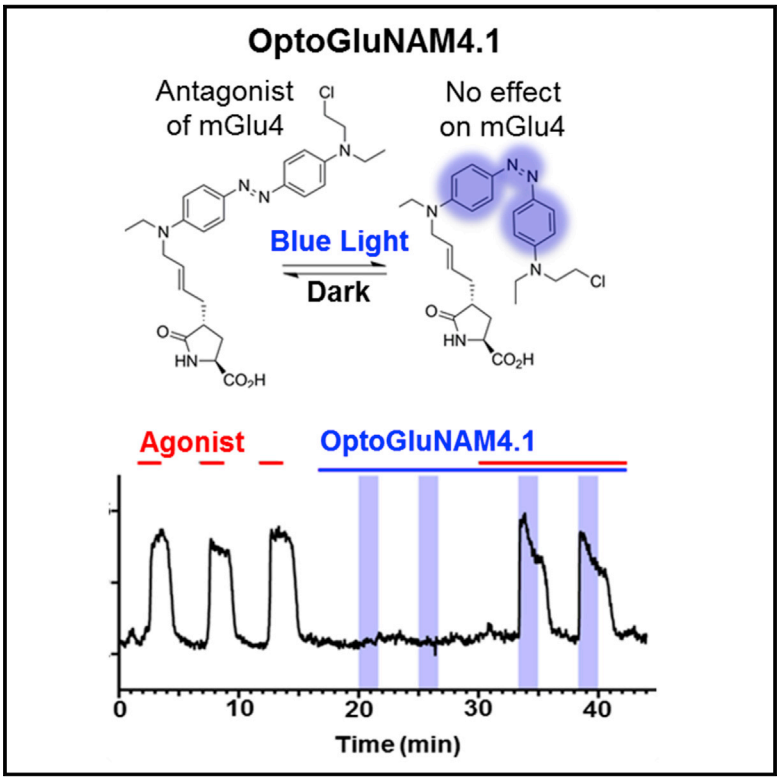
Brief Communication

Cell Chemical Biology

OptoGluNAM4.1, a Photoswitchable Allosteric Antagonist for Real-Time Control of $mGlu_4$ Receptor Activity

Graphical Abstract



Highlights

- OptoGluNAM4.1 is an azobenzene active as negative allosteric modulator of mGlu4
- OptoGluNAM4.1 is isomerized with blue-light/dark cycles with fast relaxation
- In vivo, OptoGluNAM4.1 regulates the zebrafish embryo motility with light
- OptoGluNAM4.1 mGlu4 activity is effective in a pain mouse model

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In Brief

Small molecules with photochromic properties have potential for precise drug therapeutics. Rovira et al. present OptoGluNAM4.1, allowing reversible mGlu4 activity photocontrol in vitro and in vivo that can be used to dissect the physiological roles of this receptor in the nervous system.

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OptoGluNAM4.1, a Photoswitchable Allosteric Antagonist for Real-Time Control of $mGlu_4$ Receptor Activity

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SUMMARY

OptoGluNAM4.1, a negative allosteric modulator (NAM) of metabotropic glutamate receptor 4 ($mGlu_4$) contains a reactive group that covalently binds to the receptor and a blue-light-activated, fast-relaxing azobenzene group that allows reversible receptor activity photocontrol in vitro and in vivo. OptoGluNAM4.1 induces light-dependent behavior in zebrafish and reverses the activity of the $mGlu_4$ agonist LSP4-2022 in a mice model of chronic pain, defining a photopharmacological tool to better elucidate the physiological roles of the mGlu4 receptor in the nervous system.

INTRODUCTION

Photopharmacology is based on the use of light-sensitive ligands for the optical control of specific proteins in cells, tissues, or live animals. This innovative and unconventional approach uses light for the precise control of drug activity, which in combination with spatiotemporal patterns of illumination can enable localized therapeutic effects and dosage adjustment to maximize efficacy and minimize side effects (Gorostiza and Isacoff, 2008). The photochromic ligands can rapidly and reversibly switch on or off the activity of a protein with light in delimited and specific localizations and time intervals, and have the potential for precise drug therapeutics (Reiner et al., 2015). G-protein-coupled receptors (GPCRs) represent a large family of transmembrane receptors with fertile therapeutic targets for drug development (Conn et al., 2009). Metabotropic glutamate (mGlu) receptors are class C GPCRs that play important roles in a broad range of CNS functions with promise for the treatment of various psychiatric and neurological disorders (Nicoletti et al., 2011). In particular, the $mGlu_4$ subtype has recently attracted much attention due to its involvement in several diseases, including chronic pain (Vilar et al., 2013; Huang et al., 2014).

