Molecular insights into metabotropic glutamate receptor allosteric modulation

Karen J. Gregory and P. Jeffrey Conn

Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, VIC, Australia (KJG).
Vanderbilt Center for Neuroscience Drug Discovery & Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN, USA (PJC).

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Corresponding Author:
P. Jeffrey Conn
1215 Light Hall
2215-B Garland Ave
Nashville, TN, USA
37232
jeff.conn@vanderbilt.edu

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Non-standard abbreviations: 7TMs, seven transmembrane spanning α-helical domains; ACPD, 1-Amino-1,3-dicarboxycyclopentane; AMN082, N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride; BAY36-7620: (3a*S*,6a*S*)-Hexahydro-5-methylene-6a-(2-naphthalenylmethyl)-1*H*-cyclopenta[*c*]furan-1-one; BINA, biphenyl-indanone A; cAMP: cyclic adenosine-monophosphate; CBiPES, *N*-[4'-cyano-biphenyl-3-yl)-N-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride); CPCCOEt, 7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester; CPPHA, *N*-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl)-2-hydroxybenzamide; DCG-IV, (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine; DFB, ((3-Fluorophenyl)methylene)hydrazone-3-fluorobenzaldehyde; DHPG, 3,5-dihydroxyphenylglycine; DPFE, 1-(4-(2,4-difluorophenyl)piperazin-1-yl)-2-((4-fluorobenzyl)oxy)ethanone; FITM, 4-fluoro-*N*-(4-(6-(isopropylamino)pyrimidin-4-yl)thiazole-2-yl)-*N*-methylbenzamide; GIRK, G protein-coupled inwardly-rectifying potassium channel; GPCR, G protein-coupled receptor; iCa^{2+}, intracellular Ca^{2+} mobilization; IP_1, inositol phosphate accumulation; HYDIA, (1*S*,2*R*,3*R*,5*R*,6*S*)-2-amino-3-Hydroxy-bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid; JNJ-40068782, 3-cyano-1-cyclo-propylmethyl-4-(4-phenyl-piperidin-1-yl)-pyridine-2(1*H*)-one; L-AP4, L-2-amino-4-phosphonobutyrate; LSP4-2022, (2S)-2-amino-4-([[4-(carboxymethoxy)phenyl](hydroxy)methyl](hydroxy)phosphoryl)butanoic acid; LTD, long-term depression; LTP, long-term potentiation; LY341495, (2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; LY354740, (1*S*,2*R*,5*R*,6*S*)-2-amino-bicyclo[3.1.0]hexane-2,6-dicarboxylic acid; LY379268, (-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate; LY541850, (1*S*,2*S*,4*R*,5*R*,6*S*)-2-amino-4-methylbicyclo[3.1.0]hexane2,6-dicarboxylic acid; M-5MPEP, 2-(2-(3-methoxyphenyl)ethynyl)-5-methylpyridine; MPEP, 2-Methyl-6-(phenylethynyl)pyridine; mPEPy, 3-methoxy-5-(2-pyridinylethynyl)pyridine; MMPIP, 6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo [4,5-c]pyridine-4(5*H*)-one hydrochloride; NAM, negative allosteric modulator; NCFP, *N*-(4-chloro-2-[(1,3-dioxoisoindolin-2-yl)methyl)phenyl)picolinamide; PAM, positive allosteric modulator; pERK1/2, phosphorylated extracellular-signal regulated kinases 1 and 2; PET, positron emission tomography; PHCCC, N-phenyl-7-(hydroxyimino)cyclopropa[*b*] chromen-1a-carboxamide; TM, transmembrane;

R214127, 1-(3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-2-phenylethanone; RO4988546, 5-[7-Trifluoromethyl-5-(4-trifluoromethyl-phenyl)-pyrazolo[1,5-a]pyrimidin-3-ylethynyl]-pyridine-3-sulphonic acid; RO5488608, 3′-(8-Methyl-4-oxo-7-trifluoromethyl-4,5-dihydro-3H-benzo[*b*][1,4]diazepin-2-yl)-biphenyl-3-sulphonic acid; Ro 67-4853, butyl (9H-xanthene-9-carbonyl)carbamate; Ro 67-7476, (S)-2-(4-fluorophenyl)-1-(toluene-4-sulfonyl)pyrrolidine; VFT: venus flytrap domain; VU0092273, 3′-(8-Methyl-4-oxo-7-trifluoromethyl-4,5-dihydro-3H-benzo[*b*][1,4]diazepin-2-yl)-biphenyl-3-sulphonic acid; VU0155094, methyl 4-(3-(2-((4-acetamidophenyl)thio)acetyl)-2,5-dimethyl-1*H*-pyrrol-1-yl)benzoate; VU0366248, *N*-(3-chloro-2-fluorophenyl)-3-cyano-5-fluorobenzamide; VU0409551, 5-[(4-Fluorophenyl)carbonyl]-2-(phenoxymethyl)-4,5,6,7-tetrahydro[1,3]oxazolo[5,4-*c*]pyridine; VU0422288, *N*-(3-chloro-4-((5-chloropyridin-2-yl)oxy)phenyl)picolinamide.

Abstract

The metabotropic glutamate (mGlu) receptors are a group of eight family C G protein-coupled receptors that are expressed throughout the CNS and periphery. Within the CNS the different subtypes are found in neurons, both pre- and/or post-synaptically where they mediate modulatory roles and in glial cells. The mGlu receptor family provides attractive targets for numerous psychiatric and neurological disorders, with the majority of discovery programs focussed on targeting allosteric sites, with allosteric ligands now available for all mGlu receptor subtypes. However, the development of allosteric ligands remains challenging. Biased modulation, probe dependence and molecular switches all contribute to the complex molecular pharmacology exhibited by mGlu receptor allosteric ligands. In recent years we have made significant progress in our understanding of this molecular complexity coupled with an increased understanding of the structural basis of mGlu allosteric modulation.

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Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS), eliciting its effects via two distinct receptor classes. The ionotropic glutamate receptors are ligand-gated ion channels that mediate fast synaptic responses. The metabotropic glutamate (mGlu) receptors are G protein-coupled receptors (GPCRs) that play a modulatory role in the CNS. Ubiquitously expressed throughout the CNS in glia and neurons, found both pre and post synaptically, the mGlu receptors are involved in a broad range of physiological functions in the brain. Comprised of eight members, the mGlu receptor family is often sub-classified into three groups based on homology, pharmacology and G protein coupling. Group I includes $mGlu_1$ and $mGlu_5$ that preferentially couple to $G_{q/11}$ and are typically found postsynaptically. Group II, $mGlu_2$ and $mGlu_3$; and group III, $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$, members couple to $G_{i/o}$ and with the exception of $mGlu_6$, which is exclusively found in the retina, are predominantly located pre-synaptically. Multiple recent reviews have extensively discussed the roles of mGlu receptors in different neuronal circuits, modulating synaptic transmission, neuronal excitability and other cellular functions within the CNS (Anwyl, 1999; Bellone et al., 2008; Conn and Pin, 1997; Coutinho and Knopfel, 2002; Pinheiro and Mulle, 2008; Valenti et al., 2002) as well as physiology in the periphery (Julio-Pieper et al., 2011). Based on this understanding, targeting activation or inhibition of specific mGlu receptor subtypes may provide a therapeutic benefit for a range of psychiatric and neurological disorders. For neuropathic pain, inhibition of $mGlu_1$ is being pursued as a therapeutic intervention with success reported in pre-clinical models (Bennett et al., 2012; Mabire et al., 2005). Agonists of group II receptors have reached clinical trials for schizophrenia and anxiety with varying success (Dunayevich et al., 2008; Kinon et al., 2011; Patil et al., 2007; Schoepp et al., 2003). Group II mGlu receptors are also attractive targets for

addiction and depression (Chaki et al., 2013; Ma et al., 2007; Moussawi and Kalivas, 2010). Inhibitors of mGlu$_5$ have demonstrated efficacy in pre-clinical studies for anxiety, depression, and Parkinson's disease Levodopa-induced dyskinesias (Porter et al., 2005; Li et al., 2006; Belozertseva et al., 2007; Rylander et al., 2010; Hughes et al., 2012). Promisingly, multiple mGlu$_5$ inhibitors have reached (Berry-Kravis et al., 2009; Keywood et al., 2009; Zerbib et al., 2010) or are currently in phase II clinical trials (www.clinicalTrials.gov) with varying successes and failures for fragile X syndrome, Parkinson's disease Levodopa-induced dyskinesias, gastroesophogeal reflux disorder and migraine. On the other hand, activators and/or potentiators of mGlu$_5$ have efficacy in preclinical models for schizophrenia and cognition (Moghaddam, 2004; Noetzel et al., 2012a). Activation or potentiation of group III mGlu receptors, in particular mGlu$_4$, has therapeutic potential for Parkinson's disease (Jones et al., 2012; Marino et al., 2003), with mGlu$_4$ also a potential target for pain (Vilar et al., 2013) and autism (Becker et al., 2014).

Therapeutic targeting of mGlu's with allosteric modulators

The prevailing strategy for GPCR-based drug discovery has been to target the endogenous ligand binding site, referred to as an orthosteric site, to competitively block or mimic the actions of the endogenous ligand. However, multiple receptor families and transporters recognize glutamate, and as such, glutamate binding pockets are highly conserved such that orthosteric ligands often lack sufficient selectivity. Based on this and difficulties in optimizing glutamate analogs as drug candidates, mGlu receptor-based drug discovery programs have turned their attention to alternative strategies to develop more selective agents. One such approach is to target allosteric binding sites that are topographically distinct from the orthosteric site, such that the receptor can be simultaneously bound by more than one ligand. These ligands are referred to as allosteric

modulators and have the capacity to perturb the affinity and/or efficacy of an orthosteric ligand.

