HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2018 March 15.

Published in final edited form as:

Neuropharmacology. 2017 March 15; 115: 60–72. doi:10.1016/j.neuropharm.2016.07.001.

Biased allosteric agonism and modulation of metabotropic glutamate receptor 5: implications for optimizing preclinical neuroscience drug discovery

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Abstract

Allosteric modulators, that exhibit no intrinsic agonist activity, offer the advantage of spatial and temporal fine-tuning of endogenous agonist activity, allowing the potential for increased selectivity, reduced adverse effects and improved clinical outcomes. Some allosteric ligands can differentially activate and/or modulate distinct signaling pathways arising from the same receptor, phenomena referred to as 'biased agonism' and 'biased modulation'. Emerging evidence for CNS disorders with glutamatergic dysfunction suggests the metabotropic glutamate receptor subtype 5 ($mGlu_5$) is a promising target. Current $mGlu_5$ allosteric modulators have largely been classified based on modulation of intracellular calcium (iCa^{2+}) responses to orthosteric agonists alone. We assessed eight $mGlu_5$ allosteric modulators previously classified as $mGlu_5$ PAMs or PAM-agonists representing four distinct chemotypes across multiple measures of receptor activity, to explore their potential for engendering biased agonism and/or modulation. Relative to the reference orthosteric agonist, DHPG, the eight allosteric ligands exhibited distinct biased agonism fingerprints for iCa^{2+} mobilization, IP_1 accumulation and ERK1/2 phosphorylation in HEK293A cells stably expressing $mGlu_5$ and in cortical neuron cultures. VU0424465, DPFE and VU0409551 displayed the most disparate biased signaling fingerprints in both HEK293A cells and cortical neurons that may account for the marked differences observed previously for these ligands *in vivo*. Select $mGlu_5$ allosteric ligands also showed 'probe dependence' with respect to their cooperativity with different orthosteric agonists, as well as biased modulation for the magnitude of positive cooperativity observed. Unappreciated biased agonism and modulation may contribute to unanticipated effects (both therapeutic and adverse) when translating from recombinant systems to preclinical models.

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Keywords

biased agonism; positive allosteric modulator; stimulus bias; metabotropic glutamate receptor 5

1. Introduction

In recent years, the metabotropic glutamate receptor subtype 5 ($mGlu_5$), a G protein-coupled receptor (GPCR) ubiquitously expressed throughout the brain, has emerged as a promising drug target for multiple central nervous system (CNS) disorders, including schizophrenia, autism, Parkinson's disease and depression (Gregory et al., 2013c; Niswender and Conn, 2010). Drug design efforts for $mGlu_5$ are largely focused on targeting allosteric sites, that is, receptor binding sites topographically distinct from that of the endogenous ligand. Allosteric ligands offer the promise of increasing receptor selectivity, maximizing therapeutic effects and minimizing adverse effects. Further, modulation of orthosteric agonists by allosteric ligands without intrinsic agonism, allows for spatiotemporal fine-tuning of receptor responses – a favorable therapeutic mechanism within the complex CNS environment (Christopoulos and Kenakin, 2002; Leach et al., 2007; Melancon et al., 2012). Allosteric ligands that enhance receptor responses are called positive allosteric modulators (PAMs), while some allosteric compounds may also activate the receptor in the absence of endogenous ligand (PAM-agonists). Similar to orthosteric agonists, PAM-agonists do not have the benefit of temporal control of receptor activity. Allosteric ligands that diminish endogenous responses are termed negative allosteric modulators (NAMs), and those that neither increase nor decrease responses are referred to as neutral allosteric ligands (NALs) (Gentry et al., 2015). Discovery efforts for $mGlu_5$ allosteric modulators have been particularly fruitful. Diverse modulator chemotypes recognize the 'MPEP' site, an allosteric pocket located within the receptor's seven transmembrane bundle (Dore et al., 2014; Gregory et al., 2014; Gregory et al., 2013b; Wu et al., 2014). Of note, $mGlu_5$ PAMs (classified based on glutamate stimulation of $mGlu_5$-intracellular Ca^{2+} (iCa^{2+}) mobilization in recombinant cells) have demonstrated efficacy in preclinical models of psychosis and cognition enhancement (Kinney et al., 2005; Rodriguez et al., 2010; Gregory et al., 2013a). However, recent studies have also reported adverse effects for select $mGlu_5$ enhancers, such as seizure activity induced by VU0424465 (a PAM-agonist for $mGlu_5$ mediated iCa^{2+} mobilization) (Rook et al., 2013) and neurotoxicity in rats treated with 5PAM523 (a pure PAM for $mGlu_5$-iCa^{2+} mobilization stimulated by glutamate) (Parmentier-Batteur et al., 2014).

It has also become increasingly evident that, like many GPCRs, the $mGlu_5$ receptor is pleiotropically coupled, activating multiple signaling pathways. In particular, while the receptor is predominantly coupled to G_q and subsequent changes in intracellular calcium (iCa^{2+}) mobilization, $mGlu_5$ couples to iCa^{2+}-independent signaling pathways, such as extracellular signal-regulated kinases (ERK) 1 and 2 phosphorylation, cAMP, mTOR/PI3K, and can interact with, and modulate the activity of, other GPCRs and ion channels (Sengmany and Gregory, 2015). It is conceivable therefore that $mGlu_5$ activation by chemically diverse ligands may engender unique receptor conformations, such that the receptor favors distinct subsets of physiological responses. This concept is known as 'biased

agonism' (Kenakin and Christopoulos, 2013). Biased agonism offers the opportunity to design drugs that are tailored to activate the desired complement of receptor responses linked to therapeutic outcomes while avoiding those linked to adverse effects. Biased agonism is operative for orthosteric agonists at the related Group I $mGlu_1$ receptor between G-protein and β-arrestin pathways in both recombinant and natively expressing cells (Emery et al., 2010; Emery et al., 2012; Hathaway et al., 2015). Several studies have suggested that $mGlu_5$ biased agonism may be linked to distinct physiological responses (Gregory et al., 2013a; Gregory et al., 2012; Noetzel et al., 2013; Zhang et al., 2005). However, the vast majority of $mGlu_5$ allosteric modulators have been classified as PAMs, PAM-agonists, NAMs or NALs, based on modulation of glutamate-mediated iCa^{2+} mobilization alone. To date, there have been no rigorous analyses of the $mGlu_5$ signaling fingerprints of diverse allosteric modulators across different pathways. It is possible that preferential activation or inhibition of distinct $mGlu_5$ signaling pathways could lead to different behavioral and/or toxicological outcomes.

One means to avoid the neurotoxicity and seizure liability associated with certain $mGlu_5$ PAM and/or PAM-agonists (Rook et al., 2013; Parmentier-Batteur et al., 2014) and retain anti-psychotic and pro-cognitive effects may be via the development of biased $mGlu_5$ modulators (Rook et al., 2015). Moreover, hitherto unappreciated bias may contribute to observations that markedly different doses of the same modulator are required for efficacy in different preclinical behavioral models. For example, DPFE, an $mGlu_5$ PAM-agonist of glutamate-mediated iCa^{2+} mobilization and ERK1/2 phosphorylation in HEK293A cells, required vastly different doses to elicit cognition-enhancement versus reversal of amphetamine-induced hyperlocomotion in rats (Gregory et al., 2013a). Therefore, in the current study, we undertook the first rigorous analysis of $mGlu_5$ bias for diverse PAMs in both HEK293A cells and primary cortical neuron cultures. Allosteric ligands were selected based on i) known interaction at the 'MPEP' allosteric site, ii) previous classification as PAMs or PAM-agonists in iCa^{2+} assays and iii) existing data in preclinical behavioral models. Propensity for biased allosteric agonism was determined relative to the orthosteric agonist DHPG across four signaling pathways that were amenable to assessment in both recombinant and neuronal cell cultures. We demonstrate that $mGlu_5$ allosteric modulators display biased agonism and cooperativity, providing proof-of-concept that the development of biased $mGlu_5$ allosteric modulators may be a means to tailor enhancement of $mGlu_5$ activity to elicit therapeutically beneficial effects, such as anti-psychotic and cognition enhancement, while avoiding adverse effects.

