Positive Allosteric Modulators of Metabotropic Glutamate Receptor 5 as Tool Compounds to Study Signaling Bias

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ABSTRACT

Positive allosteric modulation of metabotropic glutamate subtype 5 (mGlu5) receptor has emerged as a potential new therapeutic strategy for the treatment of schizophrenia and cognitive impairments. However, positive allosteric modulator (PAM) agonist activity has been associated with adverse side effects, and neurotoxicity has also been observed for pure PAMs. The structural and pharmacological basis of therapeutic versus adverse mGlu5 PAM in vivo effects remains unknown. Thus, gaining insights into the signaling fingerprints, as well as the binding kinetics of structurally diverse mGlu5 PAMs, may help in the rational design of compounds with desired properties. We assessed the binding and signaling profiles of N-methyl-5-(phenylethynyl)pyrimidin-2-amine (MPPA), 3-cyano-N-(2,5-diphenylpyrazol-3-yl)benzamide (CDPPB), and 1-[4-(4-chloro-2-fluoro-phenyl)piperazin-1-yl]-2-(4-pyridylmethyl)thio-ethenone [compound 2c, a close analog of 1-[4-(2-chloro-4-fluorophenyl)piperazin-1-yl]-2-(pyridin-4-ylmethyl)ethanone] in human embryonic kidney 293A cells stably expressing mGlu5 using Ca2+ mobilization, inositol monophosphate (IP1) accumulation, extracellular signal–regulated kinase 1/2 (ERK1/2) phosphorylation, and receptor internalization assays. Of the three allosteric ligands, only CDPPB had intrinsic agonist efficacy, and it also had the longest receptor residence time and highest affinity. MPPA was a biased PAM, showing higher positive cooperativity with orthosteric agonists in ERK1/2 phosphorylation and Ca2+ mobilization over IP1 accumulation and receptor internalization. In primary cortical neurons, all three PAMs showed stronger positive cooperativity with (S)-3,5-dihydroxyphenylglycine (DHPG) in Ca2+ mobilization over IP1 accumulation. Our characterization of three structurally diverse mGlu5 PAMs provides further molecular pharmacological insights and presents the first assessment of PAM-mediated mGlu5 internalization.

SIGNIFICANCE STATEMENT

Enhancing metabotropic glutamate receptor subtype 5 (mGlu5) activity is a promising strategy to treat cognitive and positive symptoms in schizophrenia. It is increasingly evident that positive allosteric modulators (PAMs) of mGlu5 are not all equal in preclinical models; there remains a need to better understand the molecular pharmacological properties of mGlu5 PAMs. This study reports detailed characterization of the binding and functional pharmacological properties of mGlu5 PAMs and is the first study of the effects of mGlu5 PAMs on receptor internalization.

Introduction

The involvement of metabotropic glutamate (mGlu) receptors in central nervous system disorders such as Parkinson disease, schizophrenia, and major depressive disorder has made these receptors interesting targets for drug discovery research (Nicoletti et al., 2015; Foster and Conn, 2017). Metabotropic glutamate receptor subtype 5 (mGlu5) is a group I mGlu receptor that is primarily coupled to Gq/11 proteins. mGlu5 is generally found postsynaptically and is important in neuronal development and synaptic plasticity—for instance, in memory formation and cognition (Valenti et al., 2002; Dhami and Ferguson, 2006; Waung and Huber, 2009). High sequence similarity in the orthosteric glutamate binding site between the eight mGlu receptor subtypes makes the discovery of selective orthosteric ligands challenging (Wellendorph and Bräuner-Osborne, 2009). Hence, mGlu5 discovery efforts have focused on targeting topographically distinct sites with allosteric modulators; many diverse scaffolds have been...
identified that interact with a common site within the seven-transmembrane domains (Doré et al., 2014; Christopher et al., 2018). Allosteric modulators offer higher subtype receptor selectivity and the ability to spatiotemporally regulate pre-existing receptor responses; in this way, allosteric modulators potentially avoid unwanted side effects (Melancon et al., 2012; Changeux and Christopoulos, 2017). Allosteric modulators may enhance [termed positive allosteric modulators (PAMs)] or diminish receptor activation [termed negative allosteric modulators (NAMs)] (Gentry et al., 2015). PAMs can have intrinsic agonist activity and are referred to as PAM agonists (Foster and Conn, 2017; Sengmany et al., 2017).

The first bioavailable mGlu5 PAM, 3-cyano-N-(2,5-diphenylpyrazol-3-yl)benzamide (CDPPB), had antipsychotic-like and procognitive effects in preclinical models, establishing mGlu5 PAMs as promising interventions for schizophrenia (Kinney et al., 2005; Horio et al., 2013). Subsequently, mGlu5 PAMs have also been associated with serious adverse effects such as neurotoxicity and seizure induction (Bridges et al., 2013; Rook et al., 2013; Parmentier-Batteur et al., 2014). These adverse side effects were initially attributed to PAM agonist activity, e.g., VU424465 (5-[2-(3-fluorophenyl)ethynyl]-N-[2(R)-3-hydroxy-3-methylbutan-2-yl]pyridine-2-carboxamide) (Rook et al., 2013) and VU403602 (N-cyclobutyl-5-(3-fluorophenylethenyl)picolinamid) (Bridges et al., 2013). However, some pure PAMs may also lead to neurotoxicity, indicating that PAM agonist activity is not the only predictor of adverse effect liability (Parmentier-Batteur et al., 2014). In many drug discovery paradigms, PAM agonist activity is only tested in a single functional assay (i.e., Ca2+ mobilization). Such approaches do not detect pleiotropic mGlu5 signaling; therefore, some “pure” PAMs may in fact be agonists for different cellular responses. Investigation of biased mGlu5 signaling has thus emerged as a means to avoid unwanted side effects (Sengmany et al., 2017). Relative to a reference agonist, a “biased agonist” preferentially activates select responses relative to others activated through the same receptor (Trinh et al., 2018). Biased agonism is believed to be achieved through the stabilization of unique receptor conformations that have higher affinity for certain effector proteins over others (Kenakin and Christopoulos, 2013; Smith et al., 2018). Biased allosteric modulation is also possible, manifesting as different apparent affinities or magnitudes of cooperativity with the same orthosteric agonist depending upon the response measured (Sengmany et al., 2017; Hellyer et al., 2019; Sengmany et al., 2019).

Alongside the conformational theory for ligand bias, ligand binding kinetics are also implicated in signaling bias (Klein Herenbrink et al., 2016; Lane et al., 2017). The duration of the ligand-receptor complex is proposed to be proportional to agonist efficacy (Copeland, 2016); compounds that occupy receptors longer potentially catalyze more effector protein activation cycles (Lane et al., 2017). Therefore, increasing receptor residence time has been exploited as a strategy in rational drug design to increase ligand affinity and efficacy (Lindstrom et al., 2007; Tummino and Copeland, 2008). However, long residence times may also lead to on-target toxicity (Kapur and Seeman, 2001). To date, the contribution of ligand binding kinetics to mGlu5 biased agonism and potentiation has remained unexplored.

Here, we evaluated the signaling profiles of three structurally diverse mGlu5 PAMs using four different functional assays: Ca2+ mobilization, IP3 accumulation, ERK1/2 phosphorylation, and real-time receptor internalization. N-methyl-(5-phenylethenyl)pyrimidin-2-amine (MPPA) is a potent PAM of glutamate stimulation of intracellular Ca2+ mobilization and has efficacy in reversing amphetamine-induced hyperlocomotion in rats (Sharma et al., 2009). Discovered alongside the in vivo efficacious PAM 1-(4-(2-chloro-4-fluorophenyl)piperazin-1-yl)-2-(pyridin-4-ylmethyl)ethenehene, compound 2c has previously only been evaluated as a PAM of glutamate in mGlu5-Ca2+ mobilization assays (Xiong et al., 2010). The intrinsic efficacy and potentiation (of DHPG and L-glutamate) by these two PAMs were compared with CDPPB, a well characterized PAM agonist of glutamate activation of mGlu5 (Kinney et al., 2005; Sengmany et al., 2017). Moreover, we determined kinetics of PAM binding to mGlu5 and compared these parameters to affinity estimates obtained with functional assays and inhibition binding experiments.

Materials and Methods

The experiments presented in this paper were planned based on the availability of compounds and established assays and cell lines in the two laboratories in which the experiments were performed. The experiments were exploratory (i.e., not designed to test a prespecified statistical null hypothesis), and the reported P values should therefore be viewed as descriptive. The minimum number of independent experiments was decided beforehand based on our previous experiences with the assays and cell lines.