Since allosteric sites are often located in regions of the receptor that show greater diversity

between subtypes, allosteric modulators offer the potential for greater subtype selectivity than

their orthosteric counterparts. Allosteric modulators may potentiate or inhibit the affinity and/or

efficacy of an orthosteric ligand a property referred to as "cooperativity". A potentiator that has

positive cooperativity with the orthosteric ligand is a positive allosteric modulator (PAM); while

an inhibitor is a negative allosteric modulator (NAM). Cooperativity is saturable, therefore

allosteric modulators have the theoretical advantage of being safer in the case of overdose, as

there is a limit to the modulatory effect. A third class of ligands that interact with allosteric sites

but have neutral cooperativity with the orthosteric ligand are called neutral allosteric ligands

(NAL) and represent excellent pharmacological tools for dissecting allosteric modulator

pharmacology (Christopoulos et al., 2014). A third advantage of allosteric modulators is the

potential to maintain spatial and temporal aspects of receptor activation, i.e. modulation will only

occur when and where the orthosteric agonist is present. However, it is important to note that

allosteric ligands may possess intrinsic efficacy (either positive or inverse) in addition to, or

exclusive of, cooperativity with orthosteric ligands (Annoura et al., 1996; Duvoisin et al., 2010;

Gregory et al., 2013a; Gregory et al., 2012; Lavreysen et al., 2013; Litschig et al., 1999;

Niswender et al., 2008; Noetzel et al., 2012b; Rook et al., 2013). The first allosteric modulator

discovered for the mGlu family was CPCCOEt, an mGlu$_1$ NAM of multiple orthosteric agonists,

including glutamate (Annoura et al., 1996; Litschig et al., 1999). The past fifteen years has seen

the discovery of selective allosteric modulators for most of the mGlu family and for many

subtypes multiple chemotypes and different classes of allosteric ligands have been disclosed

(Tables 1-3).

Complexities of mGlu receptor allosteric modulator pharmacology

Stimulus-bias.   Despite definite success in the discovery of mGlu allosteric ligands, the pharmacology of allosteric modulators is inherently more complex than their orthosteric counterparts. The overall pharmacological effect of an allosteric ligand includes affinity and efficacy as well as the cooperativity between the allosteric and orthosteric ligands. With respect to ligand efficacy it has become increasingly accepted that occupancy and efficacy are not necessarily linearly linked. The concept that orthosteric ligands may differentially impact the G-protein coupling and other functional responses mediated by activation of the receptor has come to the fore in recent years (Figure 1). This phenomenon referred to as 'stimulus-bias' (although has had many other monikers including 'agonist-directed trafficking of receptor stimulus', 'functional selectivity' and 'biased agonism') has now been observed for many GPCRs, including $mGlu_1$, $mGlu_4$, $mGlu_7$ and $mGlu_8$ (Emery et al., 2012; Gregory et al., 2012; Jalan-Sakrikar et al., 2014). For example stimulus-bias is evident when comparing orthosteric agonists L-AP4 and LSP4-2022 with glutamate between Ca^{2+} mobilization and GIRK channel activation at a single group III subtype (Jalan-Sakrikar et al., 2014). Interestingly, when these orthosteric agonist bias profiles are compared between $mGlu_7$ and either $mGlu_4$ or $mGlu_8$ there is a complete reversal in the direction of bias for the same ligands across the same two pathways. This finding highlights the important concept that the endogenous ligand itself will have a 'natural bias', revealed in this study by differences between different group III mGlu subtypes. Stimulus-bias is thought to arise from ligands stabilizing different complements of receptor conformations that favour one suite of receptor activation outcomes over others. It is perhaps not surprising then that there is preliminary evidence for stimulus-bias when comparing allosteric agonist activity to that

with glutamate. For example, VU29 and CDPPB are PAM-agonists of $mGlu_5$ for both intracellular Ca^{2+} mobilization and pERK1/2 and notably have higher intrinsic efficacy for pERK1/2 relative to Ca^{2+} signalling (Gregory et al., 2012). Glutamate, however, shows the reverse, with higher efficacy for Ca^{2+} signalling over pERK1/2. Similar observations have been reported for $mGlu_1$ PAM-agonists relative to glutamate between Ca^{2+} mobilization and cAMP accumulation (Sheffler and Conn, 2008). Moreover, many of the $mGlu_5$ PAM-agonists for pERK1/2 are super-agonists, capable to achieving higher maximal responses than glutamate (Gregory et al., 2012). Although it should be noted that both ligand and system kinetics may contribute to these observations of biased agonism. Negative allosteric modulators are also often reported to be inverse agonists (Tables 1-3), however, these observations are assay dependent, likely a result of receptor constitutive activity and assay resolution. Comprehensive investigation into stimulus-bias that factors in these contributing mechanisms and confounds is sorely needed.

An additional mechanism that may also contribute to observations of stimulus-bias relates to receptor compartmentalisation. For example, a large pool of intracellular $mGlu_5$ is located on the nuclear membrane (Jong et al., 2005; Kumar et al., 2008). Activation of $mGlu_5$ at the cell surface versus the nuclear membrane results in the activation of different signalling cascades, as demonstrated through the use of permeable/transported and impermeable/non-transported orthosteric and allosteric ligands of $mGlu_5$ (Jong et al., 2009; Kumar et al., 2012). Furthermore, activation of cell surface $mGlu_5$ leads to long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus, whereas activation of intracellular $mGlu_5$ only mediates LTD (Purgert et al., 2014). These findings highlight that stimulus-bias may also arise from a differential ability of different chemotypes to access distinct subcellular compartments where

9

receptors may be located. Therefore the lipophilicity or transportability of an allosteric ligand may impact the overall functional outcome observed.

Biased modulation. A related concept to biased agonism is the phenomenon of biased modulation (Figure 1). If we consider that the conformations engendered by the simultaneous occupation of a receptor with an orthosteric and allosteric ligand that give rise to cooperativity differ from those engendered by binding of orthosteric ligand alone. Therefore this raises the possibility that the 'natural bias' of an endogenous ligand may be influenced by an allosteric modulator. Conceptually, this may manifest as a change in the magnitude and/or direction of the cooperativity factors that describe the allosteric interaction depending on the receptor activation outcome measured. Alternatively, allosteric modulators may also show differential apparent affinity estimates depending upon the receptor activation outcome measured. In recent years, there have been a growing number of reports of biased mGlu allosteric modulation. For example at $mGlu_5$, CPPHA and DFB are both PAMs for glutamate-mediated Ca^{2+} mobilization in rat cortical astrocytes; however, DFB potentiates whereas CPPHA inhibits DHPG-stimulated pERK1/2 (Zhang et al., 2005). Further, multiple $mGlu_5$ modulators have lower positive cooperativity for glutamate-mediated pERK1/2 versus intracellular Ca^{2+} mobilization (Gregory et al., 2012). Recent studies suggest that biased modulation has relevance in more physiological systems, raising the possibility that biased modulation may have clinical implications. For example, a novel $mGlu_5$ selective modulator from the same chemotype as CPPHA, NCFP, potentiated DHPG-induced depolarization of subthalamic nucleus neurons but lacked the ability to potentiate induction of long-term potentiation or long-term depression in the hippocampus (Noetzel et al., 2013). Importantly, another $mGlu_5$ allosteric modulator, VU0092273 that shared

a similar pharmacological mGlu$_5$ PAM profile in recombinant cells, potentiated all three slice electrophysiological responses (Noetzel et al., 2013), suggesting that NCFP is showing true biased modulation in a native environment. Another novel mGlu$_5$ allosteric modulator, VU0409551, was recently disclosed that potentiates multiple glutamate-induced mGlu$_5$ signalling responses in cell-based assays, but has no efficacy in modulating hippocampal LTP or NMDA receptor currents (Rook et al., 2015). However, VU0409551 has efficacy in preclinical rodent models of psychosis and cognition (Rook et al., 2015), suggesting that biased allosteric modulators of mGlu$_5$ can possess preclinical efficacy.

Biased modulation is not limited to mGlu$_5$ PAMs, both positive and negative modulators of group III receptors have been suggested to induce biased receptor signaling (Niswender et al., 2010a; Jalan-Sakrikar et al., 2014). The extent and prevalence of biased modulators is likely to be highly underappreciated. This is due to the fact that the vast majority of mGlu allosteric modulators are characterized in a single cell-based assay, generally Ca^{2+} mobilization, to classify and rank compounds on the basis of potency. Further, the therapeutic relevance of biased pharmacology has yet to be realized, however, it is tempting to speculate that unappreciated bias may contribute to differential efficacy of allosteric modulators in behavioural models. For example DPFE, an mGlu$_5$ PAM of glutamate, requires far lower doses for efficacy in cognitive models compared with reversing amphetamine-induced hyperlocomotion, a model for anti-psychotic efficacy (Gregory et al., 2013a). Moreover, there is the potential that biased modulation may be exploited to develop therapeutics that are not only subtype selective but also pathway selective; modulating receptor responses that yield therapeutic effects and avoiding those that give rise to on-target adverse effects.

