.

Massive effort has been devoted to the development of an extensive repertoire of synthetic compounds for manipulating GPCRs in both a basic research and clinical context [1]. However, classical pharmacology has substantial shortcomings which limit the biological insight that can be gleaned from its use. For instance, pharmacological compounds often lack complete subtype specificity within their receptor subfamily. In the case of mGluRs, while many drugs can distinguish between groups there is a paucity of drugs that target a single subtype with strong specificity [3]. Furthermore, pharmacological compounds also lack the ability to be applied and removed with spatial and temporal control. This is an especially crucial point for class C GPCRs which respond to precise patterns of neurotransmitter release and whose native signaling may not be well-modeled by tonic application of drugs on slow time scales. In addition, pharmacological approaches lack genetic targeting, preventing the study of cell-type specific responses to receptor activation within tissue. Genetic knockouts have been used to study receptor function, but they also lack temporal control and cell-type targeting, and developmental compensation can occur and confound interpretation. Thus, to determine GPCR function it has become widely accepted that tools to facilitate spatiotemporally precise, genetically targeted activation with subtype specificity are needed [8].

As a way to improve spatiotemporal precision, caged compounds for neurotransmitter receptors have been developed. Caged compounds, including those for glutamate and GABA, are ligands masked with a photo-labile group which prevent the compound from functioning as an agonist until it is ‘uncaged’ with illumination by a specific wavelength of light over a given area [9, 10]. A related family of “reversibly caged” compounds has also been developed, including those for glutamate, which can be reversed with a second wavelength of light [10, 11]. While especially useful for studies of synaptic plasticity at individual dendritic spines, a number of limitations exist for these approaches. Most importantly, the specificity of the caged compound is limited by the specificity of the compound that is being uncaged. Thus, for example, caged glutamate is able to potentially activate any ionotropic or metabotropic GluR in the vicinity of the illumination which requires a cocktail of antagonists if a single receptor is to be isolated. Even when more subtype-specific ligands are caged, as has been done for orthosteric and allosteric compounds targeting mGluRs [4], full subtype specificity remains elusive. In addition, caged compounds are soluble molecules whose binding and un-binding kinetics are subject to

diffusion, limiting their temporal fidelity. Finally, while it is advantageous that such compounds work on native receptors, they are not genetically targeted in any way, limiting their utility for cell-type-specific probing.

A powerful approach to manipulating GPCRs with genetic targeting has been through designer receptors exclusively activated by designer drugs (“DREADDs”) [12]. This approach takes advantage of a combination of synthetic, non-native drugs, and an engineered GPCR that can bind the synthetic drugs but are no longer sensitive to their native ligand. Upon activation, the DREADD activates G proteins to drive the relevant signaling pathways. For example, the m4 muscarinic acetylcholine receptor was converted into a DREADD by a combination of Y113C and A203G mutations and can be activated with the ligand clozapine N-oxide (CNO), but not by acetylcholine [13]. Genetically encoding and expressing the DREADDs allows cell-type specificity, but reliance on a soluble drug precludes efficient subcellular targeting or temporal control. Importantly, because this approach relies on mutated receptors, it is unclear how much receptor identity is maintained, challenging the use of DREADDs for understanding specific receptors rather than the general consequences of activation of a particular G protein family. Last but not least, careful control experiments have to be conducted with DREADDs to delineate the role of the introduced receptors versus background effects of the CNO ligand which is hydrolyzed to clozapine and can have effects on native receptors [14].

An alternative to DREADDs that allows both genetic targeting and optical control has been to use intrinsically light-sensitive GPCRs from the opsin family, including rhodopsin and melanopsin [8, 15]. Similar to DREADDs, exogenous expression of opsins, which are typically not expressed outside of the retina, allows general G protein pathways to be manipulated but does not facilitate the study of specific GPCRs. A number of studies have used chimera-based approaches to try to mimic the native signaling of specific GPCRs. These proteins maintain the light-sensitive transmembrane core of rhodopsin or melanopsin but are combined with the cytoplasmic loops and/or C-termini of a GPCR of interest [16–19]. However, it remains unclear if the signaling initiated by these proteins is able to fully recapitulate the complex signaling properties of specific receptors and many photophysical properties of opsins, including desensitization and slow kinetics [20, 21], challenge the widespread use of these tools.

The development of Photoswitchable Tethered Ligands (PTLs) in the late-2000s provided a new way to activate various types of receptors that addresses the spatiotemporal limitations of classical pharmacology while maintaining the ability to manipulate full length receptors with subtype specificity [10, 22]. PTLs contain a chemical handle for tethering, a photoswitchable molecule, usually an azobenzene, and a ligand head group. As such, a maleimide (M) for attachment to a cysteine was first conjugated to an azobenzene (A) bearing a quaternary ammonium group (Q), resulting in MAQ which was used for the light-dependent blockage of a voltage-gated potassium channel termed “SPARK” [23]. Since the attachment mechanism uses a genetically encoded cysteine substitution on the receptor of interest, it can thus afford both cell-type and receptor subtype specificity. However, the apparent reactivity of maleimides toward other free thiols can be considered as a drawback. The first PTLs for glutamate receptors used a maleimide-azobenzene-glutamate (MAG)

family of PTLs (see Table 2) to covalently bind a cysteine residue introduced close to the binding site of an ionotropic GluR of the kainate receptor family (“LiGluR”) [24]. In 2013, this approach was successfully expanded to GPCRs with the development of light-activated mGluRs (“LimGluRs”) [20]. Again, a maleimide-azobenzene-glutamate (“D-MAG_n”) PTL was used, however, this version had different stereochemistry than the compounds used for LiGluR. Depending on the position of the cysteine substitution on the LBD and the length of the D-MAG variant, photoagonism was established for mGluR2 (“LimGluR2”) and mGluR3 (“LimGluR3”) and photoantagonism was established for mGluR2 (“LimGluR2-Block”) and mGluR6 (“LimGluR6-Block”).

While a powerful approach to manipulating mGluRs that has seen application in a number of biophysical and physiological contexts [10, 25–28], PTLs have shortcomings including, most importantly, their reliance on cysteine-maleimide chemistry which is inefficient in aqueous solutions, is prone to promiscuity and is incompatible with the reducing cytosolic environment for intracellular applications. In addition, identifying an optimal attachment position for cysteine substitution can be extremely challenging, providing a limiting step for the development of PTL-based control of other GPCRs. This issue has been tackled by *in silico* predictions [20, 29], but remains extremely difficult. To overcome these issues, photoswitchable orthogonal remotely tethered ligands (PORTLs) were recently developed with a focus on their application to mGluRs [30, 31]. Rather than attaching the photoswitchable ligand directly to the receptor of interest, PORTLs attach to the receptor through a self-labeling protein tag, such as SNAP or CLIP, which is typically fused to the extracellular N-terminus of the receptor of interest located next to the LBD (Fig. 1). These tags offer several advantages over maleimide-thiol chemistry, including high selectivity and orthogonality to native reactions, high rate constants ($k_2 \sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$), and the extreme stability of the tethering group [32]. Figure 1 outlines the general design of PORTLs and a depiction of the principle in the context of mGluRs. SNAP-targeting PORTLs attach to a receptor via the covalent binding between a genetically encoded SNAP tag and an *O*⁶-benzylguanine (BG) moiety with high selectivity. Following the BG attachment moiety, a PORTL is made up of a polyethylene glycol (PEG) linker, which provides flexibility and appropriate length and is both highly water-soluble and chemically inert, an azobenzene group, which mediates photoisomerization, and a short-linker attached to a ligand, such as glutamate. The configuration of the azobenzene determines whether the ligand at the end of the photoswitchable compound is able or unable to serve as an agonist (Fig. 1a). The family of compounds developed for SNAP-mGluRs are called “BGAGs” for the *benzylguanine-azobenzene-glutamate* components (Fig. 1b, c). The most robust photoswitching observed with the currently available tools has been with BGAG variants in combination with SNAP-mGluR2, producing the “SNAG-mGluR2” tool which provides rapid, reversible, and high-efficiency optical control of mGluR2 (Fig. 1d). PORTL-based optical control has also been established for other mGluR subtypes with a range of BGAG variants (Table 1).

The modularity of the synthesis of PORTLs has allowed for developing and testing versions that vary in linker length, protein tag, and azobenzene modifications. The linker lengths that have been developed for BGAG range from “short-BGAG” (i.e. direct urea linkage between BG and a glycine-spaced azobenzene) to 0, 4, 8, 12, or 28 PEG repeat units (Table 2). SNAP-mGluR2 shows a tolerance for linker lengths 0 and 12, but exhibits reduced

photoswitching efficiency with lengths of 4, 8, and 28, as well as completely absent linker. As such, the BGAG compound with linker length 12 (BGAG₁₂) has served as a benchmark. Based on the success of SNAP-targeting PORTLs, a CLIP-binding version of BGAG has also been developed, termed BCAG, which uses an *O*²-benzylcytosine (BC) moiety to attach the photoswitchable compound to the receptor [31]. These compounds show similar photoswitching efficiency to their BG counterparts. Finally, spectral tuning can also be achieved with PORTLs, as has also been done with PTLs. Modified azobenzenes that permit photoagonism under 460 nm light and spontaneously relax in the dark back to their inactive form have been developed for both BGAG (“BGAG_{n,460}”) and BCAG (“BCAG_{n,460}”). Apart from the advantage that laboratories can now utilize these tools with simple illumination of a single wavelength, these compounds bear other advantages such as the ability to use visible light which is less toxic and penetrates tissue deeper. The development of both orthogonal PORTL labeling, with SNAP or CLIP-targeting variants, and orthogonal wavelengths, with both standard azobenzene and red-shifted azobenzene cores, permits experiments targeting multiple receptor subtypes or variants within the same preparation [31].