2. Materials and Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Neurobasal and Opti- modified Eagle's medium (Opti-MEM), Fluo-4-AM and antibiotics were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Thermo Electron Corporation (Melbourne, Australia). IP-One HTRF® assay kit was purchased from Cisbio Assays, Genesearch (Arundel, QLD, Australia) and AlphaScreen detection beads were from PerkinElmer Life and Analytical Sciences. Select allosteric modulators *N*-cyclobutyl-5-((3-

fluorophenyl)ethynyl)picolinamide (VU0403602), *N*-cyclobutyl-6-((3-fluorophenyl)ethynyl)picolin-amide (VU0360172), *(R)*-5-((3-fluorophenyl)ethynyl)-*N*-(3-hydroxy-3-methylbutan-2-yl)picolinamide (VU0424465), 1-(4-(2,4-difluorophenyl)piperazin-1-yl)-2-((4-fluorobenzyl)oxy)ethanone (DPFE), (5-((3-fluorophenyl)ethynyl)pyridin-2-yl)(3-hydroxyazetidin-1-yl)methanone (VU0405398) (4-fluorophenyl)(2-(phenoxymethyl)-6,7-dihydrooxazolo[5,4-*c*]pyridin-5(4*H*)-yl)methanone (VU0409551) were synthesized at Vanderbilt Centre for Neuroscience Drug Discovery as described previously (Gregory et al., 2013a; Gregory et al., 2012; Rook et al., 2013; Bridges et al., 2013; Rodriguez et al., 2010; Rook et al., 2015). *N*-(1,3-Diphenyl-1*H*-pyrazolo-5-yl)-4-nitrobenzamide (VU29), 3-Cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide (CDPPB) and 7-(Hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester (CPCCOEt) were purchased from Tocris Bioscience (Melbourne, Australia). Unless stated otherwise, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade.

2.2. Animals

All animal experiments and procedures were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee (Protocol no. MIPS.2014.37). Asmu:Swiss outbred 8-week female wild-type mice were provided by the Monash Animal Research Platform (Clayton, Victoria, Australia). Time-mated females were humanely sacrificed and E16 embryos were recovered for primary cell culture.

2.3. Cell culture

HEK293A cells stably expressing wild-type rat $mGlu_5$ at low levels (HEK293A-$mGlu_5$-low), equivalent to those observed in primary cortical astrocytes (Noetzel et al., 2012) were maintained at 5% CO_2, 37°C in DMEM supplemented with 5% FBS, 16mM HEPES and 500 μg/mL G418. The day before assays, cells were seeded onto poly-D-lysine coated, clear-bottom 96-well plates in assay medium (glutamine-free DMEM supplemented with 5% dialyzed FBS, 16 mM HEPES and 500 μg/mL G418) at a density of 40,000 cells/well, unless otherwise specified.

2.4. Primary cell culture

E16 Asmu:Swiss wild-type mice were decapitated, cortices dissected and neurons mechanically dissociated in Hank's Balanced Salt Solution (HBSS; KCl 5.33 mM, KH_2PO_4 0.44 mM, $NaHCO_3$ 4.17 mM, NaCl 137.93 mM, Na_2HPO_4 0.34 mM, D-glucose 5.56 mM). Cortical neurons were then immediately plated on poly-D-lysine, FBS-coated clear-bottom 96-well plates in Neurobasal media, supplemented with 2 mM L-glutamine, 1 × B-27®, 50 U/mL penicillin, 50 μg/mL streptomycin, 1.25 μg/mL Fungizone® Antimycotic, at a density of 100 000 cells/well. Plates were maintained at 37°C and 5% CO_2 for 5–7 days before experimentation, to allow growth of neurons.

2.5. Intracellular calcium mobilization

Intracellular calcium flux was measured as a change in fluorescence of the Ca^{2+} indicator dye, Fluo-4-AM, using a FlexStation I or III as described previously using a double-add

paradigm, where allosteric ligands were added 1 min prior to orthosteric agonist (Gregory et al., 2012), with the exception of allosteric agonists, which were added simultaneously with orthosteric compound to avoid confounding effects of desensitization. Intracellular calcium mobilization was measured at room temperature (RT) for HEK293A-mGlu$_5$-low cells and at 37°C for cortical neurons. For extracellular Ca^{2+}-free experiments, $CaCl_2$ was omitted from Ca^{2+} assay buffer (HBSS as above, with 2.5 mM probenecid, pH 7.4) and also supplemented with 1 mM EDTA. A 5-point smoothing function was applied to the raw fluorescence traces and peak fluorescence values (within 60 sec post-addition of ligand) normalized to the maximal response to either glutamate (HEK293A cells) or DHPG (cortical neurons).

2.6. Inositol monophosphate (IP_1) accumulation assay

HEK293A-mGlu$_5$-low or cortical neurons were washed with PBS and incubated for 1 h at 37°C with stimulation buffer (HBSS as above, with 20 mM HEPES, 30 mM $LiCl_2$, 1.2 mM $CaCl_2$, pH 7.4) before compound addition. After 1 h incubation, cells were lysed with Lysis Buffer (HTRF® IP-one assay kit) and IP_1 levels determined using the HTRF® IP-one assay kit as per manufacturer's instructions and fluorescence measured using the PHERAstar. Data were expressed as either fold over basal, or as a percentage of the maximal DHPG response.

2.7. ERK1/2 Phosphorylation

Receptor-mediated ERK1/2 phosphorylation was determined using an AlphaScreen-based ERK SureFire kit as described previously (Gregory et al., 2012). Cells were serum-starved using DMEM supplemented with 16 mM HEPES, for a minimum of 4 h for cortical neurons, and 6 h for recombinant cells. In HEK293A-mGlu$_5$-low cells, the peak time for mGlu$_5$-mediated ERK phosphorylation by all compounds was 5–7 min (Gregory et al., 2012; Rook et al., 2015b), while in cortical neurons, DPFE and VU0405398 peaked at 5 min, and all remaining modulators and DHPG peaked at 20 min (Supplementary Information). For interaction experiments in cortical neurons, allosteric modulators or vehicle were added 1 min prior to 20 min stimulation with DHPG. Assays were terminated by aspiration of ligand-containing medium and addition of 50 μL/well lysis buffer. Following 5 min shaking, 4 μL of lysate was transferred to a white 384 well ProxiPlate (PerkinElmer). Under low-light conditions, 7 μL AlphaScreen detection mixture (1:7 (v/v) activation buffer: reaction buffer; with 1:240 (v/v) acceptor and donor beads) was added to each well and incubated 1.5h at 37°C. AlphaScreen signal was measured using an Envision with standard AlphaScreen settings. Data were expressed as fold over basal levels of phosphorylated ERK.

2.8. Data analysis

Agonist-concentration response curves were fitted to a variable four-parameter logistic equation:

$$y=\frac{bottom+(top-bottom)}{(1+10^{(logEC_{50}-Log[A])n}} \quad (1)$$

where bottom and top are lower and upper plateau levels of the concentration response curve respectively, *n* is the Hill coefficient, [A] is the molar concentration of agonist, and EC_{50} is the agonist concentration required to produce a half maximal response between top and bottom values (potency).

Allosteric modulation of glutamate or DHPG-mediated responses were fitted to the operational model of allosterism (Leach et al., 2007):

$$Effect=\frac{E_m(\tau_A[A](K_B+\alpha\beta[B])+\tau_B[B]K_A)^n}{([A]K_B+K_AK_B+K_A[B]+\alpha[A][B])^n+(\tau_A[A](K_B+\alpha\beta[B])+\tau_B[B]K_A)^n} \quad (2)$$

where [A] and [B] are the molar concentrations of orthosteric agonist, glutamate or DHPG, and allosteric modulator respectively. K_A and K_B are the equilibrium dissociation constants of the orthosteric agonist and allosteric modulator respectively, and α represents affinity cooperativity and β is a scaling factor that denotes the magnitude of effect an allosteric modulator has on the efficacy to an orthosteric agonist. Parameters τ_A and τ_B represent the respective ligand's intrinsic ability to activate the receptor, while E_m and *n* represent the maximal system response and the transducer slope respectively. K_A values for orthosteric agonists were constrained to affinity estimates previously determined from inhibition binding assays (Gregory et al., 2012). Affinity cooperativity (α) was assumed to be neutral as validated previously (Gregory et al., 2012) and thus constrained to a value of 1, allowing estimates of β as a measure of cooperativity, that is the magnitude of effect a modulator has on orthosteric agonist potency and/or efficacy.