Probe dependence. A related phenomenon to biased modulation is that of probe dependence, where allosteric interactions are dependent upon the chemical nature of the two ligands under investigation. The result being that the direction and/or magnitude of cooperativity may be significantly different. There are multiple examples of probe dependence at the group II mGlu receptors. BINA potentiates Ca^{2+} oscillations in cortical neurons induced by the mGlu$_2$ selective orthosteric agonist, LY541850, but not the mixed group II orthosteric agonist, LY379268 (Sanger et al., 2012; Sanger et al., 2013). JNJ-40068782, a PAM-agonist of glutamate-stimulation of [^{35}S]GTPγS binding and Ca^{2+} mobilization at mGlu$_2$, has neutral cooperativity with the orthosteric antagonist radioligand [^{3}H]LY341495 and positive cooperativity with the orthosteric agonist radioligand [^{3}H]DCG-IV (Lavreysen et al., 2013). For group I and III mGlu receptors there are also select examples of probe dependence. Affinity modulation occurs between the orthosteric agonist quisqualate and mGlu$_5$ PAMs (Bradley et al., 2011); however, a separate study found no evidence for affinity modulation (i.e. neutral cooperativity) between glutamate and mGlu$_5$ PAMs of glutamate in functional assays (Gregory et al., 2012). At mGlu$_4$ and mGlu$_7$, VU0155094 and VU0422288 have different degrees of cooperativity depending upon both the orthosteric agonist and the assay endpoint used (Jalan-Sakrikar et al., 2014). For example, at mGlu$_7$ VU0155094 is neutral with respect to the apparent affinity of LSP4-2022 and L-AP4 but a positive modulator of glutamate apparent affinity (Jalan-Sakrikar et al., 2014). Clearly, probe dependence is evident for the mGlu family and should be taken into consideration when designing screening paradigms and characterizing mGlu allosteric modulators. The choice of assay and/or probe can result in a very different pharmacological profile. This is of particular importance for mGlu$_7$ where L-AP4 is most commonly used as the orthosteric agonist given the low affinity of this subtype for glutamate (in the millimolar range). Moreover, extracellular Ca^{2+}

12

ions can activate $mGlu_1$ influencing the responses to orthosteric agonists, indicating that two endogenous orthosteric agonists need to be considered with respect to probe dependence but also that different endogenous ligands can allosterically interact at $mGlu_1$ (Jiang et al., 2014). Importantly, extracellular Ca^{2+} activation of $mGlu_1$ can be modulated by small molecule positive and negative allosteric modulators of glutamate (Ro67-4853 and CPCCOEt) (Jiang et al., 2014). Probe dependence can also be an important consideration when assessing allosteric modulators in native systems (brain slice electrophysiology or primary neuronal cultures) where surrogate agonists are typically used to selectively activate a particular mGlu subtype and avoid the confounds of glutamate activating ionotropic glutamate receptors and being subject to endogenous transporters.

Protein-protein interactions. Another important consideration in assessing allosteric modulator pharmacology in native systems is the fact that the mGlu family are obligate dimers (El-Moustaine et al., 2012) that also have the capacity to form heteromers with other subtypes (Doumazane et al., 2011; Kammermeier, 2012; Sevastyanova and Kammermeier, 2014; Yin et al., 2014) as well as with other unrelated GPCRs (Cabello et al., 2009; Gama et al., 2001; Gonzalez-Maeso et al., 2008). Unsurprisingly, such complexity in receptor configurations can influence the pharmacology of small molecules. Heterodimerization of $mGlu_4$ and $mGlu_2$ has differential effects on the pharmacology of different allosteric ligands (Kammermeier, 2012; Yin et al., 2014). For example at the $mGlu_{2/4}$ heteromer PHCCC has lower cooperativity with L-AP4, whereas Lu AF21934 and VU0155041 have decreased affinity but higher cooperativity, compared with $mGlu_4$ homodimers (Yin et al., 2014). This perturbation in the pharmacology of allosteric ligands has consequences in vivo at synapses where $mGlu_2$ and $mGlu_4$ are co-

expressed. VU0155041 and Lu AF21934 are both effective at potentiating the actions of L-AP4 at corticostriatal synapses (Gubellini et al., 2001; Yin et al., 2014). However, PHCCC does not potentiate the actions of L-AP4 at corticostriatal synapses (Yin et al., 2014), despite being an efficacious mGlu$_4$ PAM of L-AP4-induced responses at other synapses (Jones et al., 2008; Marino et al., 2003; Valenti et al., 2005). Direct protein-protein interactions are not necessarily required for a co-expressed receptor to impact the pharmacological effect of an mGlu ligand. Co-expression and co-activation of G$_q$-coupled receptors enhances mGlu$_4$ stimulus-response coupling to orthosteric and allosteric agonists in intracellular Ca^{2+} mobilization but not cAMP accumulation assays (Yin et al., 2012). It is apparent that cellular context, measure of receptor function and co-expression of other GPCRs can impact the pharmacology of small molecule allosteric ligands, leading to unanticipated consequences in native systems. However, this complexity extends further to the structure-activity relationships of allosteric modulators themselves.

Molecular switches. Given the complexities of allosteric modulation surrounding stimulus-bias and probe dependence it is perhaps not surprising that structure-activity relationships (SAR) for mGlu receptor allosteric modulators are notoriously challenging. SAR is often reported to be 'steep' with minimal changes resulting in a complete loss of activity (Conn et al., 2014; Lindsley, 2014). In addition, mGlu allosteric chemotypes often show 'molecular switches' where the same scaffold can give rise to modulators with negative, positive and neutral cooperativity with the same orthosteric agonist (Wood et al., 2011). The phenomenon was first observed during the development of DFB, an mGlu$_5$ PAM for glutamate-mediated intracellular Ca^{2+} mobilization (O'Brien et al., 2003). Recently, this propensity for molecular switching was

14

exploited with the development of a photoswitchable allosteric modulator for mGlu$_5$, where negative cooperativity between with glutamate could be controlled by exposure to different light wavelengths (Pittolo et al., 2014). However, unexpected changes in cooperativity continue to be a challenge for medicinal chemistry programs and interpretation of SAR (Hammer et al., 1980; Lamb et al., 2011; Rodriguez et al., 2010; Sams et al., 2011; Schann et al., 2010; Sharma et al., 2009; Sheffler et al., 2012; Zhou et al., 2010). These difficulties in SAR interpretation can in part be attributed to a paucity in our understanding of the structural basis of allosterism. The majority of discovery programs rely on modulator potency curves in the presence of a single concentration of agonist in order to classify allosteric ligands as active/inactive and ascribe either positive or negative cooperativity. However, potency represents a composite measure of efficacy, affinity and cooperativity (Gregory et al., 2012). As such, modifications to a chemotype may influence any one of these three parameters. Therefore, more quantitative analysis of allosteric chemotypes is required to truly understand how SAR relates to efficacy, affinity and cooperativity. Going forward a key challenge will be coupling SAR interpretation as we improve our understanding of the structural basis of allosterism.

Structural basis of allosteric modulation

The mGlu's are family C GPCRs typified by their large extracellular N-terminal Venus flytrap domain (VFT), linked via a cysteine-rich domain to the seven transmembrane spanning α-helical domains (7TMs) that are the hallmark of the GPCR superfamily. The orthosteric site is located in the N-terminal domain and to date all small molecule allosteric modulators are thought to bind within the 7TMs. Historically, receptor chimeras proved valuable to 1) definitively demonstrate an allosteric mechanism of action for small molecule ligands and 2) to localise allosteric binding

15

sites to the 7TM domains. Chimeric constructs swapping the VFT of one subtype onto another, or even exchange with non-mGlu family C GPCRs have been utilised. Chimeras between mGlu$_1$ and mGlu$_5$, mGlu$_4$ and the Calcium-sensing receptor were used to validate the allosteric mechanism of action of mGlu$_1$ selective allosteric modulator CPCCOEt (Brauner-Osborne et al., 1999; Gasparini et al., 2001; Litschig et al., 1999). Subsequent studies used mGlu$_{1/5}$ chimeras to demonstrate MPEP (an mGlu$_5$ selective NAM) and multiple mGlu$_1$ PAMs recognised an overlapping binding site with that of CPCCOEt (Knoflach et al., 2001; Pagano et al., 2000). Chimeric constructs were also used to validate an allosteric mechanism of action for PHCCC, AMN082 and BAY36-7620 at mGlu$_4$, mGlu$_7$ and mGlu$_1$ respectively (Carroll et al., 2001; Maj et al., 2003; Mitsukawa et al., 2005). Another approach to achieve gross localisation of allosteric binding sites is through the generation of N-terminal truncated receptors. The extracellular VFT can be removed to generate a "headless" mGlu, yielding a receptor construct that does not respond to glutamate, but can couple to intracellular signalling pathways (Goudet et al., 2004). Headless mGlu$_1$ and mGlu$_5$ are constitutively active such that negative allosteric modulators behave as inverse agonists (Chen et al., 2007; Goudet et al., 2004; Suzuki et al., 2007). Further, headless mGlu$_5$ can be stimulated by small molecule positive allosteric modulators that do not necessarily have intrinsic efficacy at the full-length receptor (Chen et al., 2007; Goudet et al., 2004; Noetzel et al., 2013). Interestingly, NCFP had little or no efficacy for stimulating headless mGlu$_5$, despite having a similar magnitude of positive cooperativity as VU0092273, which can activate headless mGlu$_5$ (Noetzel et al., 2013). The headless mGlu 7TM is thought to behave as a monomer (Goudet et al., 2004), so perhaps this discrepancy relates to receptor dimerization in the full-length receptor being important either for NCFP binding or cooperativity.

