




Detailed In Vitro Pharmacological Characterization of Clinically Tested Negative Allosteric Modulators of the Metabotropic Glutamate Receptor 5[§]

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ABSTRACT

Negative allosteric modulation of the metabotropic glutamate 5 (mGlu₅) receptor has emerged as a potential strategy for the treatment of neurologic disorders. Despite the success in pre-clinical studies, many mGlu₅ negative allosteric modulators (NAMs) that have reached clinical trials failed due to lack of efficacy. In this study, we provide a detailed in vitro pharmacological characterization of nine clinically and preclinically tested NAMs. We evaluated inhibition of L-glutamate-induced signaling with Ca²⁺ mobilization, inositol monophosphate (IP₁) accumulation, extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, and real-time receptor internalization assays on rat mGlu₅ expressed in HEK293A cells. Moreover, we determined association rates (k_{on}) and dissociation rates (k_{off}), as well as NAM affinities with [³H]methoxy-PEPy binding experiments. k_{on} and k_{off} values varied greatly between the nine NAMs (34- and 139-fold, respectively) resulting in long receptor residence times (>400 min) for basimglurant and mavoglurant, medium residence times (10–30 min) for AZD2066, remeglurant, and (RS)-remeglurant, and low residence times (<10 mins) for dipraglurant, F169521, F1699611, and STX107. We found that all NAMs inhibited L-glutamate-induced mGlu₅ receptor internalization,

generally with a similar potency to IP₁ accumulation and ERK1/2 phosphorylation, whereas Ca²⁺ mobilization was less potently inhibited. Operational model of allosterism analyses revealed that dipraglurant and (RS)-remeglurant were biased toward (affinity) receptor internalization and away (cooperativity) from the ERK1/2 phosphorylation pathway, respectively. Our study is the first to measure mGlu₅ NAM binding kinetics and negative allosteric modulation of mGlu₅ receptor internalization and adds significant new knowledge about the molecular pharmacology of a diverse range of clinically relevant NAMs.

SIGNIFICANCE STATEMENT

The metabotropic glutamate 5 (mGlu₅) receptor is important in many brain functions and implicated in several neurological pathologies. Negative allosteric modulators (NAMs) have shown promising results in preclinical models but have so far failed in human clinical trials. Here we provide the most comprehensive and comparative molecular pharmacological study to date of nine preclinically/clinically tested NAMs at the mGlu₅ receptor, which is also the first study to measure ligand binding kinetics and negative allosteric modulation of mGlu₅ receptor internalization.

Introduction

Glutamate is the main excitatory neurotransmitter in the brain, exerting its action by activating ionotropic glutamate receptors and metabotropic glutamate (mGlu) receptors. Belonging to class C G protein-coupled receptors (GPCRs), there are eight mGlu receptor subtypes, which are important in central

nervous system functions, such as learning and locomotion (Aiba et al., 1994; Anwyl, 1999; Ayala et al., 2008; Niswender and Conn, 2010). Metabotropic glutamate receptor 5 (mGlu₅) is classified as a group I mGlu receptor, together with mGlu₁, and is mainly expressed on postsynaptic membranes, although it can also be found on presynaptic membranes and glial cells (Shigemoto et al., 1997; Aronica et al., 2003; Kuwajima et al., 2004; Leach and Gregory, 2017). mGlu₅ is ubiquitously expressed throughout the brain, and is implicated in several pathologies, such that inhibitors of mGlu₅ are potential therapeutics for Alzheimer's disease, fragile X syndrome, Parkinson's disease, and major depressive disorder (Huber et al., 2002; Michalon et al., 2012; Hughes et al., 2013; Hu et al., 2014; Nicoletti et al., 2015).

Due to high sequence similarity in the orthosteric binding site with other mGlu receptors, targeting allosteric binding pockets in the seven-transmembrane (7TM) domain of mGlu₅ has emerged as a more promising drug discovery strategy

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(Harpsøe *et al.*, 2015; Leach and Gregory, 2017). Allosteric modulators offer the possibility for spatiotemporal control by modulating receptor activity only in the presence of the orthosteric ligand. In the central nervous system, preservation of preexisting signaling patterns is crucial for many cognitive processes and is important in the maintenance of balanced long-term potentiation and long-term depression (Ayala *et al.*, 2009). Negative allosteric modulators (NAMs) of mGlu₅ diminish L-glutamate-induced receptor responses, and pharmacological inhibition of mGlu₅ activity in animal models has led to the suggestion that mGlu₅ inhibition is a viable therapeutic strategy for the treatment of major depressive disorder, fragile X syndrome, and L-DOPA-induced dyskinesia (Dolen *et al.*, 2007; Michalon *et al.*, 2012; Hughes *et al.*, 2013; Lindemann *et al.*, 2015). Multiple mGlu₅ NAMs have progressed through to phase II clinical trials, however, have failed due to either lack of efficacy or concerns over adverse effects (Scharf *et al.*, 2015; Barnes *et al.*, 2018; Sebastianutto and Cenci, 2018).

The failure of mGlu₅ NAMs to show efficacy in clinical studies raises questions about the translatability of results obtained in preclinical data and their power to predict drug behavior in humans (Berry-Kravis *et al.*, 2018). Therefore, a detailed pharmacological characterization of these NAMs may provide a deeper insight into events that take place at the molecular level, which may be crucial to drug efficacy *in vivo*. Kinetics of compound binding has attracted increasing attention in pharmacological research, and studies on association rates (k_{on}) and dissociation rates (k_{off}) of GPCR ligands are on the rise (Klein Herenbrink *et al.*, 2016; Doornbos *et al.*, 2017; Strasser *et al.*, 2017). The duration of biologic effect of a drug depends not only on the affinity of the drug for the receptor but also on the temporal stability of this ligand-protein complex. With this in mind, the dissociation rate of a drug from the receptor could be a valuable indicator of the duration of a biologic action of the drug *in vivo* (Tummino and Copeland, 2008; Lu and Tonge, 2010). Receptor residence time, calculated as $1/k_{off}$, has been shown to correlate with drug activity *in vivo*, in some cases increasing therapeutic effects, such as in the examples of the neurokinin 1 receptor antagonist aprepitant (Lindström *et al.*, 2007) and the muscarinic acetylcholine M₃ receptor antagonist tiotropium (Dowling and Charlton, 2006). On the other hand, a faster dissociation rate was beneficial in the prevention of side effects associated with dopamine D₂ receptor antagonism (Kapur and Seeman, 2001).

In this study, we performed a detailed *in vitro* pharmacological characterization of a range of clinically and preclinically tested mGlu₅ NAMs (Fig. 1), as studied using four different functional assays in HEK293A cells expressing

physiologic levels of rat mGlu_{5a} (HEK293A-mGlu_{5-low}) (Noetzel *et al.*, 2012). This is the first study to measure mGlu₅ NAM binding kinetics and negative modulation of L-glutamate-induced mGlu₅ receptor internalization, demonstrates the importance of testing receptor kinetics and a range of pathway assays when profiling clinical candidates, and provides a molecular pharmacological basis to advance future drug development.

Materials and Methods

Materials. 3-fluoro-5-[5-[2-(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridin-2-yl]benzotrile (STX107), 1-(3-chloro-4-fluoro-phenyl)-5-methyl-N-(2-methyl-4-pyridyl)triazole-4-carboxamide (referred to as F169521), 1-(3-chloro-4-fluoro-phenyl)-N-(2-chloro-4-pyridyl)-5-methyl-triazole-4-carboxamide (referred to as F1699611), (6-bromopyrazolo[1,5-a]pyrimidin-2-yl)-[(1R)-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl]methanone (remeglurant), (6-bromopyrazolo[1,5-a]pyrimidin-2-yl)-[(1RS)-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl]methanone ((RS)-remeglurant), methyl (3aR,4S,7aR)-4-hydroxy-4-[2-(3-methylphenyl)ethynyl]-3,3a,5,6,7,7a-hexahydro-2H-indole-1-carboxylate (mavoglurant), 6-fluoro-2-[4-(2-pyridinyl)-3-butyn-1-yl]-imidazo[1,2-a]pyridine (dipraglurant), 2-chloro-4-[(1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl)ethynyl]pyridine (basimglurant), and 4-[5-[(1R)-1-[5-(3-chlorophenyl)-3-isoxazolyl]ethoxy]-4-methyl-4H-1,2,4-triazol-3-yl]pyridine (AZD2066) were obtained from H. Lundbeck A/S (Copenhagen, Denmark). [³H]3-methoxy-5-(2-pyridinylethynyl)pyridine ([³H]methoxy-PEPy) was custom synthesized by Pharmaron (Manchester, UK). IP-One assay kit, Advanced phospho-ERK1/2 (Thr202/Tyr204) assay kit, and SNAP-Lumi4-Tb were purchased from Cisbio (Codolet, France). Probenecid, Pierce BCA Protein Assay kit, and Fluo-4 acetoxymethyl no wash kit were purchased from Thermo Fisher Scientific (Waltham, MA). Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM) GlutaMAX-I, dialyzed fetal bovine serum (dFBS), and penicillin-streptomycin solution were purchased from Invitrogen (Carlsbad, CA). 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) was purchased from Tocris (Bristol, UK). MicroScint-20 was purchased from PerkinElmer (Waltham, MA). pcDNA3.1(+) plasmid encoding human β 2-adrenoceptor with N-terminal Flag and SNAP tags (Flag-ST- β 2AR) was previously described (Roed *et al.*, 2014). pRK5 plasmids encoding rat mGlu_{5a} with N-terminal hemagglutinin (HA) and SNAP tags (HA-ST-rmGlu_{5a}) and excitatory amino acid transporter 3 (EAAT3) were gifts from Laurent Prézeau (Institut de Génétique Fonctionnelle, Montpellier, France) and previously described (Brabet *et al.*, 1998; Doumazane *et al.*, 2011). All of the other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture. HEK293A cells stably expressing wild-type rat mGlu_{5a} (HEK293A-mGlu_{5-low}) were cultured in DMEM GlutaMAX-I supplemented with 10% dFBS, 1% penicillin-streptomycin, 16 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 500 μ g/ml geneticin (G418). The cells were a gift from P. J. Conn (Vanderbilt University, Nashville, TN). The HEK293A cell line was cultured in DMEM GlutaMAX-I supplemented with 10% dFBS and

