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

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Abstract

Modulators of metabotropic glutamate receptor subtype 5 (mGluR5) may provide novel treatments for multiple central nervous system (CNS) disorders, including anxiety and schizophrenia. Although compounds have been developed to better understand the physiological roles of mGluR5 and potential usefulness for the treatment of these disorders, there are limitations in the tools available, including poor selectivity, low potency, and limited solubility. To address these issues, we developed an innovative assay that allows simultaneous screening for mGluR5 agonists, antagonists, and potentiaters. We identified multiple scaffolds that possess diverse modes of activity at mGluR5, including both positive and negative allosteric modulators (PAMs and NAMs, respectively). 3-Fluoro-5-(3-(pyridine-2-yl)-1,2,4-oxadiazol-5-yl)(4-phenylethynyl)phenyl)methanone (VU0092273) was identified as a novel mGluR5 PAM that also binds to the MPEP site. VU0092273 was chemically optimized to an orally active analog, N-cyclobutyl-6-((3-fluorophenyl)ethynyl)nicotinamide hydrochloride (VU0360172), which is selective for mGluR5. This novel mGluR5 PAM produced a dose-dependent reversal of amphetamine-induced hyperlocomotor activity. 67207/3650626

Abbreviations: CNS, central nervous system; mGluR, metabotropic glutamate receptor; PAM, positive allosteric modulator; ESI, electrospray ionization; NAM, negative allosteric modulator; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MPEP, 2-methyl-6-(phenylethynyl)pyridine

Introduction

Glutamate, the major excitatory neurotransmitter in the central nervous system (CNS), exerts its effects through the activation of both glutamate-gated cation channels and eight distinct subtypes of G protein-coupled metabotropic glutamate receptors (mGluRs), termed mGluR1 to mGluR8 (Niswender and Conn, 2010). Previous studies suggest that selective agonists and antagonists of the mGluR5 subtype could be beneficial in the treatment of a number of CNS disorders (Gasparini et al., 2008; Conn et al., 2009a). For instance, a large number of preclinical studies and preliminary reports of clinical studies suggest that mGluR5 antagonists may be effective in the treatment of anxiety disorders (Porter et al., 2005; Swanson et al., 2005), Parkinson’s disease (Marino and Conn, 2002a; Marino et al., 2002), and Fragile X syndrome (Bear et al., 2004; Gasparini et al., 2008).
In addition to mGluR5 antagonists, selective activators of mGluR5 have been proposed as having potential usefulness in the treatment of schizophrenia and disorders of cognitive function (Marino and Conn, 2002b; Kinney et al., 2003; Moghaddam, 2004; Conn et al., 2009b). This is largely based on a number of investigations suggesting that mGluR5 is a closely associated signaling partner with the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptor that plays a key role in regulating NMDA receptor function in a variety of forebrain regions (Marino and Conn, 2002b; Lindsley et al., 2006). Based on this and a large number of cellular studies suggesting that activation of mGluR5 and NMDA receptors could have robust effects in forebrain circuits believed to be disrupted in schizophrenia, it has been postulated that activators of mGluR5 could provide novel therapeutic agents that may be useful for treating this disorder (Marino and Conn, 2002b; Moghaddam, 2004; Conn et al., 2009b).

A major breakthrough in the area of mGluR5 biology came with the discovery of highly selective allosteric activators of mGluR5, including 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and related compounds (Gasparini et al., 1999, 2002). These compounds do not interact with the orthosteric glutamate binding site but bind to an allosteric site in the seven transmembrane-spanning domain of mGluR5 to inhibit coupling of the receptor to GTP binding proteins (Knoflach et al., 2001). These mGluR5-selective negative allosteric modulators (NAMs) have had a major effect on our understanding of the physiological roles of this receptor and have allowed studies that suggest that antagonists of mGluR5 have potential as novel therapeutic agents. We have reported discovery of compounds that act as highly selective positive allosteric modulators (PAMs) of mGluR5 (O’Brien et al., 2003, 2004; Lindsley et al., 2004, 2005; Conn et al., 2009b; Engers et al., 2009). These compounds have no agonist activity by themselves but act at an allosteric site to potentiate glutamate-induced activation of mGluR5 in transfected cell lines. Two of these compounds, termed CDPPB and S-(4-fluoro-phenyl)-[3-[3-(4-fluoro-phenyl)-1,2,4-oxadiazol-5-yl]-piperidin-1-yl]-methanone (ADX-47273), display efficacy in animal models commonly used to test for potential antipsychotic-like activity and cognition-enhancing effects of novel compounds but have limited use when administered systemically (Lindsley et al., 2004; Kinney et al., 2005; Liu et al., 2008; Ayala et al., 2009; Gass and Olive, 2009).

Although progress in the discovery of novel mGluR5-selective PAMs and NAMs has been exciting, there are a number of limitations to the currently available mGluR5 modulators. For instance, prototypical mGluR5 NAMs such as MPEP and MTEP are inverse agonists (Gasparini et al., 1999; Roppe et al., 2004; Lea and Faden, 2006) and have disruptive effects on cognitive function and effects in animal models that predict potential psychotomimetic activity (Porter et al., 2005; Rodriguez et al., 2005). Furthermore, CDPPB and other mGluR5 PAMs such as ADX-47273 have limited aqueous solubility and relatively low potencies at the receptor (de Paulis et al., 2006; Liu et al., 2008; Engers et al., 2009). These deficiencies do not allow for full exploration of the functional effects of these compounds or sufficient dosing required for more extensive behavioral studies. To address these issues, we developed and used an innovative high-throughput screening assay that allows simultaneous identification of mGluR5 agonists, antagonists, and potentiators. We now report the discovery of multiple novel scaffolds possessing diverse activities as mGluR5 allosteric modulators. These novel molecules reveal a rich diversity of mGluR5 modulators, in terms of diverse chemical scaffolds, distinct modes of efficacy, and interactions with multiple allosteric sites. Furthermore, these novel compounds are active in vivo and show that some of the liabilities associated with previous mGluR5 modulators are not intrinsic to the target but can be avoided with compounds that provide efficacy in animal models.

Materials and Methods

The Vanderbilt High-Throughput Screening Center compound collection was obtained from ChemBridge Corporation (San Diego, CA) and ChemDiv, Inc. (San Diego, CA) and stored in bar-coded, 384-well, U-bottomed, standard volume polypolyethylene plates (Corning Life Sciences, Lowell, NY). The plates were thermally sealed with peelable seals using a PlateLoc (Velocity 11, Santa Clara, CA). Groups of 10 plates were vacuum-packed in thermally sealed freezer bags (Food-Saver, Jarden Corp., Rye, NY) and stored frozen at –80°C. t-|Glutamate, DHPG, and MPEP were obtained from Tocris Bioscience (Ellisville, MO). [3H]MethoxyPEPy was obtained from Bioscience (Ellisville, MO).
from American Radiolabeled Chemicals, Inc. (St. Louis, MO). CDPPB (Lindsley et al., 2004), CPPHA (Zhao et al., 2007), MTEP (Cosford et al., 2003b), and 5MPEP (Rodriguez et al., 2005) were synthesized as described previously. NADPH was purchased from Sigma-Aldrich (St. Louis, MO). Rat liver microsomes (20 mg/ml protein) were purchased from BD Biosciences (Woburn, MA). All the solvents were of either analytical or HPLC grade.

Chemistry

Full experimental details for mGluR5 allosteric modulators are available in the Supplemental Data section.

5-(3,5-Dimethoxyphenyl)-3-(pyridine-2-y1)-1,2,4-oxadiazole (VU0040228). \( ^1H \) NMR (400 MHz, \( d_7 \)-MeOH) 8.77 (dd, \( J = 5.0, 1.0 \) Hz, 1H), 8.29 (d, \( J = 8.0, 1.0 \) Hz, 1H), 8.07 (td, \( J = 8.0, 1.5 \) Hz, 1H), 7.64 (d, \( J = 8.0, 1.0 \) Hz, 1H), 7.41 (d, \( J = 2.5 \) Hz, 1H), 6.82 (t, \( J = 2.5 \) Hz, 1H), 3.92 (s, 6H); liquid chromatography/mass spectrometry (214 nm) 1.45 min (>98%); MS (ESI) \( m/z = 284.1 \) (calculated 284.1035, \( C_8H_{14}O_2N_2 \); for 4-Hydroxypiperidin-1-yl(4-phenylethynyl)phenyl)methanone (VU0092273). Melting point, 157.7°C. \( ^1H \) NMR (400 MHz, CDCl3) 6.58 (d, \( J = 8.0 \) Hz, 2H), 5.66 to 7.52 (m, 2H), 7.44 to 7.34 (m, 5H), 4.21 to 4.08 (m, 1H), 4.03 to 3.96 (m, 1H), 3.81 to 3.48 (m, 1H), 3.47 to 3.16 (m, 2H), 2.08 to 1.79 (m, 3H), 1.71 to 1.42 (m, 2H); \( ^13C \) NMR (100 MHz, CDCl3) 6.169, 135.5, 131.6, 131.5, 128.5, 128.3, 126.9, 124.7, 122.8, 90.8, 88.5, 66.9, 43.8, 39.3, 34.4, 33.8; LC (214 nm) 2.91 min (>98%); MS (ESI) \( m/z = 284.1 \) (calculated 284.1035, \( C_{15}H_{14}N_3O_3 \); for 3-fluoro-5-(3,5-dimethoxyphenyl)-1,2,4-oxadiazole (VU0041106) Melting point, 157.7°C; \( ^1H \) NMR (400 MHz, CDCl3) 8.39 (dd, \( J = 2.5 \) Hz, 1H), 8.43 (s, 1H), 8.29 to 7.8 (m, 1H), 7.82 (t, \( J = 7.5 \) Hz, 1H), 6.82 (t, \( J = 7.5 \) Hz, 1H), 5.93 (s, 1H), 3.92 (s, 6H); liquid chromatography/mass spectrometry (214 nm) 2.86 min (>98%); MS (ESI) \( m/z = 306.1 \) (calculated 306.1496, \( C_{15}H_{14}NO_2F \); and for N-Cyclooctyl-6-(3-fluorophenyl)ethenyl)nicotinamide hydrochloride (VU0360172). \( ^1H \) NMR (400 MHz, DMSO) \( \delta = 0.94 \) (d, \( J = 1.2 \) Hz, 1H), 8.93 (d, \( J = 7.8 \) Hz, 1H), 8.27 (d, \( J = 7.8 \) Hz, 1H), 7.78 (d, \( J = 8.4 \) Hz, 1H), 7.57 to 7.49 (overlapped, 3H, 7.37 (m, 1H), 4.43 (m, 1H), 2.25 (m, 2H), 2.11 (m, 1H), 1.70 (m, 2H); \( ^13C \) NMR (100 MHz, DMSO) \( \delta = 163.08, 162.22 \) (d, \( J = 244.0 \) Hz, 1C), 148.82, 143.31, 136.89, 131.52 (d, \( J = 9.0 \) Hz, 1C), 129.72, 128.47 (d, \( J = 3.0 \) Hz, 1C), 127.86, 125.13 (d, \( J = 9.0 \) Hz, 1C), 120.35, 118.4 (d, \( J = 23.0 \) Hz, 1C), 117.64 (d, \( J = 21.0 \) Hz, 1C), 90.08 (d, \( J = 3.0 \) Hz, 1C), 89.01, 45.07, 30.38 (2C), 15.18, LC (214 nm) 1.35 min (>98%); MS (ESI) \( m/z = 295.10 \) (calculated 295.1246 (\( M^+ + 1 \)) + 100%); Cyclic H_2N_2O_2F; and for 5-(3,5-Dimethoxyphenyl)-3-(pyridine-2-y1)-1,2,4-oxadiazole (VU0040228).

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Putative hits from the primary screen were confirmed by testing for concentration-dependent activity on mGluR5 over a range of 4 log units. Compounds were serially diluted 1:3 into 10-point concentration-response curves (final concentration, 30 μM–1 nM), transferred to daughter plates using the Echo acoustic plate reformatter, and tested as described in the primary screen. Putative antagonists were applied for 300 s and followed by EC\(_{50}\) concentrations of glutamate while antagonists were applied for 300 s and followed by EC\(_{50}\) concentrations of glutamate. Concentration-response curves were generated using a four-parameter logistical equation in Xlfit curve fitting software (IDBS, Bridgewater, NJ) for Excel (Microsoft, Redmond, WA) or Prism (GraphPad Software, Inc., San Diego, CA).

Selectivity Studies

Muscarinic Response. HEK293 cells were plated as described above for mGluR5. Compounds were added 300 s before an EC\(_{50}\) or EC\(_{90}\) concentration of the muscarinic agonist carbachol. Raw data from the FDDS were imported into Microsoft Excel and analyzed as described above.

Rat mGluR1. Baby hamster kidney cells expressing rat mGluR1 were cultured as described previously (Hemstap et al., 2006). Calcium flux assays were used for counterscreening rat mGluR1 by measuring the glutamate concentration-response relationship in the presence and absence of a fixed concentration of test compound using a double-addition protocol adding test compound 2.5 min before varying concentrations of glutamate. mGluR1 cells were plated at 15 × 10\(^5\) cells/well in black-walled, tissue culture-treated, 384-well plates (Greiner Bio-One, Monroe, NC) in assay medium, and calcium assays were performed as described above.

