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Clickable Photoaffinity Ligands for Metabotropic Glutamate Receptor 5 Based on Select Acetylenic Negative Allosteric Modulators

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Abstract

G protein-coupled receptors (GPCRs) represent the largest class of current drug targets. In particular, small-molecule allosteric modulators offer substantial potential for selectively “tuning” GPCR activity. However, there remains a critical need for experimental strategies that unambiguously determine direct allosteric ligand–GPCR interactions, to facilitate both chemical biology studies and rational structure-based drug design. We now report the development and use of first-in-class clickable allosteric photoprobes for a GPCR based on metabotropic glutamate receptor 5 (mGlu₅) negative allosteric modulator (NAM) chemotypes. Select acetylenic mGlu₅ NAM lead compounds were rationally modified to contain either a benzophenone or an aryl azide as a photoreactive functional group, enabling irreversible covalent attachment to mGlu₅ via photoactivation. Additionally, a terminal alkyne or an aliphatic azide was incorporated as a click chemistry handle, allowing chemoselective attachment of fluorescent moieties to the irreversibly mGlu₅-bound probe *via* tandem photoaffinity labeling-bioorthogonal conjugation. These clickable photoprobes retained submicromolar affinity for mGlu₅ and negative cooperativity with glutamate, interacted with the “common allosteric-binding site,” displayed slow binding kinetics, and could irreversibly label mGlu₅ following UV exposure. We depleted the number of functional mGlu₅ receptors using an irreversibly bound NAM to elucidate and delineate orthosteric agonist affinity

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Notes

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Supporting Information

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and efficacy. Finally, successful conjugation of fluorescent dyes *via* click chemistry was demonstrated for each photoprobe. In the future, these clickable photoprobes are expected to aid our understanding of the structural basis of mGlu₅ allosteric modulation. Furthermore, tandem photoaffinity labeling-bioorthogonal conjugation is expected to be a broadly applicable experimental strategy across the entire GPCR superfamily.

Graphical Abstract

The metabotropic glutamate (mGlu) receptor family consists of eight (mGlu₁₋₈) cell surface G protein-coupled receptors (GPCRs) that respond to the neurotransmitter glutamate. mGlu receptors are well-established as drug targets for multiple central nervous system (CNS)-related disorders.¹ Among other disorders, mGlu₅ is a promising drug target for autism, depression, and schizophrenia.² Increasingly, GPCR drug discovery programs are focused on allosteric modulators, compounds that interact at sites distinct from the orthosteric (endogenous) ligand-binding site and frequently display higher receptor-subtype selectivity. Further, allosteric modulators that lack agonist activity are attractive as potential therapeutic agents, as there is the potential to “fine-tune” receptor activity by maintaining both spatial and temporal aspects of neurotransmission.³ Diverse mGlu₅ allosteric modulators with varied pharmacological phenotypes bind a “common allosteric site” within the seven transmembrane-spanning domains (7TMs).⁴ Determining precisely how a ligand’s chemical structure dictates its allosteric pharmacology (e.g., affinity, selectivity, cooperativity), *via* accurate structure–activity relationship (SAR) interpretation, is critical for future drug design and development.⁵ Allosteric modulator SAR interpretations have proven to be fundamentally more difficult relative to orthosteric compounds, namely because pharmacological characterization of allosteric modulators frequently involves multiple parameters, which may individually be altered by medicinal chemistry optimization efforts. Recently, mGlu 7TMs have been cocrystallized with select negative allosteric modulators (NAMs) bound to this “common allosteric site.”⁶⁻⁸ These structures have provided improved templates to better understand mGlu receptor SAR at the molecular level.⁴ However, allosteric modulator SAR interpretation is fundamentally difficult due to our lack of knowledge regarding the specific and dynamic ligand–receptor interactions that dictate the pharmacology of diverse mGlu₅ allosteric modulators. Therefore, there is a need for alternative experimental strategies that directly determine allosteric ligand–receptor contacts.

Photoaffinity labeling, a robust biochemistry technique commonly used to directly determine ligand–receptor contacts, has seen a recent renaissance, principally due to its coupling with bioorthogonal/click chemistry reactions.^{9,10} Tandem photoaffinity labeling-bioorthogonal conjugation has become commonplace for identifying and studying the structure and location of drug targets, principally enzymes and ion channels. In this strategy, photoprobes contain a photoreactive functional group, to allow covalent bond formation of the probe to a

target, and a bioorthogonal/click chemistry functional group, which acts as an indirect tag. After photoaffinity labeling with such “clickable” photoprobes, a bioorthogonal conjugation reaction, typically a copper-catalyzed Huisgen 1,3-dipolar cycloaddition between an azide and a terminal alkyne,¹¹ specifically attaches a chosen tag (e.g., biotin, fluorophore) to the click chemistry handle within the photoprobe. To our knowledge, this experimental strategy has not been applied to GPCRs, in particular, GPCR allosteric modulators.

