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Design of a highly bi-stable photoswitchable tethered ligand for rapid and sustained manipulation of neurotransmission

Wan-Chen Lin*, Ming-Chi Tsai, Rajit Rajappa, and Richard H. Kramer*

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, United States

Abstract

Photoswitchable neurotransmitter receptors are powerful tools for precise manipulation of neural signaling. Through the *trans-cis* isomerization of azobenzene photoswitches tethered to these receptors, their activities can be rapidly and reversibly controlled by light. However, their applications for slow or long-lasting biological events are constrained by thermal relaxation of *cis*-azobenzene, a process that renders the receptor state unsustainable in darkness after the light stimulus. We here present a simple approach to slow down this process and thereby enhance the bi-stability (i.e. the ability to stably reside in either of the two states) of photoswitchable receptors. Introducing methyl groups to the *ortho* positions of azobenzene strongly retards photoswitch thermal relaxation both in solution and on cell surface. In cultured cells and intact brain tissue, modifying inhibitory neurotransmitter receptors with one of the derivatives, **dMPC1**, allows bi-directional receptor control with 380-nm and 500-nm light. Benefited by the bi-stability of **dMPC1**, the modified receptors can be locked in either an active or an inactive state in darkness after a brief pulse of light. This strategy thus enables both rapid and sustained manipulation of neurotransmission, allowing optogenetic interrogation of neural functions over a broad range of time scales.

Graphical Abstract

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*Corresponding Author rhkramer@berkeley.edu. * wlin_mcb@berkeley.edu.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Supplemental figures, schemes, details of chemical synthesis and electrophysiological experiments, and additional information about the photoswitch compounds (PDF).

Notes

The authors declare no competing financial interests.

Unlocking biological mechanisms requires an understanding of molecular and cellular events in space and time. Optogenetic methodologies combines the advantages of optical and genetic control to enable targeted perturbation of events with superb spatial and temporal resolution.^{1–3} In the nervous system, events can occur within milliseconds (e.g. an action potential), or occur over hours or longer (e.g. synaptic plasticity encoding memories). For manipulating slow or long-lasting events, it would be useful to have “bi-stable” optogenetic tools that can be switched persistently between on and off states with a brief flash of light. Bi-stability would remove requirement for continuous irradiation, which may be impractical, invasive, or phototoxic.

The nervous system employs myriad neurotransmitter receptors to mediate fast signaling and slow neuromodulation. To decipher their distinct roles, receptors for glutamate, γ -aminobutyric acid (GABA), acetylcholine, and ATP have been reengineered to allow optogenetic manipulation.^{2,4} Photo-control is achieved via the *trans-cis* isomerization of azobenzene-based photoswitchable tethered ligands (PTLs). Most PTLs exhibit short half-lives in their metastable state (<0.5 h).^{5–7}, allowing only transient control over their conjugate receptors.

Here we present a simple strategy to enhance the thermal stability of a PTL, thereby improving the bi-stability of the receptor it controls (Figure 1). We strategically introduce steric hindrance nearby the diazo bond of azobenzene to retard thermal isomerization of the metastable *cis*-isomer (Figures 1b and 1c). We validate this approach with GABA_A receptors (GABA_ARs), the key mediators of fast inhibitory neurotransmission in the brain.⁸ GABA_ARs are ligand-gated chloride channels assembled from 19 possible subunits, with a common stoichiometry of two α , two β , and one tertiary subunit (mostly γ or δ ; Figure 1a). We previously engineered a series of light-regulated GABA_A receptors (LiGABARs), allowing optogenetic control of six different α -isoforms.⁹ Photo-control of α 1-GABA_A receptors, the most abundant subtype, has been validated *in vitro* and *in vivo* in the cerebral cortex of awake, behaving mice, demonstrating LiGABAR in neurobiology.⁹

The α 1-LiGABAR was engineered by covalently conjugating a PTL (**PAG1C**; Figure 1b) near the receptor’s GABA-binding pocket (Figure 1a). Like many other PTLs, **PAG1C** comprises a sulfhydryl-reactive maleimide group, a photoswitchable azobenzene core, and a ligand for the receptor. Its azobenzene core isomerizes between *cis* (twisted) and *trans* (extended) states upon irradiation of 380-nm and 500-nm light, respectively. In the dark, the metastable *cis*-isomer gradually relaxes back to the thermally stable *trans* state. Once

tethered to a genetically introduced cysteine (α 1T125C), **PAG1C** delivers its antagonist ligand into the GABA-binding pocket when the azobenzene is in the *trans* but not the *cis* configuration. Hence the receptor can be rapidly and reversibly switched between functional (conducting) and antagonized (non-conducting) states with UV and green light, respectively. While the α 1-LiGABAR enabled strong photo-control of inhibitory responses in neurons, we observed limited bi-stability of this receptor, as noticeable “re-antagonism” occurred within ~5 min of post-UV dark period.⁹ This issue was attributed to the thermal relaxation of *cis*-**PAG1C**.

