1 # Photoswitchable Serotonins for Optical Control of the 5-HT2A Receptor

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Abstract

The serotonin receptor family of G protein-coupled receptors (GPCRs) and ligand-gated ion channels play central roles in neuromodulation and are critical drug targets for the treatment of psychiatric disorders. Optical control of serotonin receptor subtypes has the potential to greatly enhance our understanding of the spatiotemporal dynamics of receptor function both at the cellular level and within neural circuits. While other neuromodulatory receptors have been successfully rendered photoswitchable, reversible photocontrol of serotonin receptors has not been achieved, representing a major gap in GPCR photopharmacology. Herein, by designing and screening a family of azobenzene-conjugated serotonin analogues, we developed the first photopharmacological tools that allow for such control. **Azo5HT-2** shows light-dependent 5-HT_{2A}R agonism, inducing receptor-mediated calcium signaling in the light-activated *cis*-form. Based on computational docking and test compound analysis, we also synthesize and test photoswitchable orthogonal, remotely-tethered ligands (PORTLs). **BG-Azo5HT$_n$** PORTLs provide rapid, reversible and repeatable optical control following conjugation to SNAP-tagged 5-HT_{2A}R. Overall, this study both introduces new tools for the optical control of 5-HT_{2A}Rs and provides a foundation for the broad extension of photopharmacology to the serotonin receptor family.

24 **Introduction**

25 Serotonin (5-hydroxytryptamine; 5-HT) is a neuromodulator that is released in the brain primarily by Dorsal
26 Raphe Nuclei neurons, in the gut by enterochromaffin cells, and in blood platelet cells.[1,2] Serotonin acts
27 through a large family of G protein-coupled receptor (5-HT_1Rs, 5-HT_2Rs, 5-HT_4Rs, 5-HT_5Rs, 5-HT_6Rs, 5-
28 HT_7Rs) and ion channel (5-HT_3Rs) subfamilies to regulate a plethora of neuronal and behavioral processes.[1]
29 Given the importance of 5-HT to the regulation of mood, cognition and reward, great effort has been made
30 to harness pharmacology to manipulate 5-HTRs for both basic study and therapeutic applications. Recent
31 developments establishing the potential of 5-HT_{2A}R-targeting psychedelic drugs for the treatment of
32 depression, anxiety, and addiction have further motivated the detailed study of 5-HTR signaling.[3–7] Despite
33 great attention, limitations in the ability of 5-HTR-targeting compounds in terms of subtype-specificity and
34 spatiotemporal precision and their inability to be targeted to genetically defined cell types have hindered
35 progress toward a mechanistic understanding of the physiological and therapeutic effects of 5-HTR
36 signaling.

37 As an alternative to classical pharmacology, photopharmacology has emerged as a means of gaining
38 further precision through the development of photosensitive compounds whose activity can be modified
39 depending on the wavelength of illumination[8–10]. Photopharmacological compounds have enabled the
40 optical control of a variety of GPCRs, including class A (μ-opioid receptor,[11] dopamine receptors,[12,13]
41 histamine receptors,[14] adenosine receptors,[15] muscarinic receptors,[16] adrenergic receptors[17], fatty acid
42 receptors,[18] lysophospholipid receptors,[19,20] and cannabinoid receptors[21,22]), class B (glucagon-like peptide
43 1 receptor[23,24]), and class C GPCRs (metabotropic glutamate receptors[25–28]). For further precision, including
44 the ability to target the effects of light to genetically-defined cell populations, photopharmaceuticals may
45 be covalently tethered to a genetically engineered receptor containing a labeling domain (i.e. SNAP, Halo)
46 as Photoswitchable Orthogonal Remotely Tethered Ligands (PORTLs), as has been demonstrated with
47 metabotropic glutamate receptors (mGluRs).[29,30] mGluR-targeting PORTLs have been applied for both
48 molecular biophysical studies[31] and the *in vivo* manipulation of mGluR2 in specific cell types[32–34] in behaving
49 mice, providing a template for their development and application in complex systems.

