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.
Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a neuromodulator that is released in the brain primarily by Dorsal Raphe Nuclei neurons, in the gut by enterochromaffin cells, and in blood platelet cells.\(^1,2\) Serotonin acts through a large family of G protein-coupled receptor (5-HT\(_1\)Rs, 5-HT\(_2\)Rs, 5-HT\(_3\)Rs, 5-HT\(_5\)Rs, 5-HT\(_6\)Rs, 5-HT\(_7\)Rs) and ion channel (5-HT\(_3\)Rs) subfamilies to regulate a plethora of neuronal and behavioral processes.\(^1\)

Given the importance of 5-HT to the regulation of mood, cognition and reward, great effort has been made to harness pharmacology to manipulate 5-HTRs for both basic study and therapeutic applications. Recent developments establishing the potential of 5-HT\(_2A\)R-targeting psychedelic drugs for the treatment of depression, anxiety, and addiction have further motivated the detailed study of 5-HTR signaling.\(^3\)–\(^7\) Despite great attention, limitations in the ability of 5-HTR-targeting compounds in terms of subtype-specificity and spatiotemporal precision and their inability to be targeted to genetically defined cell types have hindered progress toward a mechanistic understanding of the physiological and therapeutic effects of 5-HTR signaling.

As an alternative to classical pharmacology, photopharmacology has emerged as a means of gaining further precision through the development of photosensitive compounds whose activity can be modified depending on the wavelength of illumination\(^8\)–\(^10\). Photopharmacological compounds have enabled the optical control of a variety of GPCRs, including class A (\(\mu\)-opioid receptor,\(^11\) dopamine receptors,\(^12,13\) histamine receptors,\(^14\) adenosine receptors,\(^15\) muscarinic receptors,\(^16\) adrenergic receptors,\(^17\) fatty acid receptors,\(^18\) lysophospholipid receptors,\(^19,20\) and cannabinoid receptors\(^21,22\)), class B (glucagon-like peptide 1 receptor\(^23,24\)), and class C GPCRs (metabotropic glutamate receptors\(^25\)–\(^29\)). For further precision, including the ability to target the effects of light to genetically-defined cell populations, photopharmaceuticals may be covalently tethered to a genetically engineered receptor containing a labeling domain (i.e. SNAP, Halo) as Photoswitchable Orthogonal Remotely Tethered Ligands (PORTLs), as has been demonstrated with metabotropic glutamate receptors (mGluRs),\(^29,30\) mGluR-targeting PORTLs have been applied for both molecular biophysical studies\(^31\) and the in vivo manipulation of mGluR2 in specific cell types\(^32\)–\(^34\) in behaving 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\(^\text{35-38}\). 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\(^\text{29,30,39}\). 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\(_2\)-2, that enables optical control of 5-HT\(_2\)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\(_2\)-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\(_2\)aR, 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\(^\text{40,41}\) for the design of photoswitchable agonists based on a previously-reported covalent agonist of 5-HT2A (left).\(^\text{42}\)
The 5-HT receptor family is targeted by a variety of natural and synthetic agonists, including many with a tryptamine (indolamine) moiety (Fig. 1A). We considered several of these ligands for the design of photochromic agonists but reasoned that serotonin would be best suited because analogs would likely mimic endogenous signaling and derivatives which could be suited for incorporation of azobenzene motifs have been reported previously. These derivatives include covalent 5-HT analogs with an appended benzene ring (Fig. 1B). We considered an ‘azologization’ approach to install the azobenzene at the matching position (Azo5HT-3) and designed additional derivatives with the azobenzene moved one carbon atom closer to the pharmacophore (Azo5HT-1 and Azo5HT-2). The derivatives were synthesized through reductive amination of 5-HT with the corresponding azobenzene-aldehydes (Fig. 2 A,B). Photophysical characterization of Azo5HT-1 to Azo5HT-3 (Fig. 2 C,D and Fig. S1) revealed similar properties to classical azobenzenes. All derivatives could be reversibly switched to their respective cis and trans forms with UV-A (365 nm) and blue light (460 nm), respectively, and underwent slow thermal relaxation ($t_{1/2} > 1h$).