Materials. MPPA, CDPPB, and compound 2c were obtained from Lundbeck (Copenhagen, Denmark). DHPG, 7-(hydroxyimino)cycloprop[b]chromen-1a-carboxylic acid, MPPA, LY341495, and DL-TBOA were purchased from Tocris (Bristol, UK). DMEM GlutaMAX-I, FBS, dialyzed FBS, penicillin-streptomycin solution, B-27, Fungizone antymiotic, Neurobasal media, Fluoro-4 AM cell permeant dye, and HBSS were purchased from In Vitrogen (Carlsbad, CA). Probenecid, Pierce BCA protein assay kit, and Fluoro-4 AM No Wash kit were purchased from Thermo Fisher Scientific (Waltham, MA). [3H]Methoxy-PEPy was custom-synthesized by Pharmaron.

ABBREVIATIONS: AM, acetoxymethyl; CDPPB, 3-cyano-N-(2,5-diphenylpyrazol-3-yl)benzamide; compound 2c, 1-[(4-(4-chloro-2-fluoro-phenyl)piperazin-1-yl)-2-(4-pyridylmethoxy)ethenehene; DHPG, (S)-3,5-dihydroxyphenylglycine; DL-TBOA, DL-threo-β-benzoxylaspartic acid; DMEM, Dulbecco’s modified Eagle’s medium; CI, confidence interval; EAAT3, excitatory amino acid transporter 3; Emax, maximum response of a system; Emax-max, Maximum response to an agonist in a functional assay; ERK1/2, extracellular signal-regulated kinase 1/2; GPCR, G protein–coupled receptor; GPT, glutamic-pyruvic transaminase; HA, hemagglutinin; HBSS, Hank’s balanced salt solution; HEK293A, human embryonic kidney 293A; IP3, inositol monophosphate; LY341495, 15(S25);2-[(1S)-1-amino-1-carbonyl-2-(9H-xanthen-9-yl)ethyl]cyclopropane-1-carboxylic acid; methoxy-PEPy, 3-methoxy-5-[2-pyridinylethenyl]pyridine; mGlu, metabotropic glutamate; mGlu5, metabotropic glutamate subtype 5; MPPA, N-methyl-5-phenylethenyl)pyrimidin-2-amine; NAM, negative allosteric modulator; PAM, positive allosteric modulator; PAMmax, Maximal level of potentiation induced by a PAM when assessed in a modulator titration curve in the presence of orthosteric agonist; pEC50, negative logarithm of the half maximal effective concentration of an agonist; pERK1/2, phosphorylated extracellular signal-regulated kinases 1 and 2; pPAMmax, negative logarithm of the half maximal effective concentration of a PAM from a modulator titration curve in the presence of orthosteric agonist.
extracellular Ca\textsuperscript{2+} (Sengmany et al., 2017). PAM potentiation of the
represents both release from intracellular stores as well as influx of
low cells was measured as previously described (Arsova et al., 2020) and
HEK293A stable cell line (HEK293A-mGlu5-low) was maintained as
plate counter (PerkinElmer).

CaCl\textsubscript{2} with pH adjusted to 7.4) with 0.1% bovine serum albumin.
37°C and 5% CO\textsubscript{2} for 6 to 7 days before experimentation.
embryonic day 16 Asmu:Swiss wild-type mice sacrificed by decapita-
tured cells were routinely monitored for mycoplasma contamination.
were humanely sacrificed, and day 16 mixed-sex embryos were
Animal Committee (protocol number MIPS.2014.37). The 8-week-old Asmu:
outbred female wild-type mice were provided by the Monash Animal
Research Platform (Clayton, Victoria, Australia). Animals
were expressed as a percentage of the DHPG maximal response.
Intrinsic PAM agonist activity was measured with and without a 30-minute preincubation with 300 μM
LY341495 prior to PAM addition. Potentiation of orthosteric agonist
activity was measured in the presence of 500 nM L-glutamate or
DHPG. Cortical neurons were starved for 4 hours in starvation media.
Compounds were diluted in IP\textsubscript{1} assay buffer to 0.3% final DMSO
concentration. Concentrations were incubated for 1 hour at 37°C before
IP\textsubscript{1} levels were determined.

ERK1/2 Phosphorylation Assay. ERK1/2 phosphorylation in
HEK293A-mGlu5-low cells was measured with either the Advanced
phospho-ERK1/2 (Thr202/Tyr204) assay kit (Cisbio) or AlphaScreen
SureFire\textsuperset{TM} kit (TGR Biosciences) as previously described after a 3-
hour incubation in serum-free DMEM supplemented with 10 μM
glutamate and 10 mM sodium pyruvate to eliminate ambient glutamate (Sengmany et al., 2017; Arsova et al., 2020). Intrinsic PAM agonist activity
(5-minute stimulation) was measured with and without a 30-minute preincubation with 300 μM
LY341495. Potentiation of orthosteric agonist activity was measured in the presence of
500 nM L-glutamate (5-minute stimulation) or DHPG (20-minute stimulation).

Receptor Internalization Assay. mGlu\textsubscript{5} internalization in tran-
siently transfected HEK293A cells was measured with a time-resolved
Forster resonance energy transfer assay after labeling the receptor
with SNAP-Lumi4-Tb (Cisbio) as previously described (Arsova et al.,
2020). Intrinsic PAM agonist activity was measured with and without a 30-minute preincubation with 300 μM
LY341495. Potentiation of orthosteric agonist activity was measured in the presence of
30 μM DL-TBOA (to measure potentiation of glutamate) or 1 μM DHPG.

Data Analysis. Data were analyzed using GraphPad Prism
software version 8 (San Diego, CA) as previously described (Arsova et al., 2020). Briefly, inhibition binding data were fitted to either a competitive binding model,
\begin{equation}
Y = \frac{\text{Top} - \text{Bottom}}{1 + \frac{\text{IC}_{50}}{\text{Dil}}},
\end{equation}
or to an allosteric binding model,
\begin{equation}
K_{\text{app}} = K_p \frac{1 + \frac{\text{modulator}}{K_a}}{1 + \frac{\text{modulator}}{K_a}} + K_0.
\end{equation}
the top and bottom plateaus. The IC_{50} was used to estimate the K_i (equilibrium dissociation constant of the unlabeled inhibitor) using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Competition association binding was fitted to the kinetics of the competitive binding model:

\[ K_A = k_1[\text{radioligand}] + k_2 \]
\[ K_0 = k_3[\text{ligand}] + k_4 \]
\[ S = \sqrt{(K_A - K_0)^2 + 4k_1k_3[\text{radioligand}][\text{ligand}]} \]
\[ K_{F} = 0.5(K_A + K_0 + S) \]
\[ K_{S} = 0.5(K_A + K_0 - S) \]
\[ Q = B_{max} \frac{K_A[\text{radioligand}]}{K_{F} - K_{S}} \]
\[ Y = Q \frac{k_4(K_F - K_0)}{K_F - K_{S}} + \frac{k_4 - K_F}{K_F - K_{S}}e^{-k_4 - K_F - K_{S}} \]

where \( k_1 \) and \( k_2 \) are the radioligand kinetic association and dissociation rates, respectively; \( k_3 \) and \( k_4 \) are the unlabeled ligand kinetic association and dissociation rates, respectively; and \( B_{max} \) is the maximum binding.

Concentration-response curves from functional assays were fitted with a four-parameter sigmoidal concentration-response curve to derive EC_{50} and E_{max} values:

\[ Y = \text{Top} - \text{Bottom} \frac{\text{Top} - \text{Bottom}}{1 + 10^{\log(\text{EC}_{50}) - \log(\text{ligand})}} \]

Biased agonism was determined by fitting to the operational model of agonism (Black et al., 1985):

\[ Y = \text{basal} + \frac{(E_m - \text{basal})}{\left(1 + \frac{[A]}{K_A}\right)^n} + \frac{1}{\left(1 + \frac{[B]}{K_B}\right)^n} \]

where \([A]\) is the agonist concentration, \(E_m\) is the maximal response of the system, \(n\) is the transducer slope, and \(\tau\) is the coupling efficiency.

System and observation bias were nullified by subtraction of the transduction coefficient \(\log(\tau/K_A)\) of a compound from the transduction coefficient of a reference agonist to obtain \(\Delta\log(\tau/K_A)\).

