DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Mapping the Molecular Determinants of Positive Allosteric Modulators of the mGlu2 Receptor

Belisa Russo

2013

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Mapping the Molecular Determinants of Positive Allosteric Modulators of the mGlu2 Receptor

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, especialização em Neurociências, realizada sob a orientação científica da Doutora Hilde Lavreysen (Janssen Pharmaceutica) e do Professor Doutor Carlos Duarte (Universidade de Coimbra)

Belisa Russo

2013

The work described in this thesis resulted from a partnership between the University of Coimbra and Janssen Pharmaceutica NV. All experimental activities were performed at Janssen Pharmaceutica NV Beerse I, a Johnson and Johnson pharmaceutical research and development facilitie in Beerse, Belgium.

Beerse, 2013

Acknowledgements

I would like to express my gratitude to my supervisor Hilde Lavreysen for giving me the opportunity to perform this internship in her group, for the useful comments, remarks and engagement through the learning process of this master thesis.

A special appreciation to Luc Peeters for all the support, helping, advice and caring. Thank you so much!

I would also like to acknowledge Ilse Biesmans and Luc Gabriels for all the help in the laboratory through this year! To all the staff of Neuroscience department a big thank you for making the students feel like part of the team.

To University of Coimbra and Janssen Pharmaceutica for this cooperation that allowed me to perform this internship.

To all the students, for the friendship and all the amazing moments throughout the year!

Finally, a big thank you to my parents, sister, brother and friends for all the constant and unconditional love, support and encouragement!

Table of Contents

Abbreviations

[^{35}S]GTPγS – Guanosine 5'-(γ-thio)triphosphate [^{35}S]-

1GZM – rhodopsin receptor

2RH1 – β_2-adrenergic receptor

5-HT$_2$ – serotonine receptor

aa – amino acid

ALS - amyotrophic lateral sclerosis

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

B_{max} – total number of binding sites

cAMP - cyclic adenosine monophosphate

CHO – chinese hamster ovary

CNS - central nervous system

CPM – counts per minute

CRD – cysteine rich domain

DMSO – dimethyl sulphoxide

DPM – disintrigations per minute

EAAT - excitatory amino acid transporters

EC_{100} – concentration of compound producing 100% of stimulation

EC_{20} – concentration of compound producing 20% of stimulation

EC_{50} – concentration of compound producing 50% of stimulation

EGFP-N1 – Enhanced Green Fluorescent Protein

EL – extracellular loop

E_{max} – relative maximal stimulation

EPS – extrapyramidal symptoms

ERK/MAPK – extracellular receptor kinase/ mitogen-activated protein kinase

FBS – fetal bovine serum

GABA - γ-amino butyric acid

GDP – guanosine 5' diphosphate

GMP – guanosine moniphosphate

GPCR - G-protein-coupled receptor

GRK – G-protein coupled receptor kinases

GTP - guanosine 5’ triphosphate

HIV - human immunodeficiency virus

hmGluR – human metabotropic glutamate receptor

HRP – horseradish peroxidase

iGluR - ionotropic glutamate receptors

IL – intracellular loop

KA – kainate

K_D – apparent equilibrium dissociation constant

LSD - lysergic acid diethylamide

LTP - long-term potentiation

mGluR - metabotropic glutamate receptor

MOE – Molecular Operating Environment

NAM - negative allosteric modulator

NFDM – non-fat dry milk

NMDA - N-methyl-D-aspartate

PAM - positive allosteric modulator

PBP – periplasmatic binding protein

PBS – phosphate-buffered saline

PCP – phencyclidine

pEC_{50} – negative logarithm of the half-maximal effective concentration

pIC_{50} - negative logarithm of the half-maximal inhibitory concentration

PKA – protein kinase A

PKC – protein kinase C

PLC – phospholipase C

PTX - pertussis toxin

rmGluR – rat metabotropic glutamate receptor

RT – room temperature

TBS-T – tris-buffered saline and tween 20

TM – transmembrane

VFD - venus flytrap domain

vGluT - vesicular glutamate transporters

Resumo

Glutamato é o principal neurotransmissor excitatório no cérebro, sendo bastante importante em várias funções do sistema nervoso central. Alterações no sistema glutamatérgico estão envolvidas em doenças como esquizofrenia e ansiedade. Tem vindo a ser demonstrado que a activação de receptores mGlu2 reduz a transmissão glutamatérgica nas regiões cerebrais associadas a estas doenças. Por esta mesma razão, a activação destes receptores tem sido alvo de investigação para desenvolvimento de novas técnicas terapêuticas, especialmente com o uso de modeladores alostericos positivos (PAMs); estes modeladores ligam-se a uma zona do receptor diferente do local de ligação do glutamato.

Dado que a área de estudo dos PAM do receptor mGlu2 se encontra em expansão e o primeiro ensaio clínico está a decorrer é bastante importante obter mais informacão sobre o correcto local de ligacão destes ligandos. Esta informacão também pode suportar e facilitar os esforços da investigação química. Com o objectivo de identificar os aminoácidos responsáveis pela interação entre os PAMs e o receptor mGlu2, foi efectuada modulação molecular e docking de PAMs de receptores mGlu2 em paralelo com mutagénese dirigida. Nos receptores mGlu2 mutantes foi avaliado o impacto das mutações na actividade e afinidade dos PAMs. Este estudo confima a importância de aminoácidos previamente demonstrados como importantes na actividade de PAMs estruturalmente diferentes nestes receptores. É tambem demonstrado que adicionais aminoácidos seleccionados com base na comparação de sequencias entre mGlu2/3 parecem não ser importantes na actividade dos PAMs. A informação obtida neste estudo tambem demonstra que a actividade dos modeladores testados é reduzida devido à diminuição da afinidade de ligação. Toda esta informação oferece um melhor entendimento sobre o 'binding pocket' para PAMs do receptor mGlu2.

Palavras-chave: receptor mGlu2, local de ligação alostérico, modulador alostérico positivo, mutagénese

Abstract

Glutamate is the major excitatory neurotransmitter in the brain and plays an important role in a wide variety of central nervous system functions. Alterations in the glutamatergic system are involved in disorders like schizophrenia and anxiety. It has been shown that activation of the metabotropic glutamate 2 receptor reduces the glutamatergic transmission in brain regions associated with these disorders. Therefore, activation of mGlu2 receptor is being pursued as a novel therapeutic approach, specially using positive allosteric modulators (PAMs), which bind to a site other than that of the endogenous mGlu2 receptor agonist glutamate.

Since the field of mGlu2 PAMs is expanding and the first clinical studies are ongoing with mGlu2 PAMs, it will be important to get more insight into the actual binding site of these ligands. This knowledge may also facilitate and support future chemistry endeavors. In order to identify the amino acids important for the activity of mGlu2 PAMs, homology modeling and docking of mGlu2 receptor PAMs were performed in parallel with site-directed mutagenesis. Mutant mGlu2 receptors were generated and the impact of these mutations on activity and affinity of PAMs was evaluated. This study confirms the importance of several amino acids previously shown as crucial for the activity of structurally diverse mGlu2 receptor PAMs. It furthermore demonstrates that additional amino acids that were selected based on mGlu2/3 comparison did not seem to be important for PAM activity. Our data also suggest that their activity is reduced due to lower binding affinity. All this sheds further light on the mGlu2 PAM binding pocket.

Keywords: mGlu2 receptor, allosteric binding site, positive allosteric modulator, mutagenesis

1. Introduction

1.1. G Protein-Coupled Receptors

The majority of transmembrane signal transduction responses to hormones and neurotransmitters is mediated by G protein-coupled receptors (GPCRs) (Gether & B. K. Kobilka 1998). GPCRs are the largest class of cell-surface receptors (Schwalbe, H. and Wess 2002), being extensively expressed in the body and playing an important role in virtually every organ system. These receptors have also been implicated in a multitude of human disorders and numerous diseases have been linked to mutations and polymorphisms in GPCRs (P Jeffrey Conn et al. 2009). It has been estimated that over than 800 genes encode for a GPCR. Of those, almost half are likely to encode sensory receptors, leaving about 400 receptors that are potentially druggable, of which around 30 are targets of currently marketed drugs (Caterina Bissantz et al. 2004; Wise et al. 2002).

For approximately 210 receptors, the natural ligand has been identified, leaving around 160 orphan receptors with no known ligand or function (Wise et al. 2002).

Though all GPCRs have a common structural architecture, they display multiple orthosteric binding modes due to the substantial diversity of endogenous ligands that they recognize (Gether & B. K. Kobilka 1998).

1.1.1. GPCR structure

GPCRs are composed of a single peptide, between 400-500 amino acids, however the length can go up until 1200 amino acids. Sequence comparison between the different GPCRs revealed the existence of different families sharing no sequence similarity (Bockaert, J; Pin 1999). In general, these receptors have in common a central core domain composed of seven transmembrane-spanning α helices (TM domains) connected by alternating 3 intracellular (IL1-IL3) and 3 extracellular (EL1-EL3) loops, with the amino terminus located on the extracellular side and the carboxy terminus on the intracellular side (Gether 2000; Vauquelin & von Mentzer 2007). GPCRs differ in the length and function of their N-terminal extracellular domain, their C-terminal intracellular domain and their intracellular loops, providing specific properties (Figure 1) (Bockaert, J; Pin 1999).

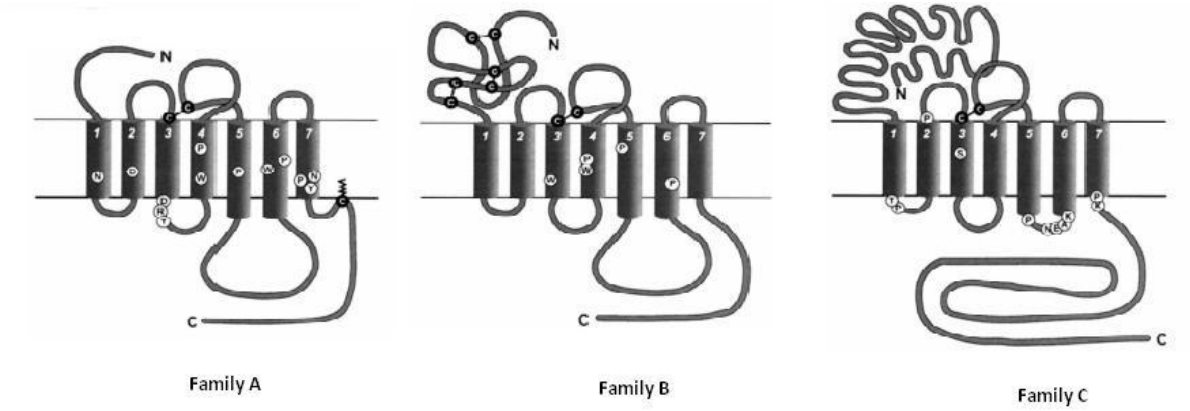


Figure 1. Structural differences between the different GPCRs families [Image adapted from (Gether 2000)]

1.1.2. GPCR mode of action

GPCRs become active when coupled to G proteins. In a resting state, G proteins form a heterotrimer, consisting of a guanine nucleotide binding α subunit (38-52 KDa), a β subunit (35 KDa) and a γ subunit (8-10 KDa). The α subunits are hydrophilic and are largely involved in the recognition of "effector components", being anchored to the plasma membrane due to their coupling to the β-γ complexes. The β and γ subunit are always closely associated and the complex formed by these two subunits is presumed to be interchangeable from one G protein to another (Vauquelin & Von Mentzer 2007).

The specificity of GPCR signaling relies on the existence of closely related molecular species of the G protein subunits. Based on the sequence of the α subunits, G proteins have been grouped into four families; at least 6 and 12 different β and γ subunits have been described, respectively (see Table 1) (Hermans 2003; Vauquelin and Von Mentzer 2007).

Receptor activation, upon agonist binding, leads to an exchange of a molecule of GDP by a molecule of GTP occurs within the active site of the α-subunit. After the binding of GTP, the heterotrimeric complex is dissociated and the different subunits are able to interact with intracellular or membrane effectors. (Gether & B. K. Kobilka 1998)

The intrinsic GTPase activity of the αsubunit hydrolyses GTP into GDP, restoring its initial inactive conformation (Hermans 2003). Receptor inactivation or desensitization can also be mediated by protein kinase A (PKA) or C (PKC), or by G protein-coupled kinases (GRKs). It is important to note that arrestin can also prevent the interaction between GPCRs and G protein (Lundstrom & Chiu 2006).

GPCRs can interact with other proteins rather than G proteins. The interaction of tyrosine kinase with the receptor result in activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade. Furthermore, interaction between β-arrestin and c-Src facilitate GPCR-dependent activation of the ERK/MAPK pathway (Lundstrom & Chiu 2006).

The complexity of intracellular response is a consequence of the diversity in the signal handling at multiple levels of the response process. It can be due to receptor subtypes that show distinct G protein coupling specificities (Figure 2A). Another level of signaling diversity is related to the ability of a single G protein subtype to elicit the activation of a variety of intracellular cascades (Figure 2B). Moreover, the divergence in cell signaling may result from secondary modulation of intracellular effectors (Figure 2C). Additionally, a further complexity in cell signaling can be related to the possible or successive coupling of a given receptor with distinct G proteins (Figure 2D)(Hermans 2003).

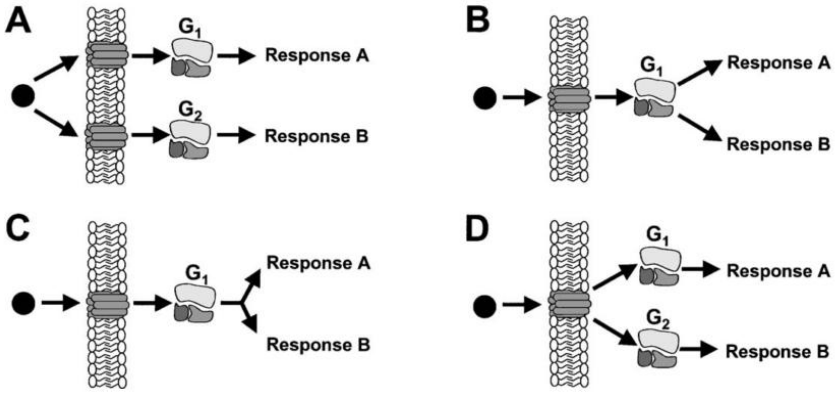


Figure 2. Intracellular signaling divergence in response to a single transmitter. (A) Transmitter binds to different receptor subtypes, showing distinct G protein coupling specificities. (B) Transmitter binds to a single receptor and triggers the direct activation of distinct intracellular effectors through a single G protein. (C) Transmitter binds to a single receptor that shows selectivity for a single intracellular effector through a single G protein, but divergence occurs at downstream levels in the signaling cascade. (D) Transmitter binds to a single receptor that mediates distinct signaling through direct interaction with multiple G proteins [image taken from (Hermans 2003)].

Table 1. G protein subunits and their primary effector [Adapted from (Hermans 2003; Vauquelin & von Mentzer 2007)]

Subunit	Family	Main subtypes	Primary effector
α	α_s	$G\alpha_s$, $G\alpha_{olf}$	Adenylate cyclase ↑
	$\alpha_{i/o}$	$G\alpha_{i1-3}$, $G\alpha_{oA-B}$, $G\alpha_{t1-2}$, $G_{\alpha z}$	Adenylate cyclase ↑, K^+ channels↑, Ca^{2+} channels↓, Cyclic GMP, Phosphodiesterase↑
	$\alpha_{q/11}$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14-16}$	Phospholipase C ↓
	$\alpha_{12/13}$	$G\alpha_{12-13}$	?
β	$\beta_{1-5\ (6)}$	Different assemblies of β/y subunits	Adenylyl cyclase ↑↓, Phospholipases ↑, Phosphatidylinositol 3-kinase ↑
y	$y_{1-11\ (12?)}$		Protein Kinase C and D ↑, GPCR kinases ↑, Ca^{2+}, K^+ (and Na^+) channels

1.1.3. Families of GPCRs

GPCRs have been divided into diverse families, sharing around 20% of sequence identity in their TM domains. There are 3 major families: Family A, also called class I, is the rhodopsin-like receptor family with ligands such as neuropeptides, chemokines and prostaniods (Figure 1); family B (class II) is also called the secretin/glucagon/VIP family; family C or class III are metabotropic glutamate receptor-like (Vauquelin & Von Mentzer 2007).

1.1.3.1. Family A

Family A GPCRs is the largest and most studied family. Receptors of this family can be divided into six subgroups (Gether 2000). In almost all of these receptors, a disulfide bridge connects the second and third extracellular loop. Moreover, they possess a palmitoylated cysteine in the carboxy-terminal tail causing formation of a putative fourth intracellular loop. The homology between all family A receptors is low and restricted to highly conserved key residues, which suggests an essential role for the structural and/or functional integrity of the receptors (Gether 2000; Vauquelin & Von Mentzer 2007). The only residue that is conserved among all family A is the arginine in the Asp-Arg-Tyr (DRY) motif at the cytoplasmatic side of transmembrane segment 3 (Gether 2000). This motif is

not present in the others GPCRs families and has been subject of much work attempting to understand the mechanisms of receptor activation and interaction with G-proteins (Flanagan 2005). Presumably mediates interactions with both G proteins and arrestins and serves to maintain the receptor transmembranes in an inactive conformation in the absence of ligand (Marion et al. 2006) . In general, endogenous ligands of this family bind to the 7TM (Vauquelin & Von Mentzer 2007), which is interesting since, as it will be discuss later, for family C this is the allosteric binding site.

1.1.3.2. Family B

This family includes about 65 members and represents an ancient signaling system that appears to play an important role in many biological processes. For that, they represent an interesting pharmaceutical target. If we look to their sequence, these GPCRs can be divided into three subfamilies (Vauquelin & Von Mentzer 2007). Despite of having the disulfide bridge connecting the EL2 and EL3, this family does not contain any of the structural features that characterize family A. The major characteristic of these receptors is the presence of a large extracellular amino terminus containing several cysteines, probably forming a network of disulfide bridges (Gether 2000).

1.1.3.3. Family C

Family C includes the receptors for the main neurotransmitters (glutamate and GABA), for Ca^{2+}, for sweet and amino acid taste compounds, for some pheromone molecules and odorants in fish. Of all genes enconding for GPCRs, 22 encode just for this class (Rondard et al. 2011). The metabotropic receptors for glutamate are the focus of this thesis.

The Family C GPCR family presents a long amino terminus (500-600 aa), where the othosteric binding site can be found and displays remote sequence homology with bacterial periplasmatic binding proteins (PBPs), specially with the leucine/isoleucine/valine binding protein (Gether 2000) .

Like the other families, Family C has putative disulfide-forming cysteines, but do not share any conserved residues with the other families (Vauquelin & Von Mentzer 2007).

The C-terminus is important for modulating G protein coupling and is also a target for alternative splicing, regulation by phosphorylation and modulatory protein-protein interactions (Niswender & P Jeffrey Conn 2010).

1.1.3.4. Family D, E and F

Yeast pheromone receptors make up two minor unrelated subfamilies, Family D also called STE2 receptors and Family B (STE3 receptors). In the amoeba *Dictyostelium Discoideum* four different cAMP receptors constitute another minor subfamily – Family F (Gether 2000).

1.2. Glutamatergic system

Glutamate is the primary excitatory neurotransmitter in the brain. This neurotransmitter accounts for 100% of pyramidal neurons, virtually all cortico-cortical neurotransmission and approximately 60% of total brain neurons (Kantrowitz & Javitt 2010).

Glutamate plays key roles in physiological processes including learning and memory and central pain transduction mechanisms. These processes are mediated by diverse families of receptors and transporters (C. J. Swanson et al. 2005; Kantrowitz & Javitt 2010).

Glutamate transporters are divided in plasma membrane glutamate transporters (excitatory amino acid transporters - EAAT) and vesicular glutamate transporters ($vGluT_1$ and $vGluT_2$) (C. J. Swanson et al. 2005). Vesicular glutamate transporters are important to transport glutamate into synaptic vesicles after its production. Glutamate is stored in synaptic vesicles at high concentrations, and protected from degradation before being released in a Ca^{2+} dependent manner into the synaptic cleft by exocytosis (Sanacora et al. 2008).

Glutamate is also involved in pathological processes such as excitotoxic neuronal injury which follows central nervous system (CNS) trauma or ischemia (Hudspith 1997). Therefore, a tight control of glutamatergic neurotransmission is required in order to maintain optimal neuronal function and prevent overactivation of the system. For that, multiple levels of regulatory processes have evolved to ensure that glutamatergic excitation is maintained within narrow boundaries. These regulatory processes are important because abnormal function of the glutamatergic system has been implicated in the pathophysiology of many different disorders including amyotrophic lateral sclerosis (ALS), Huntington's chorea, epilepsy, Alzheimer's disease, schizophrenia, and anxiety disorders. Thus, dysfunction of glutamatergic neurotransmission may be a common pathophysiological mechanism, aspects of which are shared between several disorders (Sanacora et al. 2008).

Hence, pharmacological manipulation of glutamate receptors is likely to be beneficial in important and common diseases of the nervous system (Tsai & Coyle 2002; Sanacora et al. 2008).

1.2.1. Glutamate receptors

There are two major categories of glutamate receptors (Figure 3): the ionotropic glutamate (iGlu) receptors which are ligand-gated ion channel receptors that modulate synaptic excitability and plasticity and the metabotropic glutamate (mGlu) receptors, which regulate glutamate release and modify postsynaptic excitability to glutamate (C. J. Swanson et al. 2005).

For a visual on the distribution of the transporters and receptors see Figure 4.

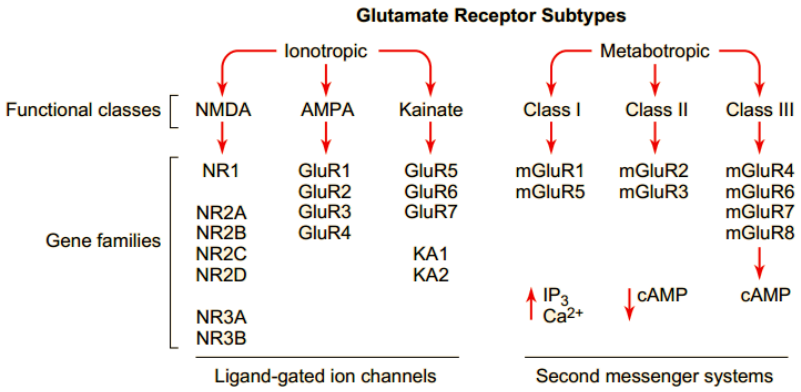


Figure 3. Molecular families of glutamate receptors (Siegel et al. 2006)

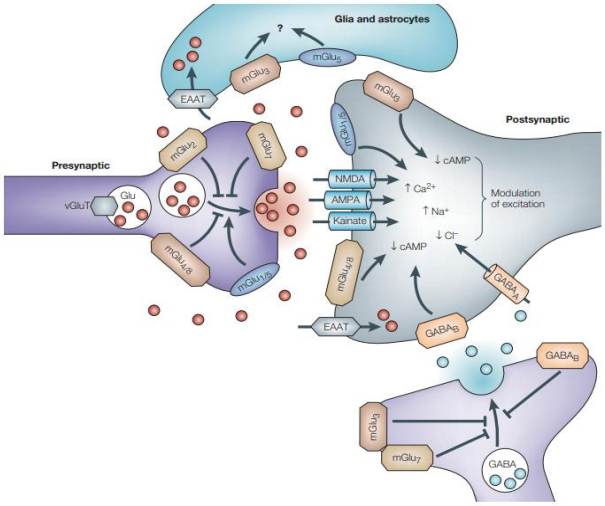


Figure 4. Hypothetical synapse illustrating the general synaptic localization and function of glutamatergic receptors and transporters (C. J. Swanson et al. 2005)

1.2.1.1. Ionotropic glutamate receptors

In the case of iGlu receptors, the agonist binding sites and associated ion channel are incorporated into the same macromolar complex. Agonist binding leads to a conformational change in the receptors that increases the probability of channel opening. There are three classes of iGlu receptors: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors, in accordance with differential sensitivity to these synthetic glutamate analogs. It is important to note that the affinity for glutamate is different for the different glutamate receptors (Siegel et al. 2006).

AMPA receptors mediate fast, rapidly desensitizing excitation at most synapses, and are responsible for the initial reaction to glutamate in the synapse. Their activation opens the pore which results in the inward flow of sodium, leading to the depolarization of the neuronal membrane. These receptors comprise a homo or heteromeric complex of four subunits (GluR1-4) and are functionally diverse since they have differences in individual subunit expression, posttranscriptional

modifications, and alternative splicing modifications. At mature synapses, AMPA receptors are usually co-expressed with NMDA receptors (Siegel et al. 2006).

NMDA receptors are believed to exist primarily as tetrameric complexes that have two obligatory NR1 subunits and two NR2 subunits. There are at least eight splice variants of the NR1 subunit, four NR2 genes (NR2 A-D), and two NR3 subunits (NR3A and NR3B) (Sanacora et al. 2008). The binding sites of these receptors consist of recognition sites for two different agonists (glutamate and glycine) and a polyamine regulatory site, all of which promote receptor activation (Siegel et al. 2006). The binding site for glutamate has been found in the NR2 subunit and the site for the co-agonist glycine has been localized to the NR1 subunit (Sanacora et al. 2008). Also, there are separate recognition sites for Mg^{2+}, Zn^{2+} and H^{+} (Siegel et al. 2006). NMDA receptors are normally blocked under resting conditions by the obstructing effects of Mg^{2+} ; membrane depolarization and the combined binding of two molecules of glutamate and two molecules of glycine or D-serine is needed for NMDA receptor activation (Sanacora et al. 2008). Thus, NMDA receptor activation serves as a functional marker of converging excitatory input and produces excitation over longer periods of time (Sanacora et al. 2008).

The other type of iGlu receptors, KA receptors, are coded by two gene families coding for the low affinity GluR5-7 subunits and the high affinity KA1 and KA2 subunits. KA receptors are associated with voltage-dependent channels, like AMPA receptors, that allow the influx of Na^{+} ions that mediate fast excitatory neurotransmission; however they appear to have a distinct distribution, when compared to AMPA receptors (Sanacora et al. 2008).

1.2.1.2. Metabotropic glutamate (mGlu) receptors

The discovery of mGlu receptors dramatically altered the traditional view of glutamatergic neurotransmission since activation of mGlu receptors can modulate activity in glutamatergic circuits which previously was associated with other neuromodulators like dopamine, serotonine, acetylcholine and norepinephrine (P J Conn & J. P. Pin 1997).

It is known that mGlu receptor-mediated glutamate activity influences Ca^{2+} and K^{+} ion channels, NMDA and AMPA receptor currents, glutamate and GABA release (P J Conn & J. P. Pin 1997). One of the most prominent physiologic effects of this type of receptors is reduction of transmission at glutamatergic synapses. This effect is typically mediated by presynaptic mGlu receptors that serve as autoreceptors to reduce glutamate release (P J Conn & J. P. Pin 1997).

Since iGlu receptors are expressed by almost every type of neurons and mediate fast excitatory neurotransmission, direct pharmacological manipulation of these receptors is not a good idea because inhibition could produce disruption of brain function. Therefore, mGlu receptors that

activate intracellular signaling cascades offer an opportunity for developing drugs that regulate glutamate neurotransmission (B. A. Rowe et al. 2008).

1.2.2. mGlu receptor family

The mGlu receptor family is divided into three major groups according to their amino acid sequence homology, pharmacology and the preferred signal transduction mechanisms they couple to when expressed *in vitro* (C. J. Swanson et al. 2005). Group I mGlu receptors ($mGlu_1$ and $mGlu_5$) are located primarily postsynaptically where they couple to $G\alpha_q/G_{11}$. Activation of group I results in phospholipase C (PLC) stimulation, an increase in phosphoinositides hydrolysis and increases in intracellular calcium (Vinson & P Jeffrey Conn 2012). Group II ($mGlu_2$ and $mGlu_3$) and group III ($mGlu_4$ and $mGlu_{6-8}$) receptors are coupled to $G\alpha_i/G\alpha_o$ (Vinson & P Jeffrey Conn 2012) and typically inhibit adenylyl cyclase activity when expressed *in vitro* (C. J. Swanson et al. 2005) and modulate voltage-dependent ion channels. Despite the same mode of action, the distribution of these two groups of receptors (group II and III) is different. While group II mGlu receptors are localized mainly presynaptically and are primarily distributed in forebrain regions (this topic will be discuss later), group III mGlu receptors are expressed both presynaptically and postsynaptically. The distribution of this group is more restricted, some of the members of this group are expressed in the cerebellum and striatum and other members are present in the hippocampus. (Vinson & P Jeffrey Conn 2012).

A variety of *in vitro* and *in vivo* studies suggest that specific mGlu receptors subtypes play neuromodulatory roles in different central nervous system circuits and that specific subtypes may provide targets for novel treatment strategies for neurological and psychiatric disorders, including anxiety (C. J. Swanson et al. 2005) , pain (Fisher et al. 2002), schizophrenia (P Jeffrey Conn et al. 2008) and cognitive disorders (Campbell et al. 2004).

In the case of group II, their activation leads to a reduction in transmission at glutamatergic synapses in brain regions where excessive glutamatergic neurotransmission may be implicated in anxiety and schizophrenia, principally, in the prefrontal cortex and hippocampus. Hence, activation of these receptors may provide anxiolytic and/or antipsychotic effects (P Jeffrey Conn et al. 2008; C. J. Swanson et al. 2005).

1.2.2.1. Structure

mGlu receptors as the other GPCRs are divided in three major domains, as mentioned in section 1.1.1 (Figure 5). These receptors, in the particular, have an extracellular N-terminal domain exceptionally large and is linked to the 7TM domain by an amino acid stretch rich in cysteine residues (Urwyler 2011).

The extracellular ligand recognition N-terminal domain has a so-called bi-lobed structure (Venus flytrap domain; VFD) that can adopt an open or closed configuration in the absence or

presence of an agonist, respectively (P J Conn & J. P. Pin 1997; F Gasparini & Spooren 2007; Bessis et al. 2000). This change, caused by glutamate binding at the VFD is transmitted via the cysteine-rich domain (CRD).

The N-terminal recognition site is a well conserved site probably because it has to accommodate the natural ligand glutamate (Urwyler 2011).

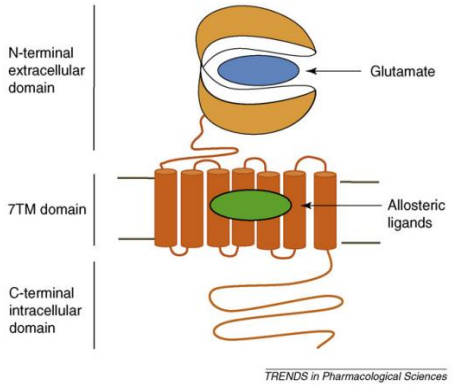


Figure 5. Schematic illustration of the mGlu receptor structure (P Jeffrey Conn et al. 2008)

The identification of the glutamate binding site or orthosteric site in the N-terminal extracellular domain of mGlu receptors was possible by X-ray crystallography, ligand binding studies and mutagenesis (Kunishima et al. 2000), which will be discussed further in another section.