For the current study, we sought to develop clickable photoprobes for mGlu₅ based on select acetylenic NAM lead compounds. Herein, we report three novel clickable photoprobes that display submicromolar binding affinity for mGlu₅ and functionally retain negative cooperativity with glutamate. Additionally, we demonstrate irreversible attachment of these compounds to mGlu₅ *via* tandem photoaffinity labeling-bioorthogonal conjugation. In turn, these clickable photoprobes are expected to serve as valuable tool compounds for identifying direct allosteric modulator-receptor interactions *via* future mGlu₅ structure–function studies. Moreover, these probes also have the potential to be used as novel imaging agents of mGlu₅ in native cells and living systems.

RESULTS AND DISCUSSION

Irreversible ligands represent an important class of compounds for studying GPCR structure and function. For family A and B GPCRs, such compounds, including those that do and do not require photoactivation (i.e., affinity labels), have been extensively used to characterize endogenous ligand-binding sites and cell surface expression.^{12,13} Indeed, cocrystallization of GPCRs with low-affinity orthosteric ligands can be facilitated by incorporation of functional groups that covalently modify a receptor.^{14,15} Recently, these approaches have been extended to allosteric ligands for the M₁ muscarinic acetylcholine receptor.^{16,17} Given the recent re-emergence of photoaffinity labeling coupled with bioorthogonal/click chemistry, we sought to develop clickable photoreactive mGlu₅ allosteric modulators as novel tools to study this clinically significant drug target.

Photoreactive Functional Groups and Click Chemistry Handles Are Well Tolerated within Select Acetylenic mGlu₅ NAMs

We first sought to confirm the activity of known acetylenic mGlu₅ NAMs **1**¹⁸ and **2**¹⁹ (Figure 1). Concentration–response curves were generated in the absence (Figure 2a) or presence (Figure 2b) of an EC₈₀ concentration of glutamate (~200 nM). There was no evidence of intrinsic agonist activity for either compound at mGlu₅. Additionally, functional potency estimates (Table 1) for inhibition of EC₈₀ glutamate for lead compounds **1**, **2**, and MPEP (**6**, the prototype acetylenic mGlu₅ NAM) at mGlu₅ were in good agreement with previous reports.^{18–20} Analysis of these data with an operational model of allosterism, where affinity modulation was assumed to be neutral and efficacy modulation approached zero, yielded affinity estimates (pK_B) that were in good agreement with pK_i estimates (Table 1) derived from radioligand binding assays (Figure 2c). As expected, lead compounds **1** and **2** fully displaced [³H]mPEPy binding to mGlu₅, indicating competition for the “common allosteric site” within the mGlu₅ 7TMs.

Toward identifying clickable allosteric photoprobes for mGlu₅ photoaffinity labeling experiments, we first assessed the functional potency and binding affinity of a benzyl ether derivative (**3**) of lead acetylenic mGlu₅ NAM **2**. Based on previous SAR studies of MPEP²¹ and mGlu₅ NAM imaging probes,^{22–24} we hypothesized that large substitutions would be tolerated on the phenyl ring of lead NAM **1**. Benzyl ether **3** retained high negative cooperativity with glutamate, completely abolishing the response to glutamate at concentrations above 3 μ M (Figure 2b). Compared to lead compound **2**, addition of the large benzyl ether group had little to no effect on potency (inhibition of glutamate EC₈₀) or affinity for mGlu₅ as determined by radioligand binding assays (Table 1). As expected, benzyl ether **3** was able to fully inhibit [³H]mPEPy binding to mGlu₅, indicating this compound is also competitive with the “common allosteric site” within mGlu₅ (Figure 2c). Since benzyl ether **3** retained submicromolar affinity to mGlu₅, which is consistent with previous SAR studies,^{22–24} we next sought to introduce “all-in-one” photoreactive click chemistry moieties at this position to produce putative mGlu₅ clickable photoprobes.

Benzophenone-Alkyne and Diazido Conjugates of a Lead Acetylenic mGlu₅ NAM (**1**) Are mGlu₅ NAMs