ABBREVIATIONS: 7TM, seven-transmembrane; [³H]methoxy-PEPy, [³H]3-methoxy-5-(2-pyridinylethynyl)pyridine; AUC, area under the curve; AZD2066, 4-[5-[(1R)-1-[5-(3-chlorophenyl)-3-isoxazolyl]ethoxy]-4-methyl-4H-1,2,4-triazol-3-yl]pyridine; basimglurant, 2-chloro-4-[(1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl)ethynyl]pyridine; BSA, bovine serum albumin; dFBS, dialyzed fetal bovine serum; dipraglurant, 6-fluoro-2-[4-(2-pyridinyl)-3-butyn-1-yl]-imidazo[1,2-a]pyridine; DL-TBOA, DL-threo- β -benzyloxyaspartic acid; DMEM, Dulbecco's modified Eagle's medium; EAAT3, excitatory amino acid transporter 3; ERK1/2, extracellular signal-regulated kinase 1/2; F169521, 1-(3-chloro-4-fluoro-phenyl)-5-methyl-N-(2-methyl-4-pyridyl)triazole-4-carboxamide; F1699611, 1-(3-chloro-4-fluoro-phenyl)-N-(2-chloro-4-pyridyl)-5-methyl-triazole-4-carboxamide; FRET, Förster resonance energy transfer; GPCR, G protein-coupled receptor; GPT, glutamic-pyruvic transaminase; HA, hemagglutinin; HBSS, Hanks' balanced salt solution; IP₁, inositol monophosphate; k_{off} , dissociation rate; k_{on} , association rate; mavoglurant, methyl (3aR,4S,7aR)-4-hydroxy-4-[2-(3-methylphenyl)ethynyl]-3,3a,5,6,7,7a-hexahydro-2H-indole-1-carboxylate; mGlu, metabotropic glutamate; MPEP, 2-methyl-6-(phenylethynyl)pyridine hydrochloride; NAM, negative allosteric modulator; RFU, relative fluorescence unit; remeglurant, (6-bromopyrazolo[1,5-a]pyrimidin-2-yl)-[(1R)-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl]methanone; (RS)-remeglurant, (6-bromopyrazolo[1,5-a]pyrimidin-2-yl)-[(1RS)-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl]methanone; STX107, 3-fluoro-5-[5-[2-(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridin-2-yl]benzotrile.

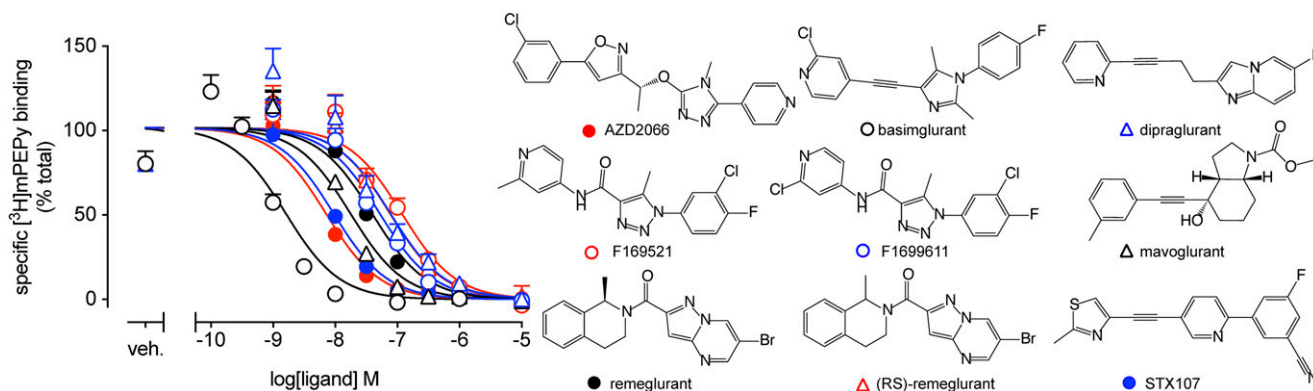


Fig. 1. Inhibition of [³H]methoxy-PEPy binding to HEK293A-mGlu₅-low cell membranes by mGlu₅ NAMs. Data are means ± S.E.M. from three independent experiments performed in duplicate. All compounds completely displaced [³H]methoxy-PEPy binding. Veh. denotes vehicle (1% DMSO), which was kept constant for all concentrations of NAMs. Chemical structures for each ligand are shown, showing the chemical diversity sampled within this study. Error bars not shown lie within the dimensions of the symbol.

1% penicillin-streptomycin. Cells were maintained in a humidified incubator at 37°C and 5% CO₂.

HEK293A Cell Membrane Preparation. HEK293A-mGlu₅-low cells were harvested and snap frozen on dry ice for 5 minutes, after which they were resuspended in ice-cold homogenization buffer (50 mM Tris-Cl, 0.9% NaCl, 10 mM EDTA, pH adjusted to 7.4). Cells were homogenized with a polytron in 3 × 30 second pulses, followed by centrifugation for 10 minutes at 1000g at 4°C. Next, the supernatant was centrifuged at 30,000g at 4°C for 60 minutes using a Sorvall Evolution RC ultracentrifuge (Thermo Fisher Scientific), and the pellet was resuspended in binding buffer (HBSS supplemented with 20 mM HEPES, 1.2 mM CaCl₂, 2 mM NaHCO₃, pH adjusted to 7.4). Protein concentration was quantified with Pierce BCA Protein Assay kit as per manufacturer's instructions. Membranes were stored at -80°C before use.

Inhibition Radioligand Binding. HEK293A-mGlu₅-low cell membranes were diluted in binding buffer. Compounds were diluted in binding buffer with 1% DMSO final concentration. Compounds, [³H]methoxy-PEPy (specific activity 85 Ci/mmol), and membranes (50 μg/well) were added to a transparent 96-well plate and incubated at room temperature while being shaken for 1 hour. Membranes were harvested on GF/C filter plates using a 96-well FilterMate harvester (PerkinElmer) to separate bound and free radioligand. Filter plates were dried at room temperature overnight before addition of MicroScint-20 scintillation liquid. Scintillation spectrometry was measured on a MicroBeta² microplate counter (PerkinElmer) after incubation for 2 hours at room temperature.

Competition Association and Dissociation Binding. HEK293A-mGlu₅-low membranes (50 μg/well) diluted in buffer were added to a transparent 96-well plate. For the competition association binding experiments, compound and [³H]methoxy-PEPy mixture were added to the plate at different time points. For the dissociation binding experiments, membranes were preincubated with [³H]methoxy-PEPy for 1 hour at room temperature, after which 1 μM MPEP was added to each well in a reverse time course. At t = 0, membranes were harvested on GF/C filter plates using a 96-well FilterMate harvester (PerkinElmer), and filter plates were dried overnight at room temperature. Radioligand binding was determined by scintillation counting as described above.

Ca²⁺ Mobilization Assay. HEK293A-mGlu₅-low cells were seeded on a poly-D-lysine-coated black-walled clear-bottom Falcon 96-well plate (Corning Inc., Corning, NY) at a density of 40,000 cells per well 24 hours before the assay. On the day of the assay, cells were incubated for 1 hour at 37°C in assay buffer (HBSS buffer supplemented with 20 mM HEPES, 1 mM MgCl₂, and 1 mM CaCl₂ with pH adjusted to 7.4) supplemented with 0.1% bovine serum albumin (BSA) followed by 3 hours in assay buffer supplemented with 10 U/ml glutamic-pyruvic transaminase (GPT), 10 mM pyruvic acid, and 0.1% BSA. Compounds

were diluted in assay buffer supplemented with 2.5 mM probenecid to 1% final DMSO concentration. Fluo-4 acetoxymethyl cell permeant dye diluted in assay buffer supplemented with 2.5 mM probenecid was added to each well, and the plate was incubated for 1 hour at 37°C. After dye loading, wells were washed with assay buffer, and plates were preincubated with NAMs for 30 minutes at 37°C. Fluorescence was measured on a FlexStation 3 plate reader (Molecular Devices, San Jose, CA) at 37°C with a single addition of L-glutamate (320 nM L-glutamate final concentration corresponding to the EC₈₀ concentration).

Inositol Monophosphate Accumulation Assay. HEK293A-mGlu₅-low cells were seeded on a poly-D-lysine-coated Falcon 96-well cell culture plate at a density of 25,000 cells per well 24 hours before the assay. On the day of the assay, plates were incubated at 37°C for 1 hour with wash buffer (HBSS buffer supplemented with 20 mM HEPES, 1 mM MgCl₂, and 1 mM CaCl₂ with pH adjusted to 7.4) supplemented with 0.1% BSA followed by 3 hours in wash buffer supplemented with 10 U/ml GPT, 10 mM pyruvic acid, and 0.1% BSA. Compounds were diluted in assay buffer (HBSS buffer supplemented with 20 mM HEPES, 1 mM MgCl₂, and 1 mM CaCl₂, 40 mM LiCl with pH adjusted to 7.4) to 1% final DMSO concentration. Cells were preincubated with compounds for 30 minutes at 37°C, after which 3.2 μM of L-glutamate final concentration (corresponding to the EC₈₀ concentration) was added and the plate was incubated for 1 hour at 37°C. Cells were lysed with 30 μl lysis buffer (IP-One assay kit) for 30 minutes at room temperature. 10 μl of lysate and 10 μl of detection solution (IP-One assay kit) were transferred to a white 384-well plate and incubated at room temperature for 1 hour, as previously explained (Nørskov-Lauritsen et al., 2014). Fluorescence emission was measured at 615 and 665 nm using an EnVision 2104 Multilabel Reader (PerkinElmer) after excitation at 340 nm. Förster resonance energy transfer (FRET) ratios were calculated as 665 nm/615 nm, and inositol monophosphate (IP₁) concentrations were obtained using the IP₁ standard curve of the assay kit.

Extracellular Signal-Regulated Kinase 1/2 Phosphorylation Assay. HEK293A-mGlu₅-low cells were seeded on a poly-D-lysine-coated Falcon 96-well cell culture plate at a density of 25,000 cells per well. Eight hours after seeding, cell media was substituted with starvation media (DMEM GlutaMAX-I supplemented with 1% penicillin-streptomycin and 16 mM HEPES), and cells were starved for approximately 16 hours at 37°C and 5% CO₂. On the day of the assay, plates were incubated at 37°C for 1 hour in assay buffer (HBSS buffer supplemented with 20 mM HEPES, 1 mM MgCl₂, and 1 mM CaCl₂ with pH adjusted to 7.4) supplemented with 0.1% BSA followed by 3 hours in assay buffer supplemented with 10 U/ml GPT, 10 mM pyruvic acid, and 0.1% BSA. Compounds were diluted in assay buffer to 1% DMSO final concentration, and plates were preincubated with the compounds for 30 minute at 37°C prior to addition of 3.2 μM

L-glutamate final concentration (corresponding to the EC₈₀ concentration). After L-glutamate addition, cells were incubated for 5 minutes at 37°C. The assay was terminated by aspiration of compounds and addition of lysis buffer [Advanced phospho-ERK1/2 (Thr202/Tyr204) assay kit]. Phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) was determined using the Advanced phospho-ERK1/2 (Thr202/Tyr204) assay, as per manufacturer's instructions. Fluorescence was measured at 615 and 665 nm using an EnVision 2104 Multilabel Reader after excitation at 340 nm. FRET ratios were calculated as 665 nm/615 nm.