Owing to the difficulty in developing subtype selective orthosteric ligands, significant effort has recently been directed toward identifying drugs that act at mGlu receptor allosteric sites (Conn et al., 2009; Wenthur et al., 2014). Azobenzene-containing photoswitchable molecules have been used for optical control of mGlu receptors at their orthosteric (Levitz et al., 2013) and allosteric (Pittolo et al., 2014) sites.

RESULTS AND DISCUSSION

In the present work, OptoGluNAM4.1 (compound **1**, Figure 1A) emerged from a screening of photoisomerizable azobenzene molecules as an antagonist, decreasing both $mGlu_4$ basal-mediated and orthosteric agonist-mediated activities in a dose-dependent manner (Figure 1B, left top panel, and Figure S1). Saturation of the inhibitory effect indicated either allosteric behavior of the drug or solubility issues. In order to test the allosteric properties of OptoGluNAM4.1, we performed a series of experiments with the full receptor (Figure 1B, right top panel), the receptor containing a mutation that renders $mGlu_4$ insensitive to glutamate (Figure 1B, left bottom panel), and a headless truncated version that lacks the extracellular domain where orthosteric agonists bind (Figure 1B, right bottom panel). These receptor constructs were activated by the known allosteric agonist VU0155041 (Rovira et al., 2015) and competed with increasing concentrations of OptoGluNAM4.1, which reduced $mGlu_4$ activation. Overall, these results confirmed the interaction of the molecule in an allosteric site located in the transmembrane domain and having a typical profile of a negative allosteric modulator (NAM), which to our knowledge is the second $mGlu_4$ NAM described so far (Utley et al., 2011), in contrast with the high number of positive allosteric modulators (PAMs) discovered for this receptor (Rovira et al., 2015). OptoGluNAM4.1 showed no PAM