Photoprobe **4**, featuring a known “all-in-one” photoreactive benzophenone unit containing a terminal alkyne click chemistry handle,²⁵ displayed 2-fold higher mGlu₅ binding affinity relative to lead NAM **2**, as estimated from radioligand inhibition binding assays (Table 1); however, it should be noted that compound **4** produced an incomplete displacement of [³H]mPEPy binding under these initial assay conditions (Figure 2c). This observation could potentially be attributed to nonequilibrium assay conditions, or the possibility that compound **4** interacts noncompetitively with [³H]mPEPy. Therefore, we repeated the [³H]mPEPy inhibition binding assay for photoprobe **4**, extending the ligand–receptor equilibration time to 4 h (Figure 2d). Under these secondary assay conditions, photoprobe **4** fully displaced [³H]mPEPy binding to mGlu₅, thus suggesting this clickable photoprobe competitively interacts with the “common allosteric site” within mGlu₅, albeit with slower binding kinetics. Additionally, benzophenone-alkyne **4** displayed no intrinsic agonist activity at mGlu₅ (Figure 2e) and concentration-dependently inhibited the glutamate-mediated intracellular Ca²⁺ mobilization response (Figure 2f). Compared to lead NAM **2**, benzophenone-alkyne **4** was ~5-fold less potent at inhibiting the EC₈₀ glutamate functional response (Table 1). High negative cooperativity was retained for photoprobe **4** with glutamate, showing complete inhibition of the glutamate maximal functional response in allosteric interaction assays (Figure 3a). Application of the operational model of allostery to these data yielded an affinity estimate (pK_B) within ~5-fold of benzyl ether derivative **3** (Table 1).

We next explored diazide **5** (Figure 1) as an alternative “all-in-one” clickable photoprobe derivative of lead acetylenic mGlu₅ NAM **2**. The rational design of conjugate **5** features incorporation of a well-known 1,3,5-substituted benzene unit at position R that contains a photoreactive aryl azide functional group and an aliphatic azide click chemistry handle.²⁶ After chemical synthesis and compound characterization, diazide **5** showed ~3-fold lower mGlu₅ binding affinity (K_i) when compared to benzophenone-alkyne **4** in the [³H]mPEPy radioligand binding assay (Figure 2c, Table 1). Diazide **5** had no intrinsic mGlu₅ agonist

activity in the Ca^{2+} mobilization assay (Figure 2e) and inhibited the EC_{80} glutamate response with ~2-fold lower potency when compared to benzophenone **4** (Figure 2f, Table 1). Interestingly, the EC_{80} glutamate inhibition curve for diazide **5** displayed a shallow Hill slope, despite fully displacing [^3H]mPEPy binding in a manner consistent with one-site binding (where slope = 1; Table 1). Glutamate concentration–response curves for intracellular Ca^{2+} mobilization in the absence and presence revealed a markedly different allosteric modulation profile for diazide **5** (Figure 3b). With 1 min of pre-exposure, diazide **5** was a low cooperativity mGlu₅ NAM, unable to completely abolish the maximal functional response to glutamate and approached the limit to its cooperativity ($\log \beta$: -0.42 ± 0.12 , β : 0.38). Under these assay conditions, the mGlu₅ affinity estimate ($\text{p}K_{\text{B}}$) for diazide **5** was ~13-fold lower than that derived from the radioligand binding assay (Table 1). These initial pharmacological profiles for benzophenone-alkyne **4** and diazide **5** demonstrated that all-in-one clickable photoprobes based on a NAM pharmacophore could be designed to retain acceptable affinity for mGlu₅ and maintain negative cooperativity with glutamate.

A Compact Azido-Alkyne Derivative of MPEP Is an mGlu₅ NAM

Conjugate-type photoprobes may be viewed as having an inherent disadvantage when mapping binding site amino acids, or optimally modeling a ligand–protein complex, *via* a binding ensemble profiling with the (f)photoaffinity labeling (BEProFL) experimental approach.²⁷ That is, the covalent point of photoprobe attachment may be removed from the pharmacophore due to a variable-length linker. Therefore, in parallel with photoprobes **4** and **5**, which feature conjugation of different “all-in-one” photoreactive click chemistry moieties *via* a relatively short linker to the NAM pharmacophore, we also explored direct substitution of MPEP (**6**) with a photoreactive group and a click chemistry handle. In particular, direct embedment or attachment of a photoreactive functional group to a pharmacophore would be expected to covalently attach the photoprobe to residue/s directly within the ligand-binding site.

Rational pursuit of azido-alkyne **8** was initiated based on known acetylenic mGlu₅ NAM **7**, an MPEP derivative reported to have ~13-fold higher mGlu₅ binding affinity than MPEP.²⁸ We validated lead compound **7** as a mGlu₅ NAM of glutamate-mediated intracellular Ca^{2+} mobilization with similar potency (within 4-fold) to MPEP (**6**; Table 1, Figure 2b). Photoprobe features replacement of the aromatic nitrile in lead NAM **7** with a photoreactive aryl azide, and slight extension of the methyl ether into a propargyl ether as a click chemistry handle. Photoprobe **8** had 3-fold lower potency than MPEP for EC_{80} glutamate inhibition (Table 1, Figure 2f). However, azido-alkyne was able to completely displace [^3H]mPEPy in radioligand binding assays with ~20-fold higher binding affinity than MPEP for the common allosteric site in mGlu₅ (Table 1). Further analysis of the interaction between azido-alkyne **8** and glutamate demonstrated concentration-dependent leftward shifts in the glutamate concentration–response curve for Ca^{2+} mobilization coupled with a complete depression in the maximal response, indicative of high negative cooperativity (Figure 3c).