Surprisingly, 5-HTRs have received limited attention in terms of photopharmacology. Photocaged variants of serotonin have enabled light-induced release of 5-HT through removal of photocleavable protecting groups.[35–38] However, these tools do not offer reversible control, lack 5-HTR subtype targeting, and have not been paired with genetic targeting as can be done with PORTLs.[29,30,39] Thus, the development of a photoswitchable ligand platform for the 5-HTR family would enable the study of these receptors with unprecedented spatiotemporal control, which could facilitate new insight into the dynamics of neural signaling. Herein, we describe the first development of a series of photoswitchable ligands for the 5-HT_2 receptor family. We identify an azobenzene-conjugated 5-HT lead compound, **Azo5HT-2**, that enables optical control of 5-HT_{2A}Rs with activity which is increased approx. 10-fold in the *cis* form upon irradiation. Computational structural analysis suggests that the 5-HT moiety of *cis*-**Azo5HT-2** binds with a canonical pose and enables access to the azobenzene ring from the extracellular face of the receptor, motivating the design and synthesis of a first generation of 5-HT PORTLs. Finally, **BG-Azo5HT$_n$** PORTLs of variable linker length enable repeatable optical control of SNAP-tagged 5-HT_{2A}R, opening the door to genetically targeted, receptor-specific optical control of serotonergic signaling.

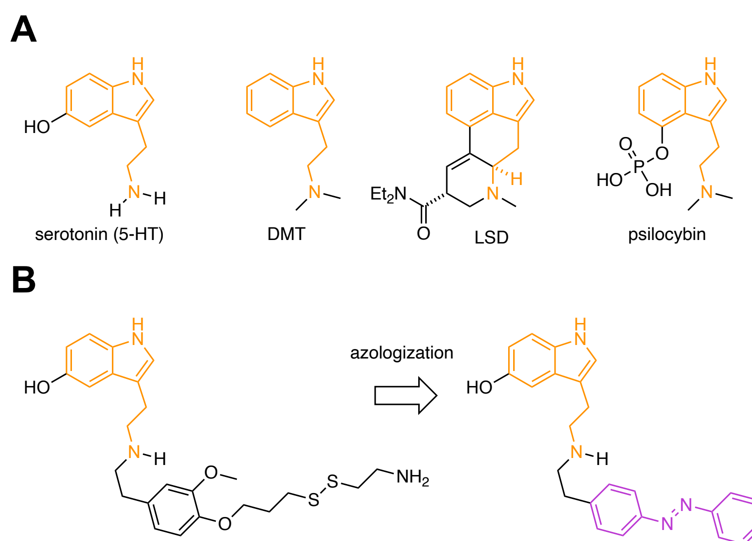
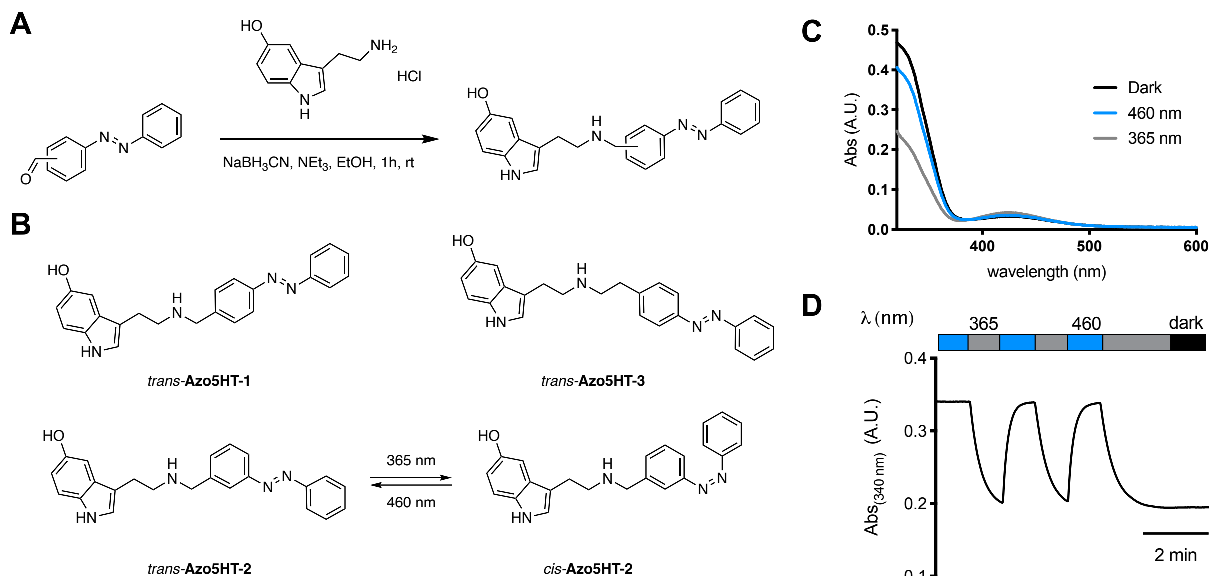