In general, the PORTL technique should be adaptable beyond mGluRs to other GPCRs and to other forms of pharmacology other than agonism (i.e. antagonism or allosteric modulation). Soluble photoswitchable ligands have been developed for a number of class A and B GPCRs [33–39] and recently SNAP-tethered, non-photoswitchable agonists have been developed for both the GLP-1R (class B) and the GHS-R1a (class A) [40]. In addition, “*drugs acutely restricted by tethering*” (DART) describes an approach where a non-photoswitchable antagonist is coupled to a protein tag fused to a transmembrane domain for the manipulation of a subset of neurons [41]. These studies point to the feasibility of PORTL-based designs on a wide range of GPCRs. Finally, a major long-term goal of the PORTL approach is to adapt the technique for optical control of native receptors without the need for heterologous receptor (over)expression. As such, genome editing techniques like CRISPR/Cas9 offer the possibility of fusing labeling tags to endogenous proteins [42]. Most recently we have shown in a proof-of-principle study that BGAG-conjugated SNAP-tagged anti-GFP nanobodies can target GFP-tagged mGluR2 with low nanomolar binding constants and allow them to be optically controlled [43]. This exciting result indicates that the PORTL attachment domain can be further outsourced beyond the target receptor itself, opening up many exciting design possibilities that can allow targeting of native receptors.

While PORTL-mediated control of GPCRs can be applied in various experimental conditions, the protocol below uses an ion channel reporter (i.e. GIRK) in conjunction with patch clamp electrophysiology as a readout for G_{i/o} activation of a GPCR. This approach provides a robust, repeatable, receptor-proximal readout with high temporal resolution that is orthogonal to light. These properties make such experiments ideally suited for PORTL development and refinement [30, 31], biophysical studies of receptor mechanisms [26], and is easily adapted to primary cells and tissues for studies of receptor physiology [44]. However, PORTL-mediated control of GPCRs can be applied in various experimental conditions, including imaging-based readouts and experiments in native systems, such as cultured neurons or brain slices, where endogenous reporters (i.e. ion channels, kinases, gene expression) or physiological responses (i.e. neuronal firing, synaptic strength) further downstream of receptor activation may be assayed. Wherever possible, we will point out

modifications that allow other GPCRs, PORTLs, downstream reporters, or cell types to be used to allow the full breadth of potential applications of these tools to be explored.

The focus of this protocol is on N-terminally SNAP-tagged mGluR2 and labeling with BGAG_n variants, the current standard for high-efficiency PORTL-mediated optical control of a GPCR. Notably, PORTL-based optical control of mGluR2 has been comprehensively characterized in vivo in the retina in a mouse model of blindness using ex vivo electrophysiology in whole-mounted retinas and visually guided behaviors in freely moving animals [44]. Ongoing work is aimed at optimizing this approach in the central nervous system of awake, behaving mice using optical fiber-based manipulation.

2 Materials

2.1 Cell Culture

1. HEK 293 or 293T cells. *See* Note 1 for alternative cellular models.
2. Biosafety cabinet (laminar flow hood).
3. 37 °C Incubator with 5% CO₂ and >50% relative humidity.
4. 37 °C Water bath.
5. Table top centrifuge.
6. Inverted light microscope with 10× or 20× objective to visualize cells.
7. “T25” Flasks: 25 cm² sterile polystyrene cell culture flasks with filter cap.
8. Polystyrene 12-well tissue culture-treated plates.
9. 15 or 18 mm diameter round glass coverslips (thickness ~0.1 mm).
10. Thin metal forceps for handling coverslips.
11. Sterile distilled water.
12. Culture Media: Dulbecco’s Modified Eagle’s Medium (DMEM) with 5% Fetal Bovine Serum. Filter after making 500 mL stock and store at 4 °C.
13. 0.25% Trypsin with EDTA
14. Borate Buffer: For a 500 mL solution, use 1.55 g Boric Acid (50.14 mM), 2.375 g Sodium Borate (23.61 mM), 500 mL dH₂O, pH 8.5, store at 4 °C.
15. 20× Poly-L-Lysine (PLL) Stock Solution (10 mg/mL) diluted in water.
16. Dulbecco’s buffered saline (DPBS) without Ca²⁺ and Mg²⁺ (Optional).

2.2 DNA and Transfection

1. Lipofectamine (2000 or 3000) or other lipid-based transfection systems. *See* Note 2 for alternatives transfection options for other cell lines.
2. DNA Construct for SNAP-tagged GPCR of interest (in this protocol, SNAP-mGluR2). *See* Notes 3 and 4 for alternatives and other considerations.

3. DNA construct for reporter of GPCR activation (in this protocol, GIRK1). *See* Notes 3 and 5 for alternatives and other considerations.
4. DNA Construct for reporter of transfection efficiency (in this protocol, tdTomato). *See* Note 3 for considerations.

2.3 Solutions for Labeling and Electrophysiology

1. Extracellular Solution (“EX”): 135 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂. Adjust pH to 7.4 with NaOH. Filter and store at room temperature. *See* Note 6 for additional usages for these solutions.
2. High Potassium Solution (“HK₁₂₀”): 120 mM KCl, 25 mM NaCl, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂. Adjust pH to 7.4 with KOH. Filter and store for up to 1 week at room temperature. *See* Note 7 for usage.
3. Intracellular Solution: 140 mM KCl, 10 mM HEPES, 5 mM EGTA, 3 mM MgCl₂, 3 mM Na₂ATP, 0.2 mM Na₂GTP (Na₂ATP and Na₂GTP are added last and maintained on ice). Adjust pH to 7.4 with KOH. Filter, make 1–2 mL aliquots, and store at –20 °C.
4. Glutamate Stock Solution: 100 mM high purity L-glutamic acid in HK₁₂₀ Solution. Store at 4 °C. *See* Note 8 for details and alternatives.

2.4 PORTLs and Related Compounds

1. Materials for Synthesis (*see* ref. 30). Semi-preparative or preparative reverse-phase C18 HPLC is of vital importance.
2. Spectrophotometer. *See* Note 9 for usage.
3. PORTLs (available compounds are shown in Table 1, *see* Note 10): Powder stocks are solubilized in a minimal volume of dry DMSO. PORTL aliquots are stored at 10–100 mM in DMSO at –20 °C in dark tubes with desiccant. *See* Note 11 for storage recommendations. *See* Note 12 for determining PORTL concentration.
4. Soluble test compounds (*see* Note 13).

2.5 Patch Clamp Electrophysiology

1. Vibration isolation table attached to house air.
2. Inverted Microscope (Ex. Olympus IX73) that is suitable for basic fluorescence imaging. 20× or 40× objectives with large working distances are ideal for patch clamp experiments.
3. Filter cubes/Filters for fluorescence imaging and optical manipulation. *See* Note 14 for considerations.
4. Micromanipulator (Ex. Scientifica PatchStar Micromanipulator).
5. Faraday Cage.

6. Amplifier (Ex. Axopatch 200B from Molecular Devices).
7. Digitizer (Ex. Axon Digidata 1550B from Molecular Devices).
8. Silver wire (>99% purity) for microelectrode and ground electrodes, respectively. Silver wire is chlorinated by incubating it in bleach for 30–60 min.
9. Microelectrode holder and headstage (Ex. Axopatch CV-203BU).
10. Illumination system. *See* Note 15 for options.
11. Borosilicate, thin wall capillary glass with filament for patch pipettes (Ex. G150TF-3 from Warner Instruments).
12. Micropipette Puller (Ex. Sutter Instruments Model P-97).
13. 1 mL Syringe to fill pipette with Intracellular Solution. Syringe tips for filling micropipettes (Ex. Microfil 28 AWG, MF28G67–5, World Precision Instruments).
14. Tubing. *See* Note 16 for how to set up tubing system for controlling pipette pressure.
15. Salt bridges made from bent glass capillaries and filled with 3 M KCl in 1% agarose and stored at 4 °C in 3 M KCl.
16. Desktop computer with Clampex (or similar) software for voltage clamp control and current recording.
17. Gravity-Driven Perfusion System mounted on a support ring stand containing syringe holders, 60 mL syringes, a stopcock for each syringe, tygon tubing (Ex. Outer diameter: 1/8 in; Inner diameter: 1/16 in) and a manifold (Ex. Warner MP series with 8 inputs). A flow regulator may be placed after the manifold to allow for modulation of the flow rate for all channels.
18. Vacuum line connecting suction reservoir of chamber to large flask for collection of perfusion output throughout experiment.
19. Recording/Imaging Chamber (Ex. Warner Instruments RC-25F) and Platform (Ex. Warner Instruments PM-4). *See* Note 17 for considerations.

3 Methods

For synthetic schemes of PORTLs we refer the reader to Broichhagen et al. [30] and Levitz et al. [20]. *See* Note 18 on CuAAC, SpAAC, peptide coupling, and NHS-ester handling.

3.1 Coverslip Preparation

1. To prepare the 1× PPL Solution, combine 1.5 mL of PLL 20× stock to 30 mL Borate Buffer (final working concentration of PLL is 0.5 mg/mL). For a 12-well plate, add ~2 mL to each well; add coverslips to well and ensure it is completely covered by the PLL solution by pushing down with metal forceps.