All Three Putative Clickable mGlu₅ NAM Photoprobes Irreversibly Bind to mGlu₅

Close interrogation of the binding and functional data for all three photoprobes revealed large disconnects (>25-fold) between functional potency and affinity estimates (pK_B) compared to affinity estimates determined *via* radioligand inhibition binding assays. Informed by the slow binding kinetics for photoprobe **4** (Figure 2d), we repeated the Ca^{2+} mobilization assay with the photoprobe preincubation time extended to 30 min (Figure 3d–f). Under these conditions, the affinity estimates (pK_B) for photoprobes **4** and **5** were significantly increased by ~10-fold (**4**: 7.44 ± 0.23 , 36 nM; **5**: 7.38 ± 0.18 , 41 nM) and in good agreement with those estimated from the radioligand inhibition binding assay (25 and 71 nM, respectively). Longer preincubation with azido-alkyne **8** yielded a ~30-fold higher mGlu₅ binding affinity estimate (pK_i : 9.60 ± 0.15 , 0.3 nM) that was in good agreement with the value obtained from the [³H]mPEPy radioligand binding assay (0.5 nM). Discrepancies between functional potencies and binding affinities greater than 10-fold have been noted within the SAR for multiple mGlu₅ NAM chemotypes;^{28–31} however, the underlying mechanisms giving rise to such discrepancies have remained relatively unexplored. Collectively, these data suggest that the nonequilibrium nature of the intracellular Ca^{2+} mobilization functional assay (i.e., the most common primary screening assay for identifying mGlu₅ allosteric modulators), coupled with slow ligand-binding kinetics, are likely key contributing factors to such discrepancies observed between functional potencies and binding affinities. Additionally, with the longer preincubation time, diazide **5** fully abolished the glutamate maximal functional response, suggesting that ligand-binding kinetics contributed to the observed limited negative cooperativity. Previously, mGlu₅ NAMs with limited cooperativity for Ca^{2+} mobilization were reported to fully abolish glutamate-mediated ERK1/2 phosphorylation.^{20,32} We previously attributed this to different system coupling efficiencies; however, the data for diazide **5** demonstrate that allosteric ligand-binding kinetics influence the apparent degree of cooperativity, thus highlighting a potential pitfall for allosteric modulator discovery when relying on nonequilibrium functional assays to classify allosteric modulators.

Importantly, we next assessed whether putative photoprobes **4**, **5**, and **8** were capable of irreversibly labeling mGlu₅. Pretreatment with 1 μ M of benzophenone **4**, diazide **5**, or azido-alkyne **8**, followed by three (5 min) washes, significantly reduced [³H]mPEPy binding (Figure 4a). However, 1 μ M MPEP (**6**) also reduced [³H]mPEPy binding to a similar extent, leading us to conclude that the wash paradigm was insufficient. Following an extensive wash protocol (five 1 h washes), benzophenone **4**, diazide **5**, and azido-alkyne **8**, but not MPEP (**6**), concentration-dependently inhibited [³H]mPEPy binding (Figure 4b), suggesting all three clickable compounds irreversibly bind to mGlu₅ in the absence of UV irradiation. We next assessed the impact of irreversible probe binding (10 min exposure followed by five extended washes) on mGlu₅ activity (Figure 4c and d). Pre-exposure to MPEP had no effect on the potency or maximal response to either glutamate or VU0424465 (an allosteric agonist), confirming the reversibility of MPEP binding. Treatment with azido-alkyne **8** depleted the number of functional mGlu₅ receptors, reducing the efficacy (log τ) of both glutamate and VU0424465 (Table 2). Interestingly, the apparent affinity of glutamate for rat mGlu₅ was 7-fold higher than the previous report using radioligand-based approaches.³³ These data indicate that irreversible mGlu NAMs can be employed to deplete functional

receptors to directly delineate the affinity and efficacy of both orthosteric and allosteric agonists in a whole cell, functional (physiological) context.

