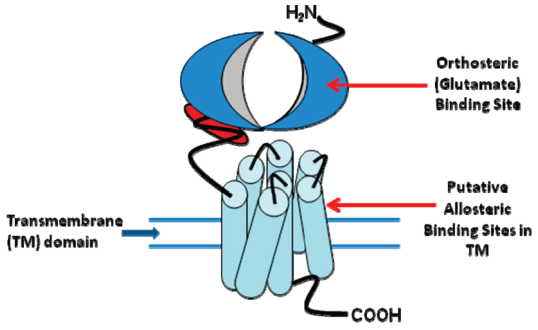
Discovery of a Novel Chemical Class of $mGlu_5$ Allosteric Ligands with Distinct Modes of Pharmacology

Alexis S. Hammond,[†,‡] Alice L. Rodriguez,[‡,∥] Steven D. Townsend,[§] Colleen M. Niswender,[‡,∥] Karen J. Gregory,[‡,∥] Craig W. Lindsley,[‡,§,∥] and P. Jeffrey Conn*[,‡,§,∥]

[†]Department of Neuroscience and Pharmacology, Meharry Medical College, Nashville, Tennessee 37208, [‡]Department of Pharmacology, [§]Department of Chemistry, and [∥]Vanderbilt Program in Drug Discovery, Vanderbilt University, Nashville, Tennessee 37232

Abstract



We previously discovered a positive allosteric modulator (PAM) of the metabotropic glutamate receptor subtype 5 ($mGlu_5$) termed 4 *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide (CPPHA) that elicits receptor activation through a novel allosteric site on $mGlu_5$, distinct from the classical $mGlu_5$ negative allosteric modulator (NAM) MPEP allosteric site. However, a shallow structure−activity relationship (SAR), poor physiochemical properties, and weak PAM activity at rat $mGlu_5$ limited the utility of CPPHA to explore allosteric activation of $mGlu_5$ at a non-MPEP site. Thus, we performed a functional high-throughput screen (HTS) and identified a novel $mGlu_5$ PAM benzamide scaffold, exemplified by VU0001850 (EC_{50} = 1.3 μM, 106% Glu_{max}) and VU0040237 (EC_{50} = 350 nM, 84% Glu Max). An iterative parallel synthesis approach delivered 22 analogues, optimized $mGlu_5$ PAM activity to afford VU0357121 (EC_{50} = 33 nM, 92% Glu_{max}), and also revealed the first non-MPEP site neutral allosteric ligand (VU0365396). Like CPPHA, PAMs within this class do not appear to bind at the MPEP allosteric site based on radioligand binding studies. Moreover, mutagenesis studies indicate that VU0357121 and related analogues bind to a yet uncharacterized allosteric site on $mGlu_5$, distinct from CPPHA, yet share a functional interaction with the MPEP site.

Keywords: $mGlu_5$, metabotropic, glutamate, allosteric, potentiator

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). It plays important roles in virtually all major CNS circuits by acting through ionotropic glutamate receptors (iGluRs), as well as metabotropic glutamate (mGlu) receptors. The eight mGluR subtypes are family C G-protein coupled receptors (GPCRs) that are divided into three groups based on sequence homology and coupling to signal transduction cascades. Group I includes $mGlu_1$ and $mGlu_5$; these receptors couple to $G_{\alpha q}$ and induce increases in intracellular calcium. Group II ($mGlu_2$ and $mGlu_3$) and group III ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$) receptors couple to $G_{\alpha i/o}$ and inhibit cAMP formation (*1*). The family C GPCRs are characterized by a large bilobed (termed venus fly trap) extracellular ligand binding domain where the endogenous ligand glutamate and other orthosteric ligands bind (Figure 1A). Because of the highly conserved nature of the orthosteric binding site, ligands that modulate receptor function at this site have poor subtype selectivity among the mGlu receptors. An alternative approach to targeting the highly conserved orthosteric glutamate site is to develop compounds that act at allosteric sites on mGlu receptors that are removed from the orthosteric site in the heptahelical transmembrane domain and may be less highly conserved. This approach is proving highly successful for multiple GPCRs. Negative allosteric modulators (NAMs) were the first allosteric ligands reported for the mGlu receptors and have been described for $mGlu_1$ and $mGlu_5$ (*2*, *3*); moreover, positive allosteric modulators (PAMs), molecules that bind at an allosteric site, do not activate the receptor directly but potentiate activation of the receptor by the orthosteric agonist glutamate, have been reported for $mGlu_2$, $mGlu_4$, and $mGlu_5$ (*4*).

Recent anatomical, cellular, molecular, and behavioral findings suggest that selective activators of the metabotropic glutamate receptor subtype 5 ($mGlu_5$) may provide a novel strategy for the treatment of the psychotic symptoms and cognitive impairments observed

Received Date: May 24, 2010
Accepted Date: August 12, 2010
Published on Web Date: August 19, 2010

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

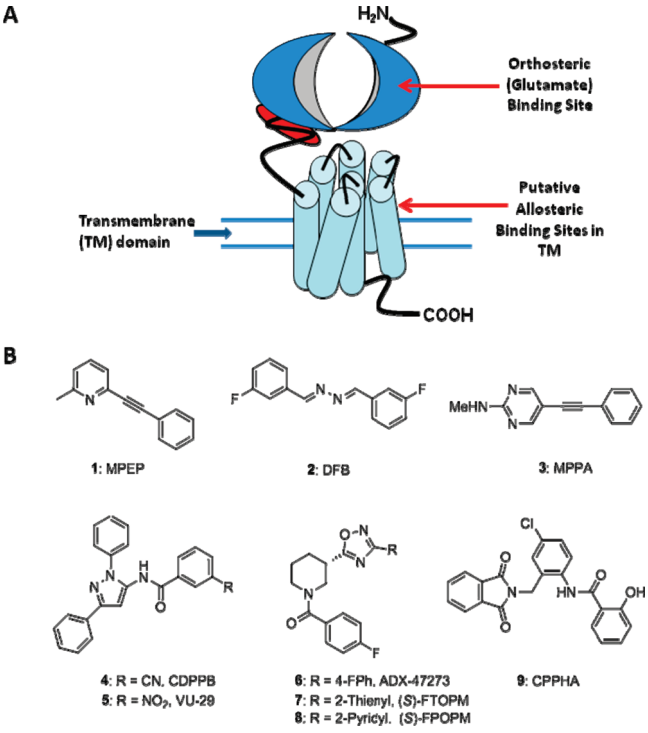


Figure 1. (A) Schematic illustration of the structure of a metabotropic glutamate receptor. (B) Structures of known $mGlu_5$ allosteric ligands (NAMs and PAMs).

in individuals with neuropsychiatric disorders (*5*−*7*). However, it has been historically difficult to produce agonists of $mGlu_5$ that are selective for this subtype as compared to other mGlu receptors. Recently, our laboratory and others have made major breakthroughs in this area by developing selective positive allosteric modulators (PAMs) of this receptor. MPEP **1** is the prototypical $mGlu_5$ NAM with a well characterized allosteric binding site (Figure 1B; see references *2*, *8*, and *9*). Several distinct chemotypes have been identified that function as $mGlu_5$ PAMs and are exemplified by DFB **2**, MPPA **3**, CDPPB **4**/VU-29 **5**, and the ADX series (ADX47273 **6**, (*S*)-FTOPM **7**, and (*S*)-FPOPM **8**) (*9*−*12*). All of these PAMs potentiate $mGlu_5$-mediated responses by binding to the well-characterized, negative allosteric modulator MPEP binding site. Interestingly, CPPHA **9** is the only known $mGlu_5$ PAM that does not bind to the MPEP site and acts via a unique site on the receptor to potentiate $mGlu_5$ responses (*13*, *14*). These data suggest that multiple allosteric sites exist on $mGlu_5$. Also, CDPPB, ADX47273, and (*S*)-FTOPM display intrinsic agonist activity at moderate to high concentrations in Chinese hamster ovary (CHO) or human embryonic kidney (HEK293) cells expressing $rmGlu_5$, whereas MPPA, DFB, (*S*)-FPOPM, and CPPHA have no effect on $mGlu_5$ alone, and only potentiate responses of the receptor to glutamate (i.e., pure PAM) (*10*, *11*, *13*, *15*). In addition, we have shown that CPPHA and DFB display differential

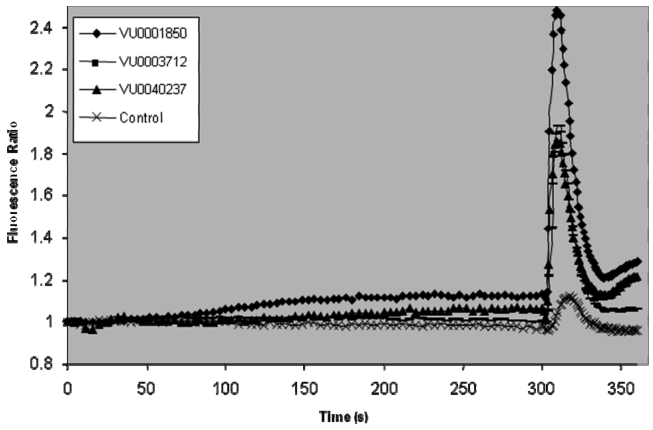


Figure 2. Benzamide compounds are able to potentiate the calcium mobilization response of $mGlu_5$ to glutamate. Raw traces show the effect of a fixed concentration (10 μM) of test compound or vehicle when added to calcium-sensitive dye-loaded cells and allowed to incubate for 5 min. A suboptimal (EC_{20}) concentration of glutamate was added and the calcium response measured by the FDSS plate reader. Responses are expressed as a fluorescence ratio.

modulatory function on $mGlu_5$-mediated phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (*16*). Together, these findings indicate that $mGlu_5$ PAMs can have differential effects on downstream receptor trafficking by virtue of the allosteric site the ligand occupies to exert conformational changes leading to potentiation. Thus, it will prove critically important to identify additional novel classes of $mGlu_5$ PAMs and allosteric binding sites to further our understanding of differential $mGlu_5$ potentiation and receptor trafficking.

CPPHA, due to its unique allosteric binding site and pharmacology, has the potential to serve as an important pharmacological tool to further investigate functional selectivity among classes of $mGlu_5$ PAMs. Unfortunately, due to diminished potency on rodent $mGlu_5$, shallow SAR, and poor physiochemical properties, CPPHA is of limited utility to translate into in vivo studies (*17*). Here, we describe the identification, SAR and in vitro pharmacological characterization of a novel chemical class of $mGlu_5$ PAMs that are reminiscent of, yet distinct from, CPPHA that activate $mGlu_5$ at a non-MPEP and non-CPPHA allosteric site. Moreover, this new series of $mGlu_5$ PAMs, represented by VU0357121 (EC_{50} = 33 nM, 92% Glu_{max}), possess potent activity on the rat receptor. This effort also identified VU0365396, the first non-MPEP neutral allosteric ligand.