Table 2. Classification of the metabotropic glutamate (mGlu) receptors (C. J. Swanson et al. 2005)

Family receptor	Coupling	Key localization and actions	Group/subtype-selective pharmacological agents
Group I			
$mGlu_1$	Excitatory Gq-coupled	Most often postsynaptic at glutamatergic synapses. Involved in synaptic plasticity, including long-term potentiation/depression. Cerebellar localization in granular cell and parallel fibre layers	Agonists: DHPG, 1S,3R-ACPD, quisqualate Antagonist: LY393675 Inverse agonist (or allosteric antagonist): LY367385
$mGlu_3$	Excitatory Gq-coupled	Most often postsynaptic at glutamatergic synapses, also found in glial cells. High expression in several forebrain regions including hippocampus and amygdala. Involved in synaptic plasticity specially long-term potentiation	Agonists: DHPG, 1S,3R-ACPD, quisqualate, CHPG Inverse agonist (or allosteric antagonist): MPEP
Group II			
$mGlu_2$	Inhibitory Gi/Go coupled	Localization largely presynaptic on glutamatergic and other neurotransmitter synapses. High expression in forebrain regions including hippocampus and amygdala, can also be find in certain layers with the cortex and cerebellum. Linked to hippocampal LTD and regulation on medial perforant path	Agonists: DCG-IV, 2R,4R-APDC, 1S,3R-ACPD, LY354740, LY379268 Antagonist: LY341495 Potentiator: 4-MPPTS (LY487379), 4-APPES, CBiPES
$mGlu_3$	Inhibitory Gi/Go coupled	Widely expressed in glial cells but also discrete localization both pre and postsynaptic on glutamatergic and other neurotransmitter synapses. Expression within forebrain regions including hippocampus and thalamus. Linked to neurotropin release from glial cells	Agonists: DCG-IV, 2R,4R-APDC, 1S,3R-ACPD, LY354740, LY379268 Antagonist: LY341495
Group III			
$mGlu_4$	Inhibitory Gi/Go coupled	Localization both pre and postsynaptic on glutamatergic and other neurotransmitter synapses. Presynaptic in cerebellar fibres and linked to cerebellar plasticity and motor learning	Agonist: L-SOP, ACPT-a, L-AP4 Antagonists: MSOP, MAP4, CPPG
$mGlu_6$	Inhibitory Gi/Go coupled	Expression confirmed only in retinal bipolar ON cells. Knockout animals reported to have visual acute deficits	Agonists: L-SOP, L-AP4 Glutamate-site antagonist: MSOP, MAP4
$mGlu_7$	Inhibitory Gi/Go coupled	Localization both pre and postsynaptic on glutamatergic and other neurotransmitter synapses in limbic and cortical regions. Has lower affinity for glutamate than other mGlu subtypes and only presynaptic inhibitory mGlu localized to active zone of synapses. Thought to serve a classical autoreceptor function	Agonists: L-SOP, L-AP4 Antagonists: MSOP, MAP4, LY341495 (100-fold lower affinity than group II)
$mGlu_8$	Inhibitory Gi/Go coupled	Localization largely presynaptic on glutamatergic and other neurotransmitter synapses. High expression un forebrain regions including hippocampus and amygdala. Linked to regulation of lateral perforant path	Agonists: L-SOP, L-AP4, 3,4-DCPG Antagonists: MSOP, MAP4

1.3. Glutamate and diseases

1.3.1. Schizophrenia

Schizophrenia is a devasting psychiatric illness that has a prevalence of approximately 0.8% of the population and a lifetime prevalence of approximately 1% (Lodge & A. a Grace 2011; P Jeffrey Conn et al. 2008; Siegel et al. 2006; Stilo & Murray 2010). Although causes of schizophrenia remain unknown, the disease has been extensively characterized from both a symptomatic and neurocognitive perspective, and much information has accumulated about elements such as genetic causation and longitudinal course (Javitt 2010).

Even though this disease was once seen as a disorder that affects only a few brain regions and regionally discrete neurotransmitter systems such as dopamine, more recent findings implicate widespread cortical and subcortical dysfunction, suggesting a more generalized etiology (Javitt 2010).

1.3.1.1. Clinical aspects

Schizophrenia is characterized by three partially independent symptom clusters. These symptoms are designated as positive symptoms that include hallucinations, delusions, thought disorder, paranoia, in general reflect features of the schizophrenia experience that are not shared by the general population; negative symptoms like social withdrawal, anhedonia, apathy, paucity of speech, basically features of normal experience that are reduced in individuals with schizophrenia. The other group of symptoms is cognitive impairments. Cognitive deficits are typically associated with deficits in perception, attention, learning, short- and long-term memory and executive function (P Jeffrey Conn et al. 2008; Javitt et al. 2001; Lodge & A. a Grace 2011; Siegel et al. 2006). The cognitive impairment in this disease is one of the major disabilities associated with the illness and is considered a reliable predictor of long-term disability and treatment outcome (P Jeffrey Conn et al. 2008).

1.3.1.2. Neurotransmitter hypothesis

Dopamine hypothesis

For many years, the dopamine hypothesis has driven the primary line of analysis into schizophrenia. This hypothesis is based on a variety of observations linking dopamine dysregulation to the pathophysiology of the disease. This includes the finding that enhanced activity within the subcortical dopamine system is associated with the positive symptoms of this disorder and the fact that most of the antipsychotic medications are dopamine D2 receptor antagonists (A. A. Grace 2011; Lodge & A. a Grace 2011). Also, there is pharmacological data showing that drugs that augment dopamine transmission exacerbate psychosis in schizophrenia patients and mimic it in controls (A. A.

Grace 2011). In other words, these drugs like amphetamines, precipitate psychotic episodes in normal individuals that are virtually indistinguishable from the acute psychotic episode observed in schizophrenia patients. Imaging studies in these patients demonstrate significantly increased release of dopamine in the striatum in response to amphetamine administration with the amplitude of increased dopamine release corresponding to the exacerbation of positive symptoms (Lodge & A. a Grace 2011; Vinson & P Jeffrey Conn 2012). The increased levels of dopamine were measured by a decrease in D_2/D_3 receptor binding by [^{11}C]raclopride and [^{123}I]iodobenzamide with the latter study showing this difference specifically in patients with active symptoms of the disease.

Regardless of these observations, there has been little direct evidence from neurochemical post-mortem studies to confirm an abnormality of central dopamine neuronal function in non treated schizophrenic patients (Rowley et al. 2001).

Serotonin hypothesis

The lesser known hypothesis in the pathophysiology of schizophrenia is hyperfunction of 5-HT_2 receptors (serotonin receptors). This hypothesis was suggested because reports have found changes in expression of serotoninergic receptors. Furthermore, atypical antipsychotic drugs like clozapine exhibit high affinity for 5-HT_2 and display efficacy on negative symptoms and cognitive deficits despite the low affinity for D2 receptors (Rowley et al. 2001).

Support for the 5-HT hypothesis has also been provided by the known hallucinogenic effect of 5-HT receptor agonists, such as lysergic acid diethylamide (LSD), mescaline and psilocin (Aghajanian & G.J. Marek 1999; Rowley et al. 2001). This hypothesis suggests that selective blockade of 5-HT2A receptors may be sufficient as a monotherapy in schizophrenia (Chavez-noriega et al. 2005).

Glutamate hypothesis

Beyond these hypotheses the now widely supported glutamate hypothesis of schizophrenia, often called the glutamate hypofunction hypothesis, is providing significant impetus in the field of schizophrenia research (Chavez-noriega et al. 2005). This model was based on the observation that phencyclidine (PCP “angel dust”), ketamine (non-competitive NDMA antagonists) and similarly acting psychotomimetic compounds, like MK-801 (Vinson & P Jeffrey Conn 2012), induced their unique behavioral effects by blocking neurotransmission at NMDA receptors. The action of these compounds uniquely reproduce the symptomatic, neurocognitive and neurochemical aspects of the disorder which led to the concept that symptoms in schizophrenia may reflect underlying dysfunction or dysregulation of NMDA receptor-mediated neurotransmission (Kantrowitz & Javitt 2010; Javitt 2010). Furthermore, these compounds exacerbate all three types of symptoms in individuals with schizophrenia (Vinson & P Jeffrey Conn 2012). The involvement of glutamate receptors in

schizophrenia is also supported by observation, in *post mortem* studies, of altered glutamate receptor (in particular NMDA receptors) density in some brain regions, in schizophrenic patients (Rowley et al. 2001).

The NMDA receptor hypofunction hypothesis is furthermore supported by the fact that administration of NMDA receptor agonists improved negative symptoms and cognitive deficits in schizophrenia patients (Heresco-Levy 2002; Javitt et al. 2001; Tsai & Coyle 2002).

All these observations suggest a possible role for decreased NMDA receptor signaling in schizophrenia (Vinson & P Jeffrey Conn 2012) and because glutamate/NMDA receptors are located throughout the brain with notable density in cortical and subcortical regions, glutamatergic models predict widespread cortical dysfunction with particular involvement of NMDA receptors throughout the brain (Javitt 2010).

This does, however, not necessarily imply a primary deficit in NMDA receptor function in the etiology of schizophrenia, there are a wide range of possible mechanisms by which NMDA receptor function could be down regulated in a manner that could contribute to the pathology. While the expression or functioning of the receptor itself may be compromised, NMDA receptor signal transduction could also be affected by changes in the level or activity of a number of proteins as well as any factor influencing glutamate availability at the postsynaptic site or the occurrence of coincident membrane depolarization (Vinson & P Jeffrey Conn 2012).

Further, NMDA receptors are located on brain circuits that regulate dopamine release, suggesting that dopaminergic deficits in schizophrenia may also be secondary to underlying glutamatergic dysfunction (Javitt 2010). In addition, with use of imaging approaches it has been found that glutamatergic and dopaminergic neurotransmission seem to interplay with each other producing the observed symptoms (Vinson & P Jeffrey Conn 2012).

In resume dopaminergic models of schizophrenia account well only for positive symptoms of the disease. In contrast, glutamatergic models account much more fully for both negative and cognitive symptoms, and thus may serve as an etiological model for the syndrome as a whole (Javitt 2010).

The diversity of observations has led to the hypothesis that schizophrenia is more than a result of a change in magnitude of neurotransmitter signaling, but is also a change in the underlying brain circuit (Vinson & P Jeffrey Conn 2012).

1.3.1.3. Glutamatergic circuitry

Further investigation has revealed the involvement of glutamatergic pathways and signaling in ways that are not mutually exclusive to the hypothesized involvement of the dopaminergic system (Vinson & P Jeffrey Conn 2012).

Glutamate hypofunction is relevant to the positive and cognitive symptoms observed in schizophrenia because NMDA receptor activation and function is a process that is critically involved in synaptic plasticity, a mechanism required for learning and memory formation. This process begins with NMDA receptor activation which requires the agonist glutamate and glycine in addition to membrane depolarization, resulting in calcium influx through the receptor channel (Vinson & P Jeffrey Conn 2012).

NMDA receptors are located on GABAergic neurons in subcortical regions such as the nucleus accumbens and on glutamatergic neurons projecting from the mediodorsal thalamus to pyramidal neurons in the prefrontal cortex. NMDA receptors on the GABAergic neurons receive excitatory input from glutamatergic afferents and their activation results in an inhibitory regulation of the thalamocortical pathway. A reduction in NMDA receptor function on these GABAergic neurons result in disinhibition of thalamocortical glutamatergic signaling to the prefrontal cortex and therefore an increase in excitatory glutamatergic input to pyramidal neurons in the prefrontal cortex (Figure 6). This model is supported by the fact that psychotomimetic NMDA receptors antagonists have been shown to cause an increase in extracellular glutamate levels in the prefrontal cortex which has been hypothesized to be linked to the effects of these agents on certain aspects of cognitive function and locomotor activity (Vinson & P Jeffrey Conn 2012).

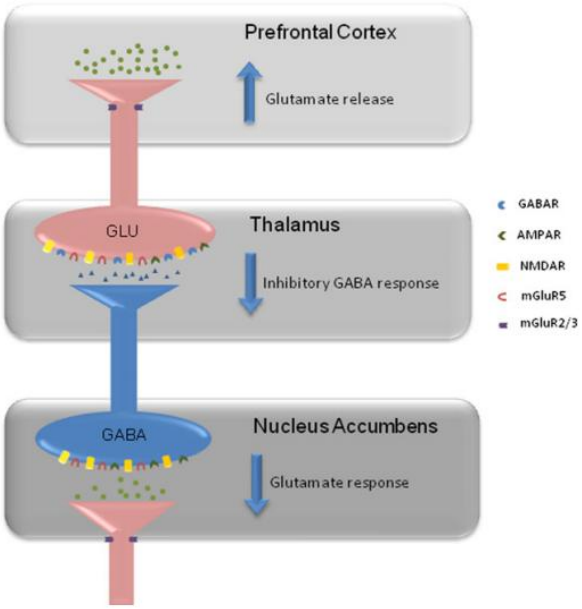


Figure 6. Simplified schematic illustration of glutamatergic-GABAergic microcircuitry between subcortical and cortical regions (Vinson & P Jeffrey Conn 2012)

1.3.1.4. Current treatment

Current antipsychotic treatments exert their function by blocking dopamine D_2 receptors. There are two classes of these antipsychotics: the typical antipsychotics that include haloperidol and chlorpromazine and the atypical antipsychotics like olanzapine and clozapine. These two classes differentiate themselves in their degree of specificity for D_2 over other neurochemical targets, occupancy time at D_2 and their resulting side effect profile. Generally, the atypical group of drugs is less selective for, bind with lower affinity to, and has a faster off rate from the D_2 receptor compared to the typical antipsychotics (Vinson & P Jeffrey Conn 2012).

As said before, the currently available antipsychotic medication is only efficient at treating positive symptoms, in a subset of patients, but do not show a high level of efficacy towards the other classes of symptoms and exhibit significant side effects such as extrapyramidal symptoms (EPS) and tardive dyskinesia (Chavez-noriega et al. 2005). Antipsychotic treatments are not treating the disease, but rather produce an abnormal state that offsets the downstream dopamine dysfunction associated with positive symptoms (Lodge & A. a Grace 2011). Furthermore, there is a high prevalence of patients discontinuing treatment (reported to be >74%). Collectively, this demonstrates the need for improved therapies (Lodge & A. a Grace 2011).

The disruption in glutamatergic signaling via NMDA receptors in cortical and midbrain circuits in schizophrenia, (see above), has convinced investigators to follow this concept for therapeutic development (Vinson & P Jeffrey Conn 2012). It has been seen that agents that stimulate NMDA receptor-mediated neurotransmission, including glycine-site agonists and glycine transport inhibitors, have shown encouraging results in preclinical studies and are currently in clinical development (Javitt 2010; Javitt et al. 2001). However, targeting NMDA receptor or other ionotropic glutamate receptors directly is not considered to be a viable approach because of their widespread role in fast synaptic transmission throughout the central nervous system and the potential toxicity of overactivation of NMDA receptors. Another option to regulate transmission through these circuits is to target **mGlu receptors** which function to modulate synaptic transmission and neuronal excitability. In particular, attention has been given on targeting mGlu receptor subtypes 2,3 and 5 as a novel treatment strategies for treatment of schizophrenia (Vinson & P Jeffrey Conn 2012). Encouraging results have been observed, as well, with agents such as mGlu 2/3 receptor agonists that decrease resting glutamate levels, reversing potential disruption in firing patterns within prefrontal cortex and possibly other brain regions (Javitt 2010).

1.3.2. Cognition

mGlu receptors play an important role in a number of forms of synaptic plasticity, including induction of hippocampal long-term potentiation (LTP), that is known to be involved in mechanisms of learning and memory formation. They also elicit physiologic effects in the hippocampus that might enhance cognitive function (P J Conn & J. P. Pin 1997).

Since cognitive deficits are another key feature of schizophrenia and are the strongest predictor of long-term outcome for patients, as such, they constitute an important target for pharmacological treatment (Chavez-noriega et al. 2005; Green & Braff 2001).

There is, also, some evidence suggesting that cognitive impairments form a core element of depression (Austin et al. 1999).

These observations suggest that mGlu receptor ligands may be useful as cognitive enhancing agents in diverse disorders that cause cognitive impairments and memory deficits (P J Conn & J. P. Pin 1997). Experiments in rodents and a small clinical study suggest that group II mGlu receptor agonists may be efficacious in ameliorating the cognitive deficits in individuals with compromised NMDA receptor function (Chavez-Noriega et al. 2005).

1.3.3. Anxiety and Depression

Anxiety disorders can last at least six months and get worse if they are not treated. They commonly occur along with other mental illness or physical illness, including alcohol or substance abuse, which could mask or worsen anxiety symptoms. In some cases, the other disorders need to be treated before the treatment of the anxiety symptoms becomes effective (National institute of Health).

In general stress- and anxiety-related illnesses represent a collection of disorders, including panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, social phobia and generalized anxiety disorders (National institute of Health), which have in common excessive or inappropriate brain excitability within crucial brain circuits (C. J. Swanson et al. 2005).

There are several forms of depression: 1) major depressive disorder; 2) dysthymic disorder; 3) psychotic depression; 4) postpartum depression; 5) season affective disorder. In general these disorders are characterized by a combination of symptoms like disability of work, sleep, study and enjoy once-pleasurable activities (anhedonia). In some cases like psychotic depression, the depressive illness is accompanied by hallucinations and delusions (National institute of Health). It is important to notice that some of these symptoms like anhedonia, hallucinations and delusions are symptoms of psychiatric diseases like schizophrenia (Venzala et al. 2012).

Major depression is a mental illness very often described as a stress-related disorder since there is good evidence that both onset and relapse of depressive disorders can be precipitated by repeated stress or severe stressful experiences (Venzala et al. 2012)

In rat models for depression alterations in neurotransmitter levels, namely increased levels of glutamate and decreased levels of dopamine and GABA, have been found. Consequently, in this type of disorder there is also excessive excitatory neurotransmission (Venzala et al. 2012).

As all of these disorders have in common inappropriate brain excitability and since glutamate is the major excitatory neurotransmitter in the brain, huge research efforts are devoted to novel treatments that could modulate glutamate functions (C. J. Swanson et al. 2005). Since the mGlu2 receptor is present in areas of the brain that are thought to play a critical role in anxiety disorders (Walker & Davis 2002) and psychosis among other CNS disorders (Galici et al. 2005), this receptor in particular is considered a promising target.

1.4. mGlu2 receptor as a drug target

1.4.1. Expression

mGlu2 receptors are expressed presynaptically in most brain regions (Chavez-noriega et al. 2005) and spinal cord areas. I*n situ* hybridization, immunohistochemistry and autoradiography have confirmed expression of mGlu2/3 receptors in hippocampus, olfactory bulb, neocortical regions and cerebellar Golgi neurons, with lower levels of expression in thalamic nuclei and striatum (Figure 7) (Hervé Schaffhauser et al. 2003).

Figure 7. Immunoreactivity for mGlu 2 receptors. AOB - accessory olfactory bulb; Cx, neocortex; St, neostriatum (Shigemoto & Mizuno 2000)

mGlu2 receptors are observed not only in somatodendritic domains but also in axonal domains. They are also present in interstitial glial cells of the pineal gland. Group II receptors are often observed in extrasynaptic sites (Figure 8) remote from the active zone in preterminal portions of axons and axon terminals and about 79% of immunoparticles for the mGlu2 receptor in cerebellar Golgi cell axons (Shigemoto & Mizuno 2000).

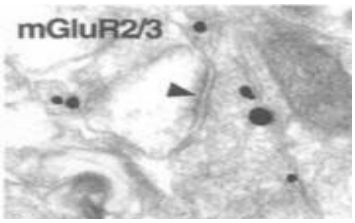


Figure 8. Subcellular localization of group II mGlu receptors (Shigemoto & Mizuno 2000)

1.4.2. Function

Generally, group I receptors potentiate glutamate function, particularly at NMDA receptors, whereas group II and III receptors decrease synaptic transmission and glutamate release in the hippocampus (Macek et al. 1996; Kantrowitz & Javitt 2010). So, pharmacological activation of group II mGlu receptors potently inhibits excitatory glutamatergic synaptic transmission in several brain areas relevant to several pathophysiologies via a presynaptic site of action (Chavez-noriega et al. 2005). It is important to note that group II mGlu receptors activation reduce transmission at inhibitory synapses in the accessory olfactory bulb (P J Conn & J. P. Pin 1997), hippocampal area CA3 in young animals (Poncer et al. 1995), and thalamus (Salt & Eaton 1995).

1.4.3. Signal transduction

As stated above, group II mGlu receptors are negatively coupled to adenylyl cyclase (C. J. Swanson et al. 2005), and inhibit cAMP formation stimulated by either forskolin (activator of adenylyl cyclase) or a G_S-coupled receptor. This effect is inhibited by pertussis toxin (PTX) treatment of the cells, which indicates the involvement of a G_i-type of G-protein (P J Conn & J. P. Pin 1997). Inhibition of the cAMP cascade occurs in neuronal and glial cells (L Prézeau et al. 1994).

Activation of mGlu2 receptors is also linked to rapid-onset regulation of various channels including calcium channels (S. Choi & Lovinger 1996) and G-protein-coupled inwardly rectifying K^+ channels (Knoflach & J. a Kemp 1998) depending on neuronal cell types (Shigemoto & Mizuno 2000).

1.4.4. Pharmacology

Agonists

The structural analogy to glutamate, with the presence of a distal carboxylic acid to the amino acid functionality, strongly influences the properties of competitive or orthosteric ligands, which bind to the same site as glutamate, and limits considerably their capacity to cross membranes by passive diffusion. As a consequence, most of these compounds have a poor oral bioavailability, and do not readily cross the blood-brain-barrier (F Gasparini & Spooren 2007).

Examples of these glutamate analogs (in rank of order of potency) are (2S, 1'R, 2'R, 3'R)-2-(2, 3-dicarboxycyclo-propyl) glycine (DCG-IV, Figure 9) = L-CCG-I > 2R,4R-4-aminopyrrolidine-2, 4-dicarboxylate (APDC, Figure 9) > glutamate > 1S, 3S-ACPD > 1S, 3R-ACPD > 4C3HPG (Figure 9) > ibotenate (P J Conn & J. P. Pin 1997). Only compound 2R, 4R - APDC is a specific group II agonist (D.D Schoepp et al. 1995). See Table 3 for compounds potency.

More recently identified orthosteric agonists for the mGlu2/3 receptor are an exception for the cases mentioned above. These small molecular weight ligands (LY354740, LY379268 and LY404039) (Rorick-Kehn et al. 2007) display potent agonist activity at mGlu2/3 receptors, are orally

bioavailable and enter the brain in preclinical models (J A Monn et al. 1999). It was also showed that these molecules have efficacy in preclinical models of psychosis and anxiety (Gregory et al. 2011; C. J. Swanson et al. 2005; Trabanco et al. 2011). The neurochemical evidence for the ability of group II activation to reverse the effects of psychotomimetic agents has been supplemented by their ability to reverse the behavioral effects in several animal models that are used to predict efficacy of potential antipsychotic agents although there are some exceptions to this effect depending on which compound is being studied, which behavioral paradigm is being tested and what strain of animal is being used (Vinson & P Jeffrey Conn 2012).

LY2140023 (Figure 9) which is the oral prodrug of the agonist LY404039 (Figure 9), has shown beneficial effects on positive and negative symptoms of schizophrenia without the side effects associated with typical and atypical antipsychotics (Patil et al. 2007). Recently this compound has entered phase II/III trials for schizophrenia. Other compounds, LY354740 (Figure 9) and LY379268 (Figure 9) have clinical efficacy in treating panic attacks and generalized anxiety disorders (Gregory et al. 2011), which can be seen by preclinical animal models of anxiety (Trabanco et al. 2011). Another group II agonist is MGS0028 (Hervé Schaffhauser et al. 2003). Nevertheless, only LY404039 is currently still in clinical development.

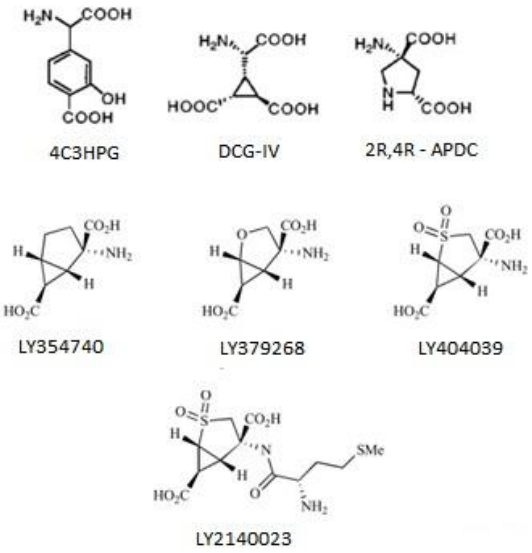


Figure 9. Structures of some glutamate group II receptors agonists [adapted from (P Jeffrey Conn et al. 2009; Trabanco et al. 2011)]

Antagonists

The rank order of potency of mGlu2 receptor antagonists is 2S-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl) propionic acid (LY341495) = (1R,2R,3R,5R,6R)-2-amino-3-(3,4-dichlorobenzyloxy)-6-fluorobicyclo [3.1.0] hexane-2,6- dicarboxylic acid (MGS0039) > (2S, 1'S, 2'S, 3'R)- 2-(2'-carboxy-3'-phenylcyclopropyl) glycine (PCCG-IV, Figure 10) > 2S, 4S- 2-amino-4-(4, 4-diphenylbut-1-yl)-pentane-1, 5-dioic acid (ADPD) > α-methyl-L-CCG-I (MCCG-I, Figure 10) > α-methyl-4-phosphonophenylglycine (MPPG) > α-methyl-4-sulfonophenylglycine (MSPG) > α-methyl-4-tetra- zoylphenylglycine (MTPG) (P J Conn & J. P. Pin 1997). See Table 3 for compounds potency.

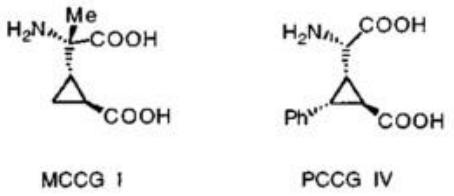


Figure 10. Structures of some glutamate group II receptors antagonists [adapted from (Trabanco et al. 2011)]

The therapeutic significance of mGlu2 receptor antagonists has not been widely investigated. However, studies of some selective competitive group II mGlu receptor antagonists, which include LY341495 and MGS0039, have suggested that these kind of compounds exhibit antidepressant-like activity and anti-obsessive-compulsive disorder-like effects in animal models (Chaki et al. 2004; Hemstapat et al. 2007).

Table 3. Summary of mGlu2 receptor agonists and antagonists potency (EC_{50} and IC_{50})

Agonist	Potency (μM)	Antagonist	Potency (μM)
Glutamate	4-20	MPPG	100
Ibotenate	35-250	MSPG	250
1S, 3R-ACPD	18	MTPG	450
1S, 3S-ACPD	13	MCCG-I	84
L-CCG-1	0.3-0.4	PCCG-IV	8
DCG-IV	0.3	ADPD	18.1
2R, 4R-APDC	3	LY341495	0.02
4C3HPG	20-50	MGS0039	0.02

1.5. Allosteric modulation

The majority of GPCR-based drug discovery programs have failed to yield highly selective compounds. This is due to the traditional approach that targets the endogenous ligand (orthosteric)-binding site, to either mimic or block the actions of the endogenous neurotransmitter or hormone in a competitive manner (Gregory et al. 2011). Some studies showed that orthosteric agonists used activate both mGlu2 and mGlu3 receptors, and induced tolerance in one rodent model (Galici et al. 2005). Preclinical studies indicate that the mGlu2 receptor is likely to be responsible for clinical efficacy (Fell et al. 2008) showing that sub-type specificity is needed. Therefore it is important to search for alternative structural compound classes to inhibit or activate mGlu receptor function (F Gasparini & Spooren 2007). Though, an alternative approach is to target allosteric sites. These sites are different from the orthosteric site, and their activation leads to enhancement or inhibition of receptor activation (Gregory et al. 2011).

The validity of GPCR allosteric modulators was demonstrated with two compounds that have entered the market. In 2004, one allosteric enhancer (cinacalcet) of the calcium-sensing receptor (CaSR) was approved for the treatment of hyperparathyroidism (Lindberg et al. 2005), and in 2007 an allosteric inhibitor (maraviroc) of the chemokine receptor CCR5 was approved for the treatment of HIV infections (Dorr et al. 2005).

One of the best known GPCR allosteric modulator is benzodiazepine. This is an allosteric modulator of $GABA_A$ receptors and is known for its effects on the treatment of anxiety and sleep disorders (P Jeffrey Conn et al. 2009).

Nowadays, for CNS disorders , allosteric modulation receives major attention in drug discovery (Gregory et al. 2011).

1.5.1. Allosteric (PAM and NAM) vs Orthosteric

In the case of mGlu receptors the orthosteric binding site is located in the N-terminal domain. The compounds that compete with endogenous agonists for this site are called competitive agonists or antagonists (F Gasparini & Spooren 2007). While the allosteric modulators bind to an allosteric site (topographically distinct from the orthosteric site) and modulate (increase or decrease) the response of an orthosteric ligand (P Jeffrey Conn et al. 2009; Vinson & P Jeffrey Conn 2012) and for the mGlu receptors is located in the less conserved transmembrane regions (Figure 5) (Urwyler 2011; Vinson & P Jeffrey Conn 2012).

Positive allosteric modulators (PAM) can increase the response of the endogenous ligand while negative allosteric modulator (NAM) decreases the response. Also, there are neutral allosteric ligands which bind to the allosteric site but have no effects on the response of the orthosteric ligand.

Because allosteric modulators bind to sites on a given receptor different from those to which orthosteric ligands bind, they are in general structurally diverse and not at all related to orthosteric ligands, particularly the endogenous natural agonists (Urwyler 2011).

1.5.2. Mode of action

The binding of an allosteric ligand to its site will change the three-dimensional conformation of the receptor (Gregory et al. 2011; Urwyler 2011) and induce a modulation (increase or decrease) of the response to the agonist that binds to the orthosteric site (Vinson & P Jeffrey Conn 2012). Basically, a receptor occupied by an allosteric ligand can be visualized as a "new" receptor type, with a unique behavior (P Jeffrey Conn et al. 2009). There are models that explain allosteric modulation. The simplest allosteric GPCR model assumes that binding of an allosteric ligand to its site modulates only the affinity of the orthosteric ligand and vice versa. This model is called the allosteric ternary complex model and is represented in Figure 11. In this model a value of $0<\alpha<1$ indicates that the binding of an allosteric ligand inhibits the binding of the orthosteric ligand (negative cooperativity), whereas values of $\alpha>1$ indicates positive cooperativity (allosteric modulator promotes the binding of orthosteric ligand). On the other hand, $\alpha=0$ indicates neutral cooperativity (the two sites are conformationally linked) (Gregory et al. 2011). It is important to know that cooperativity refers to the binding of two or more molecules of the same ligand to a receptor complex to initiate a response. It is also used in a less strict sense to describe the allosteric interaction between more than one molecule of any chemical type on a receptor complex (P Jeffrey Conn et al. 2009).