Rat mGluRs 3 and 4. Compound activity at the rat group II and III mGluRs, mGluR3 and mGluR4, respectively, was assessed using thallium flux through G protein-coupled inwardly rectifying potassium channels, a method that has been described in detail in (Niswender et al., 2008a). These cell lines were grown in growth medium containing 45% DMEM, 45% Ham’s F-12, 10% FBS, 20 mM HEPES, 2 mM 1-glutamine, antibiotic/antimycotic, nonessential amino acids, 700 μg/ml G418, and 0.6 μg/ml puromycin at 37°C in the presence of 5% CO\(_2\). In brief, mGluR3 or 4 G protein-coupled inwardly rectifying potassium channels, a method that has been described in detail in (Niswender et al., 2008a). These cell lines were grown in growth medium containing 45% DMEM, 45% Ham’s F-12, 10% FBS, 20 mM HEPES, 2 mM 1-glutamine, antibiotic/antimycotic, nonessential amino acids, 700 μg/ml G418, and 0.6 μg/ml puromycin at 37°C in the presence of 5% CO\(_2\). The following day, the medium was removed from the cells, and 20 μl/well of 1 μM concentration of the indicator dye FluorO (Invitrogen, Carlsbad, CA) in assay buffer was added.
Cells were incubated for 1 h at room temperature, and the dye was replaced with 20 μl/well of assay buffer. For these assays, compounds were added at 2× final concentration, and then 2.5 min later, varying concentrations of glutatione were added using the FDSS 6000.

Glutatione was diluted in thallium buffer (125 mM sodium bicarbonate, 1 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, 12 mM thallium sulfate, and 10 mM HEPEs) at 5× the final concentration to be assayed. Data were analyzed as described previously (Niswender et al., 2008a).

Calcium Assays on the FlexStation. Follow-up experiments with VU02856683 were performed using the FlexStation II (Molecular Devices, Sunnyvale, CA) using the following protocols. HEK cells stably expressing rat mGluR5 were plated at 6 × 10⁴ cells per well in medium containing DMEM, 10% dialyzed FBS, 20 mM HEPEs, 1 mM sodium pyruvate, and antibiotic/antimycotic in clear-bottomed, black-walled, polystyrene-coated 96-well plates (BD BioCoat, Bedford, MA) 24 h before assay and were incubated overnight at 37°C in 5% CO². On the day of the assay, the medium was removed and replaced with Hanks’ balanced salt solution containing 20 mM HEPEs, 2.5 mM probenecid, and 2 μl Flu-o-4/acetoxymethyl ester dye, pH 7.4. Cells were incubated for 45 min (37°C, 5% CO²) for dye-loading. This medium was removed and replaced with 40 μl of calcium assay buffer (Hanks’ balanced salt solution, 20 mM HEPEs, and 2.5 mM probenecid, pH 7.4). For measurement of antagonist potency by calcium fluorescence, vehicle or concentration-response curves (CRCs) of antagonist made in calcium assay buffer were added (40 μl) at the 17-s time point, and an EC₅₀ concentration of glutamate (20 μM) was added at the 107-s time point via a FlexStation II (Molecular Devices). Fluorescence was monitored for a total of 137 s using an excitation wavelength of 488 nm, an emission wavelength of 525 nm, and a cutoff wavelength of 515 nm. For fold shift assays, vehicle or fixed concentrations of antagonist were added (40 μl), and a glutamate CRC (20 μM) was added via a FlexStation II. For 5MPEP assays, calcium assay buffer (20 μl) and 5MPEP (20 μl) were added by hand and allowed to incubate for 30 min before the standard calcium fluorescence assay protocol described above. All calcium response data were normalized to the EC₅₀ response to a glutamate. Data were transformed and fit with GraphPad Prism to determine EC₅₀ values.

Mutagenesis and Transient Transfection. HEK cells were plated on 100-mm cell culture dishes in media containing DMEM, 10% FBS, 20 mM HEPEs, and antibiotic/antimycotic. Cells were transfected with cDNA for rat wild-type mGluR5 or A590V mutant mGluR5. cDNA (5 μg) and PuGENE 6 (25 μl) were added to 970 μl of Opti-MEM (Invitrogen) and allowed to incubate at room temperature for 15 min. After incubation, the cDNA/PuGENE mixture was applied dropwise to HEK cells. Twenty-four hours later, cells were plated and assayed using the same protocol described above.

Radioligand Binding Assays. The allosteric antagonist MPEP analog [³H]methoxyPEPy was used to evaluate the ability of test compounds to interact with the MPEP site on mGluR5 (Cosford et al., 2003a). Membranes were prepared from rat mGluR5 HEK293 cells (Rodriguez et al., 2005). Membranes were diluted in assay buffer (50 mM Tris/0.9% NaCl, pH 7.4) to a 5× stock, and 100 μl of test compound was added to each well of a 96-well deep-well plate. Aliquots (300 μl) of membranes diluted in assay buffer (45 μg/well) were added to each well. [³H]methoxyPEPy (100 μl; final concentration, 2 nM) was added, and the reaction was incubated at room temperature for 1 h with shaking. After the incubation period, the membrane-bound ligand was separated from free ligand by filtration through glass-fiber 96-well filter plates (Unifilter-96 GF/B; PerkinElmer Life and Analytical Sciences, Waltham, MA). The contents of each well were transferred simultaneously to the filter plate and washed three to four times with assay buffer using a cell harvester (Brandel Inc., Gaithersburg, MD). Scintillation fluid (40 μl) was added to each well, and the membrane-bound radioactivity was determined by scintillation counting (TopCount; PerkinElmer Life and Analytical Sciences). Nonspecific binding was estimated using 5 μM MPEP. Concentration-response curves were generated using a four-parameter logistical equation in GraphPad Prism.

Plasma Protein Binding. Plasma protein binding assays on the test compounds were performed in a high-throughput mode. Plasma samples spiked with 5 μM concentration of the test compound in DMSO was added to the cis chambers of Rapid Equilibrium Dialysis plates (Thermo Fisher Scientific, Waltham, MA) and Dulbecco’s phosphate-buffered saline was added to the corresponding trans sides. The samples were dialyzed for 4 h at 37°C with shaking. The dialyzed samples from the buffer and plasma compartments were extracted by a protein precipitation method using ice-cold acetonitrile containing 0.1% formic acid and an internal standard (VU0366031) having a final concentration of 50 ng/ml.

The extracts from the plasma and buffer compartments were measured by means of HPLC/MS/MS using a Thermo Finnigan TSQ Quantum Ultra (Thermo Fisher Scientific) mass spectrometer in the positive-ion mode by selective reaction monitoring. The chromatographic separation was achieved on an Acquity ultra-performance liquid chromatography BEH C18 column (1.7 μm; 2.1 × 50 mm) at a flow rate of 0.8 ml/min. A gradient program was used with the mobile phase, combining solvent A (95.5:0.1% formic acid in water/acetonitrile) and solvent B (95.5 acetonitrile/0.1% formic acid in water) as follows: 20% solvent B up to 0.5 min, ramped from 20 to 100% solvent B by 1 min, and held at 100% solvent B until 2 min. The composition was returned to 20% solvent B by 2.2 min. The total run time was 5 min. The column temperature was maintained at 50°C. The software Xcalibur version 2.2 was used to control the instrument and collect data. The electrospray ionization source was fitted with a stainless steel capillary (100 μm internal diameter). Nitrogen was used as both the sheath gas and the auxiliary gas. The ion-transfer tube temperature was maintained at 350°C. The spray voltage, tube lens voltage, and pressure of sheath gas and auxiliary gas were optimized to achieve maximal response using the test compounds mixing with the mobile phase A (50%) and B (50%) at a flow rate of 0.8 ml/min. Collision-induced dissociation was performed on test compounds and internal standard under 1.5 mTorr of argon. Percentage of unbound was calculated based on the percentage of compound in the phosphatase-buffered saline chamber (trans) compared with that in the plasma chamber.

Metabolic Stability Assays. Micromolar stability of the test compounds was also performed using an HTS platform. The test compounds (1 μM in DMSO) were incubated for 15 min at 37°C with shaking, in medium containing microsomes, phosphate buffer, and the cofactor NADPH. After incubation, the samples were extracted using ice-cold acetonitrile containing 0.1% formic acid and 50 mg/ml internal standard (VU0366031). The extracts were analyzed by means of HPLC/MS/MS using methods identical with those used for the plasma protein binding assay (detailed above). Percentage of test compound remaining after incubation was calculated based on the amount of compound in the incubated samples compared with similarly prepared unincubated controls.

In Vivo Pharmacokinetic Study. Compound was formulated as 20% hydroxypropyl β-cyclodextrin in sterile water at the concentration of 1 mg/ml and administered orally to male Sprague-Dawley rats weighing 225 to 250 g (Harlan, Indianapolis, IN) at the dose of 10 mg/kg. The rat blood (hepatic portal vein and cardiac puncture) and brain were collected at 0.5, 1, 3, and 6 h. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed in ice-cold phosphate-buffered saline, and immediately frozen on dry ice. Plasma was separated by centrifugation and stored at −80°C until analysis.

On the day of analysis, frozen whole-rat brains were weighed and homogenized in 1.3 (w/w) parts of ice-cold phosphate-buffered saline, pH 7.4. The sample extraction of plasma (100 μl) and brain homogenate (100 μl) was performed by a method based on protein precipitation using three volumes of ice-cold acetonitrile containing 0.1% formic acid and an internal standard (VU0366031) having final
The supernatants of plasma and brain homogenate extracts were analyzed by means of HPLC/MS/MS as described above. Selected reaction monitoring was carried out using the transitions from m/z 295 to 194 for VU0360172 and m/z 310 to 223 for VU0366031 (internal standard). The calibration curves were constructed, and linear response was obtained in the range of 20 to 10,000 ng/ml by spiking known amounts of VU0360172 in blank brain homogenates and plasma. The final PK parameters were calculated by noncompartmental analysis using WinNonlin software (version 5.1; Pharm- sight Inc., Mountain View, CA).

**In Vivo Behavioral Studies.** All experiments were conducted in accordance with the National Institutes of Health regulations of animal care covered in the Institute of Laboratory Animal Resources’ (1996) *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee.

**Conflict Paradigm**

**Subjects.** The subjects were 24 male Sprague-Dawley rats (225–249 g) (Harlan) individually housed and food-deprived to 90% of their free-feeding weight 1 week before the onset of training. The rats had continuous access to water, except during training, and were given enough food to maintain their weights at 90% of their expected nondeprived weights immediately after training and on weekends. All rats were maintained on a 12-h light/dark cycle with light onset at 6:00 AM.

**Apparatus.** Six commercially available operant chambers (MED Associates, St. Albans, VT), each housed in a sound-attenuating chamber, were used. The operant chambers were equipped with two response levers, a pellet dispenser centered between the levers, stimulus lights, a tone generator, and a grid floor for delivery of foot shock. The start of the session was signaled by illumination of the house light (7.5-W bulb). All equipment was controlled by programs written with MED Associates software.

**Training Procedure.** Rats were trained to lever-press for food reinforcement (Bioserv 45-mg pellets) on an FR-1 schedule that also included delivery of a free pellet every 60 s during daily 2-h sessions. All animals acquired the lever-press response within 3 days. After acquisition of the FR-1, the rats were switched to a variable interval (VI) schedule of reinforcement that began as a VI 10-s schedule and increased by 10 s each week until arriving at a final VI 30-s schedule of reinforcement. This phase of training was conducted with the house light on and later became the unpunished component of a multiple schedule. Once stable responding had occurred on the VI 30-s schedule, two components were added to training: a punished component, and a time-out. In the presence of a 70-dB tone and lever stimulus light, both of which remained on for the duration of the punished component, responses continued to be reinforced on a VI 30-s schedule; however, in addition, every 10th response was punished with a brief (500-ms) foot shock. The shock intensity was individually adjusted (0.25–0.5 mA) to produce an approximate 80% reduction in punished response rates relative to unpunished rates. The unpunished and punished components were 3 min in duration separated by 1-min time-outs, during which the lights and tone were extinguished and responses were without consequences. This multiple schedule was repeated four times for a total of 31 min of training. Training was given 5 days per week. Once stable rates of responding had been established in both the unpunished and punished components, testing began. Training required approximately 3.5 months.

**Testing Procedure.** After stable response rates had been achieved, rats were tested either once or twice a week depending on whether the test was with vehicle or drug. Drug tests were only given once a week. Test sessions were identical with training sessions. Testing began with the mGluR5 receptor antagonist MTEP, a compound that has been shown previously to increase punished responding in the conflict paradigm (Busse et al., 2004). Forty-five minutes before testing, rats (n = 24) were given injections with MTEP (vehicle, 1, 3, 5.6, or 10 mg/kg i.p.) suspended in 10% Tween 80/90% water. All concentrations were injected in a volume of 1 ml/kg. Each animal was tested with all doses of MTEP and vehicle.

VU0255683 (vehicle, 1, 3, and 10 mg/kg i.p.), suspended in 20% β-cyclodextrin, was injected 45 min before placing the animal (n = 19) in the test chamber. All concentrations were injected in a volume of 1 ml/kg except for the 10-mg concentration, which was injected in a volume of 2 ml/kg. Each animal was tested on all doses of VU0255683 and vehicle.

**Marble Burying**

**Compounds.** Doses of MTEP (positive control) and VU0255683 were dissolved in 10% Tween 80, vortexed vigorously, heated gently with a Master Heat Gun (Master Appliance Corporation, Racine, WI), and sonicated at 37°C for 30 min. The pH was checked using 0 to 14 EMD strips (EMD Biosciences, San Diego, CA) and adjusted to approximately 7. All doses were administered in a volume of 10 ml/kg i.p. Seven dose groups were administered: vehicle, 3, 5.6, 10, and 15 mg/kg MTEP, and 3, 5.6, and 10 mg/kg VU0255683.