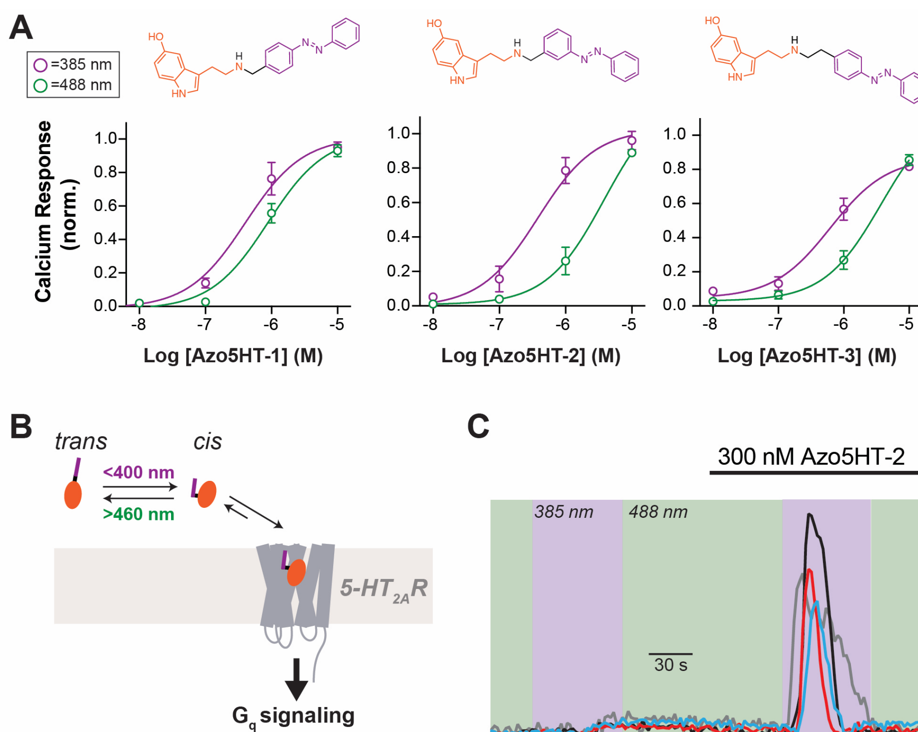
Figure 1. (A) Representative tryptamine-derived agonists of 5-HT receptors: serotonin, PNU 22394, LSD, and psilocybin. Shared tryptamine moiety highlighted in orange. (B) Azologization strategy[40,41] for the design of photoswitchable agonists based on a previously-reported covalent agonist of 5-HT2A (left).[42]

68 The 5-HT receptor family is targeted by a variety of natural and synthetic agonists, including many with a
69 tryptamine (indolamine) moiety (Fig. 1A). We considered several of these ligands for the design of
70 photochromic agonists but reasoned that serotonin would be best suited because analogs would likely
71 mimic endogenous signaling and derivatives which could be suited for incorporation of azobenzene motifs
72 have been reported previously. These derivatives include covalent 5-HT analogs with an appended
73 benzene ring (Fig. 1B).[42] We considered an 'azologization'[40,41] approach to install the azobenzene at the
74 matching position (**Azo5HT-3**) and designed additional derivatives with the azobenzene moved one carbon
75 atom closer to the pharmacophore (**Azo5HT-1** and **Azo5HT-2**). The derivatives were synthesized through
76 reductive amination of 5-HT with the corresponding azobenzene-aldehydes (Fig. 2 A,B). Photophysical
77 characterization of **Azo5HT-1** to **Azo5HT-3** (Fig. 2 C,D and Fig. S1) revealed similar properties to classical
78 azobenzenes. All derivatives could be reversibly switched to their respective *cis* and *trans* forms with UV-A
79 (365 nm) and blue light (460 nm), respectively, and underwent slow thermal relaxation ($t_{1/2}$ > 1h).