Results and Discussion

A number of significant issues have hampered the basic science efforts driving mGluR research across all receptor subtypes: (1) a lack of truly selective tool compounds to clearly elucidate the physiological role of given subtypes, (2) receptor trafficking phenomenon

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

by different allosteric ligands and by receptor activation at different allosteric binding sites, and (3) subtle molecular switches that elicit alternate modes of pharmacology. In order to develop an $mGlu_5$ PAM that could be developed as a potential therapeutic agent, we must first understand the functional relationship between distinct allosteric binding sites and downstream receptor trafficking. In order to accomplish this, it will be important to understand the similarities and differences between novel $mGlu_5$ PAMs that act at different allosteric sites.

Discovery of a Novel Benzamide Class of $mGlu_5$ PAMs by HTS

Our laboratory performed a functional high-throughput screen (HTS) (*18*−*20*) that led to the identification of about 20 structural classes of $mGlu_5$ PAMs. The present study focuses on a novel benzamide chemical scaffold that was identified in that screen. Sixty-five compounds were identified that belong to this structural class, and 10 were found to have high potencies with EC_{50} values ≤1 μM from HTS stock solutions. This class is of particular interest because these compounds share a benzamide backbone that is similar to that of CPPHA yet are distinct. These compounds display little intrinsic $mGlu_5$ agonist activity in the calcium mobilization assay and induce a robust enhancement of the response to a suboptimal concentration (EC_{20}) of glutamate (Figure 2). To test their PAM activity, HEK293 cells expressing rat $mGlu_5$ were preloaded with calcium-sensitive fluorescent dye and received vehicle or compound (10 μM), followed by an EC_{20} concentration of glutamate 5 min later. Receptor-induced calcium mobilization responses were monitored using Functional Drug Screening System (FDSS, Hamamatsu, Japan).

Design, Synthesis, and SAR of Benzamide Analogues

As previously stated, the lead optimization program that led to the discovery of CPPHA faced several challenges, making it difficult to develop compounds related to CPPHA that retained activity on rodent $mGlu_5$ (*17*). The three most potent compounds within the benzamide chemical class (VU0001850, VU0003712, and VU0040237) are shown in Figure 3. With the identification of our three main hits from the screen, we had the opportunity to determine whether slight modifications to their chemical structures would lead to tractable SAR for rat $mGlu_5$ or afford modulation in the mode of $mGlu_5$ pharmacology. For chemical lead optimization, we elected to employ a parallel synthesis approach in which we resynthesized the three initial hits along with a library of 22 analogues to obtain an initial understanding of the SAR in a 25-member library of analogues **10** (Figure 3A).

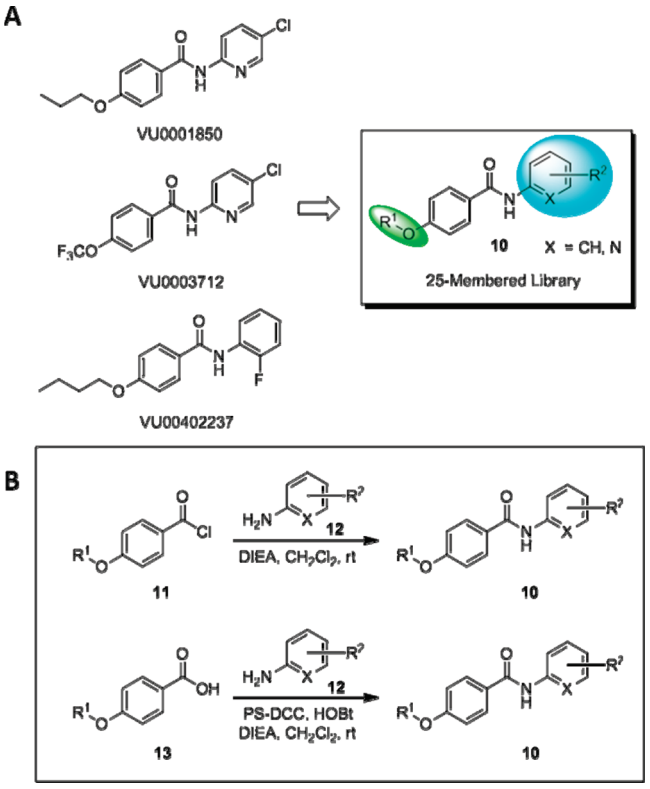


Figure 3. Design and synthetic strategy for chemical lead optimization of $mGlu_5$ PAM HTS hits. (A) Structures of $mGlu_5$ PAM hits and general library design strategy. (B) General synthetic approaches employed for iterative library synthesis.

The first set of analogues varied both the aryl/heteroaryl amide (blue) as well as different ethers (green). The chemistry involved treatment of a *p*-alkoxy benzoyl chloride **11** and an aryl/heteroaryl amine **12** to provide analogues **10** or the corresponding benzoic acid **13** could be coupled to **12** under standard carbodiimide conditions to delivers analogues **10** (Figure 3B). All compounds were purified by mass-directed preparative HPLC to >98% purity.

These novel analogues were assayed in a single-point screen, along with the three original hits, to determine their ability to potentiate a suboptimal concentration of glutamate using the FlexStation fluorometric imaging plate reader from Molecular Systems. HEK293 cells expressing $mGlu_5$ were incubated with a fixed concentration of test compound for 109 s and then stimulated with an EC_{20} concentration of glutamate. At 10 μM, all three of the original hits (VU0001850, VU0003712, and VU0040237), as well as three of the novel analogues (VU0357121, VU0365393, and VU0125936), potentiated the response to suboptimal glutamate concentrations above a 50% response, which was used as a cutoff reference (Figure 4A). Screening the compounds at a concentration of 1 μM revealed that two of the original hits (VU0001850 and VU0040237) as well as two of

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

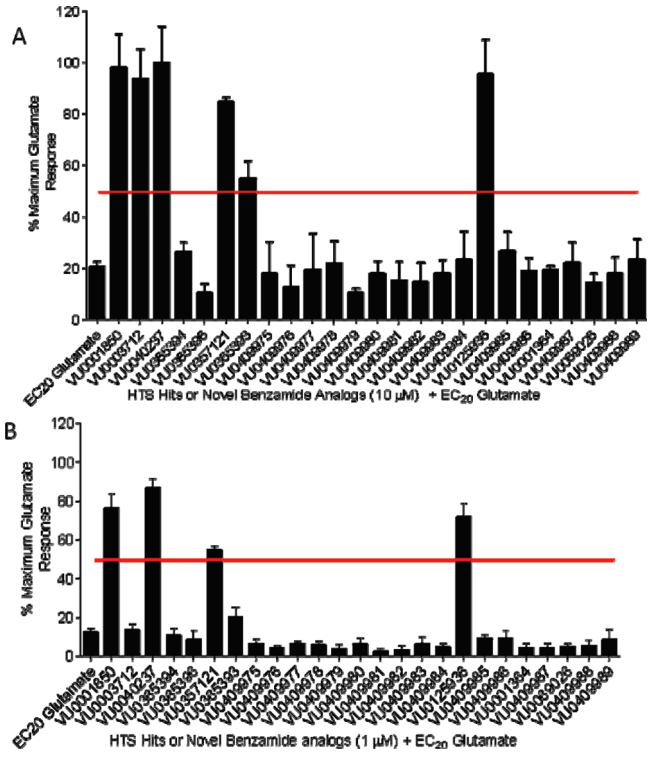


Figure 4. HTS hits and several novel analogues potentiate $mGlu_5$-mediated effects on calcium mobilization. The compounds were screened for their ability to potentiate a suboptimal concentration of glutamate when HEK cells were pretreated with a fixed concentration of test compound at either (A) 10 μM or (B) 1 μM. The red lines identify those compounds that are able to potentiate the glutamate-induced response above 50% of the maximal response. Data were normalized to the average maximal response obtained from each experiment as determined by 100 μM glutamate. Data were obtained from three separate experiments, each performed in triplicate, and are expressed as the mean ± SEM.

the newly synthesized compounds (VU0357121 and VU0125936) potentiated the response to glutamate at submicromolar concentrations (Figure 4B), and these compounds were chosen for further analysis as $mGlu_5$ PAMs. As we had previously seen with CPPHA, SAR was quite shallow for this new series, with few active PAMs.

To further evaluate the compounds that were identified as potent $mGlu_5$ PAMs (VU0001850, VU0040237, VU0357121, and VU0125936), we determined their ability to potentiate $mGlu_5$ responses to glutamate in two separate calcium mobilization assays. First, the test compound's concentration−response relationships were examined by incubating $mGlu_5$-expressing cells with increasing concentrations of compound in the presence of a fixed suboptimal concentration of glutamate (EC_{20}).

Figure 5 shows the concentration−response relationships for these compounds, and compound potencies ranged from ~33 nM to 1 μM. A second measure of potentiation was performed to assess the ability of the compounds to increase the potency of a glutamate concentration−response curve (fold-shift assay). This is measured as a leftward shift of the glutamate concentration−response curve (CRC) and decrease in the EC_{50} of glutamate in the presence of test compound as compared to a glutamate concentration−response curve in the presence of vehicle only. HEK293 cells expressing $mGlu_5$ were pretreated with vehicle or a fixed concentration (12.5 μM) of the $mGlu_5$ PAM, followed by increasing concentrations of glutamate. The test compounds caused 2- to ~6-fold shifts in the glutamate concentration−response curve at $mGlu_5$ (Figure 6), providing further evidence for their ability to potentiate $mGlu_5$-mediated responses to glutamate.

Figure 7 highlights the SAR for the benzamide series after one round of library synthesis. The initial HTS hits VU0001850 and VU0040237 were found to possess EC_{50} values of 1.3 μM and 350 nM on rat $mGlu_5$, respectively. Both VU0001850 and VU0040237 potentiated the response to the EC_{20} glutamate concentration to 106% and 84% of the response to a maximal concentration of glutamate, respectively (% Glu_{max}), and induced leftward shifts (~5.2× and ~3.9×, respectively) of the glutamate CRC (Figure 7A). The two new analogues, VU0357121 and VU0125936, represented improvements over the HTS leads. Addition of a second fluorine into the 4-position of VU0040237 provided VU0357121 (EC_{50} = 33 nM, 92% Glu_{max}, and ~2.6-fold shift) and a 10-fold increase in potency over the HTS hit. Similarly, homologation of the *n*-propyl ether in VU0001850 to an *n*-butyl ether analogue VU0125936 provided an increase in rat $mGlu_5$ potency (EC_{50} = 664 nM, 112% Glu_{max}, and ~5.8-fold shift).

