

# Illuminating Phenylazopyridines To Photoswitch Metabotropic Glutamate Receptors: From the Flask to the Animals

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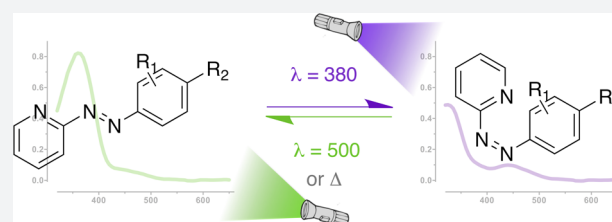
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## Supporting Information

**ABSTRACT:** Phenylazopyridines are photoisomerizable compounds with high potential to control biological functions with light. We have obtained a series of phenylazopyridines with light dependent activity as negative allosteric modulators (NAM) of metabotropic glutamate receptor subtype 5 (mGlu<sub>5</sub>). Here we describe the factors needed to achieve an operational molecular photoisomerization and its effective translation into *in vitro* and *in vivo* receptor photoswitching, which includes zebrafish larva motility and the regulation of the antinociceptive effects in mice.

The combination of light and some specific phenylazopyridine ligands displays atypical pharmacological profiles, including light-dependent receptor overactivation, which can be observed both *in vitro* and *in vivo*. Remarkably, the localized administration of light and a photoswitchable compound in the peripheral tissues of rodents or in the brain amygdalae results in an illumination-dependent analgesic effect. The results reveal a robust translation of the phenylazopyridine photoisomerization to a precise photoregulation of biological activity.



## INTRODUCTION

The therapeutic use of chemical compounds has historically driven medicine to exceptional achievements in the prevention and treatment of diseases.<sup>1</sup> Drug discovery is now a multidisciplinary activity in permanent motion to achieve new therapeutic challenges for unmet clinical needs. However, in spite of increasing R&D efforts, continuous technical progress, and outstanding scientific achievements, new drugs are scarce.<sup>2</sup> Although the reasons for this are multiple, drug discovery is facing problems associated with the increasing complexity of diseases and therapeutic targets, which require more precise therapeutics and can be connected to the lack of effective and truly innovative medicines.<sup>3</sup> Conventional pharmacology involves drug interaction with a target protein and the induction of changes in its functional activity to trigger the

therapeutic response. However, in practice after the drug is systemically administered to an organism, the precise control of its action at the target protein is lost.<sup>4</sup> Photopharmacology may provide solutions to this problem since it enables the spatiotemporal control of target proteins with light-regulated receptor-specific drugs.<sup>5,6</sup> In particular, light can restrain the drug action site and enable accurate dosing patterns<sup>5</sup> that can be adjusted in real-time mode.

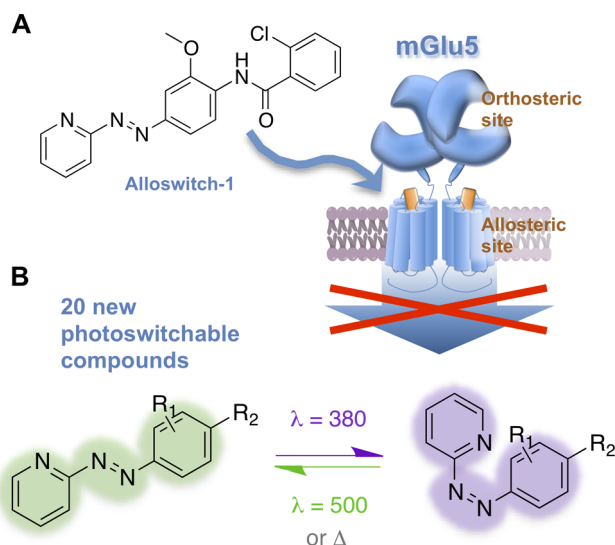
Photopharmacological strategies have proven successful in the regulation of free ligands of ion channels<sup>4,6</sup> and inhibitors of protein–protein interactions<sup>7</sup> but in many cases require genetic modification of the target receptor.<sup>8</sup> Genetic manipulation can

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be circumvented by drug azologization,<sup>9–11</sup> which is based on the insertion of azobenzene units into the chemical scaffold of existing ligands to obtain new photoswitchable molecules but maintaining the drug-like properties of the original ligand.<sup>9,10</sup> Some successful examples are bis-Q,<sup>12</sup> gluazo,<sup>13</sup> azo-propofols,<sup>14</sup> AzoTHA,<sup>15</sup> fotocaine,<sup>9</sup> JB253,<sup>16</sup> and PST-1.<sup>17</sup>

Recently, we reported on alloswitch-1 (**1a**)<sup>11</sup> (Figure 1A), a phenylazopyridine derivative as the first GPCR photoswitchable allosteric modulator with activity *in vivo*.



**Figure 1.** Design of the phenylazopyridine series. (A) Alloswitch-1 is a photoswitchable allosteric inverse agonist of mGlu<sub>5</sub>, in its *trans* configuration. (B) We designed and synthesized 20 photoswitchable derivatives of alloswitch-1, with the same phenylazopyridine scaffold. With violet light (380 nm) they switch from the thermodynamically stable *trans* isomer to the *cis* isomer and switch back to the *trans* isomer with green light (500 nm) or thermally, without illumination.

