A Novel Metabotropic Glutamate Receptor 5 Positive Allosteric Modulator Acts at a Unique Site and Confers Stimulus Bias to mGlu5 Signaling


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ABSTRACT

Metabotropic glutamate receptor 5 (mGlu5) is a target for the treatment of central nervous system (CNS) disorders, such as schizophrenia and Alzheimer’s disease. Furthermore, mGlu5 has been shown to play an important role in hippocampal synaptic plasticity, specifically in long-term depression (LTD) and long-term potentiation (LTP), which is thought to be involved in cognition. Multiple mGlu5-positive allosteric modulators (PAMs) have been developed from a variety of different scaffolds. Previous work has extensively characterized a common allosteric site on mGlu5, termed the MPEP (2-Methyl-6-(phénylthényl)pyridine) binding site. However, one mGlu5 PAM, CPPHA (N-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isindol-2-yl)methyl]phenyl)-2-hydroxybenzamide), interacts with a separate allosteric site on mGlu5. Using cell-based assays and brain slice preparations, we characterized the interaction of a potent and efficacious mGlu5 PAM from the CPPHA series termed NCFP (N-(4-chloro-2-[(4-fluoro-1,3-dioxoisoindolin-2-yl)methyl]phenyl)picolinamide). NCFP binds to the CPPHA site on mGlu5 and potentiates mGlu5-mediated responses in both recombinant and native systems. However, NCFP provides greater mGlu5 subtype selectivity than does CPPHA, making it more suitable for studies of effects on mGlu5 in CNS preparations. Of interest, NCFP does not potentiate responses involved in hippocampal synaptic plasticity (LTD/LTP), setting it apart from other previously characterized MPEP site PAMs. This suggests that although mGlu5 PAMs may have similar responses in some systems, they can induce differential effects on mGlu5-mediated physiologic responses in the CNS. Such stimulus bias by mGlu5 PAMs may complicate drug discovery efforts but would also allow for specifically tailored therapies, if pharmacological biases can be attributed to different therapeutic outcomes.

Introduction

The metabotropic glutamate receptors (mGlus) include eight subtypes (mGlu1–8) of seven transmembrane-spanning G protein–coupled receptors (7TMRs) for the neurotransmitter glutamate. The mGlus play multiple roles in regulating central nervous system (CNS) function and serve as potential therapeutic targets for a variety of brain disorders (Niswender and Conn, 2010; Vinson and Conn, 2012). A growing body of evidence suggests that selective activators of the mGlu5 subtype could provide an exciting new approach for treatment of schizophrenia and other disorders that lead to impaired cognitive function (Gregory et al., 2011; Vinson and Conn, 2012). Although discovery of selective mGlu5 agonists that have drug-like properties has been challenging, there have been major advances in development of highly selective positive allosteric modulators (PAMs) for mGlu5 (Liu et al., 2008; Conn et al., 2009; Stauffer, 2011; Varnes et al., 2011; Packiarajan et al., 2012). A diverse range of selective mGlu5 PAMs have now been identified that have efficacy in animal models used to predict potential antipsychotic and cognitive enhancing activity (Gregory et al., 2011; Vinson and Conn, 2012). In addition to providing greater subtype selectivity than competitive, orthosteric ligands, the ability of mGlu5 PAMs to maintain activity dependence of receptor activation may reduce adverse effect liability that can be associated with excessive mGlu5 activation (Conn et al., 2009).

An important property of mGlu5 PAMs that is relevant for the cognition-enhancing effects of these agents is their unique
profile in enhancing synaptic plasticity. Multiple mGlu5 PAMs enhance induction of both long-term depression (LTD) and long-term potentiation (LTP) of transmission at excitatory glutamatergic synapses in the hippocampus (Ayala et al., 2009; Auerbach et al., 2011; Popkirov and Manahan-Vaughan, 2011; Noetzel et al., 2012). Of importance, mGlu5 PAMs enhance both forms of synaptic plasticity without altering the specific pattern of afferent activity required for induction of LTD versus LTP. This ability of mGlu5 PAMs to potentiate both LTD and LTP while maintaining a strict dependence of both on specific patterns of afferent activity could provide an ideal profile for a cognition-enhancing agent. Selective mGlu5 PAMs have been developed from multiple chemical scaffolds (O’Brien et al., 2004; Kinney et al., 2005; Chen et al., 2007; Liu et al., 2008; Hammond et al., 2010; Rodriguez et al., 2010; Lamb et al., 2011; Stauffer, 2011; Varnes et al., 2011; Noetzel et al., 2012; Packiarajan et al., 2012); the majority of these mGlu5 PAMs bind to the same site as the prototypical mGlu5 negative allosteric modulator (NAM) MPEP (2-Methyl-6-(phenylethynyl)pyridine), located in the top third of the transmembrane spanning domains, involving transmembrane domains 3, 6, and 7 (Gregory et al., 2011). However, at least two mGlu5 PAMs, CPPHA (N-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl)methyl]phenyl)-2-hydroxybenzamide) (O’Brien et al., 2004; Zhao et al., 2007; Chen et al., 2008) and VU0357121 (Hammond et al., 2010), have been identified that interact noncompetitively with the MPEP site. Of interest, Zhang et al. (2005) reported that CPPHA and an MPEP site mGlu5 PAM, DBF (difluorobenzaldazine), induce similar potentiation of mGlu5-mediated calcium mobilization in cortical astrocytes. However, although DBF has similar effects on mGlu5 activation of calcium mobilization and extrasynaptic signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation in astrocytes, CPPHA inhibits ERK1/2 phosphorylation responses to maximally effective concentrations of glutamate in these cells. Similar differences in effects of allosteric modulators on different signaling pathways have also been observed in cell lines expressing other mGlu subtypes (Sheffler and Conn, 2008; Nisswender et al., 2010). Although CPPHA is a relatively efficacious and potent mGlu5 PAM, this compound is not entirely selective and has weak PAM activity at mGlu1 and weak NAM activity at mGlu5 and mGlu6 (O’Brien et al., 2004; Chen et al., 2008), which complicates the use of this compound to study functional effects of this class of mGlu5 PAM in native systems. We now report a series of studies in which we characterized a novel mGlu5 PAM, termed NCFP (N-(4-chloro-2-[(4-flouro-1,3-dioxoisodindol-2-yl)methyl]phenyl)picolinamide) (Zhao et al., 2007; patent number WO 2004087048 A2) that is structurally related to CPPHA. NCFP interacts noncompetitively with the MPEP site and likely binds instead to the same site as CPPHA. Of interest, NCFP has a fundamentally different profile from previous mGlu5 PAMs when assessing effects of hippocampal synaptic plasticity. Although NCFP potentiates multiple responses to mGlu5 activation in cell lines and potentiates mGlu5-mediated depolarization of neurons in the subthalamic nucleus, this compound does not enhance induction of either LTP or LTD at the hippocampal Schaffer collateral-CA1 (SC-CA1) synapse. These data provide strong support for the hypothesis that different mGlu5 PAMs can have fundamentally different effects on mGlu5-mediated physiologic responses in the CNS that could be important for the overall efficacy profile of these agents.