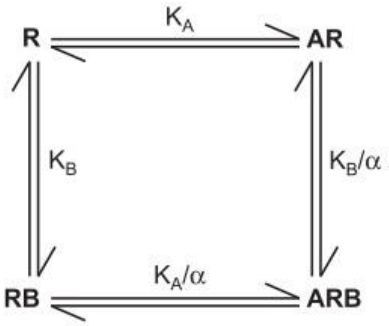


Figure 11. Allosteric ternary complex model. R – receptor; A- agonist; B – modulator; K_A – orthosteric ligand dissociation constant; K_B – allosteric ligand dissociation constant; α – cooperativity factor (Gregory et al. 2011)

In the allosteric ternary complex model the stimulus that is generated by the ARB ternary complex is assumed to be no different to that reported by the binary AR complex (Gregory et al. 2011).

However, an allosteric modulator in the GPCR can perturb signaling efficacy not only by effects on orthosteric ligand binding affinity. In the case of mGlu receptors the majority of allosteric

modulators influence orthosteric efficacy without affecting the affinity. This effect is due to the different orthosteric and allosteric binding sites localization (Gregory et al. 2011).

Furthermore, there are different modes of actions for different allosteric modulators. Some allosteric modulators can modulate orthosteric ligand affinity and/or efficacy and other allosteric ligands can directly perturb signaling in their own right (Figure 12) (P Jeffrey Conn et al. 2009). These compounds that act as agonists on their own are called allosteric agonists and add an additional layer of complexity to treatment options (Gregory et al. 2011; Hervé Schaffhauser et al. 2003).

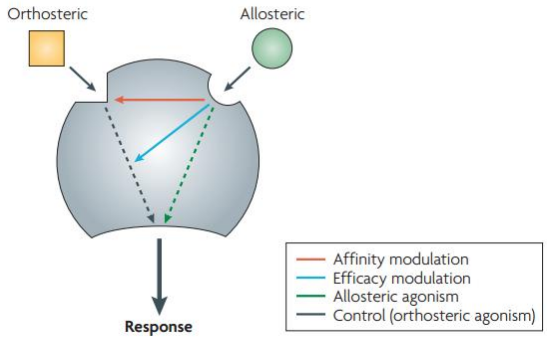


Figure 12. Modes of action of allosteric modulators (P Jeffrey Conn et al. 2009)

Since an allosteric modulator can have differential effects on affinity versus efficacy, alternative models have been developed to describe allosteric interactions (Gregory et al. 2011).

Figure 13 presents an "operational model of allosterism". This model describes both allosteric modulation of affinity and efficacy and incorporates allosteric agonism (Leach et al. 2007). While the previous models do not fit to real experimental data, this model combines both mechanistic and empirical parameters to facilitate quantification of experimentally-derived allosteric drug properties in a manner that can facilitate structure-activity studies (P Jeffrey Conn et al. 2009).

In this model, allosteric modulation is governed by two parameters (α and β) which can alter for each set of interacting ligands (Gregory et al. 2011).

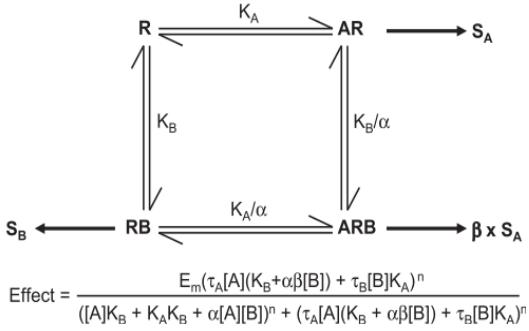


Figure 13. Operational Model of Allosterism. S – stimulus; AR – agonist bound; BR – modulator bound; ARB – ternary complex; α – cooperativity factor; β – allosteric modulation of efficacy; τ_A and τ_B – ability of the orthosteric and allosteric ligands, respectively, to engender receptor activation (incorporate the efficacy of each ligand, the total density of receptors and the stimulus-response coupling efficiency; E_m – maximal possible system response; n – slope factor (Gregory et al. 2011).

As a summary one can say that allosteric modulators exhibit one or more of the following pharmacological properties: affinity modulation: conformation change can impact the orthosteric binding site such that either association and/or dissociation rate of an orthosteric ligand is modified; efficacy modulation: allosteric effect can change intracellular responses which lead to a change in signaling capacity of an orthosteric ligand; agonism/inverse agonism: the allosteric modulator can perturb receptor signaling in a positive or negative way (P Jeffrey Conn et al. 2009; May et al. 2007; Hervé Schaffhauser et al. 2003).

1.5.3. Group II mGlu receptors NAMs and PAMs

Two main chemical classes of positive allosteric mGlu2 receptor modulators were originally described and have become standard tool compounds pyrimidylsulfonamides, represented by N-(4-(2-methoxy-phenoxy)-phenyl-N-(2,2,2-trifluoroethylsulfonyl)-pyrid-3- ylmethylamine (LY487379) and indanone compounds, represented by biphenylindanone A (BINA) (Urwyler 2011). These compounds are low molecular weight, structurally different from glutamate and are selective mGlu2 receptor positive modulators (PAMs) (B. A. Rowe et al. 2008; Hervé Schaffhauser et al. 2003). They increase the ability of the mGlu2 receptor to activate G-proteins by inducing a leftward shifts of the glutamate concentration response curve and potentiating the ability of group II selective agonists to reduce transmission at a number of glutamatergic synapses (Hervé Schaffhauser et al. 2003). BINA is a more potent and brain penetrable than LY487379, and its use *in vivo* confirmed potential anti-psychotic effects (F Gasparini & Spooren 2007).

The finding of selective mGlu2 receptor PAMs was important because it allows the development of compounds that selectively potentiate mGlu2 but not mGlu3 receptors (Hervé Schaffhauser et al. 2003).

Figure 14 shows the structure of additional mGlu2 PAM molecules, which have a similar profile as LY487379.

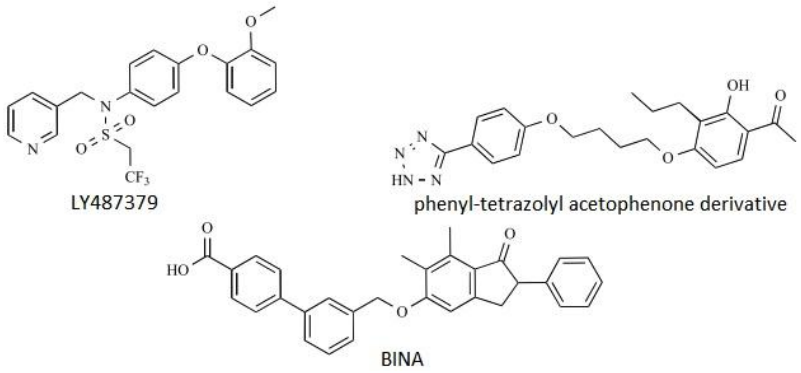


Figure 14. mGlu2 receptor PAMs [adapted from (F Gasparini & Spooren 2007)]

Other types of mGlu2 PAMs are represented in Figure 15. Compounds A, B, C and D are selective for mGlu2 receptor and increase both potency and efficacy of glutamate. On the other hand, compounds E, F, G and H are mGlu2 receptor potentiators and weak mGlu3 receptor positive allosteric modulators (B. A. Rowe et al. 2008).

There are also allosteric compounds, mGlu2 NAMs, (Figure 16) that have shown to inhibit agonist stimulated GTP-γ-[^{35}S] binding (F Gasparini & Spooren 2007).

Also Hemstapat et al. found that three compounds (MNI-135, MNI-136 and MNI-137) are selective mGlu2/3 NAMs. However, none of these compounds provided sufficient selectivity between the group II mGlu receptors to be useful for differentiating between these group subtypes.

Taking in account the effects achieved with orthosteric antagonists, it could be speculated that mGlu2 receptor NAMs could improve cognitive and memory disturbances (Higgins et al. 2004).

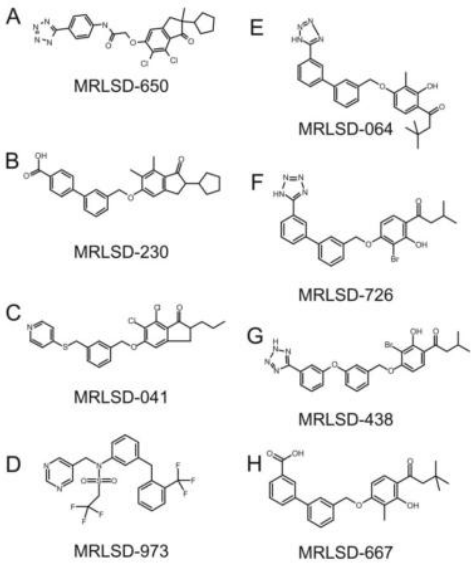


Figure 15. Chemical structures of mGlu2 PAMs described by (B. A. Rowe et al. 2008)

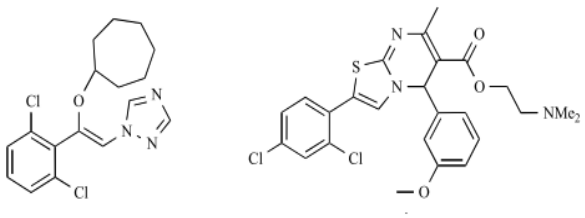


Figure 16. **mGluR2 NAMs** (F Gasparini & Spooren 2007)

1.5.3.1. Group II mGlu receptors allosteric binding site

Figure 17, shows the three-dimensional structure of the mGlu2 receptor based on X-ray crystal structure of the bovine rhodopsin receptor. The N-terminal extracellular, glutamate binding site is represented as a clamshell-shaped object. The transmembrane domains are depicted as α-helical structures. The residues (Ser 688, Gly 689 and Asn 735, respectively) depicted in the TM IV and V domain have been shown to be involved in the binding of LY487379 (Hervé Schaffhauser et al. 2003).

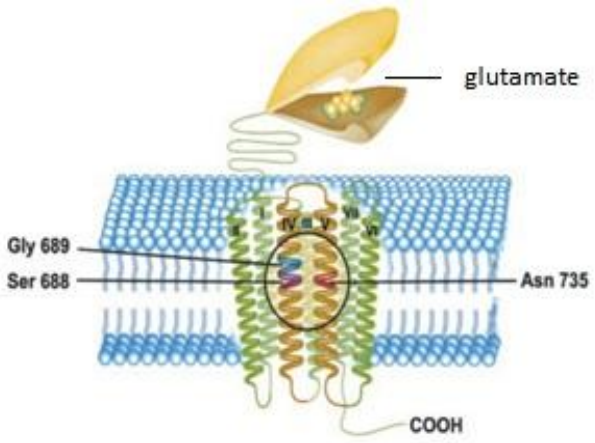


Figure 17. Schematic diagram of the three-dimensional protein structure of the mGlu2 receptor [adapted from (Hervé Schaffhauser et al. 2003)]

In addition, Hemstapat *et al.* verified that Asn 735 is also critical for the binding of BINA and Rowe *et al.* described that these residues are, as well, involved in the binding of other PAMs. Although this proves that these residues are critical it does not mean they are the only three residues involved in the activity of these compounds (B. A. Rowe et al. 2008). These studies will be discussed in more detail in section 1.6.

Previous studies demonstrate that some of the critical residues for PAM binding do not seem to affect binding of some NAM compounds (MIN-135, MNI-1366 and MNI-137), indicating that the binding site for NAMs and PAMs for mGlu2 seems to be different (Hemstapat et al. 2007).

1.5.4. Measurement of PAM and NAM in vitro

In order to identify allosteric modulators *in vitro* assays that allow the characterization of the functional activity of agents acting at the receptor have to be used. Related to group II and III mGlu receptors (coupled to Gi type of G-proteins), functional assays involving GTP-γ-^{35}S binding and determination of cAMP concentration changes can be used to identify allosteric ligands (Figure 18B) (F Gasparini & Spooren 2007).

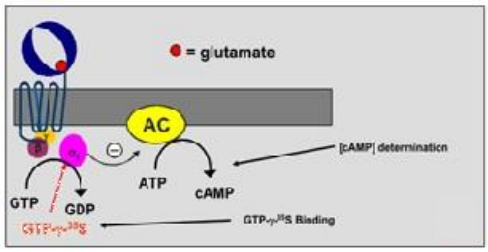


Figure 18. Group II and III mGluR signal transduction pathways and methods to see efficacy (F Gasparini & Spooren 2007)

The development of these functional assays allowed the screening of large chemical libraries and the identification of numerous ligands with no structural analogy to glutamate (natural ligand) acting as NAM or PAM (F Gasparini & Spooren 2007).

1.5.5. Advantages

PAMs offer an attractive therapeutic approach for the activation of GPCRs because they would be efficacious only in the presence of endogenous agonist (if they do not display any direct agonism) (P Jeffrey Conn et al. 2009) and for that they may elicit less tachyphylaxis (acute decrease in the response to a drug after administration) (Hervé Schaffhauser et al. 2003; Gregory et al. 2011). The use of allosteric modulators is also important because it can overcoming receptor desensitization that occurs after persistent treatment with agonists (May et al. 2007; J. P. Pin et al. 2001; Urwyler 2011). Moreover, such allosteric modulators maintain activity dependence and both temporal and spatial aspects of endogenous physiological signaling (Urwyler 2011; P Jeffrey Conn et al. 2009), and for that side effects may be reduced (Urwyler 2011). Via this way, one may expect a better therapeutic outcome compared to sustained blockade or activation achieved by orthosteric ligands (Gregory et al. 2011).

Moreover, since their binding sites are outside of the highly conserved agonist binding site, they offer the potential for highly selective ligands, which has been difficult to achieve. Alternatively, selectivity could be achieved by combining both orthosteric and allosteric pharmacophores within the same molecule to yield a novel class of bitopic GPCR ligand (P Jeffrey Conn et al. 2009; Gregory et al. 2011; Hervé Schaffhauser et al. 2003).

Also, allosteric modulators with limited positive or negative cooperativity allow a high degree of titratability of the pharmacological effect, meaning that large doses of allosteric modulator can be administered with a lower propensity towards target-based toxicity than orthosteric agonists or antagonists. Furthermore, limited cooperativity modulators can allow for a subtle re-setting of endogenous agonist activity (P Jeffrey Conn et al. 2009).

Another advantage is the fact that allosteric modulators could be useful in diseases states in which the level of the endogenous ligand is attenuated. In these cases, administration of a potentiator could achieve normal agonist responses (B. A. Rowe et al. 2008).

1.5.6. Disadvantages

It is clear that, although, both PAMs and NAMs rely upon the presence of the endogenous ligand, allosteric modulators offer a variety of advantages over orthosteric counterparts. However, the drug discovery centered on small molecules, either allosteric and orthosteric, share common problems, including solubility and formulation, generation of active metabolites, clearance and lack of brain penetrance (Gregory et al. 2011; Hervé Schaffhauser et al. 2003). Moreover, the fact that allosteric sites are not conserved, which permits receptor subtype selectivity, also may result in species differences, which may lead to problems when using rodent models in drug discovery (Urwyler 2011).

Another disadvantage lays in the activity-dependence of allosteric modulators. This fact, already discussed as an advantageous can become a handicap in some diseases like neurodegenerative disorders in which the loss of neurons can result in decreased availability of the endogenous ligand (Urwyler 2011).

1.6. Mutagenesis Studies on mGlu receptors

Mutagenesis studies of GPCRs have been extensively used to depict the molecular determinants of the receptor involved in the functional coupling, both physical interaction and activation, to G-proteins (Gether 2000; Wess 1998). This type of studies has demonstrated the existence of critical regions whose alteration differentially affects the intracellular signaling cascade triggered by the agonist. It has also been shown that distinct domains of the receptor are involved in the functional coupling with multiple G-proteins (Hermans 2003).

Site-direct mutagenesis is furthermore used to identify important residues and binding determinants for allosteric modulators (Gregory et al. 2011). The first allosteric modulator to be identified was CPCCOEt, a NAM of $mGlu_1$. Litschig *et al*. identified the two amino acids in the 7TMD of $mGlu_1$ responsible for the selective action of this compound. They were able to reach this conclusion because of the selectivity of CPCCOEt for $mGlu_1$ relatively to $mGlu_5$. Hence, they switched crucial residues in $mGlu_1$ to their corresponding residues of $mGlu_5$ resulting in a gain of function for these $mGlu_1$ selective modulators at $mGlu_5$ receptors (S Litschig et al. 1999). In other studies some amino acids were identified as important for binding of allosteric modulators in the hmGluR2 (as mentioned before in the section 1.5.3.1). One of the studies was done by Rowe *et al*., they prepared mutant receptors by exchanging either segments, single amino acids residues, or multiple amino

acids residues between hmGluR3 and hmGluR2. They determined that the exchange of the mGluR2 amino acid residues present in TMIII-V (Leu656 to Arg750) with homologous hmGluR3 sequences resulted in a complete loss of the potentiatior activity of MRLSD-650 (mGluR2 specific). This same domain was previously identified as the binding site for other mGluR2 potentiator LY487379 (Hervé Schaffhauser et al. 2003).

More mutagenesis studies on mGlu receptors followed, with the main goal of investigating the molecular determinants of the allosteric modulator-receptor interactions (Table 4). The identification of these amino acids may be important to improve selectivity and potency of allosteric compounds (Pagano et al. 2000).

In most of these studies, suggestions for amino acid mutations are based on sequence alignment, homology modeling and docking with receptors which structure has already been crystallized (Gregory et al. 2011).

Table 4. Summary of the amino acids characterized as important molecular determinants of mGlu receptors through mutagenesis studies; b.s. – binding site; hmGluR – human metabotropic glutamate receptor; rmGluT – rat metabotropic glutamate receptor

Target	Ligand	EC2	TM3	TM4	TM5	TM6	TM7	References	Remarks
hmGluR1	CPCCOEt (NAM)						Thr815 Ala818	Litschig *et al.*, 1998	
hmGluR5	MPEP (NAM)		Pro655 Ser658				Ala810	Pagano *et al.*, 2000	MPEP and CPCCOEt bind to overlapping binding pockets
rmGluR1	BAY36-7620 (NAM)			TM4 – TM7				Carrol *et al.*, 2001	
rmGluR1 **rmGluR5**	Ro 01-6128 Ro 67-4853 Ro 67-7476 (PAMs)		Ser668 Cys671		Val757 Pro654 Ser657			Knoflach *et al.*, 2001	
hmGluR2	LY487379 (PAM)			Ser688 Gly689	Asn735			Schaffhauser *et al.*, 2003	
rmGluR1	EM-TBPC (NAM)	Asn747			Val757	Trp798 Phe801 Tyr805	Thr815	Malherbe *et al.*, 2003a	
rmGluR5	MPEP (NAM)		Pro654 Tyr658		Leu743	Thr780 Trp784 Phe787 Tyr791	Ala809	Malherbe *et al.*, 2003b	
rmGluR1	2,4-dicarboxypyrroles (NAMs)			7TM				Micheli *et al.*, 2003	
rmGluR1	CTZ (NAMs)						Thr815 Ala818	Surin *et al.*, 2007	Same site as CPCCOEt
rmGluR2/3	MNI-135, MNI-136, MNI-137 (NAMs)							Hemstapat *et al.*, 2007	Asn735: not important for the binding of these NAMs
rmGlu2	RO4988546 RO5488608 (NAMs)	His723	Arg635 Arg636 Phe643		Leu732	Trp773 Phe780	Val798	Lundström *et al.*, 2011	His723 on EC2: selectivity of mGluR2 over mGluR3 PAMs/NAMs: Overlapping b.s.

1.6.1. Sequence alignment and homology modeling

High-resolution 3-dimensional structures of proteins provide detailed information of the form and function of the molecular level. This is useful to describe aspects of protein structure involved in physiological processes and to visualize the connections between their ligands and to small molecule drugs (Congreve & Marshall 2010).

For several mGlu receptors, the extracellular N-terminal domain has been crystallized. However, the structure of the hepta-helical transmembrane domain of the receptor has yet to be determined. In this case, homology modeling with class A GPCR templates has been shown to provide substantial insight into the transmembrane region of these receptors (Gregory et al. 2011).

The main goal of protein modeling is to predict a structure from its sequence with an accuracy that is comparable to the best results obtained experimentally. This allows to use rapidly generated *in silico* protein models in all the contexts where only experimental structures provide solid bases, like structre-based drug design, analysis of protein function, interactions, antigenic behavior, and rational design of proteins with increased stability or novel functions. Homology modeling is based on two major observations: a) the structure of a protein is uniquely determined by its amino acid sequence; b) the structure is more stable and changes much slower than the associated sequence, for that, similar sequences adopt identical structures, and distantly related sequences still fold into similar structures . In practice, homology modeling is a multistep process that can be summarized in seven steps: 1. Template recognition and initial alignment; 2. Alignment correction; 3. Backbone generation; 4. Loop modeling; 5. Side-chain modeling; 7. Model optimization; 8. Model validation (Krieger et al. 2003).

The Rhodopsin (class A) GPCR is a visual pigment and has for several years conferred a structural template for other GPCRs, including the assignment of secondary structural elements and the location of highly conserved amino acids. The 3D structure of this receptor was determined by Palczewski et al. from diffraction data extending to 2.8 angstroms resolution (Figure 19). The lengths of the seven transmembrane helices and of the three extracellular loops are more or less the same for most of the family members. The other regions present some variations, reflecting the specificity of each receptor for either its ligand or G protein (Palczewski et al. 2000). It is important to note that the sequence similarity between class C GPCRs and class A GPCRs is low (Lundström et al. 2011), however, by experimental work it was found that the allosteric binding site of mGlu receptors is overlapping with the retinal binding pocket of rhodopsin, which implies a conservation of ligand binding. These findings were complemented with the docking of known allosteric mGluR ligands to computationally generated models of different mGluR subtypes based on rhodopsin structure. It was also shown that positive and negative modulators docked preferentially to the active and inactive models of the receptor, respectively, suggesting that the modulators can be distinguished by their

affinities for the active or inactive conformation of the receptor (Yanamala & Klein-Seetharaman 2010).

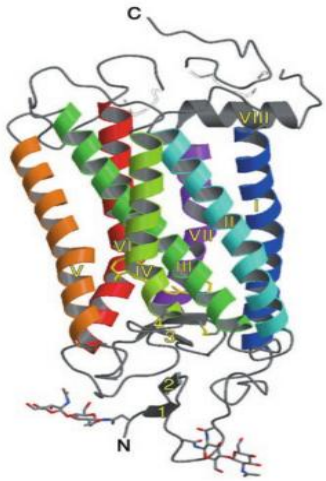


Figure 19. Three-dimensional view of rhodopsin GPCR (Palczewski et al. 2000)

Since the bovine rhodopsin GPCR crystal structure, six additional mammalian GPCR crystal structures have become available (see Table 5), which allowed the development of hight-throughput homology modeling of GPCRs, enriching the understanding of the transmembrane region of these receptors (Gregory et al. 2011).

Table 5. Summary of GPCRs which structure has been crystallized

Receptor	GPCR family	References
β_2-adrenergic	A	Cherezov *et al.* 2008
A_{2A} adenosine	A	Jaakola *et al.* 2009
CXCR4 chemokine	A	Wu *et al.* 2010
Dopamine D3	A	Chien *et al.* 2010
Histamine H1	A	Shimamura *et al*. 2012
S1P1 Sphingosine 1-phosphate	A	Hanson *et al.* 2012

1.7. Goal of the project

Janssen Pharmaceutica has an mGlu2 receptor PAM in clinical development for the treatment of schizophrenia. Additionally, as part of an internal mGlu receptor PAM program, other compounds were identified as potentially important. It is important to clarify the PAM-receptor interactions to understand the mechanisms through which these compounds produce their effects. Therefore it is of large interest to characterize the amino acids that are critical for the binding and/or activity the of PAM compounds.

In order to reveal the PAM binding site, homology modeling and docking of mGlu2 receptor PAM was performed in parallel with site-directed mutagenesis. Mutant mGlu2 receptors were generated and the impact of these mutations on activity and affinity of various structurally different PAMs was evaluated. Activity was determined using [^{35}S]GTPγS experiments, while binding was assessed with an in-house generated [^{3}H]mGlu2 PAM molecule. This study is an important contribution for the mapping of the allosteric binding site of mGlu2 receptors.

2. Materials and Methods

2.1. Materials

The 24 PAMs tested in this study were synthesized at Janssen Pharmaceutica and dissolved in 100% of dimethyl sulphoxide (DMSO). L-glutamate was purchased from Aldrich® Chemistry. The radioligand [^{3}H]-LY341495 was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), the radioligand [^{3}H]JNJ-46281222 was synthesized at Janssen Pharmaceutica and Guanosine 5'-(γ-thio)triphosphate [^{35}S]- was purchased from Perkin Elmer® (Boston, MA, USA).The monoclonal anti-metabotropic glutamate receptor 2 [mG2Na-s] antibody Ab15672 was purchase from Abcam (Cambridge, UK). Mutant mGlu2 receptor cDNA constructs were prepared by GeneArt® (Life Technologies).

2.2. Positive allosteric modulators tested

The 24 compounds tested in this study are part of an internal mGluR2 PAM program and are divided in 7 different chemical classes: 1,5-pyridone, 1,4-pyridone, azetidine, imidazopyridine, isoquinolone, pyridazine and triazolopyridine. These compounds were identified by lead optimization of hits originating from high-throughput screening through a calcium mobilization assay performed on the mGlu2 receptor. In addition, 3 reference compounds were used: BINA (Galici et al. 2006), THIIC (LY2607540) (Fell et al. 2011) and TEMPS (LY487379) (H Schaffhauser et al. 1998). Appendix 2 summarizes the chemical classes and name of each compound used in this study.

2.3. Selection of amino acid mutations: sequence alignment and building of an mGlu2 receptor homology model

The identification and selection of the amino acids as targets for mutagenesis studies was based on three hypotheses: 1) sequence alignment of the entire mGlu family; 2) sequence comparison between the mGlu2 and mGlu3 receptor; 3) binding orientation of an mGlu2 PAM with an mGlu2 receptor model generated by modeling homology. This work was performed by Gary Tresadern, head of Molecular Informatics.

1) The full length receptor sequences were aligned in a progressive manner using Molecular Operating Environment (MOE) Protein Align tool (Chemical Computing Group, Canada). In a first step, members of each mGlu subgroup were aligned to each other. Subsequently the three subgroups were aligned to each other using constraints for class C GPCRs. Figure 20A shows the aligned 7-TM's, highlighting the residues identified as important from literature mutagenesis studies.

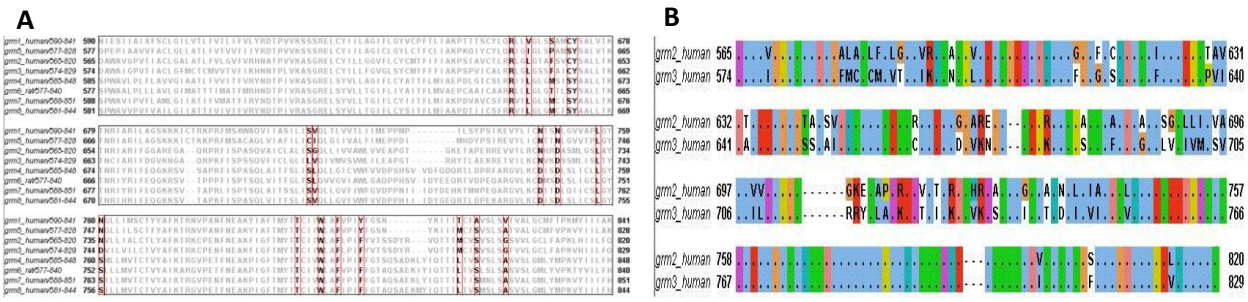


Figure 20. Alignment of mGluR 7TMs. A- Residues highlighted in red boxes have been identified as important from literature mutagenesis studies. B-Comparison between hmGluR2 and hmGluR3, with highlighted differences.

2) The mGlu2 and mGlu3 sequences were compared (Figure 20B) to identify which amino acids differ and are therefore potentially important for the selective binding of mGlu2 receptor PAM compounds. The main differences are seen in the extracellular portion of TM3, TM4, EL2 and TM5.

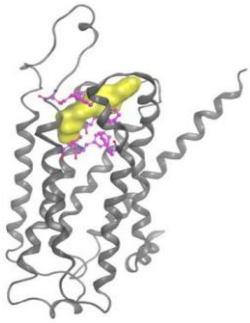


Figure 21. Ribbon diagram of the binding mode of a reference PAM compound in the mGlu2 model structure built from β2-adrenergic template

3) The mGlu2 sequence was aligned to that of the rhodopsin and β2-adrenergic receptor and the X-ray structure 2RH1 was subsequently used to build an mGlu2 receptor homology model. The 3D models of mGlu2 receptor were built using the Homology Model tool in MOE. In order to establish a possible binding mode of mGlu2 receptor PAMs, they were docked into the mGlu2 receptor model, and amino acids were selected based on their proximity to the ligand (Figure 21).

Overall, amino acids from TM2, TM4, TM5 and EL2 in a total of 40 amino acids were selected for site-direct mutagenesis (37 single point mutations, one double and two triple mutations were prepared).