Allosteric modulation of L-Glu- and DHPG-mediated responses were fitted to the operational model of allosterism:

\[ \text{Response} = \frac{E_m(t_A[A](K_a + [B]) + t_B[B]K_a)\beta}{[(A)K_a + K_a[B] + [B]K_B + [A][B] + k_0][[A][B]][K_a][K_B]} \]

where \(K_A\) and \(K_B\) are the equilibrium dissociation constants of the orthosteric ligand and allosteric modulator, respectively; \(\alpha\) represents affinity cooperativity; \(\beta\) is a scaling factor representing the effect an allosteric modulator has on orthosteric agonist efficacy; and \([A]\) and \([B]\) are the concentrations of the orthosteric agonist and the allosteric modulator, respectively. Parameters \(t_A\) and \(t_B\) represent the intrinsic ability of the orthosteric and allosteric ligand, respectively, to activate the receptor, and \(E_m\) and \(n\) represent the maximal system response and the transducer slope, respectively. \(K_a\) for DHPG and L-glutamate were constrained to values obtained from inhibition binding studies (Mutel et al., 2000; Gregory et al., 2012). Affinity cooperativity \(\alpha\) was constrained to 1, assuming neutral cooperativity.

**Results**

**Affinity, Association, and Dissociation Rates for mGlu5 PAM Binding.** Although previous studies show that CDPPB and MPPA bind to the common allosteric MPEP site on mGlu5 (Chen et al., 2007; Sharma et al., 2009), there is no binding information available for compound 2c. As such, we measured displacement of the radiolabeled MPEP analog ([\(^3\)H]methoxy-PEPy from mGlu5 to provide insight into the binding site of compound 2c and to determine PAM affinity estimates. Membranes from HEK293A cells with low expression of mGlu5 (HEK293A-mGlu5-low) were used, which have comparable mGlu5 expression to cortical astrocytes (Noetzel et al., 2012). MPPA fully displaced the radioligand, which is consistent with a competitive interaction (Fig. 1). Only partial displacement was observed for CDPPB, which may be due to either noncompetitive interaction or solubility limits of the compound (Fig. 1). Similarly, because of limited solubility, we were unable to test sufficiently high compound 2c concentrations to determine whether it can fully displace the radioligand (Fig. 1). Radioligand displacement curves were analyzed to obtain MPPA affinity (pK_I) estimates using a model of competitive binding, whereas for CDPPB, affinity (pK_B) and affinity cooperativity factor (\(\alpha\)) estimates were derived using the allosteric ternary complex model (Table 1). The compound 2c displacement curve was fitted with both models.

Binding kinetics of mGlu5 PAMs have not previously been assessed but could potentially be linked to different functional profiles. Therefore, the binding kinetics of MPPA, CDPPB, and compound 2c at mGlu5 were assessed with competition association binding experiments (Table 1). Data were fitted to the association competition binding function (Motulsky and Mahan, 1984) using kinetic parameters for ([\(^3\)H]methoxy-PEPy determined previously (k_{off}: 0.14 ± 0.01 minutes\(^{-1}\); k_{on}: 2.34 ± 0.46 × 10\(^8\) M\(^{-1}\) min\(^{-1}\); Arsova et al. (2020)) (Fig. 2). Both MPPA and compound 2c had fast binding kinetics, prohibiting accurate quantification of k_{off}. Hence, CDPPB had the longest residence time of the three PAMs, which is also reflected in a higher affinity relative to MPPA and compound 2c. MPPA had the fastest k_{on}, followed by CDPPB and compound 2c (Table 1).

**Intrinsic Agonist Activity in Signaling Assays in HEK293A-mGlu5-Low Cells.** MPPA, CDPPB, and compound 2c were assessed for intrinsic agonist activity by measuring mGlu5 activation of Ca\(^{2+}\) mobilization (release from intracellular stores and extracellular influx), IP\(_1\) accumulation, and ERK1/2 phosphorylation (Fig. 3). Each of the three PAMs showed mGlu5 agonist activity across all three measures. DHPG had similar potency (pEC_{50}) in the Ca\(^{2+}\) mobilization, IP\(_1\) accumulation, and ERK1/2 phosphorylation assays (Supplemental Table 1). We hypothesized that intrinsic agonist activity of PAMs may be due to potentiation of ambient glutamate. Therefore, experiments were repeated in the presence of 300 \(\mu\)M LY341495, a nonspecific mGlu orthosteric antagonist (Kingston et al., 1998). Treatment with LY341495 reduced the basal level of IP\(_1\) accumulation to 22.8% of the untreated control, indicative of inverse agonist activity or inhibition of ambient glutamate (Supplemental Fig. 1). LY341495 had no effect on basal responses for Ca\(^{2+}\) mobilization or ERK1/2 phosphorylation (Supplemental Fig. 1). In the presence of LY341495, only CDPPB retained agonist activity for the three measures of mGlu5 activity, indicating that apparent intrinsic agonism for MPPA and compound 2c was most likely due to modulation of ambient glutamate. CDPPB agonism was then compared with that of the orthosteric agonist DHPG. Relative to DHPG, CDPPB was a partial...
agonist for Ca\(^{2+}\) mobilization and ERK1/2 phosphorylation but achieved the same maximal response as DHPG in the absence of LY341495 in the IP\(_{1}\) accumulation assay (Fig. 3; Supplemental Fig. 1; Supplemental Table 2). CDPPB had significantly lower agonist potency in IP\(_{1}\) accumulation (12- to 30-fold) when compared with ERK1/2 phosphorylation and Ca\(^{2+}\) mobilization (Supplemental Fig. 1; Supplemental Table 2). CDPPB had similar PAM potencies (Supplemental Table 3).

The mGlu5 receptor is internalized upon stimulation with L-glutamate and DHPG in the three signaling assays (Fig. 4). All three PAMs potentiated the responses induced by both orthosteric agonists in Ca\(^{2+}\) mobilization, IP\(_{1}\) accumulation, and ERK1/2 phosphorylation signaling assays (Fig. 4). Concentration-response curves were fitted to quantify PAM potency (pPAM\(_{50}\)) and the maximum level of potentiation (PAM\(_{\text{max}}\)) (Supplemental Table 3). Compound 2c potentiated the L-glutamate and DHPG responses to the same maximum response as the orthosteric agonists alone in the Ca\(^{2+}\) mobilization and IP\(_{1}\) accumulation assays. Both compound 2c and CDPPB potentiated the L-glutamate and DHPG responses above the orthosteric agonist maximal response in the ERK1/2 phosphorylation assay (Fig. 4). Compound 2c had the lowest potency in all three assays, whereas MPPA and CDPPB had similar PAM potencies (Supplemental Table 3).

PAMs Induce mGlu\(_{5}\) Internalization in HEK293A Cells. Most GPCRs are regulated by desensitization and internalization upon agonist stimulation (Ferguson, 2001). The mGlu\(_{5}\) receptor is internalized upon stimulation with L-glutamate (Levoye et al., 2015; Arsova et al., 2020), and several PAMs can induce and/or potentiate DHPG-stimulated mGlu\(_{5}\) desensitization of Ca\(^{2+}\) mobilization (Hellyer et al., 2019). The ability of MPPA, CDPPB, and compound 2c to induce mGlu\(_{5}\) internalization was characterized using a real-time internalization assay. The assay is based on time-resolved Förster resonance energy transfer between the long lifetime donor fluorophore Lumih-Tb, covalently attached to a SNAP-tag on cell surface receptors, and the cell-impermeant acceptor fluorophore fluorescein-O-α/α-acetic acid (Roed et al., 2014; Foster and Bräuner-Osborne, 2018). The assay requires N-terminal fusion of mGlu\(_{5}\) with a SNAP-tag; therefore, HEK293A cells were transiently transfected with SNAP-tagged mGlu\(_{5}\) (HEK293A-SNAP-mGlu\(_{5}\)), which resulted in mGlu\(_{5}\) expression levels that were ~10 times higher than in the HEK293A-mGlu\(_{5}\)-low cell line (Arsova et al., 2020). Cells were cotransfected with the EAAT3 glutamate transporter to reduce the extracellular glutamate concentration during measurements. To measure PAM potentiation of glutamate transport was inhibited by adding the nontransportable EAAT3 inhibitor DL-TBOA at the same time as the PAMs. However, inhibition of EAAT3 with a saturating concentration of DL-TBOA (100 μM) resulted in ~0.9 μM extracellular L-glutamate and ~40% of the mGlu\(_{5}\) internalization induced by 100 μM L-glutamate (Arsova et al., 2020). The DL-TBOA concentration was reduced to 30 μM for the L-glutamate potentiation experiments, which resulted in 24% (95% CI 23%-25%, n = 3) of the maximum L-glutamate–induced mGlu\(_{5}\) internalization.

