

RESEARCH PAPER

Molecular determinants of positive allosteric modulation of the human metabotropic glutamate receptor 2

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BACKGROUND AND PURPOSE

The activation of the metabotropic glutamate receptor 2 (mGlu₂) reduces glutamatergic transmission in brain regions where excess excitatory signalling is implicated in disorders such as anxiety and schizophrenia. Positive allosteric modulators (PAMs) can provide a fine-tuned potentiation of these receptors' function and are being investigated as a novel therapeutic approach. An extensive set of mutant human mGlu₂ receptors were used to investigate the molecular determinants that are important for positive allosteric modulation at this receptor.

EXPERIMENTAL APPROACH

Site-directed mutagenesis, binding and functional assays were employed to identify amino acids important for the activity of nine PAMs. The data from the radioligand binding and mutagenesis studies were used with computational docking to predict a binding mode at an mGlu₂ receptor model based on the recent structure of the mGlu₁ receptor.

KEY RESULTS

New amino acids in TM3 (R635, L639, F643), TM5 (L732) and TM6 (W773, F776) were identified for the first time as playing an important role in the activity of mGlu₂ PAMs.

CONCLUSIONS AND IMPLICATIONS

This extensive study furthers our understanding of positive allosteric modulation of the mGlu₂ receptor and can contribute to improved future design of mGlu₂ PAMs.

Abbreviations

7-TM, seven-transmembrane; BINA, 4-[3-[(2-cyclopentyl-6,7-dimethyl-1-oxo-2,3-dihydroinden-5-yl)oxymethyl]phenyl]benzoic acid; JNJ-35814376, 1-(4-chloro-2-fluorobenzyl)-5-(4-methoxyphenyl)pyridin-2(1H)-one; JNJ-40068782, 3-cyano-1-cyclopropylmethyl-4-(4-phenyl-piperidin-1-yl)pyridine-2(1H)-one; JNJ-40264796, 1-butyl-4-[4-(2-methylpyridin-4-yloxy)phenyl]-2-oxo-1,2-dihydropyridine-3-carbonitrile; JNJ-40297036, 2-propyl-8-chloro-6-[4-[(6-trifluoromethyl-3-pyridinyl)methoxy]phenyl]-1(2H)-isoquinolinone; JNJ-41482012, 7-(4-benzyloxy)-phenyl-3-(2,2,2-trifluoro-ethyl)-imidazo[1,2-a]pyridine-8-carbonitrile; JNJ-42329001, 7-[3-fluoro-4-(2'-methyl-pyridin-4-yloxy)-phenyl]-8-trifluoromethyl-3-cyclopropylmethyl-1,2,4-triazolo[4,3-a]pyridine; JNJ-46281222, 3-(cyclopropylmethyl)-7-[(4-phenyl-1-piperidinyl)methyl]-8-(trifluoromethyl)-1,2,4-triazolo[4,3-a]pyridine; LY2607540 (THIC), N-({4-[3-hydroxy-4-(2-methylpropanoyl)-2-(trifluoromethyl)phenoxy]methyl}phenyl)methyl)-1-methyl-1H-imidazole-4-carboxamide; LY341495, (1S,2S)-2-[(2S)-2-amino-3-(2,6-dioxo-3H-purin-9-yl)-1-hydroxy-1-oxopropan-2-yl]cyclopropane-1-carboxylic acid;

LY487379, 2,2,2-trifluoro-N-[4-(2-methoxyphenoxy)phenyl]-N-(pyridin-3-ylmethyl)ethanesulfonamide; mGlu receptor, metabotropic glutamate receptor; MNI-135, 3-(8-iodo-5-oxo-3,4,5,6-tetrahydro-1,6-benzodiazocin-2-yl)benzotrile; MPEP, 2-methyl-6-(2-phenylethynyl)pyridine; PAM, positive allosteric modulator; VU-48, 4-nitro-N-(1-(2-bromophenyl)-3-phenyl-1H-pyrazol-5-yl) benzamide

Tables of Links

TARGETS	
β_2 -adrenoceptor	mGlu ₅ receptor
mGlu ₁ receptor	mGlu ₆ receptor
mGlu ₂ receptor	mGlu ₇ receptor
mGlu ₃ receptor	mGlu ₈ receptor
mGlu ₄ receptor	

LIGANDS	
BINA	MNI-135
Glutamate	MPEP
JNJ-40068782	[35S]-GTP γ S
LY2607540 (THIC)	VU-71
LY487379	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and is crucial for physiological functions including learning and memory, development of synaptic plasticity and motor control. Because of this ubiquitous activity in the CNS, abnormal glutamate neurotransmission has been linked to a number of neurological and psychiatric diseases (Niswender and Conn, 2010; Chiechio and Nicoletti, 2012; Vinson and Conn, 2012).

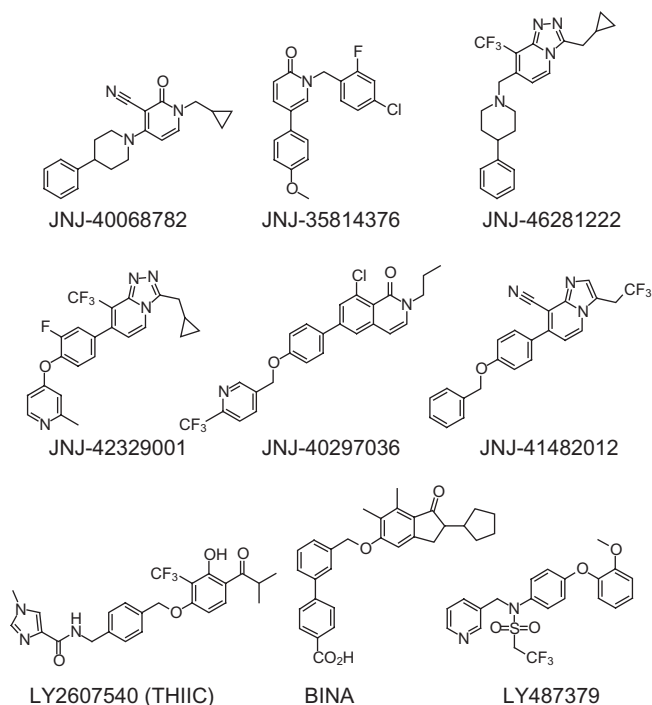
Glutamate binds to and activates metabotropic glutamate receptors (mGlu) through the extracellular amino terminal domain, the orthosteric binding site. This induces a conformational change in the receptor, resulting in activation of a G-protein and intracellular signalling pathways (Huang *et al.*, 2011). To date, eight mGlu receptors have been identified and are divided into three groups according to their amino acid sequence homology, pharmacology and their preferred signal transduction mechanism. Group I mGlu receptors (mGlu_{1/5}) preferentially activate PLC β and groups II (mGlu_{2/3}) and III (mGlu_{4/6/7/8}) inhibit AC activity (Conn and Pin, 1997).

High levels of mGlu₂ receptor are seen in brain areas such as the prefrontal cortex, hippocampus and amygdala where glutamate hyperfunction may be implicated in diseases such as anxiety and schizophrenia (Gu *et al.*, 2008; Ghose *et al.*, 2009). Receptor activation has been shown to dampen excitatory neurotransmission, and hence, the mGlu₂ receptor is being investigated as a potential target for the treatment of these diseases (Niswender and Conn, 2010). A number of group II mGlu receptor agonists have been identified from analogy with glutamate (Monn *et al.*, 1997) and have shown anxiolytic-like and antipsychotic-like effects in animal models (Shigeyuki and Hirohiko, 2011; Vinson and Conn, 2012). Efficacy has been observed in clinical studies in patients with schizophrenia or generalized anxiety disorder (Patil *et al.*, 2007; Dunayevich *et al.*, 2008).