Biased agonism was quantified using an operational model of agonism (Kenakin et al., 2012):

$$Y=basal+\frac{(Em-basal)\left(\frac{\tau}{K_A}\right)^n[A]^n}{[A]^n(\frac{\tau}{K_A})^n+\left(1+\frac{[A]}{K_A}\right)^n} \quad (3)$$

where [A] is the agonist concentration, E_m is the maximal response of the system, n is the transducer slope, and τ is the coupling efficiency of the agonist as defined by R_T/K_E, where R_T is the receptor number and K_E if the coupling efficiency of the system. From this equation the transduction coefficient $\log(\tau/K_A)$ a composite of both affinity and efficacy can be derived, which describes agonism and bias for a given pathway. A normalized transduction coefficient, $\Delta\log(\tau/K_A)$, was consequently calculated using DHPG as the reference agonist. To compare different pathways (e.g. *j*1 vs *j*2), $\Delta\log(\tau/K_A)$ values were evaluated between pathways by calculating the LogBias ($\Delta\,\Delta\log(\tau/K_A)$):

$$\Delta\Delta Log\left(\tau/K_A\right)_{j1-j2}=Log(bias)=\Delta Log\left(\tau/K_A\right)_{j1}-\Delta Log\left(\tau/K_A\right)_{j2} \quad (4)$$

Affinity, cooperativity and potency parameters were derived and represented as logarithmic mean ± SEM. Analysis of bias parameters was performed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. Comparison of operational parameters between iCa^{2+} mobilization and IP_1 accumulation in cortical neurons was determined using unpaired Student's t-test.

3. Results

Eight allosteric ligands previously classified as either PAMs or PAM-agonists based on glutamate stimulation of $mGlu_5$-mediated iCa^{2+} mobilization representing four distinct chemotypes were selected for this study to probe the potential for biased agonism and biased modulation of $mGlu_5$ (Supplementary Fig. 1). In preclinical studies, the structurally related VU0403602 and VU0424465 have known adverse effect liability (Bridges et al., 2013; Rook et al., 2013) and provide a reference for modulators with undesirable properties. We also included two other ligands from this scaffold, VU0360172 and VU0405398, both of which have lower cooperativity with glutamate in iCa^{2+} assays, and minimal agonism compared to VU0403602 or VU0424465 (Gregory et al., 2012). CDPPB and VU29 were included as structurally distinct allosteric modulators from the same chemotype class, where CDPPB has been reported to be a PAM-agonist and VU29 a 'pure' PAM (Chen et al., 2007; Lindsley et al., 2004). CDPPB has previously been tested *in vivo*, showing efficacy in models of Huntington's disease, fear extinction, psychosis and addiction (Cleva et al., 2011; Doria et al., 2015; Ganella et al., 2014; Gass et al., 2014; Uslaner et al., 2009). Recently, VU0409551 was described as a biased modulator on the basis that VU0409551 positively modulates $mGlu_5$ activity *in vitro* but does not enhance $mGlu_5$ modulation of NMDA current or NMDA receptor dependent plasticity in the hippocampus (Rook et al., 2015). Importantly, VU0409551 has efficacy in preclinical models of antipsychotic-like activity, cognition-enhancement and a genetic model of schizophrenia (Balu et al., 2016; Conde-Ceide et al., 2015; Rook et al., 2015). DPFE, a structurally distinct ligand relative to the three previous classes, was also included to offer further insight into the relationship between chemical scaffold and pharmacological outcome. As with CDPPB & VU0409551 behavioral studies have been conducted with DPFE (Gregory et al., 2013a; Peters et al., 2015), thereby allowing the potential to link *in vivo* efficacies with *in vitro* pharmacological fingerprints.

We first confirmed the pharmacological phenotypes for each of the eight allosteric modulators as potentiators of glutamate stimulation of $mGlu_5$-mediated iCa^{2+} mobilization in HEK293A-$mGlu_5$-low cells. In agreement with previous studies, compared to the orthosteric agonists glutamate and DHPG, VU0424465, VU0403602, and DPFE were efficacious partial allosteric agonists, achieving maximal responses 50–80% of the glutamate response. VU0360172, VU0409551 and CDPPB were also partial allosteric agonists. VU29 and VU0405398 had no appreciable allosteric agonist activity (Fig. 1A & B). The rank order of agonist efficacies was: glutamate = DHPG > VU0424465 > VU0403602 > DPFE ≥ VU0409551 > VU0360172 > CDPPB (see Supplementary Table 1 for E_{max} values). The rank order of agonist potencies (Supplementary table 1) was: VU0403602 > CDPPB ≥ VU0424465 > glutamate > VU0360172 > DHPG = VU0409551 > DPFE.

All eight compounds had different degrees of positive cooperativity (logβ) with glutamate for iCa^{2+} mobilization, causing concentration-dependent increases in glutamate potency that approached a limit (Supplementary Figure 1). Modulator effects on glutamate concentration-response curves for iCa^{2+} mobilization were fitted to an operational model of allosterism to quantify the functional affinity (pK_B) and efficacy modulation (logβ) for each of the ligands (Table 1). The resulting affinity and cooperativity estimates agree with those determined in previous studies (Bridges et al., 2013; Gregory et al., 2013a; Gregory et al., 2012; Manka et al., 2010).

3.1. Select $mGlu_5$ PAMs show 'probe dependence' for potentiation of $mGlu_5$-iCa^{2+} mobilization

Although glutamate is the cognate agonist, it is a sub-optimal ligand to study the effects of $mGlu_5$ alone in primary neuronal cultures due to the presence of multiple glutamate receptor subtypes and transporters in these native cells. Thus, we chose the more selective DHPG as the reference orthosteric agonist for assessing ligand pharmacology in primary neuronal cultures. We first confirmed that all eight allosteric ligands potentiated DHPG-mediated iCa^{2+} mobilization in HEK293A-$mGlu_5$-low cells. DHPG concentration-response curves for iCa^{2+} mobilization in the absence or presence of different concentrations of each allosteric ligand (Fig. 2) were analyzed using the operational model of allosterism. As expected, affinity estimates were similar, irrespective of which orthosteric agonist was used (Table 1). Cooperativity estimates (logβ) for the interaction with DHPG were also similar to those determined with glutamate for VU0403602, VU0360172, VU0409551 and CDPPB. In contrast, the cooperativity between VU0424465, DPFE or VU0405398 with DHPG was ~2-fold higher than with glutamate. VU29 had ~5-fold higher positive cooperativity with DHPG compared to glutamate.

3.2. All allosteric ligands have direct agonist activity for $mGlu_5$-mediated IP_1 accumulation and ERK1/2 phosphorylation in HEK293A-$mGlu_5$-low cells

We next assessed IP_1 accumulation and ERK1/2 phosphorylation in response to the $mGlu_5$ allosteric ligands, as alterations in these two pathways have been linked to diverse physiological outcomes (Bezprozvanny and Hayden, 2004; Ribeiro et al., 2010b; Tang et al., 2005); IP_1 accumulation assay was assessed over a 1 h period. Glutamic pyruvic transaminase (GPT) 10 U/mL was added to reduce the effect of any ambient extracellular glutamate, which may confound observations of allosteric agonism (Supplementary Fig. 2). Inclusion of GPT reduced baseline levels of IP_1 accumulation and phosphorylated ERK1/2 (pERK1/2) (Supplementary Fig. 2).

Relative to the reference orthosteric agonist, DHPG, all eight allosteric modulators were agonists for $mGlu_5$-IP_1 accumulation, achieving a similar maximal response to DHPG (Fig. 1C–D). All eight allosteric ligands stimulated pERK1/2, characterized by a transient peak (5–7min post-addition) that returned to baseline levels within 30min (Supplementary Fig. 2). Concentration-response curves for $mGlu_5$-pERK1/2 constructed at the peak time point revealed VU0409551 and VU0405398 to be equally as efficacious agonists as DHPG (Fig. 1E–F). Consistent with a previous report (Gregory et al., 2012), VU29, CDPPB, DPFE, VU0360172, VU0424465 and VU0403602 elicited higher maximal responses for pERK1/2

than the orthosteric agonist. DHPG had significantly lower potency (7-fold) for IP_1 accumulation relative to pERK1/2 and iCa^{2+} mobilization (Supplementary Table 1). Conversely, VU0424465 was 10–30 fold more potent in pERK1/2 and IP_1 relative to iCa^{2+} assays, and VU0403602 had similar potencies for IP_1 and iCa^{2+}, but was ~5-fold more potent for pERK1/2. The potencies for VU0360172, CDPPB, VU0409551 and DPFE were similar across all three measures of receptor activation, as were VU29 and VU0405398 between pERK1/2 and IP_1 accumulation. These differences in the rank orders of ligand potency for between different signaling pathways are suggestive of biased agonism.

