Differential Pharmacology and Binding of mGlu₂ Receptor Allosteric Modulators

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ABSTRACT

Allosteric modulation of metabotropic glutamate receptor 2 (mGlu₂) has demonstrated efficacy in preclinical rodent models of several brain disorders, leading to industry and academic drug discovery efforts. Although the pharmacology and binding sites of some mGlu₂ allosteric modulators have been characterized previously, questions remain about the nature of the allosteric mechanism of cooperativity with glutamate and whether structurally diverse allosteric modulators bind in an identical manner to specific allosteric sites. To further investigate the in vitro pharmacology of mGlu₂ allosteric modulators, we developed and characterized a novel mGlu₂ positive allosteric modulator (PAM) radioligand in parallel with functional studies of a structurally diverse set of mGlu₂ PAMs and negative allosteric modulators (NAMs). Using an operational model of allosterism to

analyze the functional data, we found that PAMs affect both the affinity and efficacy of glutamate at mGlu₂, whereas NAMs predominantly affect the efficacy of glutamate in our assay system. More importantly, we found that binding of a novel mGlu₂ PAM radioligand was inhibited by multiple structurally diverse PAMs and NAMs, indicating that they may bind to the mGlu₂ allosteric site labeled with the novel mGlu₂ PAM radioligand; however, further studies suggested that these allosteric modulators do not all interact with the radioligand in an identical manner. Together, these findings provide new insights into the binding sites and modes of efficacy of different structurally and functionally distinct mGlu₂ allosteric modulators and suggest that different ligands either interact with distinct sites or adapt different binding poses to shared allosteric site(s).

Introduction

The group II metabotropic glutamate receptors, mGlu₂ and mGlu₃, have emerged as attractive pharmacological targets for the treatment of multiple brain disorders. These G

protein-coupled receptors (GPCRs) function by binding their endogenous agonist, glutamate, in a large Venus Fly Trap orthosteric domain. This extracellular signal is then transduced through the receptors' seven transmembrane domain (7TM) to G_{i/o} G proteins to inhibit adenylyl cyclase and regulate ion channels, among other functions (Niswender and Conn, 2010). Selective orthosteric mGlu_{2/3} agonists and antagonists have efficacy in preclinical models of neurologic and neuropsychiatric disorders (Nickols and Conn, 2014). However, these agonists and antagonists target the highly conserved glutamate orthosteric binding pocket and do not provide mGlu₂ or mGlu₃ receptor subtype specificity. In addition, most of these agents are amino acid analogs and do not display optimal central nervous system drug properties.

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ABBREVIATIONS: 7TM, seven transmembrane domain; α , affinity cooperativity; ANOVA, analysis of variance; AZ12559322, *N*-(3-(2-isopropyl-7-methyl-1-oxoisindol-5-yl)phenyl)methanesulfonamide; AZD8418, 5-(7-chloro-2-[(1*S*)-1-cyclopropylethyl]-1-oxo-2,3-dihydro-1*H*-isindol-5-yl)-*N,N*-dimethyl-1,2-oxazole-3-carboxamide; AZD8529, 7-methyl-5-[3-(piperazin-1-ylmethyl)-1,2,4-oxadiazol-5-yl]-2-[[4-(trifluoromethoxy)phenyl]methyl]-3*H*-isindol-1-one; β , efficacy cooperativity; B_{max} , concentration of specific binding sites for the radioligand; BINA, 3'-[[2-(cyclopentyl-6,7-dimethyl-1-oxo-2,3-dihydro-1*H*-inden-5-yl)oxy]methyl]biphenyl-4-carboxylic acid; DMSO, dimethylsulfoxide; GIRK, G protein inwardly rectifying potassium channel; GPCR, G protein-coupled receptor; GTP γ S, 5'-3-*O*-(thio)triphosphate; HEK, human embryonic kidney; JNJ-42491293, 8-chloro-3-(cyclopropylmethyl)-7-[4-(3,6-difluoro-2-methoxy-phenyl)-1-piperidinyl]-1,2,4-triazolo[4,3-*a*]pyridine; LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; mGlu, metabotropic glutamate receptor; MNI-137, 4-(7-bromo-4-oxo-4,5-dihydro-3*H*-benzo[*b*]1,4-diazepin-2-yl)-pyridine-2-carbonitrile; MRK-8-29, 4-(2-fluoro-4-methoxyphenyl)-7-(2-(2-methylpyrimidin-5-yl)ethyl)quinoline-2-carboxamide; decoglutrant, 5-[2-(7-trifluoromethyl)-5-[4-(trifluoromethyl)phenyl]pyrazolo[1,5-*a*]pyrimidin-3-yl]ethynylpyridin-2-amine; NAM, negative allosteric modulator; NMR, nuclear magnetic resonance; PAM, positive allosteric modulator; P-gp, P-glycoprotein; RmGlu, rat metabotropic glutamate receptor; VU6001192, 6-(*cis*-2,6-dimethylmorpholino)methyl)-1-(4-fluorophenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide; WT, wild type.

Allosteric modulation of GPCRs has emerged as a new pharmacological strategy to selectively target individual receptor subtypes (Leach et al., 2007; Conn et al., 2009, 2014; Christopoulos, 2014). These modulators bind to sites within the 7TM domain that are less highly conserved than the orthosteric binding site, providing mGlu₂ positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) that can increase and decrease mGlu₂ activity, respectively, with good receptor subtype selectivity and improved central nervous system exposure (Hemstapat et al., 2007; Lundström et al., 2011; Andrés et al., 2012; Justinova et al., 2015). mGlu₂ PAMs and mGlu_{2/3} NAMs have efficacy in animal models of neurologic and neuropsychiatric disorders, providing further impetus for optimizing highly selective allosteric modulators (Galici et al., 2005; Benneyworth et al., 2007; Campo et al., 2011; Goeldner et al., 2013; Caprioli et al., 2015; Justinova et al., 2015). To further advance efforts to optimize and develop mGlu₂-specific allosteric modulators, it is critical to develop a clear understanding of the molecular mechanisms by which these compounds modulate mGlu₂ signaling.

Allosteric modulators of GPCRs regulate receptor-mediated signaling by altering the agonist's affinity (α) and/or efficacy (β) (Leach et al., 2007; Conn et al., 2009; Kenakin, 2013, 2016, 2017; Zhang and Kavana, 2015). Various modes of allosteric modulation have been described for mGlu receptor allosteric modulators. Neither mGlu₁ nor mGlu₅ NAMs appear to exhibit affinity cooperativity with glutamate (Litschig et al., 1999; Lavreysen et al., 2003; Bradley et al., 2011; Gregory et al., 2012). Similarly, the affinities of mGlu₅ PAMs are unaffected by glutamate, suggesting that they function by modulating the efficacy of endogenous ligands (Gregory et al., 2012). Conversely, mGlu₄ allosteric modulators alter agonist affinity for mGlu₄ (Poutiainen et al., 2015; Rovira et al., 2015). Previous studies of mGlu₂ ligands demonstrated that both mGlu₂ PAMs and mGlu_{2/3} NAMs affect agonist affinity (Lundström et al., 2011; Lavreysen et al., 2013). However, studies have yet to directly test how mGlu₂ PAMs and NAMs affect the affinity and efficacy of the endogenous ligand, glutamate, for mGlu₂. Due to potential probe-dependent effects and the possible influence of pharmacological mode on *in vivo* effects (Gregory and Conn, 2015; Rook et al., 2017), it is critical to fully characterize the allosteric modulators' mode of cooperativity with glutamate.

Additionally, previous studies have proposed the existence of multiple, topographically unique allosteric binding sites in the 7TM region of mGlu₁ and mGlu₅ (O'Brien et al., 2004; Hemstapat et al., 2006) and interactions with nonidentical binding sites on mGlu₄ have been suggested to be associated with distinct modes of allosteric modulator pharmacology (Rovira et al., 2015). Previous studies of mGlu₂ allosteric modulators have suggested that mGlu₂ PAMs and mGlu_{2/3} NAMs bind to an overlapping site (Lundström et al., 2011; Farinha et al., 2015; Doornbos et al., 2016). However, it is unclear whether this overlapping allosteric site on mGlu₂ is identical for all mGlu₂ allosteric modulators. Moreover, homology models for mGlu₂ predict that PAMs form a hydrogen bond with Asn735 (Farinha et al., 2015; Doornbos et al., 2016); however, mGlu_{2/3} NAMs are not predicted to use this residue, suggesting that some NAMs could bind to a topographically unique site (Lundström et al., 2011). Thus, further studies are needed to determine whether all known

mGlu₂ allosteric modulators share a single allosteric site or can bind to distinct sites.