Select Clickable Acetylenic NAMs Are Able to Undergo Tandem mGlu₅ Photoaffinity Labeling-Bioorthogonal Conjugation

Finally, we investigated the utility of the click chemistry handles incorporated into photoprobes **4**, **5**, and **8**. We began with benzophenone-alkyne **4** and azido-alkyne **8**, as these probes retained higher mGlu₅ binding affinity compared to diazide **5**. In turn, membrane preparations from HEK293 cells stably expressing high levels of mGlu₅ (HEK-mGlu₅-high) were incubated with the alkyne-containing photoprobes (i.e., **4** or **8**), and UV irradiated to ensure formation of a covalent photoprobe-mGlu₅ adduct. Afterward, the samples were subjected to click chemistry to attach near-infrared azide fluorophores, followed by separation of probe-labeled proteins using SDS-PAGE. In the absence of alkyne-containing photoprobes **4** or **8**, we observed little or no incorporation of the azide fluorophore (Figures 5a and b). However, in the presence of alkyne-containing photoprobes **4** or **8**, concentration-dependent click chemistry incorporation of select azide fluorophores to the terminal alkyne within the covalent photoprobe-mGlu₅ adduct was observed *via* in-gel fluorescence. Subsequent immunoblotting revealed that the fluorescent band at >250 kDa colabeled with a mGlu₅ dimer; however, a high degree of nonspecific interactions were evident. Indeed, membranes from nontransfected HEK293 cells showed a similar degree and pattern of fluorescent labeling, indicating that benzophenone-alkyne **4** and azido-alkyne **8** can photoaffinity label multiple non-mGlu₅ targets (Supporting Information Figure 2). Interestingly, the nonspecific labeling patterns of benzophenone-alkyne **4** and azido-alkyne **8** differ between one another, indicating that not all nonspecific targets are shared between these different photoreactive ligands, implicating the linkers and/or photoreactive groups in mediating nonspecific interactions. A high degree of nonspecific labeling is not unusual for photoreactive ligands.^{34,35}

Covalent Photoincorporation of Select Clickable Acetylenic NAMs into mGlu₅ Is Inhibited by an Alternative Irreversible NAM

Given the high degree of nonspecific-labeling observed in HEK-mGlu₅-high cells, we next moved our efforts to tandem photoaffinity labeling-bioorthogonal conjugation involving purified mGlu₅. In particular, fluorophores were concentration-dependently incorporated into purified mGlu₅ using click chemistry after photoaffinity labeling employing benzophenone-alkyne **4**, diazide **5**, and azido-alkyne **8** (Supporting Information Figure 3). Interestingly, a saturating concentration (10 μ M) of MPEP (**6**) did not inhibit mGlu₅ photoaffinity labeling of any of the clickable photoprobes (Supporting Information Figure 3). With these somewhat unique experimental observations in hand, we hypothesized that the inability of MPEP to competitively inhibit the covalent attachment of our probes to mGlu₅ may be due to the known reversible binding nature of MPEP. Further, MPEP and closely related analogs are known to have fast binding kinetics,³⁶ findings that have been recapitulated in our laboratory (data not shown). As a result, we tested whether covalently photoaffinity labeling mGlu₅ with one photoreactive allosteric modulator could competitively inhibit the photoaffinity labeling of another one of our clickable photoprobes. To do this, we took advantage of different click chemistries that can be applied to these

photoprobes (Figure 6a). In turn, purified mGlu₅, prephotolabeled with diazide **5** (10 μ M), showed no significant incorporation of Cy5.5-azide upon click chemistry after photoaffinity labeling involving 300 nM of either benzophenone-alkyne **4** or azido-alkyne **8** (Figure 6b). Similarly, purified mGlu₅, prephotolabeled with benzophenone-alkyne **4** (10 μ M), showed no significant incorporation of Cy5.5-alkyne upon click chemistry after photoaffinity labeling involving 600 nM of diazide **5** (Figure 6c).

In summary, this study reports the first successful demonstration of tandem photoaffinity labeling-bioorthogonal conjugation applied to a GPCR. In particular, the clickable photoprobes described herein represent an exciting new class of tools for probing mGlu₅ structure and function. Since mGlu receptor allosteric ligands interact with a 7TM-binding site analogous to the orthosteric site of family A GPCRs, tandem photoaffinity labeling-bioorthogonal conjugation is likely to be translatable to many other GPCRs, including orthosteric and allosteric pharmacophores. Moreover, the same clickable photoreactive ligand can be clicked with a different reporter tag depending upon the application desired (e.g., introducing biotin for streptavidin-based purification, or a fluorophore for mass spectrometry or receptor localization). Our future research will explore different applications of these novel pharmacological tools for mGlu₅.

METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich and were of an analytical grade.

Cell Culture and Protein Preparation

Stable HEK293-mGlu₅ cell lines, generated as described previously,²⁰ were maintained in complete DMEM supplemented with 5% FBS, 2 mM L-glutamine, 20 mM HEPES, 0.1 mM Non-Essential Amino Acids, 1 mM sodium pyruvate, and 500 μ g/mL G418 at 37 °C in a humidified incubator containing 5% CO₂ and 95% O₂. Sf9 insect cells (Expression Systems) were maintained in a shaking incubator (120–140 rpm) at 27 °C in ESF921 growth medium (Expression Systems). Sf9 cells were infected with baculovirus to express full-length mGlu₅ and N-terminally truncated mGlu₅ constructs (see Supporting Information), to purify the mGlu₅ receptor with an N-terminal FLAG tag and C-terminal 8xHis tag (full details in the Supporting Information).

