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Title Page

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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Nonstandard abbreviations:

CNS – central nervous system

LTD – long-term depression

LTP – long-term potentiation

PAMs – positive allosteric modulators

MPEP- 2-Methyl-6-(phenylethynyl)pyridine

CPPHA - *N*-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl)-2-hydroxybenzamide

NCFP – *N*-(4-chloro-2-((4-fluoro-1,3-dioxoisindolin-2-yl)methyl)phenyl)picolinamide

mGluR – metabotropic glutamate receptors

7TMRs – 7 transmembrane-spanning G protein-coupled receptors

NAM – negative allosteric modulator

DFB – difluorobenzaldazine

ERK1/2 – extracellular signal-regulated kinases 1 and 2

SC-CA1 synapse – Schaffer collateral – CA1 synapse

DMEM - Dulbecco's Modified Eagle's Medium

FBS - fetal bovine serum

DMSO - dimethyl sulfoxide

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methoxyPEPy – 3-methoxy-5-(2-pyridinylethynyl)pyridine

HEK293 cells - human embryonic kidney 293 cells

GIRK – G protein-coupled inwardly-rectifying potassium channels

HBSS – Hank's balanced salt solution

FDSS – functional drug screening system

CRC, concentration response curve

L-AP4 – L-(+)-2-Amino-4-phosphonobutyric acid

AGM – assay growth media

ACSF – artificial cerebral spinal fluid

fEPSP – field excitatory postsynaptic potential

DHPG – (S)-3,5-dihydroxyphenylglycine

STN – subthalamic nucleus

VU0092273 – (4-hydroxypiperidin-1-yl)(4-phenylethynyl)phenyl)methanone

5MPEP – 5-methyl-2-phenylethynyl-pyridine

TBS – theta burst stimulation

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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 binding site. However, one mGlu₅ PAM, CPPHA, 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. 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. Interestingly, 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 physiological 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.

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Introduction

The metabotropic glutamate receptors (mGlu) include eight subtypes (mGlu₁₋₈) of 7 transmembrane-spanning G protein-coupled receptors (7TMRs) for the neurotransmitter glutamate. The mGlu 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 (for reviews, (Gregory et al., 2011; Vinson and Conn, 2012). While 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 (Conn et al., 2009; Liu et al., 2008; Packiarajan et al., 2012; Stauffer, 2011; Varnes et al., 2011). 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 (for reviews, (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 (Auerbach et al., 2011; Ayala et al., 2009; Noetzel et al., 2012; Popkirov and Manahan-Vaughan, 2011). Importantly, 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

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potentiate both LTP and LTD 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 (Chen et al., 2007; Hammond et al., 2010; Kinney et al., 2005; Lamb et al., 2011; Liu et al., 2008; Noetzel et al., 2012; O'Brien et al., 2004; Packiarajan et al., 2012; Rodriguez et al., 2010; Stauffer, 2011; Varnes et al., 2011); the majority of these mGlu₅ PAMs bind to the same site as the prototypical mGlu₅ negative allosteric modulator (NAM) MPEP, 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 (Chen et al., 2008; O'Brien et al., 2004; Zhao et al., 2007) and VU0357121 (Hammond et al., 2010), have been identified that interact non-competitively with the MPEP site. Interestingly, Zhang et al (2005) reported that CPPHA and an MPEP site mGlu₅ PAM, DFB, induce similar potentiation of mGlu₅-mediated calcium mobilization in cortical astrocytes. However, while DFB has similar effects on mGlu₅ activation of calcium mobilization and 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 (Niswender et al., 2010; Sheffler and Conn, 2008). While 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 mGlu₄ and mGlu₈ (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 characterize a novel mGlu₅ PAM, termed NCFP (Zhao et al 2007, patent number WO 2004087048 A2 20041014) that is structurally related to CPPHA. NCFP interacts non-competitively with the MPEP site, and likely binds instead to the same site as CPPHA. Interestingly, NCFP has a fundamentally different profile from previous mGlu₅ PAMs when assessing effects of hippocampal synaptic plasticity. While NCFP potentiates

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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 physiological responses in the CNS that could be important for the overall efficacy profile of these agents.

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Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Life Technologies (Carlsbad, CA). DHPG was obtained from Ascent Scientific (Bristol, UK). CPPHA, NCFP (Zhao et al., 2007), VU0092273 (Rodriguez et al., 2010), 5-MPEP (Rodriguez et al., 2005) and MPEP (Tocris) were synthesized in house. [³H]methoxyPEPy (76.3 Ci/mmol) was custom synthesized by PerkinElmer Life and Analytical Sciences (Waltham, MA). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of an analytical grade.

Cell culture

Human embryonic kidney 293 (HEK293) cells stably expressing rat mGlu₅ or rat mGlu₁ were maintained in complete DMEM supplemented with 10% FBS, 2 mM L-glutamine, 20 mM HEPES, 0.1 mM Non-Essential Amino Acids, 1 mM sodium pyruvate, antibiotic-antimycotic (Invitrogen, Carlsbad, CA) and G418 (500 µg/mL; Mediatech, Manassas, VA) at 37°C in a humidified incubator containing 5% CO₂/95%O₂. HEK293 cells stably expressing G protein-coupled inwardly-rectifying potassium channels (HEK293-GIRK) 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, non-essential 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

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previously (Goudet et al., 2004). Transfection of HEK293A cells with wild type mGlu₅ and mutant constructs in pCI:Neo were performed using Fugene6 (Promega, Madison, WI), and passaged five times in the presence of 1ug/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, 20 mM HEPES, 0.1 mM Non-Essential 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.

Fluorescence-based calcium signaling

Measurements of compound-evoked increases in intracellular calcium were performed as described previously (Noetzel et al., 2012). Briefly, 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 Ca²⁺ indicator dye, Fluo-4AM using a FlexStation II (Molecular Devices, Sunnyvale, CA). Either vehicle or test compound was added 60 sec prior to the addition of glutamate. Compound addition 120 sec prior to the addition of glutamate was also assessed. There was no difference observed in the potency for CPPHA or NCFP between the two time points (pEC₅₀ CPPHA, 60 sec -6.14, 120 sec -6.14; NCFP 60 sec -6.30, 120 sec -6.23), suggesting that the maximum effect of the PAMs has been achieved with this preincubation period. The change in fluorescence over basal was determined before normalization to the maximal response elicited by glutamate. Experiments utilizing 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-

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response curves by allosteric modulators were globally fitted to an operational model of allosterism (Leach et al., 2007) using equation 2 from Gregory et al (2012).

