“Selective” Class C G Protein-Coupled Receptor Modulators Are Neutral or Biased mGlu₅ Allosteric Ligands[§]

Shane D. Hellyer, Sabine Albold, Taide Wang, Amy N. Y. Chen, Lauren T. May, Katie Leach, and Karen J. Gregory

Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, Victoria, Australia

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ABSTRACT

Numerous positive and negative allosteric modulators (PAMs and NAMs) of class C G protein-coupled receptors (GPCRs) have been developed as valuable preclinical pharmacologic tools and therapeutic agents. Although many class C GPCR allosteric modulators have undergone subtype selectivity screening, most assay paradigms have failed to perform rigorous pharmacologic assessment. Using mGlu₅ as a representative class C GPCR, we tested the hypothesis that allosteric modulator selectivity was based on cooperativity rather than affinity. Specifically, we aimed to identify ligands that bound to mGlu₅ but exhibited neutral cooperativity with mGlu₅ agonists. We additionally evaluated the potential for these ligands to exhibit biased pharmacology. Radioligand binding, intracellular calcium (iCa^{2+}) mobilization, and inositol monophosphate (IP₁) accumulation assays were undertaken in human embryonic kidney cells expressing low levels of rat mGlu₅ (HEK293A-mGlu₅-low) for diverse allosteric chemotypes.

Numerous “non-mGlu₅” class C GPCR allosteric modulators incompletely displaced allosteric mGlu₅ radioligand [³H]-methoxy-PEPy binding, consistent with a negative allosteric interaction. Affinity estimates for CPCCOEt (mGlu₁ ligand), PHCCC (mGlu₄ ligand), GS39783 (GABA_B ligand), AZ12216052 (mGlu₈ ligand), and CGP7930 (GABA_B ligand) at mGlu₅ were within 10-fold of their target receptor. Most class C GPCR allosteric modulators had neutral cooperativity with both orthosteric and allosteric mGlu₅ agonists in functional assays; however, NPS2143 (calcium-sensing receptor (CaSR) NAM), cinacalcet (CaSR PAM), CGP7930, and AZ12216052 were partial mGlu₅ agonists for IP₁ accumulation, but not iCa^{2+} mobilization. By using mGlu₅ as a model class C GPCR, we find that for many class C GPCR allosteric modulators, subtype selectivity is driven by cooperativity and misinterpreted owing to unappreciated bias.

Introduction

Class C G protein-coupled receptors (GPCRs) comprise the metabotropic glutamate receptors (mGlu₁ through mGlu₈), calcium-sensing receptor (CaSR), GPRC6A, γ -aminobutyric acid receptor B (GABA_B), and numerous taste, pheromone, and orphan receptors (Leach and Gregory, 2017). Given the important physiologic and pathophysiologic roles that class C

GPCRs play and their putative therapeutic potential, extensive drug discovery efforts have been undertaken (Conn et al., 2014; Leach and Gregory, 2017). Most drugs targeting class C GPCRs interact with allosteric binding sites that are topographically distinct from orthosteric sites, allowing for better spatiotemporal control over receptor function and greater subtype selectivity (Conn et al., 2014). Allosteric modulators that enhance or inhibit orthosteric ligand binding and/or efficacy, a property known as cooperativity, are defined as positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs), respectively. Neutral allosteric ligands (NALs) bind to allosteric sites but display no cooperativity with orthosteric ligands. NALs are an interesting and important class of allosteric ligands as, although they may appear to display no

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ABBREVIATIONS: AC265347, 1-(1,3-benzothiazol-2-yl)-1-(2,4-dimethylphenyl)ethanol; AZ12216052, 2-[[[4-bromophenyl]methyl]thio]-N-[4-(1-methylpropyl)phenyl]acetamide; BINA, 3'-[[[2-Cyclopentyl-2,3-dihydro-6,7-dimethyl-1-oxo-1H-inden-5-yl]oxy]methyl]-[1,1'-biphenyl]-4-carboxylic acid; CaSR, calcium-sensing receptor; CCh, carbachol; CGP7930, 3,5-bis(1,1-dimethylethyl)-4-hydroxy- β , β -dimethylbenzenepropanol; CPCCOEt, 7-(hydroxyimino)cyclopropa[b] chromen-1a-carboxylate ethyl ester; DHPG, (S)-3,5-dihydroxyphenylglycine; DMEM, Dulbecco's modified Eagle's medium; EC₂₀, 20% effective concentration; EC₈₀, 80% effective concentration; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GS39783, N₄,N₆-dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine; HBSS, Hank's balanced salt solution; HEK293A, human embryonic kidney 293; iCa^{2+} , intracellular calcium; IP₁, inositol 1-phosphate; mGlu, metabotropic glutamate receptor; ML 337, [2-fluoro-4-[2-(4-methoxyphenyl)-ethynyl]phenyl] [(3R)-3-hydroxy-1-piperidinyl]methanone; MPEP, 5-methyl-6-(phenylethynyl)-pyridine; MPEP, 2-methyl-6-(phenylethynyl)pyridine; NAL, neutral allosteric ligand; NAM, negative allosteric modulator; NPS2143, 2-chloro-6-[(2R)-3-[(1,1-dimethyl-2-(2-naphthalenyl) ethyl]amino)-2-hydroxypropoxy]-benzonitrile; PAM, positive allosteric modulator; PHCCC, (E)-1,1a,7,7a-tetrahydro-7-(hydroxyimino)-N-phenylcyclopropa[b]chromene-1a-carboxamide; SAR, structure-activity relationship; 7TM, 7 transmembrane domain; VU0424465, (R)-5-[(3-fluorophenyl)ethynyl]-N-(3-hydroxy-3-methylbutan-2-yl)picolinamide; VU0483605, 3-chloro-N-[3-chloro-4-(4-chloro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)phenyl]-2-pyridinecarboxamide.

cooperativity with orthosteric ligands in screening assays, they may exhibit unappreciated activity in other pathways or against other ligands.