Again, further libraries resulted in no significant active $mGlu_5$ PAMs, and the SAR was highly reminiscent of the CPPHA SAR. As depicted in Figure 7B, alternate chain lengths and/or cyclic constraints in the ether linkage abolished PAM activity. Minor modifications, such as N-alkylation of the secondary amide, led to inactive analogues. Finally, aryl substitution other than fluorine and alternative heterocycles for the 2-pyridyl moiety also resulted in no measurable PAM activity. These data, coupled with the structural similarities of these compounds to CPPHA, led us to speculate that this series of $mGlu_5$ PAMs may bind at either the CPPHA site or an overlapping, shallow allosteric site, as MPEP-site PAMs generally afford more robust SAR.

VU0357121 and Analogues Are Selective for $mGlu_5$ Relative to Other mGluR Subtypes

Before using these compounds to explore $mGlu_5$ allosteric sites, it was necessary to ensure that they were in fact selective for $mGlu_5$ relative to other mGluR subtypes. All four compounds were tested against mGlu receptors 1−4, 6, 7, and 8, and displayed similar selectivity profiles;

DOI: 10.1021/cn100051m | ACS Chem. Neurosci. (2010), 1, 702–716

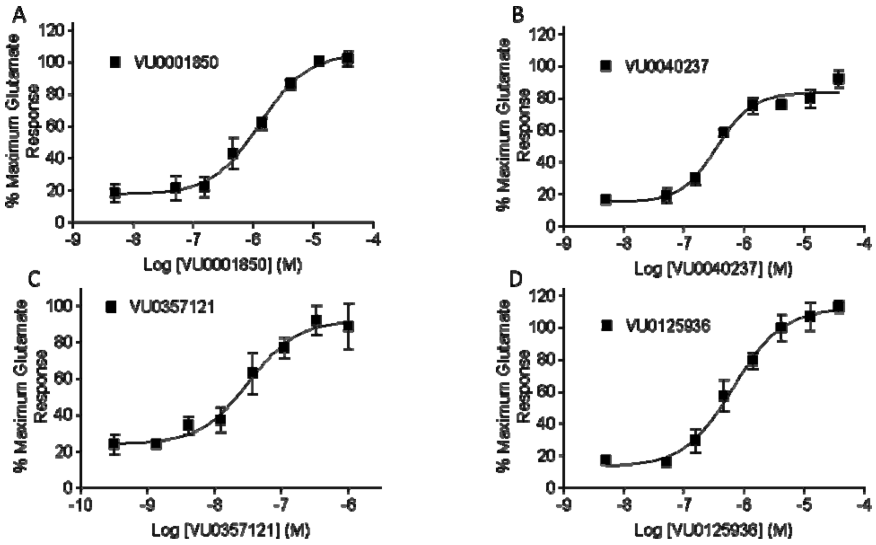


Figure 5. Test compounds are able to potentiate the calcium flux response of $mGlu_5$ to a suboptimal (EC_{20}) concentration of glutamate. Concentration−response relationships were generated by incubating cells with increasing concentrations of test compound, followed by stimulation with EC_{20} glutamate. Data were obtained from three separate experiments, each performed in duplicate, and are expressed as the mean ± SEM. Data were normalized to the average maximum response obtained from each experiment as determined by 100 μM glutamate. Concentration−response curves were generated by nonlinear curve fitting.

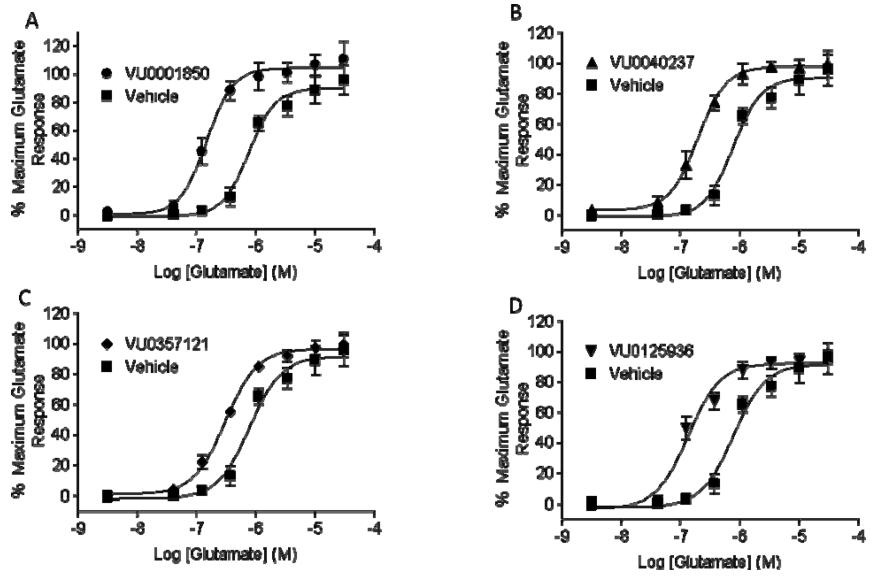
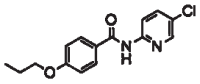
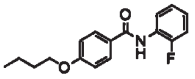
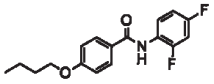
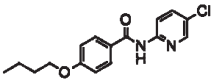


Figure 6. Benzamide PAMs induce a leftward shift in the glutamate response curve at $mGlu_5$. The 2- to 6-fold leftward shifts induced by these compounds indicate an enhancement of the response of $mGlu_5$ to glutamate. Concentration−response relationships were generated by adding a fixed concentration (12.5 μM) of test compound to cells, followed by increasing concentrations of glutamate. Data were analyzed using nonlinear regression, providing EC_{50} values for each curve. Data were obtained from three separate experiments, each performed in duplicate, and are expressed as the mean ± SEM. Data were normalized to the average maximum response obtained from each experiment as determined by 100 μM glutamate.

therefore, data for the most potent PAM, VU0357121, is highlighted as representative (Figure 8). First, we looked at the effects of the compounds on $mGlu_1$ because this receptor has the closest homology to $mGlu_5$ (*1*), which leads to a high degree of cross-activity of compounds. Fold-shift studies were performed as described above, except that HEK293 cells expressing rat Glu_1 were used. These compounds did not cause any shift in the glutamate concentration−response curve when added at 30 μM and only induced a slight depression in the maximum glutamate response, demonstrating a lack of PAM activity at $mGlu_1$ (Figure 8). Our laboratory recently developed an assay that detects the activity of compounds that interact with $G_{i/o}$-coupled GPCRs, such as the group II and III mGlu receptors (*21*). This assay measures thallium flux mediated

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

A

Compound	Structure	mGlu5 EC_{50}	% Glu Max	Fold-Shift
VU0001850		1,300 nM	106	5.2x
VU0040237		350 nM	84	3.9x
VU0357121		33 nM	92	2.6x
VU0125936		664 nM	112	5.8x

B

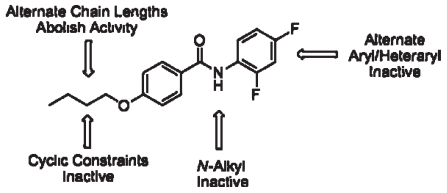


Figure 7. Structures and SAR of $mGlu_5$ PAMs from Initial Library Synthesis. (A) Diverse subset of synthetic analogues representative of the comprehensive SAR observed during lead optimization showing EC_{50} potency and Glu_{max} efficacy values. (B) Summary of observed SAR around VU0357121 showing western alkyl and eastern aryl amide functional handles.

by G-protein-regulated inwardly rectifying potassium channels (GIRKs). By performing fold-shift assays using HEK/GIRK cells that stably express either mGlu 2, 3, 4, 6, 7, or 8, we found that the test compounds did not modulate the response of any of these receptors to agonist, providing more evidence for their subtype selectivity (Figure 8). These studies confirmed that these compounds are highly selective as PAMs of $mGlu_5$ and are in fact inactive or very weakly antagonizing at other mGlu receptor subtypes.

VU0357121 and Analogues Do Not Bind at the MPEP Allosteric Site

Because of the structural similarity between these novel $mGlu_5$ PAMs and CPPHA and the shallow SAR, we hypothesized that they might interact with a site on the receptor that is distinct from the MPEP binding site. In order to test this hypothesis, we assessed the ability of these test compounds to compete for binding of the radiolabeled MPEP analogue [^{3}H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine) ([^{3}H]methoxy-PEPy) (*22*) to evaluate their interaction with the allosteric MPEP site on $mGlu_5$. The radioligand was added to membranes prepared from HEK293 cells stably expressing $mGlu_5$ with or without the test compound, and reactions were allowed to incubate for 1 h. It was found that, unlike MPEP ($K_i = 35 \pm 10.4$ nM), test compounds did not inhibit steady state [^{3}H]methoxyPEPy binding at concentrations of up to 100 μM, 3 orders of magnitude higher than those required for their PAM activity, suggesting that the ability of these agents to enhance glutamate sensitivity of $mGlu_5$ is likely due to the interaction at a site on the receptor distinct from the MPEP binding site (Figure 9). Slight inhibition of radioligand binding at higher concentrations may indicate some interaction between the MPEP site and the putative site of action of these PAMs.

