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Title Page

Functional impact of allosteric agonist activity of selective positive allosteric modulators of mGlu5 in regulating CNS function.

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Running Title Page

Running Title: Functional effects of $mGlu_5$ PAMs and ago-PAMs

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Nonstandard abbreviations:

mGlu, metabotropic glutamate receptor

CNS, central nervous system

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$mGlu_5$, metabotropic glutamate receptor subtype 5

NMDA, *N*-methyl-D-aspartate

PAM, positive allosteric modulator

Ago-PAM, allosteric agonist coupled with PAM activity

DMEM, Dulbecco's modified Eagle's medium

FBS, fetal bovine serum

VU0360172, *N*-cyclobutyl-6-((3-fluorophenyl)ethynyl)nicotinamide

VU0361747, (6-((3-fluorophenyl)ethynyl)pyridin-3-yl)(4-hydroxypiperidin-1-yl)methanone

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

VU0240382, 6-(2-phenylethynyl)-1,2,3,4-tetrahydroisoquinolin-1-one

MTEP, 3-((2-methyl-4-thiazolyl)ethynyl)pyridine

HEK, human embryonic kidney

GIRK, G protein-coupled inwardly-rectifying potassium channels

DMSO, dimethyl sulfoxide

HBSS, Hank's balanced salt solution

DHPG, dihydroxyphenylglycine

AGM, assay growth media

FDSS, functional drug screening system

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

aCSF, artificial cerebrospinal fluid

fEPSP, field excitatory postsynaptic potential

LTD, long-term depression

STN, subthalamic nucleus

ESI, electrospray ionization

i.p., intraperitoneally

MRM, multiple reaction monitoring

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CRC, concentration response curve

SC-CA1, Schaffer collateral-CA1

VU29, *N*-(1,3-DIPHENYL-1*H*-pyrazol-5-yl)-4-nitrobenzamind

CDPPB, 3-cyano-*N*-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide

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

LTP, long-term potentiation

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Abstract

Positive allosteric modulators (PAMs) of metabotropic glutamate receptor subtype 5 ($mGlu_5$) have emerged as an exciting new approach for the treatment of schizophrenia and other CNS disorders. Interestingly, some $mGlu_5$ PAMs act as pure PAMs, only potentiating $mGlu_5$ responses to glutamate whereas others (ago-PAMs) potentiate responses to glutamate and have intrinsic allosteric agonist activity in $mGlu_5$–expressing cell lines. All $mGlu_5$ PAMs previously shown to have efficacy in animal models act as ago-PAMs in cell lines, raising the possibility that allosteric agonist activity is critical for *in vivo* efficacy. We have now optimized novel $mGlu_5$ pure PAMs that are devoid of detectable agonist activity and structurally related $mGlu_5$ ago-PAMs that activate $mGlu_5$ alone in cell lines. Studies of $mGlu_5$ PAMs in cell lines revealed that ago-PAM activity is dependent on levels of $mGlu_5$ receptor expression in HEK293 cells whereas PAM potency is relatively unaffected by levels of receptor expression. Furthermore, ago-PAMs have no agonist activity in the native systems tested, including cortical astrocytes, subthalamic nucleus neurons, and in measures of long-term depression at the hippocampal Schaffer collateral-CA1 synapse. Finally, studies with pure PAMs and ago-PAMs chemically optimized to provide comparable CNS exposure revealed that both classes of $mGlu_5$ PAMs have similar efficacy in a rodent model predictive of antipsychotic activity. These data suggest that, the level of receptor expression influences the ability of $mGlu_5$ PAMs to act as allosteric agonists *in vitro* and that ago-PAM activity observed in cell-based assays may not be important for *in vivo* efficacy.

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Introduction

Metabotropic glutamate receptors (mGlus) are G protein-coupled receptors consisting of eight subtypes, termed $mGlu_{1-8}$. mGlu receptors participate in a wide range of functions throughout the central nervous system (CNS) and are thought to play roles in multiple disease states (Niswender and Conn, 2010). In recent years, one mGlu receptor subtype, metabotropic glutamate receptor subtype 5 ($mGlu_5$) has emerged as a novel target for treatment of schizophrenia and other disorders involving cognitive deficits. Current evidence suggests that schizophrenia may involve changes in activity through specific brain circuits involving glutamatergic transmission (Lisman et al., 2008; Marek et al., 2010) and that reduced activity of the *N*-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptor may induce similar circuit changes to those observed in patients with schizophrenia. Conversely, it has been postulated that increased NMDA receptor activation could reduce symptoms observed in schizophrenic patients (Ghoneim et al., 1985; Krystal et al., 1994). However, direct targeting of NMDA receptors is not practical due to potential toxicity that can occur with over activation of these ion channels (Hirose and Chan, 1993). Interestingly, $mGlu_5$ and the NMDA receptor are closely related signaling partners and activation of $mGlu_5$ potentiates NMDA receptor signaling in multiple brain circuits (Awad et al., 2000; Doherty et al., 2000; Mannaioni et al., 2001; O'Brien et al., 2004). Based on this observation, selective activation of $mGlu_5$ may provide an approach to compensate for the circuitry disruption that occurs in schizophrenia and similar disorders without inducing the adverse effects that occur with direct NMDA receptor activation.

Unfortunately, the orthosteric binding site of mGlu receptors is highly conserved across all eight mGlu receptor subtypes, making it difficult to develop selective $mGlu_5$ agonists (Conn and Pin, 1997). A major breakthrough came with discovery of $mGlu_5$-selective positive allosteric modulators (PAMs) that bind to a site on $mGlu_5$ that is spatially removed from the orthosteric glutamate site (Conn et al., 2009). These novel $mGlu_5$ PAMs provide high selectivity for $mGlu_5$

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relative to other mGlu receptor subtypes and have efficacy in a number of animal models used to predict antipsychotic and cognition-enhancing effects (for review, (Vinson and Conn, 2011)).

A broad range of $mGlu_5$ PAMs have been identified (Chen et al., 2007; Kinney et al., 2005; Liu et al., 2008; O'Brien et al., 2004; Rodriguez et al., 2010; Varnes et al., 2011) that induce robust increases in responses of $mGlu_5$ to glutamate. Interestingly, $mGlu_5$ PAMs can differ in ways that could have an important impact on their overall physiological and behavioral effects. For instance, some $mGlu_5$ PAMs behave as pure PAMs and have no intrinsic agonist activity (i.e., no activity in the absence of orthosteric agonist) when assessed using *in vitro* cell line assays, whereas others can serve as ago-PAMs to both potentiate responses to glutamate and directly activate $mGlu_5$ when added in the absence of orthosteric agonists. At present, the functional relevance of pure PAM versus ago-PAM activity is not known. It has been suggested that pure PAMs may be preferred to ago-PAMs in that the requirement for activation of $mGlu_5$ by endogenous glutamate would maintain normal patterns of receptor activity in response to neurotransmitter released from presynaptic terminals (Conn et al., 2009). However, all $mGlu_5$ PAMs that have been previously optimized for *in vivo* use have weak intrinsic agonist activity (Kinney et al., 2005; Liu et al., 2008; Rodriguez et al., 2010; Schlumberger et al., 2009), therefore, it is possible that this ago-PAM activity is critical for efficacy in animal models.

In order to directly determine the potential relevance of pure PAM versus ago-PAM activity in native systems, we chemically optimized $mGlu_5$ ago-PAMs that have maximal intrinsic agonist activity in cell lines along with close structural analogs that act as pure $mGlu_5$ PAMs and are devoid of intrinsic agonist activity. Maintaining these activity profiles while optimizing for appropriate pharmacokinetic profiles and brain penetration provided unique tools that allowed studies of the functional relevance of ago-PAM versus pure PAM activity. Using these compounds with cell lines engineered to have different levels of $mGlu_5$ expression, we found that ago-PAM activity is only seen in cell lines with high levels of $mGlu_5$ expression. Furthermore, all compounds examined behaved as pure PAMs in native systems studied,

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regardless of whether they exhibited agonist activity in overexpressing cell lines. Finally, pure PAMs and ago-PAMs showed identical efficacy in reversing amphetamine-induced hyperlocomotor activity, a model used to predict potential antipsychotic activity. Together, these data suggest that the presence of ago-PAM activity in $mGlu_5$-expressing cell lines is not a major determinant of activity in the systems studied and that pure PAM and ago-PAMs have similar physiological and behavioral effects.

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Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Invitrogen (Carlsbad, CA). DHPG was obtained from Ascent Scientific (Bristol, UK). VU0360172, VU0361747, VU0092273 (Rodriguez et al., 2010), VU0240382 (Williams et al., 2011) and MTEP were synthesized in house. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of an analytical grade.

Cell culture

Two different human embryonic kidney (HEK) cells lines stably expressing rat $mGlu_5$ (high and low expression) were maintained in complete DMEM supplemented with 10% FBS, 2 mM L-glutamine, 20 mM HEPES, 0.1 mM Non-Essential Amino Acids, 1 mM sodium pyruvate, antibiotic-antimycotic and G418 (500 µg/ml; Mediatech, Manassas, VA) at 37°C in a humidified incubator containing 5% CO_2. HEK293 cells stably expressing rat $mGlu_1$ were maintained in the same media as the $mGlu_5$ cells. HEK293 cells stably expressing G protein-coupled inwardly-rectifying potassium channels (HEK293-GIRK) along with the individual group II and group III mGlu receptors were maintained in growth media containing 45% DMEM, 45% F-12, 10% FBS, 20 mM HEPES, 2 mM L-glutamine, antibiotic/antimycotic, non-essential amino acids, G418 (700 µg/ml), and puromycin (0.6 µg/ml). HEK cells expressing human $mGlu_5$ under the control of a tetracycline promoter were maintained in the same media as the rat $mGlu_5$ cells except blasticidin (5 µg/ml) and zeocin (150 µg/ml) were substituted for G418. These cells were treated with increasing concentration of tetracycline hydrochloride (1-10 ng/ml) in media for 22 hours to induce receptor expression prior to their use in fluorescence-based calcium assays.

Fluorescence-based calcium assays

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For measurement of compound-evoked increases in intracellular calcium, HEK293 cells stably expressing rat $mGlu_5$ were plated in 96-well, poly-D-lysine coated, black-walled, clear-bottomed plates in 100 µL of assay medium (DMEM supplemented with 10% dialyzed fetal bovine serum, 20 mM HEPES and 1 mM sodium pyruvate) at a density of 40-50,000 cells/well. Cells were grown overnight at 37°C/5% CO_2. The next day, medium was removed from the cells and they were incubated with 75 µl/well of 2 µM Fluo-4AM (Invitrogen, Carlsbad, California) prepared as a 2.3 mM stock in dimethyl sulfoxide (DMSO) and mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and diluted in calcium assay buffer (Hank's Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA) supplemented with 20 mM HEPES and 2.5 mM probenecid, pH 7.4) for 1 hr at 37°C. Dye loading solution was removed and replaced with 90 µl/well of assay buffer. For PAM potency curves, $mGlu_5$ compounds were diluted at a 3X concentration and added to the cells at 20 seconds followed by the addition of a 10X concentration of an EC_{20} of glutamate or dihydroxyphenylglycine (DHPG) at 80 seconds into a 140 second protocol. The EC_{20} concentration was determined daily due to some variability (glutamate, high expression 150-200 nM, low expression 40-50 nM; DHPG, high expression 500 nM). For fold shift experiments multiple fixed concentrations of $mGlu_5$ compound (final concentrations of 30 nM - 30µM) or vehicle were added (3X stock) at 20 seconds followed by the addition of a concentration-response curve (CRC) of glutamate (10X stock) at 80 seconds into a 140 second protocol. All experiments were matched in solvent (DMSO) concentration. Calcium flux was measured over time as an increase in fluorescence using a FlexStation II (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 488 nm, an emission wavelength of 525 nm, and a cutoff wavelength of 515 nM. The increase in fluorescence over basal was determined for the peak and plateau phases of the response before being normalized to the maximal peak response elicited by glutamate (10-100 µM). Data were transformed and fitted using GraphPad Prism (Graph-Pad Software, Inc., San Diego, CA).