Intracellular Ca²⁺ Mobilization

Prior to assay, HEK293A-mGlu₅-low cells were seeded at 50 000 cells/well in poly-D-lysine coated 96-well plates in assay medium (DMEM with 5% dialyzed FBS, 20 mM HEPES, 1 mM sodium pyruvate). The cell permeable Ca²⁺ indicator dye Fluo-4 (Invitrogen) was used to assay receptor-mediated Ca²⁺ mobilization as described previously²⁰ using a Flexstation II (Molecular Devices).

Radioligand Binding

Membranes (prepared from HEK293A-mGlu₅-low as described previously)²⁰ were incubated with ~2 nM [³H]mPEPy in the presence of 0.3 nM–30 μ M of known and novel mGlu₅ allosteric modulators at RT for 1–4 h in Ca²⁺ assay buffer. Nonspecific binding was defined by 10 μ M MPEP. Assays were terminated by rapid filtration through GF/B filter paper or Unifilter plates, followed by three washes with ice-cold binding buffer (0.9% NaCl, 50 mM Tris, pH 7.4). Plates/filters were allowed to dry overnight prior to the addition of Microscint20 (40 μ L/well). After 2 h incubation, scintillation was counted using a TopCount (PerkinElmer).

To assess irreversible binding, cells were plated into 24-well poly-D-lysine coated plates in an assay medium at a density of ~200 000 cells/well. Cells were incubated with photoprobes for 10 min at RT, followed by aspiration of ligand-containing media and replacement with Ca²⁺ assay buffer. Cells were then washed 5 $\times$ 1 h (a total of 5 h) at RT with a Ca²⁺ assay buffer. Cells were incubated with ~2 nM [³H]mPEPy for 1 h at RT and radioligand-containing buffer aspirated, and the cells were washed three times with ice-cold 0.9% NaCl. Cells were lysed with 0.2 M NaOH overnight prior to transfer to scintillation vials. Samples were incubated with an UltimaGold scintillation cocktail for a minimum of 2 h prior to detection using a TriCarb liquid scintillation analyzer (PerkinElmer).

Photoaffinity Labeling and in-Gel Fluorescence

Membrane preparations (diluted at least 1:10 in PBS (phosphate buffered saline) to 1 mg mL⁻¹) or purified protein (~6 μ g/sample diluted 1:25 in PBS to final reaction volume of 25 μ L) were incubated with photoprobes, competitive ligands, or vehicle controls (1% DMSO) at RT. To covalently bind to the receptor, benzophenone-alkyne **4** was UV irradiated (365 nm) for 30 min at 4 °C; azide-containing ligands **5** and **8** were irradiated (254 nm) for 3 $\times$ 1 min with 1 min resting at 4 °C. All samples were then subjected to click chemistry. Click reagents were added in the following order: fluorescent azide/alkyne (Cy5.5 or Cy7.5 at a minimum 1:1 ratio to click photoprobe), 1 mM ascorbic acid (made immediately prior), TBTA/*t*-butanol, and 1 mM CuSO₄. The reagents were incubated for 1 h at RT with intermittent vortexing. The sample buffer was added and proteins separated by SDS-PAGE (200 V, 40 min, RT). In-gel fluorescence was detected using an Odyssey scanner (LiCOR). Proteins were then transferred to nitrocellulose (overnight, 30 V, 4 °C), membranes blocked with Odyssey blocking buffer, and incubated with anti-mGlu₅ (1:5000, rabbit polyclonal, Millipore) overnight at 4 °C. Membranes were washed three times with PBS-T (PBS with 0.1% Tween-20) and incubated with goat antirabbit-800 (1:15000; LiCOR) or donkey antirabbit-680 (1:15000; LiCOR) for 1 h at RT. Following three washes with PBS-T, membranes were imaged using the Odyssey. Signal intensity of infrared (Cy5.5 or Cy7.5) click tags or secondary antibody were quantified using ImageStudio (LiCOR). In-gel fluorescence was normalized to relative mGlu₅ levels in each sample.