2. Leave coverslips in PLL solution 12 h prior to seeding cells. PLL solution is reusable.

3.2 Cell Preparation and Seeding (Day 1)

1. Place coverslips in sterile 12-well plate with ~2 mL H₂O in each well to wash poly-L-lysine. Thoroughly aspirate dH₂O (optional: wash a second time with dH₂O) and let coverslips air dry in the biosafety cabinet. Once dry (10–15 min), add 1.5 mL culture media to each well and place in incubator.
2. Aspirate media from T25 flask of confluent cells and add 3 mL pre-warmed trypsin and place in incubator for 3 min.
3. Harvest cells and spin in table-top centrifuge at $1500 \times g$ for 3 min to produce a small pellet. Aspirate supernatant, add 6 mL media and resuspend cells by pipetting up and down.
4. Seed cells on coverslips (Fig. 2). Density should be sparse to have single cells or small clusters (3 cells) for electrophysiology recordings 36–72 h later. To achieve this density, ~50 μ L of cell suspension are added to each well. The amount of cells added can be adjusted empirically taking into account the density of the parent flask, the rate of cell growth, and the timing of seeding and subsequent experiments. Seeding at higher densities can be used for imaging experiments when using optical readouts. *See Note 19* for additional considerations.

3.3 Transfection (Day 2)

1. Transfect cells ~12 h after seeding according to the manufacturer's protocol (Fig. 2). For Lipofectamine transfection in a 12-well plate, 0.75 μ g SNAP-tagged GPCR, 0.75 μ g GIRK1-F137S, and 0.1 μ g tdTomato are used per well. Less DNA is typically used for optical reporters, such as GCaMP, because soluble proteins express more easily than membrane proteins.
2. Allow 24–48 h for expression or longer for primary cells (3–5 days). *See Note 20* for optional strategies to ensure cell health. *See Note 21* for strategies to circumvent low expression issues.

3.4 PORTL Labeling (Day 3)

1. Prepare dilution of PORTLs for labeling cells in EX solution (Fig. 2). 1–10 μ M is typically sufficient to provide saturating labeling at 37 °C for 45–50 min [30]. *See Note 22* for determining optimal labeling conditions.
2. Following a brief wash in 1–2 mL of EX, coverslips are labeled in 400 μ L of PORTL solution (i.e. BGAG) in a 12-well plate (Fig. 2). Due to the stability of the BG moiety in aqueous buffer, the same dilution of BGAG can be re-used during a full day of experiments with multiple coverslips.

3.5 Whole Cell Patch Clamp Recording (Day 3)

1. While a coverslip is being labeled with PORTLs, thaw an Intracellular Solution aliquot on ice.
2. Load perfusion system with HK₁₂₀ and relevant compounds based on the particular experiment (see Note 23).
3. Pull pipettes using the parameters necessary to achieve a resistance of 3–7 MΩ when pipette is placed in the bath.
4. Turn on equipment on patch electrophysiology rig: microscope illumination, amplifier, digidata, computer, and micromanipulator. Using a Faraday cage will reduce electrical noise, and draping a black sheet over it will prevent excess light from reaching the sample. Additionally, a vibration isolation table attached to house air will minimize vibration-induced noise in patch clamp experiments.
5. Following 45–50 min of labeling, wash cells with EX solution. An efficient way to do this is to move the entire coverslip to a new well within the 12-well plate containing 1–2 mL EX. Repeat this 2–3 times.
6. Prepare the cells for recording by setting the coverslip in the recording chamber and attaching it to the perfusion system. Set up perfusion system to achieve a flow rate of ~1–2 mL/min (using flow regulators) and position end of vacuum line in chamber to maintain a constant flow of HK₁₂₀ from perfusion Inlet port, through chamber, and out the suction reservoir. Ensure that the ground electrode, or salt bridges connected to a bath containing HK₁₂₀, is also in the suction reservoir connected to the bath.
7. Select a healthy cell (i.e. spread, flat, isolated, not round) that is expressing the fluorescent reporter construct and adjust stage/objective so that it is in the center of the field of view (Fig. 2). It is important to be aware that the light used to excite the fluorescent reporter might impact the photoswitch. Ideally, the excitation light used for the fluorescent reporter should not fall under the spectral profile for the azobenzene state that activates the receptor. For example, tdTomato can be excited at 550 nm, which will maintain BGAG_n in the inactive *trans* state for SNAG-mGluR2. In the absence of an orthogonal fluorescent reporter that falls outside of the emission range of the active configuration of the PORTL, illuminating the sample for the minimal amount of time is crucial to prevent excessive receptor activation which can lead to desensitization. For example, when using tdTomato in conjunction with group III SNAG-mGluRs (where *trans*-agonism is observed) brief (<10 s) 550 nm illumination can be used to visualize tdTomato and a short pulse at 380 nm can be used to switch the PORTL back to the inactive *cis* state.
8. Fill micropipette with Intracellular Solution using a syringe (be sure to remove all bubbles from micropipette by flicking it with fingers) and attach the micropipette to the pipette holder. Make sure that the AgCl electrode is in contact with intracellular solution and that the pipette is not filled more than is necessary to make a connection with the electrode.

9. Bring micropipette tip near the cell, apply and lock positive pressure into the pipette tip, and bring pipette offset to 0 pA while voltage clamping. Resistance of the pipette in the bath should be 3–7 M Ω as calculated in software via a 10 ms, –5 mV test pulse.
10. Approach cell slowly until resistance increases by ~0.1–0.3 M Ω , which indicates that the glass pipette has touched the cell. Immediately release the positive pressure and apply gentle suction. Ramp voltage down to ~–20 mV to facilitate seal formation. Allow resistance to increase until a >1 G Ω seal is formed. This may be quick for healthy cells with optimized conditions (<10 s) or may take a few minutes otherwise.
11. While in the cell-attached mode, turn perfusion and vacuum on, allowing HK₁₂₀ to perfuse through the chamber to maintain a steady level. Because patch stability is higher in the cell-attached mode than in whole-cell mode, adjust filters to the desired settings for the subsequent experiment before breaking in (*see step 9*).
12. Once a stable G Ω seal is formed in cell-attached mode, apply brief suction to break the cell membrane at the tip of the pipette to enter whole-cell mode. The test pulse should show an increase in the time constant of the exponential decay due to the increased capacitance due to gaining electrical access to the entire cell. A decrease in resistance is also expected.
13. Begin recording in the gap-free mode with a holding potential of –60 mV at a sampling rate of 10 kHz. *See Note 24* for more details on voltage-dependence.
14. Record for 30–60 s to achieve a steady baseline and allow cell dialysis with intracellular solution to occur. At a holding potential of –60 mV, the current level should be in the –100 to –500 pA range. Basal currents levels are due to basal GIRK activity and basal receptor activity as well as the leakiness of the patch. Generally, as long as the baseline is less than 1 nA and is stable, one can proceed with the stimulation protocol.
15. Turn on light to drive photoisomerization of the azobenzene to photoactivate receptors. For BGAG_n with SNAP-mGluR2, the optimal wavelength to drive the *cis* state for photoagonism is ~380 nm. Either brief pulses or constant illumination can be used to induce receptor photoactivation to the same level. Photoactivation of SNAP-mGluR2 with BGAG_n typically produces anywhere from 100 pA to 1–2 nA. *See Note 25* for details on timing and wavelength of illumination for different PORTLs. Photoactivation of the receptor will produce an inward current from K⁺ ion influx which is induced downstream of G protein activation via binding of G $\beta\gamma$ to the GIRK channel. Once current has reached a steady-state plateau (typically within 5–10 s), turn on light to drive photoisomerization back to the *trans* state (>480 nm for BGAG_n PORTLs). Current readout will return to baseline within 10–20 s.
16. Repeat photoactivation multiple times (at least 2–3) to assess repeatability and then apply a saturating agonist to allow for quantification of photoswitch

efficiency (see Fig. 3). See Note 26 for consideration about desensitization. Many variations of this protocol with variable illumination timing and frequency can be devised depending on the precise application. See Note 27 for further controls and characterization. See Note 28 for using optical readouts and Note 29 for consideration when imaging and photoswitching simultaneously. Generally, the variability in photoswitch efficiency relative to saturating agonist will be around 10–15%, where at least four cells on at least two separate coverslips should be patched to calculate reliable photoswitching efficiencies.

4 Notes

1. HEK 293T cells are well-suited to these experiments because of the ease of handling them for splitting, seeding, transfecting, and imaging or patch clamping and their lack of endogenous metabotropic glutamate receptors and other class C GPCRs (with the exception of GABA_B1R) [45] and low levels of endogenous ion channels. HEK 293T cells, which contain the SV40 large T-antigen, generally show higher levels of expression than HEK 293. Other mammalian cell lines also work for similar experiments, including COS-7, CHO, and HeLa cells. Primary cells, such as hippocampal or cortical neurons or glia that tolerate transfection or viral infection may also be used for experiments aimed at studying class C GPCRs in a more physiological context.
2. For primary cells, calcium phosphate-based transfection is generally more effective and better tolerated.
3. DNA is extracted and purified from competent *E. coli* cells using a miniprep kit. The concentration of the DNA is determined via absorbance at 260 nm using a spectrophotometer or nanodrop. Concentrations over 600 ng/μL are desirable. Purity of the DNA constructs is confirmed by measuring the ratio of the absorbance at 260 nm to the absorbance at 280 nm (A_{260}/A_{280}), where ratios between 1.7 and 2.0 are acceptable. DNA constructs should be fully sequenced to confirm their identity and identify any mutations. For HEK cell experiments, constructs with a CMV promoter to drive strong expression of relevant proteins in mammalian cells are used. Other DNA constructs can also be used with this protocol.
4. Any cDNA with a SNAP- or CLIP-tagged GPCR of interest can be used. N-terminally SNAP-tagged mGluRs were developed by Doumazane et al. [46]. All existing work on PORTLs has been done using N-terminal SNAP- or CLIP-tagged GPCRs that contain a minimal linker (“TR” in the case of SNAP-mGluR2) between the tag and the start of the GPCR [46]. For GPCRs with defined signal sequences, the SNAP tag should be placed following this. For those without defined signal sequence, the tag placement will have to be empirically optimized, but the N-terminus is probably a good starting point. Assessing the prior use of GFP (or other fluorescent protein) tags should guide SNAP or CLIP attachment site as positions that tolerate GFP are likely to tolerate SNAP or CLIP, which are ~70% as large. In principle, tags may be placed at

either the N-terminus (extracellular for GPCRs) or C-terminus (intracellular for GPCRs) or, potentially, at intramolecular sites. However, for every new construct, labeling and receptor function should be carefully characterized to assess efficiency and orthogonality. In addition, to SNAP and CLIP, other protein tags should be amenable to the PORTL approach, including Halo tags [32].