Instead of the classical photoisomerizable azobenzene, we used a structurally related phenylazopyridine, which includes

several potential advantages such as a better solubility and a faster thermal decay of the *cis* to the *trans* isomer enabling a potentially better spatiotemporal control of the activity of the compound. There are some drugs containing a phenylazopyridine scaffold, with different biological activities,<sup>18–24</sup> but they have not been described or exploited as photoswitchable entities or light-dependent drugs. In contrast, alloswitch-1 selectively exhibited a potent negative allosteric modulation (NAM) activity of mGlu<sub>5</sub> receptor, which belongs to the metabotropic glutamate (mGlu) GPCR family and controls important neuronal and glial functions.<sup>25</sup> Indeed, the *trans* isomer of alloswitch-1 inhibited mGlu<sub>5</sub> agonist response at nanomolar concentrations, whereas it was inactive in the *cis* configuration. Another phenylazopyridine (SIB-1757)<sup>26</sup> was previously reported as an mGlu<sub>5</sub> NAM with an IC<sub>50</sub> in the nanomolar range, but its photoswitching properties were never studied. Two other potent mGlu<sub>5</sub> NAMs, MPEP and XGS-RC-009, maintain a high structural resemblance to SIB-1757 and alloswitch-1, but they include a phenylethynylpyridine moiety instead of the phenylazopyridine<sup>27</sup> (Chart 1), maintaining similar mGlu<sub>5</sub> NAM activity. Taking advantage from this structural parallelism and as many potent mGlu<sub>5</sub> NAMs preserve the 2-arylethynylpyridine structure, such as MPEP, GRN-529, STX107, and Raseglurant<sup>28</sup> (Chart 1), we designed a family of potent mGlu<sub>5</sub> NAMs based on the 2-phenylazopyridine scaffold. With these compounds we intended to determine the molecular and photochemical features that define an efficient photoreversible ligand for operating in cells and living animals. We also investigated whether these molecules can be used to effectively control temporal dosing patterns with light in biological systems.

Interestingly, while exploring the photoswitching properties of these phenylazopyridines, we found that some compounds induced an overactivation of the receptor activity *in vitro* and increased animal motility *in vivo*. We also report on these light-dependent atypical pharmacological profiles.

**Chart 1.** mGlu<sub>5</sub> NAMs with 2-Arylethynylpyridine, SIB-1757, and Fenobam

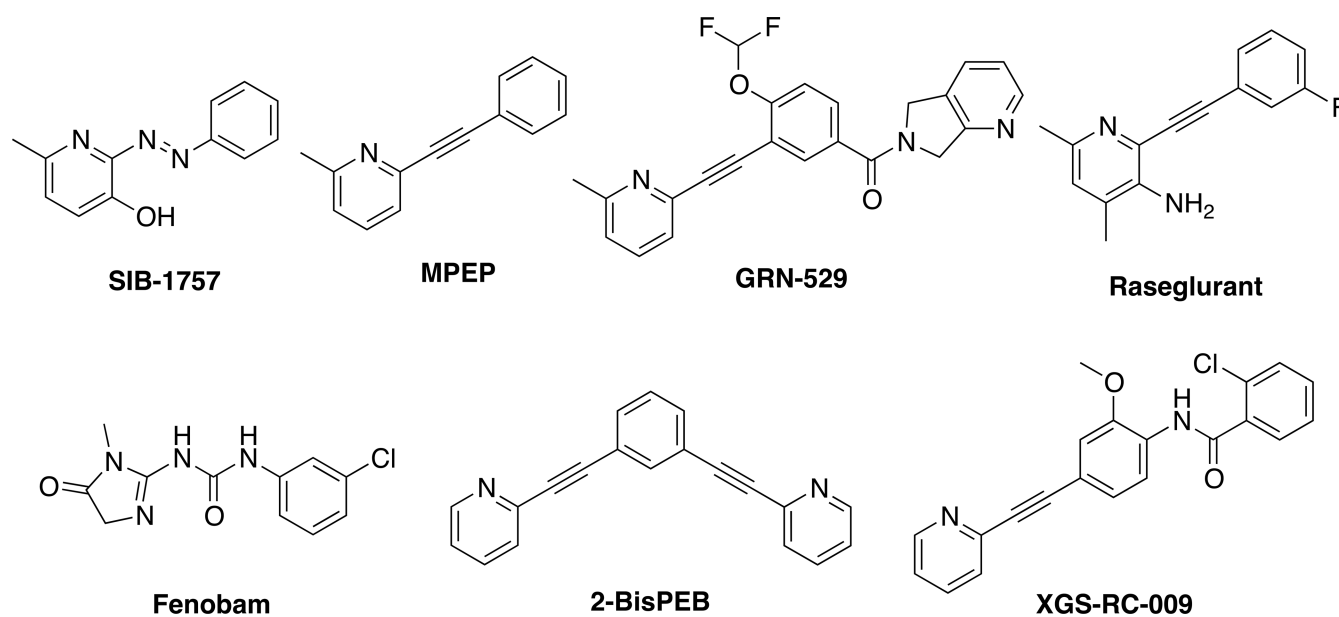


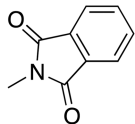
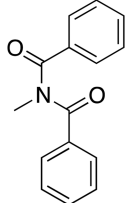
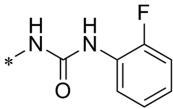
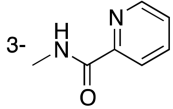
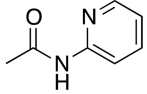
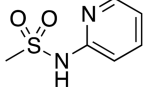
Table 1. Structure–Activity Relationship and Photoswitching Properties of the Phenylazopyridine Series<sup>a</sup>

Comp.	R <sub>1</sub>	Max abs /nm	illum λ /nm	PIS	t <sub>1/2</sub> / s	Dark IC <sub>50</sub> ± SEM/nM	PPS
1a		374	380	5	3	297±77	5.1
1b		372	380	4	3	904±82	7.1
1c		380	380	3	2	1884±44	1.7
1d		374	380	4	4	183±36	3.7
1e		374	380	4	3	30±4	3.7
1f		377	380	5	3	76±12	4.8
1g		377	380	4	3	180±28	4.1
1h		375	380	3	2	308±43	4.1
1i		377	380	4	3	328±18	2.5
1j		382	380	4	4	309±5	2.9
1k		373	380	1	1	356±57	1.3
1l		372	380	1	0	1025±257	1.2
2		366	380	5	4	4667±392*	2.5

Comp.	R	Max abs /nm	illum λ /nm	PIS*	t <sub>1/2</sub> / s	Dark IC <sub>50</sub> ± SEM/nM	PPS*
1j	3-OMe	382	380	4	4	309±5	2.9
3a	2-OMe	385	380	5	4	31661±3601	n.m.
3b	3-CN	348	380	4	0	2002±334	3.6
3c	H	358	380	5	4	3209±269	n.m.