We sought to enhance the bi-stability of LiGABAR with minimal alterations in the construction and photochemical properties of the PTL. This may be achieved by modifying the positions on azobenzene that are not involved in module assembly. Previous studies showed that azobenzene derivatives carrying two or more *ortho*-substituents exhibit higher thermal stability in the *cis* state, compared to the unmodified version.^{10–12} The choice of substituent can be as small as a methyl group, and if only two *ortho*-substituents are introduced, they should be placed on the same side of azobenzene in order to slow down thermal relaxation.^{10,11} This “*ortho*-effect” is likely due to steric hindrance between the *ortho*-substituents and the lone pair electrons of nitrogen atoms, causing retardation in the *cis*-to-*trans* conversion.¹⁰

Intrigued by its simplicity, we applied this chemical strategy to enhance the bi-stability of α 1-LiGABAR. Because **PAG1C** is tethered nearby the GABA-binding pocket, introducing additional groups to azobenzene might cause unfavorable interactions between the PTL and the receptive site. To minimize adverse effects on photo-control, we added only two methyl groups to **PAG1C** (Figure 1c), either at 2',6'-positions (**dMPC1**; close to the ligand) or at 2,6-positions (**dMPC2**; close to maleimide). Methyl group was chosen for its small size and minor impacts on the spectroscopic properties of azobenzene. The three PTLs were synthesized by sequential coupling of a glycine unit and 3-maleimidopropionic acid to 4,4'-diamino azobenzene (unsubstituted or 2,6-dimethylated), followed by guanidinylation of the glycine amino group (SI Schemes 1 and 2).

We first validated this strategy by comparing the thermal stability of *cis*-PTLs in solution. UV-VIS absorption spectra for *trans*- and *cis*-PTLs (250 μ M in DMSO) were measured after illuminating the samples with 500-nm and 380-nm light, respectively. To measure thermal relaxation, the UV-irradiated samples were stored in darkness, and their spectral changes were monitored over time. As exemplified by **PAG1C**, the characteristic π - π^* band of *trans* azobenzene shifted significantly after UV irradiation, resulting in a large absorbance difference at 376 nm between the two isomers (Figure 2a). During post-UV dark period, the absorbance at 376 nm gradually increased over time, indicating that *cis*-**PAG1C** underwent thermal relaxation to the *trans* isomer.

The di-*o*-methylated PTLs exhibited a similar spectral separation for the two isomers, providing the maximal absorbance difference at 370 nm (Figures 2b and 2c). Distinct from **PAG1C**, however, the progression of thermal relaxation was much slower for these derivatives (Figures 2b–2d). Compared to a ~90% conversion of *cis*-**PAG1C** over 22 h, relaxation of *cis*-**dMPC1** and *cis*-**dMPC2** was <10% over the same period (Figure 2d). To

estimate thermal stability under a physiologically relevant condition, we monitored the relaxation of *cis*-PTLs (as adducts of glutathione; SI Figure 1) in saline. The half-life of *cis*-**PAG1C**, *cis*-**dMPC1**, and *cis*-**dMPC2** are 16 min, 11 h, and 12 h at room temperature and 10 min, 6.2 h, and 7.6 h at 37 °C. Accordingly, di-*o*-methylation of azobenzene enhanced the thermal stability of a *cis*-PTL at least 40-fold relative to the unmodified prototype.

We next assessed the performance of **dMPC1** and **dMPC2** in photo-controlling $\alpha 1$ -GABA_A receptors. The parent PTL (**PAG1C**), when installed onto the receptor, is functionally inert in the *cis* state but strongly antagonizes the receptor in the *trans* state. Hence receptor antagonism is rapidly triggered by green light and relieved by UV light. To test whether **dMPC1** and **dMPC2** preserve this functional profile, we examined the photosensitivity of GABA-elicited currents from dMPC-conjugated receptors. We expressed the mutant receptors ($\alpha 1$ T125C together with the wild-type $\beta 2$ and $\gamma 2$ subunits) in HEK cells and treated them with **PAG1C**, **dMPC1** or **dMPC2** to generate the LiGABAR variants. GABA-elicited currents were acquired by whole-cell voltage-clamp recording, during which cells were held at -70 mV and alternately illuminated with 500 nm and 380 nm (Figure 3a).