**Subjects.** This study was conducted using male Harlan CD-1 mice weighing 30 to 35 g. Subjects were housed in a large colony room under a 12-h light/dark cycle (lights on at 6:00 AM) with food and water provided ad libitum. Test sessions were performed between 10:00 AM and 4:00 PM. All dose groups consisted of 9 to 11 mice. All experiments were conducted in accordance with Institute of Laboratory Animal Resources’ (1996) *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee.

**Procedure.** Eight small Plexiglas cages (32 × 17 × 14 cm) were arranged in two rows of four cages on top of a large, round table. Mice were transported from the colony room to the testing room and were allowed to habituate for 30 min. Mice were pretreated with a dose of MTEP (15-min pretreatment) or VU0255683 (30-min pretreatment) and individually placed in the cages in which 12 black glass marbles (14 mm diameter) had been evenly distributed (space 6.4 cm vertically and 4.25 cm horizontally from each other and the walls of the cage) on top of 2.5 cm Diamond Soft Bedding (Harlan Teklad, Madison, WI). The compound and comparator were evaluated in a counterbalanced design, in which all doses of compounds were tested in each session. Mice receiving the same dose were placed in cages on opposite sides of the table to control for effects of lighting and context. Clear, perforated plastic lids were set on top of each cage, and the amount of marble burying was recorded over a 30-min interval. The mice were then removed from the cages, and the number of buried marbles was counted using the criteria of greater than two thirds covered by bedding. Each session was videotaped with a Sony MiniDV camcorder equipped with a Sony wide-angle lens mounted on a 1.5-m tripod (Sony, Tokyo, Japan).

**Data Analysis.** The data for the dose-response studies were analyzed by a between-group analysis of variance. If there was a main effect of dose, then each dose group was compared with the vehicle control group using a Dunnett’s comparison. The calculations were performed using JMP IN 8 (SAS Institute, Cary, NC) statistical software and graphed using SigmaPlot9 (Systat Software, San Jose, CA).

**Amphetamine- and Phencyclidine-Induced Hyperlocomotion**

**Subjects.** All behavioral studies were conducted using male Sprague-Dawley rats (Harlan) weighing 270 to 300 g. Subjects were housed in pairs in a large colony room under a 12-h light/dark cycle (lights on at 6:00 AM) with food and water provided ad libitum. Test sessions were performed between 6:00 AM and 6:00 PM. Dose groups consisted of 8 to 16 rats per dose group. All doses of test compounds were injected in a 1.0 ml/kg volume. Test compound was dissolved in vehicle and double-deionized water with the pH adjusted to approximately 7.0 using 1 N NaOH.

**Testing Procedure.** After stable response rates had been achieved, rats were tested either once or twice a week depending on whether the test was with vehicle or drug. Drug tests were only given once a week. Test sessions were identical with training sessions. Testing began with the mGluR5 receptor antagonist MTEP, a compound that has been shown previously to increase punished responding in the conflict paradigm (Busse et al., 2004). Forty-five minutes before testing, rats (n = 24) were given injections with MTEP (vehicle, 1, 3, 5.6, or 10 mg/kg i.p.) suspended in 10% Tween 80/90% water. All concentrations were injected in a volume of 1 ml/kg. Each animal was tested with all doses of MTEP and vehicle.

VU0255683 (vehicle, 1, 3, and 10 mg/kg i.p.), suspended in 20% β-cyclodextrin, was injected 45 min before placing the animal (n = 19) in the test chamber. All concentrations were injected in a volume of 1 ml/kg except for the 10-mg concentration, which was injected in a volume of 2 ml/kg. Each animal was tested on all doses of VU0255683 and vehicle.
Apparatus. Amphetamine- and phencyclidine (PCP)-induced hyperlocomotor activity studies were conducted using a SmartFrame Open Field System (KinderScientific, San Diego, CA) equipped with 32 horizontal (x- and y-axes) infrared photobeams located 1 cm above the floor of the chamber. Changes in ambulation or locomotor activity were measured as the number of total photobeam breaks, expressed in 5-min intervals, and were recorded with a Pentagon I computer equipped with the Motor Monitor System software (KinderScientific).

Procedure for Amphetamine-Induced Hyperlocomotor Activity. Rats were placed in the open-field chambers for a 30-min habituation period, followed by a pretreatment with vehicle or an oral or intraperitoneal dose of test compound for an additional 30 min. Next, all rats received an injection of 1 mg/kg s.c. amphetamine or saline, and locomotor activity was measured for an additional 60 min.

PCP-Induced Hyperlocomotor Activity. Rats were placed in the open-field chambers for a 30-min habituation period, followed by a pretreatment with vehicle or a dose of either 10 mg/kg i.p. VU0285683 or MTEP for an additional 30 min. Next, all rats received an injection of 2.5 mg/kg s.c. PCP or saline, and locomotor activity was measured for an additional 120 min. Data were analyzed by a one-way analysis of variance with comparison with the vehicle + amphetamine control group with JMP version 8 (SAS Institute Inc.) statistical software.

Results

Novel Modulators of mGluR5 Are Identified via High-Throughput Screening. A major challenge in the discovery of novel compounds that fall into each of the three major functional classes of mGluR5 modulators is that assays for each of these pharmacological modes often require different conditions that are optimized for each activity. However, we were interested in the discovery of novel compounds that act as mGluR5 agonists, antagonists, and allosteric potentiators. To accomplish this, we developed a three-part assay that includes multiple compound additions with defined intervals that allows simultaneous detection of compounds with each of these activities. We measured receptor-induced mobilization of intracellular calcium by using an imaging-based plate reader that makes simultaneous measurements of calcium levels in each well of a 384-well microplate. Either vehicle or a test compound (10 μM final concentration) was added to cells expressing rat mGluR5 that were loaded with calcium-sensitive fluorescent dye. After a 2.5-min incubation period, a submaximally effective (EC_{50}) concentration of glutamate was added followed by a nearly maximal (EC_{80}) concentration added 1 min later. These intervals were chosen after extensive systematic testing of different compound addition intervals and based on the time required for maximal effects of known allosteric ligands and lack of desensitization between additions of EC_{20} and EC_{80} glutamate. Using this triple-add protocol, we were able to screen for agonists, antagonists, and potentiators simultaneously, maximizing the efficiency of the screen. Figure 1 depicts representative traces showing the effect of the different categories of mGluR5 ligands. The addition of an EC_{20} concentration of glutamate followed by an EC_{80} glutamate addition with this interval did not lead to substantial desensitization of the EC_{80} glutamate response (Fig. 1A). As can be seen in Fig. 1B, the addition of a maximally effective concentration of the mGluR5 agonist DHPG as the test compound induced a robust response upon the addition and desensitized responses to EC_{20} and EC_{80} concentrations of glutamate. The mGluR5 NAM MPEP elicited no response alone but completely blocked responses to both EC_{20} and EC_{80} concentrations of glutamate (Fig. 1C). Finally, the known mGluR5 PAM CPPHA elicited no response when added alone but potentiated the response to EC_{20} glutamate application (Fig. 1D). These data suggest that this assay is capable of detecting compounds belonging to each of these categories. It is important to note that inverse agonist activity of mGluR5 NAMs that has been described in earlier studies has never been observed using the calcium fluorescence assay used here. Thus, this assay cannot be used to determine whether novel NAMs have inverse agonist activity.

Raw kinetic data were normalized in a multistep process: 1) differences in cell number, nonuniform illumination/imaging, and dye-loading were controlled for based on the initial readings for the well by dividing each time point by the fluorescence reading at the initial time point; 2) three measurement windows were defined surrounding the addition of compound, glutamate EC_{20}, and glutamate EC_{80}; and 3) the signal amplitude for the minimum data point of each window was subtracted from each point on the trace. Wells in which

Fig. 1. High-throughput screen identifies compounds exhibiting agonist, antagonist, or potentiator activity. Traces show effect of either vehicle (A), a representative agonist (DHPG) (B), antagonist (MPEP) (C), or potentiator (CPPHA) (D) on mGluR5 calcium mobilization response to glutamate. Compounds (1 or 10 μM) were added to cells loaded with a calcium-sensitive dye and incubated for 2.5 min. A submaximal (EC_{20}) followed by a nearly maximal (EC_{80}) concentration of glutamate was added, and the calcium response was measured by the FDSS plate reader. Responses are expressed as a fluorescence ratio.
responses that differed from vehicle wells by 3 S.D. were selected as hits for further study. In addition, data from each plate were visually inspected to ensure the selection of hits. This assay was used to screen an internal Vanderbilt library consisting of 160,000 small molecules selected based on maximal chemical diversity as reported for previous screens performed at Vanderbilt (Niswender et al., 2008b; Marlo et al., 2009; Sheffler et al., 2009). Compounds were screened (n = 1) at a nominal concentration of 10 μM diluted from 10 mM stocks that were maintained in 100% DMSO (final DMSO concentration, 0.1%).

Primary hits were selected for retest using full CRC analysis for the appropriate activity. Thus, CRC analysis for agonist hits was performed in the absence of glutamate, CRC analysis of potentiators was performed in the presence of an EC_{20} concentration of glutamate, and CRC analysis for antagonist hits was performed in the presence of an EC_{80} concentration of glutamate. Finally, all primary hits were screened for activity at an endogenous Gq-coupled receptor present in a parental, untransfected HEK cell line not containing mGluR5 using an identical protocol except that EC_{20} and EC_{80} concentrations of carbachol were used in place of glutamate. The rationale for this counterscreen was that compounds acting by a nonspecific mechanism should have no effect in cells that do not express mGluR5. Thus, they should have no agonist activity in the parental cell line and should not potentiate or inhibit the response to an agonist of an unrelated G protein-coupled receptor. Compounds for which clear CRC relationships were demonstrated in mGluR5-expressing cells but which had no activity in the parental cell line were selected as verified hits.

Table 1 provides a summary of primary and secondary screening results. The primary screen of 160,000 compounds for activity at mGluR5 at 10 μM yielded 2877 (2403 available for retesting) potentiator hits, 784 (624 available for retesting) antagonist hits, and 22 agonist hits. A total of 1387 compounds were confirmed as having potentiator activity, producing a retest rate of approximately 60% and an overall hit rate of 0.9%. Likewise, 345 antagonists (55%) and all 22 agonists were confirmed. The industry standard is generally at or below 50% for retests and 0.1 to 0.5% for overall hit rate (Hodder et al., 2003). Thus, we had a hit rate for allosteric potentiators that was relatively high, a hit rate for antagonists that was consistent with this roughly defined industry standard, and a relatively low hit rate for mGluR5 agonists. Of the verified potentiator hits, 9 compounds had EC_{50} values lower than 100 nM, 63 compounds had EC_{50} values lower than 500 nM, and 156 compounds had EC_{50} values lower than 1 μM. The verified antagonists demonstrated increased potency compared with potentiators in which 33 compounds had EC_{50} values lower than 100 nM, 109 compounds had EC_{50} values lower than 500 nM, and 173 compounds had EC_{50} values lower than 1 μM. A wide range of efficacies was present for potentiators, represented by the percentage of maximal glutamate values of a test compound at 30 μM, with some nearing that of the maximal glutamate response. Approximately 10% of verified hits belonging to each hit category affected the agonist response of carbachol or untransfected HEK cells, demonstrating initial selectivity for mGluR5 among the majority of the compounds.

Compounds Discovered in the Screen Exhibit Multiple Functional Effects. CRC analysis of the confirmed hits in each category revealed that these compounds exhibited a broad range of functional activities, including full and partial antagonism and allosteric potentiator activity. Figure 2 depicts the structures, concentration-response curves, and raw data traces of examples of hits discovered in the screen highlighting the various categories. Figure 2, A and B, illustrates examples of two structurally and pharmacologically distinct
verified antagonist hits. The example shown, VU0040228, acts as a full antagonist, completely blocking the glutamate response to mGluR5 in a concentration-dependent manner (IC_{50} = 62 ± 28 nM, mean ± S.E.M., n = 3 experiments). In addition, 13 of the confirmed antagonist compounds (3.8%) proved to have “partial antagonist” activity when assessed for their ability to inhibit the response to an EC_{50} concentration of glutamate. We reported previously the discovery and detailed characterization of MPEP analogs that have partial antagonist activity (Rodriguez et al., 2005). However, it has not been clear whether it would be possible to identify partial antagonists based on other chemical scaffolds. In addition, compounds in this category had an unprecedented range of partial antagonism of mGluR5 ranging from 10 to 80%, with EC_{50} values in the range of 62 nM to 2 μM. This suggests that these compounds exhibit a range of negative cooperativities for regulation of glutamate. We reported previously the discovery and characterization of these novel mGluR5 PAMs and NAMs.