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Rat cortical astrocytes

Primary rat cortical astrocytes were received from Lonza (Basel, Switzerland) and stored in liquid nitrogen until use. Astrocytes were thawed following the protocol provided by Lonza and plated on CD Falcon Primaria dishes in assay growth media (AGM; assay basal media supplemented with AGM Singlequots from Lonza). Astrocytes were fed with AGM 4-5 hours after initial plating and then every 3-4 days until confluent. Astrocytes were plated in 96-well, poly-D-lysine coated, black-walled, clear-bottomed plates in 100 µl of AGM at a density of approximately 50,000 cells/well. The next day astrocytes were supplemented with G5 diluted 1:100 in AGM. The calcium flux assay was performed the following day using assay conditions and compound preparation identical to those used for the $mGlu_5$ HEK293 cell assay.

Radioligand Binding

HEK-293 cells expressing high or low levels of rat $mGlu_5$ or rat cortical astrocytes were assayed to determine the level of $mGlu_5$ receptor expression. Cells were harvested by trypsinization and pelleted by centrifugation for 3 min at 300 x*g*. Cells were re-suspended in calcium assay buffer. For saturation binding experiments cells were incubated with two concentrations of [^{3}H]methoxyPEPy (6 or 90 nM) for 1 hour at room temperature in calcium assay buffer. The K_d of [^{3}H]methoxyPEPy for rat $mGlu_5$ has been previously determined to be 5.7 nM. Non-specific binding was determined using 10 µM MPEP. Binding assays were terminated by rapid filtration through GF/B Unifilter plates (PerkinElmer Life and Analytical Sciences, Boston, MA) using a Brandel 96-well plate Harvester (Brandel Inc., Gaithersburg, MD), followed by three washes with cold (4°C) Binding Buffer (50 mM Tris-HCl, 0.9% NaCl, pH7.4), separating bound from free radioligand. Plates were allowed to dry overnight prior to addition of MicroScint 20 (40 µl/well; PerkinElmer). Radioactivity of bound radioligand was counted at least 2 hours after scintillation addition using a TopCount Scintillation Counter (PerkinElmer Life and Analytical Sciences, Boston, MA).

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Selectivity Screening

$mGlu_1$. HEK293 cells stably expressing rat $mGlu_1$ were plated in black-walled, clear-bottomed, poly-D-lysine coated 384-well plates (Greiner Bio-One, Monroe, NC) in 20 µl of assay medium at a density of 20,000 cells/well. Assays were performed the following day utilizing the High-Throughput Screening Center at Vanderbilt University. The medium was removed and replaced with assay buffer (HBSS, 20 mM HEPES pH 7.4, 2.5 mM probenecid) containing 1 µM Fluo4-AM using a Biotek ELX washer. The cells were incubated for 45 min at 37°C/5% CO_2 followed by a second ELX wash with assay buffer containing no dye. After a 10 min equilibration period, cell plates were introduced into the Functional Drug Screening System 6000 (FDSS 6000, Hamamatsu, Japan) for calcium flux measurements. To assess the effect of the modulator, either vehicle or a fixed concentration of test compound (10 µM) was added followed 140 seconds later by a concentration-response curve of glutamate. The change in relative fluorescence over basal was calculated before normalization to the maximal response to glutamate.

Group II and Group III mGlu's. The functional activity of the compounds of interest was assessed at the rat group II and III mGlu receptors by measuring thallium flux through GIRK channels as previously described in detail (Niswender et al., 2008). Briefly, HEK293-GIRK cells expressing mGlu subtype 2, 3, 4, 6, 7 or 8 were plated into 384-well, black-walled, clear-bottom poly-D-lysine coated plates at a density of 15,000 cells/well in 20 µl of assay medium. The following day, the medium was removed and replaced with assay buffer (HBSS, 20 mM HEPES, pH7.4) supplemented with 0.16 µM FluoZin2-AM (Invitrogen, Carlsbad, CA). (See methods above for further details.) A single concentration of test compound (10 µM) or vehicle was added followed 140 seconds later by a concentration response curve of glutamate (or L-AP4 for $mGlu_7$) diluted in thallium buffer (125 mM $NaHCO_3$, 1 mM $MgSO_4$, 1.8 mM $CaSO_4$, 5 mM

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glucose, 12 mM thallium sulfate, 10 mM HEPES) and fluorescence was measured using a FDSS 6000. Data were analyzed as described previously (Niswender et al., 2008).

Brain slice electrophysiology

Extracellular field potential recordings. All animals used in these studies were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Young adult (5-6 week) male Sprague–Dawley rats (Charles River, Wilmington, MA) were anesthetized with isoflorane, decapitated and the brains were quickly removed and submerged into ice-cold cutting solution (in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 5 D-glucose, 0.6 (+)-sodium-L-ascorbate, 0.5 $CaCl_2$, 7 $MgCl_2$) continuously bubbled with 95% O_2/5% CO_2. The brains were then hemisected and 400 µm transverse slices were made using a vibratome (Leica VT100S; Leica Microsystems, Nussloch, Germany). Individual hippocampi were microdissected from the slice and transferred to a room temperature mixture containing equal volumes of cutting solution and artificial cerebrospinal fluid (aCSF; in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 2 $CaCl_2$, 1 $MgCl_2$) where they were allowed to equilibrate for 30 minutes. The hippocampi were then transfered to aCSF continuously bubbled with 95% O_2/5% CO_2 for an additional hour. Slices were transferred to a submersion recording chamber and allowed to equilibrate for 5-10 minutes at 30-32°C with a flow rate of 2 ml/min. A bipolar-stimulating electrode was placed in the stratum radiatum near the CA3-CA1 border in order to stimulate the Schaffer collaterals. Recording electrodes were pulled with a Flaming/Brown micropipette puller (Sutter Instruments, CA), fillled with aCSF and placed in the stratum radiatum of area CA1. Field potential recordings were acquired using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and pClamp 9.2 software (Molecular Devices). Input–output curves were generated to determine the stimulus intensity that produced 40–60% of the maximum field excitatory postsynaptic potential (fEPSP) slope before each experiment. This submaximal fEPSP slope was used as the

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baseline stimulation for the remainder of the individual experiment. $mGlu_5$ compounds were diluted to the appropriate concentrations in DMSO (0.1%) in aCSF and applied to the bath for 10-20 minutes using a perfusion system. Chemically induced mGlu long-term depression (LTD) was initiated by the application of DHPG in aCSF (25–75 μM) for 10 min. Sampled data was analyzed offline using Clampfit 9.2. Three sequential fEPSPs were averaged and their slopes calculated. All fEPSP slopes were normalized to the average slope calculated during the predrug period (percent of baseline). Data were analyzed using GraphPad Prism.

Whole-cell patch-clamp recordings. Whole-cell patch-clamp recordings were performed using midbrain slices prepared from 15- to 19-day-old Sprague-Dawley rats (Charles River). After decapitation, brains were removed quickly and submerged into ice-cold cutting solution (in mM: 200 sucrose, 1.9 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 10 D-glucose, 0.5 $CaCl_2$, 6 $MgCl_2$) and equilibrated with 95% O_2/5% CO_2. Brains were hemisected and sagittal brain slices containing the subthalamic nucleus (STN) were cut at 300 μm using a vibratome (Leica VT 100S). Slices were transferred to a holding chamber containing aCSF (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 D-glucose, 2 $CaCl_2$, 1 $MgCl_2$) supplemented with 5 μM glutathione, to increase slice viability, continuously bubbled with 95% O_2/5% CO_2 at 32°C and incubated for 30 minutes. Slices were then maintained at room temperature in aCSF equilibrated with 95% O_2/5% CO_2 until they were transferred to the submersion recording chamber and perfused with room temperature aCSF at a rate of 2 ml/min. The same protocol was used for striatal slices, except slices were cut coronally. Neurons in the STN or striatum were visualized with a 40X water immersion lens with Hoffman modulation contrast optics coupled with an Olympus BX50WI upright microscope (Olympus, Lake Success, NY). Borosilicate glass patch electrodes were pulled using a Flaming/Brown micropipette puller (Sutter Instruments, CA) with a resistance of 2-5 MΩ when filled with an intracellular solution containing (in mM: 123 potassium gluconate, 7 KCl, 0.025 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 0.1

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EGTA, 2 sodium-ATP, 0.2 sodium-GTP; pH adjusted to 7.3 with 1 N KOH; mOsm 290). All whole-cell patch-clamp recordings were performed using a MultiClamp 700B amplifier (Molecular Devices). Data were digitized with a DigiData 1331 system (Molecular Devices), filtered at 2 kHz and acquired using a pClamp9.2 program (Molecular Devices). After formation of a whole-cell configuration cells were current-clamped to -60 mV for STN neurons; medium spiny neurons were maintained at their normal resting potential (around -75 mV) and changes in membrane potential were recorded. Data were analyzed using Clampfit 9.2 (Molecular Devices).

Brain Homogenate Binding.

The brain homogenate binding of each compound was determined in rat brain homogenates via equilibrium dialysis employing RED Plates (ThermoFisher Scientific, Rochester, NY) as previously described (Wan et al., 2007). Briefly, brain tissue homogenate from rats was prepared by diluting one volume whole brain tissue with three volumes of phosphate buffer (25 mM, pH 7.4). The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products, Bartlesville, OK). Brain homogenate (220 µL) was added to the 96-well plate containing test compound (5 µL) and mixed thoroughly for a final concentration of 5 µM of test compound. Subsequently, 200 µL of the brain homogenate-compound mixture was transferred to the *cis* chamber (red) of the RED plate, with an accompanying 350 µL of phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated for 4 hours at 37°C with shaking. At completion, 50 µL aliquots from each chamber were diluted 1:1 (50 µL) with either brain homogenate (*cis*) or buffer (*trans*) and transferred to a new 96-well plate, at which time ice-cold acetonitrile containing 50 nM carbamazapine as an internal standard was added to extract the matrices. The plate was centrifuged (3000 rpm, 10 minutes) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96-well plate, which

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was then sealed in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. Brain homogenate binding samples were analyzed using LC/MS/MS techniques on a Thermo Electron TSQ Quantum Ultra triple quad detector via electrospray ionization (ESI) with two Themo Electron Accella pumps (San Jose, CA), and a Leap Technologies CTC PAL autosampler. Analytes were separated by gradient elution on a dual column system with two Waters acquity BEH C18, 2.1x50 mm, 1.7 µm columns (Milford, MA) heated at 40°C. HPLC mobile phase A was 0.1 % formic acid in water and mobile phase B was 0.1 % formic acid in acetonitrile. Pump 1 runs the gradient: 90:10 (A:B) at 800 µL/min hold 0 to 0.3 minutes, linear ramp to 5:95 (A:B) 0.3 to 0.8 minutes, 5:95 (A:B) hold 0.8 to 1.2 minutes, return to 90:10 (A:B) at 1.3 minutes. While pump 1 runs the gradient method, pump 2 equilibrates the other column isocratically with 90:10 (A:B). The total run time is 1.5 minutes per injection. All compounds are optimized using Thermo Electron's QuickQuan software.

$$f_{\mathrm{u-tissue}} = \frac{1/D_{\mathrm{f}}}{(1/f_{\mathrm{u-hom}} - 1) + 1/D_{\mathrm{f}}} = \frac{f_{\mathrm{u-hom}}}{(D_{\mathrm{f}} - (D_{\mathrm{f}} - 1)f_{\mathrm{u-hom}})}$$

where $f_{\mathrm{u\text{-}hom}}$ and D_{f} represent the measured fraction unbound in dilute homogenate and the dilution factor, respectively (Wan et al., 2007).