Two recent x-ray crystal structures were solved for the 7TMs of group I mGlu's with allosteric modulators bound (PDB ID's: 4OO9 & 4OR2; (Dore et al., 2014; Wu et al., 2014)). Prior to this, research relied on family A 7TM templates to perform homology modelling and interpret site-directed mutagenesis data. Despite low sequence homology (<20%), these approaches proved fruitful, localising allosteric binding pockets of $mGlu_1$, $mGlu_2$, $mGlu_4$ and $mGlu_5$ to a region analogous to that of the biogenic amine binding sites in family A GPCRs (Gregory et al., 2014; Gregory et al., 2013b; Lundstrom et al., 2011; Malherbe et al., 2003a; Malherbe et al., 2003b; Molck et al., 2012; Ott et al., 2000; Pagano et al., 2000; Rovira et al., 2015). In light of the new crystal structures, while alignments and secondary structure prediction of TM5-7 deviated from that observed in the $mGlu_1$ and $mGlu_5$ structures, the general localisation of the pocket was consistent with earlier reports. Many residues that had previously been identified in mutagenesis-based studies were recapitulated in the crystal structures (discussed in detail below). Ongoing research continues to refine our understanding of binding pocket/s within the 7TM bundle in particular in relation to determinants that govern selectivity, affinity, cooperativity and efficacy of allosteric modulators.

The common mGlu allosteric site

The concept for a common allosteric site between mGlu subtypes arises from three lines of evidence: 1) allosteric modulators that lack subtype selectivity; 2) competitive interactions between diverse chemotypes and allosteric radioligands; 3) overlapping binding pockets based on mutagenesis studies and observed in x-ray crystal structures. While attaining selectivity with allosteric modulators is considerably more effective than for orthosteric ligands, there are a number of examples of allosteric modulators that have efficacy at multiple subtypes. MPEP is

both a negative allosteric modulator of glutamate at mGlu$_5$ and a positive allosteric modulator of L-AP4 at mGlu$_4$ (Mathiesen et al., 2003). Conversely, DFB and CPPHA, which potentiate glutamate signalling at mGlu$_5$ are weak negative allosteric modulators of glutamate at mGlu$_4$ and mGlu$_8$ (O'Brien et al., 2003; O'Brien et al., 2004). In addition, PHCCC, an mGlu$_4$ PAM for glutamate and L-AP4, negatively modulates glutamate at mGlu$_1$ (Annoura et al., 1996; Maj et al., 2003). The recent report of a molecular switch to mGlu$_3$ NAM from an mGlu$_5$ PAM scaffold (Sheffler et al., 2012), reveals that the subtleties of interactions within this common pocket that dictate subtype selectivity are not limited to cross-over between group I and group III mGlu receptors alone. This lack of selectivity within the common binding pocket is also associated with differential cooperativity between subtypes, highlighting that the small molecule interactions within the common allosteric site can elicit either positive or negative cooperativity.

Identification of allosteric ligands that interact with this common mGlu allosteric site has been facilitated by the development of radiolabelled mGlu allosteric modulators. Multiple selective tritiated and positron emission tomography (PET) allosteric ligands have been developed for mGlu$_1$, mGlu$_2$ and mGlu$_5$ (Ametamey et al., 2007; Anderson et al., 2003; Anderson et al., 2002; Baumann et al., 2010; Cosford et al., 2003b; Gasparini et al., 2002; Kohara et al., 2005; Lavreysen et al., 2003; Malherbe et al., 2006; Treyer et al., 2007). Through the use of inhibition binding assays with these allosteric ligands a direct assessment of competitive versus non-competitive binding modes can be performed (Chen et al., 2007; Hemstapat et al., 2006; Kinney et al., 2005; Lavreysen et al., 2003; Lavreysen et al., 2004; Lundstrom et al., 2011; Malherbe et al., 2006). Such experiments are often the first step towards identifying allosteric modulators that bind to alternative allosteric sites ((Chen et al., 2008; Hammond et al., 2010) discussed in further

detail later). However, competition binding experiments may also be used to identify neutral allosteric ligands that interact with the common allosteric site but have neutral cooperativity with agonist/s in functional screening assays (Rodriguez et al., 2005; Sams et al., 2011).

Early mutational studies investigating group I mGlu receptor allosteric binding pockets exploited non-conserved residues within the 7TMs. Substitution of mGlu$_1$ non-conserved amino acids onto the equivalent positions in mGlu$_5$, and vice versa, resulted in gain-of-function of selective allosteric modulators (Goudet et al., 2005; Knoflach et al., 2001; Litschig et al., 1999; Pagano et al., 2000; Surin et al., 2007). These mutagenesis studies pointed to a common location for allosteric binding pockets for group I mGlu receptors. Additional studies have shown that residues at positions 3.40 and 7.38 are important for allosteric modulation of both mGlu$_1$ and mGlu$_5$ (Ballesteros-Weinstein numbering (Ballesteros and Weinstein, 1995) adopted from mGlu$_1$ structural alignment to family A GPCRs in Wu et al., 2014; (Fukuda et al., 2009; Gregory et al., 2014; Gregory et al., 2013b; Gregory et al., 2012; Malherbe et al., 2006; Malherbe et al., 2003a; Malherbe et al., 2003b; Muhlemann et al., 2006; Suzuki et al., 2007; Turlington et al., 2014; Wu et al., 2014). Subsequently, key residues for the potency and/or binding of mGlu$_1$, mGlu$_2$, mGlu$_4$ and mGlu$_5$ allosteric modulators from diverse chemical scaffolds have been mapped to TMs 3-7. Across the four subtypes multiple residues have repeatedly been reported as contributing to allosteric modulation. For all four subtypes, residues in positions 3.36, 5.43 and 6.48 are crucial for both positive and negative allosteric modulators (Fukuda et al., 2009; Gregory et al., 2014; Gregory et al., 2013b; Gregory et al., 2012; Lundstrom et al., 2011; Malherbe et al., 2003a; Malherbe et al., 2006; Molck et al., 2012; Muhlemann et al., 2006; Pagano et al., 2000; Rovira et al., 2015; Turlington et al., 2014; Wu et al., 2014). Position 7.45 has also been implicated in

mGlu$_4$ and mGlu$_5$ allosteric modulation (Gregory et al., 2014; Gregory et al., 2013b; Molck et al., 2012; Rovira et al., 2015; Turlington et al., 2014); there are no reports investigating the influence of this amino acid on selective allosteric modulators of mGlu$_1$ or mGlu$_2$. Between group I mGlu receptors and mGlu$_2$, three residues are common: 5.44, 5.47 and 6.55 (Fukuda et al., 2009; Gregory et al., 2014; Gregory et al., 2013b; Gregory et al., 2012; Hemstapat et al., 2006; Knoflach et al., 2001; Lundstrom et al., 2011; Malherbe et al., 2006; Malherbe et al., 2003b; Molck et al., 2012; Muhlemann et al., 2006; Rowe et al., 2008; Schaffhauser et al., 2003). Group 1 receptors and mGlu$_4$ share a common determinant in F6.51 (Fukuda et al., 2009; Gregory et al., 2014; Malherbe et al., 2003a; Malherbe et al., 2006; Malherbe et al., 2003b; Muhlemann et al., 2006; Rovira et al., 2015; Suzuki et al., 2007). Two residues at the top of TM3 (3.28 and 3.29) are important for allosteric modulation of both mGlu$_2$ and mGlu$_4$ (Lundstrom et al., 2011; Rovira et al., 2015), with position 3.29 also implicated in the activity of some mGlu$_5$ allosteric modulators (Malherbe et al., 2006; Malherbe et al., 2003b; Molck et al., 2012). Therefore these mutagenesis data reinforce the notion of overlapping binding pockets across the mGlu family.

Comparison of the recently solved mGlu$_1$ and mGlu$_5$ crystal structures reveals a similar architecture for the backbone of the transmembrane-spanning helices (Figure 2A). Overlay of the 7TM binding pockets for FITM and mavoglurant in the mGlu$_1$ and mGlu$_5$ respectively confirmed that these subtypes have overlapping allosteric binding pockets within the 7TMs (Dore et al., 2014; Wu et al., 2014). Examination of binding pockets for mavoglurant and FITM reveals that mavoglurant binds deeper within the 7TM bundle (Figure 2B). Side chain conformations of two key non-conserved residues in TM3 of mGlu$_5$: P655$^{3.36}$ and S658$^{3.39}$ (mGlu$_1$ equivalents are: S668 and C671 respectively) create greater space within the pocket that

20

allows mavoglurant to bind deeper within the 7TM bundle (Figure 2C). These crystal structures of closely related mGlu receptors have yielded structural insights with respect to how selectivity can be achieved in the common mGlu allosteric site.