In this study, we characterized a chemically diverse set of mGlu₂ PAMs and NAMs, demonstrating that mGlu₂ PAMs act through affinity and efficacy modulation, whereas the mGlu₂ NAMs that were tested act predominantly through efficacy modulation. Further mutagenesis studies demonstrated that some mGlu₂ PAMs, as well as NAMs, require the Asn735 residue for their functional activity whereas others do not, suggesting that these allosteric modulators could either exhibit different binding modes or bind to distinct sites. To test for distinct modes of binding among these allosteric modulators, we developed a novel mGlu₂ PAM radioligand and used this radioligand along with functional studies to show that mGlu₂ allosteric modulators can bind to mGlu₂ with different binding poses or at distinct allosteric sites.

Materials and Methods

Cell Culture. Unless otherwise noted, cell culture reagents from Invitrogen (Carlsbad, CA) were used for the below studies. To establish stably expressing wild-type (WT) rat mGlu₂ cell lines, the WT construct (Yin et al., 2014) was cloned into the pIRESpuo3 vector, transfected into human embryonic kidney (HEK) cells expressing the G protein inwardly rectifying potassium (GIRK) channel, and stable clones were selected using puromycin. For transient transfections, WT or N735D rat mGlu₂ DNA was cloned into the pIRESpuo3 vector and transfected into HEK cells expressing the GIRK channel as outlined below. Cells were cultured as previously described (Niswender et al., 2008) with the addition of nonessential amino acids.

Drugs and Radioligands. All drugs were synthesized in house through previously described methods unless otherwise noted. The mGlu_{2/3} NAMs (MNI-137 [4-(7-bromo-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-pyridine-2-carbonitrile] and decogurant [5-[2-[7-(trifluoromethyl)-5-[4-(trifluoromethyl)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]ethynyl]pyridin-2-amine]) and mGlu₂ NAMs (MRK-8-29 [4-(2-fluoro-4-methoxyphenyl)-7-(2-(2-methylpyrimidin-5-yl)ethyl)quinoline-2-carboxamide] and VU6001192 [6-((cis-2,6-dimethylmorpholino)methyl)-1-(4-fluorophenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide]) were synthesized as described previously (Hemstapat et al., 2007; Felts et al., 2015; Walker et al., 2015). Likewise, the mGlu₂ PAMs, AZD8529 (7-methyl-5-[3-(piperazin-1-ylmethyl)-1,2,4-oxadiazol-5-yl]-2-[[4-(trifluoromethoxy)phenyl]methyl]-3H-isoindol-1-one), AZD8418 (5-[7-chloro-2-[(1S)-1-cyclopropylethyl]-1-oxo-2,3-dihydro-1H-isoindol-5-yl]-N,N-dimethyl-1,2-oxazole-3-carboxamide), BINA (3'-[[2-(cyclopentyl-6,7-dimethyl-1-oxo-2,3-dihydro-1H-inden-5-yl)oxy]methyl]biphenyl-4-carboxylic acid), JNJ-42491293 (8-chloro-3-(cyclopropylmethyl)-7-[4-(3,6-difluoro-2-methoxy-phenyl)-1-piperidinyl]-1,2,4-triazolo[4,3-a]pyridine), and AZ12559322 [N-(3-(2-isopropyl-7-methyl-1-oxoisindolin-5-yl)phenyl)methanesulfonamide], were synthesized per previously described routes (Galici et al., 2006; Empfield et al., 2007; Andrés et al., 2012; Justinova et al., 2015). Glutamate and LY341495 [(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid] were purchased from Tocris Biosciences (Bristol, UK), and [³H]-LY341495 was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO).

Synthesis of the novel AZ12559322 radioligand was performed as described below. A solution of AZ12559322 (23 mg, 0.065 mmol) and *N*-iodosuccinimide (284 mg, 1.26 mmol) in dimethylsulfoxide (DMSO) (0.5 ml) was stirred at room temperature for 4 days, at which time an additional portion of *N*-iodosuccinimide (147 mg, 0.652 mmol) was added. The solution was stirred for an additional week and was then diluted with CH₂Cl₂ (5 ml). The solution was washed with saturated aqueous Na₂S₂O₃ (5 ml) and twice with brine (5 ml). The organic layer was concentrated to dryness and the residue was purified by semi-preparative high-performance liquid chromatography (19 × 250 mm, XBridge C18 5 μ OBD, with gradient elution from 10% to 75% MeCN-0.1% aqueous trifluoroacetic acid over 30 minutes; Waters, Milford,

MA). The fractions containing di-iodinated products were combined and concentrated to dryness via lyophilization. The solid was then purified by a second semi-preparative high-performance liquid chromatography analysis using the same conditions to give two di-iodinated compounds. Each fraction was concentrated to dryness, taken up in CH_2Cl_2 , and passed through a phase separator. The resulting CH_2Cl_2 solutions were concentrated to dryness to give 9.2 mg of the early eluting peak *N*-(2,4-diiodo-5-(2-isopropyl-7-methyl-1-oxoisindolin-5-yl)phenyl)methanesulfonamide. ^1H nuclear magnetic resonance (NMR) (400 MHz, CD_3OD) δ 1.33 (s, 3H), 1.35 (s, 3H), 2.69 (s, 3H), 3.09 (s, 3H), 4.46 (s, 2H), 4.52–4.64 (m, 1H), 5.49 (s, 1H), 6.94 (s, 1H), 7.10 (s, 1H), 7.26 (d, J = 8.5 Hz, 1H), 7.99 (d, J = 8.5 Hz, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 170.2, 153.3, 152.6, 144.1, 141.1, 140.6, 138.5, 131.9, 130.8, 127.5, 122.7, 101.2, 95.0, 46.1, 44.3, 41.6, 20.8, 17.6. Liquid chromatography-mass spectrometry (LCMS) ($M+H$) = 611.2, LCMS($M-H$) = 609.2 and 7.6 mg of the late eluting peak *N*-(2,4-diiodo-3-(2-isopropyl-7-methyl-1-oxoisindolin-5-yl)phenyl)methanesulfonamide. ^1H NMR (400 MHz, DMSO) δ 1.23 (d, J = 6.8 Hz, 6H), 2.65 (s, 3H), 3.05 (s, 3H), 4.38–4.47 (m, 3H), 7.16 (s, 1H), 7.27 (s, 1H), 7.32 (s, 1H), 8.44 (s, 1H), 9.42 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 166.9, 148.1, 146.2, 144.3, 142.3, 139.8, 135.8, 130.3, 129.3, 127.1, 121.3, 98.5, 44.3, 42.1, 41.3, 20.4, 16.8. LCMS($M+1$) = 611.2, LCMS($M-1$) = 609.3 (Scheme 1).

5'-O-(3-[^{35}S]thio)triphosphate Binding to mGlu₂. To measure activity of AZ12559322 at mGlu₂, we used 5'-3'-O-(thio)triphosphate (GTP γ S) binding assays in membranes from a Chinese hamster ovary cell line expressing human mGlu₂ as described previously (Justinova et al., 2015). Briefly, membranes from Chinese hamster ovary cells expressing human mGlu₂ were incubated for 15 minutes at 30°C with AZ12559322 in 500 μl assay buffer containing 20 mM HEPES, 100 mM NaCl, 10 mM MgCl_2 with 10 μM guanosine diphosphate, and 0.1 nM 5'-O-(3-[^{35}S]thio)triphosphate. After this preincubation, an EC_{10} concentration of glutamate was added and allowed to incubate for 30 minutes at 30°C. Reactions were terminated through vacuum filtration from a Packard 96-well harvester onto Unifilter-96, GF/B filter microplates (Perkin Elmer, Waltham, MA). After washes with ice-cold water, plates were dried and then 40 μl scintillation fluid (MicroScint 20) was added to the plate. Radioactive counts per minute were then counted on a TopCount plate reader.

mGlu Selectivity. To assess the selectivity of AZ12559322 in the mGlu receptor subfamily, we used a fluorescence-based cell assay in HEK293 cells expressing human mGlu constructs as described previously (Justinova et al., 2015). Briefly, for all mGlu receptors other than mGlu₂, HEK293 cells expressed a chimeric mGlu receptor fused to the human calcium receptor. In these chimeric proteins, the extracellular and transmembrane domains were composed of the human mGlu receptor, whereas the intracellular domain was made up of the human calcium receptor fused to the promiscuous Gq15 protein. Agonist-induced changes in intracellular calcium levels were measured using a fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA) as described previously (Levinthal et al., 2009).