5. The reporter of GPCR activation needs to be altered for the GPCR of interest. For *G_{i/o}-coupled receptors* (Ex. Group II/III mGluRs and GABA_BRs), a good reporter is G protein-coupled Inward Rectifying Potassium Channel (GIRK1). The GIRK1-F137S homo-tetramerization mutant is used to reduce the number of constructs needed to simplify transfection and expression [20]. For *G_q-coupled receptors* (Ex. Group I mGluRs): to detect receptor activation with GIRK channels a chimeric Gα_{i/o} protein can be co-transfected. This chimera contains the three C-terminal acids of Gα_q [47] which allows a G_q-coupled receptor to initiate Gα_{i/o}-type signaling (i.e. GIRK activation). An alternative to electrophysiology is to use calcium sensors, such as genetically encoded GCaMP6 [48] or synthetic dyes [49], as an optical readout. *See Note 28* for other potential fluorescent reporters that may be used in conjunction with PORTLs. While GIRK channels offer a simple, robust readout of G_{i/o}-coupled receptors and other G protein pathways via chimeric Gα proteins, other ion channels may be used to assess receptor activation and provide an alternative vantage point compared to GIRK since unique signaling mechanisms are employed for each. For example, inhibition of voltage-gated calcium channels [50] or potentiation of TREK channels [51] may be used for G_{i/o}-coupled receptors and activation of TrpC3 channels [52] may be used for G_q-coupled receptors. Different voltage protocols and ionic conditions are needed depending on the channel reporter of choice (*see* referenced papers for details). In primary neurons, a GPCR activation reporter is not necessary since native effectors exist. A number of GPCR-sensitive ion channels are expressed in neurons, providing a native-like complex response that can be detected electrophysiologically. For G_{i/o}-coupled receptors activation typically results in hyperpolarization, while G_q-coupled receptors typically produce depolarization.
6. EX Solution is used for labeling cells with PORTLs or fluorophores and can be used for imaging studies or electrophysiology with activity reporters other than GIRK channels.
7. High Potassium Solution (“HK₁₂₀”) is used as a recording solution when performing GIRK reporter experiments. The high extracellular potassium allows large inward currents to be evoked at negative holding potentials.
8. Glutamate stock (100 mM) should be diluted to the desired working concentration in fresh HK₁₂₀ for experiment. For other GPCRs, a suitable stock of an agonist is required.
9. Spectrophotometer is used for determining PORTL concentration via absorbance measurements.

10. It is worth noting that there are a variety of PORTLs available and their photopharmacological profiles vary based on the receptor they are attached to. Table 1 shows a current list of PORTLs and how they function in various receptor combinations (*see* Table 2 for chemical structures). BGAG₁₂ has been modified to photoisomerize at red-shifted wavelength, resulting in BGAG_{12,460} (due to *cis*-photoisomerization occurring with 460 nm light, instead of 380 nm). This PORTL maintains the agonism profile of BGAG₁₂ (*cis* state is an agonist, *trans* state is not an agonist) on group II mGluRs. However, the pharmacological unit that activates a given receptor can be inverted, such as with group III mGluRs. For these receptors, the photopharmacology of BGAG₁₂ is inverted (*cis* state is not an agonist, *trans* state is an agonist). Although this is still unclear, the current thought is that the precise shape of the linker-azobenzene moiety plays a role in whether the glutamate is able to fit into the LBD of a specific receptor.
11. For PORTL solubilization, 0.75 mL DMSO-d₆ from ampules is recommended—do not use DMSO that has been standing on the lab bench for extended periods. The concentration of PORTL in DMSO may be determined using a dilution of the PORTL in EX Solution. The absorbance of the dilution can be measured using a spectrophotometer, and the concentration can be calculated using the extinction coefficient and peak wavelength given below (*see* Table 3 and Note 12). The BG moiety is extremely stable if stored properly. Individual batches have shown robust labeling and photoswitching for years following many rounds of freeze/thaw cycles.
12. The Beer–Lambert Law ($A = \ell * \epsilon * c$) is used for the calculation of concentration where A is absorbance, ℓ is path length in cm, ϵ is extinction coefficient in $M^{-1} \text{ cm}^{-1}$, and c is concentration in M . *See* Table 3 for values used for BGAG PORTLs.
13. When developing PORTLs a simple, iterative approach can be used to design an effective compound. First, a screen of the pharmacological literature can provide valuable information about what attachments are tolerated in the parent ligand. Such a literature search led to the observation that 4'D attachment to L-glutamate is tolerated in group II and III mGluRs [20], a key first step toward the development of D-MAG PTLs and BGAG PORTLs. Test compounds may be synthesized and tested that contain a substituted parent ligand and the first ring of the azobenzene (*see* “Tether Models” in Volgraf et al. [24] and Levitz et al. [20]). If these test compounds are functional, soluble photochromic ligands (PCL) can be developed. PCLs contain all parts of the PORTL except for the attachment moiety, allowing them to be tested as soluble drugs in both *cis* and *trans*, by alternating the illumination wavelength, on the receptor of interest. For example, AG₁₂ (Table 2) serves as a *cis*-agonist on mGluR2 [30]. This approach can serve as a way to screen photoswitchable compounds and ensure their photo-agonist activity before incorporating attachment with a PORTL, which will require additional considerations outside the pharmacology of the compound, such as linker length and flexibility. In all cases tested so far, the directionality of photoswitching of a PCL is predictive of a PORTL based on its design. In

addition, weak PCL photoswitching does not preclude high-efficiency PORTL photoswitching since the enhanced effective concentration following ligand attachment can drastically improve this. For example, AG₁₂ shows <20% photoactivation but BGAG₁₂ shows >60% on mGluR2 [30].

14. When selecting a filter cube for assessing expression markers, standard GFP or RFP cubes may be used to visualize GFP or tdTomato or other fluorophores. For photoswitching a filter cube with either a mirror or far-red (~650 nm) high-pass dichroic filter should be used to maximize the illumination that reaches the sample. No excitation or emission filters are needed. *See Note 29* for considerations needed for imaging experiments.
15. Various illumination systems can work well. LED-based systems or Xenon lamp-based systems allow for rapid switching between different wavelengths via TTL pulses from digitizer. Laser illumination in full field modes or scanning can be used as well for more targeted applications.
16. Tubing should be connected from the pipette holder to a syringe or mouth tip with a valve for opening and closing. This will allow the application of negative and positive pressure to the sample. A pressure meter may be attached for assessing positive and negative pressure values.
17. The recording chamber must have an inlet for perfusion and ideally an outlet or separate reservoir for ground electrodes and vacuum. A glass-bottom petri dish is the simplest option and can work fine, but has limitations in terms of perfusion speed and stability.
18. In the original BGAG design, a modular approach was chosen for the connection of attachment site (BG), linker, and azobenzene-containing pharmacophore. Accordingly, Cu(I)-catalyzed alkyne-azide click chemistry (CuAAC) was envisioned to serve as a late-stage modification to introduce the attachment molecule in an orthogonal way. However, in our initial studies [30] we realized that the reaction needs high temperatures to proceed together with high, over-stoichiometric catalyst loadings. In addition, the CuAAC on the red-shifted BGAG_{12,460} did not proceed at all, and was therefore circumvented by the use of a strain-promoted alkyneazide click (SpAAC) using DBCO as an alkyne. Albeit resulting in the formation of two stereoisomers (with neglectable influence on tether length), this approach was further expanded in a follow-up study [31], where BCAGs were made using SpAAC chemistry. As a first example, alkyne-azide click chemistry was completely abolished by making BGAG₂₈, as standard Fmocamine deprotection and subsequent peptide coupling employing TSTU proved to be beneficial with respect to being less labor intensive, less toxic, enabling shorter reaction times and facilitating a more straightforward purification. Furthermore, acids activated by TSTU to their NHS-ester counterparts can be chromatographed and isolated by means of reverse-phase high-performance liquid chromatography (RP-HPLC) when the mobile phase is buffered with an acid (usually MeCN/H₂O with 0.1% formic acid or TFA).

Given these experiences, we recommend focusing on peptide couplings for building PORTLs rather than designing click partners.