As illustrated by **PAG1C**, light switching caused fast and reversible changes in the current amplitude, with 380-nm light amplifying and 500-nm light reducing the response (Figure 3a). The photoswitching effect was quantified as the percent current reduction caused by green light (compared to the current peak in UV). At 3 μ M GABA, **PAG1C** exerted $77 \pm 3\%$ photoswitching of the response ($n = 5$ cells). Analogously, both **dMPC1** and **dMPC2** enabled fast and reversible photo-control of GABA_A-receptor activity (Figure 3a). Photoswitching by **dMPC1** ($67 \pm 3\%$, $n = 5$ cells) was stronger than that by **dMPC2** ($56 \pm 6\%$, $n = 6$ cells). Because these two compounds show similar photochemical profiles in solution (Figure 2), the weaker photoswitching by **dMPC2** indicates that substituting at 2,6- (but not 2',6'-) positions might cause steric interference which disfavors ligand binding.

We next tested whether the bi-stability of LiGABAR is improved by di-*o*-methylation of the PTL. We compared **PAG1C** with **dMPC1**, the derivative that exerts a similar degree of photo-control. HEK cells expressing PTL-conjugated receptors were held at -70 mV, and currents were elicited by 10 μ M GABA every 3 min. In each experiment, a pair of responses, one under 500-nm and the other under 380-nm illumination, was first recorded to measure the size of “photoswitchable current.” The experiment then proceeded in darkness for 30 min and ended with a response elicited under 380-nm light (Figure 3b). In **PAG1C**-treated cells, current amplitude gradually decreased over the 30-min dark period. This current reduction was caused by thermal relaxation of *cis*-**PAG1C** rather than other changes in the receptor or the cell, as the subsequent UV flash completely restored the current back to its initial level. We plotted the progression of PTL thermal relaxation as the time-dependent reduction in the fraction of “photoswitchable current” (Figure 3c). Fitting this plot with a single-exponential decay yielded the half-life of *cis*-**PAG1C** in the context of LiGABAR ($t_{1/2} = 34 \pm 2$ min at room temperature; $n = 4$ cells).

In contrast, current amplitude reduced very slowly in **dMPC1**-treated cells (Figures 3b and 3c). Because the current decline was too slow, we were unable to estimate the half-life for *cis*-**dMPC1**. Instead, we compared **dMPC1** with **PAG1C** using the percent of

photoswitchable current lost at the end of 30-min dark period (Figure 3d). When receptors were equipped with **dMPC1**, current loss was only $10 \pm 2\%$ ($n = 6$ cells), far less than the $46 \pm 3\%$ loss when receptors were installed **PAG1C** ($n = 4$ cells). Thus after relieving antagonism by the initial UV pulse, **dMPC1**-conjugated receptors were virtually locked in the fully functional state. Together with the fact that antagonism is mediated by the thermally stable *trans* isomer, implanting **dMPC1** makes LiGABAR a highly bi-stable and fast-acting optogenetic device.

Finally, we validated the use of **dMPC1** in neural circuitry from the brain. We used the bi-stability of **dMPC1** to gain new insights into the dynamics of membrane turnover of GABA_A receptors at inhibitory synapses, where the $\alpha 1$ -isoform is enriched^{8,13}. Brain slices from mice that had been virally transduced with $\alpha 1$ (T125C) were treated with **PAG1C** or **dMPC1**. Electrical stimulation of presynaptic neurons in the slice elicits GABA release, which result in inhibitory postsynaptic currents (IPSCs) that we measured by voltage-clamping a postsynaptic neuron to 0 mV. Excitatory synapses were blocked by adding pharmacological blockers of glutamate receptors.

Like the parent PTL (**PAG1C**), treatment with **dMPC1** conferred light-sensitivity, with IPSC amplitude increasing dramatically upon exposure to 380 nm light, as receptor antagonism is relieved, and decreasing in 500 nm light, when antagonism is reinstated (decrease of $57 \pm 6\%$ for **PAG1C** and $51 \pm 2\%$ for **dMPC1**; $n = 4$ and 5 cells; Figures 4b). However unlike the parent PTL, relief of **dMPC1** antagonism by 380 nm persisted long after light offset (Figure 4b). At 30 min after the light, **dMPC1**-treated neurons exhibited only a $9 \pm 6\%$ decline in the photoswitchable IPSC component ($n = 5$ cells), compared to a $71 \pm 4\%$ decline in **PAG1C**-treated neurons ($n = 4$ cells).

Inhibitory synapses are dynamically regulated, and insertion or removal of GABA_A receptors may be important for synaptic plasticity.¹⁵ To gain insight into the regulation of GABA_A receptor turnover, we exploited the enhanced bi-stability of **dMPC1** and measured the degree of photosensitivity remaining at inhibitory synapses hours after treatment with **dMPC1**.

GABA_A receptors conjugated to **dMPC1** are antagonized in darkness, therefore inhibition caused by ambient GABA should be suppressed. In contrast, UV light should relieve antagonism, unveiling inhibition. By suppressing or unveiling the effects of inhibition, we can reveal whether neuronal activity affects the rate of GABA_A receptor turnover. Because of the enhanced bi-stability of **dMPC1**, nearly complete relief from antagonism can be achieved for hours with minimal exposure to UV light, which can be damaging.

