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Glutamate Receptors: From the Flask to the Animals

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

Xavier Gómez-Santacana, Silvia Pittolo, Xavier Rovira, Marc Lopez, Charlene Zussy, James A. R. Dalton, Adèle Faucherre, Chris Jopling, Jean-Philippe Pin, Francisco Ciruela, Cyril Goudet, Jesús Giraldo, Pau Gorostiza, and Amadeu Llebaria

INTRODUCTION

The therapeutic use of chemical compounds has historically driven medicine to exceptional achievements in the prevention and treatment of diseases. 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. 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. 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. Photopharmacology may provide solutions to this problem since it enables the spatiotemporal control of target proteins with light-regulated receptor-specific drugs. In particular, light can restrain the drug action site and enable accurate dosing patterns that can be adjusted in real-time mode.

Photopharmacological strategies have proven successful in the regulation of free ligands of ion channels and inhibitors of protein–protein interactions but in many cases require genetic modification of the target receptor. Genetic manipulation can

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 (mGlu5). 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.

Supporting Information

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be circumvented by drug azologization,9−11 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.9,10 Some successful examples are bis-Q,12 gluazo,13 azo-propofols,14 AzoTHA,15 fotocaine,9 JB2S3,16 and PST-1.17

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

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,18−24 but they have not been described or exploited as photo-switchable entities or light-dependent drugs. In contrast, alloswitch-1 selectively exhibited a potent negative allosteric modulation (NAM) activity of mGlu5 receptor, which belongs to the metabotropic glutamate (mGlu) GPCR family and controls important neuronal and glial functions.25 Indeed, the trans isomer of alloswitch-1 inhibited mGlu5 agonist response at nanomolar concentrations, whereas it was inactive in the cis configuration. Another phenylazopyridine (SIB-1757)26 was previously reported as an mGlu5 NAM with an IC50 in the nanomolar range, but its photoswitching properties were never studied. Two other potent mGlu5 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 phenylazopyridine27 (Chart 1), maintaining similar mGlu5 NAM activity. Taking advantage from this structural parallelism and as many potent mGlu5 NAMs preserve the 2-arylethynylpyridine structure, such as MPEP, GRN-529, STX107, and Rasegurant28 (Chart 1), we designed a family of potent mGlu5 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.

Figure 1. Design of the phenylazopyridine series. (A) Alloswitch-1 is a photoswitchable allosteric inverse agonist of mGlu5 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.

Chart 1. mGlu5 NAMs with 2-Arylethynylpyridine, SIB-1757, and Fenobam
Table 1. Structure–Activity Relationship and Photoswitching Properties of the Phenylazopyridine Series

| Comp. | R₁          | Max.abs /nm | illum λ /nm | PIS * | t₁/₂ /s | Dark IC₅₀ ± SEM /nM | PPS * |
|-------|-------------|-------------|--------------|-------|---------|---------------------|-------|
| 1a    | Cl          | 374         | 380          | 5     | 3       | 54                  | 297±77 | 5.1                |
| 1b    | Cl₂         | 372         | 380          | 4     | 3       | 25                  | 904±82 | 7.1                |
| 1c    |             | 380         | 380          | 3     | 2       | 19                  | 1884±44 | 1.7             |
| 1d    | Me          | 374         | 380          | 4     | 4       | 489                 | 183±36 | 3.7                |
| 1e    | + Me        | 374         | 380          | 4     | 3       | 556                 | 30±4  | 3.7                |
| 1f    |             | 377         | 380          | 5     | 3       | 353                 | 76±12 | 4.8                |
| 1g    |             | 377         | 380          | 4     | 3       | 71                  | 180±28 | 4.1             |
| 1h    | F           | 375         | 380          | 3     | 2       | 65                  | 308±43 | 4.1             |
| 1i    | N=S         | 377         | 380          | 4     | 3       | 41                  | 328±18 | 2.5             |
| 1j    | N           | 382         | 380          | 4     | 4       | 134                 | 309±25 | 2.9             |
| 1k    |             | 373         | 380          | 1     | 1       | 15                  | 356±57 | 1.3             |
| 1l    |             | 372         | 380          | 1     | 0       | 13                  | 1025±257 | 1.2    |
| 2     | Cl          | 366         | 380          | 5     | 4       | 187                 | 4667±392 | 2.5   |