19. The specific timing and methodology will differ for different cell lines or for primary cells. For a protocol on cultured neuron preparation, *see* Ref. 53.
20. To ensure cell health, ~8 h after transfection, cell media may be changed. Some GPCRs are toxic to cells and this can be alleviated by adding an antagonist at this stage. For example, 5 μ M LY341495, a pan-mGluR competitive antagonist, can help maintain the health of mGluR-transfected cells. It is crucial that the reversibility and the effects of an antagonist on receptor expression are thoroughly characterized before using.
21. If the DNA of interest requires longer expression time, transfected cells may be re-seeded 24–48 h following transfection. For re-seeding, media is aspirated and 0.5 mL calcium-free DPBS solution is added to each well. Following a 20-min incubation, 100–200 μ L (amount determined empirically) is added to freshly washed coverslips in 1.5 mL culture media in a new 12-well plate. Typically, at least 12 h are needed following re-seeding for cells to re-adhere and develop a normal, spread-out morphology.
22. Previous studies of SNAP-mGluRs have shown saturating labeling for BGAG_n and BCAG_n at concentrations as low as 1 μ M for 45 min at 37 °C [30, 31]. However, sufficient labeling concentrations may depend on the PORTL, the receptor itself, the expression system, and may be sensitive to the state of the PORTL. To ensure sufficient labeling, the surface labeling efficiency of PORTLs may be tested using a competition-based assay with cell-impermeable BG-conjugated fluorophores (such as Surface SNAP-Alexa-546 from New England Biolabs). By initially labeling cells with the PORTL at a given concentration and then labeling with saturating amounts of the cell-impermeable BG-conjugated fluorophore, the residual fluorescence can be compared to cells incubated only with the fluorophore to calculate the PORTL labeling efficiency. These results can be used to determine the saturating concentration of a PORTL needed for photoswitching experiments and can give a reasonable estimate of the overall labeling efficiency. *See* Levitz et al. [31] for an example of this experiment where BGAG_n labeling of SNAP-mGluR2 was estimated to be 80–90%.
23. For any quantification of photoswitch efficiency, a saturating concentration of an agonist is applied for normalization. For mGluR PORTL experiments, 1 mM glutamate (or 10 mM for mGluR7) is used as a standard saturating concentration. In addition to assessing PORTL efficiency as part of the tool engineering and characterization processes, this quantification can also be used for testing the effects of different mutations, especially in the context of ligand occupancy-dependent cooperativity (*see* Ref. 26).
24. While native currents from GIRK channels [54] are typically outward, when using GIRK as an exogenous reporter of GPCR activity the inward rectification of these channels allows larger currents to be obtained by measuring inward

currents. To permit inward currents at physiological membrane potentials (~ -60 mV), a high potassium extracellular solution is used (HK₁₂₀). To confirm that potassium-selective GIRK currents are being recorded, a current–voltage (I – V) relationship can be calculated by measuring the current evoked following steps to different voltages (Ex. 500 ms steps from a baseline of -60 ranging from $+40$ to -100). Subtracting a baseline I – V curve from the I – V curve obtained in the presence of ligand or PORTL photoactivation should reveal an inward rectifying current with a reversal potential that matches what is expected for potassium in the ionic conditions of the experiment (~ 0 mV). Alternatively, a blocker can be used to confirm that currents are mediated by GIRK channels (*see* Note 27g).

25. Light requirements vary depending on whether the PORTL being used contains the standard (i.e. BGAG_{*n*}) or red-shifted (i.e. BGAG_{*n*,460}) azobenzene core. Data on intensity and time requirements of light have been characterized in HEK 293T cells for BGAG₁₂ and BGAG_{12,460} on SNAP-mGluR2 [44]. Similar results are expected for any PORTL with either a standard or red-shifted azobenzene core. BGAG₁₂ and BGAG_{12,460} vary in their photophysical profile and, thus, require very different light exposures to drive and reverse photoisomerization. BGAG_{*n*} is bistable, achieving a maximal photostationary state under ~ 380 nm light (*cis* state) or >480 nm light (*trans* state), without reverting to *trans* spontaneously in the dark [11]. BGAG_{*n*,460} photoisomerizes to the *cis* state under ~ 460 nm illumination (*see* Ref. 55 for full spectral profile of a related azobenzene PTL), but this red-shift results in a loss of bistability so that red-shifted photoswitches revert to *trans* spontaneously in the dark with a time constant of ~ 700 ms [55]. The bistability of standard azobenzenes, and the subsequent lack of relaxation from *cis* to *trans* on the time scale of receptor activation, allows BGAG₁₂ to require much lower light intensities than BGAG_{12,460}. BGAG₁₂ produces near maximal photocurrents with light intensity levels as low as ~ 10 mW/cm², while BGAG_{12,460} requires ~ 1000 mW/cm² for maximal photocurrents [44]. Light intensities should be measured post-objective at the sample's location with a power meter (Ex. ThorLabs PM100D) and should be monitored over time. For standard azobenzene cores, decreased light intensity does not decrease photoswitching efficiency but only alters the time it takes to reach the steady state with less intense light producing slower currents. With typical light intensities of ~ 1000 mW/cm², ~ 200 – 300 ms of 380 nm illumination are sufficient for maximal photoswitching amplitude. For red-shifted azobenzene cores, the intensity of light determines both the amplitude and speed of the photoactivation. For SNAP-mGluR2 + BGAG_{12,460} in HEK 293T cells with GIRK as a readout, ~ 5 s of 470 nm illumination at 1000 mW/cm² are required for a maximal response.
26. Although mGluR2 is resistant to desensitization [56] other GPCRs will need to be characterized for their desensitization kinetics. Typically, photoswitch efficiency is stable from cell to cell due to minimal desensitization typically observed with mGluR2, but this may vary from receptor to receptor.

27. To properly characterize the photoswitching of PORTL/SNAP-GPCR combinations, there are some key controls and further experiments that should be done:
- a. *Labeling Specificity Control*: An important negative control is to perform the experiment either without application of the PORTL or without transfecting cells with the SNAP- or CLIP-tagged receptor. Photocurrent should be completely dependent on the presence of both the PORTL and the tagged receptor. This condition is especially important in neurons and tissue, where native receptors are present and PORTL washout may be more complicated.
 - b. *Competitive antagonist*: A saturating concentration of a competitive antagonist may be used to block photoactivation of the receptor. For example, BGAG₁₂ photoactivation is completely blocked by LY341495, a competitive mGluR antagonist [30]. This blockage confirms that the PORTL ligand is binding at the orthosteric site. Additionally, this experiment can be used to calculate basal activity due to potential *trans* (or *cis* in the case of a *trans*-agonist, i.e. group III mGluRs) state activation of the receptor. This can be observed using electrophysiology by recording a baseline and then adding the antagonist to block basal activity; if there is any basal activity, an outward current is induced due to the termination of GIRK activation. The measured basal activity can be due to either spontaneous basal activity of the receptor or *trans* activation from the photoswitch. To separate these, basal activity of the receptor with or without the PORTL attached can be measured to determine the contribution of each.
 - c. *Action Spectra*: The wavelength-dependence of the PORTL can be described by measuring photoactivation and de-activation at various wavelengths. As previously described using azobenzene-glutamate PTLs [11], maximal photoisomerization of BGAG_n occurs at 380 nm (to *cis* state) and 500 nm (to *trans* state) and deviations from this results in sub-optimal photoswitching. Similar characterization experiments can be performed to determine an action spectra for any PORTL by simply modifying the wavelengths used for activation or de-activation (see Ref. 11 or Ref. 55 for examples with PTLs). It is important to use the same bandwidth for each wavelength and to make sure that long enough illumination times are used to reach steady state since sub-optimal wavelengths may take longer to photoactivate. While the general action spectra can be predicted based on PORTL structure, subtle effects may be produced by different ligands or binding to its target [57] and further engineering of the azobenzene core to tweak photophysical properties is an active area of research [58, 59].
 - d. *Bistability*: As described above, PORTLs with a standard azobenzene core (Ex. BGAG_n) will display bistability. Following

photoisomerization to the *cis* state they will maintain the *cis* configuration in the dark with a time constant that is typically >20 min [11]. Exposure to a second wavelength of light (i.e. ~500 nm) is required to drive its relaxation to the *trans* state. In contrast, PORTLs with a red-shifted azobenzene core (ex. BGAG_{n,460}) spontaneously relax in the dark to the *trans* state. Thus, constant illumination is required to drive such PORTLs. To determine the stability profile of a particular PORTL, shine the light expected to drive photoisomerization to the *cis* state for a short amount of time (typically <5 s to produce maximal population of the *cis* state) and return to the dark; after reaching a stable level of activation, shine light to drive photoisomerization to the *trans* state. Repeat photoactivation but without returning to dark between the two light exposures. Compare these two results; if a compound is bistable throughout the time of the recording, the results should be identical. If a compound is not bistable, there will be a full or partial return to baseline conditions while the sample is in the dark. Varying the dark time will allow for the relaxation rate to be estimated. Crucially, following full photoactivation with 380 nm light and incubation in the dark, a second pulse of 380 nm can be used to see if significant relaxation back to the *trans* state has occurred. If relaxation has occurred, the second 380 nm pulse should increase the inward current. For BGAG_n PORTLs, following complete photoactivation at least a minute or two in the dark is required before any response to 380 nm can be observed.

- e. *Effective concentration*: The effective local concentration of the PORTL ligand can be probed using low-affinity receptor mutants. Depending on the effective ligand concentration, lowering the affinity of the receptor will either reduce photoswitch efficiency or have no effect. For example, the low-affinity R57A mGluR2 mutation, which reduces glutamate affinity by ~30 ×, abolishes photoactivation of SNAG-mGluR2 [31]. However, with BGAG₀, but not BGAG₁₂, some photocurrent is rescued in the presence of glutamate, indicating that the glutamate moiety of BGAG₀ is in a higher effective concentration than in BGAG₁₂ and that there is intersubunit cooperativity in mGluR2. In contrast, the same mutation in LimGluR2 (mGluR2–300C, R57A + D-MAG-0) does not alter photoswitch efficiency [26], indicating that PTLs are in a much higher local concentration than PORTLs. Comparing photoswitch efficiency to a dose–response curve of a soluble compound that models the ligand moiety of the PORTL, as was done for LiGluR with different PTLs [11], can allow an estimate of the actual effective concentration. Based on such experiments it is generally estimated that azobenzene-glutamate PTLs are in a >10 mM local concentration while PORTLs are in the hundreds of μM to single digit mM range.