Transient Transfections. On the day before transfections, cells expressing GIRK1 and GIRK2 channels were plated in dishes for either

functional assays (10-cm dish with 2 million cells/dish) or membrane preparations (10-mm dish with 5 million cells/dish). The following day, transfections were performed using FuGENE 6 Transfection Reagent (Promega, Madison, WI) at a ratio of 6 μl reagent to 1 μg DNA per Promega's optimized protocol. Briefly, FuGENE 6 Transfection Reagent and Opti-MEM media were combined, mixed, and incubated at room temperature for 5 minutes. The appropriate amount of DNA was then added, mixed, and incubated for 15 minutes at room temperature. The FuGENE/DNA mixture was then added to dishes (600 μl /10 cm dish and 1500 μl /10 mm dish) and immediately returned to the 37°C incubator. For the functional assay, cells were plated on 384-well plates with poly(D-lysine) (Corning, Corning, NY) as described below. For membrane preparations, cells were incubated for 48 hours and then membranes were prepared as described below.

Functional GIRK-Mediated Thallium Flux Assay. mGlu₂-mediated thallium flux was measured as previously described using HEK293 cells transiently transfected with WT or N735D mGlu₂ (Niswender et al., 2008). Briefly, HEK293 cells transiently transfected with a mGlu₂ construct were plated onto 384-well plates with poly(D-lysine) (Corning) 24 hours after transfection with 30,000 cells in 20 μl per well in plating media containing Dulbecco's modified Eagle's medium, 10% dialyzed fetal bovine serum, 20 mM HEPES, 1 mM sodium pyruvate, and penicillin/streptomycin (all reagents from Life Technologies, Carlsbad, CA). The following day, using an ELX405 microplate washer (BioTek, Winooski, VT), plating media were exchanged for 20 μl /well assay buffer containing 1 $\times$ Hanks' balanced salt solution (Gibco, Grand Island, NY) and 20 mM HEPES (pH 7.4) with FluoZin-2 AM dye (330 nM final, prepared as a DMSO stock and mixed as a 1:1 ratio with pluronic acid F-127). After a 1 hour incubation at room temperature, this assay buffer containing dye was exchanged for 20 μl /well assay buffer containing only 1 $\times$ Hanks' balanced salt solution (Gibco) and 20 mM HEPES. A Functional Drug Screening System 7000 (Hamamatsu, Hamamatsu City, Japan) was then used to measure thallium flux at room temperature. After two baseline images were taken at 1 Hz (excitation, 470 ± 20 nm; emission, 540 ± 30 nm), 20 μl test compound (2 $\times$) was added to each well and incubated for 140 seconds. Then, 10 μl glutamate concentrations (5 $\times$) diluted in thallium buffer were added to each well. Data were collected for an additional 2.5 minutes and analyzed post hoc in Microsoft Excel (Microsoft Corp., Redmond, WA) as described previously (Niswender et al., 2008). Functional data were then graphed into GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) and fitted to the operational model of allosterism as described below.

Operational Modeling of Allosterism. Functional data for NAMs were fitted to the operational model of allosterism as defined by the equation from Leach et al. (2007):

$$y = \text{basal} + \frac{(E_m - \text{basal})(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n + ([A]K_B + K_A K_B + K_A[B] + \alpha[A][B])^n}$$

where A is the agonist concentration, B is the allosteric modulator concentration, K_A is the agonist's equilibrium dissociation constant,

Scheme 1. Synthesis of novel AZ12559322 radioligand.

and K_B is the allosteric modulator's equilibrium dissociation constant. Allosteric modulation was defined by both affinity cooperativity (α) and efficacy cooperativity (β), wherein the NAMs' affinity cooperativity (α) variable was set to equal 1 due to their lack of affinity cooperativity with glutamate in radioligand binding assays. The terms τ_A and τ_B quantitate the ability of the agonist and allosteric modulator to induce a response on their own, respectively. Basal, E_m , and n represent the basal response of the system, maximal system response, and transducer coefficient relating occupancy to a response, respectively.

Functional data for PAMs were fitted to a simplified version of the operational model of allosterism calculating a composite cooperativity term ($\alpha\beta$):

$$y = \text{basal} + \frac{(E_m - \text{basal})(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n + (K_A(K_B + [B]))^n}$$

Using this equation, a composite cooperativity term ($\alpha\beta$) was calculated that combined both affinity (α) and efficacy (β) cooperativity. All other parameters were the same as defined above.

Functional Potencies of PAMs and NAMs. Functional potencies for PAMs (EC_{50}) and NAMs (IC_{50}) were determined using the curve fits generated from the operational model of allosterism. For each allosteric modulator, the EC_{20} (PAMs) or EC_{80} (NAMs) glutamate concentration was interpolated from the curve fit of the DMSO control. At each subsequent concentration of allosteric modulator (1 nM to 1 μ M), the response at this EC_{20} (PAMs) or EC_{80} (NAMs) glutamate concentration was interpolated from the curve fits in GraphPad Prism software. Data were plotted in GraphPad Prism and a concentration-response curve was fitted to a four-point parameter logistic equation to determine potencies for each PAM (EC_{50}) and NAM (IC_{50}).

Membrane Preparation. Once cells reached approximately 80%–90% confluence, dishes were washed four times with ice-cold phosphate-buffered saline and then cells were scraped off of the dishes. Cells were collected using a 5-minute centrifugation at 1000g, the supernatant was removed, and the cell pellet was stored in an -80°C freezer. To prepare membrane protein, cell pellets were thawed, suspended in 10 ml membrane binding buffer (50 mM Tris-HCl with 5 mM MgCl_2 and 0.9% NaCl), and then homogenized using three 10-second bursts from a TR-10 polytron (Tekmar, Vernon, BC, Canada). After this, a 10-minute centrifugation at 1000g in an Avanti JE Beckman centrifuge (Beckman Coulter, Brea, CA) was performed at 4°C and the pellet was discarded. The resultant supernatant was centrifuged for 30 minutes at 20,000g at 4°C and the supernatant was discarded. The remaining pellet was resuspended in 1 ml membrane binding buffer and the protein concentration was determined for each sample in triplicate using a BCA assay (Bio-Rad, Hercules, CA) measured by a SmartSpec Plus Spectrophotometer (Bio-Rad). Samples were then stored in an -80°C freezer until used for one of the radioligand binding studies described below.

[^3H]-AZ12559322 Binding. Saturation binding experiments were performed to determine whether [^3H]-AZ12559322 binds in a saturable manner to rat mGlu₂ (RmGlu₂) and/or any other mGlu receptor. Briefly, for initial saturation binding assays, 10 μ g RmGlu₂-expressing membrane was incubated with 25 μ M glutamate at room temperature for 15 minutes prior to adding a range of radioligand concentrations (0.05–20 nM). For saturation binding experiments testing the effect of a glutamate concentration-response curve on binding, 10 μ g RmGlu₂-expressing membrane was incubated with a fixed concentration of glutamate (0, 1, 3, 10, 30, or 100 μ M) prior to adding a range of radioligand concentrations (0.05–20 nM). For selectivity studies, 10 μ g mGlu receptor-expressing membrane was incubated with a supersaturating concentration of glutamate (100 μ M) prior to adding a range of radioligand concentrations (0.05–20 nM). After radioligand addition, the reaction was allowed to incubate at room temperature for 60 minutes based on association binding studies that demonstrate that this allows for the reaction to

reach equilibrium (data not shown). For all of these saturation binding experiments, nonspecific binding was assessed using the mGlu₂ PAM, AZD8529. For these assays and all subsequent radioligand binding studies, the reactions were terminated by vacuum filtration through a 96-well harvester (Packard) with ice-cold membrane buffer (50 mM Tris-HCl with 5 mM MgCl_2 and 0.9% NaCl). Plates were then dried overnight and 50 μ l scintillation fluid (MicroScint 20) was added to the plate. Radioactive counts per minute were then counted on a Top-Count plate reader. Saturation binding data were fitted to a one-site binding (hyperbola) in GraphPad Prism.

For displacement binding assays, 10 μ g RmGlu₂-expressing membrane was incubated with 25 μ M glutamate and a concentration-response curve of allosteric modulator at room temperature for 15 minutes. After this incubation, approximately 1 nM [^3H]-AZ12559322 was added and incubated with this reaction at room temperature for 60 minutes. Nonspecific binding was assessed using the mGlu₂ PAM, AZD8529. Data were fitted to a one-site competition binding curve in GraphPad Prism to determine each allosteric modulator's affinity (K_i).

Dissociation binding studies were performed at 4°C since the radioligand dissociated too rapidly at room temperature (data not shown). Ten micrograms of RmGlu₂-expressing membrane was incubated with a fixed concentration of glutamate (25 μ M) prior to adding approximately 6 nM [^3H]-AZ12559322. The reaction was allowed to equilibrate for at least 4.5 hours prior to initiating radioligand dissociation. For time points between 5 and 180 minutes before terminating the reaction, 10 μ M AZD8529 with or without allosteric modulator (1–10 μ M as outlined in the text) experiments were used to compare dissociation rates between control (10 μ M AZD8529) and plus allosteric modulator conditions (10 μ M AZD8529 plus 1–10 μ M allosteric modulator). Data were fitted in GraphPad Prism to either a one-phase or two-phase exponential decay depending on goodness of fit (F-test) as determined in GraphPad Prism. An F-test was used to compare the dissociation constants (k_{off}) between the control and plus allosteric modulator conditions.