A neutral allosteric ligand or silent allosteric modulator (SAM) binds to an allosteric site but does not have positive or negative allosteric modulator activity when measuring a defined response. However, a SAM competes for binding with PAMs and NAMs and thereby blocks the effects of positive and negative allosteric modulators without affecting the action of the orthosteric ligand through its binding to an allosteric site (*10*, *23*). Our laboratory previously reported that the neutral ligand 5-MPEP binds to the same site as its analogue MPEP and is able to inhibit the function of the NAM MPEP as well as PAMs such as VU-29 and CPPHA (*14*, *23*). Interestingly, 5-MPEP causes a competitive inhibition of VU-29 activity, whereas it induces a noncompetitive blockade of CPPHA function, providing further evidence for multiple modulatory sites on $mGlu_5$, and distinct sites of action for MPEP-like compounds and CPPHA. To further test the hypothesis that the novel benzamide class of $mGlu_5$ PAMs described here acts at a site that is distinct from the MPEP site, we utilized the SAM 5-MPEP as a tool to investigate its effects on VU0357121 activity in two separate assays. First, we examined 5-MPEP activity on the CRC of VU0357121-induced potentiation of glutamate-induced increases in calcium mobilization. The $mGlu_5$-expressing HEK293 cells were treated with fixed concentrations (333 nM to 10 μM) of 5-MPEP or vehicle and increasing concentrations of VU0357121, followed by the addition of an EC_{20} concentration of glutamate. Interestingly, in this kinetic assay 5-MPEP induces progressive rightward shifts and reductions in the maximal response to VU0357121 potentiation of glutamate responses (Figure 10A), suggesting that 5-MPEP is likely to block the response to VU0357121 by a noncompetitive mechanism of action. To further explore the interaction between these allosteric modulators in an end-point assay, we performed phosphoinositide (PI) hydrolysis studies to measure the effects of 5-MPEP on the VU0357121 agonist CRC. $mGlu_5$-expressing HEK293 cells loaded with [^{3}H]myo-inositol were treated with vehicle or a fixed concentration (10 μM) of 5-MPEP, followed by various concentrations of VU0357121. The resulting radiolabeled phosphoinositides were measured and normalized to the response elicited by 1 mM glutamate. Consistent with our findings in the calcium assay, this concentration of 5-MPEP

DOI: 10.1021/cn100051m | ACS Chem. Neurosci. (2010), 1, 702–716

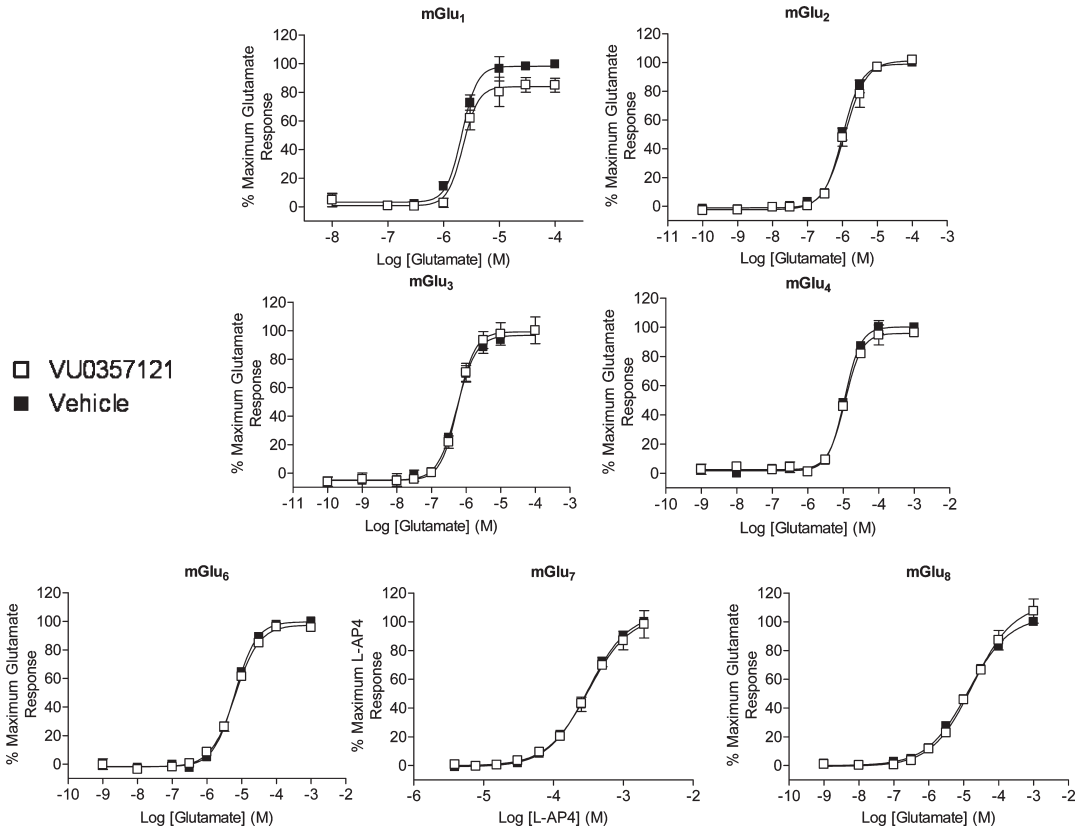


Figure 8. Novel PAMs are selective for $mGlu_5$ as compared to other subtypes. Despite weak inhibition of glutamate responses at $mGlu_1$, VU0357121 does not have PAM activity at other mGluR subtypes. Concentration−response relationships were generated by adding a fixed concentration (30 μM) of test compound to cells expressing the specific mGluR subtype, followed by increasing concentrations of agonist. Concentration−response curves were generated by nonlinear curve fitting. Data were obtained from three separate experiments, each performed in duplicate or triplicate, and are expressed as the mean ± SEM. Data were normalized to the average maximum response obtained from each experiment as determined by a maximum concentration of agonist.

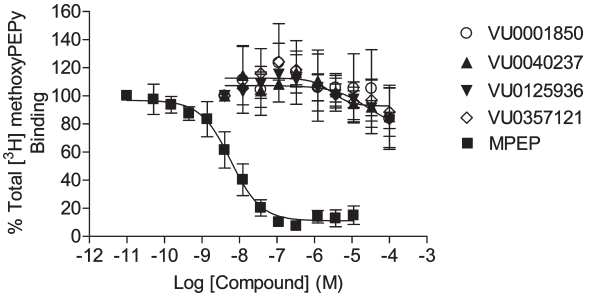


Figure 9. Unlike MPEP, test compounds do not displace radioligand binding. These novel PAMs have no effect on radioligand binding at concentrations necessary for their function. Membranes prepared from HEK cells expressing $mGlu_5$ were incubated with the radiolabeled MPEP analogue [^{3}H] methoxyPEPy (2 nM final concentration in 50 mM Tris and 0.9% NaCl, pH 7.4) for 60 min at room temperature in the presence of varying concentrations of test compound. Equilibrium binding was terminated by rapid filtration. Data are plotted as a percentage of total [^{3}H]methoxyPEPy binding. Data were obtained from three separate experiments, each performed in triplicate, and are expressed as the mean ± SEM.

caused a rightward shift in the VU0357121 CRC, coupled with a decrease in maximal potentiation (Figure 10B). As a control, we also investigated the effects of the same concentration of 5-MPEP on the VU-29 agonist curve. 5-MPEP (10 μM) induced a 20-fold parallel rightward shift of the VU-29 CRC at a magnitude consistent with the binding affinity for 5-MPEP (K_i = 388 ± 48 nM) and with the effect observed in the kinetic assay. These data are consistent with our previous studies and suggest that 5-MPEP acts in a competitive manner with VU-29 ((*14*) data not shown). These findings are consistent with our hypothesis that the benzamide class of $mGlu_5$ PAMs acts at an allosteric site other than the MPEP site and provide further evidence that there is a functional interaction between these two sites.

VU0357121 and Analogues Do Not Possess $mGlu_5$ NAM Activity

Studies with previous series of $mGlu_5$ allosteric modulators reveal that slight structural changes within a series can lead to striking changes in the modes of $mGlu_5$ pharmacology of these compounds (PAM to NAM and NAM to PAM) (Figure 11). In fact, close analogues of the first $mGlu_5$ PAM described, DFB, displayed a wide-range of modes of pharmacology with only slight structural

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

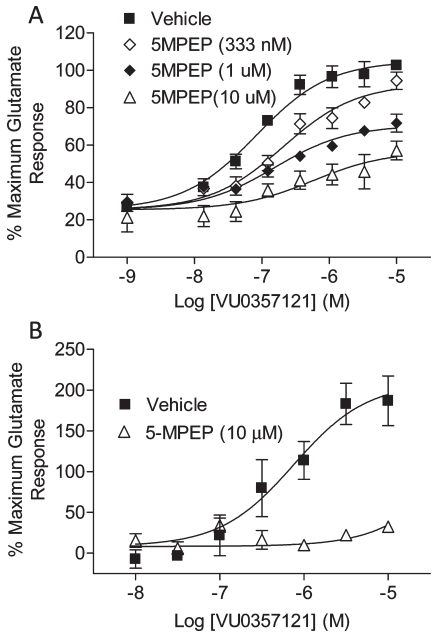


Figure 10. Neutral ligand 5-MPEP causes rightward shifts and decreases in the maximal response to VU0357121-mediated potentiation of glutamate-induced calcium mobilization, as well as effects of VU0357121 on PI hydrolysis. (A) Increasing concentrations of 5-MPEP induce progressive rightward shifts and decreases in the maximal potentiation by VU0357121 on glutamate-induced calcium flux, indicating a noncompetitive interaction between the two allosteric compounds. Concentration−response relationships were generated by incubating cells with increasing concentrations of VU0357121 and either vehicle (■) or 5-MPEP, followed by stimulation with EC_{20} glutamate. (B) 5-MPEP (10 μM) also induced a rightward shift and decrease in the maximal response to VU0357121-induced PI hydrolysis. In this assay, cells received either vehicle (■) or 5-MPEP (10 μM, △), followed by various concentrations of VU0357121, and the resulting phosphoinositides were measured. Data for each assay were obtained from three separate experiments, each performed in duplicate, and are expressed as the mean ± SEM. Data were normalized to the average maximum response obtained from each experiment as determined by a maximal concentration of glutamate. Concentration−response curves were generated by nonlinear curve fitting.

changes (*10*). Replacement of the 3,3′-diF with a 3,3′-diOMe, DOMEB **14**, provided an equipotent $mGlu_5$ NAM, while the 3,3′-diCl congener, DCB **15**, was the first neutral $mGlu_5$ ligand. Subsequently, a series of $mGlu_5$ partial antagonists **16** and **17** have also led to neutral ligands such as 5-MPEP **18** (*23*). More recently, another $mGlu_5$ partial antagonist 5-PEP **20** was converted into a potent, full NAM by the addition of a methyl group in the 3-position **19**; in contrast, addition of either a 4-methyl group **21** or an aminomethyl moiety **22** afforded potent $mGlu_5$ PAMs (Figure 11). Similar switches in the mode of $mGlu_5$ pharmacology have been noted in multiple $mGlu_5$ NAM series and in the ADX $mGlu_5$ PAM series (*15*, *24*, *25*). Notably, this has only been observed in MPEP site allosteric ligands and never observed in the non-MPEP site CPPHA series. To avoid potential

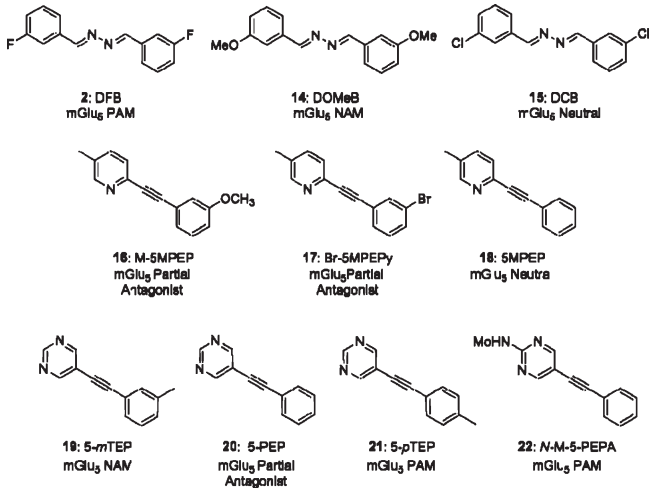


Figure 11. Structure of MPEP-site allosteric ligand chemotypes that display modes of pharmacology switches with slight structural modifications.

metabolites in vivo that might possess switches in the mode of pharmacology, it is imperative to establish whether subtle structural changes in compounds in this series of non-MPEP site ligands have a similar propensity to display fundamentally different modes of pharmacology.