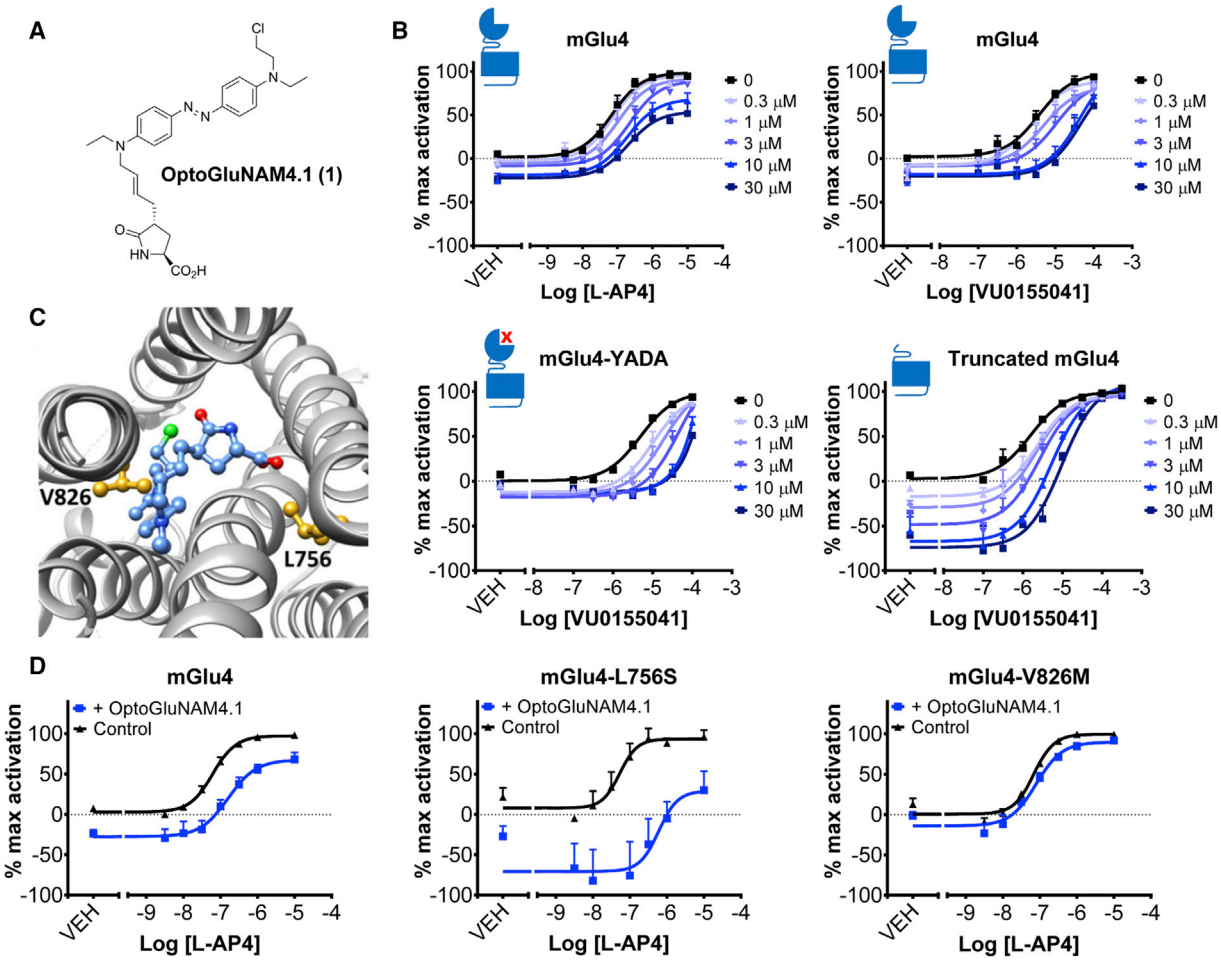

Figure 1. OptoGluNAM4.1 Is a Negative Allosteric Modulator of mGlu4

(A) The chemical structure of OptoGluNAM4.1 compound containing the azobenzene moiety.

(B) Dose-response curves of the orthosteric agonist L-AP4 or the allosteric agonist VU015041 in the presence of different doses of OptoGluNAM4.1 in wild-type, a mutated $mGlu_4$ receptor (which is insensitive to glutamate), and an ECD-truncated $mGlu_4$ receptor.

(C) Localization of the OptoGluNAM4.1 binding site within a model of the transmembrane domain proposed by a protein docking study. Two amino acid positions (L756S and V826M) topologically delimit the allosteric binding pocket.

(D) Dose-response curves for wild-type and mutated receptors after stimulation with the agonist both in the presence and in the absence of OptoGluNAM4.1. Each data point corresponds to the mean ± SEM of at least three experiments performed.

activity over any mGlu subtype at high concentrations, and, besides the $mGlu_4$ NAM activity, only a partial NAM effect on $mGlu_7$ was observed (Figure S1). A computational study performed with a molecular model of the $mGlu_4$ receptor (Figure 1C) suggests an OptoGluNAM4.1 interaction in the transmembrane domain, similar to that of PAMs of $mGlu_4$ (Rovira et al., 2015) and NAMs of $mGlu_1$ and $mGlu_5$ (Harpsøe et al., 2015). The mutation of two amino acid positions (L756S and V826M) was aimed at topologically delimiting the boundaries of the putative allosteric binding site in the $mGlu_4$ protein (Figure S1 and Movie S1). When OptoGluNAM4.1 was tested on L756S $mGlu_4$ cells, its inhibitory potency was increased over that of wild-type (WT) protein, while the V826M mutation was less sensitive to the ligand than WT $mGlu_4$, indicating that the molecule occupies the whole allosteric pocket (Figure 1D) and further supporting the binding mode proposed. Consistently, these mutations did not alter the potency of the $mGlu_4$ orthosteric agonist L-AP4 (Figure S1).