- f.** *Partial versus Full Agonism:* an important aspect of PORTL characterization is to gain a full understanding of the basis of the efficiency of the photoswitching. In even the best cases, PORTL photoswitching is well below 100% relative to saturating concentrations of the native ligand. For SNAP-mGluR2 with BGAG_n this incomplete photoswitching is largely explained by incomplete labeling, incomplete *cis* occupancy in the photostationary state and receptor cooperativity (see discussion in Ref. 31). However, one alternative possibility is that the PORTL ligand can serve as a partial agonist. To test this possibility, use the protocol described in this chapter to set up your experimental setup. Use the perfusion system to apply a saturating amount of a full agonist (be sure to not be oversaturating) and once the response has stabilized, use light to photoactivate the receptor with the PORTL ligand. The competition between the full agonist and the PORTL ligand will result in some LBDs being bound by the PORTL ligand instead of the agonist. If the PORTL ligand is a partial agonist of sufficient effective concentration, this will result in a decrease in readout response (i.e. apparent antagonism). Generally, photoswitching in the presence of a wide-range of agonist concentrations may be a valuable way to characterize the system and the agonism profile of the PORTL ligand.
- g.** *Channel Block:* To confirm that the observed photocurrent is due to GIRK channel opening, either barium [60] or tertiapin-q [61] blockers may be applied. Typically, one round of photoactivation is performed, then the drug is applied and optical stimulation should be occluded. Following washout, the photocurrent should return. For alternative ion channel readouts (see Note 5), different blockers may be used to confirm the basis of the light response.
- h.** *Light Timing:* Playing with the timing of illumination can be a valuable way to learn both about the PORTL system and the GPCR itself, especially in the context of comparing related receptors. Optical control with PORTLs allows the ON and OFF kinetics, frequency-dependence and minimal activation and de-activation times to be tested. For ion channel-based readouts of GPCRs it is important to keep in mind that the photocurrent kinetics are not a direct readout of receptor activity but are also dependent on a number of downstream processes, such as G protein activation, GTP exchange, and G protein-effector binding.
- i.** *PORTL Multiplexing:* The labeling specificity of SNAP and CLIP tags for *O*⁶-benzylguanine and *O*²-benzylcytosine, respectively, allows for separate control of SNAP- and CLIP-tagged receptors. It is critical for such experiments that the two PORTLs used have spectral differences between them to allow for separate control. BGAG₁₂, BGAG_{12,460}, and the related BCAG₁₂ and BCAG_{12,460} have previously been shown to have the necessary spectral differences due to the red-shifted azobenzene in the 460 variants [31]. This allows for co-expression of a

SNAP-tagged receptor and a CLIP-tagged receptor, labeling each with a BG-based PORTL or a BC-based PORTL, respectively, each controlled by different light exposures. *See* Fig. 8 in ref. 31 for an example. Light intensities and wavelengths must be determined empirically for a given set up, but in the previously reported experiments low intensity 380 and 590 nm light was used to activate and de-activate standard azobenzene core photoswitches (i.e. BGAG_n or BCAG_n) without co-activating red-shifted azobenzene core photoswitches (i.e. BGAG_{n,460} or BCAG_{n,460}).

- j. *Agonist Titration:* Dose–response curves for the native ligand are a useful test to *see* if either SNAP (or other protein tags) attachment or PORTL conjugation alter receptor function. Simply adding in and washing out a given concentration of a native ligand (ex. glutamate) and quantifying the ensuing current amplitude relative to a saturating concentration of the same ligand allows a dose–response curve to be constructed. For a new PORTL/GPCR combination, dose–response curves should be produced for the wild-type receptor, the SNAP-tagged receptor, and the PORTL-labeled receptor (in the inactive state of the PORTL). Any shift induced by just the SNAP tag may motivate further modification to the construct and any shift induced by the PORTL attachment indicates some effect of the less active form of the ligand. For instance, it is easy to imagine that a PORTL may serve as an agonist in the *cis* state but as an antagonist in the *trans* state (or vice-versa) which would produce a right-shifted dose–response curve.

28. In addition to sensors for calcium, other fluorescent sensors can also be used to observe GPCR activity. Both G_{i/o} and G_s result in a change in cAMP concentration, making a cAMP sensor [62–64] a potential optical readout for these receptors. G_q activation can also be sensed using PH domain-based phosphatidylinositol sensors, which can detect the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) [65]. Other pathways for which optical sensors exist have also been implicated in GPCR-induced G protein and β-arrestin-mediated signaling, including ERK [66, 67], mTOR [68], and CaMKII [69]. These sensors may be ideal in native contexts (i.e. cultured neurons or glia) to dissect the spatial and temporal signaling patterns induced by subcellular activation of GPCRs with PORTLs. *See* Note 29 for considerations when designing dual photoswitching/imaging experiments.
29. Similar to the important considerations needed when choosing a fluorescent reporter, it is crucial to account for the effects of imaging light on the *cis–trans* isomerization of PORTLs. In this note we focus on imaging while using a photoswitch that reverts to the *trans* state upon application of visible light (>480 nm), such as BGAG_n. When trying to photoactivate *cis*-active PORTLs while imaging, the major concern is that the imaging light will revert the photoswitch to *trans*, preventing sufficient population of the active *cis* state. To circumvent this, there are a few options including spatially separating the imaging and

photoactivation illumination areas, which is possible in scanning confocal microscopy, or lowering the frequency and intensity of imaging light to allow the photoswitching light to dominate. For instance, in wide-field epifluorescence imaging, G_q-coupled calcium signals can often easily be imaged at frequencies less than 1 Hz with low illumination intensities and short exposures. For example, 50 ms exposure at 1 Hz can be used to get a baseline (with the PORTL in *trans*) and photoactivation can be produced by constant illumination at ~380 nm. The constant UV should be able to maintain the PORTL in *cis* despite the competing effects of the imaging light. Alternatively, to minimize photon exposure a sufficient ~380 nm pulse can be given just after the imaging light is applied to ensure that the *cis* state is maintained for the entire period in the dark until the next image is captured. It is ideal to use a sensor, such as GCaMP6, that requires low levels of excitation light and gives a large F/F response upon activation. Red-shifted sensors which are excited at wavelengths longer than 580 nm should be used when available since standard azobenzene cores will only weakly absorb in this range. It is important to confirm that the optical sensor being used is not sensitive to photostimulation light, as has been reported for fluorescent protein-based sensors [70], which can introduce artifacts that confound interpretation of experiments. Finally, red-shifted BGAG variants (i.e. BGAG_{12,460}) require additional considerations given the broad spectra of this azobenzene [55] which makes it difficult to image without directly isomerizing the PORTL unless very dim or red-shifted light is used.

Acknowledgements

We thank the Levitz and Broichhagen labs for useful discussion, and all previous and current members of the Isacoff and Trauner labs for their contributions to the development and application of photoswitchable ligands and receptors. J.B. thanks Kai Johnsson for constant support. J.L. is supported by an R35 grant from the National Institute of General Medical Science (1 R35 GM124731).

References

1. Lagerstrom MC, Schioth HB (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov* 7(4):339–357 [PubMed: 18382464]
2. Hilger D, Masureel M, Kobilka BK (2018) Structure and dynamics of GPCR signaling complexes. *Nat Struct Mol Biol* 25(1):4–12 [PubMed: 29323277]
3. Niswender CM, Conn PJ (2010) Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu Rev Pharmacol Toxicol* 50:295–322 [PubMed: 20055706]
4. Pin JP, Bettler B (2016) Organization and functions of mGlu and GABAB receptor complexes. *Nature* 540(7631):60–68 [PubMed: 27905440]
5. Riccardi D, Kemp PJ (2012) The calciumsensing receptor beyond extracellular calcium homeostasis: conception, development, adult physiology, and disease. *Annu Rev Physiol* 74:271–297 [PubMed: 22017175]
6. Clemmensen C, Smajilovic S, Wellendorph P, Bräuner-Osborne H (2014) The GPCR, class C, group 6, subtype A (GPCR6A) receptor: from cloning to physiological function. *Br J Pharmacol* 171(5): 1129–1141 [PubMed: 24032653]
7. Sutton LP, Orlandi C, Song C, Oh WC, Muntean BS, Xie K, Filippini A, Xie X, Satterfield R, Yaeger JDW, Renner KJ, Young SM Jr, Xu B, Kwon H, Martemyanov KA (2018) Orphan receptor GPR158 controls stress-induced depression. *Elife* 7 10.7554/eLife.33273