[^3H]-LY341495 Binding. For glutamate affinity fold-shift experiments, 20 μ g RmGlu₂-expressing membrane was incubated for 60 minutes in the absence or presence of allosteric modulator (1 or 10 μ M), a concentration-response curve of glutamate (1.5 nM to 1 mM), and approximately 2 nM [^3H]-LY341495. Nonspecific binding was determined using a saturating concentration of LY341495 (10 μ M). Data were fitted to a one-site competition binding curve to determine glutamate's affinity (K_i) in the absence and presence of allosteric modulator using GraphPad Prism.

Data Analysis. All data represent the mean $\pm$ S.E.M. from three or more independent experiments unless otherwise noted in the figure legends. Data were analyzed and graphed using Microsoft Excel and GraphPad Prism. Statistical significance was determined using either an unpaired t test or one-way analysis of variance (ANOVA) as outlined in the text unless otherwise noted, and a P value less than 0.05 was considered statistically significant.

Results

mGlu₂ PAMs, But Not mGlu_{2/3} or mGlu₂ NAMs, Modulate Glutamate Affinity for mGlu₂. Previous studies have demonstrated that mGlu₂ allosteric modulators can exhibit affinity cooperativity (α) with mGlu₂ agonists (Lundström et al., 2011; Lavreysen et al., 2013). However, allosteric modulators of other mGlu receptor subtypes often mediate their primary actions by efficacy cooperativity (β) and it is not known whether mGlu₂ allosteric modulators affect both the affinity and efficacy of the endogenous orthosteric agonist, glutamate. Moreover, it is not clear whether a range of structurally diverse PAMs and NAMs act through similar or distinct mechanisms. To assess how a range of mGlu₂ allosteric modulators bind to mGlu₂ and affect its downstream

Fig. 1. Chemical structures of mGlu_{2/3} NAMs (A), mGlu₂ NAMs (B), and mGlu₂ PAMs (C).

signaling, we identified a set of chemically diverse, previously described mGlu_{2/3} NAMs, mGlu₂ NAMs, and mGlu₂ PAMs for further study (Bonnefous et al., 2005; Hemstapat et al., 2007; Lundström et al., 2011; Andrés et al., 2012; Felts et al., 2015; Justinova et al., 2015; Walker et al., 2015) (Fig. 1). To assess affinity cooperativity between allosteric modulators and glutamate, we measured mGlu₂ affinity for glutamate ($\log K_i$) by assessing its ability to displace the mGlu₂ antagonist radioligand, [³H]-LY341495, in the absence or presence of allosteric modulator. Neither the mGlu_{2/3} NAMs (MNI-137 and decoglurant) nor the mGlu₂-specific NAMs (MRK-8-29 and VU6001192) altered glutamate's affinity for mGlu₂, suggesting that these NAMs do not exhibit any appreciable affinity cooperativity with glutamate at mGlu₂ (Fig. 2, A–D). In direct contrast to the NAMs, each of the three structurally distinct mGlu₂ PAMs caused robust increases in glutamate's affinity for mGlu₂, demonstrating that these PAMs exhibit strong affinity cooperativity with glutamate at mGlu₂ (Fig. 2, E–G; unpaired *t* test comparing $\log K_i$ in absence and presence of PAM; $P < 0.001$ for each PAM; $n = 3$ experiments). Fold shifts were calculated to determine the change of glutamate's affinity in the presence of each PAM (BINA: 5.68 ± 0.66 ; AZD8418: 5.79 ± 0.91 ; and JNJ-42491293: 10.47 ± 1.15). Importantly, in the absence of glutamate (Fig. 2; vehicle condition), none of the allosteric modulators completely, or even significantly, displaced binding of [³H]-LY341495 to mGlu₂, thereby confirming that these compounds do not directly bind to the orthosteric binding site.

Functional Activity of Structurally Distinct mGlu₂ NAMs. Previous studies suggest that Asn735 is a critical residue in the binding site for mGlu₂ PAMs; however, some mGlu_{2/3} NAMs may not use this residue, instead using a different binding mode or a distinct binding site (Schaffhauser et al., 2003; Galici et al., 2006; Hemstapat et al., 2007; Lundström et al., 2011; Farinha et al., 2015). Interestingly, distinct binding sites have been posited to account for different modes of pharmacology for mGlu₁ and mGlu₄ (Hemstapat et al., 2006; Rovira et al., 2015). Based on this, it is possible that mGlu₂ NAMs and PAMs bind to distinct sites, and that this could be important for the difference between NAMs and PAMs in terms of their affinity cooperativity with glutamate. To further verify the necessity of Asn735 for functional activity on mGlu_{2/3} NAMs and extend this to novel mGlu₂ NAMs, we performed a progressive fold-shift experiment assessing NAM activity using the validated GIRK thallium flux assay in cells transiently transfected with either WT or the N735D mGlu₂

(Fig. 3). Fitting the data to the operational model of allosterism (see Table 1 for operational model parameters and functional potencies in WT mGlu₂) suggested that the mGlu_{2/3} NAMs, MNI-137 (Fig. 3A) and decoglurant (Fig. 3C), acted as partial NAMs at WT mGlu₂ through efficacy modulation. Moreover, consistent with previous studies of mGlu_{2/3} NAMs (Hemstapat et al., 2007; Lundström et al., 2011), both MNI-137 (Fig. 3B) and decoglurant (Fig. 3D) retained functional activity at N735D mGlu₂. Fitting data to the operational model (Table 1) also suggested that the mGlu₂-selective NAMs, MRK-8-29 (Fig. 3E) and VU6001192 (Fig. 3G), functioned through efficacy modulation, but as full NAMs. However, whereas MRK-8-29 did retain full, albeit less potent, NAM activity at the N735D mutant (Fig. 3F), VU6001192 completely lost activity at this mutant receptor (Fig. 3H). Thus, not all mGlu₂ NAMs retain their functional activity at this mutant receptor, demonstrating that an interaction with Asn735 is not necessary for their negative allosteric modulation.

Functional Activity of Structurally Distinct mGlu₂ PAMs. Based on prior studies, all previously characterized mGlu₂ PAMs require Asn735 for their functional activity (Schaffhauser et al., 2003; Galici et al., 2006; Hemstapat et al., 2007; Rowe et al., 2008; Farinha et al., 2015). Further homology modeling studies predict that mGlu₂ PAMs form a hydrogen bond with this Asn735, indicating that this residue may be critical for binding affinity of PAMs to the allosteric pocket (Farinha et al., 2015; Doornbos et al., 2016). Thus, to validate our previous findings with BINA and extend them to structurally distinct mGlu₂ PAMs, we performed further progressive fold-shift experiments assessing PAM activity in cells transiently transfected with either WT or the N735D mGlu₂. At WT mGlu₂, BINA (Fig. 4A) and AZD8418 (Fig. 4C) exhibited PAM activity that appeared to be through both efficacy and affinity cooperativity based on a comparison of the cooperativity ($\alpha\beta$) from the operational model to the glutamate affinity fold shifts (see Table 2 for operational model parameters and functional potencies in WT mGlu₂; BINA fold shift: 5.68 ± 0.66 and AZD8418 fold shift: 5.79 ± 0.91). In contrast, JNJ-42491293 (Fig. 4E) exhibited PAM activity that, based on a comparison between the cooperativity ($\alpha\beta$) from the operational model to the glutamate affinity fold shifts, may result predominantly from affinity cooperativity (see Table 2 for operational model parameters; JNJ-42491293 fold shift: 10.47 ± 1.15). Moreover, all PAMs demonstrated agonist-like activity in the absence of glutamate, indicating that, in this

Fig. 2. mGlu₂ PAMs, but not mGlu₂ NAMs, significantly affect glutamate's affinity for mGlu₂. (A–D) The effect of mGlu_{2/3} NAMs (A and B) and mGlu₂ NAMs (C and D) on glutamate affinity was tested in mGlu₂-expressing cells by measuring the K_i of glutamate to compete off the antagonist radioligand, [³H]-LY341495, in the absence or presence of a maximally effective concentration of mGlu₂ NAM (1 or 10 μ M). (E–G) The effect of mGlu₂ PAMs on glutamate affinity was tested in mGlu₂-expressing cells by measuring the K_i of glutamate to compete off the antagonist radioligand, [³H]-LY341495, in the absence or presence of a maximally effective concentration of mGlu₂ PAM (1 μ M). All data are normalized to total binding in the absence of allosteric modulator (Veh) condition and represent the mean $\pm$ S.E.M.

assay, they may function as agonist-PAMs (Fig. 4, A, C, and E). Consistent with previous findings, we found that BINA completely loses its functional activity in cells expressing the N735D mGlu₂ mutant (Hemstapat et al., 2007) (Fig. 4B). Likewise, AZD8418 completely lost functional activity in the cells expressing the N735D mutant, suggesting that this residue is necessary for AZD8418 PAM activity (Fig. 4D). In contrast, JNJ-42491293 retained its functional activity in cells expressing the N735D mutant, providing evidence of an

mGlu₂ PAM that either acts through a different binding mode or a distinct, nonidentical binding site (Fig. 4F). Taken together, the NAM and PAM functional activity in the N735D mutant suggests either distinct binding modes or multiple binding sites for allosteric modulators on mGlu₂.