DHPG induced a concentration-dependent increase in mGlu\(_{5}\) internalization that reached a plateau around

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**TABLE 1**

Affinity and kinetics of binding estimates for mGlu\(_{5}\) PAMs obtained from competition binding experiments with \(^{3}H\)methoxy-PEPy in HEK293A-mGlu\(_{5}\)-low cells

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pK(_{B}) (95% CI)</th>
<th>pK(_{A}) (95% CI)</th>
<th>Log a' (95% CI)</th>
<th>n</th>
<th>k(_{\text{on}}) (\times 10^{7}) M(^{-1}) min(^{-1}) (95% CI)</th>
<th>k(_{\text{off}}) (min(^{-1})) (95% CI)</th>
<th>RT(<em>{p}^f) pK(</em>{A}) n</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPPA</td>
<td>n.d.</td>
<td>n.d.</td>
<td>−0.64 (−1.10 to −0.24)</td>
<td>4</td>
<td>11.0 (3.7–18.2)</td>
<td>1</td>
<td>n.d.</td>
</tr>
<tr>
<td>CDPPB</td>
<td>7.27 (6.79–7.74)</td>
<td>n.d.</td>
<td>−0.64 (−1.10 to −0.24)</td>
<td>4</td>
<td>1.91 (–0.5 to 4.1)</td>
<td>0.211 (0.067–0.356)</td>
<td>4.7</td>
</tr>
<tr>
<td>Compound 2c</td>
<td>5.17 (5.08–5.27)</td>
<td>5.36 (5.19–5.53)</td>
<td>−0.96 (−1.17 to −0.75)</td>
<td>6</td>
<td>0.16 (−0.01 to 0.34)</td>
<td>1</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., not determined due to no fit with the given model.

\(^{a}\)Negative logarithm of the equilibrium dissociation constant determined with a competitive binding model.

\(^{b}\)Logarithm of the cooperativity factor.

\(^{c}\)Association rate constant.

\(^{d}\)Disassociation rate constant. For ligands with fast k\(_{\text{on}}\) global analyses could not derive k\(_{\text{off}}\); therefore, the value was constrained to 1 to enable estimation of k\(_{\text{on}}\).

\(^{e}\)RT\(_{p}\), residence time defined as 1/k\(_{\text{off}}\).

\(^{f}\)Negative logarithm of the equilibrium dissociation constant determined from kinetic parameters (k\(_{\text{on}}\)/k\(_{\text{off}}\)).
60 minutes after agonist addition (Supplemental Fig. 2), similar to the previously observed temporal profile for L-glutamate (Arsova et al., 2020). In the absence of added orthosteric agonist or antagonist, CDPPB and compound 2c induced mGlu5 internalization, although to a lower level than that achieved by DHPG (Fig. 5, A–C). When 300 μM LY341495 was added to block activation by ambient/released L-glutamate, only CDPPB remained a partial agonist for inducing mGlu5 internalization (Fig. 5, D–F), consistent with intrinsic efficacy in the three signaling assays (Fig. 3). LY341495 alone had no effect on the baseline level of internalization (Supplemental Fig. 1). Internalization concentration-response curves were calculated by determining the area under the curves from the 60-minute time courses (Fig. 6) and fitted to determine the E_{max} and pEC_{50} values (Supplemental Table 2). The agonist potency of CDPPB for internalization was >30-fold lower than for Ca^{2+} mobilization and pERK1/2, but within 3-fold of IP_{1} accumulation. DHPG potency for internalization was within 4-fold of the three signaling measures. Compound 2c curves were not well defined, precluding the estimation of E_{max} and pEC_{50} values (Fig. 6A).

All three PAMs potentiated L-glutamate– and DHPG-induced internalization with kinetics similar to DHPG (Fig. 5, G–L; Supplemental Fig. 3). Potentiation of both L-glutamate and DHPG by MPPA induced a lower maximum internalization than CDPPB and compound 2c (Fig. 6, C–D). Similar to the other signaling assays, compound 2c had the...
lowest pPAM$_{50}$ value of the three PAMs in the internalization assay. CDPPB and compound 2c potentiated to similar or greater levels of internalization compared with each orthosteric ligand alone (Fig. 6, C–D).

Quantification and Comparison of PAM Affinity and Cooperativity in HEK293A Cells. Comparisons of PAM$_{\text{max}}$ and pPAM$_{50}$ values between the four measures of mGlu$_5$ function revealed assay-dependent differences for each PAM (Supplemental Fig. 4) but no evidence for probe dependence when comparing values derived from DHPG versus L-glutamate (Supplemental Fig. 5). However, PAM$_{\text{max}}$ and pPAM$_{50}$ values are assay-dependent composite values comprising allosteric ligand affinity, cooperativity, and efficacy. Assay-independent measures of affinity (pK$_A$ or pK$_B$), intrinsic efficacy ($\tau$), and cooperativity ($\alpha\beta$) can be derived from fitting of concentration-response curves of PAMs and a reference agonist to operational models of agonism or allosterism.

CDPPB concentration-response curves in the presence of LY341495 were fitted with the operational model of agonism, in which DHPG was the reference agonist, to determine pK$_A$ and $\tau$ for different mGlu$_5$ functional measures (Table 2). The apparent pK$_A$ was lower for the internalization pathway than for Ca$^{2+}$ mobilization and ERK1/2 phosphorylation (Table 2). However, the model is limited to partial agonists, so for IP$_1$ accumulation, in which CDPPB behaved as a full agonist, we were only able to determine the composite transduction coefficient log($\tau$/K$_A$). By comparing these with the transduction coefficient of DHPG, we calculated $\Delta$log($\tau$/K$_A$) values for each pathway and found that CDPPB did not show any significant bias between functional measures (Fig. 7A; Table 2).

We used an operational model of allosterism to determine the affinity (pK$_B$) and cooperativity (log $\beta$) from concentration-response curves of PAM potentiation of either L-glutamate or DHPG (Tables 3 and 4). The intrinsic agonist activity ($\tau$) of CDPPB was constrained to the values determined for CDPPB in the presence of LY341495 when fitting the Ca$^{2+}$ mobilization and ERK1/2 phosphorylation curves. Since $\tau$ could not be determined for IP$_1$ accumulation of CDPPB, it was not possible to use the operational model of allosterism to analyze the corresponding potentiation curves. Furthermore, compound 2c potentiated the activity of both orthosteric agonists in IP$_1$ accumulation and of DHPG in ERK1/2 phosphorylation above the maximum response of the orthosteric agonist. This prohibited fitting compound 2c data with the operational model of allosterism, as there was no independent means to estimate the maximal system response (E$_{\text{max}}$). With the assumption that PAMs were neutral with respect to affinity cooperativity (log $\alpha$), comparing the efficacy cooperativity scaling factor (log $\beta$) across the four functional pathways showed that log $\beta$ was highest in the Ca$^{2+}$ mobilization pathway for MPPA with L-glutamate and for the Ca$^{2+}$...
mobilization and ERK1/2 phosphorylation pathways for MPPA with DHPG (Fig. 7B). The pK_B values were in general agreement between each functional measure and with pK_I values derived from radioligand inhibition binding, although the pK_B values for CDPPB and compound 2c derived from the internalization experiment were 5- to 8-fold lower than the corresponding pK_I values (Supplemental Fig. 6). There was no indication of probe bias when comparing the pK_B values obtained in presence of L-glutamate and DHPG, but the log β values were on average 2.09-fold (95% CI 1.70–2.48) higher in the presence of DHPG (Supplemental Fig. 5, C and D).

Fig. 5. Real-time measurement of mGlu5 internalization. HEK293A cells were transiently transfected with HA-SNAP-mGlu5 and EAAT3 (HEK293A-SNAP-mGlu5), and internalization was measured as a change in fluorescence over time. (A–C) Indicated concentrations of each PAM were added at t = 0 minutes, and surface mGlu5 levels were tracked for 66 minutes. (D–F) PAM-induced mGlu5 internalization in the presence of 300 μM LY341495. (G–I) Potentiation of L-glutamate–induced mGlu5 internalization by indicated PAMs. The L-glutamate concentration was increased by partially blocking the EAAT3 glutamate transporter with 30 μM DL-TBOA (DL-threo-β-Benzoxoaspartic acid). (J–L) Potentiation of 1 μM DHPG-induced mGlu5 internalization by indicated PAMs. Data points are means ± S.D. (triplicate measurements) from three independent experiments, and solid lines are nonlinear regression fit to an exponential model of one-phase association.

mobilization and ERK1/2 phosphorylation pathways for MPPA with DHPG (Fig. 7B). The pK_B values were in general agreement between each functional measure and with pK_I values derived from radioligand inhibition binding, although the pK_B values for CDPPB and compound 2c derived from the internalization experiment were 5- to 8-fold lower than the corresponding pK_I values (Supplemental Fig. 6). There was no indication of probe bias when comparing the pK_B values obtained in presence of L-glutamate and DHPG, but the log β values were on average 2.09-fold (95% CI 1.70–2.48) higher in the presence of DHPG (Supplemental Fig. 5, C and D).