Table 6. mGlu2 receptor mutagenesis constructs. The wild type (WT) and the substituting amino acids are indicated, as well as their position and the receptor region where they are located. TM – transmembrane; EL – extracellular loop. 1 – Sequence alignment and 1.1-mGlu2/3 comparison; 2-Homology modeling and docking and 2.1 – mGlu2/3 comparison

Receptor region	Name	WT AA	Position	Mutation	WT codon	mutant codon	
TM2	$C_{616}S$	Cys	616	Ser	TGC	TCC	1.1
	$I_{622}F$	Ile	622	Phe	ATC	TTC	
TM3	$T_{641}S$	Thr	641	Ser	ACC	TCC	1.1
	$A_{642}S$	Ala	642	Ser	GCC	TCC	
	$R_{636}A$	Arg	636	Ala	CGT	GCT	1 and 2
	$L_{639}A$	Leu	639	Ala	TTG	GCG	2
	$F_{643}A$	Phe	643	Ala	TTC	GCC	1 and 2
	$S_{644}A$	Ser	644	Ala	TCT	GCT	2.1
	$R_{635}A$	Arg	635	Ala	AGA	GCG	1
TM4	$S_{688}L$	Ser	688	Leu	TCG	TTG	1
	$G_{689}V$	Gly	689	Val	GGC	GTC	
	$V_{700}L$	Val	700	Leu	GTC	CTC	2.1
	$A_{681}F$	Ala	681	Phe	GCC	TTC	1.1
	$I_{693}M$	Ile	693	Met	ATT	ATG	1.1
	$V_{695}S$	Val	695	Ser	GTG	TCG	
	$A_{696}V$	Ala	696	Val	GCC	GTC	
	$S_{688}L$ $G_{689}V$	Ser	688	Leu	TCG	TTG	1
EL2	$H_{723}V$	Gly	689	Val	GGC	GTC	1 and 2.1
	$G_{706}R$	Gly	706	Arg	GGA	CGA	1.1
	$E_{708}Y$	Glu	708	Tyr	GAG	TAC	
	$A_{710}L$	Ala	710	Leu	GCC	CTC	
	$P_{711}A$	Pro	711	Ala	CCC	GCC	
	$V_{716}T$	Val	716	Thr	GTG	ACG	
	$T_{718}I$	Thr	718	Ile	ACC	ATC	
TM5	$N_{735}D$	Asn	735	Asp	AAT	GAT	1
	$D_{725}A$	Asp	725	Ala	GAT	GCT	2
	$L_{732}A$	Leu	732	Ala	CTG	GCG	1 and 2
	$M_{728}A$	Met	728	Ala	ATG	GCG	1
	$S_{731}A$	Ser	731	Ala	AGC	GCC	
	$V_{736}A$	Val	736	Ala	GTG	GCG	
	$A_{726}S$	Ala	726	Ser	GCC	TCC	1.1
	$G_{730}I$	Gly	730	Ile	GGC	ATC	
	$A_{733}T$	Ala	733	Thr	GCC	ACC	
	$A_{740}I$	Ala	740	Ile	GCC	ATC	
TM6	$W_{773}A$	Trp	773	Ala	TGG	GCG	1 and 2
	$F_{776}A$	Phe	776	Ala	TTC	GCC	2
	$F_{780}A$	Phe	780	Ala	TTC	GCC	1 and 2
TM7	$V_{798}A$	Val	798	Ala	GTG	GCG	1
TM3/4/EL2	$S_{644}A$	Ser	644	Ala	TCT	GCT	2.1
	$V_{700}L$	Val	700	Leu	GTC	CTC	
	$H_{723}V$	His	723	Val	CAC	GTC	
TM4/5	$S_{688}L$	Ser	688	Leu	TCG	TTG	1
	$G_{689}V$	Gly	689	Val	GGC	GTC	
	$N_{735}D$	Asn	735	Asp	AAT	GAT	

2.4. Cell culture

CHO-K1 cells (ATCC: CCL-61) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS: HyClone®, Thermo Scientific, Cramlington, UK) and 2% (v/v) Solution A. This solution is composed of Penicillin G (Serva, Bioconnect, Huissen, The Netherlands) 5.1E6 IU/L, Streptomycin sulphate (Serva) 5 g/L, Pyruvic acid (Sigma) 5.5 g/L and L-Glutamine (Sigma, St. Louis, MO, USA) 14.6 g/L. Cells were kept at 37^{o}C in a humidified atmosphere with 5% CO_2.

2.5. Transient Transfection of human mGlu2 receptor cDNA into CHO-K1

Transfections were done using a liposome-based method (Lipofectamine), which delivers DNA into cells. The cationic part of the lipid molecule associates with the negatively charged nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex. For cultured cells, an overall net positive charge of the liposome/nucleic acid complex normally results in higher efficiency, because this allows closer association of the complex with the negatively charged cell membrane. After endocytosis the complex appears in the endosomes and later in the nucleus (Chesnoy & Huang 2000).

Procedure

CHO-K1 cells were seeded in 145 cm^2 Petri dishes at a density of 2.9E6 cells per plate (20000 cells/cm^2) in DMEM supplemented with 10% heat-inactivated FBS and 2% solution A. After 24h, confluence of 50-70% was reached and human mGlu2 (hmGlu2) receptor cDNA, both WT (native form of the hmGlu2 receptor) and mutated, was transiently transfected into cells using Lipofectamine (Invitrogen^{TM}), followed by incubation overnight in the same medium, at 37^{o}C and in an atmosphere of 5% CO_2. One Petri dish was used for the transfection of Enhanced Green Fluorescent Protein (EGFP-N1) used as control of transfection efficiency. After a period of 20-24h the medium was replaced by fresh medium and 48h after transfection butyrate (f.c. 5 mM) was added to each Petri dish. The transfection efficiency was qualitatively evaluated, 48h after transfection through observation of transfected cells with EGFP-N1, using a fluorescence microscope (Axiovert 135, Zeiss, N.V., S.A.). One day after butyrate addition, the Petri dishes with recombinant cells were washed twice with ice-cold phosphate-buffered saline (PBS) and stored at -20^{o}C or used immediately for membrane preparation.

2.6. Crude membrane preparation

Receptor-containing membranes are prepared by successive washing, centrifugation, homogenizing/rehomogenizing and resuspending of cells. The purpose of centrifugation steps is to separate membranes from other cell parts as well as to remove any soluble substance that can interfere with the receptor, such as endogenous neurotransmitters and guanine nucleotides which may interfere with the *in vitro* pharmacological assays.

Procedure

Transfected CHO-K1 cells expressing the wild-type or mutant hmGlu2 receptors were collected with a rubber scraper and resuspended in ice-cold 50mM Tris-HCl buffer, pH 7.4. The cell suspension was always kept on ice. After collection, the cell suspension was centrifuged for 10 minutes at 16000 rpm in a Sorvall RC 5B/RC 28S SS34 at 4°C. The cell pellet was resuspended in ice-cold 5mM Tris-HCl buffer, pH 7.4, and homogenized using an Ultra Turrax homogenizer (IKA TE5) at 24000 rpm. Additional ice-cold 5mM Tris-HCl buffer was added and one more centrifugation was performed for 20 minutes at 18000 rpm in a Sorvall RC 5B/RC 28S centrifuge at 4°C. The final pellet was resuspended and homogenized in ice-cold 50mM Tris-HCl buffer, using an Ultra Turrax homogenizer at 24000 rpm. The resulting membrane suspension was aliquoted in cryovials and frozen at -80°C.

Protein determination was performed by the Bradford method, using bovine serum albumin as standard.

2.7. Western Blot

By using a western blot, it is possible to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task: separation by size, transfer to a solid support and marking target protein using a proper primary and secondary antibody to visualize.

Protein separation is done based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The transfer is done using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. The membrane is then incubated with labels antibodies specific to the protein of interest (Mahmood & Yang 2012).

Procedure

hmGlu2 receptor membranes were thawed and homogenized using the Ultra Turrax homogenizer. 200 µl of membrane suspension was transferred to a tube and 400 µl of RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0, Sigma) complemented with phosphatase and protease inhibitors (Roche) was added. The supernatant fraction was recovered to a fresh tube and the protein concentration was determined by the BCA™ Protein Assay (Sigma-Aldrich).

18-well Bio-rad gels were loaded with 3 µg of protein and in each gel 4µl of MagicMark was also loaded used for accurate molecular weight estimation directly on western blots. NuPAGE® MOPS SDS Running Buffer (Life Technologies) was added. The electrophoresis ran initially at 90 V, after which the voltage was increased to 150-190 V. Proteins were blotted for 10 minutes on a nitrocellulose membrane through a dry blotting system (Bio-rad). Membranes were blocked for 1h at RT with Non-Fat Dry Milk (NFDM) (Santa Cruz, Technology) (5% w/v) diluted in Tris-buffered saline and Tween 20 (TBS-T; 10 mM Tris pH 8.0, 150 mM NaCl and 0.05% v/v Tween 20). After, the membranes were incubated with the primary antibody (anti-metabotropic glutamate receptor 2 antibody; final concentration 0.75 µg/ml) diluted in 5% NFDM in TBS-T, overnight at 4^{o}C, with gentle agitation. Membranes were washed in TBS-T, 3 to 5 times (5 min) and incubated for 1h at RT with the secondary antibody. Primary antibodies were detected through horseradish peroxidase (HRP) -linked secondary antibody (1:10000 in TBS-T, Amersham Biosciences), via SuperSignal® West Dura Extended Duration Substrate (Pierce, Thermoscientific). Signals were captured and quantified by chemiluminescence (G-box Syngene, Syngene). For reprobing, membranes were stripped of secondary antibody with Restore™ Western Blot Stripping Buffer for 15 min with agitation at RT. Before incubation with anti-actin (dilution 1:10000), the membranes were washed 3 times (5 min) in TBS-T and blocked 1h in 5% NFDM. Actin was used as a housekeeping gene. It works as a loading control which is essential for proper interpretation of Western blots. These controls are used to normalize the levels of protein detected by confirming that protein loading is the same across the gel.

2.8. GTPyS binding assay

Background Theory

[^{35}S]GTPyS binding is a functional assay which measures the level of G-protein activation after the binding of an agonist to a GPCR. The activation of these receptors leads to an increase in guanine nucleotide exchange at the Gα-subunit of G-protein heterotrimers, resulting in the creation of G_{α}-[^{35}S]GTPyS and $G_{\beta\gamma}$ subunits. For this, the binding of [^{35}S]GTPyS is monitored in the presence of

unlabeled GDP. Quantification of G_{α}-[^{35}S]GTPyS subunit is possible because [^{35}S]GTPyS is relatively resistant to hydrolysis by the intrinsic GTPase of Gα. A slow dissociation from Gα occurs with a consequent accumulation in the membrane, enabling the quantification of Gα-[^{35}S]GTPyS. Filtration is used to remove the excess of free [^{35}S]GTPyS.

This is an attractive assay because guanine nucleotide exchange is a proximal event to receptor activation and is not subject to amplification or regulation by other cellular processes (W. Thomsen et al. 2005).

This assay can, however, not be used to evaluate receptor activation in intact cells because [^{35}S] does not cross cell membranes.

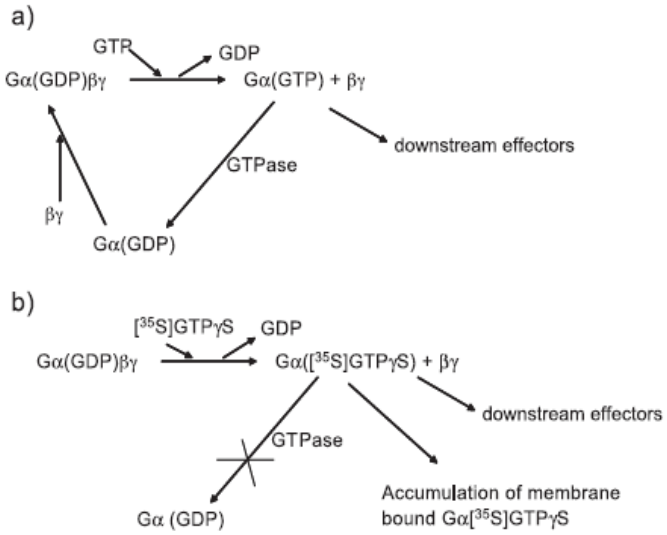


Figure 22. Scheme of the [^{35}S]GTPyS binding assay principle. a) When an agonist binds to the receptor, GTP is exchanged for GDP on the α subunit of the G protein and the complexes Gα-GTP and Gβγ activate their cellular effectors. The GTPase activity of the Gα subunit hydrolyses GTP to GDP, Gα and Gβγ reassemble and the system in turned off. b) When [^{35}S]GTPyS is present, the exchange for GDP also occurs, but the GTPase activity of the Gα subunit is unable to hydrolyze [^{35}S]GTPyS, which accumulates during the assay period (Harrison & Traynor 2003)

Procedure

hmGluR2-CHO-K1 membranes were thawed on ice and homogenized using the Ultra Turrax homogenizer and the protein concentration was measured using the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Munich, Germany). After, the membranes were diluted to a concentration of 10 µg/assay in incubation buffer supplemented with 1 mg/ml saponin. The incubation buffer is a mix of 10 mM HEPES acid, 10 mM HEPES sodium salt, 100 mM NaCl, 3 mM $MgCl_2.6H_2O$ and 10 µM GDP, pH 7.4.

The volume of 140 µl of membranes was added to a 18 µl of assay buffer, 2 µl of test compound (at 100-fold final concentration) and 20 µl of glutamate. To test compounds for their PAM effect, 4 µM glutamate (corresponding to the EC_{20} of glutamate) was used. After membrane addition, of the mixture was incubated for 30 minutes at 30°C. At last, 20 µl of [^{35}S]GTPyS (0.1 nM f.c.) was added and the total assay mixture was incubated for another 30 min at 30°C. The reaction was

stopped through filtration using Unifilter 96-well PerkinElmer filtermate harvester, in order to remove the unbound [^{35}S] GTPyS. The filters were washed 3 times with ice-cold 10 mM NaH_2PO_4/ 10 mM Na_2HPO_4 buffer, pH 7.4, and dried overnight at room temperature. 40 µl of Microscint™O (Perkin Ekmer, MA, USA) was added to each well and the plates were sealed. After 15-30 min, radioactivity was counted in a Microplate scintillation and luminescence counter (Packard).

2.9. Radioligand Binding Assay

Background Theory

Radiolabeled agonists and/or antagonists can be used as ligands in binding studies to characterize ligand binding sites of receptors. One of the most important considerations in radioligand binding assays is the determination of specific binding. Specific binding can be defined as the binding to the receptor of interest and is the total binding minus the non-specific binding, which is the binding observed in the presence of an excess of unlabeled drug (blank) that fully blocks the receptor of interest. A filtration step (or centrifugation) is required to separate bound and unbound radioligand and the radioactivity is measured as disintegrations per minute (DPM) or counts per minute (CPM).

A good radioligand has the following properties: i) high affinity (to favor specific over non-specific binding); ii) low non-specific binding; iii) high specific activity (to detect low receptor densities); iv) receptor specificity.

Radioligand binding studies can be done through different approaches depending on the goal of the experiment:

Saturation binding

Two important parameters can be obtained from this type of experiment, the affinity (dissociation constant – K_D) and the density (maximum number of binding sites - Bmax). By definition, K_D is the concentration of ligand that will occupy 50% of the receptors.

In saturation binding experiments, increasing concentrations of the radioligand are added. Typically, a range of concentrations equivalent to 10 times below and 10 times higher the (expected) K_D is used. The high concentrations should result in saturation of the binding site.

Competition binding

Competition binding assays can be used to determine the ability of unlabelled compounds to compete with binding of a fixed concentration of the labeled ligand (which is set after K_D determination). Hence, these experiments are used to estimate the affinity of unlabeled ligands for

the receptor of interest. Competition curves are obtained by plotting specific binding as a percentage of total binding against the log concentration of the competing ligand.

Procedure

Saturation binding

hmGluR2-CHO-K1 membranes were thawed and homogenized using the Ultra Turrax homogenizer and the protein concentration was measured using the Bradford Bio-Rad Protein Assay kit. Membranes were diluted in ice-cold binding buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and 2 mM $CaCl_2$. Table 7 summarizes the different protein amounts, radioligands and their concentration as well as the unlabeled competitor ligand (to determine non-specific binding) used for the various saturation binding experiments.

Table 7. Protein amount, radioligand and respective concentrations and unlabeled competitor ligand used for the saturation binding experiments.

Radioligand	Radioligand concentrations (nM)	Unlabeled competitor ligand	Protein concentration (μg/assay)	
			Stably transfected WT	Transiently transfected WT and hmGlu2 mutants
[^{3}H]-LY341495	0.25/0.5/0.75/1/1.25/1.5/2/3/6/9/10	1 mM glutamate	10	20
[^{3}H]JNJ-46281222	0.25/0.5/0.75/1/2/3/4/5/7.5/10/15/20	10 μM JNJ 42341806	75	150

The reaction mixture contains membrane protein (400 μl) and the radioligand (50 μl) in a total volume of 500 μl. To measure total binding, 50 μl of buffer, supplemented with DMSO as a control (1% DMSO f.c.) where needed, was added. To measure non-specific binding 50 μl of the unlabeled competitor ligand was added. The reaction mixture was incubated at room temperature (RT) for 60 minutes. To stop the incubation, a filtration step was performed over Whatman Filters in the Brandel Harvester system (Biomedical Research and development laboratories, USA). With the radioligand [^{3}H]-LY341495 the filters used were GF/B and with the radioligand [^{3}H]JNJ-46281222 GF/C filters presoaked in 0.1% polyethylenimine (Sigma-Aldrich), for 1h, were used. After filtration, 3 ml of scintillation fluid was added and shake for 10 minutes. Radioactivity was measure in a Liquid Scintillation Analyzer Tri-Carb 2810TR (PerkinElmer), after incubation overnight.

Competition Binding

Competition binding was only performed with [^{3}H]-LY341495. Similarly as above, the reaction mixture contained 10 µg of membrane protein, 3 nM of [^{3}H]-LY341495 and 1 mM of glutamate to determine non-specific binding, in a total volume of 500 µl. To assess the affinity of glutamate, increasing concentrations were added in order to determine the concentration of glutamate needed to inhibit 50% of binding (=IC_{50}). The reaction mixture was incubated for 60 minutes at RT and the subsequent filtration step was done using a Unifilter-96 GF/B filter plates in a 96-well PerkinElmer filtermate harvester and the plates were dried overnight. The remaining radioactivity was measured in a Microplate scintillation and luminescence counter (Packard), after addition of 40 µl of Microscint™ O.

2.10. Data Analysis

Data analysis was performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, USA). Concentration-response curves were fitted using non-linear regression analysis fitting the equation: Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope)). Saturation binding experiments were analyzed using a non-linear regression analysis.

3. Results

3.1. Orthosteric binding site integrity

In a previous study, mGlu2 receptor mutations selected based on sequence alignment and homology modeling and docking (see Table 6; Methods section), some of which were already described in literature, were tested (Master thesis of Farinha, 2012). After transient transfection with the mutated hmGlu2 receptor DNA, the integrity of the orthosteric binding site was validated through radioligand and functional binding assays. . Also for the additional set of mutant receptors, that was only applied in this thesis, similar assays were performed to verify that the orthosteric binding site was not compromised.

3.1.1. Transfection efficiency

After DNA purification and confirmation of the sequence of WT and mutant hmGlu2 receptor DNA, CHO-K1 cells were transiently transfected. Every time a transfection was performed, enhanced green fluorescent protein (EGFP-N1) was also included to evaluate the transfection efficiency. Overall, transfection efficiency was in the range of 35-45% (Figure 23).

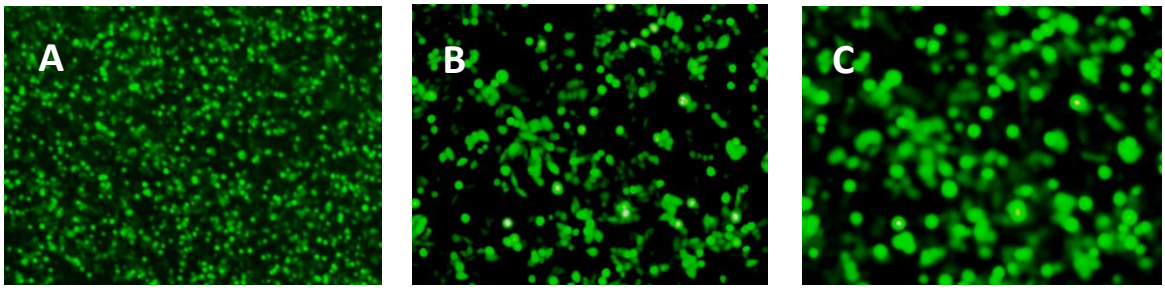


Figure 23. Visualization of EGFP-N1 expression. Cells were transiently transfected with EGFP-N1 each time that a transfection was done, in order to check transfection efficiency. The images represent an example of this evaluation and correspond to the transfection of: A: Mutation V736A; B: Mutation V700L; C: Mutation F776A

3.1.2. Receptor expression and orthosteric ligand binding

To verify receptor expression in the membranes of the CHO-K1 cells, Western blots were performed using an antibody that recognizes a 47 amino acid sequence of the C-terminal tail of the mGlu2 receptor. The controls used were non-transfected CHO-K1 cells (referred to as CHO-K1) and CHO-K1 cells either stably or transiently transfected with the WT form of the receptor. Figure 24 shows, with exception of the non-transfected cells, one immunoreactive band running at ~100 kDa corresponds to the monomeric form of the mGlu2 receptor and one band running at ~200 kDa that corresponds to the homodimer form. This analysis shows that although the receptor has been mutated, it is correctly expressed at the cell membrane.

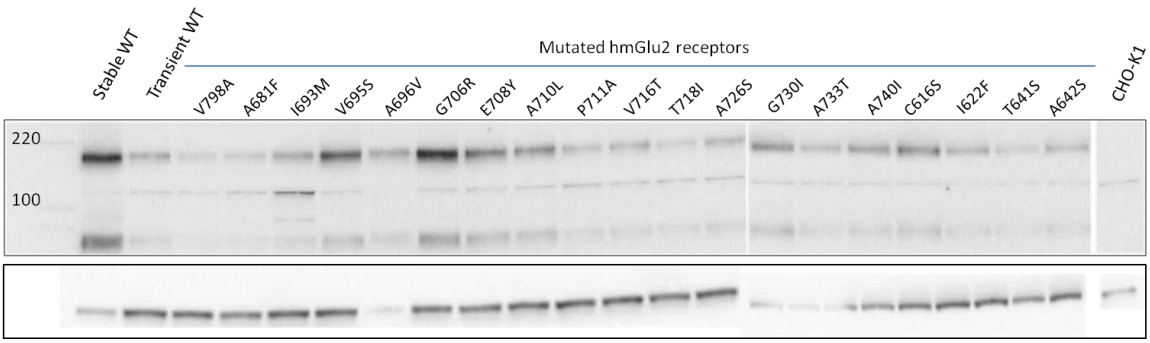


Figure 24. Western blot analysis on mGlu2 WT and mutant receptors to illustrate the expression of the receptors in CHO-K1 cells after stable (for WT hmGlu2) or transient transfection (for WT and mutant hmGlu2). Mutants are indicated with the corresponding mutation number (See Table 6, Methods section). As expected, the mGlu2 monomer (~100 kDa) and dimer (~200 kDa) are observed and this staining pattern is the same for WT and mutant mGlu2. Actin (antibody diluted 1:10000 in TBS-T) is presented as a loading control. Molecular weight markers are indicated in kDa.

To further confirm the receptor expression, as well as the orthosteric binding site integrity in the mutated receptors, a set of binding experiments with the orthosteric mGlu2/3 antagonist [^{3}H]-LY341495 was performed. All the mutants showed the same specific [^{3}H]-LY341495 binding indicating that the mutations do not affect the orthosteric pocket binding. Glutamate is also able to displace the antagonist which indicates that the mutated receptors are able to bind glutamate in the same way as the WT (Figure 25). Moreover, glutamate inhibited binding with a similar pIC_{50} value (Table 8) indicating that the affinity for glutamate does not seem to be altered in the mutated receptors compared to the WT receptor.

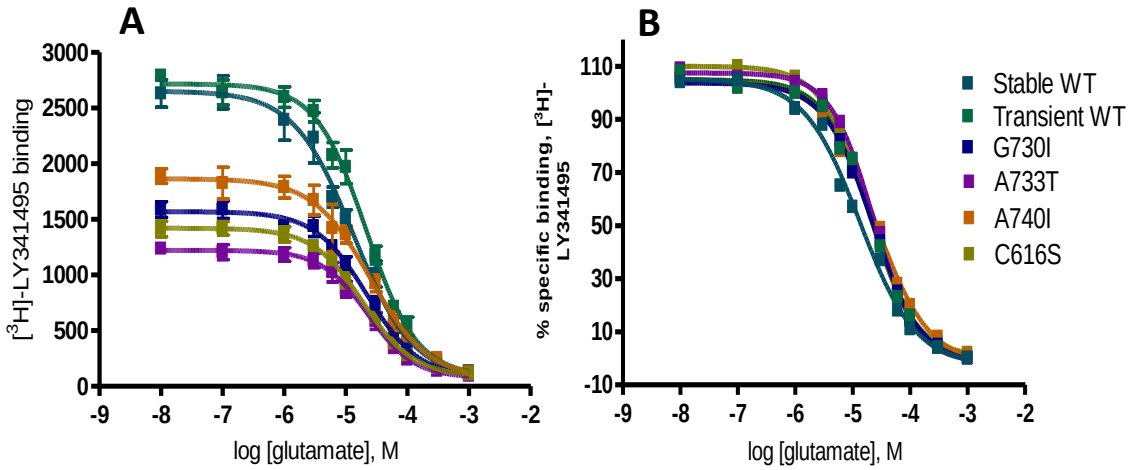


Figure 25. Representative graphs of [^{3}H]-LY341495 binding, showing the displacement of LY341495 (orthosteric antagonist) by glutamate, on WT (transiently and stably transfected) and mutated hmGlu2 receptors expressed in CHO-K1 cells. Results are presented as mean±SD of one experiment performed in triplicate. A – Total binding results are expressed in dpm (disintegrations per minute); B – Results are presented as percentage of specific binding. Specific binding is calculated as: total binding (absence of glutamate) minus non-specific binding which correspond to the binding in the presence of 1 mM glutamate.

As can be seen in Figure 25A the absolute binding values are different for every mutation; however this is an expected result taking into account that the levels of receptor expression can vary between each transient transfection.

Table 8. Summary of pIC50 values for displacement of [^{3}H]-LY341495 binding by glutamate on WT (stable and transiently transfected) and mutated hmGlu2 receptors. Data is presented as mean ± SD for WT. For the mutated receptors one concentration-response curve was performed in triplicate.

Receptor region	Mutation	pIC50	n
----	Stable WT	4.9 ± 0.09	12
----	Transient WT	4.7 ± 0.06	13
TM2	C616S	4.7	1
	I622F	4.7	1
TM4	T641S	4.7	1
	A6742	4.7	1
	A681F	5.2	1
	I693M	4.7	1
	V695S	4.7	1
	A696V	4.7	1
EL2	G706R	4.7	1
	E708Y	4.6	1
	A710L	4.7	1
	P711A	4.7	1
	V716T	4.7	1
	T718I	4.7	1
TM5	A726S	4.6	1
	G730I	4.7	1
	A733T	4.6	1
	A740I	4.6	1
TM7	V798A	4.4	1

3.1.1. Glutamate potency

Table 9 and Figure 26 show the results for the functional studies using [^{35}S]GTPyS which were performed to evaluate the glutamate potency of the WT and hmGlu2 mutated receptors. The aim of these experiments was to verify the effect of the mutations on glutamate–mediated receptor activation. The potency of glutamate (pEC_{50}) was obtained from concentration-response curves of glutamate in CHO-K1 cells expressing the mutated hmGlu2 receptors and WT, both stably and transiently transfected. On the WT receptor, glutamate exerts effects with a pEC_{50} of about 5.1 (EC50 values of about 9 µM), and as seen in Table 9 all the mutants present similar potencies. It can be seen on Figure 26A that the response obtained for the transient WT and mutated hmGlu2 receptors is lower than for the stable WT.

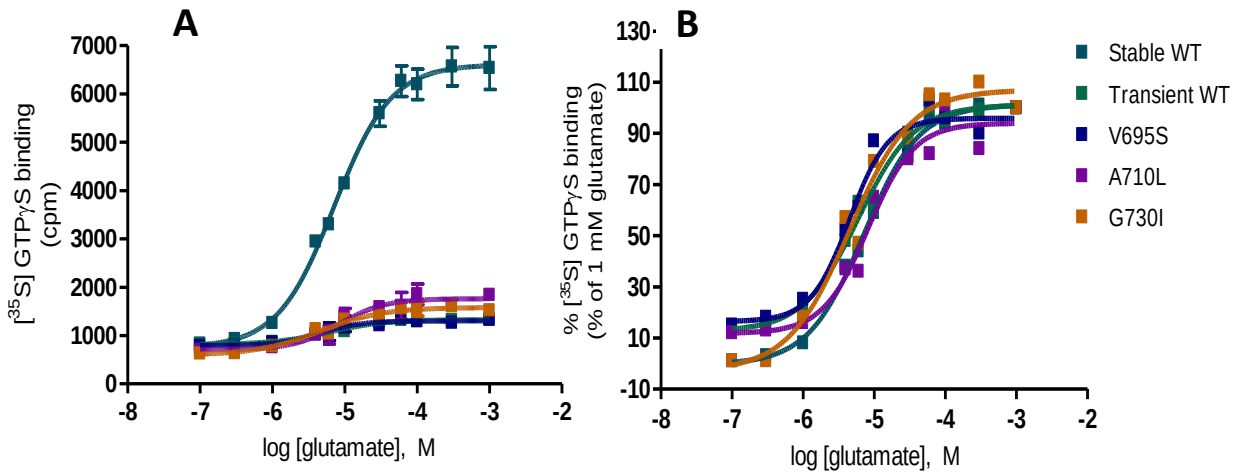


Figure 26. Representative graphs of glutamate-induced [^{35}S]GTPyS binding. Results are presented as mean±SD of one experiment performed in triplicate. A – Total binding results are expressed in cpm (counts per minute); B – Results are presented as percentage of specific binding (total binding (absence of glutamate) minus non-specific binding which correspond to the binding in the presence of 1 mM glutamate).

The response amplitude was calculated as a ratio between the response obtained with 1 mM glutamate and the response obtained under basal conditions (only using assay buffer), taking the basal condition as 100% of the response. The results (Table 9) indicate lower response amplitude for the transient transfections (WT and mutated receptors) when compared with the stable line. Furthermore, between all the mutated hmGlu2 receptors, mutation V798A shows a rather low amplitude response although glutamate pEC_{50} was unchanged compared to WT.

Table 9. Summary of the results obtained for glutamate-induced [^{35}S]GTPγS binding to WT (transiently and stably transfected) and mutated hmGlu2 receptors transiently transfected in CHO-K1 cells. pEC50 values are presented as mean±S.D (for the mutations with n≥2). The response amplitude is defined as the ratio between the response obtained with 1 mM glutamate and the response obtained under basal conditions (only using buffer), taking the basal condition as 100% and is presented, in percentage, as mean±S.D. (for the mutation with n≥2), indicating the glutamate stimulation. The concentration-curves were performed in triplicate with increasing glutamate concentrations.

Receptor region	Mutation	pEC50	Response amplitude (%)	n
----	Stable WT	5.1 ± 0.07	656% ± 154%	11
----	Transient WT	5.3 ± 0.07	223% ± 59%	8
TM2	C616S	5.4 ± 0.03	185% ± 0	2
	I622F	5.3	205%	1
TM4	T641S	5.2	181%	1
	A6742S	5.3	225%	1
	A681F	5.4	237%	1
	I693M	5.3	199%	1
	V695S	5.3 ± 0.09	180% ± 16%	2
	A696V	5.9 ± 0.54	184% ± 26%	2
EL2	G706R	5.3 ± 0.07	212% ± 35%	3
	E708Y	5.1 ± 0.02	178% ± 5%	2
	A710L	5.2 ± 0.14	293% ± 58%	2
	P711A	5.4 ± 0.16	214% ± 28%	2
	V716T	5.2	217%	1
	T718I	5.2	176%	1
TM5	A726S	5.3 ± 0.01	192% ± 8%	2
	G730I	5.3 ± 0.1	231% ± 19%	3
	A733T	5.2 ± 0.14	174% ± 18%	2
	A740I	5.3 ± 0.13	165% ± 36%	2
TM7	V798A	5.2	132%	1

3.2. Effect of mutations on PAM's activity – [^{35}S]GTPγS binding

3.2.1. Expansion of previous studies: evaluation of additional mutations

In a previous study, it was observed that mutations F643A, G689V, W773A, S688L/G689V, S688L/G689V/N735D and S644A/V700L/H723V had a general impact on the activity of structurally distinct mGlu2 PAM compounds (Master thesis of Farinha, 2012).