Selectivity between mGlu₂ and mGlu₃ receptors at the conserved orthosteric site is a challenge. This has motivated the search for positive allosteric modulators (PAMs) that

specifically potentiate glutamate-induced mGlu₂ receptor activity by binding to an alternative site from the orthosteric agonist. Early examples of mGlu₂ receptor PAMs include BINA and LY487379 (Schaffhauser *et al.*, 2003; Galici *et al.*, 2006). These compounds are selective for mGlu₂ receptors and have efficacy in animal models of antipsychotic activity similar to that seen with mGlu_{2/3} receptor agonists (Galici *et al.*, 2005). The number of reports on mGlu₂ receptor PAM chemical series has increased over recent years (Trabanco *et al.*, 2011a, Trabanco and Cid, 2013) including multiple lead series from our laboratories (Cid-Nuñez *et al.*, 2008b; 2010b; Tresadern *et al.*, 2010; Trabanco *et al.*, 2011c; 2012; Cid *et al.*, 2012a,b).

The interaction of allosteric ligands with different mGlu receptors has been shown to be disturbed by mutation of seven-transmembrane (7-TM) amino acids (Litschig *et al.*, 1999; Pagano *et al.*, 2000; Knoflach *et al.*, 2001; Malherbe *et al.*, 2003; Hemstapat *et al.*, 2006; 2007; Lundström *et al.*, 2011). This suggests that allosteric modulators bind within the 7-TM region and a binding site involving TM4 and TM5 has been hypothesized (Schaffhauser *et al.*, 2003). The same mutations often affect the activity of all ligands, although there are exceptions. For example, V757 was important for the mGlu₁ receptor PAMs Ro 67-7476 and VU-71, but the very close analogue VU-48 was not affected by the same mutation (Knoflach *et al.*, 2001; Hemstapat *et al.*, 2006). In the case of the mGlu₂ receptor, the two PAMs BINA and LY487379 were both affected by mutation N735D whereas the negative allosteric modulator (NAM) MNI-135 was not (Hemstapat *et al.*, 2007). Similarly, S688, G689 and N735 mutants all disrupted LY487379 PAM activity; however, no alteration of the MNI-135 NAM activity was seen (Schaffhauser *et al.*, 2003; Hemstapat *et al.*, 2007). For the mGlu₅ receptor, a recent study elegantly demonstrated the molecular interactions that contribute to PAM versus NAM pharmacology. Using a combination of site-directed mutagenesis and computational modelling six residues (P654, Y658, T780, W784, S808 and A809) were identified as key affinity determinants across several allosteric modulator scaffolds of the mGlu₅ receptor

**Figure 1**

Structures of mGlu₂ PAMs used in this study.

(Gregory *et al.*, 2013; 2014). The 7-TM binding site for allosteric modulators was recently confirmed by X-ray crystallographic structures of the mGlu₁ and mGlu₅ receptor 7-TM domains bound to NAMs (Dore *et al.*, 2014; Wu *et al.*, 2014).

In the present study, we set out to clarify where our internal mGlu₂ PAM lead series bind on the receptor and which amino acids are critical for their activity. Six molecules representing different chemical series were selected for study (Figure 1). Three mGlu₂ PAM reference compounds were also included: LY487379, BINA, LY2607540 (THIIC) (Johnson *et al.*, 2003; Bonnefous *et al.*, 2005; Fell *et al.*, 2011, respectively; Figure 1). A total of 39 mutant mGlu₂ receptors were prepared by site-directed mutagenesis, and the effect of the receptor mutations on the functional activity of the nine PAM molecules was evaluated. Binding displacement experiments using a tritiated radioligand were also performed to evaluate whether the molecules act at a common site. We identified several new amino acids crucial for the activity of mGlu₂ PAMs. We discuss these new findings in the context of a potential allosteric binding site at the mGlu₂ receptor.

Methods

Plasmids, cell transfection and cell culture

cDNA encoding human non-mutated and mutated mGlu₂ receptors was synthesized by GeneArt® (Life Technologies, Carlsbad, CA, USA) and subcloned to the mammalian expression vector pcDNA3.1(+) (Life Technologies). Plasmid DNA was amplified through *Escherichia coli* transformation and used for transient transfection in CHO-K1 cells. All

transfections were performed using the cationic lipid transfection reagent Lipofectamine™ (Life Technologies). Cells expressing mutated and non-mutated mGlu₂ receptors were maintained in DMEM supplemented with 10% heat-inactivated FBS, penicillin G, streptomycin sulphate, pyruvic acid and L-glutamine.

Membrane preparation

Confluent cells were washed in ice-cold 50 mM Tris-HCl buffer, pH 7.4, and stored at –20°C until membrane preparation. After thawing, cells were resuspended in 50 mM Tris-HCl buffer, pH 7.4, and the cell suspension was centrifuged for 10 min at 23 500× g at 4°C. The resulting cell pellet was resuspended and homogenized in ice-cold 5 mM hypotonic Tris-HCl, pH 7.4, using an Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany) and the homogenate was centrifuged again for 20 min at 30 000× g at 4°C. The final pellet was resuspended and homogenized in 50 mM Tris-HCl. Protein concentration was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany) using BSA as standard and membranes were stored at –20°C.

[³H]-LY341495 binding

Membranes were thawed on ice and diluted in ice-cold binding buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂ and 2 mM CaCl₂. Assay mixtures contained 10 µg of membrane protein and 3 nM of [³H]-LY341495 in a total volume of 0.5 mL. In order to measure the non-specific binding, 1 mM glutamate was used. Assay mixtures were incubated for 60 min at room temperature (RT). The incubation was stopped by filtration using Unifilter-96 GF/B filter plates in a 96-well Perkin Elmer filtermate harvester, and the plates were dried overnight at RT.

[³H]-JNJ-40068782 binding

After thawing, membranes were homogenized using an Ultra Turrax homogenizer and suspended in ice-cold binding buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 2 mM CaCl₂; 75 µg membrane protein, test compound and 10 nM [³H]-JNJ-40068782 were incubated for 60 min at RT, in a total volume of 0.5 mL. Non-specific binding (about 30% of total binding) was determined in the presence of 10 µM JNJ-40264796 (a mGlu₂ PAM structurally related to JNJ-40068782, molecule 34 from Cid *et al.*, 2012a). Filtration was performed using Unifilter-96 GF/C filters pre-soaked in 0.1% PEI and a 40-well manifold or 96-well Brandell harvester.

For both [³H]-LY341495 and [³H]-JNJ-40068782, assay conditions including radioligand concentrations were chosen such that <10% of the free radioligand concentration was receptor-bound. After the addition of scintillation liquid, radioactivity on the filters was counted in a Microplate Scintillation and Luminescence Counter or Liquid Scintillation Analyzer from Perkin Elmer.