Slices kept in darkness showed almost no loss of IPSC photosensitivity over 12 hours, consistent with retention of PTL-conjugated receptors at the synapse. However, slices that received only a few brief flashes of 390-nm light (15 sec, every 3 h) showed a significant decrease in IPSC photosensitivity ($55 \pm 6\%$ loss, $p = 0.0021$, $n = 5$ cells; SI Figure 3), indicating loss of PTL-conjugated receptors. This finding suggests that turnover of synaptic GABA_A receptors is activity-dependent, a homeostatic mechanism of potential importance for synaptic development and plasticity.

In summary, we have designed bi-stable PTLs for optogenetic control of receptors. We exploited steric hindrance of *ortho* substituents of azobenzene (the “*ortho* effect”), to slow the *cis-to-trans* thermal isomerization, thereby stabilizing the *cis* state. This strategy should apply to other PTL-regulated neurotransmitter receptors⁴ or voltage-gated ion channels¹⁴. By allowing transient light flashes to yield persistent effects, bi-stable switches minimize photodamage and enable multiplexing with other optical techniques in neurobiology, such as fluorescent Ca²⁺ imaging. Bistable photoswitches like dMPC1 are ideal for perturbing slow biological processes, including neuronal development and long-term plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Rapid versus sustained manipulation of an inhibitory neurotransmitter receptor through a photoswitchable tethered ligand (PTL). (a) Schematic illustration of the light-regulated GABA_A receptor (LiGABAR) studied in this work. The GABA-binding pocket (located at the interface of α and β subunit) is indicated by a dashed curve. Isomerization of the PTL prevents or allows GABA binding, thereby halting or triggering chloride influx through the transmembrane channel. In an ideal scenario, the state of a LiGABAR can either be rapidly switched by two different wavelengths of light or persist in darkness after the termination of light stimulus. (b) Photoisomerization and thermal relaxation processes of **PAG1C**, the parent PTL for α 1-LiGABAR. (c) The di-*o*-dimethylated derivatives of **PAG1C** tested in this work.

Figure 2.

Thermal relaxation is retarded by di-*o*-methylation of azobenzene. (a-c) Thermal relaxation of *cis*-PTLs in DMSO. Absorption spectra for each PTL (250 μ M) were measured at room temperature from samples illuminated with 500 nm (green; *trans*-dominant), illuminated with 380 nm (violet; *cis*-dominant), and in darkness (grey; post-380 nm). (d) Time course of thermal relaxation derived from panels a-c. Fraction of *cis*-PTL is calculated as $(A_{500} - A_{\text{dark}})/(A_{500} - A_{380})$ at 376, 370, and 370 nm for **PAG1C**, **dMPC1**, and **dMPC2**, respectively.

Figure 3.

Di-*o*-methylation allows the PTL to rapidly control LiGABAR activity with light (a) while exerting sustained receptor modulation in darkness (b). (a) Representative recording traces from HEK cells showing fast and reversible manipulation of receptor activity by **PAG1C**, **dMPC1**, and **dMPC2**. (b) Representative traces showing enhanced bi-stability of LiGABAR when the receptor is modified by **dMPC1**. The grey arrow indicates the fraction of current lost at the end of the dark period. (c) Time-dependent changes in photoswitchable current amplitude ($I_t - I_{500}$; t = time in darkness) since the initial 380-nm illumination. (d) Quantification of thermal relaxation over 30 minutes. Currents were elicited by 3 and 10 μ M GABA for (a) and (b), respectively, and were acquired via voltage-clamp recording (held at -70 mV). Data in (c) and (d) are shown as mean $\pm$ SEM (n = 4 cells for **PAG1C** and 6 cells for **dMPC1**).

Figure 4.

(a, b) **dMPC1** enables robust photo-control and sustained manipulation of inhibitory postsynaptic currents (IP-SCs). (a) Schematic illustration for the measurement of IPSCs. (b) Representative recording traces showing enhanced bi-stability of LiGABAR when **dMPC1** was used as the PTL. (c, d) **dMPC1** enables optogenetic interrogation of activity-dependent receptor turnover in brain slices. (c) Schematic illustration of the experiments. See SI Figure 3 for details. (d) IPSC photosensitivity was significantly lost when LiGABARs were antagonized (i.e. causing less neuronal inhibition) during the 12-hour period and more retained when LiGABARs were relieved from antagonism, suggesting that enhancing neuronal activity accelerates the turnover of synaptic GABA_A receptors. Wholecell recordings were carried out in cortical neurons at $33 \pm 2^\circ\text{C}$ (a, b) and hippocampal neurons at room temperature (c, d), with cells held at 0 mV.