Further selections of amino acids, based on mGlu2/3 comparison, were made in order to further delineate the molecular interaction of mGlu2 PAMs and verify whether some of these additional amino acids contribute to the binding pocket previously hypothesized.

In order to evaluate the effect of the different mutations on the activity of the various mGlu2 PAMs, a functional assay ([^{35}S]GTPγS binding assay) was performed. Since a large number of compounds and mutants had to be tested, a preliminary analysis was performed. For that an initial screening comparing the mutant and WT receptor was performed, using only two concentrations of PAM compounds: one concentration that displays 50% (EC_{50}) of the PAM activity and a concentration that produces 100% (EC_{100}) of PAM activity. These values were previously determined with the use of stably transfected cells. The rationale for selecting these concentrations was as follows: while for some compound-mutant pairs, there may be an effect on both potency and Emax (example given in Figure 27A), for others the compound concentration-response curve may have shifted to the right, indicating a lower potency, leaving the Emax (detected with the use of EC_{100}) unchanged compared to WT (example in Figure 27B). In the latter example, using just the EC_{100}-equivalent concentration would have masked the effect of the mutation.

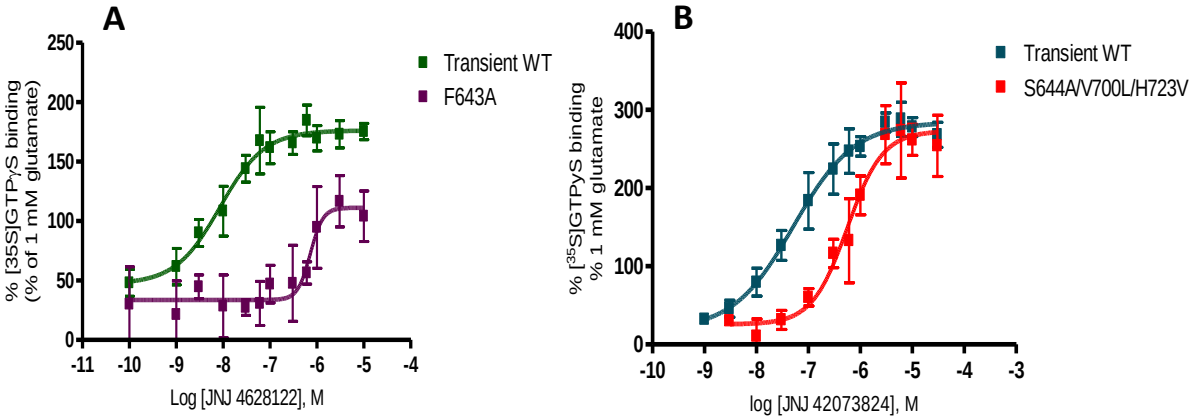


Figure 27. A: Graphic representation of the effect of JNJ 46281222 on glutamate-induced [^{35}S]GTPγS on WT and one mutated hmGlu2 receptor. Mutation F643A affects both potency and Emax; B: Graphic representation of the effect of JNJ 42073824 on glutamate-induced [^{35}S]GTPγS binding on WT and one mutated hmGlu2 receptor. The compound shows a lower potency on the triple mutation when compared to the WT (possible to see with low concentrations, as the EC_{50}). When observing the maximal effect (EC_{100}) the compound exhibits the same effect on the mutated and on the WT receptor, masking the effect that this mutation has on the compound activity.

A set of 14 compounds of different chemical classes (1,4-Pyridone, 1,5-Pyridone, Imidazopyridine, Isoquinolone, Triazolopyridine) and 3 reference PAMs (see appendix 2) was tested via this screening approach on mutations selected based on mGlu2/3 comparison (Table 6; Methods section).

PAM compounds were tested using 4 µM glutamate (correspondent to the glutamate EC_{20}) and for each PAM compound, [^{35}S]GTPγS binding was calculated as a percentage of the response obtained with 1 mM glutamate in the absence of a PAM. The stimulation produced by each compound on the different mutated receptors was compared against the stimulation produced on the WT, and values below 75% of the response obtained on the WT receptor were considered to give a meaningful difference. This screening approach was repeated twice and the results are summarized

in Table 10, mutations are sorted by receptor region and the compounds are grouped by chemical class. As can be seen in Table 3, only a few mutations seem to interfere with the activity of some PAMs and in those cases, the PAM's response is quite close to the cut-off of 75% of the WT receptor. Moreover, a small number of differences between the two screenings were detected. Therefore, to further evaluate the effect of these mutations on the activity of the PAM compounds, potency (EC_{50}) and relative efficacy (Emax) values were measured for three compounds representing three different chemical classes, as well as one reference compound on three different mutations and on mGlu2 WT. Results are shown, graphically, in Figure 28.

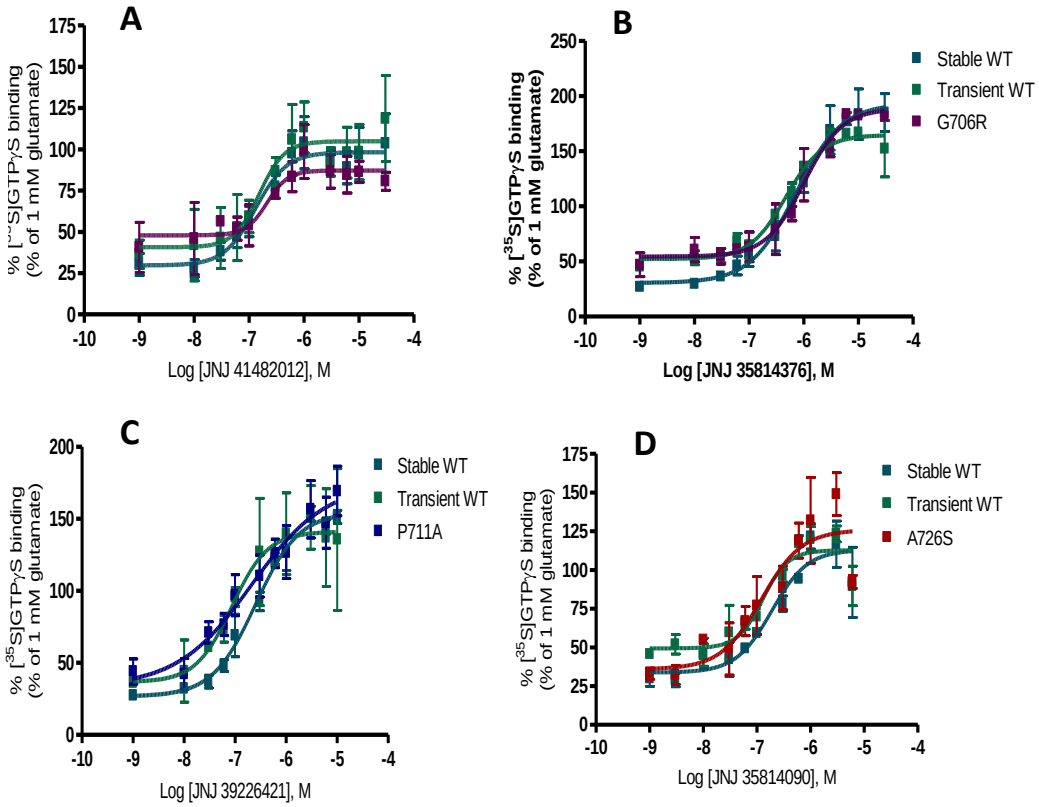


Figure 28. Effect of PAMs on glutamate-induced [^{35}S]GTPγS binding in CHO-K1 cells expressing WT and mutated hmGlu2 receptor. 4 µM glutamate (EC_{20}) was added to induce [^{35}S]GTPγS binding by the tested PAMs. Results were normalized as percentage of the response to 1 mM glutamate. Data is presented as mean±SD of one experiment performed in triplicate. A-B: glutamate-induced [^{35}S]GTPγS binding in WT (stably and transiently transfected) and mutation G706R (localized in ECL2) by one PAM representative of imidazopyridines (A) and one representative of 1,5-pyridones (B); C: glutamate-induced [^{35}S]GTPγS binding in WT (stably and transiently transfected) and mutation P711A (localized in ECL2) by one PAM compound representative of the isoquinolone series; D: glutamate-induced [^{35}S]GTPγS binding in WT (stably and transiently transfected) and mutation A726S (localized in TM5) by one reference PAM (TEMPS)

Table 10. Results from the screening approach on mutations selected based on mGlu2/3 comparison. Mutations are sorted by receptor region and PAM compounds are grouped by chemical class. EC_{50} and EC_{100} (M) values for each PAM are indicated. The screening was performed in quadruplicate (n=1 or 2) with addition of 4 µM glutamate. Effects lower than 75% of the effect seen on the WT receptor (transiently transfected) were considered significantly different and are highlighted in red. NT – not tested.

		1,4-Pyridone									1,5-Pyridone			Imidazopyridine			Isoquinolone					
	PAM	JNJ 40411813			JNJ 40068782			JNJ 41329782			JNJ 35814376			JNJ 41482012			JNJ 39226421			JNJ 40297036		
	[PAM] (M)	1.E-07	1.E-05	n	1.E-07	1.E-05	n	1.E-07	3.E-06	n	3.E-07	3.E-06	n	3.E-07	3.E-06	n	1.E-07	3.E-06	n	3.E-07	3.E-06	n
	Stable WT	110%	204%	10	107%	205%	10	168%	206%	9	90%	164%	10	73%	110%	10	55%	122%	10	102%	140%	10
	Transient WT	114%	179%	8	121%	186%	8	145%	187%	7	92%	152%	8	75%	114%	8	69%	122%	8	113%	135%	8
	75% of WT	86%	134%		91%	140%		109%	125%		69%	114%		56%	86%		52%	92%		85%	101%	
TM2	C616S	110%	149%	2	104%	160%	2	163%	187%	1	97%	143%	2	72%	78%	1	57%	111%	2	83%	68%	2
TM2	I622F	135%	199%	1	114%	181%	1	155%	161%	1	107%	147%	1	83%	97%	2	92%	151%	1	105%	140%	1
TM4	T641S	135%	204%	1	130%	183%	1	193%	181%	1	127%	151%	1	84%	128%	1	88%	142%	1	121%	162%	1
TM4	A642S	107%	155%	2	111%	173%	2	147%	180%	2	87%	139%	2	98%	157%	1	71%	120%	2	96%	116%	2
TM4	A681F	140%	190%	1	115%	188%	1	N.T.	N.T.	-----	129%	193%	1	111%	159%	1	74%	141%	1	100%	146%	1
TM4	I693M	128%	163%	1	133%	176%	1	N.T.	N.T.	-----	100%	158%	1	108%	145%	1	62%	139%	1	121%	141%	1
TM4	V695S	140%	205%	2	143%	193%	2	84%	120%	2	124%	187%	2	94%	108%	2	77%	157%	2	147%	173%	2
TM4	A696V	103%	166%	2	122%	173%	2	137%	163%	2	107%	155%	2	67%	100%	2	72%	121%	2	106%	137%	2
ECL2	G706R	113%	186%	2	105%	168%	2	150%	169%	2	73%	126%	2	61%	82%	2	75%	130%	2	89%	125%	2
ECL2	E708Y	118%	193%	2	104%	164%	2	139%	163%	2	78%	141%	2	67%	79%	2	69%	120%	2	103%	135%	2
ECL2	A710L	121%	182%	2	131%	183%	2	99%	120%	2	95%	145%	2	71%	98%	2	64%	141%	2	120%	133%	2
ECL2	P711A	139%	231%	2	111%	190%	2	189%	214%	2	105%	144%	2	63%	108%	2	24%	65%	2	116%	136%	2
ECL2	V716T	127%	194%	2	112%	186%	2	146%	172%	2	99%	159%	2	82%	104%	2	39%	74%	2	108%	132%	2
ECL2	T718I	135%	207%	2	96%	191%	2	143%	309%	2	111%	160%	2	58%	127%	2	68%	139%	2	93%	132%	2
TM5	A726S	116%	180%	2	127%	169%	2	138%	182%	2	115%	150%	2	86%	120%	2	75%	126%	2	109%	173%	2
TM5	G730I	142%	217%	2	117%	173%	2	145%	175%	2	117%	149%	2	80%	124%	2	53%	140%	2	110%	131%	2
TM5	A733T	184%	302%	1	171%	298%	1	171%	243%	1	94%	133%	1	106%	115%	1	49%	108%	1	144%	132%	1
TM5	A740I	161%	272%	1	137%	157%	1	265%	352%	1	120%	218%	1	202%	332%	1	78%	156%	1	148%	197%	1

		Triazolopyridine															THIIC			BINA			TEMPS		
	PAM	JNJ 46281222			JNJ 46356479			JNJ 42153605			JNJ 42329001			JNJ 43245046			JNJ 52149617			JNJ 35815013			JNJ 35814090		
	[PAM] (M)	3.E-07	3.E-06	n	1.E-07	3.E-06	n	3.E-08	3.E-06	n	3.E-08	3.E-06	n	3.E-09	3.E-07	n	1.E-07	3.E-06	n	1.E-08	3.E-06	n	3.E-07	3.E-06	n
	Stable WT	193%	214%	10	121%	209%	10	134%	216%	10	111%	204%	10	79%	215%	10	96%	178%	10	42%	174%	10	74%	121%	10
	Transient WT	187%	206%	8	135%	194%	8	146%	197%	8	120%	185%	8	95%	190%	8	102%	156%	8	48%	158%	8	92%	123%	8
	75% of WT	140%	155%		101%	146%		110%	148%		90%	139%		71%	143%		77%	117%		36%	119%		69%	92%	
TM2	C616S	156%	164%	2	129%	163%	2	127%	165%	2	101%	169%	2	106%	191%	2	101%	121%	2	55%	144%	2	74%	119%	2
TM2	I622F	192%	222%	1	147%	196%	1	149%	176%	1	142%	180%	1	95%	184%	1	95%	130%	1	60%	160%	1	85%	126%	1
TM4	T641S	241%	237%	1	154%	162%	1	187%	222%	1	150%	223%	1	140%	223%	1	116%	150%	1	78%	170%	1	105%	124%	1
TM4	A642S	162%	190%	1	111%	165%	2	118%	173%	2	96%	165%	2	78%	182%	2	79%	135%	2	46%	124%	2	72%	111%	2
TM4	A681F	205%	191%	1	128%	185%	1	170%	193%	1	128%	176%	1	103%	174%	1	98%	127%	1	62%	136%	1	89%	119%	1
TM4	I693M	171%	182%	1	124%	168%	1	165%	219%	1	130%	176%	1	113%	176%	1	104%	149%	1	62%	168%	1	89%	111%	1
TM4	V695S	205%	225%	2	158%	194%	2	157%	202%	2	168%	215%	2	88%	175%	2	93%	134%	2	44%	146%	2	82%	124%	2
TM4	A696V	157%	185%	2	110%	187%	2	132%	170%	2	109%	178%	2	79%	164%	2	91%	135%	2	60%	128%	2	89%	111%	2
ECL2	G706R	169%	199%	2	138%	185%	2	129%	182%	2	97%	186%	2	75%	185%	2	65%	136%	2	36%	142%	2	70%	115%	2
ECL2	E708Y	166%	200%	2	116%	190%	2	115%	181%	2	107%	199%	2	80%	174%	2	70%	140%	2	45%	143%	2	78%	105%	2
ECL2	A710L	184%	186%	2	125%	174%	2	142%	191%	2	129%	168%	2	85%	179%	2	81%	147%	2	54%	164%	2	96%	112%	2
ECL2	P711A	212%	233%	2	137%	213%	2	158%	216%	2	123%	214%	2	115%	250%	2	113%	172%	2	70%	215%	2	112%	159%	2
ECL2	V716T	179%	211%	2	132%	196%	2	149%	202%	2	120%	182%	2	83%	197%	2	77%	135%	2	53%	164%	2	78%	113%	2
ECL2	T718I	157%	216%	2	139%	202%	2	129%	211%	2	130%	198%	2	94%	201%	2	83%	152%	2	47%	161%	2	88%	148%	2
TM5	A726S	181%	233%	2	119%	173%	2	154%	214%	2	126%	210%	2	79%	158%	2	78%	127%	2	46%	130%	2	62%	87%	2
TM5	G730I	183%	213%	2	136%	195%	2	164%	240%	2	115%	215%	2	72%	150%	2	55%	136%	2	38%	138%	2	55%	93%	2
TM5	A733T	282%	306%	1	118%	165%	1	182%	272%	1	74%	167%	1	113%	283%	1	143%	192%	1	83%	325%	1	102%	177%	1
TM5	A740I	326%	341%	1	202%	332%	1	225%	335%	1	153%	335%	1	117%	409%	1	119%	243%	1	69%	242%	1	110%	205%	1

The potency and relative Emax calculated from the concentration-response curves are presented in Table 11, as well as the difference in compound potency calculated as a ratio of EC_{50} between WT and mutant mGlu2 receptors.

As shown in Figure 28 and Table 11 these tested mutations did not seem to affect the activity of the PAM compounds that were tested.

Table 11. Potency (EC_{50}) and relative Emax of the enhancement of glutamate-induced [^{35}S]GTPγS binding by 4 PAMs on WT and mutant receptors after transient transfection. Data is presented as EC_{50} (nM)) and Emax (percentage) of one experiment performed in triplicate.

		Stable		WT		EL2				TM5	
						$G_{706}R$ (#27)		$P_{711}A$ (#30)		$A_{726}S$ (#33)	
Chemical class	**JNJ number**	**EC_{50}**	**E_{max}**	**EC_{50}**	**E_{max}**	**EC_{50}**	**E_{max}**	**EC_{50}**	**E_{max}**	**EC_{50}**	**E_{max}**
TEMPS(LY487379	35814090	188	113	129	113					120	126
Imidazopyridine	41482012	131	98	152	105	212	87				
1,5-Pyridone	35814376	716	193	471	164	1019	189				
Isoquinolone	39226421	252	155	86	141			210	175		

3.1.3. Evaluation of binding pocket for novel chemical structures

To test if the results previously obtained are verified for other structurally different compounds, a second set of [^{35}S]GTPγS binding screening was performed with 10 compounds, belonging to the chemical classes pyridazine, azetidine, triazolopyridine and 1,4-pyridone (Appendix 2). For these compounds, the whole set of 40 mutants was tested. The screening was done once as described before and the results are shown in Appendix 3.

For the mutants that appeared to affect the PAM's activity based on the pre-screening method, concentration-response curves were generated with representatives (generally the most potent) compounds of each chemical class (pyridone, azetidine, pyridazine and triazolopyridine).

Figure 29-33 display the results obtained for the 5 representative PAMs on some mutations localized in a specific receptor region. Potency (EC_{50}) and relative efficacy (Emax) were calculated for the tested PAMs on WT mGlu2 and mutant receptors and are shown in Table 12.

Results of the screening approach and glutamate-induced [^{35}S]GTPγS binding are described by receptor region.

Transmembrane 2

Mutations localized in this receptor region did not affect PAM's activity when the compounds were tested in [^{35}S]GTPγS binding screening. Therefore no further analysis was performed with these mutations (see Appendix 3).

Transmembrane 3

Figure 29 displays the results obtained from the concentration-dependent enhancement of glutamate-induced [^{35}S]GTPyS binding on mutations localized in transmembrane 3. Mutation F643A elicited the biggest effects (EC_{50} > 10000 nM) (Figure 29B, C and E) with exception of compound JNJ-42073824 which shows an increase of the EC_{50} of about 64-fold (Table 12). For the compounds only tested in screening, this mutation also seems to have a large effect (see Appendix 3). Mutations R635A and L639A showed some effect on PAM's activity when tested through screening approach, but when the most potent compounds were tested on concentration-response curves, no alterations were observed (Table 12, Figure 29). Mutation R636A reduced the activity of compound JNJ-42073824 (triazolopyridine), with an increase of the EC_{50} of 4 fold and mutation S644A produced an increase of about 3-fold in the EC_{50} of compound JNJ-54768636 (pyridone) and for compound JNJ-54757027 (triazolopyridine) no curve was possible to generated. Also, through screening analysis, this mutation elicit a response lower than 75% of the WT for all the compounds tested. The remaining mutations localized in this region (T641S and A642S) did not show any effect when the screening was performed.

Transmembrane 4

[^{35}S]GTPyS screening showed that some of the mutations localized in TM4 elicit effect on PAM's activity with exception of mutations S688L, A681F, I693M, V695S and A696V, and thus these particular mutations were not further tested. For the compounds tested in concentration-response curves the triple mutation N735D/G689V/S688L showed the most prominent results, causing a decrease in potency and in Emax (20-fold increased in EC_{50}) for compound JNJ-54768636 (Table 12) and for the other compounds tested elicited values of EC_{50} > 10000 nM (Table 12, Figure 30A-C,E). The other triple mutation S644A/V700L/H723V and mutation G689V/S688L caused a similar effect, decreasing potency and Emax for almost every compound tested with exception of the representative of the chemical class azetidine (Figure 30, Table 12). Mutation V700L shows an increase of 3-fold on the EC_{50} of compound JNJ-54757027, while for the other compounds this mutation does not elicit any effect. In the case of mutation G689V no effect was observed on the azetidine or pyridazine series, as exemplified with JNJ- 54800681 and JNJ-54445001, while for the other classes an increase of 3 to 7 in EC_{50} was observed.

Extracellular loop 2

The preliminary analysis showed that mutations localized in the EL2 did not influence PAM's activity with exception of mutation H723. However when tested in concentration-dependent enhancement of glutamate-induced [^{35}S] GTPγS binding, this mutation only showed a decrease on

potency of compound JNJ-54768636 (4-fold increase on EC_{50}), while Emax was not affected (Figure 31, Table 12).

Although mutation V716T showed some effect on screening, this could not be confirmed after further analysis (Figure 31, Table 12).

Transmembrane 5

In this receptor region, mutation M728A, S731A, L732A and N735D decreased compound's activity when these were tested with 2 concentrations (screening approach). This could, however, not be confirmed for mutation M728A when was tested in glutamate-induced [^{35}S] GTPyS binding. On the other hand, mutation N735D showed dramatic effects, eliciting values of EC_{50} > 10000 nM (Figure 32A and C, Table 12). When the concentration-response curve was possible to generate, this mutation elicited a decrease in potency, with an increase of the EC_{50} of 14 fold (Table 12). Although mutation S731 showed an increase of 4 and 5-fold on EC_{50} for compound JNJ-54445001 and JNJ-42073824, respectively, for compound JNJ 54800681, this mutation did not affect potency but a decrease on Emax was observed. Mutation L732A elicits an effect so large on the activity of compound JNJ 54757027 given values of EC_{50} > 10000 nM. Mutations D725A, A733T and A740I did not show any effect when tested in screening while for V723A, A726S and G730I a slight decrease in the compound's activity was observed.

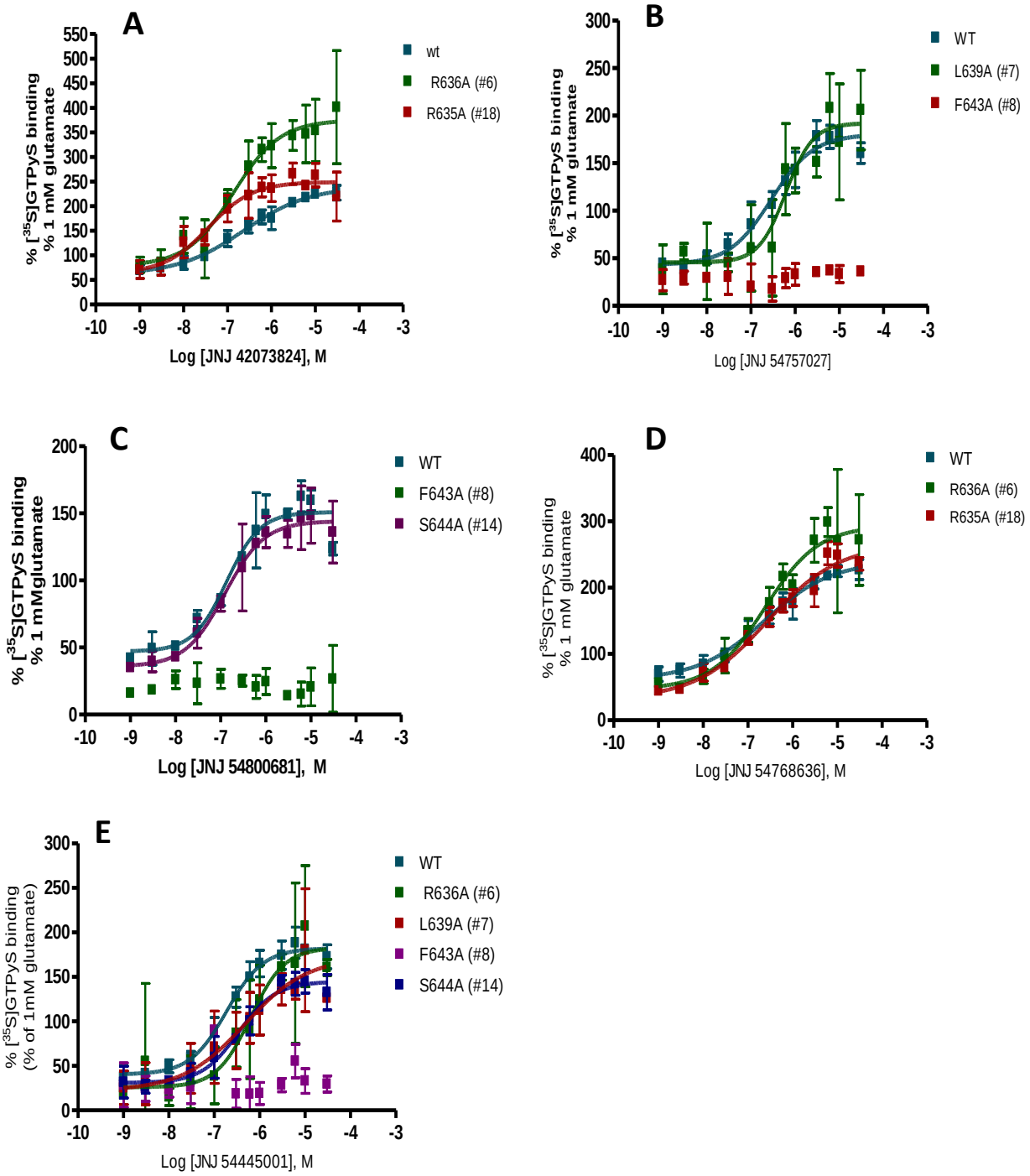


Figure 29. Effect of PAMs on glutamate-induced [^{35}S]GTPyS binding in WT mGlu2 and mGlu2 with mutations located in **transmembrane 3** (TM3). Concentration-dependent enhancement of 4 µM glutamate-induced [^{35}S]GTPyS binding by 5 PAMs, representative of 4 chemical classes. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate.

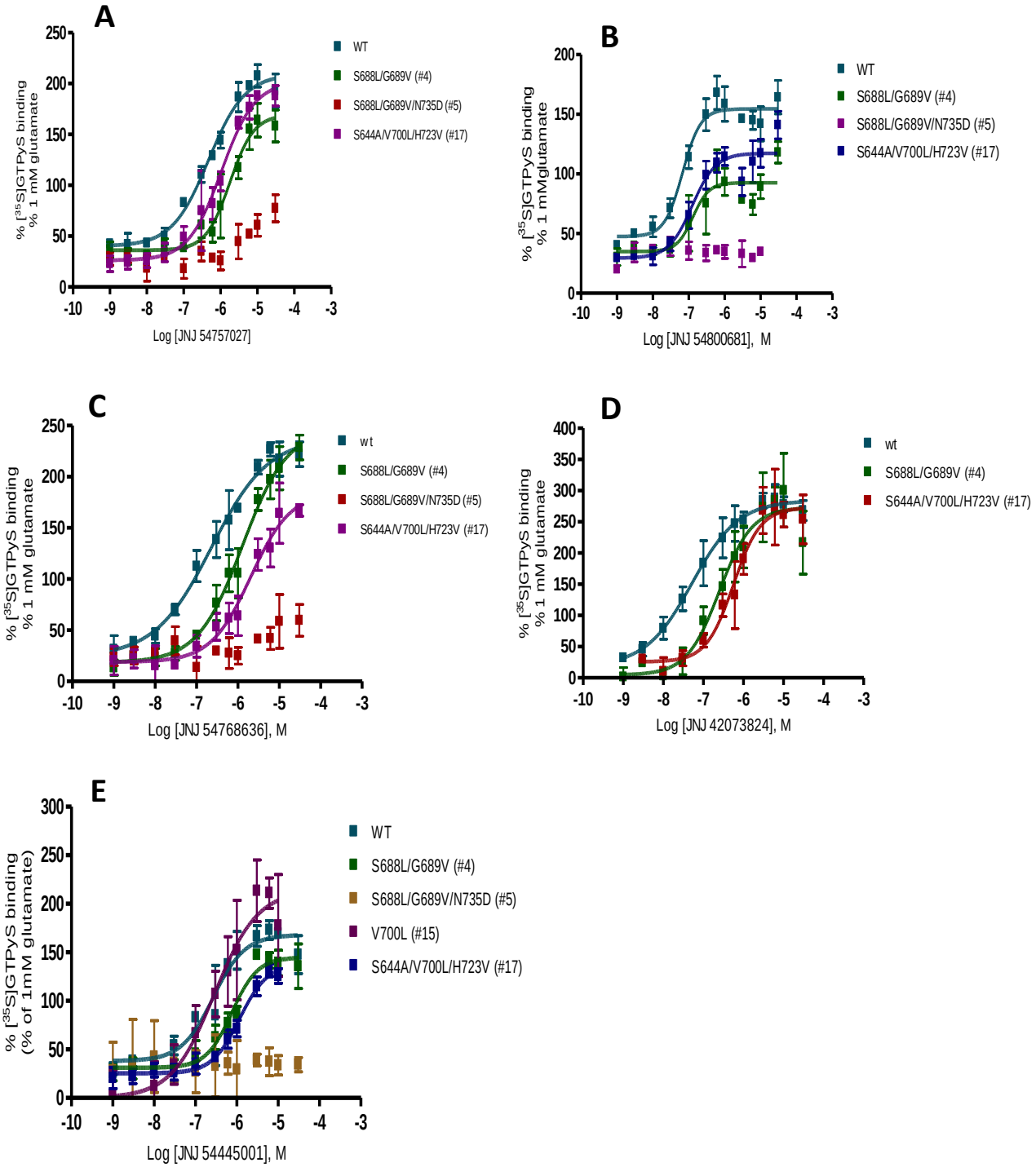


Figure 30. Effect of PAMs on glutamate-induced [^{35}S]GTPyS binding in WT mGlu2 and mGlu2 with mutations located in **transmembrane 4** (TM4). Concentration-dependent enhancement of 4 µM glutamate-induced [^{35}S]GTPyS binding by 5 PAMs, representative of 4 chemical classes. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate.