Novel mGluR5 NAMs and PAMs Are Selective for mGluR5 Relative to Other mGluR Subtypes. One of the most exciting aspects of the current findings is that they provide multiple allosteric modulators of mGluR5 that are structurally distinct from previous compounds. For instance, the majority of mGluR5 NAMs reported previously are based on the biaryl acetylene scaffold represented by MPEP. These novel compounds provide tools to allow further testing of the hypothesis that structurally diverse molecules exerting different modes of efficacy on mGluR5 have predicted effects in animal models. For a compound to be useful as a proof of concept tool in behavioral models relevant to mGluR5, it must be selective for this receptor relative to other mGluR subtypes. We examined the selectivity profiles of mGluR5 NAM, VU0040228, and PAM, VU0092273, by determining the activities of each compound at a representative example of each of the three subgroups of mGluRs: group I (mGluR1), group II (mGluR3), and group III (mGluR4). We initially measured the agonist concentration-response relationship of

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### Figure 3

**Novel mGluR5 modulators exhibit multiple binding profiles to mGluR5.** Competition binding curves for allosteric modulators were obtained in the presence of 2 nM [3H]methoxyPepy by membranes harvested from mGluR5-expressing HEK293 cells. A, antagonist VU0040228 (△) fully competes with the equilibrium of [3H]methoxyPepy (K_i = 82 ± 12 nM), whereas partial antagonist VU0029251 (●) only reaches the level of approximately 50% total binding (K_i = 1.07 ± 0.15 μM). B, PAM VU0092273 (■) fully competes with the equilibrium of radioligand (K_i = 970 ± 140 nM), whereas PAM VU0029316 (△) has no effect. Data represent the mean ± S.E.M. of three independent experiments performed in triplicate. Data are plotted as a percentage of total [3H]methoxyPepy binding.
the other mGluRs in the presence and absence of 10 μM NAM or PAM. In this manner, potential negative or positive allosteric modulator activity could both be assessed in a single assay. VU0040228 (10 μM), a concentration capable of producing maximal effect on mGluR5 responses, had no significant effect on the agonist response of mGluRs 1 and 4 but was found to slightly decrease the maximal effect of the agonist response of mGluR3 whereas having no significant effect on the potency of glutamate (see Supplemental Fig. S1). The mGluR5 PAM, VU0092273, also had no effect on the agonist response to mGluRs 1 and 4 but was found to significantly block the mGluR3 response to glutamate at 10 μM compound (see Supplemental Fig. S2). We then determined the potency of VU0092273 at mGluR3 by examining the effects of a range of concentrations of compound in the presence of an EC50 concentration of glutamate. VU0092273 inhibited the mGluR3 EC50 response to glutamate with an IC50 value of 6.3 ± 1.6 μM (mean ± S.E.M., n = 3 experiments), a value significantly higher than the potency at which the compound potentiates the mGluR5 EC20 response to glutamate (EC50 = 10 ± 5 nM, mean ± S.E.M., n = 3 experiments). Based on their selectivity profiles, interaction with the MPEP binding site, and potency values, VU0040228 and VU0092273 were selected for further optimization and characterization.

### Chemical Optimization of Novel mGluR5 NAM VU0040228

In an effort to optimize the HTS hit VU0040228 for enhanced functional potency and binding affinity, and to dial out undesirable mGluR3 antagonist activity, we prepared a small library that held the pyridyl-oxadiazole moiety constant while varying the phenyl region of the molecule. The chemistry was carried out in a single reaction by coupling 2-pyridyl amidoxime with various substituted benzoic acids to generate the oxadiazole library (see Supplemental Data for syntheses). 3-(Pyridine-2-yl)-5-(3-trifluoromethyl)phenyl)-1,2,4-oxadiazole (VU0255038) and 3-cyano-4-fluoro (VU0255036) analogs retained activity, but these substitutions led to a significant loss in potency (IC50 values of 2.4 and 6.3 μM, respectively; Table 2). Introduction of a halide in the 3-position was well tolerated but led to a slight loss in potency [5-(3-chlorophenyl)-3-(pyridine-2-yl)-1,2,4-oxadiazole (VU0067144), IC50 = 240 nM; and 5-(3-bromophenyl)-3-(pyridine-2-yl)-1,2,4-oxadiazole (VU0255037), IC50 = 215 nM]. As described previously (Kulkarni et al., 2009), both potency and affinity were enhanced with the introduction of a 3-cyano-5-fluoro substitution pattern (VU0285683). VU0285683 acts as a full antagonist, completely blocking the glutamate response to mGluR5 in a concentration-dependent manner (IC50 = 24.4 ± 3.6 nM, mean ± S.E.M., n = 3 experiments) and was found to fully compete with the equilibrium binding of [3H]mepoxycPepy (Kp = 16.9 ± 1.1 nM, mean ± S.E.M., n = 3 experiments; see Supplemental Fig. S5). This provides a nonacetylene mGluR5 NAM with an in vitro potency that is comparable with that of the prototypical biaryl acetylene mGluR5 NAM, MPEP. As in the case of VU0040228, VU0285683 had no significant effect on the agonist response of mGluRs 1 and 4, and in contrast to VU0040228 had no effect on the agonist response of mGluR3, indicating that this optimized NAM was selective for mGluR5 compared with these other mGluR subtypes (see Supplemental Fig. S3).

### The Newly Identified mGluR5 NAM VU0285683 Induces Rightward Shifts in the Glutamate Concentration-Response Curves and Reduces the Maximal Effect of Glutamate

We further evaluated the question of whether VU0285683 inhibits mGluR5 via a noncompetitive mechanism by examining the effects of this antagonist on the activation of mGluR5 by measuring the concentration-response relationship of glutamate in the presence and absence of a fixed concentration of test compound. We predicted that if the antagonist acted via a site other than the glutamate binding site, increasing concentrations of the compound would shift the curve to the right and decrease the maximal signal of glutamate, as opposed to inducing a parallel rightward shift seen in the presence of an orthosteric antagonist. Preincubation of mGluR5 cells with increasing concentrations (10, 30, and 100 nM) of VU0285683 induced robust rightward shifts of the glutamate concentration response curve and decreased the maximal response to glutamate, a pattern suggesting noncompetitive inhibition (Fig. 4). These data are consistent with the hypothesis that VU0285683 does not interact with the glutamate binding site and instead acts as a noncompetitive allosteric antagonist.

### 5MPEP Blocks VU0285683 Inhibition of mGluR5 Activity in a Competitive Manner

The finding that VU0285683 fully competes with [3H]mepoxycPepy for binding to mGluR5 and inhibits the mGluR5 concentration-response relationship in a noncompetitive manner suggests that it interacts with the MPEP site of mGluR5. The neutral

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**TABLE 2**

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<tr>
<td>VU0285683</td>
<td>F</td>
<td>H</td>
<td>CN</td>
</tr>
</tbody>
</table>

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**Fig. 4.** VU0285683 induces a rightward shift of the glutamate concentration-response curve and decreases the maximal response. DMSO-matched vehicle or 10, 30, or 100 nM VU0285683 was applied to mGluR5-expressing HEK293 cells before the addition of a range of glutamate concentrations, and the response was measured via calcium mobilization. Incremental rightward shifts of the glutamate CRC and a decrease of the maximal glutamate signal were observed. Data represent the mean ± S.E.M. of three independent experiments performed in triplicate. Data are plotted as a percentage of maximal response to glutamate.
site ligand 5MPEP is a positional isomer of MPEP that binds to the MPEP site in a competitive manner but does not alter the glutamate concentration-response relationship (Rodriguez et al., 2005). Therefore, if VU0285683 acts by binding to the MPEP site, 5MPEP should compete with VU0285683 and induce a parallel rightward shift of the concentration-response curve. Figure 5A shows the effect of a range of concentrations of VU0285683 alone and in the presence of 5MPEP (10 μM). Consistent with our predictions, the neutral MPEP site ligand 5MPEP induces a rightward shift in the VU0285683 CRC (11.8 ± 0.9-fold, mean ± S.E.M., n = 3 experiments), suggesting that 5MPEP blocks the inhibitory effect of VU0285683 in a competitive manner.

A Mutation that Reduces the Effect of MPEP at mGluR5 Also Blocks VU0285683 Inhibition of mGluR5 Activity. Mutation of alanine to valine at the 809 position of mGluR5 (A809V) significantly reduces the ability of MPEP to bind to the receptor and diminishes the potency of MPEP as a negative allosteric modulator of mGluR5 (Pagano et al., 2000; Malherbe et al., 2003). If our novel allosteric antagonist VU0285683 acts via the MPEP binding site, we would expect the A809V mutation to significantly reduce the activity of this compound in a similar manner. Figure 5B depicts the concentration-response relationship of VU0285683 in the presence of an EC₅₀ concentration of glutamate on wild-type mGluR5 and on the A809V mutant receptor. The MPEP-sensitive mutant clearly reduces the potency of VU0285683 inhibition as seen by the parallel rightward shift of the concentration-response curve of the A809V mutant. 5.6 ± 0.1-fold (mean ± S.E.M., n = 3 experiments) compared with wild type, consistent with the hypothesis that binding to the MPEP site is necessary for the antagonist activity of VU0285683. Taken together, these radioligand binding, mutagenesis, and molecular pharmacology data provide strong evidence that VU0285683 inhibits mGluR5 responses by binding to the same allosteric site as the prototypical mGluR5 antagonist MPEP.

The Novel mGluR5 NAM VU0285683 Produces Dose-Dependent Effects in Rodent Models of Anxiolytic Activity. Previous studies have shown that the mGluR5 antagonists MPEP and MTEP produce anxiolytic effects in a wide variety of animal models (Spooren et al., 2000; Busse et al., 2004; Gasparini et al., 2008), including a modified version of the Geller-Seifter conflict paradigm, a classic measure of anxiolytic activity in rodents (Busse et al., 2004). To determine whether our conflict paradigm was sensitive to the anxiolytic effects of mGluR5 antagonists, we first tested MTEP as a positive control. Pretreatment with MTEP at 1, 3, 5.6, and 10 mg/kg produced a significant dose-related increase in punished responding (F₄,₂₃ = 24.8, p < 0.0001) (Fig. 6A) and a significant decrease in unpunished responding (F₄,₂₃ = 15.4, p < 0.0001) (Fig. 6C), suggesting anxiolytic effects. Post hoc analysis indicated that punished responses were significantly higher relative to vehicle treatment at the 3, 5.6, and 10 mg/kg doses of MTEP (p < 0.05, Newman-Keuls). Unpunished response rates declined significantly at the 10 mg/kg dose of MTEP (p < 0.05, Newman-Keuls). The results of this study indicated that the modified conflict paradigm is sensitive to the anxiolytic effects of mGluR5 antagonists. We then investigated the novel mGluR5 antagonist, VU0285683, for anxiolytic activity. Rats were pretreated with 1, 3, and 10 mg/kg VU0285683 45 min before being tested in the conflict paradigm. VU0285683 produced a significant dose-dependent increase in punished responding (F₃,₁₈ = 9.63, p < 0.0001) (Fig. 6B), as well as a significant decrease in unpunished responding (F₃,₁₈ = 16.78 p < 0.0001) (Fig. 6D), suggesting anxiolytic effects. Post hoc analysis indicated that both the 3 and 10 mg/kg doses of VU0285683 significantly increased punished responding relative to vehicle treated animals (p < 0.05, Newman-Keuls), whereas unpunished responding was significantly decreased at the 10 mg/kg dose (p < 0.05, Newman-Keuls).

The effect of VU0285683 was also evaluated in marble burying, a second rodent behavioral model predictive of anxiolytic activity. Previous studies reveal that MPEP decreases a rodent’s tendency to bury marbles, and this is believed to be indicative of the compound’s anxiolytic activity (Spooren et al., 2000). We again validated our assay using MTEP as a positive control (Fig. 7A). Treatment with MTEP (10 and 15 mg/kg) produced a significant dose-dependent decrease in marbles buried compared with treatment with vehicle, suggesting anxiolytic effects (p < 0.005 and p < 0.0001, respectively). These results validated that our marble-burying protocol was sensitive to the effects of mGluR5 antagonists. Mice were then treated with 3, 5.6, and 10 mg/kg doses of VU0285683 as seen in Fig. 7B. Marble bury-
ing was significantly decreased relative to vehicle-treated animals at the 10 mg/kg dose ($p = 0.0001$), suggesting anxiolytic activity in a second model predictive of such an effect.

Next we evaluated the effects of VU0285683 on potentiation of PCP-induced hyperlocomotion, a preclinical model of psychotomimetic-like activity. Previous studies have demonstrated that MPEP and MTEP potentiate the psychotomimetic-like effects of PCP in animals over a dose range that overlaps with the anxiolytic effects of these compounds. It is believed that this may predict an adverse effect of mGluR5 NAMs based on the MPEP scaffold. In the present study, we evaluated the effects of a 10 mg/kg dose of VU0285683 or MTEP alone or in combination with a subthreshold dose of PCP (2.5 mg/kg) on locomotor activity. As shown in Fig. 8A, VU0285683 did not potentiate the effects of PCP-induced hyperlocomotion at a dose that produced robust anxiolytic-like effects in the punished responding and marble-burying assays. In contrast, MTEP produced a robust potentiation of PCP-induced hyperlocomotion over the time course tested (Fig. 8B).

Chemical Optimization of Positive Allosteric Modulator VU0092273 Reveals Interesting Structure Activity Relationships. In addition to providing a major advance in identifying and establishing a unique behavioral profile of structurally novel mGluR5 NAMs, discovery of novel mGluR5 PAMs provides an important advance. One mGluR5...
PAM, VU0092273, bears close structural resemblance to the prototypical mGluR5 NAM MPEP (Fig. 9A). Although we reported previously that allosteric modulators that occupy the MPEP binding site can display a range of activities from PAM to NAM to neutral cooperativity with slight structural modifications (O’Brien et al., 2003; Rodriguez et al., 2005; Sharma et al., 2008), in previous studies, individual scaffolds tend to be strongly biased toward dominant PAM or NAM activity. Previous allosteric modulators based on the MPEP scaffold are predominantly biased toward robust and potent NAMs. Thus, it was interesting to identify VU0092273 as one of the most potent mGluR5 PAMs identified to date.