An ideal drug discovery program will identify chemotypes that display potency at a target receptor, with limited activity at closely related subtypes. The most common approach used to assess allosteric modulator structure-activity relationships (SAR) is determination of modulator concentration-response curves in the presence of a single agonist concentration (typically EC_{20} for PAMs and EC_{80} for NAMs) to derive modulator potency estimates (Lindsley et al., 2016); however, allosteric modulator potency reflects a combination of allosteric ligand affinity, cooperativity, and intrinsic efficacy and is influenced by orthosteric agonist concentration (Gregory et al., 2010). As such, determining both ligand affinity and cooperativity at target receptors and related subtypes during drug discovery are vital to ensure optimal selectivity (Lindsley et al., 2016). A lack of selective radioligands for many class C GPCRs has hampered efforts to determine ligand affinity via traditional radioligand binding-based methods. As such, allosteric modulator optimization often relies solely on functional assays to derive affinity and cooperativity estimates, inform compound selection, and optimize selectivity (Melancon et al., 2012). Unfortunately, many studies only explore allosteric ligand pharmacology with a single orthosteric ligand for a single signaling pathway, thereby failing to appreciate the full scope of pharmacology. Indeed, biased modulation, where modulator affinity and/or cooperativity (magnitude or direction) is pathway-dependent, is operative at multiple class C GPCRs (Jalan-Sakrikar et al., 2014; Cook et al., 2015; Leach et al., 2016; Haas et al., 2017; Sengmany et al., 2017). Neutral allosteric ligands would also go largely undetected, as neutral cooperativity with an orthosteric ligand results in classification as “inactive,” despite potential receptor affinity. This is exemplified in the discovery of multiple neutral allosteric ligands across different chemotypes for mGlu₅ (Rodriguez et al., 2005; <http://www.ncbi.nlm.nih.gov/books/NBK280039>; Hammond et al., 2010; Haas et al., 2017).

Class C GPCR allosteric modulator drug discovery has proven particularly fruitful (Leach and Gregory, 2017). Numerous selective PAMs, NAMs, and NALs have been developed, with the CaSR PAM cinacalcet being one of the first GPCR allosteric modulators approved by the FDA, and others advancing to clinical trials (Leach and Gregory, 2017). Whereas some class C GPCR allosteric modulators have undergone subtype selectivity screening during their optimization process, most screening paradigms have failed to rigorously assess the full scope of modulator pharmacology. Additionally, many mGlu allosteric modulators are derived from modifications to existing mGlu modulator scaffolds (Annoura et al., 1996; Maj et al., 2003; Wenthur et al., 2013; Cho et al., 2014). Therefore, “selective” class C allosteric ligands may bind to other class C receptors and possess unappreciated neutral cooperativity or biased pharmacology.

Metabotropic glutamate receptor 5 (mGlu₅) is one of the few class C GPCRs with well defined allosteric radioligands and couples well to multiple functional outputs that can be used as measures of receptor function, allosteric modulation, and bias (Cosford et al., 2003; Sengmany et al., 2017). Therefore, we used mGlu₅ to test the hypothesis that class C GPCR allosteric modulators exhibit unappreciated neutral or biased allosteric activity at other class C receptors. Radioligand binding and

functional assays revealed that multiple class C GPCR allosteric ligand chemotypes bind to mGlu₅ and can activate this receptor in a biased manner but are NALs against mGlu₅ orthosteric and allosteric agonists, revealing that class C GPCR allosteric modulator activity and selectivity appear to be driven by cooperativity and bias rather than receptor affinity.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and Fluo-4-AM were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was sourced from Thermo Electron Corporation (Melbourne, Australia). IP-One HTRF assay kit was purchased from Cisbio Assays, Genesearch (Arundel, QLD, Australia). [2-Fluoro-4-[2-(4-methoxyphenyl)ethynyl]phenyl][(3R)-3-hydroxy-1-piperidinyl]methanone (ML 337), 3-chloro-N-[3-chloro-4-(4-chloro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)phenyl]-2-pyridinecarboxamide (VU0483605), 2-[[[4-(4-bromophenyl)methyl]thio]-N-[4-(1-methylpropyl)phenyl]acetamide (AZ12216052), 7-(hydroxyimino)cyclopropa[b]chromene-1a-carboxylate ethyl ester (CPCCOEt) and 2-chloro-6-[(2R)-3-[(1,1-dimethyl-2-(2-naphthalenyl)ethyl]amino)-2-hydroxypropoxy]benzonitrile (NPS2143) were purchased from Tocris Bioscience (Melbourne, Australia). 3'-[[[2-(Cyclopentyl-2,3-dihydro-6,7-dimethyl-1-oxo-1H-inden-5-yl)oxy]-methyl]-[1,1'-biphenyl]-4-carboxylic acid (BINA) was purchased from HelloBio (Avonmouth, Bristol, UK). 3,5-Bis(1,1-dimethylethyl)-4-hydroxy-ββ-dimethylbenzenepropanol (CGP7930), (E)-1,1a,7,7a-tetrahydro-7-(hydroxyimino)-N-phenylcyclopropa[b]chromene-1a-carboxamide (PHCCC), and N₄,N₆-Dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine (GS39783) were purchased from Abcam (Melbourne, Australia). (R)-N-[1-(1-naphthyl)ethyl]-3-[3-(trifluoromethyl)phenyl]propan-1-amine (cinacalcet HCl) was synthesized in-house at Monash Institute of Pharmaceutical Sciences according to previously published methods (Davey et al., 2012). (R)-5-((3-fluorophenyl)ethynyl)-N-(3-hydroxy-3-methylbutan-2-yl)picolinamide (VU0424465) was synthesized in-house at Vanderbilt Center for Neuroscience Drug Discovery. [³H]methoxy-PEPy was custom synthesized by Quotient Bioresearch (Rushden, Northamptonshire, UK) using the previously reported synthetic route (Cosford et al., 2003). AC-265347, (±)-2-(p-methoxyphenoxy)propionic acid (lactisole) and all other reagents (unless otherwise stated) were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. All animal experiments and procedures were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee (Protocol no. MIPS.2014.37). Eight-week-old Asmu:Swiss outbred female wild-type mice were provided by the Monash Animal Research Platform (Clayton, VIC, Australia). Animals were humanely sacrificed and embryonic day 16 embryos were recovered for primary cell culture.

Cell Culture. Nontransfected HEK293A cells or HEK293A cells stably expressing wild-type rat mGlu₅ at low levels (HEK293A-mGlu₅-low), with comparable expression levels to primary rat cortical astrocytes (Noetzel et al., 2012), were maintained at 5% CO₂, 37°C in DMEM, supplemented with 5% FBS and 16 mM HEPES. 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 and 16 mM HEPES) at a density of 40,000 cells/well.

Primary Cell Culture. Embryonic day 16 Asmu:Swiss wild-type mouse embryos were decapitated, cortices dissected, and neurons mechanically dissociated in sterile Hank's balanced salt solution (HBSS; 5.33 mM KCl, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, 137.93 mM NaCl, 0.34 mM Na₂HPO₄, 5.56 mM D-glucose). Cortical neurons were then immediately plated on poly-D-lysine, FBS-coated clear-bottom 24-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% CO₂ for 6 to 7 days before assay.