Materials and Methods

Human embryonic kidney 293 (HEK293) cells stably expressing rat mGlu5, or rat mGlu5 were maintained in complete DMEM supplemented with 10% FBS, 20 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotic-antimycotic (Invitrogen, Carlsbad, CA), and G418 (500 µg/mL). HEK293 cells stably expressing G protein–coupled inwardly rectifying potassium channels (HEK293-GIRQ) along with the individual group II and group III mGlu receptors were maintained in growth media containing 45% DMEM, 45% F-12, 10% FBS, 20 mM HEPES, 2 mM L-glutamine, antibiotic/antimycotic, nonessential amino acids, G418 (700 µg/mL), and puromycin (0.6 µg/mL).

Mutagenesis and Transfection

Single-point mutations of mGlu5 were generated using QuikChange II site-directed mutagenesis kits (Agilent Technologies, Santa Clara, CA) and confirmed by sequencing. Construction of the HA-tagged N-terminal truncated mGlu5 mutant was performed as described previously (Goudet et al., 2004). Transfection of HEK293A cells with wild-type mGlu5 and mutant constructs in pClNeo were performed using Fugene® (Promega, Madison, WI), and passaged five times in the presence of 1 µg/mL G418 to generate stable polyclonal cell lines. Stably transfected cell lines were subsequently maintained in complete DMEM supplemented with 10% FBS, 2 mM L-glutamine, 

ABBREVIATIONS: AGM, assay growth media; ACF, artificial cerebral spinal fluid; CNS, central nervous system; CPPHA, N-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl)methyl]phenyl)picolinamide; DFB, difluorobenzaldazine; DHPG, (S)-3,5-dihydroxyphenylglycine; DMEM, Dulbecco’s modified Eagle’s medium; ERK1/2, extracellular signal-regulated kinases 1 and 2; FBS, fetal bovine serum; FEPSP, field excitatory postsynaptic potential; GIRK, G protein–coupled inwardly rectifying potassium channels; HEK293 cells, human embryonic kidney 293 cells; L-AP4, L-(-)-2-Amino-4-phosphonobutyric acid; LTD, long-term depression; LTP, long-term potentiation; methoxyPEPy, 3-methoxy-5-(2-pyridinylethynyl)pyridine; mGlus, metabotropic glutamate receptors; 5MPEP, 5-methyl-2-phenylethynyl-pyridine; MPEP, 2-Methyl-6-(phenylethynyl)pyridine; NAM, negative allosteric modulator; NCFP, N-(4-chloro-2-[(4-flouro-1,3-dioxoisodindol-2-yl)methyl]phenyl)picolinamide; PAMs, positive allosteric modulators; SC-CA1 synapse, Schaffer collateral-CA1 synapse; STB, theta burst stimulation; TMRs, 7 transmembrane-spanning G protein–coupled receptors; VU0092273, (4-hydroxy-piperidin-1-yl)(4-phenylethynyl)phenyl)methanone.
Fluorescence-Based Calcium Signaling

Measurements of compound-evoked increases in intracellular calcium were performed as described previously (Noetzel et al., 2012). In brief, HEK293 cells stably expressing rat mGlu5 and mutants thereof were plated in 96-well, poly-D-lysine-coated, black-walled, clear-bottomed plates in assay medium (DMEM supplemented with 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) at a density of 40–50,000 cells/well. Calcium flux was measured over time as an increase in fluorescence of the Ca" indicator dye, Fluo-4 AM, using a FlexStation II (Molecular Devices, Sunnyvale, CA). Either vehicle or test compound was added 60 seconds before the addition of glutamate. Compound addition 120 seconds before the addition of glutamate was also assessed. There was no difference observed in the potency for CPPHA or NCFF between the two time points (pEC50 CPPHA: 60 seconds, −6.14; 120 seconds, −6.14; NCFF: 60 seconds, −6.30; 120 seconds, −6.20), suggesting that the maximum effect of the PAMs has been achieved within this preincubation period. The change in fluorescence over basal was determined before normalization to the maximal response elicited by glutamate. Experiments using the N-terminal truncated mGlu5 receptor were normalized to the maximal response elicited by 1 μM ionomycin. Data were transformed and fitted using GraphPad Prism 5.01 (Graph-Pad Software, Inc., San Diego, CA). As described in Gregory et al. (2012), shifts of glutamate concentration-response curves by allosteric modulators were globally fitted to an operational model of allostery (Leach et al., 2007) with use of Eq. 2 from Gregory et al. (2012).

Selectivity

mGlu1. To assess the effect of test compounds at mGlu1, Ca" mobilization assays were performed as described previously (Hammond et al., 2010; Noetzel et al., 2012). In brief, HEK293 cells stably expressing rat mGlu5 were plated in black-walled, clear-bottomed, poly-D-lysine-coated 384-well plates (Greiner Bio-One, Monroe, NC) in assay medium at a density of 20,000 cells/well. Calcium flux was measured over time as an increase in fluorescence of the Ca" indicator dye, Fluo-4 AM, with use of a Functional Drug Screening System 6000 (Hamamatsu, Tokyo, Japan). Either vehicle or a fixed concentration of test compound (10 μM, final concentration) was added followed 140 seconds later by a concentration-response curve of glutamate. Data were analyzed as described above.

Group II and Group III mGlus. The functional activity of the compound of interest was assessed at the rat group II and III mGlu receptors by measuring thiallum flux through GIRQ channels as described previously (Niswender et al., 2008). In brief, HEK293/GIRQ cells expressing mGlu 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/well in assay medium. A single concentration of test compound (10 μM) or vehicle was added followed 140 seconds later by a concentration-response curve of glutamate (or L(+)-2-Amino-4-phosphonobutyric acid for mGlu7) diluted in thallium buffer (125 mM NaHCO3, 1 mM MgSO4, 1.8 mM CaSO4, 5 mM glucose, 12 mM thallium sulfate, 10 mM HEPES), and fluorescence was measured using a Functional Drug Screening System 6000. Data were analyzed as described previously (Niswender et al., 2008).