Table 1. continued

Comp.	R <sub>2</sub>	R <sub>3</sub>	Max abs /nm	illum λ /nm	PIS	t <sub>1/2</sub> / s	Dark IC <sub>50</sub> ± SEM/nM	PPS
4	3-OMe		319	380	3 2	245	2496±32	3.2
5	3-OMe		329	380	3 2	46	1477±265	3.3
6	3-OMe		387	380	2 1	10	1204±220	4.4
				400	5 4	10	2184±663	12.3
7		H	309	380	2 1	62	14029±1826	1.5
8	H		326	380	1 0	-	7785±640	2.1
9	H		323	380	1 0	-	n.a.	

<sup>a</sup>PIS corresponds to photoisomerization score,  $t_{1/2}$  corresponds to the *cis* isomer thermal half-life, and PPS corresponds to photoinduced potency shift (see text).

## RESULTS AND DISCUSSION

**Initial Considerations.** In the effort of designing pharmacologically useful compounds for receptor activity photoswitching, we considered three necessary conditions: (a) biocompatible wavelengths of illumination for photo-switching, preferably in the visible range; (b) a significant difference on the relative populations of the isomers between the dark and illuminated conditions, which can be afforded with a suitable thermal relaxation of the *cis* isomer; and (c) a large difference in affinity/functional activity on the target protein between the *trans* and *cis* photoisomers.

Alloswitch-1 (**1a**) reasonably fulfilled these conditions and allowed us to control mGlu<sub>5</sub> activity with light and to induce light-dependent activity in behavioral experiments with living animals, as detailed in our previous communication.<sup>11</sup> In contrast, SIB-1757,<sup>26</sup> a potent phenylazopyridine mGlu<sub>5</sub> NAM, showed neither photoisomerization in solution nor light-induced receptor activity switch despite the structural analogy with alloswitch-1 in our hands (Supporting Information). This lack of detection of photoisomerization may be due to an azo-hydrazone tautomeric equilibrium induced by the hydroxyl of SIB-1757, which prompts a fast thermal relaxation of its *cis* isomers on the order of milliseconds<sup>29</sup> and suggests that not all the azobenzene ligands are well suited for controlling protein

activity with light. In addition, strong electron-donating substituents in phenylazopyridines induce a push–pull effect, since the pyridine acts as an electron-withdrawing group, and this leads to a further decrease of the *cis* isomer lifetime,<sup>30</sup> especially in polar solvents.<sup>31</sup> Overall, these results confirmed that the identification of an azocompound with a suitable pharmacological activity profile is a necessary but not sufficient condition to define a useful photoswitchable ligand.

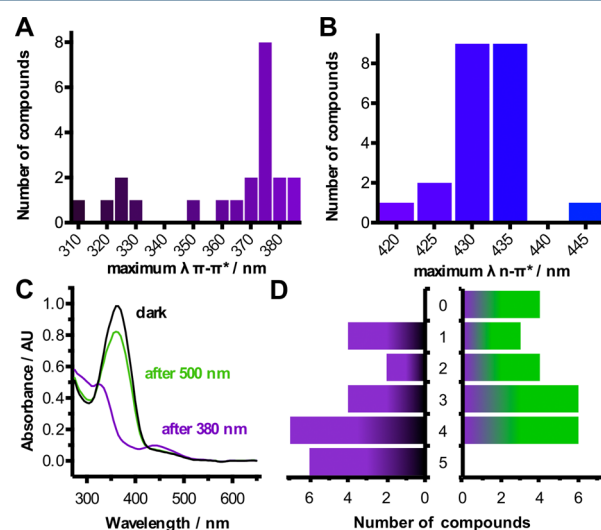
Therefore, to design compounds for mGlu<sub>5</sub> allosteric photoregulation, we maintained the amide bond present in alloswitch-1 for most of the compounds of the series and we explored the structural determinants for reaching effective receptor control with light, by changing the carboxamide substituents (Table 1). Thus, we synthesized (Supporting Information) more bulky derivatives (**1b,c**) and others with aliphatic amide substituents (**1d–f**), less bulky aromatic carboxamides (**1g,h**), or heterocyclic rings (**1i,l**) (Table 1).

To investigate the effect of the pyridine ring in alloswitch-1 (**1a**), we replaced it by a phenyl (**2**). We were also interested in the effects of the substitution in the central ring; therefore we changed the position of the methoxy group (**3a**) or replaced it by cyano (**3b**) or hydrogen (**3c**) substitutions.

Finally, we explored some changes on the amide group functionality, obtaining the imides **4** or **5** or the urea **6**. We also

explored the positional isomers **7** and **8** and also synthesized the sulfonamide **9**. All these groups were expected to confer different electronic and structural properties to the phenylazopyridine moiety, thereby altering their photoisomerization response.

**UV–Vis Absorption Spectra.** We measured the UV–vis absorption spectra of the *cis* and *trans* phenylazopyridine isomers after chromatographic separation with HPLC-PDA-MS (Supporting Information). The *trans* isomers presented typical light absorption for the  $\pi$ – $\pi^*$  band, in the range between 310 and 400 nm, even though for most of the phenylazopyridine compounds the maximum of this band was found around 380 nm (Figure 2A). The azobenzene *cis* isomers displayed a typical  $n$ – $\pi^*$  band with maxima in the range 420–450 nm (Figure 2B)



**Figure 2.** Spectroscopic and photoisomerization properties. (A, B) Distribution of wavelengths of absorption in the UV–vis spectrum of compounds in the series, detected by HPLC-PDA-MS (see Supporting Information). Maximum of the band corresponding to (A)  $\pi$ – $\pi^*$  transition for the *trans* isomers and (B)  $n$ – $\pi^*$  transition for the *cis* isomers. (C) Example of UV–vis absorption spectra corresponding to compound **3c**, 25  $\mu$ M in DMSO. The black line corresponds to the initial spectrum in dark conditions, the violet line to the spectrum after illumination at 380 nm, and the green line after illumination at 500 nm. Comparison of these curves with the spectra of pure *trans* and *cis* isomers, obtained from HPLC-PDA-MS analysis (see Supporting Information), results in a full conversion from *trans* to *cis* isomer and near complete back-isomerization from *cis* to *trans* isomer. (D) Distribution of photoisomerization scores (PIS) for phenylazopyridines under illumination at 380 nm (violet bars) and at 500 nm (green bars) (see text and Supporting Information).