[³⁵S]-GTPγS binding

Membranes were thawed on ice and diluted in assay buffer (10 mM HEPES acid and 10 mM HEPES salt, pH 7.4, containing 100 mM NaCl, 3 mM MgCl₂, 10 µM guanosine 5'-diphosphate and 14.3 µg·mL⁻¹ saponin). Assay mixtures

containing 10 µg of membrane protein and test compound were pre-incubated with buffer (to detect agonist effects) or an EC₂₀-equivalent concentration of glutamate (to detect PAM effects) for 30 min at 30°C. Finally, 0.1 nM [³⁵S]-GTPγS was added and the assay mixture was incubated for another 30 min at 30°C. The reaction was stopped through filtration using Unifilter-96 GF/B filter plates (Perkin Elmer Life Sciences) in a 96-well Perkin Elmer filtermate harvester. The filters were washed three times with ice-cold 10 mM NaH₂PO₄/10 mM Na₂HPO₄ buffer, pH 7.4, and dried overnight at RT. The remaining radioactivity was counted in a Microplate scintillation and luminescence counter from Packard.

Data analysis

Data analysis was performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA). Concentration–response curves were fitted using non-linear regression analysis fitting the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / [1 + 10^{-(\text{LogEC}_{50} - X) \times \text{Hill Slope}}]$. pEC₅₀ values obtained at the non-mutated receptor were compared with those at the mutant receptors by one-way ANOVA analysis.

Amino acid selection for site-directed mutagenesis

Amino acids previously shown to be important for only mGlu₂ receptor allosteric modulators were prioritized for mutation in our experiments. Therefore, the following amino acids were chosen: R635, R636, F643, S688, G689, H723, M728, S731, L732, N735, V736, W773 and F780.

The compounds being studied have been reported as selective for mGlu₂ versus mGlu₃ receptors. The mGlu₂ and mGlu₃ sequences were compared and different amino acids were identified. These are potentially important for the selective binding of mGlu₂ receptor PAMs. Amino acids from the extracellular side of TM2, TM4, TM5 and ECL2 were selected for mutation. This resulted in many potential mutations so preference was given to amino acids with a more significant change in properties between mGlu₂ and mGlu₃: C616, I622, T641, A642, A681, I693, V695, A696, G706, E708, A710, P711, V716, T718, A726, G730, A733 and A740. In these cases, all amino acids were mutated to their mGlu₃ equivalent.

At the time of initiating this work, the newly solved structures of mGlu₁ and mGlu₅ receptors were not available. Hence, an initial mGlu₂ receptor model was built based on precedent for the alignment of class C GPCRs with class A. The β₂-adrenoceptor X-ray structure 2RH1 was used as a template and this model used for docking to suggest amino acids for mutation. Molecules such as JNJ-40068782 were docked and plausible interacting amino acids were identified: R636, F643, L732, W773, F780, L639, D725, F776, S644, V700 and H723. Some of these had not been selected by the approaches discussed earlier and were therefore added to the list for experimental mutation. Subsequently, a second mGlu₂ receptor model was built from the mGlu₁ X-ray structure template. This model was used for interpretation of the experimental results and further docking to propose binding modes. The method for building the mGlu₂ receptor homology model and performing protein ligand docking are provided in Sup-

porting Information Appendices S1 and S2 respectively. We highlight that caveats exist for GPCR homology modelling. The difference in sequence identity, loop lengths, loop conformations and correct amino acid side chain orientation present a challenge for structure prediction. Use of the inactive states of the β₂-adrenoceptor and mGlu₁ receptors to model the mGlu₂ PAM bound receptor is also problematic. The activated state of the β₂-adrenoceptor (Rasmussen *et al.*, 2011a,b) showed large structural shifts in the intracellular G-protein binding region but only subtle changes in the 7-TM binding site, suggesting the effect may be small. The recent mGlu₁ X-ray suggests interactions at the intracellular side of helices 3 and 6 play a role in stabilizing the inactive state, but a full understanding of the activation mechanism and plausible active state is some way off. Overall, modelling an mGlu₂ receptor structure is improved by the availability of the mGlu₁ receptor crystal structure but the limitation of relying upon an inactive receptor conformation remains.

In summary, 36 amino acids were identified as potentially important for the PAM-mGlu₂ receptor interaction and chosen for site-directed mutagenesis. We prepared 36 single-point mutations along with one double and two triple mutations (Table 1).

Materials

JNJ-35814376 (Imogai *et al.*, 2006; Cid *et al.*, 2010), JNJ-40068782 (Imogai *et al.*, 2007; Cid-Nuñez *et al.*, 2008a; Lavreysen *et al.*, 2013), JNJ-40297036 (Trabanco *et al.*, 2011b), JNJ-41482012 (Trabanco-Suárez *et al.*, 2009; Tresadern *et al.*, 2010), JNJ-42329001 (Cid-Nuñez *et al.*, 2010a) and JNJ-46281222 (Cid-Nuñez *et al.*, 2010a) are original products of Janssen Research and Development, a division of Janssen Pharmaceutica. LY487379, BINA and LY2607540 (Johnson *et al.*, 2003; Bonnefous *et al.*, 2005; Fell *et al.*, 2011, respectively) were synthesized for in-house use as reference compounds. All PAMs were dissolved in 100% DMSO, and serially diluted in assay buffer with final concentrations of 1% DMSO. L-glutamate was purchased from Aldrich® Chemistry. The radioligand [³H]-LY341495 was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA) and [³⁵S]-GTPγS was obtained from Perkin Elmer® (Boston, MA, USA). The radioligand [³H]-JNJ-40068782 is an original product of Janssen Research and Development, a division of Janssen Pharmaceutica (Lavreysen *et al.*, 2013). All cell culture and transfection reagents were obtained from Invitrogen (Carlsbad, CA, USA), except for FBS, which came from HyClone® (Thermo Scientific, Cramlington, UK). CHO cells (CHO-K1, ATCC: CCL-61) were obtained from ATCC®. Drug and receptor nomenclature follows Alexander *et al.* (2013).

Results

Expression of mGlu₂-wt and mutant mGlu₂ receptors in CHO-K1 cells

Radioligand-binding studies using the orthosteric mGlu_{2/3} receptor antagonist [³H]-LY341495 were performed to assess the expression of non-mutated (defined as 'mGlu₂-wt')

Table 1mGlu₂ receptor mutagenesis constructs

WT aa	Position	Mutation	WT codon	Mutant codon	Name	Region
Cys	616	Ser	TGC	TCC	C616S	TM2
Ile	622	Phe	ATC	TTC	I622F	
Arg	635	Ala	AGA	GCG	R635A	TM3
Arg	636	Ala	CGT	GCT	R636A	
Leu	639	Ala	TTG	GCG	L639A	
Thr	641	Ser	ACC	TCC	T641S	
Ala	642	Ser	GCC	TCC	A642S	
Phe	643	Ala	TTC	GCC	F643A	
Ser	644	Ala	TCT	GCT	S644A	
Ala	681	Phe	GCC	TTC	A681F	TM4
Ser	688	Leu	TCG	TTG	S688L	
Gly	689	Val	GGC	GTC	G689V	
Ile	693	Met	ATT	ATG	I693M	
Val	695	Ser	GTG	TCG	V695S	
Ala	696	Val	GCC	GTC	A696V	
Val	700	Leu	GTC	CTC	V700L	
Gly	706	Arg	GGA	CGA	G706R	ECL2
Glu	708	Tyr	GAG	TAC	E708Y	
Ala	710	Leu	GCC	CTC	A710L	
Pro	711	Ala	CCC	GCC	P711A	
Val	716	Thr	GTG	ACG	V716T	
Thr	718	Ile	ACC	ATC	T718I	
His	723	Val	CAC	GTC	H723V	
Asp	725	Ala	GAT	GCT	D725A	TM5
Ala	726	Ser	GCC	TCC	A726S	
Met	728	Ala	ATG	GCG	M728A	
Gly	730	Ile	GGC	ATC	G730I	
Ser	731	Ala	AGC	GCC	S731A	
Leu	732	Ala	CTG	GCG	L732A	
Ala	733	Thr	GCC	ACC	A733T	
Asn	735	Asp	AAT	GAT	N735D	
Val	736	Ala	GTG	GCG	V736A	
Ala	740	Ile	GCC	ATC	A740I	
Trp	773	Ala	TGG	GCG	W773A	TM6
Phe	776	Ala	TTC	GCC	F776A	
Phe	780	Ala	TTC	GCC	F780A	
Ser/Gly	688/689	Leu/Val	TCG/GGC	TTG/GTC	S688L/G689V	TM4
Ser/Val/His	644/700/723	Ala/Leu/Val	TCT/GTC/CAC	GCT/CTC/GTC	S644A/V700L/H723V	TM3/4/ECL2
Ser/Gly/Asn	688/689/735	Leu/Val/Asp	TCG/GGC/AAT	TTG/GTC/GAT	S688L/G689V/N735D	TM4/5