The inclusion of an azobenzene group within the chemical structure of a drug confers potential photochromic activity to the molecule (Broichhagen et al., 2015). Two common properties of reported azobenzene ligands impose limitations for biological applications. First, the light source used to induce the *trans* to *cis* isomerization is usually in the UV-violet range, which is arguably not optimal for in vivo applications because of reduced tissue penetration and potential cell damage. The second limitation derives from the thermal stability of *cis*-azobenzene in the dark, which may require a second wavelength illumination to return to the *trans* isomer for efficient switching. In the present work, we could circumvent these possible drawbacks since OptoGluNAM4.1 isomerizes with blue light and shows a quick back isomerization when dark conditions are recovered (Figure 2A). OptoGluNAM4.1 exhibits a wide visible absorption band, and light sources from 420 nm to 460 nm were found to produce efficient *trans* to *cis* photoisomerization (Figures 2B

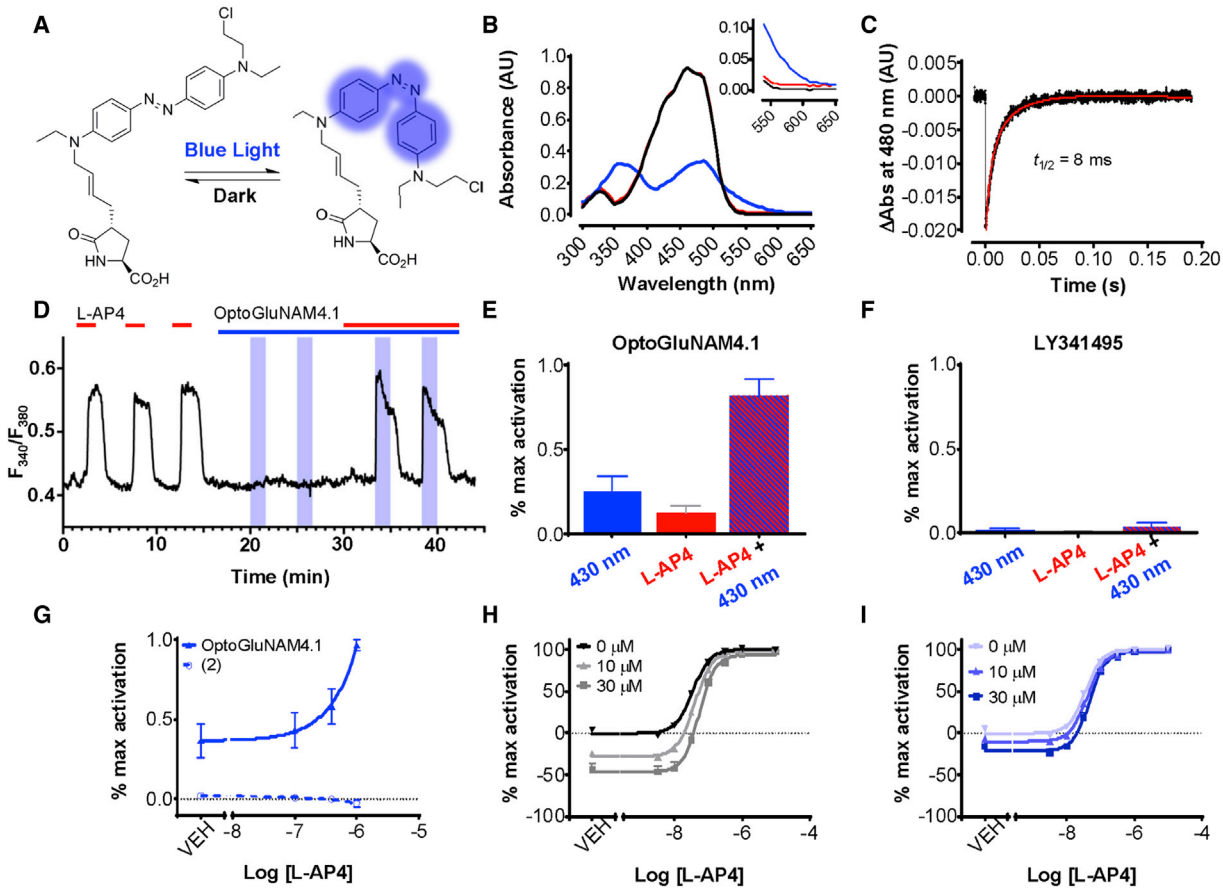

Figure 2. OptoGluNAM4.1 Can Be Dynamically Regulated by Blue Light

(A) OptoGluNAM4.1 photoisomerization from *trans* to *cis* configuration after illumination with blue light.

(B) Absorbance spectrum of OptoGluNAM4.1 (50 μM in DMSO) in the dark (black line) and after illumination with 430 nm light (blue line) for 3 min or 630 nm light (red line) for 1 min.