ERK Phosphorylation

Receptor-mediated ERK1/2 phosphorylation was determined using the AlphaScreen-based ERK SureFire kit (PerkinElmer, Waltham, MA). HEK293A cells stably expressing mGlu5 were plated at a density of 40,000 cells/well in clear 96-well poly-D-lysine-coated plates in assay medium 16–24 hours before assay. Media was aspirated and cells serum starved in serum-free media (DMEM supplemented with 16 mM HEPES) for 6 hours before assay. Serum-free media was exchanged for fresh at the start of the experiment (30 minutes before termination of the response). Cells were treated with modulators or glutamate for the indicated amount of time at room temperature. The assay was terminated by aspiration of ligand-containing media and addition of a 50 μl/well of glycogen buffer. Lysates were processed as described previously (Gregory et al., 2012) and AlphaScreen signal measured using an Enspire (PerkinElmer) with standard AlphaScreen settings. Data are expressed as fold increase over basal levels of phosphorylated ERK.

Radioligand Binding

Membranes were prepared from HEK293 cells expressing rat mGlu5 and mutants as previously described (Gregory et al., 2012). For inhibition binding experiments, membranes (20–50 μg/well) were incubated with 2 nM [3H]methylxPETY and a range of concentrations of test ligand (100 pM to 100 μM) for 1 hour at room temperature with shaking in calcium assay buffer with 1% dimethylsulfoxide final; 10 μM MPEP was used to determine nonspecific binding. Binding assays were terminated by rapid filtration through GF/B Unifilter plates (PerkinElmer Life and Analytical Sciences, Boston, MA) using a Brandel 96-well plate Harvester (Brandel Inc., Gaithersburg, MD) and three washes with ice-cold Binding Buffer (50 mM Tris-HCl, 0.9% NaCl; pH 7.4). Plates were allowed to dry overnight before addition of MicroScint20 (40 μl/well; PerkinElmer). Radioactivity was counted after at least 2 hours incubation with use of a TopCount Scintillation Counter (PerkinElmer Life and Analytical Sciences). Inhibition [3H]methylxPETY binding data sets were fitted to a one-site inhibition binding model, and estimates of inhibitor dissociation constants (Ki) were derived using the Cheng-Prusoff equation for competitive ligands (Cheng and Prusoff, 1973) and the allosteric ternary complex model for ligands that did not fully displace radioligand (Lazareno and Birdsell, 1995).

Electrophysiology

Extracellular Field Potential Recordings. All animals used in these studies were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals; 400 μm hippocampal slices were prepared from young adult (age, 30–40 days) male Sprague-Dawley rats (Charles River, Wilmington, MA) with use of standard techniques and buffers as previously described (Noetzel et al., 2012; Ayala et al., 2009) A bipolar-stimulating electrode was placed in the stratum radiatum near the CA3–CA1 border to stimulate the Schaffer collaterals. Recording electrodes were pulled with a Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA), filled with ACSF (artificial cerebral spinal fluid), and placed in the stratum radiatum of area CA1. Field potential recordings were acquired using a Multi-Clamp 700B amplifier (Molecular Devices) and pClamp 10.2 software (Molecular Devices). A stimulus intensity that produced 50–60% [long-term depression (LTD)] and 40–50% [long-term potentiation (LTP)] of the maximum field excitatory postsynaptic potential.

20 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotic-antimycotic, and 500 μg/ml G418 at 37°C in a humidified incubator containing 5% CO2/95% O2.
Clampfit 10.2 (Molecular Devices). Membrane potential were recorded. Data were analyzed using Clamp 700B amplifier (Molecular Devices). Data were digitized with a resistance of 2 MΩ when filled with intracellular solution. Borosilicate glass patch electrodes were pulled using a Flaming/Brown micropipette puller (Sutter Instrument Company) with a resistance of 2–5 MΩ when filled with intracellular solution. All whole-cell patch-clamp recordings were performed using a Multi-Clamp 700B amplifier (Molecular Devices). Data were digitized with a DigiData 1331 system (Molecular Devices), filtered at 2 kHz, and acquired using pClamp10.2 (Molecular Devices). After formation of a whole-cell configuration, cells were held at −60 mV and changes in membrane potential were recorded. Data were analyzed using Clampfit 10.2 (Molecular Devices).

Results

NCFP Acts as an mGlu5 PAM. NCFP (Zhao et al., 2007) is a close structural analog of the previously reported mGlu5 PAM CPPHA (Fig. 1A) (O’Brien et al., 2004; Zhao et al., 2007). To assess the activity of NCFP as an mGlu5 PAM, we compared the effects of NCFP on agonist-induced calcium mobilization in HEK293A cells expressing mGlu5. In common with CPPHA, NCFP had no effect on calcium mobilization when added alone but induced a concentration-dependent potentiation of the response to an EC20 concentration of glutamate (Fig. 1B; Table 1). In agreement with previous results (O’Brien et al., 2004), CPPHA also potentiated the response to an EC20 concentration of glutamate (Fig. 1B; Table 1). The ability of NCFP to potentiate the response to glutamate was also assessed in rat cortical astrocytes, a native system that expresses mGlu5 (Peavy and Conn, 1998; Zhang et al., 2005; Chen et al., 2008). In agreement with the HEK293A cell assays, NCFP and CPPHA potentiated the response to an EC20 concentration of glutamate in cortical astrocytes. However, NCFP had a slightly lower potency, compared with HEK293A cells (Fig. 1C; Table 1). Previous studies suggest that some mGlu5 PAMs can possess intrinsic mGlu5 agonist activity; we recently reported that this allosteric agonist activity is most readily observed when mGlu5 PAMs are assessed in cells expressing high levels of mGlu5 (Noetzel et al., 2012). Thus, we evaluated the effects of NCFP and CPPHA in a previously characterized cell line expressing high levels of mGlu5 (Noetzel et al., 2012). Neither NCFP nor CPPHA exhibited any agonist activity when assessed in the high mGlu5 expression cell line, and both compounds potentiated the EC20 response to glutamate in a similar manner to that observed in the lower expressing cell line (Supplemental Fig. 1A; Table 1). In the lower mGlu5-expressing cell line, both NCFP and CPPHA induced a concentration-dependent leftward shift in the concentration-response relationship of glutamate, with NCFP inducing a slightly greater shift in glutamate potency, compared with CPPHA (Fig. 1, D and E; Table 1). Allosteric modulator affinity and cooperativity estimates were determined by fitting the data to an operational model of allosterism, as described in Gregory et al. (2012) (details in Materials and Methods). Of interest, NCFP had lower affinity (pKb) and increased cooperativity (log β), compared with CPPHA (Table 2).