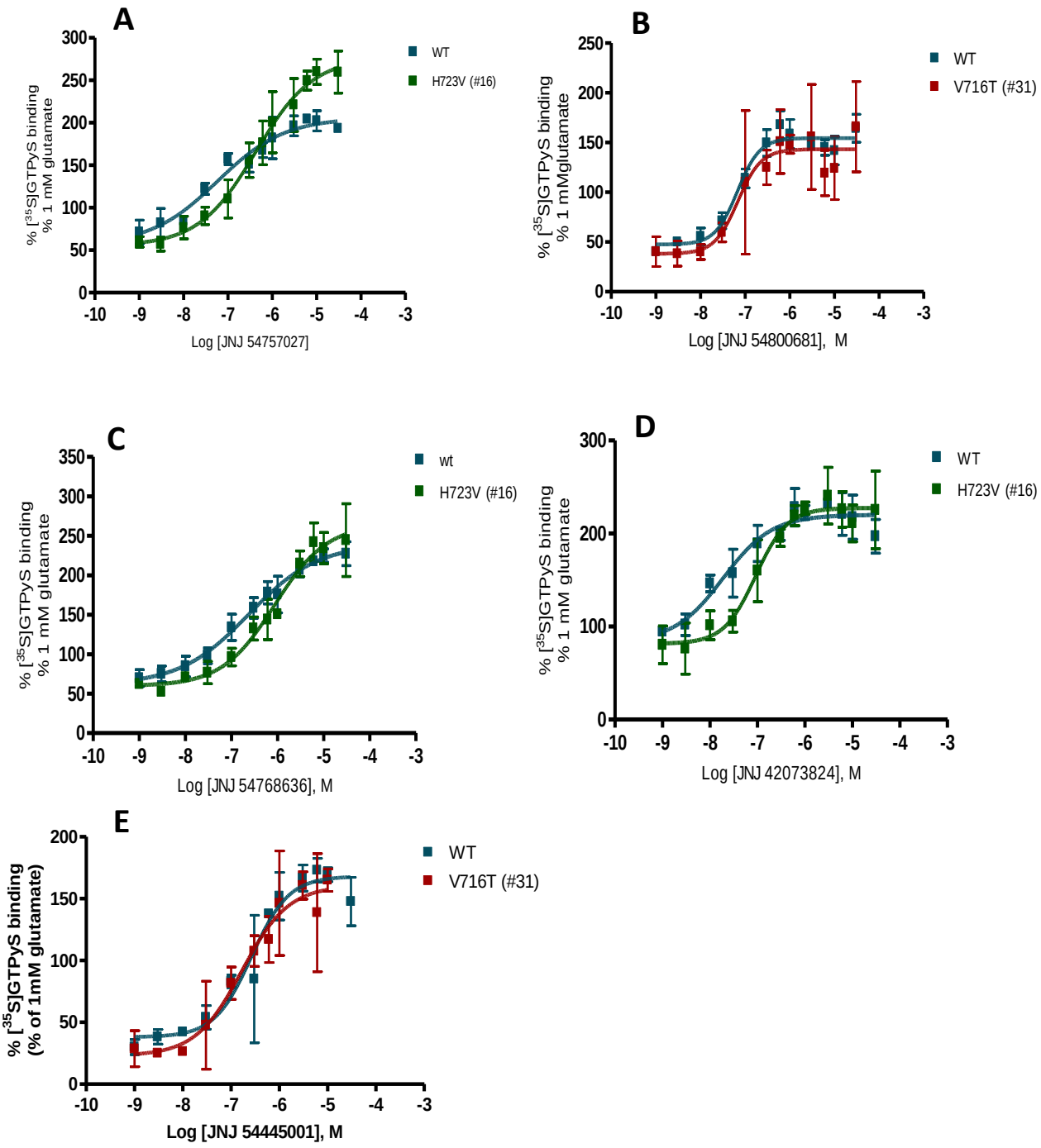
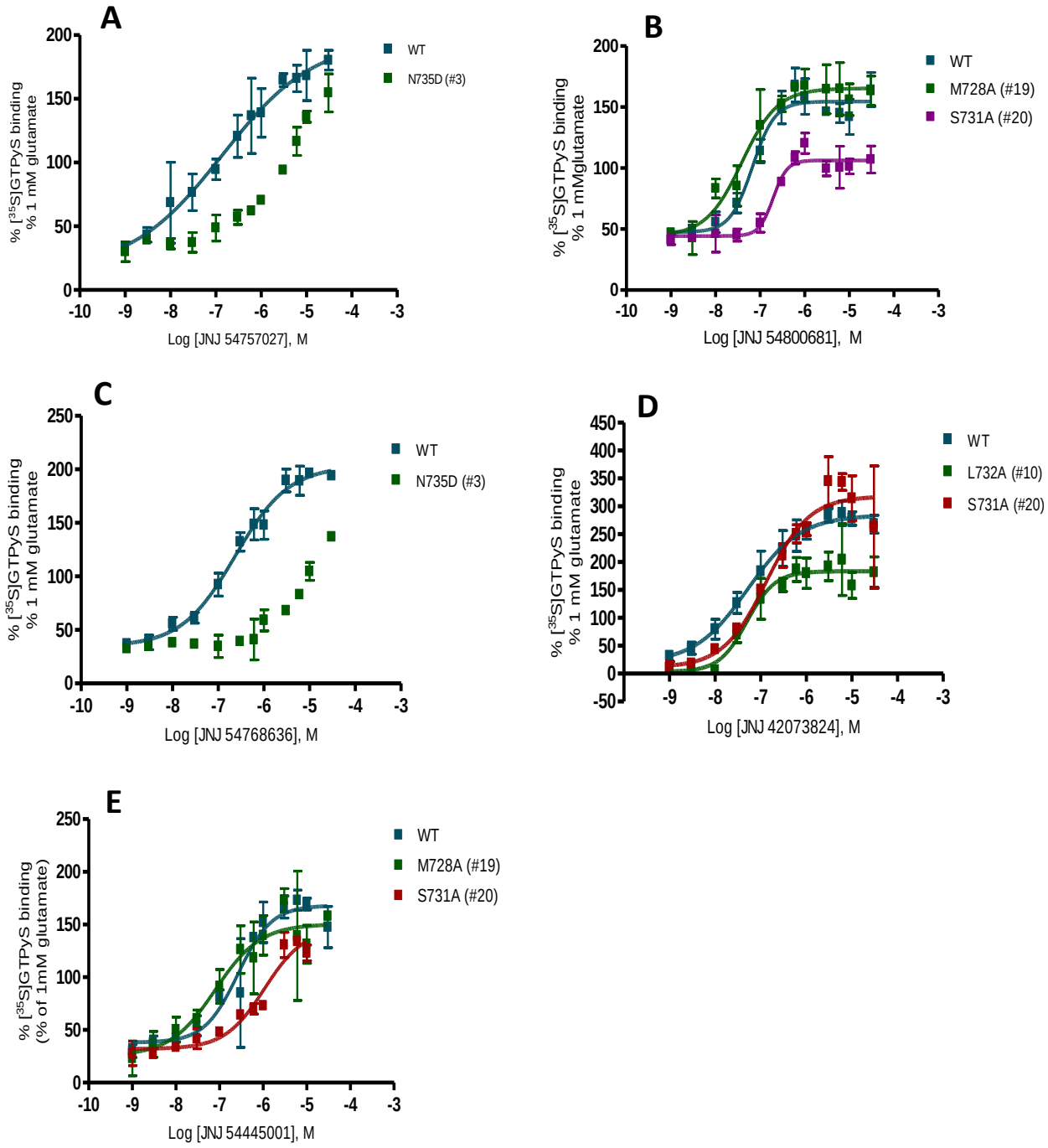


Figure 31. Effect of PAMs on glutamate-induced [^{35}S]GTPγS binding in WT mGlu2 and mGlu2 with mutations located in **extracellular loop 2** (EL2). Concentration-dependent enhancement of 4 μM glutamate-induced [^{35}S]GTPγS binding by 5 PAMs, representative of 4 chemical classes. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate.



Fig**ure 32.** Effect of PAMs on glutamate-induced [^{35}S]GTPγS binding in WT mGlu2 and mGlu2 with mutations located in **transmembrane 5** (TM5). Concentration-dependent enhancement of 4 µM glutamate-induced [^{35}S]GTPγS binding by 5 PAMs, representative of 4 chemical classes. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate.

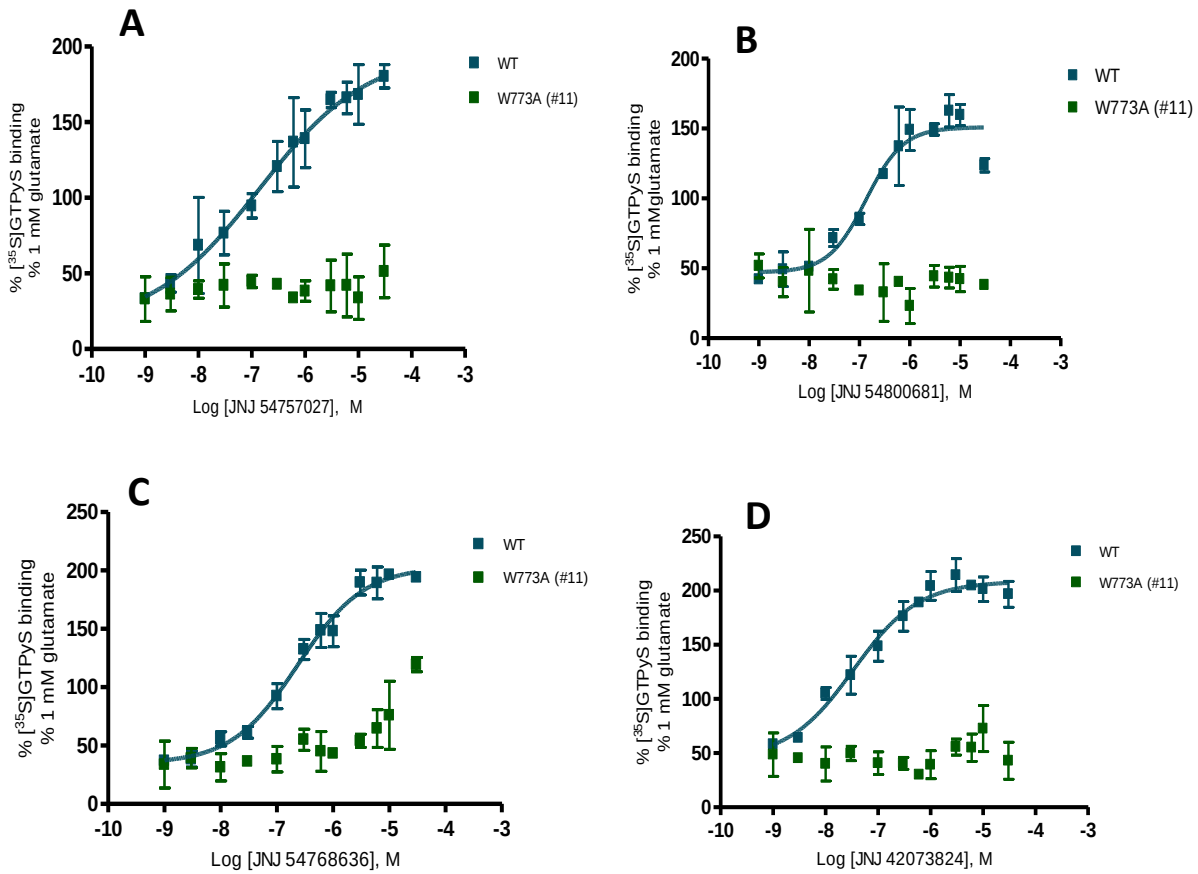


Figure 33. Effect of PAMs on glutamate-induced [^{35}S]GTPγS binding in WT mGlu2 and mGlu2 with mutations located in **transmembrane 6** (TM5). Concentration-dependent enhancement of 4 μM glutamate-induced [^{35}S]GTPγS binding by 4 PAMs, representative of 3 chemical classes. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate.

Transmembrane 6

Mutation W773A affects the activity of all compounds tested, disrupting completely their ability to generate a concentration-response curve (Figure 33). Mutation F776A showed a small effect in two compounds when tested on screening but mutation F780A did not affect PAM's activity.

Transmembrane 7

The only mutation of this receptor region was not tested in concentration-response curves because of the fact that this mutation presents rather low response amplitude (see Table 9) even though some effect was seen in screening (Table 12).

In the cases that just a small effect was observed when mutations were analyzed by screening with two concentrations it was considered as not having an effect.

Table 12. Summary of screening results and potency (EC_{50}, nM) and relative Emax (%) of the enhancement of glutamate-induced [^{35}S]GTPyS by 5 PAMs on WT and mutant receptors after transient transfection into CHO-K1 cells. n.e. – no effect in initial screening; ▢ - only tested in screening, effect observed (<75% WT); ▢. - effect observed in glutamate-induced [^{35}S]GTPyS binding, EC50 mutant/EC50 WT > 3

		WT		Transmembrane 2		Transmembrane 3											
				C616S (#37)	I622F (#38)	T641S (#39)	A642S (#40)	R635A (#18)		R636A (#6)		L639A (#7)		F643A (#8)		S644A (#14)	
		EC_{50}	Emax					EC_{50}	Emax	EC_{50}	Emax	EC_{50}	Emax	EC_{50}	Emax	EC_{50}	Emax
Pyridazine	JNJ-54445001	225	175	n.e.	n.e.	n.e.	n.e.			631	183	478	172	**>10000.**	26	344	145
Azetidine	JNJ-54352272	Sc	Sc	n.e.	n.e.	n.e.	n.e.					n.e.					
	JNJ-54750592	Sc	Sc	n.e.	n.e.	n.e.	n.e.					n.e.					
	JNJ-54469805	98	202	n.e.	n.e.	n.e.	n.e.					n.e.		**>10000**	36		
	JNJ-54800681	78	158	n.e.	n.e.	n.e.	n.e.	156	196	81	199	n.e.		**>10000**	23	123	144
Pyridone	JNJ-54768636	246	227	n.e.	n.e.	n.e.	n.e.	268	266	242	293	467	204	**>10000**	88	**841**	202
	JNJ-54768597	Sc	Sc	n.e.	n.e.	n.e.	n.e.			n.e.							
	JNJ-54752997	Sc	Sc	n.e.	n.e.	n.e.	n.e.										
Triazolopyridine	JNJ-54757027	296	199	n.e.	n.e.	n.e.	n.e.	138	273			614	192	**>10000**	35	**n.v.**	248
	JNJ-42073824	33	234	n.e.	n.e.	n.e.	n.e.	37	249	**136**	375	27	189	**2109**	69	63	188

Table 12. (cont)

		WT		Trasmembrane 4													
				S688L (#1)	G689V (#2)		G689V/S688L (#4)		N735D/G689V/S688L (#5)		V700L (#15)		S644A/V700L/H723V (#17)		A681F (#23)	I693M (#24)	V695S (#25)	A696V (#26)
		EC_{50}	Emax		EC_{50}	Emax	EC_{50}	Emax	EC_{50}	Emax	EC_{50}	Emax	EC_{50}	Emax				
Pyridazine	JNJ-54445001	225	175	n.e.	598	128	**757**	145	**>10000**	35	286	214	**1159**	138	n.e.	n.e.	n.e.	n.e.
Azetidine	JNJ-54352272	Sc	Sc	n.e.											n.e.		n.e.	n.e.
	JNJ-54750592	Sc	Sc	n.e.									n.e.		n.e.		n.e.	n.e.
	JNJ-54469805	98	202	n.e.					**>10000**	26					n.e.		n.e.	n.e.
	JNJ-54800681	78	158	n.e.	185	96	130	93	**>10000**	35	145	234	125	117	n.e.	n.e.	n.e.	n.e.
Pyridone	JNJ-54768636	246	227	n.e.	**723**	181	**1282**	247	**4797**	62	668	373	**2023**	185	n.e.	n.e.	n.e.	n.e.
	JNJ-54768597	Sc	Sc	n.e.									n.e.		n.e.	n.e.	n.e.	n.e.
	JNJ-54752997	Sc	Sc										n.e.				n.e.	n.e.
Triazolopyridine	JNJ-54757027	296	199	n.e.	**2183**	167	**1782**	169	**>10000**	87	**1014**	395	**1059**	202	n.e.	n.e.	n.e.	n.e.
	JNJ-42073824	33	234	n.e.	**106**	179	**249**	273	**>10000**	195	63	344	**561**	273	n.e.	n.e.	n.e.	n.e.

Table 12. (cont)

		WT		Extracellular loop 2									
				H723V (#16)		G706R (#27)	E708Y (#28)	A710L (#29)	P711A (#30)	V716T (#31)		T718I (#32)	
		EC_{50}	Emax	EC_{50}	Emax					EC_{50}	Emax	EC_{50}	Emax
Pyridazine	JNJ-54445001	225	175			n.e.	n.e.	n.e.	n.e.	218	159	n.e.	
Azetidine	JNJ-54352272	Sc	Sc			n.e.		n.e.	n.e.	n.e.		n.e.	
	JNJ-54750592	Sc	Sc			n.e.	n.e.	n.e.	n.e.	n.e.			
	JNJ-54469805	98	202			n.e.	n.e.	n.e.	n.e.				
	JNJ-54800681	78	158	121	180	n.e.	n.e.	n.e.	n.e.	68	143	n.e.	
Pyridone	JNJ-54768636	246	227	**861**	265	n.e.		n.e.	n.e.	n.e.		n.e.	
	JNJ-54768597	Sc	Sc			n.e.	n.e.	n.e.	n.e.			n.e.	
	JNJ-54752997	Sc	Sc										
Triazolopyridine	JNJ-54757027	296	199	427	277	n.e.	n.e	n.e.	n.e.	n.e.		n.e.	
	JNJ-42073824	33	234	92	228	n.e.	n.e	n.e.	n.e.	n.e.			

Table 12. (cont)

		WT		Trasmembrane 5											Transmembrane 6			TM 7
				D725A (#9)	M728A (#19)		S731A (#20)		L732A (#10)		N735D (#3)		V736A (#21)	A726S (#33)	G730I (#34)	A733T (#35)	A740I (#36)	W773A (#11)		F776A (#12)	F780A (#13)	V798A (#22)
		EC_{50}	Emax		EC_{50}	Emax	EC_{50}	Emax	EC_{50}	Emax	EC_{50}	Emax						EC_{50}	Emax			
Pyridazine	JNJ-54445001	225	175	n.e.	80	151	**1067**	146	n.e.		**>10000**	31	n.e.	n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.
Azetidine	JNJ-54352272	Sc	Sc	n.e.					n.e.				n.e.	n.e.		n.e.	n.e.			n.e.	n.e.	
	JNJ-54750592	Sc	Sc	n.e.									n.e.	n.e.	n.e.	n.e.	n.e.				n.e.	n.e.
	JNJ-54469805	98	202	n.e.					n.e.				n.e.	n.e.		n.e.	n.e.			n.e.	n.e.	
	JNJ-54800681	78	158	n.e.	39	165	195	106	148	137	**>10000**	39		n.e.	n.e.	n.e.	n.e.	**>10000**	232	n.e.	n.e.	
Pyridone	JNJ-54768636	246	227	n.e.	n.e.		710	219	n.e.		**>10000**	189	n.e.	n.e.	n.e.	n.e.	n.e.	**>10000**	855	n.e.	n.e.	
	JNJ-54768597	Sc	Sc	n.e.	n.e.								n.e.	n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	
	JNJ-54752997	Sc	Sc	n.e.					n.e.				n.e.		n.e.	n.e.	n.e.			n.e.	n.e.	
Triazolopyridine	JNJ-54757027	296	199	n.e.	n.e.		564	172	**>10000**	98	**>10000**	198	n.e.	n.e.	n.e.	n.e.	n.e.	**>10000**	41	n.e.	n.e.	
	JNJ-42073824	33	234	n.e.	n.e.		**132**	318	50	187	**454**	209	n.e.	n.e.	n.e.	n.e.	n.e.	**>10000**			n.e.	n.e.

3.2. Radioligand binding assays

After assessing the impact of the mutated amino acids on efficacy, through use of a functional assay ([^{35}S] GTPγS binding) it is important to verify the impact on binding of the compound (affinity). For that reason, binding assays were done to assure that the compound is able to bind to the receptor. For these experiments, a tritiated form of a PAM compound ([^{3}H] JNJ-46281222) was used and the mutations chosen were N735S, S688L/G689V, S688L/G689V/N735D, F643A, S644A/V700L/H723V, M728A and V736A.

To make sure that potential differences observed in the binding levels are due to alterations in binding affinity rather than differences in receptor densities, it was first important to use membrane pools with similar receptor densities. For this, we used the orthosteric antagonist [^{3}H]-LY341495.

3.2.1. Choosing the optimal protein amount

[^{3}H]LY341495 binding experiments were done with diverse concentrations of WT (stable and transiently transfected) hmGlu2 receptors with the aim of knowing the optimal range of protein amount that should be used in further binding experiments. Figure 34 shows that the specific binding of 3 nM [^{3}H] LY341495 was proportional to the amount of membrane protein and increased linearly between 2.5 and 30 µg of membrane protein/assay.

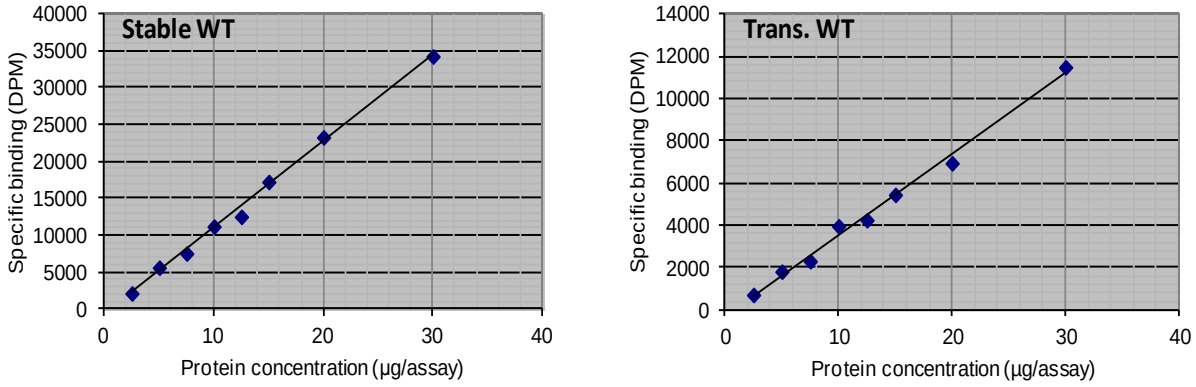


Figure 34. Specific [^{3}H] LY341495 binding is linear with amount of membrane protein in Stable WT (left) and in Transient WT (right). Increased concentrations of these pools were tested, namely, 2.5, 5, 7.5, 10, 12.5, 15, 20 and 30 µg/assay. Data is presented as mean of triplicate determinations from one experiment.

For the stably transfected CHO-K1 cells the use of 10 µg of protein/assay was taken as optimal for experiments with this radioligand. To have equivalent protein amounts, 20 µg of protein/assay was chosen for transiently transfected WT. This amount was also chosen for mutant forms of the mGlu2 receptor that were transiently expressed in CHO-K1 cells.

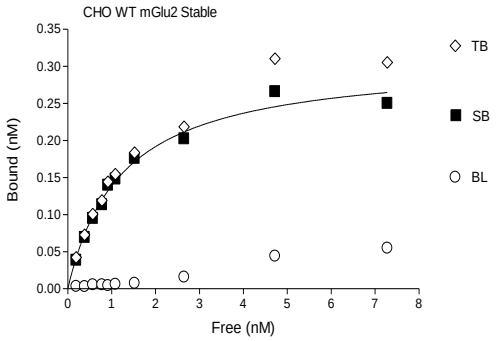
3.2.2. Determination of receptor density

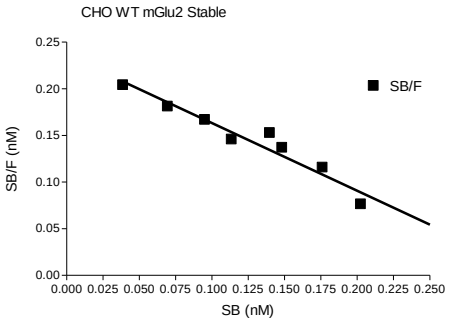
After determining which protein amount to use, saturation binding experiments using [^{3}H]-LY341495 were done with the main aim of knowing the density (B_{max}) of mGlu2 receptors expressed in cell membranes of WT (stable and transiently transfected) and mutated receptors. The results can be seen in Figure 35.

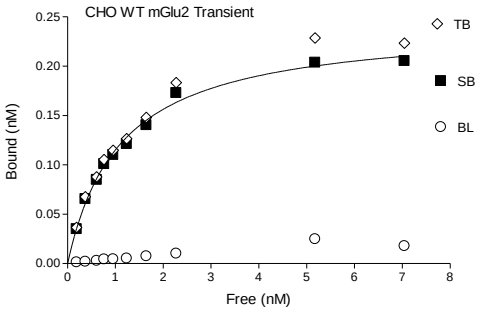
As seen in Table 13 the B_{max} is quite similar for every mutated receptor with exception for mutation L639A. For that reason this mutation was not chosen for further analysis.

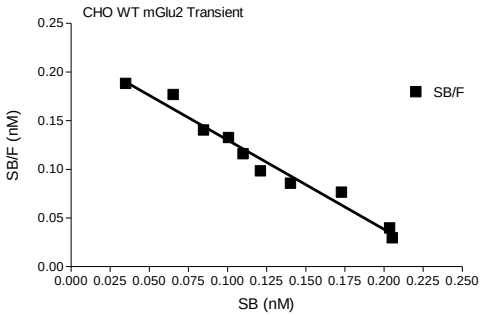
Table 13. Summary of receptor density (B_{max}) and of the equilibrium dissociation constants (K_D) of [^{3}H]-LY341495 binding to WT (stably and transiently transfected) and mutant hmGlu2 receptors; Experiments were done once, in triplicate, with exception for both WT (n=2)

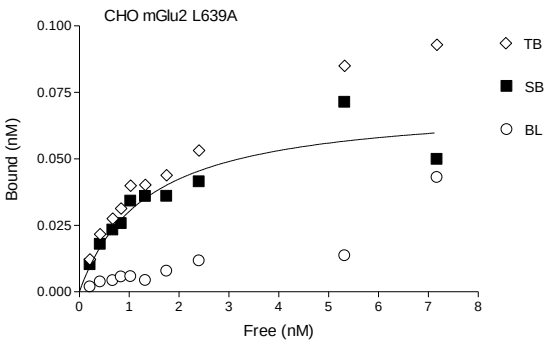
	K_D (nM)	B_{max} (fmol/mg of protein)
mGlu2 Stable WT	1.51 ± 0.41	15898 ± 619
mGlu2 Transient WT	1.31 ± 0.27	6673 ± 838
S688L/G689V (#4)	0.97	9060
S688L/G689V/N735D (#5)	1.2	6633
L639A (#7)	1.35	1775
F780A (#13)	1.23	4128
M728A (#19)	1.07	8408
V736A (#21)	1.25	6393

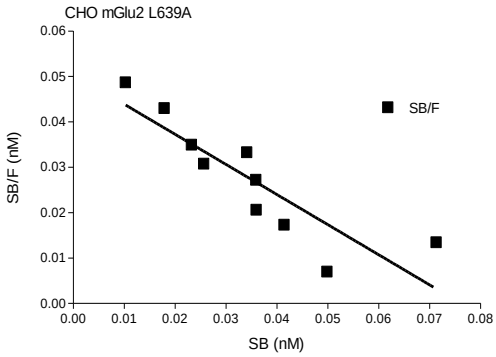

CHO WT mGlu2 Stable
Bound (nM)
Free (nM)
TB
SB
BL

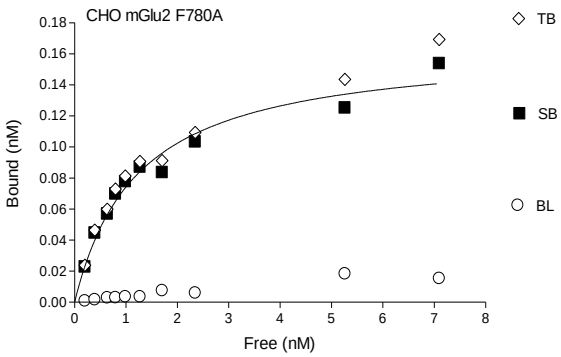

CHO WT mGlu2 Stable
SB/F (nM)
SB (nM)
SB/F

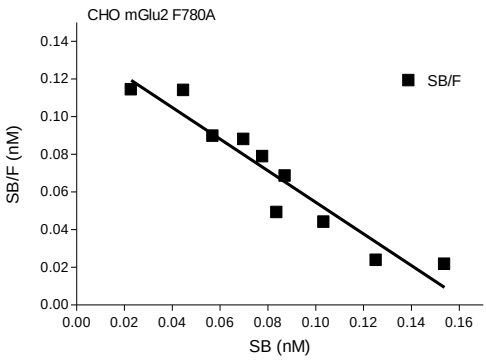

CHO WT mGlu2 Transient
Bound (nM)
Free (nM)
TB
SB
BL


CHO WT mGlu2 Transient
SB/F (nM)
SB (nM)
SB/F


CHO mGlu2 L639A
Bound (nM)
Free (nM)
TB
SB
BL


CHO mGlu2 L639A
SB/F (nM)
SB (nM)
SB/F


CHO mGlu2 F780A
Bound (nM)
Free (nM)
TB
SB
BL


CHO mGlu2 F780A
SB/F (nM)
SB (nM)
SB/F

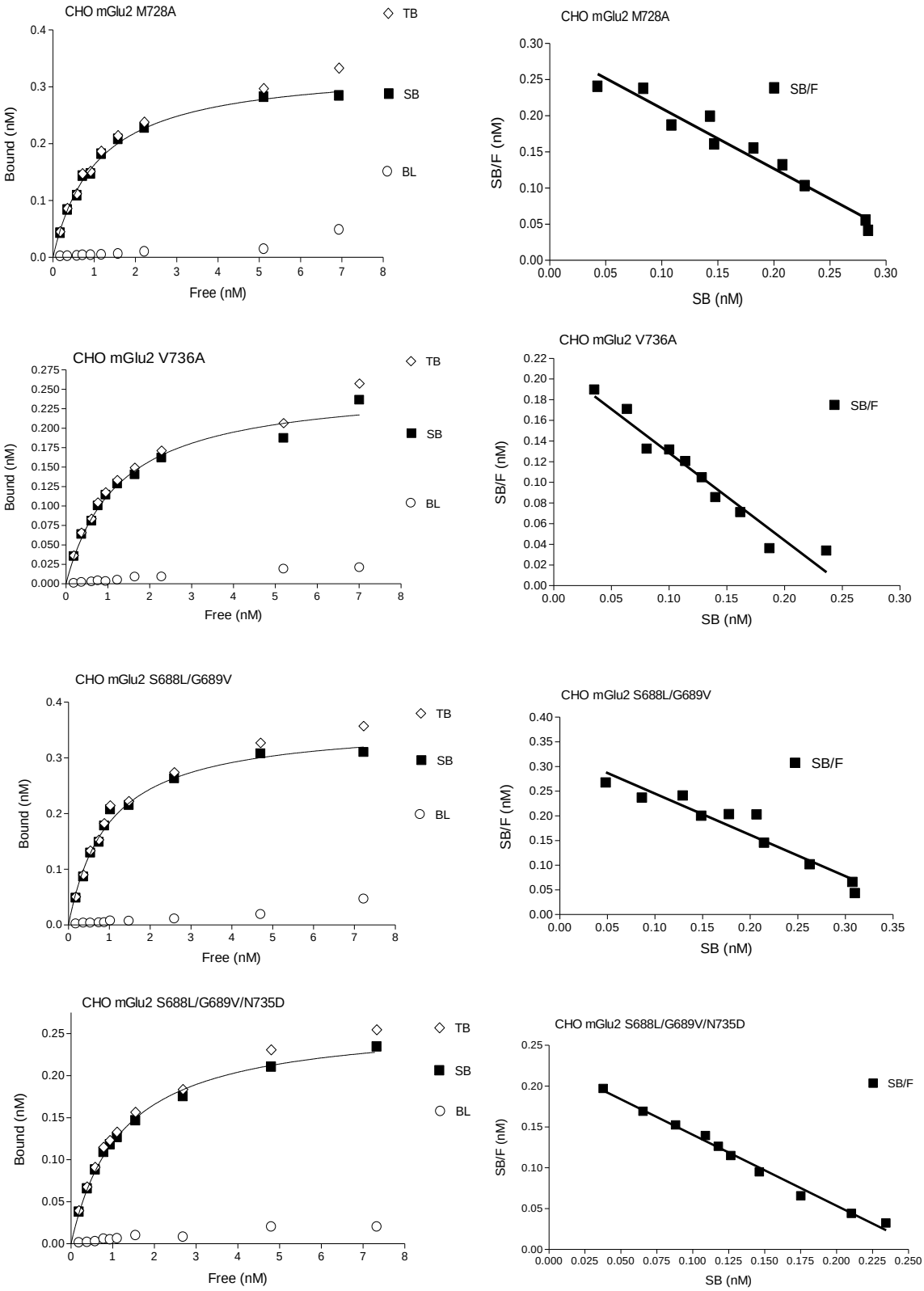


Figure 35. Saturation binding curves (on the left) and their correspondent scatchard plots (on the right) of [^{3}H]-LY341495 binding to WT (transiently and stably transfected) and some of the mutated hmGlu2 receptors. Mutation L639A belongs to TM3 of the receptor, S688L/G689V and S688L/G689V/N735D are part of the TM4, M728A and V736A part of TM5 and F780A belongs to TM6. Data is presented as nanomolar specifically bound. Data points were determined in triplicate (n=1).