Agonism and Potentiation of DHPG in Cortical Neurons. We used primary cortical neurons to study PAM Ca^{2+} mobilization and IP_1 accumulation in cells with endogenous expression of mGlu5. In these experiments, we used DHPG as the orthosteric agonist (pEC_{50} values given in Supplemental Table 1), as glutamate was unsuitable because of the presence of other mGlu receptors and ionotropic
glutamate receptors in cortical neurons. We preincubated cells with 30 μM 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester to inhibit activation of mGlu1, which is also a receptor for DHPG (Ito et al., 1992). In the absence of orthosteric ligand, only compound 2c induced a weak agonist response in Ca2+ mobilization (Fig. 8A). In contrast, all PAMs acted as partial agonists (50%–60% of the maximum DHPG response) in the IP1 accumulation assay (Fig. 8B; Supplemental Table 3). Although neurons endogenously express glutamate transporters, it is possible that glutamate released during the IP1 accumulation experiment could contribute to the observed stimulation, similar to what we observed in HEK293A cells, in which it was necessary to block the orthosteric binding site with a competitive antagonist to determine the agonist activity of the PAMs. However, there are no mGlu5-selective orthosteric antagonists, and since cortical neurons also express other mGlu receptors, the inclusion of a nonselective orthosteric antagonist such as LY341495 could be a further confounding factor. GPT was included to minimize the influence of ambient glutamate.

All PAMs potentiated a low concentration (120 nM) of DHPG-stimulated mGlu5 Ca2+ mobilization and IP1 accumulation (Fig. 8, C and D). In Ca2+ mobilization assays, the maximum response of compound 2c potentiation of DHPG (Emax) was similar to the Emax of DHPG, whereas MPPA and CDPPB induced 40%–50% of the DHPG Emax (Supplemental Table 3). In the IP1 accumulation pathway, all three PAMs potentiated the DHPG response to 70%–80% of the DHPG Emax (Fig. 8D). CDPPB had the highest potency (pPAM50) in both assays in the presence of DHPG and the highest pEC50 in the IP1 accumulation assay without added agonist.

Again, we used operational models to derive the affinities (pKA) and transduction coefficients log(\(t/KA\)) from PAM concentration-response curves in the absence of orthosteric ligand and the affinities (pKB) and cooperativities (log \(b\)) from DHPG potentiation curves (Table 5). Only compound 2c elicited a response in both pathways in the absence of

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pK(A^c) (95% CI)</th>
<th>Log((t/KA)) (95% CI)</th>
<th>n</th>
<th>pK(A^c) (95% CI)</th>
<th>Log((t/KA)) (95% CI)</th>
<th>n</th>
<th>pK(A^c) (95% CI)</th>
<th>Log((t/KA)) (95% CI)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPG</td>
<td>n.d.</td>
<td>6.78 (6.64–6.93)</td>
<td>3</td>
<td>n.d.</td>
<td>5.89 (5.80–5.98)</td>
<td>5</td>
<td>n.d.</td>
<td>6.06 (5.99–6.13)</td>
<td>11</td>
</tr>
<tr>
<td>MPPA</td>
<td>n.r.</td>
<td>6.18 (5.89–6.47)</td>
<td>5</td>
<td>n.r.</td>
<td>6.18 (5.89–6.47)</td>
<td>5</td>
<td>n.r.</td>
<td>7.03 (6.58–7.61)</td>
<td>5</td>
</tr>
<tr>
<td>CDPPB</td>
<td>7.20 (6.36–7.94)</td>
<td>6.71 (5.88–7.55)</td>
<td>4</td>
<td>n.d.</td>
<td>6.58 (6.58–7.61)</td>
<td>5</td>
<td>n.r.</td>
<td>5.57 (5.22–5.96)</td>
<td>5</td>
</tr>
<tr>
<td>Compound</td>
<td>n.r.</td>
<td>n.r.</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>3</td>
<td>n.r.</td>
<td>n.r.</td>
<td>3</td>
</tr>
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</table>

n.d., not determined; n.r., no response.
\(^c\)pKA, negative logarithm of the equilibrium dissociation binding constant determined with the operational model of agonism.
\(^d\)Log(\(t/KA\)), transduction coefficient; r, intrinsic efficacy.
\(^f\)P < 0.05 when compared with estimates from Ca2+ mobilization or IP1 accumulation by one-way ANOVA with Tukey’s multiple comparisons test.
orthosteric ligand, with similar transduction coefficients for the Ca\(^{2+}\) mobilization and IP\(_1\) accumulation pathways. In the presence of DHPG, the cooperativity factors (log \(\beta\)) of MPPA, CDPPB, and compound 2c were indistinguishable from 0 in the IP\(_1\) accumulation pathway. Therefore, log \(\beta\) was higher for all three PAMs in the Ca\(^{2+}\) mobilization pathway. Affinity estimates (pK\(_B\)) were similar for the two pathways for MPPA, CDPPB, and compound 2c (Table 5).

**Discussion**

Positive allosteric modulation of mGlu\(_5\) shows promise as a potential therapeutic strategy for schizophrenia. However, undesirable on-target side effects associated with mGlu\(_5\) PAMs have stalled development (Rook et al., 2013; Parmentier-Batteur et al., 2014; Foster and Conn, 2017). Development of safer and more efficacious mGlu\(_5\) PAMs is hampered by the lack of in-depth molecular pharmacological characterization to accurately link in vitro profiles to in vivo effects. We provided a detailed characterization of the mGlu\(_5\) PAMs CDPPB, MPPA, and compound 2c, representing distinct structural scaffolds; MPPA and compound 2c had previously only been assessed in mGlu\(_5\)-mediated Ca\(^{2+}\) mobilization assays. CDPPB had a longer receptor residence time than either MPPA or compound 2c, which correlated with higher mGlu\(_5\) affinity. In addition to rigorously profiling the agonist and potentiator activity of each modulator at three measures of acute mGlu\(_5\) signaling, we assessed the influence of mGlu\(_5\) PAMs on receptor internalization for the first time. We found no evidence for biased agonism; however, MPPA was a biased modulator, with different magnitudes of positive cooperativity with L-glutamate or DHPG depending on the measure of mGlu\(_5\) function. Importantly, this biased cooperativity also translated to natively expressed mGlu\(_5\) in primary cortical neurons.

Ligand binding kinetics have been correlated with compound affinity and efficacy in vivo (Tummino and Copeland, 2008; Copeland, 2016). We report the first assessment of mGlu\(_5\) PAM binding kinetics. CDPPB has the longest receptor residence time of the three PAMs. A slower rate of dissociation may be linked to the fact that CDPPB alone showed intrinsic efficacy at all four measures of mGlu\(_5\) activity in recombinant cells. For some GPCRs, compounds with longer residence times or fast k\(_{on}\) rates have higher efficacy and, thus, have been considered as more desirable lead compounds (Vauquelin and Charlton, 2010; Guo et al., 2014; Doornbos et al., 2017). Indeed, the efficacy (but not affinity) of select mGlu\(_2\) PAMs

![Fig. 7. Assessment of biased agonism or modulation in HEK293A-mGlu\(_5\)-low or HEK293A-SNAP-mGlu\(_5\) cells. (A) For CDPPB intrinsic agonism, Δlog(\(\psi_0\)) values (relative to DHPG) were derived from concentration-response curves in the presence of LY341495. (B) Cooperativity factors for each functional response are presented relative to the value calculated from Ca\(^{2+}\) mobilization. Cooperativity with DHPG is depicted in squares. Cooperativity with t-glutamate is depicted in triangles. For select PAMs and functional outputs, cooperativity could not be determined (not applicable: n.a.) or was indistinguishable from neutral because of intrinsic PAM agonist activity. Data are means and 95% CI from three to five independent experiments (refer to Tables 2 and 4 for exact numbers). *P < 0.05, one-way ANOVA with Tukey’s multiple comparisons test.](image-url)
Indeed, MPPA behaved similarly here; a fast kon rate correlated with increased affinities (Doornbos et al., 2017).

...ligands.

...for the receptors (Unett et al., 2013; Klein Herenbrink et al., 2016). 