Figure 2. Synthesis and photophysical characterization of photoswitchable 5-HT derivatives Azo5HT-1-3. (A) Synthesis of Azo5HT series. (B) Chemical structures of Azo5HTs. (C) The UV-Vis spectra of Azo5HT-2 in the dark-adapted (black, trans), 365 nm adapted (grey, cis) and 460 nm adapted (blue, trans) photostationary states (50 mM, DMSO). (D) Reversible cycling between Azo5HT-2 photoisomers with alternating illumination at 365/460 nm (50 mM, DMSO).
To assess the ability of Azo5HT molecules to serve as 5-HT agonists, we tested each compound across the human 5-HT family (5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R). As these receptors are all G_{q}-coupled and produce intracellular Ca^{2+} release via phospholipase C-β activation, we performed live cell Ca^{2+} imaging with the fluorescent sensor, GCaMP6f. Using this assay, all three receptors showed the expected 5-HT responses with nM EC_{50} values (Table 1). Compounds Azo5HT-1-3 were tested independently either under standard conditions with 488 nm illumination for GCaMP6f excitation, which maintain them in the trans state, or with interweaved 385 nm illumination to convert them to the cis state. All three compounds showed dose-dependent activation of 5-HT_{2A}R in the trans and cis states with a leftward shift in the cis state (Fig. 3A; Fig. S2). For Azo5HT-1 there was a ~2-fold shift, while a larger 5-10-fold shift was seen for Azo5HT-2 and Azo5HT-3 (Table 1). It’s worth noting that 385 nm likely does not maximally occupy the cis state, so the relative difference between cis and trans may be underestimated using this approach. In contrast to the 5-HT_{2A}R, no or very modest differences were observed between cis and trans for each molecule on 5-HT_{2B}R and 5-HT_{2C}R (Table 1; Fig. S2).

**Figure 3.** Photoactivation of 5-HT2AR by Azo5HT-2. (A) Dose-response curves for Azo5HT compounds showing enhanced agonism for cis versus trans for all compounds using a Ca^{2+} imaging assay. (see Table
Schematic of Azo5HT-2 mediated optical control. (C) Representative Ca\(^{2+}\) imaging traces showing photoactivation of 5-HT\(_{2A}\)R by Azo5HT-2. In the absence of Azo5HT-2, no 385 nm light response is seen but a clear response is seen in the presence of 300 nM Azo5HT-2 with similar on and desensitization kinetics compared to 5-HT application (see Fig. S4).

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

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

Motivated by our docking and Boc-Azo5HT-2 test compound analysis, we designed PORTLs with the goal of enabling tethered optical control of SNAP-5HT\(_{2A}\)R (Fig. 4D). To this end, BOC-Azo5HT-2 was deprotected and various PEG-linkers and benzyl guanine (BG) were attached through successive amide 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-HT₂AR ("SNAP-5HT₂AR") (Fig. S7A, B). We then tested the ability of each PORTL to produce optically-evoked Ca²⁺ responses following conjugation to SNAP-5HT₂R and 385 nm illumination. Reversible and repeatable 385 nm light-evoked Ca²⁺ transients were seen with all 3 PORTL variants in 10-40% of cells with a higher proportion of cells showing photoactivation with BG-Azo5HT₂₄ and BG-Azo5HT₁₂ compared to BG-Azo5HT₆ (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-Azo5HT₂₄ and smaller for shorter variants (Fig 4F). Importantly, 385 nm light responses were blocked by the 5-HT₂R antagonist ketanserin (Fig. S7F). A subset (<10%) of cells showed Ca²⁺ 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-HT₂AR and provide a strong foundation for both further engineering and application.
Figure 4. Docking and test compound analysis enable PORTL development. (A-B) Docking analysis showing that both 5-HT (A) and the 5-HT moiety of Azo5HT-2 (B) show identical poses, with the azobenzene moiety occupying a water-filled cavity at the extracellular face of 5-HT2A receptor. Residues associated with canonical 5-HT binding are highlighted in (A) and position T134, which was previously substituted for conjugation of a covalent 5-HT2A agonist, is highlighted in (B). Top view (B, right) shows that the para position (yellow) is positioned facing toward the extracellular solution. C) Chemical structure (top; para position circled in yellow) and dose response curve (bottom) showing light-dependent (purple=385 nm illumination; green=488 nm illumination) activation of 5-HT2A receptor by Boc-Azo5HT-2. (D) Chemical structure, top, and schematic, bottom, of BG-Azo5HT, PORTL-mediated optical control of SNAP-tagged 5-HT2AR. (E-F) Representative traces (F) and summary bar graph (G) showing photoactivation of SNAP-5-HT2AR by BG-Azo5HT, PORTLs. The numbers of cells analyzed are shown in parentheses. * indicates statistical significance (1-way ANOVA with Tukey-Kramer Multiple Comparisons; p=0.0068 for BG-Azo5HT6 vs. BG-Azo5HT12, p<0.0001 for BG-Azo5HT6 vs. BG-Azo5HT24, p=0.036 for BG-Azo5HT12 vs. BG-Azo5HT24).