Selectivity

mGlu₁. To assess the effect of test compounds at mGlu₁, Ca²⁺ mobilization assays were performed as described previously (Hammond et al., 2010; Noetzel et al., 2012). Briefly HEK293 cells stably expressing rat mGlu₁ 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 Ca²⁺ indicator dye, Fluo-4AM using a Functional Drug Screening System 6000 (FDSS 6000, Hamamatsu, Japan). Either vehicle or a fixed concentration of test compound (10 μ M, final concentration) was added followed 140 sec later by a concentration-response curve (CRC) of glutamate. Data were analyzed as described above.

Group II and Group III mGlu's. The functional activity of the compounds of interest was assessed at the rat group II and III mGlu receptors by measuring thallium flux through GIRK channels as previously described (Niswender et al., 2008). Briefly, HEK293-GIRK 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 μ M) or vehicle was added followed 140 sec later by a concentration response curve of glutamate (or L-AP4 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 FDSS 6000. Data were analyzed as described previously (Niswender et al., 2008).

Rat cortical astrocytes

Primary rat cortical astrocytes (from rats of mixed gender) were purchased from Lonza (Basel, Switzerland) and maintained as previously described (Noetzel et al 2012). Astrocytes

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were grown in assay growth media (AGM; assay basal media supplemented with AGM Singlequots from Lonza). Two days prior to the assay, astrocytes were plated in 96-well, poly-D-lysine coated, black-walled, clear-bottomed plates in AGM at a density of approximately 50,000 cells/well. The next day astrocytes were supplemented with G5 diluted in AGM. The calcium flux assay was performed the following day using assay conditions and compound preparation identical to those used for the mGlu₅ HEK293A cell assay.

ERK phosphorylation

Receptor-mediated ERK1/2 phosphorylation was determined using the AlphaScreen™-based ERK SureFire™ kit (PerkinElmer, TGR Biosciences). 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 hr prior to assay. Media was aspirated and cells serum starved in serum-free media (DMEM supplemented with 16 mM HEPES) for 6 hr prior to assay. Serum-free media was exchanged for fresh at the start of the experiment (30 min prior to 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 50 µL/well of lysis buffer. Lysates were processed as described previously (Gregory et al 2012) and AlphaScreen signal measured using an Enspire (PerkinElmer, Waltham Massachusetts) 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]methoxyPEPy and a range of concentrations of test ligand (100 pM-100 µM) for 1 hr at room temperature with shaking in calcium assay buffer with

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1% DMSO final. 10 μ M MPEP was used to determine non-specific 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, pH7.4). Plates were allowed to dry overnight prior to addition of MicroScint20 (40 μ l/well; PerkinElmer). Radioactivity was counted after at least 2 hr incubation using a TopCount Scintillation Counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Inhibition [3 H]methoxyPEPy binding data sets were fitted to a one-site inhibition binding model and estimates of inhibitor dissociation constants (K_i) 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 Birdsall, 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 μ m hippocampal slices were prepared from young adult (30-40 day old) male Sprague–Dawley rats (Charles River, Wilmington, MA) using 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 in order to stimulate the Schaffer collaterals. Recording electrodes were pulled with a Flaming/Brown micropipette puller (Sutter Instruments, CA), filled with ACSF and placed in the stratum radiatum of area CA1. Field potential recordings were acquired using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) 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 (fEPSP) slope was set before each experiment. mGlu₅ compounds were diluted to the appropriate concentrations in DMSO (0.1% final) in ACSF

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and applied to the bath for 10-20 min using a perfusion system. Chemically induced mGlu LTD was initiated by the application of (S)-3,5-dihydroxyphenylglycine (DHPG) in ACSF (25–75 μ M) for 10 min. Threshold LTP was induced by one train of theta burst stimulation (TBS) (nine bursts of four pulses at 100 Hz, 230ms interburst interval). Saturated LTP was induced by four trains of 10 Hz TBS (nine bursts of four pulses at 100 Hz, 100 ms interburst interval). Data were analyzed using Clampfit 10.2 and GraphPad Prism 5.0 as described previously (Noetzel et al 2012).

Whole-cell patch-clamp recordings. Whole-cell patch-clamp recordings were performed using midbrain slices prepared from 15- to 19-day-old Sprague-Dawley rats of mixed gender (Charles River). 300 μ m sagittal slices were prepared using standard techniques and buffers described previously (Noetzel et al 2012). Whole-cell recordings were made from visually identified subthalamic nucleus (STN) neurons. Borosilicate glass patch electrodes were pulled using a Flaming/Brown micropipette puller (Sutter Instruments, CA) with a resistance of 2-5 M Ω when filled with intracellular solution. All whole-cell patch-clamp recordings were performed using a MultiClamp 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).

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Results

NCFP acts as an mGlu₅ PAM.

NCFP (Zhao et al., 2007) is a close structural analog of the previously reported mGlu₅ PAM CPPHA (O'Brien et al., 2004; Zhao et al., 2007) (Figure 1A). 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 (Table 1, Figure 1B). In agreement with previous results (O'Brien et al., 2004), CPPHA also potentiated the response to an EC₂₀ concentration of glutamate (Table 1, Figure 1B). The ability of NCFP to potentiate the response to glutamate was also assessed in rat cortical astrocytes, a native system that expresses mGlu₅ (Chen et al., 2008; Peavy and Conn, 1998; Zhang et al., 2005). 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 to HEK293A cells (Table 1; Figure 1C). 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 (Supplementary Figure 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 when compared to CPPHA (Table 1, Figure 1 D, E).

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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 methods). Interestingly, NCFP had lower affinity (pK_B) and increased cooperativity ($\log\beta$) compared to 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 min (3.1 ± 0.8 fold/basal). When added alone, both NCFP (Figure 1F) and CPPHA (data not shown) also induced robust increases in ERK1/2 phosphorylation peaking at 7 min 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 (Noetzel et al., 2012; Rodriguez et al., 2010). VU0092273 alone also induced an increase in ERK1/2 phosphorylation that was similar to the responses to NCFP and CPPHA (Figure 1F; 3.9 ± 1.1 fold/basal at the 7 min). Similar results were observed with other MPEP-site PAMs (Gregory et al., 2012). We further tested whether the ERK1/2 phosphorylation response observed following 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 (Supplementary Figure 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

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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 μ M (Figure 2). In addition, NCFP had no effect on [³H]methoxyPEPy binding under these conditions (Figure 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 (Table 2, Figure 3A). 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 ; data not shown). 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 (Figure 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, 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 (Chen et al., 2007; Rodriguez et al., 2005). 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 μ M) caused a small reduction in potency (~2 fold) and reduced the maximal response (~20%) when measuring potentiation of the response