Structure-activity relationship (SAR) development of allosteric potentiators has proven extremely challenging, with

![Graph showing the effects of VU0285683 and MTEP on locomotor activity](image)

**Fig. 8.** VU0285683 does not potentiate the psychotomimetic effects of PCP on locomotor activity in contrast to MTEP. The effects of VU0285683 (A) and MTEP (B) on locomotor activity were evaluated at a dose of 10 mg/kg alone or in combination with a 2.5 mg/kg dose of PCP. Data are expressed as mean ± S.E.M. of the number of beam breaks per 5 min; S.E.M. are not shown if less than the size of the point (n = 6–8 animals per dose). *, p < 0.05 versus vehicle + PCP, Dunnett’s test. Note, the Veh/Veh, VU0285683, and MTEP-alone dose groups were significantly different from the Veh + PCP group across the 70- to 120-min interval; * are not shown for clarity.

**Fig. 9.** A, structures of phenyl acetylene mGluR5 NAMs and PAMs. B, regions of VU0092273 targeted for chemical optimization. C, optimization of phenyl acetylene VU0366026 to nicotinamide VU0360172.
many chemical series displaying no tractable SAR (Lindsley et al., 2004, 2005; Kinney et al., 2005; Zhao et al., 2007). An iterative analog library synthesis approach was used to quickly establish SAR, determine chemical tractability for VU0092273, and eliminate the significant unwanted mGluR3 activity exhibited by the lead. In preliminary SAR studies, we found that the 3’F, 5-CN substitution that was useful in the NAM series leads to complete loss of activity of the mGluR5 PAMs. Thus, our focus, as outlined in Fig. 9B was the evaluation of alternative aryl/heteroaryl rings for the phenyl rings, diverse amide analogs for the piperidine nucleus, and alternative linkers to replace the acetylenic spacer.

To accomplish this, we synthesized several libraries of functionalized aryl and heteroaryl analogs of VU0092273. In parallel, we focused on exploring a diverse array of amide analogs to diminish both oxidative metabolism and P-glycoprotein susceptibility. The scaffolds for conducting the amide library synthesis were generated via Sonogashira coupling to install the bi-phenyl or nicotinamide acetylene backbone, followed by hydrolysis of the ethyl ester to afford the acid. Standard amide formation chemistry was used to generate a library using polymer-supported reagents and scavengers (see Supplemental Data for representative syntheses). Table 3 highlights selected examples from this effort, which afforded a number of highly active mGluR5 PAMs. However, the efficacy of some compounds was relatively low in which the percentage of the maximal glutamate response was typically less than 50%. In addition, as observed with CDPPB (Lindsley et al., 2004; Chen et al., 2007), a degree of partial agonism was observed at higher compound concentrations when tested in our calcium assay using HEK293 cells expressing rat mGluR5. This is similar to what we reported previously for mGluR5 PAMs in the CDPPB series (Chen et
al., 2007). Initially, morpholino amide derivative (4-morpholino)(4-phenylethynyl)phenyl)methanone (VU0240381) provided an interesting profile (EC$_{50}$ = 2.3 ± 1.0 nM, mean ± S.E.M., n = 3 experiments; cLog $P$ = 3.6) relative to previous mGluR5 PAMs in that this compound has substantially improved potency and anticipated increased solubility in aqueous vehicles that could be used for in vivo studies. Alternative linker elements, such as amides and oxadiazoles, were evaluated as replacements for the acetylene moiety in an effort to avoid potential metabolic liabilities of the acetylene linker. Unfortunately, data from 2 24-member libraries demonstrated that these alternative linkers were generally inactive (data not shown).

Finally, we synthesized a two-dimensional library to combine the optimal findings from each of the libraries prepared using the synthetic routes described herein to produce derivatives including compounds shown in Table 4. This library generated the most potent and efficacious (reaching a maximum response of 72 to 105% maximal glutamate) mGluR5 PAMs ever described. Of particular interest once again were the morpholino amide congeners such as (4-morpholino)(4-(3-fluorophenyl)ethyl)phenyl)methanone (VU0366031), which structurally represents an added 3-fluoro substituent on the pendant aryl group relative to VU0240381. The incorporation of the 3-fluoro substituent in particular improves both maximal glutamate response and affinity at the MPEP site. In addition, it was anticipated that fluorination of the pendant aryl ring may have a beneficial effect on metabolic stability.

### Table 4
Chemical optimization of VU0092273 combining optimal findings from each library

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<td>25 ± 5</td>
<td>103 ± 6</td>
<td>390 ± 50</td>
</tr>
<tr>
<td>VU0366029</td>
<td>3-F</td>
<td>CH</td>
<td>65 ± 16</td>
<td>104 ± 3</td>
<td>980 ± 20</td>
</tr>
<tr>
<td>VU0360175</td>
<td>3-F</td>
<td>N</td>
<td>49 ± 7</td>
<td>105 ± 4</td>
<td>N.D.</td>
</tr>
<tr>
<td>VU0360172</td>
<td>3-F</td>
<td>N</td>
<td>16 ± 6</td>
<td>87 ± 3</td>
<td>195 ± 20</td>
</tr>
</tbody>
</table>
Based on metabolite structure identification studies using related nonfluorinated compounds (data not shown). Overall, VU0366031 displays the best combination of potency (EC\textsubscript{50} = 3.8 ± 1.1 nM, mean ± S.E.M., n = 3 experiments) and efficacy (glutamate max = 98 ± 8\%, mean ± S.E.M., n = 3 experiments) among all of the mGluR5 PAMs generated in this effort and generally provides a major improvement in affinity at the MPEP site. As mentioned earlier, the prototypical mGluR5 PAM CDPPB has an EC\textsubscript{50} of 113 nM and a K\textsubscript{i} at the MPEP site of 2.6 μM. VU0366031 (EC\textsubscript{50} = 3.8 ± 1.1 nM, mean ± S.E.M., n = 3 experiments) has a K\textsubscript{i} of 40 ± 12 nM (mean ± S.E.M., n = 3 experiments), and VU0240381 (EC\textsubscript{50} = 2.3 ± 1.0 nM, mean ± S.E.M., n = 3 experiments) has a K\textsubscript{i} of 55 ± 4 nM (mean ± S.E.M., n = 3 experiments). Thus, the biphenyl acetylene mGluR5 PAMs described herein represent a significant advance over CDPPB in terms of functional potency and binding affinity for mGluR5. Efforts to incorporate polarity in the core aryl group resulted in identification of (6-((3-fluorophenyl)ethynyl)pyridin-3-yl)(4-hydroxyperipedin-1-yl)methanone (VU0361747), however, at a potency loss of ~50-fold (Fig. 9C and Table 3).

Despite the apparent loss in potency upon incorporation of the nicotinamide nitrogen, this modification allowed for the first time the preparation of a salt form of the final compound that has not been possible for previously investigated mGluR5 PAMs. The ability to generate a salt can be an important factor in enhancing drug solubility and its dissolution rate for in vivo studies using neutral to weakly acidic nontoxic vehicles. As such, we incorporated the potency- and efficacy-enhancing morpholino amide substructure within the nicotinamide core to arrive at compound (6-((3-fluorophenyl)ethyl)pyridin-3-yl)morpholine)methanone (VU0360175). Although a potency loss was noted for VU0360175 relative to VU0366031 (EC\textsubscript{50} = 49 versus 3.8 nM), the magnitude was 5-fold less than that observed for (4-hydroxyperipedin-1-yl)4-(3-fluorophenethyl)phenyl)methanone (VU0366026) and VU0361747. Further modification identified that small branched secondary amines and in particular, VU0360172 offers excellent potency (EC\textsubscript{50} = 16 ± 6 nM, mean ± S.E.M., n = 3 experiments) and efficacy (glutamate maximum = 87 ± 3\%, mean ± S.E.M., n = 3 experiments). More importantly, as described previously, we anticipate the presence of the nicotinamide functionality to be advantageous in terms of having optimal physiochemical properties for in vivo studies. It is noteworthy that VU0360172 had no significant effect on the agonist response of mGluR5.

VU0360172 In Vitro and In Vivo Pharmacokinetic Profile. A significant drawback of CDPPB and other mGluR5 PAMs is poor pharmacokinetic and physiochemical properties that limit in vivo dosing. Free fraction of a compound in the presence of plasma proteins and the stability of a compound in liver microsomes are important qualities in a desirable drug metabolism and pharmacokinetic profile. VU0360172 was tested for plasma protein binding in the presence of rat plasma proteins and was found to be 98.9\% protein-bound. Although 4\% free fraction is desirable, >1% demonstrates a degree of free fraction and is therefore greater than a minimum cutoff that we typically apply for a proof-of-concept compound. The compound was also tested for stability in both rat and human liver microsomes and was found to have excellent stability with 87 and 86\% parent compound remaining after a 15-min incubation period (data not shown). In vivo pharmacokinetics of VU0360172 were studied in male Sprague-Dawley rats after oral administration of 10 mg/kg dose in 20% hydroxypropyl β-cyclodextrin (BCD) solution. At different time points including 0.5, 1, 3, and 6 h after dosing, the concentrations of VU0360172 were measured in hepatic portal vein plasma, systemic plasma (cardiac puncture), and whole-brain tissues. The compound was rapidly and very significantly absorbed, as evident from systemic plasma concentrations (see Supplemental Data, Fig. S6). The C\textsubscript{max} of 7432.98 ng/ml (~21 μM) was achieved in systemic plasma within 1 h of dosing. There is very little or no first-pass hepatic effect, as indicated by the AUC\textsubscript{sysplasma}/AUC\textsubscript{hypplasma} ratio of 0.93. Although characterized by low to moderate CNS penetration (AUC\textsubscript{brain}/AUC\textsubscript{sysplasma} = 0.13), more than acceptable levels (C\textsubscript{max} ~2 μM) were achieved in brain after oral dosing of this compound.

Novel mGluR5 PAM Produced Dose-Dependent Activity in a Rodent Model Predictive of Antipsychotic-Like Activity. Both typical and atypical antipsychotic drugs are known to reduce amphetamine-induced hyperlocomotion; this effect is believed to have predictive value for determining the antipsychotic efficacy of a compound (Kinney et al., 2003). Previous studies with CDPPB and ADX-47273 have demonstrated that these mGluR5 PAMs also have efficacy in this behavioral model (Kinney et al., 2005; Liu et al., 2008). However, although these compounds provided an important advance, they are not highly aqueous soluble and are therefore not optimal for in vivo dosing. Thus, previous studies with these earlier mGluR5 PAMs required dosing in vehicles containing DMSO, which is unpleasant for animals and makes more extensive behavioral studies difficult. Discovery of VU0360172 as a potent and efficacious mGluR5 PAM with improved aqueous solubility and a favorable PK profile represents a significant advance and may provide the first mGluR5 PAM that could be used for in vivo studies using standard aqueous vehicles. Thus, we determined the effect of the optimized mGluR5 PAM, VU0360172, on amphetamine-induced hyperlocomotor activity to determine whether this novel mGluR5 PAM has antipsychotic-like activity in this animal model. Administration of VU0360172 significantly reversed amphetamine-induced hyperlocomotion when analyzed across the 5-min intervals from the time of amphetamine delivery to the end of testing (t = 60–120 min). In the study using injections with the nontoxic vehicle 20% BCD, post hoc analysis revealed that doses of 30, 56.6, and 100 mg/kg i.p. VU0360172 produced significantly fewer ambulations than the group receiving vehicle and amphetamine across the time course (Fig. 10A). Again, the Veh/Veh and 56.6 mg · kg\textsuperscript{-1}/Veh were also significantly different from the Veh/amphetamine-treated rats; however, the 56.6 mg · kg\textsuperscript{-1}/Veh group was not different from the Veh/Veh group. When dosed orally in the 20% BCD vehicle, doses of 56.6 and 100 mg/kg VU0360172 also significantly reduced amphetamine-induced hyperlocomotor activity with no effect when administered alone 30 min before amphetamine addition (Fig. 10B). These data provide further support for the hypothesis that multiple structurally distinct mGluR5 PAMs can have antipsychotic-like activity in a rodent preclinical model and rep-
resent the first example of efficacy of an mGluR5 PAM when dosed in a vehicle that lacks short-term adverse effects. In addition, this provides a major advance in demonstration of an orally active mGluR5 PAM.

**Discussion**

Using an innovative method of functional high-throughput screening capable of detecting multiple categories of activity, we have greatly expanded the structural and functional diversity of mGluR5 allosteric modulators. Structurally distinct novel molecules were discovered for each class of mGluR5 modulators, including NAMs with full antagonist activity (VU0285683), partial antagonists (VU0029251), agonist-potentiators (VU0092273), and pure potentiators (VU0028316). One of the advances provided by these studies is the finding that some of liabilities of previously identified mGlu5 PAMs are not intrinsic to the target or mechanism and can be avoided with new chemical scaffolds. We and others reported previously that mGluR5 NAMs in the MPEP class induce significant potentiation of behavioral effects of NMDA receptor antagonists that are believed to reflect the psychotomimetic and cognition-impairing effects of these compounds (Henry et al., 2002; Kinney et al., 2003). This, coupled with findings that a member of a different chemical class of mGluR5 NAM, fenobam, induced adverse effects that seem to be similar to those of NMDA receptor antagonists in patients (Friedmann et al., 1980; Pecknold et al., 1982), led to a concern that mGluR5 NAMs may have psychotomimetic effects that could limit their clinical use. However, the mechanism of this effect of mGluR5 NAMs is not known and may not be intrinsic to all mGluR5 NAMs. Indeed, MPEP is known to interact with NMDA receptors, and this
could contribute to this action in animal models (Homayoun et al., 2004). The finding that the novel mGluR5 NAM VU0285683 has anxiolytic-like activity in two rodent models but does not potentiate PCP-induced hyperlocomotor activity provides an exciting advance and suggests that it is possible to achieve full efficacy of mGluR5 NAMs in animal models of anxiety activity in the absence of potentiation of responses to NMDA receptor antagonists. In addition, the current finding that both MTEP and VU0285683 have efficacy in reducing marble-burying provides another model, lending support to the hypothesis that mGluR5 PAMs have anxiolytic-like activity.