As a second measure of mGlu5 activation, NCFP and CPPHA were evaluated for their ability to stimulate an increase in ERK1/2 phosphorylation in mGlu5-expressing HEK293 cells. Treatment of HEK293A cells with 1 mM glutamate resulted in an increase in ERK1/2 phosphorylation that peaked at 7 minutes (3.1 ± 0.8 fold/basal). When added alone, both NCFP (Fig. 1F) and CPPHA (unpublished data) also induced robust increases in ERK1/2 phosphorylation peaking at 7 minutes and were comparable to the responses to glutamate (3.0 ± 0.9 and 2.8 ± 0.5 fold/basal, respectively). In parallel, we evaluated the response to VU0092273, a known MPEP-site mGlu5 PAM (Rodriguez et al., 2010; Noetzel et al., 2012). VU0092273 alone also induced an increase in ERK1/2 phosphorylation that was similar to the responses to NCFP and CPPHA (Fig. 1F; 3.9 ± 1.1 fold/basal at 7 minutes). Similar results were observed with other MPEP-site PAMs (Gregory et al., 2012). We further tested whether the ERK1/2 phosphorylation response observed after the addition of compound alone could be blocked with an orthosteric antagonist, LY341495. Incubation with antagonist, followed by compound addition, resulted in a blockade of the ERK1/2 phosphorylation response (Supplemental Fig. 2), suggesting that observed response to the compounds alone is not the result of direct activation of the receptor by the modulator. Thus, each of these compounds induces ERK1/2 phosphorylation in HEK293A cells that is dependent on mGlu5 activation.

NCFP Interacts with the CPPHA Site on mGlu5. Previous studies demonstrated that CPPHA has little or no effect on [3H]methoxyPEPy binding (O’Brien et al., 2004) in membranes from cells expressing a high level of mGlu5, suggesting that CPPHA does not bind at the MPEP site. We performed similar binding studies using the mGlu5 cell line expressing high levels of mGlu5 (Table 1; Noetzel et al., 2012). Consistent with O’Brien et al. (2004), MPEP induced a concentration-dependent inhibition of [3H]methoxyPEPy binding, whereas CPPHA was without effect at concentrations up to 30 μM (Fig. 2). In addition, NCFP had no effect on [3H]methoxyPEPy binding under these conditions (Fig. 2), suggesting that neither NCFP nor CPPHA act by competitive binding to the MPEP binding site.

A single point mutation in the first transmembrane domain of mGlu5, mGlu5-F585I, has been shown to inhibit the ability of CPPHA to potentiate the response to glutamate but has no effect on responses to PAMs that act at the MPEP site (Chen et al., 2008). In agreement with these previous results, we found that CPPHA was unable to potentiate the response to glutamate in HEK293A cells expressing mGlu5-F585I (Fig. 2; Table 2). In contrast, the MPEP site mGlu5 PAM, VU0092273 induced a robust potentiation of the response to glutamate in cells expressing the mGlu5-F585I mutant receptor (maximum fold-shift 6.8 ± 0.6; unpublished data).
As for CPPHA, introduction of the F585I mutation resulted in a loss of the ability of NCFP to potentiate the response to glutamate (Table 2), suggesting that NCFP interacts with mGlu5 in a similar manner to CPPHA (Fig. 3A). To further support the hypothesis that NCFP potentiates mGlu5 responses via interaction with a second allosteric site, we performed Schild regression analysis of NCFP potentiator concentration–response curves in the absence and presence of 5MPEP (5-methyl-2-phenylethynyl-pyridine), a neutral MPEP site ligand. As previously shown, 5MPEP will induce parallel rightward shifts in the concentration-response curves of PAMs that act competitively at the MPEP site, with no change in the maximum response (Rodriguez et al., 2005; Chen et al., 2007). Previous studies with CPPHA suggest that the actions of this PAM are not mediated by interactions at the MPEP site; 5MPEP has minimal effects on CPPHA potency and depresses the maximal response to CPPHA (Chen et al., 2007). Consistent with these studies, treatment with increasing concentrations of 5MPEP (3–30 μM) caused a small reduction in potency (∼2-fold) and reduced the maximal response (∼20%) when measuring potentiation of the response to an EC_{20} concentration of glutamate (Fig. 3B).

Fig. 1. NCFP potentiates the response to glutamate in a manner similar to that of CPPHA. (A) Structures of CPPHA and NCFP. (B) The potencies of CPPHA (black squares) and NCFP (open triangles) were determined by adding increasing concentrations of each PAM to HEK293 mGlu5 cells 60 seconds before the addition of a concentration of glutamate eliciting a 20% maximal response (EC_{20}, 40–60 nM). The calcium response was normalized to the response induced by a maximally effective concentration of glutamate (10 μM). (C) Potencies of the compounds were determined as in B, except rat cortical astrocytes were used. (EC_{20}, 600–650 nM). (D and E) Progressive fold shift values were determined by treating HEK293 mGlu5 cells with fixed concentrations (300 nM, black triangles, 1 μM black circle, 3 μM open square or 10 μM open triangle) of CPPHA (D) or NCFP (E), followed by the addition of a concentration response curve to glutamate. Calcium responses were normalized to the response induced by a maximally effective concentration of glutamate (10 μM). (F) The level of ERK1/2 phosphorylation (fold/basal) was determined by treating HEK293 mGlu5 cells with a fixed concentration of mGlu5 compounds (3 μM; control black square, NCFP open triangle, VU0092273 black circle) or glutamate (1 mM; black diamond) for the times indicated. Data represent the mean ± S.E.M. of 3–4 independent experiments performed in duplicate.
These data provide strong evidence that the functional response to NCFP is not mediated via competitive interaction with the MPEP binding site; instead, an allosteric interaction between the two sites is potentially occurring.

**CPPHA and NCFP Can Allosterically Inhibit Binding to the MPEP Site under Conditions of Low Receptor Expression.** Although these studies provide clear evidence that NCFP and CPPHA do not bind directly to the MPEP site, it is somewhat surprising that these compounds have no effect on [3H]methoxyPEPy binding. The functional studies outlined here suggest that neutral ligands that act at the MPEP site can induce weak noncompetitive inhibition of actions of CPPHA and NCFP, suggesting that ligands that act at one of these distinct sites can regulate interactions of ligands at the other allosteric site. Of note, the radioligand binding studies were performed using membranes from cell lines that intentionally express high levels of mGlu5 to provide high specific binding and reduce the signal to noise ratio of the assay. It is possible that this high expression and other differences in the cellular background may influence the ability to detect an interaction between these two sites. With the ability to detect interactions between the CPPHA and MPEP site under conditions where receptor expression is low, we next sought to quantify the influence of the F585I mutation on modulator affinity. Receptor expression levels were lower in mGlu5-F585I-expressing cells than in the high mGlu5-expressing cell line (Table 1). Of interest, introduction of the F585I mutation into the mGlu5 receptor had no effect on affinity of [3H]methoxyPEPy binding (Gregory et al., 2012) (Fig. 4A). Similarly, NCFP induced a weak partial inhibition of [3H]methoxyPEPy binding with a maximal inhibition of 31.4% ± 8.5%. This partial inhibition of [3H]methoxyPEPy binding is consistent with an allosteric interaction between the modulators at CPPHA/NCFP site and [3H]methoxyPEPy (Fig. 4A) and in agreement with the results from Fig. 3 B and Bradley et al. (2011). These studies suggest that receptor expression level or other differences in the cellular background may influence the ability to detect an interaction between these two sites. With the ability to detect interactions between the CPPHA and MPEP site under conditions where receptor expression is low, we next sought to quantify the influence of the F585I mutation on modulator affinity. Receptor expression levels were lower in mGlu5-F585I-expressing cells than in the high mGlu5-expressing cell line (Table 1). Of interest, introduction of the F585I mutation into the mGlu5 receptor had no effect on affinity of [3H]methoxyPEPy or VU0092273 (unpublished data) but completely abolished the ability of NCFP to inhibit [3H]methoxyPEPy binding (Fig. 4B), providing further support for the hypothesis that this effect is attributable to interactions with the CPPHA/NCFP binding site.