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to an EC₂₀ concentration of glutamate (Figure 3B). These data provide strong evidence that the functional response to NCFP is not mediated via competitive interaction with the MPEP binding site, rather 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. While 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 suggest that neutral ligands that act at the MPEP site can induce weak non-competitive 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. It is important to 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 cell background used for binding versus functional studies could introduce an unexpected change in results of the radioligand binding studies. Thus, we evaluated ability of CPPHA and NCFP to inhibit [³H]methoxyPEPy binding to the MPEP site using the same physiological buffer system used in the calcium mobilization assays and using membranes from the lower expressing mGlu₅ cell line. Interestingly, under these conditions, CPPHA induces weak partial (64.2 ± 1.7 percent) inhibition of [³H]methoxyPEPy binding (Gregory et al., 2012) (Figure 4A). Similarly, NCFP induced a weak partial inhibition of [³H]methoxyPEPy binding with a maximal inhibition of 31.4 ± 8.5 percent. This partial inhibition of [³H]methoxyPEPy binding is consistent with an allosteric interaction between the modulators at CPPHA/NCFP site and [³H]methoxyPEPy (Figure 4A) and in agreement with the results from Figure 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

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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 compared to the high mGlu₅ expressing cell line (Table 1). Interestingly, introduction of the F585I mutation into the mGlu₅ receptor had no effect on affinity of [³H]methoxyPEPy or VU0092273 (data not shown) but completely abolished the ability of NCFP to inhibit [³H]methoxyPEPy binding (Figure 4B), providing further support for the hypothesis that this effect is due to interactions with the CPPHA/NCFP binding site.

NCFP has minimal efficacy at the N-terminal truncated receptor

Glutamate binds to and activates mGlu₅ via the orthosteric site located in the N-terminal domain of the receptor (Goudet et al., 2004). Truncation of the N-terminal domain prevents mGlu₅ from being activated by orthosteric ligands (Goudet et al., 2004). While N-terminal truncation eliminates responses to glutamate and other orthosteric ligands, previous studies have demonstrated that mGlu₅ PAMs that act at the MPEP site retain their activity and act as agonists of the N-terminal truncated receptor (Chen et al., 2008; Chen et al., 2007; Goudet et al., 2004). Based on these observations, we tested the hypothesis that NCFP would also stimulate a response in the N-terminal truncated mGlu₅ construct by measuring NCFP-induced calcium mobilization. Ionomycin was used in place of glutamate to determine the maximum response, since it is able to stimulate calcium release independent of mGlu₅ activation. Surprisingly, 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 mGlu₅ compared to the MPEP-site PAM VU0092273 (Figure 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

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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. Since calcium mobilization and ERK1/2 phosphorylation can occur through different pathways, we tested the ability of NCFP to induce ERK1/2 phosphorylation using the N-terminal truncated mutant. In this case, FBS was utilized as a positive control as 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 to control (0.9 ± 0.04 fold/basal; Figure 5B), whereas VU0092273 induced a robust increase in ERK1/2 phosphorylation (2.1 ± 0.4 fold/basal; Figure 5B) that was similar to the level of phosphorylation observed with the wild-type receptor (Figure 1F). In addition to greatly reduced or no effects in functional assays, NCFP was no longer able to inhibit the binding of [3 H]methoxyPEPy to the N-terminal truncated receptor (2.2 ± 4.8 percent inhibition; Figure 5C). In contrast, MPEP retained the ability to completely inhibit [3 H]methoxyPEPy binding in a concentration dependent manner (Figure 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 responsible for the lack of [3 H]methoxyPEPy inhibition by NCFP. Additionally, VU0092273 also maintained the ability to completely inhibit [3 H]methoxyPEPy binding (Figure 5C). While 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

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To utilize mGlu₅ allosteric modulators in brain slice assays, it is important to determine if they are selective for mGlu₅ compared to other mGlu subtypes. We previously reported that CPPHA has weak PAM activity at mGlu₁ and weak NAM activity at mGlu₄ and mGlu₈ (Chen et al., 2008; O'Brien et al., 2004). Based on 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 μ M NCFP to determine if this compound could modulate the response to glutamate (or L-AP4 for mGlu₇). NCFP did not shift the concentration response curves for glutamate in cells expressing any of the mGlu receptor subtypes (Supplementary Figure 3). Thus, while NCFP and CPPHA behave in a very similar manner in potentiating responses to mGlu₅ activation, NCFP is more selective for mGlu₅ than 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 since glutamate will modulate multiple targets including other mGlu subtypes, ionotropic glutamate receptors and glutamate transporters. Also, 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 physiological effects of activation of group I mGlu_s. However, previous work has shown that some allosteric modulators can potentiate responses of other 7TMRs 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

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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, Supplementary Figure 4). Thus, NCFP does not exhibit probe-dependence for glutamate relative to DHPG, allowing this combination of allosteric and orthosteric ligands to be employed 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 subthalamic nucleus (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; Chen et al., 2007; Noetzel et al., 2012; O'Brien et al., 2004; Rodriguez et al., 2010; Rodriguez et al., 2005). In agreement with previous results, treatment of STN neurons with 100 μM DHPG resulted in a robust membrane depolarization (Figure 6; 10.7 ± 1.3 mV), whereas 1 μM DHPG induced a small depolarization that was just above threshold for detection (Figure 6; 2.4 ± 0.8 mV). Treatment of midbrain slices with 10 μM NCFP alone had no effect on STN membrane voltage (Figure 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 (Figure 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.

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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 (Auerbach et al., 2011; Ayala et al., 2009; Noetzel et al., 2012; Popkirov and Manahan-Vaughan, 2011). In addition, selective mGlu₅ PAMs potentiate induction of LTP at this synapse in response to a weak, threshold theta burst stimulation (TBS) protocol (Ayala et al., 2009). To assess the effects of mGlu₅ PAMs on DHPG-induced LTD, field excitatory postsynaptic potentials (fEPSPs) were recorded from the dendritic layer of CA1 following stimulation of the Schaffer collaterals. In agreement with our previous studies (Noetzel et al., 2012), 75 μ M DHPG induced robust LTD at the hippocampal SC-CA1 synapse measured 55 min after washout of the compound (Figure 7A,B; 52.2 ± 3.9 % of baseline fEPSP slope). In contrast, a lower concentration of DHPG (25 μ M) induced only slight depression of fEPSPs at this synapse (Figure 7A,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 (Auerbach et al., 2011; Ayala et al., 2009; Noetzel et al., 2012; Popkirov and Manahan-Vaughan, 2011). 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 μ M VU0092273 significantly potentiated the response to 25 μ M DHPG, inducing LTD that lasted more than 55 min after washout of the compound (Figure 7B; VU0092273 51.6 ± 5.8 % baseline, $p < 0.05$). In contrast, NCFP (10 μ M) did not induce a significant potentiation of the LTD response to 25 μ M DHPG (Figure 7A,B; NCFP 79.2 ± 6.0 % baseline). Interestingly there was a small effect observed with CPPHA (70.9 ± 4.7 % of baseline). However, mGlu₁ 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 mGlu₁ 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 following stimulation of the Schaffer collaterals. In agreement with previous results from our lab (Ayala et al., 2009), maximal LTP