The wavelength of the maximum of these bands fundamentally depends on the nature of the substituents on the aromatic rings of the azocompound,<sup>32</sup> in particular electron-donating substituents in *ortho* or *para* position.<sup>32</sup> However, in our cases, the 2-pyridyl group, which is a  $\pi$ -electron-deficient heterocycle, induces a certain push–pull character to the azobenzene, especially in polar solvents.<sup>29</sup> Then, to achieve a proper  $\pi$ – $\pi^*$  band suitable for our interests, mild electron-donating substituents are needed to shift the maximum to longer wavelengths. An example of such is the high electron-withdrawing sulfonamide of compound **9** shifting the band to energetically higher wavelengths (310 nm), which are not suitable for biological experiments. On the other hand, the urea from compound **6**, having a more electron-donating character

than the amides, shifts the band to the visible range of the spectrum (387 nm). Moreover, we found a noticeable correlation between this shift in the  $\pi$ – $\pi^*$  band and the electron density of the azo moiety (Supporting Information).

**Photoisomerization Studies.** To measure the extent of photoisomerization, we measured the UV–vis absorption spectra of all compounds in the dark, after illumination with violet light ( $\lambda = 380$  nm) and after illumination with green light ( $\lambda = 500$  nm) under light conditions similar to those used in pharmacological experiments (Figure 2C, Supporting Information). In the dark, we practically detected only the *trans* isomer. We selected 380 nm illumination (violet) to induce the *trans* to *cis* isomerization, according to the position of the *trans* isomer  $\pi$ – $\pi^*$  bands and the *cis* isomer  $\pi$ – $\pi^*$  and  $n$ – $\pi^*$  (Supporting Information). After that, we used green light ( $\lambda = 500$  nm) to accelerate the opposite isomerization to the thermodynamically stable *trans* isomer.

With the information obtained from these spectra we calculated a “photoisomerization score” (PIS), to account for the effectiveness of the photoswitch and compare the molecules (5, efficient switching; 0, poor switching, see Supporting Information). A large part of the compounds of the series have photoisomerization efficiency similar to or better than that of alloswitch-1 (**1a**) (Table 1, Figure 2D). However, in some cases, compounds with a similar structure, such as pyridylamides **1k** and **1l**, have a very poor photoswitching behavior, possibly due to the fast thermal decay from the *cis* to the *trans* isomer (Table 1, Supporting Information). Moreover, we found a trend correlation in between the photoswitching effectiveness and the thermal relaxation rate (Supporting Information).

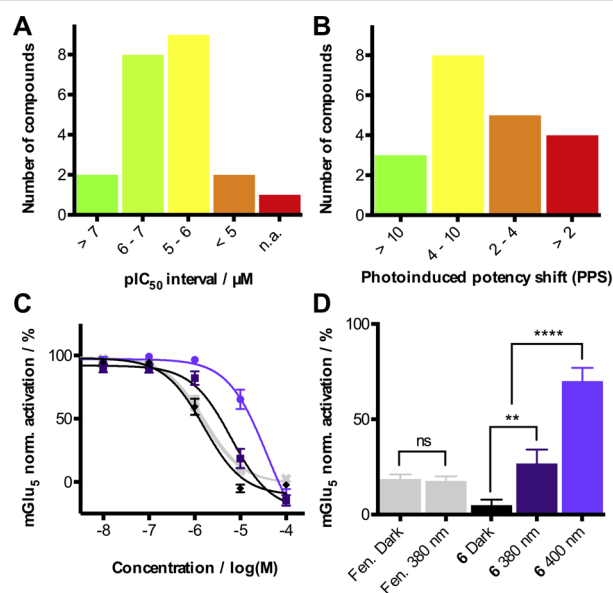
Additionally, the efficacy of the observed photoisomerization also depends on the selection of a suitable wavelength. For example, some compounds such as **2** or **3c**, despite having the maximum of absorption at 366 and 358 nm respectively, display the maximal absorbance difference between the *cis* and *trans* isomers around 380 nm (375 and 370 nm, respectively). This indicates that the optimal wavelength for illumination does not necessarily correspond to that of maximum absorption, but to the wavelength that maximizes the difference of absorption between the *trans* and the *cis* isomers (Figure 2C). An illustrative example is the urea **6**, which has its  $\pi$ – $\pi^*$  band maximum at 387 nm and a maximal *cis*–*trans* absorbance difference at 395 nm. This compound has a PIS of 2 when illuminated at 380 nm, but PIS reaches up to 5 at 400 nm achieving a practically complete isomerization, despite the fast relaxation of the *cis* isomer ( $t_{1/2} = 10$  s).

In summary, we have shown that compounds like alloswitch-1, **1f**, **2**, **3a**, **3c**, or **6** have photoisomerization properties that make them suitable potential candidates for mGlu<sub>5</sub> photoswitching applications. However, other compounds like **7**, **8**, or **9** require harmful short UV wavelengths to achieve a proper photoisomerization whereas some others such as **1k**, **1l**, or SIB-1757 have too fast *cis*–*trans* relaxation rates that limit their practical use in biological applications.