3.3. $mGlu_5$-mediated calcium mobilization is a composite of intracellular and extracellular calcium sources

Upon activation of G_q-coupled receptors, inositol phosphate hydrolysis is widely known to result in mobilization of intracellular calcium. Therefore it was unexpected that ligands that were agonists for IP_1 had lower potency or efficacy in iCa^{2+} assays. However, it is also well-known that $mGlu_5$ couples to various calcium ion channels (Sengmany and Gregory, 2015). Hence, iCa^{2+} mobilization detected upon receptor activation may be the combination of both extracellular calcium entering the cell via ion channels, and the calcium released from internal stores. To differentiate the sources of calcium, extracellular calcium was excluded from the buffer, thereby allowing measurement of calcium arising from intracellular stores. The maximal responses to both glutamate and DHPG were significantly reduced in the absence of 1.2 mM extracellular calcium, although potencies were unaffected (Fig. 1G). With the exception of CDPPB, which had increased efficacy in the absence of extracellular Ca^{2+}, there was no significant change in the potency or maximal responses to DPFE, VU0409551, VU0403602 or VU0424465 between the differing calcium conditions with the $mGlu_5$ allosteric ligands studied (Fig. 1G–H, Supplementary Table 1).

3.4. Assessment of agonist activity of $mGlu_5$ allosteric ligands in cortical neurons

The same four measures of $mGlu_5$ activity that we investigated in recombinant cells were next examined in cortical neurons to assess the potential of biased allosteric agonist activity in a native system (Fig. 3). Cortical neurons represent a physiologically relevant system to assess $mGlu_5$ allosteric ligand activity, and can serve as a vital translational link for validating allosteric modulator pharmacology prior to preclinical assessments. To eliminate the influence of $mGlu_1$, which is also expressed in the cortex, the $mGlu_1$ selective negative allosteric modulator CPCCOEt 30 μM, was used in conjunction with DHPG for all experiments in cortical neurons. GPT had no effect on basal IP_1 levels or allosteric agonism (Supplementary Fig. 3) and was not included in these experiments.

DHPG was ~10 fold less potent for IP_1 accumulation relative to iCa^{2+} (in absence and presence of 1.2 mM extracellular Ca^{2+}) and ERK1/2 phosphorylation (Supplementary Table 3). In iCa^{2+} mobilization assays relative to DHPG, VU0424465 and VU0403602 were nearly full agonists, whereas VU0360172, DPFE, VU29 and CDPPB were all partial agonists (Fig. 3A, B, Supplementary Table 2). VU0405398 also showed agonist activity, however, the maximal response was highly variable and restricted to concentrations above 3 μM. VU0409551 had no appreciable agonist activity for iCa^{2+} mobilization. The rank order

of agonist efficacy: DHPG > VU0424465 = VU0403602 > CDPPB = VU29 = VU0360172 > VU0405398 ≫ VU0409551, was different to that observed in HEK293A cells.

For IP_1 accumulation the relative efficacies of the allosteric ligands were similar to that observed for iCa^{2+} assays, with the exception of VU0409551 which had appreciable agonist activity for IP_1 accumulation (Fig. 3C–D). The time course for $mGlu_5$-mediated ERK1/2 phosphorylation by both orthosteric and allosteric ligands was characterized by sustained elevations or inhibition in cortical neurons, a markedly different profile to the transient response observed in HEK293A cells (Supplementary Figure 4). VU0424465, VU0403602, VU0360172, VU29, CDPPB and VU0409551 were all agonists for pERK1/2 in cortical neurons eliciting maximal responses to similar to DHPG (Fig. 3E–F). Allosteric agonism (iCa^{2+}, pERK1/2 and IP_1) in cortical neurons was inhibited by pre-exposure to the $mGlu_5$ selective neutral allosteric ligand 5MPEP confirming that agonism was mediated via interaction with $mGlu_5$ (Supplementary Fig. 5). Interestingly, while DPFE and VU0405398 were partial agonists for IP_1 accumulation, they exhibited a bell-shaped concentration-response relationship for pERK1/2, elevating pERK1/2 at 1μM and dropping below basal at concentrations above 1μM (Fig. 3E–F). Similar to observations in HEK293A-$mGlu_5$-low cells, the DHPG maximal response was significantly reduced in the absence of extracellular calcium (Fig. 3G). There were no significant differences in potency or maximal response of VU0424465, VU0403602, VU0360172, or CDPPB between differing calcium conditions (Fig. 3G–H). However, agonist activity by VU29, DPFE and VU0405398 was lost.

3.5. $mGlu_5$ allosteric ligands are biased agonists

In order to rigorously and quantitatively assess the apparent bias of $mGlu_5$ allosteric agonists in HEK293A-$mGlu_5$-low cells and cortical neurons, observational bias between assays, and systems bias of the cell background should be minimized – thereby resulting in a more faithful estimate of true (conformationally-driven) biased agonism (Kenakin et al., 2012). From each assay, a transduction coefficient $\log(\tau/K_A)$ (Supplementary Table 4), that is, the composite of ligand efficacy and affinity, was derived as previously described (Kenakin et al., 2012) and normalized to the reference ligand DHPG ($\Delta\log(\tau/K_A)$). Subsequent normalization of $\Delta\log(\tau/K_A)$ between pathways, $\Delta\Delta\log(\tau/K_A)$, allows for quantification of the degree of bias between pathways (Figure 4). Each allosteric ligand displayed a unique signaling bias fingerprint in HEK293A-$mGlu_5$-low cells and neurons, although there were some notable trends. Relative to DHPG, VU0424465 showed significant bias away from iCa^{2+} mobilization and toward IP_1 accumulation (110-fold) and ERK1/2 phosphorylation (9-fold) in HEK293A-$mGlu_5$-low cells, a profile that was largely retained in neurons. VU0403602 was biased toward IP_1 over pERK1/2 (11-fold) and iCa^{2+} mobilization (14-fold) in HEK293A-$mGlu_5$-low cells, however this wasn't reflected in the neurons. VU29 and CDPPB were biased (3–25 fold) toward IP_1 over pERK1/2 and/or iCa^{2+} mobilization in both HEK293A cells and neurons. DPFE, VU0409551 and VU0405398 showed no discernible bias in HEK293A cells, but had distinct bias fingerprints in cortical neurons. VU0405398 and DPFE were biased toward IP_1 over iCa^{2+} (12 and 26-fold respectively) coupled with biphasic pERK1/2 responses that could not be analyzed using the operational model. VU0409551 was the only ligand to show significant bias (24-fold) between IP_1 and pERK1/2 in cortical neurons.

3.6. CDPPB, DPFE, VU0405398 are biased allosteric modulators of DHPG in cortical neurons

We were unable to perform interaction studies between the modulators with DHPG to quantify affinity and cooperativity in HEK293A-$mGlu_5$-low cells, because of the robust agonism for IP_1 accumulation and ERK1/2 phosphorylation displayed by each allosteric ligand in their own right. However, in cortical neurons, a number of allosteric ligands had minimal or partial agonist effects, such that a sufficient response window remained to allow for allosteric interaction experiments with DHPG in this physiologically relevant system. Thus, the resulting DHPG concentration-response curves for IP_1 and iCa^{2+} in the absence and presence of increasing concentrations of VU0360172, VU29, CDPPB, DPFE, VU0409551 or VU0405398 were constructed and analyzed using an operational model of allosterism (Fig. 5, Supplementary Fig. 6). Affinity estimates were similar for each allosteric ligand across the two pathways, with the exception of VU0405398, which had higher affinity in the IP_1 assay (Table 1). For all six allosteric modulators, the magnitude of logβ was lower for IP_1 versus iCa^{2+} (Fig. 5D); this reached significance for DPFE (3.8-fold), VU0360172 (3.6-fold), VU0409551 (6.5-fold) and VU0405398 (2.8-fold). Given that VU0360172, VU0405398 and DPFE were also partial agonists for ERK1/2 phosphorylation, we assessed modulation of the DHPG concentration-response curve by these three modulators. In cortical neurons, VU0360172 was a PAM-agonist for DHPG-stimulated ERK1/2 phosphorylation with similar affinity and cooperativity estimates (pK_B: 6.49±0.20 and logβ: 0.69±0.18) to those determined in iCa^{2+} assays (Fig. 5D). In contrast, DPFE and VU0405398 showed a unique bell-shaped modulatory response in ERK1/2 phosphorylation. While 1 μM of each ligand potentiated DHPG responses, 10 μM of each ligand resulted in decreased DHPG maximal responses for ERK1/2 phosphorylation (Fig. 5).