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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 to baseline, when measured 35 min after stimulation (Figure 8B). In order to study the ability of mGlu₅ PAMs to potentiate LTP, a threshold TBS protocol was employed to induce submaximal potentiation (Ayala et al 2009). Using this protocol, a single train of lower frequency bursts resulted in a slight potentiation of the fEPSP slope (Figure 8A,B; 118.5 ± 4.1 % of baseline). Treatment of hippocampal slices with 10 μ M NCFP for 20 min, followed by threshold TBS, had no effect on fEPSP slope compared to threshold TBS alone (Figure 8A,B; NCFP 112.8 ± 2.5 % of baseline). Similar results were observed with CPPHA (112.8 ± 1.8 % of baseline). In contrast, 1 μ M VU0092273 for 20 min, followed by threshold TBS, resulted in a significant increase in the fEPSP slope (Figure 8A,B; 134.9 ± 6.8 % of baseline, $p < 0.05$). It should be noted that, in a subset of slices, there was an increase in fEPSP slope with VU0092273 alone, although this did not appear to affect the level of potentiation. Also, 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.

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Discussion

Preclinical studies suggest that mGlu₅ are viable candidates as drug targets for treatment of a variety of CNS disorders. Much of the current research around mGlu₅ is focused on the development of allosteric modulators due to 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 (Gregory et al., 2012; Niswender et al., 2010; Sheffler and Conn, 2008), a phenomenon referred to as functional selectivity or stimulus bias. We found that a novel mGlu₅ PAM, NCFP, potentiates multiple responses to mGlu₅ activation but fails to enhance hippocampal synaptic plasticity. These data provide a striking example of the potential impact of biased signaling on physiological responses that have been postulated to be critical for the cognition-enhancing effects of mGlu₅ PAMs.

Allosteric modulators have now been identified for each of the eight mGlu subtypes, and selective PAMs for mGlu₂ and mGlu₄ are now progressing rapidly for clinical testing in schizophrenia and Parkinson's patients respectively (for review, (Gregory et al., 2011)). The potential impact of modulators with stimulus bias with respect to in vivo and/or clinical efficacy has yet to be realized; however, understanding this could ultimately prove to be critical in optimizing allosteric modulators as therapeutic agents. Conceivably, neglect to recognize biased effects on specific responses to 7TMR activation could lead to an unexpected failure to observe the predicted in vivo or clinical effects. Consider the lack of efficacy exhibited by NCFP to

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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 therapeutic effects. Importantly, 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 (Chen et al., 2007; Noetzel et al., 2012; O'Brien et al., 2004; Rodriguez et al., 2005). 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. Additionally, mGlu₅ has been shown to mediate effects in other brain regions such the striatum where it has been shown to modulate NMDA receptor responses in medium spiny neurons (Pisani et al., 2001) and activation of mGlu_{1/5} 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 since 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 physiological 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 findings). 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 physiological responses to mGlu₅ activation. Provision of such tools will be key in elucidating

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whether biased modulation is a desirable trait for a particular therapeutic outcome, or alternatively, is 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 CPPHA, 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 utilized 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. While 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., 2009; Sharma et al., 2008; Sheffler et al., 2012; Wood et al., 2011). 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, due to a lack of in depth assessment of their physiological 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

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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; Chen et al., 2007) and is replicated here with the previously characterized MPEP site mGlu₅ PAM VU0092273. 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. Interestingly, CPPHA has been shown to act as an agonist in activating inositol phosphate production via N-terminal truncated mGlu₅ (Chen et al., 2008), suggesting that this does not entirely reflect differences between ligands interacting with the MPEP site versus the CPPHA site. In the absence of a radioligand to assess binding of NCFP to mGlu₅, it is impossible to determine whether this reflects a loss of affinity of the N-terminal truncated receptor for NCFP or a difference in the mechanism by which NCFP regulates receptor function.

In summary, we have demonstrated that NCFP is a potent and selective mGlu₅ PAM that interacts with the mGlu₅ receptor in a manner similar to the non-MPEP site ligand, CPPHA. While NCFP potentiates multiple responses to mGlu₅ activation in both recombinant and native systems, this novel mGlu₅ PAM does not have similar effects to previously described mGlu₅ PAMs in enhancing hippocampal LTD and LTP. These results suggest that different mGlu₅ allosteric modulators may behave similarly in multiple assays used to assess their effects on mGlu₅ function but could have fundamentally different effects in specific circuits in the CNS. This functional selectivity could complicate drug discovery efforts but could also be exploited to develop functionally selective ligands that can be used to tailor drug therapies.

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Authorship contributions

Participated in research design: Noetzel, Gregory, Vinson, Niswender, Xiang, Conn

Conducted experiments: Noetzel, Gregory, Vinson

Contributed new reagents: Manka, Stauffer, Lindsley

Performed data analysis: Noetzel, Gregory, Vinson

Wrote or contributed to the writing of the manuscript: Noetzel, Niswender, Conn

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References

- Auerbach BD, Osterweil EK and Bear MF (2011) Mutations causing syndromic autism define an axis of synaptic pathophysiology. *Nature* **480**(7375): 63-68.
- Awad H, Hubert GW, Smith Y, Levey AI and Conn PJ (2000) Activation of metabotropic glutamate receptor 5 has direct excitatory effects and potentiates NMDA receptor currents in neurons of the subthalamic nucleus. *J Neurosci* **20**(21): 7871-7879.
- Ayala JE, Chen Y, Banko JL, Sheffler DJ, Williams R, Telk AN, Watson NL, Xiang Z, Zhang Y, Jones PJ, Lindsley CW, Olive MF and Conn PJ (2009) mGluR5 positive allosteric modulators facilitate both hippocampal LTP and LTD and enhance spatial learning. *Neuropsychopharmacology* **34**(9): 2057-2071.
- Bradley SJ, Langmead CJ, Watson JM and Challiss RA (2011) Quantitative analysis reveals multiple mechanisms of allosteric modulation of the mGlu5 receptor in rat astroglia. *Mol Pharmacol* **79**(5): 874-885.
- Chen Y, Goudet C, Pin JP and Conn PJ (2008) N-{4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide (CPPHA) acts through a novel site as a positive allosteric modulator of group 1 metabotropic glutamate receptors. *Mol Pharmacol* **73**(3): 909-918.
- Chen Y, Nong Y, Goudet C, Hemstapat K, de Paulis T, Pin JP and Conn PJ (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**(5): 1389-1398.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**(23): 3099-3108.