**Pharmacological Activity on mGlu<sub>5</sub> Receptor in Cell-Based Assays.** To study the activity of the phenylazopyridines, we generated dose–response curves of the complete series (IP accumulation assay with HEK293 expressing mGlu<sub>5</sub> receptor) to quantitatively evaluate the antagonistic activity of the series to quisqualate. We excluded compound **9**, which was found inactive in an initial screening (Supporting Information). To evaluate the light-dependent effects, we simultaneously generated two curves for each compound, by incubating the

cells with the azocompounds in the dark and under illumination at 380 nm (Supporting Information). As a result, we obtained the potencies ( $IC_{50}$ ) for each compound under both conditions. Exceptionally, compound **6** was also tested under illumination at 400 nm.

In the dark, the phenylazopyridines displayed  $IC_{50}$ s in the range from low nanomolar to micromolar (Table 1 and Figure 3A). The aliphatic carboxamides **1e** and **1f** displayed low



**Figure 3.** Pharmacological properties. (A) Distribution of compounds with the  $IC_{50}$  values obtained with an IP accumulation assay, with fenobam as the control. Approximately one-half of the compounds have potencies in the nanomolar range in dark conditions, and most of the compounds are more potent than fenobam ( $IC_{50} = 1.6 \mu M$ ). (B) Distribution of compounds with the photoinduced potency shift (PPS) for the phenylazopyridines tested (see text). (C) IP accumulation dose–response curve of compound **6** with 100 nM of quisqualate in dark conditions (black), under illumination at 380 nm (dark violet), under illumination at 400 nm (bright violet). Gray curves correspond to the inverse agonist control (fenobam). (D) Percentage of activation of the  $mGlu_5$  receptor with 10  $\mu M$  **6** and 100 nM quisqualate. Analysis of variance (one-way ANOVA with Šidák correction for multiple comparisons;  $**p < 0.01$ ,  $****p < 0.0001$ ) showed significant differences between dark bar and 380 nm values, and also of 400 nm values with both dark and 380 nm values.

nanomolar  $IC_{50}$  (nearly 10- and 4-fold more potent than alloswitch-1 **1a**). Some other compounds were found to display potencies comparable to that of alloswitch-1, such as **1d** and **1g–k**, whereas **1b**, **1c**, **1l**, **3b**, **3c**, **4**, **5**, and **6** displayed micromolar  $IC_{50}$ , and some other even lower potencies (**3a**, **7**, and **8**). Singularly, compound **2**, a derivative of alloswitch-1 where the phenylazopyridine scaffold is replaced by an azobenzene, only partially inhibits the response of  $mGlu_5$  (Supporting Information) despite its micromolar potency. A plausible explanation for this different behavior might be attributed to a different binding mode to the receptor, which would prevent the full antagonistic activity found for most of the phenylazopyridines. To investigate this point we performed a computational docking study for compounds **2**, **1e**, and **1f** in an  $mGlu_5$  crystal structure.<sup>33</sup> The results obtained showed different binding modes for the azobenzene **2** compared to phenylazopyridines **1e** and **1f**, which resulted to be very similar to the docking previously reported for alloswitch-1 (**1a**).<sup>34</sup>

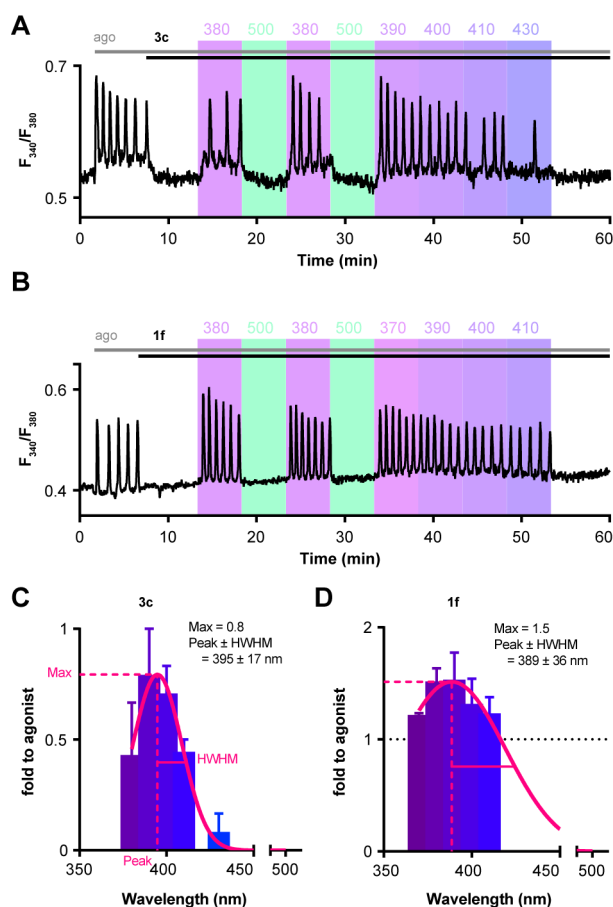
These differences in the binding modes might affect  $mGlu_5$  functionality despite not affecting considerably the binding energies, which are directly related to the affinity to  $mGlu_5$  receptor (Supporting Information).

In these assays, the illumination at 380 nm induced a right shift of the dose–response curves when compared to the nonilluminated controls, compatible with a loss of the NAM potency of the *cis* isomers. Therefore, to quantify the effectiveness of the compound photoswitching, we introduced the term “photoinduced potency shift” (PPS) as the ratio of the  $IC_{50}$ s under illumination and in dark conditions. We obtained a wide variety of PPS (Table 1, Figure 2B), which are not correlated with the potency of each compound (Supporting Information), but have a significant correlation with the PIS ( $r = 0.77$ ,  $P < 0.0001$ ), extracted from the UV–vis absorption spectra (Figure 2A,B). This would indicate that the functional photoswitching of the receptor in living cells is similar to that observed in solution for the isolated compounds (Supporting Information).