Receptor Internalization Assay. HEK293A cells were transiently transfected in 10-cm dishes (9 million cells/dish) with 24 μl Lipofectamine 2000, 1.2 μg EAAT3, 4.5 μg HA-ST-rmGlu₅, and 3.9 μg pcDNA3.1(+) plasmids 48 hours before the assay. Twenty-four hours after transfection, cells were seeded on a white poly-D-lysine-coated Falcon 384-well culture plate (Corning Inc.) at a density of 20,000 cells per well, and plates were incubated for 24 hours at 37°C and 5% CO₂. For determination of NAM interference with the internalization assay, HEK293A cells were transiently transfected with 1.33 μl/ml Lipofectamine 2000, 133 ng/ml Flag-ST-β2AR, and 400 ng/ml pcDNA3.1(+) and seeded directly in the plate at a density of 20,000 cells per well in 30 μl. On the day of the assay, plates were incubated for 2 hours at 37°C in assay buffer (HBSS buffer supplemented with 20 mM HEPES, 1 mM MgCl₂, and 1 mM CaCl₂ with pH adjusted to 7.4) supplemented with 0.1% BSA followed by labeling of surface receptors with 100 nM SNAP-Lumi4-Tb diluted in assay buffer supplemented with 0.1% BSA for 1 hour at 37°C. After labeling, plates were washed twice with assay buffer supplemented with 0.1% BSA and twice with assay buffer. Compounds were diluted in assay buffer to 1% DMSO final concentration. Plates were preincubated with the compounds for 30 minutes at 37°C. Next, 50 μM fluorescein-O'-acetic acid, 11.6 μM L-glutamate (corresponding to the EC₈₀ concentration), and 100 μM DL-threo-β-benzyloxyaspartic acid (DL-TBOA) were added. For NAM interference experiments, assay buffer or 100 μM isoprenaline was added at the same time as the compounds. Receptor internalization was measured using an EnVision 2104 Multilabel Reader every 6 minutes for 66 minutes at 37°C. The donor was excited at 340 nm, and donor and acceptor emissions were measured at 615 nm and 520 nm, respectively. Internalization ratios were calculated as 615 nm/520 nm. The assay method has been described previously (Levoye et al., 2015; Foster and Bräuner-Osborne, 2018). NAM interference was analyzed by calculating the area under the curve of 66-minute real-time isoprenaline internalization curves in presence of vehicle or NAMs after subtraction of the basal internalization. NAM concentrations that reduced the signal more than 25% compared with vehicle were excluded from further analysis.

Data Analysis. Data were analyzed in GraphPad Prism software version 7 (GraphPad, San Diego, CA). For radioligand binding experiments, nonspecific binding was subtracted from each data point, and 100% was defined as the mean of the total specific radioligand binding. Inhibition binding data were fitted to a one-site binding function with the following equation:

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

where Y is the specific binding (%), Top and Bottom are the maximal and the minimal asymptotes, respectively, and IC₅₀ is the concentration of ligand that induces a response midway between Top and Bottom and is reported as the negative logarithm within the text (pIC₅₀). Obtained IC₅₀ values were converted to K_i estimates with the Cheng-Prusoff equation (Cheng and Prusoff, 1973), where the

concentration of radioligand (1.9–2.1 nM) was slightly below the K_D determined from saturation binding (see Table 1).

k_{off} of [³H]methoxy-PEPy was calculated with a one-phase exponential decay function using the following equation:

$$k_{\text{off}} = \frac{\ln 2}{t_{1/2}} \quad (2)$$

k_{on} of [³H]methoxy-PEPy was calculated using a single-phase association function with the following equation:

$$k_{\text{on}} = \frac{k_{\text{ob}} - k_{\text{off}}}{[\text{radioligand}]} \quad (3)$$

where k_{off} was calculated with eq. 2 and k_{on} was calculated with an exponential association analysis.

Competition association binding data were fitted to the model for competitive binding (Motulsky and Mahan, 1984) to obtain kinetic rates for unlabeled ligand with the following equations:

$$\begin{aligned} K_A &= k_1[L] \cdot 10^{-9} + k_2 \\ K_B &= k_3[I] \cdot 10^{-9} + k_4 \\ S &= \sqrt{(K_A - K_B)^2 + 4 \cdot k_1 \cdot k_3 \cdot [L] \cdot [I] \cdot 10^{-18}} \\ K_F &= 0.5 \cdot (K_A + K_B + S) \\ K_S &= 0.5 \cdot (K_A + K_B - S) \\ Q &= \frac{B_{\text{max}} \cdot K_1 \cdot [L] \cdot 10^{-9}}{K_F - K_S} \\ Y &= Q \cdot \left(\frac{k_4 \cdot (K_F - K_S)}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} \cdot e^{-K_F X} - \frac{k_4 - K_S}{K_S} \cdot e^{-K_S X} \right) \quad (4) \end{aligned}$$

where X represents time (minutes), L is the concentration of [³H]methoxy-PEPy, I is the concentration of the unlabeled ligand, and k₁ (M⁻¹min⁻¹) and k₂ (min⁻¹) are the k_{on} and k_{off} rates of [³H]methoxy-PEPy, respectively. The k_{on} and k_{off} rates of [³H]methoxy-PEPy were calculated with eqs. 2 and 3 and fitted to the model to obtain the maximal number of receptors, or B_{max}, and k_{on} and k_{off} rates of unlabeled ligand represented in the equation as k₃ (M⁻¹min⁻¹) and k₄ (min⁻¹), respectively. Receptor residence time was calculated as 1/k_{off} (minutes).

Data obtained from functional assays were normalized to 0% defined by the mean for the buffer value and 100% defined by the mean of the maximal orthosteric agonist response. Concentration-response curves were fitted to a four-parameter function with the following equation:

$$\text{Response} = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{(1 + 10^{(\text{LogEC}_{50} - \text{Log}[\text{agonist}])^n})} \quad (5)$$

where EC₅₀ is the concentration of agonist that is required to give a half-maximal response and Top and Bottom are the maximal and minimal asymptotes, respectively, of the concentration-response curve. EC₅₀ values are reported as negative logarithms (pEC₅₀).

Allosteric modulation of glutamate-mediated responses was fitted to the following operational model of allosterism:

where [A] is the molar concentration of orthosteric ligand, [B] is the concentration of allosteric modulator, K_A and K_B are the equilibrium dissociation constants of the orthosteric ligand and allosteric modulator, respectively, α represents affinity cooperativity, and β is an empirical scaling factor defining the effect of a modulator on

$$\text{Response} = \frac{E_m(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{([A]K_B + K_A K_B + K_A[B] + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n} \quad (6)$$

TABLE 1

Affinity estimates and kinetic binding parameters for mGlu₅ NAMs based on inhibition of [³H]methoxy-PEPy binding. Data are represented as means ± S.E.M. from indicated number (*n*) of independent experiments performed in duplicate.

	pK _I ^a	<i>n</i>	k _{on} ^b (× 10 ⁶) M ⁻¹ min ⁻¹	k _{off} ^c min ⁻¹	Residence time ^d min	<i>n</i>	pK _D ^e
[³ H]methoxy-PEPy	8.24 ± 0.09 ^f	4	14.2 ± 4.3	0.133 ± 0.006	7.6 ± 0.4	6	8.04
AZD2066	8.53 ± 0.13	3	12.6 ± 1.3	0.045 ± 0.007	26.0 ± 6.2	5	8.50
Basimglurant	9.29 ± 0.06	3	13.6 ± 2.9	0.005 ± 0.002	491 ± 136	8	9.44
Dipraglurant	7.67 ± 0.11	3	33.8 ± 16.0	0.691 ± 0.235	2.2 ± 0.5	6	7.69
F169521	7.21 ± 0.11	3	10.6 ± 3.2	0.471 ± 0.063	2.3 ± 0.4	4	7.35
F1699611	7.58 ± 0.05	3	21.3 ± 7.4	0.496 ± 0.091	2.2 ± 0.4	4	7.63
Mavoglurant	8.10 ± 0.06	3	1.1 ± 0.2	0.006 ± 0.002	478 ± 157	8	8.26
Remeglurant	7.74 ± 0.07	3	4.5 ± 1.0	0.072 ± 0.016	19.9 ± 5.3	7	7.81
(RS)-remeglurant	7.48 ± 0.07	3	2.4 ± 0.5	0.078 ± 0.019	23.1 ± 11.9	5	7.48
STX107	8.32 ± 0.08	3	37.9 ± 6.5	0.166 ± 0.011	6.2 ± 0.4	6	8.35

^aNegative logarithm of the equilibrium dissociation constant.

^bAssociation rate constant.

^cDissociation rate constant.

^dResidence time is defined by 1/k_{off}, where individual k_{off} values approached zero for basimglurant and mavoglurant; these were limited to 0.001.

^eNegative logarithm of the equilibrium dissociation constant determined from kinetic parameters (k_{off}/k_{on}).

^fpK_D derived from saturation binding paradigm.

orthosteric efficacy. Parameters τ_A and τ_B represent the intrinsic ability of the orthosteric and allosteric ligand, respectively, to activate the receptor; E_m is the maximal system response; and *n* represents the transducer slope. As validated previously (Gregory et al., 2012), we constrained the glutamate K_A based on a previously determined affinity estimate from radioligand binding studies (Mutel et al., 2000); we also made the assumption that none of the NAMs modulated glutamate affinity (α = 0) or had intrinsic efficacy (τ_B = 0).

Real-time receptor internalization data were analyzed as area under the curve (AUC), and the data were normalized to 0% being the AUC of 1% DMSO and to 100% being the AUC of maximum L-glutamate activation. Normalized data of L-glutamate was fitted to a four-parameter function (eq. 5), whereas NAM data were fitted to the operational model of allosterism (eq. 6).

Fluorescence traces obtained with Ca²⁺ mobilization experiments were quantified in relative fluorescence units (RFUs) and represented as ΔRFU = RFU_{max} - RFU_{min}, where RFU_{max} is the peak value of agonist stimulation and RFU_{min} is the mean of the basal fluorescence that is measured for 20 seconds before agonist addition.