80 

81 **Figure 2.** Synthesis and photophysical characterization of photoswitchable 5-HT derivatives **Azo5HT-1-3**.
82 (A) Synthesis of **Azo5HT** series. (B) Chemical structures of **Azo5HT**s. (C) The UV-Vis spectra of **Azo5HT-**
83 **2** in the dark-adapted (black, *trans*), 365 nm adapted (grey, *cis*) and 460 nm adapted (blue, *trans*)
84 photostationary states (50 mM, DMSO). (D) Reversible cycling between **Azo5HT-2** photoisomers with
85 alternating illumination at 365/460 nm (50 mM, DMSO).

86 To assess the ability of **Azo5HT** molecules to serve as 5-HTR agonists, we tested each compound
87 across the human 5-HT_2R family (5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R). As these receptors are all G_q-coupled and
88 produce intracellular Ca^{2+} release via phospholipase C-β activation, we performed live cell Ca^{2+} imaging
89 with the fluorescent sensor, GCaMP6f. Using this assay, all three receptors showed the expected 5-HT
90 responses with nM EC_{50} values (Table 1). Compounds **Azo5HT-1-3** were tested independently either under
91 standard conditions with 488 nm illumination for GCaMP6f excitation, which maintain them in the *trans*
92 state, or with interweaved 385 nm illumination to convert them to the *cis* state. All three compounds
93 showed dose-dependent activation of 5-HT_{2A}R in the *trans* and *cis* states with a leftward shift in the *cis*
94 state (Fig. 3A; Fig. S2). For **Azo5HT-1** there was a ~2-fold shift, while a larger 5-10-fold shift was seen for
95 **Azo5HT-2** and **Azo5HT-3** (Table 1). It's worth noting that 385 nm likely does not maximally occupy the *cis*
96 state, so the relative difference between *cis* and *trans* may be underestimated using this approach. In
97 contrast to the 5-HT_{2A}R, no or very modest differences were observed between *cis* and *trans* for each
98 molecule on 5-HT_{2B}R and 5-HT_{2C}R (Table 1; Fig. S2).



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100 **Figure 3.** Photoactivation of 5-HT2AR by **Azo5HT-2**. (A) Dose-response curves for **Azo5HT** compounds
101 showing enhanced agonism for *cis* versus *trans* for all compounds using a Ca^{2+} imaging assay. (see Table