8. Spangler SM, Bruchas MR (2017) Optogenetic approaches for dissecting neuromodulation and GPCR signaling in neural circuits. *Curr Opin Pharmacol* 32:56–70 [PubMed: 27875804]
9. Callaway EM, Katz LC (1993) Photostimulation using caged glutamate reveals functional circuitry in living brain slices. *Proc Natl Acad Sci U S A* 90(16):7661–7665 [PubMed: 7689225]
10. Reiner A, Levitz J, Isacoff EY (2015) Controlling ionotropic and metabotropic glutamate receptors with light: principles and potential. *Curr Opin Pharmacol* 20:135–143 [PubMed: 25573450]
11. Gorostiza P, Volgraf M, Numano R, Szobota S, Trauner D, Isacoff EY (2007) Mechanisms of photoswitch conjugation and light activation of an ionotropic glutamate receptor. *Proc Natl Acad Sci U S A* 104:10865–10870 [PubMed: 17578923]
12. Roth BL (2016) DREADDs for neuroscientists. *Neuron* 89(4):683–694 [PubMed: 26889809]
13. Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A* 104(12):5163–5168 [PubMed: 17360345]
14. Gomez JL, Bonaventura J, Lesniak W, Mathews WB, Sysa-Shah P, Rodriguez LA, Ellis RJ, Richie CT, Harvey BK, Dannals RF, Pomper MG, Bonci A, Michaelides M (2017) Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science* 357(6350):503–507 [PubMed: 28774929]
15. Rost BR, Schneider-Warme F, Schmitz D, Hegemann P (2017) Optogenetic tools for subcellular applications in neuroscience. *Neuron* 96(3):572–603 [PubMed: 29096074]
16. Morri M, Sanchez-Romero I, Tichy AM, Kainrath S, Gerrard EJ, Hirschfeld PP, Schwarz J, Janovjak H (2018) Optical functionalization of human Class A orphan G-proteincoupled receptors. *Nat Commun* 9(1):1950 [PubMed: 29769519]
17. Airan RD, Thompson KR, Fenno LE, Bernstein H, Deisseroth K (2009) Temporally precise in vivo control of intracellular signalling. *Nature* 458(7241):1025–1029 [PubMed: 19295515]
18. Siuda ER, Copits BA, Schmidt MJ, Baird MA, Al-Hasani R, Planer WJ, Funderburk SC, McCall JG, Gereau RWt, Bruchas MR (2015) Spatiotemporal control of opioid signaling and behavior. *Neuron* 86(4):923–935 [PubMed: 25937173]
19. Oh E, Maejima T, Liu C, Deneris E, Herlitze S (2010) Substitution of 5-HT_{1A} receptor signaling by a light-activated G protein-coupled receptor. *J Biol Chem* 285(40):30825–30836 [PubMed: 20643652]
20. Levitz J, Pantoja C, Gaub B, Janovjak H, Reiner A, Hoagland A, Schoppik D, Kane B, Stawski P, Schier AF, Trauner D, Isacoff EY (2013) Optical control of metabotropic glutamate receptors. *Nat Neurosci* 16(4):507–516 [PubMed: 23455609]
21. Masseck OA, Spoida K, Dalkara D, Maejima T, Rubelowski JM, Wallhorn L, Deneris ES, Herlitze S (2014) Vertebrate cone opsins enable sustained and highly sensitive rapid control of Gi/o signaling in anxiety circuitry. *Neuron* 81 (6):1263–1273 [PubMed: 24656249]
22. Kramer RH, Mourot A, Adesnik H (2013) Optogenetic pharmacology for control of native neuronal signaling proteins. *Nat Neurosci* 16(7):816–823 [PubMed: 23799474]
23. Banghart M, Borges K, Isacoff E, Trauner D, Kramer RH (2004) Light-activated ion channels for remote control of neuronal firing. *Nat Neurosci* 7(12):1381–1386 [PubMed: 15558062]
24. Volgraf M, Gorostiza P, Numano R, Kramer RH, Isacoff EY, Trauner D (2006) Allosteric control of an ionotropic glutamate receptor with an optical switch. *Nat Chem Biol* 2(1):47–52 [PubMed: 16408092]
25. Levitz J, Popescu AT, Reiner A, Isacoff EY (2016) A toolkit for orthogonal and in vivo optical manipulation of ionotropic glutamate receptors. *Front Mol Neurosci* 9:2 [PubMed: 26869877]
26. Levitz J, Habrian C, Bharill S, Fu Z, Vafabakhsh R, Isacoff EY (2016) Mechanism of assembly and cooperativity of homomeric and heteromeric metabotropic glutamate receptors. *Neuron* 92(1):143–159 [PubMed: 27641494]
27. Carroll EC, Berlin S, Levitz J, Kienzler MA, Yuan Z, Madsen D, Larsen DS, Isacoff EY (2015) Two-photon brightness of azobenzene photoswitches designed for glutamate receptor optogenetics. *Proc Natl Acad Sci U S A* 112(7): E776–E785 [PubMed: 25653339]
28. Li D, Herault K, Zylbersztejn K, Lauterbach MA, Guillon M, Oheim M, Ropert N (2015) Astrocyte VAMP3 vesicles undergo Ca²⁺-independent cycling and modulate glutamate transporter trafficking. *J Physiol* 593(13):2807–2832 [PubMed: 25864578]

29. DuBay KH, Iwan K, Osorio-Planes L, Geissler PL, Groll M, Trauner D, Broichhagen J (2018) A predictive approach for the optical control of carbonic anhydrase II activity. *ACS Chem Biol* 13(3):793–800 [PubMed: 29357237]
30. Broichhagen J, Damijonaitis A, Levitz J, Sokol KR, Leippe P, Konrad D, Isacoff EY, Trauner D (2015) Orthogonal optical control of a G protein-coupled receptor with a SNAP-tethered photochromic ligand. *ACS Cent Sci* 1(7):383–393 [PubMed: 27162996]
31. Levitz J, Broichhagen J, Leippe P, Konrad D, Trauner D, Isacoff EY (2017) Dual optical control and mechanistic insights into photoswitchable group II and III metabotropic glutamate receptors. *Proc Natl Acad Sci U S A* 114 (17):e3546–e3554 [PubMed: 28396447]
32. Gautier A, Juillerat A, Heinis C, Correa IR Jr, Kindermann M, Beaufils F, Johnsson K (2008) An engineered protein tag for multiprotein labeling in living cells. *Chem Biol* 15 (2):128–136 [PubMed: 18291317]
33. Schonberger M, Trauner D (2014) A photochromic agonist for mu-opioid receptors. *Angew Chem Int Ed Engl* 53(12):3264–3267 [PubMed: 24519993]
34. Donthamsetti PC, Winter N, Schonberger M, Levitz J, Stanley C, Javitch JA, Isacoff EY, Trauner D (2017) Optical control of dopamine receptors using a photoswitchable tethered inverse agonist. *J Am Chem Soc* 139 (51):18522–18535 [PubMed: 29166564]
35. Broichhagen J, Podewin T, Meyer-Berg H, von Ohlen Y, Johnston NR, Jones BJ, Bloom SR, Rutter GA, Hoffmann-Roder A, Hodson DJ, Trauner D (2015) Optical control of insulin secretion using an incretin switch. *Angew Chem Int Ed Engl* 54(51):15565–15569 [PubMed: 26585495]
36. Broichhagen J, Johnston NR, von Ohlen Y, Meyer-Berg H, Jones BJ, Bloom SR, Rutter GA, Trauner D, Hodson DJ (2016) Allosteric optical control of a class B G-protein-coupled receptor. *Angew Chem Int Ed Engl* 55 (19):5865–5868 [PubMed: 27059784]
37. Agnetta L, Kauk M, Canizal MCA, Messerer R, Holzgrabe U, Hoffmann C, Decker M (2017) A photoswitchable dualsteric ligand controlling receptor efficacy. *Angew Chem Int Ed Engl* 56(25):7282–7287 [PubMed: 28510314]
38. Westphal MV, Schafröth MA, Sarott RC, Imhof MA, Bold CP, Leippe P, Dhopeswarkar A, Grandner JM, Katritch V, Mackie K, Trauner D, Carreira EM, Frank JA (2017) Synthesis of photoswitchable delta(9)-tetrahydrocannabinol derivatives enables optical control of cannabinoid receptor 1 signaling. *J Am Chem Soc* 139(50):18206–18212 [PubMed: 29161035]
39. Hauwert NJ, Mocking TAM, Da Costa Pereira D, Kooistra AJ, Wijnen LM, Vreeker GCM, Verweij EWE, De Boer AH, Smit MJ, De Graaf C, Vischer HF, de Esch IJP, Wijnmans M, Leurs R (2018) Synthesis and characterization of a bidirectional photoswitchable antagonist toolbox for real-time GPCR photopharmacology. *J Am Chem Soc* 140 (12):4232–4243 [PubMed: 29470065]
40. Podewin T, Ast J, Broichhagen J, Fine NHF, Nasteska D, Leippe P, Gailer M, Buenaventura T, Kanda N, Jones BJ, M'Kadmi C, Baneres JL, Marie J, Tomas A, Trauner D, Hoffmann-Roder A, Hodson DJ (2018) Conditional and reversible activation of class A and B G protein-coupled receptors using tethered pharmacology. *ACS Cent Sci* 4 (2):166–179 [PubMed: 29532016]
41. Shields BC, Kahuno E, Kim C, Apostolides PF, Brown J, Lindo S, Mensh BD, Dudman JT, Lavis LD, Tadross MR (2017) Deconstructing behavioral neuropharmacology with cellular specificity. *Science* 356(6333). 10.1126/science.aaj2161
42. Nishiyama J, Mikuni T, Yasuda R (2017) Virus-mediated genome editing via homology-directed repair in mitotic and postmitotic cells in mammalian brain. *Neuron* 96(4):755–768.e5 [PubMed: 29056297]
43. Farrants H, Acosta-Ruiz A, Gutzeit VA, Trauner D, Johnsson K, Levitz J, Broichhagen J (2018) SNAP-tagged nanobodies enable reversible optical control of a G protein-coupled receptor via a remotely tethered photoswitchable ligand. *ACS Chem Biol* 13(9):2682–2688 [PubMed: 30141622]
44. Berry MH, Holt A, Levitz J, Broichhagen J, Gaub BM, Visel M, Stanley C, Aghi K, Kim YJ, Cao K, Kramer RH, Trauner D, Flannery J, Isacoff EY (2017) Restoration of patterned vision with an engineered photoactivatable G protein-coupled receptor. *Nat Commun* 8 (1):1862 [PubMed: 29192252]
45. Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* 12:14 [PubMed: 21214938]