A critical question for optimizing novel $mGlu_5$ PAMs as potential therapeutic agents or *in vivo* tools is whether ago-PAM activity is important for *in vivo* efficacy. To address this important question, it is essential to achieve adequate pharmacokinetic and physiochemical properties that provide sufficient CNS exposure to assess *in vivo* efficacy following systemic dosing. In addition to optimizing these $mGlu_5$ PAMs to achieve pure PAM versus ago-PAM activity, chemistry efforts were also made to optimize the molecules to achieve high CNS exposure for representatives of each class. Free fraction of novel CNS-active compounds in the brain is a critical parameter for achieving high brain exposure. In general, we set a criterion of achieving at least 1% free fraction in assays of brain homogenate binding before advancing to *in*

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vivo pharmacokinetic studies. Incubation of compound with brain homogenate samples resulted in a free fraction of 2.9% for VU0360172, 9.6% for VU0361747 and 2.5% for VU0092273 (Supplemental Figure 1). In contrast, the free fraction of VU0240382 was very limited at 0.01%, which prohibited it from being assessed further for use in behavioral studies (Supplemental Figure 1).

***In Vivo* Pharmacokinetic Analysis.**

Test compound was formulated as 10% Tween 80 in sterile water at the concentration of 3.33 mg/ml and administered intraperitoneally (i.p.) to male Sprague-Dawley rats weighing 275 to 300 g (Harlan, Indianapolis, IN) at a dose of 10 mg/kg. Rat blood (cardiac puncture) and brain were collected at 0.25, 0.5, 1, 3, and 6 hours. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed in 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 diluted with 1:3 (w/w) parts of 70:30 isopropanol:water. The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products). The sample extraction of plasma (20 µl) and brain homogenate (20 µl) was performed by a method based on protein precipitation using three volumes of ice-cold acetonitrile containing an internal standard (carbamazepine) with a final concentration of 50 ng/ml. Extracts were centrifuged at 4000 x g for 5 minutes. The supernatants of plasma and brain homogenate extracts were then diluted 1:1 with water.

Samples were analyzed via ESI on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 2.1 x 50 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH

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unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 10% B after a 0.2 minute hold and was linearly increased to 90% B over 0.8 minutes; held at 90% B for 0.5 minutes and returned to 30% B in 0.1 minute followed by a re-equilibration (0.9 min). The total run time was 2.5 minutes and the HPLC flow rate was 0.5 ml/min. The source temperature was set at 50°C and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage). 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, VU0361747, or VU0092273 in blank brain homogenates and plasma. All data were analyzed using AB Sciex Analyst 1.5.1 software. The final pharmacokinetic parameters were calculated by noncompartmental analysis using WinNonlin software (version 5.1; Pharsight Inc., Mountain View, CA).

In vivo pharmacokinetic analysis revealed that VU0360172, VU0361747 and VU0092273 demonstrated rapid and significant absorption following i.p. dosing (Supplemental Figure 1). Maximum plasma concentrations of 4450 ng/ml of VU0360172 were observed at 0.5 hour post administration. VU0361747 also reached peak plasma levels (4690 ng/ml) at 0.5 hour, while VU0092273 obtained a maximum concentration of 4863 ng/ml at 1 hour. All three allosteric modulators also had rapid and significant uptake into the brain following systemic administration (Supplemental Figure 1). VU0360172 reached maximum levels of 2000 ng/g in whole-brain tissues with a t_{max} of 0.5 hour and AUC_{brain}:AUC_{plasm} of 0.46. The t_{max} of VU0092273 was 0.5 hour with C_{max} of 19261 ng/g and AUC_{brain}:AUC_{plasm} of 4.47. Similar to the t_{max} of plasma, VU0361747 reached maximum brain concentrations of 5739 ng/g at 0.5 hour with 1.12 AUC_{brain}:AUC_{plasm}. These pharmacokinetic properties, including brain to plasma ratios and acceptable free fractions, of each of these compounds support their use for *in vivo* behavioral testing and provide an optimal dose regimen for behavioral studies.

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Amphetamine-Induced Hyperlocomotion

Subjects. Studies were conducted using male Sprague-Dawley rats (Harlan) weighing 280 to 310 g. Subjects were housed three per cage under a 12-h light/dark cycle (lights on at 6:00 AM) with access to food and water *ad libitum*. Testing procedures were performed between 7:00 AM and 6:00 PM. Dose groups consisted of 5 to 12 rats per group. All test compounds were dissolved in 10% Tween 80 and double-deionized water and the pH was adjusted to approximately 7.0 using 1 N NaOH. Test compounds were administered i.p. at a dose of 56.6 mg/kg in a 3 ml/kg volume. All experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee.

Apparatus. A SmartFrame Open Field System (KinderScientific, San Diego, CA) equipped with 32 horizontal (*x*- and *y*-axes) infrared photobeams positioned 1 cm above the floor of the chamber was used to conduct the studies. Ambulation or locomotor activity was measured as the number of total photobeam breaks per 5-minute interval, and was recorded with a Pentium I computer equipped with Motor Monitor System software (KinderScientific).

Procedure. Rats were placed in the open-field chambers and allowed to habituate for 30 minutes followed by i.p. pretreatment with vehicle or test compound. After an additional 30 minutes, rats received a saline vehicle or 1 mg/kg amphetamine subcutaneous injection. Locomotor activity was measured for an additional 60 minutes.

Analysis. Main effects of test compound treatment on the locomotor activity area under the time course curve were evaluated using one-way analysis of variance. Comparisons of treatment group effects relative to the vehicle + amphetamine group were completed across the time interval from t = 60 to 120 min using Dunnett's post hoc tests. $p < 0.0001$ versus vehicle + amphetamine group.

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Results

Discovery of structurally related pure mGlu5 PAMs and mGlu5 ago-PAMs.

We recently identified a novel series of $mGlu_5$ PAMs that is derived from a biaryl acetylene scaffold (Rodriguez et al., 2010; Williams et al., 2011) (Figure 1). We chose to examine two scaffolds within the biaryl acetylene class based upon the identity of the eastern aryl ring with the presence of either a phenyl or nicotinamide-based structure. In initial experiments, we used an established cell line that expresses a high density of $mGlu_5$ (2.3 +/-0.4 pmol/mg) and has been used for discovery and extensive characterization of $mGlu_5$ PAMs in our previous studies (Chen et al., 2007; Rodriguez et al., 2010; Rodriguez et al., 2005; Williams et al., 2011). This cell line was used to support an extensive chemical optimization and pharmacological characterization effort in which we identified VU0360172 and VU0092273 as $mGlu_5$ PAMs that exhibit robust ago-PAM activity. This profile can be seen in Figure 2A where VU0360172 and VU0092273 (10 μM) induce strong agonist responses upon addition in the absence of glutamate and also potentiate the response to glutamate (EC_{20}) that is added 60 seconds after addition of the test compound. We also identified two close structural analogs of VU0360172 and VU0092273 termed VU0361747 and VU0240382 (Figure 1). In contrast to VU0360172 and VU0092273, VU0361747 and VU0240382 behaved as pure PAMs *in vitro* (Figure 2B) and did not activate $mGlu_5$ when added alone (10 μM) but potentiated the response to a subsequent addition of an EC_{20} concentration of glutamate (Figure 2B). Figure 2C shows a full concentration-response relationship for agonist activity of each compound in the absence of glutamate addition. Consistent with the responses to addition of 10 μM concentrations of each PAM, VU0360172 and VU0092273 induced concentration-dependent activation of $mGlu_5$ with potencies of 220 ± 25 nM and 1.3 ± 0.2 μM, respectively. In contrast, VU0361747 and VU0240382 induced little or no activation of $mGlu_5$ at any concentration tested (Figure 2C). We then established the potencies of each compound as a PAM by evaluating the effects of multiple concentrations of each compound in the presence of an EC_{20} concentration of glutamate (Figure

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2D). Each of the $mGlu_5$ PAMs tested induced a concentration-dependent potentiation of the response to glutamate with EC_{50} values of 13 ± 2 nM (VU0360172), 35 ± 5 nM (VU0092273), 126 ± 23 nM (VU0361747), and 39 ± 10 nM (VU0240382) (mean ± SEM, n=3-6). While the agonist activity of these compounds has not been previously evaluated, these potencies for the PAM response agree with values that were reported previously by Rodriguez et al. (2010) for VU0360172, VU0092273, and VU0361747.

$mGlu_5$ allosteric modulators are selective for $mGlu_5$

Discovery of structurally related $mGlu_5$ PAMs that have robust ago-PAM activity versus pure PAM activity could provide important new tools for understanding the functional impact of these two classes of compounds. However, to use these as tools in later physiological or behavioral assays, it is important to establish whether they are selective for $mGlu_5$ relative to other mGlu receptor subtypes. We evaluated the effects of 10 μM of each compound on its ability to shift the glutamate concentration-response curve (CRC) in calcium mobilization ($mGlu_1$) or thallium flux ($mGlu_{2, 3, 4, 6, or 8}$) assays using cell lines that have been extensively used to characterize the effects of allosteric modulators of each of the other mGlu receptor subtypes (Niswender et al., 2008; Rodriguez et al., 2010). For $mGlu_7$, L-AP4 was employed as the agonist in the thallium flux assay due to the low potency of glutamate at this mGlu receptor subtype. All of the compounds were inactive in shifting the concentration response curves for glutamate in cells expressing other mGlu receptor subtypes (Supplemental Figure 2). The one exception was VU0092273, which slightly shifted the glutamate CRC at $mGlu_3$ to the right and downward, suggesting that VU0092273 may have weak antagonist activity at $mGlu_3$. This is in agreement with the previously reported effects of VU0092273 including very weak inhibition of the $mGlu_3$ response (Rodriguez et al., 2010).

Receptor expression level influences the shift from pure PAM to ago-PAM activity

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We next assessed the influence of receptor expression level on the responses to these $mGlu_5$ ago-PAMs and pure PAMs. To accomplish this, we developed a new HEK293 cell line that was selected for expression of lower levels of $mGlu_5$. After extensive characterization of this cell line to assess $mGlu_5$ density and to insure appropriate responses to orthosteric ligands (data not shown), we compared the activity of these four compounds in our original cell line ($mGlu_5$ B_{max} = 25.2 +/- 1.5 fmol/100,000 cells) to the phenotypes induced in a second cell line that was developed to provide lower $mGlu_5$ expression ($mGlu_5$ B_{max} = 7.8 +/- 0.6 fmol/100,000 cells). In contrast to the effects observed with the high expressing cell line, none of the compounds tested induced an agonist response in the cell line with a lower density of $mGlu_5$ receptors (Figure 3A, 3B). However, glutamate induced a concentration-dependent activation of $mGlu_5$ (Figure 3B) with a potency similar to that observed in the higher expressing cell lines (glutamate EC_{50} = 200 ± 42 nM (n=4) vs. 360 ± 70 nM (n=6) in the high expressing cell line, Figure 2C). Furthermore, all compounds potentiated the response to an EC_{20} concentration of glutamate (Figure 3C) with potencies similar to those observed for potentiation of glutamate responses in the high expression line (EC_{50} values: VU0360172 39 ± 5 nM; VU0092273 122 ± 19 nM; VU0240382 64 ± 10 nM; VU0361747 200 ± 5 nM; mean ± SEM, n=3-4). These data suggest that the ability of $mGlu_5$ ago-PAMs to activate the receptor in the absence of orthosteric agonist may be highly sensitive to levels of receptor expression.