MOL #82891

- Conn PJ, Christopoulos A and Lindsley CW (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov* **8**(1): 41-54.
- Fazio F, Lionetto L, Molinaro G, Bertrand HO, Acher F, Ngomba RT, Notartomaso S, Curini M, Rosati O, Scarselli P, Di Marco R, Battaglia G, Bruno V, Simmaco M, Pin JP, Nicoletti F and Goudet C (2012) Cinnabarinic acid, an endogenous metabolite of the kynurenine pathway, activates type 4 metabotropic glutamate receptors. *Mol Pharmacol* **81**(5): 643-656.
- Goudet C, Gaven F, Kniazeff J, Vol C, Liu J, Cohen-Gonsaud M, Acher F, Prezeau L and Pin JP (2004) Heptahelical domain of metabotropic glutamate receptor 5 behaves like rhodopsin-like receptors. *Proc Natl Acad Sci U S A* **101**(1): 378-383.
- Gregory KJ, Dong EN, Meiler J and Conn PJ (2011) Allosteric modulation of metabotropic glutamate receptors: structural insights and therapeutic potential. *Neuropharmacology* **60**(1): 66-81.
- Gregory KJ, Noetzel MJ, Rook JM, Vinson PN, Stauffer SR, Rodriguez AL, Emmitte KA, Zhou Y, Chun AC, Felts AS, Chauder BA, Lindsley CW, Niswender CM and Conn PJ (2012) Investigating mGlu5 Allosteric Modulator Cooperativity, Affinity and Agonism: Enriching Structure-function Studies and Structure-activity Relationships. *Mol Pharmacol*.
- Hammond AS, Rodriguez AL, Townsend SD, Niswender CM, Gregory KJ, Lindsley CW and Conn PJ (2010) Discovery of a Novel Chemical Class of mGlu(5) Allosteric Ligands with Distinct Modes of Pharmacology. *ACS Chem Neurosci* **1**(10): 702-716.
- Jung KM, Sepers M, Henstridge CM, Lassalle O, Neuhofer D, Martin H, Ginger M, Frick A, DiPatrizio NV, Mackie K, Katona I, Piomelli D and Manzoni OJ (2012) Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome. *Nature communications* **3**: 1080.
- Keov P, Sexton PM and Christopoulos A (2011) Allosteric modulation of G protein-coupled receptors: a pharmacological perspective. *Neuropharmacology* **60**(1): 24-35.

MOL #82891

- Kinney GG, O'Brien JA, Lemaire W, Burno M, Bickel DJ, Clements MK, Chen TB, Wisnoski DD, Lindsley CW, Tiller PR, Smith S, Jacobson MA, Sur C, Duggan ME, Pettibone DJ, Conn PJ and Williams DL, 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**(1): 199-206.
- Lamb JP, Engers DW, Niswender CM, Rodriguez AL, Venable DF, Conn PJ and Lindsley CW (2011) Discovery of molecular switches within the ADX-47273 mGlu5 PAM scaffold that modulate modes of pharmacology to afford potent mGlu5 NAMs, PAMs and partial antagonists. *Bioorg Med Chem Lett* **21**(9): 2711-2714.
- Lazareno S and Birdsall NJ (1995) Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol Pharmacol* **48**(2): 362-378.
- Leach K, Sexton PM and Christopoulos A (2007) Allosteric GPCR modulators: taking advantage of permissive receptor pharmacology. *Trends Pharmacol Sci* **28**(8): 382-389.
- Liu F, Grauer S, Kelley C, Navarra R, Graf R, Zhang G, Atkinson PJ, Popiolek M, Wantuch C, Khawaja X, Smith D, Olsen M, Kouranova E, Lai M, Pruthi F, Pulicicchio C, Day M, Gilbert A, Pausch MH, Brandon NJ, Beyer CE, Comery TA, Logue S, Rosenzweig-Lipson S and Marquis KL (2008) ADX47273 [S-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone]: a novel metabotropic glutamate receptor 5-selective positive allosteric modulator with preclinical antipsychotic-like and procognitive activities. *J Pharmacol Exp Ther* **327**(3): 827-839.
- Niswender CM and Conn PJ (2010) Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu Rev Pharmacol Toxicol* **50**: 295-322.
- Niswender CM, Johnson KA, Luo Q, Ayala JE, Kim C, Conn PJ and Weaver CD (2008) A novel assay of Gi/o-linked G protein-coupled receptor coupling to potassium channels provides

MOL #82891

new insights into the pharmacology of the group III metabotropic glutamate receptors.

Mol Pharmacol **73**(4): 1213-1224.

Niswender CM, Johnson KA, Miller NR, Ayala JE, Luo Q, Williams R, Saleh S, Orton D, Weaver CD and Conn PJ (2010) Context-dependent pharmacology exhibited by negative allosteric modulators of metabotropic glutamate receptor 7. *Mol Pharmacol* **77**(3): 459-468.

Noetzel MJ, Rook JM, Vinson PN, Cho HP, Days E, Zhou Y, Rodriguez AL, Lavreysen H, Stauffer SR, Niswender CM, Xiang Z, Daniels JS, Jones CK, Lindsley CW, Weaver CD and Conn PJ (2012) Functional impact of allosteric agonist activity of selective positive allosteric modulators of metabotropic glutamate receptor subtype 5 in regulating central nervous system function. *Mol Pharmacol* **81**(2): 120-133.

O'Brien JA, Lemaire W, Wittmann M, Jacobson MA, Ha SN, Wisnoski DD, Lindsley CW, Schaffhauser HJ, Rowe B, Sur C, Duggan ME, Pettibone DJ, Conn PJ and Williams DL, 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**(2): 568-577.

Packiarajan M, Mazza Ferreira CG, Hong SP, White AD, Chandrasena G, Pu X, Brodbeck RM and Robichaud AJ (2012) N-Aryl pyrrolidinonyl oxadiazoles as potent mGluR5 positive allosteric modulators. *Bioorg Med Chem Lett* **22**(17): 5658-5662.