(C) Thermal isomerization of OptoGluNAM4.1 from *cis* to *trans* in the dark (50 μM, PBS:DMSO 98:2) after a single nanosecond laser pulse (t = 0) at λ_{exc} = 355 nm and 25°C. The solid thick red line corresponds to monoexponential fitting of the experimental data.

(D) Time course of intracellular calcium in an $mGlu_4$-expressing HEK cell, challenged with the $mGlu_4$ agonist L-AP4 (0.3 μM, red bar) in the absence or in the presence of OptoGluNAM4.1 (10 μM, blue bar). Blue boxes indicate illumination at 430 nm.

(E and F) Quantification of agonist-dependent (1 μM L-AP4) and light-dependent (λ = 430 nm) $mGlu_4$ activation in the presence of OptoGluNAM4.1 (E, 10 μM, n = 12) or LY341495 (F, 100 μM, n = 3) from the results indicated in (D). Differences in receptor activation are significant compared with L-AP4 alone (maximum response) during application of OptoGluNAM4.1 under blue light ($p < 0.05$) and after the addition of L-AP4 ($p < 0.001$) but not when both L-AP4 and blue light are applied. No response was observed during LY341495 application in any condition.

(G) Dose response of L-AP4 during exposure to 430 nm light in individual $mGlu_4$-expressing cells, 30 min after treatment (and a subsequent thorough washing) with 30 μM of either OptoGluNAM4.1 or the –OH substituted compound **2**. Mean ± SEM from at least two independent experiments indicated in Figure S2C. Data normalized to the maximum response obtained in each cell ($n \geq 5$).

(H and I) Dose-response curves of L-AP4 after pre-treatment of cells with different concentrations of OptoGluNAM4.1 and subsequent washing both in the dark and under blue light exposure. Each data point corresponds to the mean ± SEM of at least three experiments.

and S2A). This change was reversed by applying red light (630 nm) or by spontaneous thermal relaxation when the blue illumination was discontinued. Absorption was maintained in several light-switching cycles (Figure S2A). Interestingly, *cis* to *trans* relaxation in the dark was very fast (Figures 2C and S2A), and it should enable the photocontrol of $mGlu_4$ activity with a single illumination wavelength (Chi et al., 2006).

Single-cell calcium imaging experiments demonstrated the ability of OptoGluNAM4.1 to optically control $mGlu_4$. Indeed, the application of OptoGluNAM4.1 in the dark impeded the activation of the receptor by L-AP4, and 430 nm illumination restored L-AP4 induced intracellular calcium responses (Figures 2D and S2B and Movie S2). The OptoGluNAM4.1 activity was only abolished upon illumination conditions, and it was rapidly reestablished in the dark as expected from its fast relaxation dynamics. The quantification of the single-cell experiments showed significant differences between illuminated and dark situations (Figure 2E), whereas no light effect was found for the antagonism of L-AP4-induced-activity by LY341495, a general non-photoswitchable orthosteric antagonist of mGlu receptors (Figure 2F).

The OptoGluNAM4.1 molecule contains a 2-chloroethyl substituent at nitrogen, similar to that present in drugs such as