Data Analysis

All nonlinear regression curve fits were performed using Prism 6 (GraphPad Software). Inhibition of [³H]mPEPy binding data sets were fitted to a one-site inhibition binding model and estimates of inhibitor equilibrium dissociation constants (*K_i*) were derived using the

Cheng-Prusoff equation.³⁷ Allosteric modulator concentration–response curves as well as glutamate interaction experiments were fitted to a four-parameter logistic equation and operational models of agonism or allosterism as described previously.^{20,38} Equations, parameters, and constraints are described in full in the Supporting Information. All affinity, cooperativity, and potency parameters are expressed as mean $\pm$ SEM and were estimated as logarithms. Statistical analyses were performed where appropriate using one-way ANOVA with Dunnett's post-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Select acetylenic mGlu₅ NAM lead compounds **1–3**, **6**, and **7** were derivatized to provide clickable photoprobes **4**, **5**, and **8**. Click chemistry handles are highlighted in blue; photoreactive functional groups are highlighted in red.

Figure 2.

Pharmacological characterization of select acetylenic mGlu₅ NAM lead compounds, and clickable photoreactive derivatives thereof, in radioligand binding and functional assays. (a) No evidence of agonist activity for lead compounds **1–3**, **6**, and **7** for mGlu₅-intracellular Ca²⁺ mobilization; the glutamate concentration–response curve run in parallel is shown for reference. (b) Acetylenic mGlu₅ NAM lead compounds **1–3**, **6**, and **7** concentration-dependently inhibit the functional response to a submaximal (EC₈₀) glutamate concentration in a mGlu₅ intracellular Ca²⁺ mobilization assay. (c) Inhibition of [³H]mPEPy binding to membrane preparations from HEK293A-mGlu₅-low. (d) Increasing incubation time to 4 h resulted in complete displacement of [³H]mPEPy binding by benzophenone **4**, whereas there was no effect on MPEP (**6**) binding. (e) No evidence of agonist activity is observed for clickable photoprobes **4**, **5**, and **8** in the mGlu₅ intracellular Ca²⁺ mobilization assay. (f) Novel clickable probes **4**, **5**, and **8** concentration-dependently inhibit the response to a submaximal (EC₈₀) concentration of glutamate in an mGlu₅ intracellular Ca²⁺ mobilization assay. Data represent the mean ± SEM from *n* = 3 performed in duplicate, with the exception of panel D, where individual replicates from *n* = 2 are plotted.

Figure 3.

Estimation of mGlu₅ affinities for clickable photoprobes **4**, **5**, and **8** using an intracellular Ca²⁺ mobilization assay. Progressive shifts of the glutamate concentration–response curve for intracellular Ca²⁺ mobilization were observed with 1 min (**a–c**) and 30 min (**d–f**) preincubation with increasing concentrations of benzophenone-alkyne **4** (**a** and **d**), diazide **5** (**b** and **e**), and azido-alkyne **8** (**c** and **f**). Data are mean ± SEM from *n* = 3 performed in duplicate. Curves represent the best fit of the data to eq 2 (see Supporting Information).

Figure 4.

Novel mGlu₅ NAM photoprobes irreversibly bind to mGlu₅. (a) HEK293A-mGlu₅-low cells were pretreated with indicated mGlu₅ NAM (1μM) with or without UV exposure. The cells were then washed (3 × 5 min) before conducting a [³H]mPEPy binding assay. (b) Photoprobes 4, 5, and 8 irreversibly bind to mGlu₅ in the absence of UV irradiation, as evidenced by their ability to inhibit [³H]mPEPy binding following an extended wash paradigm (five 1 h washes). Effect of acute exposure to MPEP or photoprobe 8, followed by extensive washes, on mGlu₅-intracellular Ca²⁺ mobilization in response to glutamate (c) or VU0424465 (d). Data represent the mean ± SEM from *n* = 3 performed in duplicate, with the exception of # where data are *n* = 1.

Figure 5.

Concentration-dependent incorporation of Cy5.5 and Cy7.5 azide is evident upon tandem mGlu₅ photoaffinity labeling-bioorthogonal conjugation employing select clickable photoreactive acetylenic NAMs. HEK293-mGlu₅-high membrane preparations were incubated with benzophenone-alkyne **4** (a) and azido-alkyne **8** (b), or vehicle (1% DMSO), exposed to UV irradiation, and subjected to click chemistry with Cy5.5 or Cy7.5 azide (using a 1:1 ratio) to attach fluorophores to the terminal alkyne groups within the photoprobes. In the absence of photoprobes **4** or **8**, little or no in-gel fluorescence was observed when membrane preparations were separated by SDS-PAGE (top). Immunoblotting for mGlu₅ was performed, wherein bands corresponding to mGlu₅ monomer (~140 kDa) and dimer (~280 kDa) are evident in all samples (middle). Co-labeling (yellow) of Cy7.5 (panel **a**; green) or Cy5.5 (panel **b**; red) and mGlu₅ immunoreactivity (panel **a**, red; panel **b**, green) is evident on nitrocellulose membranes (bottom).

Figure 6.