**NCFP Has Minimal Efficacy at the N-Terminal Truncated Receptor.** Glutamate binds to and activates mGlus via the orthosteric site located in the N-terminal domain of the receptor (Goudet et al., 2004). Truncation of the N-terminal domain prevents mGlus from being activated by orthosteric ligands (Goudet et al., 2004). Although N-terminal truncation eliminates responses to glutamate and other orthosteric ligands, previous studies have demonstrated that mGlus PAMs that act at the MPEP site retain their activity and act as agonists of the N-terminal truncated receptor (Goudet et al., 2004; Chen et al., 2007, 2008). On the basis of these observations, we tested the hypothesis that NCFP would also stimulate a response in the N-terminal–truncated mGlus construct by measuring NCFP-induced calcium mobilization. Ionomycin was used in place of glutamate to determine the maximum response, because it is able to stimulate calcium release independent of mGlus activation. Of surprise, in contrast to the response induced by the MPEP site PAM VU0092273, NCFP has low efficacy for calcium mobilization in cells expressing the N-terminal–truncated mGlus compared to wild-type mGlus cell line. Of interest, under these conditions, CPPHA induces weak partial (64.2% ± 1.7%) inhibition of [3H]methoxyPEPy binding (Gregory et al., 2012) (Fig. 4A). Similarly, NCFP induced a weak partial inhibition of [3H]methoxyPEPy binding with a maximal inhibition of 31.4% ± 8.5%. This partial inhibition of [3H]methoxyPEPy binding is consistent with an allosteric interaction between the modulators at CPPHA/NCFP site and [3H]methoxyPEPy (Fig. 4A) and in agreement with the results from Fig. 3 B and Bradley et al. (2011). These studies suggest that receptor expression level or other differences in the cellular background may influence the ability to detect an interaction between these two sites. With the ability to detect interactions between the CPPHA and MPEP site under conditions where receptor expression is low, we next sought to quantify the influence of the F585I mutation on modulator affinity. Receptor expression levels were lower in mGlu5-F585I-expressing cells than in the high mGlu5-expressing cell line (Table 1). Of interest, introduction of the F585I mutation into the mGlu5 receptor had no effect on affinity of [3H]methoxyPEPy or VU0092273 (unpublished data) but completely abolished the ability of NCFP to inhibit [3H]methoxyPEPy binding (Fig. 4B), providing further support for the hypothesis that this effect is attributable to interactions with the CPPHA/NCFP binding site.

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**TABLE 1**
Expression level and potency values for mGlu5-expressing cell lines

Data represent the mean ± S.E.M. from a minimum of three individual experiments conducted in duplicate or triplicate.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Expression level&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Wild-type-mGlu5 (high expression)</td>
<td>2.3 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.69 ± 0.09 (214 nM)</td>
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<tr>
<td>Wild-type-mGlu5 (low expression)</td>
<td>0.6 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.67 ± 0.10 (225 nM)</td>
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<td>F585I-mGlu5</td>
<td>1.8 ± 0.6</td>
<td>ND</td>
</tr>
<tr>
<td>Rat cortical astrocytes</td>
<td>3.4 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.04 ± 0.10 (969 nM)</td>
</tr>
</tbody>
</table>

CPPHA, N-(4-chloro-2-[[1,3-dioxo-1,3-dihydro-2H-isindol-2-yl][methyl]phenyl]-2-hydroxybenzamide; mGlu, metabotropic glutamate receptor; NCFP, N-(4-chloro-2-(4-fluoro-1,3-dioxoisindolin-2-yl)[methyl]phenyl)picolinamide; ND, not determined.

<sup>a</sup> Receptor expression level determined by [3H]methoxyPEPy binding.

<sup>b</sup> Negative logarithm of the concentration of modulator that resulted in half-maximal potentiation of an EC<sub>20</sub> concentration of glutamate.

<sup>c</sup> Expression level previously reported in Gregory et al., 2012.

---

**TABLE 2**
Operational model parameters and fold shift values for NCFP and CPPHA modulation of intracellular Ca<sup>2+</sup> mobilization

Data represent the mean ± S.E.M. from a minimum of three individual experiments conducted in duplicate.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type-mGlu5</th>
<th>F585I-mGlu5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCFP</td>
<td>CPPHA</td>
</tr>
<tr>
<td>pK&lt;sub&gt;i&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.56 ± 0.13</td>
<td>6.09 ± 0.09d</td>
</tr>
<tr>
<td>Log&lt;sub&gt;i&lt;/sub&gt;</td>
<td>0.99 ± 0.05</td>
<td>0.59 ± 0.08d</td>
</tr>
<tr>
<td>Fold shift (max)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.7 ± 0.1</td>
<td>5.9 ± 1.6</td>
</tr>
<tr>
<td>Fold shift (3μM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.9 ± 0.1</td>
<td>4.0 ± 0.7</td>
</tr>
</tbody>
</table>

CPPHA, N-(4-chloro-2-[[1,3-dioxo-1,3-dihydro-2H-isindol-2-yl][methyl]phenyl]-2-hydroxybenzamide; mGlu, metabotropic glutamate receptor; NCFP, N-(4-chloro-2-(4-fluoro-1,3-dioxoisindolin-2-yl)[methyl]phenyl)picolinamide; ND, not determined; P<sub>K</sub>, protein kinase B.

<sup>a</sup> Negative logarithm of the allosteric modulator dissociation constant.

<sup>b</sup> Negative logarithm of the efficacy cooperativity factor.

<sup>c</sup> Fold shift values were determined for the maximum fold shift determined for multiple concentrations or at a single (3 μM) concentration.

<sup>d</sup> Operational model parameters were previously reported in Gregory et al., 2012. ND values not determined.
the results observed in the calcium mobilization assay, NCFP phosphorylation in the absence of receptor activation. Similar to a positive control, because it will stimulate ERK1/2 phosphorylation.