Radioligand Binding. HEK293A-mGlu₅-low cells or primary cortical neurons were incubated with ~3 nM [³H]methoxy-PEPy in the presence of increasing concentrations of allosteric modulators on a shaker at RT for 1 hour in binding buffer (HBSS, as described above, with 20 mM HEPES and 1.2 mM CaCl₂). Non-specific binding was determined using 10 μM 2-methyl-6-(phenylethynyl)pyridine (MPEP). Assays were terminated by buffer aspiration, followed by three washes with ice-cold 0.9% NaCl. Cells were then lysed with 0.2 M NaOH for 1–3 hours at 50°C. Lysates were transferred to scintillation vials and incubated with 4 ml of UltimaGold scintillant for a minimum of 1 hour. Scintillation was counted using a TriCarb 2900TR liquid scintillation counter (PerkinElmer, Waltham, MA).

iCa²⁺ Mobilization. All iCa²⁺ mobilization assays were carried out in calcium assay buffer (binding buffer, as in the preceding, with 4 mM probenecid). The cell-permeable Ca²⁺ indicator dye Fluo-4 was used to assay receptor-mediated iCa²⁺ mobilization using a FlexStation I or III (Molecular Devices, San Jose, CA) as described previously (Gregory et al., 2012). Initially, a double-add paradigm was used, where allosteric ligands were added 1 minute before orthosteric agonist (DHPG/carbachol (CCh)/ATP) or PAM-agonist (VU0424465). For assays with a preincubation step, allosteric ligands were added 30 minutes before the addition of agonist. A five-point smoothing function was applied to the raw calcium fluorescence traces. The baseline fluorescence of each individual well was determined (mean first 15 seconds) before the addition of agonists/modulators. Peak fluorescence was defined as the change from corresponding baseline, and values were normalized to the maximal response to orthosteric or allosteric agonist in vehicle-treated cells.

IP₁ Accumulation Assay. HEK293A-mGlu₅-low cells were washed with phosphate-buffered saline (137 mM NaCl, 8.1 mM Na₂HPO₄, 1.7 mM KH₂PO₄, 2.7 mM KCl, pH 7.4) and incubated for 1 hour with stimulation buffer (HBSS with 20 mM HEPES, 30 mM LiCl₂, 1.2 mM CaCl₂, pH 7.4). For competition studies with 5-methyl-6-(phenylethynyl)-pyridine (5MPEP), cells were incubated with vehicle or 10 μM 5MPEP for 30 minutes before compound addition. Orthosteric and allosteric compounds were then added for 1 hour before cell lysis with lysis buffer (HTRF IP-one assay kit). IP₁ levels were determined using the HTRF IP-one assay kit according to manufacturer's instructions, and fluorescence was measured using the Envision plate reader (PerkinElmer). Data are expressed as fold over basal IP₁ accumulation or percentage inhibition of VU0424465-induced IP₁ accumulation.

Data Analysis and Statistics. All nonlinear regression analyses were performed using Prism 7.02 (GraphPad Software Inc., San Diego, CA). Inhibition of [³H]methoxy-PEPy binding data were fitted to a either a one-site inhibition binding model or an allosteric interaction model (eq. 1). An extra sum-of-squares *F* test was used to determine the preferred model for each data set:

$$\frac{Y}{Y_{max}} = \frac{[A]}{[A] + \frac{K_D \left(1 + \frac{[B]}{K_B}\right)}{\left(1 + \frac{\alpha[B]}{K_B}\right)}} \quad (1)$$

where Y/Y_{max} is the fractional specific binding, $[A]$ is the radioligand concentration, $[B]$ is the concentration of the allosteric modulator, K_D is the radioligand equilibrium dissociation constant, K_B is the allosteric modulator equilibrium dissociation constant, and α is the binding cooperativity factor. An α value of $\alpha > 1$ denotes positive cooperativity, values of $0 > \alpha < 1$ denote negative cooperativity, and $\alpha = 1$ denotes neutral cooperativity.

Agonist-concentration response curves in the presence and absence of allosteric modulators were fitted to a three-parameter logistic equation (eq. 2):

$$y = \frac{bottom + (top - bottom)}{(1 + 10^{(\log EC_{50} - \log[A])n})} \quad (2)$$

where *top* and *bottom* are the upper and lower plateaus of the concentration response curve, respectively, $[A]$ 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).

Orthosteric agonist concentration-response curves in the absence and presence of increasing concentrations of class C allosteric ligand were fitted to an operational model of allosterism in eq. 3:

$$Effect = \frac{E_m(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n} \quad (3)$$

where E_m is the maximal response, τ_A , and τ_B are the efficacy of orthosteric (A) and allosteric (B) ligands, respectively, α and β denote allosteric effects on orthosteric ligand-binding affinity and efficacy, respectively, K_A and K_B represent the functional affinities of orthosteric and allosteric ligands, respectively, and $[A]$ and $[B]$ denote their respective concentrations, and n is the slope factor of the transducer function.

Affinity, cooperativity, and potency parameters were estimated as logarithms and are presented as mean $\pm$ S.E.M. Functional potency and basal/maximal response values for orthosteric ligands in the presence of allosteric modulators were compared with vehicle controls using one-way analysis of variance with Dunnett's post-hoc test.

Results

Class C Allosteric Ligands Noncompetitively Displace [³H]methoxy-PEPy from mGlu₅ in HEK293A-mGlu₅-Low Cells and Primary Cortical Neurons. Initially, 17 class C GPCR allosteric ligands representing a variety of PAMs and NAMs for mGlu₁₋₈, GABA_B, CaSR, and taste receptors were screened in a five-point binding assay for their ability to displace [³H]methoxy-PEPy binding from HEK293A-mGlu₅-low cells (Supplementary Fig. S1). [³H]methoxy-PEPy is a NAM that binds to a "common" allosteric binding site in the mGlu₅ seven-transmembrane (7TM) domain, also used by several other mGlu₅ NAMs and PAMs, including VU0424465 (Rook et al., 2013). Although 7 of the 17 ligands did not significantly displace [³H]methoxy-PEPy (<10% displacement at the highest concentrations), 10 compounds displaced [³H]methoxy-PEPy ~20% or more (Supplemental Fig. 1). These ligands represent diverse allosteric chemotypes with reported selectivity across a spectrum of class C GPCR family members, including NAMs of mGlu₁ (CPCCOEt), mGlu₃ (ML337) and the CaSR (NPS2143), and PAMs of mGlu₁ (VU0483605), mGlu₂ (BINA), mGlu₄ (PHCCC), mGlu₈ (AZ12216052), GABA_B (GS39783, CGP7930), and the CaSR (cinacalcet) (Fig. 1). We therefore sought to further characterize the activity of these mGlu₅ binders.