3.2.3. Effect of mutations on PAM's affinity – [^{3}H]PAM binding

Further saturations experiments were done with [^{3}H]-JNJ 46281222, which is a PAM of the triazolopyridine chemical class, with final concentrations of 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 7.5, 10, 15 and 20 nM. Since previous work determined the use of 75 µg/assay for the stable WT as optimal (data not shown), this protein amount was chosen for the stable WT, while – in analogy with the higher amounts needed for [^{3}H] LY341495 binding, 150µg/assay was used for the transiently transfected pools. Also, looking at the Bmax results obtained in section 3.1.1., one can see that receptor expression for transient transfections seems more or less half of the stable transfection.

Firstly, the mutants not affecting JNJ-46281222 activity on [^{35}S] GTPγS binding were evaluated for [^{3}H]-JNJ 46281222 saturation binding and as can be seen in Figure 36, the selected mutants M728A and V736A are able to bind this PAM compound confirming the lack of effect on the glutamate-induced [^{35}S] GTPγS binding.

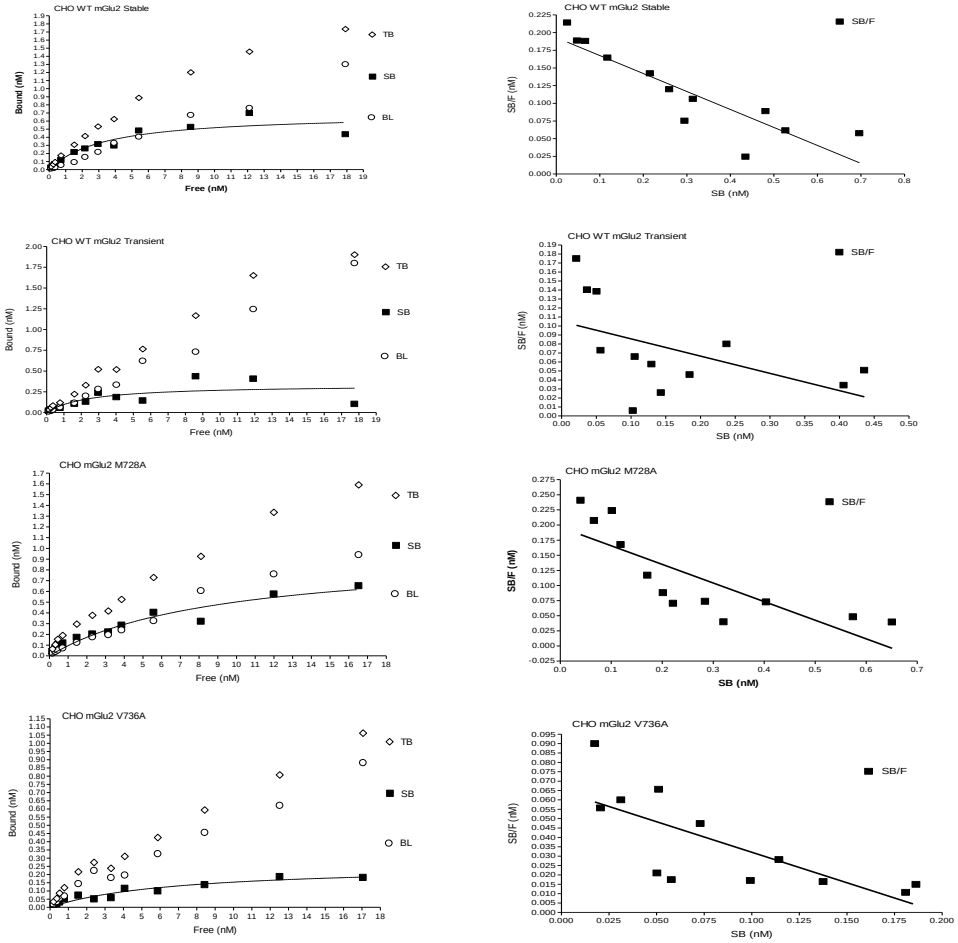


Figure 36. Saturation binding curves and correspondent Scatchard plots of [^{3}H]-JNJ 46281222 binding to WT (stably and transiently transfected) and mutant mGlu2 receptors (M728A and V736A) expressed in CHO-K1 membranes. 75 µg/assay for stable WT and 150 µg/assay for transient WT and mutated hmGlu2 receptors were used. Data are presented as nanomolar specifically bound. Data points were determined in triplicate (n=1).

It is important to note, however, that the saturation binding experiments shows for the stable WT a specific binding (calculated with 4 nM of radioligand) of 30%, while transient WT showed 23% of specific binding and for mutation M728A and V736A the percentage was only 22% and 6% respectively. Because of this low specific binding it was not possible to calculate the K_D. Therefore one concentration of this radioligand, equivalent to the K_D (4nM) previously determined on stable mGlu2 WT cells (data not shown), was chosen and further experiments were performed. In this case, additionally to the mutations already tested in [^{3}H]-JNJ 46281222 saturation binding, mutations N735S, S688L/G689V, S688L/G689V/N735D, F643A and S644A/V700L/H723V were included, to verify whether binding of PAMs was affected. Figure 37 shows that mutations S644A/V700L/H723V, M728A and V736A are still able to bind the PAM compound as demonstrated before. As for the other mutations (N735D, S688L/G689V, S688L/G689V/N735D and F643A) the binding was disrupted. Figure 37B shows a clear effect for the triple mutation (S688L/G689V/N735D) which totally prevented the binding of the PAM to the receptor, as well as for mutation N735D.

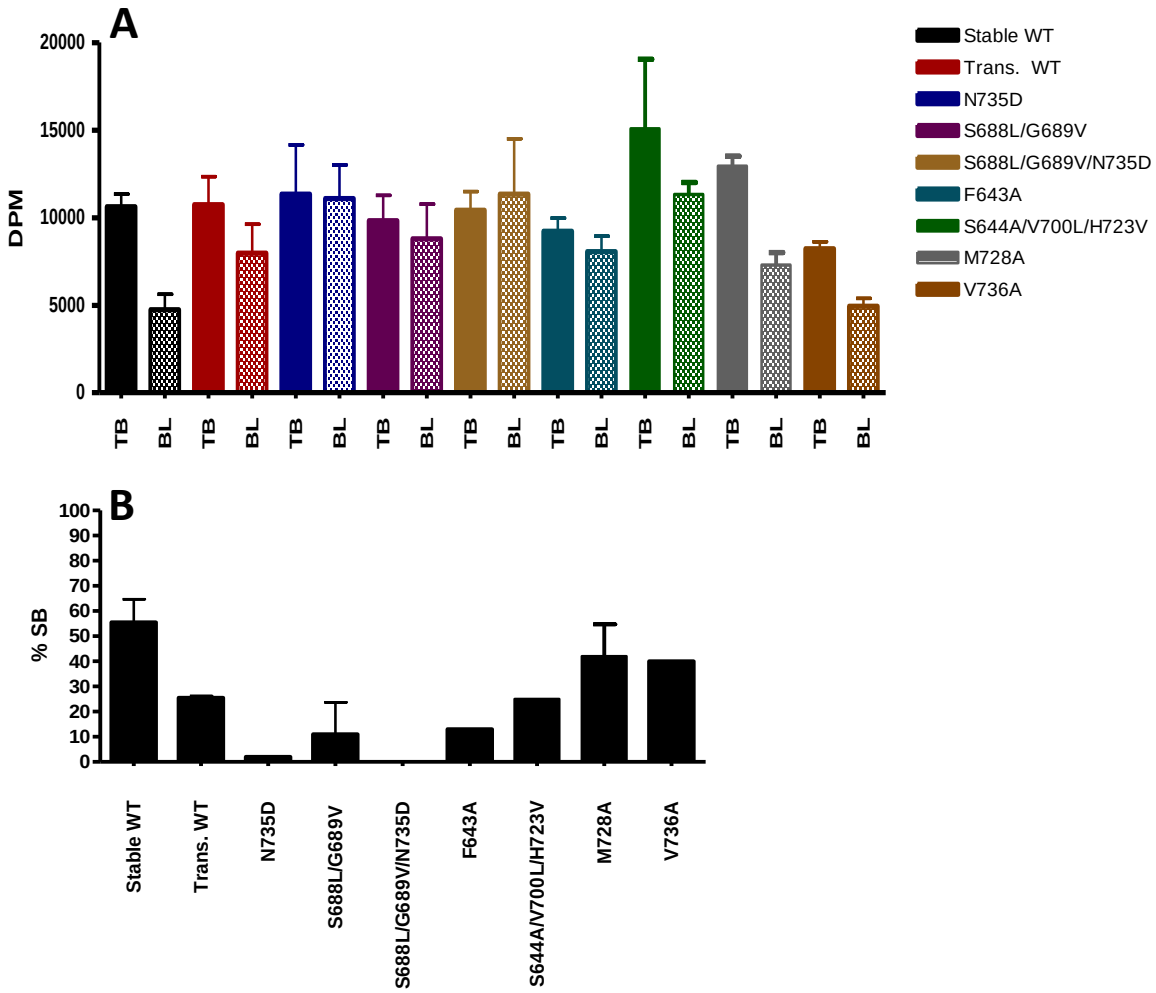


Figure 37. Effect of mutated mGlu2 receptors on [3H]JNJ 46281222 (PAM) binding (4 nM). Data is presented as mean ± SD of two independent experiments performed in triplicated (exception for mutation N735D, F643A, S644A/G689V/N735D, S644A/V700L/H723V; n=1). A: Results are presented as DPM; TB – Total binding (only used buffer), BL – Blank (non-specific binding), determined with addition on 10 µM JNJ-42341806 (mGlu2 PAM). B: Results are presented as percentage of specific binding.

The experiment was also done in the presence of 1 mM glutamate. Interestingly, the addition of the agonist increased the binding in both stable and transient WT. The specific binding of the stable WT is 55% in the absence of glutamate against 80% with 1 mM glutamate. For the transient WT the values are 25% against 55% without and with glutamate, respectively. For the mutated receptors the binding also increased with addition of glutamate. However mutations N735D, S688L/G689V/N735D, F643A and S644A/V700L/H723V still show reduced specific binding compared to WT (Figure 38B).

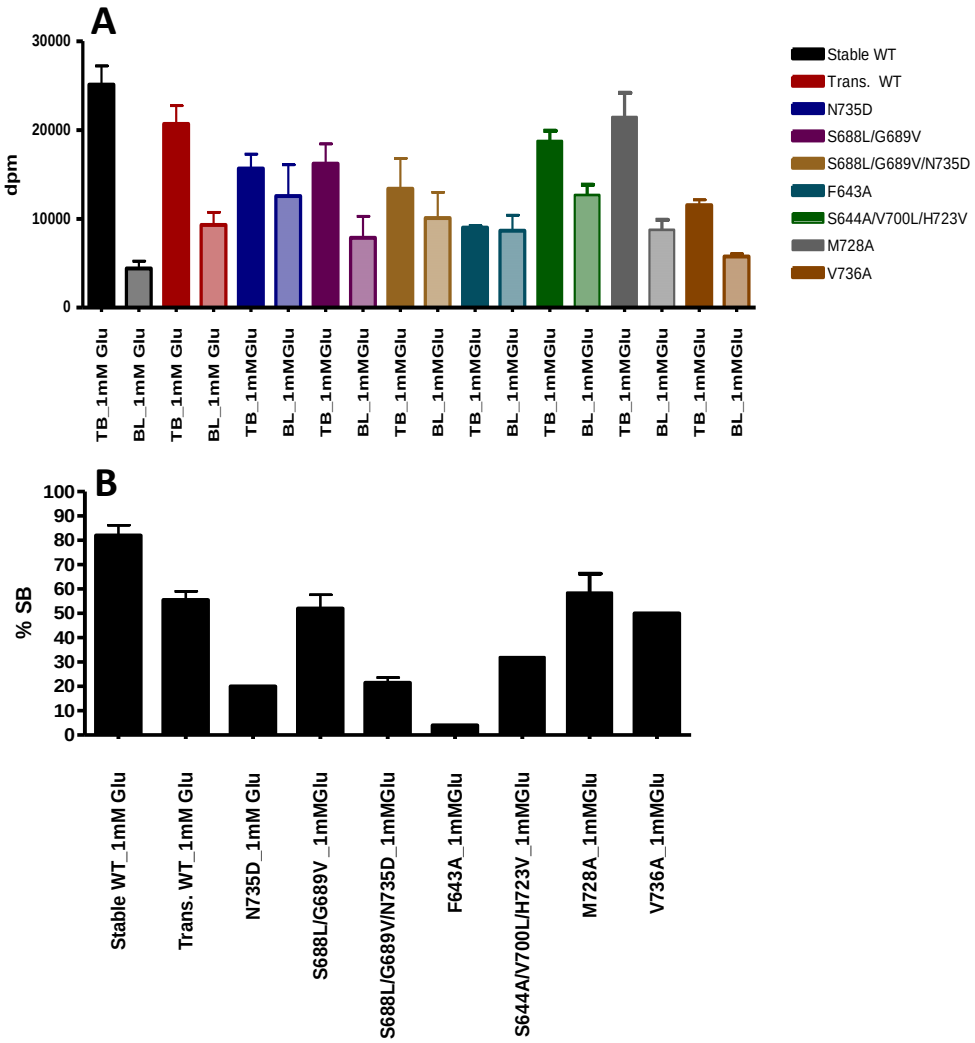


Figure 38. Effect of mutated mGlu2 receptors on [^{3}H]JNJ 46281222 (PAM) binding. 4nM of Radioligand were incubated with 1 mM glutamate. Data is presented as mean ± SD of two independent experiments performed in triplicated (exception for mutation N735D, F643A, S644A/G689V/N735D; n=1). A: Results are presented as DPM; TB – Total binding (only used buffer), BL – Blank (non-specific binding), determined with addition on 10 µM JNJ-42341806 (mGlu2 PAM). B: Results are presented as percentage of specific binding.

4. Discussion

To overcome the disadvantages associated to the use of orthosteric ligands as therapeutic approaches, alternative approaches are being looked into. One of these alternatives is the use of allosteric modulators. As already mentioned, targeting mGlu2 with PAMs may improve selectivity, tractability and tolerability. Thus, the emerging field of positive allosteric modulation of mGlu2 offers exciting potential for novel therapeutics in neurological and psychiatric disorders (Trabanco et al. 2011).

The presence of different modulatory sites on mGlu2 and mGlu3 receptors allows for the development of compounds that selectively potentiate mGlu2 but not mGlu3 or vice versa. Therefore it is important to investigate the molecular factors involved in the interaction between PAM compounds and the receptor. In this way it is possible to understand how these compounds bind and activate the receptor and may help the development of sub-type selective compounds.

In the present study, the molecular interaction of 24 PAMs with the mGlu2 receptor was clarified. In order to asses these interactions, the activity and affinity of these compounds on WT and point-mutated mGlu2 receptors was compared, aiming the confirmation or identification of new amino acids important for the interaction between PAMs and the mGlu2 receptor.

The ultimate goal of these studies is to generate a putative binding pocket for mGlu2 PAMs, which may help future chemistry efforts to identify druggable mGlu2 PAMs.

4.1. Expression of WT and mutated mGlu2 receptors

The Western Blot analysis revealed that the WT and mutated forms of the mGlu2 receptor were expressed and that the levels of expression are variable between the forms transiently transfected, while in the case of CHO-K1 cells stably transfected with WT receptor exhibit higher levels of expression.

4.2. Comparison between the affinity and potency for glutamate between WT and mutated hmGlu2 receptors

Besides the confirmation that mGlu2 receptors are being expressed by CHO-K1 cells it is also important to know if the receptor is still able to bind glutamate (orthosteric agonist) to know if the mutations do not have an effect on the receptor conformation that is needed to bind glutamate. It was possible to observe that glutamate was able to displace [^{3}H]-LY341495 in a concentration-dependent manner. The pIC_{50} values obtained were similar between the WT and tested mutated hmGlu2 receptors, which indicate that the mutated receptors are expressing the receptor and are able to bind glutamate in a similar way as the WT receptor.

To evaluate functional proprieties of the mutated receptors, concentration-response curves for glutamate-induced [^{35}S] GTPγS binding were generated. With this assay it was possible to

compare the functional activity of the WT and mutated receptors. Results showed that the response amplitude was variable within the mutated receptors, which is likely due to differences in receptor expression or transfection efficiency. Nevertheless all mutated receptors elicited concentration-response curves with similar potencies as the WT receptor (stably and transiently transfected), indicating that the mutated receptors were functional active and that mutations did not affect glutamate potency towards the receptor.

These results were expected since mutations were introduced outside of the extracellular domain; therefore no changes in the affinity or potency of orthosteric ligand were expected to be observed when compared to the WT.

4.3. Effect of mutations on positive allosteric modulators activity

Schaffhauser *et al*. (2003) identified amino acids present in TM4 and TM5 (S688, G689 and N735) as critical for the binding of LY487379. Studies with other compounds confirm that these amino acids seem to form a binding pocket (Hemstapat et al. 2007; B. A. Rowe et al. 2008). A recent study performed at Janssen Pharmaceutica showed that in addition to those already mentioned, amino acids present in TM3, 5 and 6 are also important for receptor-PAM interaction. These additional residues were: F643, W773, R635, L639, H723, L732, and F776.

The present study greatly expands this study both in terms of testing additional chemical classes as well as in further delineating the mGlu PAM binding pocket via the evaluation of additional compound-amino acid interactions. The results confirm the information about the critical amino acids for PAM's activity. In addition, it was shown that residues localized in TM2, not tested previously, do not affect the PAM's activity, and therefore do not contribute to the mGlu2 binding pocket.

Overall, effects of mutations F643A, S644A, G689V, G689V/S688L, S644A/V700L/H723V, H723V, S731A, L732A, N735D and W773A were similar across all the different compounds tested. This was especially surprising for the novel azetidine series as the compounds within this class did not seem to overlay in an mGlu2 PAM pharmacophore model that was generated previously. Interestingly, the mutations that showed to be important for the activity of these compounds were already reported as critical for the formation of the binding pocket: F643A, N735D/G689V/S688L, N735D ad W773A.

Overall, this study shows that structurally different mGlu2 PAMs appear to share a common group of amino acids to which they bind.

It is important to note that some amino acids reported for Lundstrom *et al*. (2011) as important for NAM's activity also have an effect on PAM's activity (F643A, H723V, L732A and W773A), indicating an overlap in the binding site on mGlu2 receptor for PAMs and NAMs. However,

mutation F780A shown a large effect on the binding of the NAMs Ro 4988546 and Ro 5488608, but no effect was observed on the activity of the tested PAMs. On the other hand, mutation S644A and S688L/G689V did not affect NAM's activity (Lundström et al. 2011) but was observed a big effect on PAM's activity, especially regarding the double mutation.

Allosteric binding studies have been performed for other mGlu receptors. Residue P655 on mGlu5 was shown to be important for the binding of a mGlu5 receptor NAM (MPEP) (Pagano et al. 2000) and the residue V757 on mGlu1 receptor was shown to be crucial for the binding of PAM compounds. In this study it was shown that the correspondent residues on mGlu2, F643 and L732, have a large effect on the tested PAMs.

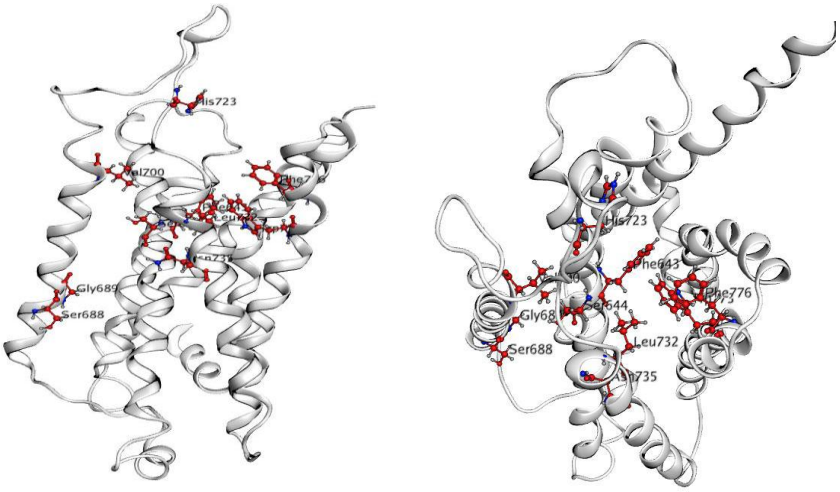


Figure 39. 3D representation of the receptor and the mutations that seem to affect the binding of the tested PAMs

mGlu2/3 comparison

Analysis of differences between mGlu2 and mGlu3 is important since these differences are what give compound selectivity.

In a previous study it was observed that the amino acids S644, V700 and H723 form an important binding pocket for the activity of PAMs and are responsible for their subtype-specificity (Master Thesis of Farinha, 2012). The results obtained in this study, when different compounds were tested, confirm this information. As shown, the triple mutation (S644A/V700L/H723V) caused the major effects, though the activity of compounds of the chemical class azetidine was not affected by this mutation. Furthermore, the other mutations selected based on sequence comparison between the mGlu2 and mGlu3 receptor (Table 6; Methods Section) do not affect the binding of any PAM compounds. These data give additional information about the critical amino acids for the subtype specificity highlighting the importance of the ones previously mentioned (S644, V700 and H723).

Amino acids indentified in literature that were also selected based on this criteria, namely, Ser688, Gly689 and Asn735 (B. A. Rowe et al. 2008; Hemstapat et al. 2007), were also tested in this

study and in the previous one. Once again the results obtained in this study confirm the ones previously obtained. In both studies was observed that mutation S688L did not affect PAM's activity. However mutation G689V, N735D and the double and triple mutation of these three amino acids had a high impact on the activity of all PAMs tested, with exception for the chemical class azetidine. In this case just the triple mutation and N735D had an effect when tested on glutamate-induced [^{35}S]GTPyS binding (EC_{50} > 10000 nM), which confirms the already reported importance of this amino acids in mGlu2 receptor subtype specificity.

4.4. Effect of mutations on positive allosteric modulators affinity

Saturation binding experiments with [^{3}H] JNJ-46281222 confirm it can still bind to the mutants at which it do not exerts a functional effect. Since the specific binding is reduced in the transient transfected pools it was impossible to calculate the K_D. Therefore it may be useful to do saturation bindings with addition of the agonist (glutamate).

The use of this tritiated form of PAM with one concentration confirm, that the mutations M7287A and V736A (previously tested in [^{3}H] JNJ-46281222 saturation binding), are still able to bind this compound in a similar way as the WT. Thus it is confirmed that the lack of effect on [^{35}S]GTPyS binding assay is due to the fact that these mutations do not elicit any change in the physical PAM-receptor interaction. On the other hand, this experiment shows in fact, that mutations that elicit a substantial decrease in the potency of JNJ-46281222 when tested in glutamate-induced [^{35}S]GTPyS binding (mutations N735D, S688L/G689V/N735D and F643A) are not able to bind the tritiated version of this compound in the same way as the WT. The triple mutation did abolish the binding ability when no glutamate was added. These data suggest that the activity of the PAM is reduced due to a lower binding affinity. This was confirmed when testing binding in the presence of glutamate, which was shown to increase binding at the WT receptor. Mutation S644A/V700L/H723V showed a reduction in the specific binding when glutamate is present, however this effect was not as big as seen for the mutations N735D, S688L/G689V/N735D and F643A. Nevertheless it is important to note that this mutation elicits a larger increase of EC_{50} of 13, when tested in the functional assay, and the other ones show an increase of 56 to 135-fold. Therefore these results confirm the data obtained when glutamate-induced [^{35}S]GTPyS assay was performed, with exception of mutation S688L/G689V. In the presence of glutamate the double mutation showed the same specific binding levels as the WT; however it was expected to have a similar binding pattern as mutation S644A/V700L/H723V. In general, these results seem to be in accordance with the data obtained from the functional assay. Nevertheless this was just a modest set of experiments and further investigation with other tritiated PAMs and mutants should be performed.

Conclusion Remarks

This study showed that the impact of several mutations is consistent between structurally different molecules indicating that mGlu2 PAMs may share a common group of amino acids to which they bind. Nevertheless is important to note that an apparent overlap in the binding site for NAMs and PAMs exists. It was also demonstrated that additional amino acids that were selected based on mGlu2/3 comparison did not seem to be important for PAM activity. Moreover it is suggested that the activity of the tested PAMs is reduced due to lower binding affinity.

The knowledge obtained from this study help gives further insight in the receptor regions involved in the formation of the binding pocket for allosteric modulators, contributing to the development of novel, potentially improved mGlu2 PAMs.