4. Discussion

Allosteric modulators of $mGlu_5$ offer considerable therapeutic potential for the treatment of numerous psychiatric and neurological disorders. In particular, $mGlu_5$ PAMs have demonstrated efficacy in preclinical models of psychosis, cognition-enhancement and NMDA receptor hypofunction (Ayala et al., 2009; Gregory et al., 2013a; Horio et al., 2013; Rook et al., 2015; Uslaner et al., 2009). However, a number of recent studies have reported adverse effects with select $mGlu_5$ allosteric modulators (Rook et al.,, 2013; Parmentier-Batteur et al.,, 2014). Therefore in order to realize the therapeutic potential of $mGlu_5$ allosteric modulators, there is a need to better understand the full scope of allosteric drug action. We tested the hypothesis that structurally diverse PAMs had unappreciated biased pharmacology. We found that $mGlu_5$ allosteric ligands had diverse biased signaling fingerprints in both recombinant and native systems. Furthermore, this bias extended to modulation of orthosteric agonist activity, where the magnitude of cooperativity between allosteric and orthosteric ligands differed depending upon the measure of receptor activation. The prevalence of biased agonism and modulation for diverse $mGlu_5$ PAM chemotypes highlights the paucity in our understanding of the functional consequences of $mGlu_5$ allosteric modulation, and raises new avenues for therapeutic discovery. Armed with a better appreciation of the full scope of $mGlu_5$ allosteric modulator action, it may be possible to

link activation (and/or enhancement) of specific pathways to different physiological outcomes to promote therapeutic effects and avoid adverse effects.

In particular, many of the studied $mGlu_5$ allosteric ligands, which previously were largely classified based on modulation of glutamate-stimulated iCa^{2+} mobilization, showed significant differences in coupling efficiency to iCa^{2+} mobilization, IP_1 accumulation and ERK1/2 phosphorylation. Most strikingly, each allosteric ligand tested had greater efficacy for IP_1 accumulation relative to iCa^{2+} mobilization. Therefore the ligands tested cannot be considered as pure $mGlu_5$ PAMs, and do not posses the safety advantages of pure allosteric modulators, i.e., the advantage of spatiotemporal fine-tuning of receptor responses and saturable effects. Classically, iCa^{2+} mobilization is considered to be downstream of inositol phosphate hydrolysis, however, our data show that IP_1 accumulation does not necessarily result in iCa^{2+} mobilization via $mGlu_5$. It is possible that these differences between IP_1 and iCa^{2+} are related to the different kinetics of response coupling measurements for these two assays. A lack of equilibrium being achieved by allosteric ligands relative to orthosteric agonists in the transient iCa^{2+} assay may also be a contributing factor. Indeed, underlying temporal differences between assays and ligands influences bias at the dopamine D2 receptor, such that kinetic context may also play a crucial role in understanding biased agonism (Klein Herenbrink et al., 2016). However, it is widely accepted that the $mGlu_5$ receptor is coupled to various calcium channels (Gao et al., 2013; Kammermeier et al., 2000; Lu et al., 1999; McCool et al., 1998; Ribeiro et al., 2010a; Tu et al., 1999), thereby resulting in Ca^{2+} mobilization from both intracellular and extracellular stores. In order to differentiate the sources of Ca^{2+}, we compared iCa^{2+} mobilization in the presence and absence of 1.2 mM Ca^{2+} in HEK293A-$mGlu_5$-low cells. Interestingly, while glutamate and DHPG had reduced maximal responses in the absence of extracellular Ca^{2+}, all studied $mGlu_5$ allosteric ligands were unaffected. By applying a rigorous analytical approach to quantify bias, we found that VU0424465 showed significant bias between IP_1 and extracellular Ca^{2+}-free iCa^{2+} assays (20-fold) and between IP_1 and iCa^{2+} assays (110-fold). These data suggest that allosteric agonists couple to a different complement of effectors than orthosteric agonists to influence intracellular Ca^{2+} levels.

Our data clearly demonstrate that allosteric modulator activity in an $mGlu_5$-iCa^{2+} mobilization assay is not necessarily predictive of activity for other $mGlu_5$-mediated effects. Our data contribute to a growing body of evidence for biased agonism at metabotropic glutamate receptors including for $mGlu_1$ (Emery et al., 2012; Hathaway et al., 2015) and group III mGlu receptors (Jalan-Sakrikar et al., 2014). Further, we observed probe-dependence for potentiation of $mGlu_5$-iCa^{2+} mobilization in HEK293A cells, where the magnitude of cooperativity for select allosteric ligands was dependent upon the orthosteric agonist present. Probe dependence has not previously been observed for $mGlu_5$, however, is known to be prevalent for other mGlu subtypes (Jalan-Sakrikar et al, 2014). The phenomenon of probe dependence is a crucial consideration, particularly when translating findings from *in vitro* assays to native preparations, such as slice electrophysiology, where surrogate agonists are often used. Interestingly, while VU29 had higher positive cooperativity with DHPG compared to glutamate, CDPPB potentiated both agonists to a similar extent. Similarly, for the three picolinamide acetylenes studied, VU0424465 and VU0405398 had differential cooperativities with DHPG and glutamate whereas VU0403602

did not. These data demonstrate that the probe dependence observed is not linked to a particular chemotype, or consistent between ligands from the same scaffold.

While probe dependence is an important consideration when moving from recombinant to physiologically relevant systems, the change in cell background in itself may be a confounding factor in understanding drug pharmacology. Indeed, in this study, we observed clear differences in the temporal profile for $mGlu_5$-ERK1/2 phosphorylation between HEK293A-$mGlu_5$-low and cortical neurons. While ERK1/2 phosphorylation was transient in HEK293A-$mGlu_5$-low cells, in cortical neurons pERK1/2 levels remained elevated over 30min. These data suggest that distinct intracellular effectors are mediating pERK1/2 in response to $mGlu_5$ activation in cortical neurons versus HEK293A cells. Moreover, sustained ligand-induced responses suggest that the consequences of receptor activation will continue after a ligand has vacated the receptor. It is also possible that cellular context is driving differential compartmentalization and/or internalization of $mGlu_5$ in these two cell types. Of note, 50–80% of $mGlu_5$ receptors are thought to be located on intracellular membranes (Hubert et al., 2001; Kumar et al., 2008), and subcellular localisation of $mGlu_5$ can dictate the overall cellular response (Jong et al., 2009; Kumar et al., 2012). It remains to be determined whether or not the $mGlu_5$ allosteric ligands tested cross the plasma membrane, induce receptor internalization or are internalized with the receptor. DHPG is not actively transported across the plasma membrane, only activating receptors at the cell surface (Jong et al., 2009), this suggests that the sustained pERK1/2 response in cortical neurons originates from cell surface receptors, however, it is possible that the continued signaling may be driven by internalised cell surface receptors within endosomes.

Comparison of the biased agonism signaling fingerprints for all eight allosteric ligands revealed that VU0424465 had the most divergent profile in both HEK cells and neuronal cultures. Our earlier study linked VU0424465 intrinsic efficacy (for $mGlu_5$-iCa^{2+} in HEK cells) to seizure activity and behavioral convulsions (Rook et al., 2013). However, comparison of the bias signaling fingerprint of VU0424465 with that of DPFE, VU0409551 and CDPPB, three ligands that have efficacy in models of cognition and psychosis but no reported adverse effects (Gregory et al., 2013a; Uslaner et al., 2009; Balu et al., 2016; Rook et al., 2015), suggests that the pronounced bias (10–100 fold) of VU0424465 toward IP_1 and ERK1/2 relative to iCa^{2+} in HEK293A-$mGlu_5$-low cells may be a better prediction of adverse effect liability. It is worth noting that all experiments in cortical neurons were performed in the presence of CPCCOEt (an $mGlu_1$ negative allosteric modulator) to eliminate DHPG activation of $mGlu_1$. However, $mGlu_1$ and $mGlu_5$ are known to heteromerize (Sevastyanova and Kammermeier, 2014). Heteromerization of metabotropic glutamate receptors ($mGlu_2$ and $mGlu_4$) can influence allosteric modulator pharmacology (Yin et al., 2014). In the future it would be of interest to determine if $mGlu_1$/$mGlu_5$ heteromers influence the biased agonism observed for $mGlu_5$ selective PAM-agonists. Understanding the bias profile of different ligands, and linking this to known preclinical profiles, offers the opportunity to design therapeutics that avoid signaling pathways associated with adverse effects.