Peavy RD and Conn PJ (1998) Phosphorylation of mitogen-activated protein kinase in cultured rat cortical glia by stimulation of metabotropic glutamate receptors. *J Neurochem* **71**(2): 603-612.

Pisani A, Gubellini P, Bonsi P, Conquet F, Picconi B, Centonze D, Bernardi G and Calabresi P (2001) Metabotropic glutamate receptor 5 mediates the potentiation of N-methyl-D-aspartate responses in medium spiny striatal neurons. *Neuroscience* **106**(3): 579-587.

MOL #82891

- Popkirov SG and Manahan-Vaughan D (2011) Involvement of the metabotropic glutamate receptor mGluR5 in NMDA receptor-dependent, learning-facilitated long-term depression in CA1 synapses. *Cereb Cortex* **21**(3): 501-509.
- Rodriguez AL, Grier MD, Jones CK, Herman EJ, Kane AS, Smith RL, Williams R, Zhou Y, Marlo JE, Days EL, Blatt TN, Jadhav S, Menon UN, Vinson PN, Rook JM, Stauffer SR, Niswender CM, Lindsley CW, Weaver CD and Conn PJ (2010) Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and antipsychotic activity. *Mol Pharmacol* **78**(6): 1105-1123.
- Rodriguez AL, Nong Y, Sekaran NK, Alagille D, Tamagnan GD and Conn PJ (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**(6): 1793-1802.
- Sharma S, Kedrowski J, Rook JM, Smith RL, Jones CK, Rodriguez AL, Conn PJ and Lindsley CW (2009) Discovery of molecular switches that modulate modes of metabotropic glutamate receptor subtype 5 (mGlu5) pharmacology in vitro and in vivo within a series of functionalized, regioisomeric 2- and 5-(phenylethynyl)pyrimidines. *J Med Chem* **52**(14): 4103-4106.
- Sharma S, Rodriguez AL, Conn PJ and Lindsley CW (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**(14): 4098-4101.
- Sheffler DJ and Conn PJ (2008) Allosteric potentiators of metabotropic glutamate receptor subtype 1a differentially modulate independent signaling pathways in baby hamster kidney cells. *Neuropharmacology* **55**(4): 419-427.

MOL #82891

- Sheffler DJ, Wenthur CJ, Bruner JA, Carrington SJ, Vinson PN, Gogi KK, Blobaum AL, Morrison RD, Vamos M, Cosford ND, Stauffer SR, Daniels JS, Niswender CM, Conn PJ and Lindsley CW (2012) Development of a novel, CNS-penetrant, metabotropic glutamate receptor 3 (mGlu3) NAM probe (ML289) derived from a closely related mGlu5 PAM. *Bioorg Med Chem Lett* **22**(12): 3921-3925.
- Stauffer SR (2011) Progress toward Positive Allosteric Modulators of the Metabotropic Glutamate Receptor Subtype 5 (mGlu(5)). *ACS Chem Neurosci* **2**(8): 450-470.
- Varnes JG, Marcus AP, Mauger RC, Throner SR, Hoesch V, King MM, Wang X, Sygowski LA, Spear N, Gadiant R, Brown DG and Campbell JB (2011) Discovery of novel positive allosteric modulators of the metabotropic glutamate receptor 5 (mGlu5). *Bioorg Med Chem Lett* **21**(5): 1402-1406.
- Vinson PN and Conn PJ (2012) Metabotropic glutamate receptors as therapeutic targets for schizophrenia. *Neuropharmacology*.
- Wood MR, Hopkins CR, Brogan JT, Conn PJ and Lindsley CW (2011) "Molecular switches" on mGluR allosteric ligands that modulate modes of pharmacology. *Biochemistry* **50**(13): 2403-2410.
- Zhang Y, Rodriguez AL and Conn PJ (2005) Allosteric potentiators of metabotropic glutamate receptor subtype 5 have differential effects on different signaling pathways in cortical astrocytes. *J Pharmacol Exp Ther* **315**(3): 1212-1219.
- Zhao Z, Wisnoski DD, O'Brien JA, Lemaire W, Williams DL, Jr., Jacobson MA, Wittman M, Ha SN, Schaffhauser H, Sur C, Pettibone DJ, Duggan ME, Conn PJ, Hartman GD and Lindsley CW (2007) Challenges in the development of mGluR5 positive allosteric modulators: the discovery of CPPHA. *Bioorg Med Chem Lett* **17**(5): 1386-1391.

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Footnotes

a) This work is supported by the National Institute of Mental Health [Grant 2R01 MH062646-13]; National Institute of Neurological Disorders and Stroke [Grant 2R01NS031373-16A2]; National Institute of Drug Abuse [Grant 1R01DA023947]; Molecular Libraries Probe Production Centers Network [Grant 5 u54 MH84659-03, 5 u54 MH84659-03S1]; National Research Service Award from National Institute of Neurological Disorders and Stroke [MJN: F32 NS071746]; NARSAD Young Investigator Award 2011 (KJG); American Australian Association Merck Foundation Fellowship 2010 (KJG); National Health and Medical Research Council (Australia) Overseas Biomedical Postdoctoral Training Fellowship (KJG). The content is solely the responsibility of the authors and does not necessarily represent the official view of the organizations listed above.

b) The work was presented in abstract form:

Noetzel, MJ, K.J. Gregory, P.N. Vinson, J. Manka, S.R. Stauffer, Z. Xiang, J.S. Daniels C.M. Niswender, C.W. Lindsley, and P.J. Conn. (October 2012) "Characterization of a potent and efficacious metabotropic glutamate receptor 5 positive allosteric modulator from the CPPHA series." *2012 Neuroscience Meeting Planner*. Society for Neuroscience 2012. New Orleans, LA.

c) Reprint Requests should be addressed to Prof. P. Jeffrey Conn, Department of Pharmacology & Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University Medical Center, 1215 Light Hall, 2215-B Garland Ave, Nashville, TN, USA, 37232-0697
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Figure captions

Figure 1. NCFP potentiates the response to glutamate in a manner similar to 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 sec prior to the addition of a concentration of glutamate eliciting a 20% maximal response (EC₂₀; 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 utilized. (EC₂₀; 600-650 nM) D,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 ± SEM of 3-4 independent experiments performed in duplicate.

Figure 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 one hr prior to termination. Non-specific binding was determined using 10 μM MPEP. Data represent the mean ± SEM of 3 individual experiments conducted in duplicate.