Fig. 2. NCFP does not inhibit [3H]methoxyPEPy binding in cells expressing high levels of mGlu5. Cells expressing high levels of mGlu5 were treated with increasing concentrations of NCFP (open triangles), CPPHA (black squares), or MPEP (black circles) and 2 nM [3H]methoxyPEPy. Reactions were allowed to incubate for 1 hour before termination. Nonspecific binding was determined using 10 μM MPEP. Data represent the mean ± S.E.M. of three individual experiments conducted in duplicate.

with the MPEP-site PAM VU0092273 (Fig. 5A; NCFP, 1.3% ± 0.5% of ionomycin; VU0092273, 19.9% ± 2.7% of ionomycin).

As discussed above, the MPEP site PAMs that have been tested for agonist activity at the N-terminal–truncated receptor can have also weak agonist activity at wild-type mGlu5 when measuring calcium mobilization, whereas CPPHA and NCFP have no agonist activity at the wild-type receptor when measuring the calcium mobilization response. Thus, it is possible that this difference in agonist activity for calcium mobilization is simply reflected in the weak partial agonism of NCFP at the N-terminal–truncated receptor. Because calcium mobilization and ERK1/2 phosphorylation can occur through different pathways, we tested the ability of NCFP to induce ERK1/2 phosphorylation with use of the N-terminal–truncated mutant. In this case, FBS was used as a positive control, because it will stimulate ERK1/2 phosphorylation in the absence of receptor activation. Similar to the results observed in the calcium mobilization assay, NCFP had no effect on the level of ERK1/2 phosphorylation, compared with control (0.9 ± 0.04 fold/basal; Fig. 5B), whereas VU0092273 induced a robust increase in ERK1/2 phosphorylation (2.1 ± 0.4 fold/basal; Fig. 5B) that was similar to the level of phosphorylation observed with the wild-type receptor (Fig. 1F). In addition to greatly reduced or no effects in functional assays, NCFP was no longer able to inhibit the binding of [3H]methoxyPEPy to the N-terminal–truncated receptor (2.2% ± 0.4% inhibition; Fig. 5C). In contrast, MPEP retained the ability to completely inhibit [3H]methoxyPEPy binding in a concentration-dependent manner (Fig. 5C). The level of receptor expression was similar for the wild-type mGlu5 cells (Table 1) and the N-terminal–truncated receptor construct (0.69 ± 0.16 pol/mg protein), suggesting that receptor expression level was not likely to be responsible for the lack of [3H]methoxyPEPy inhibition by NCFP. In addition, VU0092273 also maintained the ability to completely inhibit [3H]methoxyPEPy binding (Fig. 5C). Although the precise molecular mechanisms underlying the differences between NCFP and MPEP site ligands, such as VU0092273, are not yet understood, these results further support the hypothesis that NCFP interacts with mGlu5 in a manner that is distinct from MPEP site ligands.

NCFP Is Selective for mGlu5 and Does Not Exhibit Probe Dependence for Glutamate Versus the Orthosteric mGlu5 Agonist DHPG. To use mGlu5 allosteric modulators in brain slice assays, it is important to determine whether they are selective for mGlu5, compared with other mGlu subtypes. We previously reported that CPPHA has weak PAM activity at mGlu1 and weak NAM activity at mGlu4 and mGlu8 (O’Brien et al., 2004; Chen et al., 2008). On the basis of the lack of selectivity of CPPHA, we initiated chemistry efforts to develop a CPPHA analog with a better selectivity profile that could be used to assess mGlu5 function in a more complex system (Zhao et al., 2007). Of the CPPHA analogs that came from this effort, NCFP had properties that prompted us to characterize this compound more extensively. To assess the selectivity of NCFP, cells expressing each mGlu subtype were treated with 10 μM NCFP to determine whether this compound could modulate the response to glutamate (or L(+)-2-Amino-4-phosphonobutyric acid for mGlu7). NCFP
did not shift the concentration-response curves for glutamate in cells expressing any of the mGlu receptor subtypes (Supplemental Fig. 3). Thus, although NCFP and CPPHA behave in a very similar manner in potentiating responses to mGlu5 activation, NCFP is more selective for mGlu5 than is CPPHA. Thus, NCFP provides an improved tool for studies in native preparations and was used for all subsequent experiments.

In addition to determining compound selectivity for use in brain slice-based assays, it is also important to consider the choice of agonist used for these experiments. It is not practical to use glutamate in brain slice–based electrophysiology.
experiments when studying mGlu₅ activation, because glutamate will modulate multiple targets, including other mGlu subtypes, ionotropic glutamate receptors, and glutamate transporters. In addition, glutamate is a substrate for glutamate transporters that are abundant in brain slice preparations, therefore making it difficult to achieve reliable and stable glutamate concentrations within the slice. For these reasons, the group I orthosteric agonist DHPG is routinely used for studies of the physiologic effects of activation of group I mGlu. However, previous work has shown that some allosteric modulators can potentiate responses of other TMRs to some, but not all orthosteric agonists, a phenomenon termed probe-dependence (Keov et al., 2011). Therefore, before using DHPG to study effects of NCFP in rat brain slices, it was critical to assess the ability of NCFP to potentiate the response to DHPG. Similar to the results observed with glutamate, NCFP potentiated the response to an EC₂₀ concentration of DHPG with a potency of pEC₅₀ 6.94 ± 0.16 (133 nM; Supplemental Fig. 4). Thus, NCFP does not exhibit probe dependence for glutamate relative to DHPG, allowing this combination of allosteric and orthosteric ligands to be used in brain slice preparations.

**NCFP Potentiates DHPG-Induced Depolarization of Subthalamic Nucleus Neurons.** One of the most well-established responses to mGlu₅ activation in the CNS is depolarization of projection neurons in the STN (Awad et al., 2000). DHPG-induced depolarization of these neurons is completely blocked by MPEP and other mGlu₅ NAMs and is potentiated by previously characterized mGlu₅ PAMs (Awad et al., 2000; O'Brien et al., 2004; Rodriguez et al., 2005; Chen et al., 2007; Rodriguez et al., 2010; Noetzel et al., 2012). In agreement with previous results, treatment of STN neurons with 100 µM DHPG resulted in a robust membrane depolarization (Fig. 6; 10.7 ± 1.3 mV), whereas 1 µM DHPG induced a small depolarization that was just above threshold for detection (Fig. 6; 2.4 ± 0.8 mV). Treatment of midbrain slices with 10 µM NCFP alone had no effect on STN membrane voltage (Fig. 6; 0.5 ± 0.7 mV). However, 10 µM NCFP, followed by co-addition of 10 µM NCFP and 1 µM DHPG, resulted in a robust potentiation of the depolarization relative to that observed with 1 µM DHPG alone (Fig. 6; 5.7 ± 0.6 mV). We observed similar results with CPPHA, in agreement with data published by Chen et al. (2007) demonstrating the potentiation of the DHPG response in STN neurons by CPPHA. These results suggest that NCFP potentiates mGlu₅-mediated depolarization of STN neurons in a manner similar to that observed in HEK293 cells and in cortical astrocytes.