Therefore, these compounds were also screened in a manner to identify NAMs or noncompetitive antagonists of $mGlu_5$. For the antagonist screen, we determined whether the test compounds could inhibit the effect of glutamate on $mGlu_5$-expressing HEK293 cells. The calcium mobilization assay was performed as described above, with cells being treated with test compounds at a fixed concentration of 10 μM, except that they were stimulated with a submaximal concentration of glutamate (~EC_{80}). In contrast to the blockade induced by the $mGlu_5$ NAM MPEP, VU0357121 and analogues did not inhibit the effect of glutamate on $mGlu_5$; in fact, the majority of compounds induced a mild potentiation of the EC_{80} glutamate response (Figure 12).

Identification of a Non-MPEP Site Neutral Ligand

To date, the only known neutral allosteric ligands or SAMs for mGlu receptors are ligands at the MPEP site on $mGlu_5$. To expand our investigation of putative non-MPEP allosteric sites on $mGlu_5$, it would be helpful to have a neutral ligand that acts at a different site on the receptor and possibly at the same site as VU0357121. We utilized the benzamide analogues that were synthesized earlier to determine whether any neutral ligands were present among these compounds. To screen our compounds for this activity, we determined their ability to block the VU0357121-induced potentiation of $mGlu_5$-mediated responses to glutamate. To accomplish this, we

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

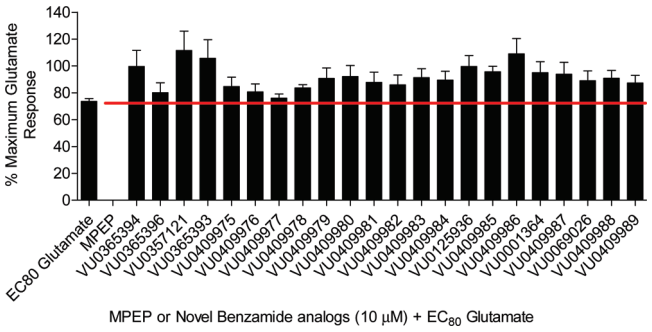


Figure 12. Unlike MPEP, no benzamide analogues were able to antagonize a submaximal (EC_{80}) concentration of glutamate. HEK cells were pretreated with a fixed concentration of test compound (10 μM) or vehicle, followed by EC_{80} glutamate. Data were normalized to the average maximal response obtained from each experiment as determined by 100 μM glutamate. Data were obtained from three separate experiments, each performed in triplicate, and are expressed as the mean ± SEM.

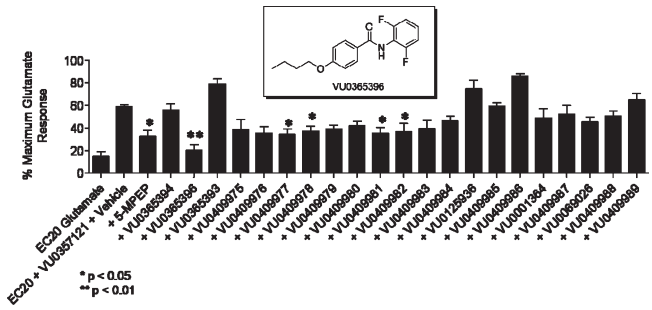


Figure 13. VU0365396 is the first non-MPEP site neutral allosteric ligand. Several benzamide analogues, as well as the previously identified neutral ligand 5-MPEP, significantly attenuate the potentiation of $mGlu_5$-mediated responses to glutamate by VU0357121. VU0365396 induces the most robust inhibition of VU0357121 effects ($p < 0.01$), and its chemical structure is shown in the inset. The calcium mobilization assay was used to screen benzamide analogues for neutral ligand activity. To establish the level of VU0357121 potentiation, HEK cells expressing $mGlu_5$ were pretreated with VU0357121 (50 nM), vehicle, and EC_{20} glutamate. To determine whether test compounds could inhibit the effect of VU0357121, cells were treated with VU0357121 (50 nM) and 5-MPEP (10 μM) or a benzamide analogue (10 μM) made up in the same solution, followed by EC_{20} glutamate. Data were normalized to the average maximal response obtained from each experiment as determined by 100 μM glutamate. Data were obtained from three separate experiments, each performed in triplicate, and are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$.

again used a calcium assay, but cells were pretreated with the test compound (10 μM) and VU0357121 (50 nM) followed by stimulation with an EC_{20} concentration of glutamate. Several compounds significantly inhibited the PAM activity of VU0357121 (Figure 13), but VU0365396 caused the most robust inhibition and was therefore chosen for further characterization. Interestingly, VU0365396 (30 μM) induced a parallel rightward shift in the VU0357121 CRC (Figure 14A), suggesting that this compound is a neutral ligand that acts in a competitive manner at the same allosteric site as VU0357121. In contrast, VU0365396 (30 μM) had no effect on CPPHA-induced potentiation of $mGlu_5$ responses (Figure 14B). The lack of effect on CPPHA PAM activity was somewhat surprising but provides clear evidence that these $mGlu_5$ PAMs do not act at the same site and raises the possibility that these sites may not interact functionally. However, it is possible that limited affinity of the neutral ligand at its own site or limited negative cooperativity led to a lack of effect on CPPHA responses. Unfortunately, limitations with solubility precluded studies with higher concentrations of VU0365396.

Mutations Differentially Alter Responses to VU0357121 and CPPHA

We previously reported the effects of single point mutations in $mGlu_5$ which can inhibit the responses of $mGlu_5$ to specific PAMs and NAMs (*8*, *14*, *20*). These mutants were tested for their ability to alter responses to VU0357121 (1 μM) in transiently transfected HEK293 cells using the calcium mobilization assay. Mutation of the alanine at the 809 position of rat $mGlu_5$ to a valine ($A809V/rmGlu_5$) reduces the binding and function of MPEP and inhibits functional responses to PAMs that act at the MPEP site, such as VU-29 (*8*, *14*, *20*) but has no effect on responses to CPPHA (*14*). In contrast, the $F585I/rmGlu_5$ mutation inhibits the PAM activity of CPPHA but has no effect on responses to VU-29 activity (*14*). We replicated these previous findings and found that the response to VU-29 but not CPPHA is lost in the $A809V/rmGlu_5$ mutant, whereas the response to CPPHA but not VU29 is lost in the $F585I/rmGlu_5$ mutant (data not shown). Interestingly, we found that the $A809V/rmGlu_5$ mutation inhibited the ability of VU0357121 to shift the glutamate concentration–response curve (Figure 15A), whereas the response to VU0357121 was not altered by the $F585I/rmGlu_5$ mutation (Figure 15B). The finding that the F585I mutation was without effect is consistent with studies above suggesting that CPPHA and VU0357121 do not act at identical sites. The finding that the response was inhibited by the A809V mutation is somewhat surprising in light of the results from the molecular pharmacology studies and further illustrates the complexity of the relationships between putative allosteric sites on $mGlu_5$. In addition, these data highlight the fact that mutagenesis studies must be interpreted with caution. While previous studies and the current data provide strong evidence that multiple $mGlu_5$ modulators do not bind to identical binding sites, it is possible that they interact with overlapping sites that share critical residues. Also, in the absence of direct structural information about the class C GPCRs, models of class C GPCR structure and functional effects of specific mutations do not provide

DOI: 10.1021/cn100051m | ACS Chem. Neurosci. (2010), 1, 702–716

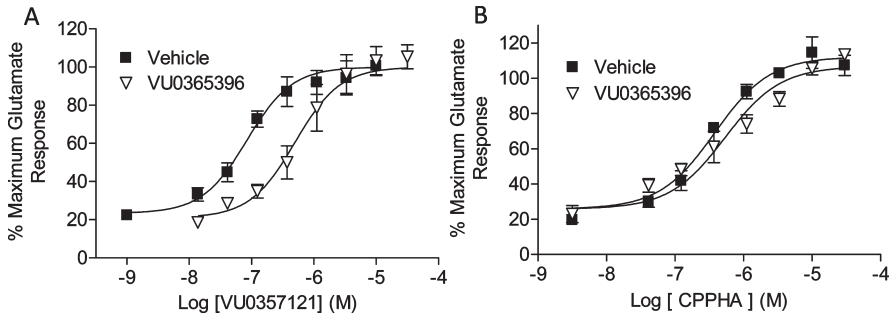


Figure 14. VU0365396 competitively inhibits VU0357121 function but has no effect on CPPHA activity. (A) VU0365396 induces a parallel rightward shift in the VU0357121 concentration−response curve, indicating a competitive interaction between the two compounds. Cells expressing $mGlu_5$ were treated with increasing concentrations of VU0357121 and either vehicle (■) or VU0365396 (30 μM, △) followed by EC_{20} glutamate. (B) The calcium mobilization response produced when $mGlu_5$-expressing HEK293 cells were treated with increasing concentrations of CPPHA and EC_{20} glutamate do not differ in the absence (■) or presence (△) of VU0365396 (30 μM). Data were obtained from three separate experiments, each performed in duplicate, and are expressed as the mean ± SEM. Data were normalized to the average maximum response obtained from each experiment as determined by 100 μM glutamate. Concentration−response curves were generated by nonlinear curve fitting.