Results

Affinity, Association Rate, and Dissociation Rate of Binding for mGlu₅ NAMs. To date, all small molecule mGlu₅ NAMs are thought to bind to a common pocket in the 7TM domain of the receptor, also known as the MPEP site (Harpsøe et al., 2015). Indeed, crystal structures of the 7TM domain of mGlu₅ with different NAM chemotypes bound support this concept (Dore et al., 2014; Christopher et al., 2019). However, mGlu₅ allosteric ligands, including NAMs, can possess complex binding isotherms, biased pharmacology, and differential effects in preclinical models (Trinh et al., 2018; Sengmany et al., 2019). Here we sought to undertake an in-depth assessment of the molecular pharmacological properties of diverse mGlu₅ NAM chemotypes, including ligands that have progressed into clinical trials (basimglurant, mavoglurant, dipraglurant). We first measured the affinity (pK_I) of each ligand based on inhibition of [³H]methoxy-PEPy binding to HEK293A-mGlu₅-low cell membranes that express similar levels of mGlu₅ as cortical astrocytes (Noetzel et al., 2012). All ligands fully displaced the radioligand, consistent with a competitive interaction with the MPEP binding site (Fig. 1). Of those tested, basimglurant had the highest affinity, and

F169521 had the lowest, with K_I estimates ranging from 0.5 to 62 nM (Table 1).

The binding kinetics of mGlu₅ NAMs have not been previously described; however, for other GPCRs, ligand binding kinetics has improved predictions of in vivo efficacy (Copeland, 2016; Guo et al., 2016). To determine the k_{on} and k_{off} of these chemically diverse NAMs, we first determined the k_{on} and k_{off} rates of the radioligand [³H]methoxy-PEPy (Supplemental Fig. 1; Table 1). Kinetics of mGlu₅ NAM binding was then measured using competition association experiments to generate time curves for [³H]methoxy-PEPy displacement (Fig. 2), which were globally analyzed to estimate k_{on} and k_{off} values for each ligand (Table 1). To confirm the robustness of our kinetic parameters, we compared the NAM affinities calculated from the kinetic parameters (pK_D) to the values obtained with inhibition binding experiments (pK_I) (Table 1). Here, we observed comparable affinity values across the two different experimental setups, with an R² = 0.99 (Supplemental Fig. 2A).

We observed a 34.5-fold difference in k_{on} between mavoglurant and STX107, which were the NAMs with the slowest and the fastest association rate, respectively. Comparison of k_{off} parameters revealed greater differences (138-fold) between the NAM with the slowest k_{off} (basimglurant) and the NAM with the fastest k_{off} (dipraglurant). These k_{off} rates were further converted into residence times, calculated as the reciprocal value of the dissociation rate (1/k_{off}) (Table 1). Based on these data, the NAMs could be grouped into three classes, where basimglurant and mavoglurant had slow k_{off}/long receptor residence times (>400 minutes); medium residence times (10–30 minutes) for AZD2066, remeglurant, and (RS)-remeglurant; and fast k_{off}/low residence times (<10 minutes) for dipraglurant, F169521, F1699611, and STX107. Furthermore, we investigated the linear relationship between k_{on} and k_{off} rates and the calculated NAM affinity pK_D. Here, we observed a moderate correlation (R² = 0.57) between affinity pK_D and dissociation rate k_{off} and no correlation between affinity pK_D and association rate k_{on} (Supplemental Fig. 2, B and C).

NAMs Inhibit Glutamate-Induced Ca²⁺ Mobilization, IP₁ Accumulation, and ERK1/2 Phosphorylation. We next measured the inhibitory effect of these NAM on mGlu₅

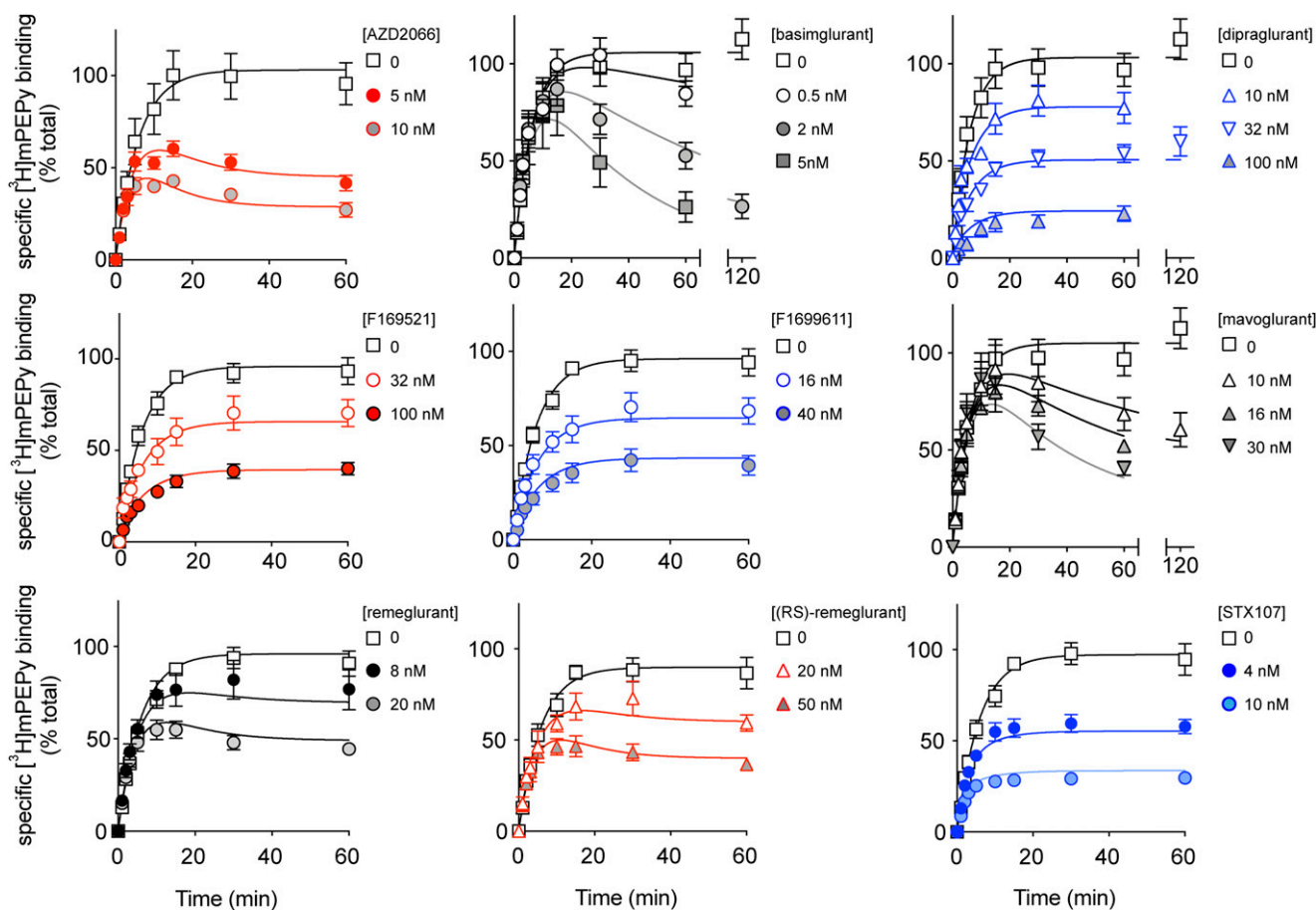


Fig. 2. Competition association binding of mGlu₅ NAMs at HEK293A-mGlu₅-low membranes. [³H]methoxy-PEPy specific binding measured at different time points in the absence or presence of indicated concentrations of different NAMs added simultaneously. Data are means ± S.E.M. from three to eight independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol. Curves shown are the best fit of the entire data set with eq. 4.

activation of Ca²⁺ mobilization, IP₁ accumulation, and ERK1/2 phosphorylation in response to an EC₈₀ concentration of L-glutamate in HEK293A-mGlu₅-low cells (Fig. 3). To diminish the effect of ambient/released glutamate, HEK293A cells were incubated with GPT before each assay (Sengmany et al., 2017). L-glutamate has greater potency in Ca²⁺ mobilization compared with IP₁ accumulation and ERK1/2 phosphorylation assays (Supplemental Table 1); therefore different L-glutamate concentrations were employed to achieve ~EC₈₀ responses: 320 nM for Ca²⁺ mobilization and 3.2 μM for IP₁ accumulation and ERK1/2 phosphorylation. As expected, all compounds inhibited L-glutamate-induced responses in all three functional assays. With the exception of (RS)-remerglurant in the ERK1/2 phosphorylation assay, all NAMs completely inhibited L-glutamate responses in all three measures of mGlu₅ activity (Fig. 3). NAMs were consistently more potent at inhibiting L-glutamate stimulated IP₁ accumulation and ERK1/2 phosphorylation than Ca²⁺ mobilization (Supplemental Table 2), with the exception of basimglurant, which had similar pIC₅₀ values (within 2-fold) across all three measures.

NAMs Inhibit Glutamate-Induced mGlu₅ Internalization. Beyond acute activation of intracellular signal transduction pathways, allosteric ligands may also differentially influence receptor regulatory processes (Hellyer et al., 2019). Therefore, we next sought to assess the effect of mGlu₅ NAMs

on L-glutamate-induced receptor internalization. HEK293A cells were transiently transfected with SNAP-tagged mGlu₅, as well as the EAAT3 glutamate transporter to reduce ambient glutamate. Transient mGlu₅ expression resulted in ~10 times higher mGlu₅ levels compared with the stable cell line (4.6 pmol/mg vs. 0.3 pmol/mg, respectively; Supplemental Fig. 3). Introduction of a N-terminal SNAP tag did not affect orthosteric agonist potency for IP₁ accumulation (data not shown). During the assay, glutamate transport was blocked with the nontransportable EAAT3 inhibitor DL-TBOA. This internalization assay relies on a time-resolved FRET technique that enables real-time measurement of receptor internalization with the help of a FRET donor Lumi4-Tb attached to the SNAP tag located at the N terminus of the receptor and a cell impermeant acceptor fluorescein-O'-acetic acid (Roed et al., 2014). There was appreciable (39% ± 4% of the AUC for 100 μM L-glutamate) increase in internalization when blocking glutamate transport with DL-TBOA without adding additional agonist (Fig. 4A). Upon stimulation with L-glutamate, we observed an increased response over unstimulated levels, indicative of agonist-induced mGlu₅ internalization. Maximum mGlu₅ internalization was reached around 60 minutes after stimulation with L-glutamate concentrations above 10 μM, in accordance with a previous study (Levoye et al., 2015). L-glutamate induced mGlu₅ receptor internalization with a similar potency (Fig. 4B, pEC₅₀: 5.47 ± 0.11;