We found that allosteric ligand bias profiles differed between HEK293A-$mGlu_5$-low cells and cortical neuronal cultures. Of note, VU0409551 was the only ligand to show significant

bias away from pERK1/2 (relative to IP_1) and a lack of agonist efficacy for iCa^{2+} mobilization in cortical neurons. These data are consistent with the previous report that VU0409551 acting at $mGlu_5$ does not potentiate NMDA channel currents (Rook et al., 2015) or stimulate pERK1/2 levels in the hippocampus or prefrontal cortex when dosed chronically (Balu et al., 2016). Interestingly, DPFE and VU0405398 had distinctly different concentration-response relationships for ERK1/2 phosphorylation in cortical neurons, eliciting a bi-phasic response. While VU0405398 has not yet been tested *in vivo,* DPFE has a unique preclinical efficacy profile. Considerably lower doses of DPFE (0.56mg/kg) are required for cognition enhancement, whereas >10mg/kg is needed for efficacy in reversing amphetamine-induced hyperlocomotion (Gregory et al., 2013). In contrast, cognition studies with CDPPB (Horio et al., 2013; Stefani and Moghaddam, 2010) have demonstrated efficacy at doses similar those required for efficacy in reversing amphetamine-induced hyperlocomotion (Kinney et al., 2005). It is tempting to speculate that the distinct coupling profiles for DPFE and VU0409551 with respect to pERK1/2 may be predictive of *in vivo* efficacy that is biased toward cognition enhancement.

In addition to biased agonism, biased modulation was also demonstrated for different $mGlu_5$ allosteric ligands in cortical neurons. Biased modulation may be more favorable for CNS drugs, rather than direct activation of receptors, as it allows for greater control over basal glutamatergic tone. Multiple studies have demonstrated that different $mGlu_5$ effectors and second messengers can be perturbed in different CNS disorders. For example, disrupted mTOR and Akt signaling is associated with major depressive disorder and suicide (Dwivedi et al., 2010; Jernigan et al., 2011) whereas increases in basal pERK1/2, Akt and altered IP_3/iCa^{2+} signaling are observed in a mouse model of Huntington's disease (Bezprozvanny and Hayden, 2004; Ribeiro et al., 2010b; Tang et al., 2005). Therefore, biased modulation may provide the means to tailor enhancement or inhibition of $mGlu_5$ activity to effectors associated with different disease states.

In summary, we have determined the signaling fingerprint of several $mGlu_5$ allosteric ligands at iCa^{2+} mobilization, IP_1 accumulation and ERK1/2 phosphorylation in both recombinant and native systems. Probe dependence, biased agonism and biased modulation were operative at this receptor. Importantly, the most divergent bias profiles were associated with ligands previously shown to possess distinct *in vivo* efficacy profiles. Drug discovery for neuroscience-related disorders continues to suffer from high attrition rates, in large part due to an inability to translate promising preclinical pharmacological profiles to clinically effective therapeutics. Our findings suggest that unappreciated biased agonism and/or modulation may contribute to these failures and highlight the need to examine multiple measures of receptor activity to best classify the pharmacology of ligands. In the future, these biased signaling fingerprints may provide a framework that can be used to rationally develop $mGlu_5$ allosteric ligands with optimal *in vitro* profiles that translate to desirable activity in preclinical models and avoid on-target adverse effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Health & Medical Research Council of Australia (NHMRC): CJ Martin Overseas Biomedical postdoctoral training Fellowship (KJG: APP1013709), Project Grant APP1084775 (KJG), Program Grant APP1055134 (AC) and Senior Principal Research Fellowship APP1102950 (AC). Work within the Vanderbilt Center for Neuroscience Drug Discovery on $mGlu_5$ PAMs was supported by the NIH/NIMH R01MH062646. PJC is an inventor on patents that protect multiple classes of mGlu5 PAMs and receives research support from AstraZeneca. ERK SureFire kits were a kind gift from Dr. Michael Crouch and TGR Biosciences (Thebarton, Australia). We thank Ms. Sabine Albold for expert technical assistance.

Abbreviations

cAMP	cyclic adenosine monophosphate
CDPPB	3-Cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide
CNS	central nervous system
CPCCOEt	7-(hydroxyimino)cyclopropa[*b*] chromen-1a-carboxylate ethyl ester
DHPG	(*S*)-3,5-dihydroxyphenylglycine
DMEM	Dulbecco's modified Eagle's medium
DPFE	1-(4-(2,4-difluorophenyl)piperazin-1-yl)-2-((4-fluorobenzyl)oxy)ethanone
eCa^{2+}	extracellular calcium
ERK1/2	extracellular signal-regulated kinases 1 and 2
FBS	fetal bovine serum
GPCR	G protein-coupled receptor
GPT	glutamic pyruvic transaminase
HBSS	Hank's Balanced Salt Solution
HEK293A	human embryonic kidney 293
iCa^{2+}	intracellular calcium
IP_1	inositol 1-phosphate
$mGlu_5$	metabotropic glutamate receptor subtype 5
MPEP	2-Methyl-6-(phenylethynyl)pyridine
mTOR	mammalian target of rapamycin
NAL	neutral allosteric ligand
NAM	negative allosteric modulator
Opti-MEM	Opti- modified Eagle's medium
PAM	positive allosteric modulator

PI3K	phosphoinositide 3-kinase
VU0360172	*N*-cyclobutyl-6-((3-fluorophenyl)ethynyl)picolinamide
VU0403602	*N*-cyclobutyl-5-((3-fluorophenyl)ethynyl)picolinamide
VU0424465	*(R)*-5-((3-fluorophenyl)ethynyl)-*N*-(3-hydroxy-3-methylbutan-2-yl)picolinamide
VU0405398	(5-((3-fluorophenyl)ethynyl)pyridin-2-yl)(3-hydroxyazetidin-1-yl)methanone
VU0409551	((4-fluorophenyl)(2-(phenoxymethyl)-6,7-dihydrooxazolo[5,4-*c*]pyridin-5(4*H*)-yl)methanone)
VU29	*N*-(1,3-diphenyl-1*H*-pyrazolo-5-yl)-4-nitrobenzamide

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Highlights

- mGlu5 enhancers differentially affect signaling and are biased agonists/modulators
- Biased mGlu5 agonism is evident in both recombinant cells and neuronal cultures
- Differences noted in vivo may be due to biased mGlu5 allosteric agonism/modulation

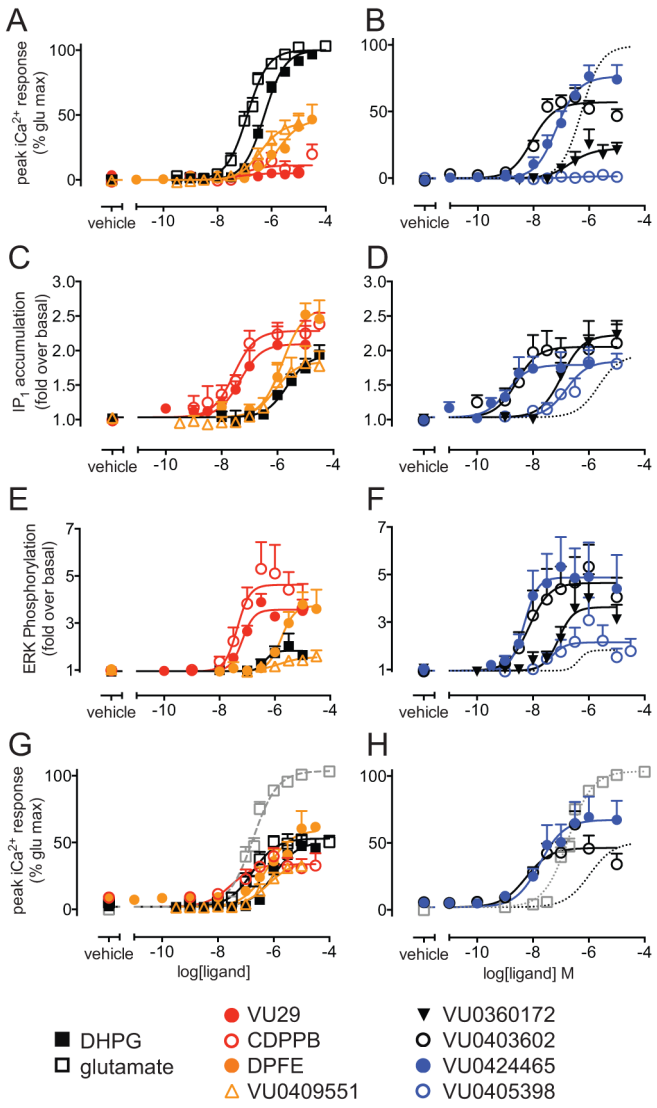

Figure 1. $mGlu_5$ allosteric ligands have agonist activity for iCa^{2+} mobilization, IP_1 accumulation and ERK phosphorylation in HEK293A-$mGlu_5$-low

Concentration-response curves for indicated $mGlu_5$ orthosteric and allosteric ligands for iCa^{2+} mobilization (A & B), IP_1 accumulation (C & D), ERK1/2 phosphorylation (E & F) and iCa^{2+} mobilization in the absence of 1.2mM $CaCl_2$ (G & H; % glu max represents maximal glutamate response in the presence of 1.2mM $CaCl_2$, curve from panel A shown in grey). In panels B, D, F and H, the DHPG curve is plotted as dotted lines for ease of reference. Responses were normalized to either the glutamate maximal response (iCa^{2+} mobilization) or represented as fold over basal (IP_1 accumulation and ERK phosphorylation). IP_1 and ERK1/2 phosphorylation experiments were performed in the presence of 10 U/mL GPT to minimize contribution of ambient glutamate. Data are expressed as mean + SEM of 3–17 experiments performed in duplicate, error bars not shown lie within the dimensions of the symbols.