throughout the text) and mutated mGlu₂ receptors in membranes of transiently transfected CHO-K1 cells. All mutated receptors showed similar specific [³H]-LY341495 binding (in the range of 90%, data not shown), thereby confirming that the orthosteric site had remained intact. Furthermore, glutamate was able to displace [³H]-LY341495

from mGlu₂-wt (stably and transiently transfected) and mutant receptors with similar pIC₅₀ values (pIC₅₀ ~5, see Supporting Information Appendix S3 and Figure 2A). Proper receptor expression was also confirmed via [³H]-LY341495 B_{max} determinations as well as immunoblotting (data not shown).

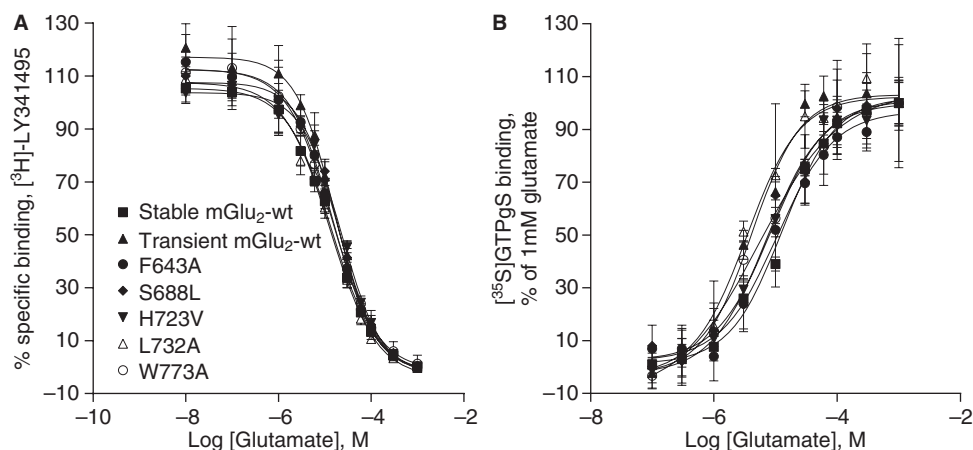


Figure 2

(A) Displacement of [³H]LY341495 binding from mGlu₂-wt (stably and transiently transfected) and mGlu₂ mutants by glutamate. Results are presented as percentage of specific binding. The affinity of glutamate is not altered by any of the mutations; total binding levels varied for the different membrane pools. (B) Glutamate-induced [³⁵S]-GTPγS binding. Glutamate concentration–response curves were determined on membranes from CHO-K1 cells expressing mGlu₂-wt (stably and transiently transfected) and five representative mutant receptors (transiently transfected). Results are expressed as % ± SD of the response to 1 mM glutamate. Both data sets are from one experiment performed in triplicate. Similar data were found for the additional set of mutants.

Glutamate potency for mGlu₂-wt and mutated mGlu₂ receptors

The potency of glutamate was assessed using [³⁵S]-GTPγS assays permitting further verification of the effect of the mutations on glutamate-mediated receptor signalling. Mutated receptors elicited concentration–response curves for glutamate resulting in potencies that were similar to those of the stably and the transiently transfected mGlu₂-wt receptor (see Supporting Information Appendix S3 and Figure 2B). The amplitude of the response to glutamate, that is the ratio between the response obtained under basal conditions (only using assay buffer) and the response obtained with 1 mM glutamate, was however considerably lower in cells transiently expressing the mGlu₂ receptor compared with the stable cell line (Supporting Information Appendix S3).

Effect of mGlu₂ mutations on the activity of mGlu₂ PAMs

Initial screening. Given the large number of compounds and mutants being studied, an initial screening approach was applied to confirm if a mutation was likely to affect PAM activity. In this preliminary step, two concentrations of PAM were tested: a concentration equivalent to the compound's EC₅₀ and a concentration producing a maximal PAM response (typically 3 or 10 μM). Both were previously determined with the use of stably transfected cells. The resulting PAM effect at each concentration was calculated as a percentage of the response to 1 mM glutamate and was then compared between mGlu₂ mutants and the transiently transfected mGlu₂-wt receptor. The results from these experiments, including details on the reliability assessment of this initial screening are added as Supporting Information Appendix S4. Supporting Information Fig. S1 provides a useful graphical comparison of the pre-screen results at the two concentrations. Mutants showing

effects at either of the two concentrations were carried forward for further study.

Through this screening approach, 26 mutations were considered not to have an effect on the activity of any of the compounds. The others were evaluated in more detail by performing concentration–response analyses. Corresponding pEC₅₀ values are shown in Table 2 along with concentration–response curves for a subset in Figure 3.

Overall results for all mutations (for screening data see Supporting Information Appendix S4)
Mutations located in TM2. The mutations located in this receptor region (C616S and I622F), which were selected based on sequence alignment and comparison of mGlu₂ and mGlu₃ receptor sequence, did not affect the response to either concentration of compound tested in the screening phase.

Mutations located in TM3. The mutations located in TM3 caused distinct effects on the activity of the compounds tested. Mutation F643A showed the most prominent results (Table 2, Figure 3). For the different compounds tested, the effects of this mutation ranged from no change (for JNJ35814376) to a complete loss of compound's activity for JNJ-40297036, JNJ-41482012 and LY487379 (i.e. pEC₅₀ < 5; Table 2). Mutation L639A affected the activity of JNJ-40068782, JNJ-35814376, JNJ-40297036, LY2607540, BINA and LY487379 albeit to a lesser extent compared with mutation F643A (2- to 18-fold potency decrease, Table 2); no change was observed for compounds JNJ-46281222, JNJ-42329001 and JNJ-41482012. Mutation R635A had no effect on the majority of the compounds tested; it did cause a slight decrease in potency of JNJ-40068782 (fivefold), JNJ-35814376 (twofold) and BINA (eightfold) (Table 2). Finally, mutations S644A, R636A, T641S and A642S had no effect on the responses of any of the molecules tested.