We found very potent compounds with high PPS, such as **1e** and **1f** (PPS (**1e**) = 3.7 and PPS (**1f**) = 4.8), but the higher value of PPS of compound **1f** defines it as a stronger candidate to control  $mGlu_5$  activity with light. In addition, we could find interesting compounds with  $IC_{50}$ s in the micromolar range and very high PPS, such as **1b**, **3c**, or **6**. Specifically, compound **6** displayed large photoswitching capacities when it was illuminated at 400 nm (PPS = 12.3), much better than those observed at 380 nm (PPS = 4.4) (Figure 3C). Again, these results are consistent with the wavelength dependence observed in the *trans*–*cis* isomerization experiments of compound **6**. This effect was further quantified at a single 10  $\mu M$  dose of **6** in the same cell-based assay (Figure 3D). In the dark, compound **6** induced a practically complete inhibition of  $mGlu_5$ , and under 380 nm light, it still induced a 75% inhibition. However, when 400 nm light was applied, the inhibition was reduced to 25%, compatible with the improved photoisomerization at this wavelength, as shown above. This particular experiment opens the possibility of inducing wavelength-dependent effects to fine-control the activity of the receptor with light, which in conventional pharmacology would require applying different concentrations of ligand.

**Real-Time Photoswitching in Individual Cells.** We next evaluated the possible application of the observed photoswitching to a reversible real-time control of  $mGlu_5$  receptor. Hence, we selected the most representative phenylazopyridine compounds and tested their effects by time course of intracellular calcium in individual HEK293 cells overexpressing  $mGlu_5$  receptor. In the dark, all tested compounds antagonized the calcium oscillations evoked by the activation of  $mGlu_5$  with quisqualate, although the inhibition with **5** and **6** was not complete, in accordance with the low potency found in IP accumulation assays (Table 1, Supporting Information).

After a period in the dark, illumination with 380 nm light induced the photoisomerization of the phenylazopyridines and reestablished the calcium oscillations observed in response to the orthosteric agonist (Figures 4AB). This effect was reversed by green illumination and could be repeated over two light cycles for all compounds, demonstrating a reversible photo-switch in the cellular assays as well as a real-time control of the biological activity of  $mGlu_5$  receptor with light (Figures 4AB). For some compounds we observed that, under 380 nm light, the frequency of calcium oscillations was increased compared to that observed with the orthosteric agonist alone (Figure 4B),

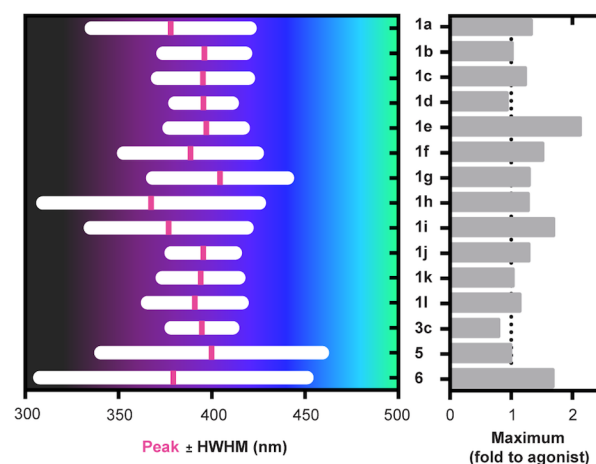


**Figure 4.** Calcium imaging in individual cells illustrates the light-dependency of mGlu<sub>5</sub> inhibition by compounds **1f** and **3c**. (A, B) Fluorescence ratio ( $F_{340}/F_{380}$ ) over time of calcium indicator Fura-2 loaded in mGlu<sub>5</sub>-expressing HEK293 cells. Cells were challenged with an mGlu<sub>5</sub> agonist (ago, gray line, 3  $\mu$ M quisqualate), 1  $\mu$ M **3c** (A) or 1  $\mu$ M **1f** (B) (black lines), and different illumination wavelengths (six, ranging between 370 and 500 nm) indicated by color boxes and corresponding numbers above. (C, D) Quantification of the light-induced receptor activity in the presence of **3c** (C) or **1f** (D) at indicated illumination wavelengths. Data are presented as mean  $\pm$  SEM of the normalized calcium oscillation frequency. The frequency of calcium oscillations during an illumination period (5 min) was calculated as number of oscillations per minute, and normalized to the initial response to the agonist. Peak, half-width at half-maximum (HWHM), and maximum (Max) values were inferred by fitting the data shown in the graph to a Gaussian function (magenta curve).

suggesting an overactivation or an increased signaling of mGlu<sub>5</sub> receptors.

After violet–green light cycles, we additionally illuminated the cells with several wavelengths around 380 nm, to identify the best wavelength for restoring mGlu<sub>5</sub> activity (Figure 4AB). The oscillatory frequencies for each illumination period were normalized to the response to the agonist alone, plotted versus the wavelength used for illumination, and we fitted a Gaussian curve (Figures 4CD, Supporting Information). These results demonstrate that phenylazopyridines not only are able to switch *on* and *off* the mGlu<sub>5</sub> receptor upon green and violet illumination wavelengths but also can induce a fine-tuning of its activity. In addition, each compound of the series depends on different wavelengths for optimal photoswitching (peak), has broader or sharper “functional” absorption bands (HWHM), and presents varying degrees of receptor overactivation (Figure

5). For example, compound **1e**, which was found to be the most potent in the IP accumulation assay, is the most effective



**Figure 5.** Summary of wavelength–activity relationships in single-cell experiments. In the left graph, magenta bars indicate the illumination wavelength (peak) at which the maximum light-induced receptor activity is obtained for the compounds indicated. White bars represent the range of wavelengths at which the light-induced activity is equal to half the maximum response or more (half-width at half-maximum, HWHM). The maximum amplitude of the light-induced receptor activity is reported in the graph on the right, and expressed as times the response to agonist of the naive receptors. These three parameters (peak, HWHM, and maximum) describe the Gaussian fitting performed for all compounds on the original data from single-cell experiments, as exemplified for **1f** and **3c** in Figure 4C,D. Original data were obtained from calcium imaging experiments in individual cells (done as described in Figure 4A,B).

in inducing an overactivation under violet illumination (>2-fold to agonist activation), whereas compound **3c** only recovered the response to quisqualate alone (Figure 5). The spectral range to achieve this activation also depends on the compound. Some have a narrow range, such as **1d** or **3c**, whereas others display a wider wavelength range, as **1h**, **5**, or **6**.