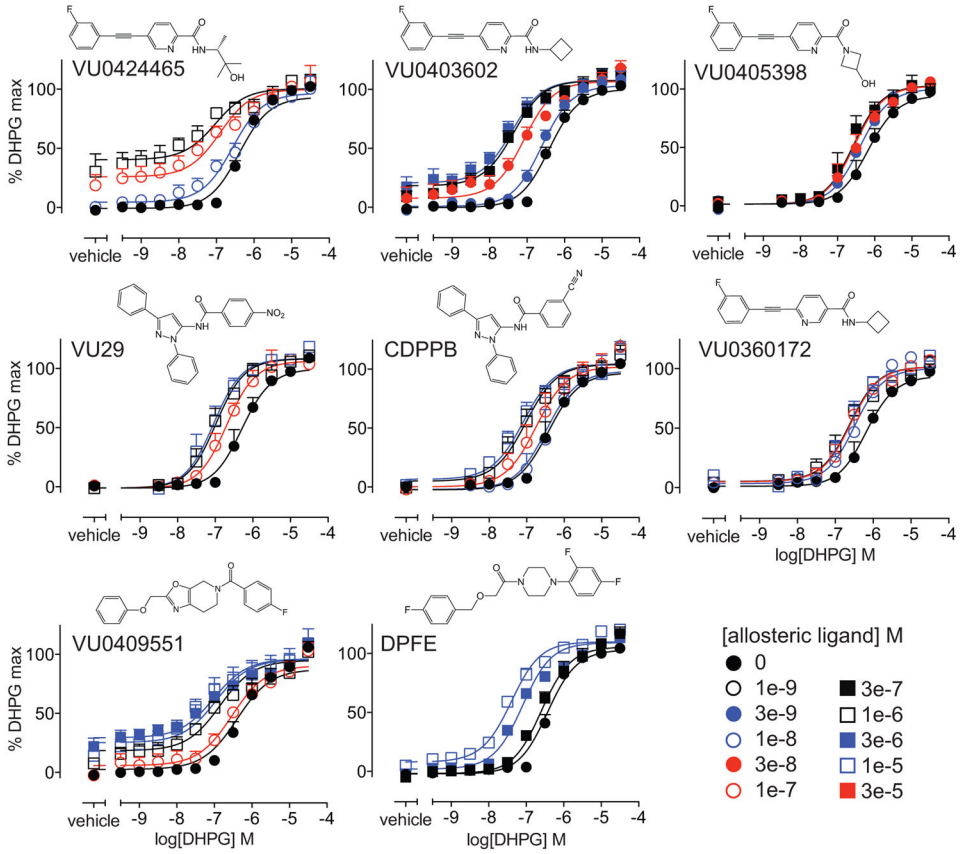

Figure 2. Allosteric modulation of DHPG-stimulated iCa^{2+} mobilization in HEK293A-$mGlu_5$-low cells

DHPG concentration-response curves for iCa^{2+} mobilization in the absence and presence of indicated concentrations of allosteric ligands. Interaction studies for DPFE, VU0409551, VU0424465, and VU0403602 were performed using simultaneous addition of both ligands, to minimize allosteric ligand-induced acute desensitization due to intrinsic agonist activity. VU29, VU0405398, CDPPB and VU0360172 were added 1 min prior to addition of glutamate. Data sets were globally fitted to an operational model of allosterism to estimate affinity and cooperativity. Curves represent the best fit of the data. Data are mean + SEM of n=3–10 experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

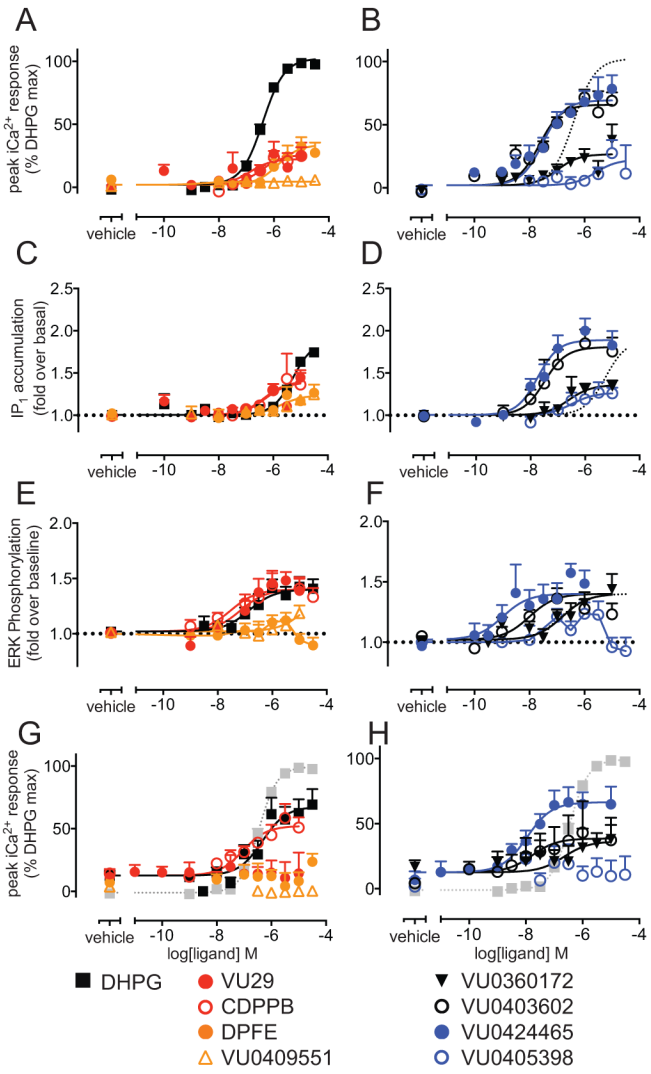

Figure 3. $mGlu_5$ allosteric ligands have agonist activity for iCa^{2+} mobilization, IP_1 accumulation and ERK phosphorylation in cultured cortical neurons

Concentration-response curves for indicated $mGlu_5$ orthosteric and allosteric ligands for iCa^{2+} mobilization (A&B), IP_1 accumulation (C & D), ERK1/2 phosphorylation (E&F) and iCa^{2+} mobilization in the absence of 1.2mM $CaCl_2$ (G & H; % DHPG max represents maximal DHPG response in the presence of 1.2mM $CaCl_2$, curve from panel A is shown in grey). In panels B, D, F and H, the DHPG curve is plotted as a dotted line for ease of reference. Responses were normalized to either the glutamate maximal response (iCa^{2+} mobilization) or represented as fold over basal (IP_1 accumulation and ERK phosphorylation). All assays were performed in the presence of 30 μM CPCCOEt to eliminate the contribution of $mGlu_1$. Data are expressed as mean + SEM of 3–22 experiments performed in duplicate, error bars not shown lie within the dimensions of the symbols.