To directly test the hypothesis that these mGlu₂ modulators exhibit distinct binding interactions with mGlu₂, we sought to develop an mGlu₂ allosteric modulator radioligand. Based on functional affinities (K_B) and potencies (EC_{50} and IC_{50})

Fig. 3. mGlu_{2/3} and mGlu₂ NAMs act primarily by modulating glutamate efficacy and some, but not all, retain activity in a BINA binding site mutant. (A–D) The effect of mGlu_{2/3} NAMs (MNI-137 and decoglurant) and selective mGlu₂ NAMs (VU6001192 and MRK-8-29) was tested using the GIRK thallium flux in HEK293 cells transiently transfected with either WT (A and C) or N735D mGlu₂ mutant (B and D) as outlined in the *Materials and Methods*. (E–H) The effect of selective mGlu₂ NAMs (VU6001192 and MRK-8-29) was tested using the GIRK thallium flux in HEK293 cells transiently transfected with either WT (E and G) or N735D mGlu₂ mutant (F and H) as outlined in the *Materials and Methods*. Data represent the mean ± S.E.M. and were fitted to the operational model of allosterism using the equation described in the *Materials and Methods* to determine allosteric modulator affinity (K_B) and efficacy cooperativity (β) (see Table 1). Veh, vehicle.

determined using the operational model of allosterism (see Tables 1 and 2 for K_B , EC_{50} , and IC_{50} values), none of the allosteric modulators possessed the low nanomolar affinity necessary for a radioligand. Although functional affinities and

potencies do not necessarily correspond to binding affinity (Kenakin, 2014), functional affinity has been shown to directly relate to affinity for other mGlu receptors (Gregory et al., 2012). Yet previous findings demonstrated that

TABLE 1
mGlu₂ and mGlu_{2/3} NAMs function predominantly through glutamate efficacy modulation

	MRK-8-29	VU6001192	MNI-137	Decoglurant
Log β (β) ^a	−100 (0)	−100 (0)	−0.43 ± 0.01 (0.37)	−0.62 ± 0.05 (0.24)
Log K_B (K_B) ^a	−6.46 ± 0.04 (347 nM)	−5.76 ± 0.04 (1.74 μM)	−6.18 ± 0.05 (661 nM)	−6.02 ± 0.08 (955 nM)
<i>n</i>	2.6 ± 0.2	2.7 ± 0.3	8.3 ± 0.8	3.9 ± 0.5
Basal	−2.4 ± 0.7	1.5 ± 1.2	2.9 ± 1.0	0.2 ± 1.4
Maximum	110.9 ± 4.4	124.1 ± 11.5	97.8 ± 1.2	108.9 ± 5.2
Log IC_{50} ^b	−6.51 ± 0.01 (307 nM)	−5.98 ± 0.02 (1.05 μM)	−5.93 ± 0.02 (1.18 μM)	−6.14 ± 0.01 (731 nM)

^aData for efficacy modulation (β) and allosteric modulator affinity (K_B) obtained from fitting functional data to operational model of allosterism as outlined in the *Materials and Methods*.
^bFunctional potencies (IC_{50}) were obtained from interpolating data from curve fits at the EC_{80} glutamate concentration and plotting a concentration-response curve as outlined in the *Materials and Methods*.

Fig. 4. mGlu₂ PAMs exhibit intrinsic agonist activity primarily through modulating glutamate affinity, but not all are affected by a BINA binding site mutant. (A–F) The effect of mGlu₂ PAMs on a glutamate concentration-response curve was tested using the GIRK thallium flux assay in HEK cells transiently expressing either WT (A, C, and E) or N735D mGlu₂ mutant (B, D, and F) as outlined in the *Materials and Methods*. Data represent the mean $\pm$ S.E.M. and were fitted to the operational model of allosterism using the equation described in the *Materials and Methods* to determine allosteric modulator affinity (K_B) and cooperativity ($\alpha\beta$) (see Table 2).

JNJ-42491293 exhibited low nanomolar affinity for mGlu₂, suggesting that functional affinity may not correspond to binding affinity for mGlu₂ (Andrés et al., 2012). Although JNJ-42491293 was developed as a radiotracer, it was recently shown to exhibit nonspecific binding *in vivo*, suggesting that it may not be a suitable for radioligand binding studies (Laurquin-Sterk et al., 2017). Thus, further chemical optimization efforts were undertaken to develop a more suitable mGlu₂ allosteric modulator for radiolabeling.

AZ12559322 Is a Potent and Selective mGlu₂ PAM. Chemical optimization efforts led to the discovery of AZ12559322 (Fig. 5A) as a novel mGlu₂ PAM that has properties suggesting that it may be suitable for use as a radioligand. Complementary functional and binding experiments were conducted in cells expressing mGlu₂ to further study this compound. We performed progressive fold-shift experiments assessing the PAM activity of AZ12559322 in

cells transiently transfected with either WT or the N735D mGlu₂ using the validated GIRK thallium flux assay. Similar to the structurally distinct PAMs described above, AZ12559322 exhibited apparent agonist-PAM activity in cells transiently expressing WT mGlu₂ (Fig. 5B; see Table 3 for operational model parameters and its functional potency derived as described in the *Materials and Methods*). Moreover, its functional activity was completely abrogated in cells expressing the N735D mGlu₂ mutant, demonstrating that, like BINA and AZD8418, AZ12559322 requires this residue for PAM activity (Fig. 5C). In membranes expressing WT mGlu₂, AZ12559322 increased glutamate's affinity for mGlu₂ as evidenced by the shift in a glutamate's ability to compete off the mGlu₂ antagonist radioligand, [³H] LY341495 (Fig. 5D, unpaired *t* test comparing log K_i in absence and presence of PAM; $P < 0.001$ for AZ12559322; $n = 3$ experiments). The affinity fold shift was calculated to determine the change of

TABLE 2
mGlu₂ PAMs function predominantly through glutamate affinity modulation

	BINA	AZD8418	JNJ-42491293
Log $\alpha\beta$ ($\alpha\beta$) ^a	1.58 $\pm$ 0.22 (38.01)	1.50 $\pm$ 0.19 (31.62)	1.11 $\pm$ 0.19 (12.88)
Log K_B (K_B) ^a	-6.16 $\pm$ 0.21 (692 nM)	-5.69 $\pm$ 0.19 (2.04 μ M)	-6.91 $\pm$ 0.13 (123 nM)
<i>n</i>	1.3 $\pm$ 0.1	1.2 $\pm$ 0.1	1.5 $\pm$ 0.2
Basal	4.0 $\pm$ 1.7	2.4 $\pm$ 1.6	0.6 $\pm$ 2.1
Maximum	98.1 $\pm$ 1.0	100.8 $\pm$ 0.9	94.8 $\pm$ 0.9
Log EC ₅₀ (nM) ^b	-7.26 $\pm$ 0.01 (55)	-6.70 $\pm$ 0.01 (200)	-7.76 $\pm$ 0.01 (17)

^aData for cooperativity ($\alpha\beta$) and allosteric modulator affinity (K_B) obtained from fitting functional data to operational model of allosterism as outlined in the *Materials and Methods*.

^bFunctional potencies (EC₅₀) were obtained from interpolating data from curve fits at the EC₈₀ glutamate concentration and plotting a concentration-response curve as outlined in the *Materials and Methods*.

Fig. 5. AZ12559322 is a novel mGlu₂ PAM acting through glutamate affinity modulation and is dependent on N735 for its activity. (A) Chemical structure of AZ12559322. (B and C) The effect of AZ12559322 on glutamate responses was tested in HEK cells transiently expressing WT mGlu₂ (B) or N735D mGlu₂ (C) by measuring mGlu₂-induced GIRK thallium flux. DMSO or 1 nM to 1 μM AZ12559322 was added to cells 140 seconds prior to a serial dilution of glutamate. Data from measured GIRK thallium flux responses are normalized to the maximal response to 1 mM glutamate. Symbols represent vehicle, 1 μM, and 300, 100, 30, 10, and 3 nM. (D) The effect of AZ12559322 on glutamate affinity was tested in mGlu₂-expressing cells by measuring the K_i of glutamate to compete off the antagonist radioligand, [³H]-LY341495, in the absence or presence of a maximally effective concentration of AZ12559322 (1 μM). Binding data are normalized to total binding in the absence of allosteric modulator (Veh) condition. For all panels, data are graphed as the mean ± S.E.M.