In addition to the advance provided by discovery of this novel mGluR5 NAM, these studies represent a major step forward in the discovery of VU0360172 as an mGluR5 PAM with greatly improved properties relative to previous mGluR5 PAMs. A significant drawback of available mGluR5 PAMs is a lack of physiochemical and PK properties suitable for optimal in vivo dosing. Extensive medicinal chemistry efforts based on the CDPPB, ADX-47273, and CPPHA scaffolds (Lindsay et al., 2004; de Paulis et al., 2006; Zhao et al., 2007; Engers et al., 2009) have failed to produce compounds with the aqueous solubility and PK profiles required for optimal in vivo studies. Although limited in vivo studies have been performed with these earlier compounds, they have required dosing in DMSO, which can compromise the interpretation of in vivo data. Chemical optimization of HTS lead PAM VU0092273 led to the discovery of nictinamide VU0360172 and the preparation of a salt form of the final compound, an important factor in enhancing drug solubility for in vivo studies. VU0360172 has activity in the animal model of psychosis, amphetamine-induced hyperlocomotion, validating the potential usefulness of mGluR5 potentiators in the treatment of schizophrenia. VU0360172 exhibited activity in this model not only when dosed intraperitoneally, but when dosed orally as well, providing the first orally bioavailable mGluR5 PAM.

The current studies also provide important advances in our understanding of the extent of structural diversity and modes of efficacy of mGluR5 allosteric modulators. VU0029251 provides the first example of a compound exhibiting mGluR5 partial antagonist activity that is not based on the MPEP scaffold (Rodriguez et al., 2005). Partial antagonists have potential as therapeutic agents in a setting in which there is a need to block receptor function but maintain some level of receptor activity. VU0029251 represents the first departure of partial antagonists from MPEP-related scaffolds. Both MPEP and structurally related MTEP function as inverse agonists, blocking all mGluR5 activity, including constitutive activity, a quality that could contribute to adverse side effects such as cognitive deficits and psychotomimetic effects. Divergence from this scaffold while maintaining partial antagonist activity may lead to an improved drug profile in the treatment of chronic disorders including pain and anxiety. However, examples of partial antagonists are rare, and the majority of MPEP-related mGluR5 NAMs that have been characterized fully block or act as inverse agonists at mGluR5. Thus, discovery of novel structural classes of mGluR5 NAMs that possess this profile provides further support for the potential usefulness of this approach.

It is noteworthy that although mGluR5 PAMs are known to interact at multiple allosteric sites, most NAMs identified to date are believed to act at the MPEP site (Gasparini et al., 2008). However, Zhang et al. (2010) recently identified a novel mGluR5 NAM with partial antagonist activity that did not fully displace [3H]MPEP binding. Likewise, it was interesting to find that another partial antagonist reported here, VU0029251, does not completely displace [3H]methoxyPEPy binding. This may suggest that VU0029251 does not compete directly for binding to this site but instead inhibits MPEP site binding by an allosteric mechanism. Previously identified partial antagonists that interact with the MPEP site fully displace binding and their apparent Kᵢ values are consistent with the IC₅₀ values in functional assays (Rodriguez et al., 2005). Furthermore, a broad range of structurally distinct mGluR1 NAMs all interact with a single allosteric site that is homologous with the MPEP site on mGluR5 (Pagano et al., 2000; Lavreysen et al., 2003), although mGluR1 PAMs have been identified that do not interact with this site (Chen et al., 2008). Based on this, there has been a growing view that multiple allosteric sites may exist but that it may be difficult to identify mGluR NAMs that do not act by competitive interactions with the MPEP site or a homologous site on other mGluR subtypes. The present data provide the first evidence that it may be possible to develop mGluR5 NAMs that interact with multiple allosteric sites.

Both the full antagonist VU0285683 and the agonist-potentiatior VU0092273 fully compete with the equilibrium binding of the MPEP site ligand. However, reduction of MPEP binding does not necessarily imply a competitive interaction. We have reported previously that CDPPB and related compounds act by binding in a competitive manner at the MPEP site (Chen et al., 2007), and our current studies provide evidence suggesting that the novel NAM VU0285683 also acts competitively at the MPEP site. Based on the structural similarities of the new PAMs derived from VU0092273 and MPEP, along with the potent and full displacement of MPEP ligand binding, it is likely that these novel PAMs also act at the MPEP site. However, previous studies suggest that the structurally distinct mGluR5 PAM CPPHA does not act at this site (Chen et al., 2008), and in this report we have also identified VU0028316 as another example of an mGluR5 PAM that does not inhibit MPEP site binding. It is noteworthy that VU0028316 bears no obvious structural resemblance to either MPEP or CPPHA. In future studies, it will be important to determine whether this compound is likely to act at the same site as CPPHA or at a new distinct PAM site.

Chemical optimization of VU0092273 has provided us with clear advances over available PAMs such as CDPPB and ADX-47273 in terms of potency and binding affinity. However, it is important to note that although both potency and affinity values are left-shifted compared with CDPPB and ADX-47273, the binding affinity of these novel PAMs to the MPEP site is still considerably lower than the potencies of the compounds when assessed as PAMs in our functional assay. We have also observed this phenomenon in the CDPPB series, and this disconnect suggests a strong positive cooperativity between the allosteric modulator and glutamate when both ligands are present (Chen et al., 2007; Conn et al., 2009a). A greater leftward shift of potency compared with binding affinity implies a larger degree of positive cooperativity, as seen in the case of VU0092273.

In summary, we developed a screening strategy that allowed us to identify potent and efficacious mGluR5 modulators that possessed diverse chemical scaffolds. These modu-
lators exhibited unique binding modes to the receptor, providing support for mGlu5 modulator activity acting via both the MPEP site and a distinct site. These ligands showed selectivity for mGlu5 versus other mGluR5 subtypes and belonged to scaffolds distinct from known compounds. Finally, both NAM VU0285683 and PAM VU0360172 demonstrated in vivo activity in rodent models of anxiety and anti-psychoactive activity, respectively, validating the potential of mGlu5 NAMs and PAMs for the treatment of CNS disorders, including anxiety and schizophrenia.

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References


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Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 demonstrates robust chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and antipsychotic activity


Molecular Pharmacology

Supporting Information

Chemistry

General. All NMR spectra were recorded on a Bruker 400 mHz instrument. $^1$H chemical shifts are reported in $\delta$ values in ppm downfield from DMSO as the internal standard in DMSO. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constant (Hz). $^{13}$C chemical shifts are reported in $\delta$ values in ppm with the DMSO carbon peak set to 39.5 ppm. Low resolution mass spectra were obtained on an agilent 1200 series 6130 mass spectrometer. High resolution mass spectra were recorded on a Waters Q-TOF API-US. Analytical thin layer chromatography was performed on Analtech silica gel GF 250 micron plates. Analytical HPLC was performed on an agilent 1200 series. Preparative purification was performed on combi-flash companion. Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification. All polymer-supported reagents were purchased from Argonaut Technologies.
Experimental Procedures for mGluR5 PAMs:

**Ethyl 4-(phenylethynyl)benzoate (2).** To a solution of ethyl 4-Iodobenzoate 1 (5.0 g, 18.2 mmol) in DMF (8 mL) was added phenylacetylene (2.25 g, 22.1 mmol), Pd(Ph₃P)₄ (502 mg, 0.45 mmol), CuI (172 mg, 0.91 mmol) and diethylamine (2 mL). The reaction vessel was sealed and heated at 60 °C for 1h in a microwave reactor. The reaction was cooled to rt, diluted with EtOAc:hexanes (2:1, 150 mL) and washed with water (2 x 100 mL) and brine (100 mL). The organic phase was dried over MgSO₄, filtered and concentrated under vacuum. The crude product was purified by column chromatography (silica gel) using 0 to 10 % EtOAc/hexanes to afford ester 2 (7.89 g, 86%) as a pale yellow solid: ¹H-NMR (400 MHz, CDCl₃) δ 8.05 (d, J = 8.0 Hz, 2H), 7.61 (d, J = 8.0 Hz, 2H), 7.56 (dd, J = 8.0, 2.0 Hz, 2H), 7.41-7.37 (m, 3H), 4.41 (q, J = 7.0 Hz, 2H), 1.44 (t, J = 7.0 Hz, 3H); LC (214 nm) 5.79 min (>98%); MS (ESI) m/z = 250.9.

**4-(phenylethynyl)benzoic acid.** To a solution of ester 2 (7.81 g, 31.2 mmol) in THF (80 mL) was added MeOH (15 mL) and a solution of LiOH (5.24 g, 124 mmol) in water (15 mL). The reaction was stirred at room temperature and for 4h. The reaction was acidified with 1 N HCl (50 mL) and isolated benzoic acid (5.78 g, 83%) as a white solid: mp 190.1 °C; ¹H-NMR (400 MHz, CDCl₃) δ 8.11 (d, J = 8.0 Hz, 2H), 7.61 (d, J = 8.0 Hz, 2H), 7.56 (dd, J = 8.0, 2.0 Hz, 2H), 7.41-7.37 (m, 3H), 4.41 (q, J = 7.0 Hz, 2H), 1.44 (t, J = 7.0 Hz, 3H); ¹³C-NMR (100 MHz, d₆-DMSO) δ 167.3, 138.0, 134.5, 131.9, 131.4, 130.9, 130.6, 130.2, 130.0, 129.6, 127.0, 122.15, 101.6, 92.3, 89.0; LC (214 nm) 5.12 min (>98%); MS (ESI) m/z = 222.9.
(4-hydroxypiperidin-1-yl)(4-phenylethynyl)phenyl)methanone, VU0092273 (3a). To a solution of acid 9 (1.40 g, 6.30 mmol) and DIPEA (2.70 g, 20.8 mmol) in DMF (25 mL) was added EDC (1.41 g, 7.56 mmol), HOBt (850 mg, 6.30 mmol) and 4-hydroxypiperidine hydrochloride (1.29 g, 9.46 mmol). The reaction was stirred at room temperature for 18 h. The reaction was diluted with water (100 mL) and isolated amide 3a (1.84 g, 98%) as a white solid by vacuum filtration: mp 157.7 °C; \(^1\)H-NMR (400 MHz, CDCl\(_3\)) δ 7.58 (d, \(J = 8.0\) Hz, 2H), 7.56-7.52 (m, 2H), 7.44-7.34 (m, 5H), 4.21-4.08 (m, 1H), 4.03-3.96 (m, 1H), 3.81-3.48 (m, 1H), 3.47-3.16 (m, 2H), 2.08-1.79 (m, 3H), 1.71-1.42 (m, 2H); \(^{13}\)C-NMR (100 MHz, CDCl\(_3\)) δ 169.7, 135.5, 131.6, 131.5, 128.5, 128.3, 126.9, 124.7, 122.8, 90.8, 88.5, 66.9, 44.8, 39.3, 34.4, 33.8; LC (214 nm) 2.86 min (>98%); MS (ESI) \(m/z = 306.1\); HRMS = 306.1496 (calc. 306.1494), C\(_{20}\)H\(_{20}\)N\(_1\)O\(_2\).

(4-methanolpiperidin-1-yl)(4-phenylethynyl)phenyl)methanone, VU0366024 (3b). Amide 3b (1.26 g, 73%) isolated as a white solid was prepared in a manner similar to 3a: mp 144.8 °C; \(^1\)H-NMR (400 MHz, CDCl\(_3\)) δ 7.57-7.50 (m, 4H), 7.40-7.31 (m, 5H), 4.76 (br s, 1H), 3.77 (br d, 1H), 3.54-3.49 (m, 2H), 3.12-2.70 (m, 2H), 1.95 (br s, 4H), 1.22 (br s, 2H); \(^{13}\)C-NMR (100 MHz, CDCl\(_3\)) δ 169.7, 135.8, 131.6, 131.5, 128.5, 128.3, 126.9, 124.6, 122.8, 120.2, 90.7, 88.6, 67.1, 47.7, 42.2, 38.8, 38.4, 36.9, 29.4, 28.8, 28.3; LC (214 nm) 3.02 min (>98%); MS (ESI) \(m/z = 320.0\); HRMS = 320.1645 (calc. 320.1651), C\(_{21}\)H\(_{22}\)N\(_1\)O\(_2\).

(4-methanolpiperidin-1-yl)(4-phenylethynyl)phenyl)methanone, VU0240381 (3c).
(4-morphonyl)(4-phenylethynyl)phenyl)methanone, VU0240381 (3c). Amide 3c (2.01 g, 86%) isolated as a white solid was prepared in a manner similar to 3a: mp 118.4 °C; $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ 7.60-7.53 (m, 4H), 7.43-7.34 (m, 5H), 3.65 (br s, 8H); $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ 169.7, 134.7, 131.7, 131.6, 128.5, 128.3, 127.2, 125.0, 122.7, 90.9, 88.4, 66.8; LC (214 nm) 3.11 min (>98%); MS (ESI) $m/z$ = 292.2; HRMS = 292.1332 (calc. 292.1338), C$_{19}$H$_{18}$N$_1$O$_2$.