Full inhibition binding curves for the 10 selected compounds revealed incomplete displacement of [³H]methoxy-PEPy binding for all, with a maximum of 20%–60% displacement at 100–300 μM (Fig. 2, A and B). This is consistent with a noncompetitive interaction between the various ligands and [³H]methoxy-PEPy. Indeed, inhibition binding titration curves were best fitted to an allosteric ternary complex model versus a competitive binding model ($P < 0.05$, extra sum-of-squares *F* test), with the exception of cinacalcet (CaSR PAM) and BINA (mGlu₂ PAM), where displacement of [³H]methoxy-PEPy binding did not reach a plateau at the highest concentrations tested. Due to insolubility at higher concentrations, we were unable to discern unambiguously the mode of action for both BINA and cinacalcet. Given the lack of a plateau response we compared non-linear

Fig. 1. Chemical structures of class C GPCR allosteric ligands included in this study.

regression analyses for competitive (where Hill slope = 1 and bottom plateau = 0) versus allosteric (where bottom plateau ≥ 0) inhibition binding of the data using an extra sum-of-squares F test. Both BINA and cinacalcet inhibition binding data were best fitted to a competitive model. As a control, full inhibition binding curves were also generated for the mGlu₅ PAM-agonist VU0424465 (Fig. 2), which fully displaced [³H]methoxy-PEPy binding in a competitive manner. Affinity (pK_B) and cooperativity ($\log \alpha$) estimates are summarized in Table 1.

To establish the possible physiologic relevance of the allosteric interaction between the class C ligands and [³H]methoxy-PEPy, full inhibition binding assays were carried out for a subset of allosteric ligands in mouse primary cortical neurons. Compounds were chosen to represent a broad range of reported selectivity profiles that encompass group 1 mGlu (CPCCOEt), group 2 mGlu (ML337), group 3 mGlu (AZ12216052, PHCCC), GABA_B (CGP 7930), and CaSR (cinacalcet) ligands. All compounds displaced [³H]methoxy-PEPy binding in cortical neurons, with maximum [³H]methoxy-PEPy displacement and

Fig. 2. Inhibition of [³H]methoxy-PEPy binding to HEK293A-mGlu₅-low cells and primary mouse cortical neurons. Class C GPCR allosteric modulators incompletely displaced [³H]methoxy-PEPy binding to HEK293A-mGlu₅-low cells (A and B) and mouse cortical neurons (C). Data represent the mean + S.E.M. from three to six independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbols.

TABLE 1

Summary of affinity and cooperativity estimates for class C GPCR allosteric modulators binding to mGlu₅ in HEK293A-mGlu₅-low cells and cortical neurons, determined from [³H]methoxy-PEPy inhibition binding assays

Data represent the mean ± S.E.M. from the indicated number (*n*) of independent experiments performed in duplicate. pK_B is the negative logarithm of the allosteric modulator equilibrium dissociation constant; log α is the logarithm of the cooperativity factor for the interaction between indicated ligands and [³H]methoxy-PEPy.

Ligand	Target	Target pK _B	Target Selectivity ^a	pK _B ^b				Log α	
				HEK293A-mGlu ₅ -Low	<i>n</i>	Cortical Neurons	<i>n</i>	HEK293A-mGlu ₅ -Low	Cortical Neurons
VU0424465	mGlu ₅ PAM-agonist	8.27	n.d.	8.27	2	7.54 ± 0.42	6	n.a.	-0.59 ± 0.14
CPCCOEt	mGlu ₁ NAM	5.31 ^c	2.2	4.90 ± 0.10	6	5.03 ± 0.37	3	-0.54 ± 0.06	-0.64 ± 0.07
VU0483605	mGlu ₁ PAM	6.02 ± 0.14 ^d	0.35	6.48 ± 0.17	3	n.d.		-0.17 ± 0.06	n.d.
BINA	mGlu ₂ PAM	6.48 ^e	30	4.69 ± 0.25	6	n.d.		n.a.	n.d.
ML337	mGlu ₃ NAM	7.10 ^e	23	5.67 ± 0.22	3	6.29 ± 0.16	3	-0.23 ± 0.07	-0.23 ± 0.01
PHCCC	mGlu ₄ PAM	5.59 ^e	0.85	5.63 ± 0.11	6	6.23 ± 0.26	3	-0.33 ± 0.03	-0.46 ± 0.06
AZ12216052	mGlu ₈ PAM	5.43 ± 0.12 ^d	0.81	5.38 ± 0.13	5	5.81 ± 0.09	3	-0.38 ± 0.08	-0.59 ± 0.09
Cinacalcet	CaSR PAM	6.39 ± 0.05 ^e	81	4.35 ± 0.10	6	5.12 ± 0.23	4	n.a.	n.a.
NPS2143	CaSR NAM	6.66 ± 0.04 ^e	37	5.08 ± 0.02	3	n.d.		-0.36 ± 0.09	n.d.
GS39783	GABA _B PAM	4.68 ^e	0.08	6.27 ± 0.33	6	n.d.		-0.18 ± 0.04	n.d.
CGP7930	GABA _B PAM	4.48 ^e	0.63	4.78 ± 0.11	6	4.90 ± 0.29	3	-0.34 ± 0.11	-0.48 ± 0.08

n.a. denotes not applicable, as data were best fitted to a competitive model; n.d., not determined.

^aRatio of affinity for target relative to mGlu₅, where a ratio <1 indicates greater affinity for mGlu₅ than target receptor.

^bAffinity values for cinacalcet, BINA, and VU0424465 (in HEK293A-mGlu₅-low) are pK_i.

^cLiterature-reported pK_i estimate for CPCCOEt (Lavreysen et al., 2003) and pK_B estimates for cinacalcet and NPS2143 (Leach et al., 2016).

^dpK_B estimates from analysis of iCa²⁺ mobilization curves in mGlu₁ or mGlu₈ expressing HEK293A cells (see Supplementary Fig. S2).

^epK_B estimates generated from reanalysis of previously reported data (see Supplementary Fig. S2).

affinity/cooperativity estimates similar to those observed in HEK293A-mGlu₅-low cells (Fig. 2C; Table 1). All inhibition binding curves were again found to best fit to an allosteric ternary complex model, except cinacalcet, which fit a one-site competition model. These similarities were expected given that rat and mouse mGlu₅ have 100% sequence identity within 7TM domain. Surprisingly, VU0424465 was also unable to fully displace [³H]methoxy-PEPy in neurons, possibly indicating allosteric interaction between the two modulators or binding to non-mGlu₅ targets in these cells. GABA_B receptors and all mGlu subtypes, except for mGlu₆, are expressed in mouse embryonic cortex (Han et al., 2009). A lack of selectivity at the level of affinity could contribute to both the radioligand and competing allosteric modulators binding to multiple targets in cortical neurons.

Taken together, the above data suggest that several class C GPCR ligands bind to mGlu₅ receptors in recombinant and native cells, at a site/s distinct from the common allosteric binding site used by [³H]methoxy-PEPy. The CaSR PAM, cinacalcet, on the other hand, appeared to bind in a manner that was either competitive with [³H]methoxy-PEPy or allosteric with such high negative cooperativity that the interaction between the two ligands could not be distinguished from a competitive one; however, our studies may be limited by compound solubility and the inability to reach high enough receptor occupancy levels to completely displace [³H]methoxy-PEPy.