To increase confidence that the difference between responses to the $mGlu_5$ PAMs in the two cell lines is related to differences in receptor expression, we engineered a third HEK293 cell line in which human $mGlu_5$ expression is under control of a tetracycline-inducible promoter. Cells were treated with vehicle or increasing concentrations of tetracycline (1-10 ng/ml) to induce $mGlu_5$ expression. Radioligand binding studies revealed that cells treated with vehicle had low expression of $mGlu_5$ and that increasing concentrations of tetracycline induced corresponding increases in $mGlu_5$ density (Supplemental Figure 3). VU0360172 is the most potent compound with both agonist and PAM activity and, therefore, was chosen for these

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studies. As in the previous studies with the lower expressing cell line, VU0360172 induced robust potentiation of the response to glutamate in this inducible line when no tetracycline was added and $mGlu_5$ expression was low. However, VU0360172 had no agonist activity in cells where $mGlu_5$ expression was not induced (Supplemental Figure 3). With increasing concentrations of tetracycline and corresponding increases in receptor expression there was a shift from pure PAM activity to agonist activity coupled with PAM activity for VU0360172 (Supplemental Figure 3). This provides strong evidence that increased expression of $mGlu_5$ can confer ago-PAM activity that is not present in cells expressing lower levels of the receptor. Importantly, the degree of agonism was not as pronounced in this cell line as was observed with the high expression rat $mGlu_5$ cell line (Figure 2). One possible explanation for the less robust agonism is species differences between rat and human. Since our further studies rely on use of rodent tissue, we focused further studies on use of the rat lower-expressing cell line.

We further evaluated the ability of the $mGlu_5$ compounds to shift the glutamate concentration-response curve. Incubation of the low expression rat $mGlu_5$ cell line with increasing fixed concentrations of $mGlu_5$ modulators (30 nM – 30 μM) followed by a concentration-response curve of glutamate (10 nM - 100 μM) resulted in progressive leftward shifts of the glutamate concentration-response curve for each of the compounds with maximal fold shift values of 6.8 fold at 3 μM VU0360172, 1.9 fold at 30 μM VU0361747, 5.4 fold at 10 μM VU0092273 and 4 fold at 10 μM VU0240382 (Figure 4, n=3-8). In this case, the lower fold shift value for VU0361747 compared to the other three compounds corresponds with the lower potency of this compound compared to the other $mGlu_5$ compounds studied. Slightly higher fold shift values were observed for all of the compounds with the high expression $mGlu_5$ cell line (VU0360172, 8.7 fold at 100 nM; VU0092273, 9.1 fold at 300 nM; VU0361747, 8.4 fold at 10 μM; and VU0240382, 10.4 fold at 10 μM; Supplemental Figure 4, n=3-5). It was necessary to run the fold shift experiments with VU0360172 and VU0092272 at lower concentrations to avoid the complications of agonist activity when assessing the response in the high $mGlu_5$ expression

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cell line. The difference in fold shift values could be a result of differences in coupling efficiency or signaling between the two cell lines. The potential for differences in coupling efficiency or signaling between the cell lines could also explain why in the high expression $mGlu_5$ cell line the compounds elevate the maximum response to glutamate above one hundred percent (Figure 2D), but are unable to do so in the lower expressing cell lines (Figure 3C).

Ago-PAM activity is not observed in cortical astrocytes.

The studies of effects of $mGlu_5$ PAMs and ago-PAMs in HEK293 cells raise the possibility that ago-PAM activity for these compounds may be a function of overexpression of $mGlu_5$ or other factors that could impact responses in recombinant systems. Availability of new $mGlu_5$ PAMs that are optimized for robust agonist activity provides tools to allow us to determine whether this translates into agonist activity in native systems. As a first method to evaluate effects of these compounds in a native system, we determined the effects of these pure PAMs and ago-PAMs in rat cortical astrocytes, where $mGlu_5$–mediated responses have been extensively characterized (Chen et al., 2008; Peavy and Conn, 1998; Zhang et al., 2005). Radioligand binding studies revealed that the level of $mGlu_5$ expression in rat cortical astrocytes was similar to that of the low expression $mGlu_5$ HEK cell line ($mGlu_5$ B_{max} = 3.4 +/- 0.3 fmol/100,000 cells). Interestingly, responses to the $mGlu_5$ PAMs in astrocytes were virtually identical to those observed in the HEK293 cells expressing lower levels of $mGlu_5$ (Figure 5). Thus, none of the compounds tested induced an $mGlu_5$ agonist response when added alone (Figure 5A). However, each of the $mGlu_5$ PAMs induced a concentration-dependent potentiation of the response to an EC_{20} concentration of glutamate with potencies similar to those observed in HEK293 cells (Figure 5B; EC_{50} VU0360172 80 ± 6 nM; VU0092273 168 ± 12 nM; VU0240382 215 ± 48 nM; VU0361747 504 ± 98 nM; mean ± SEM, n=3).

$mGlu_5$ PAMs do not exhibit probe-dependence for DHPG.

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Studies in astrocytes suggest that members of this class of biaryl acetylene $mGlu_5$ PAMs do not have agonist activity in at least one native system. To further assess activity of these on CNS function, it will be important to evaluate their effects in brain slice preparations. However, it is not practical to use glutamate as an $mGlu_5$ agonist in brain slice electrophysiology studies since it has multiple targets; mGlu receptors, ionotropic glutamate receptors, and glutamate transporters. For studies of $mGlu_5$ responses in brain slices, we routinely use DHPG, a group I orthosteric agonist that selectively activates $mGlu_1$ and $mGlu_5$. However, before advancing to studies with DHPG in brain slices, it is important to determine whether these compounds induce similar potentiation of responses to DHPG as they do to glutamate. Previous studies have shown that allosteric modulators can display strong probe-dependence and potentiate responses to some but not all orthosteric agonists (Keov et al., 2011). Thus, we used calcium fluorescence assays to determine if there is a probe-dependent effect of the ability of the compounds to potentiate DHPG compared to glutamate in the high expression $mGlu_5$ cell line. In all cases, the $mGlu_5$ PAMs potentiated the EC_{20} response to DHPG in a similar manner to glutamate (Supplemental Figure 5; EC_{50} VU0360172 6.6 ± 2.7 nM; VU0092273 34 ± 14 nM; VU0240382 42 ± 5 nM; VU0361747 124 ± 16 nM; mean ± SEM, n=4). These results support the use of DHPG, in place of glutamate, in electrophysiology experiments to study the potentiation of the $mGlu_5$ response by these allosteric modulators.

$mGlu_5$ PAMs potentiate DHPG-induced LTD at the hippocampal SC-CA1 synapse.

One of the most well characterized responses to $mGlu_5$ activation is induction of a form of synaptic plasticity termed long-term depression (LTD) at the Schaffer collateral – CA1 (SC-CA1) synapse in the hippocampal formation (Ayala et al., 2009; Huber et al., 2001; Malenka and Bear, 2004). Orthosteric $mGlu_5$ agonists induce profound LTD at this synapse and this can be potentiated by previously reported $mGlu_5$ PAMs (Ayala et al., 2009). If the novel ago-PAMs used in this study have agonist activity at the SC-CA1 synapse, they will be expected to induce LTD

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when added alone. In contrast, if these compounds act as pure PAMs, they should potentiate responses to DHPG but would not be expected to induce LTD in the absence of agonist. To assess this, extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from the dendritic layer of CA1 following stimulation of the SC-CA1 synapse. In agreement with our previous studies (Ayala et al., 2009), we found that 75 μM DHPG induced robust depression of synaptic responses that persisted at least 55 minutes following washout (Figure 6A). LTD was defined as the level of depression 55 min after washout of DHPG (55.3 ± 4.2 % of baseline). In contrast to DHPG, 3 μM VU0360172 did not induce a LTD response when added alone (Figure 6B), with a mean fEPSP slope of 97.1 ± 4.3% of baseline 55 min after compound washout. Similarly, treatment of slices with 10 μM VU0360172 alone did not result in the induction of LTD (fEPSP slope 91.6 ± 5.1% of baseline) and there was only a small transient decrease in fEPSP slope in two of the seven slices assessed (Supplemental Figure 6A).

We next evaluated the effects of each of the $mGlu_5$ PAMs on fEPSP slope both alone and in the presence of a low concentration of DHPG that was below the concentration needed to elicit LTD. Consistent with our previous report (Ayala et al., 2009), 25 μM DHPG induced an acute depression of fEPSP slope but did not induce LTD measured 55 minutes after washout (Figure 7A; fEPSP slope = 95.7 ± 4.8% of baseline). Based on these findings, all subsequent potentiation experiments were conducted with 25 μM DHPG. Each of the $mGlu_5$ PAMs was applied for 10 minutes followed by co-application of the PAM and 25 μM DHPG for 10 minutes. In each case, 10μM of the $mGlu_5$ PAM alone (VU0361747, VU0092273 or VU0240382) failed to significantly reduce fEPSP slope during the 10 minute incubation before DHPG. This is consistent with the results with VU0360172 and suggests that these compounds do not have intrinsic agonist activity in inducing LTD. However, each $mGlu_5$ PAM induced a significant ($p < 0.001$) enhancement of LTD induced by 25 μM DHPG measured 55 minutes after washout of compound (Figure 7; B) VU0360172 39.2 ± 6.2 % of baseline, n=7; C) VU0361747 63.1 ± 5.0 % of baseline, n=6; D) VU0092273 51.6 ± 5.8 % of baseline, n=7; E) VU0240382, 68.6 ± 4.7 %

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of baseline, n=8; mean ± SEM). The $mGlu_5$ antagonist MTEP (10 μM) blocked induction of LTD when VU0360172, 3 μM (97.3 ± 6.0 % of baseline) or 10 μM (80.9 ± 3.2 % of baseline), was administered in combination with 25 μM DHPG assessed 55 minutes after washout of the compound (Supplemental Figure 6B). These results confirm the role of $mGlu_5$ in mediating this response.

VU0360172 has no agonist activity at $mGlu_5$ in multiple brain regions assessed using slice electrophysiology

VU0360172 has the most potent agonist activity of the compounds tested in the high $mGlu_5$ expression cell line. Therefore, we further evaluated this $mGlu_5$ PAM to see if it has agonist activity at inducing other known $mGlu_5$-mediated responses. $mGlu_5$ is localized postsynaptically on STN neurons and activation of $mGlu_5$ in these neurons leads to depolarization (Awad et al., 2000). Previous work has shown that $mGlu_5$ PAMs VU29, CDPPB and CPPHA potentiate membrane depolarization induced by DHPG in STN neurons (Chen et al., 2007; O'Brien et al., 2004; Rodriguez et al., 2005). Consistent with our previous reports, 100 μM DHPG resulted in a robust depolarization of STN neurons (12.1 ± 2.1 mV; mean ± SEM, n=6; Supplemental Figure 7). In contrast, bath application of 10 μM VU0360172 had no effect on membrane voltage (0.6 ± 0.7 mV, mean ± SEM, n=5). Additionally, 10 μM VU0360172 had no effect on spontaneous firing of STN neurons whereas DHPG induced a robust increase in spontaneous firing in STN neurons (data not shown). Further experiments were undertaken in medium spiny neurons in striatum, which have been shown to express higher levels of $mGlu_5$ compared to STN (Kerner et al., 1997; Pisani et al., 2001; Testa et al., 1995). Once again 10 μM VU0360172 did not mimic the established effects of DHPG in these cells (data not shown). Taken together these results suggest that VU0360172 does not have intrinsic $mGlu_5$ agonist activity in any of the native systems studied.