Delineating affinity versus cooperativity determinants

The vast majority of research on allosteric modulator SAR and structure-function has relied on measures of allosteric modulator potency; however, potency values represent a composite of affinity and cooperativity. In recent years the application of rigorous analytical pharmacological methods has facilitated delineation of the impact of mutations on allosteric modulator affinity, cooperativity and efficacy based on functional interaction data alone. By combining this research with earlier studies using radiolabelled allosteric modulators where affinity was determined or a loss of binding was observed we can start to map affinity determinants in the binding pocket. Residues known to be crucial affinity determinants are summarised in Table 4 (also see Supplementary Table 1) where the most well-characterised representatives from individual allosteric modulator scaffolds were included. These affinity determinants are also mapped onto the $mGlu_1$ and $mGlu_5$ structures (Figure 2A, B), Significant overlap is observed between $mGlu_1$ and $mGlu_5$, although there is limited affinity data for $mGlu_1$. For $mGlu_5$, six residues have been implicated in the binding of most PAMs and NAMs: $P655^{3.36}$, $Y659^{3.40}$, $T781^{6.44}$, $W785^{6.48}$, $S809^{7.45}$ and $A810^{7.46}$ within the common allosteric site. Mapping the known affinity determinants for MPEP, the most well studied $mGlu_5$ allosteric ligand with respect to structure-function, onto the $mGlu_5$ crystal structure yields additional insights (Figure 4; (Gregory et al., 2014; Gregory et al., 2013b; Gregory et al., 2012; Malherbe et al., 2003b; Pagano et al., 2000)). Coloring residues on the basis of the impact of mutations reveals that substitutions that cause a

moderate (<10-fold) reduction in affinity are located on the fringes of the mavoglurant binding pocket or are immediately adjacent to key residues (Figure 4, Supplementary Table 1).

In addition to effects on affinity, molecular determinants for a large set of allosteric modulators spanning multiple scaffolds with both positive and negative cooperativity have been explored at mGlu$_5$. Some residues that are important for modulator affinity also contribute to cooperativity; however, there are select amino acids that only influence cooperativity. Negative cooperativity with glutamate of multiple allosteric modulators at mGlu$_5$ is decreased at P655[3.36] mutants (Gregory et al., 2014; Gregory et al., 2013b). Additionally mutations in TM3-7 (Y659[3.40], N747[5.47], T781[6.44], W785[6.48], S809[7.45]; (Gregory et al., 2014; Molck et al., 2012)) reduced cooperativity for negative modulators of glutamate at mGlu$_5$. Notably, in mGlu$_1$ mutations of T794[6.44] and S822[7.45] had no effect on the interaction between glutamate and FITM (Wu 2014), suggesting that the structural determinants that govern cooperativity can be subtype selective even for conserved amino acids.

Negative cooperativity with glutamate at mGlu$_5$ was increased for M-5MPEP and/or VU0366248 when mutations were introduced to S658[3.39], P743[5.43], G748[5.48] and V789[6.52] (Gregory et al., 2014). Both M-5MPEP and VU0366248 have limited negative cooperativity at the wild type receptor, with saturating concentrations unable to completely abolish the glutamate response (Felts et al., 2010; Rodriguez et al., 2005). The cooperativity of other mGlu$_5$ negative allosteric modulators tested at these mutations was not discernibly different to wild-type (Gregory et al., 2014). However, given that these all have strong negative cooperativity at wild-type, fully abolishing the maximal glutamate response, further increases in negative cooperativity were likely beyond the limits of detection. For P743[5.43] this increase is opposite to

MOL #97220

that observed for the equivalent mGlu$_1$ mutation (Wu et al., 2014), again highlighting the subtype selective nature of cooperativity. Interestingly, two point mutations (N747[5.47] and S809[7.45]) had differential effects on allosteric ligands, actually increasing negative cooperativity of M-5MPEP and VU0366248 respectively (Figure 3C; Gregory et al., 2014). It is clear that the amino acids within the common allosteric site can differentially contribute to affinity and the manifestation of negative cooperativity in a manner that is very much ligand dependent.

With respect to positive allosteric modulators, no mutations have yet been demonstrated to unequivocally reduce cooperativity. However, there are multiple examples of mutations that increase the magnitude of positive cooperativity with glutamate (Figure 3C). Shared with the low cooperativity negative modulators, mutation of P743[5.43] and V789[6.52] also increased positive cooperativity of diverse allosteric modulators with glutamate at mGlu$_5$ (Gregory et al., 2014; Gregory et al., 2013b). Four residues (L744[5.44], Y792[6.55], A810[7.46] and C816[7.52]) are implicated in positive but not negative cooperativity (Gregory et al., 2014; Gregory et al., 2013b). Interestingly, compared to negative allosteric modulators, mutation of T781[6.44] and W785[6.48] had differential effects, increasing positive cooperativity with glutamate at mGlu$_5$ (Gregory et al., 2014; Gregory et al., 2013b). The complicated nature of cooperativity determinants is further highlighted through evidence of mutations that engender 'molecular switches' in allosteric modulator cooperativity (Figure 3D). Substitution of Y6.51 at mGlu$_5$ switches DFB from a PAM of glutamate to a NAM, whereas the reverse is true for YM298198 at mGlu$_1$ (Fukuda et al., 2009; Muhlemann et al., 2006). Multiple additional point mutations in TMs 3, 6 and 7 can also switch acetylenic PAMs to have either neutral or negative cooperativity with glutamate at mGlu$_5$ (Gregory et al., 2013b; Turlington et al., 2014). Mutation of conserved W785[6.48] in mGlu$_5$ can

23

switch the cooperativity of select NAMs of glutamate to positive (Gregory et al., 2014). Collectively, these data highlight the subtleties and complexities of interactions within the common allosteric site that dictate whether or not the binding of a ligand will result in enhancement or inhibition of mGlu receptor function. Further, developing a better understanding of the dynamic ligand-receptor interactions that give rise to cooperativity will likely improve interpretation of allosteric modulator SAR and perhaps inform as to why 'molecular switches' are prevalent for some chemical scaffolds.

Additional mGlu allosteric sites

As mentioned earlier, the development of radiolabelled allosteric ligands has identified allosteric ligands that are non-competitive. The first such ligand identified was CPPHA, a group I mGlu selective positive allosteric modulator of glutamate. CPPHA is unable to fully displace the mGlu$_5$ radioligands [^{3}H]mPEPy and [^{3}H]MPEP or compete with [^{3}H]R214127 for binding to mGlu$_1$ (Bradley et al., 2011; Chen et al., 2008; Noetzel et al., 2013; O'Brien et al., 2004). Another structurally related selective mGlu$_5$ PAM of glutamate, NCFP, is also thought to bind to this alternate allosteric site (Noetzel et al., 2013). Potentiation of glutamate by both NCFP and CPPHA is non-competitively inhibited by the neutral allosteric ligand, 5MPEP (Chen et al., 2008; Noetzel et al., 2013). This indicates that the second allosteric site is conformationally linked to the common allosteric site such that ligand binding to these sites can allosterically influence one another. Isoleucine substitution of F1.42 reduces potentiation by CPPHA (both mGlu$_1$ and mGlu$_5$) and NCFP (mGlu$_5$ only) (Chen et al., 2008; Noetzel et al., 2013). Interestingly, NCFP also loses either affinity or the capacity to allosterically modulate [^{3}H]mPEPy binding and has very little efficacy as an agonist at an N-terminus truncated mGlu$_5$

(Noetzel et al., 2013). This suggests that the intact full-length receptor is required for this alternate allosteric site and/or receptor dimerization is also necessary. Also for mGlu$_5$, VU0357121 and VU0400100 do not completely displace [^{3}H]mPEPy binding (Gregory et al., 2012; Hammond et al., 2010; Rodriguez et al., 2012). Multiple mGlu$_1$ PAM scaffolds have also been flagged as binding to alternate allosteric site/s based on no or incomplete displacement of the allosteric radioligand [^{3}H]R214127 (Hemstapat et al., 2006). Overall, these data strongly support the hypothesis that multiple allosteric sites are present on the group I mGlu receptors.

In the absence of an allosteric radioligand, multiple allosteric sites have also been proposed for mGlu$_4$. The concentration-response curve for VU0155041 as an allosteric agonist at mGlu$_4$ was unaffected by co-application of PHCCC, an mGlu$_4$ PAM of glutamate and L-AP4 (Niswender et al., 2008). Recent mutational analysis of mGlu$_4$ allosteric modulators suggests that these two allosteric ligands interact with the common allosteric pocket made up of TM3-7 but in a non-overlapping manner (Rovira et al., 2015). Based on homology modelling and mutational data, PHCCC is proposed to bind deep within the allosteric site, analogous to the binding pose of mavoglurant in the mGlu$_5$ structure (Rovira et al., 2015). On the other hand, VU0155041 binds higher in the pocket, interacting with residues at the very top of the TM domains as well as extracellular loops 1 and 2 (Rovira et al., 2015). This suggests that two allosteric ligands can be accommodated simultaneously by mGlu$_4$; however, at least for PHCCC and VU0155041, there is neutral cooperativity between the binding of these ligands. Interestingly, VU0415374 appears to be bitopic, with homology modelling proposing it can span both binding sites. It is tempting to speculate that the reason VU0415374 has ten-fold (or more) higher apparent affinity than other mGlu$_4$ positive allosteric modulators is due to its ability to engage more residues within the pocket. Further, it is interesting to consider that CPPHA has weak activity at mGlu$_4$ (Chen et al.,

2008), as such there is the possibility that allosteric ligands may interact with mGlu$_4$ in three distinct binding modes. The extensive literature surrounding orthosteric and allosteric binding pockets of class A GPCRs may offer some insights into the location of alternative allosteric sites (Christopoulos, 2014). As confirmed in the recent co-crystal structure of the M2 muscarinic acetylcholine receptor, class A GPCR allosteric modulators recognise a binding pocket defined by the top of the TMs and the second and third extracellular loops (Kruse et al., 2013). Given the parallels between the common allosteric site of class C GPCRs and the orthosteric site in class A GPCRs, there is the possibility that alternate allosteric sites in class C GPCRs may also involve the extracellular surfaces of the receptor. Allosteric modulators that interact with different binding pockets are likely to engender a different complement of receptor conformational states than those that interact with the common allosteric site. Therefore the functional consequences of allosteric modulation through different binding pockets are likely to be divergent. Indeed, CPPHA and related compounds have biased pharmacology at mGlu$_5$ when compared to common site modulators (Noetzel et al., 2013; Zhang et al., 2005). We are just beginning to scratch the surface with respect to our understanding of what drives efficacy, cooperativity and affinity through the different allosteric sites across the entire mGlu family.