Affinity of Class C Allosteric Ligands for Target Receptors. Previous reports on the class C GPCR ligands studied herein generally reported functional potency estimates (EC₅₀/IC₅₀) for allosteric agonism or modulatory activity against a single concentration of an orthosteric ligand as measures of modulator selectivity; however, we sought to determine their binding selectivity by comparing the affinity of these compounds at mGlu₅ with their affinity at their target receptors (i.e., by comparing allosteric modulator equilibrium dissociation constants, pK_B). For CPCCOEt, NPS2143, and cinacalcet, affinity estimates were readily available in the literature (Lavreysen et al., 2003; Leach et al., 2016). For those compounds for which affinity estimates were not reported, pK_B

estimates were determined by applying an operational model of allosterism (eq. 3) to previously published interaction studies with orthosteric agonists. Where previous data were unavailable, interaction studies with the orthosteric agonist were performed using iCa²⁺ mobilization assays in HEK cells stably expressing the target receptor (see Supplementary Methods; Supplementary Fig. S2). Affinity estimates revealed a range of selectivity profiles (Table 1). Surprisingly, both GABA_B PAMs had a higher affinity for mGlu₅ versus GABA_B, whereas the group 3 mGlu PAMs, PHCCC and AZ12216052, had similar affinities for mGlu₅ and their reported targets (mGlu₄ and mGlu₈ respectively). VU0483605 displayed 3-fold greater affinity for mGlu₅ versus mGlu₁. All other ligands had a higher relative affinity for their target receptors over mGlu₅ ranging from 2-fold for CPCCOEt at mGlu₁ to ~80-fold for cinacalcet at the CaSR.

Select Class C GPCR Allosteric Ligands Stimulate IP₁ Accumulation in HEK293A-mGlu₅-Low Cells. To determine whether there were functional consequences for the binding of class C GPCR allosteric ligands to mGlu₅, modulator effects on mGlu₅ signaling were initially assessed using an IP₁ accumulation assay. At 10 μ M, none of the allosteric ligands had a significant effect on the pEC₅₀ or E_{max} of the mGlu₅ orthosteric agonist, DHPG, or allosteric agonist, VU0424465, in IP₁ accumulation assays (Fig. 3, A–D; Table 2). A small (~2-fold) but nonsignificant shift in VU0424465 pEC₅₀ was evident in the presence of PHCCC, CPCCOEt, and ML337. Increasing the concentration of PHCCC, CPCCOEt, and ML337 (30 μ M) reduced VU0424465-induced IP₁ accumulation (Fig. 4A). Interestingly, NPS2143 alone significantly increased IP₁ levels to ~86% of the maximal DHPG response at 10 μ M (Fig. 3, A and C). Cinacalcet, AZ12216052, and CGP7930 also increased IP₁ accumulation over baseline at 10 μ M (Fig. 3, A and C). Importantly, 10 μ M cinacalcet, AZ12216052, CGP7930, and NPS2143 induced no such increase in basal IP₁ accumulation in nontransfected HEK293A cells and had no significant effect on the potency of carbachol (CCh) for activating endogenous muscarinic receptors (Fig. 3E). Concentration-response curves for NPS2143, cinacalcet,

Fig. 3. Effect of class C GPCR allosteric modulators on agonist concentration-response curves for IP₁ accumulation in HEK293A-mGlu₅-low and nontransfected HEK293A cells. Class C GPCR modulators (10 μ M) had no effect on the potency of DHPG (A and B) or VU0424465 (C and D) in HEK293A-mGlu₅-low cells. NPS2143 (10 μ M) significantly increased basal IP₁ accumulation in HEK293A-mGlu₅-low cells (A and C). Cinacalcet, NPS2143, CGP7930, and AZ12216052 did not induce IP₁ accumulation alone nor affect CCh concentration-response curves for IP₁ accumulation in nontransfected HEK293A cells (E). Data represent the mean $\pm$ S.E. M. from three to six independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbols.

AZ12216052, and CGP7930 in HEK293A-mGlu₅-low revealed that these ligands were all low-potency agonists relative to DHPG and VU0424465, with pEC₅₀ values similar to pK_B estimates derived from binding assays (Fig. 4B; Table 3). Therefore, these allosteric agonists have lower intrinsic

efficacy than DHPG and VU0424465, which have pEC₅₀ values for IP₁ accumulation that are >10-fold higher than binding pK_B estimates (Table 1) (Mutel et al., 2000).

The mGlu₅ allosteric ligand, 5MPEP, is a NAL with respect to mGlu₅ orthosteric agonists, but it interacts competitively with other mGlu₅ allosteric modulators that bind to a “common” allosteric site (Rodriguez et al., 2005). Concentration-response curves to allosteric agonists were therefore generated in the presence and absence of 5MPEP, to determine whether the class C GPCR allosteric ligands were acting through the common allosteric site. As expected, preincubation with 5MPEP significantly reduced VU0424465 pEC₅₀ from 9.29 ± 0.34 to 7.84 ± 0.21 ($P < 0.05$, students *t* test) with no change in the maximal response, indicative of a competitive interaction (Fig. 5A). 5MPEP had no significant effect on the concentration-response curves to AZ12216052, NPS2143, cinacalcet, and CGP7930, although there was a trend toward a reduction in E_{max} for cinacalcet and AZ12216052 (Fig. 5, B–E). This finding suggests that these latter ligands do not bind to the common 7TM domain allosteric site in mGlu₅.

Class C GPCR Allosteric Ligands Are Inactive in iCa²⁺ Mobilization Assays in HEK293A-mGlu₅-Low Cells. Class C GPCRs are pleiotropically coupled to multiple intracellular signaling pathways and biased agonism and

TABLE 2

Effect of class C GPCR allosteric modulators (10 μ M) on potency and maximal response to DHPG and VU0424465 in IP₁ accumulation in HEK293A-mGlu₅-low cells

Data represent the mean $\pm$ S.E.M. from four independent experiments performed in duplicate.

Ligand	DHPG		VU0424465	
	pEC ₅₀ ^a	E _{max} ^b	pEC ₅₀	E _{max}
Vehicle	6.41 $\pm$ 0.18	1.62 $\pm$ 0.11	9.40 $\pm$ 0.29	1.54 $\pm$ 0.08
CPCCOEt	6.45 $\pm$ 0.34	1.58 $\pm$ 0.11	9.26 $\pm$ 0.26	1.58 $\pm$ 0.14
ML337	6.23 $\pm$ 0.12	1.60 $\pm$ 0.11	9.14 $\pm$ 0.19	1.52 $\pm$ 0.08
PHCCC	6.46 $\pm$ 0.27	1.54 $\pm$ 0.07	9.00 $\pm$ 0.14	1.53 $\pm$ 0.08
AZ12216052	6.70 $\pm$ 0.27	1.56 $\pm$ 0.11	9.33 $\pm$ 0.25	1.55 $\pm$ 0.10
Cinacalcet	6.75 $\pm$ 0.18	1.63 $\pm$ 0.09	9.18 $\pm$ 0.22	1.62 $\pm$ 0.09
NPS2143	7.09 $\pm$ 0.35	1.59 $\pm$ 0.12	9.54 $\pm$ 0.30	1.58 $\pm$ 0.10
CGP7930	6.70 $\pm$ 0.33	1.56 $\pm$ 0.08	9.40 $\pm$ 0.28	1.55 $\pm$ 0.09

^aNegative logarithm of the concentration of agonist required to generate the half-maximal response.