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VU0360172, VU0361747 and VU0092273 significantly reduced amphetamine-induced hyperlocomotion

The primary impetus for discovery and characterization of $mGlu_5$ PAMs has been their potential use as a treatment for schizophrenia. A well-characterized *in vivo* behavioral assay that has been used to evaluate potential antipsychotic efficacy of $mGlu_5$ PAMs is assessing their ability to reverse amphetamine-induced hyperlocomotor activity in rodents (Kinney et al., 2005; Liu et al., 2008; Rodriguez et al., 2010). Interestingly, all $mGlu_5$ PAMs that have previously been evaluated for activity in animal models that predict antipsychotic activity have possessed ago-PAM activity. Thus, it is not clear whether agonist activity as measured in cell lines is an important property to confer *in vivo* efficacy in animal models. Therefore, we determined the effects of the $mGlu_5$ ago-PAMs, VU0360172 and VU0092273 and the pure PAM, VU0361747 on amphetamine-induced hyperlocomotor activity. Consistent with the findings of Rodriguez et al. (2010), administration of VU0360172 significantly (p <0.0001) reversed amphetamine-induced hyperlocomotion when analyzed from the time of amphetamine delivery to the end of testing period (60–120 minutes). Thus, the group receiving 56.6 mg/kg i.p. VU0360172 prior to amphetamine administration produced significantly fewer ambulations (p < 0.0001) than the group receiving vehicle and amphetamine. This represents a 56% reduction in the hyperlocomotor response (Figure 8A). Furthermore, VU0092273, another ago-PAM in *in vitro* assays, also significantly (p < 0.0001) reduced the increase in locomotor activity caused by subcutaneous administration of amphetamine with a maximum reversal of 60% (Figure 8C). Finally, the pure $mGlu_5$ PAM, VU0361747 (56.6 mg/kg), significantly reversed amphetamine-induced hyperlocomotion in a manner similar to that observed with the compounds that possess ago-PAM activity in HEK cells expressing high levels of $mGlu_5$. Ambulations in rats receiving VU0361747 were reduced by 53% (Figure 8B). These data suggest that compounds optimized as pure PAMs have similar activity in this animal model when compared to compounds optimized to have robust ago-PAM activity in overexpressing cell lines.

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Discussion

Preclinical studies suggest that selective activation of mGlu receptors may be beneficial for the treatment of various disorders of the CNS. Current focus has been on identifying compounds that act at allosteric sites on these receptors, which are less conserved across subtypes, in order to achieve a greater degree of subtype selectivity. Previously, we reported that allosteric modulators of $mGlu_5$ can display a range of activities from PAM to NAM to neutral cooperativity with slight structural modifications (O'Brien et al., 2004; Rodriguez et al., 2010; Rodriguez et al., 2005). In some cases, individual scaffolds can be strongly biased towards dominant PAM or NAM activity; however, additional chemical exploration has shown that for some series it is possible to identify novel opposing molecular switches that can completely alter the ligand's pharmacological function (Schann et al., 2010; Sharma et al., 2009). We recently reported results of an $mGlu_5$ high-throughput screen resulting in the identification of VU0092273 (Rodriguez et al., 2010) and related monocyclic and bicyclic biaryl acetylene compounds, some of which are the most potent $mGlu_5$ PAMs to date. In addition, these agents can have different profiles in different cell populations, such as pure PAM or ago-PAM activity. This intrinsic agonist or ago-PAM activity has been observed for a number of $mGlu_5$ PAMs across multiple laboratories, but the functional relevance of the agonist activity of $mGlu_5$ PAMs has not been clear.

In the present studies, we took advantage of novel $mGlu_5$ PAMs that were systematically optimized and evaluated to favor strong agonist activity and closely related compounds that did not display robust agonist activity in the primary cell lines (high expression) that have been used for discovery of novel $mGlu_5$ PAMs. Using these compounds and new cell lines engineered to have lower levels of $mGlu_5$ expression, we found that ago-PAM activity is only observed in cell lines expressing higher levels of $mGlu_5$. Furthermore, we utilized a separate cell line in which human $mGlu_5$ is expressed under an inducible promoter and found that, consistent with our observations in the high and low expression rat $mGlu_5$ cell lines, the most-potent ago-PAM,

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VU0360172 has agonist activity when $mGlu_5$ expression is induced to high levels but has pure PAM activity when $mGlu_5$ expression is maintained at lower levels. These studies suggest that overexpression of $mGlu_5$ in cell lines may be one factor that contributes to the presence ago-PAM activity for some allosteric modulators. However, while overexpression of $mGlu_5$ may be an important factor in the observation of ago-PAM activity, other factors may also contribute to the ability of some compounds to directly activate the receptor along with other factors that strictly depend on activation by glutamate.

While it is impossible to definitively relate expression levels to individual neuronal populations or subcellular compartments in neurons, it is possible that certain locations in the brain may exhibit higher levels of $mGlu_5$ relative to other areas. Also, other factors that may influence the ability of some compounds to act as ago-PAMs and others as pure PAMs could exist in native systems. Thus, it will be important to evaluate whether the agonist activity observed *in vitro* under conditions of high receptor expression may be relevant and may be critical for *in vivo* activity. Further experiments revealed that optimized $mGlu_5$ ago-PAMs and pure PAMs behave similarly in multiple native systems, including studies in rat cortical astrocytes, measures of effects on hippocampal LTD, and depolarization of STN neurons. In these native systems, all compounds behaved as $mGlu_5$ PAMs but none showed evidence of direct $mGlu_5$ agonist activity. Finally, one of the most important findings of the present studies is that a compound that behaved as a pure PAM in $mGlu_5$ cell lines, VU0361747, exhibited similar efficacy in at least one animal model compared to other compounds that have robust agonist activity in the cell line assays (VU0360172 and VU0092273). Taken together, these data suggest that agonist activity of $mGlu_5$ PAMs may be influenced by high expression in recombinant cell lines and does not necessarily predict agonist activity in native systems. Furthermore, agonist activity in expression systems may not be an important factor in determining *in vivo* efficacy.

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Despite the finding that each of these compounds behaves in a similar manner in the native systems studied here, it would not be appropriate to conclude that allosteric agonist activity will not be observed in any native system. It is possible that there are specific neuronal populations or subcellular compartments where $mGlu_5$ levels are higher than those studied here or where other factors provide a context by which some PAMs can display ago-PAM activity in native systems. Also, it is important to remain open to the possibility that compounds with agonist activity in overexpressing cell lines will differentiate from pure PAMs in other behavioral assays. Previous studies have shown that $mGlu_5$ PAMs have a number of *in vivo* effects in multiple animal models that are relevant for schizophrenia and cognition-enhancing activity. It is possible that these compounds will have differential responses when assessed across a broad range of physiological responses and behavioral models. However, the studies presented here clearly suggest that the predominant effect of $mGlu_5$ PAMs in native systems is to potentiate responses to glutamate rather than directly activating the receptor. Importantly, these compounds provide new tools that can be used to fully address these important questions across a broader range of measures of $mGlu_5$ function.

An especially interesting component of these studies was the finding that compounds classified as strong ago-PAMs versus pure PAMs did not differentiate in studies of LTD. DHPG-induced LTD at the SC-CA1 synapse in hippocampus has been shown to be mediated primarily by $mGlu_5$ (Faas et al., 2002; Huang and Hsu, 2006; Huber et al., 2001). In addition, $mGlu_5$ plays an important role in the induction of long-term potentiation (LTP) at the same synapse (Ayala et al., 2009; Cohen et al., 1998; Lu et al., 1997). A balance of LTP and LTD is thought to be critical for normal cognitive function and any manipulation that disrupts this balance could impair cognition. This is especially critical in considering development of $mGlu_5$ activators as therapeutic agents since orthosteric $mGlu_5$ agonists induce profound LTD and disrupt this critical balance between LTD and LTP. Based on this finding, over activity of $mGlu_5$ has been postulated to play an important role in impairments in cognitive function in Fragile X syndrome,

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where signaling of this receptor is altered (Bear et al., 2004; Huber et al., 2002; Lauterborn et al., 2007). Interestingly, we previously reported that other $mGlu_5$ PAMs, such as VU29, enhance both LTD and LTP but maintain a strict dependence of both on specific patterns of afferent activity and maintain the balance between these opposing forms of synaptic plasticity (Ayala et al., 2009). This unique profile may be critical for cognition-enhancing effects of these agents and is likely to depend on the mechanism of these compounds in potentiating but not directly activating $mGlu_5$. However, there has been a concern that compounds that have strong ago-PAM activity *in vitro* may not provide this important advantage and could induce strong LTD in a manner similar to orthosteric agonists. The current finding that compounds classified from *in vitro* studies as strong ago-PAMs had similar effects on LTD to those classified as pure PAMs was encouraging and suggests that this difference in activity profile in cell lines may not predict adverse effects on cognitive function. In future studies, it will be important to evaluate these $mGlu_5$ PAMs in animal models of learning and memory to determine whether they have similar effects in enhancing hippocampal-dependent cognitive function.

In conclusion, the current studies provide initial evidence that the $mGlu_5$ ago-PAM activity that is often observed in cell-based assays may not reflect a clear distinction between pure PAMs and ago-PAMs that is relevant to the activity of these compounds in native systems or to their *in vivo* efficacy. The apparent discrepancy between *in vitro* and *in vivo*, in terms of the mode of action (PAM versus ago-PAM), may be due in part to the artificial nature of cell expression systems in which overexpression of the receptor may lead to differences in interactions between receptors, receptor coupling and the signaling pathways activated. It is important to mention that this does not appear to be a strict example of the well-known phenomenon of receptor reserve in that the orthosteric agonist glutamate exhibited similar potencies in cell lines expressing different $mGlu_5$ densities. If the high expressing cell lines had high receptor reserve according to the classical view, the glutamate CRC would be expected to be shifted to the left with higher $mGlu_5$ expression. However, this could represent an analogous

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phenomenon that influences the ability to detect agonist responses of $mGlu_5$ PAMs. In future studies, it will be important to gain a further understanding of the mechanistic underpinnings of these differences in $mGlu_5$ PAM activity and to also further evaluate the relevance of these differences in other native systems.

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Authorship Contributions

Participated in research design: Noetzel, Rook, Days, Rodriguez, Lavreysen, Niswender, Xiang, Weaver, Daniels, Lindsley, Conn

Conducted experiments: Noetzel, Rook, Vinson, Cho

Contributed new reagents of analytical tools: Zhou, Stauffer

Performed data analysis: Noetzel, Rook, Vinson

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

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Footnotes

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b) This work was presented in abstract form;
Noetzel, M.J.,H.P. Cho, E. Days, Y Zhou, A.L. Rodriguez, T. Steckler, H. Lavreysen, S.R. Stauffer, C.M. Niswender, C.W. Lindsley, C.D. Weaver, and P.J. Conn. (November 2010) "Receptor expression level influences the effect of allosteric modulators at metabotropic glutamate receptor 5." Society for Neuroscience 2010. San Diego, California.

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d) [1]Meredith Noetzel and Jerri Rook contributed equally to this work.