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Figure 3. NCFP activity is blocked by a mutation in the CPPHA site, but not affected by an MPEP site ligand. A) Cells expressing the F585I mutant mGlu₅ receptor were treated with a fixed concentration (3 μ M) of NCFP (open triangles) or CPPHA (black triangles) followed by the addition of increasing concentrations of glutamate. B) mGlu₅ cells were first treated with fixed concentrations of 5MPEP (3 μ M black triangles, 10 μ M black circles or 30 μ M black diamonds), then increasing concentrations of NCFP were added, and finally an EC₂₀ concentration of glutamate was applied. In all cases, the calcium response was normalized to the response induced by a maximally effective concentration of glutamate (10 μ M). Data represent the mean \pm SEM of 4 independent experiments performed in duplicate.

Figure 4. NCFP inhibits [³H]methoxyPEPy binding in a manner suggestive of a noncompetitive interaction. A) Cell membranes from HEK293A cells expressing a low level of mGlu₅ were treated with increasing concentrations of CPPHA (black squares), NCFP (open triangles) or MPEP (black circles) and 2 nM [³H]methoxyPEPy. Reactions were allowed to incubate for one hr prior to termination. Non-specific binding was determined using 10 μ M MPEP. B) F585I cell membranes were treated the same as in A. Data represent the mean \pm SEM of 3-7 independent experiments performed in duplicate or triplicate.

Figure 5. NCFP acts differently at the N-terminal truncated receptor compared to MPEP-site ligands. A) Cells expressing the N-terminal truncated mGlu₅ receptor were treated with increasing concentrations of NCFP (open triangles) or VU0092273 (black diamonds). The calcium response was normalized to the response induced by a maximally effective concentration of ionomycin (1 μ M). B) The level of ERK1/2 phosphorylation (fold/basal) was determined by treating cells with a fixed concentration of mGlu₅ compound (3 μ M; NCFP open triangles, VU0092273 black diamonds), FBS (10%, black triangles) or control (black squares) for the times indicated. C) Cell membranes were treated with increasing concentrations of

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NCFP (open triangles), VU0092273 (black diamonds) or MPEP (black circles) and 2 nM [³H]methoxyPEPy. Reactions were allowed to incubate for one hr prior to termination. Non-specific binding was determined using 10 μ M MPEP. Data represent the mean \pm SEM of 3-6 independent experiments conducted in duplicate.

Figure 6. NCFP potentiates the DHPG-induced change in membrane voltage in

subthalamic nucleus neurons. Bath application of 100 μ M DHPG induced a robust change in membrane voltage, whereas application of 1 μ M DHPG induced a small change in membrane voltage. Application of 10 μ M NCFP for 10 min had no effect on membrane voltage alone. Co-application of 10 μ M NCFP and 1 μ M DHPG resulted in a significant enhancement in the change in membrane voltage compared to 1 μ M DHPG alone. Data represent the mean \pm SEM for 5 individual experiments for each treatment. * denotes $p < 0.5$ when compared to 1 μ M DHPG. Sample traces from individual experiments are presented on the right.

Figure 7. NCFP does not significantly enhance DHPG-induced -LTD at the Schaffer

collateral-CA1 synapse in hippocampus. A stimulus intensity that produced 50-60 percent of the maximal fEPSP response was used as the baseline response and was determined for each individual experiment. Insets are sample fEPSP traces measured pre-drug (black) and 55 min after drug washout (grey). A) Bath application of 75 μ M DHPG for 10 min (open circle, solid line) resulted in LTD of the fEPSP slope ($n=8$). In contrast, bath application of 25 μ M DHPG for 10 min (black circles, solid line) resulted in a slight decrease in fEPSP slope 55 min after compound washout ($n=9$). Application of 10 μ M NCFP for 10 min (dashed line) first alone and then in combination with 25 μ M DHPG (solid line) for 10 min (grey circles), resulted in no significant change in fEPSP slope ($n=7$). B) Quantification of the change in fEPSP slope measured 55 min after compound washout. Error bars represent SEM. * denotes $p < 0.5$ when compared to control (25 μ M DHPG).

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Figure 8. NCFP has no effect on potentiation of threshold TBS LTP at the Schaffer

collateral-CA1 synapse in hippocampus. A stimulus intensity that produced 40-50 percent of the maximal fEPSP response was used as the baseline response and was determined for each individual experiment. Insets are sample fEPSP traces measured pre-drug (black) and 35 min after TBS stimulation for LTP (grey). A) Threshold TBS (black circles) resulted in a small increase in fEPSP slope measured 35 min after stimulation (n=11). Bath application of 10 μ M NCFP for 20 min (solid line) followed by threshold TBS (light grey circles) resulted in no change in fEPSP slope from threshold TBS alone (n=8). In contrast, bath application of 1 μ M VU0092273 for 20 min (solid line) followed by threshold TBS (dark grey circles), resulted in a significant enhancement of fEPSP slope measured 35 min after stimulation (n=9). B) Quantification of the change in fEPSP slope measured 35 min after TBS stimulation. Error bars represent SEM. * denotes $p < 0.5$ when compared to control (TBS threshold).

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Table 1: Expression level and potency values for mGlu₅ expressing cell lines. Data

represent the mean \pm SEM from a minimum of 3 individual experiments conducted in duplicate or triplicate.

Cell line	Expression level ^a	pEC ₅₀ ^b	
	(pmol/mg protein)	NCFP	CPPHA
Wild-type-mGlu ₅ (high expression)	2.3 \pm 0.4 ^c	6.69 \pm 0.09 (214 nM)	6.44 \pm 0.04 (370 nM)
Wild-type-mGlu ₅ (low expression)	0.6 \pm 0.1 ^d	6.67 \pm 0.10 (225nM)	6.16 \pm 0.01 (689nM)
F585I-mGlu ₅	1.8 \pm 0.6	ND	ND
Rat cortical astrocytes	3.4 \pm 0.3 ^{ce} fmol/10 ⁵ cells	6.04 \pm 0.10 (969 nM)	6.33 \pm 0.28 (470 nM)

^a receptor expression level determined by [³H]methoxyPEPy binding

^b negative logarithm of the concentration of modulator that resulted in half-maximal potentiation of an EC₂₀ concentration of glutamate. Values below are conversion to molar concentration

^c expression level previously reported in Noetzel et al 2012

^d expression level previously reported in Gregory et al 2012

^e expression level reported as fmol/10⁵ cells due to methodological difficulties in membrane binding

ND values not determined

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Table 2: Operational model parameters and fold shift values for NCFP and CPPHA modulation of intracellular Ca^{2+} mobilization. Data represent the mean \pm SEM from a minimum of 3 individual experiments conducted in duplicate.