(3-hydroxyazetidin-1-yl)(4-phenylethynyl)phenyl)methanone, VU0366025 (3d). Amide 3d (1.68 g, 88%) isolated as a white solid was prepared in a manner similar to 3a: mp 161.2 °C, $^1$H-NMR (400 MHz, $d_6$-DMSO) $\delta$ 7.68-7.61 (m, 4H), 7.49-7.41 (m, 3H), 7.34-7.26 (m, 1H), 5.78 (br s, 1H), 4.52-4.43 (m, 2H), 4.27-4.23 (m, 1H), 4.09-4.00 (m, 1H), 3.82-3.76 (m, 1H); $^{13}$C-NMR (100 MHz, $d_6$-DMSO) $\delta$168.5, 133.5, 131.9, 131.7, 129.5, 129.2, 128.5, 125.1, 122.3, 91.5, 89.0, 63.1, 60.8, 58.9; LC (214 nm) 2.81 min (>98%); MS (ESI) $m/z$ = 278.1; HRMS = 278.1189 (calc. 278.1181), C$_{18}$H$_{16}$N$_1$O$_2$.

4-((3-fluorophenyl)ethynyl) benzoic acid (4). To a solution of ethyl 4-iodobenzoate 1 (10.0 g, 36.2 mmol) in DMF (30 mL) was added 3-fluorophenylacetylene (5.22 g, 43.4 mmol), Pd(Ph$_3$P)$_4$ (1.04 g, 0.91 mmol), CuI (346 mg, 1.82 mmol) and diethylamine (6 mL). The reaction vessel was sealed and heated at 60 °C for 1h in a microwave reactor. The reaction was cooled to room temperature, diluted with EtOAc:hexanes (2:1, 250 mL) and washed with water (2 x 200 mL) and brine (200 mL). The organic phase was dried
over MgSO₄, filtered and concentrated under vacuum. The crude product was dissolved in THF (130 mL), MeOH (28 mL) and a solution of LiOH (3.04 g, 72.4 mmol) in water (28 mL) added. The reaction was stirred at room temperature and for 4h. The reaction was acidified with 1 N HCl (100 mL) and isolated benzoic acid 4 (6.89 g, 79%) as a light tan solid: mp 157.1 °C (with decomp.); ¹H-NMR (400 MHz, d₆-DMSO) δ 7.97 (d, J = 8.0 Hz, 2H), 7.88 (d, J = 8.0 Hz, 1H), 7.71-7.63 (m, 2H), 7.52-7.40 (m, 2H), 7.36-7.29 (m, 1H); ¹³C-NMR (100 MHz, d₆-DMSO) δ 167.0, 163.5, 138.0, 132.0, 131.4, 130.6, 129.9, 128.3, 126.5, 124.1, 118.6, 117.0, 101.6, 90.9, 89.8; LC (214 nm) 3.29 min (>85%); MS (ESI) m/z = 241.1.

(4-hydroxypiperidin-1-yl)(4-(3-fluorophenyl)ethynyl)phenyl)methanone, VU0366026 (5a). Amide 5a (2.35 g, 91%) isolated as a white solid and prepared in a manner similar to 3a: mp 143.3 °C; ¹H-NMR (400 MHz, CDCl₃) δ 7.57 (d, J = 8.5 Hz, 2H), 7.41 (d, J = 8.5 Hz, 2H), 7.38-7.31 (m, 2H), 7.28-7.21 (m, 1H), 7.11-7.03 (m, 1H), 4.21-4.08 (m, 1H), 4.03-3.96 (m, 1H), 3.81-3.48 (m, 1H), 3.47-3.15 (m, 2H), 2.14 (s, 1H), 2.05-1.78 (m, 2H), 1.71-1.42 (m, 2H); ¹³C-NMR (100 MHz, CDCl₃) δ 169.6, 163.5, 161.1, 135.8, 131.7, 130.0, 127.5, 127.2, 126.9, 124.7, 124.6, 124.2, 118.3, 115.6, 89.4, 66.8, 44.8, 39.4, 34.4, 33.8; LC (214 nm) 2.98 min (>98%); MS (ESI) m/z = 324.2; HRMS = 324.1399 (calc. 324.1400), C₂₀H₁₉N₁O₂F₁.

(4-((3-fluorophenyl)ethynyl)phenyl)(4-hydroxymethyl)piperidin-1-yl)methanone, VU0366027 (5b). Amide 5b (2.32 g, 78%) light cream solid as prepared in identical fashion to 3a; mp 144.9 °C; ¹H-NMR (400 MHz, CDCl₃) δ 7.56 (d, J = 8.5 Hz, 2H), 7.40 (d, J = 8.5 Hz, 2H), 7.38-7.30 (m, 2H), 7.28-7.21 (m, 1H), 7.09-7.02 (m, 1H), 3.89-3.71 (m, 1H), 3.61-3.52 (m, 2H), 3.12-2.71 (m, 2H), 1.96-1.69 (m, 3H), 1.41-1.12 (m, 3H); ¹³C-NMR (100 MHz, CDCl₃) δ 169.6, 163.5, 136.1, 131.6, 129.9, 127.5, 127.0, 124.0, 118.5, 115.8, 89.4, 89.3, 67.2, 48.2, 42.1, 37.8, 29.4, 28.3; LC (215 nm) 4.98 min (>98%); MS (ESI) m/z = 338.1; HRMS = 338.1556 (calc. 338.1556), C₂₁H₂₁N₁O₂F₁.
(4-morphonyl)(4-(3-fluorophenyl)ethynyl)phenyl)methanone, VU0366031 (5c). Amide 5c (3.11 g, 97%) white solid as prepared in identical fashion to 3a; mp 97.0 °C; \(^1\)H-NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.57 (d, \(J = 8.5\) Hz, 2H), 7.41 (d, \(J = 8.5\) Hz, 2H), 7.38-7.31 (m, 2H), 7.28-7.21 (m, 1H), 7.13-7.05 (m, 1H), 4.00-3.31 (bd s, 8H); \(^13\)C-NMR (100 MHz, CDCl\(_3\)) \(\delta\) 169.6, 163.5, 161.1, 135.0, 131.8, 130.0, 129.9, 127.5, 127.4, 124.5, 118.3, 115.9, 89.6, 89.5, 66.8; LC (214 nm) 3.18 min (>98%); MS (ESI) \(m/z = 310.3\); HRMS = 310.1236 (calc. 310.1243), C\(_{19}\)H\(_{17}\)N\(_1\)O\(_2\)F\(_1\).

Synthesis of (3-hydroxyazetidin-1-yl)(4-(3-fluorophenyl)ethynyl)phenyl)methanone, VU0366029 (5d). Amide 5d (2.12 g, 83%) white solid as prepared in identical fashion to 3a; mp 151.6 °C (with decomp.); \(^1\)H-NMR (400 MHz, d\(_6\)-DMSO) \(\delta\) 7.68-7.61 (m, 4H), 7.49-7.41 (m, 3H), 7.34-7.26 (m, 1H), 5.78 (br s, 1H), 4.52-4.43 (m, 2H), 4.27-4.23 (m, 1H), 4.09-4.00 (m, 1H), 3.82-3.76 (m, 1H); \(^13\)C-NMR (100 MHz, d\(_6\)-DMSO) \(\delta\) 168.4, 163.5, 161.0, 137.6, 133.8, 131.8, 131.4, 131.3, 130.0, 128.5, 128.3, 124.6, 124.3, 118.4, 118.3, 117.0, 90.1, 89.9, 63.1, 60.8, 58.9; LC (215 nm) min (>98%); MS (ESI) \(m/z = 296.1\); HRMS = 296.1088 (calc. 296.1087), C\(_{18}\)H\(_{15}\)N\(_1\)O\(_2\)F\(_1\).

(4-((3-fluorophenyl)ethynyl)phenyl)(4-hydroxy-4-methylpiperidin-1-yl)methanone, VU0366030 (5e). Amide 5e (120 mg, 73%) white solid as prepared in identical fashion to 3a; mp 143.3 °C; \(^1\)H-NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.58 (d, \(J = 8.0\) Hz, 2H), 7.42 (d, \(J = 8.0\) Hz, 2H), 7.39-7.32 (m, 2H), 7.25 (dd, \(J = 9.5, 2.0\) Hz, 1H), 7.11-7.05 (m, 1H), 4.37 (s, 1H), 3.57-3.48 (m, 1H), 2.81-2.42 (m, 3H), 1.74-1.52 (m, 3H), 1.32 (s, 3H); \(^13\)C-NMR (100 MHz, CDCl\(_3\)) \(\delta\) 169.9, 163.4, 135.5, 132.5, 131.7, 130.0, 129.9, 127.5, 126.9, 124.3,
4-((3,4-fluorophenyl)ethynyl) benzoic acid (6). To a solution of ethyl 4-Iodobenzoate 1 (5.0 g, 18.1 mmol) in DMF (20 mL) was added 3,4-difluorophenylacetylene (2.97 g, 21.5 mmol), Pd(Ph₃P)₄ (520 mg, 0.45 mmol), CuI (173 mg, 0.91 mmol) and diethylamine (3 mL). The reaction vessel was sealed and heated at 60 °C for 1h in a microwave reactor. The reaction was cooled to room temperature, diluted with EtOAc:hexanes (2:1, 130 mL) and washed with water (2 x 100 mL) and brine (100 mL). The organic phase was dried over MgSO₄, filtered and concentrated under vacuum. The crude product was dissolved in THF (70 mL) added MeOH (14 mL) and a solution of LiOH (870 mg, 36.2 mmol) in water (14 mL). The reaction was stirred at room temperature and for 4h. The reaction was acidified with 1 N HCl (60 mL) and isolated benzoic acid 6 (3.45 g, 74%) as a light tan solid; ¹H-NMR (400 MHz, d₆-DMSO) δ 7.97 (d, J = 8.0 Hz, 2H), 7.76-7.70 (m, 1H), 7.66 (d, J = 8.0 Hz, 1H), 7.55-7.42 (m, 2H); ¹³C-NMR (100 MHz, d₆-DMSO) δ 167.0, 151.7, 150.9, 149.2, 148.4, 132.0, 131.1, 129.9, 129.6, 126.4, 121.1, 119.5, 118.7, 90.1, 89.4; LC (214 nm) 3.35 min (>98%); MS (ESI) m/z = 259.1.
Synthesis of (4-((3,4-difluorophenyl) ethynyl)phenyl)(morpholino)methanone VU0366028, (7a). Amide 7a (2.91 g, 96%) white solid was prepared in identical fashion to 3a; mp 132.8 °C; ¹H-NMR (400 MHz, CDCl₃) δ 7.56 (d, J = 8.5 Hz, 2H), 7.35 (t, J = 8.0 Hz, 1H), 7.31-7.25 (m, 1H), 7.15 (dd, J = 18.0, 8.0 Hz, 1H), 4.00-3.31 (bd s, 8H); ¹³C-NMR (100 MHz, CDCl₃) δ 169.6, 151.9, 149.4, 135.1, 131.7, 128.2, 127.2, 124.3, 120.6, 119.6, 117.5, 88.8, 88.7, 66.8; LC (215 nm) 5.10 min (>98%); MS (ESI) m/z = 327.9; HRMS = 328.1126 (calc. 328.1149), C₁₉H₁₆N₁O₂F₂.

6-Bromo-Ν-cyclobutynicotinamide (8). Commercially available 6-bromonicotinic acid (4.71 g, 23.4 mmol), cyclobutanamine (2.0 mL, 23.4 mmol) and HATU (10.6 g, 28.1 mmol) were mixed in DMF (50.0 mL) at room temperature. Ν,Ν-Diisopropylethylamine (12.2 mL, 70.3 mmol) was then added into the mixture. The reaction was allowed to stir at room temperature for 4 h. Analysis of the reaction mixture after 4 h by LC-MS indicated full conversion. The reaction was quenched with H₂O (100 mL). White precipitate was formed upon the addition of H₂O and was filtered. NMR and LC-MS indicated the white precipitate was the desired pure product 8 in 76% yield. ¹H NMR (400MHz, CDCl₃): δ 8.71 (d, J = 2.4 Hz, 1H), 7.97 (dd, J = 8.2, 2.4 Hz, 1H), 7.59 (d, J = 8.2 Hz, 1H), 6.23 (br s, NH), 4.60 (m, 1H), 2.48 (m, 2H), 1.99 (m, 2H), 1.84 (m, 2H); LC-MS (ESI), single peak, 1.10 min, m/z = 255.0 ([M+1]+).

N-Cyclobutyl-6-((3-fluorophenyl)ethynyl)nicotinamide hydrochloride, VU0360172 (9). N-Cyclobutyl-6-((3-fluorophenyl)ethynyl)nicotinamide hydrochloride (9) was prepared by Sonogashira coupling. 6-Bromo-Ν-cyclobutynicotinamide (8) (1.33g, 5.26 mmol), 1-ethynyl-3-fluorobenzene (0.61 mL, 5.26 mmol), Pd(PPh₃)₄ (0.30 g, 0.26 mmol), Cul (0.10 g, 0.53 mmol), and diethylamine (1.64 mL, 15.8 mmol) were mixed in 13.0 mL of DMF in a sealed 10 – 20 mL microwave tube. The reaction was subjected to microwave irradiation at 80 °C for 1 h. Analysis of the reaction by LC-MS indicated 93% conversion. The crude reaction was diluted by EtOAc (60.0 mL), and the resulting mixture was passed through a celite pad. The filtrate was extracted with H₂O (40.0 mL x
3), and the organic layer was separated and washed with brine (50 mL). The volatile component was removed under reduced pressure to give the crude residue. The crude mixture was purified by column chromatography and afforded product N-cyclobutyl-6-((3-fluorophenyl)ethynyl)nicotinamide as an orange color powder in 77% yield.