Table 2

pEC₅₀ of the enhancement of glutamate-induced [³⁵S]-GTPγS binding by nine PAMs on mGlu₂-wt and the mutant receptors that were selected for further evaluation, after transient transfection into CHO-K1 cells

		JNJ-40068782	JNJ-35814376	JNJ-46281222	JNJ-42329001	JNJ-40297036	JNJ-41482012	LY2607540 (THIIC)	BINA	LY487379
WT	Stable	6.9 ± 0.1	6.19 ± 0.12	8.09 ± 0.23	7.41 ± 0.18	6.79 ± 0.17	6.74 ± 0.23	6.97 ± 0.08	7.03 ± 0.14	6.78 ± 0.13
	Transient	7.1 ± 0.1	6.36 ± 0.25	8.22 ± 0.29	7.55 ± 0.2	6.75 ± 0.15	6.83 ± 0.15	6.97 ± 0.14	7.11 ± 0.30	6.85 ± 0.20
TM3	R635A	6.42 ± 0.03***	6.07 ± 0.04****	n.c.	n.c.	n.c.	n.c.	n.c.	6.21****	n.c.
	L639A	6.27 ± 0.16***	5.83 ± 0.03**	n.c.	n.c.	6.28 ± 0.35****	n.c.	6.78 ± 0.06**	5.86 ± 0.01****	6.16****
	F643A	5.64 ± 0.18***	n.c.	6.32 ± 0.25****	5.24 ± 0.08****	<5***	<5***	6.08****	5.85 ± 0.08****	<5***
TM4	G689V	6.27 ± 0.20***	5.74 ± 0.12***	7.21 ± 0.30*	6.84 ± 0.18**	6.30 ± 0.29*	<5***	6.22 ± 0.22**	6.20 ± 0.37*	6.58 ± 0.34
ECL2	H723V	6.27 ± 0.06***	5.79 ± 0.11**	n.c.	n.c.	6.46 ± 0.12	6.72 ± 0.10	6.56 ± 0.10	n.c.	n.c.
TM5	S731A	n.c.	5.92 ± 0.03*	n.c.	n.c.	n.c.	n.c.	6.56 ± 0.12	n.c.	6.08 ± 0.03***
	L732A	7.03 ± 0.06	6.22 ± 0.16	7.31 ± 0.33	6.25 ± 0.09****	6.51 ± 0.18	<5***	6.49 ± 0.24*	7.80 ± 0.03 ^a	<5***
	N735D	5.52****	<5***	6.67 ± 0.23****	5.97 ± 0.06****	<5***	<5***	5.53 ± 0.04****	5.13****	<5***
TM6	W773A	<5***	<5***	7.11 ^{ab} ****	5.9****	<5***	<5***	<5***	<5***	<5***
	F776A	6.38 ± 0.23***	n.c.	n.c.	7.16 ± 0.07 ^b	n.c.	n.c.	6.93 ± 0.07	6.71 ± 0.06	<5***
S688L/G689V		6.29 ± 0.39***	5.82****	7.15 ± 0.41*	6.54 ± 0.46***	6.58 ± 0.06 ^b	6.70 ± 0.30 ^b	6.22 ± 0.18**	6.30 ± 0.23*	6.48 ± 0.14*
S688L/G689V/N735D		<5***	<5***	6.38 ± 0.27****	5.95 ± 0.51****	<5***	<5***	<5***	<5***	<5***
S644A/N700L/H723V		5.91 ± 0.05****	5.67 ± 0.11****	7.10 ± 0.24*	6.96 ± 0.12*	6.40 ± 0.05	6.64 ± 0.08	6.21 ± 0.05**	7.11 ± 0.15	6.18 ± 0.06****

pEC₅₀ ± SD values were determined based on three independent experiments, each performed in triplicate. Activity at transient mGlu₂-wt receptors was always tested in parallel as a control.

n.c., no change (in the pre-screening, the mutation did not have an effect on the activity of the compound).

^aFor one or two experiments, pEC₅₀ was <5.

^bn = 2.

*P < 0.05, **P < 0.01, ***P < 0.001 significantly different from transient mGlu₂-wt value, one-way ANOVA. It is of note that by applying the Dunnett's post-test, some results lost statistical significance (e.g. mutant G689V and compound JNJ-46281222).

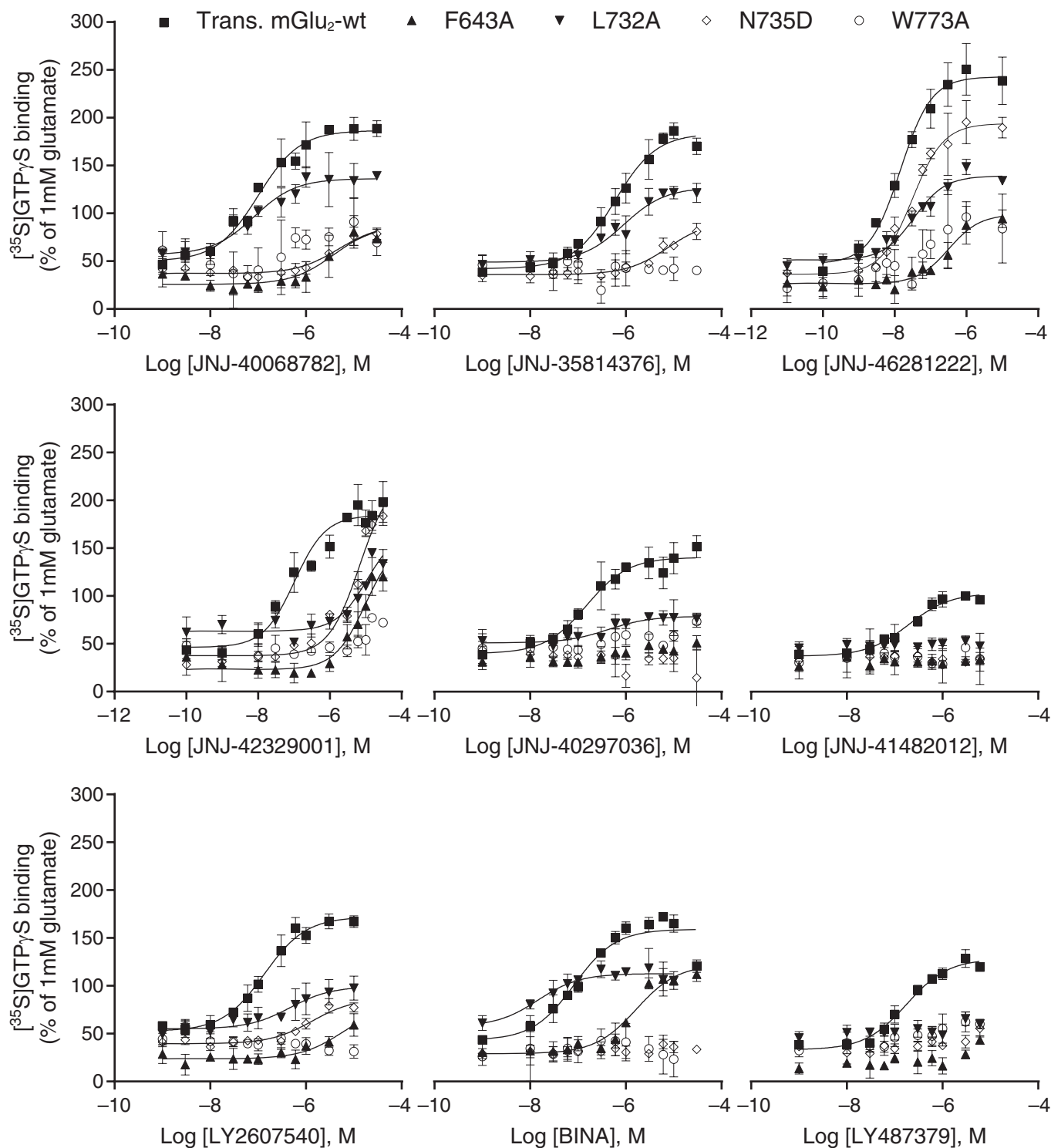


Figure 3

Effect of the PAMs used in this study on glutamate-induced $[^{35}\text{S}]\text{-GTP}\gamma\text{S}$ binding in $\text{mGlu}_2\text{-wt}$ and a subset of mGlu_2 mutants. Concentration-dependent enhancement of 4 μM glutamate-induced $[^{35}\text{S}]\text{-GTP}\gamma\text{S}$ binding by nine PAMs used in this study. Results are expressed as % \pm SD of the response to 1 mM glutamate, and refer to one experiment performed in triplicate.