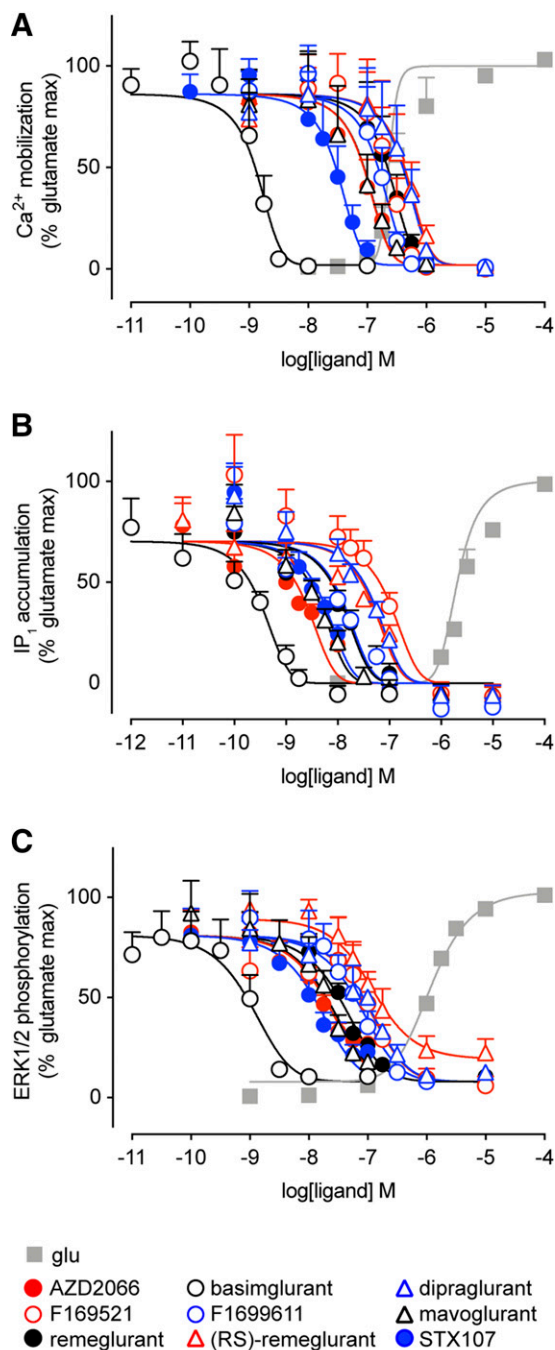


Fig. 3. Inhibition of L-glutamate-induced signaling in HEK293A-mGlu₅-low cells. Effect of NAMs on Ca²⁺ mobilization response to 320 nM L-glutamate (A), IP₁ accumulation in response to 3.2 μM L-glutamate (B), and ERK1/2 phosphorylation measured after 5-minute incubation with 3.2 μM L-glutamate (C). Data are means + S.E.M. from three to five independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol. Data were normalized to 0% as the mean of the vehicle response and 100% as the mean of maximal L-glutamate response.

Supplemental Table 1) to that measured for IP₁ accumulation in HEK293A-mGlu₅-low cells (Supplemental Table 1). To account for ambient extracellular glutamate levels induced by inclusion of DL-TBOA in the assay, we refitted the glutamate concentration-response curve such that the bottom plateau was equal to that observed in the absence of DL-

TBOA, removing responses for glutamate concentrations below the inflection point (dashed line in Fig. 4B). In doing so, we estimate that in the presence of DL-TBOA the ambient L-glutamate concentration is ~0.9 μM.

Next, we investigated the effect of 30-minute preincubation with mGlu₅ NAMs on receptor internalization induced by a submaximal concentration of L-glutamate (11.6 μM). High concentrations of AZD2066, remeglurant, (RS)-remeglurant, and STX107 interfered with the assay detection in a non-specific manner (Supplemental Fig. 4), limiting the concentration ranges tested. From the real-time internalization traces (Supplemental Fig. 5), we calculated the AUC for each NAM (Fig. 4B). All NAMs reduced the L-glutamate-induced response to below the unstimulated condition (in the presence of DL-TBOA). AUC values were normalized to the maximum induced by L-glutamate (Fig. 4, C and D), with the baseline defined by the response in the absence of DL-TBOA. In contrast to results from acute signaling assays, none of the NAMs completely inhibited L-glutamate-induced mGlu₅ internalization. NAM pIC₅₀ values (Supplemental Table 2) for internalization were similar (within 3-fold) to those derived from Ca²⁺ mobilization assays, with the exception of basimglurant (10-fold lower) and dipraglurant (5-fold higher).

Quantification and Comparison of mGlu₅ NAM Affinity and Cooperativity Estimates across Different Measures. To quantify the affinity of NAMs (pK_B) as well as apparent cooperativity with L-glutamate across the four functional measures of mGlu₅ activity, we fitted the NAM titration curves in parallel with a control L-glutamate concentration-response curve to the operational model of allosterism (Gregory et al., 2012). To best fit the internalization data, the extrapolated curve with the true basal of the system was used, and the L-glutamate concentrations were corrected by subtracting the estimated level of ambient glutamate present. We first compared pK_B values (Table 2) with pK_I estimates obtained from equilibrium radioligand inhibition binding experiments (Fig. 5A). In all cases pK_B values were lower than pK_I estimates. However, we observed a high correlation between NAM pK_B values in the Ca²⁺ mobilization ($R^2 = 0.82$), IP₁ accumulation ($R^2 = 0.95$), and ERK1/2 phosphorylation ($R^2 = 0.87$) assays to pK_I values. On the other hand, we observed a weaker correlation of NAM pK_B values in the real-time receptor internalization assay ($R^2 = 0.63$) to pK_I values. Indeed, linear regression of these data revealed that the slope for the internalization assay data was significantly different from 1 (Fig. 5A). To appreciate how each individual NAM compared across the four functional measures, we plotted the pK_B estimates to visualize an affinity bias fingerprint (Fig. 5B).

A common fingerprint was evident across different scaffolds [AZD2066, dipraglurant, F1699611, mavoglurant, remeglurant, (RS)-remeglurant], which was that pK_B estimates from Ca²⁺ mobilization assays were significantly lower (ranging from 4- to 21-fold) than those derived from IP₁ accumulation and ERK1/2 phosphorylation studies. The exceptions to this fingerprint were basimglurant, STX107, and F169521, where pK_B estimates were all within 4-fold of one another (Table 2). For the majority of NAMs, pK_B from Ca²⁺ mobilization was similar to that derived from internalization assays; the exceptions were remeglurant and dipraglurant, where pK_B values were higher for internalization than for Ca²⁺ (5- and 22-fold, respectively). Beyond apparent affinity, there was high

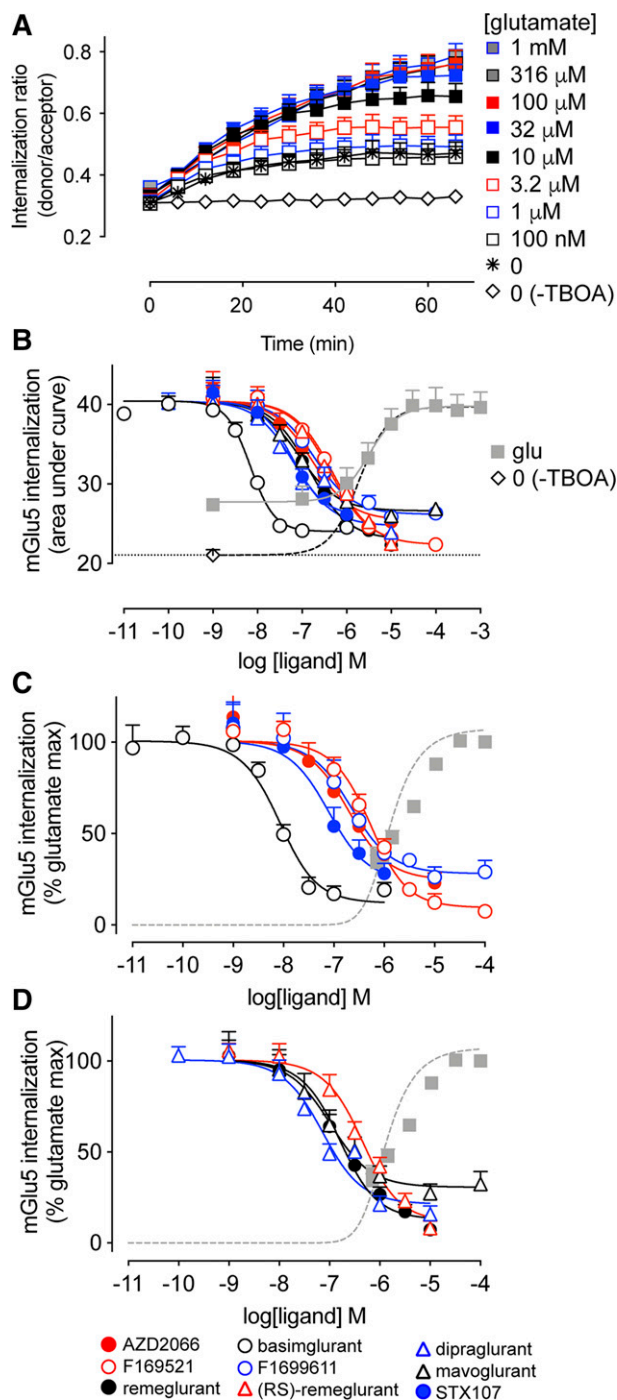


Fig. 4. L-glutamate-induced mGlu₅ internalization and modulation by NAMs. (A) L-glutamate (added at $t = 0$) concentration-dependently increases mGlu₅ internalization over time (based on FRET between fluorescein-O'-acetic acid and Lumi4-Tb-labeled SNAP-tagged mGlu₅), achieving a maximal level within 60 minutes. DL-TBOA was included in these experiments to prevent glutamate transport and isolate changes in surface mGlu₅ due to extracellular stimulation; however, this also results in apparent mGlu₅ internalization over time in the absence of exogenously applied L-glutamate. In the absence of DL-TBOA, mGlu₅ surface levels remain constant. (B) The area under the curve of real-time internalization ratios (ratio of donor and acceptor emissions) over 66 minutes were calculated to plot concentration-response curves. Cells were incubated with NAMs for 30 minutes prior to addition of a submaximal concentration (11.6 μ M) of L-glutamate. The dashed line is the nonlinear fit of the L-glutamate response when the bottom plateau is constrained to equal the basal condition (no DL-TBOA, plotted here at -9) and glutamate concentrations below the EC₅₀ excluded from the fit. (C and D) Data in panel B

negative cooperativity for the vast majority of compounds (Table 3). However, there were two notable exceptions. (RS)-remeglurant showed limited inhibition and therefore weaker negative cooperativity with L-glutamate for ERK1/2 phosphorylation relative to all other NAMs. In the internalization assay, the cooperativity with L-glutamate was limited for all NAMs. For (RS)-remeglurant, the limited cooperativity with L-glutamate between ERK1/2 phosphorylation and internalization was not significantly different (Student's unpaired t test).