...modulation of low concentrations of orthosteric ligand (l-glutamate or DHPG) by mGlu5 PAMs in HEK293A-mGlu5- low or HEK293A cells

Data represent the means and 95% CI of n independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ca\textsuperscript{2+} Mobilization</th>
<th>IP\textsubscript{1} Accumulation</th>
<th>pERK1/2</th>
<th>Internalization\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Glutamate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPPA</td>
<td>0.73 (0.57–0.89) \textsuperscript{bc}</td>
<td>0.19 (0.15–0.24) \textsuperscript{ef}</td>
<td>0.34 (0.28–0.41)</td>
<td>0.18 (0.16–0.21) \textsuperscript{f}</td>
</tr>
<tr>
<td>CDPPB</td>
<td>0.39 (0.20–0.58) \textsuperscript{d}</td>
<td>n.a. \textsuperscript{d} \textsuperscript{f}</td>
<td>0.16 (0.04–0.28)</td>
<td>0.44 (0.29–0.75) \textsuperscript{f}</td>
</tr>
<tr>
<td>Compound 2c</td>
<td>0.87 (0.63–1.11) \textsuperscript{d}</td>
<td>n.a. \textsuperscript{d} \textsuperscript{f}</td>
<td>0.48 (0.42–0.55)</td>
<td>0.48 (0.41–0.59) \textsuperscript{f}</td>
</tr>
<tr>
<td>DHPG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPPA</td>
<td>1.04 (0.88–1.24) \textsuperscript{d}</td>
<td>0.45 (0.28–0.62) \textsuperscript{ef}</td>
<td>0.94 (0.80–1.09)</td>
<td>0.40 (0.35–0.45) \textsuperscript{ef}</td>
</tr>
<tr>
<td>CDPPB</td>
<td>0.74 (0.54–0.91) \textsuperscript{f}</td>
<td>n.a. \textsuperscript{f} \textsuperscript{e}</td>
<td>0\textsuperscript{f}</td>
<td>0\textsuperscript{f}</td>
</tr>
<tr>
<td>Compound 2c</td>
<td>1.40 (1.14–1.99) \textsuperscript{d}</td>
<td>1.23 (0.76–1.70) \textsuperscript{f}</td>
<td>0.98 (0.89–1.08)</td>
<td>3 \textsuperscript{f}</td>
</tr>
</tbody>
</table>

n.a., not applicable.

\textsuperscript{a}Log β, logarithm of the efficacy cooperativity factor.

\textsuperscript{b}Log β, logarithm of the efficacy cooperativity factor.

\textsuperscript{c}Because of the full agonist activity of CDPPB in IP\textsubscript{1} accumulation assays, it was not possible to fit the operational model of allosterism to PAM titration curves in the presence of EC\textsubscript{50} orthosteric agonist. Compound 2c potentiated orthosteric agonist activity for IP\textsubscript{1} accumulation above the maximal agonist response, prohibiting accurate estimation of log β, as there was no independent means to determine the maximal system response (Em).

\textsuperscript{d}P < 0.05 when compared with estimate from pERK1/2 by one-way ANOVA with Tukey’s multiple comparisons test.

\textsuperscript{e}P < 0.05 when compared with 0 (one-sample t test).

\textsuperscript{f}An F-test revealed that log β was not different from 0, and log β was therefore constrained to 0. As such, the observed response is assumed to be a combination of the orthosteric ligand and the intrinsic agonist activity of CDPPB, where log(β) was equal to −0.49 (Ca\textsuperscript{2+}), −0.11 (pERK1/2), and −0.02 (internalization) based on intrinsic agonism in the presence of LYS41495.

Correlates with residence time, whereas increased k\textsubscript{on} rates correlated with increased affinities (Doornbos et al., 2017).

Indeed, MPPA behaved similarly here; a fast k\textsubscript{on} rate compensated for a short residence time, giving rise to submicroscopic affinity. Ligand kinetics can also determine the extent of signaling bias for the serotonin 5-HT\textsubscript{2B} and the dopamine D\textsubscript{2} receptors (Unett et al., 2013; Klein Herenbrink et al., 2016) but not for the μ opioid receptor (Pedersen et al., 2020). In the future, it would be of interest to explore the relationship between receptor residence time and PAM agonist efficacy and biased agonism for additional structurally diverse mGlu\textsubscript{5} ligands.

CDPPB alone retained PAM agonist activity across all four measures of mGlu\textsubscript{5} activity in the presence of both GPT/EAAT3 and LYS41495 to negate the confounding influence of ambient glutamate. These data are in keeping with previous evidence for CDPPB PAM agonist activity as well as induction of receptor desensitization and tolerance development with respect to changes in sleep architecture, although such effects are known to be context- and model-dependent (Kinney et al., 2005; Parmentier-Batteur et al., 2012; Hellyer et al., 2019).

CDPPB showed robust agonist efficacy for IP\textsubscript{1} accumulation while being a weak partial agonist for Ca\textsuperscript{2+} mobilization and ERK1/2 phosphorylation, consistent with previous work.

Fig. 8. PAM agonism and potentiation of DHPG in cortical neurons. Intrinsic agonist activity of PAMs in primary cortical neurons for Ca\textsuperscript{2+} mobilization (A) and IP\textsubscript{1} accumulation (B). (C) PAM potentiation of 120 nM DHPG was assessed in Ca\textsuperscript{2+} mobilization assays with simultaneous measurement of intracellular Ca\textsuperscript{2+} and veh. denotes vehicle.
Application of the operational model of agonism found that CDPPB was not biased relative to DHPG, in direct contrast to earlier findings in which CDPPB and a number of structurally diverse mGlu5 PAMs preferentially activated IP$_1$ accumulation over Ca$^{2+}$ mobilization to l-glutamate or DHPG (Sengmany et al., 2017; Hellyer et al., 2018). One possible explanation for this discrepancy may be the inclusion of the orthosteric antagonist LY341495. Notably, the same concentration of LY341495 had very different effects on the DHPG concentration-response curve, right-shifting DHPG potency in Ca$^{2+}$ mobilization as expected for a competitive antagonist; however, the DHPG response was completely abolished in IP$_1$ accumulation and pERK1/2. Moreover, LY341495 markedly reduced basal IP$_1$ accumulation, suggestive of inverse agonist activity. The lack of apparent inverse agonism for other mGlu$_5$ activity measures may reflect observational bias or an LY341495-specific accumulation over Ca$^{2+}$ mobilization relative to l-glutamate or DHPG (Sengmany et al., 2017; Hellyer et al., 2018). One possible explanation for this discrepancy may be the inclusion of the orthosteric antagonist LY341495. Notably, the same concentration of LY341495 had very different effects on the DHPG concentration-response curve, right-shifting DHPG potency in Ca$^{2+}$ mobilization as expected for a competitive antagonist; however, the DHPG response was completely abolished in IP$_1$ accumulation and pERK1/2. Moreover, LY341495 markedly reduced basal IP$_1$ accumulation, suggestive of inverse agonist activity. The lack of apparent inverse agonism for other mGlu$_5$ activity measures may reflect observational bias or an LY341495-specific effect. Certain mGlu$_5$ NAMs are biased modulators (Jong et al., 2019; Sengmany et al., 2019; Arsova et al., 2020). Future experiments should explore this possibility for orthosteric antagonists. Therefore, it is possible that the receptor conformations sampled when simultaneously occupied by LY341495 and CDPPB are distinct from those sampled by CDPPB alone or CDPPB with a small population occupied by the low ambient glutamate levels.

Probe dependence is operative at mGlu$_5$, manifesting as differences in the magnitude of cooperativity depending on the orthosteric agonist used (Sengmany et al., 2017; Hellyer et al., 2018). Probe-dependent PAMs include 1-(4-(2,4-difluorophenyl)piperazin-1-yl)-2-(4-fluorobenzyl)oxy)ethanone (structurally related to compound 2c) and acetylenic PAMs (which share an overlapping pharmacophore with MPPA). Herein, CDPPB did not show probe dependence; affinity and cooperativity estimates derived from interactions with either DHPG or l-glutamate were similar, consistent with our earlier report (Sengmany et al., 2017). Further, affinity and cooperativity estimates for MPPA and compound 2c determined from potentiation curves of DHPG or l-glutamate were also similar, despite belonging to structural classes of mGlu$_5$ PAMs that show probe dependence. These findings build on the evidence base that structurally similar mGlu$_5$ allosteric ligands can differentially exhibit probe dependence, representing an important consideration when interpreting structure-activity relationships within a discovery program.