In summary, we have developed first-in-class photoswitchable analogs of serotonin that allow for the optical control of 5-HT2AR. Interestingly, all three test compounds, Azo5HT1-3 showed preferential agonism in cis over trans on the 5-HT2AR, but no clear difference between states on the 5-HT2AR or 5-HT2C receptor, providing a powerful chemical lead for further molecular pharmacological analysis. At the appropriate concentrations (100-300 nM), our lead compound Azo5HT-2 is inactive in the dark and becomes an effective agonist for 5-HT2AR following illumination. While Azo5HT-2 offers the advantage of being based on the endogenous 5-HT ligand, the design employed likely provides a template for azologization of other 5-HTR agonists, including psilocin and LSD which all contain a shared tryptamine motif. Furthermore, this study establishes the proof-of-principle of photopharmacology for 5-HTRs and should provide a basis for extension of this approach to other 5-HTR subfamilies, including those that are Gi/o (5-HT1Rs, 5-HT5Rs) or Gs (5-HT4R, 5-HT5R, 5-HT7R)-coupled.

Most importantly, our screen of azobenzene-conjugated 5-HT analogs lays the groundwork for their proximity photopharmacology. BG-Azo5HT PORTLs enable reversible, repeatable optical control of
SNAP-5HT2\textsubscript{A}R, opening the door to spatiotemporally precise and genetically-targeted control of this biologically important receptor. As an intriguing possibility, the PORTL technique enables incorporation of mutations to the SNAP-tagged receptor that alter transducer coupling (e.g. G protein versus arrestin) or regulation (e.g. phosphorylation or scaffold sites) to test their roles in a biological context. This approach has long-term potential for untangling the pleiotropic antidepressant, anxiolytic, anti-addictive and hallucinogenic effects of 5-HT2\textsubscript{A}R agonism. Finally, the establishment of a core PORTL for the 5-HT2\textsubscript{A}R may enable the application of next-generation PORTL approaches including branched PORTLs for dual imaging and manipulation, spectrally fine-tuned PORTLs or PORTL-based strategies for targeting native receptors.\textsuperscript{44,45}

**Associated Content**

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

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

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Acknowledgment

We thank New York University for financial support. We thank Jordana Thibado for preliminary functional studies and SNAP-5HT_{2A}R cloning. NMR spectra were acquired using the TCI cryoprobe supported by the NIH (OD016343). J.M. thanks the New York University for a Margaret and Herman Sokol fellowship, and the NCI for a K00 award (4K00CA253758). G.R. is supported by the Weill Cornell Molecular Biophysics Training Grant (T32GM132081). A.P. gratefully acknowledges support from NSF grant BIGDATA: IA: Collaborative Research: In Situ Data Analytics for Next Generation Molecular Dynamics Workflows (NSF #1740990) and computational resources from (project BIP109) of the Oak Ridge Leadership Computing Facility under Contract DE-AC05-00OR22725. J.L. and D.T. are supported by an R61 (R61 DA051529) grant from NIDA. J.L. is supported by an R35 grant (R35 GM124731) from NIGMS, the Rohr Family Research Scholar Award and the Irma T. Hirschl/Monique Weill-Caulier Research Award. DT thanks the McKnight Endowment Fund for Neuroscience for a McKnight Memory and Cognitive Disorders Award.

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https://doi.org/10.1016/j.chembiol.2013.11.005.


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