Discussion

Several mGlu₅ NAMs have been tested in preclinical studies and clinical trials for different indications, but none has been approved for clinical use due to lack of efficacy or adverse effects. Central nervous system adverse effects, e.g., dizziness and attention deficits (Rohof et al., 2012) are associated with mGlu₅ NAMs as well as psychoactive potential for AZD2066, which has been described as a class effect (Swedberg and Raboisson, 2014). For example, mavoglurant has entered clinical trials several times but failed to show efficacy in the treatment of fragile X syndrome (ClinicalTrials.gov NCT01357239; NCT01253629) and L-DOPA-induced dyskinesia (Trenkwalder et al., 2016). Basimglurant, a very potent mGlu₅ NAM with long half-life in rats, has reached phase II clinical trials for the treatment of fragile X syndrome and as an adjunctive therapy in major depressive disorder (Jaeschke et al., 2015; Lindemann et al., 2015). Nevertheless, basimglurant did not show significant improved efficacy in patients with major depressive disorder and fragile X syndrome when compared with placebo (Quiroz et al., 2016; Youssef et al., 2018). Interestingly, dipraglurant showed efficacy in phase II clinical trials for the treatment of L-DOPA-induced dyskinesia in patients with Parkinson's disease, granting it a status of an orphan drug by the US Food and Drug Administration and progression to phase III clinical trials (Emmitte, 2017). Despite these multiple clinical studies targeting mGlu₅, there is currently a lack of comparative molecular pharmacological data on these NAM compounds, which could potentially explain some of the differences observed in clinical studies and inspire future drug design. Accordingly, we have performed a comprehensive pharmacological characterization of nine preclinically/clinically tested NAMs: AZD2066, basimglurant, dipraglurant, F169521, F1699611, mavoglurant, remeglurant, (RS)-remeglurant, and STX107 (Fig. 1).

Ligand binding kinetics are becoming increasingly recognized as a critical factor in drug development (Copeland, 2016; Guo et al., 2016). Here we showed that all of the mGlu₅ NAMs fully displaced the radioligand [³H]methoxy-PEPy (Fig. 2), with affinity estimates comparable to previous studies where available (Kågedalet et al., 2013; Dore et al., 2014; Lindemann et al., 2015; Westmark et al., 2018). For the first time, we

were normalized to the L-glutamate maximum response (100%) with the basal system response (no DL-TBOA) set to zero and fitted with an operational model of allosterism. In all panels, data are means + S.E.M. from three independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol.

TABLE 2

Affinity estimates (pK_B) for mGlu₅ NAMs from functional assaysCollated data were fitted to the operational model of allosterism. Data represented as means ± S.E.M. from the indicated number (*n*) of independent experiments performed in triplicate.

	Ca ²⁺ mobilization		IP ₁ accumulation		ERK1/2 phosphorylation		Receptor internalization	
	pK _B	<i>n</i>	pK _B	<i>n</i>	pK _B	<i>n</i>	pK _B	<i>n</i>
AZD2066	6.49 ± 0.15	3	7.82 ± 0.12	4	7.34 ± 0.14	3	6.75 ± 0.14	3
Basimglurant	8.33 ± 0.15	3	8.69 ± 0.13	4	8.66 ± 0.15	4	8.06 ± 0.12 ^a	3
Dipraglurant	5.83 ± 0.15	3	6.50 ± 0.12 ^b	4	6.67 ± 0.14 ^b	3	7.17 ± 0.13 ^{a,b}	3
F169521	6.09 ± 0.15	3	6.19 ± 0.15	4	6.69 ± 0.14	3	6.24 ± 0.12	3
F1699611	6.25 ± 0.15	3	7.08 ± 0.12 ^b	4	6.82 ± 0.14	3	6.71 ± 0.13	3
Mavoglurant	6.49 ± 0.15	3	7.55 ± 0.11 ^b	4	7.30 ± 0.14 ^b	3	7.04 ± 0.13	3
Remeglurant	6.09 ± 0.15	3	7.12 ± 0.12 ^b	4	7.09 ± 0.14 ^b	3	6.79 ± 0.13 ^b	3
(RS)-remeglurant	5.80 ± 0.15	3	6.54 ± 0.13 ^b	4	7.04 ± 0.13 ^b	3	6.33 ± 0.13 ^c	3
STX107	6.97 ± 0.15	3	7.46 ± 0.11	4	7.52 ± 0.14	3	7.14 ± 0.19	3

^aSignificantly different from estimate derived from IP₁ accumulation assays, *P* < 0.05, one-way ANOVA with Tukey's multiple comparisons post-test.^bSignificantly different from estimate derived from Ca²⁺ mobilization assays, *P* < 0.05, one-way ANOVA with Tukey's multiple comparisons post-test.^cSignificantly different from estimate derived from ERK1/2 phosphorylation assays, *P* < 0.05, one-way ANOVA with Tukey's multiple comparisons post-test.

determined the kinetic rates of association and dissociation for the NAMs. The observed k_{off} values were spread over two orders of magnitude, whereas k_{on} values ranged within one and a half orders of magnitude. There was a correlation between the affinity and the dissociation rate, but not the association rate (Supplemental Fig. 2), suggesting that affinity is k_{off} - rather than k_{on} -driven for the NAMs used in this study. We identified basimglurant and mavoglurant as NAMs with residence times longer than 7 hours, which is more than 200-fold higher than the NAMs with the lowest residence time, dipraglurant and F1699611. These data are intriguing, especially as there are few reports regarding pharmacokinetics of mGlu₅ NAMs. The notable exception is that studies in rodents and clinical data in healthy subjects show that basimglurant has a much longer half-life relative to mavoglurant (Levenga et al., 2011; Gantois et al., 2013; Walles et al., 2013; Fowler et al., 2017). In our data, the association rate of basimglurant is 10-fold higher than mavoglurant; therefore k_{on} rather than k_{off} may be one contributing factor to the longer half-life of basimglurant. In the clinic, there are few examples where different mGlu₅ NAMs have been assessed for the same indication. In patients with fragile X syndrome, neither basimglurant nor mavoglurant showed efficacy in reversing behavioral deficits (Berry-Kravis et al., 2016; Youssef et al., 2018). For L-dopa-induced dyskinesia in patients with Parkinson's disease, although mavoglurant was no better than placebo and associated with more adverse events (Trenkwalder et al., 2016), dipraglurant, which has a much lower residence time, was well tolerated and improved dyskinesia (Tison et al., 2016). Further studies exploring the pharmacokinetics/pharmacodynamics relationships of mGlu₅ NAMs are required to establish if ligand kinetics contributes to preclinical/clinical efficacy.

We used the operational model of allosterism to determine functional pK_B affinity estimates for the NAMs. Generally, the rank-order of NAM affinities were similar across all measures. Basimglurant had the highest affinity in radioligand displacement and all four functional assays, consistent with previously reported pharmacology (Lindemann et al., 2015). We speculate that basimglurant recognizes a larger complement and/or

more stable inactive receptor conformations than the other NAMs, which gives rise to its higher affinity. Dipraglurant, F16952, F1699611, and (RS)-remeglurant grouped as the NAMs with the lowest affinity in these five assays with the exception of the internalization assay. In the internalization assay, dipraglurant showed relatively high affinity, ranking second only to basimglurant. NAMs generally displayed higher affinity in the IP₁ and ERK1/2 assays compared with Ca²⁺ mobilization and internalization assays (Fig. 5A). Accordingly, there was a strong correlation between affinities obtained by radioligand displacement and in the functional assays, although it was weakest for receptor internalization ($R^2 = 0.631$ for internalization vs. $R^2 \geq 0.816$ for the other assays; Fig. 5A). These data highlight dipraglurant as an NAM with affinity bias toward the internalization pathway and are consistent with our previous report, where the apparent affinity of dipraglurant was dependent on the mGlu₅ signaling response and cell type measured (HEK293A-mGlu₅-low vs. mouse cortical neurons) (Sengmany et al., 2019). Dipraglurant also had the shortest receptor residence time (Table 1), which could potentially be a cause of the observed bias as binding kinetics have been previously shown to influence apparent bias of the dopamine D₂ receptor (Klein Herenbrink et al., 2016). However, as F169521 and F1699611 displayed very similar receptor residence times without showing bias toward the internalization pathway, this explanation appears less likely. The bias could instead be caused by other mechanisms such as receptor conformational-driven bias, but more studies are needed to elucidate the mechanism.

All NAMs fully inhibited L-glutamate activation of the mGlu₅ receptor in the Ca²⁺ mobilization and IP₁ accumulation functional assays (Fig. 3, A and B). All NAMs, except (RS)-remeglurant, also fully inhibited ERK1/2 phosphorylation, whereas none of the NAMs fully inhibited receptor internalization (Fig. 3C; Fig. 4). Accordingly, the log β cooperativity estimates derived from the operational model of allosterism showed strong negative cooperativity for NAMs, with full inhibition in the functional assays but weaker log β values for (RS)-remeglurant in the ERK1/2 phosphorylation and for all

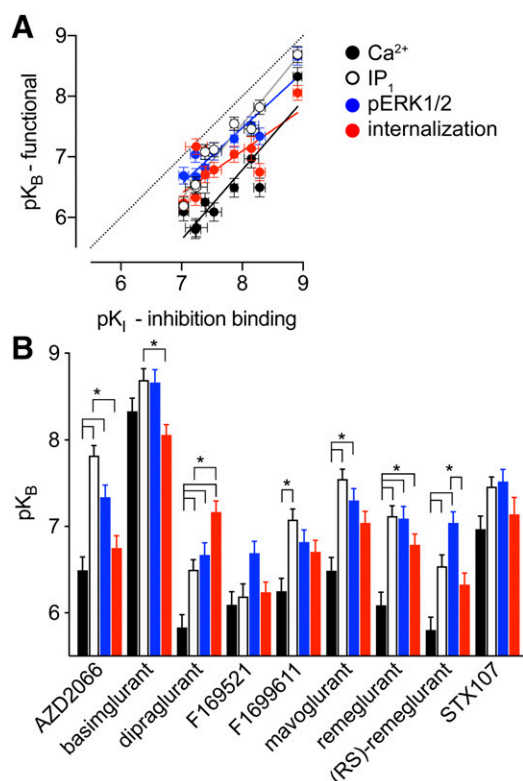


Fig. 5. Comparison of mGlu₅ NAM affinity estimates between binding and functional measures. (A) Correlation plot for functional p_{K_B} estimates relative to inhibition binding derived p_{K_I} values for nine mGlu₅ NAMs. In all cases, p_{K_B} estimates were lower than p_{K_I} values; however, there were significant correlations between binding p_{K_I} and p_{K_B} values from Ca²⁺ ($R^2 = 0.816$; $P = 0.0008$), IP₁ ($R^2 = 0.946$; $P < 0.0001$), ERK1/2 phosphorylation ($R^2 = 0.866$, $P = 0.0003$), and internalization ($R^2 = 0.631$, $P = 0.0106$). The 95% confidence intervals of the slopes of the linear regressions for Ca²⁺ (black line), IP₁ (gray line), and ERK1/2 phosphorylation (blue line) all included 1, but internalization (red line) was significantly different (slope = 0.70, 95% confidence interval = 0.44–0.96). Unity is represented by the dotted line. (B) Affinity estimates for each ligand across the four measures of mGlu₅ function. * $P < 0.05$, one-way ANOVA Tukey's multiple comparisons post-test.