TABLE 3
AZ12559322 is a potent and selective mGlu₂ PAM

Target	PAM EC ₅₀ (Maximum Effect)	Agonist EC ₅₀
	μM	μM
HmGlu ₂ (%) ^a	0.01 (77.5)	>25
HmGlu ₁ ^b	>25	N/A
HmGlu ₃ ^b	>25	>25
HmGlu ₄ ^b	>25	>25
HmGlu ₅ (%) ^b	0.7692 (66.8)	>25
HmGlu ₆ ^b	>25	>25
HmGlu ₇ ^b	>25	>25
HmGlu ₈ ^b	>25	>25
Operational model parameters		
Log αβ (αβ) ^c	0.99 ± 0.12 (9.77)	
Log K _B (K _B , nM) ^c	-7.17 ± 0.12 (68)	
n	1.4 ± 0.2	
Basal	2.9 ± 2.0	
Maximum	100.9 ± 0.8	
Log EC ₅₀ (nM) ^d	-7.78 ± 0.01 (17)	

hCaR, human calcium receptor; HmGlu, human metabotropic glutamate receptor; N/A, not available.

^aData from 5'-O-(3-[³⁵S]thio)triphosphate binding in Chinese hamster ovary cell line expressing human mGlu₂.

^bmGlu receptor selectivity data from fluorescence-based binding assays wherein HEK293 cell lines express chimeric hmGlu receptor/hCaR.

^cData for cooperativity (αβ) and allosteric modulator affinity (K_B) obtained from fitting functional data from the GIRK assay in WT mGlu₂ (Fig. 5B) to the operational model of allosterism as outlined in the *Materials and Methods*.

^dFunctional potencies (EC₅₀) were obtained from interpolating data from the GIRK assay curve fit in Fig. 5B at the EC₂₀ glutamate concentration and plotting a concentration-response curve as outlined in the *Materials and Methods*.

glutamate's affinity in the presence of AZ12559322 (fold shift = 3.42 ± 0.73). Importantly, AZ12559322 did not significantly impact the binding of the antagonist radioligand, [³H]-LY341495, on its own, thereby verifying its binding to an allosteric site (Fig. 5D, vehicle treatment).

Further experiments were performed to assess whether AZ12559322 was selective for mGlu₂ over other mGlu receptor subtypes. In vitro GTPγS binding studies on cells expressing human mGlu₂ showed that AZ12559322 potentiated an EC₂₀ concentration of glutamate with potency of 10 nM and produced 77.5% of the maximal glutamate effect (Table 3, mean of $n = 2$ experiments). To determine whether AZ12559322 was selective for mGlu₂, we performed further in vitro binding studies. As outlined in Table 3, AZ12559322 demonstrated no PAM or agonist activity at any other mGlu receptor subtype (mGlu₁, mGlu₃, mGlu₄, mGlu₆, mGlu₇, mGlu₈; Table 3, EC₅₀ > 25 μM, $n = 2$ experiments). However, AZ12559322 did exhibit weak PAM activity at mGlu₅ with an EC₅₀ of 769 nM and produced 66.8% of the maximal glutamate effect (Table 3, mean of $n = 2$ experiments). Thus, comparing its PAM potencies at mGlu₂ and mGlu₅, AZ12559322 exhibited 76.9-fold selectivity for mGlu₂ over mGlu₅. Interestingly, although AZ12559322 did exhibit robust PAM activity in the mGlu₅ assay, no agonist-PAM activity was noted up to 25 μM.

[³H]-AZ12559322 Is a Suitable In Vitro mGlu₂ PAM Radioligand Tool Compound. Due to its functional potency, selectivity, and amenable physicochemical properties, further studies were conducted to radiolabel and validate AZ12559322 as a suitable radioligand for mGlu₂. After successful radiolabeling, we confirmed that, under experimental conditions, the binding of [³H]-AZ12559322 to mGlu₂ reached equilibrium in the presence of a supersaturating (25 μM) concentration of the orthosteric agonist, glutamate (data not shown). Under these conditions in membranes stably

Fig. 6. [³H]-AZ12559322 binds with high affinity to mGlu₂ in a glutamate-dependent manner and selectively binds to mGlu₂ over other mGlu receptors. (A) Representative saturation binding experiment with [³H]-AZ12559322 demonstrates saturable binding in the presence of 25 μM glutamate ($B_{\max}$ = 5306 ± 174 fmol/mg and K_d = 0.80 ± 0.07; n = 3 experiments). (B) Representative saturation binding experiment with [³H]-AZ12559322 with varying concentrations of glutamate (0–100 μM) demonstrates that glutamate concentration affects K_d (D), but not $B_{\max}$ (C) (data from n = 3 to 4 experiments,

Fig. 7. Affinities (K_i) of structurally distinct mGlu₂ PAMs and NAMs for mGlu₂. (A) Determination of selective mGlu₂ PAM affinities using competition binding studies with the mGlu₂ PAM radioligand, [³H]-AZ12559322. (B) Determination of mGlu_{2/3} NAMs (decogluturant and MNI-137) and selective mGlu₂ NAM (VU6001192 and MRK-8-29) affinities using competition binding studies with the mGlu₂ PAM radioligand, [³H]-AZ12559322. Data are representative of three experiments expressed as the mean $\pm$ S.E.M.

expressing mGlu₂, saturation binding experiments demonstrated that [³H]-AZ12559322 bound with a high affinity ($K_d = 0.80 \pm 0.07$ nM, $n = 3$ experiments) and to a significant number of binding sites on mGlu₂ ($B_{max} = 5306 \pm 174$ fmol/mg, $n = 3$ independent experiments) (Fig. 6A). Previous studies of mGlu₂ and other GPCRs illustrated that the binding of such PAM radioligands is affected by concentration of an orthosteric agonist; however, whether the agonist affects the affinity and/or number of binding sites has not been consistent across radioligands even at mGlu₂ (Lavreysen et al., 2013; Doornbos et al., 2016; Smith et al., 2016). Thus, in further saturation binding studies, [³H]-AZ12559322 affinity and number of binding sites were measured in the presence of different concentrations of the endogenous orthosteric agonist, glutamate (1–100 μ M). As evidenced in the representative saturation binding experiment depicted in Fig. 6B, increasing concentrations of glutamate enhanced the affinity of [³H]-AZ12559322 for mGlu₂ without affecting the number of binding sites. Data from three to four independent experiments

TABLE 4
mGlu₂ affinities (K_i) of mGlu₂ PAMs and NAMs determined by [³H]-AZ12559322 displacement experiments
Data represent the mean $\pm$ S.E.M. of three individual experiments performed in triplicate.

Allosteric Modulator	Log K_i (K_i)
	<i>nM</i>
BINA	-8.35 ± 0.11 (4.5)
AZD8418	-7.59 ± 0.10 (28.2)
JNJ-42491293	-9.12 ± 0.07 (0.77)
Decogluturant	-8.55 ± 0.18 (2.85)
VU6001192	-6.87 ± 0.08 (136)
MNI-137	-7.53 ± 0.09 (29.3)
MRK-8-29	-8.54 ± 0.11 (2.91)

are quantified in Fig. 6, C and D, illustrating that glutamate did not alter the number of binding sites (B_{max} , Fig. 6C; one-way ANOVA) but did increase the affinity of [³H]-AZ12559322 for mGlu₂ (Fig. 6D; one-way ANOVA with a Dunnett post-test compared with 0 μ M glutamate, $P < 0.05$). To ensure that [³H]-AZ12559322 bound specifically to mGlu₂ over other mGlu receptors, additional saturation binding studies were performed using a supersaturating concentration of glutamate (100 μ M) in membranes expressing different mGlu receptors. As in Fig. 6, A and B, [³H]-AZ12559322 bound with a high affinity to mGlu₂; however, it showed no appreciable binding to any of the other mGlu receptors (Fig. 6E).