Photoincorporation of select acetylenic clickable photoprobes into purified mGlu₅ is inhibited by preincubation with an alternative acetylenic clickable photoprobe. (a) Competitive click photolabeling schematic: 1 = no competitor, 2 = with competitor. (b) Photoaffinity labeling of purified mGlu₅ with a saturating concentration of either benzophenone-alkyne **4** or diazide **5** inhibits incorporation of an alternative clickable photoprobe. A representative in-gel fluorescence image and corresponding immunoblot is shown. (c) Quantification of Cy5.5 in-gel fluorescence of the mGlu₅ dimer band normalized to relative mGlu₅ levels. Benzophenone-alkyne **4**, diazide **5**, and azido-alkyne **8** in the absence of a competitor significantly increase Cy5.5 fluorescence over vehicle treated levels, * $p < 0.05$, one-way ANOVA, Dunnett's post-test. Data are mean $\pm$ SEM from $n = 3$.

Summary of Functional Potency and Binding Affinity Estimates for Lead Acetylenic mGlu₅ NAMs and Clickable Photoprobe Derivatives Thereof^a

Table 1

compound	EC ₅₀ glutamate inhibition			[³ H]-mPEPy binding		modulation of glutamate	
	pIC ₅₀ ^b (nM)	hill slope	pK _B ^c (nM)	pK _i ^d (nM)	pK _B ^e (nM)		
1	8.24 ± 0.10 (5.8)	-1.07 ± 0.05	8.01 ± 0.16 (9.8)	8.21 ± 0.05 (6.2)	n.d.		
2	6.86 ± 0.21 (138)	-1.37 ± 0.21	6.73 ± 0.06 (186)	7.23 ± 0.05 (59)	n.d.		
3	6.71 ± 0.23 (195)	-1.68 ± 0.23	6.55 ± 0.22 (282)	7.32 ± 0.07 (48)	7.04 ± 0.15 (91)		
4	6.19 ± 0.28 (647)	-0.91 ± 0.23	6.05 ± 0.20 (891)	7.60 ± 0.02 (25)	6.24 ± 0.09 (575)		
5	5.98 ± 0.18 (1047)	-0.63 ± 0.11	6.05 ± 0.09 (902)	7.15 ± 0.07 (71) ^f	6.62 ± 0.05 ^f (240)		
6 (MPEP)	8.23 ± 0.16 (5.8)	-1.53 ± 0.18	7.97 ± 0.16 (11)	8.02 (9.5) ^g	8.55 (2.8) ^g		
7	7.64 ± 0.14 (23)	-2.05 ± 0.32	7.53 ± 0.16 (30)	n.d.	8.09 ± 0.09 (8.1)		
8	7.73 ± 0.11 (19)	-2.36 ± 0.10	7.41 ± 0.05 (39)	9.29 ± 0.06 (0.5)	8.04 ± 0.20 (9.1)		

^aData are mean ± SEM from a minimum of three independent experiments. n.d., not determined.^bNegative logarithm of the concentration of modulator required to yield half maximal inhibition of EC80 glutamate response.^cNegative logarithm of the equilibrium dissociation constant for the indicated allosteric modulator determined by analysis of concentration response curves for inhibition of EC80 glutamate using the operational model of allosterism.²⁰^dNegative logarithm of the equilibrium dissociation constant for modulators as determined from inhibition of [³H]mPEPy binding using the Cheng-Prusoff equation.³⁷^eNegative logarithm of the equilibrium dissociation constant for the indicated compound determined from glutamate concentration response curves for Ca²⁺ mobilization in the absence and presence of modulators added 1 min prior to glutamate addition.^fUnder nonequilibrium conditions, diazide **5** did not fully inhibit the maximal response to glutamate.^gData previously reported in ref 20 are provided for comparison.

Impact of Irreversible Binding of Azido-Alkyne 8 (1uM) on mGlu₅ Agonists Coupling to Intracellular Ca²⁺ Mobilization in HEK293A-mGlu₅-low Cells^a

Table 2

	pEC ₅₀ (untreated) ^b	pEC ₅₀ (treated)	E _{max} ^c	log τ ^d	pK _A ^e
glutamate	7.31 ± 0.28	7.00 ± 0.24	-46.3 ± 18.3	-0.53 ± 0.18	7.00 ± 0.20
VU0424465	6.60 ± 0.18	6.11 ± 0.23	-18.3 ± 10.8	-0.46 ± 0.17	6.17 ± 0.25

^aData are mean ± SEM from three independent experiments.^bThe negative logarithm of the molar concentration of agonist required to yield a half maximal response.^cThe change in agonist maximal response (E_{max}), expressed as a percentage of the maximum response to glutamate.^dThe change in the coupling efficiency (τ) of agonist, determined by globally fitting treated and untreated data sets to an operational model of agonism.^eThe negative logarithm of the equilibrium dissociation constant of the agonist.