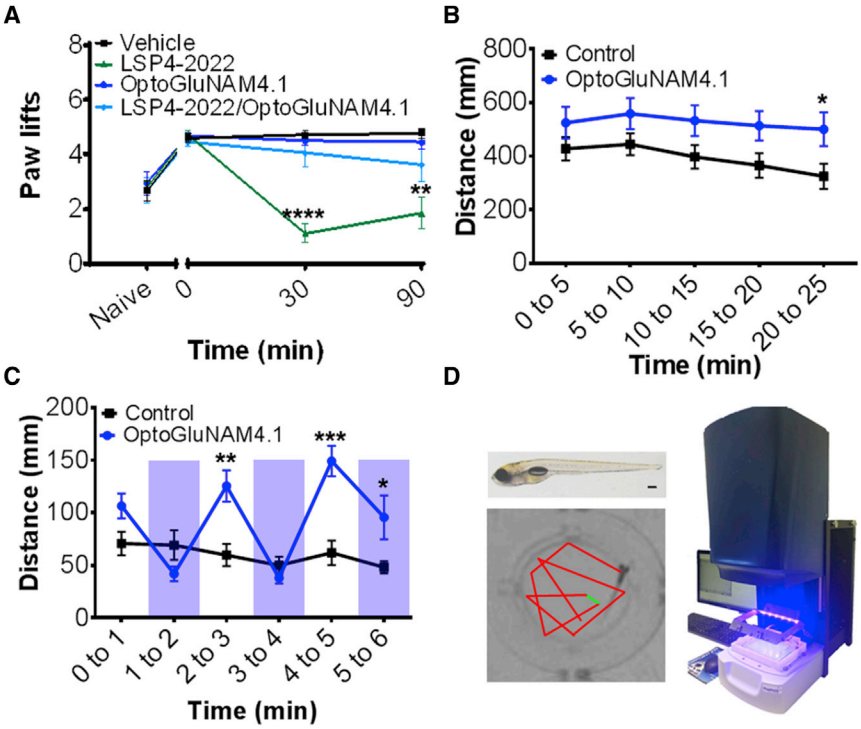

Figure 3. OptoGluNAM4.1 Is Active in Mouse and Zebrafish Animal Models

(A) Pharmacological modulation of spinal $mGlu_4$ receptors in chronic inflamed C57BL/6 mice by OptoGluNAM4.1 evaluated by the Von Frey method. The number of paw lifts from five stimulations using a noxious Von Frey filament was counted before and after inflammation (induced by CFA) and intrathecal treatment. Animals were treated with vehicle (1% DMSO), LSP4-2022 (10 μg/mice), OptoGluNAM4.1 (100 μM), and LSP4-2022/OptoGluNAM4.1 (10 μg/mice and 100 μM, respectively). All the results are expressed as means ± SEM.

(B) Quantification of the total accumulated free-swimming distance of the zebrafish larvae for every 5 min in the presence or in the absence of OptoGluNAM4.1.

(C) Quantification of the accumulated free-swimming distance of the zebrafish larvae for cycles of 1 min in the dark or under blue light (430 nm) upon OptoGluNAM4.1 (10 μM) treatment.

(D) Zebrabox setup with the optogenetic module allowing for very precise control of the light conditions during the behavioral experiments. Individual zebrafish larvae can be independently tracked in each well (scale bar, 200 μm). For all zebrafish experiments, each data point corresponds to the mean ± SEM of 12 animals from two independent experiments.

Statistical differences from control are denoted for adjusted p values as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

chlorambucil or bendamustine, which belong to the nitrogen mustard family of anticancer agents. It is well known that the mechanism of action of these molecules involves an electrophilic alkylation and cross-linking of DNA (Gandhi, 2002), where the *N*-2-chloroethyl substituent plays an essential role, generating a highly reactive aziridinium electrophile that reacts with nucleophiles present in the biomolecules after binding. This chemical reactivity has also been employed in the development of GPCR covalent ligands (Weichert and Gmeiner, 2015). We hypothesized that a similar mechanism could take place in our case and could explain some of the pharmacological effects observed with OptoGluNAM4.1. A light-dependent NAM effect was maintained in $mGlu_4$-expressing cells even after extensive washout of the allosteric ligand (Figure S2B). This could indicate conjugation of the compound to the protein receptor, compatible with the precedents of the reactivity of 2-chloroethyl anilines after binding to DNA or proteins. To better study this phenomenon, single-cell experiments were conducted in which a meticulous washing protocol was applied (Figures 2G and S2C). Again, we observed that the antagonistic effect persisted and that this could be switched off after illumination and restored in the dark, indicating photoreversible receptor activation after removal of the ligand in solution. These results were in contrast with those obtained with the closely related compound **2** (Figures 2G and S2C) containing a hydroxyl group instead of the chlorine atom at the nitrogen substituent (Figure S2B). Of note, compound **2** is unable to chemically react with the protein residues, but it conserves antagonist properties similar to OptoGluNAM4.1 albeit at higher concentration (Figure S2B). We independently confirmed this permanent effect in inositol phosphate (IP) endpoint assays. When OptoGluNAM4.1 was incubated for 20 min and after a thorough washing protocol, the dose response of the $mGlu_4$ agonist L-AP4 corroborated a remaining NAM effect, evidenced by the inhibition of the constitutive activity (Figure 2H). This effect was decreased by the application of blue light, thus suggesting that the activity was due to the presence of covalently bound OptoGluNAM4.1 (Figure 2I). GPCR covalent allosteric ligands are scarce (Davie et al., 2014) in spite of their singular pharmacological profile.