	Wild-type-mGlu ₅		F585I-mGlu ₅	
	NCFP	CPPHA	NCFP	CPPHA
pK_B^a	5.56 ± 0.13	6.09 ± 0.09^d	ND	ND
$\text{Log}\beta^b$	0.99 ± 0.05	0.59 ± 0.08^d	ND	ND
Fold shift (max) ^c	7.7 ± 0.1	5.9 ± 1.6	ND	ND
Fold shift (3 μM) ^c	5.9 ± 0.1	4.0 ± 0.7	$1.6 \pm 0.1^*$	$1.3 \pm 0.1^*$

^a negative logarithm of the allosteric modulator dissociation constant

^b negative logarithm of the efficacy cooperativity factor

^c fold shift values were determined for the maximum fold shift determined for multiple concentrations or at a single (3 μM) concentration

^d operational model parameters were previously reported in Gregory et al., 2012

ND values not determined

* denotes significantly different ($p < 0.05$) to wild-type value

Figure 1

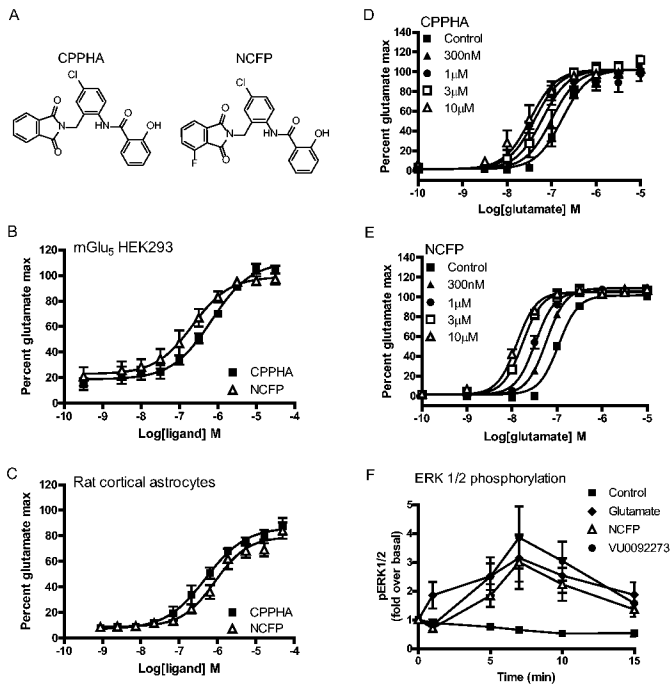


Figure 2

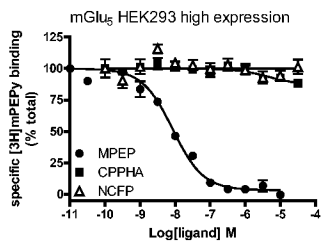
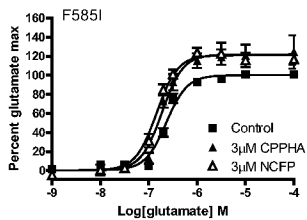


Figure 3

A



B

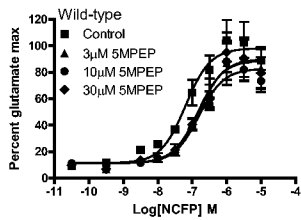
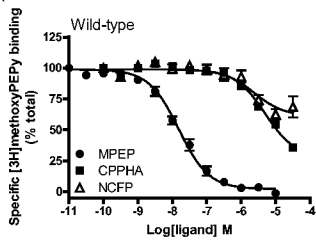


Figure 4

A



B

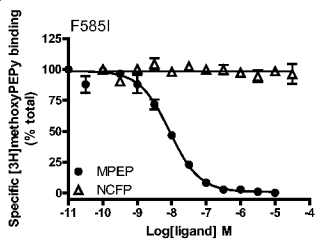


Figure 5

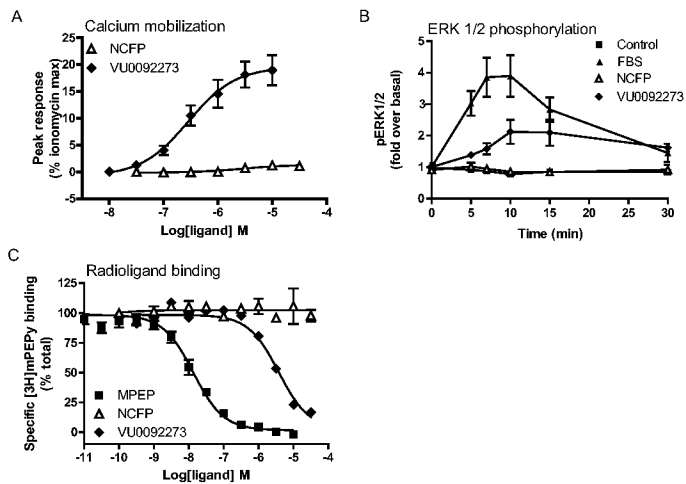


Figure 6

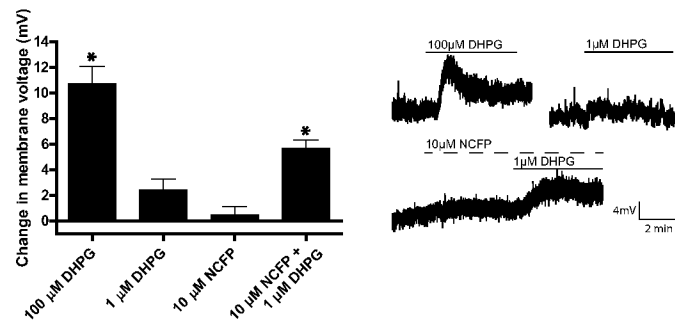
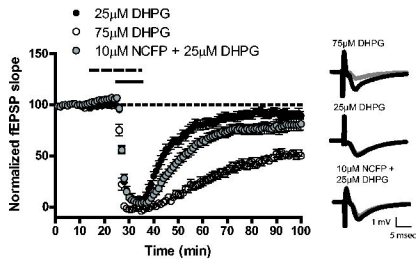


Figure 7

A



B

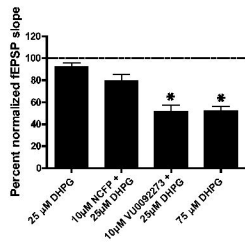
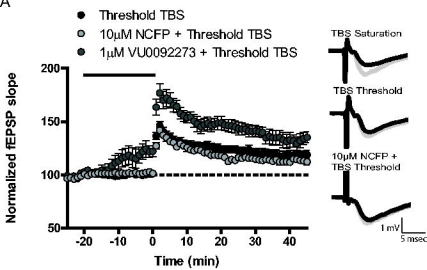


Figure 8

A



B