Related to probe dependence is the idea that allosteric modulators can engender biased modulation, as evidenced by different magnitudes of affinity or cooperativity depending on the measure of receptor activity in the presence of the other orthosteric agonist. For mGlu$_5$, biased modulation has been observed for allosteric ligands classified as NAMs or PAMs based on Ca$^{2+}$ mobilization assays (Sengmany et al., 2017, 2019; Arsova et al., 2020). Here, we show that MPPA is a biased mGlu$_5$ PAM for which the magnitude of cooperativity with orthosteric agonists is lower/neutral when measured in IP$_1$ accumulation or receptor internalization when compared with Ca$^{2+}$ mobilization and ERK1/2 phosphorylation in HEK293A-mGlu$_5$ cells. These data are in agreement with previous reports for mGlu$_5$ in which cooperativity was lower when measured in IP$_1$ accumulation assays over Ca$^{2+}$ mobilization (Sengmany et al., 2017, 2019). Although mGlu$_5$ couples predominantly to G$_{q/11}$ proteins to elevate inositol trisphosphate levels and release of Ca$^{2+}$ from intracellular stores, mGlu$_5$ also couples to G$_{q}$ and modulates the activity of multiple ion channels (enabling extracellular Ca$^{2+}$ influx) in a G$_{q/11}$-independent fashion [reviewed in Gregory and Goudet (2021)]. Both intracellular release and extracellular influx of Ca$^{2+}$ were measured in the assays used herein. Therefore, the biased agonism and modulation observed for mGlu$_5$ PAMs between two measures that are traditionally considered linked likely arises because of stabilizing receptor conformations that differentially favor these different effectors. A key difference between the responses for which MPPA cooperativity is greater is the temporal nature of the assays. Ca$^{2+}$ mobilization and ERK1/2 phosphorylation are short-lived in comparison with IP$_1$ accumulation and receptor internalization, which are both measured over 1 hour. Measuring a non-equilibrium response can influence how signaling bias is observed, as previously shown for the dopamine D$_2$ receptor.
The biased agonism profiles for mGlur5 PAM agonists can be different between recombinant and native systems as well (Sengmany et al., 2017). In native systems, mGlur5 forms oligomeric complexes with 1) other GPCRs, 2) surface proteins, and 3) scaffolding proteins via the C-tail (Pin and Bettler, 2016); differing complements of effector, regulatory, and scaffolding proteins have the capacity to shape mGlur5 signaling in a cell type–dependent manner. In addition, mGlur5 is found on intracellular membranes such that the cellular response to mGlur5 activation may differ depending on where it is generated from and accessibility of ligands to different subcellular compartments (Jong et al., 2014, 2019). Recombinant versus native cells may have different ambient glutamate levels, or glutamate may be released in an activity-dependent manner. All of these factors may contribute to the mechanisms that drive biased mGlur5 modulation. Future experiments could employ selective inhibitors of coexpressed channels and transporters to decipher these underlying mechanisms.

Compound 2c consistently had the greatest degree of positive cooperativity, independent of the orthosteric ligand or response measured. Although compound 2c has not yet been tested for in vivo efficacy, related compounds have demonstrated antipsychotic efficacy and preclinical effects in preclinical models (Xiong et al., 2010; Gregory et al., 2013). The magnitude of mGlur5 PAM cooperativity with l-glutamate based on Ca2+ mobilization was recently shown to correlate with efficacy in the amphetamine hyperlocomotion assay (Gregory et al., 2019). However, whether such correlations extend to structurally diverse mGlur5 PAMs remains to be tested. Indeed, MPPA has higher positive cooperativity than CDPPB, yet a lower CDPPB dose is required for efficacy in reducing amphetamine-induced hyperlocomotion in rats relative to MPPA (Kinney et al., 2005; Sharma et al., 2009). It is unknown how the pharmacokinetics of MPPA compares with CDPPB. Additionally, differences in receptor residency times may also be linked to in vivo efficacy; further investigation is warranted.

In summary, we have determined the binding and signaling profiles of three mGlur5 PAMs from distinct scaffolds at four measures of mGlur5 function in recombinant cells. Key differences in vitro pharmacological profiles translated to natively expressed mGlur5 in primary cortical neurons. By assessing the kinetics of PAM binding to the mGlur5 receptor, we reveal previously unappreciated differences that may contribute to observations of PAM agonist activity, as well as biased cooperativity. Improved molecular characterization provides a better basis to understand the pharmacological properties of mGlur5 PAMs, which can be implemented in the future for improved structure-activity relationship interrogation and rational drug discovery.
Positive Allosteric Modulation of the mGlu<sub>5</sub> Receptor


Supplemental material

Positive allosteric modulators of metabotropic glutamate receptor 5 as tool compounds to study signaling bias

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Current address for S.R.F: QIMR Berghofer Medical Research Institute, Brisbane 4006, QLD, Australia
Supplemental Fig. 1. Influence of LY341495 on mGlu5 activity and responses to CDPPB in HEK293A cells. (A) Effect of 300 µM LY341495 pre-treatment (+LY) on basal mGlu5 activity. Raw data from each individual experiment are expressed as a percentage of the overall mean of the corresponding untreated responses. Error bars show the mean ± SD. (B and C) CDPPB agonist activity in presence of LY341495. E_{max} values relative to the maximal DHPG response (B) and pEC_{50} values (C). Data points represent values from individual experiments performed in duplicate (Ca^{2+}, IP1, pERK1/2) or triplicate (internalization) and horizontal lines show the mean. *P = 0.01-0.05, one-way ANOVA with Tukey’s multiple comparisons test.
**Supplemental Fig. 2.** DHPG-induced real-time internalization of mGlus. Symbols and error bars represent the mean and the SD, respectively, of 3 independent experiments performed in triplicate.
Supplemental Fig. 3. Concentration-response relationships for mGlu₅ internalization kinetics induced by PAMs in the presence of orthosteric agonists (DHPG or L-glutamate) or antagonists (LY341495). The internalization half-life was obtained from fitting an exponential model of one-phase association to the real-time internalization curves (Fig. 5) after subtraction of the vehicle curves. (A) CDPPB in the presence of 300 μM LY341495 to eliminate ambient glutamate effects. (B) PAM potentiation of ambient L-glutamate relative to exogenously introduced L-glutamate. (C) PAM potentiation of 1 μM DHPG. Symbols represent the mean and error bars represent SD from 3 independent experiments performed in triplicate.
Supplemental Fig. 4. Comparison of mGlu$_5$ PAM potencies and maximal responses in HEK293A cells across the four functional assays. Data points represent values from individual experiments performed in triplicate and vertical lines show the mean. *$P < 0.05$, one-way ANOVA with Tukey’s multiple comparisons test.
Supplemental Fig. 5. Assessment of probe dependence of mGlu5 PAMs. (A, B and D) No probe dependence was evident when comparing the $E_{\text{max}}$, $pE_{50}$ and $pK_B$ values obtained from PAM potentiation of L-glutamate and DHPG. (C) Log$\beta$ is approximately double for potentiation of DHPG EC$_{20}$ compared to L-glutamate EC$_{20}$. Data represent the mean and 95% CI. The dotted line represents unity.
Supplemental Fig. 6. Comparison of pK_I estimates from radioligand inhibition binding and pK_B values from functional assays for mGlu_5 PAMs. Data points represent the mean and error bars show the 95% CI from 3-6 independent experiments (refer to Tables 1 and 3 for exact n values). *P = 0.01-0.05, **P = 0.001-0.01, ***P < 0.001, t-test with Holm-Sidak’s multiple comparisons test.
**Supplemental Table 1: pEC$_{50}$ values from functional assays for orthosteric ligands in HEK293A cells or cortical neurons.** Data represent the mean and 95% CI of $n$ independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Cell type/ligand</th>
<th>Ca$^{2+}$ mobilization</th>
<th>IP$_1$ accumulation</th>
<th>pERK1/2</th>
<th>Internalization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC$_{50}$ (95% CI)</td>
<td>pEC$_{50}$ (95% CI)</td>
<td>pEC$_{50}$ (95% CI)</td>
<td>pEC$_{50}$ (95% CI)</td>
</tr>
<tr>
<td><strong>HEK293A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-glutamate</td>
<td>6.52 (5.88 - 7.16)</td>
<td>5.44 (5.29 - 5.59)</td>
<td>6.05 (5.93 - 6.17)</td>
<td>5.76 (5.61 - 5.90)</td>
</tr>
<tr>
<td>DHPG</td>
<td>6.09 (5.54 - 6.63)</td>
<td>5.89 (5.74 - 6.03)</td>
<td>6.09 (5.90 - 6.27)</td>
<td>5.45</td>
</tr>
<tr>
<td><strong>Cortical neurons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHPG</td>
<td>6.32 (6.20 - 6.44)</td>
<td>5.44 (5.16 - 5.72)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., not determined.
Supplemental Table 2: CDPPB pEC\textsubscript{50} and \(E_{\text{max}}\) values from functional assays in HEK293A cells in the presence of 300 µM LY341495. Data represent the mean and 95% CI of \(n\) independent experiments performed in duplicate (Ca\textsuperscript{2+} mobilization, IP\textsubscript{1} accumulation and ERK1/2 phosphorylation in presence of LY341495) or triplicate (internalization).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ca\textsuperscript{2+} mobilization</th>
<th>IP\textsubscript{1} accumulation</th>
<th>pERK1/2</th>
<th>Internalization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pEC_{50}) (95% CI)</td>
<td>(E_{\text{max}}) (95% CI)</td>
<td>(n)</td>
<td>(pEC_{50}) (95% CI)</td>
</tr>
<tr>
<td>CDPPB</td>
<td>7.38 (6.56 - 8.21)</td>
<td>5.93 (5.42 - 6.47) (b)</td>
<td>20  4</td>
<td>87  5</td>
</tr>
</tbody>
</table>

\(a\)% of the DHPG maximal response.