A related control of the frequency of mGlu<sub>5</sub> calcium oscillations was previously reported applying different concentrations of a conventional NAM<sup>35</sup> in different cell cultures. In contrast, the use of photoswitchable NAMs allows adjusting the effective ligand concentration by illuminating a single dose with specific wavelengths. This suggests a way to regulate the receptor response with a high spatiotemporal precision.

**Zebrafish *in Vivo* Screening.** We finally selected the active phenylazopyridines with a suitable efficacy in IP accumulation assays and tested on zebrafish larvae behavioral assays.

The activity of all compounds was first evaluated by monitoring zebrafish locomotion in dark conditions. Thus, after 10  $\mu$ M administration of each compound in the swimming medium, we recorded the larva track during 30 min and the free-swimming distance of every animal was integrated at 5 min intervals (Figure 6B, Supporting Information). As a non-photoswitchable control, we used 2-BisPEB, which is a potent NAM of mGlu<sub>5</sub><sup>36</sup> because fenobam, previously used in cell-based assays, did not show significant differences with the vehicle. Otherwise, 2-BisPEB significantly inhibited the zebrafish motility, in line with the best phenylazopyridines of this series, such as **1a** and **1f** (Figure 6B).

In line with their mGlu<sub>5</sub> NAM activities, all the tested phenylazopyridines induced inhibition of the zebrafish motility



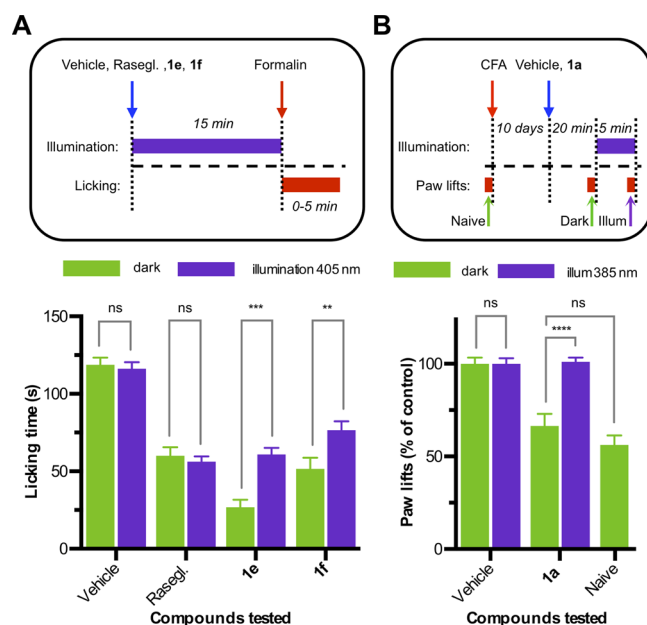


present, its electron density is low due to the presence of electron-withdrawing groups, like fluorine, or they have pyridines, which are intrinsically electron poor. Compound 7 constitutes a particular case, since it also induces overactivation under illumination and includes a 2-pyridylcarboxamide ring as the third aromatic ring. However, it is the only compound tested with 1,3 substitution in the central ring, which is expected to induce a lower influence on the  $\pi$  electron density of the azo bond. Intriguingly, not all the compounds that induce this overactivation do it in real-time cell assays. For example, compounds **1e**, **1f**, and **1i**, with a higher overactivation effect in cell-based assays, did not induce a measurable overactivation in animal motility. In contrast, **1b**, unable to produce the receptor overactivations in cell-based assays, induced the highest overactivation *in vivo*. This may be due to differences in these two assays, which use different illumination protocols and test mammalian and zebrafish mGlu<sub>5</sub> receptors respectively, whose functionalities can differ considerably. In any case, this atypical effect appears to be light dependent, could only be detected with photoswitchable compounds, and will be subject of future studies.

Regarding the *in vivo* photoswitchable properties of the phenylazopyridines, all of them induce photoswitching in zebrafish motility (Figure 6D) to different extents. The compounds that promoted an overactivation of the animal motility under violet light show higher statistical significances in their photoswitching. Even so, differences for the rest of compounds were still significant, except for compound **1d**, whose *in vivo* activity was abnormally weak, and **1k** and **1l**, which also performed poorly in cell assays (PPS(**1k**) = 1.3, PPS(**1l**) = 1.2), probably as a consequence of their limited intrinsic low photoisomerization capacity (PIS<sub>380</sub>(**1k**) = 1, PIS<sub>500</sub>(**1k**) = 1; PIS<sub>380</sub>(**1l**) = 1 PIS<sub>500</sub>(**1l**) = 0). In fact, if compound **1d** and the compounds with overactivation behavior are not taken into account, the difference between the violet and dark values of each compound in Figure 6D correlates with photoisomerization score (PIS) ( $r = 0.70$ ,  $P = 0.025$ ) and with photoinduced potency shift (PPS) logarithm ( $r = 0.83$ ,  $P = 0.0031$ ) (Supporting Information), showing a remarkable consistency of the photoisomerization throughout the different assays, from compound in solution to cell-based *in vitro* assays and *in vivo* behavioral experiments.

**Rodent *in Vivo* Pain Assay.** Next, we aimed to assess photoswitching in rodent tissue. We used a classical animal model of pain based on the intraplantar administration of formalin (Supporting Information) since local plantar blockade of group I mGluRs was reported to reduce the spontaneous formalin behavior.<sup>37</sup> Thus, we monitored the local efficacy of compounds **1e** and **1f** to modify the formalin-mediated nociception in dark and upon illumination with violet light (Figure 7A). As expected, both compounds showed antinociceptive properties in dark conditions since the mouse licking paw time was significantly reduced when compared to the vehicle condition (Figure 7A).