| Comp. | R          | Max.abs /nm | illum λ /nm | PIS * | t₁/₂ /s | Dark IC₅₀ ± SEM /nM | PPS * |
|-------|------------|-------------|--------------|-------|---------|---------------------|-------|
| 1j    | 3-OMe      | 382         | 380          | 4     | 4       | 134                 | 309±5  | 2.9               |
| 3a    | 2-OMe      | 385         | 380          | 5     | 4       | 75                  | 3166±3601 | n.m.           |
| 3b    | 3-CN       | 348         | 380          | 4     | 0       | 25                  | 2002±334 | 3.6             |
| 3c    | H          | 358         | 380          | 5     | 4       | 322                 | 3209±269 | n.m.           |
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 mGlu5 activity with light and to induce light-dependent activity in behavioral experiments with living animals, as detailed in our previous communication. In contrast, SIB-1757, a potent phenylazopyridine mGlu5 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 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, especially in polar solvents. 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 mGlu5 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 π−π* 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−π* 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) π−π* transition for the trans isomers and (B) n−π* transition for the cis isomers. (C) Example of UV−vis absorption spectra corresponding to compound 3c, 25 μM 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-isomerisation 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).](https://example.com/figure2)

The wavelength of the maximum of these bands fundamentally depends on the nature of the substituents on the aromatic rings of the azo-compound, in particular electron-donating substituents in ortho or para position. However, in our cases, the 2-pyridyl group, which is a π-electron-deficient heterocycle, induces a certain push−pull character to the azobenzene, especially in polar solvents. Then, to achieve a proper π−π* 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 π−π* 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 (λ = 380 nm) and after illumination with green light (λ = 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 π−π* bands and the cis isomer π−π* and n−π* (Supporting Information). After that, we used green light (λ = 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 pyridylamines 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 π−π* 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 mGlu5 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 mGlu5 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 mGlu5 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 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} receptor (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.35 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).34

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} 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 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 μM 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 photosomerization 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 photosomerization 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 photoswitch 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),
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on switch demonstrate that phenylazopyridines not only are able to curve (Figures 4CD, Supporting Information). These results data shown in the graph to a Gaussian function (magenta curve). ± indicated illumination wavelengths. Data are presented as mean (HWHM), and maximum (Max) values were inferred by initial response to the agonist. Peak, half-width at half-maximum calculated as number of oscillations per minute, and normalized to the of calcium oscillations during an illumination period (5 min) was SEM of the normalized calcium oscillation frequency. The frequency the wavelength used for illumination, and we normalized to the response to the agonist alone, plotted versus 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).

Figure 4. Calcium imaging in individual cells illustrates the light-dependency of mGlu5 inhibition by compounds 1f and 3c. (A, B) Fluorescence ratio (F_{340}/F_{380}) over time of calcium indicator Fura-2 loaded in mGlu5-expressing HEK293 cells. Cells were challenged with an mGlu5 agonist (ago, gray line, 3 μM quisqualate), 1 μM 3c (A) or 1 μM 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 ± 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 mGlu5 receptors.

After violet–green light cycles, we additionally illuminated the cells with several wavelengths around 380 nm, to identify the best wavelength for restoring mGlu5 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 mGlu5 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 4).

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).

5). For example, compound 1e, which was found to be the most potent in the IP accumulation assay, is the most effective 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 mGlu5 calcium oscillations was previously reported applying different concentrations of a conventional NAM35 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 μM 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 mGlu5 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 mGlu5 NAM activities, all the tested phenylazopyridines induced inhibition of the zebrafish motility.
normalizing the distances by the lower distance mean of the set (0%) and the mean corresponding to the vehicle-treated animals (100%). The subtraction of the 2-BisPEB effect was done to minimize the effect of the light not corresponding to the effect of the administered compounds. The error bars correspond to the associated SEM.