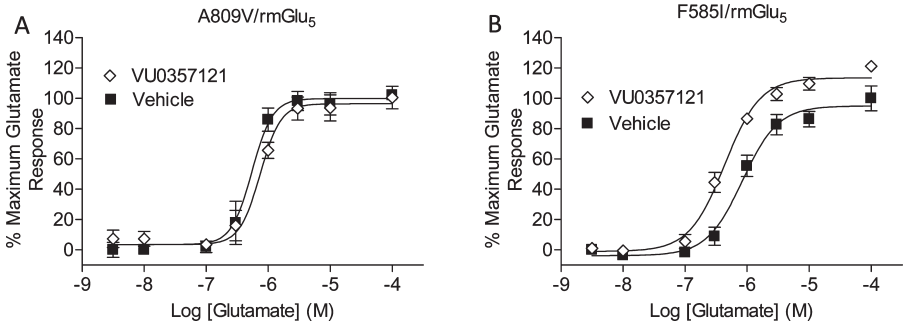


Figure 15. Mutations that inhibit the function of MPEP/VU-29 also inhibit VU0357121 function, but mutations that inhbit CPPHA activity do not. The ability of VU0357121 (1 μM) to function when specific $mGlu_5$ mutants were transiently expressed in HEK cells was assessed. The calcium mobilization assay was used to determine whether the $mGlu_5$ PAM was able to enhance the glutamate concentration−response relationship at either the A809V/$rmGlu_5$ mutant or the F585I/$rmGlu_5$ mutant. The A809V mutation led to an inhibition of VU0357121 activity (A), whereas the F585I mutation had no effect on its function (B). Concentration−response relationships were generated by adding either vehicle or a fixed concentration (1 μM) of VU0357121 to transiently transfected HEK cells, followed by increasing concentrations of glutamate. Data were obtained from three separate experiments, each performed in duplicate, and are expressed as the mean ± SEM. Data were normalized to the average maximum response obtained from each experiment as determined by 100 μM glutamate.

definitive information about the nature of the binding of these ligands. However, the results of these mutagenesis studies combined with the current and previous molecular pharmacology studies provide further evidence that there is some interaction between the putative binding sites of CPPHA, VU0357121, and the MPEP binding site that are important for the action of these structurally and functionally diverse allosteric modulators.

In summary, using a functional HTS screen and subsequent library synthesis approach, we have discovered a novel series of highly selective $mGlu_5$ PAMs and a neutral ligand that have properties that suggest that they do not act as traditional MPEP site ligands. Also, our results suggest that these compounds do not interact with the receptor in a manner that is identical to that of CPPHA. Our present and previous studies suggest the presence of distinct allosteric sites or modes of action for VU0357121, CPPHA, and MPEP-site ligands such as VU-29. If this is the case, it is not yet clear whether these sites physically overlap or whether they are physically distinct but functionally interact through allosteric mechanisms. While the weight of evidence suggests that these allosteric modulators interact with different binding sites, it is important to consider that until the binding pockets for each of these compounds is fully elucidated, it is impossible to definitively determine whether these compounds bind to the same or distinct binding sites. For instance, if the interaction of each of the allosteric modulators at $mGlu_5$ has not reached equilibrium, this could influence the nature of the shifts in PAM CRCs that are observed with neutral ligands (*26*). Also, the lack of apparent binding to the MPEP site could be explained by very high cooperativity such that occupancy of less than 0.1% of the receptor leads to full potentiation of the response to glutamate. Finally, allosteric ligands could bind to a single monomer in a functional homodimer, and this could impact the pharmacological profile. While unlikely, it is important to consider these possibilities until we have definitive

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

structural information on $mGlu_5$ and the allosteric binding sites.

Methods

Compounds

The Vanderbilt High Throughput Screening Center compound collection was obtained from ChemBridge Corporation (San Diego, CA) and ChemDiv, Inc. (San Diego, CA). L-Glutamate and MPEP were obtained from Tocris Bioscience (Ellisville, MO). [^{3}H]methoxyPEPy was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). CPPHA (*17*) and 5-MPEP (*23*) were synthesized as previously described.

Chemical Synthesis

General. All NMR spectra were recorded on a Bruker 400 MHz instrument. ^{1}H chemical shifts are reported in δ values in ppm relative to $CDCl_3$ at 7.27 ppm. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, and m = multiplet), coupling constant (Hz), and integration. Low resolution mass spectra were obtained on an agilent 1200 series 6130 mass spectrometer. Analytical thin layer chromatography was performed on Analtech silica gel GF 250 μm plates. Analytical HPLC was performed on an agilent 1200 series. Preparative purification was performed on a combi-flash companion. Solvents for extraction, washing, and chromatography were of HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification.

VU0001850

To a solution of 2-amino-5-chloro-pyridine (129 mg, 1.0 mmol) in dichloromethane (5 mL) was added *N*,*N*-diisopropylethylamine (174 μL, 1.0 mmol), 4-propoxybenzoic acid (180 mg, 1.0 mmol), and chlorodipyrrolidinocarbenium hexafluorophosphate (PyClU) (365 mg, 1.0 mmol) under an argon atmosphere. The reaction was stirred for 1 h at ambient temperature. Water (5 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Flash Column Chromatography (hexane/ethyl acetate, gradient = 50% to 100%) to afford the benzamide as an off white crystalline solid (110 mg, 38%) upon concentration. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, J = 8.8, 2H), 8.00 (s, 1H), 7.72 (d, J = 8.8, 1H), 6.98 (d, J = 8.8, 2H), 6.51 (d, J = 8.8, 1H), 4.00 (t, J = 6.8, 6.4, 2H), 1.90−1.77 (m, 2H), 1.06 (t, J = 7.2, 7.4, 3H); LC-MS (214 nM) 2.0 min, m/z 292.1.

VU0040237

To a solution of 2-fluoroaniline (101 μL, 1.0 mmol) in dichloromethane (5 mL) was added *N*,*N*-diisopropylethylamine (174 μL, 1.0 mmol) and 4-butoxybenzoyl chloride (189 μL, 1.0 mmol), and the reaction was stirred for 45 min at ambient temperature. Saturated sodium bicarbonate (1 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Gilson HPLC to afford the benzamide as a white crystalline solid (280 mg, 97%) upon concentration. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (bs, 1H), 7.88−7.86 (d, J = 8, 2H), 7.20−7.04 (m, 4H), 7.01−6.99 (d, J = 8, 2H), 4.04 (t, J = 6.8, 6.4, 2H), 1.84−1.77 (m, 2H), 1.56−1.47 (m, 2H), 1.00 (t, J = 7.6, 7.2, 3H); LC-MS (214 nM) 2.0 min, m/z 288.2.

VU0365396

To a solution of 2,6-difluoroaniline (101 μL, 1.0 mmol) in dichloromethane (5 mL) was added *N*,*N*-diisopropylethylamine (174 μL, 1.0 mmol) and 4-butoxybenzoyl chloride (189 μL, 1.0 mmol), and the reaction was stirred for 45 min at ambient temperature. Saturated sodium bicarbonate (1 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Gilson HPLC to afford the benzamide as a white crystalline solid (285 mg, 93%) upon concentration;. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 8.8, 2H), 6.99−6.88 (m, 5H), 4.04 (t, J = 6.4, 6.4, 2H), 1.84−1.77 (m, 2H), 1.57−1.47 (m, 2H), 0.99 (t, J = 7.6, 7.2, 3H); LC-MS (214 nM) 2.0 min, m/z 306.2.

VU0357121

To a solution of 2,4-difluoroaniline (101 μL, 1.0 mmol) in dichloromethane (5 mL) was added *N*,*N*-diisopropylethylamine (174 μL, 1.0 mmol) and 4-butoxybenzoyl chloride (189 μL, 1.0 mmol), and the reaction was stirred for 45 min at ambient temperature. Saturated sodium bicarbonate (1 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Gilson HPLC to afford the benzamide as a white crystalline solid (290 mg, 95%) upon concentration. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, J = 8, 2H), 7.01−6.95 (m, 5H), 4.06 (t, J = 6.4, 6.4, 2H), 1.85−1.75 (m, 2H), 1.57−1.46 (m, 2H), 1.00 (t, J = 7.6, 7.2, 3H); LC-MS (214 nM) 2.0 min, m/z 306.2.

VU0125936

To a solution of 2-amino-5-chloro-pyridine (129 mg, 1.0 mmol) in dichloromethane (5 mL) was added *N*,*N*-diisopropylethylamine (174 μL, 1.0 mmol) and 4-butoxybenzoyl chloride (189 μL, 1.0 mmol), and the reaction was stirred for 45 min at ambient temperature. Saturated sodium bicarbonate (1 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Gilson HPLC to afford the benzamide as a white crystalline solid (260 mg, 86%) upon concentration. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 1H), 7.21 (d, J = 8, 2H), 7.75 (d, J = 8.8, 2H), 6.85 (d, J = 8.8, 2H), 6.46 (d, J = 8.8, 1H), 4.50 (bs, 1H), 3.98 (t, J = 6.4, 6.4, 2H), 1.80−1.73 (m, 2H), 1.51−1.46 (m, 2H), 0.98 (t, J = 7.6, 7.4, 3H); LC-MS (214 nM) 2.0 min, m/z 305.2.

Cell Culture

HEK293 cells stably expressing rat $mGlu_5$ were grown in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1× antibiotic-antimycotic (all from Invitrogen, Carlsbad, CA), and 500 μg/mL G418 sulfate from Mediatech, inc. (Manassas,VA), at 37 °C in the presence 5% CO_2.

Fluorescence-Based Calcium Flux Assay

Screening assays were completed within Vanderbilt University's HTS laboratory and were performed as previously described (*18*−*20*). Hits from the primary screen including VU0001850, VU0003712, and VU0040237 were confirmed by testing for concentration-dependent activity on $mGlu_5$ over a range of 4 log units including 10 μM, as depicted in Figure 2. Compounds were serially diluted 1:3 into 10-point