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Legends for Figures

Figure 1. Structures of $mGlu_5$ allosteric modulators used in this study. Biphenyl acetylines: VU0092273 and VU0240382. Nicotinamde acetylines: VU0360172 and VU0361747.

Figure 2. $mGlu_5$ allosteric modulators exhibit distinctions *in vitro* in their ability to modulate glutamate-induced calcium mobilization. A,B) Representative raw calcium traces from one experiment show the effect of 10 μM $mGlu_5$ modulators alone (compound; 20-80 seconds; agonist) and in response to an EC_{20} concentration of glutamate (80-140 seconds; PAM); the response to an EC_{20} concentration of glutamate in the presence of vehicle alone (control) is shown for comparison. A) Compounds classified as ago-PAMs. B) Compounds classified as pure PAMs. C, D) Potencies of the compounds were assessed by adding a concentration response curve of the $mGlu_5$ compound to cells before the addition of an EC_{20} concentration of glutamate. The calcium response was measured and normalized to a maximally effective concentration of glutamate (100 μM). C) A concentration-dependent increase in agonist activity was observed for VU0360172 (■) and VU0092273 (▲). A glutamate CRC is included as a positive control (●). D) All compounds enhanced the response to an EC_{20} concentration of glutamate in a concentration-dependent manner. Data represent the mean ± SEM of three to six independent experiments performed in duplicate.

Figure 3. In contrast to results seen with a cell line expressing a high density of $mGlu_5$, $mGlu_5$ allosteric modulators act exclusively as PAMs in a cell line with lower receptor expression. A) Representative raw calcium traces from one experiment show that 10 μM $mGlu_5$ modulator alone (compound; 20-80 seconds; agonist) induces no agonist response, but potentiates an EC_{20} concentration of glutamate (80-140 seconds; PAM). The response to an EC_{20} concentration of glutamate in the presence of vehicle alone (control) is shown for comparison. B, C) Potencies of $mGlu_5$ allosteric modulators were determined by adding a CRC of test

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compound followed by the addition of an EC_{20} concentration of glutamate. The calcium response was normalized to a maximally effective concentration of glutamate (10 μM). B) None of the compounds tested stimulated an agonist response. A glutamate CRC is included as a positive control (●). C) All compounds potentiated the response to a suboptimal (EC_{20}) concentration of glutamate in a concentration-dependent manner. Data represent the mean ± SEM of three to four independent experiments performed in duplicate.

Figure 4. $mGlu_5$ allosteric modulators induce a leftward shift in the glutamate concentration-response curve in a low expression $mGlu_5$ cell line. Progressive fold shift values were determined by treating cells with increasing fixed concentrations of PAMs followed by the addition of a glutamate CRC. The calcium response was measured and normalized to a maximally effective concentration of glutamate (10 μM). A) VU0360172, B) VU0361747, C) VU0092273, and D) VU0240382. Data represents the mean ± SEM of 3-8 independent experiments performed in duplicate.

Figure 5. $mGlu_5$ allosteric modulators stimulate a functional calcium response in rat cortical astrocytes that is similar to the response observed in the low expression $mGlu_5$ cell line. A,B) To assess the potency of $mGlu_5$ PAMs in rat cortical astrocytes, astrocytes were treated with increasing concentrations of $mGlu_5$ compound prior to the addition of an EC_{20} concentration of glutamate. Calcium flux was measured and normalized to a concentration of glutamate that stimulated a maximal response (10 μM). A) An agonist response was not observed with any of the $mGlu_5$ PAMs tested. B) All compounds were able to potentiate the response to an EC_{20} concentration of glutamate in a concentration-dependent manner. Data represent the mean ± SEM of three independent experiments performed in duplicate.

Figure 6. Long-term depression is induced by DHPG, but not VU0360172 alone, at the Schaffer collateral-CA1 synapse in the hippocampus. fEPSPs in which a response of 40-60% of the maximal response were used to assess the effect of bath administration of DHPG or VU0360172 at the SC-CA1 synapse in rat hippocampal slices. fEPSP slopes were quantified as described in the methods. Insets are sample fEPSPs traces measured predrug (black) and 55 minutes after drug washout (grey). A) Bath application of 75 μM DHPG (solid line) for 10 minutes resulted in induction of LTD measured 55 minutes after dug washout (n=6). B) Treatment of slices with 3 μM VU0360172 (solid line) had no effect on fEPSPs (n=7). Error bars represent SEM.

Figure 7. $mGlu_5$ allosteric modulators potentiate DHPG-induced long-term depression at the Schaffer collateral-CA1 synapse in hippocampus. fEPSPs were measured in rat hippocampus at the SC-CA1 synapse with a stimulus intensity that produced 40-60% of the maximum fEPSP response, determined at the start of each individual experiment. fEPSP slopes were quantified as described in the methods. Insets are sample fEPSPs traces measured predrug (black) and 55 minutes after drug washout (grey). A) Application of 25 μM DHPG (solid line) for 10 minutes resulted in a slight decrease in fEPSP slope measured 55 minutes after drug washout (n=6). B-E) Application of $mGlu_5$ compound to the slice for 20 minutes (dashed line), first alone and then in combination with 25 μM DHPG (solid line; 10 minutes), resulted in significant enhancement of DHPG-induced LTD (VU0360172 n=7; VU0361747 n=6; VU0092273 n=7; VU0240382 n=8), while having no effect on acute depression. Error bars represent SEM.

Figure 8. $mGlu_5$ PAMs reverse amphetamine-induced hyperlocomotion. Rats were placed in the open-field chambers for a 30-minute habituation interval, followed by a pretreatment with a 10 % Tween 80 vehicle or a 56.6 mg/kg dose of test compound i.p. for an additional 30 minutes. Rats then received an injection of saline or 1 mg/kg amphetamine s.c. and locomotor activity

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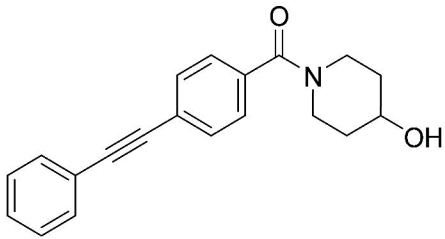
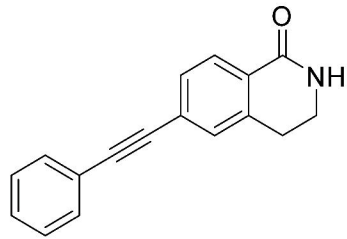
was measured for an additional 60 minutes. Statistical analyses were performed using JMP8 (SAS Inc., Cary, NC). A) VU0360172, B) VU0361747 and C) VU0092273 significantly reduced amphetamine-induced hyperlocomotion. Data are reported as mean ± SEM of the total number of beam breaks per 5-minute intervals (n = 5-12 per dose).

Figure 1

Biphenyl acetylenes:



VU0092273



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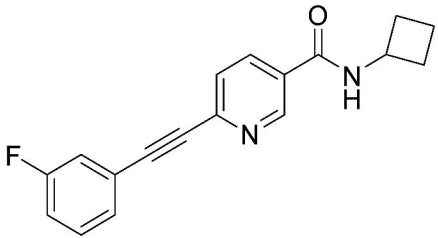

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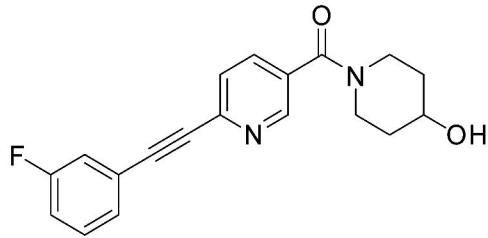
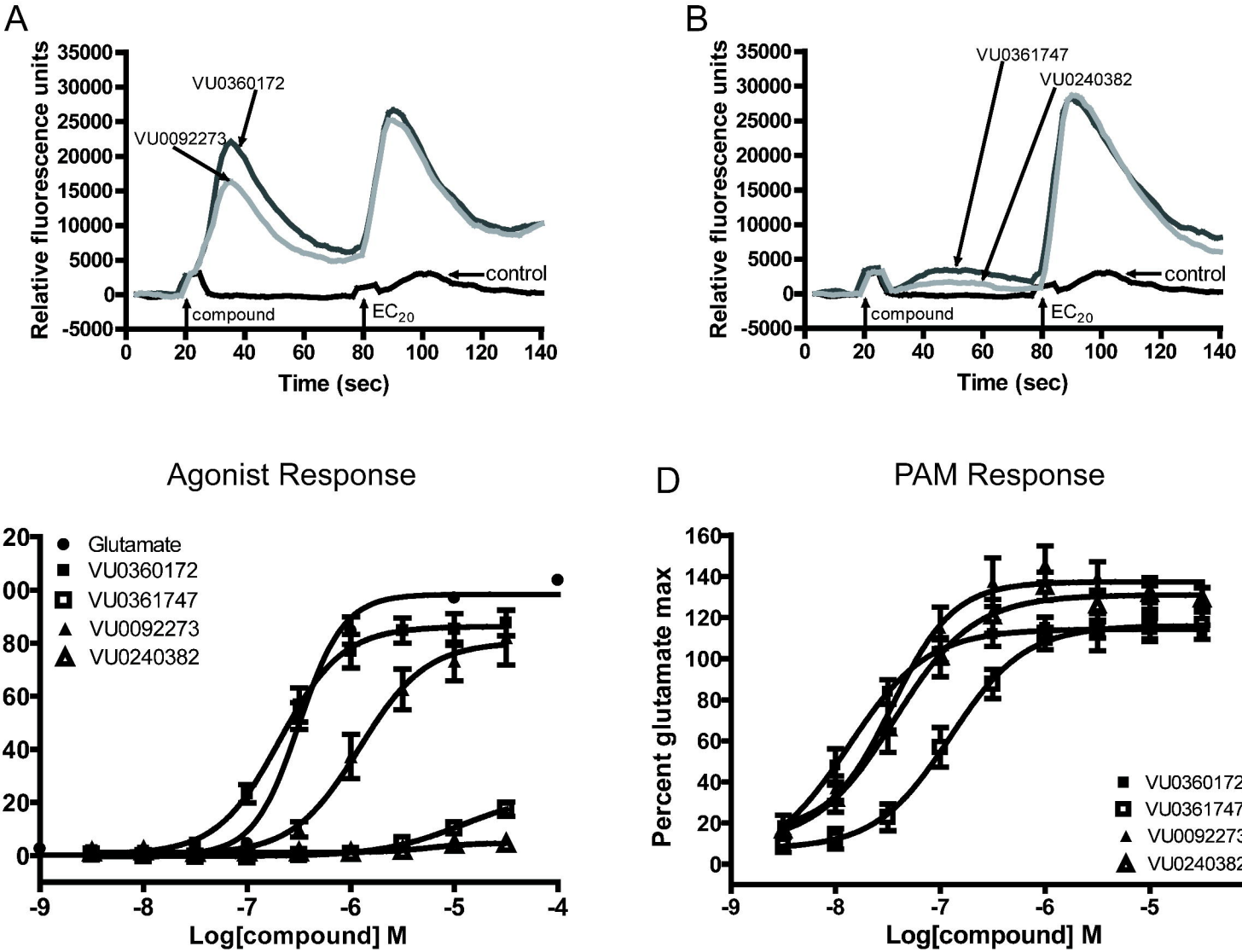