46. Doumazane E, Scholler P, Zwier JM, Trinquet E, Rondard P, Pin JP (2011) A new approach to analyze cell surface protein complexes reveals specific heterodimeric metabotropic glutamate receptors. *FASEB J* 25 (1):66–77 [PubMed: 20826542]
47. Conklin BR, Farfel Z, Lustig KD, Julius D, Bourne HR (1993) Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha. *Nature* 363 (6426):274–276 [PubMed: 8387644]
48. Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS (2013) Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* 499(7458):295–300 [PubMed: 23868258]
49. Deo C, Lavis LD (2018) Synthetic and genetically encoded fluorescent neural activity indicators. *Curr Opin Neurobiol* 50:101–108 [PubMed: 29454295]
50. Li X, Gutierrez DV, Hanson MG, Han J, Mark MD, Chiel H, Hegemann P, Landmesser LT, Herlitze S (2005) Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin. *Proc Natl Acad Sci U S A* 102(49):17816–17821 [PubMed: 16306259]
51. Sandoz G, Levitz J, Kramer RH, Isacoff EY (2012) Optical control of endogenous proteins with a photoswitchable conditional subunit reveals a role for TREK1 in GABA (B) signaling. *Neuron* 74(6):1005–1014 [PubMed: 22726831]
52. Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature* 397(6716):259–263 [PubMed: 9930701]
53. Peng Y, Xiong WC, Mei L (2013) Culture of dissociated hippocampal neurons. *Methods Mol Biol* 1018:39–47 [PubMed: 23681615]
54. Lüscher C, Slesinger PA (2010) Emerging concepts for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. *Nat Rev Neurosci* 11(5):301–315 [PubMed: 20389305]
55. Kienzler MA, Reiner A, Trautman E, Yoo S, Trauner D, Isacoff EY (2013) A red-shifted, fast-relaxing azobenzene photoswitch for visible light control of an ionotropic glutamate receptor. *J Am Chem Soc* 135(47):17683–17686 [PubMed: 24171511]
56. Iacovelli L, Molinaro G, Battaglia G, Motolese M, Di Menna L, Alfiero M, Blahos J, Matriciano F, Corsi M, Corti C, Bruno V, De Blasi A, Nicoletti F (2009) Regulation of group II metabotropic glutamate receptors by G protein-coupled receptor kinases: mGlu2 receptors are resistant to homologous desensitization. *Mol Pharmacol* 75(4):991–1003 [PubMed: 19164443]
57. Barber DM, Liu SA, Gottschling K, Sumser M, Hollmann M, Trauner D (2017) Optical control of AMPA receptors using a photoswitchable quinoxaline-2,3-dione antagonist. *Chem Sci* 8(1):611–615 [PubMed: 28451208]
58. Beharry AA, Woolley GA (2011) Azobenzene photoswitches for biomolecules. *Chem Soc Rev* 40(8):4422–4437 [PubMed: 21483974]
59. Dong M, Babalhavaeji A, Samanta S, Beharry AA, Woolley GA (2015) Red-shifting azobenzene photoswitches for in vivo use. *Acc Chem Res* 48(10):2662–2670 [PubMed: 26415024]
60. Alagem N, Dvir M, Reuveny E (2001) Mechanism of Ba²⁺ block of a mouse inwardly rectifying K⁺ channel: differential contribution by two discrete residues. *J Physiol* 534 (Pt 2):381–393 [PubMed: 11454958]
61. Jin W, Lu Z (1998) A novel high-affinity inhibitor for inward-rectifier K⁺ channels. *Biochemistry* 37(38):13291–13299 [PubMed: 9748337]
62. Hackley CR, Mazzoni EO, Blau J (2018) cAMPr: a single-wavelength fluorescent sensor for cyclic AMP. *Sci Signal* 11(520). 10.1126/scisignal.aah3738
63. Klarenbeek J, Goedhart J, van Batenburg A, Groenewald D, Jalink K (2015) Fourth-generation epac-based FRET sensors for cAMP feature exceptional brightness, photostability and dynamic range: characterization of dedicated sensors for FLIM, for ratiometry and with high affinity. *PLoS One* 10(4): e0122513 [PubMed: 25875503]
64. Harada K, Ito M, Wang X, Tanaka M, Wongso D, Konno A, Hirai H, Hirase H, Tsuboi T, Kitaguchi T (2017) Red fluorescent protein-based cAMP indicator applicable to optogenetics and in vivo imaging. *Sci Rep* 7(1):7351 [PubMed: 28779099]

65. Stauffer TP, Ahn S, Meyer T (1998) Receptor- induced transient reduction in plasma membrane PtdIns(4,5)P₂ concentration monitored in living cells. *Curr Biol* 8(6):343–346 [PubMed: 9512420]
66. Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, Davis RJ, Svoboda K (2008) A genetically encoded fluorescent sensor of ERK activity. *Proc Natl Acad Sci U S A* 105(49):19264–19269 [PubMed: 19033456]
67. de la Cova C, Townley R, Regot S, Greenwald I (2017) A real-time biosensor for ERK activity reveals signaling dynamics during *C. elegans* cell fate specification. *Dev Cell* 42(5):542–553.e4 [PubMed: 28826819]
68. Zhou X, Clister TL, Lowry PR, Seldin MM, Wong GW, Zhang J (2015) Dynamic visualization of mTORC1 activity in living cells. *Cell Rep*. 10.1016/j.celrep.2015.02.031
69. Lee SJR, Escobedo-Lozoya Y, Szatmari EM, Yasuda R (2009) Activation of CaMKII in single dendritic spines during long-term potentiation. *Nature* 458(7236):299–304 [PubMed: 19295602]
70. Ai M, Mills H, Kanai M, Lai J, Deng J, Schreiter E, Looger L, Neubert T, Suh G (2015) Green-to-red photoconversion of GCaMP. *PLoS One* 10(9):e0138127 [PubMed: 26382605]

Fig. 1.

Principle of PORTL-mediated Optical Control of Class C GPCRs. **(a)** Schematic showing general PORTL design. The relaxed *trans* state (top) of an azobenzene-based PORTL can isomerize to the *cis* state (bottom) panel by exposure to light of wavelength λ_1 . Exposure to light of wavelength λ_2 or thermal relaxation drives isomerization back to the *trans* state. **(b)** Chemical structure of BGAG_n, a PORTL for SNAP-tagged mGluRs in the *trans* (top) and *cis* (bottom) configurations. **(c)** Schematic showing the general PORTL and SNAP-tagged mGluR design. In principle, SNAP tags may be replaced with CLIP-tags, Halo-tags, or other protein tags, and mGluRs may be replaced with any GPCR. **(d)** Schematic showing general principle of PORTL-mediated optical control of a class C GPCR. Light-induced (~380 nm) isomerization of BGAG_n to the *cis* state allows the glutamate moiety to bind and activate the mGluR, which is reversed by either thermal relaxation or visible light (~500 nm) illumination.

Fig. 2.

Workflow for PORTL-mediated optical control of SNAP-tagged GPCRs in cultured cells. On day 1 cells are cultured in a 12-well plate and are transfected with relevant DNA on day 2. After 24–48 h of expression, cells are labeled with PORTL, washed and mounted on an inverted microscope for electrophysiology. Light is transmitted through the objective to illuminate the sample and cells are patch-clamped in whole cell mode using a glass patch micropipette with an electrode connected to digitization and acquisition equipment. The inset gray box shows a representative result, where UV light (~380 nm) is used to drive photoactivation and visible light (>480 nm) is used to reverse the photoswitch and terminate photoactivation

Fig. 3.

Photoactivation of SNAP-tagged mGluRs with various PORTLs. **(a)** Representative trace showing SNAP-mGluR2 photoactivation via BGAG₁₂ in HEK 293T cells co-expressing GIRK channels. This combination averages a ~60% photoswitching efficiency relative to saturating glutamate upon photoactivation by 380 nm light and termination of photoactivation by 500 nm light. **(b)** Representative trace showing SNAP-mGluR2 photoactivation via BGAG_{12,460}. This combination averages ~40% photoswitching efficiency upon photoactivation by 460 nm light and termination of photoactivation in the dark. **(c)** Representative trace of SNAP-mGluR6 activated by BGAG₁₂. This combination averages a ~45% photoswitching efficiency upon photoactivation by >480 nm light and termination of photoactivation by ~380 nm light

Table 1

Summary of existing PORTL/GPCR combinations

Receptor	Construct	Photoswitch	Efficiency %	λ (nm)	Notes
mGluR2	SNAP-mGluR2	BGAG _n ($n = 0, 4, 8, 12$)	~60	~380: ON ~500: OFF	"SNAG-mGluR2"
		BGAG _{12,460}	~50	~460: ON Dark: OFF	"SNAG ₄₆₀ -mGluR2"
	CLIP-mGluR2	BCAG ₁₂	~60	~380: ON ~500: OFF	Orthogonal to SNAP
		BCAG _{12,460}	~50	~460: ON Dark: OFF	Orthogonal to SNAP
mGluR3	SNAP-mGluR3	BGAG ₀	~20	~380: ON ~500: OFF	SNAG-mGluR3
mGluR6	SNAP-mGluR6	BGAG ₁₂	~50	~500: ON ~380: OFF	SNAG-mGluR6
mGluR7	SNAP-mGluR7-N74K	BGAG ₁₂	~40	~500: ON ~380: OFF	SNAG-mGluR7
mGluR8	SNAP-mGluR8	BGAG ₁₂	~25	~500: ON ~380: OFF	SNAG-mGluR8

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2

Chemical structures of mGluR-targeting PTLs, PORTLs and related compounds

PTLs	
L-MAG _n
D-MAG _n
PORTLs	
AG ₁₂ (untethered control compound)
BGAG _{short}
BGAG _{short,460}
BGAG ₀
BGAG ₄
BGAG ₈
BGAG ₁₂
BGAG ₂₈
BGAG _{12,460}

BCAG _{12,460}
BCAG ₁₂
BCAG _{short,460}

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 3Extinction coefficient (ϵ) and peak absorption wavelength (λ) for BGAG PORTLs

Compound	ϵ (M ⁻¹ cm ⁻¹)	λ (nm)
BGAG _n	19,600	378
BGAG _{n,460}	24,600	420