Interestingly, compounds **1f** and **1e** were equipotent or even more effective, respectively, when compared to the mGlu<sub>5</sub> NAM reference compound used (raseglurant, Chart 1, Figure 7A). Importantly, the intrinsic antinociceptive efficacy of both compounds was significantly reduced upon external paw illumination with violet light, thus suggesting that local photoisomerization was achieved while monitoring nociception (Figure 7A).



**Figure 7.** Photoswitching of pain-like behavior in mice. (A) Local photoswitching in the mouse hindpaw. The mouse hindpaw was injected with vehicle (20% DMSO + 20% Tween-80 in saline), 5 mM raseglurant (Rasegl.), 5 mM compound **1e** or 5 mM compound **1f** and then illumination at 405 nm (or dark) during 15 min was performed ( $n = 6$  for each condition). Subsequently, the total hindpaw licking (in seconds) was measured during 5 min after the intraplantar injection of formalin solution (2.5% paraformaldehyde). The scheme of the protocol used is depicted in the upper inset. Analysis of variance (one-way ANOVA with Šidák correction for multiple comparisons; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) showed significant differences between dark and 405 nm values for phenylazopyridines **1e** and **1f**, but not for vehicle and raseglurant. (B) Local photoswitching in mouse amygdala. Persistent inflammatory pain was induced by unilateral intraplantar injection of 30  $\mu$ L of complete Freund's adjuvant (CFA) in the left hind paw. Mechanical allodynia was measured by stimulating the CFA-treated hindpaw with a 1.4 g von Frey filament after intra-amygdala injection of vehicle (0.003% DMSO in PBS,  $n = 11$ ) or compound **1a** (300 nM,  $n = 13$ ) in dark condition and with amygdala illumination at 385 nm. Naive-mouse mechanical sensitivity was measured before CFA injection ( $n = 11$ ). The scheme of the protocol used is also depicted in the upper inset. Analysis of variance (one-way ANOVA with Šidák correction for multiple comparisons; \*\*\*\* $p < 0.0001$ ) showed significant differences between dark and 385 nm values for **1a** but not for vehicle and showed nonsignificant differences between the naive mice and those treated with **1a** with no illumination.

We next examined the *in vivo* phenylazopyridine photoswitching in mouse CNS, since mGlu<sub>5</sub> receptor is expressed in amygdala and its activation is related to pain-like effects.<sup>38</sup> We evaluated the mechanical allodynia in a CFA-induced persistent inflammatory pain model by using the von Frey technique (Supporting Information). Mice were stereotaxically implanted with hybrid optic and fluid cannulas in the amygdala allowing the controlled administration of compounds and light in freely behaving animals. Amygdala injection of alloswitch-1 (**1a**) restored the mechanical sensitivity to the level of naive mice (before inflammation), compatible with the *trans* active form of the compound (Figure 7B). The observed analgesic effect was abolished after amygdala illumination at 385 nm (Figure 7B). That suggests that the local photoisomerization of **1a** from *trans* to *cis* configuration in the amygdala can control the analgesic effect of the compound in peripheral tissues. In

contrast, 385 nm illumination induced no effect with vehicle conditions (Figure 7B).

These results confirm that phenylazopyridines can be used for local control of mGlu<sub>5</sub> with light in behaving rodents, both peripherally and in the CNS, opening uses of photopharmacology in disease models and behavioral assays.

## CONCLUDING REMARKS

We synthesized a series of photoswitchable mGlu<sub>5</sub> NAMs based on the phenylazopyridine scaffold, with a robust SAR, since almost all the *trans* isomers were active on mGlu<sub>5</sub> and half of them significantly inhibited zebrafish motility at 10 μM concentration. We obtained compounds with different photoisomerization properties, and their translation to *in vitro* and *in vivo* assays was also robust, since very few compounds lost their photoswitching properties in any of the different subsequent assays in spite of the increasing complexity of the biological tests. From these compounds, we obtained excellent photoswitching behavior in all the assays performed with compounds **1a**, **1f**, **1i**, **1j**, and **3c** as well as **6**, in which we afforded a more powerful effect at more cell-compatible blue-shifted wavelengths. To account for the efficiency in the photoswitching we have proposed two parameters: the photoisomerization score (PIS) and the photoinduced potency shift (PPS).

Additionally, these phenylazopyridines allowed us to define a way to perform a fine control of mGlu<sub>5</sub> receptor activity by tuning the wavelength of illumination between 360 and 500 nm, and, depending on the compound used, we were able to control an overactivation of the receptor. This functional overactivation was also detected *in vivo* after treating zebrafish larvae with some specific compounds, but not with others. The reasons for this light-dependent atypical effect could be linked to the potentiation of calcium responses in single cell assays and require further investigation. Finally, mGlu<sub>5</sub> NAM phenylazopyridines showed analgesic effects in rodents, which can be regulated by peripheral violet illumination or directly in the CNS, thus validating the potential usefulness of allosteric mGlu<sub>5</sub> photopharmacology *in vivo*. Overall, photoswitchable phenylazopyridines reveal new paradigms on the ligand-induced protein responses with potential to add a new modulatory dimension to drug therapeutics.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acscentsci.6b00353](https://doi.org/10.1021/acscentsci.6b00353).

Experimental details, chemical and photo- and biochemical characterization of **1–9**, and computational details (PDF)

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## Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

GPCR, G protein-coupled receptor; mGlu<sub>5</sub>, metabotropic glutamate receptor subtype 5; NAM, negative allosteric modulator; HPLC-PDA-MS, high performance liquid chromatography coupled to photodiode array detector and a mass spectrometer; UV-vis, ultraviolet-visible; HEK293, human embryonic kidney 293; IP, myo-inositol phosphate; ANOVA, analysis of variance; SAR, structure-activity relationship; CNS, central nervous system; CFA, complete Freund's adjuvant

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