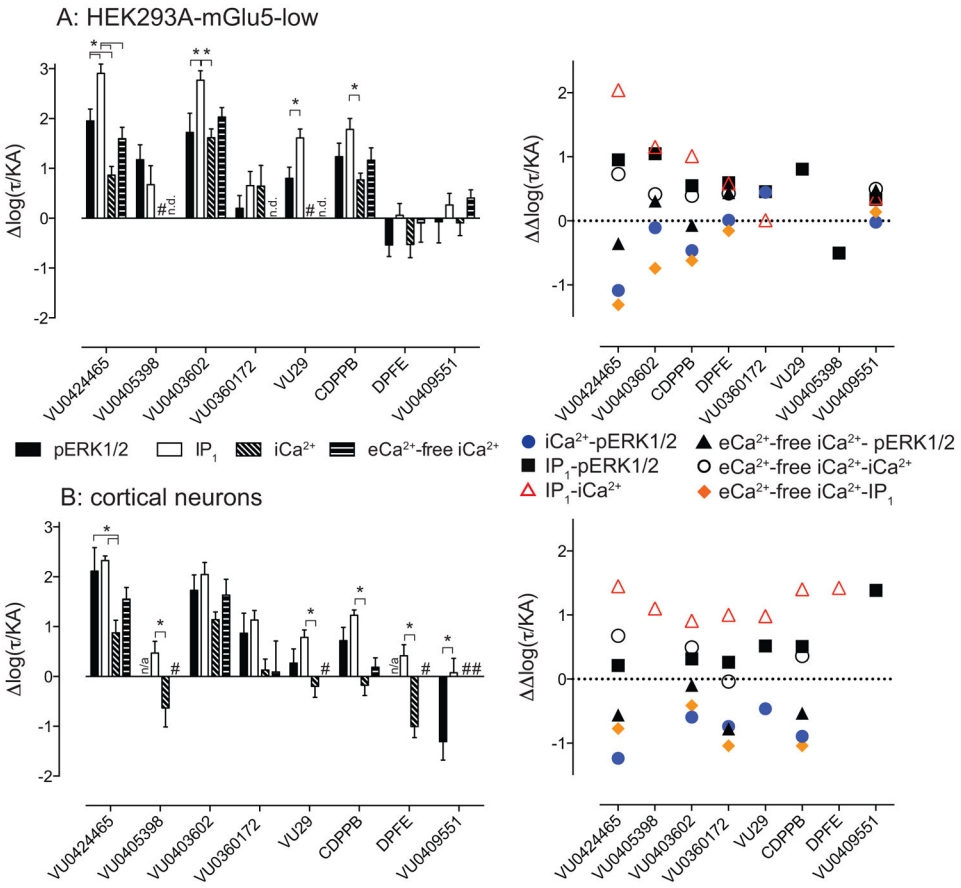

Figure 4. $mGlu_5$ allosteric ligands are biased agonists relative to DHPG in HEK293A-$mGlu_5$-low and cortical neurons

The transduction coefficient ($\log(\tau/K_A)$) was derived by applying equation 3 to agonist concentration-response curves in HEK293A-$mGlu_5$-low (A, curves in Figure 1) and cortical neurons (B, curves in Figure 2) and normalized to DHPG ($\Delta\log(\tau/K_A)$). To calculate the degree of bias evident for different ligands between different pathways, $\Delta\log(\tau/K_A)$ values were subtracted from one another $\Delta\Delta$ ($\log(\tau/K_A)$) to determine Log bias factors. Data for $\Delta\log(\tau/K_A)$ represent the mean ± SEM, whereas Log bias estimates are mean only. * denotes significantly different comparisons, $p<0.05$, one-way ANOVA with Tukey's post-test.

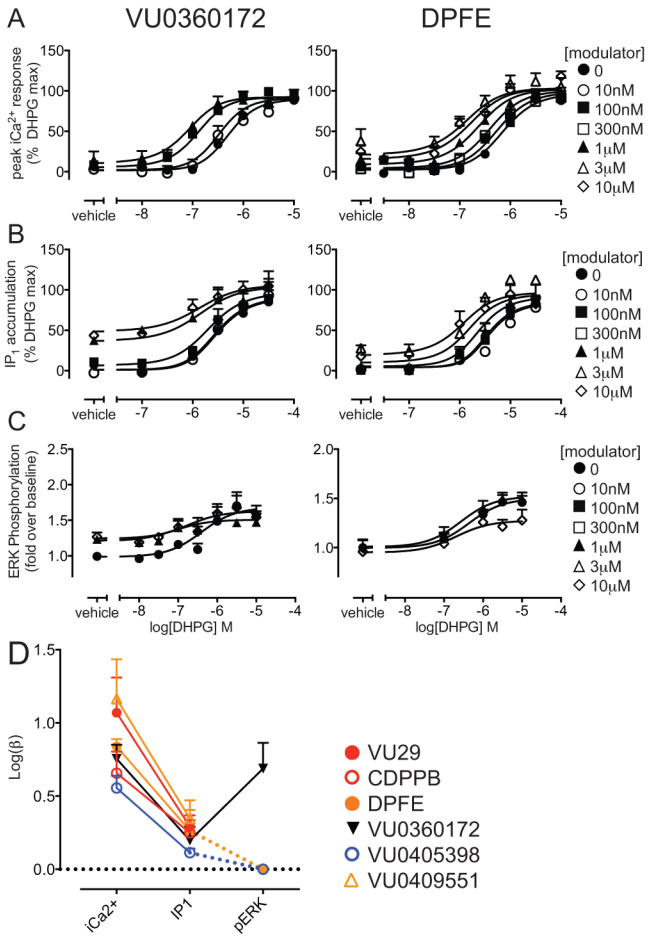

Figure 5. $mGlu_5$ allosteric ligands are biased modulators of DHPG responses in native cortical neurons

DHPG concentration response curves for iCa^{2+} mobilization (A), IP_1 accumulation (B) and ERK1/2 phosphorylation (C) in the absence and presence of VU0360172 and DPFE. Both VU0360172 and DPFE produced leftward shifts in DHPG potency for iCa^{2+} and IP_1 assays. Data sets were globally fitted to an operational model of allosterism to estimate affinity and cooperativity. Curves represent the best fit of the data. In ERK1/2 phosphorylation assays, DPFE produced a leftward shift with 1 μM however reduced the maximal response of DHPG at 10 μM. D) Cooperativity estimates (Logβ) of allosteric ligands were plotted to enable comparison of modulation across pathways. For DPFE and VU0405398, which showed mixed modulatory activity, Log β values were plotted as zero to highlight the implicit bias; the absolute numerical value in this instance has no meaning. Data are mean ± SEM of n=3–9 experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

Table 1

Affinity and cooperativity estimates for allosteric modulation of orthosteric agonist-mediated iCa^{2+} mobilization and IP_1 accumulation in HEK293A-$mGlu_5$-low and cortical neurons. Data are mean ± SEM of 3–9 independent experiments performed in duplicate.

	HEK293A: glu-iCa^{2+}		HEK293A: DHPG-iCa^{2+}		Cortical: DHPG-iCa^{2+}		Cortical: DHPG-IP_1	
	pK_B[a]	logβ[b]	pK_B	logβ	pK_B	logβ	pK_B	logβ
VU0424465	6.94±0.19	0.37±0.11	7.00±0.11	0.84±0.13[c]	n/a	n/a	n/a	n/a
VU0403602	7.62±0.17	0.97±0.15	7.23±0.34	1.06±0.12	n/a	n/a	n/a	n/a
VU0360172	6.67±0.18	0.53±0.13	7.24±0.36	0.52±0.07	7.11±0.29	0.75±0.10	6.55±0.15	0.20±0.09[d]
VU29	6.87±0.22	0.13±0.09	6.67±0.28	0.81±0.08[c]	5.70±0.45	1.07±0.24	6.51±0.19	0.29±0.04
CDPPB	6.58±0.25	0.69±0.16	6.87±0.17	0.72±0.08	6.61±0.25	0.66±0.15	6.81±0.41	0.25±0.12
DPFE	6.04±0.27	0.60±0.13	5.35±0.25	1.07±0.11[c]	5.45±0.22	0.84±0.05	5.50±0.23	0.26±0.14[d]
VU0405398	7.00±0.12	0.31±0.06	6.97±0.24	0.57±0.06[c]	5.37±0.29	0.56±0.08	6.46±0.13[d]	0.11±0.03[d]
VU0409551	5.96±0.43	1.00±0.27	5.88±0.20	1.06±0.09	6.04±0.24	1.16±0.27	6.14±0.16	0.35±0.13[d]

[a] pK_B, negative logarithm of the equilibrium dissociation constant determined using an operational model of allosterism.

[b] logβ, logarithm of the efficacy modulation factor.

n/a denotes operational parameters not determined due to confounding agonism.

[c] denotes $p<0.05$, comparing logβ values between glutamate and DHPG-stimulated iCa^{2+} mobilization.

[d] denotes $p<0.05$, comparing respective operational parameters between iCa^{2+} mobilization and IP_1 accumulation for cortical neurons, using an unpaired Student's t-test.

Neuropharmacology. Author manuscript; available in PMC 2018 March 15.