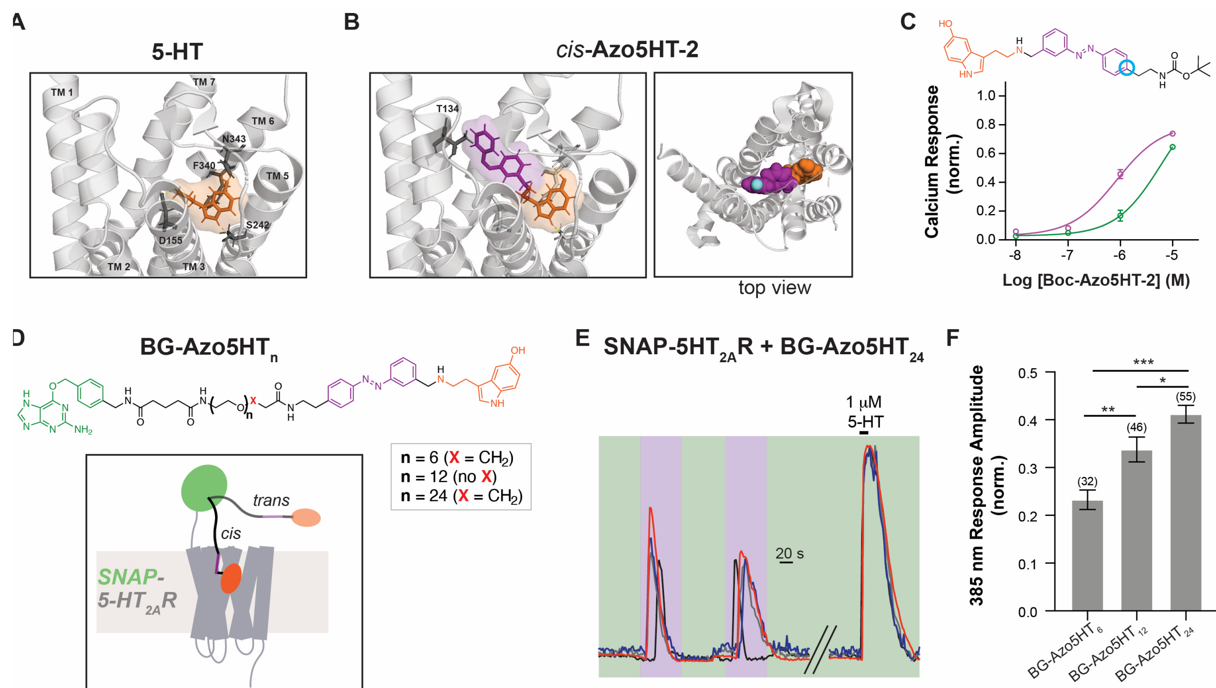
102 S1). (B) Schematic of **Azo5HT-2** mediated optical control. (C) Representative Ca^{2+} imaging traces showing
103 photoactivation of 5-HT_{2A}R by **Azo5HT-2**. In the absence of **Azo5HT-2**, no 385 nm light response is seen
104 but a clear response is seen in the presence of 300 nM **Azo5HT-2** with similar on and desensitization
105 kinetics compared to 5-HT application (see Fig. S4).

106 Next, we asked if **Azo5HT-2** photoconversion could be harnessed for optical activation of 5-HT_{2A}R
107 (Fig. 3B). Application of 100-300 nM **Azo5HT-2** produced minimal responses under 488 nm illumination
108 but following application of 385 nm light, clear responses were observed that were up to 50% in amplitude
109 relative to saturating 5-HT (Fig. 3C; Fig. S4A-B). Together, these data indicate that **Azo5HT-2** enables
110 reversible photoagonism of 5-HT_{2A}R with similar signaling properties to the endogenous agonist 5-HT.

111 We next used computational ligand docking to gain insight into the binding mode of **Azo5HT-2**
112 using the recently reported LSD-bound crystal structure of the 5-HT_{2A}R.[43] *Cis*-**Azo5HT-2** showed similar
113 binding of the 5-HT moiety compared to 5-HT alone (Fig. 4A, B) and the azobenzene moiety showed
114 occupancy of a pocket toward the extracellular face of the receptor with likely solvent accessibility from
115 the cell surface (Fig. 4B, Fig. S5A). In contrasts, *trans*-**Azo5HT-2** showed variable docking results with a
116 lower proportion of docks containing a canonical pose (see SI for details) for the 5-HT moiety (24/78 for
117 *cis*-**Azo5HT-2** versus 7/41 for *trans*-**Azo5HT-2** versus 25/95 for 5-HT) (Fig. S5B, C). Based on the potential
118 binding pose of *cis*-**Azo5HT-2**, we reasoned that extension of this molecule would be tolerated and,
119 ultimately, enable tethering to a labeling site (e.g. SNAP-tag) outside the core of the transmembrane helix
120 bundle and extracellular loops of the receptor. To test this, we synthesized the extended photoswitch **Boc-**
121 **Azo5HT-2** (Fig. 4C) using our established reductive amination conditions (Fig. S2). **Boc-Azo5HT-2** showed
122 clear agonism of 5-HT_{2A}R and maintained enhanced apparent affinity in the *cis* state (Fig. 4C; Table 1),
123 enabling photo-activation of Ca^{2+} responses (Fig. S6).