**Unlike Other mGlu₅ PAMs, NCFP Does Not Potentiate Induction of LTD and LTP in the Hippocampus.** Activation of mGlu₅ plays an important role in regulating hippocampal synaptic plasticity, and previous studies have shown that mGlu₅ PAMs induce a robust potentiation of DHPG-induced LTD at the hippocampal SC-CA1 synapse (Ayala et al., 2009; Auerbach et al., 2011; Popkirov and Manahan-Vaughan, 2011; Noetzel et al., 2012). In addition, selective mGlu₅ PAMs potentiate induction of LTD at this synapse in response to a weak threshold TBS protocol (Ayala et al., 2009). To assess the effects of mGlu₅ PAMs on DHPG-induced LTD, fEPSPs were recorded from the dendritic layer of CA1 after stimulation of the Schaffer collaterals. In agreement with our previous studies (Noetzel et al., 2012), 75 µM DHPG induced robust LTD at the hippocampal SC-CA1 synapse measured 55 minutes after washout of the compound (Fig. 7, A and B; 52.2% ± 3.9% of baseline fEPSP slope). In contrast, a lower concentration of DHPG (25 µM) induced only slight depression of fEPSPs at this synapse (Fig. 7, A and B; 92.3% ± 3.7% of baseline fEPSP slope). Previous work has shown that mGlu₅ PAMs can potentiate mGlu-LTD induced by low concentrations of DHPG (Ayala et al., 2009; Auerbach et al., 2011; Popkirov and Manahan-Vaughan, 2011; Noetzel et al., 2012). All of the compounds that have been tested thus far are thought to bind at or near the MPEP site. Consistent with our previous report (Noetzel et al., 2012), 10 µM VU0092273 significantly potentiated the response to 25 µM DHPG, inducing LTD that lasted more than 55 minutes after washout of the compound (Fig. 7B; VU0092273 51.6% ± 5.8% baseline, P < 0.05). In contrast, NCFP (10 µM) did not induce a significant potentiation of the LTD response to 25 µM DHPG (Fig. 7, A and B; NCFP 79.2% ± 6.0% baseline). Of interest, there was a small effect observed with CPPHA (70.9% ± 4.7% of baseline). However, mGlu₅ has been established to play a potential role in inducing LTD at this synapse; thus, this small effect may be a result of activation of mGlu₅ rather than mGlu₅.

To determine the effects of mGlu₅ PAMs on induction of LTP at the SC-CA1 synapse, fEPSPs were recorded from the dendritic layer of CA1 after stimulation of the Schaffer collaterals. In contrast to the LTD results, we observed that NCFP does not potentiate LTP induction at the SC-CA1 synapse. Treatment of midbrain slices with 10 µM NCFP alone had no effect on STN membrane voltage (Fig. 6; 0.5 ± 0.7 mV). However, 10 µM NCFP, followed by co-addition of 10 µM NCFP and 1 µM DHPG, resulted in a significant potentiation of the LTP response relative to that observed with 1 µM DHPG alone (Fig. 6; 5.7 ± 0.6 mV). We observed similar results with CPPHA, in agreement with data published by Chen et al. (2007) demonstrating the potentiation of the DHPG response in STN neurons by CPPHA. These results suggest that NCFP potentiates mGlu₅-mediated depolarization of STN neurons in a manner similar to that observed in HEK293 cells and in cortical astrocytes.

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collaterals. In agreement with previous results from our laboratory (Ayala et al., 2009), maximal LTP was initiated using a standard TBS protocol (saturation TBS; four trains of 10Hz TBS), which resulted in a 153.4 ± 4.9% increase in fEPSP slope, compared with baseline, when measured 35 minutes after stimulation (Fig. 8B). To study the ability of mGlu5 PAMs to potentiate LTP, a threshold TBS protocol was used to induce submaximal potentiation (Ayala et al., 2009). With use of this protocol, a single train of lower frequency bursts resulted in a slight potentiation of the fEPSP slope (Fig. 8, A and B; 118.5% ± 4.1% of baseline). Treatment of hippocampal slices with 10 μM NCFP for 20 minutes, followed by threshold TBS, had no effect on fEPSP slope, compared with threshold TBS alone (Fig. 8, A and B; NCFP 112.8% ± 2.5% of baseline). Similar results were observed with CPPHA (112.8% ± 1.8% of baseline). In contrast, 1 μM VU0992273 for 20 minutes, followed by threshold TBS, resulted in a significant increase in the fEPSP slope (Fig. 8, A and B; 134.9% ± 6.8% of baseline, P < 0.05). Of note, in a subset of slices, there was an increase in fEPSP slope with VU0992273 alone, although this did not appear to affect the level of potentiation. In addition, in previous studies, another MPEP site PAM (VU29) potentiated threshold TBS LTP while having no effect on fEPSPs when added alone (Ayala et al., 2009). Collectively, these results suggest that NCFP shows a clearly distinct profile in its effects on hippocampal synaptic plasticity and does not share the ability of other mGlu5 PAMs that have been characterized to potentiate either hippocampal LTD or LTP.

Discussion

Preclinical studies suggest that mGlus are viable candidates as drug targets for treatment of a variety of CNS disorders. Much of the current research around mGlus is focused on the development of allosteric modulators because of their potential for subtype selectivity and ability to maintain activity dependence of receptor activation. At present, the most advanced efforts have been in discovery and development of mGlu5 allosteric modulators; both mGlu5 PAMs and NAMs are now progressing through preclinical and clinical development. A broad range of mGlu5 modulators from a variety of different scaffolds have been discovered and characterized. Many of these compounds have been shown to have efficacy in animal models used to predict efficacy in treatment of CNS disorders. However, the mechanisms through which they mediate their different in vivo effects are not fully understood. Furthermore, it is now becoming increasingly clear that distinct allosteric modulators can differentially modulate coupling of a 7TMR to different signaling pathways of functional responses (Sheffler and Conn, 2008; Niswender et al., 2010; Gregory et al., 2012), signaling pathways of functional responses (Sheffler and Conn, 2008; Niswender et al., 2010; Gregory et al., 2012), and that these response biases could lead to an unexpected failure to observe the predicted in vivo or clinical effects. Consider the lack of efficacy exhibited by NCFP to enhance hippocampal synaptic plasticity. If the ability of mGlu5 PAMs to potentiate induction of LTP and LTD is important for the effects of previous agents on cognitive function, compounds that have a profile similar to that of NCFP may not have the desired profile in its effects on hippocampal synaptic plasticity and does not share the ability of other mGlu5 PAMs that have been characterized to potentiate either hippocampal LTD or LTP.