Mutations located in TM4. Mutations A681F, I693M, V695S, A696V and V700L showed no effect on the activity of the tested compounds. In addition, mutation S688L yielded similar results as the mGlu₂-wt receptor for every compound. With the exception of LY487379, all the other compounds seemed to be affected by G689V (Table 2).

Mutations located in ECL2. In ECL2, mutations G706R, E708Y, A710L, P711A, V716T and T718I did not change the response to the two concentrations of compound tested initially; only H723V caused an increase in EC₅₀ of JNJ-40068782 (sevenfold) and JNJ-35814376 (fourfold) (Table 2).

Mutations located in TM5. Mutants D725A, A726S, M728A, G730I, A733T, A740I and V736A yielded similar responses as the mGlu₂-wt receptor. Mutation S731A also had a minor impact on the activity of the compounds tested, with the exception of JNJ-35814376 (threefold decrease in potency) and LY487379 (sixfold decrease in potency). Mutation L732A affected the potency of JNJ-46281222, JNJ-42329001 and LY2607540 and caused a complete loss of activity of JNJ-41482012 and LY487379. It is of note that changing this amino acid lowered the efficacy of these and other compounds (see e.g. LY2607540 in Figure 3). Finally, mutation N735D dramatically impacted the activity and potency of all the molecules (Table 2, Figure 3).

Mutations located in TM6. For the mutations localized in TM6, changing the tryptophan 773 residue (W773A) seemed to affect the potency of all compounds (Table 2, Figure 3). Although to a lesser extent, mutation F776A also significantly affected the activity of JNJ-40068782, and led to the complete loss of the receptor's ability to respond to increasing concentrations of LY487379. Mutation F780A showed similar results as the mGlu₂-wt receptor.

Double and triple mutations. Mutation G689V/S688L caused significant shifts in the activity of all the tested molecules (3- to 11-fold potency decrease) with the exception of JNJ-40297036, JNJ-41482012 and LY487379. Mutation G689V/S688L/N735D had a major impact on the concentration-response curves for all the compounds. Finally, the triple mutation S644A/V700L/H723V decreased the activity of the majority of the tested compounds, with the exception of JNJ-40297036, JNJ-41482012 and BINA (Table 2).

Binding displacement of mGlu₂ PAMs on mGlu₂-wt receptor. We showed previously that JNJ-40068782, BINA, LY487379 and LY2607540 can fully displace [³H]-JNJ-40068782 (Lavreysen *et al.*, 2013) and likely bind within the same pocket. We tested the additional mGlu₂ PAMs included in this study for competition with [³H]-JNJ-40068782. Membranes prepared from stably mGlu₂-wt receptor transfected CHO-K1 cells were used. JNJ-42329001 and JNJ-46281222 fully displaced [³H]-JNJ-40068782 with relatively high affinity (IC₅₀ values of 23 and 30 nM, *n* = 2 for JNJ-42329001 and 19.5 ± 0.4 nM, *n* = 3 for JNJ-46281222), in line with these compounds being most potent in the [³⁵S]-GTPγS assay (pEC₅₀ values of 7.41 ± 0.18 for JNJ-42329001 and 8.09 ± 0.23 for JNJ-46281222; Table 2). Although the least potent JNJ-35814376 also inhibited binding with lower affinity (IC₅₀ values of 417 and 676 nM,

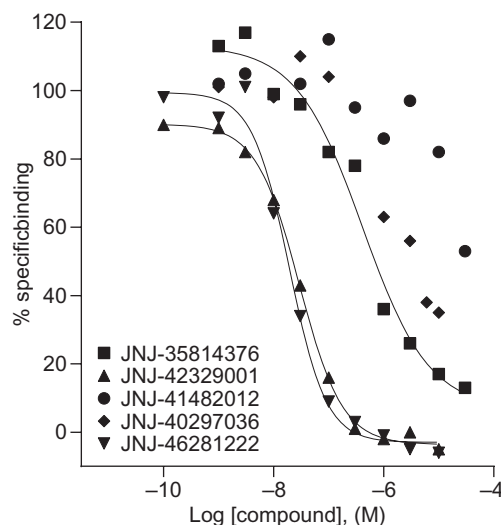


Figure 4

Displacement curves of [³H]-JNJ-40068782 binding to membranes prepared from CHO-K1 cells stably expressing the hmGlu₂ receptor. Results are expressed as a % of specific binding and are from one representative experiment. The same results were found in at least one independent experiment.

n = 2), the moderately potent JNJ-41482012 and JNJ-40297036 displaced the radioligand 36 ± 13% (*n* = 3) and 58 ± 9% (*n* = 2) at 10 μM respectively (Figure 4). So while some compounds may reside in an overlapping binding site, this suggests potential differences in binding for other allosteric modulators.

Docking mGlu₂ PAMs. The alignment of the transmembrane domains of human mGlu receptors 1–8 as well as rat and mouse mGlu₂ receptor sequences are provided as Supporting Information Figure S2.

Molecules were docked into the homology model of the mGlu₂ receptor built using mGlu₁ as template (Figure 5). Important amino acids identified in the mutagenesis experiments are seen to cluster around the 7-TM binding site (Figure 5A). A consistent binding mode was identified among the top-ranked docking poses and four examples JNJ-40068782, JNJ-40297036, JNJ-46281222 and LY2607540 are shown in Figures 5B to E.

JNJ-40068782 (Figure 5B) binds in the 7-TM site interacting with the hydrophobic cluster formed between amino acids L639, F643, L732, W773 and F776 from TM3, TM5 and TM6. The cyclopropyl methyl substituent enters furthest into the receptor towards TM3 and TM5. The hydrophobic residues cluster tightly around the pyridone scaffold and the cyclopropyl methyl substituent. Space exists beyond the cyclopropyl methyl to go deeper towards W773 located on TM5, allowing larger groups in other mGlu₂ PAMs such as the halogenated benzyl in JNJ-35814376. The carbonyl of the pyridone scaffold forms a hydrogen bond with N735. S644 forms an H-bond to the sidechain carbonyl oxygen of N735 suggesting that mutation of this residue may alter the position or orientation of N735 and disturb its interaction with the ligand. The 4-phenylpiperidine substituent in JNJ-