Concluding remarks

Since the discovery of the first mGlu receptor allosteric modulator in 1996, the mGlu allosteric modulator pharmacological toolbox has continued to expand with selective allosteric ligands now available for the majority of mGlu family members. This toolbox includes chemically diverse allosteric ligands that may possess positive, negative or neutral cooperativity and/or positive or inverse agonism. At least two allosteric sites have been proposed, one of which is

shared across mGlu receptor subtypes, but can still be selectively targeted. As to which allosteric site is best targeted for therapeutic efficacy remains unknown. Given the complexity introduced by the phenomenon of biased modulation, the optimal allosteric ligand pharmacological profile and the relative contribution of affinity, efficacy and cooperativity will likely be both pathophysiology and mGlu subtype dependent. With two x-ray crystal structures of the 7TMs for group I mGlu receptors now available, the field is now entering an exciting new era in our understanding of the dynamic interactions that govern allosteric ligand affinity, cooperativity and efficacy.

MOL #97220

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MOL #97220

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Footnotes

b) Reprint requests should be addressed to P. Jeffrey Conn, Department of Pharmacology & Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University Medical Center, 1215 Light Hall, 2215-B Garland Ave, Nashville, TN, USA, 37232-0697; jeff.conn@vanderbilt.edu

Legends for Figures

Figure 1: Schematic depicting the concepts of biased agonism and modulation.
A) The endogenous ligand (blue triangle) activates the receptor resulting in intracellular signal transduction. However, not all pathways will be activated to the same extent, this is the natural bias of the receptor/system. **B**) A surrogate orthosteric agonist (red triangle) may engender a different suite of active receptor conformations resulting in biased signalling when compared to the endogenous ligand, in this case the strength of signalling to response 1 versus 2 is reversed. **C**) The binding of an allosteric modulator (orange star) potentiates the response to the endogenous agonist for all signal pathways to the same extent. **D**) In contrast, a biased allosteric modulator (purple star) may have different magnitude or direction on cooperativity depending on the response measured, in this case, response 1 is potentiated while response 2 is inhibited.

Figure 2: Comparison of allosteric pockets within the 7TM domains of mGlu$_1$ and mGlu$_5$.
A) X-ray crystal structures of 7TMs of mGlu$_5$ (grey, PDB ID: 4OO9) and mGlu$_1$ (teal, PDB ID: 4OR2) were aligned using MacPyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). **B**) Inside surface rendering of the allosteric binding pockets of mavoglurant (black) in mGlu$_5$ (grey) and FITM (blue) in mGlu$_1$ (teal). **C**) Zoomed in image of the bottom of the allosteric pockets with mavoglurant shown. Two amino acids differ between mGlu$_1$ and mGlu$_5$ in this region, the differences in the side chains (depicted as sticks) create more space in mGlu$_5$, allowing mavoglurant to bind deeper within the 7TM bundle. All images created using MacPyMOL.

Figure 3: Affinity and cooperativity determinants for allosteric modulation of metabotropic glutamate receptors.
A) X-ray crystal structure of mGlu$_1$ 7TMs with FITM (rendered in blue sticks) bound (PDB ID: 4OR2) with known mGlu$_1$ allosteric ligand affinity determinants highlighted in dark blue. **B**) X-ray crystal structure of mGlu$_5$ 7TMs with mavoglurant (rendered in black sticks) bound (PDB ID: 4OO9), affinity determinants for mGlu$_5$ allosteric ligands are highlighted in red. **C**) X-ray crystal structure of mGlu$_5$ and mGlu$_1$ 7TM domains were aligned, only the mGlu$_5$ structure is represented in cartoon. The binding poses of FITM and mavoglurant show the overlapping allosteric pockets of mGlu$_1$ and mGlu$_5$. Highlighted are residues that when mutated change allosteric ligand cooperativity: dark red: reduce mGlu$_5$ NAM cooperativity; light red: increase NAM cooperativity; orange: differential effects on NAM cooperativity; dark blue: increase PAM cooperativity; light blue: increase cooperativity of NAMs and PAMs; purple: differential effects on cooperativity of NAMs versus PAMs. **D**) Structures of mGlu$_1$ and mGlu$_5$ were aligned as per panel C, highlighted are residues that when mutated switch allosteric modulator cooperativity: blue: PAM to either NAM or neutral switch; red: NAM to PAM switch; purple: PAM to NAM and vice versa for NAMs. All images created using MacPyMOL.

Figure 4: Relative contribution of 7TM residues to the binding affinity of prototypical mGlu$_5$ allosteric modulator, MPEP.
Known affinity determinants for MPEP are mapped onto the mGlu$_5$-7TM x-ray crystal structure with mavoglurant bound (PDB ID: 4OO9). The relative contribution of pocket residues are colour-coded with side chains rendered as sticks based on mutational data as indicated in the key. All images created using MacPyMOL.

Table 1: Molecular pharmacology of selected examples of allosteric ligands for group I metabotropic glutamate receptors.

Ligand/selectivity		Allosteric pharmacology	Ref.
CPCCOEt	1	**NAM**: glutamate, DHPG, quisqualate and ACPD mediated inositol phosphate accumulation (IP_1); glutamate mediated intracellular Ca^{2+} mobilization (iCa^{2+}); quisqualate induced changes in cell impedance (xCelligence); extracellular Ca^{2+} induced changes in iCa^{2+}. **NAL**: [^{3}H]glutamate binding.	1-4
JNJ-16259685	1>>5	**NAM**: glutamate mediated iCa^{2+} and IP_1; quisqualate induced changes in cell impedance (xCelligence). **Inverse agonist**: IP_1. **NAL**: [^{3}H]quisqualate binding.	5-6
R214127	1/5	**NAM**: glutamate mediated iCa^{2+}. **Inverse agonist**: IP_1.	7
Ro 67-7476	1	**PAM**: glutamate efficacy for mediating $iCa^{2+}$and cAMP accumulation. **Agonist**: pERK1/2.	8-9
Ro0711401	1	**Agonist**: iCa^{2+}. **PAM**: glutamate mediated K^+ currents via GIRK activation.	10
YM-298198	1	**NAM**: glutamate induced IP_1; quisqualate induced changes in cell impedance (xCelligence). **NAL**: orthosteric agonists and affinity of radiolabelled ligand.	3, 11
5MPEP	5	**NAL**: glutamate efficacy for iCa^{2+}; glutamate and DHPG efficacy for IP_1; quisqualate affinity.	12-17
5PAM523	5	**PAM**: glutamate efficacy for iCa^{2+}. **NAL**: [^{3}H]quisqualate binding.	18
ADX47273	5	**PAM**: quisqualate affinity; quisqualate efficacy for IP_1.	17
CDPPB	5	**PAM**: glutamate efficacy for iCa^{2+}; quisqualate affinity; quisqualate efficacy for IP_1. **PAM-agonist**: glutamate efficacy for iCa^{2+}, pERK1/2 and p-Akt.	13, 17-20
CPPHA	5>1 >4/8	**mGlu$_5$ PAM**: glutamate efficacy for iCa^{2+}; quisqualate efficacy for IP_1 and iCa^{2+}. **mGlu$_5$ NAL**: quisqualate and glutamate affinity. **mGlu$_5$ PAM**-agonist: glutamate efficacy for pERK1/2. **NAM-agonist**: DHPG efficacy for pERK1/2 (cortical astrocytes). **mGlu$_4$ and mGlu$_8$ NAM**: glutamate-mediated Ca^{2+} signalling.	14, 17, 19, 21, 22
DPFE	5	**PAM-agonist**: glutamate efficacy for iCa^{2+}& pERK1/2.	23
M-5MPEP	5	**NAM (low cooperativity**): glutamate efficacy for iCa^{2+}; quisqualate & DHPG efficacy for IP_1. **NAM**: glutamate, DHPG and quisqualate mediated Ca^{2+} oscillations; glutamate efficacy for pERK1/2. **NAL**: quisqualate affinity.	12, 17, 19
MPEP	5>>4	**mGlu$_5$ NAM**: glutamate efficacy for $iCa^{2+}$and pERK1/2; quisqualate and DHPG stimulated IP_1; glutamate, DHPG and quisqualate induced Ca^{2+} oscillations. **mGlu$_5$ inverse agonist**: IP_1. **mGlu$_5$ NAL**: quisqualate and glutamate affinity. **mGlu$_4$ PAM-agonist**: L-AP4 mediated iCa^{2+}. **mGlu$_4$ PAM**: L-AP4 induced changes in VFT.	13, 17, 19, 24, 25
VU0357121	5	**NAL**: glutamate affinity. **PAM**: glutamate efficacy for iCa^{2+}. **PAM-agonist**: glutamate efficacy for pERK1/2	19, 16
VU0360172	5	**PAM**: glutamate efficacy for iCa^{2+}. **NAL**: glutamate affinity. **PAM-agonist**: glutamate efficacy for pERK1/2	19

References: 1, Annoura et al., 1996; 2, Litschig et al., 1999; 3, Scandroglio et al., 2010; 4, Jiang et al., 2014; 5, Lavreysen et al., 2004; 6, Scandroglio et al., 2010; 7, Lavreysen et al., 2003; 8, Hemstapat et al., 2006; 9, Sheffler et al., 2008; 10, Vieira et al., 2009; 11, Kohara et al., 2005; 12, Rodriguez et al., 2005; 13, Chen et al., 2007; 14, Chen et al., 2008; 15, Ayala et al., 2009; 16, Hammond et al., 2010; 17, Bradley et al., 2011; 18, Parmentier-Batteur et al., 2014; 19, Gregory et al., 2012; 20, Doria et al., 2013; 21, O'Brien et al., 2004; 22, Zhang et al., 2005; 23, Gregory et al., 2013b; 24, Gasparini et al., 1999; 24, Rovira et al., 2015.