We next implemented in vivo assays to determine OptoGluNAM4.1 activity in animal behavior. We first tested its $mGlu_4$ NAM activity in a mouse model of inflammatory chronic pain. One week after injection of complete Freund's adjuvant (CFA), an increase of mechanical hypersensitivity was observed for all animal groups, which was reduced by activation of spinal $mGlu_4$ receptors following intrathecal injection of the $mGlu_4$ orthosteric agonist LSP4-2022 (Vilar et al., 2013). OptoGluNAM4.1 was able to block the analgesic effect induced by LSP4-2022 (Figures 3A and S3) in line with its $mGlu_4$ antagonist activity, whereas OptoGluNAM4.1 alone did not have any measurable effect. At the concentrations used here, the LSP4-2022 inhibition of mechanical hypersensitivity is lost in $mGlu_4$ knockout animals (Vilar et al., 2013), and therefore this compound is believed to selectively exert its effects through the $mGlu_4$ receptor. The reversal of LSP4-2022 physiological effects by OptoGluNAM4.1 suggests that its action is likely exerted through $mGlu_4$, although a partial contribution of $mGlu_7$ antagonism cannot be ruled out (Figure S1). Overall, these

results confirmed that OptoGluNAM4.1 is active and suitable for in vivo experimentation in mice.

To test the light-dependent activity in vivo, we set up behavioral assays in transparent zebrafish larvae, which are amenable to illumination (Portugues et al., 2013). In comparison with mammals, the mGlu family of receptors in zebrafish have similar expression patterns and high amino acid sequence similarity (Haug et al., 2013). The activity of OptoGluNAM4.1 in water was evaluated by monitoring zebrafish locomotion. In the dark, OptoGluNAM4.1 treatment increased the free-swimming distance over untreated control animals (Figure 3B). We next compared its effects with those exerted by Alloswitch-1, an azobenzene selective NAM of $mGlu_5$ (Pittolo et al., 2014). In the absence of illumination, Alloswitch-1 was found to exert an inhibitory action on the fish locomotion (Figure S3 and Movie S5) over that of untreated controls. The opposite effects found for OptoGluNAM4.1 and Alloswitch-1 on animal behavior (Figures 3B and S3) are consistent with the opposite synaptic roles of these receptors. Indeed, the activation of $mGlu_4$ (and also $mGlu_7$) is known to inhibit synaptic glutamate release, whereas $mGlu_5$ has a positive modulatory effect on neuronal activity (Nicoletti et al., 2011). We next tested the effect of *cis-trans* isomerization by illumination of the zebrafish larvae. In vivo optical control of OptoGluNAM4.1 and Alloswitch-1 on zebrafish demonstrated reversibility (Figures 3C, S3C, and S3E and Movies S3, S4, and S5). The increase in the free-swimming distance observed in animals treated with OptoGluNAM4.1 over the untreated control group was reduced to similar levels upon illumination of the larvae with blue light. On the contrary, the reduced free-swimming distance elicited in vivo by Alloswitch-1 was not only reversed by 380 nm illumination but increased over untreated controls, in agreement with the activity rebounding previously reported in a heterologous cellular system expressing mGlu5 (Pittolo et al., 2014). It is worth noting the biological activity of these photoswitchable NAM compounds and the light-dependent effects induced in cells and living animals, thus highlighting the usefulness of photopharmacology in the discovery of new pharmacological paradigms.

SIGNIFICANCE

OptoGluNAM4.1 is the first $mGlu_4$ NAM compound with in vivo activity described to date. It can be useful as a tool to investigate the effects of $mGlu_4$ receptor (and eventually $mGlu_7$) both in cells and in living animals, and amenable to dissect the physiological roles of this receptor in the nervous system. Moreover, the OptoGluNAM4.1 advantageous photoswitch properties with blue-light isomerization and fast relaxation in the dark facilitate the safe and efficient control of receptor activity with a single illumination wavelength. OptoGluNAM4.1 permanent activity in endogenous $mGlu_4$ after washing out further increases the versatility of the photoswitchable ligand, expanding the molecular tool set in photopharmacology.