Since the functional activity of AZ12559322 was completely abolished at the N735D mGlu₂ mutant, further experiments were performed to determine whether Asn735 is necessary for AZ12559322 binding as well as its functional activity. Saturation binding experiments using [³H]-AZ12559322 in membranes transiently transfected with either WT or N735D mGlu₂ demonstrated appreciable binding of [³H]-AZ12559322 only at WT mGlu₂ (Fig. 6F; unpaired t test, $P < 0.05$). As a critical control, further experiments using the mGlu₂ orthosteric antagonist radioligand, [³H] LY341495, illustrated that the B_{max} , or number of binding sites, was comparable in the same membranes transiently transfected with WT and N735D mGlu₂ (Fig. 6G; unpaired t test, $P = 0.80$). Taken together, these radioligand binding studies show that the selective and high-affinity binding of [³H]-AZ12559322 to mGlu₂ requires Asn735.

We next tested whether all of the structurally diverse allosteric modulators could completely compete off [³H]-AZ12559322. These studies aimed to verify that binding of [³H]-AZ12559322 is reversible and to assess whether mGlu₂ allosteric modulators act at a distinct site. Interestingly, displacement binding experiments revealed that all of the structurally distinct PAMs completely inhibited [³H]-AZ12559322 binding to mGlu₂ (Fig. 7A; Table 4 for K_i values). Similarly, all structurally distinct mGlu_{2/3} and mGlu₂ NAMs completely inhibited [³H]-AZ12559322 binding to mGlu₂ (Fig. 7B; Table 4 for K_i values).

mGlu₂ Allosteric Modulator Binding to mGlu₂ Suggests Nonidentical Modes of Interaction. Although each allosteric modulator inhibited binding of the novel PAM

one-way ANOVA with a Dunnett's post hoc test, $*P < 0.05$). (E) Representative saturation binding experiment demonstrates that, in the presence of 25 μ M glutamate, [³H]-AZ12559322 binds selectively to mGlu₂ over other mGlu receptors. (F and G) Saturation binding data from three experiments show B_{max} (mean $\pm$ S.E.M.) for [³H]-AZ12559322 (F) and [³H]-LY341495 (G) in HEK cells transiently transfected with WT or N735D mGlu₂ (unpaired t tests, $*P < 0.05$).

radioligand, this does not necessarily indicate that these allosteric modulators all bind to the same site that is labeled by [³H]-AZ12559322 (Limbird, 2005). Based on the mutagenesis studies, as well as the finding that [³H]-AZ12559322 binding is lost at the N735D mGlu₂ mutant, it is possible that some allosteric modulators inhibit [³H]-AZ12559322 binding through an allosteric mechanism rather than through competitive interactions. Thus, these allosteric modulators may bind to distinct sites and ligands at these sites may exhibit mutual negative cooperativity. To test this hypothesis, we performed dissociation binding experiments to evaluate the off-rate dissociation kinetics (K_{off}) of [³H]-AZ12559322 in the absence and presence of selected allosteric modulators. For these experiments, the selected PAMs and NAMs were added to the assay buffer at concentrations far in excess of predicted saturating concentrations ($100\times$ – $1000\times$ determined K_i values; see Table 4) to determine whether their addition affects the off-rate kinetics of [³H]-AZ12559322. A decrease or increase in the [³H]-AZ12559322 off-rate would indicate positive or negative cooperativity with a nonidentical allosteric site, respectively. Consistent with data from the mutagenesis studies, 1 μ M JNJ-42491293 increased the off-rate of [³H]-AZ12559322 compared with control conditions, providing evidence that JNJ-42491293 binds to a nonidentical allosteric

site with negative cooperativity with the site defined by AZ12559322 (Fig. 8A; F-test comparing k_{off} values in Table 5, $P < 0.001$). In agreement with functional data from N735D mGlu₂, 10 μ M AZD8418 did not alter dissociation kinetics compared with control dissociation conditions, consistent with AZD8418 binding to the site in a manner that is similar to that of AZ12559322 (Fig. 8B; F-test comparing k_{off} values in Table 5, $P = 0.68$). Likewise, 1 μ M BINA did not affect dissociation kinetics compared with control conditions, consistent with AZ12559322 binding to the canonical BINA binding site (Fig. 8C; F-test comparing k_{off} values in Table 5, $P = 0.68$). In contrast, both decoglurant and MRK-8-29 increased the off-rate of [³H]-AZ12559322 compared with control conditions, providing evidence that both NAMs bind to mGlu₂ in a manner that is not identical to that of the radioligand and that they exhibit negative cooperativity with AZ12559322 (Fig. 8, D; F-test comparing k_{off} values in Table 5, $P < 0.001$ for both NAMs). Interestingly, both decoglurant and MRK-8-29 changed the kinetics of dissociation from a one-phase decay to two-phase decay (see Table 5). However, in contrast with predictions from the mutagenesis data (see Fig. 3H), VU6001192 increased the off-rate of [³H]-AZ12559322 compared with control conditions, providing evidence that VU6001192 binds to a nonidentical allosteric site and confers

Fig. 8. The dissociation rate of [³H]-AZ12559322 is affected by certain mGlu₂ PAMs or NAMs, suggesting the presence of multiple allosteric binding sites. (A) Saturating concentrations of JNJ-42491293 (1 μ M) increase the dissociation rate of [³H]-AZ12559322 from mGlu₂-expressing membranes compared with the control dissociation induced by 10 μ M AZD8529. (B and C) Neither a saturating concentration of AZD8418 (10 μ M) nor BINA (1 μ M) altered the dissociation rate of [³H]-AZ12559322 from mGlu₂-expressing membranes compared with control dissociation induced by 10 μ M AZD8529. (D–F) The mGlu₂ NAMs, decoglurant (D; 3 μ M), MRK-8-29 (E; 3 μ M), and VU6001192 (F; 10 μ M) all increase the dissociation rate of [³H]-AZ12559322 from mGlu₂-expressing membranes compared with the control dissociation induced by 10 μ M AZD8529. Data are representative of three experiments expressed as the mean $\pm$ S.E.M. (F-test, *** $P < 0.001$).

TABLE 5

Dissociation binding kinetics (K_{off}) of [3 H]-AZ12559322 in absence and presence of allosteric modulator. Data represent the mean $\pm$ S.E.M. of three individual experiments performed in triplicate. Values in parentheses are $t_{1/2}$ in minutes.

Allosteric Modulator	Control (K_{off})	Plus Allosteric Modulator (K_{off})
JNJ-42491293	0.023 $\pm$ 0.0019 (30.20)	0.027 $\pm$ 0.0021 (15.65)*
AZD8418	0.027 $\pm$ 0.0021 (19.32)	0.027 $\pm$ 0.0021 (17.78)
BINA	0.027 $\pm$ 0.0021 (25.71)	0.034 $\pm$ 0.0036 (20.40)
Decoglutrant	0.036 $\pm$ 0.0022 (19.29)	0.61 $\pm$ 0.37 (K_{fast}) (1.13)* 0.035 $\pm$ 0.0068 (K_{slow}) (19.83)
MRK-8-29 ^a	0.034 $\pm$ 0.0030 (20.11)	0.44 $\pm$ 0.12 (K_{fast}) (1.57)* 0.0085 $\pm$ 0.0023 (K_{slow}) (81.90)
VU6001192 ^a	0.024 $\pm$ 0.0017 (28.43)	0.050 $\pm$ 0.0035 (13.85)*

^aData from experiments with addition of MRK-8-29 and decoglutrant fit better to a two-phase decay model.

* $P < 0.001$ comparing Control and Plus Allosteric Modulator values using a F-test.

negative cooperativity with regard to [3 H]AZ12559322 binding (Fig. 8F; F-test comparing k_{off} values in Table 5, $P < 0.001$). This could represent negative cooperativity between two distinct binding sites for VU6001192 and AZ12559322, or it could reflect changes in dissociation due to other actions, including effects on interactions to two protomers of the mGlu₂ homodimer. However, regardless of the specific mechanism, when taken together, these data provide further evidence for nonidentical modes of interaction with allosteric sites on mGlu₂ that are not specifically defined by whether Asn735 is necessary for the allosteric modulators' function.

Discussion

In this study, we characterized the in vitro molecular pharmacology properties of a chemically diverse set of mGlu₂ PAMs and NAMs to probe allosteric mechanisms and inform further chemical optimization. We found that each of the mGlu₂ PAMs evaluated appears to exhibit intrinsic agonist activity and modulate both affinity and efficacy of glutamate at mGlu₂. Interestingly, the mGlu_{2/3} and mGlu₂ NAMs studied did not alter glutamate's affinity, and functional studies suggested that the NAMs predominantly act through efficacy modulation, in contrast to mGlu₂ PAMs. Moreover, through the characterization of a novel mGlu₂ PAM and its radioligand, we demonstrated that an mGlu₂ PAM and multiple mGlu₂ NAMs exhibit distinct modes of binding to mGlu₂, which could reflect binding to a single site with different binding poses or interactions with distinct allosteric sites from the prototypical mGlu₂ PAM, BINA. Taken together, these data further refine our understanding of mGlu₂ allosteric modulation, serving to inform further drug discovery efforts.