^bMaximal response to indicated agonist expressed as fold increase over basal IP₁ levels.

Fig. 4. Stimulation and modulation of VU0424465-induced IP₁ accumulation in HEK293A-mGlu₅-low cells. CPCCOEt, PHCCC, and ML337 inhibit IP₁ accumulation elicited by an EC₉₀ concentration of VU0424465 in HEK293A-mGlu₅-low cells (A). Cinacalcet, CGP7930, NPS2143, and AZ12216052 concentration dependently induced IP₁ accumulation alone in HEK293A-mGlu₅-low cells (B). Data represent the mean $\pm$ S.E.M. from four independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbols.

modulation is operative at multiple class C GPCRs, including mGlu₅ (Jalan-Sakrikar et al., 2014; Cook et al., 2015; Leach et al., 2016; Haas et al., 2017; Sengmany et al., 2017). Indeed, we recently showed that multiple mGlu₅ PAM agonists were biased toward IP₁ accumulation over iCa²⁺ mobilization in HEK293A-mGlu₅-low cells (Sengmany et al., 2017). As such, we examined the potential for class C GPCR allosteric ligands to exhibit biased agonism and/or biased modulation by using iCa²⁺ mobilization as a second measure of receptor function in HEK293A-mGlu₅-low cells. None of the ligands displayed intrinsic agonist activity for iCa²⁺ mobilization (Supplementary Fig. S3), indicating that the four agonists previously identified in the IP₁ accumulation assays (AZ12216052, cinacalcet, NPS2143, CGP7930) have a lower efficacy for coupling to iCa²⁺ mobilization over IP₁ accumulation. This is consistent with the activity of the PAM-agonist VU0424465, which exhibits bias toward activation of IP₁ accumulation versus iCa²⁺ mobilization, whereas the opposite is true for the orthosteric agonist, DHPG (Sengmany et al., 2017).

No significant effect was seen with 1-minute preincubation with selected allosteric ligands on the pEC₅₀ or E_{max} of the

PAM-agonist, VU0424465 (Fig. 6; Supplementary Table 1). To determine whether a lack of equilibrium contributed to the lack of modulator effect on agonist responses, the preincubation time was extended to 30 minutes before agonist addition. Extended preincubation with modulators at 10 μ M had no significant effect on VU0424465 pEC₅₀ (Fig. 6B); however, AZ12216052, NPS2143, and cinacalcet significantly decreased the maximum response to VU0424465 in HEK293A-mGlu₅-low cells (Fig. 6B; Table 4). We next determined whether the class C ligands had a similar effect on mGlu₅ orthosteric agonist-induced iCa²⁺ mobilization. Similar to VU0424465, there was no effect of modulators on DHPG pEC₅₀, but AZ12216052, NPS2143, and cinacalcet all significantly decreased the maximum response to DHPG (Fig. 6C). Given that both CaSR ligands exhibited an effect on mGlu₅ responses, mGlu₅ ligands were tested for their ability to alter CaSR orthosteric (extracellular Ca²⁺) and allosteric (AC265347) agonist mediated iCa²⁺ mobilization (see Supplementary Methods). There was no effect of mGlu₅ PAMs or NAMs from diverse chemotypes on the pEC₅₀ or maximum response to either CaSR agonist (Supplementary Fig. S4).

To determine whether the effects of 10 μ M AZ12216052, NPS 2143, and cinacalcet were mGlu₅-mediated, the impact on responses to nonglutamatergic agonists CCh and ATP, which activate endogenously expressed G_q-coupled class A GPCRs in HEK293A-mGlu₅-low cells, were assessed. AZ12216052, NPS 2143, and cinacalcet had no significant effect on ATP and CCh potency (Fig. 6, D and E; Table 4). Although AZ12216052 and NPS2143 significantly reduced the maximum response elicited by both ATP and CCh, cinacalcet only significantly reduced the maximum response elicited by ATP (Fig. 6; Table 4). These experiments were repeated in nontransfected HEK293A cells, to establish whether mGlu₅ was required for these three ligands to influence endogenous GPCRs, possibly via heterologous desensitization. There was a nonsignificant trend toward a reduction in CCh or ATP maximal responses in the presence of 10 μ M NPS2143, AZ12216052, or cinacalcet in nontransfected HEK293A cells (Supplementary Fig. S5; Supplementary Table S2). Therefore, the reduction in the maximal ATP, CCh, DHPG-, and VU0424465-elicited iCa²⁺ mobilization response in the presence of NPS2143, AZ12216052, and cinacalcet in HEK293A-mGlu₅-low cells may be partially mGlu₅ mediated. Prolonged exposure to NPS2143, AZ12216052, and cinacalcet could be resulting in a slow, low-level, sustained mGlu₅-mediated release of Ca²⁺ from intracellular stores, reducing the pool available for subsequent release upon receptor activation. We have previously shown a similar mechanism reduces agonist-mediated iCa²⁺ mobilization at a constitutively active naturally occurring CaSR mutant (Leach et al., 2013).

Discussion

Here we reveal that class C GPCR allosteric ligands previously classified as selective for the GABA_B receptor, mGlu₁, mGlu₄, or mGlu₂, have hitherto unappreciated affinity for mGlu₅ (within 10-fold of the target receptor). Furthermore, most ligands had negative cooperativity with the allosteric mGlu₅ radioligand but displayed neutral cooperativity with allosteric agonists in functional assays, suggesting that cooperativity was probe-dependent and mediated via non-competitive interactions with well characterized common site

TABLE 3

Summary of potency and maximal response of class C GPCR allosteric modulator-mediated agonism for IP₁ accumulation in HEK293A-mGlu₅-low cells

Data represent the mean $\pm$ S.E.M. from four independent experiments performed in duplicate.

Ligand	pEC ₅₀	E _{max} ^a
AZ12216052	5.56 $\pm$ 0.35	1.20 $\pm$ 0.06
Cinacalcet	5.08 $\pm$ 0.35	1.43 $\pm$ 0.06
NPS2143	4.97 $\pm$ 0.12	1.44 $\pm$ 0.06
CGP7930	4.46 $\pm$ 0.25	1.29 $\pm$ 0.17

^aMaximal response to indicated agonist expressed as fold increase over basal IP₁ levels.