DOI: 10.1021/cn100051m | ACS Chem. Neurosci. (2010), 1, 702–716

concentration−response curves (30 μM−1 nM final), transferred to daughter plates using the Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA), and tested as described in the primary screen except that test compounds were applied for 300 s followed by addition of EC_{20} concentrations of glutamate. All other $mGlu_5$ calcium assays were performed in the following manner. Human embryonic kidney (HEK293) cells stably expressing $mGlu_5$ were plated in black-walled, clear-bottomed, poly-D-lysine coated, 96-well plates (BD Biocoat, Bedford, MA) in 100 μL glutamate/glutamine-free medium (glutamine-free DMEM plus 10% dialyzed fetal bovine serum; Invitrogen) at 30,000 cells per well at least 24 h prior to the assay. Cells were incubated overnight at 37 °C in the presence of 5% CO_2. The next day, the medium was manually removed, and the cells were incubated with 50 μL of Hank's balanced salt solution (HBSS; Invitrogen) containing 2.5 mM Probenecid, 20 mM HEPES, and 2 μM calcium-sensitive Fluo4-AM dye (Molecular Probes) at pH 7.4 for 45 min at 37 °C and 5% CO_2. The dye was removed, and 60 μL of assay buffer containing HBSS, 20 mM HEPES, and 2.5 mM Probenecid (Sigma), adjusted to pH 7.4, was added. Ten minutes later, cells were placed into the Flexstation II (Molecular Devices, Sunnyvale, CA) where they received 20 μL of vehicle or test compound diluted into assay buffer to a 4× stock after 19 s, followed by 20 μL of glutamate diluted to a 5× stock at 109 s. Specifically, for the PAM screen, cells received a fixed concentration of test compound (10 or 1 μM) or vehicle, followed by a concentration of glutamate that evoked about a 20% response as compared to the maximum glutamate response (EC_{20}). To screen for antagonists, cells received a fixed concentration of test compound (10 μM) or vehicle, followed by a concentration of glutamate, which elicited a response that was 80% of the maximum glutamate response (EC_{80}). To determine the concentration−response relationships of the $mGlu_5$ PAMs, cells received increasing concentrations of the test compound, followed by stimulation with EC_{20} glutamate. To determine the effect of the $mGlu_5$ PAMs on the glutamate dose−response relationship, cells received the test compound (12.5 μM) or vehicle followed by increasing concentrations of glutamate (with a top concentration of 100 μM). To screen for neutral allosteric site ligand activity, cells received the test compound (10 μM) or vehicle and VU0357121 (50 nM) made up in the same 4× solution followed by EC_{20} glutamate. To determine the effect of the neutral ligands on $mGlu_5$ PAM activity, cells received increasing concentrations of 5-MPEP (333 nM to 10 μM), a fixed concentration of VU0365396 (30 μM), or vehicle prepared in the same 4× solution as increasing concentrations of VU0357121 or CPPHA, followed by EC_{20} glutamate. Fluorescence was measured for a total of 160 s per well for each assay. Data were transformed and fit with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) to determine EC_{50} values. For the neutral ligand screen, data were analyzed by a one-way ANOVA with comparison to the EC_{20} + VU0357121 + vehicle control group using a Dunnett's test. Calculations were performed using GraphPad Prism.

Selectivity Studies

Rat $mGlu_1$. HEK293 cells stably expressing $mGlu_1$ were cultured and assayed as described above for $mGlu_5$-expressing cells. During the calcium fluorescence assay, cells were incubated with vehicle or test compound at a fixed concentration of 30 μM, followed by increasing concentrations of glutamate.

Rat mGlu Receptors 2, 3, 4, 7, and 8, and Human $mGlu_6$. Compound activity at the group II and III mGlu receptors was assessed using thallium flux through G-protein-coupled inwardly rectifying potassium (GIRK) channels, a method that has been described in detail (*21*). These cell lines were grown in growth media containing 45% DMEM, 45% F-12, 10% FBS, 20 mM HEPES, 2 mM L-glutamine, antibiotic/antimycotic, nonessential amino acids, 700 μg/mL G418, and 0.6 μg/mL puromycin at 37 °C in the presence of 5% CO_2. Briefly, GIRK cells expressing the mGluR subtype 2, 3, 4, 6, 7, or 8 were plated into 384-well, black-walled, clear-bottom poly-D-lysine coated plates at a density of 15,000 cells/20 μL/well in assay medium and incubated overnight at 37 °C in the presence of 5% CO_2. The following day, the medium was removed from the cells, and 20 μL/well of 1 μM concentration of the indicator dye Fluozin2-AM (Invitrogen, Carlsbad, CA) in assay buffer was added. Cells were incubated for 1 h at room temperature, and the dye was replaced with 20 μL/well of assay buffer. For these assays, vehicle or test compound (2× final concentration) was added, followed by varying concentrations of glutamate 2.5 min later using FDSS 6000. Glutamate was diluted in thallium buffer (125 mM sodium bicarbonate, 1 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, 12 mM thallium sulfate, and 10 mM HEPES) at 5× the final concentration to be assayed. Data were analyzed as previously described (*21*).

Radioligand Binding Assay

The allosteric antagonist MPEP analogue [^{3}H]methoxyPEPy was used to evaluate the ability of test compounds to interact with the MPEP site on $mGlu_5$ (*22*). Membranes were prepared from rat $mGlu_5$ HEK293 cells (*23*). Compounds were diluted in assay buffer (50 mM Tris/0.9% NaCl, pH 7.4) to a 5× stock, and 100 μL of the test compound was added to each well of a 96 deep-well assay plate. Three hundred microliter aliquots of membranes diluted in assay buffer (20 μg/well) were added to each well. One hundred microliters of [^{3}H]methoxyPEPy (2 nM final concentration) was added, and the reaction was incubated at room temperature for 1 h with shaking. After the incubation period, the membrane-bound ligand was separated from free ligand by filtration through glass-fiber 96-well filter plates (Unifilter-96, GF/B, PerkinElmer Life and Analytical Sciences, Boston, MA). The contents of each well were transferred simultaneously to the filter plate and washed 3−4 times with assay buffer using a cell harvester (Brandel Cell Harvester, Brandel Inc., Gaithersburg, MD). Forty microliters of scintillation fluid was added to each well and the membrane-bound radioactivity determined by scintillation counting (TopCount, PerkinElmer Life and Analytical Sciences). Nonspecific binding was estimated using 5 μM MPEP. Concentration−response curves were generated using a four parameter logistical equation in GraphPad Prism.

Phosphoinositide (PI) Hydrolysis Assay

HEK cells expressing $mGlu_5$ were plated at 150,000 cells per well in the glutamate/glutamine-free assay medium detailed above in 24-well plates 24 h prior to assay. Sixteen hours

DOI: 10.1021/cn100051m | ACS Chem. Neurosci. (2010), 1, 702–716

prior to experiments, the cells received new assay media containing 1 μCi/mL [^{3}H]myo-inositol (Perkin-Elmer LAS). On the day of the assay, cells were treated with either vehicle or a fixed concentration of 5-MPEP (10 μM) in HBSS (containing 110 mM NaCl, 5.4 mM KCl, 1.8 mM$CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, and 58.4 mM sucrose at pH 7.4) supplemented with 20 mM HEPES and 30 mM LiCl, replacing the [^{3}H]myo-inositol-containing media. Following vehicle or neutral ligand addition, a range of concentrations of VU0357121 were added, and cells were incubated for 1 h at 37 °C and 5% CO_2. The accumulation of phosphoinositides was terminated by aspiration of ligand-containing buffer, followed by the addition of 10 mM formic acid (1 mL/well) and incubation for 1 h at room temperature to ensure the extraction of phosphoinositides. Columns packed with a 1 mL bed of AG 1-X8 Resin 100−200 mesh anion-exchange resin (formate form) (Bio-Rad; Hercules, CA) were washed twice with 10 mL of water. Following the water washes, the entire 1 mL sample volumes were added to the columns, avoiding the transfer of any cells. Columns were washed with 10 mL of water, followed by 10 mL of 5 mM myo-inositol. Total phosphoinositides (PIs) were eluted with 10 mL of 0.1 M formic acid/0.2 M ammonium formate into vials containing 3a70B liquid scintillation cocktail (Research Products International; Elk Grove Village, IL), and the radioactivity was measured by liquid scintillation counting. Following use, columns were regenerated with 10 mL of 0.1 M formic acid/1 M ammonium formate and washed with 30 mL of water. Data were corrected for baseline levels, normalized to the maximum response to 1 mM glutamate, and analyzed with GraphPad Prism to determine EC_{50} values.

Transient Transfections

HEK293 cells were grown in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, and 1× antibiotic−antimycotic. Cells were collected and plated in 100 mm tissue culture dishes (Costar; Corning Life Sciences, Acton, MA) in normal growth medium overnight before transfection. The next day, cells were transiently transfected with mutant forms of $mGlu_5$ cDNA using FuGENE 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions (2 μg of DNA, 24 μL of FuGENE 6, and 776 μL of Opti-MEM (Invitrogen, Carlsbad, CA)). The next day, cells were plated in black-walled, clear-bottomed, 96-well plates (Costar; Corning Life Sciences, Acton, MA) pretreated with poly-D-lysine (Sigma, St. Louis, MO). Calcium assays were performed on FlexStation II, as described above, on the following day to study the effect of VU0357121 (1 μM) on the glutamate concentration−response activity of each mutant.

Author Information

Corresponding Author

*Corresponding author. Department of Pharmacology, Vanderbilt Program in Drug Discovery, Vanderbilt University Medical Center, Nashville, TN 37232-0697. Phone: (615) 936-2478. Fax: (615) 343-3088. E-mail: jeff.conn@vanderbilt.edu.

Author Contributions

P.J.C. and C.W.L. oversaw and designed the molecular pharmacology and chemistry, respectively. A.R. oversaw the high-throughput screening and compound confirmation, as well as the molecular pharmacology. C.N. provided the selectivity data for mGluRs 2, 3, 7 and 8. A.H. performed all of the other in vitro pharmacology assays and the initial medicinal chemistry synthesis. K.G. performed PI hydrolysis studies. S.T. performed additional medicinal chemistry and characterized the compounds.

Funding Sources

P.J.C. receives funding from NIH, NIMH, NINDS, NIDA, the Michael J. Fox Foundation, Seaside Therapeutics, and Johnson&Johnson. C.W.L. receives funding from NIH, NIMH, NIDA, the Alzheimer's Association, the Michael J. Fox Foundation, Seaside Therapeutics, and Johnson&Johnson.

Acknowledgment

We thank NIMH, NINDS, and NIDA, for generous support of these studies. A.S.H. acknowledges a Public Health Service award T32 GM07347 from the National Institute of General Medical Sciences for the Meharry-Vanderbilt Alliance Medical-Scientist Training Program, as well as a National Research Service award F30 MH086219 from the NIMH.