124 Motivated by our docking and **Boc-Azo5HT-2** test compound analysis, we designed PORTLs with
125 the goal of enabling tethered optical control of SNAP-5HT_{2A}R (Fig. 4D). To this end, BOC-Azo5HT-2 was
126 deprotected and various PEG-linkers and benzyl guanine (BG) were attached through successive amide
127 couplings (Fig. S3). We first used a previously established fluorophore competition labeling assay[30] to

confirm that all PORTLs efficiently label N-terminally SNAP-tagged 5-HT_{2A}R ("SNAP-5HT_{2A}R") (Fig. S7A, B). We then tested the ability of each PORTL to produce optically-evoked Ca^{2+} responses following conjugation to SNAP-5HT_{2A}R and 385 nm illumination. Reversible and repeatable 385 nm light-evoked Ca^{2+} transients were seen with all 3 PORTL variants in 10-40% of cells with a higher proportion of cells showing photoactivation with BG-Azo5HT_{24} and BG-Azo5HT_{12} compared to BG-Azo5HT_{6} (Fig. S7C, D). Light responses were not seen in the absence of PORTL labeling (Fig. S7E) labeling and were as large as 60% in amplitude relative to saturating 5-HT for BG-Azo5HT_{24} and smaller for shorter variants (Fig 4F). Importantly, 385 nm light responses were blocked by the 5-HT_2R antagonist ketanserin (Fig. S7F). A subset (<10%) of cells showed Ca^{2+} transients in the absence of 385 nm illumination (Fig. S7G), likely indicative of some activation via the PORTL in *trans*. This potential *trans* activation was more pronounced in shorter variants, suggesting that the decreased local concentration associated with longer PORTLs enhances the relative *cis* versus *trans* agonism via the Azo5HT moiety. Together, these data establish genetically targetable, PORTL-mediated optical control of 5-HT_{2A}R and provide a strong foundation for both further engineering and application.



144 **Figure 4.** Docking and test compound analysis enable PORTL development. (A-B) Docking analysis
145 showing that both 5-HT (A) and the 5-HT moiety of **Azo5HT-2** (B) show identical poses, with the
146 azobenzene moiety occupying a water-filled cavity at the extracellular face of 5-HT_{2A}R. Residues
147 associated with canonical 5-HT binding are highlighted in (A) and position T134, which was previously
148 substituted for conjugation of a covalent 5-HT_{2A}R agonist[42], is highlighted in (B). Top view (B, right) shows
149 that the para position (yellow) is positioned facing toward the extracellular solution. C) Chemical structure
150 (top; para position circled in yellow) and dose response curve (bottom) showing light-dependent
151 (purple=385 nm illumination; green=488 nm illumination) activation of 5-HT_{2A}R by **Boc-Azo5HT-2**. (D)
152 Chemical structure, top, and schematic, bottom, of **BG-Azo5HT$_n$** PORTL-mediated optical control of
153 SNAP-tagged 5-HT_{2A}R. (E-F) Representative traces (F) and summary bar graph (G) showing
154 photoactivation of SNAP-5-HT_{2A}R by **BG-Azo5HT$_n$** PORTLs. The numbers of cells analyzed are shown in
155 parentheses. * indicates statistical significance (1-way ANOVA with Tukey-Kramer Multiple Comparisons;
156 $p=0.0068$ for BG-Azo5HT_6 vs. BG-Azo5HT_{12}, $p<0.0001$ for BG-Azo5HT_6 vs. BG-Azo5HT_{24}, $p=0.036$ for BG-
157 Azo5HT_{12} vs. BG-Azo5HT_{24})