Analysis of variance (two-way (compound, light conditions) ANOVA with light conditions as a repeated measure and including the Sidák correction for multiple comparisons; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

25 min after their administration with different extents, except compounds 6 and 7, which induced a slight increase in the animal motility (Figure 6A) when comparing to the vehicle treated animals. In contrast, compounds 1e, 1g, and 1h, which display nanomolar potency in IP accumulation assays, inhibited zebrafish motility with a lower efficacy than expected. The reasons for these deviations are presently unknown and can be related to the multiple factors influencing the in vivo activity and the characteristics of the assay. Remarkably, the magnitude of the inhibition of the animal motility for most of the compounds was comparable to the potencies in cell-based assays (Supporting Information).

To evaluate the photoswitching effects in vivo we performed an extension of the assay with the same animals right after the experiments done in dark conditions. We applied six repetitive light cycles consisting of 1 min in dark conditions and 1 min under violet illumination (λ = 380 nm), and the animal motility was tracked with an integration of the free-swimming distance every 60 s cycle. Between the violet illumination and the dark conditions, the fish were also illuminated during 5 s with white light to accelerate the phenylazopyridine relaxation from cis to trans configuration. Violet light prompted an increment of the motility respective to the dark conditions for all the zebrafish tested, including vehicle and 2-BisPEB-treated ones. However, 2-BisPEB-treated animals displayed a sustained inhibition of their motility compared to the vehicle treated animals independently of the light conditions (Figure 6C, Supporting Information).

As a general trend, all phenylazopyridines showed inhibitory effects in dark conditions that disappeared after applying 380 nm light, and it was recovered in the following dark cycle, defining a fully reversible process (Supporting Information). In addition, we found two different behaviors under 380 nm illumination that are dependent on the compound used: (a) a motility in line with vehicle-treated larvae or (b) an overactivation of the animal motility, which exceeded the levels displayed by vehicle-treated larvae. Compounds 1a and 1f constitute a prototypical example of these behavioral modes (Figure 6C), while in dark conditions they equally inhibit the animal motility with a high efficacy.

This in vivo overactivation after applying violet illumination could be related to the overactivation observed in real-time cell-based assays and was evident for compounds 1b, 1c, 1g, and 7, or for compounds 4, 5, and 6, as a milder effect (Figure 6D, Supporting Information). All these phenylazopyridine derivatives comprise a set of compounds with very different potencies, from low nanomolar ranges to micromolar IC50s in IP accumulation assays, and structurally have three aromatic rings with a phenyl group at the amide end. In contrast, either the group of compounds that did not prompt this overactivation does not have this third aromatic ring or, if it is...
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 π 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 mGlu5 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(1l) = 1.3, PPS(1k) = 1.2), probably as a consequence of their limited intrinsic low photoisomerization capacity (PIS500(1k) = 1, PIS500(1l) = 0.5, PIS500(1k) = 1; PIS500(1l) = 1 PIS500(1k) = 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. 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 mGlu5 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 photosomization was achieved while monitoring nociception (Figure 7A).

We next examined the in vivo phenylazopyridine photo-switching in mouse CNS, since mGlu5 receptor is expressed in amygdala and its activation is related to pain-like effects. 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 photosomization 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 mGlu5 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 mGlu5, NAMs based on the phenylazopyridine scaffold, with a robust SAR, since almost all the trans isomers were active on mGlu5, 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, 1b, 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 photoswitching score (PIS) and the photoinduced potency shift (PPS).

Additionally, these phenylazopyridines allowed us to define a way to perform a fine control of mGlu5 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, mGlu5, 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 mGlu5 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.

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; mGlu5, 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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