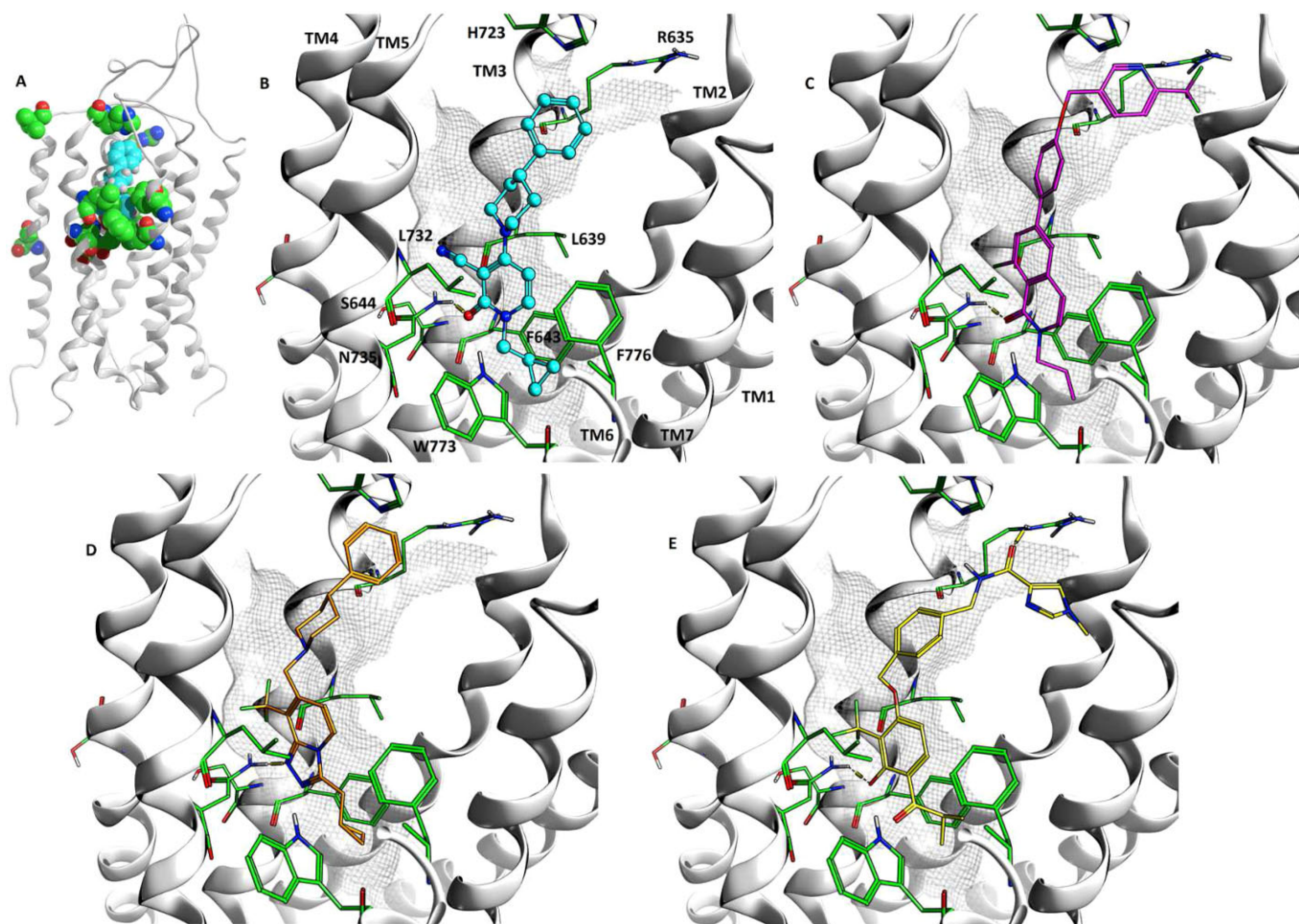


Figure 5

(A) Amino acids, R635, L639, F643, S644, S688, G689, V700, H723, L732, N735, W773, F776, highlighted in the mGlu₂ receptor model built using the mGlu₁ receptor X-ray structure as template, the docked binding mode of JNJ-40068782 shown in sphere representation, coloured turquoise. View between TM5 and TM6 (cut-away) of the docked binding modes of (B) JNJ-40068782, (C) JNJ-40297036, (D) JNJ-46281222, (E) LY2607540 (THIIC). Selected important amino acids from experimental mutagenesis are shown in green. Amino acids and TM annotation is provided in (B) and consistent for (C), (D) and (E).

40068782 is directed towards the extracellular side of the binding site and the distal phenyl sits between R635 and H723. As the binding site becomes larger in this region it permits bigger groups seen in this position for many PAMs. This greater space and flexibility is consistent with not all ligands being affected by the same mutations in this region. Also, the presence and importance of positively charged amino acids such as R635 in this vicinity is in agreement with structure-activity relationships (SAR), which allow distal carboxylic acids such as in BINA.

LY2607540 (Figure 5E) occupies the same 7-TM binding site interacting above the hydrophobic cluster of amino acids L639, F643, L732, W773 and F776. The lipophilic scaffold substituent, in this case isopropyl, again goes deepest into the receptor towards TM3 and TM5. It extends on top of F643 and forms a hydrophobic interaction with the edge of the phenylalanine. The phenolic scaffold sits in a similar space to the pyridone of JNJ-40068782 and also forms an H-bond with N735. The large ether substituent with the

distal imidazole is directed perpendicular to the 7-TM helices towards the extracellular side. The active site has a bent shape and sufficient space, which is again consistent with the greater structural diversity in this region of the molecules.

Discussion

The present work represents the most extensive study to date of mGlu₂ receptor amino acids important for allosteric activity. The activity of nine mGlu₂ receptor PAMs was assessed on 39 human mGlu₂ receptors carrying 7-TM mutations. The mutations were introduced outside of the extracellular domain and no change was observed in the affinity or potency of glutamate compared with mGlu₂-wt receptor, confirming the integrity of the orthosteric site and the receptor. Amino acids N735 and W773 were identified as crucial for the

activity of all the PAMs tested. In addition, F643, R635, L639, H723, L732 and F776 were shown to be important for multiple mGlu₂ PAMs.

Effect on functional activity

Many factors can influence the potency of PAMs, including the binding affinity of the PAM, the affinity cooperativity between the PAM and glutamate, the efficacy cooperativity between PAM and glutamate and the level of receptor expression. Our data show that all mutant receptors get expressed at the cell membrane, but do not allow quantitative comparison of expression. Changes in [³⁵S]-GTPγS effect because of either compound binding affinity and/or cooperativity can also not be excluded. Efforts are ongoing to assess potential effects on binding affinity. Unfortunately, [³H]-JNJ-40068782 binding to transiently transfected cells was too weak and novel radioligands are being evaluated. In addition, validating an operational model (according to Leach *et al.*, 2007; Gregory *et al.*, 2012) to analyze shifts of glutamate concentration–response curves by mGlu₂ modulators may help delineate determinants for cooperativity versus affinity.

With regards to differences in functional or binding effects induced by the mutants it is of interest to highlight S688 and G689. These amino acids have been reported to influence binding of allosteric modulators at mGlu₂ receptors, for instance LY487379 (Schaffhauser *et al.*, 2003) and MRSLD-650 (a BINA analogue) (Rowe *et al.*, 2008). However, it is clear from the homology model and the docking results (Figure 5) that S688 and G689 are not directly in the allosteric binding site. This is consistent with a study of mGlu₂ receptor allosteric antagonists (Lundström *et al.*, 2011) and suggests these amino acids cause indirect effects related to signalling. Alternatively, they may play a role in dimerization as the adjacent amino acids, I687 and Q690, may be at the dimer interface (Bruno *et al.*, 2009).