NAMs in the internalization assay (Table 3). Given that all NAMs showed a similar partial inhibitory profile in the internalization assay, where transient expression yielded 10 times higher mGlu₅ levels, and that these experiments were performed in the presence of the glutamate transporter inhibitor DL-TBOA to prevent cellular uptake of exogenous L-glutamate during the experiment, we cannot rule out that

TABLE 3

Cooperativity estimates ($\log\beta$) for mGlu₅ NAMs from functional assays

Collated data were fitted to the operational model of allostery. Data represented as means \pm S.E.M. from the indicated number (n) of independent experiments performed in triplicate.

	Ca ²⁺ mobilization		IP ₁ accumulation		ERK1/2 phosphorylation		Receptor internalization	
	$\log\beta$	n	$\log\beta$	n	$\log\beta$	n	$\log\beta$	n
AZD2066	full NAM	3	full NAM	4	full NAM	3	-0.30 ± 0.04	3
Basimglurant	full NAM	3	full NAM	4	full NAM	4	-0.41 ± 0.06	3
Dipraglurant	full NAM	3	full NAM	4	full NAM	3	-0.32 ± 0.04	3
F169521	full NAM	3	full NAM	4	full NAM	3	-0.44 ± 0.07	3
F1699611	full NAM	3	full NAM	4	full NAM	3	-0.28 ± 0.03	3
Mavoglurant	full NAM	3	full NAM	4	full NAM	3	-0.26 ± 0.03	3
Remeglurant	full NAM	3	full NAM	4	full NAM	3	-0.39 ± 0.05	3
(RS)-remeglurant	full NAM	3	full NAM	4	-0.24 ± 0.04	3	-0.41 ± 0.07	3
STX107	full NAM	3	full NAM	4	full NAM	3	-0.30 ± 0.05	3

the partial inhibition is (in part) caused by this experimental condition. However, (RS)-remeglurant has a unique profile in the ERK1/2 phosphorylation assay, where it had weaker negative cooperativity than the other NAMs, indicating bias away from this pathway. It is very interesting to note the (R)-enantiomer remeglurant did not show this bias profile, suggesting that the bias is driven by the presumably less potent (S)-enantiomer. Unfortunately, (S)-remeglurant was unavailable and thus was not tested directly in the present study.

The different magnitudes of negative cooperativity by structurally diverse NAMs suggests that the different chemotypes stabilize distinct complements of receptor conformations, rather than a single inactive receptor state. Stabilization of different inactive conformations by different NAMs is consistent with limited structure-function analyses of the common class C GPCR allosteric binding pocket, where single point mutations can engender a “switch” in modulator cooperativity from positive to negative/neutral or vice versa in a chemotype-dependent manner (Petrel et al., 2004; Hu et al., 2006; Fukuda et al., 2009; Gregory et al., 2013, 2014).

Overall, this study has significantly increased our knowledge of the molecular pharmacological profiles of nine clinically and preclinically tested mGlu₅ NAMs. Unfortunately, the NAMs have not been tested in comparative (pre-)clinical studies, so it is not possible to use our data to rationalize the lack of animal to human translation. Our results show that the affinities and residence times span two orders of magnitude. We also show that kinetic binding parameters k_{on} and k_{off} are not well correlated with binding affinities. Binding kinetics are becoming increasingly recognized as important parameters in drug development programs as, e.g., the ligand-receptor residence time can have profound effect on the pharmacodynamic effect in vivo (Copeland, 2016; Guo et al., 2016). The large differences in binding kinetics of clinically relevant NAMs tested in this study underscore the importance of taking this into consideration in future drug development programs. Finally, we show that dipraglurant and (RS)-remeglurant are biased toward the receptor internalization (i.e., relatively high affinity) and away from the ERK1/2 phosphorylation pathway (i.e., relatively low negative cooperativity), which emphasizes the importance of using a range of pathway assays when profiling clinical candidates to assess their potential signaling bias.

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Authorship Contributions

Participated in research design: Arsova, Vedel, Hansen, Foster, Gregory, Bräuner-Osborne.

Conducted experiments: Arsova, Møller.

Contributed new reagents or analytical tools: Hansen.

Performed data analysis: Arsova, Møller, Gregory.

Wrote or contributed to drafting the manuscript: Arsova, Møller, Gregory, Bräuner-Osborne.

Editing and approval of final manuscript: All authors.

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Supplementary Material

Detailed *in vitro* pharmacological characterization of clinically tested negative allosteric modulators of the metabotropic glutamate receptor 5 (mGlu₅)

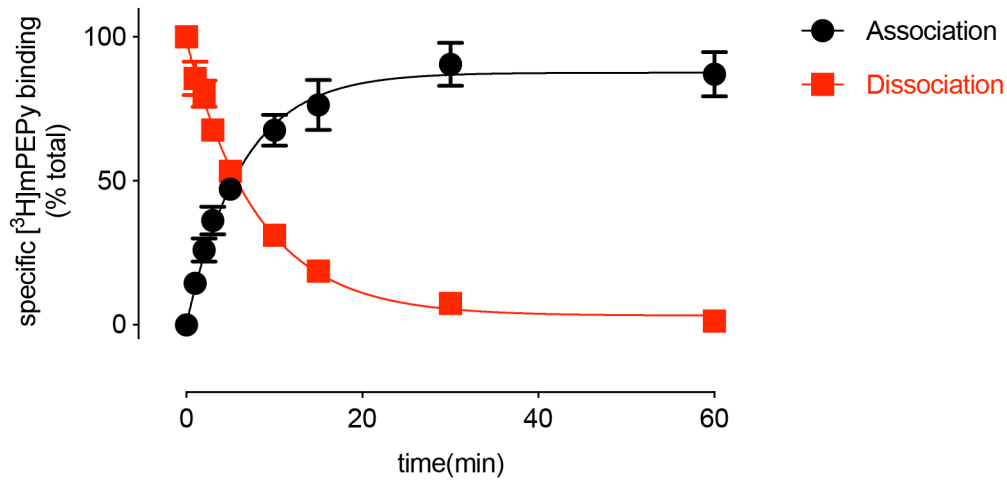
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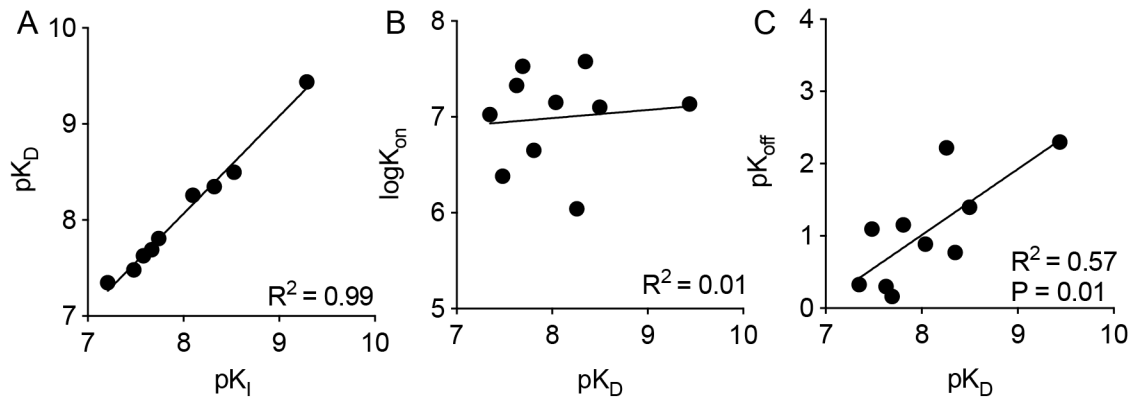
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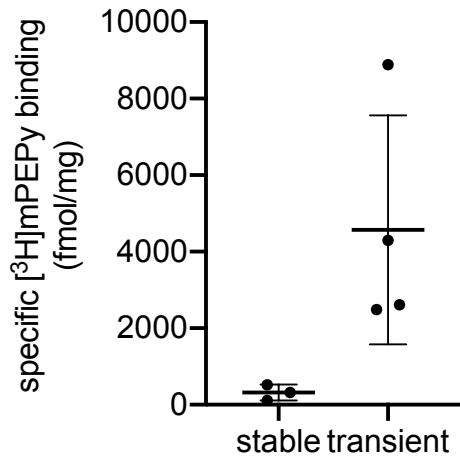
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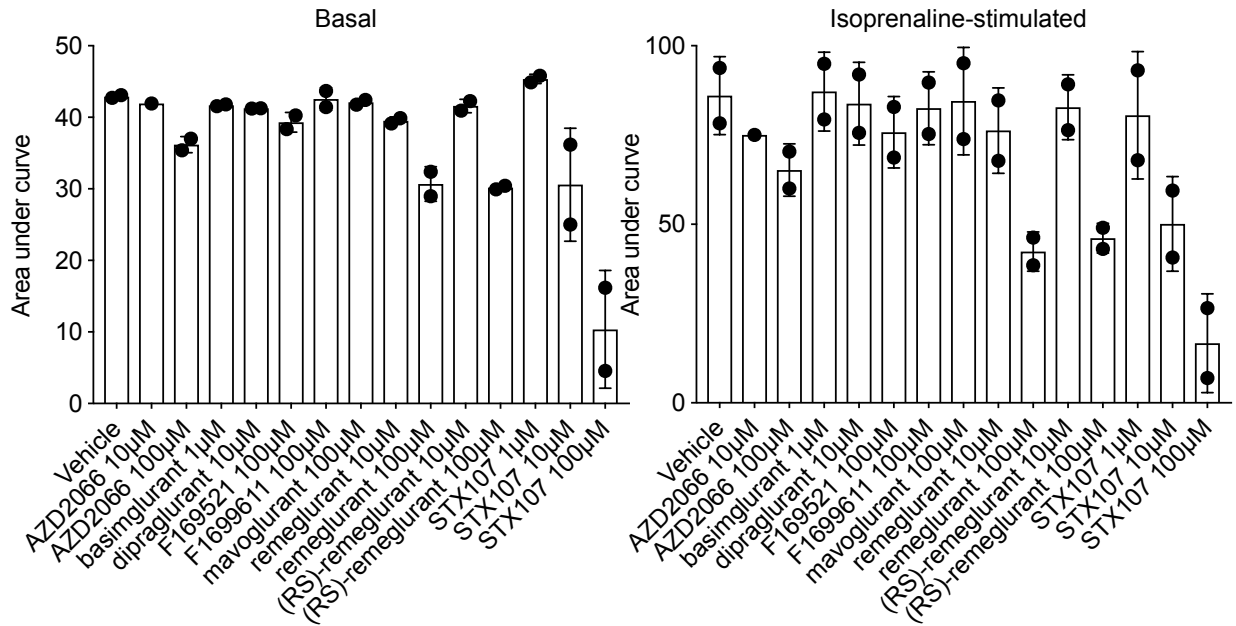
Supplementary Figure 1. Association and dissociation kinetics of [³H]methoxy-PEPy binding in HEK293A-mGlu₅-low cell membranes. The dissociation rate was determined by simultaneous addition of a saturating concentration of unlabeled competitive ligand (1 μ M MPEP). Data are represented as mean \pm SEM of 7 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.