VU0361747

Figure 2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VU0360172
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EC20
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40
60
80
100
120
140
Time (sec)
B
Relative fluorescence units
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VU0240382
control
compound
EC20
Time (sec)
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VU0092273
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-8
-7
-6
-5
-4
Log[compound] M
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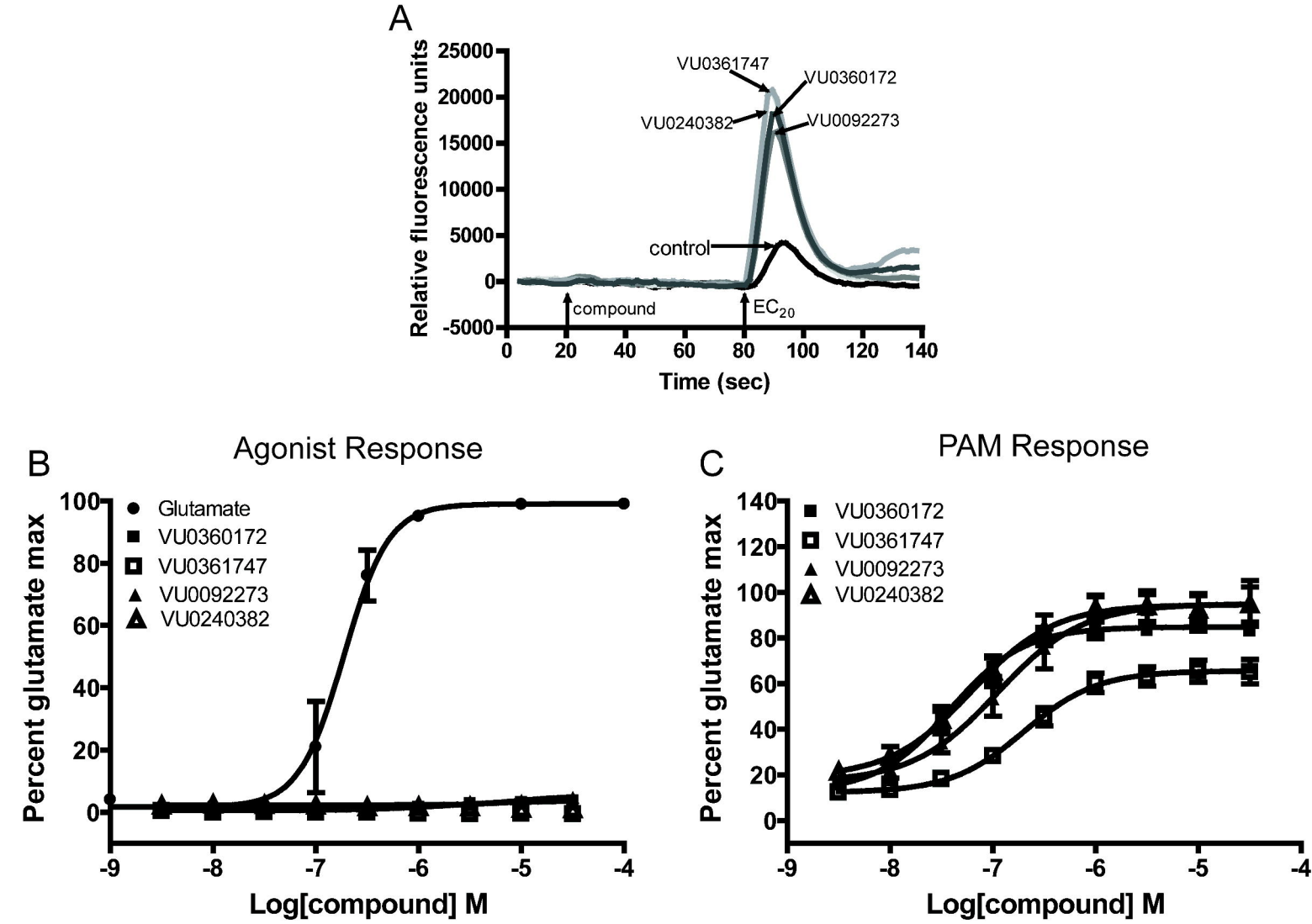
Figure 3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Agonist Response
Percent glutamate max
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0
Glutamate
VU0360172
VU0361747
VU0092273
VU0240382
-9
-8
-7
-6
-5
-4
Log[compound] M
C
PAM Response
Percent glutamate max
140
120
100
80
60
40
20
0
VU0360172
VU0361747
VU0092273
VU0240382
-9
-8
-7
-6
-5
-4
Log[compound] M

Figure 4

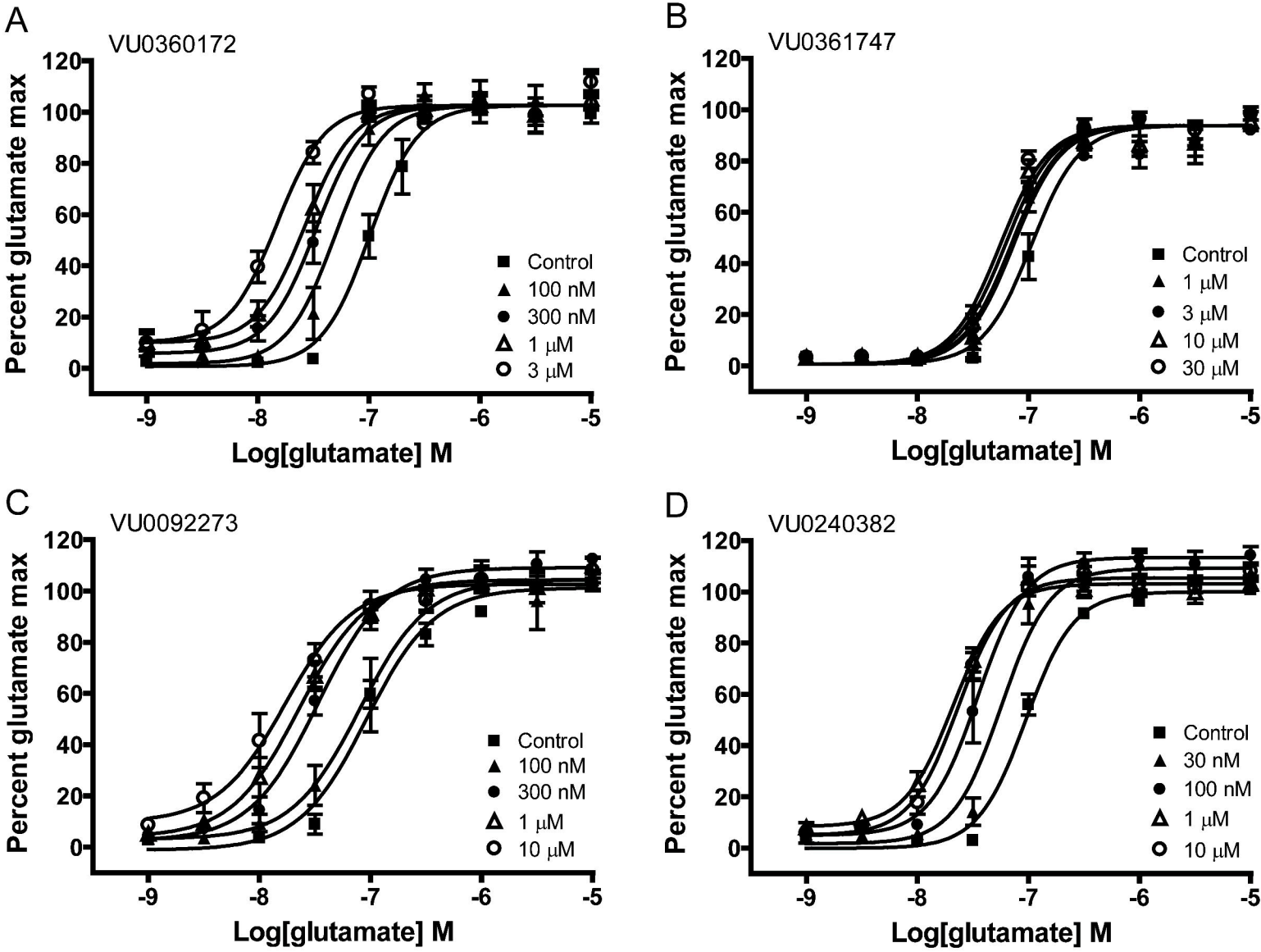
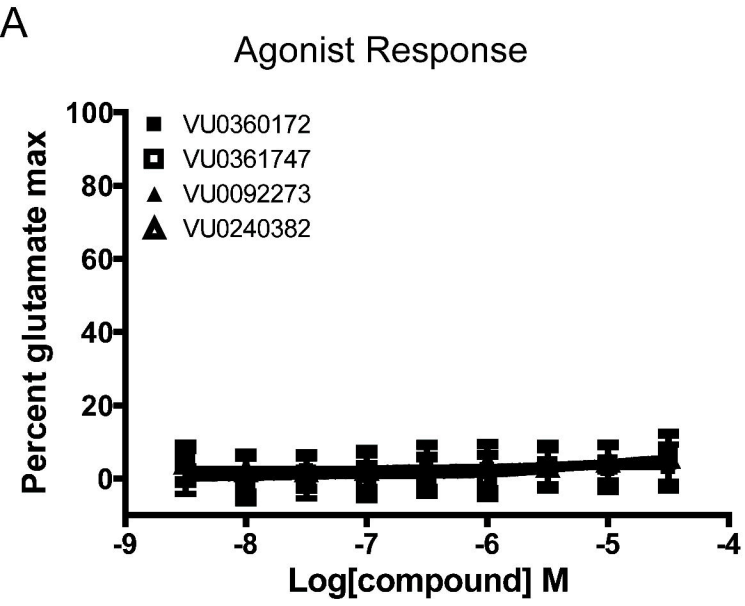

A
VU0360172
Percent glutamate max
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
Log[glutamate] M
Control
100 nM
300 nM
1 μM
3 μM
B
VU0361747
Percent glutamate max
Log[glutamate] M
Control
1 μM
3 μM
10 μM
30 μM
C
VU0092273
Percent glutamate max
Log[glutamate] M
Control
100 nM
300 nM
1 μM
10 μM
D
VU0240382
Percent glutamate max
Log[glutamate] M
Control
30 nM
100 nM
1 μM
10 μM

Figure 5


A
Agonist Response
VU0360172
VU0361747
VU0092273
VU0240382
Percent glutamate max
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log[compound] M

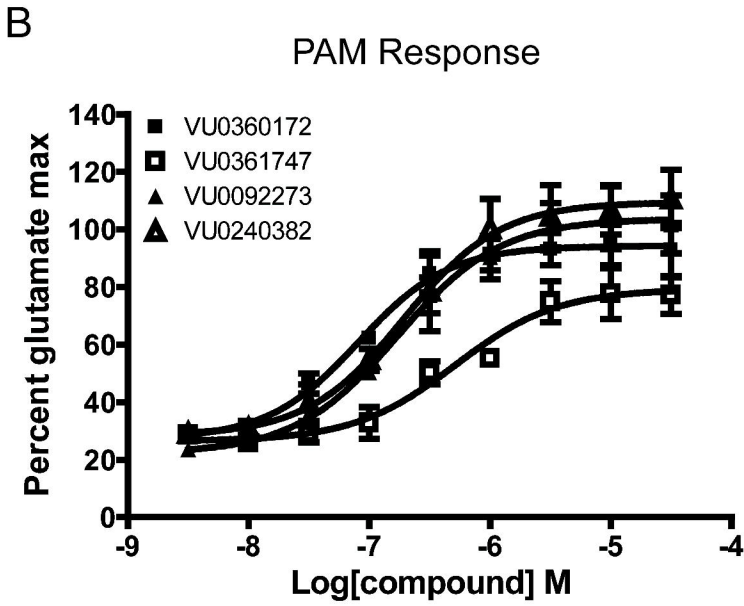

B
PAM Response
VU0360172
VU0361747
VU0092273
VU0240382
Percent glutamate max
140
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log[compound] M