\(bP < 0.05\) when compared with the estimate from Ca\textsuperscript{2+} mobilization by one-way ANOVA with Tukey’s multiple comparisons test.

\(cP < 0.05\) when compared with the estimate from IP\textsubscript{1} accumulation by one-way ANOVA with Tukey’s multiple comparisons test.
Supplemental Table 3: Potency and maximal potentiation estimates for mGlu5 PAM potentiation of the indicated orthosteric agonist at ~EC20 in HEK293A cells. Data represent the mean and 95% CI of n independent experiments performed in duplicate (Ca\textsuperscript{2+} mobilization, IP\textsubscript{1} accumulation and ERK1/2 phosphorylation) or triplicate (internalization).

<table>
<thead>
<tr>
<th>L-glutamate</th>
<th>(p\text{PAM}_{50}) (95% CI)(^a)</th>
<th>(\text{PAM}_{\text{max}}) (95% CI)(^b)</th>
<th>n</th>
<th>(p\text{PAM}_{50}) (95% CI)</th>
<th>(\text{PAM}_{\text{max}}) (95% CI)</th>
<th>n</th>
<th>(p\text{PAM}_{50}) (95% CI)</th>
<th>(\text{PAM}_{\text{max}}) (95% CI)</th>
<th>n</th>
<th>(p\text{PAM}_{50}) (95% CI)</th>
<th>(\text{PAM}_{\text{max}}) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamate</td>
<td>(p\text{PAM}_{50}) (95% CI)(^a)</td>
<td>(\text{PAM}_{\text{max}}) (95% CI)(^b)</td>
<td>n</td>
<td>(p\text{PAM}_{50}) (95% CI)</td>
<td>(\text{PAM}_{\text{max}}) (95% CI)</td>
<td>n</td>
<td>(p\text{PAM}_{50}) (95% CI)</td>
<td>(\text{PAM}_{\text{max}}) (95% CI)</td>
<td>n</td>
<td>(p\text{PAM}_{50}) (95% CI)</td>
<td>(\text{PAM}_{\text{max}}) (95% CI)</td>
</tr>
<tr>
<td><strong>MPPA</strong></td>
<td>7.28 (6.62 - 7.95)</td>
<td>94 (78 - 110)</td>
<td>4</td>
<td>6.71 (6.19 - 7.23)</td>
<td>79 (59 - 99)</td>
<td>3</td>
<td>7.50 (6.77 - 8.24)</td>
<td>116 (53 - 178)(^c)</td>
<td>3</td>
<td>7.17 (6.96 - 7.38)</td>
<td>72 (56 - 87)(^c,d)</td>
</tr>
<tr>
<td>CDPPB</td>
<td>6.38 (6.01 - 6.75)</td>
<td>69 (44 - 94)</td>
<td>4</td>
<td>6.94 (5.98 - 7.90)</td>
<td>77 (70 - 84)</td>
<td>3</td>
<td>7.10 (6.00 - 8.20)</td>
<td>179 (145 - 213)(^c,e)</td>
<td>3</td>
<td>5.99 (5.78 - 6.21)(^c,d)</td>
<td>143 (94 - 193)(^c,e)</td>
</tr>
<tr>
<td>compound 2c</td>
<td>5.93 (5.43 - 6.43)</td>
<td>102 (85 - 119)</td>
<td>4</td>
<td>5.65 (5.26 - 6.03)</td>
<td>105 (62 - 148)</td>
<td>3</td>
<td>6.00 (5.24 - 6.76)</td>
<td>182 (86 - 278)(^c,e)</td>
<td>3</td>
<td>5.09 (4.48 - 5.69)(^d,e)</td>
<td>141 (92 - 190)</td>
</tr>
<tr>
<td><strong>DHPG</strong></td>
<td>7.02 (6.34 - 7.69)</td>
<td>76 (39 - 113)</td>
<td>4</td>
<td>6.65 (6.36 - 6.94)</td>
<td>90 (70 - 111)</td>
<td>3</td>
<td>7.38 (6.49 - 8.27)</td>
<td>128 (104 - 151)(^e)</td>
<td>3</td>
<td>5.91 (5.19 - 7.31)</td>
<td>62 (39 - 85)(^d)</td>
</tr>
<tr>
<td>CDPPB</td>
<td>6.66 (6.07 - 7.25)</td>
<td>64 (37 - 91)</td>
<td>4</td>
<td>6.95 (6.40 - 7.49)</td>
<td>94 (68 - 120)</td>
<td>3</td>
<td>7.23 (6.96 - 7.49)</td>
<td>191 (45 - 336)(^c,e)</td>
<td>3</td>
<td>6.68 (5.78 - 7.59)</td>
<td>86 (8 - 164)(^d)</td>
</tr>
<tr>
<td>compound 2c</td>
<td>5.88 (5.29 - 6.46)</td>
<td>99 (90 - 108)</td>
<td>4</td>
<td>5.85 (5.65 - 6.05)</td>
<td>112 (86 - 137)</td>
<td>3</td>
<td>5.57 (4.11 - 7.02)</td>
<td>231 (33 - 428)</td>
<td>3</td>
<td>4.47 (3.10 - 5.83)(^c,e)</td>
<td>210 (-32 - 452)</td>
</tr>
</tbody>
</table>

\(^a\)Negative logarithm of the concentration of PAM required to give half-maximal potentiation of the orthosteric agonist response.

\(^b\)Maximal level of potentiation of the DHPG or L-glutamate response maximal responses, expressed as % maximal agonist response in the absence of PAM.

\(^c\)P < 0.05 when compared with the estimate from IP\textsubscript{1} accumulation by one-way ANOVA with Tukey’s multiple comparisons test.

\(^d\)P < 0.05 when compared with the estimate from ERK1/2 phosphorylation by one-way ANOVA with Tukey’s multiple comparisons test.

\(^e\)P < 0.05 when compared with the estimate from Ca\textsuperscript{2+} mobilization by one-way ANOVA with Tukey’s multiple comparisons test.
Supplemental Table 4: Potency and maximal response of mGlu5 PAMs as agonists or potentiators of DHPG in cortical neurons. Data represent the mean and 95% CI of n independent experiments performed in duplicate.

<table>
<thead>
<tr>
<th>Cortical neurons</th>
<th>Ca$^{2+}$ mobilization</th>
<th>IP$_1$ accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC$_{50}$ (95% CI)$^a$</td>
<td>$E_{\text{max}}$ (95% CI)$^b$</td>
</tr>
<tr>
<td>No agonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPPA</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>CDPPB</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>+ EC$_{20}$ DHPG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPPA</td>
<td>6.13 (4.12 - 8.14)</td>
<td>42 (14 - 70)</td>
</tr>
<tr>
<td>CDPPB</td>
<td>7.23 (6.77 - 7.69)</td>
<td>45 (37 - 53)</td>
</tr>
<tr>
<td>compound 2c</td>
<td>6.32 (5.79 - 6.85)</td>
<td>89 (73 - 105)</td>
</tr>
</tbody>
</table>

n.r., no response; n.d., not determined.

$^a$P > 0.05 when comparing the pEC$_{50}$ values for compound 2c in the two assays.

$^b$Maximal response expressed as % maximal DHPG response in the absence of PAM.

$^c$Negative logarithm of the concentration of PAM required to give half-maximal potentiation of the DHPG response. None of the compounds had P < 0.05 (two-tailed unpaired t-test) when comparing the pPAM$_{50}$ values determined in the two assays.

$^d$Maximal level of potentiation of the DHPG or L-glutamate response maximal responses, expressed as % maximal DHPG response in the absence of PAM.

$^e$P < 0.05 when compared with the estimate from Ca$^{2+}$ mobilization by two-tailed unpaired t-test.