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therapeutic effects. Of importance, the failure of NCFP to enhance synaptic plasticity does not reflect a difference in the activity of this compound in cell lines versus native systems. For instance, we found that NCFP potentiates responses to mGlu5 activation in cortical astrocytes and in recordings from STN neurons in midbrain slices. The effects of NCFP in both astrocytes and STN neurons are similar to effects of other mGlu5 PAMs that have been reported previously (O’Brien et al., 2004; Rodriguez et al., 2005; Chen et al., 2007; Noetzel et al., 2012). Thus, the ability of NCFP to stimulate some but not all responses to mGlu5 activation more likely reflects an ability of this PAM to selectively potentiate coupling of mGlu5 to some but not all downstream responses. In addition, mGlu5 has been shown to mediate effects in other brain regions, such as the striatum, where it has been shown to modulate NMDA receptor responses in medium spiny neurons (Pisani et al., 2001), and activation of mGlu5 by DHPG has been shown to induce LTD in the ventral striatum (Jung et al., 2012). Thus, studying the ability of CPPHA or MPEP site ligands at additional synapses in the brain will be critical to aid in the understanding of modulation of signaling through these two sites, because the effects cannot be generalized to all forms of synaptic plasticity or to all regions in the CNS. These data highlight a potential pitfall in many drug discovery programs, that is, the use of in vitro assays that may not be predictive of desired physiologic responses.

Unfortunately, despite significant effort, it has been impossible to achieve sufficient free brain concentrations of either NCFP or CPPHA to allow use of these compounds in behavioral studies (unpublished data). In the future, it will be important to optimize mGlu5 PAMs that have different pharmacological profiles for in vivo use to allow direct studies of the behavioral effects of mGlu5 PAMs that bind to different allosteric sites or differentially modulate physiologic responses to mGlu5 activation. Provision of such tools will be essential in elucidating whether biased modulation is a desirable trait for a particular therapeutic outcome or, alternatively, whether a particular pharmacological profile can be predictive of efficacy. The issue of functional selectivity and the potential impact of in vivo efficacy is a critical consideration for allosteric modulators of not just other mGlu but all 7TMRs.

NCFP is derived from the same scaffold as CPHA, and the current data suggest that these mGlu5 PAMs interact with mGlu5 receptor at a unique site that is distinct from the common binding site used by the prototypical mGlu5 NAM, MPEP, and many other mGlu5 modulators. It is tempting to speculate that the unique profile of NCFP on mGlu5 signaling will be shared by all mGlu5 PAMs that interact with this site and that mGlu5 PAMs that interact with the MPEP site will all share the effects on mGlu5 signaling, including effects on synaptic plasticity, that have been described for previous MPEP site PAMs. Although possible, it would be premature to assume that the differences observed between NCFP and PAMs that are competitive with the MPEP site are strictly related to the different binding sites of these agents. Previous studies have shown that small changes within a chemical scaffold can lead to fundamental changes in responses to allosteric modulators. For example, there are multiple examples of single atom changes in allosteric modulators converting a partial NAM, a full NAM, or a PAM to a robust allosteric agonist (Sharma et al., 2008, 2009; Wood et al., 2011; Sheffler et al., 2012). If subtle changes within a scaffold can have such dramatic effects on the mode of modulation of mGlu5, it is also possible that such changes could alter the signaling pathways that are most efficiently engaged by the receptor. It is possible that other biased allosteric modulators of mGlu5 have been discovered, but remain unappreciated, because of a lack of in-depth assessment of their physiologic effects. Further studies are required to fully understand the molecular basis for this pharmacological bias by NCFP.

In addition to the differential effects of NCFP on different functional responses to mGlu5 activation, there were other interesting differences between NCFP and previously characterized mGlu5 PAMs. For instance, previous work by Goudet et al. (2004) demonstrated that N-terminal truncation
of mGlu5 eliminates the orthosteric glutamate binding site and prevents glutamate from activating the receptor but that the mGlu5 PAM DFB behaves as an agonist at the N-terminal–truncated receptor (Goudet et al., 2004). This phenomenon has now been observed with multiple mGlu5 PAMs (Chen et al., 2008) and is replicated here with the previously characterized MPEP site mGlu5 PAM U0092273. Furthermore, PAMs acting at other mGlu subtypes have now been shown to behave as agonists when assessed using the N-terminal–truncated receptor (Fazio et al., 2012), suggesting that this is a common feature of mGlu PAMs. In the present studies, it was interesting to find that NCFP has minimal or no agonist activity at the N-terminal–truncated receptor when assessed by measuring mGlu5 coupling to calcium mobilization or ERK1/2 phosphorylation, respectively.

Chen et al. (2008) have shown that NCFP acts through a novel mechanism as a positive allosteric modulator of group 1 metabotropic glutamate receptors. Mol Pharmacol 73:908–918.


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (50%) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.


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Supplemental Figure 1. High expression of mGlu5 has no effect on modulator potency. The potencies of NCFP (open triangles) and CPPHA (black squares) were determined using a cell line expressing high levels of mGlu5 by adding increasing concentrations of each mGlu5 compound to cells prior to the addition of an EC20 concentration of glutamate. The calcium response was normalized to the response induced by a maximally effective concentration of glutamate (100 µM). Data represent the mean ± SEM of 3 individual experiments conducted in duplicate.
Supplemental Figure 2. The ERK1/2 phosphorylation response to mGlu5 compounds alone is blocked by the orthosteric antagonist LY341495. The level of ERK1/2 phosphorylation (fold/basal) was determined by treating HEK293 mGlu5 cells with a fixed concentration of LY341495 (100 μM; open shapes) or buffer (closed shapes) followed by addition of mGlu5 compounds (3 μM; NCFP triangle, CPPHA circle) or glutamate (1 mM; black diamond) for the times indicated. Data represent the mean ± SEM of 3 independent experiments performed in duplicate.
Supplemental Figure 3. NCFP is selective for mGlu5 over other mGlu subtypes. A fixed concentration of NCFP (10 µM, open triangles) was added to the cells prior to the addition of increasing concentrations of glutamate, or L-AP4 in the case of mGlu7. There was no shift in the glutamate concentration response curve at any of the mGlu subtypes in the presence of NCFP. A) mGlu1, B) mGlu2, C) mGlu3, D) mGlu4, E) mGlu6, F) mGlu7, G) mGlu8. Data represent the mean ± SEM of 3 individual experiments conducted in duplicate.
Supplemental Figure 4. NCFP does not exhibit probe-dependence when DHPG is used as the orthosteric agonist. The potencies of NCFP (open triangles) and CPPHA (black squares) were determined using a cell line expressing high levels of mGlu5 by adding increasing concentrations of NCFP or CPPHA followed by the addition of an EC20 concentration of DHPG. The calcium response was normalized to the response induced by a maximally effective concentration of glutamate (100 µM). Data represent the mean ± SEM of 3 individual experiments conducted in duplicate.