44

Table 2: Molecular pharmacology of selected allosteric ligands for group II metabotropic glutamate receptors.

Ligand/selectivity		Allosteric pharmacology	Ref.
BINA	2	**PAM-agonist**: glutamate stimulated [^{35}S]GTPγS binding at human mGlu$_2$. **PAM**: glutamate stimulated [^{35}S]GTPγS binding at rat mGlu$_2$ and iCa^{2+} at human and rat mGlu$_2$; [^{3}H]DCG-IV binding to human mGlu$_2$; LY541850 mediated decreases in Ca^{2+} oscillation frequency in primary neuronal cultures. **NAL**: [^{3}H]LY341495 binding to human mGlu$_2$, LY379268 mediated decreases in Ca^{2+} oscillation frequency in primary neuronal cultures.	1, 2, 3, 4
CBiPES		**PAM**: glutamate mediated iCa^{2+}; LY379268 and LY541850 mediated decreases in Ca^{2+} oscillation frequency in primary neuronal cultures.	3, 5
JNJ-40068782	2	**PAM-agonist**: glutamate stimulated [^{35}S]GTPγS binding and iCa^{2+} at human mGlu$_2$. **PAM**: glutamate stimulated [^{35}S]GTPγS binding at rat mGlu$_2$; [^{3}H]DCG-IV binding to human mGlu$_2$; glutamate affinity (displacement of [^{3}H]DCG-IV). **NAL**: [^{3}H]LY341495 binding	1
LY2607540 (THIIC)	2	**PAM-agonist**: glutamate stimulation of iCa^{2+} and [^{35}S]GTPγS binding at human receptor. **PAM**: glutamate stimulation of iCa^{2+} and [^{35}S]GTPγS binding at rat receptor	1, 6
LY487379	2	**PAM-agonist**: glutamate stimulation of [^{35}S]GTPγS binding to rat cerebral cortical membranes. **PAM**: glutamate, DCG-IV, LCCG-I and LY379268 stimulated [^{35}S]GTPγS binding and glutamate mediated iCa^{2+}; [^{3}H]DCG-IV binding to human mGlu$_2$. **NAL**: [^{3}H]LY341495 binding to human mGlu$_2$.	1, 2, 7, 8, 9
MNI-137	2/3	**mGlu$_2$ NAM**: glutamate and DCG-IV mediated iCa^{2+} and glutamate stimulated [^{35}S]GTPγS binding. **mGlu$_3$ NAM**: glutamate mediated iCa^{2+} and [^{35}S]GTPγS binding. **mGlu$_2$ NAL**: [^{3}H]LY341495 binding and glutamate affinity.	2, 10
RO4491533	2/3	**mGlu$_2$ NAM**: [^{3}H]LY354740 affinity; ACPD inhibition of cAMP accumulation; glutamate stimulation of GIRK channels and [^{35}S]GTPγS binding. **mGlu$_2$ NAL**: [^{3}H]LY341495 binding to human mGlu$_2$. **mGlu$_3$ NAM**: glutamate stimulation of GIRK channels.	11, 12
RO4988546	2/3	**mGlu$_2$ NAM**: [^{3}H]LY354740 binding; LY354740 stimulation of [^{35}S]GTPγS binding and iCa^{2+}. **mGlu$_2$ NAL**: [^{3}H]HYDIA binding.	13
RO5488608	2/3	**mGlu$_2$ NAM**: [^{3}H]LY354740 binding; LY354740 stimulation of [^{35}S]GTPγS binding and iCa^{2+}. **mGlu$_2$ NAL**: [^{3}H]HYDIA binding.	13

References: 1, Lavreysen et al., 2013; 2, Hemstapat et al., 2007; 3, Sanger et al., 2013; 4, Galici et al., 2006; 5, Johnson et al., 2005; 6, Fell et al., 2011; 7, Odagaki et al., 2013; 8, Schaffhauser et al., 2003; 9, Odagaki et al., 2011; 10: Yin et al., 2014; 11, Woltering et al., 2010; 12, Campo et al., 2011; 13, Lundstrom et al., 2011.

45

Table 3: Molecular pharmacology of selected allosteric ligands for group III metabotropic glutamate receptors.

Ligand/selectivity		Allosteric pharmacology	Ref.
ADX71743	7	**NAM**: L-AP4 efficacy for iCa^{2+} and inhibition of cAMP accumulation; glutamate in mediating inhibition of cAMP accumulation.	1
AMN082	7[#]	**NAL-Agonist**: inhibition of cAMP accumulation, stimulates pERK1/2 and receptor internalization. **NAL**: MSOP (does not block AMN082 internalization).	2-5
AZ12216052	8	**PAM-agonist**: glutamate stimulation of [^{35}S]GTPγS binding	6
MMPIP	7	**NAM**: L-AP4 efficacy for iCa^{2+} and changes in cell mass distribution; L-CCG-I and CPPG efficacy for iCa^{2+}; glutamate stimulation of GIRK channels. **Inverse/agonist**: cAMP accumulation and Epic label-free assay. **NAL**: [^{3}H]LY341495 binding	4, 7
PHCCC	4/1 6	**PAM**: glutamate for iCa^{2+} and GIRK channel activation; L-AP4 stimulated iCa^{2+} and changes in VFT. **PAM-agonist**: L-AP4 and glutamate stimulated [^{35}S]GTPγS binding. **mGlu$_1$ NAM**: glutamate mediated iCa^{2+}. **mGlu$_6$ agonist**: glutamate mediated calcium current inhibition.	10-15
VU0155041	4	**PAM-agonist**: glutamate mediated iCa^{2+} and GIRK channel activation. **PAM**: L-AP4 mediated iCa^{2+} and changes in VFT.	13, 14, 16
VU0155094	4-8	**PAM**: glutamate apparent affinity (mGlu$_{4, 7, 8}$); glutamate efficacy for stimulation of GIRK channels (mGlu$_8$) and iCa^{2+} (mGlu$_{4,7}$); L-AP4 apparent affinity (mGlu$_{7,8}$); L-AP4 efficacy for iCa^{2+} (mGlu$_4$); LSP4-2022 apparent affinity (mGlu$_{4, 7, 8}$); LSP4-2022 efficacy for iCa^{2+} (mGlu$_4$). **NAL/NAM**: L-AP4 apparent affinity (mGlu$_4$); L-AP4 and LSP4-2022 efficacy for iCa^{2+} (mGlu$_7$). **NAL**: glutamate, L-AP4, LSP4-2022 efficacy for iCa^{2+} (mGlu$_8$).	17
VU0422288	4-8	**PAM**: glutamate, L-AP4 and LSP4-2022 efficacy for iCa^{2+} and GIRK channel activation (mGlu$_{4,7,8}$); glutamate, L-AP4, LSP4-2022 apparent affinity (mGlu$_{7,8}$). **NAL**: glutamate apparent affinity (mGlu$_4$). **NAM**: L-AP4 and LSP4-2022 apparent affinity (mGlu$_4$).	17

[#]compound and/or its metabolite hits other non-mGlu targets (Ayala et al., 2008; Sukoff Rizzo et al., 2011).

References: 1, Kalinichev et al., 2013; 2, Mitsukawa et al., 2005; 3, Pelkey et al., 2007; 4, Suzuki et al., 2007; 5, Iacovelli et al., 2014; 6, Duvoisin et al., 2010; 7, Niswender et al., 2010b; 10, Beqollari and Kammermeier, 2008; 11, Maj et al., 2003; 12, Marino et al., 2003; 13, Niswender et al., 2008;14, Rovira et al., 2015; 15, Yin et al., 2014; 16, Yin et al., 2012; 17, Jalan-Sakrikar et al., 2014.

46

Table 4: Known affinity determinants for mGlu allosteric modulators interacting with the common allosteric site.