Although our data suggest the possibility that mGlu₂ PAMs possess intrinsic agonist activity, this apparent intrinsic activity of our mGlu₂ PAMs could result from potentiation of glutamate released by cells or a large receptor reserve in our overexpression system (Noetzel et al., 2012; Rovira et al., 2015). Indeed, in overexpression systems with large receptor reserves, some mGlu₅ PAMs exhibit robust agonist-PAM activity that is not seen in native tissues presumably due to lower receptor expression levels (Noetzel et al., 2012; Rook et al., 2013). However, other optimized mGlu₅ ago-PAMs have demonstrated intrinsic agonist activity in native tissues that can have a major impact in the overall in vivo activity (Rook et al., 2013). Additionally, M₁ PAMs also exhibit intrinsic agonist activity in native tissues and induce robust seizure activity (Davoren et al., 2016). In future studies, it will be important to

systematically evaluate the propensity of different mGlu₂ PAMs to display allosteric agonist activity and determine the impact of agonist activity in overall in vivo effects.

Consistent with previous findings, all mGlu₂ PAMs, including our novel PAM radioligand, demonstrated robust affinity cooperativity with the orthosteric site. Previous studies reported such affinity cooperativity for mGlu₂ PAMs with orthosteric agonists; however, the mechanism through which various PAMs affected agonist affinity differs from one PAM to another (Lavreysen et al., 2013; Doornbos et al., 2016). Similar to our reported findings, a PAM radioligand's affinity for mGlu₂ increased in the presence of agonist without a corresponding change in the number of binding sites (Lavreysen et al., 2013). In contrast, another structurally distinct mGlu₂ PAM radioligand displayed an agonist-dependent increase in the number of binding sites without any change in its affinity for the receptor (Doornbos et al., 2016). These findings may reflect differential PAM effects on receptor active conformations, wherein PAMs can either increase the agonist's affinity for the receptor or shift the population of receptors in the active state. Indeed, direct comparison of B_{max} values determined from mGlu₂ PAM, agonist, and antagonist radioligands support this hypothesis. Based on studies of mGlu receptor dimers, PAM binding to one protomer of the homodimer is sufficient to potentiate receptor-mediated downstream signaling, suggesting that PAMs may bind to only one side of the homodimer. In support of this, Lundström et al. (2011) found that an mGlu₂ PAM radioligand recognized approximately 40% of the binding sites observed with an mGlu₂ agonist radioligand, consistent with the PAM binding to one protomer of the active-state homodimer receptors or to a site spanning the dimer interface. Additionally, this group demonstrated that an mGlu₂ agonist radioligand recognizes nearly half of the binding sites on mGlu₂ compared with an mGlu₂ antagonist radioligand, suggesting that, as seen with other GPCRs, the antagonist binds both active and inactive receptor states (Lundström et al., 2009). Taken together, these data may explain how some PAMs increase the number of agonist-bound sites and our finding that [3 H]-AZ12559322 recognizes less than 20% of the sites seen with the orthosteric antagonist radioligand.

In contrast to PAMs, mGlu_{2/3} and mGlu₂ NAMs act predominantly through efficacy cooperativity, thereby delineating the allosteric modes of action for PAMs and NAMs. Based on functional and binding data, mGlu₂ PAMs appear to function through a combination of affinity and efficacy modulation since the PAM-induced fold shift of glutamate's affinity for mGlu₂

cannot account for the cooperativity ($\alpha\beta$) determined using the operational model of allostery. Conversely, neither mGlu_{2/3} nor mGlu₂ NAMs affect glutamate's affinity, indicating that they function predominantly through efficacy modulation. However, it is important to avoid generalizing the results of studies with the mGlu₂ NAMs included here to all mGlu₂ NAMs. Indeed, a previous study using two mGlu_{2/3} NAMs that were not included here found that these NAMs could reduce the affinity of the agonist radioligand, [³H]-LY354740, for the orthosteric site (Lundström et al., 2011). This could also suggest that some NAMs can act by affinity modulation. However, it is also possible that this reflects a probe dependence of NAM effects on glutamate versus LY354740 binding or the use of human mGlu₂ in this GTP γ S. Probe dependence is a common phenomenon for allosteric modulators and has been posited to account for differential affinity modulation seen with mGlu₅ PAMs (Gregory and Conn, 2015). Specifically, when assessing PAM affinity using inhibition of the [³H]-MPEP binding, quisqualate, but not glutamate, increased the affinity of mGlu₅ PAMs for the receptor (Bradley et al., 2011; Gregory et al., 2012). Indeed, for the class A GPCR M₅, both a PAM and NAM displayed probe-dependent effects on agonist affinity (Berizzi et al., 2016). Overall, these findings illustrate differences in mGlu₂ PAM and NAM pharmacology and highlight the importance of using the endogenous ligand to assess pharmacological parameters of allosteric modulators.

Understanding the modes of action for allosteric modulators is important since it varies between allosteric modulators of other GPCRs and it could influence in vivo pharmacological properties. Among the class C mGlu receptors, neither mGlu₁ nor mGlu₅ allosteric modulators that have been evaluated exhibit affinity cooperativity with the endogenous agonist (Litschig et al., 1999; Gregory et al., 2012); however, both L-(+)-2-amino-4-phosphonobutyric acid and glutamate demonstrated affinity cooperativity with mGlu₄ PAMs (Rovira et al., 2015). The affinity cooperativity seen with mGlu₄ and our mGlu₂ PAMs may be critically important in the further development of in vivo radiotracers to measure receptor occupancy since its binding could depend on local agonist concentration and thus muddle the interpretation of in vivo occupancy data. Thus, high-affinity mGlu₂ NAMs may be better tools for further development of radiotracers due to their lack of affinity cooperativity with the endogenous ligand, glutamate. This may also apply more broadly to development of allosteric modulator radiotracers for other GPCRs since allosteric modulators of the class A receptors, M₁, M₄, and M₅, all exhibit affinity cooperativity with the endogenous ligand and the binding of a recent M₁ PAM radioligand is dependent on agonist concentration (Bubser et al., 2014; Berizzi et al., 2016; Smith et al., 2016; Rook et al., 2017). The allosteric modulator mode of action may also be important for in vivo physiologic effects including adverse side effect profile. A recent study demonstrated that the seizure liability of structurally distinct M₁ PAMs varied potentially due to subtle differences in pharmacological properties including differing degrees of affinity cooperativity with acetylcholine (Rook et al., 2017). Thus, drug discovery efforts should fully characterize the cooperativity of allosteric modulators and optimize these properties based on how the cooperativity corresponds to behavioral outcomes on target-by-target basis.

The presence of multiple modes of interaction of allosteric modulators with binding sites on mGlu₂ raises further questions and complications that must be addressed to inform additional

drug discovery efforts. Previous homology modeling studies of mGlu₂ allosteric modulators posited that both PAMs and NAMs occupied a common and overlapping binding pocket (Lundström et al., 2011; Farinha et al., 2015). Interestingly, our dissociation binding data indicate that some mGlu₂ allosteric modulators bind to a nonidentical site from AZ12559322; however, this does not rule out the possibility that these distinct sites are overlapping. Regardless, the presence of multiple sites complicates future in vivo studies since Rook et al. (2015) demonstrated that an mGlu₅ NAM positron emission tomography ligand was not displaced by mGlu₅ PAMs that bind to overlapping, but non-identical, sites. Therefore, with the further development of mGlu₂ radiotracers, in vitro experiments must ensure that allosteric modulators occupy the identical allosteric site to accurately establish in vivo occupancy-efficacy relationships. Another potential consequence of multiple binding sites is that allosteric modulators that bind to one site may exhibit different pharmacological properties from those binding at another. As a recent example of this, the degree of mGlu₄ PAM cooperativity corresponded with their predicted binding to distinct allosteric sites based on homology modeling (Rovira et al., 2015). While our data do not suggest a relationship between cooperativity of these nonidentical sites, it remains possible that such a relationship between pharmacology and binding site could exist. Regardless, further development of mGlu₂ allosteric modulators must take into account the multiple binding sites and should better define these sites using mutagenesis and homology modeling.

In conclusion, this study provides a further characterization of mGlu₂ allosteric modulators and demonstrates the presence of nonidentical allosteric sites on mGlu₂. These findings should be considered in further drug discovery efforts especially for the development of novel radiotracers. Yet questions remain about the physiologic relevance of potential ago-PAM activity, cooperativity, and the distinct allosteric sites on mGlu₂. Thus, further studies with novel in vivo tool compounds are necessary to define if and how the discrete in vitro pharmacological properties translate to behavioral responses.

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Authorship Contributions

Participated in research design: O'Brien, Cross, Wesolowski, Elmore, Niswender, Conn.

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