EXPERIMENTAL PROCEDURES

Synthesis of Molecules

A description of the synthesis of compounds OptoGluNAM4.1 (**1**) and compound **2** is given in the Supplemental Information.

In Vitro mGlu Receptor Functional Assay

HEK293 cells were cultured and transfected by electroporation for expression of all rat mGlu receptors. We estimated IP accumulation using the IP-One HTRF kit (Cisbio Bioassays) both in dark and violet light illumination (LED plate; FCTecnics) as previously reported (Pittolo et al., 2014). All points were realized in triplicate and read with a RUBYstar HTRF HTS microplate reader (BMG Labtech). Dose-response curves were fitted using Prism software (GraphPad). See Supplemental Experimental Procedures for detailed procedures.

Molecular Modeling

Following the protocol described previously (Rovira et al., 2015), a model of mGlu4 was generated with Modeller 9.12 using the crystal structure of mGlu5. OptoGluNAM4.1 was introduced by docking using AutoDock Vina. Discovery studio visualizer (Accelrys Software) and Jalview 2.8 were used for protein structure and sequence analysis, respectively. See Supplemental Experimental Procedures for detailed procedures.

Photophysical Characterization

Absorption spectra were recorded on a Varian Cary 300 UV-visible spectrophotometer (Agilent Technologies) using a quartz cuvette. Photoisomerization was accomplished by irradiation with a 430 nm LED array (FCTecnics) or 630 nm LED (Photo Activation Universal Light, Geniul). Transient absorption measurements were registered in a nanosecond laser flash-photolysis system (LKII, Applied Photophysics) equipped with an Nd:YAG laser (Brilliant, Quantel) and a photomultiplier tube (Hamamatsu). See Supplemental Experimental Procedures for detailed procedures.

Single-Cell Calcium Imaging and Photostimulation

HEK tsA201 cells were cultured, transfected, and seeded as previously reported (Pittolo et al., 2014). Fura-2 AM was loaded into the cells, excited with a Polychrome V light source (Till Photonics), and the resulting images acquired with an inverted digital microscope (iMic, Till Photonics). The fluorescence ratio was calculated by the software (LA Arivis Browser 1.6, Arivis). Variations in intracellular calcium were evaluated with IgorPro 6.0.5 (WaveMetrics). Dose-response curves were fitted with Prism (GraphPad). Videos were edited from raw images using Fiji (ImageJ). See Supplemental Experimental Procedures for detailed procedures.

Mechanical Allodynia in a Mouse Pain Model

Animals were treated in accordance with the European Community Council Directive 86/609. Experimental protocols were approved by the local authorities (regional animal welfare committee (CEEA-LR) with the guidelines of the French Agriculture and Forestry Ministry (C34-172-13). All efforts were made to minimize animal suffering and number.

A Von Frey assay on 8- to 10-week-old C57BL/6 mice was used to evaluate the effect of mGlu4 compounds. We chose a chronic inflammatory pain model induced by unilateral intraplantar injection of CFA (Mycobacterium tuberculosis; Sigma). Compounds were intrathecally administered. Data were analyzed with Prism software (GraphPad). See Supplemental Experimental Procedures for detailed procedures.

Zebrafish

Zebrafish experiments were conducted in accordance with the European Communities council directive 2010/63. WT Tupfel long-fin specimens were used for these experiments. Monitoring of animal locomotion was made with the Zebrabox using the optogenetic module (Viewpoint Life Sciences). One larvae per well was placed in a 96-well plate (BD Falcon) with E3 medium. Data were analyzed with Prism software (GraphPad). See Supplemental Experimental Procedures for detailed procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2016.06.013.

AUTHOR CONTRIBUTIONS

Conceptualization, A.L., C.G., P.G., X.R.A., and A.T.; Methodology, A.L., C.G., P.G., X.R.A., A.T, C.Z., S.P., J.G., A.F., C.J., J.-P.P; Formal Analysis, J.G., S.P., X.R.A., and C.Z.; Writing – Original Draft, A.L., X.R.A., S.P., and A.T.; Writing – Review & Editing A.L., X.R.A., and A.T.; Project Administration, A.L., X.R.A., and A.T.; Supervision, C.G., P.G., and A.L.; Funding Acquisition, C.G., P.G., C.J., J.-P.P., J.G., and A.L.

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