Appendix

Appendix 1. Complete names of the compounds mentioned in the text

Abbreviation	Name
(1S, 3R) - ACPD	1S, 3R-1-aminocyclopentane-1,3,4-tricarboxylic acid
(1S, 3S) - ACPD	1S, 3S-1-aminocyclopentane-1,3-dicarboxylic acid
(2R, 4R) - APDC	(2R, 4R)-4-Aminopyrrolidine-2,4-dicarboxylic acid
(2S, 4S) - ADPD	2S, 4S-2-amino-4-(4,4-diphenylbut-1-yl)-pentane-1,5-dioic acid
4C3HPG	RS-4-carboxy-3-hydroxyphenylglycine
BAY36-7620	(3aS6aS)-6a-naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]furan-1-on
BINA	3'[[(2-Cyclopentyl-2,3-dihydro-6,7-dimethyl-1-oxo-1H-inden-5-yl)oxy]methyl]-1[1,1'-biphenyl]-4-carboxylic acid
CPCCOEt	7-hydroxyiminocyclopropan[b]chromen-1ª-carboxylic acid ethyl ester
CTZ	Cyclothiazide
DCG-IV	(2S,2'R,3'R)-2-(2',3')-Dicarboxycyclopropyl glycine
EM-TBPC	1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[d]azepin-3-yl)-1,6-dihydro-pyrimidine-5-carbonitrile
LCCG-1	(2S,1'S,2'S)-2-(carboxycyclopropyl)glycine
LY341495	2SS-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl)proprionic acid
LY354740	(1S,2S,5R,6S)-(+)-2-aminobicylco[3.1.0]hexane-2,6-dicarboxylic acid
LY379268	(-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid
LY404039	(-)-(1R,4S,5S,6S)-4-amino-2-sulfonylbicyclo[3.1.0]hexane-4,6-dicarboxylic acid
LY487379	N-[4-(2-methoxyphenoxy)phenyl]-N-(2,2,2-Trifluoroethylsulfonyl)-pyrid-3-ylmethylamine
MCCG-I	α-methyl-L-CCG-I
MGS0028	(1R,2S,5S,6S)-2-amino-6-fluoro-4-oxobicyclo[3.1.0]hexane-2,6-dicarboxylic acid
MGS0039	(1R,2R,3R,5R,6R)-2-amino-3-(3,a-dichlorobenzyloxy)-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid
MK-801	Dizocilpine
MNI-135	[3-(7-iodo-4-oxo-4,5-dihydro-3H-benzo[1,4]diazepin-2-yl)-benzonitrile]
MNI-136	[7-bromo-4-(3-pyridin-3-yl-phenyl)-1,3-dihydro-benzo[1,4]diazepin-2-one]
MNI-137	[4-(7-bromo-4-oxo-4,5-dihydro-3H-benzo[1,4]diazepin-2-yl)-pyridine-2-carbonitrile]
MPEP	2-methyl-6-(phenylethynyl)pyridine
MPPG	α-methyl-4-phosphonophenylglycine
MRLSD-650	2-[(6,7-dichloro-2-cyclopentyl-2-methyl-1-oxo-2,3-dihydro-1*H*-inden-5-yl) oxy]-*N*-[4-(1*H*-tetraazol-5-yl) phenyl] acetamide
MSPG	α-methyl-4-sulfonophenylglycine
MTPG	A-methyl-4-tetra-zoylphenylglycine
PCCG-IV	(2S, 1'S, 2'S, 3'R)-2-(2'-carboxy-3'-phenylcyclopropyl) glycine
Ro 01-6128	(Diphenylacetyl)-carbamic acid ethy ester
Ro 67-4853	(9*H*-Xanthen-9-ylcarbonyl)-carbamic acid butyl ester
Ro 67-7476	(2*S*)-2-(4-Fluorophenyl)-1-[(4-methylphenyl)sulfonyl]-pyrrolidine
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

Appendix 2. Chemical class and name of each positive allosteric modulator tested in this study

Chemical class	Compound
1,4-Pyridone	JNJ-40068782
	JNJ-41329782
	JNJ- 54768636
	JNJ- 54768597
	JNJ-54752997
1,5-Pyridone	JNJ-35814376
Azetidine	JNJ-54352272
	JNJ-54750592
	JNJ-54469805
	JNJ-54800681
Imidazopyridine	JNJ-41482012
Isoquinolone	JNJ-39226421
	JNJ-40297036
Pyridazine	JNJ-54445001
Triazolopyridine	JNJ-46281222
	JNJ-46356479
	JNJ-42153605
	JNJ-42329001
	JNJ-43245046
	JNJ-42073824
	JNJ-54757027
THIIC (LY2607540)	JNJ-52149617
BINA	JNJ-35815013
TEMPS (LY487379)	JNJ-35814090

Appendix 3. Results from the screening on all the mutation. Mutations are sorted by receptor . EC_{50} and EC_{100} values for each PAM are indicated. The screening was performed in quadruplicated (n=1) with addition of 4 µM glutamate. Effects lower than 75% of the effect seen on the WT receptor (transiently transfected) were considered significantly different and are highlighted in red. Highlighted in yellow are the results lower than 50% of the effect seen on the WT

	PAM	JNJ 54768636 - AAA			JNJ 54768597 - AAA			JNJ 54752997 - AAA			JNJ 54757027 - AAA			JNJ 54445001 - AAA		
	[PAM] (M)	3,E-07	1,E-05	n	3,E-06	3,E-05	n	3,E-06	1,E-05	n	1,E-06	1,E-05	n	3,E-07	3,E-06	n
	Stable	141%	228%	6	128%	203%	6	70%	98%	6	157%	207%	6	128%	194%	6
	WT	154%	266%	6	139%	218%	6	84%	117%	6	164%	224%	6	140%	219%	6
	75% of WT	116%	200%		104%	164%		63%	88%		123%	168%		105%	164%	
	50% of WT	77%	133%		70%	109%		42%	59%		82%	112%		70%	110%	
TM 2	C616S (#37)	160%	210%	1	153%	214%	1	98%	116%	1	183%	194%	1	153%	169%	1
	I622F (#38)	221%	306%	1	233%	266%	1	104%	164%	1	250%	278%	1	203%	250%	1
	T641S (#39)	246%	300%	1	242%	283%	1	134%	191%	1	267%	285%	1	233%	288%	1
	A642S (#40)	152%	203%	1	149%	178%	1	87%	111%	1	172%	193%	1	141%	176%	1
TM3	R635A (#18)	24%	275%	1	98%	262%	1	6%	5%	1	152%	325%	1	15%	121%	1
	R636A (#6)	98%	100%	1	142%	173%	1	19%	37%	1	172%	226%	1	80%	146%	1
	L639A (#7)	104%	199%	1	94%	170%	1	43%	55%	1	118%	186%	1	98%	158%	1
	F643A (#8)	22%	64%	1	23%	15%	1	12%	22%	1	26%	11%	1	13%	31%	1
	S644A (#14)	75%	183%	1	65%	147%	1	35%	58%	1	89%	149%	1	68%	146%	1
TM4	S688L (#1)	185%	315%	1	155%	256%	1	52%	87%	1	205%	287%	1	172%	212%	1
	G689V (#2)	100%	239%	1	76%	170%	1	42%	50%	1	93%	150%	1	85%	156%	1
	G689V S688L (#4)	84%	218%	1	70%	175%	1	36%	39%	1	82%	155%	1	89%	185%	1
	N735D G689V S688L (#5)	18%	45%	1	11%	47%	1	20%	26%	1	38%	76%	1	25%	22%	1
	V700L (#15)	75%	237%	1	63%	150%	1	14%	32%	1	82%	177%	1	75%	158%	1
	S644A V700L H723V (#17)	102%	255%	1	131%	287%	1	74%	99%	1	169%	287%	1	101%	183%	1
	A681F (#23)	147%	230%	1	131%	199%	1	51%	67%	1	169%	184%	1	140%	206%	1
	I693M (#24)	132%	224%	1	130%	173%	1	59%	71%	1	159%	209%	1	108%	177%	1
	V695S (#25)	154%	234%	1	140%	198%	1	67%	87%	1	159%	200%	1	160%	205%	1
	A696V (#26)	138%	205%	1	124%	205%	1	64%	85%	1	139%	180%	1	133%	188%	1
ECL2	H723V (#16)	71%	200%	1	94%	218%	1	32%	39%	1	127%	222%	1	67%	153%	1
	G706R (#27)	127%	224%	1	123%	182%	1	45%	66%	1	124%	175%	1	114%	169%	1
	E708Y (#28)	128%	255%	1	130%	190%	1	44%	71%	1	164%	193%	1	116%	140%	1
	A710L (#29)	127%	211%	1	134%	173%	1	46%	74%	1	134%	169%	1	130%	182%	1
	P711A (#30)	135%	207%	1	108%	160%	1	56%	81%	1	135%	177%	1	119%	181%	1
	V716T (#31)	117%	209%	1	98%	172%	1	54%	68%	1	131%	175%	1	86%	162%	1
	T718I (#32)	118%	170%	1	113%	144%	1	45%	76%	1	131%	164%	1	107%	135%	1
TM5	D725A (#9)	190%	236%	1	146%	201%	1	138%	209%	1	169%	217%	1	149%	207%	1
	M728A (#19)	144%	205%	1	106%	134%	1	63%	95%	1	127%	168%	1	103%	162%	1
	S731A (#20)	108%	260%	1	97%	195%	1	56%	64%	1	125%	209%	1	74%	131%	1
	L732A (#10)	116%	162%	1	99%	107%	1	85%	80%	1	84%	72%	1	117%	157%	1
	N735D (#3)	71%	190%	1	52%	154%	1	42%	40%	1	124%	217%	1	62%	41%	1
	V736A (#21)	165%	303%	1	154%	222%	1	71%	108%	1	170%	279%	1	147%	230%	1
	A726S (#33)	176%	226%	1	125%	216%	1	59%	111%	1	138%	191%	1	138%	197%	1
	G730I (#34)	145%	205%	1	106%	179%	1	67%	89%	1	130%	161%	1	107%	174%	1
	A733T (#35)	261%	390%	1	242%	273%	1	146%	205%	1	283%	295%	1	232%	311%	1
	A740I (#36)	225%	330%	1	185%	303%	1	91%	134%	1	238%	273%	1	208%	267%	1
TM6	W773A (#11)	60%	111%	1	73%	96%	1	53%	60%	1	67%	43%	1	69%	86%	1
	F776A (#12)	121%	242%	1	190%	266%	1	69%	142%	1	238%	326%	1	192%	250%	1
	F780A (#13)	219%	312%	1	235%	319%	1	122%	221%	1	255%	310%	1	189%	249%	1
TM7	V798A (#22)	105%	316%	1	92%	230%	1	61%	49%	1	117%	218%	1	121%	228%	1

Appendix 3 (cont).

	PAM	JNJ 54352272 - AAA			JNJ 54750592 - AAA			JNJ 42073824 - AAA			JNJ 54469805 - AAA			JNJ 54800681 - AAA		
	[PAM] (M)	1,E-07	1,E-06	n	1,E-07	3,E-06	n	1,E-07	3,E-06	n	2,E-07	3,E-06	n	3,E-07	1,E-05	n
	Stable	130%	190%	6	95%	209%	6	175%	252%	6	142%	228%	6	102%	208%	6
	WT	151%	223%	6	105%	230%	6	192%	290%	6	160%	260%	6	107%	205%	6
	75% of WT	113%	167%		79%	173%		144%	218%		120%	195%		80%	154%	
	50% of WT	76%	112%		53%	115%		96%	145%		80%	130%		54%	103%	
TM 2	C616S (#37)	137%	173%	1	116%	174%	1	198%	197%	1	156%	195%	1	118%	157%	1
	I622F (#38)	223%	291%	1	190%	277%	1	252%	315%	1	233%	278%	1	180%	268%	1
	T641S (#39)	226%	309%	1	203%	291%	1	249%	310%	1	293%	311%	1	191%	285%	1
	A642S (#40)	155%	186%	1	145%	188%	1	174%	196%	1	170%	203%	1	140%	191%	1
TM3	R635A (#18)	29%	122%	1	16%	132%	1	75%	352%	1	33%	230%	1	11%	154%	1
	R636A (#6)	83%	131%	1	53%	168%	1	128%	278%	1	102%	228%	1	89%	209%	1
	L639A (#7)	118%	175%	1	103%	188%	1	142%	228%	1	130%	203%	1	109%	171%	1
	F643A (#8)	8%	25%	1	32%	42%	1	7%	50%	1	15%	17%	1	22%	26%	1
	S644A (#14)	78%	140%	1	66%	163%	1	106%	236%	1	111%	217%	1	71%	185%	1
TM4	S688L (#1)	193%	274%	1	123%	309%	1	253%	366%	1	217%	351%	1	145%	304%	1
	G689V (#2)	87%	143%	1	59%	159%	1	121%	258%	1	82%	182%	1	54%	148%	1
	G689V S688L (#4)	97%	163%	1	49%	161%	1	108%	253%	1	85%	196%	1	59%	170%	1
	N735D G689V S688L (#5)	21%	35%	1	21%	35%	1	19%	147%	1	27%	30%	1	19%	43%	1
	V700L (#15)	81%	166%	1	38%	181%	1	117%	291%	1	82%	222%	1	49%	173%	1
	S644A V700L H723V (#17)	96%	186%	1	87%	228%	1	125%	316%	1	114%	283%	1	105%	245%	1
	A681F (#23)	149%	202%	1	103%	198%	1	175%	235%	1	159%	194%	1	102%	199%	1
	I693M (#24)	110%	148%	1	77%	179%	1	163%	220%	1	111%	174%	1	96%	184%	1
	V695S (#25)	168%	201%	1	130%	193%	1	180%	228%	1	158%	216%	1	131%	198%	1
	A696V (#26)	156%	205%	1	114%	216%	1	164%	235%	1	175%	196%	1	115%	219%	1
ECL2	H723V (#16)	79%	182%	1	48%	157%	1	85%	216%	1	83%	193%	1	47%	152%	1
	G706R (#27)	128%	172%	1	98%	198%	1	160%	224%	1	144%	227%	1	90%	187%	1
	E708Y (#28)	99%	189%	1	134%	202%	1	157%	229%	1	130%	208%	1	108%	189%	1
	A710L (#29)	127%	172%	1	113%	179%	1	145%	194%	1	132%	173%	1	99%	165%	1
	P711A (#30)	115%	177%	1	95%	179%	1	148%	216%	1	124%	174%	1	91%	173%	1
	V716T (#31)	126%	184%	1	88%	188%	1	148%	182%	1	116%	201%	1	76%	149%	1
	T718I (#32)	123%	160%	1	93%	163%	1	142%	175%	1	118%	153%	1	88%	155%	1
TM5	D725A (#9)	146%	209%	1	113%	218%	1	179%	220%	1	157%	231%	1	154%	238%	1
	M728A (#19)	80%	144%	1	51%	142%	1	199%	269%	1	88%	186%	1	79%	153%	1
	S731A (#20)	76%	141%	1	63%	178%	1	144%	253%	1	112%	213%	1	74%	175%	1
	L732A (#10)	138%	178%	1	109%	155%	1	120%	164%	1	136%	153%	1	116%	168%	
	N735D (#3)	23%	29%	1	34%	56%	1	128%	289%	1	49%	80%	1	45%	49%	1
	V736A (#21)	118%	187%	1	79%	222%	1	228%	360%	1	134%	251%	1	74%	211%	1
	A726S (#33)	148%	201%	1	95%	195%	1	193%	214%	1	153%	220%	1	97%	208%	1
	G730I (#34)	107%	150%	1	108%	188%	1	165%	216%	1	120%	174%	1	84%	156%	1
	A733T (#35)	241%	340%	1	209%	324%	1	294%	378%	1	249%	320%	1	198%	298%	1
	A740I (#36)	188%	271%	1	174%	308%	1	235%	306%	1	254%	313%	1	157%	292%	1
TM6	W773A (#11)	58%	61%	1	58%	63%	1	62%	49%	1	54%	61%	1	57%	40%	1
	F776A (#12)	115%	181%	1	73%	168%	1	140%	306%	1	136%	239%	1	100%	197%	1
	F780A (#13)	150%	259%	1	95%	245%	1	196%	319%	1	156%	246%	1	124%	236%	1
TM7	V798A (#22)	68%	146%	1	82%	220%	1	168%	437%	1	82%	290%	1	72%	188%	1

Bibliography

Aghajanian, G.K. & Marek, G.J., 1999. Serotonin and Hallucinogens. *Neuropsychopharmacology*, 21(2), pp.16–23.

Anon, National institutional of Health. Available at: http://wwwapps.nimh.nih.gov/health/publications/depression/complete-publication.shtml [Accessed July 2, 2012].

Austin, M.P. et al., 1999. Cognitive function in depression: a distinct pattern of frontal impairment in melancholia? *Psychological medicine*, 29(1), pp.73–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10077295 [Accessed July 2, 2012].

Bessis, A.S. et al., 2000. Three-dimensional model of the extracellular domain of the type 4a metabotropic glutamate receptor: new insights into the activation process. *Protein science*, 9(11), pp.2200–2209. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2144486&tool=pmcentrez&rendertype=abstract.

Bissantz, Caterina, Logean, A. & Rognan, D., 2004. High-throughput modeling of human G-protein coupled receptors: amino acid sequence alignment, three-dimensional model building, and receptor library screening. *Journal of chemical information and computer sciences*, 44(3), pp.1162–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15154786.

Bockaert, J; Pin, J.P., 1999. Molecular tinkering of G protein-coupled receptors: an evolutionary success. *The EMBO Journal*, 18(7), pp.1723–1729.

Campbell, U.C. et al., 2004. The mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) potentiates PCP-induced cognitive deficits in rats. *Psychopharmacology*, 175(3), pp.310–318.

Chaki, S. et al., 2004. MGS0039: a potent and selective group II metabotropic glutamate receptor antagonist with antidepressant-like activity. *Neuropharmacology*, 46(4), pp.457–467.

Chavez-noriega, L.E. et al., 2005. Novel Potential Therapeutics for Schizophrenia: Focus on the Modulation of Metabotropic Glutamate Receptor Function. *Current neuropharmacology*, 3, pp.9–34.

Cherezov, V. et al., 2008. High Resolution Crystal Structure of an Engineered Human B2-Adrenergic G protein-Coupled Receptor. *Science*, 318(5854), pp.1258–1265.

Chesnoy, S. & Huang, L., 2000. Structure and funtion of lipid-DNA complexes for gene delivery. *Annual Review of Biophysical Biomolecular Structure*, 19, pp.27–47.

Chien, E.Y.T. et al., 2010. Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science*, 330(6007), pp.1091–1095.

Choi, S. & Lovinger, D.M., 1996. Metabotropic Glutamate Receptor Modulation of Voltage-Gated Ca2+ Channels Involves Multiple Receptor Subtypes in Cortical Neurons. *The Journal of neuroscience*, 16(1), pp.36–45.

Congreve, M. & Marshall, F., 2010. The impact of GPCR structures on pharmacology and structure-based drug design. *British journal of pharmacology*, 159(5), pp.986–96. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2839258&tool=pmcentrez&rendertype=abstract [Accessed March 10, 2013].

Conn, P J & Pin, J.P., 1997. Pharmacology and functions of metabotropic glutamate receptors. *Annual review of pharmacology and toxicology*, 37, pp.205–237. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9131252.

Conn, P Jeffrey, Christopoulos, A. & Lindsley, C.W., 2009. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nature reviews. Drug discovery*, 8(1), pp.41–54.

Conn, P Jeffrey, Lindsley, C.W. & Jones, C.K., 2008. Activation of metabotropic glutamate receptors as a novel approach for the treatment of schizophrenia. *Trends in pharmacological sciences*, 30(1), pp.25–31.

Dorr, P. et al., 2005. Maraviroc (UK-427 , 857), a Potent , Orally Bioavailable , and Selective Small-Molecule Inhibitor of Chemokine Receptor CCR5 with Broad-Spectrum Anti-Human Immunodeficiency Virus Type 1 Activity. *Antimicrobial agents and chemotherapy*, 49(11), pp.4721–4732.

Farinha, A., 2012. *Mapping the molecular determinants of positive allosteric modulators of the mGlu2 receptor*. University of Coimbra.

Fell, M.J. et al., 2011. benzyl) -1-methyl-1H-imidazole-4-carboxamide (THIIC), a Novel Metabotropic Glutamate 2 Potentiator with Potential Anxiolytic / Antidepressant Properties□: In Vivo Profiling Suggests a Link between Behavioral and Central Nervous System Neurochemical Cha. , 336(1), pp.165–177.

Fell, M.J. et al., 2008. Evidence for the Role of Metabotropic Glutamate (mGlu) 2 Not mGlu3 Receptors in the Preclinical Antipsychotic Pharmacology of the mGlu2 / 3 Receptor (-)-(1R,4S,5S,6S)-4-Amino-2-sulfonylbicyclo[3.1.0]hexane- 4,6-dicarboxylic Acid (LY404039) Agonist. *The journal of Pharmacology and experimental therapeutics*, 326(1), pp.209–217.

Fisher, K., Lefebvre, C. & Coderre, T.J., 2002. Antinociceptive effects following intrathecal pretreatment with selective metabotropic glutamate receptor compounds in a rat model of neuropathic pain. *Pharmacology, biochemistry, and behavior*, 73(2), pp.411–418.

Flanagan, C.A., 2005. A GPCR That Is Not “ DRY ”. , 68(1), pp.1–3.

Galici, R. et al., 2005. A Selective Allosteric Potentiator of Metabotropic Glutamate (mGlu) 2 Receptors Has Effects Similar to an Orthosteric mGlu2 / 3 Receptor Agonist in Mouse Models Predictive of Antipsychotic Activity. *The journal of pharmacology and experimental therapeutics*, 315(3), pp.1181–1187.

Galici, R. et al., 2006. Biphenyl-indanone A , a Positive Allosteric Modulator of the Metabotropic Glutamate Receptor Subtype 2 , Has Antipsychotic- and Anxiolytic-Like Effects in Mice. , 318(1), pp.173–185.

Gasparini, F & Spooren, W., 2007. Allosteric modulators for mGlu receptors. *Current neuropharmacology*, 5(3), pp.187–194.

Gether, U., 2000. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrine reviews*, 21(1), pp.90–113. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10696571.

Gether, U. & Kobilka, B.K., 1998. G Protein-coupled Receptors II Mechanisms of agonist activation. *The journal of Biological Chemistry*, 273(29), pp.17979–17982.

Grace, A.A., 2011. Dopamine system dysregulation by the hippocampus: implications for the pathophysiology and treatment of schizophrenia. *Neuropharmacology*, 62(3), pp.1342–1348. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3179528&tool=pmcentrez&rendertype=abstract [Accessed March 8, 2012].

Green, M.F. & Braff, D.L., 2001. Translating the basic and clinical cognitive neuroscience of schizophrenia to drug development and clinical trials of antipsychotic medications. *Biological psychiatry*, 49(4), pp.374–84.

Gregory, K.J. et al., 2011. Allosteric modulation of metabotropic glutamate receptors: structural insights and therapeutic potential. *Neuropharmacology*, 60(1), pp.66–81.

Hanson, M.A. et al., 2012. Crystal Structure of a Lipid G protein-coupled Receptor. , 335(6070), pp.851–855.

Harrison, C. & Traynor, J.., 2003. The [35S]GTPγS binding assay: approaches and applications in pharmacology. *Life Sciences*, 74(4), pp.489–508. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0024320503008920 [Accessed May 21, 2013].

Hemstapat, K. et al., 2007. A Novel Family of Potent Negative Allosteric Modulators of Group II Metabotropic Glutamate Receptors. , 322(1), pp.254–264.

Heresco-Levy, U., 2002. Placebo-Controlled Trial of D-Cycloserine Added to Conventional Neuroleptics, Olanzapine, or Risperidone in Schizophrenia. *American Journal of Psychiatry*, 159(3), pp.480–482.

Hermans, E., 2003. Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacology & Therapeutics*, 99(1), pp.25–44. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0163725803000512 [Accessed March 13, 2013].

Higgins, G.A. et al., 2004. Pharmacological manipulation of mGlu2 receptors influences cognitive performance in the rodent. *Neuropharmacology*, 46(7), pp.907–917.

Hudspith, M.J., 1997. Glutamate: a role in normal brain function, anaesthesia, analgesia and CNS injury. *British journal of anaesthesia*, 78(6), pp.731–747.

Jaakola, V. et al., 2009. The 2.6 A Crystal Structure of a Human A2A Adenosine Receptor Bounf To an Antagonist. *Science*, 322(5905), pp.1211–1217.

Javitt, D.C. et al., 2001. Adjunctive high-dose glycine in the treatment of schizophrenia. *The International Journal of Neuropsychopharmacology*, 4(04), pp.385–391.

Javitt, D.C., 2010. Glutamatergic theories of schizophrenia. *The Israel journal of psychiatry and related sciences*, 47(1), pp.4–16.

Kantrowitz, J.T. & Javitt, D.C., 2010. N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: the final common pathway on the road to schizophrenia? *Brain research bulletin*, 83, pp.108–121.

Knoflach, F. & Kemp, J. a, 1998. Metabotropic glutamate group II receptors activate a G protein-coupled inwardly rectifying K+ current in neurones of the rat cerebellum. *The Journal of physiology*, 509(2), pp.347–354.

Krieger, E., Nabuurs, S.B. & Vriend, G., 2003. Homology modeling. In *Structural Bioinformatics*. pp. 507–521.

Kunishima, N. et al., 2000. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature*, 407(6807), pp.971–7.

Leach, K., Sexton, P.M. & Christopoulos, A., 2007. Allosteric GPCR modulators: taking advantage of permissive receptor pharmacology. *Trends in pharmacological sciences*, 28(8), pp.382–9.

Lindberg, J.S. et al., 2005. Cinacalcet HCl, an oral calcimimetic agent for the treatment of secondary hyperparathyroidism in hemodialysis and peritoneal dialysis: a randomized, double-blind, multicenter study. *Journal of the American Society of Nephrology*, 16(3), pp.800–7.

Litschig, S et al., 1999. CPCCOEt, a noncompetitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. *Molecular pharmacology*, 55(3), pp.453–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10051528.

Lodge, D.J. & Grace, A. a, 2011. Hippocampal dysregulation of dopamine system function and the pathophysiology of schizophrenia. *Trends in pharmacological sciences*, 32(9), pp.507–513.

Lundstrom, K.H. & Chiu, M.L., 2006. *G Protein-Coupled Receptors in Drug Discovery* 1st editio., Taylor and Francis.

Lundström, L. et al., 2011. Structural determinants of allosteric antagonism at metabotropic glutamate receptor 2: mechanistic studies with new potent negative allosteric modulators. *British journal of pharmacology*, 164(2b), pp.521–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21470207 [Accessed May 19, 2012].

Macek, T.A. et al., 1996. Differential involvement of group II and group III mGluRs as autoreceptors at lateral and medial perforant path synapses. *Journal of neurophysiology*, 76(6), pp.3798–806.

Mahmood, T. & Yang, P.-C., 2012. Western Blot: Technique, Theory, and Trouble Shooting. *North American Journal of Medical Sciences*, 4(9), pp.429–434.

Marion, S. et al., 2006. A beta-arrestin binding determinant common to the second intracellular loops of rhodopsin family G protein-coupled receptors. *The Journal of biological chemistry*, 281(5), pp.2932–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16319069 [Accessed May 23, 2013].

May, L.T. et al., 2007. Allosteric modulation of G protein-coupled receptors. *Annual review of pharmacology and toxicology*, 47, pp.1–51.

Monn, J A et al., 1999. Synthesis, pharmacological characterization, and molecular modeling of heterobicyclic amino acids related to (+)-2-aminobicyclo[3.1.0] hexane-2,6-dicarboxylic acid (LY354740): identification of two new potent, selective, and systemically active agonists f. *Journal of medicinal chemistry*, 42(6), pp.1027–40.

Niswender, C.M. & Conn, P Jeffrey, 2010. Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annual review of pharmacology and toxicology*, 50, pp.295–322.

Pagano, A. et al., 2000. The Non-competitive Antagonists 2-Methyl-6- (phenylethynyl) pyridine and 7-Hydroxyiminocyclopropan [b] chromen-1a-carboxylic Acid Ethyl Ester Interact with Overlapping Binding Pockets in the Transmembrane Region of Group I Metabotropic Glutamate Recep. , 275(43), pp.33750–33758.

Palczewski, K. et al., 2000. Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor. *Science*, 289(5480), pp.739–745. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.289.5480.739 [Accessed February 27, 2013].

Patil, S.T. et al., 2007. Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nature medicine*, 13(9), pp.1102–7.

Pin, J.P., Parmentier, M.L. & Prézeau, L, 2001. Positive allosteric modulators for gamma-aminobutyric acid(B) receptors open new routes for the development of drugs targeting family 3 G-protein-coupled receptors. *Molecular pharmacology*, 60(5), pp.881–4.

Poncer, J.C., Shinozaki, H. & Miles, R., 1995. Dual modulation of synaptic inhibition by distinct metabotropic glutamate receptors in the rat hippocampus. *The Journal of physiology*, 485(1), pp.121–134.

Prézeau, L et al., 1994. Pharmacological characterization of metabotropic glutamate receptors in several types of brain cells in primary cultures. *Molecular pharmacology*, 45(4), pp.570–7.

Rondard, P. et al., 2011. The complexity of their activation mechanism opens new possibilities for the modulation of mGlu and GABAB class C G protein-coupled receptors. *Neuropharmacology*, 60(1), pp.82–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20713070 [Accessed June 9, 2012].

Rorick-Kehn, L.M. et al., 2007. Pharmacological and pharmacokinetic properties of a structurally novel, potent, and selective metabotropic glutamate 2/3 receptor agonist: in vitro characterization of agonist (-)-(1R,4S,5S,6S)-4-amino-2-sulfonylbicyclo[3.1.0]-hexane-4,6-dicarboxylic acid. *The Journal of pharmacology and experimental therapeutics*, 321(1), pp.308–17.

Rowe, B.A. et al., 2008. Transposition of Three Amino Acids Transforms the Human Metabotropic Glutamate Receptor (mGluR) -3-Positive Allosteric Modulation Site to mGluR2 , and Additional Characterization of the mGluR2-Positive Allosteric Modulation Site. *The Journal of Pharmacology and Experimental Therapeutics*, 326(1), pp.240–251.

Rowley, M., Bristow, L J & Hutson, P.H., 2001. Current and novel approaches to the drug treatment of schizophrenia. *Journal of medicinal chemistry*, 44(4), pp.477–501.

Salt, T.E. & Eaton, S.A., 1995. Distinct presynaptic metabotropic receptors for L-AP4 and CCG1 on GABAergic terminals: pharmacological evidence using novel alpha-methyl derivative mGluR antagonists, MAP4 and MCCG, in the rat thalamus in vivo. *Neuroscience*, 65(1), pp.5–13.

Sanacora, G. et al., 2008. Targeting the Glutamatergic System to Develop Novel, Improved Therapeutics for Mood Disorders. *Nature reviews. Drug discovery*, 7(5), pp.426–437.

Schaffhauser, H et al., 1998. In vitro binding characteristics of a new selective group II metabotropic glutamate receptor radioligand, [3H]LY354740, in rat brain. *Molecular pharmacology*, 53(2), pp.228–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9463480.

Schaffhauser, Hervé et al., 2003. Pharmacological characterization and identification of amino acids involved in the positive modulation of metabotropic glutamate receptor subtype 2. *Molecular pharmacology*, 64(4), pp.798–810.

Schoepp, D.D et al., 1995. Selective Inhibition of Forskolin-stimulated Cyclic AMP Formation in Rat Hippocampus by a Novel mGluR Agonist , 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate. *Neuropharmacology*, 34(8), pp.843–850.

Schwalbe, H. and Wess, G., 2002. Dissecting G-Protein-Coupled Receptors: *ChemBioChem*, 3(10), pp.915–919.

Shigemoto, R. & Mizuno, N., 2000. Metabotropic glutamate receptors - immunocytochemical and in situ hybridization analyses. *Handbook of chemical neuroanatomy*, 18, pp.63–98.

Shimamura, T. et al., 2012. Structure of the human histamine H1 receptor complex with doxepin. *Nature*, 475(7354), pp.65–70.

Siegel, G.J., Albers, R.W. & Brady, S.T., 2006. *Basic Neurochemistry* seventh ed. W. Alberts, S. Brady, & Donald Price, eds.,

Stilo, S.A. & Murray, R.M., 2010. The epidemiology of schizophrenia: replacing dogma with knowledge. , pp.305–315.

Swanson, C.J. et al., 2005. Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. *Nature reviews. Drug discovery*, 4(2), pp.131–44.

Thomsen, W., Frazer, J. & Unett, D., 2005. Functional assays for screening GPCR targets. *Current opinion in biotechnology*, 16(6), pp.655–65.

Trabanco, A. et al., 2011. Progress in the developement of positive allosteric modulators of the metabotropic glutamate receptor 2. *Current medicinal chemistry*, 18(1), pp.47–68.

Tsai, G. & Coyle, J.T., 2002. Glutamatergic Mechanisms in Schizophrenia. *Annual review of pharmacology and toxicology*, 42, pp.165–179.

Urwyler, S., 2011. Allosteric Modulation of Family C G-Protein-Coupled Receptors: from Molecular Insights to Therapeutic Perspectives. *Pharmacological Reviews*, 63(1), pp.59–126.

Vauquelin, G. & Von Mentzer, B., 2007. *G Protein-coupled Receptors*,

Venzala, E. et al., 2012. Social vs. environmental stress models of depression from a behavioural and neurochemical approach. *European Neuropsychopharmacology*.

Vinson, P.N. & Conn, P Jeffrey, 2012. Metabotropic glutamate receptors as therapeutic targets for schizophrenia. *Neuropharmacology*, 62(3), pp.1461–1472.

Walker, D.L. & Davis, M., 2002. The role of amygdala glutamate receptors in fear learning, fear-potentiated startle, and extinction. *Pharmacology, biochemistry, and behavior*, 71(3), pp.379–92.

Wess, J., 1998. Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacology & therapeutics*, 80(3), pp.231–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9888696.

Wise, A., Gearing, K. & Rees, S., 2002. Target validation of G-protein coupled receptors. *Drug discovery today*, 7(4), pp.235–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11839521.

Wu, B. et al., 2010. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science (New York, N.Y.)*, 330(6007), pp.1066–71. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3074590&tool=pmcentrez&rendertype=abstract [Accessed March 9, 2013].

Yanamala, N. & Klein-Seetharaman, J., 2010. Allosteric Modulation of G Protein Coupled Receptors by Cytoplasmic, Transmembrane and Extracellular Ligands. *Pharmaceuticals*, 3(10), pp.3324–3342. Available at: http://www.mdpi.com/1424-8247/3/10/3324/ [Accessed April 2, 2013].