Fig. 5. Agonist concentration-response curves for IP₁ accumulation in the absence and presence of 10 μ M 5MPEP in HEK293A-mGlu₅-low cells. Preincubation with the neutral mGlu₅ allosteric ligand 10 μ M 5MPEP for 30 minutes significantly reduced VU0424465 potency (A) but had no significant effect on the responses to AZ12216052, NPS2143, cinacalcet, or CGP7930 (B–E). Data represent the mean $\pm$ S.E.M. from three or four independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbols.

mGlu₅ allosteric modulators. Previous reliance on single orthosteric ligand functional assays (generally iCa²⁺ mobilization) to determine selectivity may have resulted in the unintentional optimization of compounds that are biased and/or neutral modulators at other class C GPCRs. We used two different functional assays, observing differential profiles with respect to agonist efficacy and off-target effects. Of note, AZ12216052, cinacalcet, NPS2143, and CGP7930 were biased agonists relative to DHPG, showing higher efficacy in IP₁ accumulation than iCa²⁺ mobilization assays, in keeping with previous observations for mGlu₅ PAM-agonists (Sengmany et al., 2017). Collectively, our data support the notion that for diverse class C GPCR allosteric ligands subtype selectivity is driven by cooperativity and bias rather than affinity and highlight the limitations of a single functional screening assay to understand the full scope of class C GPCR allosteric ligand activity.

In the initial discovery studies of the class C GPCR allosteric ligands studied, many were screened in only one functional assay against a single off-target receptor. Some mGlu allosteric ligands underwent initial or subsequent screening at a larger number of targets, although only within the mGlu subfamily. The only allosteric ligand included in the current study that has been screened against a broader range of class C GPCRs is the CaSR NAM, NPS2143, which was tested for

activity at GABA_B, mGlu₁, and mGlu₅, albeit only at a single concentration (Nemeth et al., 2001). For those tested against mGlu₅, CPCCOEt was initially reported to have no effect on mGlu₅ receptors in IP₁ accumulation assays but was later revealed to inhibit both rat and human mGlu₅-mediated iCa²⁺ mobilization (Litschig et al., 1999; Marino et al., 2003). Further, PHCCC also antagonized mGlu₅-mediated iCa²⁺ mobilization at high concentrations, despite initial reports showing no effect (Maj et al., 2003; Marino et al., 2003). BINA, AZ12216052, CGP7930, NPS2143, and ML337 all had no effect in mGlu₅ functional assays (Binet et al., 2004; Galici et al., 2006; Duvoisin et al., 2010; Wenthur et al., 2013). The current study builds on these observations, estimating apparent affinities for mGlu₅ and determining cooperativity with multiple ligands. Clearly, class C GPCR selectivity for diverse chemotypes is driven by cooperativity over affinity. M₄ muscarinic acetylcholine receptor allosteric modulators also exhibit cooperativity-based selectivity (Lazareno et al., 2004; Valant et al., 2012), demonstrating the relevance of this phenomenon across multiple GPCR families.

Structure-function studies and computational modeling also suggest a common allosteric site shared across class C GPCRs (Wu et al., 2014; Leach et al., 2016; Harpsøe et al., 2017). Further, multiple mGlu allosteric ligands have documented activity at multiple receptor subtypes (Annoura et al.,

Fig. 6. Effect of class C GPCR allosteric ligands on iCa²⁺ mobilization in HEK293A-mGlu₅-low cells. (A) Pretreatment with allosteric modulators (100 μ M for all except cinacalcet at 30 μ M) for 1 minute before the mGlu₅ PAM agonist VU0424465 had no significant effect on VU0424465 potency or maximal response. (B–E) Preincubation for 30 minutes with AZ12216052, NPS2143, and cinacalcet (10 μ M) significantly reduced the maximum response to VU0424465 (B), DHPG (C), CCh (D), and ATP (E), whereas CGP7930, ML337, CPCCOEt, and PHCCC had no effect on VU0424465 or DHPG. Data represent the mean $\pm$ S.E.M. from a minimum of three independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbols.

1996; Maj et al., 2003; Marino et al., 2003; Mathiesen et al., 2003; O'Brien et al., 2003, 2004). Given the shared location of a 7TM binding pocket between different class C GPCRs, it is hardly surprising that we revealed many class C GPCR ligands have binding affinity for mGlu₅. With the exception of cinacalcet, none of the class C GPCR allosteric modulators

in the current study fully displaced [³H]methoxy-PEPy binding at mGlu₅, suggestive of a noncompetitive interaction. The effect of the common allosteric site mGlu₅ NAL, 5MPEP, on the IP₁ agonism of select compounds was also consistent with a noncompetitive interaction. Collectively, these data indicate that these class C GPCR allosteric modulators interact with a

TABLE 4
Effect of class C GPCR allosteric modulators (10 μ M) after 30-minute preincubation on potency and maximum response for DHPG and VU0424465-mediated iCa²⁺ mobilization in HEK293A-mGlu₅-low cells
Data represent the mean $\pm$ S.E.M. from indicated number (*n*) of independent experiments performed in duplicate. n.a. not performed.

Ligand	DHPG			VU0424465			CCh			ATP		
	pEC ₅₀	E _{max} ^a	<i>n</i>	pEC ₅₀	E _{max} ^a	<i>n</i>	pEC ₅₀	E _{max} ^a	<i>n</i>	pEC ₅₀	E _{max} ^a	<i>n</i>
Vehicle	6.98 $\pm$ 0.13	60.0 $\pm$ 2.6	3	7.68 $\pm$ 0.16	45.1 $\pm$ 4.8	4	4.75 $\pm$ 0.25	15.6 $\pm$ 1.6	4	5.49 $\pm$ 0.03	19.8 $\pm$ 2.4	4
CPCCOEt	6.98 $\pm$ 0.09	63.5 $\pm$ 7.6	3	7.55 $\pm$ 0.10	48.6 $\pm$ 5.0	4	n.a.	n.a.		n.a.	n.a.	
ML337	7.06 $\pm$ 0.12	57.2 $\pm$ 3.2	3	7.59 $\pm$ 0.10	41.7 $\pm$ 2.8	4	n.a.	n.a.		n.a.	n.a.	
PHCCC	7.04 $\pm$ 0.12	61.3 $\pm$ 12.9	3	7.52 $\pm$ 0.18	41.2 $\pm$ 2.6	4	n.a.	n.a.		n.a.	n.a.	
AZ12216052	7.22 $\pm$ 0.18	39.3 $\pm$ 3.3*	3	7.48 $\pm$ 0.10	27.6 $\pm$ 3.4*	4	4.10 $\pm$ 0.12	8.1 $\pm$ 0.9*	4	5.59 $\pm$ 0.12	7.3 $\pm$ 1.7*	4
cinacalcet	7.08 $\pm$ 0.16	34.0 $\pm$ 1.9*	3	7.88 $\pm$ 0.23	26.5 $\pm$ 3.2*	4	5.04 $\pm$ 0.16	12.13 $\pm$ 1.7	4	5.82 $\pm$ 0.17	9.3 $\pm$ 1.6*	4
NPS2143	6.77 $\pm$ 0.37	27.8 $\pm$ 4.1*	3	7.72 $\pm$ 0.38	20.4 $\pm$ 5.9*	4	4.36 $\pm$ 0.39	7.6 $\pm$ 1.8*	3	5.73 $\pm$ 0.16	5.1 $\pm$ 0.6*	4
CGP7930	7.07 $\pm$ 0.14	55.1 $\pm$ 2.6	3	7.66 $\pm$ 0.10	40.0 $\pm$ 2.2	4	n.a.	n.a.		n.a.	n.a.	