Pure product (N-cyclobutyl-6-((3-fluorophenyl)ethynyl)nicotinamide (1.19 g, 4.05 mmol) was dissolved in 1,4-dioxane (15.0 mL), and HCl (4.0 mL, 4.0 M in 1,4-dioxane) was added to the mixture dropwise. Off-white precipitate formed upon the addition of HCl. The mixture was allowed to stir at room temperature for 1 h, and filtered. The filtrand was the desired product VU0360172, 9 as an off-white powder. $^1$H NMR (400MHz, DMSO): $\delta$ 9.04 (d, $J$ = 1.2 Hz, 1H), 8.93 (d, $J$ = 7.8 Hz, 1H), 8.27 (dd, $J$ = 7.8, 1.2 Hz, 1H), 7.78 (d, $J$ = 8.4 Hz, 1H), 7.57 – 7.49 (overlapped, 3H), 7.37 (m, 1H), 4.43 (m, 1H), 2.25 (m, 2H), 2.11 (m, 2H), 1.70 (m, 2H); $^{13}$C NMR (100MHz, DMSO): $\delta$ 163.08, 162.22 (d, $J$ = 244.0 Hz, 1C), 148.82, 143.31, 136.89, 131.52 (d, $J$ = 9.0 Hz, 1C), 129.72, 128.67 (d, $J$ = 3.0 Hz, 1C), 127.68, 123.33 (d, $J$ = 9.0 Hz, 1C), 120.35, 118.82 (d, $J$ = 23.0 Hz, 1C), 117.64 (d, $J$ = 21.0 Hz, 1C), 90.08 (d, $J$ = 3.0 Hz, 1C), 89.01, 45.07, 30.38 (2C), 15.18; LC-MS (ESI), single peak, 1.35 min, $m/z$ = 295.10 ([M+1]$^+$); HRMS = 295.1248 ([M+1]$^+$, 100%) calcd for C$_{18}$H$_{16}$N$_2$OF, 295.1247.

![VU0360175](image)

(6-((3-Fluorophenyl)ethyl)pyridin-3-yl)(morpholino)methanone, VU0360175, (10).

Amide 10 was prepared in similar fashion to 9; $^1$H NMR (400MHz, CDCl$_3$): $\delta$ 8.70 (d, $J$ = 1.2 Hz, 1H), 7.82 (dd, $J$ = 8.0, 2.6 Hz, 1H), 7.62 (d, $J$ = 8.0 Hz, 1H), 7.42 (dd, $J$ = 6.6, 6.6 Hz, 1H), 7.37 (dd, $J$ = 8.0, 2.0 Hz, 1H), 7.34 (m, 1H), 7.14 (m, 1H), 7.13 (m, 1H), 3.79 (br m, 2H); LC-MS (ESI), single peak, 1.25 min, $m/z$ = 311.1 ([M+1]$^+$); HRMS = 311.1184 ([M+1]$^+$, 100%), calcd for C$_{18}$H$_{16}$FNO$_2$, 311.1196.

![VU0361747](image)

(6-((3-Fluorophenyl)ethyl)pyridin-3-yl)(4-hydroxypiperidin-1-yl)methanone, VU0361747 (11). Amide 11 was prepared in similar fashion to 9; $^1$H NMR (400MHz, CDCl$_3$): $\delta$ 8.70 (d, $J$ = 2.0 Hz, 1H), 7.81 (dd, $J$ = 8.0, 2.0 Hz, 1H), 7.61 (d, $J$ = 8.0 Hz, 1H), 7.41 (dd, $J$ = 8.2, 8.2 Hz, 1H), 7.37 (dd, $J$ = 8.0, 2.4 Hz, 1H), 7.34 (m, 1H), 7.13 (m, 1H),
4.15 (br m, 1H), 4.06 (m, 1H), 3.68 (br m, 1H), 3.53 (br m, 1H), 3.32 (br m, 1H), 2.03 – 1.90 (overlapped, 4H); LC-MS (ESI), single peak, 1.18 min, $m/z = 325.1 ([M+1]^+)\); HRMS = 325.1351 ([M+1]^+, 100%), calcd for C_{19}H_{18}FN_2O_2, 325.1352.

**Standard Experimental Procedures for mGluR5 NAMs:**

\[ \text{N} \quad \begin{array}{c}
\text{N} \\
\text{OH} \\
\text{NH}_2
\end{array} \\
\begin{array}{c}
\text{CN} \\
\text{F}
\end{array}
\]

Reaction conditions: a) i) 3-cyano-5-fluorobenzoic acid, EDC, HOBT, 1,4-dioxane, rt, 6h, ii) reflux, 18 h (65%).

**Synthesis of 3-fluoro-5-(3-(pyridine-2-yl)-1,2,4-oxadiazol-5-yl)benzonitrile, VU0285683 (12a).** To a mixture of 2-pyridylamidoxime (2.00 g, 14.6 mmol) in 1,4-dioxane (100 mL) was added 3-cyano-5-fluorobenzoic acid (2.40 g, 14.6 mmol), EDC (4.50 g, 23.3 mmol) and HOBT (2.00 g, 14.6 mmol). The reaction mixture was stirred at room temperature for 6h, then at reflux for 18 h. The reaction mixture was cool, diluted with water (250 mL) and extracted with EtOAc (3 x 150 mL). The combined organic extracts were dried over MgSO4, filtered and concentrated under vacuum. The crude product was recrystallized from EtOAc/hexanes to afford 12a as a yellow solid (2.53 g, 65%); $^1$H-NMR (400 MHz, CDCl3) $\delta$ 8.88 (d, $J = 5$ Hz, 1H), 8.43 (s, 1H), 8.29-8.24 (m, 2H), 7.94 (td, $J = 8.0, 1.5$ Hz, 1H), 7.65 (ddd, $J = 8.0, 2.5, 1.5$ Hz, 1H), 7.52 (ddd, $J = 8.0, 5.0, 1.5$ Hz, 1H); LC-MS (214 nm) 2.91 min (>98%); MS (ESI) $m/z = 267.1$; HRMS = 267.0679 (calc. 267.0682), C_{14}H_8N_4O_1F_1.

**Synthesis of 5-(3-bromophenyl)-3-(pyridine-2-yl)-1,2,4-oxadiazole, VU0255037 (12b).** Oxadiazole 12b (120 mg, 96%) white solid as prepared in identical fashion to 12a; $^1$H-NMR (400 MHz, d_4-MeOH) $\delta$ 8.76 (d, $J = 5.0$ Hz, 1H), 8.43 (t, $J = 1.5$ Hz, 1H), 8.29 (d, $J = 8.0$ Hz, 1H), 8.25 (dt, $J = 8.0, 1.5$ Hz, 1H), 8.08 (td, $J = 8.0, 1.5$ Hz, 1H), 7.89 (dd, $J = 8.0, 2.5, 1.0$ Hz, 1H), 7.64 (ddd, $J = 8.0, 5.0, 1.0$ Hz, 1H), 7.58 (t, $J = 8.0$ Hz, 1H); LC-MS (214 nm) 1.54 min (>98%); MS (ESI) $m/z = 302.0$ and 304.0; HRMS = 301.9925 (calc. 301.9929), C_{13}H_9N_3O_1Br.
Synthesis of 5-(3-chlorophenyl)-3-(pyridine-2-yl)-1,2,4-oxadiazole, VU0067144 (10c). Oxadiazole 12c (96 mg, 91%) white solid as prepared in identical fashion to 12a; \textsuperscript{1}H-NMR (400 MHz, d\textsubscript{4}-MeOH) δ 8.77 (d, J = 5.0 Hz, 1H), 8.32-8.26 (m, 2H), 8.21 (dt, J = 8.0, 1.5 Hz, 1H), 8.08 (td, J = 8.0, 1.5 Hz, 1H), 7.74 (ddd, J = 8.0, 1.5, 1.0 Hz, 1H), 7.65 (t, J = 8.0 Hz, 1H), 7.64-7.62 (m, 1H); LC-MS (214 nm) 1.51 min (>98%); MS (ESI) m/z = 258.0; HRMS = 258.0436 (calc. 258.0434), C\textsubscript{13}H\textsubscript{9}N\textsubscript{3}O\textsubscript{1}Cl.

Synthesis of 3-(pyridine-2-yl)-5-(3-(trifluoromethyl)phenyl)-1,2,4-oxadiazole, VU0255038 (12d). Oxadiazole 12d (102 mg, 93%) white solid as prepared in identical fashion to 12a; \textsuperscript{1}H-NMR (400 MHz, d\textsubscript{4}-MeOH) δ 8.77 (d, J = 5.0 Hz, 1H), 8.56 (s, 1H), 8.53 (d, J = 8.0 Hz, 1H), 8.31 (d, J = 8.0 Hz, 1H), 8.08 (td, J = 8.0, 1.5 Hz, 1H), 8.04 (d, J = 8.0 Hz, 1H), 7.89 (t, J = 8.0 Hz, 1H), 7.64 (ddd, J = 8.0, 5.0, 1.0 Hz, 1H); LC-MS (214 nm) 1.49 min (>98%); MS (ESI) m/z = 292.0; HRMS = 292.0697 (calc. 292.0698), C\textsubscript{14}H\textsubscript{9}N\textsubscript{3}O\textsubscript{1}F\textsubscript{3}.

Synthesis of 5-(3,5-dimethoxyphenyl)-3-(pyridine-2-yl)-1,2,4-oxadiazole, VU00040228 (12e). Oxadiazole 12e (98 mg, 89%) white solid as prepared in identical fashion to 12a; \textsuperscript{1}H-NMR (400 MHz, d\textsubscript{4}-MeOH) δ 8.77 (dd, J = 5.0, 1.0 Hz, 1H), 8.29 (d, J = 8.0 Hz, 1H), 8.07 (td, J = 8.0, 1.5 Hz, 1H), 7.64 (ddd, J = 8.0, 5.0, 1.0 Hz, 1H), 7.41 (d, J = 2.5 Hz, 1H), 6.82 (t, J = 2.5 Hz, 1H), 3.92 (s, 6H); LC-MS (214 nm) 1.45 min (>98%); MS (ESI) m/z = 284.1; HRMS = 284.1035 (calc. 284.1035), C\textsubscript{15}H\textsubscript{14}N\textsubscript{3}O\textsubscript{3}.
Synthesis of 2-fluoro-5-(3-(pyridin-2-yl)-1,2,4-oxadiazol-5-yl)benzonitrile, VU0255036 (12f). Oxadiazole 12f (46 mg, 78%) white solid as prepared in identical fashion to 12a; $^1$H-NMR (400 MHz, CDCl$_3$) δ 8.88 (ddd, $J = 4.8, 0.8, 0.4$ Hz, 1H), 8.64 (dd, $J = 6.0, 2.0$ Hz, 1H), 8.57 (m, 1H), 8.24 (d, $J = 7.8$ Hz, 1H), 7.93 (td, $J = 7.8, 2.0$ Hz, 1H), 7.52 (ddd, $J = 7.8, 4.8, 1.2$ Hz, 1H), 7.48 (t, $J = 8.5$ Hz, 1H); LC-MS (214 nm) 1.36 min (>98%); MS (ESI) $m/z = 267.0$; HRMS 289.0503 (calc. 289.0502), C$_{14}$H$_7$N$_4$OFNa.
Figure S1. VU0040228 Selectivity

**Figure S1.** VU0040228 is selective for mGluR5 versus other mGluR subtypes. Agonist concentration response relationship of mGluRs1, 3, and 4 in the presence and absence of 10 µM VU0040228 was measured.
Figure S2. VU0092273 Selectivity

Figure S2. VU0092273 selectivity profile. Agonist concentration response relationship of mGluRs1, 3, and 4 in the presence and absence of 10 µM VU0092273 was measured.
Figure S3. VU0285683 Selectivity

Figure S3. VU0285683 is selective for mGluR5 versus other mGluR subtypes. Effect of 10 µM VU0285683 on EC20 and EC80 agonist (top panel), or agonist concentration response relationship (bottom panels) of mGluRs1, 3, and 4 was measured.
Figure S4. VU0360172 Selectivity

Figure S4. VU0360172 is selective for mGluR5 versus other mGluR subtypes. Agonist concentration response relationship of mGluRs1, 3, and 4 in the presence and absence of 10 µM VU0360172 was measured.
Figure S5. VU0285683 competes with $[^3\text{H}]$methoxyPEPy

Figure S5. Antagonist VU0285683 fully competes with the equilibrium of $[^3\text{H}]$methoxyPEPy ($K_i = 16.9 \pm 1.1$). Competition binding curves for allosteric modulators were obtained in the presence of 2 nM $[^3\text{H}]$methoxyPEPy using membranes harvested from mGluR5-expressing HEK293 cells.
Figure S6. In vivo pharmacokinetic profile of VU0360172

Figure S6. In vivo pharmacokinetics of VU0360172 was studied in male SD rats after oral administration of 10 mg/kg dose in 20% hydroxypropyl β-cyclodextrin (BCD) solution. At times 0.5h, 1h, 3h and 6h after dosing, the concentrations of VU0360172 were measured in hepatic portal vein (HPV) plasma, systemic plasma (cardiac puncture), and whole brain tissues.