Figure 6

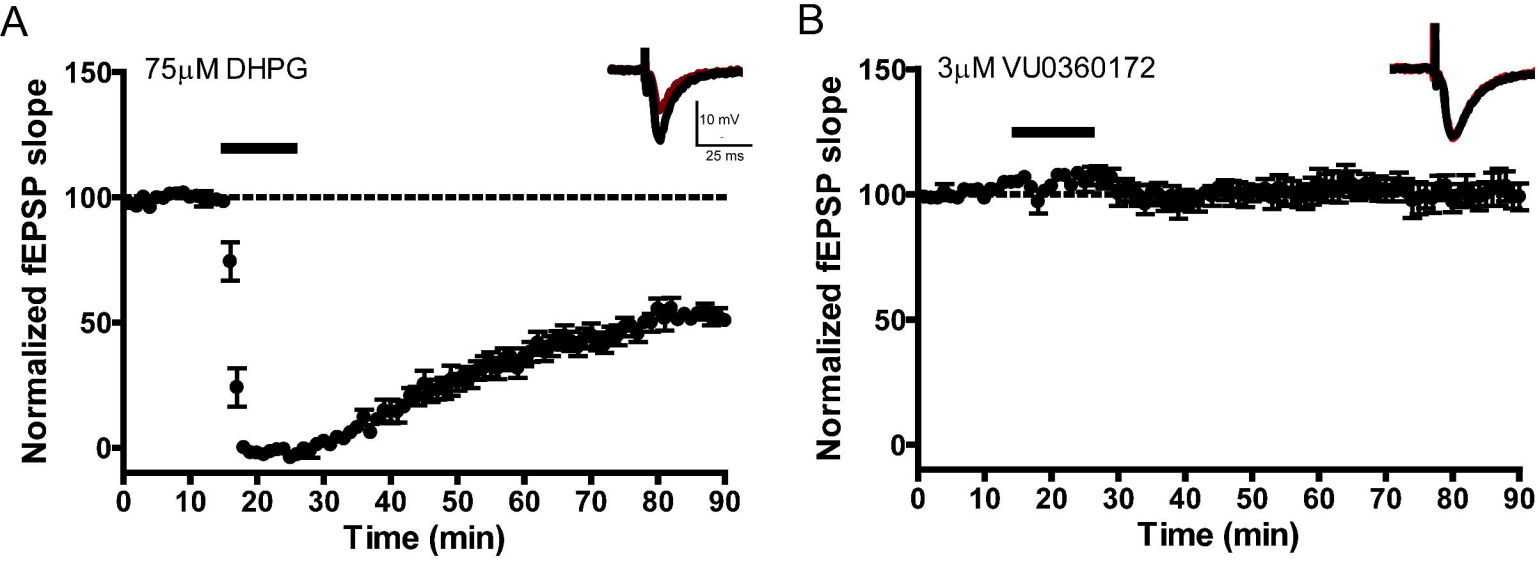

A
75μM DHPG
10 mV
25 ms
Normalized fEPSP slope
150
100
50
0
0 10 20 30 40 50 60 70 80 90
Time (min)
B
3μM VU0360172
Normalized fEPSP slope
150
100
50
0
0 10 20 30 40 50 60 70 80 90
Time (min)

Figure 7

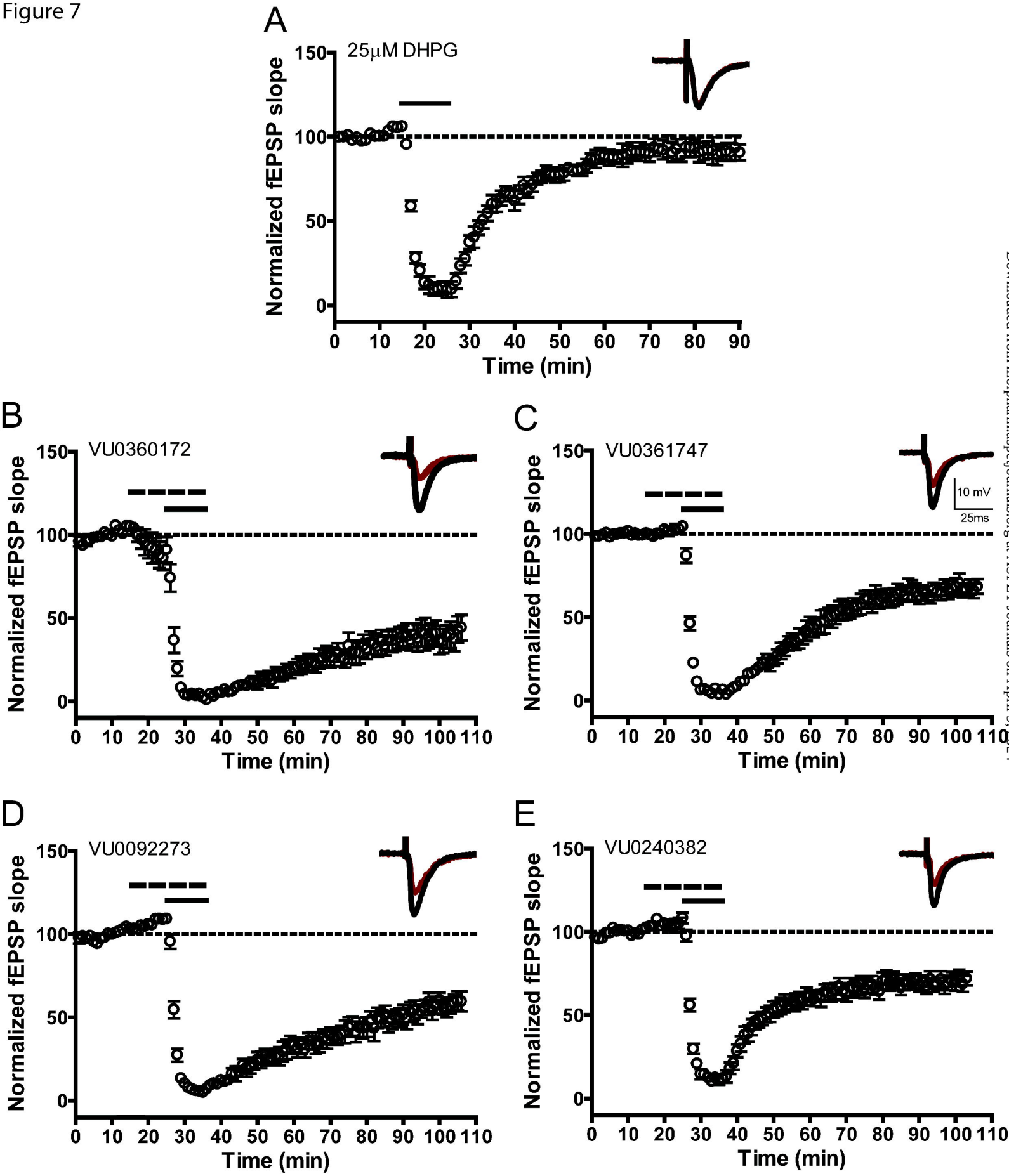
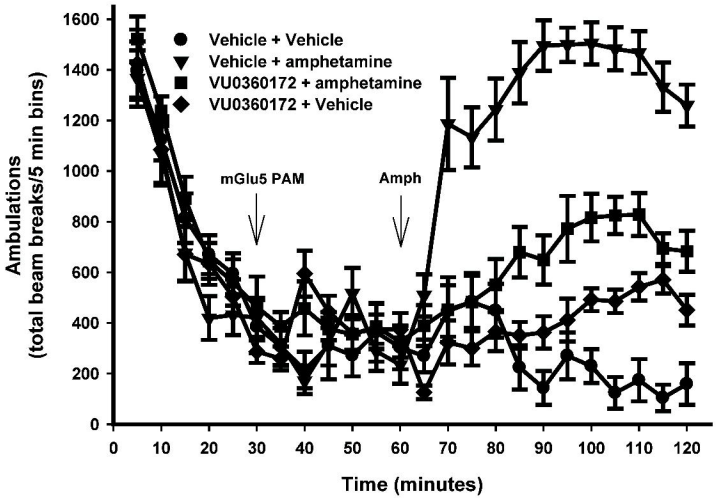
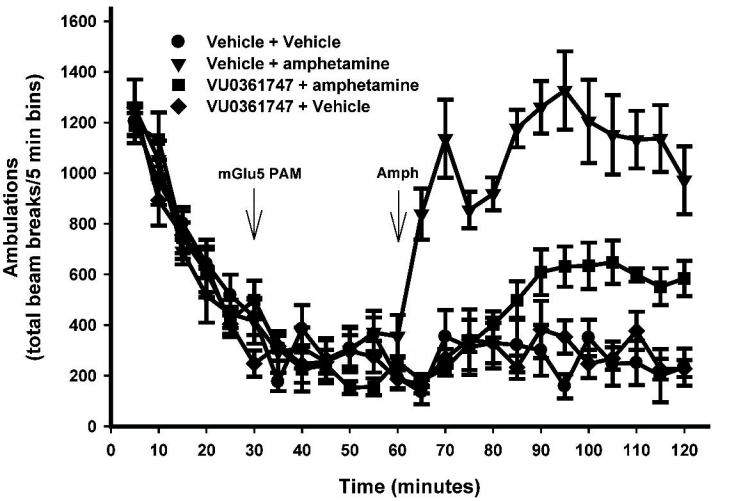

A
25μM DHPG
Normalized fEPSP slope
Time (min)
B
VU0360172
C
VU0361747
10 mV
25ms
D
VU0092273
E
VU0240382

Figure 8

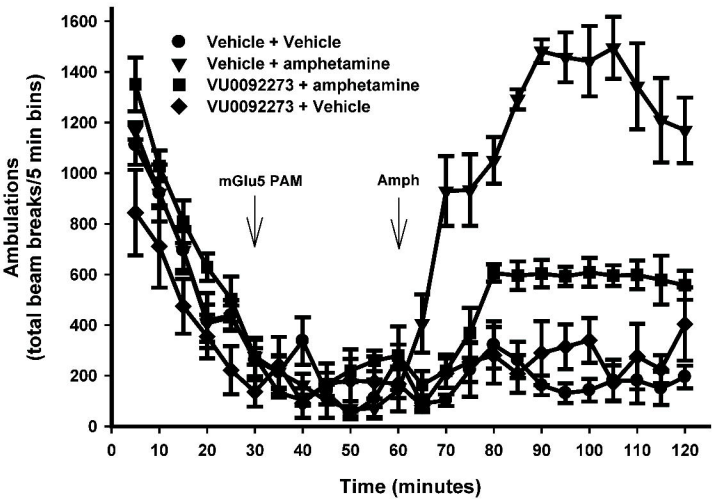
A


Vehicle + Vehicle
Vehicle + amphetamine
VU0360172 + amphetamine
VU0360172 + Vehicle
mGlu5 PAM
Amph
Ambulations
(total beam breaks/5 min bins)
Time (minutes)

B


Vehicle + Vehicle
Vehicle + amphetamine
VU0361747 + amphetamine
VU0361747 + Vehicle
mGlu5 PAM
Amph
Ambulations
(total beam breaks/5 min bins)
Time (minutes)

C


Vehicle + Vehicle
Vehicle + amphetamine
VU0092273 + amphetamine
VU0092273 + Vehicle
mGlu5 PAM
Amph
Ambulations
(total beam breaks/5 min bins)
Time (minutes)