Abbreviations

PAM, positive allosteric modulator; mGlu5, metabotropic glutamate receptor subtype 5; CPPHA, *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide; NAM, negative allosteric modulator; MPEP, 2-methyl-6(phenylethynyl)pyridine; SAR, structure−activity relationship; HTS, high-throughput screen; VU0001850, *N*-(5-chloropyridin-2-yl)-4-propoxybenzamide; VU0040237, 4-butoxy-*N*-(2-fluorophenyl)benzamide; VU0357121, 4-butoxy-*N*-(2,4-difluorophenyl)benzamide; VU0365396, 4-butoxy-*N*-(2,6-difluorophenyl)benzamide; CNS, central nervous system; iGluR, ionotropic glutamate receptor; mGluR, metabotropic glutamate receptor; GPCR, guanine nucleotide binding protein coupled receptor; cAMP, cyclic adenosine monophosphate; DFB, 3,3′-difluorobenzaldazine; MPPA, *N*-methyl-5-(phenylethynyl)pyrimidin-2-amine; CDPPB, 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; VU-29, 4-nitro-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; ADX47273, *S*-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidin-1-yl}-methanone; (*S*)-FTOPM, (*S*)-(4-fluorophenyl)(3-(3-(thiophen-2-yl)-1,2,4-oxadiazol-5-yl)piperidin-1-yl)methanone; (*S*)-FPOPM, (*S*)-(4-fluorophenyl)(3-(3-(pyridin-2-yl)-1,2,4-oxadiazol-5-yl)piperidin-1-yl)methanone; CHO, chinese hamster ovary; HEK293, human embryonic kidney; ERK1/2, extracellular signal-regulated kinases 1 and 2; FDSS, functional drug screening system; VU0003712, *N*-(5-chloropyridin-2-yl)-4-((2,2,2-trifluoroethoxy)methyl)benzamide; VU0365393, 4-butoxy-*N*-(2,5-difluorophenyl)benzamide;

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

VU0125936, 4-butoxy-*N*-(5-chloropyridin-2-yl)benzamide; GIRK, G-protein regulated inwardly rectifying potassium channel; [^{3}H]methoxy-PEPy, [^3H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine); DOMEB, 3,3′-dimethoxybenaldazine; DCB, 3,3′-difluorobenzaldazine; 5-MPEP, 5-methyl-6-(phenyl ethynyl)-pyridine; 5-PEP, 5-(phenylethynyl)pyrimidine; PI, phosphoinositide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

References

1. Conn, P. J., and Pin, J. P. (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol. 37*, 205–237.

2. Gasparini, F., Lingenhohl, K., Stoehr, N., Flor, P. J., Heinrich, M., Vranesic, I., Biollaz, M., Allgeier, H., Heckendorn, R., Urwyler, S., Varney, M. A., Johnson, E. C., Hess, S. D., Rao, S. P., Sacaan, A. I., Santori, E. M., Velicelebi, G., and Kuhn, R. (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. *Neuropharmacology 38*, 1493–1503.

3. Pagano, A., Ruegg, D., Litschig, S., Stoehr, N., Stierlin, C., Heinrich, M., Floersheim, P., Prezeau, L., Carroll, F., Pin, J. P., Cambria, A., Vranesic, I., Flor, P. J., Gasparini, F., and Kuhn, R. (2000) The non-competitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. *J. Biol. Chem. 275*, 33750–33758.

4. Shipe, W. D., Wolkenberg, S. E., Williams, D. L., Jr., and Lindsley, C. W. (2005) Recent advances in positive allosteric modulators of metabotropic glutamate receptors. *Curr. Opin. Drug Discovery Dev. 8*, 449–457.

5. Marino, M. J., and Conn, P. J. (2002) Direct and indirect modulation of the N-methyl D-aspartate receptor. *Curr. Drug Targets CNS Neurol. Disord. 1*, 1–16.

6. Moghaddam, B. (2004) Targeting metabotropic glutamate receptors for treatment of the cognitive symptoms of schizophrenia. *Psychopharmacology 174*, 39–44.

7. Conn, P. J., Lindsley, C. W., and Jones, C. K. (2009) Activation of metabotropic glutamate receptors as a novel approach for the treatment of schizophrenia. *Trends Pharmacol. Sci. 30*, 25–31.

8. Malherbe, P., Kratochwil, N., Zenner, M. T., Piussi, J., Diener, C., Kratzeisen, C., Fischer, C., and Porter, R. H. (2003) Mutational analysis and molecular modeling of the binding pocket of the metabotropic glutamate 5 receptor negative modulator 2-methyl-6-(phenylethynyl)-pyridine. *Mol. Pharmacol. 64*, 823–832.

9. Chen, Y., Nong, Y., Goudet, C., Hemstapat, K., de Paulis, T., Pin, J. P., and Conn, P. J. (2007) Interaction of novel positive allosteric modulators of metabotropic glutamate receptor 5 with the negative allosteric antagonist site is required for potentiation of receptor responses. *Mol. Pharmacol. 71*, 1389–1398.

10. O'Brien, J. A., Lemaire, W., Chen, T. B., Chang, R. S., Jacobson, M. A., Ha, S. N., Lindsley, C. W., Schaffhauser, H. J., Sur, C., Pettibone, D. J., Conn, P. J., and Williams, D. L., Jr. (2003) A family of highly selective allosteric modulators of the metabotropic glutamate receptor subtype 5. *Mol. Pharmacol. 64*, 731–740.

11. Kinney, G. G., O'Brien, J. A., Lemaire, W., Burno, M., Bickel, D. J., Clements, M. K., Chen, T. B., Wisnoski, D. D., Lindsley, C. W., Tiller, P. R., Smith, S., Jacobson, M. A., Sur, C., Duggan, M. E., Pettibone, D. J., Conn, P. J., and Williams, D. L., Jr. (2005) A novel selective positive allosteric modulator of metabotropic glutamate receptor subtype 5 has in vivo activity and antipsychotic-like effects in rat behavioral models. *J. Pharmacol. Exp. Ther. 313*, 199–206.

12. Le Poul, E. B., Lutgens, R.; Bonnet, B.; Rocher, J. P.; Epping-Jordan, M., and Mutel, V. (2005) In vitro Pharmacological Characterisation of Selective mGluR5 Positive Allosteric Modulators, 5th International Meeting on Metabotropic Glutamate Receptors, Taormina, Italy.

13. O'Brien, J. A., Lemaire, W., Wittmann, M., Jacobson, M. A., Ha, S. N., Wisnoski, D. D., Lindsley, C. W., Schaffhauser, H. J., Rowe, B., Sur, C., Duggan, M. E., Pettibone, D. J., Conn, P. J., and Williams, D. L., Jr. (2004) A novel selective allosteric modulator potentiates the activity of native metabotropic glutamate receptor subtype 5 in rat forebrain. *J. Pharmacol. Exp. Ther. 309*, 568–577.

14. Chen, Y., Goudet, C., Pin, J. P., and Conn, P. J. (2008) N-{4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-methyl]phenyl}-2-hy droxybenzamide (CPPHA) acts through a novel site as a positive allosteric modulator of group 1 metabotropic glutamate receptors. *Mol. Pharmacol. 73*, 909–918.

15. Engers, D. W., Rodriguez, A. L., Williams, R., Hammond, A. S., Venable, D., Oluwatola, O., Sulikowski, G. A., Conn, P. J., and Lindsley, C. W. (2009) Synthesis, SAR and unanticipated pharmacological profiles of analogues of the mGluR5 ago-potentiator ADX-47273. *ChemMedChem 4*, 505–511.

16. Zhang, Y., Rodriguez, A. L., and Conn, P. J. (2005) Allosteric potentiators of metabotropic glutamate receptor subtype 5 have differential effects on different signaling pathways in cortical astrocytes. *J. Pharmacol. Exp. Ther. 315*, 1212–1219.

17. Zhao, Z., Wisnoski, D. D., O'Brien, J. A., Lemaire, W., Williams, D. L., Jr., Jacobson, M. A., Wittman, M., Ha, S. N., Schaffhauser, H., Sur, C., Pettibone, D. J., Duggan, M. E., Conn, P. J., Hartman, G. D., and Lindsley, C. W. (2007) Challenges in the development of mGluR5 positive allosteric modulators: the discovery of CPPHA. *Bioorg. Med. Chem. Lett. 17*, 1386–1391.

18. Niswender, C. M., Johnson, K. A., Weaver, C. D., Jones, C. K., Xiang, Z., Luo, Q., Rodriguez, A. L., Marlo, J. E., de Paulis, T., Thompson, A. D., Days, E. L., Nalywajko, T., Austin, C. A., Williams, M. B., Ayala, J. E., Williams, R., Lindsley, C. W., and Conn, P. J. (2008) Discovery, characterization, and antiparkinsonian effect of novel positive allosteric modulators of metabotropic glutamate receptor 4. *Mol. Pharmacol. 74*, 1345–1358.

19. Marlo, J. E., Niswender, C. M., Days, E. L., Bridges, T. M., Xiang, Y., Rodriguez, A. L., Shirey, J. K., Brady,

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716

A. E., Nalywajko, T., Luo, Q., Austin, C. A., Williams, M. B., Kim, K., Williams, R., Orton, D., Brown, H. A., Lindsley, C. W., Weaver, C. D., and Conn, P. J. (2009) Discovery and characterization of novel allosteric potentiators of M1 muscarinic receptors reveals multiple modes of activity. *Mol. Pharmacol. 75*, 577–588.

20. Rodriguez, A. L., Grier, M. D., Williams, R., Smith, R. L., Kane, A. S., Jones, C. K., Niswender, C. M., Zhou, Y., Marlo, J. E., Days, E. L., Nalywajko, T., Yin, H., Lindsley, C. W., Weaver, C. D., and Conn, P. J., unpublished work.

21. Niswender, C. M., Johnson, K. A., Luo, Q., Ayala, J. E., Kim, C., Conn, P. J., and Weaver, C. D. (2008) A novel assay of Gi/o-linked G protein-coupled receptor coupling to potassium channels provides new insights into the pharmacology of the group III metabotropic glutamate receptors. *Mol. Pharmacol. 73*, 1213–1224.

22. Cosford, N. D., Roppe, J., Tehrani, L., Schweiger, E. J., Seiders, T. J., Chaudary, A., Rao, S., and Varney, M. A. (2003) [3H]-methoxymethyl-MTEP and [3H]-methoxy-PEPy: potent and selective radioligands for the metabotropic glutamate subtype 5 (mGlu5) receptor. *Bioorg. Med. Chem. Lett. 13*, 351–354.

23. Rodriguez, A. L., Nong, Y., Sekaran, N. K., Alagille, D., Tamagnan, G. D., and Conn, P. J. (2005) A close structural analog of 2-methyl-6-(phenylethynyl)-pyridine acts as a neutral allosteric site ligand on metabotropic glutamate receptor subtype 5 and blocks the effects of multiple allosteric modulators. *Mol. Pharmacol. 68*, 1793–1802.

24. Sharma, S., Rodriguez, A. L., Conn, P. J., and Lindsley, C. W. (2008) Synthesis and SAR of a mGluR5 allosteric partial antagonist lead: unexpected modulation of pharmacology with slight structural modifications to a 5-(phenylethynyl)pyrimidine scaffold. *Bioorg. Med. Chem. Lett. 18*, 4098–4101.

25. Zhou, Y., Rodriguez, A. L., Williams, R., Weaver, C. D., Conn, P. J., and Lindsley, C. W. (2009) Synthesis and SAR of novel, non-MPEP chemotype mGluR5 NAMs identified by functional HTS. *Bioorg. Med. Chem. Lett. 19*, 6502–6506.

26. Lew, M. J., Ziogas, J., and Christopoulos, A. (2000) Dynamic mechanisms of non-classical antagonism by competitive AT(1) receptor antagonists. *Trends Pharmacol. Sci. 21* (10), 376–381.

DOI: 10.1021/cn100051m | *ACS Chem. Neurosci.* (2010), *1*, 702–716