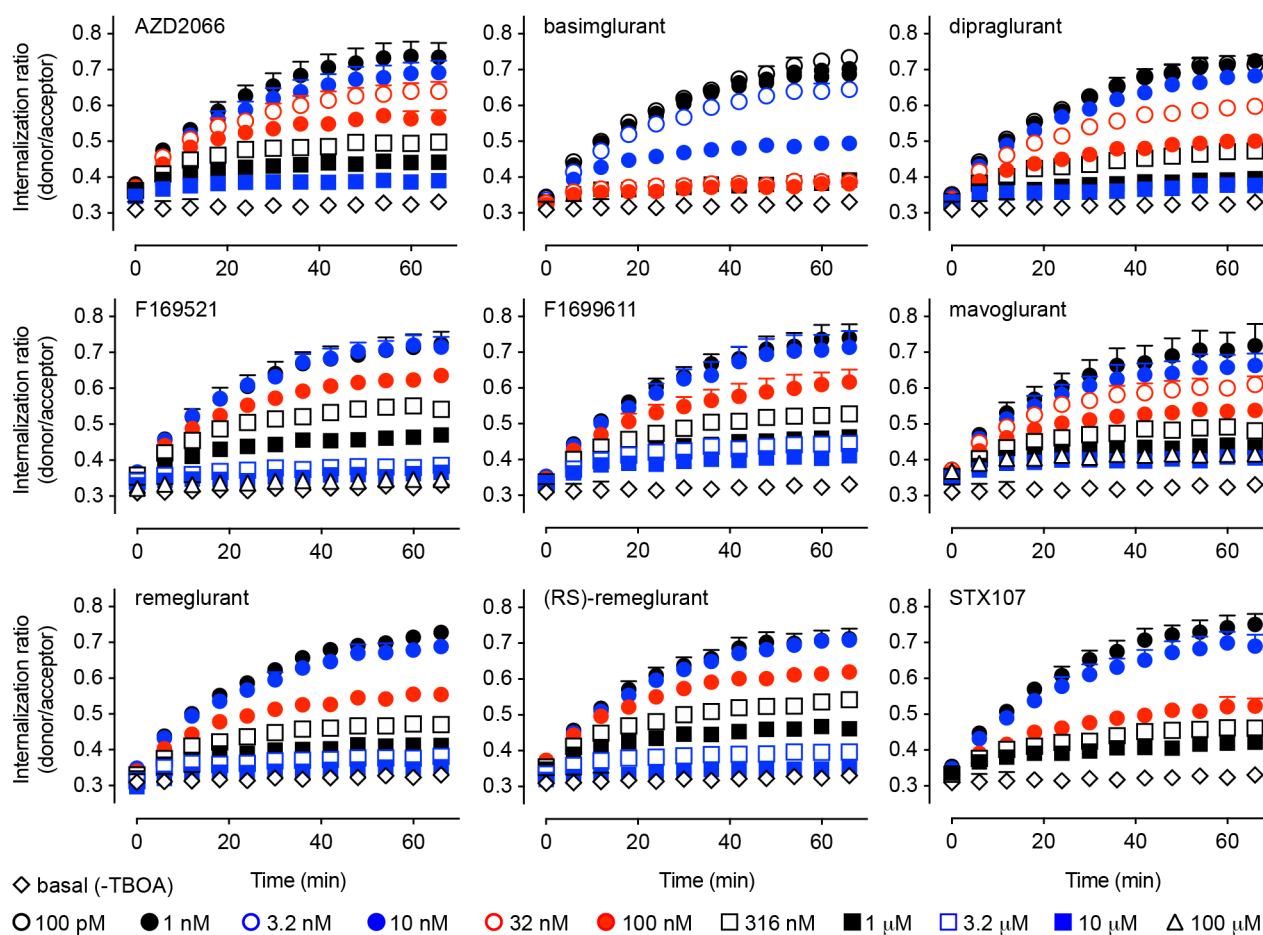
Supplementary Figure 2. Correlation plots of affinity, association rate, and dissociation rate of mGlu₅ NAMs. **A)** Affinities calculated from association (k_{on}) and dissociation (k_{off}) rates ($K_D = k_{off}/k_{on}$), where pK_D is the negative logarithm of K_D , versus pK_I estimates from inhibition binding experiments. **B)** Association rates do not correlate with pK_D . **C)** Dissociation rates correlate with pK_D .



Supplementary Figure 3. B_{\max} estimates for [^3H]mPEPy binding to mGlu $_5$ in membranes from stable and transient transfected HEK293A cells. To estimate total receptor levels (B_{\max}), two-point saturation binding experiments were performed using 2.6 ± 1.1 nM and 16.6 ± 3.6 nM [^3H]mPEPy and 10 μM MPEP to define non-specific binding. A saturation binding isotherm was fitted to the data to estimate B_{\max} , with the assumption that the N-terminal SNAP-tag had no effect on the affinity of the allosteric radioligand ([^3H]mPEPy, reported in Table 1), which binds to the 7TM domain. Independent replicates for $n=3-4$ are shown, data are mean \pm SD.



Supplementary Figure 4. High concentrations of select mGlu₅ NAMs interfere with FRET-based assay detection. The basal and agonist (isoprenaline)-stimulated internalization of SNAP-tagged β_2 -adrenoceptor measured in presence of high concentrations of mGlu₅ NAMs or vehicle (1% DMSO). Area under the curve was calculated for real time internalization ratios (ratio of donor and acceptor emissions) measured for 66 min in 6 min intervals. Data points represent the mean of individual experiments and bars represent mean \pm SEM from two independent experiments performed in triplicate.



Supplementary Figure 5. Real-time mGlu₅ internalization in response to 11.6 μ M of L-glutamate is inhibited by mGlu₅ NAMs. Indicated NAMs were pre-incubated for 30 min prior to addition of L-glutamate (11.6 μ M), fluorescein and DL-TBOA (to inhibit transport of glutamate). The ratio of donor (Lumi4-Tb-labeled SNAP-tagged mGlu₅) and acceptor (fluorescein) emissions (internalization ratio) increases over time when the receptor is internalized. Data are mean + SEM from 3 independent experiments performed in triplicate.

Supplementary Table 1: L-glutamate potency for mGlu₅ in four functional assays. Data are represented as mean \pm SEM from the indicated number (n) of independent experiments performed in triplicate.

	pEC ₅₀	n
Ca ²⁺ mobilization	6.53 \pm 0.13	4
IP ₁ accumulation	5.40 \pm 0.05	5
ERK1/2 phosphorylation	6.00 \pm 0.05	4
Receptor internalization	5.47 \pm 0.08	3
	5.80 \pm 0.19 [#]	

[#] For receptor internalization data the concentration-response curve was re-fitted such that the bottom plateau was equal to the true basal level of the system, i.e. in the absence of DL-TBOA. In order to do so, the data points below the inflection point of the original curve were removed (100 nM and 1 μ M).

Supplementary Table 2: Potency (pIC₅₀) values from functional assays for mGlu₅ NAMs, based on titrations in the presence of sub-maximal L-glutamate. Data was fitted to a four-parameter model of inhibition. Values are mean ± SEM from the indicated number (n) of independent experiments performed in triplicate.

	Ca²⁺ mobilization		IP₁ accumulation		ERK1/2 phosphorylation		Receptor internalization	
	pIC₅₀	n	pIC₅₀	n	pIC₅₀	n	pIC₅₀	n
AZD2066	7.29 ± 0.33	4	8.52 ± 0.04	4	7.88 ± 0.09	3	6.85 ± 0.03	3
basimglurant	9.13 ± 0.16	4	9.42 ± 0.07	4	8.99 ± 0.06	4	8.12 ± 0.04	3
dipraglurant	6.44 ± 0.20	4	7.37 ± 0.04	4	6.93 ± 0.12	3	7.14 ± 0.02	3
F169521	6.69 ± 0.12	4	7.07 ± 0.06	4	6.83 ± 0.03	3	6.34 ± 0.06	3
F1699611	7.16 ± 0.31	4	7.71 ± 0.10	4	7.21 ± 0.11	3	6.77 ± 0.10	3
mavoglurant	7.22 ± 0.27	4	8.43 ± 0.07	4	7.62 ± 0.05	3	7.09 ± 0.12	3
remeglurant	6.96 ± 0.29	4	7.92 ± 0.13	4	7.38 ± 0.14	3	6.78 ± 0.05	3
(RS)-remeglurant	6.63 ± 0.26	4	7.20 ± 0.04	4	7.00 ± 0.10	3	6.22 ± 0.05	3
STX107	7.81 ± 0.25	4	8.44 ± 0.08	4	8.00 ± 0.04	3	7.29 ± 0.09	3