Previously identified important amino acids were also crucial for the mGlu₂ receptor PAMs tested in this study (G689V, N735D and S688L/G689V/N735D, Schaffhauser *et al.*, 2003). Interestingly, mutations R635A (TM3), F643A (TM3), H723V (ECL2) and W773A (TM6), which were shown to affect NAM activity (Lundström *et al.*, 2011), also affected the activity of the PAMs tested. In addition, residues in TM3 (L639), TM5 (L732) and TM6 (F776) are reported for the first time as affecting the activity of mGlu₂ PAMs.

Common and unique amino acids important for allosteric mGlu modulation

Allosteric binding studies have been performed for other mGlu receptors. Mutation of two residues in TM3 of mGlu₁ receptors (S668, C671) led to a loss of effect of the mGlu₁ receptor PAMs diphenylacetyl-carbamic acid ethyl ester (Ro 01-6128) and (2S)-2-(4-fluorophenyl)-1-[(4-methylphenyl)sulfonyl]-pyrrolidine (Ro 67-7476). These amino acids were also relevant from the corresponding mGlu₅ study by Pagano *et al.* (2000). A residue in TM5 (V757 in rat mGlu₁, equivalent to L757 in human mGlu₁) was also shown to be crucial for the binding of these compounds (Knoflach *et al.*, 2001). Residues in TM3 (P655, S658) and TM7 (A810) were shown to be important for the binding of MPEP at mGlu₅ (Pagano *et al.*, 2000). Malherbe *et al.* (2003) identified eight additional

residues in TM3, TM5, TM6 and TM7 crucial for MPEP binding to mGlu₅. Y658, T780, S808, P654 and A809 in TM3, TM6 and TM7 were found to be important for mGlu₅ PAM affinity (Gregory *et al.*, 2013; 2014), and a common allosteric binding site was proposed. The mGlu receptor sequence alignment in Supporting Information Fig. S2 facilitates comparison of these amino acids with mGlu₂ receptor. Our data with [³H]-JNJ-40068782 also support an overlapping binding site for some mGlu₂ allosteric modulators. In our study, mutations of residues F643 and L732, equivalent to residues P655 in mGlu₅ and L757 in human mGlu₁, respectively, were also seen to have a great impact on the compounds tested.

The exchange of mGlu₂ receptor amino acid residues H723, G689 and N735 with the correspondent residues of mGlu₃ receptor caused a significant impact on the activity of multiple PAMs tested in this study, which suggests, as seen previously for G689 and N735 (Schaffhauser *et al.*, 2003) the importance of these residues for subtype specificity between mGlu₂ and mGlu₃.

Structural interpretation and binding mode of mGlu₂ PAMs

The homology model of the mGlu₂ receptor built from the recently solved mGlu₁ X-ray structure was used to dock the mGlu₂ receptor PAMs and suggest a possible binding mode. The mGlu₁ structure offers the chance to build a class C GPCR atomic model based on a similar class C structural template. The most significant structural difference arising from the mGlu₁ template is a tighter transmembrane binding site formed by the inwards movement of TM5 and TM7 compared with class A GPCR templates (Wu *et al.*, 2014). Previous reports of mutagenesis studies on the mGlu₂ receptor used rat sequence in the case of Lundström *et al.*, 2011 and human in the cases of Schaffhauser *et al.*, 2003 and Hemstapat *et al.*, 2007. The sequence alignment (Supporting Information Appendix S5) demonstrates only three amino acids differ between species therefore permitting direct comparison of results.

We can be quite certain in the location of amino acids in the mGlu₂ receptor model given the facile sequence alignment to the mGlu₁ X-ray structure. Highlighting the location of the important amino acids in the mGlu₂ receptor structure shows clearly how the majority: L639, F643, S644, L732, N735, W773 and F776 are closely clustered on the deep interior of the 7TM binding site (Figure 5A). However, R635 and H723 are located at the top of the binding site on the extracellular side. As commented earlier, S688, G689 and V700 do not make direct interactions with the ligand. Previous reports (Schaffhauser *et al.*, 2003; Hemstapat *et al.*, 2007; Rowe *et al.*, 2008) have suggested a role for S688 and G689 in the binding of allosteric modulators at the mGlu₂ receptor. All three studies assume direct interaction between these amino acids and the allosteric ligands. Lundström *et al.* (2011) subsequently constructed an mGlu₂ receptor homology model based on the β₂-adrenoceptor template and revealed that S688 and G689 (Ser^{4.44} and Gly^{4.45} in their work) were located outside the characterized 7-TM binding cavity. Our results based on a more accurate structural template using the mGlu₁ receptor structure confirm this. Interestingly, functional activity of all the PAMs was affected by amino acids S688, G689 and N735, which are not conserved

across the mGlu family. Therefore, they may still contribute to the subtype selectivity albeit in an indirect manner for S688 and G689.

The predicted binding mode was seen to be in good agreement with known SAR. Important pharmacophoric features and consistent interactions were found. A key feature of mGlu₂ PAMs is the hydrogen bond acceptor on the scaffold. For each of the four compounds shown in Figure 5 this is satisfied by the interaction with the sidechain of N735. This is in agreement with mutation of N735 impacting the activity of all the molecules in this study. Three additional SAR features are consistent for all four molecules and present in the docked binding poses. Firstly, the requirement for a lipophilic substituent such as the isopropylmethyl from JNJ-40068782 is consistent with the binding of this group in the hydrophobic pocket formed by amino acids F643, W773 and F776. Secondly, the small scaffold substituents adjacent to the scaffold acceptor (e.g. CF₃, -Cl, -CN, -Me) all occupy a similar space. Thirdly, the scope for broad substitution in the region of the distal phenyl in JNJ-40068782 is in accordance with this part of the binding site being larger and more open than the tighter binding pocket in the deeper 7TM region. JNJ-40068782, LY2607540 and JNJ-46281222 all showed potent displacement of [³H]JNJ-40068782 in agreement with the overlapping binding modes presented. However, some caution may be needed because the computational approach predicts a similar binding mode for JNJ-40297036 (Figure 5C), which only showed weak displacement of [³H]-JNJ-40068782, 58 ± 9% at 10 μM. Overall, the docking poses were consistent with the overlap of shared pharmacophoric features and SAR from our previously reported ligand overlay hypothesis and QSAR work (Tresadern *et al.*, 2010; 2014).

In conclusion, the present study provides further additional insight on the interactions that drive the activity of PAMs towards the mGlu₂ receptor and enabled the identification of amino acids that play a crucial role in PAM pharmacology. Multiple amino acids in TM3 (R635, L639, F643), TM5 (L732) and TM6 (W773, F776) were for the first time shown to have an effect on mGlu₂ PAM activity, with many consistent between molecules suggesting a common binding site. The knowledge obtained from the mapping of allosteric modulators can contribute to guide research towards the achievement of more selective and potent drugs.

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Author contributions

A. F., L. P., B. R., H. L. and G. T. participated in research design. A. F., L. P., B. R. and G. T. conducted experiments. A. A. T., J. C. and S. M. contributed new reagents or analytic tools. A. F., L. P. and B. R. performed data analysis. A. F., H. L., G. T., L. P., B. R., S. M., A. A. T and J. C. wrote or contributed to the writing of the paper.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13065>

Appendix S1 Building the mGlu₂ receptor homology model.

Appendix S2 Protein ligand docking.

Appendix S3 Data confirming the integrity of the orthosteric receptor binding site.

Appendix S4 Details on the initial screening of each PAM on all 39 mutant mGlu₂ receptors.

Appendix S5 Sequence alignment.