158 In summary, we have developed first-in-class photoswitchable analogs of serotonin that allow for
159 the optical control of 5-HT_{2A}R. Interestingly, all three test compounds, **Azo5HT1-3** showed preferential
160 agonism in *cis* over *trans* on the 5-HT_{2A}R, but no clear difference between states on the 5-HT_{2B}R or 5-
161 HT_{2C}R, providing a powerful chemical lead for further molecular pharmacological analysis. At the
162 appropriate concentrations (100-300 nM), our lead compound **Azo5HT-2** is inactive in the dark and
163 becomes an effective agonist for 5-HT_{2A}R following illumination. While **Azo5HT-2** offers the advantage of
164 being based on the endogenous 5-HT ligand, the design employed likely provides a template for
165 azologization of other 5-HTR agonists, including psilocin and LSD which all contain a shared tryptamine
166 motif. Furthermore, this study establishes the proof-of-principle of photopharmacology for 5-HTRs and
167 should provide a basis for extension of this approach to other 5-HTR subfamilies, including those that are
168 $Gi_{/o}$ (5-HT_1Rs, 5-HT_5Rs) or G_s (5-HT_4R, 5-HT_6R, 5-HT_7R)-coupled.

169 Most importantly, our screen of azobenzene-conjugated 5-HT analogs lays the groundwork for
170 their proximity photopharmacology. BG-Azo5HT PORTLs enable reversible, repeatable optical control of

171 SNAP-5HT$_{2A}$R, opening the door to spatiotemporally precise and genetically-targeted control of this
172 biologically important receptor. As an intriguing possibility, the PORTL technique enables incorporation of
173 mutations to the SNAP-tagged receptor that alter transducer coupling (e.g. G protein versus arrestin) or
174 regulation (e.g. phosphorylation or scaffold sites) to test their roles in a biological context. This approach
175 has long-term potential for untangling the pleiotropic antidepressant, anxiolytic, anti-addictive and
176 hallucinogenic effects of 5-HT$_{2A}$R agonism. Finally, the establishment of a core PORTL for the 5-HT$_{2A}$R may
177 enable the application of next-generation PORTL approaches including branched PORTLs for dual imaging
178 and manipulation,[33] spectrally fine-tuned PORTLs[34] or PORTL-based strategies for targeting native
179 receptors.[44,45]

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181 **Associated Content**

182 The Supporting Information is available free of charge at [weblink].

183 Experimental details, NMR spectra, photophysical characterization, and supporting data on cellular
184 imaging and computational ligand docking studies.

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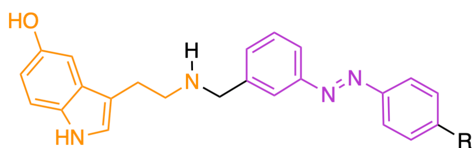
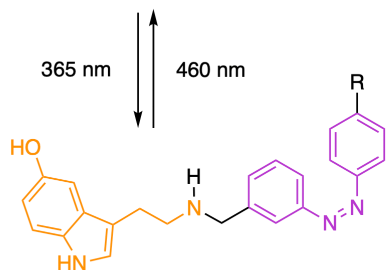
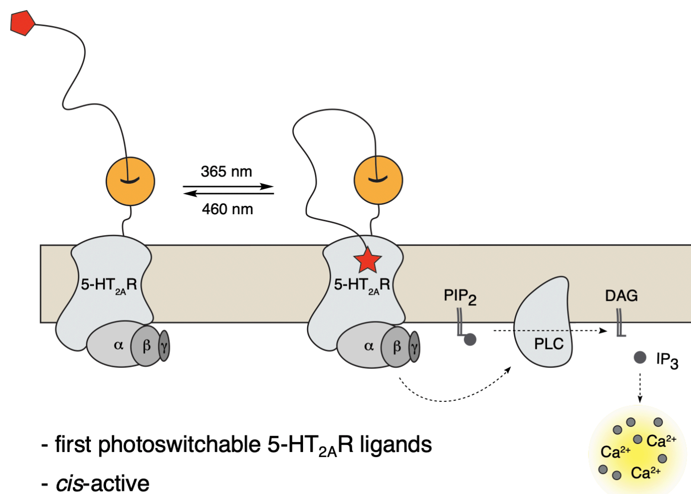
364 **TOC Figure**



365 nm 460 nm



R = H or Tether



- first photoswitchable 5-HT_{2A}R ligands
- *cis*-active
- 10-fold activity difference
- tethered variants for genetically-targeted control

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