Ligand/subtype		Amino acid residue position																
		3.29	3.36	3.39	3.40	5.43	5.44	5.47	5.48	6.44	6.48	6.51	6.52	6.55	6.56	7.38	7.45	7.46
EM-TBPC	1				Y		V/L				W+	F	--	Y		T		
FITM	1		s		P					--						T	--	
MPEP	5	R	P	S	Y	P	L	--	G	T	W	F	V	Y	f+	--	S	A
fenobam	5	R	P	S	Y					T	W	F		Y				A
VU0366248	5		P	S	Y			N	--	t	W	f#	--	Y	--	S	S+	A
VU0285683	5		p#		Y	--		N	--	T	W	F	--	Y			S	A
VU0366058	5		p#		Y			--	g+		W	F+	v+	Y+			s	A
VU0409106	5		p#		Y			N	--	T	W	F	--	Y	f		S	A
DPFE	5	--	P		Y	P	L+		--	T	W	--	V+	y+	f+		s	A
VU29	5	--	p#		--	p	l		G	T	w+	f	v	y	f+		-	A
VU0403602	5	--	P	s	Y#	p	--		--	T	W			--			S	A

Most well-characterised representatives from individual allosteric modulator scaffolds were selected for inclusion. Alignment of amino acids and assignment of Ballesteros-Weinstein numbering was adopted from the structural alignment to class A GPCRs in Wu et al., 2014. A colorised version of this table (Supplementary Table 1) is included in the online supplementary materials.

lower case: mutations caused a change (2-10 fold) in affinity estimates that did not reach significance (determined from either radioligand binding or from functional interactions with orthosteric agonists).

upper case: mutations caused a significant change in affinity (determined from either radioligand binding or from functional interactions with orthosteric agonists; not included: T6.43 for MPEP, C7.52 for VU0403602)

mutation caused a loss in allosteric modulation of orthosteric agonist functional response; impact on affinity inferred from effects across other scaffolds.

-- mutation/s had no effect on modulator affinity (determined from either radioligand binding or from functional interactions with orthosteric agonists).

+ mutation increases modulator affinity, in all other cases mutations cause a decrease (determined from either radioligand binding or from functional interactions with orthosteric agonists).

Grey scale: white: 3-10 fold change; light grey: 10-30 fold change; mid grey: 30-100 fold decrease; dark grey: >100 fold decrease or complete loss of radiolabelled allosteric modulator binding (Data summarised from: Gregory et al., 2012; Gregory et al., 2013b; Gregory et al., 2014; Malherbe et al., 2003a; Malherbe et al., 2003b; Malherbe et al., 2006; Pagano et al., 2000; Turlington et al., 2013; Wu et al., 2014).
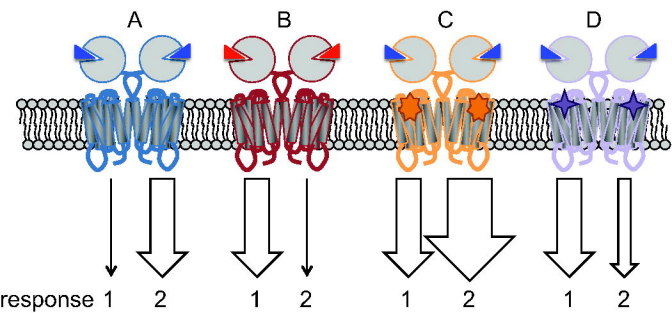
47

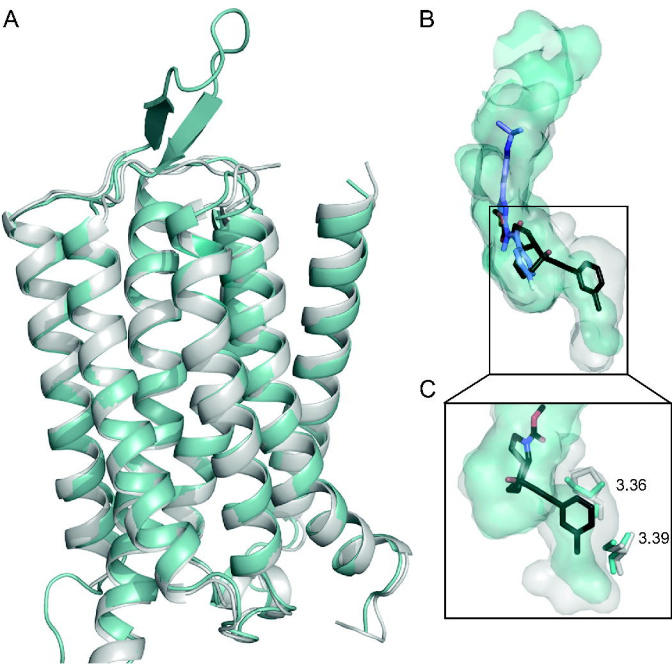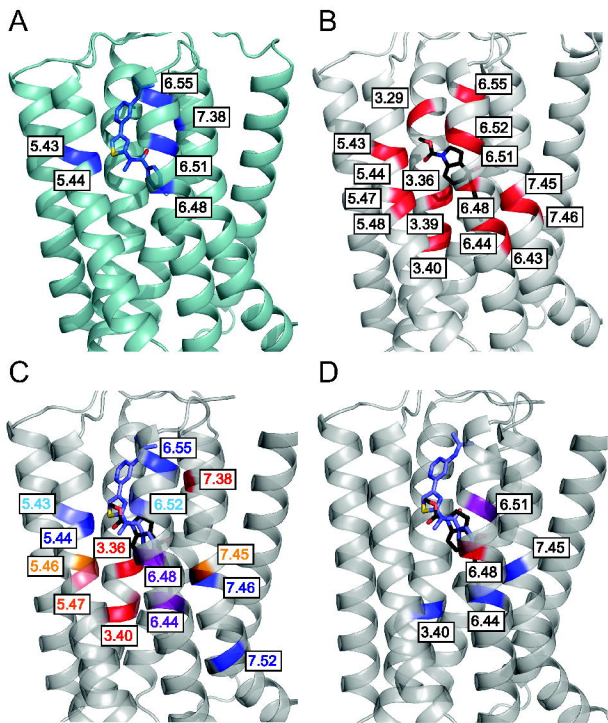response 1   2   1   2   1   2   1   2

Figure 1

A

B

C

3.36

3.39

Figure 2

Figure 3

Mutations reduce affinity 3-10 fold
Mutations reduce affinity 10-30 fold
Mutations reduce affinity 30-100 fold
Mutations reduce affinity >100 fold

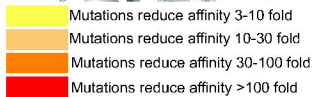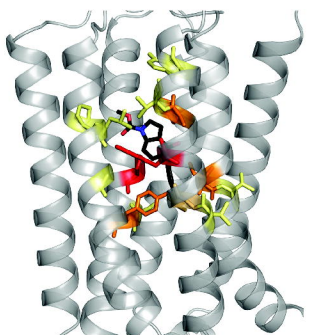Figure 4

Molecular insights into metabotropic glutamate receptor allosteric modulation

Karen J. Gregory and P. Jeffrey Conn

Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, VIC, Australia (KJG).
Vanderbilt Center for Neuroscience Drug Discovery & Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN, USA (PJC).

Supplementary Table 1: Known affinity determinants for mGlu allosteric modulators interacting with the common allosteric site.

Ligand/subtype		3.29	3.36	3.39	3.40	5.43	5.44	5.47	5.48	6.44	6.48	6.51	6.52	6.55	6.56	7.38	7.45	7.46
																	Amino acid residue position	
EM-TBPC	1				Y		V/L				W	F	--	Y		T		
FITM	1		s		P					--							T	--
MPEP	5	R	P	S	Y	P	L	--	G	T	W	F	V	Y	f	--	S	A
fenobam	5	R	P	S	Y					T	W	F		Y				A
VU0366248	5		P	S	Y			N	--	t	W	f#	--	Y	--		S S	A
VU0285683	5		p#		Y	--		N	--	T	W	F	--	Y			S	A
VU0366058	5		p#		Y				--	g	W	F	v	Y			s	A
VU0409106	5		p#		Y			N	--	T	W	F	--	Y	f		S	A
DPFE	5	--	P		Y	P	L		--	T	W	--	V	y	f		s	A
VU29	5	--	p#		--	p	l		G	T	w	f	v	y	f		-	A
VU0403602	5	--	P	s	Y#	p		--	--	T	W		--				S	A

Most well-characterised representatives from individual allosteric modulator scaffolds were selected for inclusion. Alignment of amino acids and assignment of Ballesteros-Weinstein numbering was adopted from the structural alignment to class A GPCRs in Wu et al., 2014.

lower case: mutations caused a change (2-10 fold) in affinity estimates that did not reach significance (determined from either radioligand binding or from functional interactions with orthosteric agonists).

upper case: mutations caused a significant change in affinity (determined from either radioligand binding or from functional interactions with orthosteric agonists; not included: T6.43 for MPEP, C7.52 for VU0403602)

[#] mutation caused a loss in allosteric modulation of orthosteric agonist functional response; impact on affinity inferred from effects across other scaffolds.

-- mutation/s had no effect on modulator affinity (determined from either radioligand binding or from functional interactions with orthosteric agonists).

Colour coding: yellow: 3-10 fold decrease; pale orange: 10-30 fold decrease; dark orange: 30-100 fold decrease; red: >100 fold decrease or complete loss of radiolabelled allosteric modulator binding; light blue: 3-10 fold increase; royal blue: 10-30 fold increase.

(Data summarised from: Gregory et al., 2012; Gregory et al., 2013b; Gregory et al., 2014; Malherbe et al., 2003a; Malherbe et al., 2003b; Malherbe et al., 2006; Pagano et al., 2000; Turlington et al., 2013; Wu et al., 2014)