n.a., not performed.
^aMaximal response to indicated agonist expressed as percentage of maximum response to ionomycin.
*Significantly different (*P* < 0.05) from vehicle, with one-way analysis of variance and Dunnett's post-hoc test.

site, or sites, distinct from the common allosteric site. Incomplete radioligand displacement has previously indicated that class C receptors contain multiple conformationally linked allosteric sites (O'Brien et al., 2004; Hemstapat et al., 2006; Chen et al., 2008; Hammond et al., 2010; Bradley et al., 2011; Gregory et al., 2012; Rodriguez et al., 2012; Noetzel et al., 2013). Functional studies have also revealed that multiple allosteric sites exist within mGlu₂ and mGlu₄ receptors and CaSR (Hemstapat et al., 2007; Niswender et al., 2008; Rovira et al., 2015; Leach et al., 2016); however, for mGlu receptors, the precise location of these additional sites remains elusive, and the functional consequences of modulation through multiple sites have yet to be fully determined. Moreover, the apparent nonselectivity of many class C GPCR allosteric ligands, which can interact noncompetitively with mGlu₅ allosteric ligands, highlights that allosteric binding sites within class C GPCRs may share greater conservation than previously appreciated. Indeed, these observations reconcile well with medicinal chemistry efforts that have identified or exploited "molecular-switches" to derive novel pharmacologic tools from agents targeting related GPCRs (Lindsley et al., 2016).

Although most class C GPCR allosteric ligands had negative cooperativity with [³H]methoxy-PEPy, there was little or no functional effect on the efficacy or potency of the orthosteric agonist DHPG or the PAM-agonist VU0424465. Neutral cooperativity with an orthosteric agonist was expected and is in keeping with the limited selectivity data available. CPCCOEt, PHCCC, and ML337 trended toward inhibition of VU0424465 activity, whereas VU0424465 interactions with cinacalcet, AZ12216052, CGP7930, and NPS2143 were consistent with neutral cooperativity. This probe dependence is a hallmark of allosteric interactions and is consistent with the idea that cinacalcet, AZ12216052, CGP7930, and NPS2143 interact noncompetitively with the common allosteric site in mGlu₅. Allostery within class C GPCRs can be mediated across the dimer (Goudet et al., 2005; Hlavackova et al., 2005; Jacobsen et al., 2017); therefore, the differential effect of class C allosteric ligands on mGlu₅ PAM/NAM binding could represent effects across the dimer. Whatever the mechanism, the net result of many class C allosteric ligands binding to mGlu₅ is neutral cooperativity with both orthosteric and allosteric agonists.

Multiple class C GPCR allosteric ligands engender biased agonism and/or biased modulation (Jalan-Sakrikar et al., 2014; Cook et al., 2015; Leach et al., 2016; Haas et al., 2017; Sengmany et al., 2017). Our observations that cinacalcet, AZ12216052, CGP7930, and NPS2143 activate mGlu₅-mediated IP₁ accumulation but have shown no activity in iCa²⁺ mobilization assays, indicate that these four agonists are biased relative to DHPG, which has greater efficacy for iCa²⁺ mobilization over IP₁ accumulation (Sengmany et al., 2017). The propensity of mGlu₅ allosteric agonists to favor IP₁ accumulation over iCa²⁺ mobilization has major implications for discovery programs that commonly use iCa²⁺ mobilization as the primary assay for both hit discovery and selectivity determinations. Compounds classified as inactive based solely on mGlu₅-iCa²⁺ mobilization assays may in fact be either neutral or biased allosteric modulators, likely contributing to observations of shallow SAR. Small changes to scaffolds can cause a switch or loss in cooperativity, selectivity, or efficacy (Conn et al., 2014; Johnstone and Albert, 2017). A further

contributor to misinterpretation of SAR is the reliance on functional assays with orthosteric ligands to measure changes in modulator potency. Functional modulator IC₅₀/EC₅₀ values alone are unreliable, as potency represents a composite of allosteric cooperativity, affinity and intrinsic efficacy factors (Gregory et al., 2010; Lindsley et al., 2016; Johnstone and Albert, 2017). Each factor has its own SAR to consider during compound optimization and modification. SAR efforts aiming to eliminate off-target affinity may be misinterpreted if the ligands maintain their off-target affinity but lose cooperativity. To progress subtype selective allosteric ligands there is a need to monitor each allosteric component individually (Johnstone and Albert, 2017). Modulator affinity estimates derived from functional data using operational models of allostery are well correlated with binding affinity estimates, at least for mGlu₅ (Gregory et al., 2012). In the absence of allosteric radioligands, these approaches provide reliable quantification of affinity and cooperativity to delineate changes to allosteric modulator SAR during optimization. The correct experimental design and implementation of such analytical approaches should be considered a crucial part of future drug discovery programs for class C GPCR allosteric modulators.

Cooperativity-driven selectivity could have ramifications with respect to clinical development where unanticipated activity at off-target receptors could occur as the result of clinically relevant mutations in class C GPCRs (Leach et al., 2013; Cho et al., 2014). Indeed, naturally occurring mutations in the CaSR can change CaSR PAM and NAM affinity or cooperativity in a pathway-dependent manner (Leach et al., 2013). Therefore, it is conceivable that unanticipated off-target effects for modulators targeting other class C GPCRs may manifest owing to naturally occurring mutations influencing bias or cooperativity independently of affinity.

In summary, we have revealed that a wide variety of class C GPCR allosteric modulators display previously unappreciated affinity for mGlu₅. These class C GPCR allosteric modulators displayed neutral cooperativity with both orthosteric and allosteric mGlu₅ agonists in functional studies, but some were able to stimulate IP₁ accumulation. These data indicate that the use of functional studies and efficacy-driven approaches are inadequate to appreciate allosteric modulator pharmacology and subtype selectivity more completely, and that selectivity for class C GPCR allosteric modulators at mGlu₅ appears to be largely driven by cooperativity.

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Authorship Contributions

Participated in research design: Hellyer, May, Leach, Gregory.
Conducted experiments: Hellyer, Albold, Wang, Chen, Leach.
Performed data analysis: Hellyer, Wang, Gregory.
Wrote or contributed to the writing of the manuscript: Hellyer, Leach, Gregory.

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Address correspondence to: Karen J. Gregory, Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC, 3052 Australia. E-mail: karen.gregory@monash.edu